STABILIZED ANTISENSE-LIPID PARTICLES WITH ENHANCED INTRACELLULAR DELIVERY PROPERTIES

by

LENORE JENNIFER LOUIE

B.Sc., The University of British Columbia, 1997

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES
Department of Biochemistry and Molecular Biology

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
June 2000

© Lenore Jennifer Louie, 2000
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of **Biochemistry**

The University of British Columbia
Vancouver, Canada

Date **June 16/00**
ABSTRACT

Effective systemic delivery systems for DNA and RNA are essential to the successful clinical application of genetic drugs. Liposomal formulations of genetic drugs, such as antisense oligonucleotides (AS-ODN) and plasmid DNA, are difficult to achieve as the large size and highly charged nature of these molecules mitigates against the formation of small, neutral, serum stable carriers, which are required to achieve the long circulation times necessary for efficient delivery to disease sites such as tumors. Stabilized antisense-lipid particles (SALPs) are liposomal carriers of antisense oligonucleotides that exhibit high antisense-to-lipid ratios, long circulation lifetimes and accumulate preferentially at disease sites. As a result of high encapsulation efficiencies, SALPs are able to deliver large quantities of AS-ODN to target tissues. However, SALPs are relatively ineffective at delivering antisense molecules inside target cells.

For AS-ODN to be effective in inhibiting gene expression, they must be internalized by the target cell, effectively released from endosomal compartments and translocated to the nucleus, their primary site of action. The studies conducted in this thesis examined methods to enhance the intracellular delivery of antisense oligonucleotides contained within SALP systems. In particular, we examined the effect of a surface associated cationic PEG-lipid (CPL4) on increasing cellular uptake of SALPs. Subsequent studies investigated the ability of dioleoylphosphatidylethanolamine (DOPE) in SALP systems to enhance nuclear delivery of AS-ODN sequences by promoting endosome destabilization.

It is shown that the incorporation of CPL4 into distearylphosphatidylcholine/cholesterol/1,2-dioleoyl-3-[N,N-dimethylamino]propane/PEG-CeramideC14 (20/45/25/10 mol%) SALP was successful in increasing the cellular internalization of these vesicles but
did not result in increased nuclear delivery of AS-ODN. Nuclear accumulation of oligonucleotides was achieved through the use of a novel formulation, DODAP/DOPE/PEG-Cer-C_{14}, in which the fusogenic lipid DOPE was substituted for DSPC. However, these liposomes are unstable. Reasons for SALP instability are discussed as are ways to improve the stability and intracellular delivery properties of SALP.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>x</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xi</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>xii</td>
</tr>
<tr>
<td>CHAPTER 1: INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Liposomes and the intracellular delivery of antisense oligonucleotides: Overview</td>
<td>1</td>
</tr>
<tr>
<td>1.2 ANTISENSE OLIGONUCLEOTIDES</td>
<td>2</td>
</tr>
<tr>
<td>1.2.1 Protein Expression</td>
<td>2</td>
</tr>
<tr>
<td>1.2.2 Antisense Oligonucleotides</td>
<td>2</td>
</tr>
<tr>
<td>1.2.3 Mechanisms of Action</td>
<td>4</td>
</tr>
<tr>
<td>1.2.3.1 Passive Arresting of Protein Synthesis</td>
<td>4</td>
</tr>
<tr>
<td>1.2.3.2 Active Catalytic Termination of Protein Synthesis</td>
<td>6</td>
</tr>
<tr>
<td>1.2.4 Biological Barriers</td>
<td>6</td>
</tr>
<tr>
<td>1.2.4.1 Nuclease Degradation of Antisense Oligonucleotides</td>
<td>7</td>
</tr>
<tr>
<td>1.2.4.2 Cellular Uptake of Antisense Oligonucleotides – Membrane</td>
<td>7</td>
</tr>
<tr>
<td>1.2.4.3 Toxicity of Antisense Oligonucleotides</td>
<td>8</td>
</tr>
<tr>
<td>1.2.5 Chemical Design and Modifications</td>
<td>8</td>
</tr>
<tr>
<td>1.2.6 Pharmacokinetics of Antisense Oligonucleotides</td>
<td>10</td>
</tr>
<tr>
<td>1.2.7 Potential Therapeutic Applications</td>
<td>10</td>
</tr>
<tr>
<td>1.3 CHEMICAL AND PHYSICAL PROPERTIES OF LIPIDS</td>
<td>11</td>
</tr>
<tr>
<td>1.3.1 Phospholipids</td>
<td>11</td>
</tr>
<tr>
<td>1.3.2 Lipid Polymorphism</td>
<td>13</td>
</tr>
<tr>
<td>1.3.3 Cholesterol</td>
<td>13</td>
</tr>
<tr>
<td>1.4 LIPOSOMES</td>
<td>16</td>
</tr>
<tr>
<td>1.4.1 Liposomes as Drug Carriers</td>
<td>16</td>
</tr>
<tr>
<td>1.4.2 Classification of Liposomes Based on Lamellarity</td>
<td>19</td>
</tr>
<tr>
<td>1.4.2.1 Multilamellar Vesicles (MLVs)</td>
<td>19</td>
</tr>
<tr>
<td>1.4.2.2 Small Unilamellar Vesicles (SUVs)</td>
<td>19</td>
</tr>
<tr>
<td>1.4.2.3 Large Unilamellar Vesicles (LUVs)</td>
<td>20</td>
</tr>
<tr>
<td>1.4.3 Passive versus Active Targeting of Liposomes to Disease Sites</td>
<td>20</td>
</tr>
<tr>
<td>Figure 1.1</td>
<td>Antisense Oligonucleotide Inhibition of Protein Expression</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Potential Mechanisms and Sites of Antisense Oligonucleotide Action</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>Chemical Modifications of Antisense Oligonucleotides</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>General Structure of a Phospholipid Molecule</td>
</tr>
<tr>
<td>Figure 1.5</td>
<td>Molecular Geometry and Morphological Structures of Lipids</td>
</tr>
<tr>
<td>Figure 1.6</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>Figure 1.7</td>
<td>Lamellarity of Liposomes</td>
</tr>
<tr>
<td>Figure 1.8</td>
<td>Structure of Dansylated Cationic PEG-lipid –4 (CPL4)</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Cryo-Transmission Electron Micrograph of Stablized Antisense-Lipid Particles (SALP)</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Uptake of FITC labeled EGFR AS-ODN into BHK Cells</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>Interaction of Cationic Lipid-Antisense Oligonucleotide Complexes and Stabilized Antisense-Lipid Particles (SALPs) with Cells</td>
</tr>
<tr>
<td>Figure 3.4</td>
<td>Insertion of Cationic PEG-lipids (CPLs) into Stabilized Antisense-lipid Particles (SALP)</td>
</tr>
<tr>
<td>Figure 3.5</td>
<td>Elution Profile of SALP-CPL4</td>
</tr>
<tr>
<td>Figure 3.6</td>
<td>Insertion Profile of CPL4 into DSPC/Chol/DODAP/PEG-Cer (20/45/25/10) SALP</td>
</tr>
<tr>
<td>Figure 3.7</td>
<td>Lipid Uptake of DSPC/Chol/DODAP/PEG-CerC14 SALP and SALP-CPL4 into BHK Cells</td>
</tr>
<tr>
<td>Figure 3.8</td>
<td>Fluorescence micrographs of lipid and oligonucleotide uptake of DSPC/Chol/DODAP/PEG-CerC14 SALP and SALP-CPL4 by BHK Cells</td>
</tr>
<tr>
<td>Figure 3.9</td>
<td>Insertion Profile of CPL4 into DODAP/DOPE/PEG-CerC20 SALP</td>
</tr>
<tr>
<td>Figure 3.10</td>
<td>Fluorescence micrographs of lipid and oligonucleotide uptake of DODAP/DOPE/PEG-CerC14 SALP and SALP-CPL4 by BHK Cells</td>
</tr>
</tbody>
</table>
Figure 3.11 Fluorescence micrographs comparing lipid uptake of different SALP formulations by BHK cells

Figure 3.12 Lipid Uptake of DODAP/DOPE/PEG-CerC_{20} and DODAC/DOPE/PEG-CerC_{20} SALP and SALP-CPL_{4} into BHK Cells
LIST OF TABLES

Table 3.1  AS-ODN Encapsulation of DSPC/Chol/DODAP/PEG-Cer Systems ..................43
Table 3.2  Insertion of CPL\textsubscript{4} into DSPC/Chol/DODAP/PEG-Cer ..................51
Table 3.3  AS-ODN Encapsulation of DODAP/DOPE/PEG-Cer SALP Systems ..................56
Table 3.4  Influence of Temperature on Insertion of CPL\textsubscript{4} into DODAP/DOPE/PEG-CerC\textsubscript{14} SALP .......................................................57
Table 3.5  Insertion of CPL\textsubscript{4} into DODAP/DOPE/PEG-Cer SALP ..................60
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>Cross sectional area of the acyl chains of lipids</td>
</tr>
<tr>
<td>AH</td>
<td>Cross sectional area of the headgroup of lipids</td>
</tr>
<tr>
<td>AS</td>
<td>Antisense</td>
</tr>
<tr>
<td>AS-ODN</td>
<td>Antisense oligonucleotides</td>
</tr>
<tr>
<td>BHK</td>
<td>Baby hamster kidney (cells)</td>
</tr>
<tr>
<td>Cer</td>
<td>Ceramide</td>
</tr>
<tr>
<td>Chol</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>CPL</td>
<td>Cationic PEG-lipid</td>
</tr>
<tr>
<td>CPL₄</td>
<td>Cationic PEG-lipid with four positive charges</td>
</tr>
<tr>
<td>D/L</td>
<td>Drug to lipid ratio</td>
</tr>
<tr>
<td>D/R</td>
<td>Dansyl to rhodamine fluorescence ratio</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DODAC</td>
<td>Dioleoyldimethylammonium chloride</td>
</tr>
<tr>
<td>DODAP</td>
<td>1,2-dioleoyl-3-[N,N-dimethylamino]propane</td>
</tr>
<tr>
<td>DOPA</td>
<td>Dioleoylphosphatic acid</td>
</tr>
<tr>
<td>DOPE</td>
<td>Dioleoylphosphatidylethanolamine</td>
</tr>
<tr>
<td>DSPC</td>
<td>Distearylphosphatidylcholine</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EPC</td>
<td>Egg phosphatidylcholine</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>HBSC</td>
<td>HEPES buffered saline with calcium</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]</td>
</tr>
<tr>
<td>ICR</td>
<td>Outbred strain of mice</td>
</tr>
<tr>
<td>LUV</td>
<td>Large unilamellar vesicles</td>
</tr>
<tr>
<td>MLV</td>
<td>Multilamellar vesicles</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>ODN</td>
<td>Oligodeoxynucleotides/oligonucleotides</td>
</tr>
<tr>
<td>OGP</td>
<td>n-octyl-beta-D-glucopyranoside</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEAA</td>
<td>Poly(2-ethylacrylic acid)</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PO</td>
<td>Phosphodiester</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphorothioate</td>
</tr>
<tr>
<td>RES</td>
<td>Reticuloendothelial system</td>
</tr>
<tr>
<td>Rho</td>
<td>Rhodamine</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SALP</td>
<td>Stabilized antisense-lipid particles</td>
</tr>
<tr>
<td>SPLP</td>
<td>Stabilized plasmid-lipid particles</td>
</tr>
<tr>
<td>SUV</td>
<td>Small unilamellar vesicles</td>
</tr>
<tr>
<td>t₁/₂</td>
<td>Half-life of dissociation</td>
</tr>
<tr>
<td>TX-100</td>
<td>Triton X-100</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

There are so many people who I would like to acknowledge, and for such different reasons. I would like to take this opportunity to thank you Pieter, for accepting me as one of your students and providing me with an interesting project and wonderful working atmosphere. I leave here with the knowledge that I should “work hard and party equally as hard.” I will learn “to get my priorities right,” one day.

I would like to extend a special thanks to Norbert, whose help and guidance without which would have taken me longer to get here. To Kim (for all your honest advice and never failing to “kill me”), Dave “Fenske” (who showed me that anyone can be funny), Lorne “Pomegranate” (for being such a good sport), Angel (for being “Angel”), Ismail (for your subtle humour and being “Ismail”), Ammen (for our little talks), Tabitha, and John, my sincerest thanks for your friendship and support over the years.

Last but not least, I would like to thank my “chief bottle washer and cook,” for the “little things” he does and his continuous support and encouragement. William, I couldn’t thank you enough.
DEDICATION

To my

Mom & Dad

Who taught me that nothing is too difficult if you try and that no goal is ever out of reach

and

William

For always being there
CHAPTER 1

INTRODUCTION

1.1 Liposomes and the intracellular delivery of antisense oligonucleotides: Overview

The elucidation of the molecular defects that give rise to disease conditions combined with the information derived from the human genome sequencing project will lead to a new generation of therapeutic agents. Antisense technology is a novel molecular approach to the design of therapeutics based on the specific inhibition of gene expression. Antisense oligonucleotides are short sequences of single-stranded DNA designed to bind DNA or RNA targets, consequently blocking expression of the protein. These nucleotide sequences can be used to decrease the expression of proteins involved in the initiation or progression of a disease, leading to potential treatments for a wide range of conditions including cancer, viral infections, inflammatory and cardiovascular diseases. However, the pharmacological activity of antisense oligonucleotides is restricted by their molecular weight, charge, chemistry and ability to access intracellular sites of action. To overcome these obstacles, researchers have chemically modified oligonucleotides and have used various carriers to address the problems of serum degradation and low levels of intracellular delivery. The work presented in this thesis characterizes “stabilized antisense-lipid particles” (SALP), a liposomal drug delivery system for antisense oligonucleotides, and examines the effect of these carriers, alone or with surface associated cationic PEG-lipids, on the intracellular delivery of antisense oligonucleotides. This chapter summarizes features relevant to the design of antisense-liposomal drug delivery vehicles. First I discuss antisense oligonucleotides followed by the properties of lipids that are used to form liposomes.
1.2 ANTISENSE OLIGONUCLEOTIDES

1.2.1 Protein Expression

The human genetic code is contained within our DNA by the unique arrangement of four bases: adenine, guanine, cytosine and thymine. The information encoded by nucleotide sequences specifies individual proteins. The information in one of the two DNA strands, termed the template or sense strand, is enzymatically copied by RNA polymerase generating a messenger RNA (mRNA) sequence, otherwise referred to as the sense transcript (Figure 1.1). Following post-transcriptional modifications, mRNA is translocated into the cytoplasm where the combined activity of ribosomes and transfer RNA translates the sequence, generating a polypeptide chain that assembles into a protein molecule.

