LIPID-BASED VEHICLES FOR NUCLEIC ACID DRUGS

BY

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ABSTRACT

Lipid-based carriers are anticipated to be a viable option to deliver nucleic acid drugs (NAs) for gene therapy. This thesis describes a method of adding lipids to NAs in a manner that generates a hydrophobic lipid-NA complex and bypasses the aggregation events induced by use of conventional cationic liposomes. It is anticipated that careful control of the lipid components will facilitate generation of a carrier formulation that protects, directs and delivers NAs to a target and in a form capable of mediating a therapeutic response.

The factors that govern the cationic lipid/DNA binding reaction were assessed using a hydrophobic lipid/DNA complex generated by Bligh and Dyer extraction. Efficient recovery of DNA (>95%) in an organic phase was achieved when monocationic lipids interacted with anionic phosphate groups to neutralise the DNA charge. Results indicate that the cationic lipid/DNA complex forms at the aqueous/organic interface and binding is dependent on co-operative, multivalent interactions. Based on the hydrophobic intermediate, self-assembling lipid-DNA particles (LDPs) were formed in detergent and were used to assess the role(s) of lipid components that govern the attributes of a novel lipid-based DNA transfer system. While plasmid DNA, formulated within LDPs containing phosphatidylethanolamine (PE)-lipids, was more sensitive to enzymatic cleavage than DNA in LDPs containing phosphatidylcholine (PC)-lipids, only LDPs with PE-lipids could mediate transgene expression. Another key factor effecting delivery of NAs is electrostatic mediated binding to the target cell. In an effort to model this interaction, a microelectrophoresis technique was used to provide information on LDP surface charge and binding to an anionic surface. It was

confirmed that LDPs bound anionic latex beads through electrostatic interactions, where binding involved both the NA and associated lipids.

While the previous studies assessed transfection activity of a reporter gene, a second goal of these studies was to use lipids with a therapeutically relevant NA. A particulate delivery system was formed through a hydrophobic intermediate containing cationic lipid, PE-lipid, poly(ethylene glycol)-phosphatidylethanolamine (PEG-PE) and an antisense oligonucleotide (ASO), shown to effect eradication of a B-cell lymphoma *via* downregulation of Bcl-2 protein. Although *in vitro* data did not demonstrate that lipid-ASO particles (LAPs) could mediate downregulation of Bcl-2 protein, *in vivo* data indicated that LAPs were more efficacious when compared to free ASOs. While the use of stabilised PEG-conjugated lipids may not be advantageous for delivery *in vitro*, the results suggest that LAPs can modulate the pharmacokinetic properties of an associated ASO leading to increased bioavailability.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ASO	antisense oligonucleotide
CAT	chloramphenicol acetyl transferase
Chol	cholesterol
CPA	cyclophosphamide
CSC	critical solubilization concentration
cmc	critical micellar concentration
DC-Chol	3β-[(N-dimethylaminoethane)-carbamoyl] cholesterol
DDAB	dimethyldioctadecylammonium bromide
DLPC	1,2-dilauroyl-*sn*-glycero-3-phosphocholine
DLPE	1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine
DMPC	1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
DMPE	1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine
DMPE-PEG$_{2000}$	1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol) 2000]
DMPG	1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)]
DODAC	*N,N*-dioleyl-*N,N*-dimethylammonium chloride
DODAP	1,2-dioleoyl-3-dimethylammonium propane
DOGS	dioctadecylamidoglycyl spermine
DOPC	1,2-dioleoyl-*sn*-glycero-3-phosphocholine
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
DOPS	1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine]
DOSPA	2,3-dioleyloxy-N-[2-sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate
DOTAP	1,2-dioleoyl-3-trimethylammonium propane
DPPE	1,2-dipalmitoyl-*sn*-3-phosphoethanolamine
DPPE	1,2-dipalmitoyl-*sn*-3-phosphoethanolamine
DSC	differential scanning calorimetry
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphocholine
DSPE	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine
DSPE-PEG$_{2000}$	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol) 2000]
ECL	enhanced chemiluminescence
EnASO	encapsulated antisense oligonucleotide
EtBr	ethidium bromide
H-bond(ing)	hydrogen-bond(ing)
i.p.	intraperitoneally
i.v.	intravenously
K_n	apparent dissociation constant
LAA	liposome-ASO aggregate

LAP	lipid-antisense oligonucleotide particle
LDA	liposome-DNA aggregate
LDP	lipid-DNA particle
LPI	liver phosphatidylinositol
LUV	large unilamellar vesicle
MLV	multilamellar vesicle
MMO	mismatch oligonucleotide
MWF	Monday, Wednesday and Friday
NA	nucleic acid drug
NHL	non-Hodgkin's lymphoma
NK	natural killer
OD	optical density
ODN	oligodeoxynucleotide
OGP	n-octyl β-D-glucopyranoside
PBS	phosphate buffered saline
PEG-CerC14	poly(ethylene glycol) conjugated ceramide (14 carbon chain)
PEG-PE	poly(ethylene glycol) conjugated phosphatidylethanolamine
PG	proteoglycan
QD	every day treatment
QELS	quasi-elastic light scattering
QOD	every other day treatment
RPO	reverse-polarity oligonucleotide
SCID	severe combined immunodeficient
SD	standard deviation
SEM	standard error of the mean
SM	sphingomyelin
TBS	Tris buffered saline

ACKNOWLEDGEMENTS

One sunny day a rabbit came out of her hole in the ground to enjoy the fine weather. The day was so nice that she became careless and a fox sneaked up behind her and caught her.

"I am going to eat you for lunch!" said the fox.
"Wait!" replied the rabbit; "You should at least wait a few days."
"Oh yeah? Why should I wait?"
"Well, I am just finishing my thesis on 'The Superiority of Rabbits over Foxes and Wolves.'"
"Are you crazy? I should eat you right now! Everybody knows that a fox will always win over a rabbit."
"Not really, not according to my research. If you like, you can come into my hole and read it for yourself. If you are not convinced, you can go ahead and have me for lunch."
"You really are crazy!" But since the fox was curious and had nothing to lose, it went with the rabbit. The fox never came out.

A few days later the rabbit was again taking a break from writing and sure enough, a wolf came out of the bushes and was ready to set upon her.
"Wait!" yelled the rabbit; "you can't eat me right now."
"And why might that be, my furry appetiser?"
"I am almost finished writing my thesis on 'The Superiority of Rabbits over Foxes and Wolves.'"
The wolf laughed so hard that it almost lost its grip on the rabbit. "Maybe I shouldn't eat you. You really are sick...in the head. You might have something contagious."
"Come and read it for yourself. You can eat me afterward if you disagree with my conclusions."
So the wolf went down into the rabbit's hole...and never came out. The rabbit finished her thesis and was out celebrating in the local lettuce patch. Another rabbit came along and asked, "What's up? You seem very happy."

"Yup, I just finished my thesis."
"Congratulations. What's it about?"
"'The Superiority of Rabbits over Foxes and Wolves.'"
"Are you sure? That doesn't sound right."
"Oh yes. Come and read it for yourself."

So together they went down into the rabbit's hole. As they entered, the friend saw the typical graduate student abode, albeit a rather messy one after writing a thesis. The computer with the controversial work was in one corner. To the right there was a pile of fox bones, to the left a pile of wolf bones. And in the middle was a large, well-fed lion.

The title of your thesis doesn't matter.
The subject doesn't matter.
The research doesn't matter.

All that matters is who your advisor is.

Thanks, Marcel.

DEDICATION

To my parents

and Gloria, my sister and my friend,

I trained you well.

To Derrick,

for driving me crazy and keeping me sane.

CHAPTER 1

INTRODUCTION

Since many human diseases have a genetic origin or a genetic component it follows that treatment strategies can be based on modifying gene expression in order to elicit a therapeutic response (1-4). In the foreseeable future disease treatments may focus on pre-translational pathways and will become a standard in therapy regimes; possibly these treatments will cater to an individual's genetic makeup. Based on the rapidly increasing knowledge of gene functions and molecular biology techniques, the pathogenesis of disease can be altered through specific and controlled gene manipulations (5). Those diseases that stem from a single genetic mutation or multiple alterations may be prevented or treated by: (i) long-term expression or repeated administration of a single gene (6), (ii) stimulating an immune response (7), (iii) transmitting apoptosis (death) signals (8), (iv) hindering the function of a deleterious gene (9, 10), or (v) tagging cells for destruction by producing specific proteins (11).

Identifying a genetic target and generating a nucleic acid drug that specifically repairs the damage caused by that genetic target are difficult endeavours. Mechanistic information associated with disease progression underlies the strategy for the discovery of novel sites to treat or delay disease progression. Once a target is identified and the NA generated, the ultimate goal is to maximise the nucleic acid drug's efficacy profile through specific targeting to a disease site. The methodology for enhancing therapeutic activity of NAs will involve altering their metabolism as well as their pharmacokinetic and biodistribution properties. Therefore, a critical step in the development of NAs is the optimisation of the

1

methods for achieving optimal efficacy through use of rationally designed methods for NA delivery. This thesis focuses on a new approach for designing lipid-based carriers of NAs.

1.1 Nucleic acid drugs

The Human Genome Project is a large-scale venture into deciphering the sequence of nucleotides for all 23 human chromosomes and, as a result, identifies a large pool of potential molecular targets (see Table 1.1). As the Human Genome Project nears completion (12, 13), investigators are recognising an increasing number of possible genes available for therapeutic applications. Notably, cancer is a disease that is particularly amenable to genetic alteration due to its origins in DNA mutations. However, the choice for a single effective target for cancer gene therapy remains elusive. A pharmaceutically viable NA capable of eliciting a specific therapeutic response is an important first step. While viral systems are by far a more effective means to induce efficient transfection, these vectors are limited by their small capacity for carrying genetic material and the potential for severe immunological responses (14-17). For these reasons, synthetic DNA delivery systems using plasmid DNA and ASOs are becoming increasingly desirable as pharmaceuticals.

1.1.1 Plasmid DNA

One of the most convenient vehicles by which a therapeutically relevant gene of choice is generated into a readily manipulatable, deliverable NA is through incorporation into a plasmid vector. Recombinant DNA technology has made it possible to modify plasmids for gene therapy applications and made them useful as vectors for specific copy DNA sequences (18-20). Although the design of an appropriate plasmid expression vector cancer

2

Table 1.1

The human genome sequenced by chromosome as of May 30, 2000 (12, 13)

Chromosome	Effective Size (kb)[*]	Sequence done (kb)	Percent finished	RefSNPs[#] Co-ordinate known	Mean intermarker distance (kb)
1	263,000	28,875	11	455	536
2	255,000	30,346	11.9	200	1179
3	214,000	12,375	5.8	87	2285
4	203,000	13,100	6.5	90	2170
5	194,000	19,845	10.2	92	1591
6	183,000	49,646	27.1	552	324
7	171,000	85,334	49.9	1221	132
8	155,000	9,097	5.9	72	1729
9	145,000	5,732	4.0	47	1947
10	144,000	7,469	5.2	43	2689
11	144,000	8,803	6.1	153	873
12	143,000	25,629	17.9	261	497
13	98,000	2,797	2.9	58	499
14	93,000	36,217	38.9	243	368
15	89,000	2,203	2.5	33	2372
16	98,000	20,817	21.2	218	438
17	92,000	29,277	31.8	533	142
18	85,000	4,225	5.0	18	2671
19	67,000	17,497	26.1	256	241
20	72,000	31,479	43.7	232	284
21	34,000	33,824	99.5	184	195
22	34,491	33,464	97	627	55
X	164,000	67,731	41.3	368	454
Y	35,000	12,947	37.0	15	1946
Total	3,175,491	590,161	18.6	6058	

*The "effective size" excludes repetitive DNA, such as that found on the short arms of the acrocentric chromosomes (13, 14, 15, 21, 22).
#RefSNPs: Reference single nucleotide polymorphism

for gene therapy is complicated, the progress of plasmid design has made it possible to avoid the use of viral-based systems. This, in turn, results in a number of advantages, including: (i) non-infectivity (21), (ii) low immunogenicity (22), (iii) low toxicity (23), (iv) low probability of integration with design of episomal plasmids (24, 25), (v) ease in complexing to cationic vehicles (26) and (vi) long-term storage (27).

While investigators examine and debate whether expression of a single gene will induce a therapeutically relevant response against a specific disease, the determination of transfection efficiency, through the quantification of plasmid expression vectors, is typically assessed using reporter genes. The reporter genes typically used to assess transfection efficiency are chloramphenicol acetyl transferase (CAT) (28), β-galactosidase (β-gal) (29), luciferase (30) and green fluorescent protein (31). While the choice of the copy DNA fragment inserted into the plasmid is based on its ability to demonstrate a measurable response, there are a number of structural features that have a bearing on plasmid expression. These include size, where fragments >10-15 kb are difficult to deliver to cells (32, 33), and structure, where supercoiled DNA is suggested to be the most efficacious conformation (34).

The two plasmids, pINEXCATv2.0 and pCMVβ, used in this thesis are illustrated in Figure 1.1. Included in these plasmids are the unique copy DNA encoding the reporter gene and a number of common design features that are universal in creating and optimising plasmid DNA systems (35). In order to propagate plasmid DNA for experimental purposes, one must be able to select those bacteria that are actively generating the correct plasmid. As such, plasmid DNA generated in bacteria, in the presence of antibiotics, require a bacterial DNA origin of replication and an antibiotic resistance gene (36). Further, plasmid design must consider that the expression of exogenous genes in human cells is dependent on an

4

Figure 1.1

Plasmid conformations and reporter vector systems for detection of transfection

Plasmid DNA exists as three conformations depending on the extent of nicks. The two plasmid used in this thesis are: (i) pCMVβ contains the immediate early gene promoter/enhancer from the cytomegalovirus (CMV), an intron (splice donor/splice acceptor) and polyadenylation signal from SV40 (simian virus 40), and the full-length *E. coli* β-galactosidase gene with eukaryotic translation initiation signals and (ii) pInexCATv2.0 is 4490 bp in size and includes the chloramphenicol acetyl transferase reporter gene, the CMV promoter, the SV40 intron for message processing, the alfalfa mosaic virus (AMV) enhancer and the SV40 polyadenylation signals.
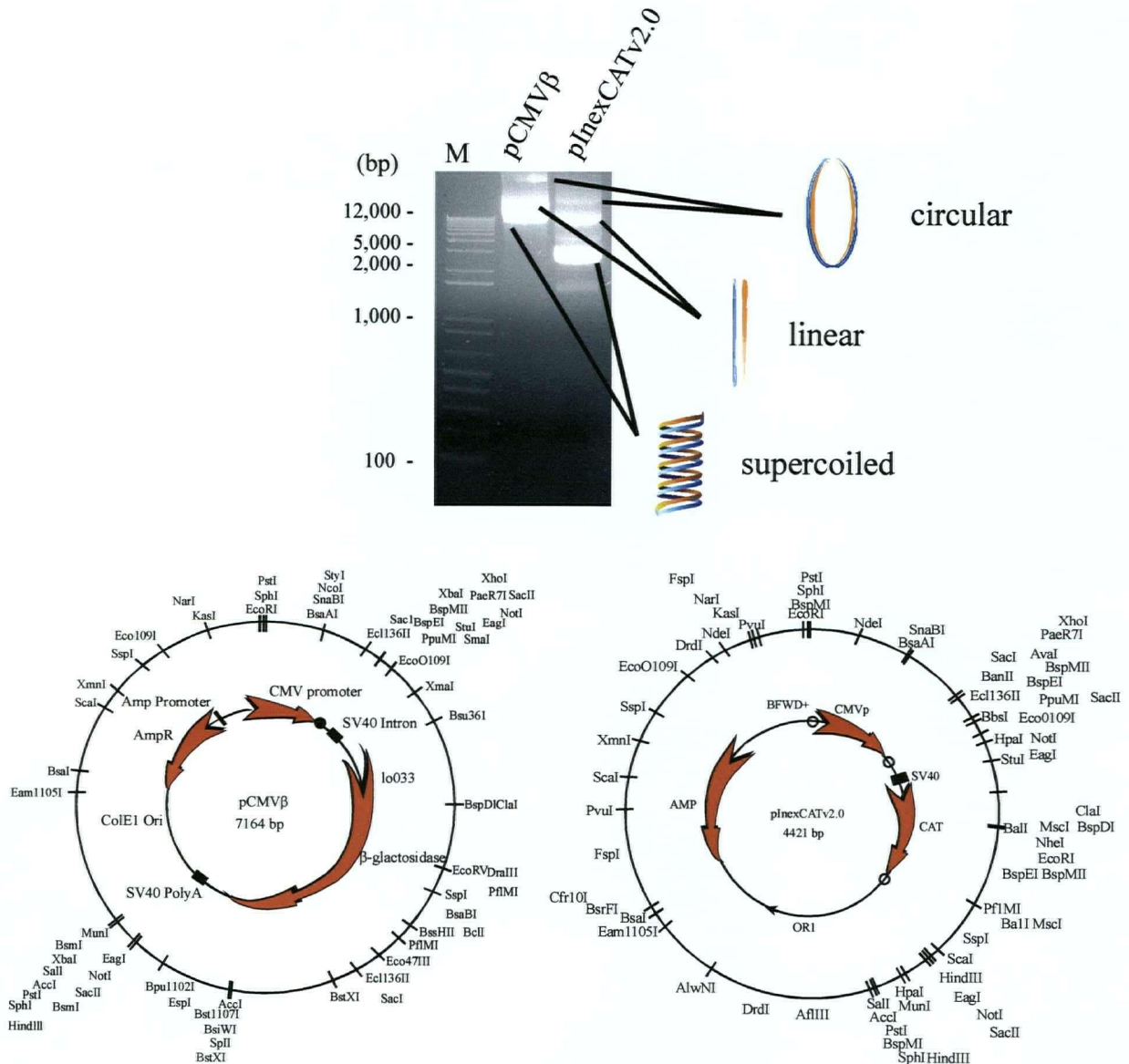
eukaryotic transcription cassette as well as transcriptional promoters and enhancers and these must be incorporated into the vector (37, 38). These may include consensus recognition sequences for transcription factors and RNA polymers. Finally a polyadenylation signal is inserted into the plasmid DNA for promotion of mRNA stability.

The expression of plasmid vectors tends to be transient since plasmid replication is not active in parallel with dividing cells (39). Further, transfected plasmids remain episomal, persisting according to their biochemical half-life (40). Gene expression decline in a transfected cell is a function of a reduction in the plasmid copy number and loss of the transgene as a consequence of recombination events or nuclease degradation. In order to maintain a sufficient plasmid copy number capable of preserving a therapeutically relevant level of expression in a transfected cell, episomal plasmids that replicate autonomously have been developed (41). Some DNA fragments have the ability to undergo semi-conservative replication during the S (synthesis)-phase of the cell cycle when incorporated into a plasmid vector (33, 42). Other methods for increasing the level and duration of gene expression after transfection require specific sequences extrapolated from DNA viruses, i.e. a viral DNA origin of replication and a viral early gene product. The viral early gene product binds to the viral DNA origin, which is then recognised by the human cell as a functional DNA origin, and thereby undergoes replication (43).

As indicated above, structural features, irrespective of the choice of the gene, are critical in defining an effective plasmid. When plasmids are isolated by cesium chloride centrifugation or agarose gel electrophoresis, three distinct tertiary structures exist. These are linear, open circular, and covalently closed circular conformations (see Figure 1.1). It is likely that the circular forms are the predominant forms generated by bacteria, while the

presence of the linear form is a result of harsh extraction procedures, which results in double-stranded nicks. The covalently closed circular DNA conformation exists as interlocking rings and thereby has the ability to renature more efficiently after extreme treatments, such as exposure to temperatures above 52°C, than the open circular and linear forms. One important observation of particular utility in molecular technology is that the binding of the intercalating dye, ethidium bromide, binds linear, open circular and covalently closed circular DNA (supercoiled) conformations in an increasing manner (44). The extent of EtBr binding to plasmid DNA is exemplified by their position on an agarose gel when plasmid DNA is separated using electrophoresis as shown in Figure 1.1. The covalently closed circular DNA exhibits negative supercoiling and is considered to be the most desirable of the plasmid forms for transfection due to its high density and poor substrate specificity for nucleases.

1.1.2 Antisense oligonucleotides

One approach to specifically preventing the function of a deleterious gene is to eliminate the production of its protein product. The downregulation or elimination of the expression of an individual gene by use of a short string of nucleotides is not only a powerful molecular biological tool but is also considered to be of therapeutic value (45-47). The concept of antisense oligonucleotides (ASOs) originated over 20 years ago when P.C. Zamecnik and M.L. Stephenson described control of the expression the Rous sarcoma virus (48, 49). However, the use of synthetic ASOs for use in therapeutic application is only in its infancy (50, 51). The essential function of ASOs is to block the transfer of information from the genetic template to decrease the amount of functional protein; ultimately, this inhibits a specific cellular activity (52). Depending on the design specificity of ASOs, the mechanism

7

of action for these molecules is quite variable and can range from forming triplex DNA to activation of RNase H (53-55) (Figure 1.2). The collective basis of the molecular action of ASO is through complementary (Watson-Crick) base-pairing with the target nucleotides.

Despite the success of ASOs in early pre-clinical work and its more recent advance into a clinical setting, there are a number of issues that limit its usage as a therapeutic (56-59). As for any NA, there are biological barriers that prevent optimal ASO activity (60). The specifc problems as related to delivery and efficacy of NAs, for both plasmids and ASOs, will be described in section *1.3*. As far as its restrictions as a pharmaceutical is concerned, ASOs have large-scale production costs (61), unknown potential for mutagenicity and immunogenicity (62), transient effects (63) and a non-specific biodistribution with a short half-life (64).

Medicinal chemistry has been widely examined as an option to enhance the pharmaceutical viability of ASOs. Chemical modifications for enhancing stability and/or potency can be made at the internucleotidic phosphate backbone, the sugar moiety, or the 3' end as shown in Figure 1.3. As phosphodiester oligonucleotides are readily amenable to nuclease digestion (65), a second generation of ASOs was developed. The most widely used method of stabilising ASOs is to replace an oxygen atom with sulphur (66). Phosphorothioates maintain their ability to hybridise *via* Watson-Crick interactions while becoming an imperfect substrate for nucleases as indicated by lower melting temperatures (67). However, their increase in stability in the plasma compartment is in conflict with their ability to act as efficacious molecules, as imperfect ASO-mRNA binding results in premature release. As well, first generation phosphorothioated ASOs have also been shown to induce non-antisense, non-sequence specific effects, some of which is due to lack of stereoregularity

Figure 1.2

ASO mechanism of action

All potential sites of action upon intracellular uptake of ASOs are dependent on complementary base-pairing (Watson-Crick) interactions with the target. Ultimately ASO act to disrupt 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 triplex; (4) acting as a substrate for RNase H which recognises RNA:DNA duplexes and cleaves the RNA sequence; (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 the translation process by preventing ribosome function, and (11) disrupting protein-RNA interactions. Adapted from (68).
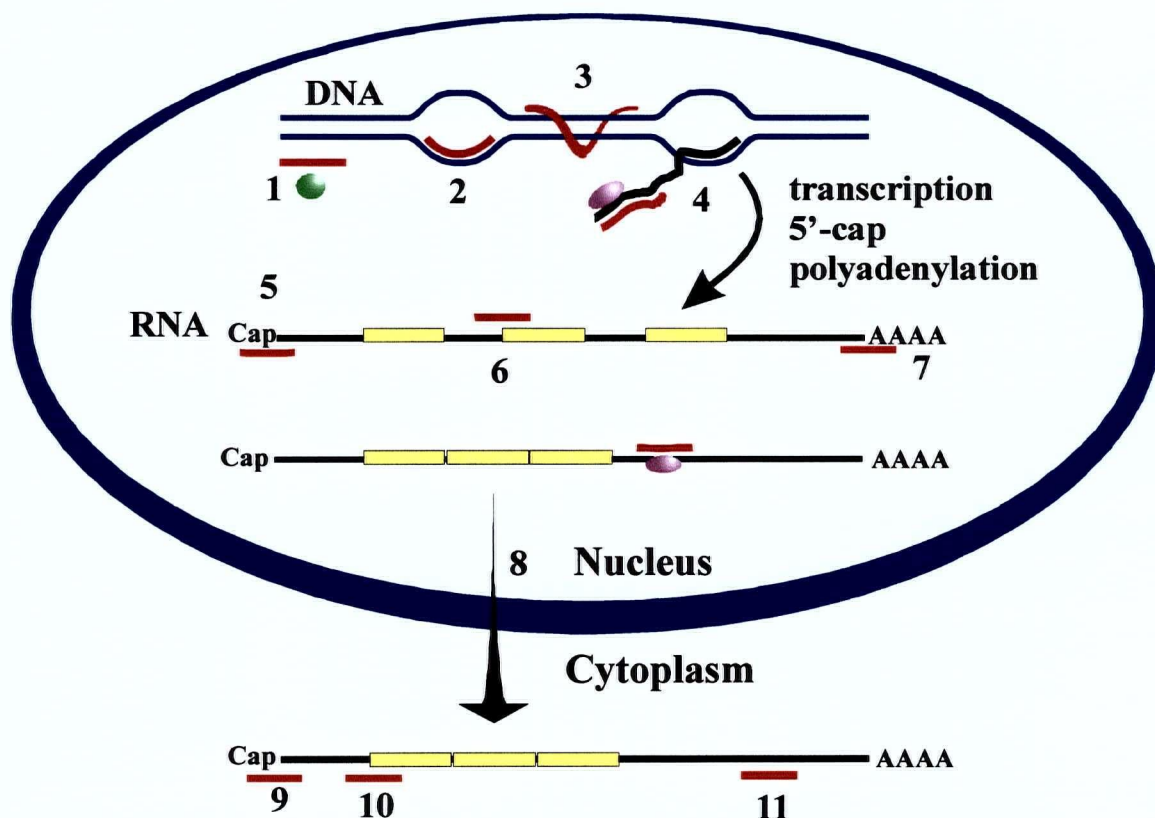
Figure 1.3

Chemical modifications of ASOs

The difficulty is to generate modified ASOs that are not good substrates for serum nucleases while maintaining Watson-Crick base pairing with the target nucleotide (mRNA or DNA). Hybrid and chimeric ASOs are becoming more popular since they combine nuclease-resistant ends with a central core that activates RNase H. Some investigators have also focused on decreasing the charge exhibited by the phosphate groups in order to enhance diffusion through the cell membrane. Modified from (69).

Backbone

Phosphodiester   Phosphorothioate   Phosphorodithioate   Methylphosphonate

Formacetal   3'-Thioformacetal   5'-Thioformacetal   N3',P5'-Phosphoramide

Peptide nucleic acid

Sugar

2'-Fluororibose   2'-O-Methylribose   2'-Propylribose   2-Methylethoxyribose

Base

C-5 Proynyl U   C-5 Pronyl C   C-5 Thiazole   Phenoxazine

10
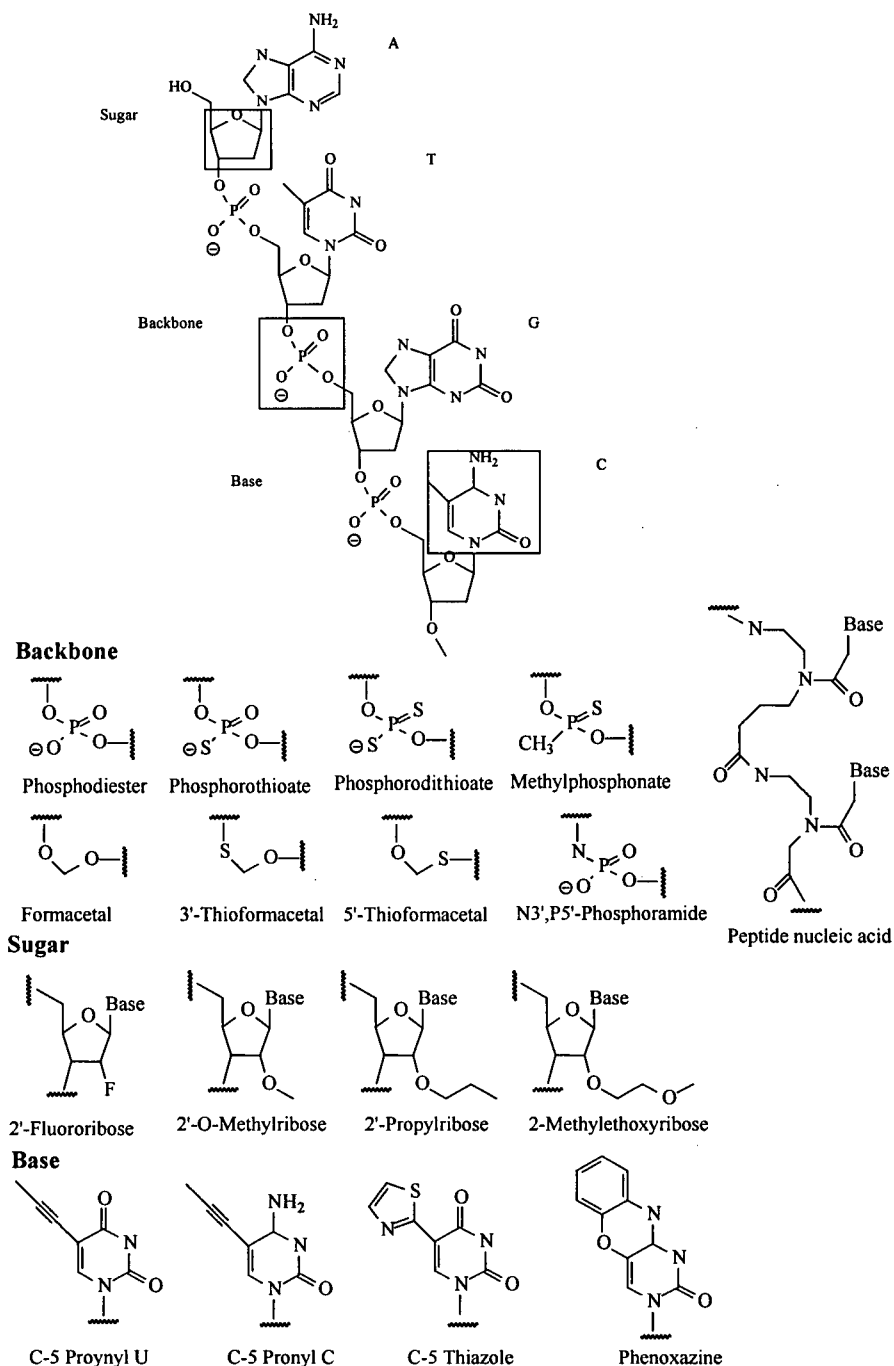
(70). It has also been suggested through work with bacterial DNA fragments that nucleotides with specific sequences also cause immunostimulation, irrespective of the number of nucleotides, and target of the ASO (71, 72). High doses of ASOs have been associated with cytokine release and inhibition of blood coagulation and other non-specific effects (73, 74). Degradation products from ASOs, especially those with backbone modifications, may also have mitogenic activity (75, 76).

What is particularly interesting for ASO chemistry is the potential to modify them to an extent where they are soluble in lipophilic carriers and can be readily manipulated in conjunction with lipid-based transfer systems. The methylphosphonates are generated such that the non-bridging oxygen atom is substituted for a methyl group (77). This provides two benefits - the resultant molecule is nuclease resistant and has no negative charge. Thereby, methylphosphonate ASOs have the ability to passively diffuse through cellular membranes and are well solubilized in lipid-based vehicles (78). Although stable with minimal toxic effects, methylphosphonates are not readily recognised by RNase H, a trigger for degradation of the mRNA target and a primary mechanism of ASO activity (see Figure 1.2). Other methods to enhance potency of ASOs are to stabilise the mRNA target-ASO duplex by attaching chemical groups able to intercalate between base-pairs and by adding RNase H activating moieties (79).

1.2 Synthetic lipid-NA carriers

Upon administration of a NA *in vivo*, there are numerous barriers encountered before that NA can achieve its optimal therapeutic activity. These include, but are not limited to, dose toxicity, nuclease degradation, elimination from bloodstream, and, if the NA manages to

11

co-localise within the disease site, a host of cellular barriers (80). In order to overcome these problems, investigators have utilised delivery vehicles that can modify, encapsulate or complex the NA. Synthetic vehicle formulations are particularly desirable based on their low antigenicity, high capacity and increased safety (81), when compared to viral vectors. There has also been little evidence to demonstrate that non-viral vectors can successfully mediate copy DNA integration into the genome. This is generally perceived to be an attractive attribute since permanent alterations in non-somatic tissues as a bystander result of treatment regimes are not favourable. As such, the use of non-viral vectors, generated with the efficiency of viruses in mind, has become a goal that has been vigorously pursued (82, 83). However, non-viral vectors are currently limited in their usage as they require much higher doses for penetration into cells (84), are less efficient in intracellular entry (85) and require extensive chemical conjugations for targeting to specific tissues or cells (86).

The use of lipids for drug delivery is a logical extrapolation of their biological function (87) and the benefit of using a carrier system that is generated using biocompatible material is clear. Membrane phospholipids are more than a biological barrier for a cell's microenvironment. The lipid composition in cellular membranes is diverse and complex and plays critical roles in natural processing such as endocytosis, intracellular processing, signalling and nuclear delivery. This can be illustrated by the role of non-bilayer lipids in membrane fusion (see section *1.3.3.2.1*). Alternatively, close contact between membranes, triggered through electrostatic interactions, can facilitate lipid phase separation, local dehydration and membrane disruption as described in sections *1.3.3.2.1-3*. Further, delivery systems using lipids are readily amenable to chemical manipulation for altering binding to

NAs or cells. These principles should be considered in the design of effective NA delivery systems and are of particular relevance in Chapters 2, 3, 4 and 6.

1.2.1 The critical micellar concentration of amphiphiles

In a diluted solution, amphipathic lipids do not associate readily into an organised bilayer structure. Upon increasing lipid concentration in an aqueous environment, free amphiphiles will ultimately aggregate at a minimal concentration called the "critical micelle concentration" (*cmc*), a value that is determined empirically. For any given lipid the *cmc* is the concentration where monomers and micelles (aggregate structures) co-exists in the medium. More exactly, the *cmc* can be measured as the total concentration of amphiphile added at which phase separation first occurs or the free concentration in solution can be measured analytically in the presence of micelles (88). Similar to bilayer structures, the driving force behind micelle formation is the segregation of the hydrophobic acyl chain from a polar region (such as water) and close contact is limited by the repulsion of the hydrophilic headgroup (89). Further, the value of the *cmc* for a particular lipid or lipid mixture is a function of the acyl chain length and degree of unsaturation, temperature and ionic strength of the medium (90). Note that at concentrations above the *cmc*, amphiphiles are organised into well-defined structures dependent on the characteristics of the individual lipid components. The *cmc* of the individual lipid components used to form lipid-based NA vehicles is of particular significance in the work described in Chapter 3 for two reasons: (i) lipid dissolution using detergents is one of the parameters that is integral to successful particle formation and (ii) the hydrophobic complex is formed by spontaneous association of cationic lipids with NAs in a manner similar to lipids at their *cmc*.

1.2.2 Liposomes

In an aqueous environment most pure lipids and lipid mixtures form highly structured, spherical bilayers that surround an aqueous core. A.D. Bangham first generated liposomes by rehydrating a thin lipid film in water and determined that at appropriate concentrations the phospholipids spontaneously formed bilayer-enclosing vesicles (91). The main force that drives the formation of liposomes is the amphipathic nature of the phospholipid components, specifically, the hydrophobic interaction among the acyl chains that excludes the hydrophilic environment. The polar headgroups are oriented towards the aqueous medium and internal core while the acyl chains interact to form a hydrophobic layer (see Figure 1.4). The ultimate size of a liposomes is limited by its curvature stress.

Liposomes were originally developed as model membranes for studying ion flux, protein insertion and molecular transport, among others, and their utility has extended to pharmaceuticals as carriers of drugs, proteins, NAs, and other molecules (92, 93). Their versatility is based on the capability to modulate their structural properties such as size, lamellarity, composition and surface properties. Functionally, these can lead to variability in the solubilization of hydrophobic and hydrophilic drugs, protection of NAs from degradative enzymes (94, 95), drug release characteristics (96, 97), intracellular delivery (98), and alterations in pharmacokinetics and biodistribution (99, 100). Generally, it is perceived that the individual lipid composition and temperature determines the organisation of lipids and these functional properties.