1.2.2 Antisense Oligonucleotides

Antisense inhibition of gene expression was first proposed by Zamecnik and Stephenson in 1978. Since then, numerous studies have examined the effect of these genetic drugs to alter protein expression in a variety of in vitro and in vivo situations and have documented their usefulness (reviewed in Stein and Cohen, 1989; Putnam, 1996).

Antisense oligonucleotides are short single-stranded sequences of DNA designed to hybridize to a specific and complementary sequence on sense mRNA transcripts through Watson and Crick base-pairing. Such binding interferes with the normal processes involved in translation by blocking the function of various proteins involved in translation, thereby ultimately inhibiting gene expression.

The selectivity of Watson-Crick base-pairing ensures the specificity of antisense molecules. One method through which conventional drugs inhibit the function of a protein
The first step of protein synthesis involves transcribing the information contained in DNA into a messenger RNA (mRNA) sequence. This intermediary sequence is then exported into the cytoplasm where it is translated by the protein synthesis machinery into peptide sequences which form protein molecules. Antisense oligonucleotides are small molecules of single stranded DNA designed to target a specific sequence contained in the mRNA of a protein. Subsequent binding of the antisense molecule to mRNA via Watson-Crick base-pairing blocks protein expression. This is a simplified model of antisense activity. A more detail account of antisense mechanisms are detailed in Figure 1.2.
or enzyme is by binding to their active sites. Such mechanisms can lack specificity and produce undesirable side effects. Specificity of antisense molecules is based on the observation that a sequence of approximately 13 bases in RNA and 17 bases in DNA are unique in the human genome (Hélène and Toulme, 1990). Thus, designing an ODN of appropriate length will ensure a high degree of selectivity. The affinity and specificity of antisense molecules can be increased by lengthening the oligonucleotide; however, long sequences can adopt secondary structures which impede binding to the target and enables partial matching to non-targeted regions. To avoid this problem, the molecules used are generally no longer than 25 bases.

1.2.3 Mechanisms of Antisense Action

There are several mechanisms by which antisense oligonucleotides can potentially interrupt or inhibit gene expression by interfering with either transcription or translation. However, little is known about the precise mode of action of antisense at the molecular level.

1.2.3.1 Passive Arresting of Protein Synthesis

Binding of ODNs to the target mRNA possibly inhibits gene expression through simple steric hindrance (Hélène and Toulme, 1990). The RNA-DNA duplex may block the RNA from physically interacting with cellular components involved in protein synthesis (Figure 1.2). In the cytoplasm, steric obstruction could inhibit processes such as translation initiation or ribosomal elongation. However, passive blocking of translation by hybridization arrest may not be very effective, as ribosomes are able to displace the antisense oligonucleotides (Uhlmann and Peyman, 1990). In the nucleus, blocking could inhibit post-
Within a cell, there are several potential sites for the binding of antisense oligonucleotides to targeted sequences which could disrupt or halt the normal mechanisms involved in RNA metabolism and protein expression. These include: (1) competing with transcription factors, (2) binding to single stranded DNA, (3) binding to double stranded DNA to form a triple helix, (4) acting as a substrate for RNase H which cleaves the RNA strand of RNA-DNA hybrids, (5) inhibiting 5'-cap formation, (6) inhibiting RNA splicing, (7) inhibiting mRNA polyadenylation, (8) inhibiting RNA export into the cytoplasm, (9), inhibiting 5'-cap recognition, (10) inhibiting translation by preventing ribosome function, (11) disrupting RNA-protein interactions.
transcriptional modifications such as pre-mRNA splicing, polyadenylation, capping or transport of mRNA into the cytoplasm (Hélène and Toulme, 1990). Additionally, oligonucleotides can arrest the transcriptional process by acting as transcription factor decoys (Sharma et al., 1996) and by binding to double stranded genomic DNA to form a triplex structure (Sun et al., 1996).

1.2.3.2 Active Catalytic Termination of Protein Synthesis

Inhibition of protein synthesis may also be achieved through enzymatic cleavage of the mRNA transcript. Upon hybridization, the RNA-DNA duplex can activate and subsequently serve as the substrate for cellular RNase H. RNase H is a ubiquitous nuclear enzyme which cleaves the RNA strand of RNA-DNA hybrids (Stein and Cheng, 1993). Once the mRNA sequence has been digested, the antisense molecule is free to hybridize with other mRNA sequences, thus acting catalytically as opposed to stoichiometrically. RNase H is sensitive to the chemistry of the RNA-DNA duplex and most of the structurally modified antisense oligonucleotides are unable to activate RNase H (Lönberg and Vuorio, 1996). Only oligonucleotides with the natural phosphodiester or modified phosphorothioate backbone act as substrates for RNase H (Agrawal et al., 1990).

1.2.4 Biological Barriers

In order for antisense oligonucleotides to be successful therapeutics, the problems of degradation and inactivation by nucleases, rapid elimination from circulation, low intracellular delivery, non-specific sequence side-effects, non-specific protein interactions and systemic toxicity must be overcome.
1.2.4.1 Nuclease Degradation of Antisense Oligonucleotides

A concern in the delivery of antisense agents is their susceptibility to degradation by enzymes in the blood and within cells. Therefore, methods to enhance antisense efficacy have focused on modifying oligonucleotides (section 1.3.5) to reduce nuclease susceptibility while retaining their affinity and specificity to bind target sites. Various studies have revealed that phosphodiester (PO) linkages of naturally occurring oligonucleotides are highly susceptible to cleavage by nucleases. A common chemical alteration replaces a non-bridging oxygen of the phosphate backbone with a sulfur atom to generate a phosphorothioate (PS) molecule which dramatically increases resistance to nuclease digestion (Eckstein, 1985).

1.2.4.2 Cellular Uptake of Antisense Oligonucleotides – Membrane Permeability Barrier

Membranes are the greatest obstacle to antisense activity. Polyanionic oligonucleotides are unable to cross cellular membranes passively due to their large, highly charged nature. Since their sites of activity are in the cytoplasm and/or nucleus, penetration into cells is essential for antisense inhibition of gene expression. The primary mechanism of internalization for charged oligonucleotides is receptor-mediated endocytosis at low concentrations and predominantly pinocytosis or fluid phase endocytosis at higher concentrations (Sharma and Narayanan, 1995). These processes are extremely inefficient and intracellular delivery is further compromised by the limited ability of oligonucleotides to escape the endo-lysosomal pathway. Although antisense sequences are internalized, the extent of uptake is not adequate for activity. Consequently, various intracellular delivery techniques have been developed.
1.2.4.3 Toxicity of Antisense Oligonucleotides

The toxicity associated with antisense oligonucleotides is mild at low concentrations. At high levels, however, the kidney, liver and bone marrow have been identified as the major sites of toxicity, with the principal hematological finding being dose-dependent thrombocytopenia (Sarmiento et al., 1994). These toxicities are independent of length or base composition but are related to oligonucleotide charge. The polyanionic nature of charged PO and PS-ODNs can result in non-specific binding to various blood proteins, which may be sequence selective (Branda et al., 1993). The association directly interferes with oligonucleotide function as well as those of the proteins. Protein-oligonucleotide interactions do not account for all of the side effects of antisense drugs. Other non-specific effects of antisense drugs include (i) immune stimulation by some nucleotide sequences and structural motifs, such as that by CpG motifs (Krieg et al., 1995), (ii) activation of Sp1 transcription factor (Perez et al., 1994), (iii) complement activation (Henry et al., 1997) and (iv) effects due to degradation products of ODNs (Stein and Cheng, 1993).

1.2.5 Chemical Design and Modifications

Chemical modification of the phosphodiester linkage of oligonucleotides can provide antisense molecules with enzymatic stability without affecting binding capacities (Figure 1.3). The modifications are designed to retain spatial configurations consistent with naturally occurring phosphodiester bonds, to maximize hybridization energy and nuclease stability while minimizing non-specific interactions causing side effects. Numerous modifications to the sugar-phosphate backbone structures, the nucleotide base and the sugar moiety have been made to attain sufficient nuclease stability and have met with variable success. These
Numerous chemical alterations to the general structure of oligonucleotides have been made in an attempt to overcome the various limitations associated with antisense therapy. The modifications have included changing the oligonucleotide backbone, saccharide moiety, and the bases while maintaining specificity and affinity to their target sequences.
modifications appear to render oligonucleotides stable towards nucleases but unfortunately, result in decreased affinity for RNA (De Mesmaeker et al., 1995). The new generation of antisense molecules that consists of combinations of chemically altered backbones and bases or sugars have generally shown increased efficacy. Mixed backbone oligonucleotides with phosphorothiate segments at 3' and 5' ends and modified oligodeoxynucleotide or oligoribonucleotide central segments have shown improved properties compared to PS-ODN with respect to affinity to RNA, RNase H activation, and activity (Agrawal et al., 1997).

1.2.6 Pharmacokinetics of Antisense Oligonucleotides

The pharmacokinetic profile of antisense sequences depends primarily on the type of bond linking the bases (Putnam, 1996). Antisense oligonucleotides with phosphodiester linkages exhibit very short half-lives, some less than 5 min (Agrawal et al., 1995). Antisense molecules with phosphorothioate linkages have perhaps the most favourable pharmacokinetic profile. PS-ODN exhibit biphasic clearance kinetics with an initial half-life of distribution of less than 1 h and an elimination-phase half-life of approximately 40 h (Agrawal et al., 1995). The oligonucleotides distribute to all major tissues throughout the body, except the brain, with the majority accumulating in the liver and kidneys. Antisense molecules are eliminated from the circulation primarily by excretion through the urine (Agrawal et al., 1995).

1.2.7 Potential Therapeutic Applications

The potential therapeutic applications of antisense drugs are immense. In theory, any disease state with a gene-expression component involving protein over-expression has the potential to be treated with antisense agents. An antisense oligonucleotide can be generated
to target any protein provided the nucleic acid sequence of the parent gene is known. This is
in contrast to the lengthy process involved in identifying protein function and defining
receptor-ligand or enzyme-substrate relationships necessary to design small molecular weight
conventional drugs. To date, viral infections and cancer have been the disease states
primarily investigated. Other applications include restenosis, rheumatoid arthritis and
allergies (reviewed in Putnam, 1996; Sharma and Narayanan, 1995).

1.3 CHEMICAL AND PHYSICAL PROPERTIES OF LIPIDS

1.3.1 Phospholipids

Lipids play an essential role in biological systems. They function as reservoirs for
energy, constitute the barriers for cellular compartmentalization and play vital roles in
signaling, fusion and transport processes among many others. The biological importance of
phospholipids to living organisms is emphasized by the paucity of genetic defects in the
metabolism of these lipids in humans. Presumably, such defects are lethal in early
developmental stages.

Phospholipids are composed of various combinations of polar (hydrophilic)
headgroups coupled to apolar (hydrophobic) tails (Figure 1.4). The specific combination
dictates the physical properties of the lipids (Cullis and de Kruijff, 1979). The acyl chain
length and the degree of saturation governs bilayer fluidity and the gel-liquid crystalline
phase transition temperature ($T_m$), which directly affect membrane permeability. In general,
long acyl chains and high degrees of saturation give rise to higher phase transition
temperatures. Cis-double bonds form “kinks” or bends in the lipid tails which decrease the
“order” in the acyl chain region resulting in a less rigid, more permeable membrane structure.
Phospholipids are composed of three principal segments: a headgroup, a glycerol backbone and acyl chains. Commonly occurring headgroups and fatty acid molecules are presented below.
1.3.2 *Lipid Polymorphism*

Phospholipids are able to form a variety of structures in aqueous media. The ratio between the cross sectional area of the lipid headgroup ($A_H$) and the fatty acyl chains ($A_C$) is a critical parameter in determining the geometry of lipid molecules and the structures these lipids adopt (Figure 1.5). When the cross sectional area of the headgroup is greater than that swept by the acyl tails, the lipids exhibit an “inverted-cone” shape (Figure 1.5a). These lipids tend to pack into micelles, a structure adopted by detergents. When this is reversed and the cross sectional area of the headgroup is less than that occupied by the acyl chains, the lipids are “cone” shaped (Figure 1.5c). These lipids form hexagonal $H_{II}$ phase structures in aqueous media, which consists of cylinders of lipids surrounding long aqueous channels (Cullis and de Kruijff et al., 1979). However, these lipids will form bilayers when stabilized by bilayer-forming lipids. Lipids exhibiting a headgroup-to-acyl chain cross sectional area ratio of unity display “cylindrical” geometry and spontaneously form bilayers (Figure 1.5b).

1.3.3 *Cholesterol*

Cholesterol is the major neutral lipid component in plasma membranes of eukaryotic cells. Due to the polar 3-β-hydroxyl group, this steroid also possesses amphipathic characteristics. Consequently, when introduced into a phospholipid bilayer, the hydroxyl group orients such that it is adjacent to the fatty acyl carbonyl esters of the phospholipids while the rigid steroid nucleus interacts extensively with the hydrocarbon chains (Huang, 1977) (Figure 1.6).
Lipids can adopt a number of bilayer morphologies depending on their molecular geometry. The molecular geometry of lipids is dictated by the ratio of the cross sectional area of the headgroup ($A_H$) to the cross sectional area of the acyl chains ($A_c$). (A) "Inverted cone" shaped lipids, (B) "Cylindrical" shaped lipids and (C) "Cone" shaped lipids.

Figure 1.5
Molecular Geometry and Morphological Structures of Lipids
Figure 1.6
Cholesterol

(a) Structure of cholesterol. (b) Positioning of cholesterol with the lipid bilayer.
Cholesterol has a number of effects in membranes. In bilayer systems, cholesterol exhibits a well-characterized ability to increase the fluidity of membranes at temperatures below $T_m$ while decreasing the fluidity at temperatures greater than $T_m$. The inclusion of 30 mol% cholesterol or higher effectively eliminates the sharp transition between gel and liquid crystalline phases (Chapman, 1975).