Figure 1.4

A liposome

A liposome is composed of amphipathic lipids arranged in a bilayer surrounding an aqueous core. Drugs, proteins, nucleotides and other possible therapeutic compounds can be associated on the outer surface, solubilized within the hydrophobic region of the bilayer or encapsulated in the inner core. The outer surface can be modified by conjugating poly(ethylene glycol) polymers and targeting moieties. Modified from (101).
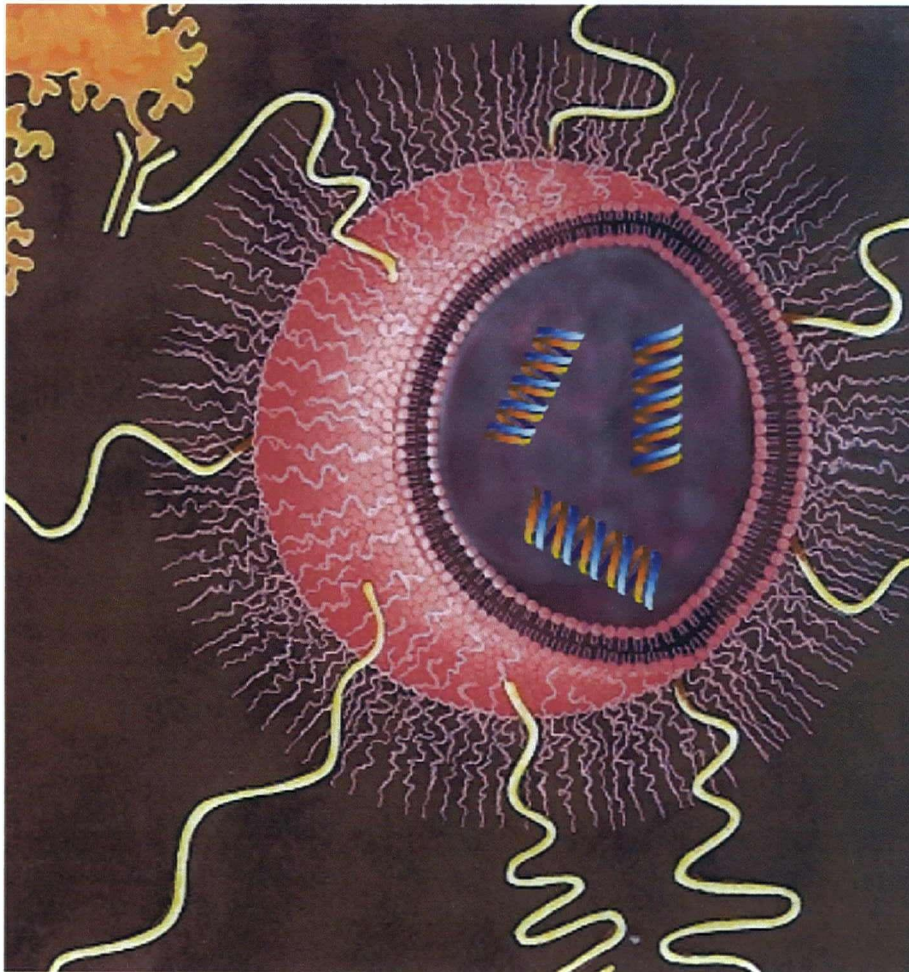
1.2.3 Lipid polymorphism – "The shape hypothesis"

The macroscopic arrangements of phospholipids in an aqueous medium can be investigated through techniques such as X-ray diffraction, freeze-fracture electron microscopy and nuclear magnetic resonance. Depending on the lipid components in an aggregate, the concentration and the temperature, the macroscopic arrangement can vary and is conditional on minimising the free energy of hydration. The inherent structure that is adopted by certain lipid components upon hydration is a function of that lipid's molecular shape (102). More specifically, it is the molecular volume ratio of the headgroup to hydrocarbon acyl chain that influences the preferred lipid macrostructure. Importantly, the shape and ultimate orientation (i.e. the polymorphic capability) of lipids within a membrane defines its biological functional role that is related to maintenance of its bilayer integrity, permeability barrier and cellular processes such as fusion (103-105), exocytosis, endocytosis, transbilayer (flip-flop) lipid movement, macromolecular transport and protein orientation and insertion (106).

The postulate that relates polymorphic phase behaviour to the three-dimensional organisation of a specific lipid species is called the "shape hypothesis of lipid polymorphism" (102, 107). The shape model is useful as a predictor of the resultant structure based on the packing parameter of an individual lipid as it reflects its molecular geometry. The polymorphic phase and the corresponding molecular shapes of lipids are described in Figure 1.5. Single-chain surfactants and lysophospholipids form micellar structures at concentrations above their *cmc*. As individual components, the molecular shape is considered be "inverted cone" whereby the cross-sectional area of the polar region is larger than that occupied by the acyl chain. The effective area of the headgroup is thus sensitive to

Figure 1.5

The "shape hypothesis" of lipid polymorphism

Lipids exhibit a fundamental shape and spontaneously adopt pre-designated configurations upon hydration. Specifically, it is the molecular volume ratio between the headgroup and acyl chains that contributes to its preferential shape assumed by the individual lipid components. The molecular shapes of component lipids are designated as either inverted cone, cylindrical or cone resulting in a polymorphic phase of micellar, bilayer or hexagonal (H_{II}), respectively. Modified from (107)

lysophospholipids
detergents
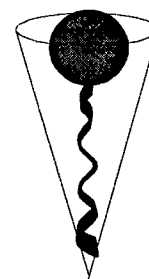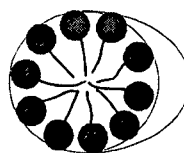
phosphatidylcholine
sphingomyelin
phosphatidylserine
phosphatidylglycerol
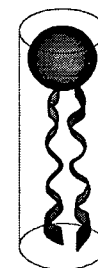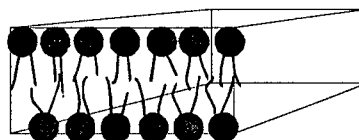
phosphatidylethanolamine
(unsaturated)
cardiolipin - Ca^{2+}
phosphatidic acid - Ca^{2+}
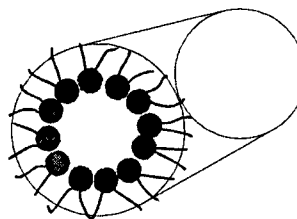
electrostatic repulsion and is orientated to minimise close proximity. Lipids, such as phosphatidyl- choline, serine and inositol, are considered to posses a "cylindrical" shape and assume the lamellar (or bilayer) configuration. It is believed that for these lipids the hydrocarbon chains are too bulky to be accommodated into a micellar inner core. Finally, lipids with an effective acyl chain volume larger than the headgroup, form hexagonal phase (H_{II}) structures where the headgroup is orientated toward the internal aqueous core [for review see (108)]. In a mixture, hexagonal shape lipids, such as unsaturated phosphatidylethanolamines, can be induced to form lamellar structures with a bilayer-forming shape (i.e. DOPC) or an opposing shaped lipid (i.e. lyso-PC). The bilayer structure in biological membranes may, in fact, call for alternating-shaped lipids to maintain the architectural state around protein conduits. Diverse lipid components are necessary to provide optimal packing for maintenance and functionality of the lipid bilayer.

1.2.4 Lipid phase transition

The forces that determine the geometry with the lowest free energy are based on temperature and composition of the lipid. The phase transition, as assessed by thermodynamic rules, is always first order and is defined as an equilibrium where two discrete entities are stable (109). At the phase transition one phase can be generated in a matrix of the other and thus it can be difficult to differentiate one lipid from another. Thus the interfacial effects of different lipid components in co-existence play a large part in lipid phase morphology. Lipid morphology and phase transitions are dependent on such forces as hydration effects, repulsion and attraction among headgroups, and the molecular shape and volume of the headgroup and hydrocarbon chains. These attributes are important in

understanding the role(s) of lipid components that govern the attributes of lipid-based NA transfer systems as described in Chapter 3 and (110).

1.2.4.1 The gel to liquid-crystalline phase transition

The specific physical state of a pure lipid or mixtures corresponds to a number of precise inherent features and external factors, including temperature. Lipids undergo increases in the conformational freedom of their hydrocarbon chains upon increasing temperature. At the endothermic transition temperature, there is a structural change of state from the gel-crystalline (L_β) state to the liquid-crystalline (L_α) state. Figure 1.6 shows the differences between the two states and the phase diagram associated with this change. The gel phase is associated with the phospholipids adopting a bilayer structure whereby the acyl chains are tightly packed in a *trans* array; there is little lateral mobility and the acyl chains occupy a minimal volume (111). In a L_α state the lipids remain in a bilayer; however, the acyl chains are disordered as a result of increased rotation about the carbon bonds. This includes the existence of gauche rotational isomers (112).

One defining property of the L_β - L_α phase transition is co-operativity. There is one temperature for pure lipids where the two phases co-exist and the phase transition is defined very sharply. This is due to co-operativity of "melting". As lipids in the bilayers are not isolated, the increased rotation in one chain will cause movement in the other acyl chains. The tendency of one molecule to become disordered is a result of the dependency of the molecules in close proximity. The resultant disordering of one lipid or a small region of lipids subsequently causes the other lipid chains to "melt". The state of the lipid bilayer is dependent on the balance between the entropy associated with order/disorder of the acyl

19

Figure 1.6

Phase transition of DOPE

Over the phase transition temperature of an amphipathic lipid, the lipids undergo an alteration in the order of the acyl chains. In the L_β state the lipids are organised by a rigid, parallel organisation of the lipids chains. Above the phase transition temperature the acyl chains are much more fluid (L_α). DOPE can also exist in a H_{II} configuration where the headgroups are oriented towards the centre. The phase diagram of DOPE:water system shows the mole % and temperature at which the three main phases exist. Solid and dash lines represent equilibrium phase border whereas dot and dot-and-dash lines are metastable equilibria. Modified from (113).

L_β gel phase L_α liquid phase H_{II} hexagonal phase

chains, the van der Waals forces between the chains and the attraction between the headgroups. The effect of intermolecular hydrogen bonding has been suggested to contribute significantly to the L_β - L_α phase transition temperature (114). Particularly, the presence of hydrogen bonding between lipid molecules that exists more prominently in the gel phase leads to closer packing densities and, as such, increases the phase transition temperatures relative to lipids with headgroups do not exhibit H-bonding.

The L_β - L_α phase transition temperatures of specific lipids are described in Table 1.2. There are general trends that can be observed corresponding to the specific lipid structure. Note that increases in phase transition temperature are indicative of stabilisation of the L_β phase. First, the transition temperature and enthalpies are increased relative to increasing length of acyl chains. As the number of carbon moeities increase, the increase to tighter packing constraints within the bilayer is induced by interactions among the hydrophobic tails (115). Second, the presence of a double bond in the hydrophobic acyl chains decreases transition temperatures and enthalpies, in addition, a *cis* double bond induces a more marked decrease in the temperature of phase transition but not enthalpy (116). The presence of unsaturated bonds decreases close-knit packing among lipid molecules. The *cis* configuration of the acyl chains, in particular, leads to a greater occupied volume in the bilayer and the interaction among the chains is less. Finally, when a methyl group is added to the headgroup or if the headgroup exhibits a charge, there is a decrease in the transition temperature. Each methyl group adds to the steric repulsion between headgroups by decreasing proximity of lipid molecules reflected by the decrease in transition temperature (117). Charged headgroups cause repulsion of the lipid molecules adding to the destabilisation of the gel phase and inducing liquid-crystalline formation (118).

Table 1.2

Phase transition data for aqueous lipid dispersions

	acyl chain configuration	ΔH (kcal/mol)	T_c (°C)	Ref
Phospholipid				
dilauroylphosphatidylcholine	12:0	4.0	-1.0	(119)
dimyristoylphosphatidylcholine	14:0	5.0	23.5	(120)
dipalmitoylphosphatidylcholine	16:0	9.7	41.9	(121)
distearoylphosphatidylcholine	18:0	10.7	58.0	(122)
dioleoylphosphatidylcholine	18:1	6.7	-22.0	(123)
dilauroylphosphatidylethanolamine	12:0	4.0	30.5	(124)
dimyristoylphosphatidylethanolamine	14:0	6.1	50.2	(124)
dipalmitoylphosphatidylethanolamine	16:0	8.8	63.1	(125)
distearoylphosphatidylethanolamine	18:0	10.5	70.4	(125)
dioleoylphosphatidylethanolamine	18:1	7.5	-8.1	(126)
Cationic lipids				
dioleyldimethylammonium chloride			-8	(127)
dimethyldioactadecylammonium bromide			47	(127)

1.2.5 Liposome/NA complexes

The generation of a liposome/NA complex is achieved through a simple protocol. This involves the incorporation of a cationic lipid with secondary lipids (i.e. DOPE) in the form of liposomes to which the NA of choice is added (26, 128). The original model describing lipoplexes, a term describing liposome/DNA complexes, suggests that there are four intact liposomes associated with a strand of plasmid DNA (26). However, studies now show that upon DNA addition to cationic liposomes, a plethora of structures is generated (Figure 1.7). These include long strands of DNA with lipids attached directly to the DNA backbone, large fused liposomes and DNA encapsulated within the aqueous core, among others (44, 129-131). It is important to note that the complexation reaction is associated with modification of the inherent pharmacokinetic and pharmacological properties of the NA (132, 133). So far, liposomal-based DNA delivery systems are effective in humans only as aerosolised particles for lung-associated diseases (134) and applications involving regional administration (135).

Protocol variations in creating liposome/NA complexes have not been extensively evaluated and this is one factor that has prevented development of consistent homogeneous liposomal-based carriers. Further, since there is a poor understanding of the complex reaction, the rational design of lipid-based NA delivery systems is difficult when liposomes are used to provide the cationic lipids. This is particularly valid when considering the wide variability in parameters that defines optimal transfection efficiency. Among them are the cationic lipid concentration, the NA concentration, mixing properties and the presence or absence of salts and serum (136).

Figure 1.7

**Cryo-transmission electron micrographs of cationic lipid-based
delivery systems for NAs**

The aggregate reaction upon addition of NAs to cationic LUVs produces a heterogeneous mix of structures including large fused liposomes, striated regions indicative of stacked structures, long thin strands of DNA coated with a bilayer of lipid and possibly some encapsulated DNA. There are also regions where cationic lipids are not associated with the plasmid DNA. Lipid-DNA particles exhibit many of the same diverse characteristics but at a smaller size. Photographs provided by E. Wasan (136). Arrows point to the edge of the polymer scaffolding. Bar indicates 100 nm.
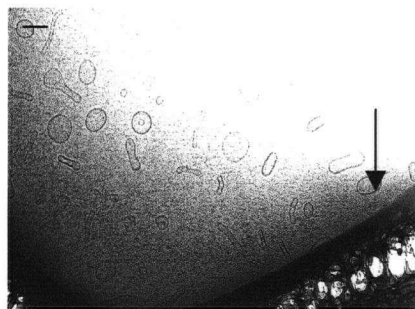
LUVs
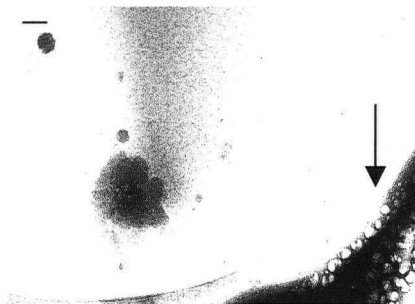
Liposome/DNA complexes
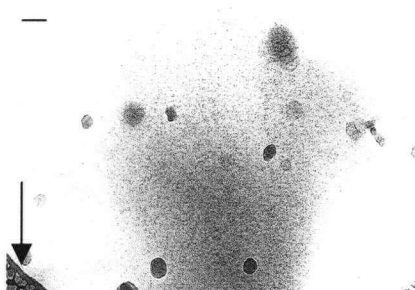
Lipid-DNA particles

Structure-function relationships have not been forthcoming for liposomes/NA complexes. The main difficulty being that it is unknown which, if any, of the multitude of structures generated upon liposome mixing with NAs facilitate transfer and/or activity (expression and/or hybridisation with a mRNA target). Homogeneous fractions of structures are metastable and it is not possible to isolate a form that is transfection competent. Currently, NA activity is correlated with biophysical attributes of an entire species of lipoplexes (137).

This thesis describes a systematic method for generating lipid-based vehicles for NAs. This can be achieved using a protocol whereby the cationic lipid is added to NA and binding interactions are monitored (as described in Chapter 2, 6). Subsequently, the addition of secondary lipids is assessed to determine the extent of interference in the cationic lipid/NA binding (Chapter 3). Only then are the lipid-NA particles evaluated for their capacity to bind to cell surfaces (Chapter 4) and effect transfection (Chapter 3) or the downregulation of a specific protein (Chapter 6).

1.3 Barriers to NA delivery and efficacy

Gene transfer by lipid-based vehicles leading to an efficacious response will involve a series of steps (93, 138, 139). These major steps are defined as the following, but are not necessarily limited to:

1)   condensation and protection of the NA

2)   association/adhesion of packaged NA to cell surface

3)   internalisation – endocytosis or fusion

4)   release of NA from carrier and endosome

5)   entry (ASOs) and expression (DNA) in nucleus

Facilitating one or some of these steps may enhance the efficacy of a NA, although it has not been determine which of these steps are limiting. However, it is anticipated that all of these steps are vital to maximise transfection or downregulation of protein levels. Many investigators have designated specific barriers that must be overcome in order to increase activity and have focused their efforts on surpassing these barriers (138, 140, 141). Note that an effective delivery system will optimise the path between administration and the pharmacological target. I suggest that gene therapy will be effective once all steps, those already recognised and those that remain to be determined, are optimised. It is believed that the utility of lipid-based vehicles will enhance one or more of these steps to facilitate NA activity.

Figure 1.8 outlines the potential steps of lipid-based NA transfer leading to effective therapeutic activity. The characteristics of the carrier system contribute to the efficiency in bypassing the specific cellular barriers. While a therapeutic response is dependent on the NA, the extent of the response is dependent on the carrier system. Understanding the biological mechanisms that govern NA activity will help guide efforts to improve the carriers.

1.3.1 Protection of the NA

One of the primary goals for developing of lipid-based gene transfer vehicles is based on protecting the NA from degradative enzymes extra- and intra- cellularly. Protection can be divided into two categories – indirect and direct. For systems that are designed for intravenous administration, lipid-based NA vehicles must be stable within the blood

Figure 1.8

Barriers to delivery or transfection of NAs

A therapeutic response of NAs must overcome the limitation at many levels from the point of administration. The key barriers associated with delivery of NAs are suggested to be: **(1)** condensation and protection; **(2)** association/adhesion to the cell surface; **(3)** internalisation including endocytosis or fusion; **(4)** release of the NA; **(5)** escape from endosomes; and **(6)** entry (ASOs) and expression (DNA) in the nucleus.
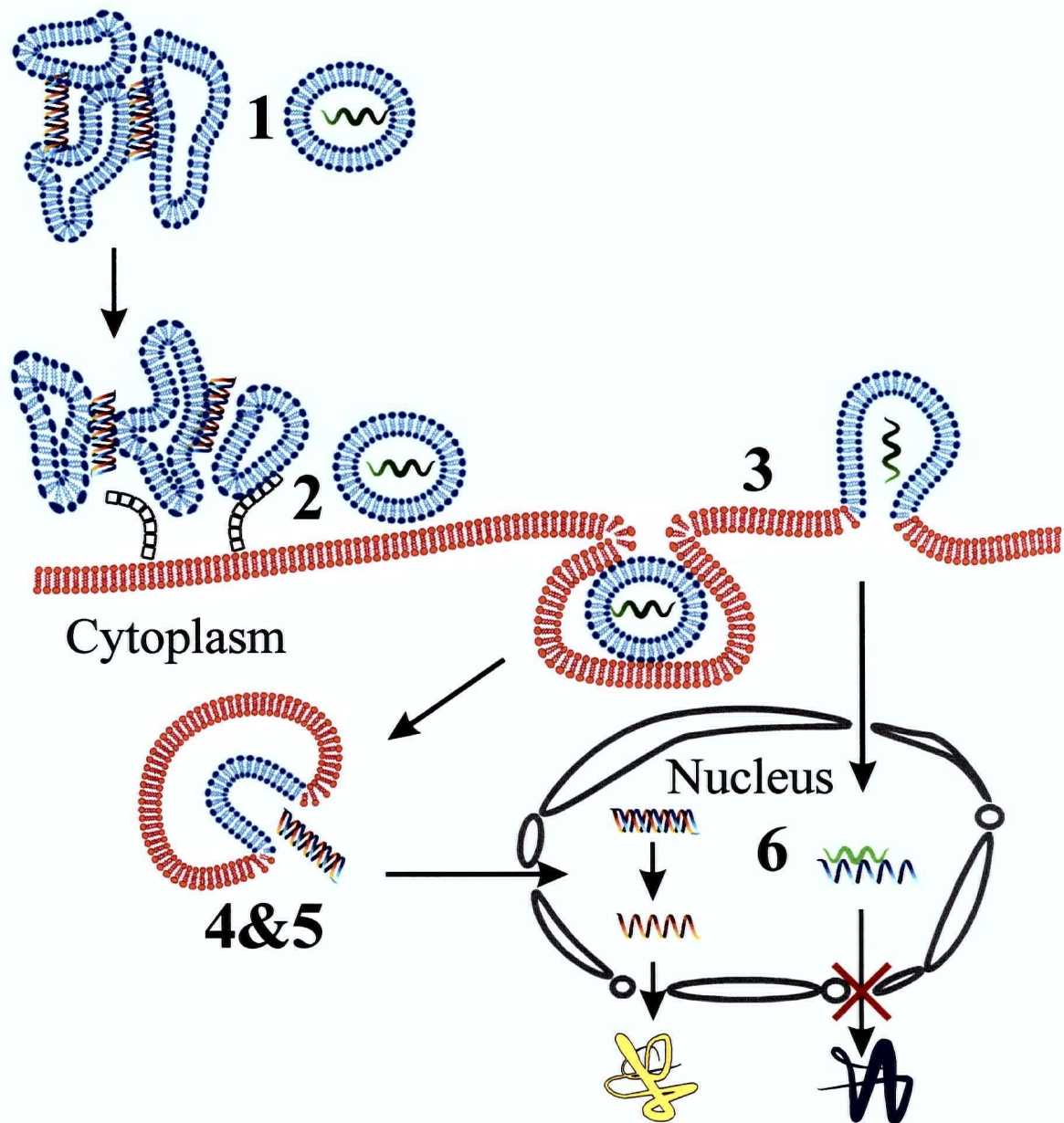
compartment for a sufficient time period to allow passive localisation or effective active (i.e. antibody-mediated) targeting to the disease site. The avoidance of elimination from circulation and cellular uptake by lysosomal pathways are examples of indirect protection. Strategies that prevent NA degradation against nucleases, small molecule binding and ultrasonic cavitation are classified as approaches to directly protect the NA. It is vital that the lipid-based vehicle must maintain an intact NA structure in order to preserve the activity of the NA. Investigators have determined that lipid formulations can increase the effective half-life of NAs by saturating intracellular nucleases and limiting size to enhance passive targeting events (99).

1.3.1.1 PEG – Indirect protection

One consideration when designing a long-circulating, systemic lipid-based NA vehicle *in vivo* is the characteristics of the exposed surface. NA delivery vehicles, especially as related to biodistribution and protection against elimination within the plasma compartment, can be made more biocompatible by decreasing the charge associated with the surface. Excessive presentation of surface charge can stimulate protein binding leading to: (i) elimination from the bloodstream (142-144), (ii) induction of aggregation (136), (iii) destabilisation of the NA delivery complex (145) and immune effects (146-149); these properties are not considered advantageous for systemic delivery of drugs. Charge shielding is based on occupying a region of space with an inert polymer with the propensity for attracting water molecules in creating a hydrophilic barrier (150). This prevents close contact between molecules stemming from charge interactions. The polymer typically used for lipid/NA delivery systems is poly(ethylene glycol) conjugated to a lipid anchor (151,

152). Poly(ethylene glycol)s are hydrophilic, flexible polymers generated through chemical reactions of poly(ethylene oxide)s. Further, the polymer is a hydrophilic, mobile chain, which lacks an ionic charge and is biocompatible (153). Many investigators are using PEG derivatives, in the absence of a lipid anchor, as a carrier system for conventional and nucleic acid drugs, i.e. nanoparticles. This thesis describes a lipid-based formulation containing PEG, conjugated to a lipid anchor with ASOs, to assess its efficacy on Bcl-2 downregulation (Chapter 6).

Lipid-based transfer systems containing poly(ethylene glycol)-conjugated lipids contribute to pharmaceutical and pharmacological benefits. PEG-lipids can prevent opsonin interactions (154), the first step in immune recognition, resulting in increased half-life of the carrier (155), decrease in the size of the NA delivery vehicle (156) and decreased non-specific uptake mediated by the mononuclear phagocytic system in order to prolong circulation longevity (96, 157). These features are particularly advantageous when considering cationic liposome/DNA complexes for intravenous administration (158). PEG-conjugated lipids incorporated into the delivery vehicle also have the capability to prevent aggregation and increase stability of lipid/NA complexes, resulting in smaller, more stable lipid-based complexes (158, 159). Further, there are indications that passive loading of ASOs is increased with the use of PEG-lipid vehicles (160). Within the lipid complex itself, PEG-lipids also can diminish fusion promoted by "helper" lipids such as DOPE by decreasing curvature stress instability and dehydration of membrane surfaces (161, 162).

The relationship of a PEG molecule in a bilayer and its ability to act as a steric barrier is dependent on the pressure distance (163-165). The PEG-polymer, based on its concentration on the bilayer, can exist in a mushroom configuration or a brush configuration

(Figure 1.9). In the mushroom configuration the polymer is folded over upon itself and is limited in its movements across the bilayer surface. The brush configuration exists as an extended polymer length that is inherently mobile and less restricted than the mushroom configuration, enabling a wider range of inert area. There are numerous considerations that determine the effectiveness of the steric barriers of the PEG polymer. One must establish the extent of the overall relative surface coverage of the polymer required in order to minimise surface interactions (166), and then the concentration and size of the PEG polymer is selected to ensure that the molecule mainly exists in a brush region (167).

The selection of the PEG-lipid may be a key issue in its flexibility as a component in lipid-based gene transgene vehicles. Two features that have been exploited in order to enhance gene delivery are the pH sensitivity of the conjugation site on the linkage between the lipid and the polymer and adding targeting moieties to the PEG polymer (168, 169). The low pH environment in the endosomal compartment can facilitate release of the polymer from its lipid anchor. Removal of the PEG-lipid from the vehicle (170) or cleavage of the PEG molecule from the lipid (169) can result in exposure of surface charge characteristics that exhibit a propensity for initial binding reactions with the cell or endosomal membrane. Another aspect of using PEG in lipid delivery systems is the ability to conjugate targeting moieties on the PEG polymer, such as portions of antibodies, thereby enhancing targeting specificity (171). Further, the presence of PEG can prevent immune reactions mediated by the presence of the foreign protein used for targeting protocols.

Figure 1.9

The surface regimes of poly(ethylene glycol)

Two configuration of PEG exists – the "mushroom" or "brush". At low density (concentration) the adjacent chains do not interact laterally (mushroom). However, brushes exist at high grafting densities where adjacent chains are mobile and can overlap. D is the distance between PEG-lipids in the plane of the bilayer; R_F is the Flory dimension of the polymer in solution; L represents the extension length from the bilayer surface. Modified from (165).

| $D > 2R_F$ | $R_F < D < 2R_F$ | $D < R_F$ |
| Interdigitated Mushroom | Mushroom | Brush |
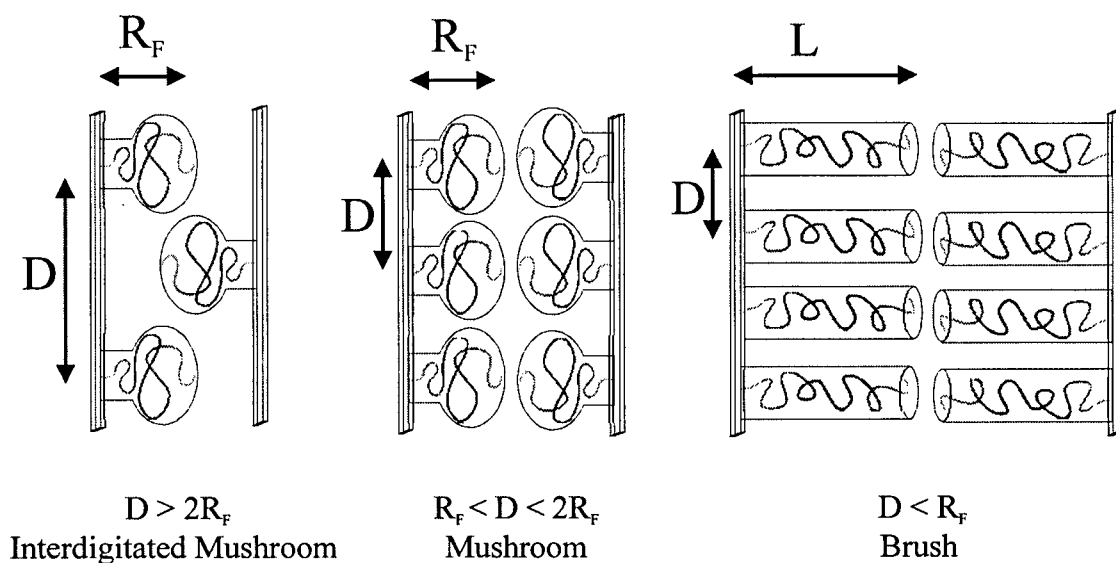
1.3.1.2 Cationic Lipids – Direct protection

For synthetic lipid-based NA delivery applications, the main lipid vehicle component typically contains a positively charged headgroup. A variety of cationic surfaces have been demonstrated to form complexes with NA, including linear and branched polymers (172, 173), glycopeptides, histones (174), dendrimers (175) and peptides (176). However, the focus of this thesis is the use of amphipathic lipids and this brief overview will focus on cationic liposomes and cationic lipids (177). Although the chemical and physical properties of positive amphiphiles that define the effectiveness of NA delivery is unclear, there are a number of key attributes associated with their use. The encapsulation or association of NA with cationic lipids/liposomes can protect polynucleotides from nuclease degradation in serum (145, 178), alter their pharmacokinetic profiles (179) and enhance their intracellular trafficking (180-182). These events can, in turn, lead to a therapeutic benefit (183-185).

From a protective perspective, the advantage of adding cationic lipids to NAs can result in a charge neutralisation of the nucleotide as well as providing a positive surface charge to augment cell binding. When cationic lipids are combined with NAs, there is a complexation reaction driven by electrostatic interactions that does not require a further input of energy. The overall cationic surface charge of the cationic lipids can spontaneously associate *via* electrostatic interactions with the anionic phosphate groups on the backbone of NAs. The DNA phosphate charge density determines the level of co-operative binding (186). The association of the cationic lipid species with the phosphate is likely to depend on ionic strength, counterion species and pH. Further, transfection protocols often call for an excess cationic lipid associated with the NA. The resultant cationic lipid/NA complex contains a residual positive surface charge, which, in turn, can facilitate adhesion and/or

association with the negative cell membrane. However, this excess in positive charge has also been attributed to lipid-based toxicity *in vitro* as well as rapid clearance *in vivo*. Investigators are aggressively pursuing the use of PEG-modified lipids to shield the charged surface (as discussed in section *1.3.1.1*).

1.3.2.1 Cationic lipid structure

All the cationic lipids used for delivery of gene-based therapeutics are synthetically generated (187, 188). Many investigators are attempting to elucidate structure-function relationships by selective changes in the portion of the lipid molecule that affects NA binding and molecular rearrangement upon complexation (189-192). The first cationic liposome-based complexation was completed by P.L. Felgner *et al.* (26) using the cationic lipid, DOTAP (Figure 1.10). Since then it has been determined that acyl chain length (187, 193), saturation and L_β - L_α phase transition temperature, among others, play a role in the effectiveness of gene delivery system as it relates to the efficiency of transgene expression. Lipids with multiple headgroups, such as those found in the commercial transfection reagents Lipofectamine™ and Transfectam™, may be useful for electrostatic association to polynucleotides as well as condensing DNA. These agents may be able to protect DNA from enzymatic degradation but they will not necessarily deliver the DNA to the target site. The usefulness of specific lipid formulations may also be dependent on the plasmid or ASO and the cell line being transfected (194, 195). Points to be considered for transfection agents are: (i) the extent of electrostatic interaction, (ii) the ease of NA release, (iii) the overall surface charge of the complex and (iv) the size of the final product. These parameters may influence

the design of hydrophilic headgroup or the hydrophobic acyl chains and, ultimately, the choice of a specific lipid (196).

While lipid components are considered to be essentially non-toxic, a few immune stimulating features mediated by cationic lipids have been reported (148). Cationic lipid-based systems can activate complement (197), deplete platelets (198) and monocytes and induce interleukins (147, 149).

The cationic lipid component used in this thesis is DODAC (Figure 1.10), a monocationic lipid that contains a quarternary ammonium headgroup with an 18 carbon unsaturated acyl chain at the Δ^{9cis} position and a chloride as the counterion species. Liposomes containing DODAC can transfect B16 melanoma with pCMVβ better than Lipofectin™ (DOTMA:DOPE), the most widely used commercial transfection product. Investigators have made extensive attempts to chemically modify cationic lipids in an effort to improve the activity of cationic lipids (187, 189-191). However, only a few have the required characteristics to achieve efficient NA transfer resulting in transgene expression for plasmid DNA or downregulation of protein when using ASOs.

1.3.2.2 The phase transitions of charged lipids

A charged lipid bilayer in an aqueous solution will attract what is described as an electrical double layer (199, 200). The electric double layer is composed of an accumulation or depletion of ions in alternating attractive and repulsive layers along the charged surface. When these charged lipid bilayers undergo phase transitions, the relative distribution of ions in the electric double layer changes and leads to alterations in the thermodynamic parameters that define the phase transition. For example, when considering the Gouy-Chapman theory,

Figure 1.10

Cationic lipids used for delivery and transfection of NAs

Lipids that exhibit a positively charge headgroup are the most widely used species for self-associated delivery of NAs. No cationic lipids exist in nature and, as such, these lipids are synthetically generated. Further, they are classified into three groups: 1) *quaternary ammonium salts*, i.e. DDAB, DODAC 2) *lipoamines*, i.e. DODAP, DC-Chol, DOGS, DOTAP and 3) *lipids containing both*, i.e. DOSPA
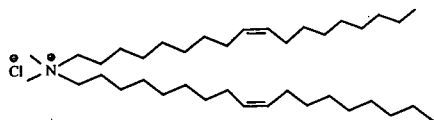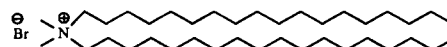
DODAC N,N-dioleyl-N,N-dimethylammonium chloride   DDAB dimethlydioctadecylammonium bromide
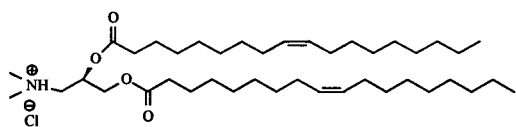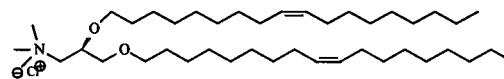
DODAP 1,2-dioleoyl-3-dimethylammonium-propane   DOTMA N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethyl ammonium chloride (Lipofectin)
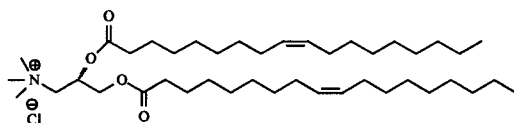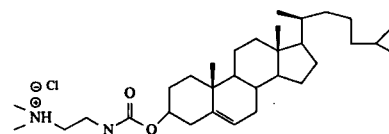
DOTAP 1,2-dioleoyl-3-trimethylammonium-propane   DC-Chol 3b[N-(N',N'-dimethylaminoethane)carbamoyl] cholesterol
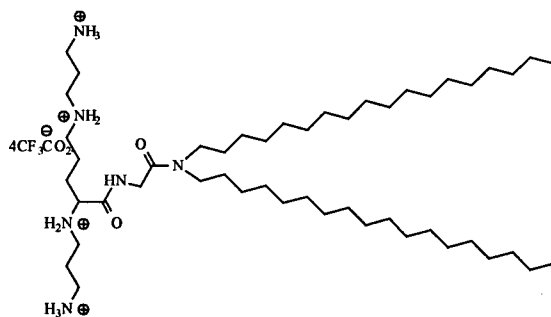
DOGS dioctadecylamidoglycyl spermine (Transfectam)
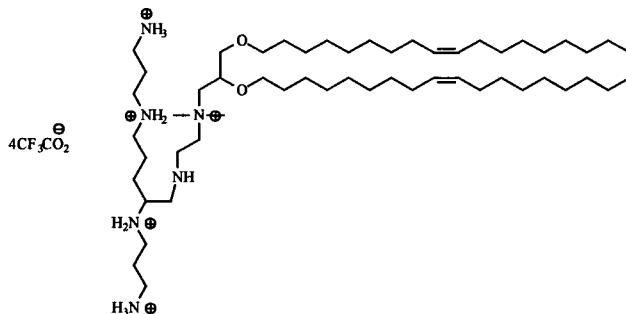
DOSPA 2,3-dioleoyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (Lipofectamine)

35

when the adsorbed layer of ions exists at absolute zero, the distribution of charges is well defined and the double layer distance is fixed. However, at a finite temperature, thermal motion disrupts the alignment of ions and alters the electrostatic potential of the surface conferred by the electric double layer. These changes can have profound effects on intermolecular interactions among lipids as well as binding with ions in the aqueous medium.

Electrostatic interactions can mediate strong association upon close cationic lipid proximity with an anionic cell membrane. Cationic lipids will interact electrostatically with the cell membrane and form discrete microdomains upon close contact. This event, where lipids are no longer completely miscible around each other and exist in separate areas, is called lateral phase separation (201). The capacity for phase separation is of particular importance when considering membrane fusion events. For example, close contact between a NA delivery system containing cationic and PE-containing lipids will initiate the separation of these lipids. Subsequently, microdomains, similar to those that exists during a phase separation event, will form. This region, rich in PE-lipids, is capable of promoting hexagonal phase intermediates, a trigger for membrane fusion; this mechanism is described in more detail in section *1.3.3.2.*

1.3.2.3 Cationic lipid induced changes in DNA structure

The protection of plasmid DNA by cationic lipids (or polyvalent cations) has been suggested to be a result of an alteration in the structure of the DNA *via* condensation. There are biological and physical implications for transfection efficiency when DNA is in a condensed form. The reaction is described as an energetically favourable co-operative event that occurs when the DNA phosphate charge is at least 90% neutralised. These resultant

structures typically yield orderly toroidal or rod-like shapes of 40 - 60 nm in diameter (202). Condensed DNA is shown to exclude intercalating dyes such as EtBr and nuclease degradation of the DNA is much less effective (95). However, it has not been determined whether EtBr exclusion and protection against nucleases are the result of DNA condensation or hindrance of the enzyme due to the presence of a bound cationic lipid. Further, there is evidence that DNA in a hydrophobic cationic lipid/DNA complex in the presence of solvents does not exist in a condensed state (203). Studies in Chapter 3 demonstrate the extent of DNA alteration upon cationic lipid binding. This is assessed using dye intercalation and the extent of protection against serum degradation.