The inclusion of cholesterol stabilizes liposomes in the circulation and reduces their uptake by the reticuloendothelial system (Gregoriadis and Davis, 1979; Kirby et al., 1980). This effect results from decreased binding of serum proteins (Dave and Patel, 1986), as cholesterol, at levels greater than 30 mol%, has shown to reduce association of opsonizing serum proteins (Gregoriadis and Davis, 1979; Kirby et al, 1980). Additionally, the large steroid nucleus of cholesterol increases the packing densities of the phospholipid acyl chains which may explain the decreased permeability of liquid crystalline lamellar systems (Demel and de Kruijff, 1976) and enhanced solute retention (Papahadjopoulos et al., 1973).

1.4 LIPOSOMES

1.4.1 Liposomes as Drug Carriers

The structures formed by lipids when dispersed in an aqueous environment were first documented by Bangham et al. in 1965. They reported that lipids adopt a bilayer configuration resulting in a closed spherical structure, later referred to as liposomes by Sessa and Weissmann (1968). The characteristic lipid bilayer forms as a consequence of the amphipathic nature of lipids (Figure 1.7a). The polar (hydrophilic) headgroup and apolar (hydrophobic) tail portions associate such that the most energetically favourable conformation is obtained. Hence, the hydrophilic regions orient toward the aqueous phase.
Figure 1.7
Lamellarity of Liposomes

(a) Illustration of the natural tendency for amphipathic lipids to form bilayer configurations. (b) Schematic representation and freeze fracture micrographs of MLVs, LUVs, and SUVs.
while the hydrophobic acyl chains associate with themselves, and are partitioned away from the aqueous environment.

Liposomes are extremely useful as models of biological membranes. Studies employing liposomes have enabled the assessment of the structural and functional roles of lipids in isolation or collectively in well-defined systems. Such studies have elucidated the roles of lipids in providing a permeability barrier (Deamer and Bramhall, 1986), as mediators of membrane fusion (Cullis et al., 1986) and as triggers of specific biological responses such as complement activation (Muller-Eberhard, 1988). Furthermore, liposomes are crucial in the characterization of membrane protein interactions with lipids (Jost and Griffith, 1982) and of how membrane proteins function (Racker, 1972).

Liposomes can also be used as drug delivery systems. The internal aqueous cavity is capable of entrapping hydrophilic molecules while the lipid bilayer allows the association of lipophilic moieties. Consequently, liposomes have been developed as drug carrier systems for the administration of numerous therapeutic agents. Since the initial reports by Sessa and Weissmann (1968) that liposomes were able to entrap ions and small molecules, these vesicles have been refined to deliver a wide range of drugs including anticancer drugs (Mayer et al., 1989), anti-viral drugs (Szebeni et al., 1990), anti-bacterial agents (Bakker-Wondenberg et al., 1989) and anti-fungal pharmaceuticals. Encapsulation of these drugs can significantly decrease drug toxicity associated with systemic injection (Mayer et al., 1990; Gregoriadis, 1995) by reducing delivery to sensitive tissues. Higher doses of therapeutic agents can be administered resulting in improved drug efficacy (Gabizon et al., 1986; Bally et al., 1990). Drugs contained in small (diameter <100 nm) liposomes that exhibit long circulation lifetimes can also exhibit enhanced efficacy due to their ability to accumulate as
sites of disease such as tumors (Gabizon and Papahadjopoulos, 1988; Chonn and Cullis, 1995).

1.4.2 Classification of Liposomes Based on Lamellarity

1.4.2.1 Multilamellar Vesicles (MLVs)

Dispersion of bilayer-forming lipids in aqueous media leads to the spontaneous formation of liposomes. Hydration of lipids from a dried film usually results in liposomes with multiple bilayers configured as concentric spherical lamellae resembling "vesicles within vesicles," commonly referred to as multilamellar vesicles (MLVs) (Figure 1.7b). MLV preparations exhibit a broad size distribution, typically possess diameters on the order of several micrometers, and are heterogeneous with respect to size and lamellarity. With the majority of lipids present in internal bilayers, MLVs have small trapped volumes of about 0.5 μl/μmol lipid, despite their large size. A freeze-thaw technique established by Mayer et al. (1985) improves trapping efficiency and is able to achieve trapped volumes in excess of 2 μl/μmol lipid by increasing interbilayer spacing. This can also be accomplished through the incorporation of charged lipids which cause electrostatic repulsion between internal lamellae, thereby increasing the distance between bilayers (Hope et al., 1986). As a result of their size, MLVs are rapidly cleared from circulation by the reticuloendothelial system. Consequently, they are rarely used in vivo.

1.4.2.2 Small Unilamellar Vesicles (SUVs)

Processes such as sonication (Huang, 1969) or high pressure homogenization
(Barenholz et al., 1979) are able to produce small unilamellar vesicles (SUVs) with diameters ranging from 20-50 nm (Figure 1.7b). Although these methods produce liposomes with small, uniform sizes, they are restricted in their application because of small trapped volumes (< 0.5 µl/µmol lipid). These liposomes represent the lower physical limit of vesicle size and as such exhibit a high surface curvature, rendering the vesicles unstable and prone to fusion (Wong et al., 1982).

1.4.2.3 Large Unilamellar Vesicles (LUVs)

The inherent instability of SUVs led to the use of large unilamellar vesicle (LUVs) (Figure 1.7b). They can be prepared by detergent dialysis, infusion and reverse phase evaporation (Gennis, 1989). LUVs have also been produced from MLVs by high-pressure extrusion wherein MLVs are forced through polycarbonate filters of defined pore sizes (Hope et al., 1985). Sizes generally range from 50-400 nm in diameter with a narrow size distribution. The extrusion method permits the formation of liposomes composed of a wide variety of lipid species at high concentrations without contamination by detergents or solvents (Hope et al., 1985).

1.4.3 Passive versus Active Targeting of Liposomes to Disease Sites

Liposomes are able to exit the circulation and extravasate through areas where the vasculature naturally allows the extravastion of particles or is made “leaky” by disease. There are three major classes of capillary endothelial vascular structure: continuous, fenestrated and discontinuous. Continuous capillaries are relatively impermeable vessels while fenestrated capillaries contain 30-80 nm gaps between endothelial cells covered by a
layer of mucoprotein. Liposome extravasation occurs mainly in areas of discontinuous endothelium where gaps are on the order of 100 nm in diameter or larger. The penetration of liposomes through endothelial vasculature and accumulation in local tissues is known as passive targeting.

Various chemical techniques enable the coupling of ligands to vesicle surfaces. Based on the affinity between paired ligands and receptors, liposomes with a defined ligand attached to the external membrane can be targeted to cells with the matching receptor. Hence, ligand-conjugated lipid vesicles should preferentially accumulate in tissues expressing the corresponding receptor. This is known as active targeting.

1.4.4 DOPE and Cationic Lipids as Membrane Destabilizers

Unsaturated phosphatidylethanolamines (PEs) are common membrane phospholipids which, in isolation, spontaneously adopt the inverted hexagonal phase (H_II phase). They have been proposed to play a key role in membrane fusion by facilitating the formation of highly curved fusion intermediates (Hope and Cullis, 1981; de Kruijff et al., 1985; Ellens et al., 1986). Unsaturated PEs can adopt a bilayer structure in the presence of stabilizing lipids such as PCs, detergents, and PEG-lipid constructs (Seddon, 1990; Madden and Cullis, 1982; Holland et al., 1996). Loss of the stabilizing function results in H_II phase formation and is, for liposomes, accompanied by leakage of contents. Formation of non-bilayer structures also results in extensive lipid mixing.

The cationic lipid component of vesicles enhances the association of liposomes with nucleic acid polyanions through electrostatic interactions, allowing “complexes” to be formed. This results in partial protection of these molecules against hydrolytic enzymes. A
net positive charge of the complexes facilitates uptake into cells by allowing efficient interaction with the negatively charged surface of cell membranes. The cationic lipid-mediated association with cell membranes may also initiate membrane fusion and disruption. Destabilization of the endosomal membrane by the complexes with concomitant displacement of the cationic lipid by ion pair formation with anionic cellular lipids may be responsible for the release of nucleic acid-based drugs from endocytic compartments into the cytosol (Zelphati and Szoka, 1996).

The use of cationic lipids for the encapsulation of nucleotide-based drugs, such as antisense oligonucleotides, within liposomes has also been investigated. The use of tertiary amines, such as DODAP, has several advantages over quaternary amines. Farhood et al. (1992) have shown that lipids bearing tertiary amines exhibit lower levels of toxicity than quaternary amine analogues. DODAP, a pH sensitive cation, is positively charged at low pH but is neutral at physiological pH and higher. As such, entrapment of antisense oligonucleotides can be performed at pH 4 when DODAP is positively charged. Unencapsulated oligonucleotides can be dissociated from their interactions with cationic lipids by neutralizing the pH and subsequently removed by anion exchange chromatography. Adjusting the pH to 7.5 renders the liposome surface charge neutral, offering the potential of minimized complications due to toxicity encountered with charged carrier systems.

1.4.5 Stabilized Antisense-Lipid Particles (SALPs)

The inability of oligonucleotides to penetrate cell membranes efficiently is a major factor limiting the efficacy of antisense therapy. Various strategies to increase oligonucleotide intracellular delivery have been examined (Lönnberg and Vuorio, 1996).
These approaches have focused on site specific delivery, augmenting the rate of cellular internalization and increasing the rate at which they are released from the endosome. Such methods have included (i) coupling of oligomers to polycations (Clarenc et al., 1993), (ii) use of transferrin/polylysine-conjugated DNA in the presence of a replication deficient adenovirus (Zatloukal et al., 1992), (iii) conjugation of oligonucleotides to fusogenic peptides (Bongartz et al., 1994), (iv) targeting of oligonucleotides to specific cell surface receptors such as folate (Wang et al., 1995) and transferrin (Citro et al., 1992) receptors, (v) conjugation to cholesterol (Ing et al., 1993), and, most successfully (vi) complexation with cationic lipids (Bennett et al., 1992).

Currently, antisense-cationic lipid complex systems deliver the largest quantities of oligonucleotides to cells. However, complexes formed from the electrostatic interaction between polynucleotides and cationic liposomes are large and highly charged. As a result, they are rapidly cleared by the reticuloendothelial system following intravenous administration (Litzinger, 1997; Litzinger et al., 1996). In vivo experiments show that the highest level of antisense activity is observed in “first pass” organs such as the lung, liver and spleen (Chonn and Cullis, 1998). Complexes also do not provide sufficient protection for associated nucleotide-based drugs against degradation by nucleases (Wheeler et al., 1998). In addition, these systems are highly toxic at higher doses (Zelphati et al., 1998). Consequently, complexes have limited utility for the treatment of systemic diseases such as cancer and inflammatory diseases.

Liposomes are one of the most promising methods for the delivery of antisense molecules as they have the potential for facilitating intracellular delivery and are able to protect nucleic acids from degradation. Initial methods used to encapsulate antisense
oligonucleotides within liposomes were based on passive entrapment techniques which gave rise to poor trapping efficiencies and low drug-to-lipid ratios. These included direct rehydration of lipid films in the presence of nucleic acids, detergent dialysis, and reverse phase encapsulation (reviewed in Semple et al., 2000b; Thierry and Takle, 1995). A recently developed procedure, in which antisense oligonucleotides are incubated with preformed cationic liposomes in ethanolic solutions, results in the efficient encapsulation of AS-ODN. This process exhibits remarkably high levels of entrapment (Maurer et al., 2000; Semple et al., 2000), approximately three orders of magnitude greater than that obtained by passive processes. The association of nucleic acids with the cationic lipid component of liposomes leads to the formation of multilamellar vesicles in which antisense molecules are entrapped between the concentric bilayers. These liposomes consist of a mixed population of unilamellar and small multilamellar vesicles, are relatively small in size (80 - 140 nm) (Semple et al., 2000) and remain in circulation for extended periods. Furthermore, “stabilized antisense-lipid particles” (SALPs) do not exhibit the levels of toxicity associated with cationic complexes.

The practical applications of liposome encapsulated antisense, however, are limited due to poor uptake of SALP systems into cells (Semple and Mui, unpublished). In order to achieve significant downregulation of protein expression, antisense sequences must reach the nucleus.

1.4.6 Cationic PEG-Lipids (CPLs)

Cellular uptake of liposomes occurs through a two-phase process which involves cell surface binding followed by endocytosis. Hence, factors that interfere with the binding
process lead to decreased levels of intracellular delivery. This is of particular importance for "sterically stabilized" liposomes. These vesicles contain poly(ethylene glycol) (PEG) which produces an outer sheath or "steric barrier" surrounding the vesicle (Torchlin et al., 1994). The presence of PEG on the outer monolayer decreases the interaction and association of blood proteins, thereby extending the circulation lifetime of these systems (Oja et al., 2000). However, this also results in decreased interactions with cell membranes, compromising cellular uptake.

One avenue to increase cellular delivery of liposomes is to incorporate moieties on the vesicle surface that are target specific. These include targeting peptides (Zalipsky et al., 1995), certain oligosaccharides (Zalipsky et al., 1997), folate (Lee and Low, 1995; Wang et al., 1995; Gabizon et al., 1999), riboflavin (Holladay et al., 1999) or antibodies (Hansen et al., 1995; Lopes de Menezes et al., 1998). An alternative approach is to explore molecules that non-specifically increase liposome-cell surface interactions. In this regard, it has been shown that a net positive charge on nucleic acid-lipid particles facilitates binding and uptake by cells in vitro (Hope et al., 1998), which is likely the result of electrostatic interactions with the negative charge exhibited by biological membranes. Hence, designing a molecule that imparts a positive charge when incorporated into liposomes would non-specifically increase vesicle-cell association thereby enhancing uptake. Previous work in this laboratory has been devoted to developing cationic PEG-lipids (CPLs) to achieve this aim.

It has been demonstrated that the presence of surface associated CPLs, in particular CPL4, can dramatically enhance the uptake of LUVs into BHK cells (Fenske et al., 2000). Subsequent work by Palmer et al. (2000) on the effects of CPL4 on "stabilized plasmid-lipid
particles" (SPLP) showed that the inclusion of CPL₄ improved uptake by 50-fold and increased transfection potency 3x10³-fold, compared to SPLP without CPL₄.

1.5 FACTORS INFLUENCING THE KINETIC BEHAVIOUR OF LIPOSOMES

Many factors influence liposome biodistribution, stability and clearance following systemic administration. In particular, it is well established that liposomes experience rapid elimination from the circulation by the reticuloendothelial system (RES). Four parameters that have major impacts on liposome clearance are size, composition, electrostatic charge, and lipid dose (Senior, 1987). These properties affect the degree to which serum proteins adsorb to the vesicle surface and hence the rate of recognition for elimination by the phagocytic monocytes of the RES (Chonn et al., 1992).