1.3.2 Association/adhesion to the cell membrane

Due to the overwhelming negative charge associated with the NA, association with the cell and subsequent penetration is a difficult venture. However, the delivery of the therapeutic agent may be as simple as inducing close contact with the target. One example of achieving close proximity in the absence of a carrier is direct injection into muscle. Plasmid DNA, in the absence of a carrier, exhibits acceptable levels of gene expression when injected at high concentrations into muscle (204). Intramuscular injection of naked plasmid DNA is one of the few examples of transfection that does not require a carrier to induce effective, long-term gene expression (205, 206). More recently, investigators have described high levels of expression of naked plasmid DNA in hepatocytes after hydrodynamic force injections (207, 208). The preparation of such systems is very simplistic as the highest degree of expression is found when using concentrated plasmid DNA in an isotonic saline solution. Interestingly, the expression of DNA when using any transfection-enhancing

reagent such as cationic lipids, polymers, or receptor-meditated systems is not improved in muscle tissue. The understanding of the mechanism associated with intramuscular injection is still limited; however, the active uptake process may be associated with caveolae (209).

While delivery of free DNA is possible, the probability for cell association increases when a vehicle for carrying NA exhibits a net positive charge. This is conferred, for example, by cationic lipids bound to NA as described in section *1.3.2*. Since the plasma membrane has an inherent negative charge, it is likely that lipid-based gene transfer is mediated, to some extent, by electrostatic attraction (210). Attracting surface potentials therefore drive close contact leading to the association and adhesion of the complex to the cell membrane (211-213). For some formulations and cell species, loss of binding leads directly to a decrease in transfection efficiency (214). Surface charge characteristics are also of particular importance when considering effects in distribution and uptake (215-217). The dilemma for investigators developing lipid-based NA vehicles is to generate systems that facilitate cell binding *via* charge interaction, while minimising surface charge exposure that causes protein binding and elimination (refer to section *1.3.1.1*).

There is evidence that electrostatic interactions can mediate close association and/or adhesion between the cell membrane and the NA carrier. Investigators have shown that there is only a small window of charge ratios that effect specific binding to cell membranes (218-220). A low valency results in insufficient affinity between the lipid carrier and the cell membrane; however, beyond optimal valency, binding and internalisation processes become saturated whereby increases in cell binding no longer correlates with delivery. A theory on adhesion of two membranes and induction membrane fusion has been described by S. Ohki *et al.* (221). Normally there exists a highly repulsive hydration force around membranes that

prevents close contact between bilayer (222). However, due to the hydrophobic nature of the membranes, this property may facilitate bilayer neighbours to minimise the interlayer separation. This close contact between bilayers is due to a resultant decrease in the hydration repulsive forces in parallel to the increase in attractive forces (223). Subsequently, the lipid molecules at the carrier/cell membrane boundary are at high stress and can form regions of high energy to create an area of hydrophobicity. This hydrophobicity, conferred by the lipids and allows molecule exchange and it is the initial step of membrane fusion.

It has also been suggested that membrane-associated proteoglycan (PG) can mediate the initial step of cationic lipid/NA complexes binding to cell membranes. These sulfated, membrane-associated PGs can serve as a receptor when using cationic lipids as carriers. PGs are large macromolecules that consist of glycosaminoglycan chains covalently attached to a protein core. They regulate cytokine activity (224, 225), enzymes (226, 227) and growth factors (228). K.A. Mislick has reported that cell carbohydrate moieties lacking PG synthesis exhibit an 80-fold decrease in reporter gene expression (229). Further, there is a recent report stating that surface PGs do not stimulate endocytosis for internalisation of liposome-DNA complexes and cells deficient in PG exhibit increased sensitivity to cationic liposome-based cytotoxicity (230). Other evidence for a receptor-mediated mechanism for cationic lipid/NA complexes is demonstrated by the existence of surface receptors specifically for DNA (231). Whether these receptors are present on all cell types or act through non-specific surface charge interactions to stimulate endocytosis remains to be determined.

1.3.3 Intracellular uptake

Cell uptake of NAs can be dependent on parameters that have no bearing on the properties of the carrier (232). For example the cell type, specifically the number and type of protein receptors, may designate the extent of endocytosis or cell association of the complexes. Design attributes of a lipid/NA complex that effect successful transfection to one cell type may not be applicable to another. It is important to bear in mind the individual requirements for a cell type when generating appropriate lipid-based NA delivery systems.

1.3.3.1 Endocytosis

The hydrophobic area of the cell membrane does not allow passive intracellular delivery of large, charged molecules such as NAs. While membrane fusion events are considered important, endocytosis is the main mode of entry for lipid/NAs complexes (233, 234). This mode of intracellular uptake is applicable for non-viral vehicles, including lipid-based and protein-based vectors, i.e. poly-lysine (235), although fusion events cannot be completely excluded. Upon delivery to cells using lipid-based vehicles, there is evidence that NAs can be recovered in endosomes and this is associated with morphological changes within the endocytic pathway (236). Further, the presence of endocytic inhibitors, i.e. to prevent uptake of NA into the cytosol, can decrease transfection rates (218). Many investigators have also correlated inhibition of endocytosis with lowered expression of a transgene or antisense downregulation (236, 237). Phagocytosis may also be a possible the mechanism associated with internalisation in cultured epithelial cells. (234)

Vector entry mediated by endocytosis is advantageous for a number of reasons. Since entry of the exogenous therapeutic compound is *via* a normal physiological pathway,

transduction is less damaging in comparison to delivery mediated by cationic lipid fusion with the plasma membrane. Cell-specific targeting, together with agents that facilitate endocytosis, may provide mechanisms by which uptake can be enhanced. For example, the addition of a cell-type specific ligand, i.e. folate, may introduce additional numbers of lipid-NA delivery systems to increase delivery and stimulate endocytosis (171, 238). While substances such as chloroquine (to prevent lysosomal degradation) and NH_4Cl can prevent the endocytic turnover (218).

1.3.3.2 Fusion

Although fusion events may be a minor component in NA delivery, there are some indications that uptake of DNA can be a result of fusogenic events mediated by cationic liposomes (239-241). Efficient NA delivery correlates with high levels of lipid mixing activity, a property typically attributed to membrane fusion (242, 243). While cationic lipid species can stimulate close cell membrane contact to initiate fusion events, there have been suggestions of other mechanisms. In the presence of cationic lipids, there is potential for the electrodiffusive transport of a highly charged, large macromolecule, such as DNA through the hydrophobic region of a lipid bilayer (244). The presence of associated cationic lipids plays a significant role in this process.

The membrane fusion reaction between two bilayers is of fundamental biological importance, not only for delivery of exogenous NA but also for cell processes such as endocytosis. Membrane fusion may be one mechanism by which two specific steps in the intracellular barrier of NA delivery - NA entry and release of the NA after endocytosis - can be overcome. A crucial step in the initiation of this event is the close contact between

bilayers; for example, this could be mediated by electrostatic interactions between the excess cationic lipid charge on lipid/NA complexes and the cell membrane. Close contact results in displacement of water molecules and dehydration of the apposed lipid bilayers. Subsequently, there is charge neutralisation and formation of non-bilayer lipid intermediates which is promoted by lipids or conditions that induce a preference for negative curvature in the regions of contacting monolayers. The following section *1.3.3.2.1* describes the role of unsaturated phosphatidylethanolamines, which have been described as inducers of the fusogenic event, to mediate fusion events by (i) initiating the H_{II}-phase (section *1.3.3.2.1.1*), (ii) (de)hydration of apposed surfaces (section *1.3.3.2.1.2*) and (iii) inter- and intra-molecular hydrogen bonding (section *1.3.3.2.1.3*).

1.3.3.2.1 PE as a mediator of fusion events

The presence of lipids that contain the phosphatidylethanolamine headgroup in biological systems is quite prevalent and very relevant when considering lipid/NA complexes. Chapter 3 describes in detail the effect of PE-lipids on formation of lipid-NA particles. While the functions of PE-containing lipids are still being studied, one consistent message is that PE-lipids have diverse physiological roles. These include assisting association of proteins (245, 246), altering transport protein properties (247), increasing the propensity for membrane fusion (248) and altering the infectivity of viruses (249). The most interesting feature relevant to NA delivery is the ability of selected PEs to induce membrane fusion when incorporated into lipid delivery systems. For example, DOPE has frequently been used as a helper lipid in cationic lipid-based NA delivery systems. While the cationic lipid is perceived to be the protective agent, the helper lipid is considered to be the "acting"

species. DOPE is the fusogenic lipid of choice for many gene delivery applications (250, 251). It has an ability to adopt hexagonal phase tube structures, a suggested fusion intermediate, that facilitate cationic lipid interaction with anionic cell membranes or increase the propensity for NA release (252-254). Transfection protocols also include DOPE as chasers after lipoplex treatment (250, 255). The rationale behind this strategy is to stimulate fusion intermediate formation and facilitate the fusion event after delivery of NAs to the target cell.

1.3.3.2.1.1 Shape concept and non-bilayer-forming lipids

While the mechanism by which PEs act to assist or effect NA delivery is well debated, many investigators suggest that it is the nature of their chemical and physical behaviour that defines their activity. Unsaturated PEs, due to their small headgroup volume relative to their acyl chains, are strongly driven towards high curvature stress (256). The smaller headgroup cross-sectional area results in a profound effect on the physical properties of membrane surfaces due to the tendency to induce strain and increases the spontaneous negative curvature of bilayers (257, 258). Following the shape concept of lipid polymorphism (described in section *1.2.3*), a molecular volume ratio favouring the acyl chain (cone-shaped) results in unsaturated PE-lipids spontaneously adopting the H_{II} phase. The H_{II} phase is one of inverted micelles or lipid particles sandwiched between two monolayers of the bilayer. The smaller headgroup also allows for closer packing constraints and leads to the restriction in the rotational dynamics of the acyl chains. While PE lipids are described to have a native H_{II} configuration, they also undergo phase transitions from L_β - L_α as well as the L_α - H_{II} phase.

The phase transition from the L_α - H_{II} to the hexagonal phase can occur at the contact surfaces between apposed bilayers. In addition to fusion, this event can be involved in many functional processes such as endocytosis, transbilayer transport of lipids and small molecules, compartmentalisation of membranes, membrane flow, and inter-organelle communication (259). Siegel and Epand (104) proposed a mechanism for fusion that mimics the L_α - H_{II} phase transition where inverted micelle intermediates form during the process of membrane fusion. The first step in the fusion process is the formation of small connections between the apposed bilayer surfaces - for example, between the cell membrane and the lipid-NA complex. When they connect, aggregates form an array of H_{II} tubes (260). Subsequently, the connection elongates into H_{II} phase domains whereby the lipids diffuse from the contiguous bilayers. The small interbilayer connections between the cell membranes are comparable to the *trans* monolayer contact.

In terms of energetics of this process, the equilibrium state between L_α and H_{II} phase is a function of the monolayer intrinsic curvature and acyl chain packing energies. The formation of non-bilayer lipids is driven by the intrinsic curvature of certain lipid monolayers (239). Two factors that contribute to the resultant stability of the fusion intermediate are the magnitude of monolayer curvature (261) as well as the degree and position of unsaturation in the acyl chains (262). Since the lamellar phase is one that rigorously resists curling, there is a large prohibitive hydrocarbon-packing energy that must be overcome (263). Although fusion events may be a minor component by which NA is delivered intracellularly, this mechanism may also describe release of the NA from the endosomes (264).

1.3.3.2.1.2 Lipid hydration

The hydration characteristics during the fusion or L_α - H_{II} phase transition undergoes drastic alterations in order to enable the shift from flat bilayer structures to one of tightly packed arrays of monolayer tubes (125). The dynamics of membrane fusion are also a function of lipid hydration properties. More specifically, the steps of the L_α - H_{II} phase transition, as described in section *1.2.4.1*, contribute to the membrane fusion mechanism partly by the energetics of hydration or, rather, dehydration. One key step in the initiation of the membrane fusion event is the local dehydration of apposed bilayers. This causes the outer lipid monolayers to compensate for the decreased water cavity by bending. Subsequently, the volume ratio of the molecular shape of the lipid molecules is altered, whereby the acyl chain volume increases corresponding to a decrease in the polar region. The resultant molecular shape is attributed to lipids forming the hexagonal phase, the shape of the intermediate in membrane fusion. In summary, the L_α - H_{II} phase transition is dependent on the bending energies associated with molecular shape changes of the apposing lipid monolayers as well as a contribution from the hydration energy of the polar headgroups.

The reason unsaturated PE-lipids have a "fusogenic" capacity is partly attributed to its hydration characteristics. The extent of hydration of PE-containing lipid bilayers is significantly less than for other lipids (i.e. PC) (265). In both gel and liquid-crystalline lamellar phases, adsorption isotherms have shown that pure PC samples take up more water than PE MLVs, and the presence of a bulk phase is observed at a lower number of water molecules for PE dispersions, as reviewed by T.J. McIntosh (266). The extent of hydration for a lipid is attributed to the area per lipid molecules and the width of the fluid phase between the apposing bilayers. As such, a decrease in either of these parameters will effect

lower hydration of the headgroup. Two explanations have been associated with the reduced hydration of PE lipids – headgroup volume and hydrogen-bonding. When comparing PC to PE headgroups, the volume decreases from 344 to 246 Å, respectively (267). Additionally, hydrogen bonding water bridges between the amine and phosphate groups inhibits lateral movement in adjacent PE molecules, thereby reducing expansion capacity by tighter packing at the lipid-water interface in and between the bilayers (114). Ultimately, the propensity for PE molecules to initiate fusion events is partly attributed to their ability to induce dehydration locally.

1.3.3.2.1.3 Hydrogen bonding

Lipids containing the PE headgroup have attributes that promote membrane fusion such as closely packed monolayers and ordered bilayer. As described in section *1.3.3.2.1.2* above, the hydration properties of the headgroup play a fundamental role in portraying a small headgroup capable of high molecular ordering. In addition, the ability of PE to interact intermolecularly with adjacent molecules *via* hydrogen bonding contributes to its propensity for close packing (268). This is a result of interactions between the negatively charged phosphate and the protonisable amine moiety, whereby the NH_3^+ "donor" and PO_2^- "acceptor" are orientated suitably for interaction. The limitation for this interaction is high stress due to curvature and steric repulsion (269). Inter- and intra- molecular interactions among PE-lipids have implications for induction of membrane fusion. Specifically, the initiation of the close contact and local dehydration of the bilayer, which are stabilised by hydrogen bonding interactions, lead to formation of the H_{II} intermediate (see Figure 1.11). In

Figure 1.11

A model of hexagonal phase (H$_{II}$) fusion intermediates

The fusion events contribute to a number of fundamental biological processes. However, electrostatic based interactions such as those governing liposome-DNA complex formation or complex-cell association can also lead to initiating H$_{II}$ formation. The rearrangement of lipids results in conformational changes in the bilayer where H$_{II}$ predominates. Adapted from (270).
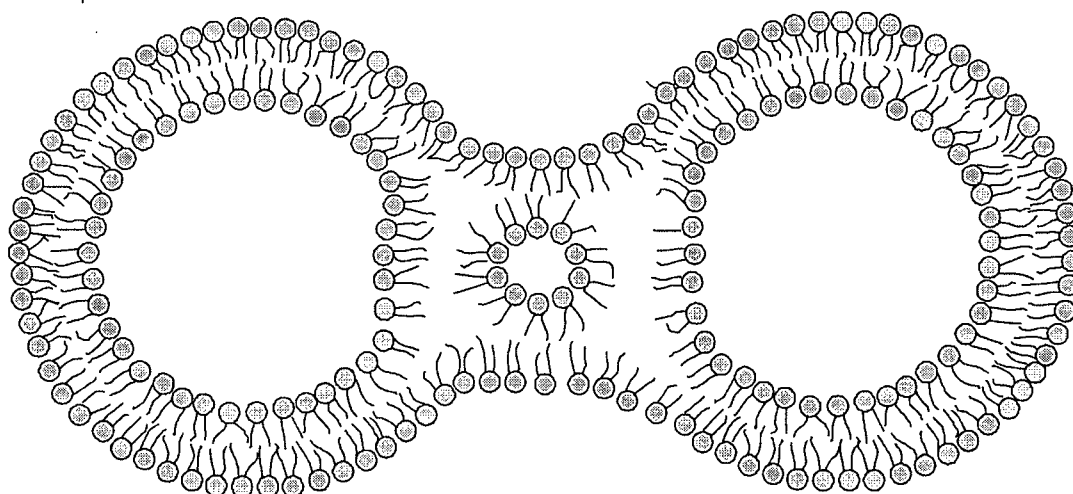
turn, the intermediate, vital in the mechanism of membrane fusion, is again stabilised by H-bonding among headgroups.

Another aspect in which hydrogen bonding can effect increased delivery of NA, besides stimulating the fusion event, is through its ability to H-bond with the phosphate groups of the NA. Due to its propensity for H-bonding, the ethanolamine headgroup may play a role in dissociating cationic lipids bound to NAs. As described in the sections above, in addition to geometry and hydration forces, H-bonding forces mediate formation of the macromolecule structure adopted by PE lipids. Specifically, these are the interactions between the amine group and the non-esterified oxygen of the phosphate groups within and between lipids (271). While the interactions among phospholipids are well described, similar H-bonding can occur between the amine groups of PE and the phosphate groups of the NAs. The propensity for H-bonding between these specific moieties would weaken the binding between cationic lipids and DNA. One potential outcome of these interactions would be that those lipid-based NA formulations containing PE would be more susceptible to factors that promote disassembly as a result of weakened binding (272). This feature is of relevance when considering the next step in intracellular delivery – release of the NA.

1.3.4 Release of the NA from the vehicle and the endosome

Although many investigators are focused on increasing intracellular delivery of DNA or ASOs, it may be that the limiting step in NA-mediated activity is a result of inefficient processing of the delivery complex after internalisation (195, 273-276). This thesis argues that effective transfection/downregulation requires the release of NAs in order to allow interaction with the cell's transcription machinery or hybridisation to mRNA targets. This

statement is supported by a poor correlation between uptake efficiency of lipid/NA complexes and NA activity (180, 277). For example, studies have demonstrated low levels of transgene expression despite direct injection of lipid/NA complexes into the nucleus (138). Another significant observation is that, while detectable levels of DNA ($>1\times10^{6}$ plasmids/cell) delivered by cationic lipid was observed in 100% of a cell population, only 50% demonstrated transgene expression using X-gal staining (138). As such, the association of cationic lipid may impede the processing of the plasmid DNA or, in the case of ASOs, hybridisation (138). Although there is evidence that DNA can dissociate from cationic lipids in the nucleus by displacement by genomic DNA (278), this process is not efficient nor does it have the potential for enhancement. It may be that processing of plasmid DNA or ASO delivery systems require two separate and specific events: (i) that all lipids incorporated into the carrier system must dissociate from the NA and (ii) free NA must co-localise with the cell's transcription machinery or, in the case of ASOs, to the target mRNA. In order to safeguard NA activity, the release of NA must ensue in a timely efficient manner prior to acidic denaturation or nuclease degradation in the endosome. Whether release events transpire in the cytoplasm or nucleus remains uncertain, as does the mechanism of release and the extent of the involvement of anionic biomolecules (274, 276).

The compositional requirement of a lipid-based carrier does not merely assist in NA uptake - they may also act to dissociate NA from the lipid complex. Investigators Y. Xu, O. Zelphati and F.C. Szoka Jr. have described a destabilisation event mediated, in part, by anionic lipids (or anionic molecules at high concentrations) and cationic lipids association *via* ion-pairing (274, 276). Consequently, these electrostatic interactions enable the cytoplasmic-facing anionic lipids to undergo flip-flop actions; therefore, promotion of interlipid ion-pairs

is vital in this process. Ion-pair formation is important for two reasons: (i) to displace the NA from the bound cationic lipids and (ii) to permit free NA entry into the cytoplasm. The latter is a result of progressive reduction of asymmetry in the lipid composition and induces instability by formation of a membrane fusion-like intermediate (i.e. lipids in H_{II} phase). While the electrostatics are important in promoting the initial steps of release, hydrophobic interactions reinforce the permeability and accessibility of the NA into the cytoplasm (279). It is unclear whether cationic lipid dissociation transpires before or after interactions with organelles such as the endosome, but it is vital for dissociation events to occur for NA activity.

Electrostatic interactions have been described in previous sections to be important for (i) binding to the NA (section *1.3.2*), (ii) fusion (section *1.3.3.2*), and (iii) dissociation leading to NA release (section *1.3.4*). While PE-lipids are considered to be the "acting" species in a lipid-based carrier system mediating events such as fusion and release, there is also evidence that cationic lipids play an active role in destabilisation of endosomal and/or lysosomal membranes (218, 277, 280). For example, cationic lipids can initiate fusion events, described in detail for intracellular delivery of NAs (section *1.3.3.2*), and they may significantly contribute to the release mechanism. This is similar to the mechanism described for fusion leading to delivery events (281) where structural intermediates, initiated by cationic lipids, are formed similar to those mediated by H_{II}-forming, PE-containing lipids. However, the intermediate is formed from lipids of the lipid carrier and the endosomal membrane. The release of NA is the result of destabilisation of the binding between cationic lipids and NA with free PE-lipids contributing to an H_{II} intermediate. The presence of PE-lipids serves two functions - to introduce instability to the carrier systems and to assist in

creation of the intermediate structure. Other investigators have attempted to incorporate drugs that disrupt the lysosome or endosome to accomplish effective NA release (282, 283).

1.3.5 Transfer into nucleus

The direct injection of naked DNA into the cytoplasm does not enhance transfection efficiency whereas injection into the nucleus does increase the amount of transgene expression (284). This leads to the strong belief that DNA expression vectors must enter the nucleus in order to access the cell's transcription machinery. In contrast, ASOs were designed to interact with mRNA in the nucleus or cytoplasm. As it is improbable that lipid/NA complexes remain intact until entry into the nucleus, likely passive events, such as diffusion, enable efficient transport of the free NA but with the risk of nuclease degradation. While the presence of associated lipids may facilitate delivery to the nuclear membrane, they would hinder, rather than augment, diffusion to the nucleus (285, 286).

The nuclear pore complex provides aqueous channels for an energy-independent transport of small metabolites and particles, whereby these molecules can bypass to the nucleus purely by a diffusive process. However, the nuclear membrane remains a significant barrier to NA delivery (287). The restricted movement of a large molecular species, such as DNA, and the limited number of nuclear pores hinder entry into the nucleus. An important observation related to gene processing is that transfection efficiency is elevated in rapidly dividing cells (288). In particular, the cell cycle status has been prioritised as an important determinant of transfection efficiency. Cells undergoing mitosis/meiosis exhibit high levels of transfection, while cells in G_o phase tend to exhibit the lowest levels of transfection (232). Nuclear localisation of NAs is enhanced upon membrane breakdown during cell division, i.e.

the M (mitotic) phase. Many studies have attempted to synchronise cell cycles in order to establish an optimal moment for induction of a therapeutic response. These treatments typically utilise various chemicals in order to induce a simultaneous M phase [for review, see (289)]. However, synchronisation of cell cycle with the time of gene transfer treatments will likely be of value only *in vitro*.

Passive diffusion may be an option for molecules <60 kDa (290, 291); however, formulations can also include nuclear localisation signal (NLS) for active transport. NLS from viruses (i.e. SV40) in the form of proteins or inserted into the plasmid vector have been utilised as a mechanism to traffic free plasmid into the nucleus (292). This must occur in the absence of a carrier system, which can limit the capacity for DNA to move across the nuclear membrane. A bound karyophilic signal peptide has the potential to dock DNA to nuclear pore filaments and initiate nuclear transport (293). Synthetic polymers, such as poly(ethylenimine), have been shown to promote nuclear entry (285) as well as nuclear-targeting peptide scaffolds for non-dividing cells, which increases gene expression by 63-fold (294). By exploiting the localisation attributes of specific proteins, it is possible to enhance delivery beyond the nuclear envelope and increase the amount of substrate at the reaction site. Efforts to enhance DNA delivery to the nucleus should proceed with caution as it is possible that naked DNA will be complexed into chromatin-like structures by histones and nuclear matrix protein and become inaccessible to cellular transcription machinery (295, 296).

1.3.5.1 Transcription and translation

It is important to note that therapeutic activity of plasmid expression vectors depend ultimately on expression of the transgene. It is unclear what level of expression is required; however, it could be argued that a therapeutic benefit will likely arise when the expression level is close to that of the normal gene. Since it is the specific protein level that modulates cell activity or induces a cascade of immunotherapeutic effects, transcription and translation need to function effectively and efficiently. However, the strategy for transcriptional optimisation will vary depending on the requirement for transient expression or stable integration into the host chromatin. At the nuclear level, where the DNA is delivered in an intact form capable of inducing gene expression, it will be the characteristics of the vector itself that defines its therapeutic activity. The difficulty lies in the use of foreign promoters or enhancers, typically used in the design of DNA vectors, which are not well recognised by eukaryote transcriptional activators (297). Further, sequences that not native to the cell may be recognised by the immune system and become inactivated. (298-300). Immune recognition may also play a part in eliminating a new or modified protein product generated as a result of the therapeutic gene.

Although the cell's own transcriptional machinery may provide more efficient expression, the identification of each cell component with an effect of exogenous gene regulation is a near-impossible task. For example, one potential limitation is the efficiency of RNA polymerase II activity. Although there is a constant presence of polymerase as a part of the nuclear content, its activity is dependent on a variety of interdependent factors irrespective of the amount of DNA, including environment and cell cycle (301). This may be overcome by co-delivery of a T7 polymerase promoter in order to enhance cytoplasmic

transcription rather than depending on local machinery (302, 303). The ultimate activity of the transcription unit is dependent on all the elements of the construct to exert a cumulative effect. Further, the role of lymphokines, cytokine and growth factors in initiating and maintaining gene expression is poorly understood but will play a significant factor in regulating protein expression.

Upon efficient transcription of an exogenous gene, the resultant transcript must be stable for transport into the cytoplasm for translation into the protein product. For example, eukaryote mRNA is stabilised through the addition of a poly-A tail at the 3' end. This is vital for transport stability and export from the nucleus. It has also been suggested that the inclusion of introns within a gene will increase stable mRNA production (304). As such, it may be an important requirement to include an intron within the transgene being delivered. Thus, in addition to providing sufficient copies of a DNA to the nucleus, one must also consider the status of the target cell. Design of lipid-based DNA systems must be considered not only on the properties of the carrier itself but also the characteristics of the cell target being transduced.

1.3.5.2 Downregulation of protein product

Protein downregulation can be achieved through two ways: (i) DNA transfer of an antisense sequence or (ii) use of ASOs. Given all the concerns listed above (section *1.3.5*) and localisation into the nucleus, it is believed that the latter strategy may be more efficient. In particular, it is possible that ASOs exert their activity directly in the cytoplasm. Alternatively, if the ASOs act within the nucleus, their small size will facilitate a greater potential to cross the nuclear membrane. In contrast to plasmid DNA delivery, ASOs are

designed to inhibit the production of a selected protein. Investigators designing ASOs have based the interaction of mRNA target with ASO on complementary (Watson-Crick) base-pairing. These interactions can be generated to be very specific. However, the question that remains is whether this is the mechanism by which ASOs mediates the therapeutic activity. The answer is dependent on the molecular mechanism of the target. This molecular target must have a key role in effecting disease. Further, the target must have a minimal redundancy within the molecular cascade such that a decrease or elimination of the protein will induce a therapeutic activity. These factors are exemplified by the following example of Bcl-2.

When considering the Bcl-2 family of apoptotic proteins (305) it is necessary to consider the ramifications of ASO therapy. Upon ASO treatment of a disease that overexpresses the Bcl-2 protein, it is anticipated that Bcl-2 levels will decrease as a result of specific targeted interaction of ASO with the mRNA target. This will lead to release of heterodimerization with Bax protein and initiation of the apoptotic pathway. This is a simplistic view of the molecular mechanism as there are other members of the protein family with significant interactions with Bcl-2 protein or those that in the signalling cascade that effect a response (306). However, there are two points that can be made to relate therapeutic activity and molecular mechanisms. First, the therapeutic activity is based on downregulation of the protein product. While mRNA is the target species, a therapeutic response can be only achieved once sufficient protein is degraded by proteases for downregulation. Thus, the Bcl-2 protein half-life is a vital factor (307). This leads us to the second point. A threshold level of Bcl-2 protein must be eliminated such that apoptosis can occur; it is possible, however, that downregulation of Bcl-2 cannot reach a level such that the

protein is no longer a functional part of the cascade. It is important to note that in ASO treatment one must consider the threshold level of protein downregulation as well as the relation between functionality and therapeutics.

1.4 Hypotheses and Rationale

The therapeutic potential of NAs is rapidly expanding due to the explosive increase in the number of target genes and proteins being discovered. While liposomal-based systems are touted to be one viable method for delivery of NAs to a target site, the widespread difficulties in generating homogeneous, pharmaceutically-useful complexes with high levels of activity makes it a difficult endeavour. As such, this thesis describes the use of lipid-based NA delivery systems created through systematic addition of cationic lipids, phospholipids, and PEG-conjugated lipids to polyanionic NAs. The reaction is mediated through simple charge interaction in the presence of solvents or detergents. It is hoped that a systematic approach protocol, based on a hydrophobic lipid-NA intermediate, will lead to these novel findings:

1) identification of the basic interactions governing lipid to NA binding

2) definition of the mechanism by which phospholipids (helper lipids) affect cationic lipid-anionic phosphate NA interactions

3) an understanding of the role of electrostatics in governing complex formation and complex delivery to cells

4) formulation of well-defined lipid-NA particles that can be used for transfection of plasmid DNA and delivery of ASOs under various conditions

5) development of lipid-NA particles with improved therapeutic activity *in vivo*.

With these aims in mind, the following hypotheses define the research developed in this thesis.

Hypothesis 1: A hydrophobic lipid-DNA complex can be generated through electrostatic-based neutralisation of charges and the addition of hydrophobic moieties (lipids) to NAs.

Hypothesis 2: Phosphatidylethanolamine-containing lipids destabilise cationic lipid-NA interactions through headgroup interactions with the cationic lipid as well as the NA phosphate groups.

Hypothesis 3: Surface charge interactions mediate the association of LDPs with an anionic surface.

Hypothesis 4: Lipid-ASO particles, generated through a hydrophobic intermediate, are useful for delivery of therapeutically active ASOs.

CHAPTER 2

CATIONIC LIPID BINDING TO DNA: CHARACTERIZATION OF

COMPLEX FORMATION*

2.1 Introduction

This chapter focuses on the development of lipid-based carriers since these synthetic formulations are the most pharmaceutically advanced (308); however, these systems are also perhaps the most difficult to characterise (129, 138, 187). Addition of DNA to pre-formed cationic liposomes triggers significant structural changes in the liposomes as well as the DNA (129), as described in more detail in Chapter 1, section *1.2.5*. Depending on the liposomal lipid composition (cationic lipid and associated secondary lipids) and lipid concentration, DNA addition engenders liposomal aggregation resulting in the formation of structures which are heterogeneous with respect to physical and chemical characteristics (129). The structural changes in the complexes are also associated with changes in the DNA structure. Plasmid DNA within the liposome aggregates is less sensitive to DNase I and is resistant to ethidium bromide binding (309). Although these changes to the DNA are consistent with polycation-induced DNA condensation (202), it is unlikely that liposomes condense DNA in a manner similar to classical condensing agents such as polylysine. For example, multivalent cation-condensed DNA has been shown to generate toroid-like structures (202, 310) that have not been observed with liposome-condensed DNA. The benefits associated with DNA structural changes induced by such polyvalent cations are

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*Wong, F.M.P., Reimer, D.L., and Bally, M.B. (1996) *Biochemistry* 35:5776-63.

comparable in that DNA adopts a structure that renders the molecule partially protected from extracellular and intracellular degradation.

D.L. Reimer *et al.* have recently demonstrated that hydrophobic lipid/DNA complexes can be prepared using monomeric lipids rather than pre-formed liposomes and that DNA in the resulting complex is not in a condensed state (203). This observation is important for a number of reasons: First, the complex can be used as a well-defined intermediate in the preparation of lipid-DNA particles (LDPs). Second, characterisation of this binding reaction will provide information on the factors that control association, dissociation and aggregation of lipids and DNA; finally, the influence of bound lipid on the DNA structure can be assessed in the absence of a membrane structure. The objective of this chapter is to determine the binding characteristics of cationic lipid to DNA. The lipid/DNA binding was assessed by evaluating the hydrophobic cationic lipid/DNA complex extracted into organic solvents. This chapter demonstrates that the binding reaction was highly co-operative and this reaction occurs at the interface between aqueous and organic phases. It is suggested that DNA-bound lipid in the organic phase adopts an inverted micelle-like structure.

2.2 Materials and Methods

2.2.1 Materials

The monocationic lipids, DODAC and ^{14}C-DODAC (specific activity ~9.0 x 10^5 dpm/μg), were synthesised and supplied by Steven Ansell of Inex Pharmaceuticals Corp. (Vancouver, B.C.). Zwitterionic lipids (DOPE, DOPC) and anionic lipids (DOPS, DMPG, PEG-PE and LPI) were obtained from Avanti Polar Lipids Inc. (Birmingham, AL) or

Northern Lipids Inc. (Vancouver, BC). Cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO). Solvable™ was obtained from Packard (Meriden, CT). The pCMVβ plasmid encoding *E. coli* β-galactosidase was obtained from Clontech Labs (Palo Alto, CA) and was propagated and purified using standard techniques (311). Methyl-^{3}H-thymidine-5'-triphosphate, obtained from Dupont NEN (Boston, MA), was used to synthesise radiolabeled pCMVβ plasmid. A specific activity of ~5.0 x 10^4 dpm/μg was typically obtained. All solvents used, chloroform and methanol, were HPLC grade (EM Science, Toronto, ON).

2.2.2 Methods

2.2.2.1 Bligh and Dyer extraction

The monocationic lipid DODAC and the pCMVβ plasmid were solubilized in a Bligh and Dyer monophase consisting of chloroform:methanol:water (1:2.1:1) (312). DNA (1 μg to 160 μg) and DODAC (10 nmoles to 640 nmoles) were combined to a total volume of 1 mL. The monophase mixture was subsequently partitioned into two phases by the addition of 250 μL each of chloroform and water. The samples were mixed vigorously by vortexing for 1 min and centrifuged at 600×g for 5 min at room temperature. The upper aqueous phase (~1.0 mL) and the lower organic phase (~0.5 mL) were removed and the amount of DNA in each phase was determined. The pH was estimated to be 6.5 in both aqueous and organic phases using pH sticks obtained from EM Science (Cherry Hill, NJ).

2.2.2.2 Quantification of DNA and DODAC

One of two methods was used to quantify the amount of DNA recovered in the aqueous and organic fractions following Bligh and Dyer extraction. First, trace quantities of

^{3}H-pCMVβ were added to unlabelled DNA in the monophase such that each sample contained 3000 to 4000 dpm (~60 pg) of ^{3}H-labelled DNA. The lipid and DNA were mixed in the monophase and separated into organic and aqueous fractions as described. The organic phase was dried down using a stream of nitrogen gas prior to the addition of 1 mL of Solvable™. This lipid-DNA film was incubated for 1 h at 50°C to solubilized the complex. Subsequently, Pico-Fluor-40 scintillation cocktail was added to the aqueous and organic fractions and the radioactivity was measured by a Packard TR 1900 Scintillation Counter. Alternatively, DNA in the aqueous phase was quantified by measuring the optical density at a wavelength of 260 nm using a Beckman UV Spectrophotometer (DU-64). Data collected by this method were presented as percentage of DNA recovered in the organic phase. No differences were observed in the values when data was collected by the radioactive labelling method or by spectrophotometric analysis. Trace amounts of ^{14}C-DODAC (~8.0 pmoles) were also evaluated (in the presence of 10 μg DNA) in organic and aqueous phases by scintillation counting following Bligh and Dyer extraction.

2.2.2.3 Effects of other lipids on the hydrophobic complex

The effects of zwitterionic (DOPE and DOPC), neutral (cholesterol) and anionic (DOPS, DMPG, PEG-PE and LPI) lipids on the formation and/or dissociation of DODAC/pCMVβ complexes were evaluated using two methods:

In one method, the DODAC/pCMVβ complexes were pre-formed prior to the addition of other lipids. These lipids were injected directly into the organic phase following Bligh and Dyer extraction and formation of the two-phase system. The samples were mixed vigorously by vortexing and separated by centrifugation as previously described. The effect

of these added lipids on the dissociation of the complex was evaluated by quantifying the DNA in the aqueous and/or organic phases.

Alternatively, the effects of the additional lipids on the formation of the complexes were evaluated by mixing them with pCMVβ prior to the addition of DODAC. As a control, DOPE was added to the DNA in the absence of DODAC to ensure that DOPE did not mediate extraction of the DNA into the organic phase.

2.3 Results

2.3.1 Formation of the hydrophobic cationic lipid/DNA complex

Evidence for the formation of a hydrophobic cationic lipid/DNA complex has been published (203) and is summarised in Figure 2.1. Specifically, when 40 nmoles of DODAC, a monovalent cationic lipid, was added to 10 μg of plasmid DNA, >95% of the DNA initially present in the monophase was extracted into the organic phase. The presence of DNA in the organic phase resulted from the formation of a hydrophobic complex generated through interactions between the cationic head group of the lipid and the negatively charged phosphate groups of the DNA backbone. In samples containing 40 nmoles of cationic lipid and 10 μg DNA the charge ratio of lipid to nucleotide phosphate (+/-) was 1.0:1.0, suggesting that charge neutralisation mediated the formation of this hydrophobic complex. One of the primary objectives of this chapter was to further characterise this binding reaction.

The significance of cationic lipid-dependent charge interactions with the DNA phosphate groups was evaluated over a broad range of DNA concentrations. The results, presented in Figure 2.2, illustrate two important points regarding the binding reactions.

Figure 2.1

Recovery of pCMVβ plasmid DNA following Bligh and Dyer extraction

Increasing amounts of DODAC were solubilized and combined with 10 μg of pCMVβ in a Bligh and Dyer monophase. Subsequently, the solution was partitioned using additional chloroform and water. Recovery of pCMVβ is shown as a percent of the total added in the aqueous (■) and organic (●) phases following extraction of the DODAC/DNA complexes and measured by spectrophotometry.
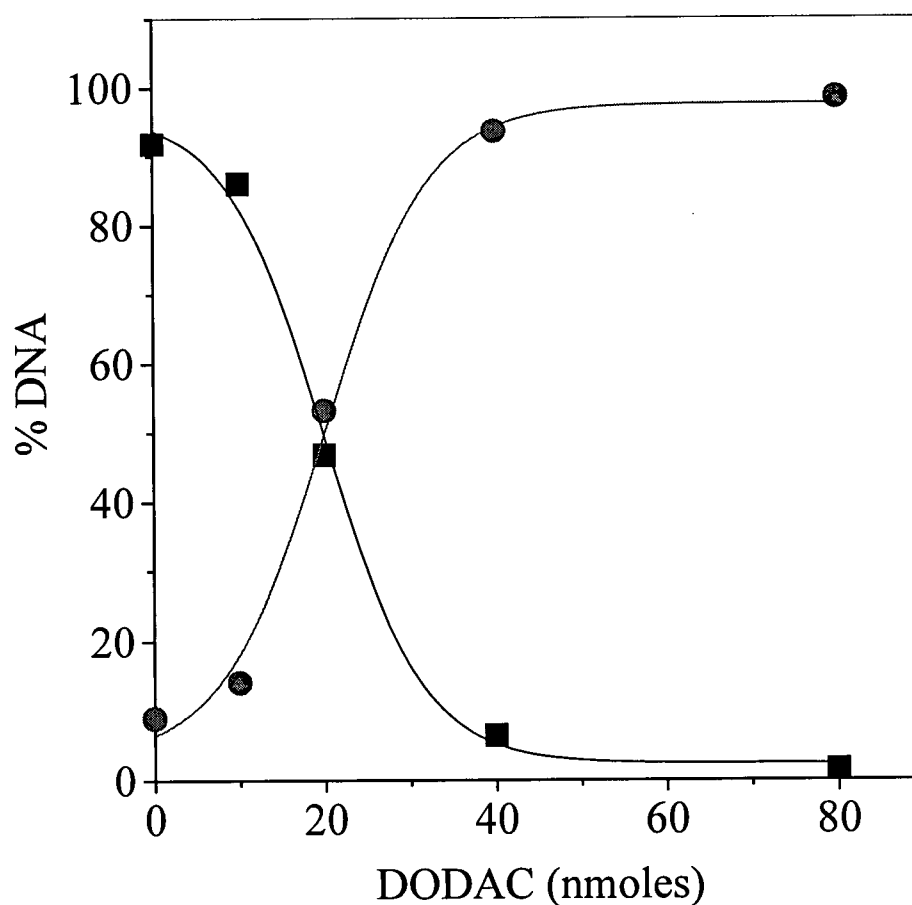
Figure 2.2

Recovery of various initial amounts of DNA as a function of increasing amounts of DODAC

Increasing amounts of DODAC were added to various initial DNA amounts followed by Bligh and Dyer extraction of the complexes (incubation time ~5 min). Amounts of plasmid was measured by spectrophotometry in the aqueous phase and expressed as % of initial added in the organic phase over amounts of DODAC in nmoles (**A, C**) or as calculated charge ratios (**B, D**). Amounts of DNA used in **A** and **B** were 5.0 µg ($\triangle$), 10.0 µg ($\blacksquare$), 20.0 µg ($\bullet$), and 40.0 µg ($\blacktriangle$). Amounts of DNA used in **C** and **D** were 1.0 µg ($\square$), 2.5 µg ($\bigcirc$), 5.0 µg ($\triangle$), 8.0 µg ($\triangledown$), 10.0 µg ($\blacksquare$). All data points are averaged from three replications and expressed ± SEM.
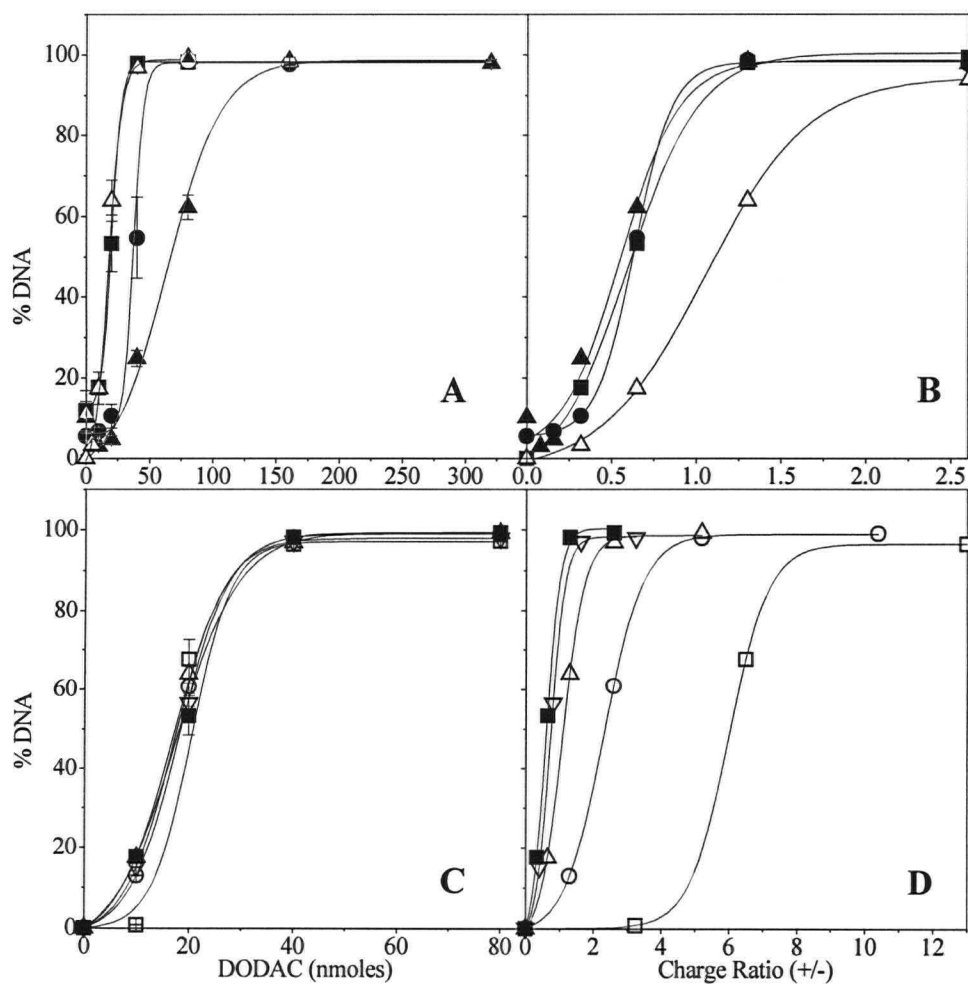
First, formation of the hydrophobic complex, as measured by an increase in the proportion of DNA isolated in the organic phase, appears to be dependent on achieving charge neutralisation when the amount of DNA in the system was in excess of 10 μg. Figure 2.2A shows that for 10, 20 and 40 μg DNA >95% of the DNA was recovered in the organic phase when 40, 80 and 160 nmoles of DODAC was added, respectively. Upon calculation of the charge ratio of cationic DODAC to anionic DNA, efficient recovery of DNA in the organic phase was achieved only when a charge ratio (+/-) of 1.0 or greater was obtained (Figure 2.2B).

The second point illustrated in Figure 2.2 is that the relationship between charge ratio and complex formation was no longer valid when the amount of DNA present in the assay was below 10 μg; specifically, when using 5 μg DNA a charge ratio (+/-) of approximately 2.6 was necessary for efficient recovery of DNA into the organic phase (Figure 2.2B and 2.2D). At the lowest amount of DNA evaluated (1 μg) even a higher charge ratio was required (>12.0) to obtain complete recovery of DNA in the organic phase. As shown in Figure 2.2C, these results suggest that a minimum concentration of lipid (40 nmoles per assay) is necessary to effect the formation of the hydrophobic complex when the DNA concentration is below 10 μg. In order to elucidate the mechanisms governing the formation of the hydrophobic complex, the rate of complex formation was evaluated. The rationale for this study lay in the possibility that at low DNA concentrations (<10 μg per assay) the rate of complex formation might be slower than that observed at higher DNA concentrations. Time-dependent formation of the complex was evaluated in two ways. First, the DNA and cationic lipids were incubated for defined periods in the Bligh and Dyer monophase prior to the creation of the two-phase system. Alternatively, the amount of DNA recovered in the

organic phase was measured as a function of time after formation of the two phases. The results demonstrated that DNA recovery in the organic phase was not affected by the length of time the samples were incubated in the monophase; however, a time-dependent increase in the amount of DNA recovered in the organic phase was obtained after formation of the two-phase system. The results presented in Figure 2.3 suggest that for assays containing 5 μg DNA and 20 nmoles DODAC the amount of DNA recovered in the organic phase increased as the incubation time following phase separation increased; thus, for low amounts of DNA a charge ratio of 1.0 was sufficient to mediate efficient extraction of DNA into the organic phase provided that there was ample time after phase separation. These results suggest that the hydrophobic complex, if formed within the monophase, is not stable and readily dissociates. As such, the process of DNA to DODAC binding and eventual complex formation occurs after preparation of the aqueous and organic phases.

A second experiment evaluating the formation of the hydrophobic complex assessed the partitioning behaviour of the cationic lipid. DNA extraction into the organic phase is dependent on lipid binding; for example, when sufficient amounts of lipids are bound, the DNA exhibits hydrophobic characteristics. If this binding reaction is strong enough to mediate the transformation of a hydrophilic molecule into a hydrophobic complex, the reverse may also be true. For this reason the effect of trace amounts of radiolabeled cationic lipid binding to excess DNA on the partitioning characteristics of the lipid was studied. The results shown in Figure 2.4 demonstrate that with excess DNA >90% of the cationic lipid was always recovered in the organic phase (Figure 2.4C). The results conclusively demonstrate that the binding reaction was not strong enough to mediate partitioning of the lipid into the aqueous phase.

Figure 2.3

Time-dependent recovery of plasmid DNA following Bligh and Dyer extraction

Five μg DNA and 20 nmoles DODAC were solubilized in a Bligh and Dyer monophase and the plasmid DNA recovered in the organic phase as a function of time following preparation of the two phases. Plasmid DNA was measured by spectrophotometry in the aqueous phase and expressed as a % of initial added in the organic phase. Data points were averaged from three experiments.
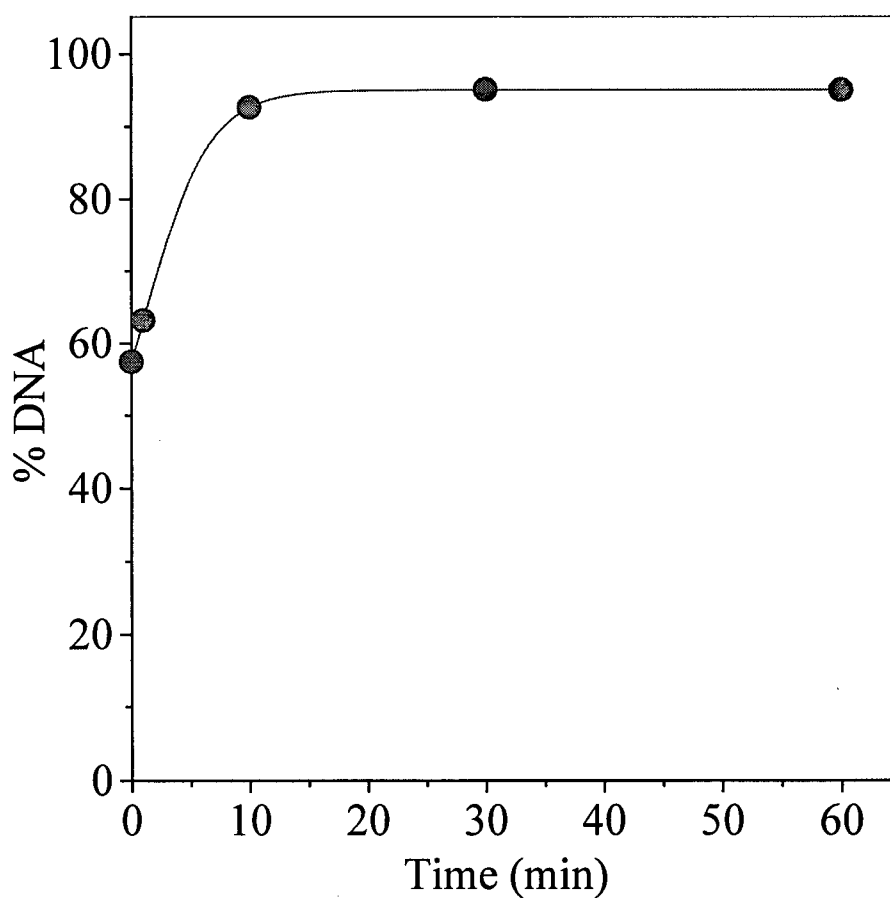
Figure 2.4

Recovery of plasmid DNA and radiolabeled lipid following Bligh and Dyer extraction

The % amount of DNA (10 μg) in the organic (**A**) and aqueous (**B**) phases was determined following Bligh and Dyer extraction in the absence of DODAC. Trace amounts of ^{14}C-DODAC (~5.0 pmoles) expressed as % of initial added in the organic (**C**) and aqueous (**D**) phases following Bligh and Dyer extraction in the presence of excess DNA (10 μg).
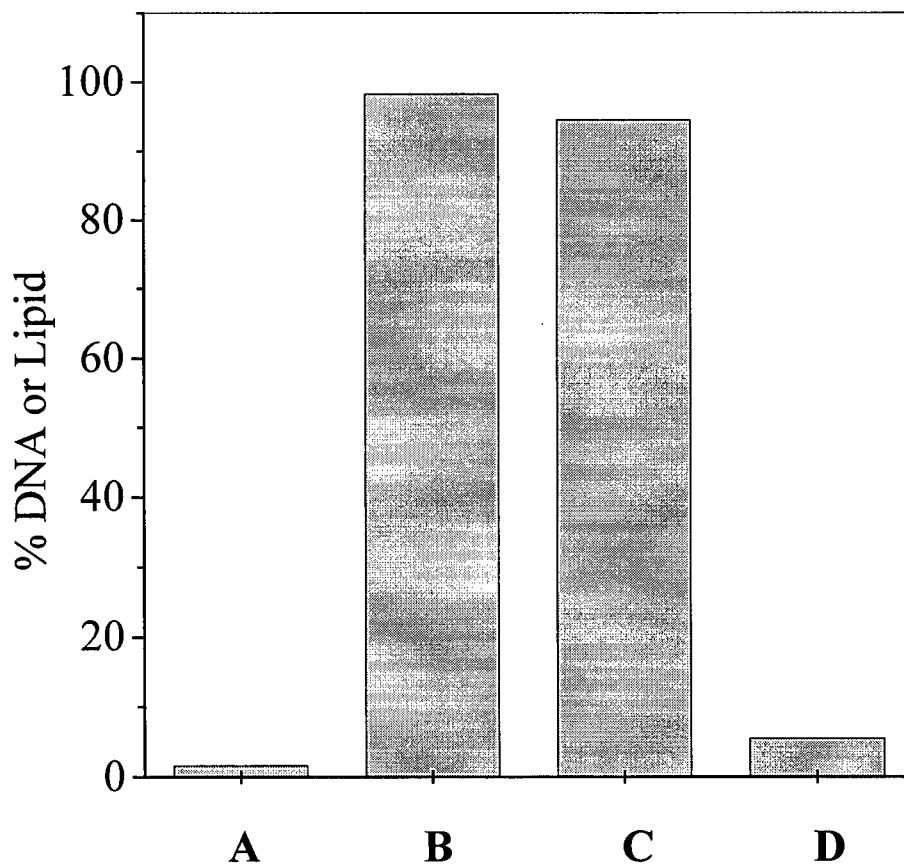
2.3.2 Analysis of lipid – DNA binding

The data presented thus far strongly suggest that the formation of the hydrophobic complex between DNA and the monovalent cationic lipid DODAC occurs at the aqueous/organic interface after phase separation. The binding of cationic lipid and DNA at this interface was evaluated and is shown in Figure 2.5. In this analysis, an estimate of bound lipid is plotted as a function of added lipid for a system that contains 40 μg DNA. The estimated bound lipid was calculated from the amount of DNA recovered in the organic phase after addition of various amounts of lipid and where the amount of lipid added was limiting. The amount of bound lipid was calculated using the assumption that each anionic phosphate charge on DNA recovered in the organic phase was complexed to one cationic lipid. The unoccupied binding sites on the DNA was considered to be free ligand whereas DNA in the organic phase was completely bound by lipids. For example, under conditions where >95% of the DNA (40 μg) was recovered in the organic phase, it was estimated that 160 nmoles lipid were bound. The sigmoidal curve obtained is indicative of a reaction that exhibits positive co-operativity and the corresponding Hill plot of the data (Figure 2.5 inset) - which is linear (r = 0.97) and has a slope of 1.73 - is also consistent with positive co-operativity. Similarly, Figure 2.6 showing DNA bound plotted as a function of DNA added reinforces this idea. Over a large range of DNA concentrations, the amount of DNA in the organic phase correlated directly with the amount of DNA added to the system, provided that the amount of lipid present was not limiting. A Scatchard analysis of these data exhibited a bell shaped curve, which is characteristic of reactions that exhibit positive co-operativity. For highly co-operative binding reactions the apparent dissociation constant (K_n) can be estimated within regions where near-saturation was achieved; for this system, the estimated

Figure 2.5

Evaluation of the extent of cationic lipid to DNA binding at the interface

The correlation between estimated lipid bound to 40 μg DNA and added lipid. The amount of bound lipid was estimated from the amount of DNA recovered in the organic phase after Bligh and Dyer extraction assuming that each nucleotide phosphate bound one cationic lipid. **Inset**: corresponding Hill Plot.
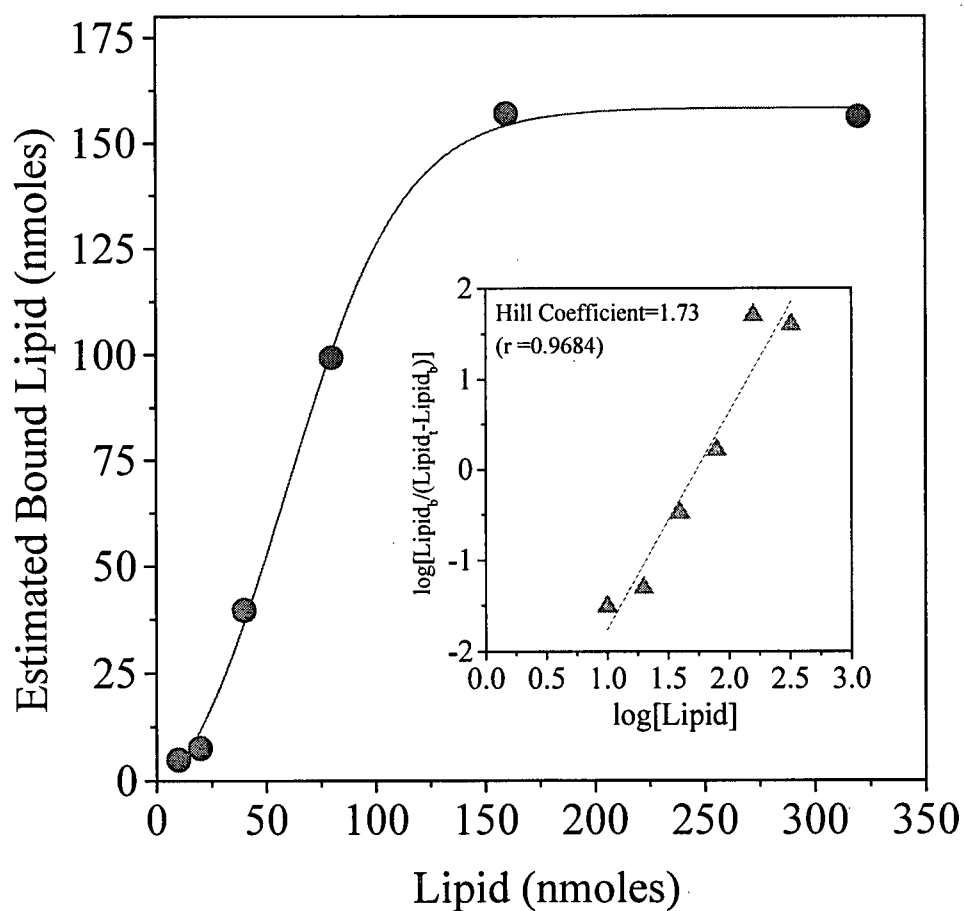
Figure 2.6

Correlation between DNA bound and DNA added

Amount of DNA bound (DNA in the organic phase) per amount of lipid (at the interface) as a function of DNA added and extracted using the Bligh and Dyer method.
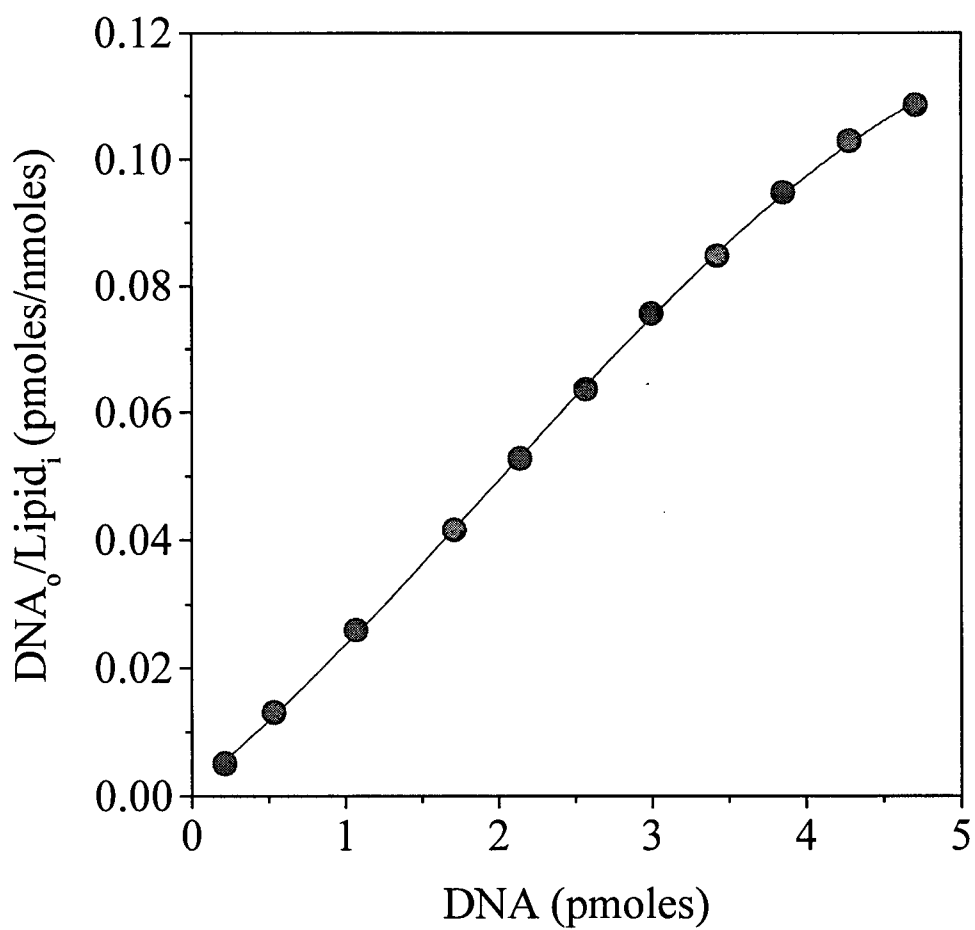
K_n was 2.03×10^{-11} mol/L, and additionally, at saturation, n (pmoles DNA/nmole lipid) was estimated to be 0.1188. The inverse of this value (1/n) was the estimate of the amount of lipid bound to each DNA molecule (8,417 moles lipid bound per mole DNA). Therefore, the charge ratio (+/-) at saturation was estimated to be 0.585 based on these binding curves. It is important to note that the Scatchard analysis required no assumptions regarding the number of lipid monomers bound to the DNA - however, the method employed was not based on direct measurement of free lipid.

2.3.3 Effect of neutral and anionic lipids on formation and dissociation

This interest in the binding reactions that resulted in formation of a hydrophobic cationic lipid/DNA complex has focused on methods for preparing well-defined lipid-DNA particles (LDPs) for use in gene therapy. As indicated in the study by D.L. Reimer *et al.*, two approaches could be considered (203): First, particles could be prepared from solutions where cationic lipids, secondary lipids and DNA were mixed in the presence of detergent as described in Chapter 3 and (313). Second, solvent-based approaches could be considered where hydrophobic cationic lipid/DNA complexes were mixed with selected lipids prior to solvent removal (lyophilization or rotoevaporation) and subsequently hydrated to achieve particle formation (see Chapter 6) and (314). For the latter approach to be viable, it is important to assess complex formation and stability in the presence of other lipids. The presence of secondary lipids can affect lipid/DNA complex formation in two ways:

In a similar manner to DODAC, added amphipathic lipids can arrange themselves at the interface such that the headgroups would be oriented towards the aqueous phase. Since the interface represents a finite surface area, these added lipids would displace cationic lipids

and effectively reduce the amount of DODAC at the interface (area between the aqueous and organic phase).

Alternatively, the added amphipathic lipid may interact directly with the cationic lipid to prevent complex formation. For these reasons, secondary lipids may affect both complex formation as well as complex stability.

Studies evaluating complex destabilisation, where 40 nmoles neutral or anionic lipids were added to pre-formed DODAC/DNA complexes, are summarised in Figure 2.7. The results in Figure 2.7A indicate that zwitterionic lipids, such as DOPE and DOPC and the neutral lipid cholesterol, had little or no impact on the stability of the DODAC/DNA complex. The anionic lipid DSPE-PEG [a poly(ethylene glycol) polymer conjugated to DSPE] also did not affect complex stability (Figure 2.7B). In contrast, the presence of anionic lipids such as DOPS, DMPG and LPI destabilised the complex (Figure 2.7B). This was most evident when DMPG was the secondary lipid which, when present in equimolar amounts to DODAC, completely dissociated the complex even when prepared at a cationic lipid to DNA charge ratio (+/-) of 2.0.

A second approach assessing the effect of added lipids on cationic lipid/DNA complex formation and/or destabilisation was based on a lipid titration where the secondary lipid amount was increased in a system containing 10 µg DNA and 40 nmoles DODAC (Figure 2.8). The second lipid, either DOPE or DOPC, was added either before or after complex formation. These lipids were selected on the basis of results shown in Figure 2.7 that indicated that DOPC and DOPE have little effect on complex stability. Three points are evident from these studies and are summarised in Figure 2.8:

Figure 2.7

Effect of other lipids added to the recovery of DNA

Neutral lipids (**A**) and anionic lipids (**B**) were added to assess the recovery of DNA (10 μg) from the organic phase following formation of DNA/DODAC complexes. Varying amounts of DNA were added to 40 nmoles of DODAC prior to addition of other lipids. Amphipathic lipids used were DOPE (●), DOPC (▲) and Cholesterol (◆). As a control, DOPE was added to DNA in the absence of DODAC (□). Anionic lipids used were DOPS (○), DMPG (▽), DSPE-PEG (▼) and LPI (△). DODAC was also added to DNA in the absence of all other lipids (■). Data points were averaged from three replications and expressed ± SEM.
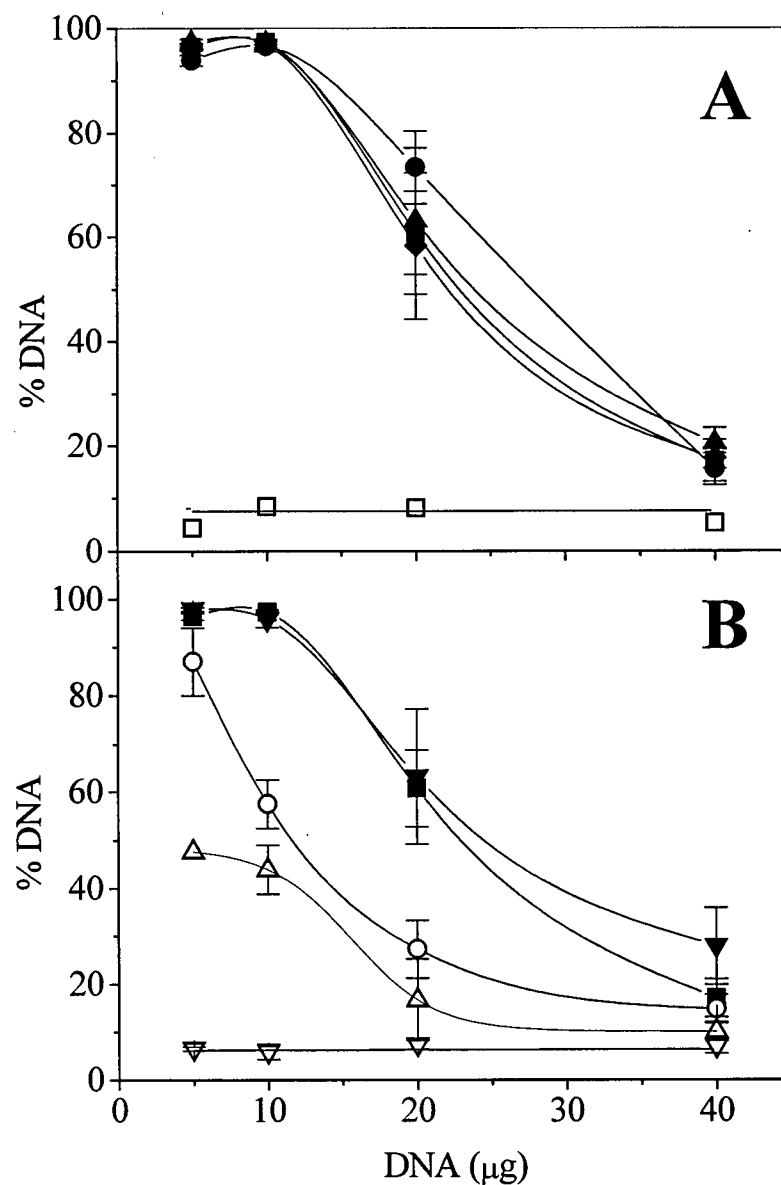
Figure 2.8

Effect of DOPC vs DOPE on formation and destabilisation of the hydrophobic lipid/DNA complex

Increasing amounts of DOPC (**A**) and DOPE (**B**) were added to assess the recovery of plasmid DNA (10 μg) in the organic phase following Bligh and Dyer extraction. Amphipathic lipids were added under conditions before (■) or after (●) formation of the complexes [i.e.. addition of cationic lipid, DODAC at (40 nmoles)].
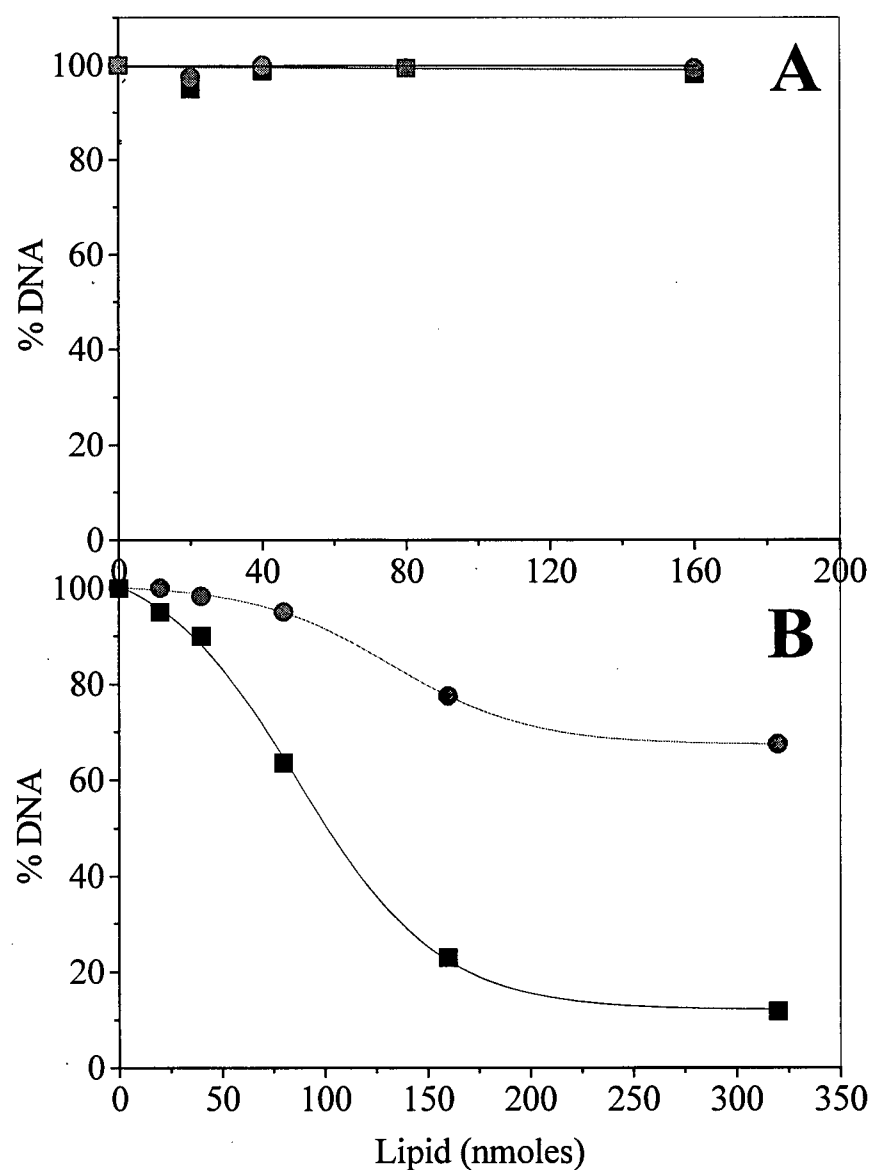
First, as shown in Figure 2.8A, the addition of DOPC had no impact on formation or stability of the DODAC/DNA complex even at levels approaching 10-fold molar excess relative to DODAC.

Second, the cationic lipid/DNA complex was destabilised by the addition of increasing amounts of DOPE (Figure 2.8B). When DOPE was added in amounts that were >2-fold molar excess relative to DODAC a reduction in DNA recovered in the organic phase was observed. Vigorous mixing of this sample in the two-phase system lead to near complete dissociation of the complex.

Third, complex formation was inhibited when DOPE was added prior to complex formation (Figure 2.8B). These results strongly indicate that DOPE influences DODAC/DNA binding, an effect that is likely a consequence of direct DOPE/DODAC interactions.

2.4 Discussion

D.L. Reimer *et al.* demonstrated that cationic lipid binding to DNA engenders formation of a hydrophobic complex which can be isolated in organic solvents (203). This previous study suggested that the complex could be formed in the presence of neutral detergents and dissociated after the addition of NaCl at concentrations as low as 1 mM. Plasmid DNA in this complex was sensitive to DNase I and susceptible to intercalation of small DNA binding fluorescent probes suggesting that it was not in a form that was condensed. I conclude that this hydrophobic complex, prepared under well-defined conditions, serves as the intermediate in the preparation of lipid-DNA particles (LDPs) for DNA delivery *in vitro* and *in vivo*. The studies described in this chapter further characterise

76

binding reactions between cationic lipids and DNA in an aqueous/organic system where the lipids exist as free monomers or in micellar form.

Other investigators have characterised the binding of mono- and di-valent cations to anionic lipids using a two phase partitioning assay similar to the one used in this report (315, 316). Binding parameters were obtained by determining the anionic lipid-dependent movement of cations from an aqueous phase to an organic phase. These previous studies suggested that the binding reaction involved formation of an inverted micelle where the cation was sequestered within a structure where the hydrophobic moieties were oriented towards the organic phase. Although the number of cationic lipids affecting redistribution of DNA into the organic phase is significantly greater than would be expected for a small cation like Ca^{2+}, an inverted micelle-like structure can also be envisioned for the hydrophobic lipid/DNA complex. Lipids that are bound to the nucleotide polymer through ionic as well as hydrogen bonding interactions will surround DNA in the organic phase.

It is evident from these results that hydrophobic cationic lipid/DNA complex formation is achieved through a binding reaction that occurs at the aqueous/organic interface following Bligh and Dyer extraction. This likely involves DNA - a multivalent anion - binding to cationic lipids that are arranged at the interface with their acyl chains extending into the organic phase and their ionic headgroups orienting toward the aqueous phase. Plasmid DNA binding to lipids at the interface is illustrated by the reaction scheme shown in Figure 2.9. This reaction involves a binding reaction between two multivalent systems, DNA (which carries 14,400 negative charges), and the charged surface at the aqueous/organic interface. The formation of the hydrophobic cationic lipid/DNA complex would progress *via* a reaction that exhibits positive co-operativity, a conclusion supported by sigmoidal lipid

Figure 2.9

Reaction scheme proposed for the DODAC/DNA binding reaction that occurs at the aqueous/organic phase interface.

The charge-mediated interaction between DODAC and DNA occurs at the interface after formation of the two-phase system. Plasmid DNA can be recovered from the organic phase if sufficient cationic lipids neutralise the phosphate charges on the DNA. It is unknown whether lipids and the lipid/DNA complex exist in equilibrium.