1.5.1 Interactions of Liposomes with the Reticuloendothelial System (RES)

Removal of particulates from the circulation is a natural function of the reticuloendothelial system (RES). The RES consists primarily of the liver and spleen but includes other organs, such as the lungs, bone marrow and lymph nodes, which contain a fixed population of phagocytic cells (macrophages) that rapidly ingest and degrade foreign matter. Recognition of liposomes as foreign is, in part, dependent on the binding of serum proteins known as opsonins. Adsorption of opsonins to vesicles marks them for elimination by stimulating macrophage recognition and phagocytosis. As a result, a primary area of liposome research has centered around refining liposomal formulations to reduce their uptake.
by the RES mainly by decreasing opsonin binding, thereby prolonging circulation (Chonn et al., 1992).

1.5.2 Liposome Size

Vesicle size is a crucial determinant of survival time in circulation. Numerous in vivo studies have shown that larger systems are cleared more rapidly than smaller liposomes (Sommerman, 1986). As early as 1975, Juliano and Stamp (1975) reported that MLVs (diameter > 1 µm) experienced an approximately 4-fold shorter circulation lifetime compared to liposomes of identical composition with a median size of 55±25 nm. The effect of size may be due to the activation of the complement system. Devine et al. (1994) found that 400 nm LUVs were more effective at activating complement pathways than 50 nm vesicles for equivalent amounts of exposed lipid.

1.5.3 Lipid Composition and Surface Charge of Liposomes

The lipid composition of a liposome is crucial in determining its circulation half-life. This is reflected in the finding of Chonn et al. (1992) who found that EPC/Chol liposomes experience relatively long circulation times compared to vesicles composed of EPC/Chol/DOPA which are rapidly cleared. The incorporation of DOPA alters the surface characteristics of the liposome, in particular the surface charge. Various studies have shown that positively and negatively charged liposomes bind more plasma protein than net neutral vesicles (Hernandez-Casselles et al., 1993; reviewed in Semple et al., 1998). Moreover, charged liposomes are known to activate the complement system. The nature of the charge
appears to dictate which pathway is stimulated. Negatively charged vesicles activate the classical pathway in a calcium dependent fashion while positively charged liposomes activate the alternative pathway (Chonn et al., 1991).

Lipid packing in the bilayer additionally influences vesicle elimination. Liposomes rich in saturated lipids and cholesterol exhibit the longest circulation times as the rigid membrane structure inhibits protein penetration into the bilayer, effectively reducing vesicle clearance (Chonn et al., 1992). Furthermore, the tight packing of fatty acyl chains provides a more effective permeability barrier, improving drug retention while in the circulation.

1.6 FACTORS THAT ENHANCE CIRCULATION LIFETIME AND CELLULAR UPTAKE OF LIPOSOMES

1.6.1 Polyethylene Glycol Lipid Conjugates – Sterically Stabilized Liposomes

Several strategies have been examined to reduce RES uptake by decreasing or inhibiting opsonization. Incorporation of specific glycolipids such as monosialo-ganglioside (GM1) has shown to result in prolonged circulation and reduced uptake by the RES (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988). The inclusion of phospholipids with polyethylene glycol (PEG) polymers grafted to the lipid headgroups has also shown to decrease binding of blood proteins (Oja et al., 2000).

PEG, a hydrophilic polymer, is proposed to form a dense polymeric "cloud" that extends above the liposome surface (Torchilin et al., 1994). The local surface concentration of PEG sterically inhibits both electrostatic and hydrophobic interactions of plasma proteins with the lipid surface. This phenomenon is known as surface steric stabilization and decreases the rate at which liposomes are cleared from the circulation.
The SALP systems described in this thesis contained PEG$_{2000}$-Cer which consists of an average of 45 ethylene glycol monomers per PEG chain conjugated to a ceramide (Cer) anchor, a sphingolipid derivative. The ceramide acyl chain can be of varying lengths. Typical lipid anchors are composed of C$_8$, C$_{14}$ or C$_{20}$ carbon chains. PEG-CerC$_{14}$ and PEG-CerC$_{20}$ are used in this thesis.

1.6.2 Cationic PEG-Lipids (CPLs)

Cationic PEG-lipids (CPLs) are derivatives of PEG-lipids synthesized to exhibit a positively charged headgroup attached to the distal end of the PEG polymer. The charge imparted by the headgroup increases liposome-cell membrane interactions, thereby enhancing vesicle internalization.

CPLs all have a basic structure consisting of four moieties conjugated in the following order (Figure 1.8): (i) a glycerolipid anchor, (ii) a lysine spacer, (iii) a PEG chain and (iv) a positively charged headgroup composed of lysine residues attached covalently to the distal end of the PEG chain. Dansylated CPLs contain a dansyl moiety linked to the lysine spacer by the ε-amino group.

CPLs may be modified by altering the groups composing the molecule. By varying the number of lysine residues composing the headgroup, the charge exhibited by the CPLs can be modified (Chen at al., 2000). To date, our laboratory has successfully synthesized CPL$_1$, CPL$_2$, CPL$_4$ and CPL$_8$ which exhibit 1, 2, 4 and 8 positive charges, respectively. The length of the PEG chain may be also be varied in conjunction with the headgroup. CPLs have been made using PEG$_{1000}$ and PEG$_{3400}$ (Wong, unpublished). Finally, the fatty acyl chains composing the lipid anchor may be changed.
Dansylated cationic PEG-lipids are composed of four major moieties: a glycerolipid anchor, a lysine spacer to which a dansyl-label is attached, a PEG spacer molecule and a positively charged headgroup comprised of lysine residues. Specifically, the CPL shown here, CPL₄, is constructed with a DSPE lipid anchor, PEG₂₄₀₀ and contains 3 lysine residue to produce 4 positive charges.
1.7 THESIS OBJECTIVE

Current stabilized antisense-lipid particle (SALP) systems exhibit poor uptake by cells \textit{in vitro} and \textit{in vivo}. The studies conducted in this thesis examine various approaches to enhancing the intracellular delivery of antisense oligonucleotides contained within SALP systems. In particular, the effect of incorporating a cationic PEG-lipid with four positive charges (CPL$_4$), for enhancing the cellular internalization of SALP liposomes is investigated. In addition, we examine the ability of DOPE in SALP systems to increase the intracellular and nuclear delivery of antisense oligonucleotides encapsulated within SALPs by promoting endosome destabilization.
CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine-N-[Lissamine-Rhodamine-B-Sulfonyle] (Rho-PE) was purchased from Avanti Polar lipids. Distearylphosphatidylcholine (DSPC) and 1,2-dioleoylphosphatidylethanolamine (DOPE) were from Northern Lipids (Vancouver, B.C.). 1,2-dioleoyl-3-[N,N-dimethylamino]propane (DODAP), dioleoyldimethylammonium chloride (DODAC) and PEG-Ceramide (C₁₄ and C₂₀) were generously donated by Inex Pharmaceuticals (Burnaby, B.C.). Cholesterol, N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] (HEPES), DEAE-Sepharose CL-6B, n-octyl-β-D-glucopyranoside (OGP) and Triton X-100 (TX-100) were obtained from Sigma Chemicals. Citric acid, sodium chloride, calcium chloride and sucrose were bought from Fisher Scientific. Dialysis tubing (Spectra/Por Membranes, MWCO 12-14,000) was purchased from Spectrum Laboratories.

Phosphorothioate antisense oligodeoxynucleotide (AS-PS-ODN) were provided by Inex Pharmaceuticals (Burnaby B.C., Canada). Originally, antisense molecules against epidermal growth factor receptor (EGFR) (5’-CCG TGG TCA TGC TCC-3’) were purchased from the Oligonucleotide Synthesis Lab at UBC (Vancouver, B.C.). 5’-FITC labeled EGFR AS-ODN was purchased from Boston Biosystems Inc. (Bedford, MA).

Dansylated CPL₄ was synthesized in our laboratory (Chen et al., 2000) by Dr. Kim Wong.
Dulbecco’s modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (UBC, Vancouver, B.C.).

2.2 Stabilized Lipid Antisense Particles (SALP): Encapsulation of Antisense Oligonucleotides within Liposomes

Rho-PE labeled liposomes were prepared using the preformed vesicle method established by Semple et al. (2000). The entrapment of oligonucleotides in liposomes relies on the presence of high concentrations of ethanol. Lipids, dissolved in ethanol or chloroform, were mixed in desired molar ratios, following which the solvents were removed under a stream of nitrogen gas and subsequently placed under high vacuum for 1 h to remove residual solvent. The lipid film was rehydrated with citrate buffer (50 mM citrate, 210 mM sucrose, pH 4.0) thereupon, ethanol was added dropwise to the MLVs while vortexing. The final ethanol concentration was 40%. The mixture was then extruded through two stacked 100 nm polycarbonate filters using an extrusion device (Lipex Biomembranes, Vancouver, B.C.) under 50-150 psi of nitrogen gas (Hope et al., 1985). This process was followed by the addition of antisense oligonucleotides (AS-ODN). AS-ODN, dissolved in distilled water, was added dropwise to the ethanolic liposome dispersion, while vortexing, to the desired drug to lipid (D/L) ratio. Typically, an initial D/L of 0.1 (1 mg ODN/10 mg lipid) was used, unless stated otherwise. The sample was immediately incubated at 40 °C for 1 h to facilitate the encapsulation of antisense oligonucleotides. The formulation was thereafter subjected to dialysis in citrate buffer to remove the ethanol. The external buffer was then exchanged for HBSC (20 mM HEPES, 70 mM NaCl, 50 mM CaCl₂, pH 7.5) by dialysis, initially for 2 h (4L) followed by fresh buffer (4L) for an additional 16 h. At pH 7.5 DODAP becomes charge-neutral and oligonucleotides bound to the external membrane are released from their
association with cationic lipid. Following dialysis, the vesicles were passed over a DEAE-Sepharose CL-6B column, equilibrated with HBSC, to remove unencapsulated antisense molecules.

2.3 Lipid Formulations

Two formulation types were used in this thesis: DSPC/Chol/DODAP/PEG-Cer (20/45/25/10 mol%) and DODAP/DOPE/PEG-Cer (45/45/10 mol%, 25/65/10 mol%). Variations of these systems were utilized in which the molar ratio of the lipids was altered or the use of PEG-CerC_{14} was substituted for PEG-CerC_{20}.

2.4 Insertion of Cationic PEG-Lipids (CPLs) into SALP

Stock solutions of cationic PEG-lipids (CPLs) were stored as micellar solutions in methanol. CPL insertion into preformed vesicles followed that detailed by Fenske et al. (2000) with some minor modifications. Briefly, CPL_{4} and SALP were combined to provide the desired molar ratio and incubated for various times at chosen temperatures. Standard conditions were 40 °C for 3 h, unless otherwise stated.

Following incubation, the samples were cooled to room temperature and applied to a DEAE-Sepharose CL-6B size exclusion column (1.5 cm x 15 cm), primarily to separate SALP-CPL from free CPL but additionally to remove antisense molecules that may have leaked from the vesicles during the incubation.

The insertion level of CPL into SALP was measured by fluorescence. All formulations contained 0.5 mol% Rho-PE, incorporated as a lipid marker, while CPLs were
synthesized to include a dansyl group. Quantifying the amount of CPL inserted was determined by measuring the dansyl to rhodamine (D/R) ratio of the initially combined sample and the D/R ratio of the isolated SALP-CPL sample, and then taking the ratio of \([\frac{[D/R]_{\text{final}}}{[D/R]_{\text{initial}}}]\) and multiplying it by the initial molar percent of CPL. The fluorescence assay was conducted as follows: to an aliquot of initial sample (10 µl) or SALP-CPL (20 µl) was added 20 µl 10% Triton X-100 followed by 2 ml of HBSC. Rhodamine fluorescence was measured with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Dansyl fluorescence was measured at 340 nm and 510 nm, respectively. The fluorescence levels of both rhodamine and dansyl were read consecutively using a wavelength program as per the parameters outlined above on a Perkin Elmer LS52 Luminescence spectrophotometer with excitation and emission slit widths maintained at 10 nm. An emission cut-off filter at 430 nm was utilized.

### 2.5 Size Determination of SALP and SALP-CPL

Vesicle diameters of SALP and SALP-CPL were determined by quasi-elastic light scattering (QELS) using a NICOMP Model 370 Submicron Particle Sizer (Nicomp Particle Sizing Inc., Santa Barbara, California). Throughout the thesis number-averaged sizes are presented, which were obtained by cumulant fit from the experimental correlation functions. The polydispersity is expressed as the half-width at half-height of a monomodal Gaussian size distribution.
2.6 Determination of Lipid Concentration

Phospholipid concentration was determined using a standard phosphate assay established by Fiske and Subbarow (Fiske and Subbarow, 1925). Measurement of lipid concentration of SALP systems first required the lipids to be extracted from the vesicles, as the oligonucleotides contain phosphate groups that interfere with the phosphate assay. Lipid extraction was accomplished using a Bligh-Dyer extraction (Bligh and Dyer, 1959). The lipid concentration of SALP-CPL₄ formulations was calculated by measuring the fluorescence ratio of rhodamine prior to and after the isolation column. The fluorescence of a known quantity of SALP is compared to the fluorescence of a sample of SALP-CPL, from which the lipid concentration is extrapolated.

2.7 Determination of Antisense Oligonucleotide Concentration

The concentrations of antisense oligonucleotide (AS-ODN) solutions were determined by measuring the absorbance at 260 nm. The following equation was used to calculate concentration.

\[
[ODN] = A_{260} \times \varepsilon \times D_f
\]

where \( \varepsilon \) = extinction coefficient (34 \( \mu \)g/ml)

\( D_f \) = dilution factor

Stock solutions of ODN were prepared in distilled water and stored at -20 °C.

The AS-ODN concentration in SALP formulations was measured by solubilizing an aliquot of SALP (10–20 \( \mu \)l) with 100 mM OGP in HBSC and measured against a blank of
HBSC containing OGP. Before measuring AS-ODN concentration of SALP-CPL4 samples, lipids and ODN were separated, as the dansyl moiety on CPL4 interferes with the absorbance readings. This was accomplished by measuring the absorbance of the methanol-buffer phase from a Bligh-Dyer extraction (Bligh and Dyer, 1959), and referenced against a buffer blank subject to identical conditions as the SALP-CPL4 sample.