Nucleotides $(DNA)_a$ + Charged Interface $(Lipids)_i \Leftrightarrow$ Lipid/DNA Complex$_o$

DNA_a + Lipids$_i \Leftrightarrow$ Lipid/DNA Complex$_i$ + Lipids$_i \Leftrightarrow$ Lipid/DNA Complex$_o$ + Lipids$_o$

$(DNA)_a$		Phase I: Aqueous (a)
+		
$(Lipids)_i$ $\Rightarrow$ $(Lipid/DNA\ Complex)_i$		Phase II: Charged Interface (i)
$\uparrow\downarrow$	$\downarrow$	
$(Lipids)_o$ $\Leftrightarrow$ $(Lipid/DNA\ Complex)_o$		Phase III: Organic (o)

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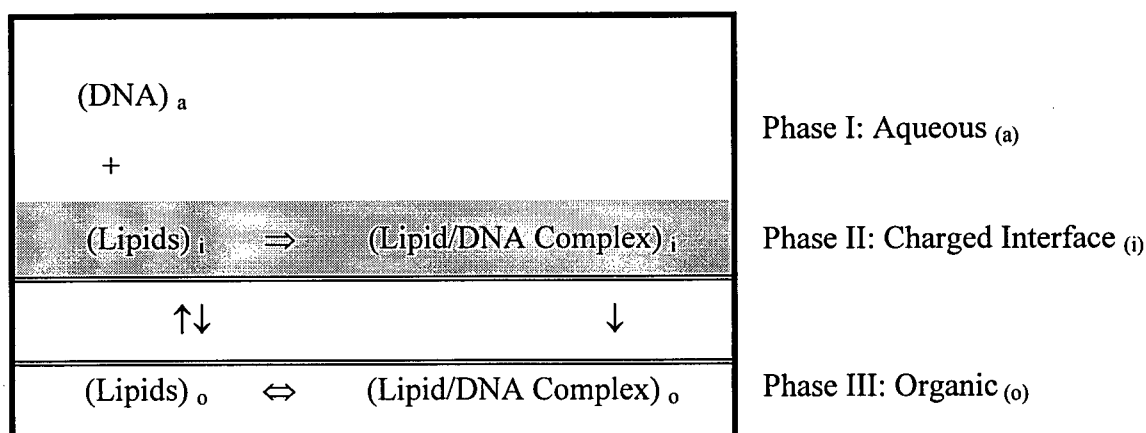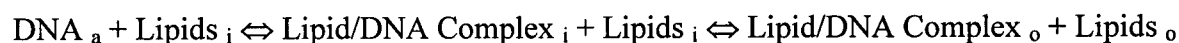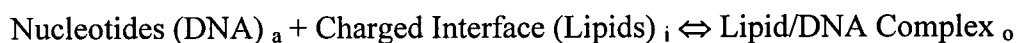
binding curves with a Hill coefficient of 1.73 (Figure 2.5) and a bell-shaped Scatchard plot. It should be noted that binding curves obtained for multivalent interactions exhibiting positive co-operativity are difficult to analyse. However, these results indicate that the formation of the hydrophobic complex requires lipid binding, partial charge neutralisation and hydrophobic interactions.

Based on the reaction mechanism proposed in Figure 2.9, I believe that the presence of secondary lipids can affect lipid/DNA complex formation and/or stability in several ways.

The added amphipathic lipids could displace cationic lipids at the interface ultimately reducing the amount of cationic lipid to DNA binding. This effect would be most pronounced when using a lipid such as the DSPE-PEG, where the large hydrophilic headgroup would orient at the interface as predicted and would also provide a charge shielding effect (165).

Alternatively, the added lipids may directly interact with the DODAC, preventing and/or inhibiting this cationic lipid from binding to the nucleotide phosphate groups. Under the conditions employed here, where the interfacial area and lipid concentration at the interface are not controlled, it is likely that lipid-lipid interactions rather than interfacial displacement would be the most significant factor affecting complex formation. Thus, the charge shielding effect of the PEG polymer likely does not prevent complex formation through steric interactions only; instead charge shielding reduces the ionic interactions between the DSPE-PEG anionic headgroup and DODAC at the interface. In contrast, anionic lipids that lack the PEG headgroup will bind DODAC and interfere with formation and stability of the complex. This is apparent when using the anionic lipids - DOPS, DMPG

and LPI - that destabilise the complex (Figure 2.7). Ionic interactions between DODAC and the added anionic lipid would result in competitive inhibition of DODAC binding to DNA.

The anionic lipid DMPG is a more effective inhibitor of complex stability then DOPS, a result that suggests that DODAC interaction with the phosphoglycerol headgroup is stronger than the interaction of DODAC with phosphoserine. The reduced effect of DOPS and LPI on the inhibition of DODAC/DNA complexes compared with that of DMPG may be explained by differences in the ability of these anionic lipids to form intermolecular hydrogen bonding [see (317) for review]. DMPG exhibits little intermolecular hydrogen bonding; therefore, DMPG may have an increased propensity to interact with the cationic DODAC solely through charge-charge interactions. DOPS and LPI, alternatively, exhibit higher levels of intermolecular bonding, reducing the propensity to interact with the cationic lipid, DODAC.

Zwitterionic lipids (such as DOPE and DOPC), cholesterol and the anionic PEG-PE are less effective in terms of destabilising pre-formed cationic lipid/DNA complexes. When evaluating the effects of the zwitterionic lipid DOPE, inhibition of the complex formation was observed if the amount of DOPE present in the system described previously increased above a DODAC/DOPE mole ratio of 2.0. This result suggests that there is an interaction between the cationic lipid DODAC and DOPE. This interaction may be due to hydrogen bonding between the phosphoethanolamine headgroup (114, 317) and the cationic headgroup of DODAC as described in Chapter 1, section *1.3.3.2.1*. Alternatively, since the ethanolamine group of DOPE has a titratable amine function there may be sufficient quantities of the anionic DOPE to affect DODAC binding to DNA. The latter is unlikely since the pH of the assay system used here is approximately 6.5 for the organic and aqueous

phases and the pK_a of the ethanolamine group is 9.5. If the pH of the system is increased one might expect an increase in the capacity for the ethanolamine headgroup of DOPE to interact with DODAC through ionic interactions. Regardless of the mechanisms controlling DODAC/DOPE interaction, these results clearly demonstrate the potential for DOPE to interact with cationic lipids and this interaction may be relevant in liposomal systems used for complexing polynucleotides. The importance of DOPE/cationic lipid interactions with respect to the development of transfection systems based on liposomes prepared from DOPE and cationic lipids is equivocal.

The ability to study lipid/DNA binding reactions using the systems described in this chapter provides a reasonable approach for characterising and selecting novel cationic lipids that are being considered for use in gene transfer applications. Furthermore, factors that can alter DNA binding to cationic lipid surfaces can be easily identified without generating large heterogeneous aggregates that typically occur when mixing DNA with cationic liposomes. D.L. Reimer *et al.*, for example, have shown that DNA does not appear to condense as a consequence of charge interactions or cationic lipid binding (203). Research programs synthesising novel cationic lipids (187) could use this assay to characterise differences in DNA binding to these cationic lipids in well-defined systems. Additionally, the presence of contaminants (such as endotoxins) in DNA preparations may be detectable through subtle changes in cationic lipid binding as measured by the procedure described. A major advantage of this assay system is its reproducibility and predictability in forming lipid/DNA complexes under controlled conditions. The interest in the binding reaction, however, is based on the fact that a hydrophobic lipid/DNA complex can be isolated in organic solvents and used subsequently for the preparation of lipid-DNA particles (LDPs). It will be possible

to make use of the hydrophobic nature of this complex to generate novel systems that may, in turn, be designed to promote gene transfer. These systems can be generated with the use of secondary lipids added to stabilise the complex following removal of solvents and/or addition of water or buffer such as that described in Chapter 6.

In summary, one cannot preclude the possibility that DODAC/DNA hydrophobic complexes are formed when the cationic lipid is added to DNA in a Bligh and Dyer monophase. However, following phase separation of the monophase into an organic and an aqueous phase this complex must dissociate with DNA being retained in the aqueous phase and lipid being extracted into the organic phase. These results indicate that after phase separation DNA binds cationic lipids at the interface between the organic and aqueous phases. This binding reaction is highly co-operative and is likely a consequence of multivalent interactions between DNA and the cationic interface consisting of a specifically oriented monolayer of cationic lipids. Complex formation can be effectively inhibited when adding secondary lipids that bind DODAC. Such effects were anticipated for the anionic lipids such as DMPG, LPI and DOPS; however, these results show that the zwitterionic lipid DOPE also affects complex formation, perhaps through an interaction with DODAC. This observation is assessed in more detail in the following chapter.

CHAPTER 3

PHOSPHATIDYLETHANOLAMINE MEDIATED DESTABILIZATION OF LIPID-BASED PLASMID DELIVERY SYSTEMS: DNA DISSOCIATION IN REGULATING TRANSGENE EXPRESSION[*]

3.1 Introduction

Structure-activity relationships of cationic liposome-DNA complexes have been difficult to assess due to the diverse range of structures that are generated when using formulation strategies involving preformed cationic liposomes. Further, it is apparent that any single preparation can contain a wide range of structures in varying proportions and it is unknown which of these morphologically distinct structures contribute to transfection activity. Following DNA addition to liposomes significant alterations in the liposome structure, which occur as a consequence of electrostatic and hydrophobic interactions, lead to transient generation of mixed lipid micelles and/or monomeric lipids (136). In turn, this may facilitate fusion, hemifusion or other bilayer destabilisation events. Alternatively, these intermediate lipid structures may be available to bind directly to DNA, leading to formation of a hydrophobic lipid/DNA complex as described in Chapter 2 and (313, 318).

Considering the incertitude of formulations relying on preformed cationic liposomes, a method has been established whereby cationic lipid and DNA are combined in a systematic manner in the presence of detergents (318, 319), thereby bypassing the steps involving liposome formation followed by DNA-mediated liposome destabilisation. In this procedure, cationic lipids and any other lipid species of interest are solubilized as mixed lipid micelles

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prior to the addition of plasmid DNA. Ion-pairing between the DNA and cationic lipids is believed to generate a hydrophobic complex (203). Additional lipid incorporation into the hydrophobic lipid DNA complex is anticipated on the basis of hydrophobic interactions. Under appropriate conditions, these intermediates spontaneously form lipid-DNA particles (LDPs), where hydrophobic effects and solvent re-organisation are the main driving forces promoting particle formation. This chapter demonstrates that it is possible to generate novel LDPs from a wide variety of lipid species, under well-defined conditions and in a stepwise manner. Studies in this chapter with the resulting LDPs were developed in an attempt to expand the understanding of the interactions that govern lipid binding and dissociation. In particular, these studies correlate lipid headgroup and lipid-DNA interactions with formulation stability, destabilisation and transfection activity both *in vitro* and *in vivo*.

3.2 Materials and Methods

3.2.1 Materials

Dimethyldioctadecylammonium bromide (DDAB) and N-octyl β-D-glucopyranoside (OGP) were purchased from Sigma Chemical Company (St. Louis, MO). DLPE, DMPE, DPPE, DSPE, DLPC, DMPC and DSPC were obtained from Northern Lipids Inc. (Vancouver, B.C.) or Avanti Polar Lipids Inc. (Alabaster, AL). The DNA intercalating fluorescent dye, TO-PRO-1, was acquired from Molecular Probes (Eugene, OR). Radiolabeled [^{14}C]-chloramphenicol was from NEN Dupont (Boston, MA). Dialysis tubing (SPECTRA/POR, mwco:12,000-14,000) was obtained from Fisher Scientific (Ottawa, ON).

All other materials used are described in Chapter 2, section *2.2.1*.

3.2.1.1 Plasmid preparation

The plasmid pINEXCATv2.0 was obtained from Roger Graham of Inex Pharmaceuticals Corp. The *Escherichia coli* chloramphenicol acetyl transferase (CAT) gene, including the alfalfa mosaic virus (AMV) translational enhancer, was cleaved from pCMV4CAT plasmid (generously provided by K. Bringham, Toronto, ON) using the *NotI* restriction endonuclease. Similarly the *NotI* fragment of pCMVβ, obtained from Clontech (Palo Alto, CA), was removed and the CAT gene inserted into this site. The resulting plasmid, pINEXCATv2.0, is 4490 bp and includes the CMV promoter, the SV40 intron for processing the message, the AMV enhancer and the SV40 polyadenylation signals. Radiolabeled pINEXCATv2.0 was prepared using methyl-[^{3}H]-thymidine-5'-triphosphate (Dupont NEN, Boston, MA). The plasmid was isolated by standard molecular techniques (311) and purified using a Qiagen Plasmid Purification Kit (Qiagen Chatsworth, CA). The nucleic acid concentration was measured by UV absorption at 260 nm and purity was verified by electrophoresis on a 0.8% agarose gel.

3.2.2 Methods

3.2.2.1 Bligh and Dyer extraction

The monocationic lipid DODAC (40 nmoles) and the pINEXCATv2.0 plasmid (10 μg) were solubilized separately in a Bligh and Dyer monophase consisting of chloroform:methanol:water (1:2.1:1) (312). The effects of zwitterionic lipids (DLPE, DMPE, DPPE, DSPE, DOPE, DMPC DSPC, DOPC) on the formation and/or dissociation of DODAC/pINEXCATv2.0 complexes were evaluated. The effects of the additional lipids on the formation of the complexes were evaluated by mixing them with pINEXCATv2.0 prior to

the addition of DODAC (final volume of 1 mL). The monophase mixture was subsequently partitioned into two phases by the addition of 250 μL each of chloroform and water. The samples were mixed vigorously by vortexing for 1 min and centrifuged at 600×g for 5 min at room temperature. In order to assess dissociation, the DODAC/pINEXCATv2.0 complexes were formed prior to the addition of other lipids. Zwitterionic lipids were injected directly into the organic phase following Bligh and Dyer extraction and formation of the two-phase system. For both procedures the upper aqueous phase (~1.0 mL) was removed and the amount of DNA in the aqueous phase was determined. The estimated pH was found to be 6.5 as determined by using pH paper obtained from EM Science (Cherry Hill, NJ). DNA in the aqueous phase was quantified by measuring the optical density at a wavelength of 260 nm using a luminescence UV Spectrophotometer (DU-64) from Beckman Instrument Inc. (Fullerton, CA). Data collected by this method were presented as percentage of DNA recovered in the organic phase. As a control, DOPE was added to the DNA in the absence of DODAC to ensure that DOPE alone did not mediate extraction of the DNA into the organic phase.

3.2.2.2 Solubilization of pre-formed vesicles

Stock solution of lipids in chloroform:methanol were evaporated under N_2 to obtain a thin lipid film and then lyophilised for 1 h at a pressure <50 mtorr in a freeze-dry system (LabConco, Kansas City, MO). The lipid films were hydrated in sterile distilled water to form multilamellar vesicles (MLVs) by mechanical agitation. The final lipid concentration was 10 mM. Lipid vesicles were then diluted to a concentration of 1 mM in water and titrated against 100 mM OGP. The titrated solutions were well mixed prior to measuring

light scattering intensity at 600 nm. The critical solubilization concentration of the lipids in OGP was determined at the minimal concentration of OGP required for the optical density at 600 nm to reach <0.01. The critical solubilization concentration of different lipids reported represents the mean from 3 different experiments ± SD.

3.2.2.3 Preparation of liposome-DNA aggregates (LDAs)

Pre-formed DODAC/DOPE liposomes were obtained from Inex Pharmaceuticals Corp. (Vancouver, B.C.). These liposomes had a mean diameter of 80±20 nm before the formation of LDAs as determined by quasi-elastic light scattering (QELS, Nicomp submicron particle sizer operating with an helium/neon laser at 675.8 nm). DNA was diluted into the same volume of water as the liposomes. Both DNA and liposomes were placed on ice prior to mixing. Following DNA addition to liposomes, liposome-DNA aggregates immediately formed as indicated by a change in sample turbidity. The samples were placed on ice for 30 min before use and sizes were determined using QELS. Diameter of conventional LDAs were consistently between 200 - 800 nm.

3.2.2.4 Preparation of lipid-DNA particles (LDPs)

A lipid film consisting of cationic and neutral lipids was prepared as described above for solubilization of pre-formed vesicles. After the film was dried to remove the residual solvent, the lipids were solubilized with at least 35 mM OGP, a non-ionic detergent. In order to ensure solubilization, the lipid solution was heated briefly at 50°C. If the sample remained turbid, 1 M OGP was added dropwise until the solution was no longer visibly cloudy. The lipid solutions (3520 µM total lipid) were incubated on ice until ready for particle formation.

The plasmid, prepared in OGP (concentration equal to that used to prepare the mixed detergent-lipid micelles), was diluted to a concentration such that mixing equal volumes of the DNA (up to 280 μg/mL) and lipid solution would achieve the desired lipid-DNA ratio. DNA was added in one addition to the lipid solution and the resulting solution immediately became turbid. Unless stated otherwise the final concentration of DNA and total lipid was 140 μg/mL and 1760 μM, respectively. The mixture was vortexed and placed in dialysis tubing. LDPs were dialysed against sterile distilled water for 72 h at 4°C with a complete change of water at 12 h intervals. Particle size distribution and homogeneity were evaluated after dialysis by QELS. The resulting LDPs, when prepared at 4°C, had mean diameters between 50 - 150 nm.

3.2.2.5 Dye exclusion assay

The cationic, cyanine dye, TO-PRO-1, fluoresces under conditions when it is intercalated to DNA (320). LDPs at charge ratios (+/-) of 2:1 and 4:1, and cationic lipid:neutral lipid ratios of 1:1 and 1:4 were generated as described above. Two μg of formulated DNA was added to a quartz cuvette containing water in a final volume of 500 μL. Subsequently, 1 μL of TO-PRO-1 was added to these samples to achieve a final concentration 1×10^{-6} M. Fluorescence was measured at room temperature using a Luminescence Spectrophotometer 50B (Perkin-Elmer, Norwalk, CT) at an excitation wavelength of 509 nm, slit width of 2.5 mm, and an emission wavelength of 533 nm, slit width of 5.0 mm. All samples containing TO-PRO-1 were maintained in the dark to minimise photobleaching. Initial fluorescence was determined as (I). In order to determine

the I_F (maximum fluorescence intensity), OGP (50 µL) was added to a final OGP concentration of 100 mM. Dye exclusion indices were calculated as: $[(I_F-I)/I_F]\times100$.

3.2.2.6 Differential scanning calorimetry

Lipid samples of DSPC, DPPE and DDAB for DSC experiments were prepared from stock solutions of pure lipids or by mixing a given amount of the solutions to achieve molar ratios of 2:1 or 1:2 for DDAB: DSPC or DPPE. The lipid solutions (typically containing 3 - 5 µmoles lipid) were dried to a thin film by blowing N_2 gas followed by drying under vacuum for 3 - 5 h. The lipid was then hydrated in 1 mL phosphate buffered saline (pH ~7.2) by vigorous vortexing at temperatures well above the phase transition temperatures of the lipids (>80°C). After cooling to room temperature, exactly 500 µL of the lipid solutions were transferred to the sample cell and placed in the calorimeter for scanning. The DSC measurement was carried out when the system reached equilibrium and DSC thermograms were recorded with a computer-controlled Microcal MC-2 high-sensitivity differential scanning calorimeter (Microcal Inc., Northampton, MA) operating at heating scan rate of 30°C/h. The data acquired were analysed with Origin software (Microcal Software Inc., Northampton, MA). Approximately 3 - 5 scans were recorded for each sample to ensure reproducibility and both heating and cooling endotherms were generated for each sample.

3.2.2.7 Serum stability assay

LDPs and LDAs were prepared as described above. Two times volume of water or normal mouse serum (Cedar Lane, Hornby, ON) were added to the each sample containing 2 µg DNA. The samples were mixed and incubated at 37°C for 2 h. Samples were removed

from the water bath and were made up to a constant volume with water. One equivalent of buffered phenol was added and each sample vortexed vigorously. The samples were centrifuged at 600×g for 15 min to separate the phases. The DNA in the resulting aqueous phase was precipitated using ethanol and the results analysed by a 0.8% agarose gel electrophoresis.

3.2.2.8 In vitro *transfection*

B16/BL6 murine melanoma cells were plated at 4×10^3 cells/well in a 96-well plate in DMEM supplemented with 10% fetal bovine serum (FBS). Twenty-four hours later, media was removed and 100 µL containing 2 µg of pINEXCATv2.0 of free DNA, LDAs or LDPs were added to the cells and incubated for 4 hours. Subsequently, media was removed and replaced with 200 µL fresh media and incubated for another 48 h. The cells were analysed for CAT activity as previously described (321). After protein extraction, 25,000 dpm of [^{14}C]-chloramphenicol and *N*-butyryl-CoA (5 mg/ml) were added to each sample and incubated for 2 h at 37°C. Mixed xylenes (Aldrich Chemical Co., Milwaukee, WI) were added followed by vigorous mixing and separation by centrifugation (8000×g). The upper aqueous phase was removed and washed with buffer. Subsequently 100 µL of the aqueous phase was removed and Pico-fluor scintillant was added to determine the amount of radioactivity. CAT units were derived from a standard curve. Each assay was evaluated three separate times with at least 3 well replicates per sample and reported as ± SEM.

3.2.2.9 In vivo *transfection and delivery*

Seven week old C57/BL6 (Charles River, Montreal, PQ) female mice were injected i.p. with 1×10^5 B16/BL6 cells. Seven days after tumour cell inoculation LDPs or free DNA was injected i.p. with 500 μL of a sample containing 70 μg/mL DNA. At 24 h post-injection the tumours were excised, frozen at -70°C for at least 24 h and subsequently analysed for CAT activity (321). For delivery studies, [^{3}H]-DNA (~25,000 dpm) was used to prepare the LDP formulations and tissues were harvested and processed by addition of Solvable™ and incubated overnight at 50°C. Scintillation fluid was added to 200 μL homogenates and radioactivity was determined by scintillation counting using a Packard TR 1900 Scintillation Counter. Each group contained 4 animals and assay results were reported as ± SEM.

3.2.2.10 Statistical analysis

All data values for transfection studies are presented as the mean ± standard error of the mean. A Student's t-test was performed on treated and untreated samples comparing independent or dependent results using Statistica software. Differences were considered significant if the *p*-value <0.05 and *p*-values are reported.

3.3 Results

3.3.1 Lipid interactions

Previous studies and Chapter 2 demonstrated that the formation of LDPs is mediated through the generation of a hydrophobic DNA/lipid complex intermediate (203, 313). The formation and destabilisation of this hydrophobic intermediate provides a useful tool to assess interactions between the lipids and DNA. This is demonstrated by the data shown in

Figure 3.1, where the influence of defined diacylphosphatidylethanolamines [DLPE (12:0),
DMPE (14:0), DSPE (18:0), DOPE (18:1)] and diacylphosphatidylcholines [DLPC (12:0),
DMPC (14:0), DSPC (18:0), DOPC (18:1)] on formation and destabilisation of the
hydrophobic cationic lipid DNA intermediate was studied. These data describe the amount
of DNA recovered in the organic phase as increasing amounts of zwitterionic lipids were
added. In order to determine whether the lipids had an effect on formation of the
hydrophobic cationic lipid/DNA complex, the zwitterionic lipids were added to solubilized
cationic lipid before addition to the plasmid DNA in the Bligh and Dyer monophase (Figure
3.1A). The effect of the phospholipids on dissociation was determined by adding them to
preformed hydrophobic cationic lipid/DNA complexes (Figure 3.1B). Regardless of whether
PC-containing lipids were added before or after lipid-DNA complex formation, all the DNA
was recovered in the organic phase, even at >8-fold molar excess of PC-lipids over cationic
lipids. This result is consistent with Chapter 2 and previous reports (272, 313) and clearly
demonstrates that diacylphosphatidylcholines have no impact on formation or destabilisation
of hydrophobic cationic lipid/DNA complexes.

When similar studies were completed in the presence of
diacylphosphatidylethanolamines, prevention of formation as well as dissociation of the
preformed lipid/DNA complex was observed. For example, when there was an 8-fold molar
excess of DSPE, there was greater than 95% inhibition of complex formation (Figure 3.1A,
▲) and complete destabilisation of pre-formed complexes (Figure 3.1B, ▲). Interestingly,
the effect of diacylphosphatidylethanolamines was dependent on the acyl chain composition.
Specifically, inhibition of complex formation by these lipids was greater as acyl chain length
increased; i.e. inhibition with DLPE was less than that observed with DMPE. In contrast,

Figure 3.1

Effect of lipids on the formation and dissociation of the hydrophobic lipid/DNA complex

Various helper lipids were added before (formation) (**A**) and after (dissociation) (**B**) addition of cationic lipid (40 nmoles) to DNA (10 μg) and extracted using the Bligh and Dyer extraction procedure (see Methods). The % DNA recovered in the organic phase was determined in the presence of increasing amount of lipids: DLPE (■), DMPE (●), DSPE (▲), DOPE (▼), DLPC (♦), DMPC (+), DSPC (x), DOPC (∗).
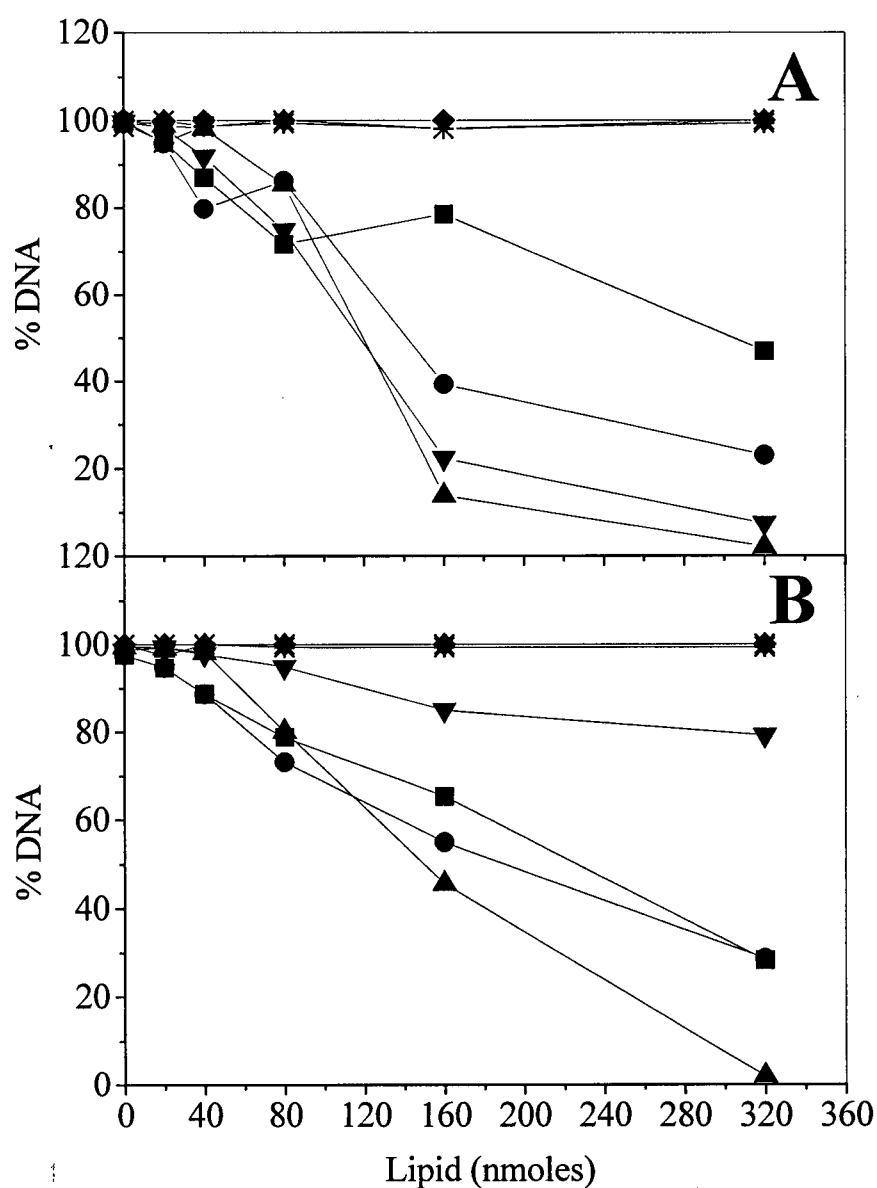
measurements of complex destabilisation suggested that DOPE was much less effective than DSPE in terms of facilitating dissociation of the hydrophobic complex. It is worth noting that phospholipid headgroup-mediated effects on complex formation and dissociation may be the result of the sum of two separate events. It has already been postulated that the potential of ethanolamine headgroup interactions with phosphate groups on the DNA may displace bound cationic lipids (272). Alternatively, the cationic lipid headgroup may interact with the headgroup of the added lipid. The role of acyl chain composition in regulating these interactions is speculative but likely requires use of lipids which are miscible. For this reason, studies were initiated using DSC to assess the miscibility of cationic lipid/phospholipid mixtures.

Differential scanning calorimetry (DSC) is a well established technique for the evaluation of the thermodynamic properties of lipids and lipid mixtures (322), where lipid-lipid interactions can be detected as shifts in the phase transition temperature as well as broadening of the width of the thermal transition and changes in the transition enthalpy (114). For the studies reported in this chapter, lipids were selected (DDAB, DSPC and DPPE) with acyl chain lengths that differed by two carbons in length or less because these lipids typically exhibit good miscibility as measured by DSC (323). Lipids were also chosen to have at least a 10°C difference in their L_β (gel) to L_α (liquid-crystalline) phase transition temperatures (T_c). For this reason, I anticipated that changes in the phase properties of the lipid mixtures would primarily be a consequence of headgroup interactions. In addition, the lipids selected exhibited distinct T_cs when analysed in isolation. The cationic lipid DDAB ($T_c = 47°C$) was utilised in place of DODAC. DODAC could not be used since its low T_c (<0°C) could not be determined using the apparatus employed. These lipids have identical

headgroup structure (DODAC is a chloride salt while DDAB is a bromide salt), but the acyl chain of DODAC is unsaturated while DDAB is not. Mixtures of DDAB and DSPC (T_c = 54°C) or DPPE (T_c = 64°C) were studied and the resulting heating endotherms are shown in Figure 3.2. It should be noted that the heating and cooling endotherms were identical for all samples studied. The gel to liquid-crystalline phase transitions for each of the pure phospholipids were comparable to those reported previously [DSPC: (324); DPPE: (325)]. The cationic lipid, DDAB, showed a sharp and highly co-operative main phase transition temperature of 47°C and a broader pre-phase transition at approximately 40°C. When evaluating mixtures of DDAB and the selected phospholipids, two points are worth noting: First, at a mole ratio of 1:2 (cationic lipid to phospholipid) the transition temperature of DDAB was completely abolished; second, at DDAB to DPPE mole ratios of 2:1 (Figure 3.2B), the thermogram was unimodal, exhibiting a broad endothermic peak at 58°C - a temperature between that of the main phase transitions of DDAB and DPPE. This suggests that these lipids are miscible. In contrast, the thermogram was bimodal for mixtures of DDAB:DSPC (2.5:1 mole ratio) where the T_c of DDAB was still present at 47°C, but a second (broad transition) was observed with a peak at 53°C - data suggesting that these lipids are less miscible. The DSC data shown in Figure 3.2 is not sufficient to conclude cationic lipid/PE headgroup interactions; however, these data do suggest, in contrast to combinations of DDAB:DSPC, that DDAB:DPPE mix better.

3.3.2 Characterisation of lipid-DNA particles

The results presented thus far demonstrate differences in cationic lipid/phospholipid interactions that depend on whether the phospholipid has a choline or ethanolamine

Figure 3.2

Differential scanning calorimetry thermogram of lipid mixtures

Lipid mixtures consisted of DSPC (**A**) or DPPE (**B**) and DDAB, a cationic lipid. Thermograms were obtained at molar ratios of 2:1 or 1:2 for DDAB: DSPC or DPPE. Exactly 500 µL of the lipid solutions was scanned at a heating scan rate of 30°C/h from 15°C - 90°C by a computer-controlled Microcal MC-2 high-sensitivity differential scanning calorimeter. The data acquired were analysed with Origin software. Each pure lipid sample or lipid mixture was scanned 3 - 5 times to ensure reproducibility. Heating and cooling thermograms were identical.
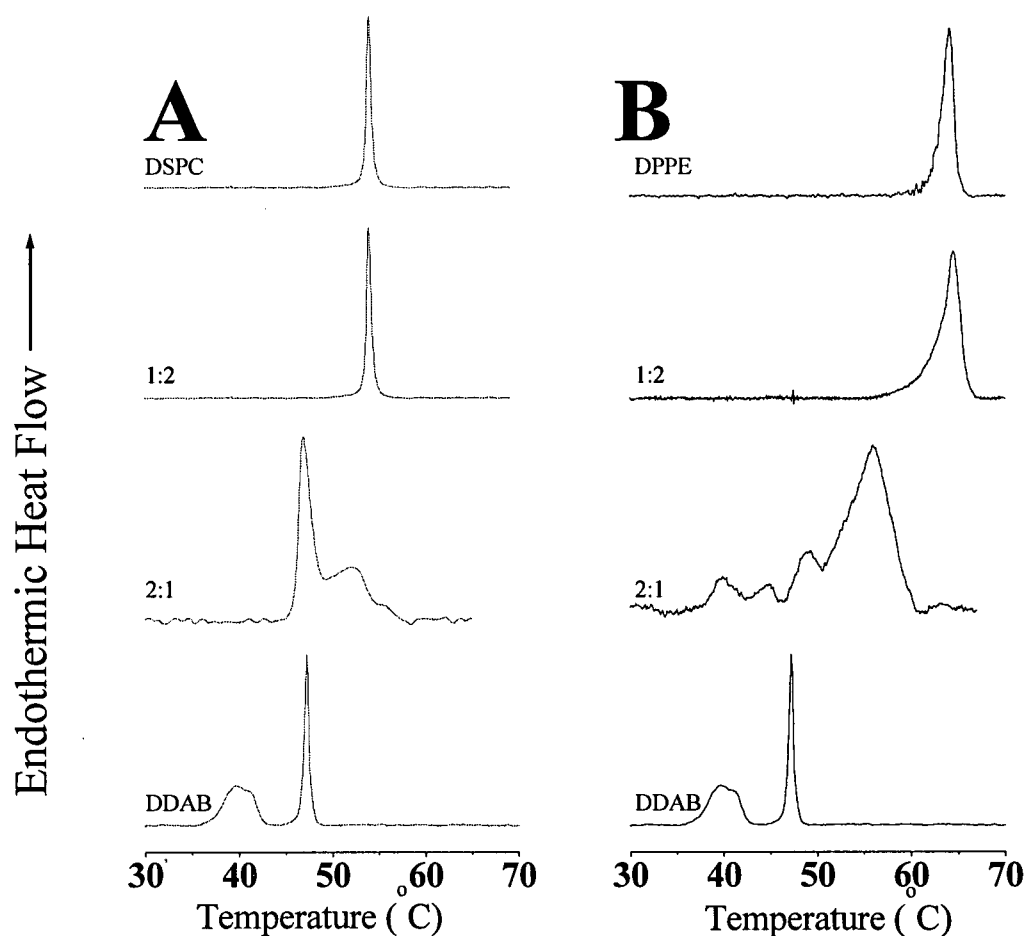
headgroup. In order to assess how PE-cationic lipid interactions affect the attributes of LDPs formed using these lipids (in contrast to diacylphosphatidylcholines-cationic lipid mixtures), particles were generated from hydrophobic lipid-DNA complex intermediates prepared from lipid-detergent mixtures. LDPs can be prepared by various means, provided that one step leading to particle formulations involves formation of hydrophobic lipid-DNA complex. As such, the lipids used must be monomers or mixed lipid-detergent micelles. Lipid solubility measurements for these lipids were defined by the critical solubilization concentration (CSC), or the minimal concentration of OGP required to decrease the optical density at 600 nm of 1 mM of lipid (initial concentration) in water to <0.01. In general, MLVs prepared from lipids containing the choline headgroup are solubilized at lower concentration of OGP than required to solubilize MLVs prepared from lipids with the ethanolamine headgroup. Based on these results, LDPs were generated at OGP concentrations higher than the critical solubilization concentrations shown in Table 3.1. On a practical level, if the concentration of OGP is below the CSC, large aggregated structures formed upon DNA addition to the OGP lipid mixtures.

The temperature at which LDPs formed proved to be a second critical parameter in the preparation of small uniform LDPs prepared when using varying lipid compositions. This is illustrated by the data shown in Table 3.2, where the effect of temperature on the size of LDPs was assessed. LDPs were prepared from lipid mixtures consisting of the cationic lipid, DODAC, co-solubilized in OGP (35 mM) with DOPE, DOPC, DLPE or DLPC. Mean diameters, measured by QELS, were between 130 - 160 nm for all formulations of LDPs prepared at 4°C. When the LDPs were prepared at room temperature (~25°C), the mean diameter of the resulting particles was >200 nm. Particle formation at 37°C produced visible

Table 3.1

Critical solubilization concentration used in LDPs preparation

Lipid vesicles at 1 mM in water were titrated against OGP. The critical solubilization concentration of the various lipids was determined as the minimal concentration of OGP required for the OD_{600} to reach <0.01.

Lipid at 1 mM	CSC of OGP (mM)[*]
DODAC	15 ± 1
SM	18.0 ± 0.0
DOPC	28 ± 1
DLPC	26.0 ± 0.0
DOPE	34.3 ± 0.5
DLPE	33 ± 1

*Value represents the mean from 3 different experiments ± SD. The critical solubilization concentration of the lipid in OGP is defined as the minimal concentration of OGP required to decrease the optical density at 600 nm.