2.8 Uptake of SALP and SALP-CPL by BHK Cells

Tissue culture experiments were conducted on a transformed (tk-) baby hamster kidney (BHK) cell line donated by Dr. Ross T.A. MacGillivray. Approximately 1x10^5 BHK cells, plated the day prior in 12 well plates, were incubated in DMEM supplemented with 10% fetal bovine serum (FBS) and either 2 μg ODN/ml or 40 μM lipid, containing either no CPL or 3-4mol% CPL. Incubations were performed for various times, typically 4, 6 and 24 h, at 37°C at which time the cells were washed twice with PBS and subsequently lysed with 600 μl lysis buffer (0.1% Triton X-100 in PBS, pH 7.5). The rhodamine or FITC fluorescence of the lysate was measured using a Perkin Elmer LS52 Luminescence spectrophotometer. Rhodamine or FITC uptake was extrapolated by measuring fluorescence intensities of the lysate compared to that of a lipid or oligonucleotide standard, respectively, and normalized against protein concentration, determined by BCA Protein Assay (Pierce, Rockford, IL.).

Complexes were formed from DOPE/DODAC/Rho-PE (50/50/0.5 mol%) LUVs and FITC-AS-ODN at a charge ratio of 1.5+ (moles of positive charges/moles of negative charges). Once combined, the vesicles and oligonucleotides were left at room temperature for at least 0.5 h to allow for sufficient formation of the complexes.
2.9 Fluorescence Microscopy

Fluorescence micrographs were taken using a Axiovert 100 Zeiss Fluorescence microscope (Carl Zeiss Jena GmbH) using a rhodamine filter ($\lambda_{ex} = 560 \pm 20$ nm, 600 nm LP, and DC 590 nm) or FITC filter ($\lambda_{ex} = 475 \pm 20$ nm, $\lambda_{em} = 535 \pm 22.5$ nm, and DC 500 nm) from Omega Opticals (Brattleboro, VT). Images were recorded on Kodak Ektachrome P1600 colour reversal film at 1600 ISO with a Zeiss MC 80 DX microscope camera.
CHAPTER 3
The Development of Stabilized Antisense-Lipid Particles (SALP) with Surface Associated Cationic PEG-Lipids (CPL)

3.1 Introduction

As indicated in Chapter 1, liposomal formulations of genetic drugs such as antisense oligonucleotides and plasmid DNA are difficult to achieve. The large size and highly charged nature of these molecules mitigates against the formation of small, neutral, serum stable carriers, which are required to achieve the long circulation times necessary for efficient accumulation at disease sites. Further, entrapment is very inefficient in the absence of interactions between the lipid components of the carrier and the nucleotide-based drugs. Complexes formed by electrostatic interactions between polynucleotides and cationic liposomes exhibit broad size distributions. These complexes efficiently transflect cells \textit{in vitro}; however, \textit{in vivo} their large size and positive charge triggers rapid clearance from the circulation. They can also be highly toxic.

The SALP process for the encapsulation of antisense oligonucleotides (AS-ODN) within liposomes permits high levels of entrapment, approximately three orders of magnitude greater than achieved by previous techniques (Semple et al., 2000). SALPs offer the potential for high levels of AS-ODN intracellular delivery. However, the practical applications of SALP systems are limited due to minimal uptake by cells observed both \textit{in vitro} and \textit{in vivo} (unpublished observations).

This chapter examines various approaches to enhancing the intracellular delivery of antisense oligonucleotides contained within SALP systems. For AS-ODN to be effective in inhibiting gene expression, they must be internalized by the target cell, effectively released
from the endosomes, and translocated to the nucleus, their primary site of action. Therefore, we examine the effect of a surface associated cationic PEG-lipid (CPL), which exhibits four positive charges (CPL₄), on increasing the cellular internalization of SALP systems. Subsequent studies investigate the ability of DOPE in SALP systems to increase nuclear delivery of antisense sequences by promoting endosome destabilization. The two basic SALP formulations investigated, DSPC/Chol/DODAP/PEG-Cer and DODAP/DOPE/PEG-Cer, were characterized with respect to antisense oligonucleotide encapsulation efficiency, size, and CPL₄ insertion efficiency.

3.2 Stabilized Antisense-Lipid Particles (SALPs)

3.2.1 Lipid Formulation of SALP

Initial studies in the development of SALP were conducted using a lipid mixture of DSPC, cholesterol (Chol), DODAP and PEG-CerC₁₄ (25/45/20/10 mol%) (Semple et al., 2000), as liposomes that are serum stable and display long circulation lifetimes are typically composed of DSPC and cholesterol. The ionizable cationic lipid DODAP was employed to provide the positive charge used to sequester negatively charged antisense oligonucleotides to liposome surfaces. PEG-CerC₁₄ was included to minimize aggregation and fusion between particles during the formulation process. The studies presented here further characterize the existing DSPC/Chol/DODAP/PEG-CerC₁₄ SALP system and examine methods to improve the efficacy of SALPs.
3.2.2 Characteristics of SALP

The interaction of cationic liposomes with polyanionic nucleotides leads to the formation of uni-, oligo- and multi-lamellar vesicles ranging in size from 80-140 nm, only slightly larger than the parent LUVs from which they originated (Semple et al., 2000, Maurer et al., 2000). The encapsulated antisense is entrapped between the concentric lamellae of the small multilamellar liposomes (Maurer et al., 2000). The structure of SALP and location of the oligonucleotides is illustrated by cryo-transmission electron microscopy (Figure 3.1). The image of a sample of SALP (D/L 0.13 w/w) reveals the coexistence of unilamellar liposomes with di-, oligo- and multilamellar vesicles. Note the close proximity between membranes of the MLVs. The inset of Figure 3.1 is an expanded view of the formation of a multilamellar liposome. It illustrates two initially separate membranes forced into close apposition by bound oligonucleotides (as indicated by the arrow). In some SALPs continuous bridging between membranes does not occur around the entire vesicle. Areas that are not continuous exhibit bulbs, which appear as if the vesicles were divided by a diaphragm.

Table 3.1 shows that antisense oligonucleotides can be efficiently encapsulated within liposomes. As the initial drug-to-lipid ratio (D/L) was increased, a concomitant decrease in the encapsulation efficiency of ~95% to ~70% was observed. However, the absolute quantity of entrapped antisense oligonucleotides (total mg ODN encapsulated) increases as the initial D/L is raised. Through this range, the D/L ratio increased from 0.08 to 0.20 mg ODN/mg lipid.
Figure 3.1 Cryo-Transition Electron Micrograph of stabilized antisense lipid particles (SALP). A drop of buffer containing SALP was applied to a standard electron microscopy grid with a perforated carbon film. Excess liquid was removed by blotting leaving a thin layer of water covering the holes of the carbon film. The grid was rapidly frozen in liquid ethane, resulting in vesicles embedded in a thin film of amorphous ice. Images of the vesicles in ice were obtained under cryogenic conditions at a magnification of 66000 and a defocus of 1/5 micron using a Gatan cryo-holder in a Philips CM200 FEG electron microscope.
Table 3.1 AS-ODN Encapsulation of DSPC/Chol/DODAP/PEG-Cer Systems

<table>
<thead>
<tr>
<th>Lipid Composition</th>
<th>Mol % Composition</th>
<th>Initial D/L</th>
<th>Final D/L</th>
<th>Percent (%) Entrapped</th>
<th>Size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSPC/Chol/DODAP/PEG-CerC&lt;sub&gt;14&lt;/sub&gt;</td>
<td>20/40/25/10</td>
<td>0.087</td>
<td>0.081</td>
<td>93.4</td>
<td>118 ± 12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.093</td>
<td>0.089</td>
<td>95.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.120</td>
<td>0.113</td>
<td>94.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.130</td>
<td>0.108</td>
<td>83.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.190</td>
<td>0.148</td>
<td>77.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.200</td>
<td>0.140</td>
<td>70.0</td>
<td></td>
</tr>
<tr>
<td>DSPC/Chol/DODAP/PEG-CerC&lt;sub&gt;20&lt;/sub&gt;</td>
<td>20/40/25/10</td>
<td>0.087</td>
<td>0.083</td>
<td>95.9</td>
<td>123±27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.101</td>
<td>0.096</td>
<td>95.0</td>
<td></td>
</tr>
</tbody>
</table>

* D/L ratio is determined as mg ODN/mg lipid

3.2.3 Uptake of SALP into Cells

Current SALP systems demonstrate minimal uptake by cells compared to antisense-cationic lipid complexes. This is illustrated in Figure 3.2 for BHK cells. BHK cells do not efficiently internalize SALPs, as the quantity of AS-ODN delivered intracellularly by complexes exceeds that achievable by SALP by greater than one order of magnitude. In particular, when 2 pg of ODN were incubated with BHK cells, FITC-AS-ODN DODAC/DOPE complexes exhibited high levels of delivery, 4.6 pg ODN/mg protein, in contrast to DSPC/Chol/DODAP/PEG-CerC<sub>14</sub> (20/45/25/10 mol%) SALP which were only able to achieve 0.4 μg ODN/mg protein after 24 h.

The ability of cationic lipids in complexes to enhance cellular internalization of liposomes is likely the result of the positive charge imparted by these lipids (Figure 3.3). DSPC/Chol/DODAP/PEG-CerC<sub>14</sub> SALPs are charge neutral at physiological pH and are therefore are not electrostatically “attracted” to cells. This may explain the difference in
Figure 3.2 Uptake of FITC labeled EGFR AS-ODN into BHK cells. Time course of the intracellular delivery of 2 μg/ml FITC-AS-ODN for (●) DODAC:DOPE (50:50 mol%) complexes (charge ratio 1.5+) and (■) DSPC/Chol/DODAP/PEG-CerC14 (20/45/25/10 mol%) SALPs (D/L = 0.11). The preparation of complexes and SALP followed that described in the Materials and Methods. BHK cells were plated in 12-well plates at 1x10^5 cells/well the day prior. To 200 μl of sample, containing complexes or SALP and HBSC (20 mM HEPES, 70 mM NaCl, 50 mM CaCl₂, pH 7.5), was added 800 μl of DMEM + 10% FBS such that the final concentration of CaCl₂ was 10 mM. Following the incubation period of 1, 2, 4, 6 and 24 h, the cells were lysed with 600 μl lysis buffer (0.1% TX-100 in PBS) at which time the FITC fluorescence was measured and BCA Assay conducted on the lysate, as outlined in the Materials and Methods.
Figure 3.3 Interaction of Cationic Lipid-Antisense Oligonucleotide Complexes and Stabilized Antisense Lipid Particles (SALPs) with Cells. (A) Charge neutral stabilized antisense-lipid particles are not electrostatically attracted to negatively charged cell membranes. (B) Positively charged cationic lipid-antisense oligonucleotide complexes are electrostatically attracted to negatively charged cell membranes. Increased associations between liposomal carriers and cells are though to increase vesicle internalization into cells.
uptake between the two systems. Therefore, in the next stages of this work, we examined whether the post insertion of a cationic ligand (CPL₄) into SALP results in enhanced intracellular delivery of antisense oligonucleotides.

3.3 Applications of Cationic PEG-lipids (CPLs)

3.3.1 Insertion of CPLs into SALP Systems

We first examined whether a similar insertion procedure for CPL₄ to that first documented by Fenske et al. (2000) could be applied to the SALP system. The protocol for the post-insertion of CPL₄ into SALPs follows that shown in Figure 3.4. The insertion process is identical to that utilized for empty LUVs (Fenske et al., 2000) and plasmid-DNA containing LUVs (SPLPs) (Palmer et al., 2000) with the exception of incubation temperature. In contrast to the original procedure, it was found that 60°C was too high a reaction temperature as it resulted in considerable loss of encapsulated antisense molecules (data not presented) as well as massive aggregation and subsequent precipitation of SALP. However, it was determined that 40 °C was more compatible with insertion with minimal leakage of SALP contents. Lower temperatures did not result in adequate insertion efficiencies. SALPs were incubated with ~4-6 mol% CPL₄ at 40°C for up to 3 h at which point SALP-CPL₄ was separated from unincorporated CPL₄ by column chromatography. To characterize this isolation process, an elution profile of a SALP-CPL₄ was examined. As illustrated in Figure 3.5, SALP-CPL₄ elutes at approximately 7 ml elution volume (equivalent to fraction 14 (0.5 ml)). This is followed by a peak representative of CPL₄ micelles. The co-elution of dansyl and rhodamine labels indicates the incorporation of CPL₄ into SALPs (Fenske et al., 2000). For the example shown in Figure 3.5, 61% CPL₄ was incorporated into
Figure 3.4 Insertion of Cationic PEG-lipids (CPLs) into Stabilized Antisense-Lipid Particles (SALP). The diagram illustrates the protocol for the insertion of CPL4 into SALP. SALP and CPL4 are mixed together and incubated for 3 hours at 40 °C. SALP-CPL4 is isolated from free unincorporated CPL4 by size exclusion chromatography using a DEAE-Sepharose CL 6B column.
Figure 3.5 Elution Profile of SALP-CPL₄. DSPC/Chol/DODAP/PEG-CerC₁₄ SALP (2.0 μmol lipid) was incubated with 0.12 μmol CPL₄ (6 mol%) at 40 °C for 3 h. Following incubation, the sample was applied to a DEAE-Sepharose CL 6B column equilibrated in HBSC (20 mM HEPES, 70 mM NaCl, 50 mM CaCl₂, pH 7.5). 0.5 ml fractions were collected and assayed for dansyl-labeled CPL₄ (●) and rhodamine-PE (■) as described in the Materials and Methods. 61% insertion was achieved for this sample, resulting in a final CPL₄ incorporation of 3.7 mol%.
DSPC/Chol/DODAP/PEG-CerC_{14} SALP, corresponding to a final insertion of 3.7 mol% of total lipid.

The effect of time on insertion of CPL_{4} into DSPC/Chol/DODAP/PEG-Cer (20/45/25/10 mol%) SALP is depicted in Figure 3.6. The two formulations examined differ in the lengths of the ceramide acyl chain composing the PEG-lipid. One sample was prepared using PEG-CerC_{14} while the other contained PEG-CerC_{20}. No significant difference in the insertion efficiency was observed between the two samples. The greatest degree of insertion was achieved between 2 to 3 h and thus, subsequent incubations were conducted for 3 h.

The effect of the initial CPL_{4} concentration on insertion efficiency into DSPC/Chol/DODAP/PEG-Cer SALP was investigated. The results of the study are presented in Table 3.2. Increasing the initial concentration of CPL_{4} resulted in decreases in the total percent of CPL_{4} inserted into the vesicle, although the absolute amount of CPL incorporated into SALP steadily increased. Furthermore, comparison between PEG-CerC_{14} and PEG-CerC_{20} systems revealed little difference in the insertion efficiencies into the two formulations. This observation is supported by the similarity of the insertion profiles (Figure 3.6). Notably, insertion of CPL_{4} into DSPC/Chol/DODAP/PEG-Cer did not significantly increase vesicle size (Table 3.2).
Figure 3.6 Insertion Profile of CPL \(_4\) into DSPC/Chol/DODAP/PEG-Cer (20/45/25/10) SALP. Insertion was performed at 40 °C for both PEG-Cer\(_{14}\) (•) and PEG-Cer\(_{20}\) (■). 8.55 µmol lipid was combined with 0.12 µmol CPL\(_4\) (6 mol%) and incubated for 0.5, 1.0, 2.0 and 3.0 h. At these times, an aliquot was removed (2.13 µmol lipid), cooled to halt insertion, and passed down a DEAE-Sepharose CL 6B column. The isolated fraction was then assayed for CPL insertion, as described in the Materials and Methods.
Table 3.2 Insertion of CPL₄ into DSPC/Chol/DODAP/PEG-Cer SALP

<table>
<thead>
<tr>
<th>Lipid Composition (Mol % Composition)</th>
<th>Initial Mol% CPL</th>
<th>% CPL Inserted</th>
<th>Final Mol% CPL</th>
<th>Size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSPC/Chol/DODAP/PEG-CerC₁₄ (20/45/25/10)</td>
<td>5.1</td>
<td>69</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>62.1 ± 1.2</td>
<td>3.7 ± 0.1</td>
<td>123 ± 6</td>
</tr>
<tr>
<td></td>
<td>6.7</td>
<td>58.2 ± 1.1</td>
<td>3.9 ± 0.1</td>
<td>(118 ± 12)</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>51.9</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>49.0 ± 1.6</td>
<td>4.9 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>DSPC/Chol/DODAP/PEG-CerC₂₀ (20/45/25/10)</td>
<td>6</td>
<td>61.6 ± 2.9</td>
<td>3.7 ± 0.2</td>
<td>130 ± 9</td>
</tr>
</tbody>
</table>

n = 2-4 for samples
Sizes in parentheses indicate average size of vesicles before CPL insertion

3.3.2 Lipid Uptake of DSPC/Chol/DODAP/PEG-CerC₁₄-CPL₄ into BHK Cells

The insertion of CPL₄ into SALPs is expected to enhance the cellular internalization of SALP liposomes. Hence, the effect of incorporating CPL₄ on lipid uptake in baby hamster kidney (BHK) cells was examined as shown in Figure 3.7. The presence of 3 mol% CPL₄ led to significant enhancement of lipid uptake after 24 h. In contrast, SALPs of identical lipid composition without CPL₄ showed minimal uptake when incubated with BHK cells. Uptake was determined by quantitating the amount of fluorescently labeled lipid associated with the cells. Intracellular distribution and location of the internalized SALP was determined by fluorescence microscopy.

The dramatic effect of CPL₄ on uptake is most clearly depicted by fluorescence microscopy, as visualized in Figure 3.8. In the absence of CPL₄, only trace amounts of lipid, identified by the lipid label rhodamine-PE, are detectable. However, the incorporation of 3.5 mol% CPL₄ into DSPC/Chol/DODAP/PEG-CerC₁₄ SALP results in high levels of uptake, as indicated by the intensity of the fluorescence. The difference in lipid uptake is more than that
Figure 3.7 Lipid Uptake of DSPC/Chol/DODAP/PEG-CerC_{14} SALP and SALP-CPL_{4} into BHK cells. Preparation of SALP and insertion of CPL_{4} was conducted as described in the Materials and Methods. 40 nmol DSPC/Chol/DODAP/PEG-CerC_{14} SALP (•) or SALP containing 3 mol% CPL_{4} (■) was applied to 1x10^{5} cells and incubated at 37 °C. 200 μl of SALP (± CPL) in HBSC (20 mM HEPES, 70 mM NaCl, 50 mM CaCl_{2}, pH 7.5) was added to 800 μl of DMEM + 10% FBS, resulting in a final CaCl_{2} concentration of 10 mM. Following incubation periods of 4, 6 and 24 h, the cells were washed with PBS and lysed with 600 μl of lysis buffer (0.1% TX-100 in PBS) and rhodamine fluorescence of the lysate was determined as described in the Materials and Methods. The fluorescence intensity was used to extrapolated lipid uptake in nmol lipid and then normalized against protein concentration, determined by a BCA Protein Assay.
Figure 3.8 Fluorescence micrographs of lipid and oligonucleotide uptake of DSPC/Chol/DODAP/PEG-Cer-C_{14} SALP and SALP-CPL₄ by BHK cells. 40 nmol lipid of DSPC/Chol/DODAP/PEG-Cer-C_{14} SALP or SALP-CPL₄ (3.5 mol%) were placed on 1x10⁵ cells in DMEM supplemented with 10% FBS containing 10 mM CaCl₂ at 37 °C. Micrographs were taken 24 h after sample application. Image descriptions are as follows. Exposure time is indicated in parentheses. (A) Phase contrast micrographs of BHK cells (I) with SALP (0.63 s) or (II) with SALP-CPL₄ (0.89 s). (B) Fluorescence micrographs of lipid uptake as illustrated by rhodamine-PE fluorescence for (III) SALP (2.24 s) or (IV) SALP-CPL₄ (0.50 s). (C) Fluorescence micrographs of antisense oligonucleotide intracellular delivery as illustrated by FITC fluorescence for (V) SALP (7.13 s) or (VI) SALP-CPL₄ (6.3 s).
reflected in the micrographs since the exposure times for SALP images were considerably longer than for SALP-CPL₄ images. The micrographs show Rho-PE intracellular fluorescence to occur as a small and punctate organization. The structures appear to be endosomal and located perinuclearly. This uptake pattern is observed for both SALP and SALP-CPL₄.

The SALP samples employed in the uptake experiments contained fluorescently labeled antisense oligonucleotides (FITC-AS-ODN). Hence, the intracellular localization of antisense molecules could be monitored employing fluorescence microscopy. Those oligonucleotides that remain trapped in endosomes appear as small punctate structures within the cytoplasm. However, those molecules released from endosomal compartments migrate toward the nucleus, manifesting as nuclear fluorescence, indicating that the AS-ODNs have reached their target, the nucleus (Zelphati and Szoka, 1996; Zelphati and Szoka, 1996b; Bennett et al., 1992). As seen in Figure 3.8 (V) and (VI), the effect of CPL₄ on the intracellular delivery of ODN is limited. After 24 h, only punctate structures are detected with only a few scarce cells exhibiting nuclear fluorescence.

In designing drug delivery vehicles, possible toxicity is an issue. An important point to note is that the cells remain healthy following incubation with both SALP and SALP-CPL₄, as seen in panel (I) and (II) (Figure 3.8), respectively.

3.4 SALP Containing DOPE

DSPC/Chol/DODAP/PEG-CerC₁₄–CPL₄ systems exhibit good uptake into BHK cells. However, based on a lack of nuclear fluorescence after 24 h, this formulation is defective in its ability to release liposomal contents from endosomes. These observations
suggest that the lipid formulation is excessively stable with respect to release of antisense oligonucleotides. DSPC/Chol/DODAP/PEG-CerC₁₄ SALP is desirable in that it is stable in the circulation. However, the system is ineffective in releasing AS-ODN from the endosome, a feature essential for a functional delivery vehicle.

Given the limitation of DSPC/Chol/DODAP/PEG-CerC₁₄ SALP and SALP-CPL₄ systems, we developed a new lipid formulation, DODAP/DOPE/PEG-CerC₁₄, designed to increase intracellular delivery of AS-ODN by enhancing the membrane destabilizing ability of SALP. DOPE is a non-bilayer forming lipid which promotes bilayer destabilization and membrane fusion (Cullis et al., 1986). It was shown to increase transfection potency of lipofectin formulations by virtue of its membrane destabilizing ability (Farhood et al., 1995). Inclusion of this lipid should therefore encourage the release of liposome contents from the endosome.

3.4.1 Physicochemical Characteristics of DODAP/DOPE/PEG-Cer SALP

Formulations of SALP containing DOPE, produced by the same process as the DSPC containing SALP, were characterized. Table 3.3 lists the encapsulation efficiencies of DODAP/DOPE/PEG-CerC₁₄ SALP for various initial antisense-to-lipid ratios. Inspection of the data shows that increasing the initial drug to lipid ratio results in a decrease in encapsulation efficiency. Analysis of the various molar compositions revealed no significant differences in encapsulation percentages, although higher DOPE concentrations or the use of PEG-CerC₂₀ over PEG-CerC₁₄ resulted in slightly larger encapsulation efficiencies.

The amount of ethanol required to prepare SALPs depended on the lipid composition. When the DOPE concentration was increased from 45 mol% to 65 mol% the ethanol
concentration had to be reduced from 40% to 30% in order to achieve sizes in the range of 100 nm (Table 3.3). In 40% ethanol, DODAP/DOPE/PEG-CerC_{14}/C_{20} (25/65/10 mol%) formed SALPs which were 200 ± 20 nm in size.

<table>
<thead>
<tr>
<th>Lipid Composition</th>
<th>Mol % Composition</th>
<th>Initial D/L</th>
<th>Final D/L</th>
<th>Percent (%) Entrapped</th>
<th>Size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DODAP/DOPE/PEG-CerC_{14}</td>
<td>45/45/10</td>
<td>0.110</td>
<td>0.093</td>
<td>84.5</td>
<td>83 ± 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.116</td>
<td>0.096</td>
<td>82.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.131</td>
<td>0.098</td>
<td>74.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.142</td>
<td>0.090</td>
<td>63.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>35/55/10</td>
<td>0.115</td>
<td>0.105</td>
<td>91.4</td>
<td>178</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.137</td>
<td>0.110</td>
<td>80.1</td>
<td>(117)*</td>
</tr>
<tr>
<td></td>
<td>25/65/10</td>
<td>0.164</td>
<td>0.100</td>
<td>60.8</td>
<td>156</td>
</tr>
<tr>
<td>DODAP/DOPE/Chol/PEG-CerC_{14}</td>
<td>30/45/15/10</td>
<td>0.130</td>
<td>0.110</td>
<td>80.4</td>
<td></td>
</tr>
<tr>
<td>DODAP/DOPE/PEG-CerC_{20}</td>
<td>45/45/10</td>
<td>0.089</td>
<td>0.079</td>
<td>88.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.14</td>
<td>0.105</td>
<td>75.0</td>
<td>72 ± 15</td>
</tr>
<tr>
<td></td>
<td>25/65/10</td>
<td>0.099</td>
<td>0.082</td>
<td>82.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.100</td>
<td>0.097</td>
<td>97.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.100</td>
<td>0.100</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.116</td>
<td>0.115</td>
<td>99.0</td>
<td>181 ± 25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.132</td>
<td>0.110</td>
<td>83.3</td>
<td>(121±10)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.207</td>
<td>0.112</td>
<td>54.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.217</td>
<td>0.121</td>
<td>55.7</td>
<td></td>
</tr>
</tbody>
</table>

* D/L ratio is measured as mg ODN/mg lipid
* Values in parentheses represent size of SALP using 30% ethanol.

All other sizes are result of using standard 40% EtOH.
3.4.2 *Insertion of CPL*₄* into DODAP/DOPE/PEG-Cer SALP*

Following the preparation of DODAP/DOPE/PEG-CerC₁₄ SALP, insertion of CPL₄ into these vesicles was examined. The procedure for the post-insertion of CPL₄ into DODAP/DOPE/PEG-CerC₁₄ SALP followed that used for insertion into the conventional formulation (Figure 3.4).

The effect of temperature on insertion of CPL₄ into DODAP/DOPE/PEG-CerC₁₄ is indicated in Table 3.4. Despite the relatively high degree of insertion observed at 60 °C, the loss of encapsulated antisense associated with the temperature was not acceptable. Lower temperatures produced better results. The difference in insertion between 37°C and 50°C is the same as between 50°C and 60°C. Incubation of SALPs and CPL₄ at 40 °C and 50 °C yielded similar insertion efficiencies. Thus, the insertion temperature was maintained at 40°C as milder conditions would less likely disturb SALP structure and not encourage leakage.

<table>
<thead>
<tr>
<th>Lipid Composition (Mol% Composition)</th>
<th>Temperature (°C)</th>
<th>Initial Mol% CPL</th>
<th>% CPL Inserted</th>
<th>Final Mol% CPL</th>
</tr>
</thead>
<tbody>
<tr>
<td>DODAP/DOPE/PEG-CerC₁₄ (45/45/10)</td>
<td>37</td>
<td>6.5</td>
<td>54.5</td>
<td>3.54</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>6.5</td>
<td>62.1</td>
<td>4.04</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6.5</td>
<td>66.6</td>
<td>4.33</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>6.5</td>
<td>74.5</td>
<td>4.84</td>
</tr>
</tbody>
</table>
An insertion profile was constructed for this system to examine whether similar CPL₄ insertion kinetics for DODAP/DOPE/PEG-Cer was observed (Figure 3.9) compared to the DSPC/Chol/DODAP/PEG-Cer formulation, and to determine the incubation time that results in optimal insertion. Two DODAP/DOPE/PEG-Cer formulations were investigated: 45/45/10 mol% and 25/65/10 mol%. The CPL₄ insertion curves follow the same trend seen for DSPC/Chol/DODAP/PEG-Cer with increasing insertion over time. Inspection of the curves finds no distinguishable difference in the insertion efficiency of CPL₄ into these two systems.

When comparing the insertion of CPL₄ into DSPC/Chol/DODAP/PEG-Cer and DODAP/DOPE/PEG-Cer systems of equal initial CPL₄ to lipid ratios, a number of differences become apparent. There are minor differences in the insertion levels, with DODAP/DOPE/PEG-Cer exhibiting slightly higher insertion efficiencies. The most notable difference occurs in the insertion kinetics of the two formulations, in particular at earlier time points. Insertion is slower for DSPC/Chol/DODAP/PEG-Cer SALP (~ 47% at 0.5 h — ~ 60% at 3 h) than DODAP/DOPE/PEG-Cer SALP (~ 62% at 0.5 h — ~ 68% at 3 h).