Table 3.2

Effect of mixing temperature on the size of LDPs

LDPs were formulated with DNA at a charge ratio (+/-) of 2:1 and with a phospholipid to DODAC ratio of 1:1. Selected lipids were solubilized in OGP as described in the methods and subsequently mixed with DNA also prepared in OGP. Samples were incubated at the indicated temperatures prior to mixing and the detergent was removed by dialysis also at the same temperatures. The resulting particles were analysed by QELS.

Temperature	Phospholipids used in combination with DODAC in the preparation of LDPs			
	DOPC	DOPE	DLPC	DLPE
4°C	138±69[*]	130±63	154±86	148±87
25°C	212±129	198±112	291±168	223±145
37°C	>1000	>1000	>1000	>1000

[*]Size in nm is reported as the mean from at least 3 different samples ± SD.

aggregates that were >1000 nm, a size distribution that could not be determined accurately using QELS. Based on these results, all LDPs used for subsequent experiments were generated at 4°C.

As indicated in the previous two paragraphs, conditions were established whereby LDPs, exhibiting comparable size distributions as evaluated by QELS measurements, could be prepared from a variety of zwitterionic phospholipids and the cationic lipid DODAC. In order to establish how effectively LDPs - consisting of diacylphosphatidylethanolamine or diacylphosphatidylcholine - protected associated DNA, two assays were used: TO-PRO-1 binding and DNA degradation in mouse serum.

TO-PRO-1 fluoresces when the dye interacts with DNA through intercalation. For example, when DNA is in a condensed form, dye binding is inhibited and no fluorescence is observed. TO-PRO-1 fluorescence was measured following its addition to LDPs prepared with DODAC plus either DOPC, DOPE, DLPC, or DLPE and the results have been summarised in Table 3.3. The formulations were prepared at cationic lipid:DNA charge ratio (+/-) of 2:1 and 4:1 and mole ratios of cationic lipid:phospholipid of 1:1 and 1:4. Dye binding measurements are summarised through use of a dye exclusion index derived as described in the Methods. When the dye exclusion index approaches 100, the DNA assumes a form that is not accessible to the dye. There are two important observations that can be made regarding the data shown in Table 3.3.

First, regardless of whether diacylphosphatidylethanolamine and diacylphosphatidylcholine are used, LDP formulations prepared at a lipid ratio of 1:1 (cationic lipid:phospholipid) show high dye exclusion indices (>77). The high dye exclusion index is observed at both charge ratios (+/-) examined (2:1 and 4:1).

Table 3.3

Dye exclusion indices of DNA in LDPs

LDPs were formulated at $4^{\circ}C$ with DNA at charge ratios (+/-) of 2:1 and 4:1 and with cationic lipid to phospholipid ratios of 1:1 and 1:4. TO-PRO-1 was added to LDP formulations and the fluorescence, I, was determined as described in the Methods. Fluorescence was also measured after addition of OGP to a final concentration of 100 mM and this value was recorded as I_F. Dye exclusion indices were calculated as $[(I_F-I)/I_F]\times100$. For reference, poly-L-lysine condensed DNA typically shows a dye exclusion index of >95.

Charge Ratio (+/-)	Lipid molar ratio (DODAC: Phospholipid)	Dye exclusion indices			
		Phospholipids used in the preparation of LDPs			
		DOPC	DOPE	DLPC	DLPE
2:1	1:1	86	87	78	93
2:1	1:4	33	61	25	77
4:1	1:1	90	88	87	97
4:1	1:4	34	63	26	85

Second, formulations prepared with a 4-fold molar excess of phospholipids (relative to the cationic lipid) exhibited lower dye exclusion indices indicative of poor DNA protection. However, as shown in Table 3.3, LDPs that are prepared with diacylphosphatidylethanolamines exhibited dye exclusion indices (63 - 85) that were substantially greater than those observed for LDPs prepared with diacylphosphatidylcholines (dye exclusion indices of <35).

Mouse serum-mediated degradation of DNA within the LDP formulations was evaluated and a representative result is shown in Figure 3.3. LDPs prepared from DODAC and either DOPE, DOPC, DLPE or DLPC were incubated with normal mouse serum for 2 h at 37°C. Plasmid DNA was recovered from these samples as described in the Methods, and DNA integrity was analysed by electrophoresis in a 0.8% agarose gel. Under the conditions used, free DNA is degraded completely as visualised by the low molecular weight band (arrow). A second control, consisting of plasmid DNA formulated with preformed DODAC/DOPE (1:1 mole ratio, 2:1 charge ratio) liposomes, was completed and, as expected, a proportion of the associated DNA was protected as indicated by the distinct high MW bands. These bands are characteristic of the linear and supercoiled forms of DNA. LDPs offer a similar (in comparison to the liposome-based formulation) level of protection; however, there was greater DNA protection provided by LDP formulations prepared using DODAC and diacylphosphatidylcholines. In this study the formulations were prepared using a cationic lipid/phospholipid ratio of 1:1 and a 2:1 charge ratio (+/-). Following incubation in mouse serum, those LDPs that contain diacylphosphatidylethanolamines (DOPE and DLPE) exhibited an increase in the low molecular weight band and decreased intensity of the characteristic plasmid bands.

Figure 3.3

Serum stability of DNA formulated in LDPs

LDPs were made up using the helper lipids - **DOPC, DOPE, DLPC,** or **DLPE. Free DNA, LDAs** and **LDPs** were incubated in normal mouse serum for 2 h at 37°C. The DNA was isolated using phenol-chloroform extraction and the integrity of the DNA was analysed using 0.8% agarose gel electrophoresis. M is the molecular weight marker. As controls, DNA was extracted in the absence (labelled as **pINEXCATv2.0**) and presence (labelled as **Free DNA**) of serum. LDP formulations, containing the cationic lipid DODAC and the indicated phospholipid, were prepared at charge ratios (+/-) of 2:1 and DODAC to phospholipid ratios of 1:1. For comparison, DNA was also prepared as **LDAs**, by mixing DNA with pre-formed liposomes prepared of DODAC and DOPE (1:1 mole ratio).
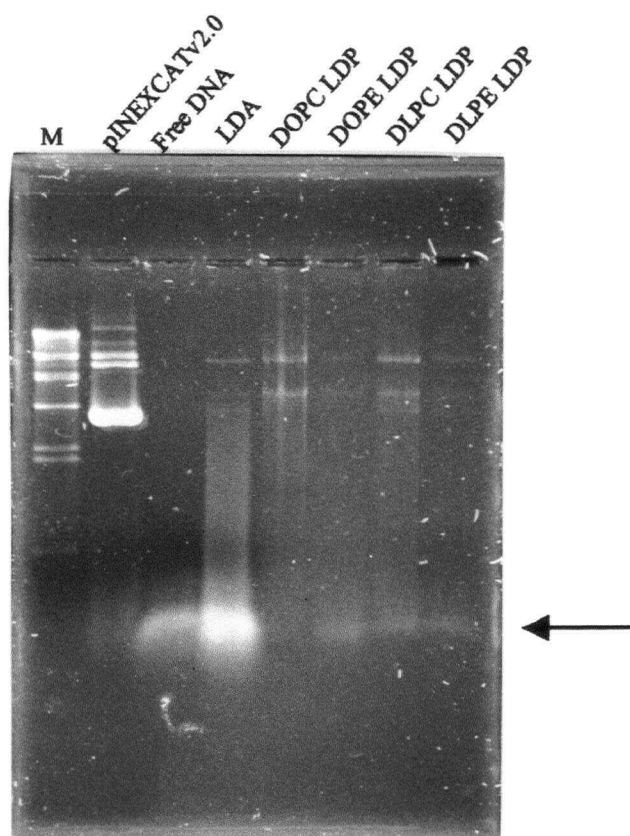
3.3.3 Transfection and delivery using LDPs

In addition to characterising DNA protection and DNA accessibility to the intercalating dye TO-PRO-1, the influence of LDP phospholipid composition on transgene expression and plasmid DNA delivery was assessed using *in vitro* and *in vivo* assays. The plasmid expression vector used throughout these studies contained the gene encoding chloramphenicol acetyl transferase (CAT). Transfection *in vitro* was assessed by measuring CAT activity in murine B16/BL6 melanoma cells that had been incubated with the various LDP formulations and free DNA as described in the Methods. The use of cationic lipids in the formulation resulted in 10 - 20% reduction in cell number. However, this was consistent for all formulations regardless of the type of secondary lipid added. The results of these *in vitro* studies are shown in Figure 3.4. Free DNA, in the absence of any carrier, did not transfect these cells as indicated by the scarcely detectable levels of CAT. CAT expression achieved with LDP formulations prepared with diacylphosphatidylcholine, although higher than that obtained using free plasmid, was relatively low. When the LDPs were prepared using diacylphosphatidylethanolamines the level of CAT expression increased to values in excess of 8 mU/μg protein. The differences in transfection achieved using DLPE and DOPE containing formulations were not significantly different and both were at least an order of magnitude greater than that achieved with LDPs containing diacylphosphatidylcholine. The level of CAT expression achieved following incubation of B16/BL6 cells with plasmid DNA formulated using preformed DODAC/DOPE [1:1 mole ratio, 2:1 charge ratio (+/-)] liposomes was not significantly different from that obtained using DODAC/DOPE LDPs. This was not a result of decreased amount of cell-associated DNA.

Figure 3.4

In vitro transfection of B16/BL6 cells

B16/BL6 melanoma cells were plated at 4×10^3 cells/well in a 96-well plate containing DMEM and 10% FBS and grown up overnight. Lipid-based formulations containing the cationic lipid DODAC and various phospholipids were made up at charge ratios (+/-) of 2:1 and DODAC to phospholipid ratios of 1:1. LDPs (containing helper lipids **DOPC, DOPE, DLPC, or DLPE**) or **free DNA** were added and incubated in DMEM and 10% FBS for 4 h. Media was removed and replaced with fresh media for a further 48 h. The level of chloramphenicol acetyltransferase (CAT) activity was measured as described in the Methods. Values were determined from three replications and expressed as mean ± SEM.
*p<0.00005 when comparing DOPC to DOPE LDPs
*p<0.005 when comparing DLPC to DLPE LDPs
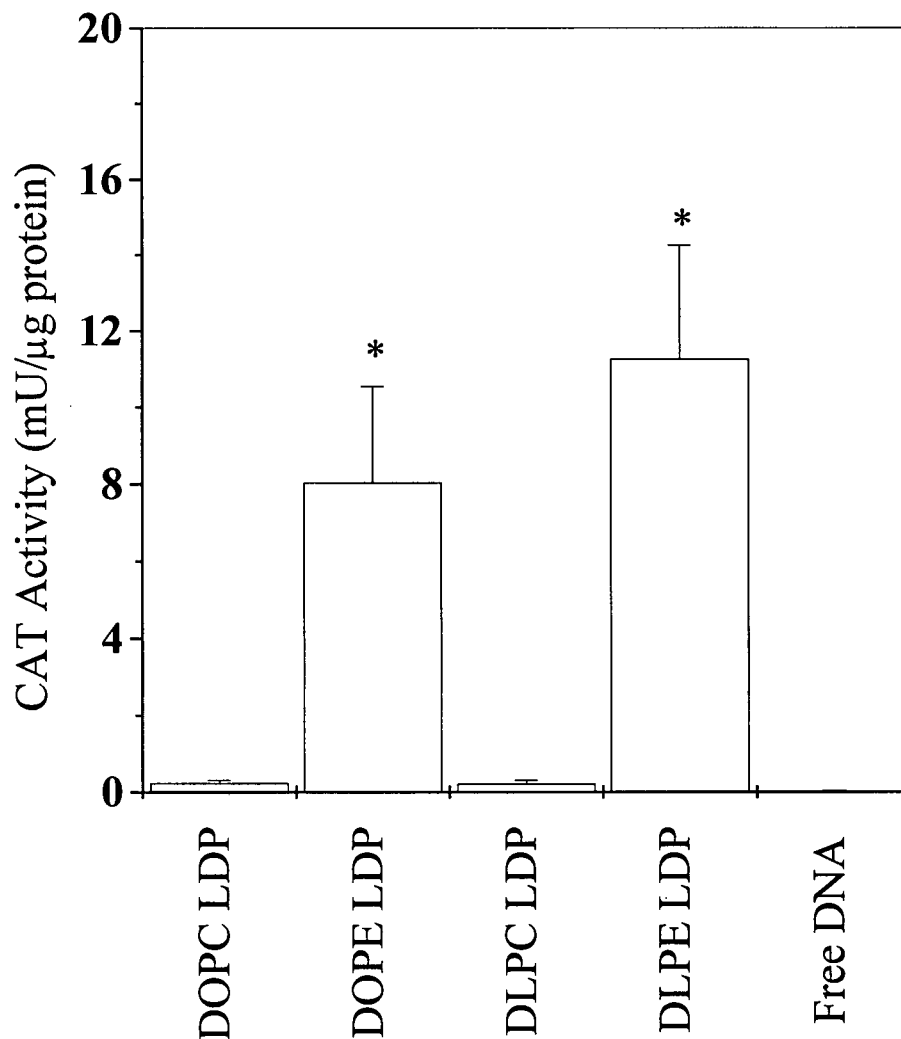
The *in vivo* transfection and biodistribution results for the same four formulations of LDPs utilized in the *in vitro* studies are shown in Figure 3.5. These data were obtained using an *in vivo* B16/BL6 model previously characterised (195). This model described in the Methods, measures transgene expression in small (<100 mg) tumours that arise after i.p. injection of B16/BL6 cells. Seven days after tumour cell inoculation, the animals were injected i.p. with LDP formulations prepared with DODAC and the indicated phospholipid. Twenty-four hours later, tumours were removed and CAT activity was determined (Figure 5A). Similar to the *in vitro* data, LDPs prepared with diacylphosphatidylethanolamines showed increased transfection activity (2- to 4- fold) in comparison to those prepared with diacylphosphatidylcholines. The latter formulations showed no significant differences in CAT expression when compared to free DNA. It is important to note that the transfection activity of the LDPs evaluated here, despite being equivalent in terms of *in vitro* transfection activity, was at least 10× less than that of formulations using LAAs (195). In order to determine whether delivery of the DNA *in vivo* was limiting the transfection activity of LDPs, radiolabeled DNA was used in the preparation of the LDPs. Radiolabeled DNA formulated within LDPs was injected i.p. into mice 7 days after implantation of B16/BL6 tumours. Twenty-four hours later the tumours were removed, processed and the amount of DNA associated per gram of tumour was determined. Figure 3.5B shows that the amount of DNA associated with tumours is significantly greater when the DNA is given as LDPs in comparison to free DNA. Further, no significant differences in tumour-associated plasmid DNA were observed when comparing LDPs prepared using DODAC and diacylphosphatidylcholines or diacylphosphatidylethanolamines although there was a

Figure 3.5

In vivo transfection of and delivery to B16/BL6 i.p. tumours in C57/BL6 mice

LDPs were administered i.p. to C57/BL6 female mice inoculated with B16/BL6 cells (i.p.) 7 days before. Transfection (**A**) and delivery (**B**) of DNA to B16/BL6 tumours was assessed using CAT activity or radioactivity [^{3}H], respectively. LDPs or **free DNA** was injected at a dose of 35 μg pINEXCATv2.0 per animal. LDP formulations used were at a charge ratio (+/-) of 2:1 and DODAC to phospholipid (**DOPC, DOPE, DLPC, DLPE**) ratio of 1:1. Tumours were harvested 24 h post-injection and analysed for chloramphenicol acetyltransferase (CAT) activity (**A**) or amount DNA (**B**) (See Methods). There were 4 animals per group and data were expressed as mean activity ± SEM.
*$p<0.05$ when comparing DOPC or DLPC to DOPE or DLPE LDPs, respectively
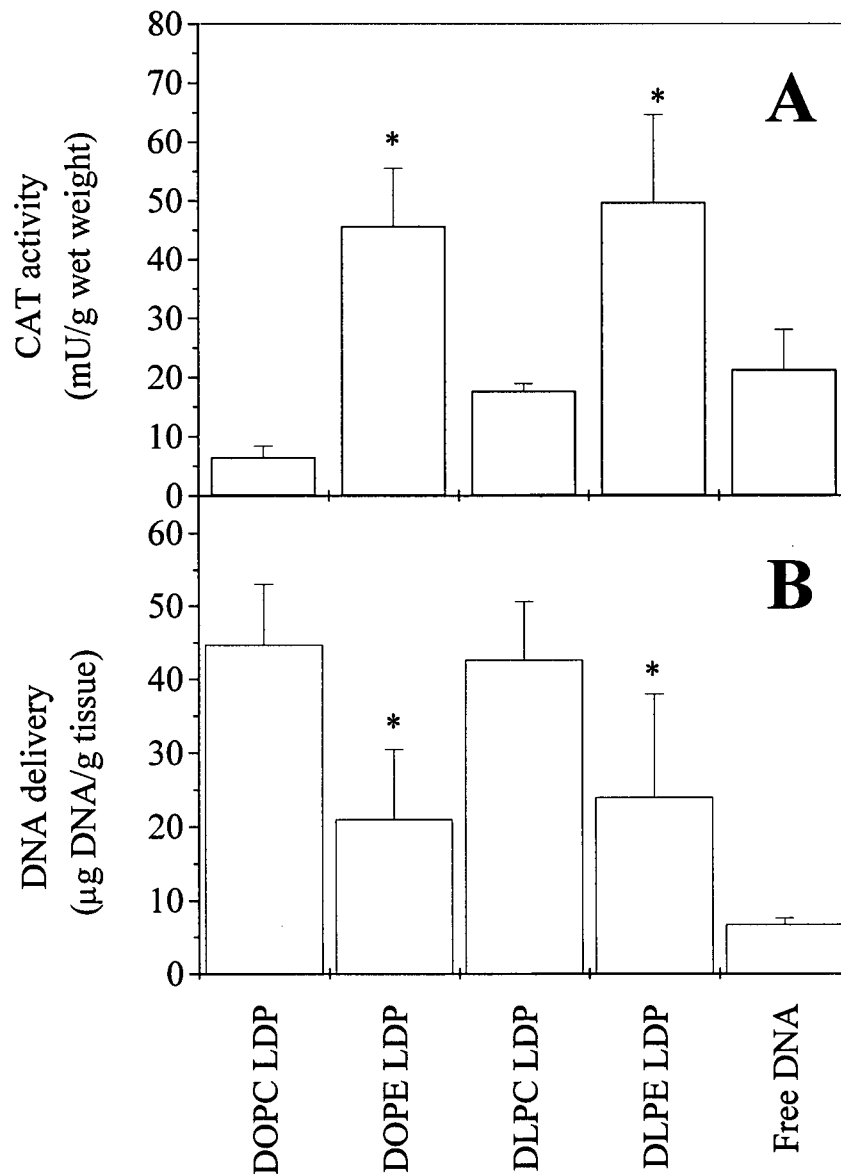
tendency to observe less DNA delivery when using LDPs prepared using diacylphosphatidylethanolamines.

3.4 Discussion

The ability to establish structure/function relationships for cationic liposome formulations designed to bind, protect and deliver plasmid expression vectors has been severely hampered by the heterogeneous structures that arise from cationic lipid-DNA interactions. Many investigators have begun to develop formulation approaches that rely on the use of a well-defined hydrophobic lipid-DNA complex intermediate which, in aqueous solutions, spontaneously adopt a heteromolecular structure (318, 319, 326, 327). This chapter characterised these formulations with the aim of developing a greater understanding of how lipid-lipid and lipid-DNA interactions can influence transfection activity.

It is important to note at the onset of this discussion that the LDP formulations described here can be prepared using a variety of lipid components. Macromolecular assembly of the lipid/DNA particle is dependent on binding of a cationic lipid, such as DODAC. As shown in Table 3.2 and Table 3.3 structures exhibiting comparable mean size distributions of <150 nm can be prepared under appropriate conditions (4°C and OGP concentrations slightly in excess of that required to solubilize all lipid components) from various lipid compositions. This allows questions, such as the role of lipid headgroup in governing transfection activity, to be addressed in a methodical fashion. Studies have focused on the lipid-mixing behaviour of LDPs in comparison to cationic liposome DNA aggregates (272). In this chapter, an assessment of changes in DNA protection and transfection was made as a function of simple changes in phospholipid headgroup and acyl

chain length. This discussion will extend the argument that the ethanolamine headgroup helps to destabilise cationic lipid-DNA interactions, a process that involves cationic/phospholipid lipid interactions as well as phospholipid/DNA interactions.

Results in Figure 3.1 show unequivocally that phospholipids with an ethanolamine headgroup, in contrast to a choline headgroup, can inhibit formation and cause dissociation of the hydrophobic cationic lipid/DNA complex. I believe that these effects are regulated by factors that involve three entities - lipid headgroup interactions, acyl chain miscibility as well as phospholipid-DNA interactions. The discussion of the first two of these factors will be described, while phospholipid-DNA interactions are described in greater detail in section *4.4* and (272).

It was observed in Figure 3.1 that inhibition of hydrophobic complex formation by combining lipids prior to the addition of DNA was maximal when using diacylphosphatidylethanolamines containing carbon chains of C16 or C18 as opposed those containing C14 and C12. These results may be explained by the schematic shown in Figure 3.6A. Based on the model of complex formation described in detail in Figure 2.9, Chapter 2 and (313), the headgroups of the cationic lipids are aligned along the interface. When modulating lipids are added in combination with DODAC prior to the addition of DNA, this interface will consist of a monolayer matrix of cationic lipid and zwitterionic lipid. The packing parameter of zwitterionic lipid at this interface will be based on its ability to intermembrane mix with the cationic lipid. This is governed by acyl chain miscibility, which, in turn is governed by directly dependent on its length and saturation (328). The presence of the modulating lipid at the interface can then prevent formation of the cationic lipid/DNA complex in one of two ways:

Figure 3.6

Model of diacylphosphatidylethanolamine on prevention of formation and destabilisation

Diacylphosphatidylethanolamines have the ability to confer a decreased electrostatic potential of the cationic lipid at the interface when diacylPEs and cationic lipids were added before DNA (**A**) and after Bligh and Dyer extraction. After hydrophobic complex formation, diacylPE can mix with cationic lipid to prevent binding to DNA (**B**). Serum access and dye binding have very different effects on DNA (**C**).

A   Aqueous Phase

Interface

Organic Phase

B

C   Serum
TO-PRO-1
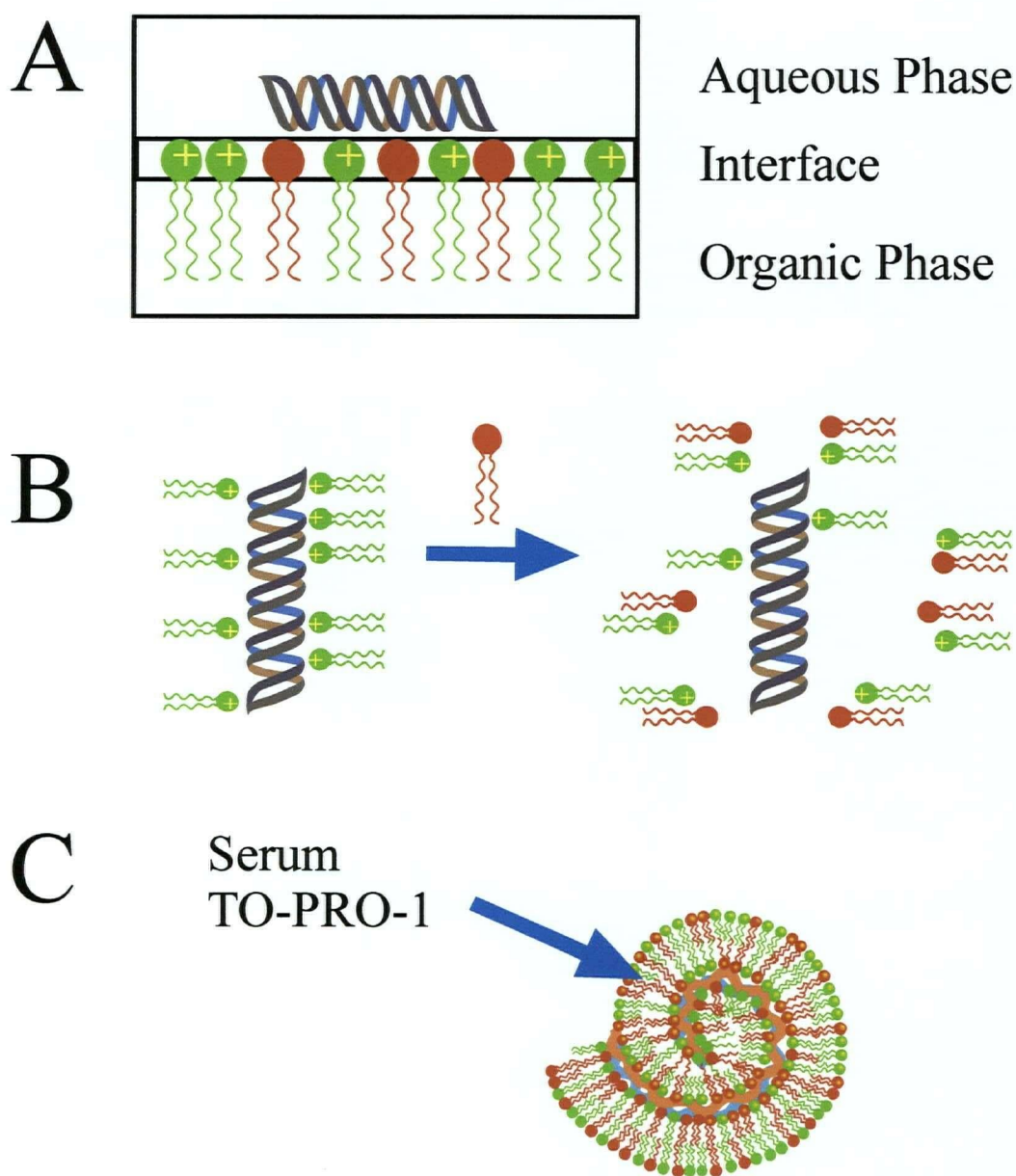
First, the modulating lipid, whose headgroup is effectively neutral, may dilute the cationic lipid charge available at the interface through steric hindrance, thereby preventing sufficient cationic lipid binding to DNA. This is unlikely to be the case since diacylPCs have a much larger headgroup due to the methyl groups on the amine moeity and would likely prevent formation of the hydrophobic lipid/DNA complex to a greater extent than diacylPE. This result was not observed.

The second explanation for diacylPE's ability to prevent hydrophobic lipid/DNA complex formation is based on the ability of the headgroup of the modulating to alter the electrostatic potential of the cationic lipid. While the net headgroup charge is neutral, both diacylPE and diacylPC have the potential to exhibit a negative charge on the phosphate moiety and a positive charge on the amine group at pH 7; with the amine on the diacylPE being titratable. As such, it appears that diacylPE may have a further advantage for interaction with the cationic lipid and contributes to decreasing the electrostatic potential of the cationic lipid. The cationic lipid:diacylPE binding may be facilitated by one or more of a number of interactions, including ionic interaction, dipole effects, hydrogen bonding or salt bridging, which then in turn can be stabilised by an appropriately miscible acyl chain. The specific interaction and the orientation of each of the species at the interface - cationic lipid, modulating lipid, and DNA - remain to be determined.

The ability for a degree of a diacylPE to destabilise the hydrophobic lipid/DNA complex relative to its acyl chain is not longer clear when the modulating lipid was added after cationic lipid and DNA. The rationale may be explained by Figure 3.6B. Penetration through the monolayer would partially depend on the size of overall size of the combined headgroup – cationic lipid and modulating lipid. Due to the smaller headgroup size of

diacylPE the combined charge density of the DODAC-diacylPE dimer is less than that of DODAC-diacylPC. The smaller combined headgroup area (and therefore increased charge density) would mediate an increase in destabilisation of the lipids with the DNA. Further assisting the penetration ability of the modulating lipid is the miscibility between the cationic lipid and modulating lipid. Lipid miscibility may be conferred by the acyl chin or the headgroup. For example, DOPE, which has an unsaturated acyl chain, has a limited ability to penetrate the bilayer when compared to DSPE, while DMPE and DLPE have a median effect.

Another aspect of miscibility may be at the level of the headgroup and results observed using DSC might contribute to an explanation of this phenomenon. While DSC is of limited value in assessing lipid-headgroup interactions, the thermotropic phase behaviour of lipid binary mixtures can be used to determine whether lipids are miscible as related to lipid order/disorder. With regard to this, the lipids selected for this analysis contained acyl chain lengths that should be miscible assuming the absence of headgroup effects. Specifically, phospholipids with acyl chains differing in length by 2 or less carbon atoms mix well (323); therefore, it was anticipated that mixtures of DPPE (16:0)/DDAB (18:1) and DSPC (18:0)/DDAB (18:1) would be highly miscible. The DSC results (Figure 3.2) demonstrate that the DPPE was miscible with DDAB. In contrast, the DSPC/DDAB mixture at a 2.5:1 ratio appeared to be less miscible as indicated by the two distinct peaks. These preliminary data indicate that diacylphosphatidylethanolamines, in contrast to diacylphosphatidylcholines, can mix better with cationic lipids where the difference in miscibility is conferred by the headgroup. Based on these data and the model described in Figure 3.6B, I suggest that in order for destabilisation to take place, the phosphate and the amine moieties on diacylPE cannot be ionised. In contrast to diacylPC, where the phosphate

and quaternary amine remains ionised, diacylPE has a titratable amine that can be deprotonated, with resultant protonisation of the phosphate group. Therefore, diacylPE has the capacity to penetrate the monolayer of cationic lipids to decrease the electrostatic potential of cationic lipid and leading interference with DNA binding.

The extent of DNA protection by the presence of PE or PC upon formation of a LDP can be determined by lipid packing parameters at the DNA/cationic lipid interface. Experimentally, these effects are correlated to accessibility of DNA to TO-PRO-1 and sensitivity to mouse serum. Dye binding data (Table 3.3) confirms that upon formation of LDPs, TO-PRO-1 is excluded to some degree from intercalation to DNA. Dye exclusion indices are similar for both PE and PC formulations when the DODAC:phospholipid ratio is 1:1, whereas DODAC:PE at 1:4 excludes TO-PRO-1 from the plasmid to a greater degree than for DODAC:PC at the same lipid ratio. However, it was observed that the LDP formulations which exhibited high dye exclusion indices, indicative of increased protection, also exhibited decreased stability in serum and DNase I (Figure 3.3 and P. Harvie, personal communication). The acyl chain composition was irrelevant when considering these observations. The data represented in Table 3.3 and Figure 3.3 appear to be contradictory. Results presented here and in previous publications may explain these disparate results (272). Other results from this chapter suggest that the presence of diacylPE can destabilise the cationic lipid/DNA binding. While the serum studies are amenable to this explanation, there are other different factors involved for a small hydrophilic molecule like TO-PRO-1 (Figure 3.6C). Differences in TO-PRO-1 binding for LDPs containing PC versus PE is observed only upon the presence of excess modulating lipid. The presence of diacylPC within LDPs may be able to stabilise TO-PRO-1 intercalation to DNA. Therefore the disparate results of

serum degradation and dye binding may be explained by the fundamental differences in access of serum or dye rather than the stability of binding between cationic lipid and DNA mediated by the different modulating lipid.

Transfection efficiencies achieved with lipid-based transfer vehicles have been attributed to the nature of the neutral lipid used (255, 327). Previous reports, for example, have emphasised the role of DOPE in mediating fusion with either the target cell plasma membrane or the endocytic vesicle membrane (240, 329). Formulations containing lipids that include DOPC induce lower transfection activity than those containing DOPE as the headgroup. These observations are consistent with those reported here, but the increases in transfection are not necessarily directly attributable to DOPE. Rather, enhanced transfection can be attributed to any phospholipid containing the PE headgroup. CAT expression achieved following addition of LDPs containing PE, regardless of the acyl chain composition, was higher than that for PC formulations. Based on the results in Figure 3.5A and 3.5B, differences in transfection efficiency observed between the diacylphosphatidylcholines or diacylphosphatidylethanolamines formulated LDPs were not a consequence of DNA delivery.

It should be noted that in comparison to data obtained using CAT plasmid formulated with pre-formed liposomes, the level of DNA delivery to the B16/BL6 tumours was at least 2- to 3- fold less. Although the liposome-DNA aggregates and LDPs result in gene transfection systems that work equally well *in vitro,* these LDP formulations are much less effective in terms of the *in vivo* transfection model used here. This is a consequence of reduced DNA delivery due in part to enhanced stability, both in terms of physical and chemical attributes of the LDPs. One contrasting property between these formulations that

114

can explain this discrepancy is formulation size. Delivery to the tumour site and subsequent internalisation of lipid-based gene transfer vehicles may be influenced by the size of the delivery system as well as by lipid composition effects.

This chapter has defined some of the basic interactions that are involved in formation of lipid-DNA particles and subsequent DNA release from bound cationic lipids. More specifically, these results further define the role of diacyl-PEs, suggesting that headgroup interactions with cationic lipids and DNA are distinct from properties promoting fusion. The use of LDPs, generated from mixed detergent/lipid micelle interaction with DNA, result in a more versatile system for preparing plasmid expression vector delivery systems. Based on the methodology described here, basic interactions involved in formation, delivery and destabilisation of lipid-based delivery systems due to individual lipid components can be systematically assessed.

CHAPTER 4

ELECTROSTATICALLY MEDIATED INTERACTIONS BETWEEN CATIONIC

LIPID-DNA PARTICLES AND AN ANIONIC SURFACE[*]

4.1 Introduction

As described extensively in Chapter 1, section *1.3*, the transfection of cells, utilising lipid-based gene transfer methods, involves a number of well-defined steps (330). Each step must be successfully achieved so that a gene may be delivered to the cell of interest. A critical first step in transfection requires delivery of the gene transfer system, a process that involves attachment of the delivery system and the associated gene to the cell membrane. Once the gene has reached the desired cell population, it must enter the cell and transfer to the nucleus where it may be efficiently transcribed and translated such that gene expression can be observed.

For lipid-based delivery systems, cationic lipids have been utilised to achieve efficient association of anionic DNA with the carriers (26, 331). Formulation strategies have mainly involved the use of lipids in the form of liposomes (132, 332); however, these strategies have not reached their full potential due to problems caused by DNA cross-linking of liposomes and liposome fusion (129). These reactions are difficult to control and result in formation of large heterogeneous aggregates. In light of the problems encountered with liposomes, novel self-assembling lipid-DNA particles for gene transfer were developed (318). These formulations are structurally distinct from liposomal-based systems and have the advantages of being small, well defined and homogeneous.

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[*] Wong, F.M.P., Bally, M.B. and Brooks, D.E. (1999) *Arch. Biochem. Biophys.* 366:31-39.

Regardless of the technology used for gene transfer, efficient transfection must involve successful binding of the delivery vehicle to the cell membrane. Delivery and attachment of cationic lipid gene transfer vehicles are suggested to be mediated by electrostatic interactions (333). Characterisation of the electrostatic interaction between the anionic surface and cationic lipid will help elucidate important parameters that determine effective binding. In addition, there have been suggestions that gene transfer into the cell requires endocytosis (236) (see Chapter 1, section *1.3.3.1*) as well as spontaneous fusion (255) (see Chapter 1, section *1.3.3.2*) and these steps may directly or indirectly involve electrostatic interactions. Surface charge properties of both the cell membrane and the vehicle will be critical in determining the efficiency and, perhaps, effectiveness of DNA transfer to a cell. Since optimum transfection occurs at cationic lipid to anionic DNA phosphate ratios of greater than 1, the entire carrier system must exhibit a positive charge (26). This has been rationalised on the basis that the cationic particles will interact with the anionic membrane of cells. If this is true then a host of important factors, which regulate surface electrostatic potential, should also be evaluated, including ionic strength, counter-ion species and pH.

In these studies I have utilised anionic latex polystyrene beads as a model charged surface to characterise the binding properties of lipid-based DNA formulations, including conventional cationic liposome-DNA aggregates (LDAs) and novel lipid-DNA particles (LDPs). Changes in electrophoretic mobility of these anionic latex beads can provide information on the surface charge of LDPs as well as binding interactions mediated by the cationic lipid species within LDPs. Ultimately these data will determine whether charge

interactions are sufficient to promote DNA binding to an anionic surface by monitoring these changes utilising the microelectrophoresis apparatus.

These studies demonstrate that charge interactions play a major role in mediating attachment of lipid-based DNA formulations to an anionic surface. At a net LDP to anionic bead charge ratio of 1:1 (+/-), charge reversal is observed. Neutralisation of the surface charge of both the cationic lipid-mediated DNA delivery system as well as the anionic surface charge of the cell membrane may play a role in transferring DNA across the cell membrane. This chapter also assesses whether additional lipids, other than the cationic lipid species, may contribute to differences in transfection capabilities by altering the electrostatic interactions of these formulations with cell membranes.

4.2 Material and Methods

4.2.1 Materials

Anionic sulphate polystyrene latex beads of $0.96\pm0.03\mu m$ containing a surface charge density of 6.2 $\mu C/cm^2$ were purchased from Interfacial Dynamics Corporation (Portland, OR).

All other materials used are described in the Chapter 2 section *2.2.1* and Chapter 3 section *3.2.1*.

4.2.2 Methods

4.2.2.1 Preparation of MLVs and LUVs

Cationic liposomes were prepared using the method of Hope *et al.* (334). Lipid stock solutions (DODAC:DOPE or DODAC:DOPC at 1:1 lipid mole ratio) in 1:1 (v/v)

chloroform:methanol were dried under a stream of nitrogen. The resulting thin films were further dried down using a vacuum aspirator for approximately 4 h. MLVs (multi-lamellar vesicles) were formed by addition of 10 mM NaCl and vortexed vigorously. In preparing LUVs (large unilamellar vesicles), the MLV solutions were subjected to 5 freeze-thaw cycles and subsequently passed through three stacked 0.08 µm pore size polycarbonate membranes (Nucleopore, Cambridge, MA) at least 10× using an Extruder (Lipex Biomembranes, Vancouver, B.C.). Extrusion was done operating at room temperature with pressures of <100 psi. Vesicle size distribution and homogeneity were evaluated by quasi-elastic light scattering (QELS) using a Nicomp 270 submicron particle sizer (Pacific Scientific, Santa Barbara, CA) equipped with an helium/neon laser operating at 632.8 nm. LUVs had mean diameters between 100 - 150 nm.