The insertion efficiency of CPL₄ as a function of the initial amount of CPL₄ is tabulated in Table 3.5. Increasing the initial mol% of CPL₄ resulted in a decrease in the fraction of CPL₄ inserted into the vesicles, although the absolute number of moles of CPL₄ incorporated increased. There is little difference in the insertion efficiencies observed between PEG-CerC₁₄ and PEG-CerC₂₀ systems.

The insertion of CPL₄ into DODAP/DOPE/PEG-Cer SALP is approximately equivalent to that observed for DSPC/Chol/DODAP/PEG-Cer SALP. The same trends, formerly mentioned, are observed for both systems.
Figure 3.9 Insertion Profile of CPL$_4$ into DODAP/DOPE/PEG-CerC$_{20}$ SALP. Insertion was performed at 40 °C for both DODAP/DOPE/PEG-CerC$_{20}$ (45/45/10 mol%) (●) and DODAP/DOPE/PEG-CerC$_{20}$ (25/65/10 mol%) (■). 8.55 μmol lipid was combined with 0.12 μmol CPL$_4$ (6 mol%) and incubated for 0.5, 1.0, 2.0 and 3.0 h. At these times, an aliquot was removed (2.13 μmol lipid), cooled to halt insertion, and passed down a DEAE-Sepharose CL 6B column. The isolated fraction was then assayed for CPL insertion, as described in the Materials and Methods.
Table 3. 5 Insertion of CPL$_4$ into DODAP/DOPE/PEG-Cer SALP

<table>
<thead>
<tr>
<th>Lipid Composition</th>
<th>Mol % Composition</th>
<th>Initial % CPL</th>
<th>% CPL Inserted</th>
<th>Final Mol% CPL</th>
<th>Size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DODAP/DOPE/PEG-CerC$_{14}$ 45/45/10</td>
<td>4</td>
<td>79.3</td>
<td>3.2</td>
<td>87 ± 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>63.2</td>
<td>3.8</td>
<td>(83 ± 6)*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>66.6</td>
<td>4.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>47.8</td>
<td>4.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>35/55/10</td>
<td>4.0</td>
<td>75.2</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>DODAP/DOPE/Chol/PEG-CerC$_{14}$ 30/45/15/10</td>
<td>5.5</td>
<td>64.1</td>
<td>3.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DODAP/DOPE/PEG-CerC$_{20}$ 45/45/10</td>
<td>4</td>
<td>83.2</td>
<td>3.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.3</td>
<td>63.3</td>
<td>3.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>69.9</td>
<td>4.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>54.7</td>
<td>4.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>25/65/10</td>
<td>4</td>
<td>61.6</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>56.2</td>
<td>2.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.3</td>
<td>56.7</td>
<td>3.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>65.4 ± 0.9</td>
<td>3.9 ± 0.1</td>
<td>129 ± 9</td>
<td>(121±10)*</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>41.4</td>
<td>3.31</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values in parentheses indicate sizes of vesicles before CPL insertion

3.4.3 Uptake of DODAP/DOPE/PEG-CerC$_{14}$ SALP and SALP-CPL$_4$ into BHK Cells

As previously discussed, the presence of the fusogenic lipid DOPE should increase the membrane destabilizing ability of SALP systems, thereby enhancing endosomal release of liposome contents, resulting in increased nuclear delivery of AS-ODN. Therefore, uptake of DODAP/DOPE/PEG-CerC$_{14}$ SALP and SALP-CPL$_4$ was examined. Fluorescence micrographs (Figure 3.10) document the significant difference in lipid uptake observed for DODAP/DOPE/PEG-CerC$_{14}$ (45/45/10 mol%) SALP in the presence and absence of 5 mol%
Figure 3.10 Fluorescence micrographs of lipid and oligonucleotide uptake of DODAP/DOPE/PEG-CerC₁₄ SALP and SALP-CPL₄ by BHK cells. 40 nmol lipid of DODAP/DOPE/PEG-CerC₁₄ (45/45/10 mol%) SALP or SALP-CPL₄ (5 mol%) were placed on 1x10⁵ cells in DMEM supplemented with 10% FBS containing 10 mM CaCl₂ at 37 °C. Micrographs were taken 24 h after sample application. Image descriptions are as follows. Exposure time is indicated in parentheses. (A) Phase contrast micrographs of BHK cells (I) with SALP (0.40 s) or (II) with SALP-CPL₄ (0.56 s). (B) Fluorescence micrographs of lipid uptake as illustrated by rhodamine-PE fluorescence for (III) SALP (6.35 s) or (IV) SALP-CPL₄ (0.20 s). (C) Fluorescence micrographs of antisense oligonucleotide intracellular delivery as illustrated by FITC fluorescence for (V) SALP (16 s) or (VI) SALP-CPL₄ (2.52 s).
CPL4. As observed with the DSPC/Chol/DODAP/PEG-CerC14 SALPs, the DODAP/DOPE/PEG-CerC14 system is taken up into BHK cells in small amounts. What lipid is internalized appears to remain within the endosome and gives rise to small punctate fluorescence. The structures are located perinuclearly, however, the majority of lipid within the cells became polarized after 24 h. DODAP/DOPE/PEG-CerC14 SALP-CPL4 exhibits remarkably high levels of lipid uptake compared to SALP alone, which is clearly indicated by the intensity of the fluorescence. The difference is more than reflected in the micrographs since the exposure time was approximately 30x less for SALP-CPL4 compared to SALP.

The primary aim of SALP-CPL systems is to increase the intracellular delivery, in particular nuclear delivery, of antisense oligonucleotides carried within liposomes. DODAP/DOPE/PEG-CerC14 SALPs are considerably more effective in satisfying this objective than DSPC/Chol/DODAP/PEG-Cer formulations. The difference in ODN delivery between DODAP/DOPE/PEG-CerC14 SALP and SALP-CPL4 is exceptional (Figure 3.10 (V) and (VI)). Significant nuclear fluorescence is observed for SALP-CPL4, indicating efficient release of FITC-AS-ODN from the endo-lysosomal pathway. Nuclear fluorescence was first detected at 8 h, evidence that DODAP/DOPE/PEG-CerC14 SALP is an effective vehicle for the delivery and nuclear targeting of AS-ODN.

Unfortunately, the DODAP/DOPE/PEG-CerC14 SALP-CPL4 system is subject to destabilization and aggregation, manifested as large clusters of Rho-PE lipid (data not shown) when introduced to cells. This is despite the addition of 10 mM CaCl2 to the media. Calcium, at concentrations of 40-50 mM, is utilized during the preparation of SALP-CPLs, SPLP-CPLs (Palmer et al., 2000) and LUV-CPLs (Fenske et al., 2000) to prevent aggregation of liposomes. Supplementing the media with calcium serves to decrease SALP-
CPL₄, SPLP-CPL and LUV-CPL aggregation in vitro. Titration of the calcium levels (10-27 mM) in the media demonstrated that increasing concentrations resulted in decreased aggregation but led to cell toxicity (data not presented). Additionally, calcium concentrations greater than 10 mM results in significant decreases in transfection efficiencies (Lam and Cullis, 2000). The variable results of repeated in vitro experiments is further evidence of SALP instability. Nuclear delivery of antisense oligonucleotides could not be achieved consistently. Furthermore, variability in the degree of aggregation was observed, in spite of uniformly maintained 10 mM CaCl₂ throughout all tissue culture studies. Aggregation was initially detected at 4 h, when the cells were first inspected and remained at the 8 h time point. DODAP/DOPE/PEG-CerC₁₄ (25/65/10 mol%) SALPs (±CPL₄) were also examined with regard to cellular uptake. The findings proved identical to those made as with the 45/45/10 mol% system, with the notable exception that larger molar amounts of DOPE resulted in a greater degree of aggregation.

3.5 Increasing the Stability of DOPE Systems

Clearly, SALPs containing DOPE increase the intracellular delivery of oligonucleotides, as indicated by the nuclear fluorescence attained by DODAP/DOPE/PEG-CerC₁₄ SALP-CPL₄. However, the stability of these systems is a problem due to the massive and variable levels of aggregation observed when these samples are introduced to tissue culture media. Furthermore, SALP systems containing DOPE are not able to consistently achieve nuclear delivery of AS-ODN. In refining the formulation to increase SALP stability, two methods were examined. First, the substitution of PEG-CerC₁₄ for PEG-CerC₂₀ was investigated. The second approach examined the incorporation of cholesterol.
DODAP/DOPE/PEG-Cer SALP systems are rich in DOPE, a non-bilayer forming lipid. It has been shown that PEG-lipid constructs are able to stabilize DOPE in bilayer structures (Holland et al., 1996). However, since PEG-CerC<sub>14</sub> exhibits such a short dissociation half-life ($t_{1/2} = 1.1 \pm 0.3$ h) (Wheeler et al., 1998), it is reasonable to assume that the diffusion of this moiety away from the SALP surface leads to SALP destabilization and subsequent aggregation. The lipid anchor of PEG-CerC<sub>20</sub> is composed of a C<sub>20</sub> carbon chain that does not readily allow the lipid to exchange out of bilayers. PEG-CerC<sub>20</sub> exhibits a half-life of dissociation greater than 13 days (Wheeler et al., 1998) and therefore is proposed to increase SALP stability by remaining associated with the vesicles for extended periods. Inclusion of cholesterol was examined as liposomes prepared from phospholipids of low transition temperatures usually require cholesterol to achieve plasma stability (Mayhew et al., 1979).

The effect of PEG-CerC<sub>20</sub> and cholesterol on aggregation was determined when the samples were applied to BHK cells (Figure 3.11). Panel (A) illustrates what was previously seen with the DODAP/DOPE/PEG-CerC<sub>14</sub> (45/45/10 mol%) formulation. Panel (B) and (C) demonstrate the effect of PEG-CerC<sub>20</sub> and cholesterol, respectively, on SALP-CPL<sub>4</sub> aggregation after 24 h. The effect of PEG-CerC<sub>20</sub> appears favourable as the aggregates observed are fewer in number and smaller in size. Aggregation was not detected after 4 h of incubation and did not become largely apparent until 7 h. At 24 h, precipitates exhibited a very regular circular shape located on the surface of the cells. In contrast, it appears that the influence of cholesterol on stability is poor as the micrograph depicts aggregates larger than that observed with the initial formulation. Aggregation was readily visible at 4 h. Increasing incubation time was paralleled by increasing precipitate size and number. Massive
Figure 3.11 Fluorescence micrographs comparing lipid uptake of different SALP-CPL formulations by BHK cells. 40 nmol lipid of (A) DODAP/DOPE/PEG-CerC₁₄ (45/45/10 mol%, 5 mol% CPL₄), (B) DODAP/DOPE/PEG-CerC₂₀ (45/45/10 mol%, 4.4 mol% CPL₄) and (C) DODAP/DOPE/Chol/PEG-CerC₁₄ (30/45/15/10 mol%, 3.5 mol% CPL₄) were placed on 1x10⁵ cells at 37 °C. DMEM media was supplemented with 10% FBS and contained 10 mM CaCl₂. Micrographs were taken 24 h after sample application.
aggregates at 24 h were noted to be spherical in shape and greatly outnumbered those seen for PEG-CerC_{20}. Aggregation was not observed for SALP formulations without CPL_{4} (data not shown). As a result of this particular series of experiments, subsequent formulations utilized PEG-CerC_{20}. DODAP/DOPE/PEG-CerC_{20} systems hereafter were composed as 25/65/10 mol\%, respectively, as higher levels of DOPE are predicted to increase the fusogenic propensity of SALPs and thus promote release of liposomal contents from the endosomes.

The modified formulation, DODAP/DOPE/PEG-CerC_{20} (25/65/10 mol\%), revised to increase vesicle stability and decrease aggregation, was tested in vitro (Figure 3.12). The observed kinetics of lipid uptake for DODAP/DOPE/PEG-CerC_{20} (25/65/10 mol\%) samples containing CPL_{4} into BHK cells was not that which was predicted. Rather, an uptake pattern similar to that determined for stabilized plasmid lipid particles (SPLPs) was expected — a steady increase in uptake over time. Instead, SALP-CPL_{4}, composed of DODAP/DOPE/PEG-CerC_{20} (25/65/10 mol\%, 3.0 mol\% CPL) and DODAC/DOPE/PEG-CerC_{20} (25/65/10 mol\%, 2.6 mol\% CPL), exhibited peak uptake after only 2 h incubation, which subsequently decreased over time. Furthermore, SALPs formulated from DODAP/DOPE/PEG-CerC_{20} (25/65/10 mol\%) were unable to achieve levels of uptake comparable to DSPC/Chol/DODAP/PEG-CerC_{14}, even though fluorescence microscopy indicated that levels of uptake are significantly greater in systems containing DOPE. The maximum lipid uptake into BHK cells of DODAP/DOPE/PEG-CerC_{20} (25/65/10 mol\%) was quantitated at 11.2 nmol lipid/mg protein after 2 h while DSPC/Chol/DODAP/PEG-CerC_{14} reached approximately 42 nmol lipid/mg protein after 24 h, nearly four times more. The reason for this is not clear.
Figure 3.12 Lipid Uptake of DODAP/DOPE/PEG-CerC<sub>20</sub> and DODAC/DOPE/PEG-CerC<sub>20</sub> SALP and SALP-CPL<sub>4</sub> into BHK Cells. 40 nmol lipid of DODAP/DOPE/PEG-CerC<sub>20</sub> (●), DODAP/DOPE/PEG-CerC<sub>20</sub>-CPL<sub>4</sub> (3.0 mol% CPL<sub>4</sub>) (■), DODAC/DOPE/PEG-CerC<sub>20</sub> (▲), and DODAC/DOPE/PEG-CerC<sub>20</sub>-CPL<sub>4</sub> (2.6 mol% CPL<sub>4</sub>) (▼) were placed on 1x10<sup>5</sup> cells in DMEM supplemented with 10 % FBS and incubated at 37 °C. 200 μl of SALP (± CPL) in HBSC (20 mM HEPES, 70 mM NaCl, 50 mM CaCl<sub>2</sub>, pH 7.5) was added to 800 μl of DMEM + 10% FBS, resulting in a final CaCl<sub>2</sub> concentration of 10 mM. Following incubation periods of 1,2,4 and 8 h, the cells were washed with PBS and lysed with 600 μl of lysis buffer (0.1% TX-100 in PBS) and rhodamine fluorescence of the lysate was determined. The fluorescence intensity was used to extrapolated lipid uptake in nmol lipid and then normalized against protein concentration, determined by a BCA Protein Assay.
Presumably, the abnormal kinetics stems from the instability of DODAP/DOPE/PEG-Cer SALP and SALP-CPL₄ systems and their tendency to aggregate.