4.2.2.2 Preparation of liposome-DNA aggregates (LDAs)

LDAs were generated as described in Chapter 3, section *3.2.2.3* in 10 mM NaCl.

4.2.2.3 Preparation of lipid-DNA particles (LDPs)

Lipid-DNA particles were generated by the method of Y.-P. Zhang *et al.* (318) and modified in Chapter 3, section *3.2.2.4* (110).

4.2.2.4 Microelectrophoresis

Latex anionic beads were suspended in 10 mM NaCl pH 7.0 at 25°C. The resulting concentration of these beads was approximately 0.1% (w/v). LDPs, LDAs or free DNA were added to the beads and mixed vigorously. The combined suspension was placed in a

cylindrical microelectrophoresis chamber (Rank Mark I, Rank Bros., Cambridge, England) and an electric field was applied at approximately 3.9 V/cm. Ten measurements were made in each direction by switching polarities between timings. Timing was measured as each visible bead passed through a pre-determined number of squares, magnified 40×, in the eyepiece graticule of a horizontal microscope focused at the stationary level. The microelectrophoresis apparatus, procedure and calculation of the electrophoretic mobility as determined from the averaged velocities, applied voltage and chamber electrical length is described elsewhere (335). Red blood cells had a mobility of $-1.1 \pm 0.1 \times 10^{-4}$ cm^2/Vs in 150 mM NaCl, pH 7.0. All other measurements using anionic beads were completed in 10 mM NaCl, pH 7.0, due to increased sensitivity in assessing changes in electrophoretic mobility and zero point of charge.

4.2.2.5 Centrifugation studies

Samples of 0.1% Beads, 0.17 pmoles ^{3}H-LDPs, Bead + ^{3}H-DNA, or Bead + ^{3}H-LDPs were made up in 10 mM NaCl pH 7.0 at 25°C. One mL was taken from each sample, placed in a 1.5 mL polypropylene tube and centrifuged in a microcentrifuge at 10,000×g for 12 min. Bead samples were separated into supernatant and pellet samples and the concentrations of the anionic beads were measured by absorbance at 259 nm. The supernatant, containing radiolabeled ^{3}H-DNA or ^{3}H-LDP, was removed from each sample and 0.5 mL of Solvable™ was added in a scintillation vial. Re-suspension of the remaining pellets was done by addition of Solvable™ (0.5 mL). Both supernatant and pellet solutions were incubated overnight. Subsequently, Pico-flour scintillant was added and radioactivity was determined.

120

4.3 Results

4.3.1 Electrophoretic mobility of LDAs

Non-viral delivery systems, specifically, lipid-based DNA carriers, appear to be dependent on simple positive-negative charge interactions to mediate efficient transfection of cells (213, 332). Microelectrophoresis was utilised to characterise the interactions between novel self-assembling lipid-DNA particles and a model anionic surface, whereby changes in the mobility of anionic latex beads can be measured as a function of binding. Figure 4.1 shows how this technique can be used to characterise particle or cell mobilities of samples. Fixed red blood cells were used to determine the reproducibility of the apparatus and these cells have an electrophoretic mobility of $-1.1\pm0.1\times10^{-4}$ cm^2/Vs, at physiological ionic strength. The membrane of any cell is negatively charged at pH 7.0 and the mobility values observed are typical. The anionic latex beads contain a significant anionic charge and move toward the anode. These beads, in 10 mM NaCl, pH 7.0, have an observed mobility of $-7.5\pm0.3\times10^{-4}$ cm^2/Vs. Conventional liposomal formulations for the delivery of DNA to cells are prepared with as much as 50 mole % of cationic lipid and a similar portion of a neutral or zwitterionic lipid. Multi-lamellar vesicles (MLVs), formulated with 1:1 lipid mole ratio of DODAC:DOPE or DODAC:DOPC, were prepared and the resulting mobilities were determined in the absence of anionic beads since MLVs are microscopically visible in the microelectrophoresis apparatus. When analysed by microelectrophoresis, the net positively charged MLVs showed movement toward the cathode. The observed mobilities were $3.7\pm0.1\times10^{-4}$ cm^2/Vs and $2.5\pm0.2\times10^{-4}$ cm^2/Vs for the DOPE and DOPC formulations, respectively.

Figure 4.1

The electrophoretic mobility of fixed red blood cells, anionic latex beads and MLVs.

Fixed red blood cells (**RBCs**), anionic latex beads (**Beads**), DODAC:DOPE (**DOPE MLVs**) and DODAC:DOPC (**DOPC MLVs**) MLVs were assessed. MLVs were formulated at a 1:1 cationic lipid:phospholipid ratio. Microelectrophoresis was done in 150 mM NaCl for RBCs and 10 mM NaCl for the others, at pH 7.0 and 25°C. Each sample was measured 10 times in each direction and the error expressed as ± SD.
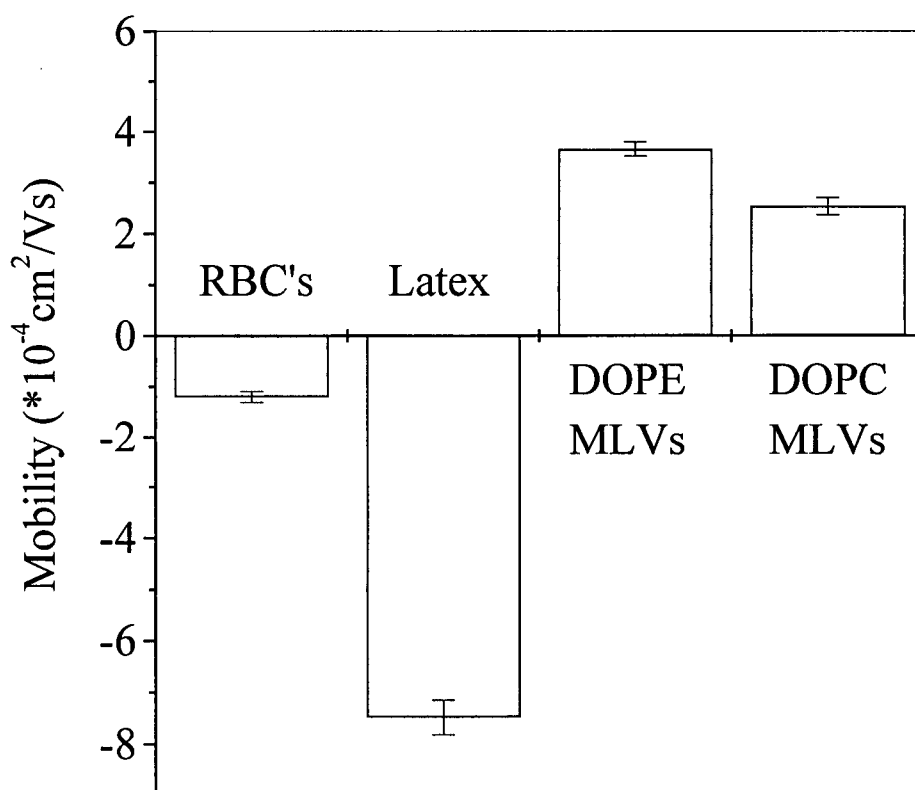
Although MLVs can be used to effect efficient transfection (336), pre-formed cationic liposomes are more frequently prepared to produce a small (<100 nm) and uniform size: this can be achieved by sonication or extrusion of MLVs. Extruded MLVs were prepared to exhibit a mean diameter as measured by QELS, of 100 - 150 nm and these liposomes, referred to as large unilamellar vesicles (LUVs), contain half the total amount of charged lipid on the outside of the vesicle. These outside surface charges have the potential to interact with the anionic latex beads. The cationic liposomes used were composed of a 1:1 lipid mole ratio of DODAC:DOPE or DODAC:DOPC. Microelectrophoretic studies were done at 25°C in the presence of 10 mM NaCl pH 7.0. Figure 4.2 shows the mobilities of the anionic latex beads as increasing amounts of cationic LUVs are added, in terms of the ratio of the number of total cationic lipid species (one positive charge per lipid moiety) to the number of point charges on the anionic bead, as calculated from the surface charge density provided by the manufacturer (6.2 $\mu C/cm^2$). At apparent charge neutralisation, where the number of positive lipid charges on the exposed surface of the LUV equals the number of negative charges on the anionic beads, the net mobility of the liposome/bead complex is zero. At this point, either the anionic beads do not move in the electrophoresis chamber or the population average mobility of the beads moving toward the anode and the cathode is zero. At net zero mobility the charge ratio of the total moles of cationic lipid species to moles of point charges on the anionic bead is 2 (+/-). Thus, a ratio of two cationic lipid moieties to one point charge on the anionic bead produced effective neutralisation. This is consistent with the fact that 50% of the cationic lipid in these systems was available in the outer monolayer that can then adsorb on the surface of the anionic latex beads. If the LUVs remained intact, the cationic moieties on the inside of the liposome bilayer were neutralised

Figure 4.2

Effect of increasing amounts of cationic liposomes on the electrophoretic mobility of anionic beads

The electrophoretic mobilities of anionic latex beads incubated with increasing amounts of 1:1 lipid ratio of DODAC:DOPE (■) or DODAC:DOPC (●) liposomes were determined. Microelectrophoresis was done in 10 mM NaCl pH 7.0 and 25°C. Each point was measured 10 times in each direction and the error expressed as ± SD.
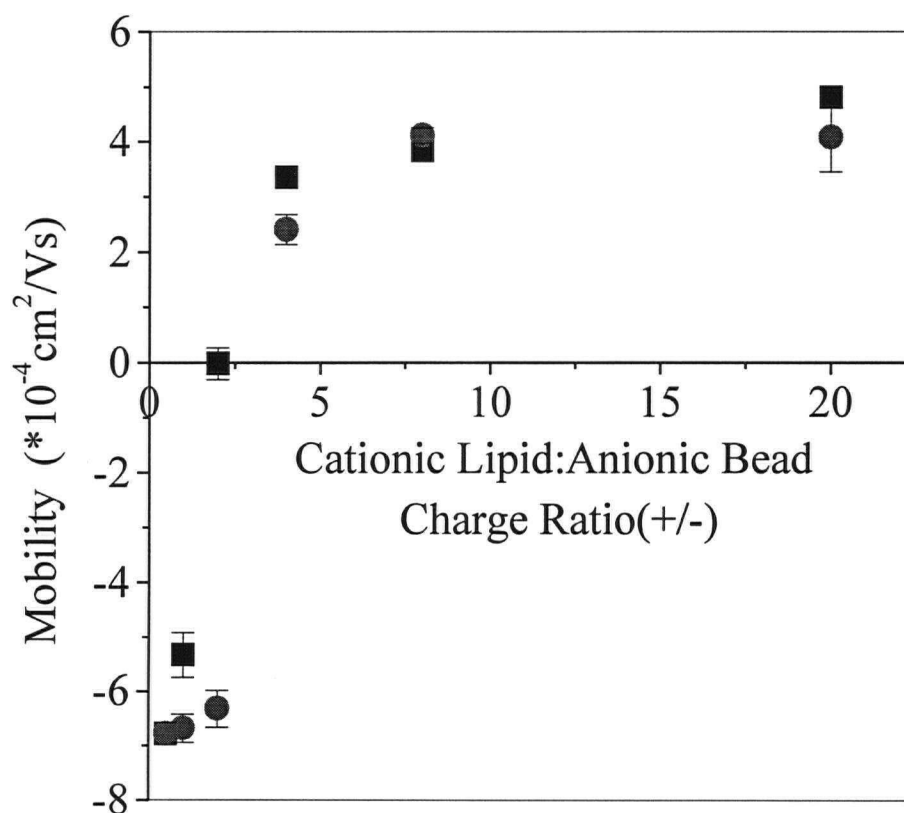
by counterions inside the vesicles and do not contribute to electrostatic interactions undergone by the exterior charges.

Upon addition of plasmid DNA to these cationic liposomes, aggregates - which can be used in the transfection of cells and selected tissues in animals - are formed (331). A net positive charge is required for optimal transfection (337) and these charge interactions mediate delivery of DNA to a cell. Previous experiments show that LDAs prepared at a charge ratio (monocationic lipid species to anionic phosphate charges of the DNA) 2:1, 5:1, and 10:1 result in the highest transfection efficiency (195). LDAs prepared at these ratios were analysed using microelectrophoresis and the results are shown in Figure 4.3. The net charge of the complex was estimated by subtracting the total number of anionic phosphate charges on the DNA from the positive charges of the cationic lipid species. Mobility was measured as a function of increasing amounts of LDAs, represented as a ratio of the net charge of the aggregate to total point charges of the anionic bead. LDAs were made such that there was always an excess of cationic lipid moieties relative to negative phosphate charges. Charge reversal was observed when sufficient liposome-DNA aggregates were added to neutralise the charges on the anionic latex beads.

At these concentrations of LDAs and anionic beads the charge reversal was achieved when the ratio of net positive charge on the liposome-DNA complex to charge on the anionic latex beads was approximately 1:1 for formulation charge ratios of 2:1 and 5:1 (cationic lipid:anionic phosphate DNA groups). Interestingly, the LDA formulation of 10:1 did not exhibit a charge reversal point of 1:1, but rather approached a 2:1 net positive to anionic bead charge ratio.

Figure 4.3

The effect of LDAs on the electrophoretic mobility of anionic latex beads

The electrophoretic mobilities of anionic latex beads incubated with increasing amounts of liposome-DNA aggregates. LDAs were made up at cationic lipid:anionic phosphate DNA charge ratios of 2:1 (+/-) (■), 5:1 (+/-) (●), and 10:1 (+/-) (▲) and a lipid ratio of 1:1 DODAC:DOPE for each formulation. Microelectrophoresis was done in 10 mM NaCl pH 7.0 and 25°C. Each point was measured 10 times in each direction and the error represented as ± SD.
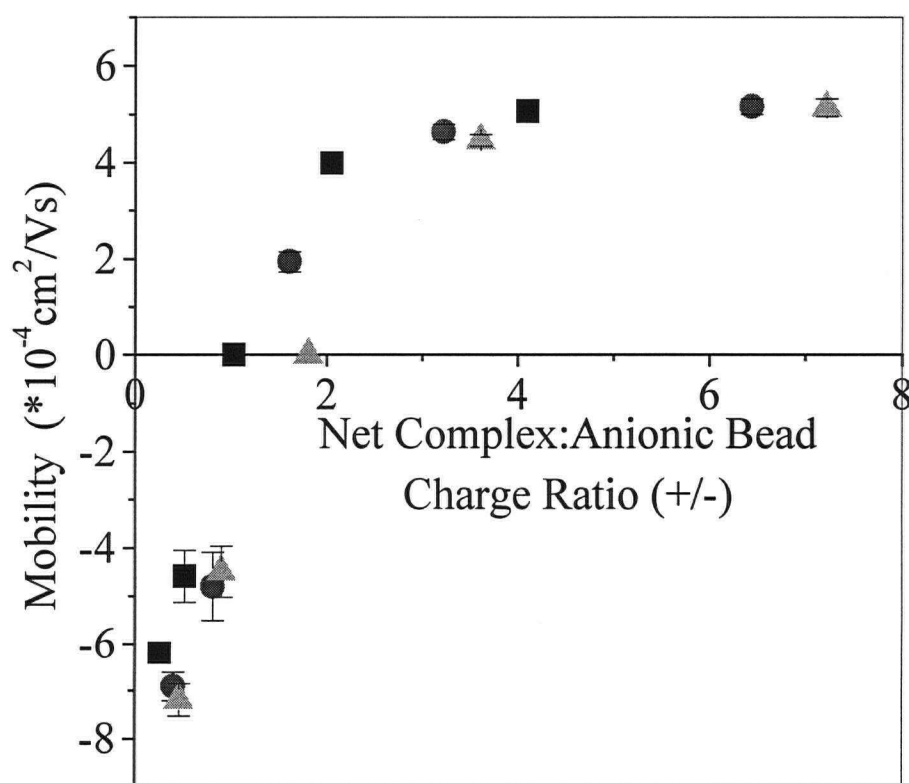
4.3.2 Electrophoretic mobility of LDPs

In developing better-defined systems for increased gene delivery a novel strategy was devised consisting of self-assembling lipid-DNA structures. These structures can be made in the presence of detergent and are different from conventional liposomal formulations since DNA binding is mediated by cationic lipids in either micellar or monomer form (318). The headgroups of the lipid associate directly with the anionic phosphate charges on the DNA resulting in a hydrophobic lipid-DNA complex which can subsequently be used to prepare LDPs. LDPs can be prepared at a variety of cationic lipid to anionic DNA phosphate charge ratios and we have excellent control over the lipid composition. Figure 4.4A shows the mobilities of anionic latex beads following addition of LDPs made up at ratios of 0.2:1 and 0.4:1 cationic lipid species to anionic DNA phosphate groups. These LDPs have an excess anionic charge and would not have an effect on the mobility of the anionic latex beads since there is no available cationic species would interact with the anionic charges on the latex beads. However, one cannot discount the possibility that the cationic lipid may dissociate from the DNA. As shown in Figure 4.4A, this does not appear to be the case. The mobilities of the anionic latex beads, even at >10 times molar excess of LDPs, remained constant at approximately -7.5×10^{-4} cm^2/Vs. This mobility is equivalent to the mobility of the anionic latex beads in the absence of any other species.

When there are cationic species in excess with respect to DNA phosphate group, mobilities of the anionic latex beads are vastly different. Figure 4.4B shows charge reversal at a net positive charge of the LDPs to anionic charge of the latex beads of approximately 1:1 when LDP formulations of 2:1 and 4:1 cationic lipid to anionic DNA phosphate groups were used. The LDP formulations prepared at a 10:1 (+/-) cationic lipid to anionic phosphate

Figure 4.4

The effect of LDPs on the electrophoretic mobility of anionic latex beads

The electrophoretic mobility of anionic latex beads incubated with increasing amounts of lipid-DNA particles formulated at a 1:1 ratio of DODAC:DOPE. Microelectrophoresis was done in 10 mM NaCl pH 7.0 and 25°C. Each point was measured 10 times in each direction and the error is represented as ± SD.
A: LDPs were made up at cationic lipid:anionic phosphate DNA charge ratios of 0.2:1 (+/-) (■) and 0.4:1 (+/-) (●) and 1:1 DODAC:DOPE lipid ratios.
B: LDPs were made up at cationic lipid:anionic phosphate DNA charge ratios of 2:1 (+/-) (▲), 4:1 (+/-) (▼), 10:1 (+/-) (◆) and 1:1 DODAC:DOPE lipid ratios.
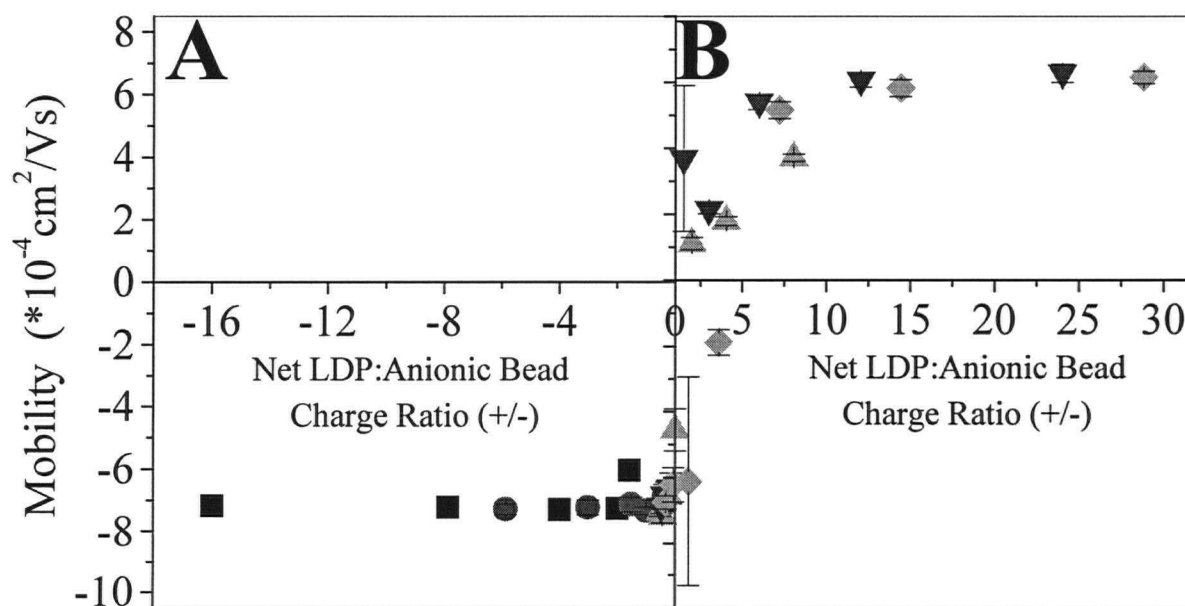
charge ratio were able to promote a charge reversal at 3 - 4:1 (+/-) net cationic LDP to anionic bead charge ratio, a result similar to that obtained with the liposome-DNA aggregates.

4.3.3 Comparison of DOPE- vs. DOPC- containing LDPs

When developing effective transfection of lipid-based DNA transfer formulations, a key element is often transfection efficiency as measured by transgene expression. It is well established that the incorporation of DOPE, a non-bilayer forming lipid, increases transfection of lipid-based delivery systems, in comparison to lipids such as DOPC (250). DOPE acts as a fusogen that promotes delivery following lipid binding to the cell membrane. In addition, the results from Chapter 2 and 3 demonstrated that the PE headgroup destabilises binding between the cationic lipid and the DNA phosphate groups (110, 313). In order to characterise differences in electrostatic binding of DOPE- and DOPC- containing LDP formulations to anionic cell membranes microelectrophoresis was employed (Figure 4.5). It should be noted that DOPC-containing LDPs are not effective transfection formulations while DOPE-containing LDPs are as efficient as systems based on use of pre-formed liposomes (see Chapter 4, section *3.3.3*). DOPC and DOPE LDPs were made up at cationic lipid to anionic phosphate DNA groups charge ratio of 2:1(+/-) and a 1:1 lipid (DODAC:neutral) ratio. The mobilities of the anionic latex beads were measured as a function of increasing amounts of LDPs added to a constant amount of anionic latex beads. Beyond the point of charge reversal, where the mobilities indicate a positive surface charge, the profiles of DODAC:DOPE and DODAC:DOPC LDPs interaction with the anionic latex beads were significantly different. Specifically, the DOPC formulations reached a saturation

Figure 4.5

The phospholipid effect on the electrophoretic mobility of anionic latex beads

The electrophoretic mobilities of anionic latex beads incubated with increasing amounts of lipid-DNA particles. LDPs were made up at a cationic lipid:anionic phosphate DNA charge ratio of 2:1 (+/-) and either DODAC:DOPC (●) or DODAC:DOPE (■) lipid ratios of 1:1. Microelectrophoresis was done in 10 mM NaCl pH 7.0 and 25°C. Each point was measured 10 times in each direction and the error is expressed as ± SD.
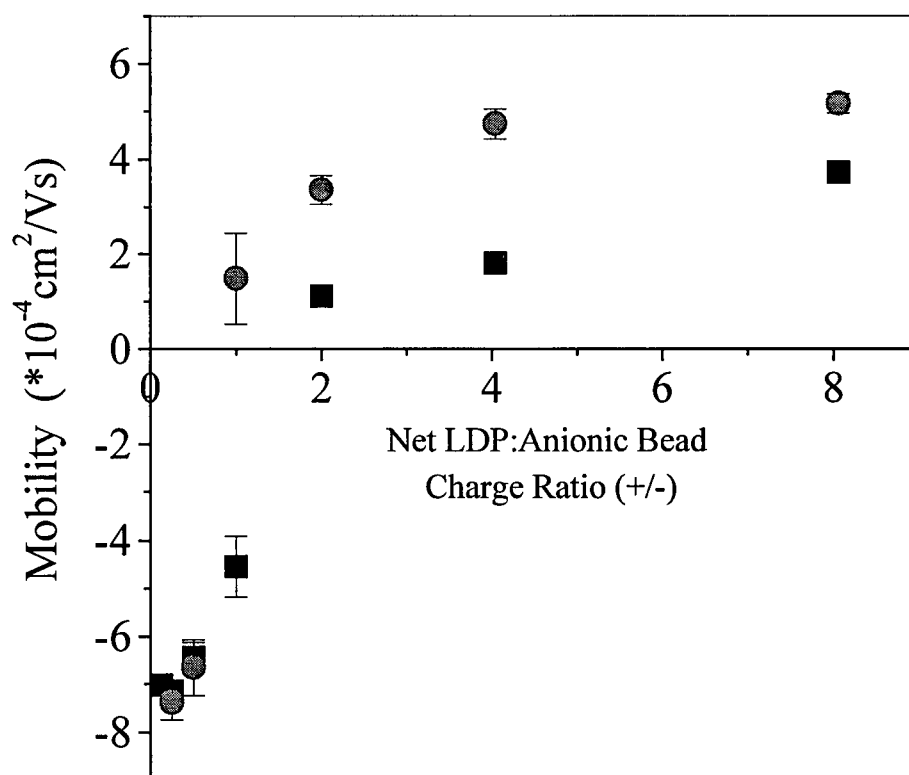
mobility of $5.2\pm0.2\times10^{-4}$ cm^2/Vs at a 10-fold molar excess to anionic latex beads while DOPE formulations show a mobility of $3.7\pm0.1\times10^{-4}$ cm^2/Vs at the same concentration.

Although the changes in mobilities of the anionic beads are significant when cationic lipid-based DNA delivery systems are added, it is important to determine whether the DNA is indeed associated with the lipid which caused the changes in bead mobilities. This is required to determine whether the lipids in the formulations involved can mediate efficient association of DNA to the bead through electrostatic interactions and, in addition, this information will help assess whether the cationic lipid remains associated with the LDP. In order to determine DNA association with the anionic latex beads centrifugation studies were performed (Figure 4.6). The recovery of ^{3}H-DNA or anionic latex beads in the supernatant and the pellet were determined through scintillation counting for the former and absorbance at 259 nm for the latter. At 10,000×g and 12 min, beads centrifuged alone were recovered at in the pellet (>86%) and LDPs without beads were recovered in the supernatant (>96%). Furthermore, ^{3}H-DNA, without a carrier lipid and incubated with the beads and centrifuged, was recovered only in the supernatant (>96%) while the beads alone were recovered in the pellet. Recovery of ^{3}H-DNA with the pellet at the appropriate speed and time would indicate that the DNA was indeed associated with the lipid and the anionic latex bead. When the LDPs were incubated with the anionic latex beads at a charge ratio (+/-) of 1:1 >88% of the ^{3}H-DNA was recovered with the anionic latex beads in the pellet.

4.4 Discussion

The electrostatic interaction between cationic lipid delivery systems and anionic latex beads may prove to be a useful model in characterising the initial step governing the gene

Figure 4.6

Association of LDPs and DNA with anionic latex beads

Recovery of Beads, ^{3}H-LDPs, or ^{3}H-DNA in the supernatant (**A**) and pellet (**B**) after centrifugation of the samples at 10,000×g for 12 min. Bead represents the recovery of anionic beads only as determined by A_{259}. LDP represents the recovery of LDPs as determined by ^{3}H-LDPs. Bead + DNA represents the recovery of ^{3}H-DNA in the presence of anionic beads. Bead + LDP represents the recovery of ^{3}H-DNA in LDP (1:1 net LDP to bead charge ratio) in the presence of anionic beads.
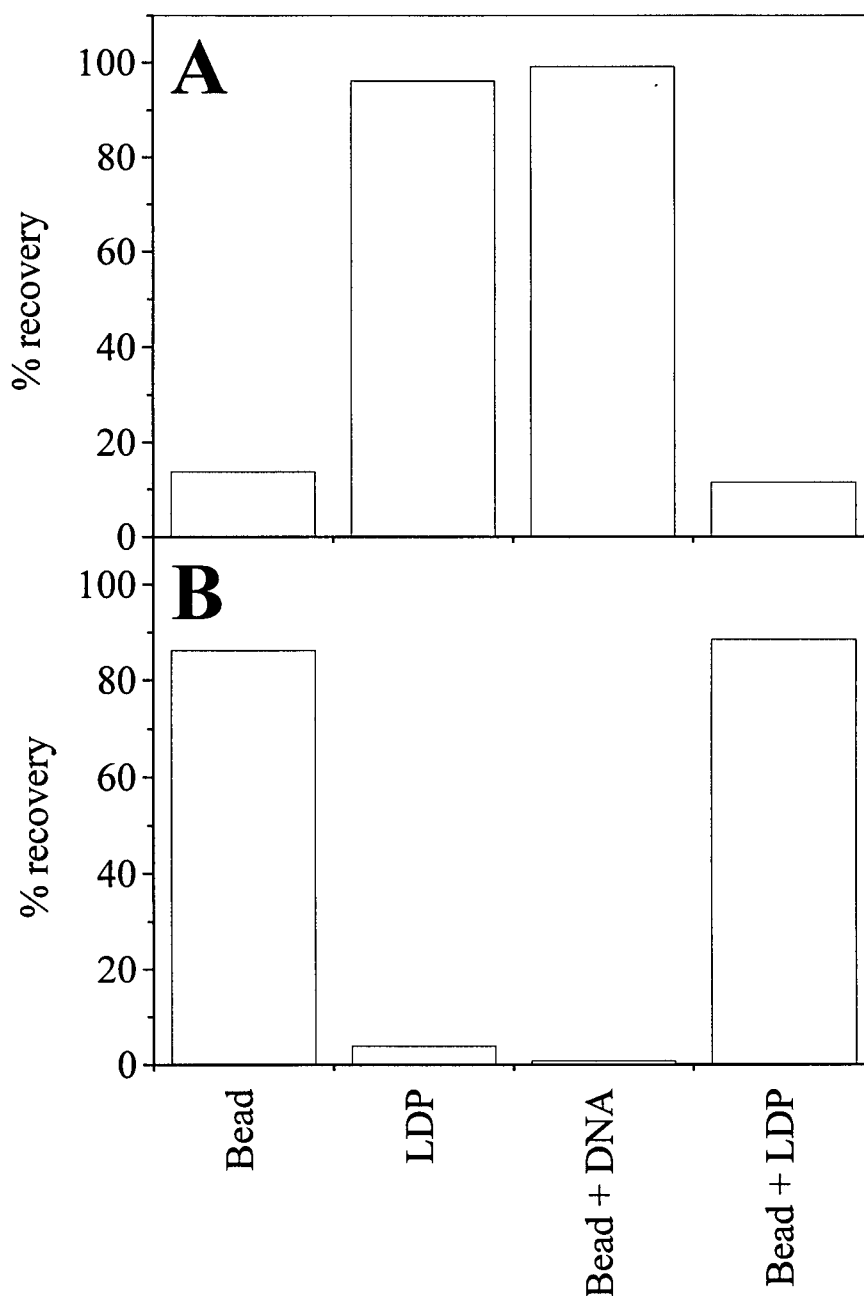
transfer activity of lipid-based carriers. This chapter utilises changes in electrophoretic mobilities of anionic latex beads in order to characterise the electrostatic interactions which mediate binding of conventional liposome-DNA aggregates as well as lipid-DNA particles (an alternative lipid-based gene transfer technology) to an anionic surface. The results are considered in light of three points: (i) that charge interaction is the driving force in cell/lipid interaction, (ii) that lipids containing the phosphatidylethanolamine (PE) headgroup may influence the nature of the binding interactions and (iii) that charge interactions may play a role beyond that of simple contact.

It is essential to demonstrate that cationic lipids that have dissociated from the LDPs or LDAs did not affect changes in anionic bead mobility. Figure 4.4A shows that LDPs formulated with a net negative charge did not alter anionic bead mobility. The electrophoretic mobility remained approximately -7×10^{-4} cm^2/Vs, even when a quantity of LDP was added such that there was a significant number of total positive charges in the solution. When a net positive charge, using LUVs, LDAs, or LDPs, was added to the anionic bead mixture, the electrophoretic mobilities became more positive as a function of increased concentration of cationic lipid species. It should be noted that net positive charge in systems containing DNA was determined by subtracting the number of anionic phosphate charges of DNA from the total number of cationic lipid charges. Importantly, as shown in Figure 4.6, when anionic beads were incubated with LDPs and centrifuged, the resultant pellet contained >88% of ^{3}H-DNA. It was only when cationic lipid was present in order to mediate interaction between the DNA and the anionic bead that a significant amount of DNA could be found in the pellet. These data demonstrate that the DNA must remain associated with the

133

cationic lipid and that adsorption of the cationic lipids to the surface of the anionic beads was required to induce changes in electrophoretic mobility.

There are significant differences in the saturation mobilities among MLVs without anionic beads ($\sim 3.5 \times 10^{-4}$ cm^2/Vs), anionic beads with LUVs ($\sim 4.5 \times 10^{-4}$ cm^2/Vs), anionic beads with liposome-DNA aggregates ($\sim 5.0 \times 10^{-4}$ cm^2/Vs) and beads with lipid-DNA particles ($\sim 6.0 \times 10^{-4}$ cm^2/Vs). The mobility of a complex in an electric field is determined primarily by two features: (i) the number of surface charges per unit area (surface charge density), and (ii) the location of the hydrodynamic surface of the particle (shear plane). It has been suggested for many lipid-based complexes that the shear plane is located a few angstroms from the plane defined by the surface charge (338). There is no evidence to indicate that a different assumption applies to the membrane systems studies here, therefore I believe that the surface charge density is the dominating factor governing the electrophoretic mobility of these various systems. If the electrophoretic mobility is determined by the surface charges displayed on the outer surface, then differences between saturation mobilities of MLVs and LUVs can be resolved. The inner bilayers of the MLVs are unlikely to contribute to the surface potential and the relatively low positive mobility of the MLVs are a consequence of positive charges distributed evenly over the MLV surface. In contrast, if it is assumed that the LUV structure remains intact and that numerous, small (<100 nm) LUVs can bind to a single one micron anionic bead a more complicated charge density results. As suggested above, there is no indication that the lipids dissociate from the liposomes as a consequence of association with the anionic beads. Therefore, increased saturation mobilities observed for LUV/anionic bead complexes must be due to significantly higher numbers of associated positive charges on the outer surface of the complex.

The cationic LUVs undergo significant structural changes following addition of DNA and these have been attributed to fusion and distortion of the liposome induced by DNA binding (130, 339). The potential for lateral phase separation of lipids in these systems is likely decreased due to these previous events. While there still exists some intact liposomes, it is likely that some of the cationic lipids normally found on the inner bilayer of the liposome can contribute to a remodelled surface exposed as a consequence of LDA formation. These results indicate that the saturation mobility produced with LDPs is greater than that observed for the LDAs, suggesting that following binding to the bead even more cationic lipids are exposed to the external medium.

When comparing MLV formulations containing DOPE or DOPC, DOPE-containing MLVs have significantly higher mobilities (Figure 4.1). Contributions to changes in electrophoretic mobility based on surface charge properties can also include pH. The amine function of PE has the ability to exhibit a slight positive potential at a bulk pH of 7.0 because the pH at the surface of the lipid/bead complex will be higher than the bulk. This is due to the decreased [H$^+$] concentration near the surface due to electrostatic repulsion by the positive surface charge. This surface pH can be readily calculated from the measured electrophoretic mobilities (340). The calculation suggested that the surface pH is 7.85 for the MLV surface, 8.1 for the bead/LUV surface, 8.3 for the bead/LDA surface and 8.5 for the bead/LDP surface. This effect will tend to increase the PE positive charge and likely is responsible for the higher mobility of the PE-containing MLVs compared to those made with PC.

In contrast, the saturation mobilities of DODAC:DOPE LDPs compared to DOPC-containing LDPs are opposite to those obtained with MLVs (Figure 4.5). The unique

properties of DOPE have been previously described as they relate to destabilisation, and these arguments may also explain discrepancies in electrophoretic mobility (272). The explanation is based on PEs hydration properties (341). The structures adopted by both PC and PE are the result of attractive van der Vaals and repulsive hydration forces. Although inter- and intra- molecular interactions and hydration-repulsion forces stabilise PE lipid into the lamellar phase, additional interactions between the amine and the nonesterified oxygen of phosphate groups can result in local dehydration and close contact between the membranes (271). In addition to the amine group of PE interacting with the nonesterified oxygen of phosphate groups of lipids, it can also interact with the phosphate charges on the DNA. Such an interaction can be expected to alter the structure of lipid-based DNA formulations containing PE compared to those containing PC. The presence of a partial positive charge on PE would tend to destabilise the membrane structure due to electrostatic repulsion between head groups and, coupled with PE-DNA interactions, could be sufficient to alter the way in which the lipid-based DNA complexes interact with the latex surface, producing the lower mobilities observed.

A second important feature of this data is the charge reversal point, which is defined as the concentration or ratio of cationic lipid to anionic bead charge resulting in a net electrophoretic mobility of zero. At this point the average mobility of the bead population is zero, although both positive and negative mobility beads may be present. Generally, the charge reversal point for LUVs, LDAs and LDPs occurred when the charge ratio of net cationic charges (determined by subtracting the number of anionic phosphate charges from DNA from the total number of cationic lipid molecules) to negative charge on the latex was near 1:1. If the lipids remain in a bilayer configuration, direct neutralisation of each latex

surface charge by a cationic lipid would require this ratio to be at least 2:1, since each lipid in the monolayer providing the neutralising lipid would have an equivalent free molecule in the opposite monolayer of the bilayer; this appeared to be the case for the LUVs (Figure 4.2). When DNA was present the charge reversal ratio varied from significantly less than 2:1 to significantly greater than 2:1, implying an uneven distribution of cationic lipids between the outermost plane of the liposome-DNA aggregate and those bound to DNA within the structure.