Collectively, the results of this work provide strong evidence to support the argument that DODAP/DOPE/PEG-Cer SALPs are currently the best intracellular delivery systems but are not stable. The requirement for a lower incubation temperature during CPL insertion, the CPL₄ insertion kinetics, the uptake of SALP-CPL₄ into BHK cells, the inconsistent nuclear delivery of AS-ODN, but especially the variability in aggregation, all indicate that DODAP/DOPE/PEG-Cer SALPs are unstable. This formulation is highly sensitive to slight changes in molar lipid composition and environmental conditions. Considerable investigation of this system must be conducted before SALPs and SALP-CPL will be successful for delivering ODN intracellularly.
Effective systemic delivery systems for DNA and RNA are essential to the successful clinical application of gene-based drugs (Rojanasakul, 1996; Friedmann, 1997; Zelphati and Szoka, 1996c). The recent development of “stabilized antisense-lipid particles” (SALP) as liposomal carriers of oligonucleotides offers great promise for antisense therapy. However, current SALP lipid formulations (Semple et al., 2000) exhibit poor cellular uptake. The studies conducted in this thesis examined whether the incorporation of cationic PEG-lipids (CPLs), in particular CPL4, and/or the fusogenic lipid, DOPE, could improve the intracellular delivery of antisense oligonucleotides in SALP systems by increasing vesicle internalization and release from the endo-lysosomal pathway, respectively. The inclusion of CPL4 into DSPC/Chol/DODAP/PEG-CerC_{14} SALP was successful in enhancing cellular uptake but did not increase the nuclear delivery of antisense molecules. Nuclear accumulation of oligonucleotides was achieved through the use of a novel formulation in which DOPE was substituted for DSPC. However, these liposomes were unstable. Here I discuss the effects of CPL4 and DOPE on antisense intracellular delivery and SALP stability. Possible ways to further improve the stability and intracellular delivery of current SALP systems are also addressed.

The results presented here clearly show that insertion of CPL4 in SALP systems significantly enhanced the cellular uptake of liposomes. Large increases were observed for both DSPC/Chol/DODAP/PEG-CerC_{14} SALP-CPL4 and DODAP/DOPE/PEG-CerC_{14}/C_{20} SALP-CPL4 compared to corresponding SALP without CPL4. This observation is consistent with the uptake results of DOPE/DODAC/PEG-CerC_{20} (84/6/10 mol%) “stabilized plasmid-
lipid particles” (SPLPs) (Palmer et al., 2000) and DOPE/DODAC/PEG-CerC$_{20}$ (84/6/10 mol%) LUVs (Fenske et al., 2000) with surface associated CPL$_4$. It has been proposed that the positive charge of the CPL$_4$ headgroup plays the key role in enhancing uptake by interacting non-specifically with negatively charged cellular membranes. Obvious candidates for interaction with cationic vectors would be the most abundant anionic cell surface molecules such as sulfated proteoglycans and sialic acids. Studies conducted by Mounkes et al. (1998) show that membrane heparin sulfate-bearing proteoglycans mediate cationic liposome-DNA complex based gene transfer and expression both in cultured cells and following intravenous delivery into ICR mice. Cationic lipid-DNA complexes, which bear a net positive charge, are unable to transfect Raji cells which lack proteoglycans, but efficiently transfect Raji cells that have been stably transfected with a proteoglycan, syndecan-1 (Mounkes et al., 1998). The treatment of HeLa cells with sodium chlorate, a potent inhibitor of proteoglycan sulfation, dramatically reduced poly-lysine mediated gene expression by 69% (Mislick and Baldeschwieler, 1996), suggesting that proteoglycans must be sulfated to mediate uptake. Subsequent experiments by Mounkes et al. (1998) demonstrated that intravenous pretreatment of mice with heparinases, which specifically cleave heparin sulfate molecules from cell surface proteoglycans, blocked in vivo transfection, supporting the concept that intact proteoglycans are required for the efficient delivery of complexes to cells. In addition, pre-treatment of cationic lipid complexes with highly anionic polysaccharides inhibited transfection in vitro and in vivo (Mounkes et al., 1998) likely by blocking the interaction between the positively charged complexes and negatively charged cell surface molecules. Proteoglycans may interact with cationic vectors/liposomes directly and be internalized as a complex, or they may serve to anchor
cationic vectors/liposomes for presentation to secondary receptors that in turn undergo specific receptor-mediated endocytosis. Nonetheless, the variable distribution of proteoglycans among tissues may explain why some cell types are more susceptible to transfection than others (Mislick and Baldeschwieler, 1996).

The presence of CPL4 on the surface of SALP increases cellular internalization of lipid vesicles, but does not increase the nuclear delivery of antisense oligonucleotides. Presumably, this is the result of inefficient release of antisense molecules from the endosomal compartments. Past studies have found that the majority of cationic lipid complexes require the addition of a fusogenic helper lipid for efficient in vitro gene transfer (Hui et al., 1996; Felgner et al., 1994). Liposomes containing fusogenic lipids, such as DOPE, are thought to facilitate the intracellular delivery of complexed nucleic acids by promoting destabilization and fusion of the endosomal membrane due to the preference of DOPE for the hexagonal H\textsubscript{II} phase. Subsequent experiments examined the effect of DOPE on increasing the endosomal release and hence, nuclear accumulation of antisense oligonucleotides delivered by SALPs.

Refining the conventional SALP formulation to contain DOPE generated a SALP system, DODAP/DOPE/PEG-CerC\textsubscript{14}, that was capable of nuclear delivery of antisense oligonucleotides when coupled with CPL4. The increase in nuclear delivery is likely the consequence of enhanced endosome destabilization induced by the presence of DOPE, in addition to destabilization due to the cationic lipid, DODAP. Interaction of DODAP with anionic lipids present in the endosomal membrane will induce bilayer destabilization as it has been shown that mixtures of cationic and anionic lipids can adopt "inverted" non-bilayer lipid phases such as the hexagonal H\textsubscript{II} phase (Hafez et al., 2000). The ability of
cationic lipids to form non-bilayer structures in combination with anionic lipids may also be related to the ability of cationic lipids to facilitate the intracellular delivery of plasmid-DNA (Hafez et al., 2000). These destabilizing effects will be augmented by the presence of DOPE, which also favours the non-bilayer hexagonal (H$_{11}$) phase conformation.

The stability of SALP and SALP-CPL$_4$ is a major factor limiting the success of these lipid-based nucleic acid delivery systems. The incorporation of CPL$_4$ and DOPE proved successful in increasing the intracellular delivery of antisense oligonucleotides but consequently rendered SALPs unstable. Insertion of CPL$_4$ into SALP induced vesicle aggregation which is remedied by the addition of 50 mM CaCl$_2$ to the incubation buffer. This behaviour was also observed for SPLP-CPL$_4$ in which optimal deaggregation occurred above 30-40 mM CaCl$_2$ or MgCl$_2$ (Palmer et al., 2000) and for LUV-CPL$_4$ (Fenske et al., 2000). The finding that CPL$_4$ induced aggregation can be inhibited or reversed by increasing the ionic strength of the sample buffer suggests that the phenomenon originates from electrostatic interactions, presumably between the cationic headgroups of CPL$_4$ and certain negatively charged residues on the SALP surface. It is conceivable that the positive charge exhibited by CPL$_4$ associates with the negatively charged phosphate groups of the phospholipids in apposing membranes. Alternatively, aggregation may be related to the ability of PEG crown ethers to act as chelators for cations, specifically, the lysine residues of CPL$_4$ (Bailey and Koleske, 1991). Currently, the cause of this aggregation is not understood. However, it is not the result of the post-insertion process itself, as aggregation is also observed for LUV-CPL$_4$ in which CPL$_4$ was contained in the initial lipid formulation in organic solvent and thus was present when the LUVs were formed (Fenske et al., 2000).
The use of DOPE greatly enhances nuclear delivery of SALP antisense molecules but achieves this at the expense of liposome stability. SALP systems containing DOPE aggregate in vitro despite the presence of 10 mM CaCl₂ in the media. This was not observed for DSPC/Chol/DODAP/PEG-CerC₁₄ SALP or SALP-CPL₄. Aggregation is likely due to an increased tendency of the vesicle bilayers to fuse as a result of the presence of DOPE. Notably, increasing the DOPE content results in aggregates of larger sizes. As mentioned previously, the tendency of DOPE to adopt “inverted” hexagonal (H₃) phases at physiological pH and ionic strength (Allen et al., 1990) increases the propensity of membranes containing DOPE to fuse.

SALP instability may also arise from polynucleotide-cationic lipid domains formed during the encapsulation of AS-ODN. Interactions of multivalent ions or polyelectrolytes with oppositely charged vesicles can result in phase separation and structural conformations such as aggregation and fusion (Maurer et al., 2000). Localized separation of DODAP from DOPE could form areas rich in DOPE which are highly prone to fusion.

Improved SALP stability may be accomplished through a number of methods. The first approach should examine the influence of additional PEG on bilayer stability. During the course of this thesis, the effect of substituting PEG-CerC₁₄ for PEG-CerC₂₀ in DODAP/DOPE/PEG-Cer systems on SALP stability and aggregation was studied and it was found that PEG-CerC₂₀ significantly decreased the degree of aggregation. PEG-CerC₂₀ exhibits an extended half-life of dissociation (t₁/₂ >13 d) compared to PEG-CerC₁₄ (t₁/₂ = 1.1 ± 0.3 h), as a result of the longer acyl chain in the ceramide lipid anchor (Wheeler et al., 1998). Hence, PEG-CerC₂₀ remains associated with SALP for prolonged periods thereby providing membrane stability for extended durations. Increasing the total PEG-lipid content
in SALP may additionally stabilize these liposomes. The maximum amount of PEG-lipids usually obtainable in liposomes is generally 7-10 mol% and is limited by difficulties in liposome generation such as foam formation (Woodle and Lasic, 1992). However, results from Palmer et al. (2000) and Fenske et al. (2000) have shown that post-insertion of cationic PEG-lipids into vesicle membranes can achieve levels of PEG equivalent to ~12 mol% of the total lipid. Other authors have reported that PEG-lipids can be incorporated into LUVs at amounts as great at 15 mol% before inducing structural changes (Edwards et al., 1997) and as high as 20 mol% before lytic effects are observed (Edwards et al., 1997; Kenworthy et al., 1995).

The problem with stabilizing SALP by additional PEG-lipid is that this is likely to inhibit fusion with, and destabilization of, the endosomal membrane. This could be countered by the use of detachable PEG-polymer coatings on the surface of vesicles. Destabilization and fusion of DOPE vesicles can be triggered by the cleavage of surface grafted PEG molecules (Kirpotin et al., 1996) once liposomes reach their destination site. Kirpotin et al. (1996) synthesized a novel, thiolytically cleavable, PEG-phospholipid conjugate that was able to demonstrate rapid and complete release of liposome contents once the disulfide-linked polymer was cleaved off the vesicle surface. Moreover, detachment of PEG from DOPE liposomes resulted in vesicle fusion. This method enables liposomes to remain intact and stable until they reach the cells, at which point the vesicles become unstable and membrane fusion occurs.

Alternatively, utilizing different cationic CPL headgroups may result in increasing SALP stability. Various cationic lipids have been shown to exhibit distinct cellular effects/characteristics in vitro (Song et al., 1997; Mahato et al, 1998). It could then be
suggested that altering the chemical structure or completely changing the cationic moiety of
the CPL headgroup could substantially reduce the problem of aggregation. However, given
that aggregation stems from electrostatic interactions, the use of various types of cationic
PEG-lipids may not prove beneficial. Replacing the cationic lysine residues of the CPL
headgroup with a neutral molecule(s) may prevent these charge interactions from occurring
and completely eliminate aggregation. However, by removing the cationic moiety, we are no
longer able to exploit uptake due to the negative charge of biological membranes. The
molecule/ligand could, however, take advantage of cell surface proteins and receptors.

In particular, the use of cell specific ligands could enable liposomes with a defined
ligand attached to the external membrane to be targeted to cells expressing the matching
receptor. Various ligands that could be used include folate (Wang et al., 1995; Wang and
Low, 1998), biotin (Loughrey et al, 1993), peptides (Zalipsky et al., 1995), oligosaccharides
(Zalipsky et al., 1997) and antibodies (Hansen et al., 1995; Lopes de Menezes et al., 1998).
The major requirement would be that the cell surface receptor be internalized following
binding of the ligand in order for intracellular delivery to occur.

Intracellular delivery occurs through a biphasic process: internalization followed by
endosomal destabilization and oligonucleotide release. Enhancing intracellular delivery may
also be achieved by increasing the release of liposomes and their contents from the endo-
lysosomal pathway. Liposomes can be designed take advantage of the considerably lower
pH of the endosomes compared to the blood. pH sensitive liposomes can be triggered to
destabilize and release encapsulated contents when exposed to lower pH environments
(Straubinger et al., 1985). More refined, “tunable” pH sensitive liposomes can undergo pH
dependent fusion by forming non-bilayer structures at specified acidic pH values depending
on the ratio of cationic to anionic lipids composing the vesicle (Hafez et al., 2000). Recently, synthetic polymers have been described that exhibit a pH-dependent effect on the lipid bilayer. Poly(2-ethylacrylic acid) (PEAA) (Thomas and Tirrell, 1992; Maeda et al., 1988) and succinylated poly(glycidol) (Kono et al., 1994) have been reported to mediate the proton-induced release of liposomal contents from SUVs under acidic conditions. Recent studies of PEAA in LUVs found that these vesicles also induced membrane destabilization release of calcein from both LUVs PEAA is linked to and from target vesicles (Chen et al., 1999). PEAA-LUVs were also able to instigate pH-dependent fusion with target membranes (Chen et al., 1999). The major advantage of utilizing pH-sensitive liposomes is that they remain stable at physiological pH, and only release their contents when an acidified environment is encountered, such as the endosomes following internalization through the endocytotic pathway.
BIBLIOGRAPHY