If the anionic bead is accepted as a model for the cell membrane, neutralisation of charge at the cell surface and the delivery system may be considered as a fundamental step in delivering the DNA of interest into the cell. For example, models of hydrophobic pores forming in the cell surface have been suggested (213). Formation of pores is dependent on charge neutralisation of the cell surface as well as the lipid gene transfer system. Hence, most investigators utilise an excess positive charge ratio (+/-) when preparing lipid-based gene transfer systems. Empirically, optimal transfection is observed when there is a slight excess positive charge. These data suggests that a system exhibiting a 2:1 (+/-) charge ratio has one half of the charges able to interact with the DNA to be delivered and the other half associated with the negative charges on the latex beads or cell surface. These charge interactions and perhaps charge reversal may be required for successful cell binding as well as cell delivery.

In the context of cell surface interactions, one of the parameters that induce effective gene transfer, besides electrostatic interactions, is destabilisation of the liposome-DNA aggregate or lipid-DNA particle. Release of the DNA may be a requirement for effective gene expression and charge interactions may be an essential step in this process (274). In

Chapters 2 and 3 I have suggested that PE may be a contributor to lipid dissociation and DNA release (110, 313).

Microelectrophoresis, as utilised here, has been useful as a technique to determine the surface charge properties of lipid-based delivery systems interacting with a negatively charged surface. This is an important attribute since electrostatic interactions are proving to be important in mediating binding to the cell membrane. LDPs are currently being developed to incorporate additional natural and modified lipids. The use of microelectrophoresis in analysing systems prepared using PEG-modified lipids may be extremely effective since surface properties of the delivery vehicle are altered dramatically through incorporation of PEG (342). PEG-lipids can be exchangeable (343) and these lipids may be used to regulate membrane fusion and subsequent delivery of the gene product to the disease site. Such formulations would be dependent on electrostatic interactions revealed after loss of the steric barrier provided by PEG.

CHAPTER 5

ERADICATION OF HUMAN NON-HODGKIN'S LYMPHOMA IN SCID MICE BY BCL-2 ANTISENSE OLIGONUCLEOTIDES COMBINED WITH LOW DOSE CYCLOPHOSPHAMIDE[*]

5.1 Introduction

While the addition of a specific gene, through vectors such as plasmid DNA, can alter disease pathogenesis, the prevention of the expression of a deleterious gene may be effective through the addition of antisense oligonucleotides (ASOs). This chapter focuses on the therapeutic relevance of an ASO. ASOs are short sequences of nucleotides complementary to coding regions of a gene of interest (48, 49). Advances in our understanding of the biological activity of ASOs have allowed development of these molecules as therapeutic agents (47, 344). Anticancer gene targeted therapy based on reducing the level of a putative oncoprotein can be achieved by ASOs designed to bind and facilitate degradation of the messenger RNA that codes for that specific protein. More specifically, appropriately designed synthetic ASOs bind RNA molecules in a sequence-specific manner and either directly impair interaction with factors in the cytoplasm that are required for translation into a protein and/or recruit endogenous RNase H to cleave the RNA backbone (345). Regardless of the mechanism of activity, ASOs have the potential to reduce a target protein overexpressed by tumourigenic cells.

Although elegant experiments have been carried out to define the mechanisms by which ASOs can specifically abrogate gene function, recent investigations have raised some puzzling questions regarding what factors govern their biological activity.

First, ASOs can be very potent immune stimulators by virtue of the unmethylated CpG motifs within the context of certain flanking sequences. Therapeutic activity can be attributed to systemic immune effects rather than to specifc ASO/mRNA interactions (73, 74, 346, 347). The development of control ODNs, containing a similar backbone, codon length and CpG motifs, have been useful to provide evidence for biological action linked specifically to a ODN sequence.

Second, in order for ASOs to be active, delivery to the inside of a target cell must be achieved efficiently. In tissue culture, for example, this requires association with a delivery system such as cationic lipids (348, 349). *In vivo*, however, ASOs are active when given in free form (350-352). Although studies assessing the mechanism of ASO activity *in vivo* are critically important to the further advancement of this technology, the primary concern remains the identification of agents with proven therapeutic activity in the clinical setting for disseminated systemic malignant disease.

An 18 mer phosphorothioated oligonucleotide, G3139, directed against the first six codons of the open reading frame of the bcl-2 gene message has been developed by Genta Inc. and is used in this chapter and the Chapter 6. The target protein, Bcl-2, is an anti-apoptotic member of a large family of genes involved in the regulation of programmed cell death (305, 306). Studies of G3139 on the Bcl-2 overexpressing lymphoma cell lines DoHH2 and SU-DHL-4 *in vitro* have shown downregulation of message and resultant decrease in protein expression (352). Tumour xenograft models in SCID mice using G3139

alone have demonstrated therapeutic activity that is specific when compared to control antisense sequences (344, 352). Pharmacokinetic as well as toxicity studies have been performed identifying a dose range with a good therapeutic index (353, 354). Used as a single agent in a Phase I study in patients with relapsed non-Hodgkin's lymphoma, G3139 has been reported to modify Bcl-2 levels in clinical samples of lymphoid cells using doses that were also associated with objective responses (355).

More recently, *in vitro* experiments have suggested that Bcl-2 plays a major role in the response of malignant cells to a variety of stresses that produce cellular damage, including chemotherapy (356-358). Malignant cell lines transfected with the bcl-2 gene, with resultant overexpression of the protein product, demonstrate increased resistance to various chemotherapeutic agents (359-362). Additionally, cell lines overexpressing Bcl-2 are rendered more sensitive to killing by chemotherapeutic agents either with introduction of antisense oligonucleotides directed at the bcl-2 message into culture or upon transfection of the cells with a vector bearing the antisense sequence (363, 364). This resultant chemosensitisation has been correlated with downregulation of Bcl-2 expression.

The studies reported in this chapter assess the *in vivo* therapeutic potential of combining ASOs targeting bcl-2 with a low dose of a cytotoxic agent commonly used in the treatment of lymphoma. It is believed that this chemosensitising effect correlates with specific ASO-mediated downregulation of bcl-2 mRNA message and subsequently, Bcl-2 protein. This is supported by PCR data and immunohistochemical evaluation of bone marrow obtained from DoHH2 bearing mice. Further, results in perforin-deficient mice demonstrate that elimination of lymphoma cells is not a result of natural killer cell function.

5.2 Materials and Methods

5.2.1 Materials

G3139 [antisense ODNs (ASO)], G3622 [reverse-polarity ODNs (RPO)] and G4126 [mismatch ODNs (MMO)] are fully phosphorothioated, linear, single-stranded 18 mer oligodeoxyribonucleotides (Genta Inc., Lexington, MA). G3139 (sequence: 5' - tct ccc agc gtg cgc cat, MW: 5764 g/mol) is complementary to the first six codons of the human bcl-2 open reading frame. G3622 (sequence: 5' - tac cgc gtg cga ccc tct; MW: 5764 g/mol) is the reverse-polarity sense control of G3139 while G4126 (sequence: 5' - tct ccc agc atg tgc cat; MW: 5683 g/mol) has a two base mismatch to G3139.

DoHH2 is an EBV-negative B cell non-Hodgkin's lymphoma cell line which carries the t(14;18) and has been extensively studied (365). Following the initial description, it was found that a complex translocation involving chromosomes 8, 14 and 18 resulted in a derivative 8 which contained both the c-myc and bcl-2 oncogenes juxtaposed to the transcriptional promoter of the immunoglobulin heavy chain locus with resultant overexpression of both protein products (366). DoHH2 cells were grown up in RPMI media containing 5% horse serum and 5% fetal bovine serum with (10 units/mL and 0.1 mg/mL) penicillin/streptomycin and (2 mM) L-glutamine. Cells were used for experiments between passages 8 - 17.

All other materials used are described Chapter 2, section 2.2.1 and Chapter 3, section 3.2.1.

5.2.2 Methods

5.2.2.1 Preparation of LUV/ODN complexes

Bcl-2 expression could not be downregulated with ASOs in the absence of a synthetic oligonucleotide delivery agent as demonstrated by these results and others (349, 367). For this reason, cationic liposomes consisting of DODAC and DOPE were used to enhance ASO intracellular delivery. ODNs in sterile water were diluted to 5 μM concentration. DODAC:DOPE LUVs (Inex Pharmaceuticals Corp., Vancouver, B.C.) were diluted in sterile water to a concentration of 4.6 mM total lipid to an equal volume to the ODNs solution. The LUVs had a mean diameter of 80±20 nm before the formation of LUV/ODN complexes as determined by QELS. ODNs were combined with the diluted LUV solution in one addition at 4°C and LUV/ODN complexes immediately formed as indicated by a change in sample turbidity. Final charge ratio of ODN:cationic lipids was 1.3:1 (+/-) and final ODN concentration was 2.5 μM. After incubation for at least 30 min, LUV/ODN complexes (200 - 400 nm using QELS) were added to DoHH2 cells.

5.2.2.2 In vitro delivery assays

DoHH2 cells were plated at a concentration of 1×10^6 cells/mL in 6-well tissue culture treated plates. All cells were plated in RPMI media in the absence of serum. Cells were incubated for 4 h at 37°C and subsequently RPMI media was replaced with 5% fetal bovine and 5% horse serum-containing media. After 72 h, cell lysates were obtained using 0.15% SDS in 50 mM Tris pH 8.0 and the level of Bcl-2 protein expression was determined using Western blot.

143

5.2.2.3 Western Blot

Western blot analysis was completed as previously described (311). Protein concentrations in the obtained cell lysates were determined by absorbance at 280 nm. Approximately 75 - 100 µg protein was separated using polyacrylamide gel electrophoresis. Western transfer was completed on nitrocellulose membrane and blocked with 5% skim milk. Bcl-2 primary antibody was diluted to 12 ng/mL and β-actin primary antibody (DAKO; Carpenteria, CA) was diluted to 10 ng/mL in TBS containing 1% skim milk and 0.1% Tween-20 for 1 h. Anti-mouse secondary antibody was diluted to 20 ng/mL in buffer and incubated at room temperature for 30 min. Bands were detected *via* chemiluminescence using ECL reagent from Amersham (Baie D'Urfe, PQ). Membrane was exposed to X-Omat Kodak film (Mandel Scientific; Guelph, ON) for 30 s - 1 min.

5.2.2.4 In vivo *model*

Male SCID/Rag-2 mice used for these studies were obtained from a breeding colony at our institution when they were 6 - 9 weeks old and weighed 22 g and were maintained in a pathogen-free environment. Viable DoHH2 cells (5×10^6 cells in 200 µL) were injected i.v. *via* the tail vein of each animal and disease was allowed to establish for 4 days. Cohorts of at least 3 animals were then treated in the following groups: (1) untreated control (injected with saline); (2) cyclophosphamide (CPA from Carter-Horner Inc., Mississauga, ON) at 35, 75, or 150 mg/kg i.p. on days 4, 8 and 12; (3) ASO, RPO or MMO at 5.0 or 12.5 mg/kg QD or QOD i.p. for 14 treatments; (4) ASO, RPO, or MMO QD or QOD i.p. for 14 treatments in combination with CPA i.p. on days 4, 8, 12. Animals were assessed for illness by non-biased

technicians and terminated at signs of illness including, but not limited to, paralysis in the hind region, scruffy coat, lethargy, weight loss of >20%, or if they survived past 90 days.

Pfp/Rag-2 male mice were obtained from Taconic (Germantown, NY). These are Rag-2 SCID mice deficient in perforin synthesis. Although NK cells are present in these animals they are not capable of cells lysis. Animals were used for experimentation within 2 weeks of arrival at our facility. Cohorts of 5 or 6 animals were injected with 5×10^6 DoHH2 cells in 200 µL i.v. *via* the tail vein. On Day 4 post-tumour cell inoculation animals began treatment with ASO alone (5 mg/kg for 14 treatments QOD) or in combination with CPA (35 mg/kg, Day 4, 8, 12) injected i.p. Animals were assessed for illness and terminated at signs of illness as described above.

5.2.2.5 Immunohistochemistry

Microscopic sections of a femur for controls or treated animals were processed routinely for hematoxylin and eosin staining and reviewed by an experienced heamatopathologist (Dr. R.D. Gascoyne). Paraffin section immunohistochemistry was performed to analyse Bcl-2 expression as previously described (368).

5.2.2.6 Molecular Genetics

High molecular weight DNA was extracted from all tissue specimens and the DoHH2 cell line with an automated DNA extractor (Applied Biosystems Model 341, Perkin-Elmer, Foster City, CA). PCR for immunoglobulin heavy chain and the presence of a bcl-2 (major breakpoint region) rearrangement was performed as previously described (369). The

presence of amplifiable DNA in the reactions was confirmed in all cases by a parallel amplification of a 155 bp and a 510 bp segment of the p53 and β-globin gene, respectively.

5.2.2.7 Statistical Analysis

Cohorts were determined by combining several experiments together. Survival analysis data were evaluated with censored regression with median survival times determined using the Cox's f-test. This was done since several of the treated groups consisted of long-term survivors (>90 days). For these groups median survival times were estimated based on the assumption that the surviving animals died on day 91. Groups with >50% 90-day median survival were labelled not obtained (NO). Cohorts with <3 uncensored data points were not determined (ND) since estimated parameters were not reliable. Survival curves were computed using the Kaplan-Meier method. Treatment groups were subsequently analysed using Statistica software and compared using a two-sample log-rank test. P-values were derived from the log-rank test comparing two samples and were reported if $p<0.05$ or were determined to be not significant (NS).

5.3 Results

5.3.1 Bcl-2 status of the DoHH2 cell line

Confirmation that the DoHH2 cell line expresses the Bcl-2 protein and that this expression can be downregulated *in vitro* with bcl-2 antisense oligonucleotides is demonstrated by Western blot analysis (Figure 5.1). Cells were treated with either ASO or two controls - ODN consisting of a full length reverse polarity sense sequence (RPO) as well as an antisense sequence with a two base mismatch to G3139 (MMO). Of note, these control

Figure 5.1

Effect of ASO on Bcl-2 expression in DoHH2 cells

Western blot (**A**) and densitometry analysis (**B**) of Bcl-2 expression in DoHH2 cells treated with ODNs. DoHH2 (concentration 1×10^6 cells/mL) were untreated or with 2.5 μM (final concentration) free ODN or LUV/ODN complexes at a 1.3:1 (+/-) charge ratio as described in the Methods. Bcl-2 protein (26 kDa) is indicated by the arrow labelled Bcl-2 and β-actin (43 kDa) was used as a loading control. Lanes are labelled as follows: **DoHH2**: untreated cells; **LAS**: LUV/ASO complex treated, **LRP**: LUV/RPO complex treated, **LMM**: LUV/MMO complex treated, **ASF**: Free ASO treated; **RPF**: RPO treated; **MMF**: Free MMO treated.

Densitometry analysis was assessed from three separate experiments and reported as mean ± SEM.

*$p<0.00005$ when comparing LAS to DoHH2

147
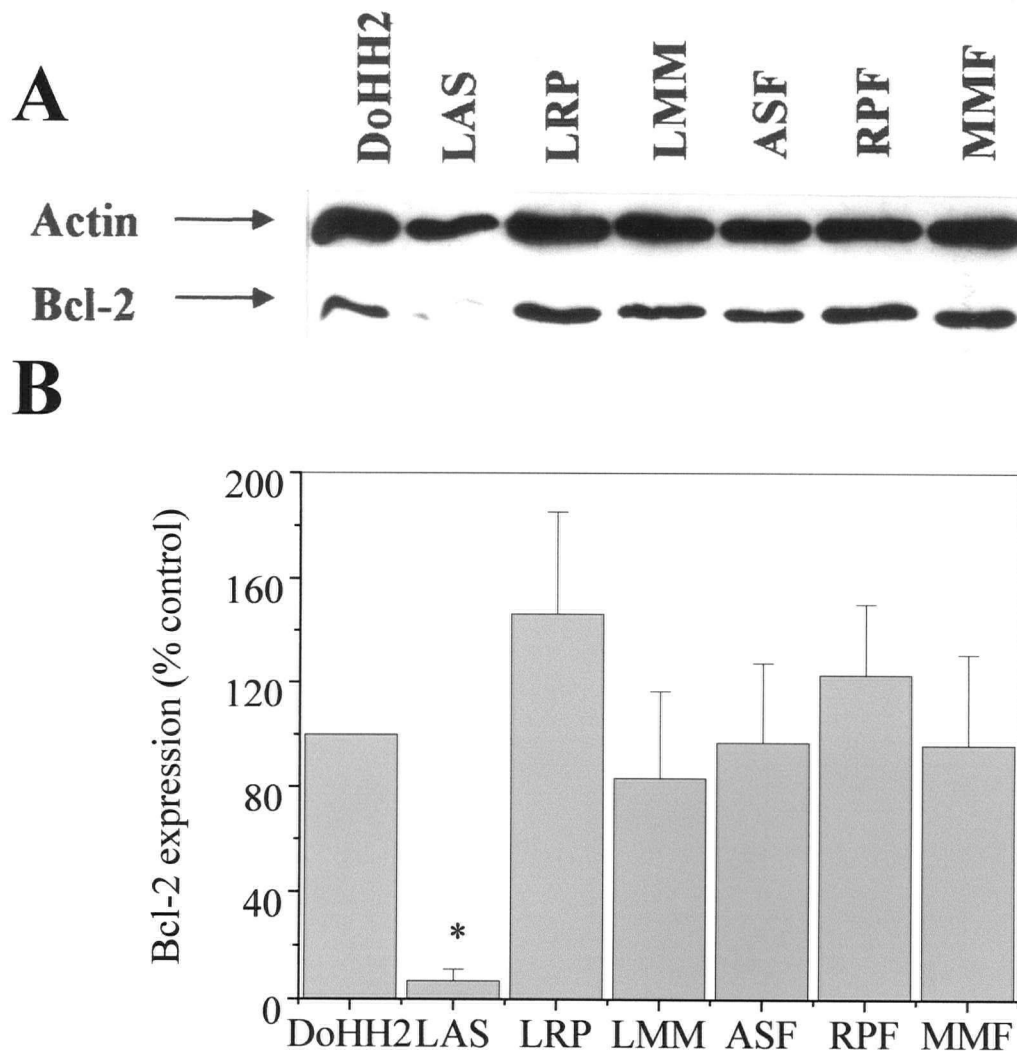
oligonucleotides contain similar backbone, codon length and CpG motifs as G3139. ODNs were delivered in free form or with cationic liposomes (DODAC:DOPE LUVs) as a carrier. The concentration of ODNs used (2.5 μM) was the minimum concentration, following complexation with cationic lipids, that clearly demonstrated downregulation of Bcl-2 protein with minimal toxicity. The cell toxicity that was observed following ASO addition was due to associated lipids. DoHH2 cells expressed the Bcl-2 protein as indicated by the 26 kDa band, while delivery of LUV/ASO complexes specifically downregulated Bcl-2 protein expression. This effect was sequence-specific as it was not seen with the control oligonucleotides where results were not significant when compared to untreated cells. This antisense effect could only be obtained when using a formulation method involving ASO complexation with cationic liposomes to engender ASO delivery.

5.3.2 Therapeutic activity of bcl-2 ASO or CPA alone

In vivo studies evaluated therapeutic activity in a SCID-human xenograft model where DoHH2 cells, a human B cell lymphoma, were injected i.v. Mice that were inoculated with 5×10^6 cells intravenously were terminated as a consequence of tumour progression within 35 days if left untreated. Cell titration data indicated that survival time is concentration-dependent, as previously reported (370). For example, these data shows that a two-log reduction in cell number (i.e. injection of 5×10^4 cells) results in 100% death by day 79. Untreated, control animals exhibited paralysis, disorientation and lethargy, among other symptoms, while gross observation during necropsy showed subcutaneous masses as well as enlarged retroperitoneal and perigastric lymph nodes in some, but not all, animals. ODN-treated animals, regardless of whether the ODN was antisense or a control, presented with

enlarged spleens (at least 2× larger than untreated animals) consistent with the mitogenic activity observed for ODNs in toxicology studies with mice. Figure 5.2A shows the representative section through a femur in untreated control animals and Figure 5.2B shows the corresponding Bcl-2 immunoperoxidase staining. Extensive infiltration with Bcl-2 expressing cells was observed where human lymphoma completely replaced murine marrow and Bcl-2 immunoperoxidase staining was strikingly positive in control femur and lymph nodes. Similarly, bcl-2 and immunoglobulin heavy chain PCR demonstrated a single dominant band in the untreated control animals of approximately 100 bp and 365 bp, respectively (lane 2, Figure 5.2E).

Treatment of DoHH2 bearing SCID mice was initiated 4 days after tumour cell injection, with both ODNs and cyclophosphamide (CPA) always given *via* i.p. injection. The animals were treated with ODNs alone, CPA alone, or ODNs together with CPA. The experiments, summarised in Tables 5.1A, 5.1B and Figure 5.3 assessed a range of doses of both ODNs and CPA. Two dosing schedules of ODNs were evaluated, each schedule consisting of 14 total i.p. injections: one given over a period of 18 days and the other over a period of 28 days. Dose scheduling was based on previous reports that phosphorothioate ODNs have elimination half-lives of ~12 - 72 h (353). CPA was given consistently i.p. 4, 8 and 12 days after tumour cell injection. Untreated control animals, as well as the RPO and MMO treated control groups were terminated or died with progressive tumour at a median of 33 to 37 days after tumour cell inoculation (see Table 5.1A). No long-term survivors were observed in these groups. CPA treated animals (Table 5.1B) showed a dose-response correlation with no effect (median survival time of 36 days) seen at a dose of 15 mg/kg and modest effect (median survival time of 47 days) at 35 mg/kg. The DoHH2 bearing animals

Figure 5.2

Immunohistochemistry and PCR analysis of Bcl-2 expression in femoral bone marrow

SCID/Rag-2 male mice inoculated with 5×10^6 DoHH2 cells. Femoral bone marrow sections were stained with hematoxylin and eosin for an untreated mouse (**A**) and a mouse treated with 35 mg/kg CPA treated on Day 4, 8, 12 and ASO at 5.0 mg/kg treated every other day for 14 treatments beginning on Day 4 (**C**) and with Bcl-2 immunoperoxidase for an untreated mouse (**B**) and a mouse treated as above for C (**D**). **E**: PCR was completed on extracted DNA and analysed by agarose gel electrophoresis on the following: 123 bp ladder (**1**), DoHH2 cell line (**2**), femoral bone marrow from untreated SCID/Rag-2 mouse (**3**) and a mouse treated as for C (**4**). The presence of amplifiable DNA was confirmed for the untreated (**5**) and treated (**6**) mice by parallel amplification of a 155 bp and a 510 bp segment of the p53 and β-globin gene, respectively.
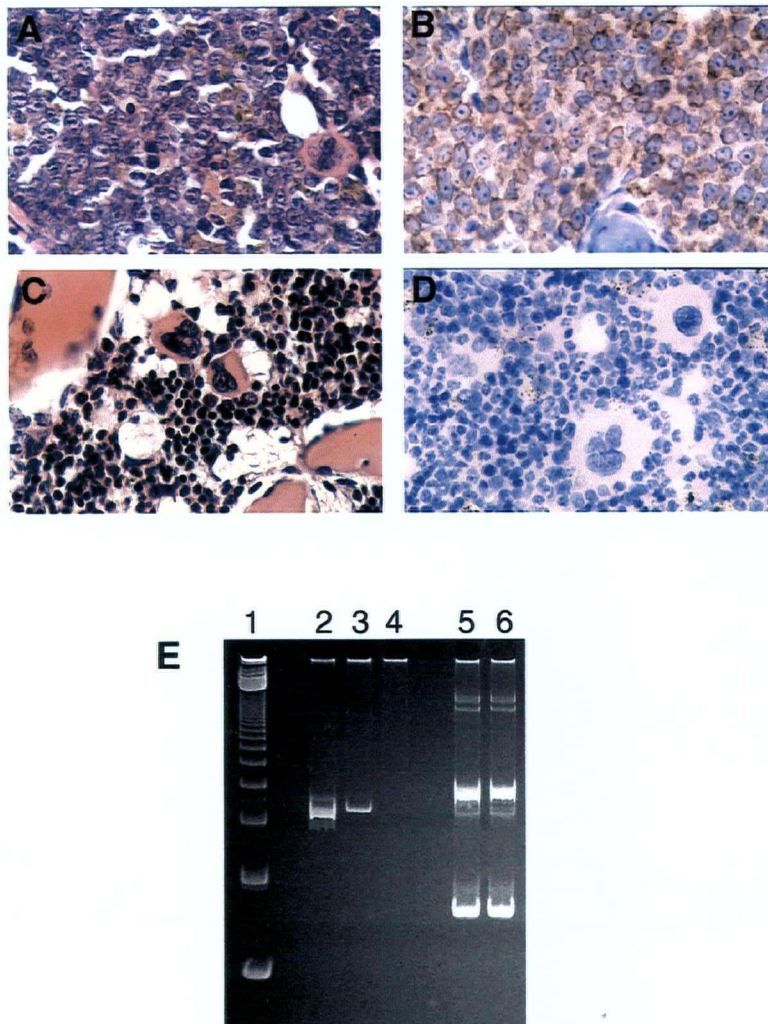
Table 5.1A

Effect of ODN treatment on SCID/Rag-2 male bearing DoHH2 tumours

SCID/Rag-2 mice were inoculated with 5×10^6 DoHH2 cells i.v. Animals were treated with None (saline only), RPO, MMO or ASO for 14 treatments i.p.

Treatment	Dose mg/kg	Schedule	N	Median Survival	% 90 day survival	p-value[5]
None	NA[1]	NA	44	33	0	NA
RPO	5.0	QOD[2]	6	37	0	NS[6]
RPO	12.5	QOD	3	35	0	NS
MMO	5.0	QOD	6	36	0	NS
MMO	12.5	QOD	3	37	0	NS
ASO	2.5	QOD	6	62	17	<0.000001
ASO	5.0	QOD	21	79	48	<0.000001
ASO	5.0	QD	9	46	0	<0.000001
ASO	12.5	QOD	12	NO[4]	58	<0.000001
ASO	12.5	QD	9	85	44	<0.000001

[1]NA: Not applicable
[2]QOD: Every other day treatment, animals were treated alternate days, including weekends.
[3]QD: Every day treatment, animals were treated every day either 14 days consecutively or excluding weekends.
[4]NO:Not obtained, if greater than 50% of the animals survived past 90 days then median survival could not be obtained.
[5]P-values were derived from using the log-rank test comparing each group vs control.
[6]NS: Not significant $p>0.05$ when compared to control animals.

151

Table 5.1B

Effect of ODN and CPA treatment on SCID/Rag-2 male mice bearing DoHH2 tumour

SCID/Rag-2 mice were injected with 5×10^6 DoHH2 cells i.v. Animals were treated with None (saline only), CPA (cyclophosphamide alone), or in combination with RPO, MMO, or ASO. CPA was injected i.p. on Days 4, 8, 12 after tumour inoculation and ODNs were given 14 treatments total i.p.

Treatment	Dose mg/kg	Schedule	N	Median Survival	% 90 day survival	p-value[5]
None	NA[1]	NA	44	33	0	NA
CPA	15	4,8,12	6	36	0	NS[6]
CPA	35	4,8,12	36	47	0	<0.000001
CPA	75	4,8,12	6	NO[4]	100	<0.000001
CPA	150	4,8,12	6	NO	100	<0.000001
RPO+CPA	5+35	QOD[2]+4,8,12	6	57	0	NS[7]
MMO+CPA	5+35	QOD+4,8,12	6	53	0	NS[7]
ASO+CPA	2.5+15	QOD+4,8,12	6	73	50	<0.005
ASO+CPA	5+15	QOD+4,8,12	6	84	50	<0.01
ASO+CPA	2.5+35	QOD+4,8,12	6	NO	67	<0.0001
ASO+CPA	5+35	QOD+4,8,12	18	NO	61	<0.000001
ASO+CPA	5+35	QD[3]+4,8,12	6	NO	100	ND[8]
ASO+CPA	12.5+35	QD+4,8,12	6	NO	83	ND

[1]NA: Not applicable

[2]QOD: Every other day treatment, animals were treated alternate days, including weekends.

[3]QD: Every day treatment, animals were treated every day either 14 days consecutively or excluding weekends.

[4]NO: Not obtained, if greater than 50% of the animals survived past 90 days then median survival could not be obtained.

[5]P-values were derived from using the log-rank test comparing each group vs the corresponding CPA concentration. CPA only treated animals were compared to the control group.

[6]NS: Not significant $p>0.05$ when compared to control.

[7]NS: Not significant $p>0.05$ when compared to the corresponding CPA concentration.

[8]ND: Not determined, there were <3 uncensored samples and estimated parameters were not reliable.

Figure 5.3

Survival curves of SCID/Rag-2 male mice bearing B cell lymphoma

SCID/Rag-2 male mice were inoculated with 5×10^6 DoHH2 cells i.v. and treatment groups
were as follows: 5 mg/kg ODN alone (injected i.p. QOD for 14 treatments) (**A**) or 35 mg/kg
CPA (injected i.p. on Days 4, 8, 12 after tumour cell inoculation) and 5 mg/kg ODN (injected
i.p. QOD for 14 treatments) (**B**). ODNs used were control (None) (-■-), ASO (-◆-), RPO
(-▲-), MMO (-▼-) and CPA only (-●-).
*$p < 0.000001$ when comparing groups treated with ASO alone or in combination with CPA
to untreated controls or CPA only, respectively

153
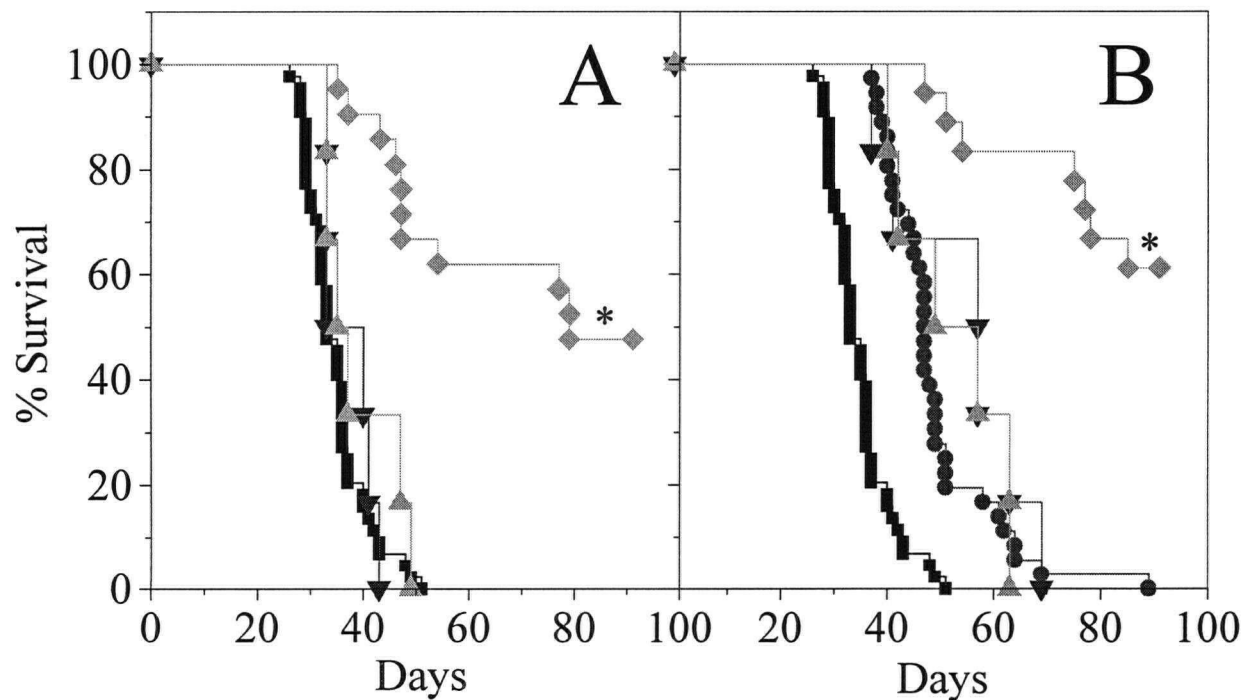
could be treated at higher dose of CPA (75 and 150 mg/kg) and at these doses 100% long-term (>90 days) survival was observed. Therefore, the two lower doses (15 and 35 mg/kg) were selected for studies evaluating combinations with ASO.

Free ASOs were given at doses of 2.5, 5 and 12.5 mg/kg/day for 14 doses every other day (QOD) starting 4 days after cell inoculation (Table 5.1A). Even at the lowest dose of ASO there was a significant ($p < 0.000001$) increase in median survival - from 33 days to at least 62 days, when compared to control. Dose dependent increases in therapeutic efficacy were most easily observed by monitoring the percentage of long-term (>90 days) survivors following treatment. As the dose increased from 2.5 to 5 to 12.5 mg/kg/day, the number of long-term survivors increased from 17% to 48 to 58%, respectively. The differences in response between the 5 and 12.5 mg/kg dose levels were not significant. If the dosing schedule was changed to daily injections (QD) there was a slight, but not significant, decrease in therapeutic activity noted. The QD schedule at 12.5 mg/kg gave a long-term survival rate of 44% and a median survival time of 85 days, while the QOD schedule resulted in a long-term survival rate of 58%. There was no statistically significant difference between results when animals were treated QD or QOD.

It is worth noting that pathological evaluation at necropsy was completed in all long-term survivors and those animals showed no evidence of tumour either on gross inspection or on histological examination of tissues. Molecular studies, designed to detect the human bcl-2 gene by PCR, failed to detect residual disease in these mice (lane 4, Figure 5.2E). In contrast, those animals that were terminated during the course of these studies because of disease progression, whether in an ASO or a CPA alone treatment group, were found to have enlarged lymph nodes and tumour nodules consistent with those of untreated mice as well as

molecular evidence of disease as demonstrated by PCR (lane 2, Figure 5.2E). Immunohistochemistry was used to confirm the presence of bcl-2 expressing human lymphoma in the lymph nodes, spleen and femoral bone marrow. The findings established that the progression of the human lymphoma was the cause of death in these animals. It was concluded on the basis of these results that death during the 90-day time course was always associated with progression of the DoHH2 tumour and that animals surviving beyond 90 days were free of any residual disease. Figures 5.2C&D show a representative femoral bone marrow of a combination treated animal (5.0 mg/kg ASO and 35.0 mg/kg CPA) that survived past 90 days. It was observed that there was no human lymphoma cell infiltrate (Figure 5.2C) and Bcl-2 staining was negative (Figure 5.2D). Regardless, immunohistochemistry was routinely performed on samples from all animals that survived or were terminated as a consequence of tumour development. Some animals were maintained in the vivarium for time periods in excess of 120 days. In addition, selected mice that survived as a consequence of treatment were re-inoculated with 5×10^6 DoHH2 cells to assess whether the SCID animals had developed immune resistance to tumour cell challenge. These animals died within 35 days, typical of control animals, and exhibited Bcl-2 expressing human lymphoma in spleen, lymph nodes, bone marrow and elsewhere.

It is clear, as assessed by immunohistochemistry and PCR, that ASO treatment of lymphoma-bearing mice eliminated Bcl-2 expression associated with the DoHH2 cell. This is most likely a consequence of eradication of the tumour cells and may not be directly linked to downregulation of bcl-2 expression. It is possible that the therapeutic activity is linked to ASO induced immune stimulation and tumour cell loss by activated killer cells. Reports from many investigators have suggested that the mechanism of action for ASOs may be, in

155

fact, a result of their potency as immune stimulators rather than specific downregulation of protein expression (73, 74, 346, 347). Although the SCID/Rag-2 mice used in these studies were deficient in B and T cell maturation, the effect of ASOs on immunostimulation was further examined by treating lymphoma-bearing mice also deficient in perforin production (Figure 5.4 and Table 5.2). Pfp/Rag-2M mice were injected with DoHH2 i.v. and treated with ASO at 5 mg/kg alone for 14 treatments (QOD) or in combination with CPA (35 mg/kg, 3 treatments, day 4, 8, 12). Median survival of animals treated with ASOs increased from 27 days (control) to 37 days ($p<0.01$). When mice were treated with CPA in addition to ASO, median survival times increased to 61 when compared to animals treated with ASO only ($p<0.01$). Further, 17% of the animals in the combination treated cohort survived past 90 days.

5.3.3 Therapeutic activity of bcl-2 ASO in combination with CPA

The aim of these studies was to determine whether there was a clear therapeutic advantage provided when ASOs targeting the bcl-2 gene product were used in combination with CPA. As the experimental model was sensitive to the activity of each agent alone, doses where minimal activity was defined (CPA at 15 and 35 mg/kg and ASOs at 2.5 and 5 mg/kg/injection) were utilised in the combination studies. These studies have been summarised in Table 5.1B and Figure 5.3B. The benefits achieved by using the bcl-2 ASOs in combination with CPA are best illustrated at the lowest doses. In the absence of ASOs, a dose of CPA of 15 mg/kg resulted in no measurable increase in the life span of mice bearing the DoHH2 tumours. Alternatively, ASO given alone at a dose of 2.5 mg/kg/injection increased median survival to 62 days and one out of six animals survived beyond 90 days. In

Figure 5.4

Survival curves of pfp/Rag-2 male mice bearing a B-cell lymphoma

Pfp/Rag-2 mice were inoculated with 5×10^6 DoHH2 cells i.v and treated with ASO and CPA. Cohorts of mice were control (-■-), treated with 5 mg/kg ASO i.p. (-●-) or treated with 5 mg/kg ASO and 35 mg/kg CPA i.p. (-▲-). ASO treatment began on day 4 and continued QOD for 14 treatments and CPA treatments were on days 4, 8, 12.
*$p<0.01$ when comparing groups treated with ASO to untreated controls
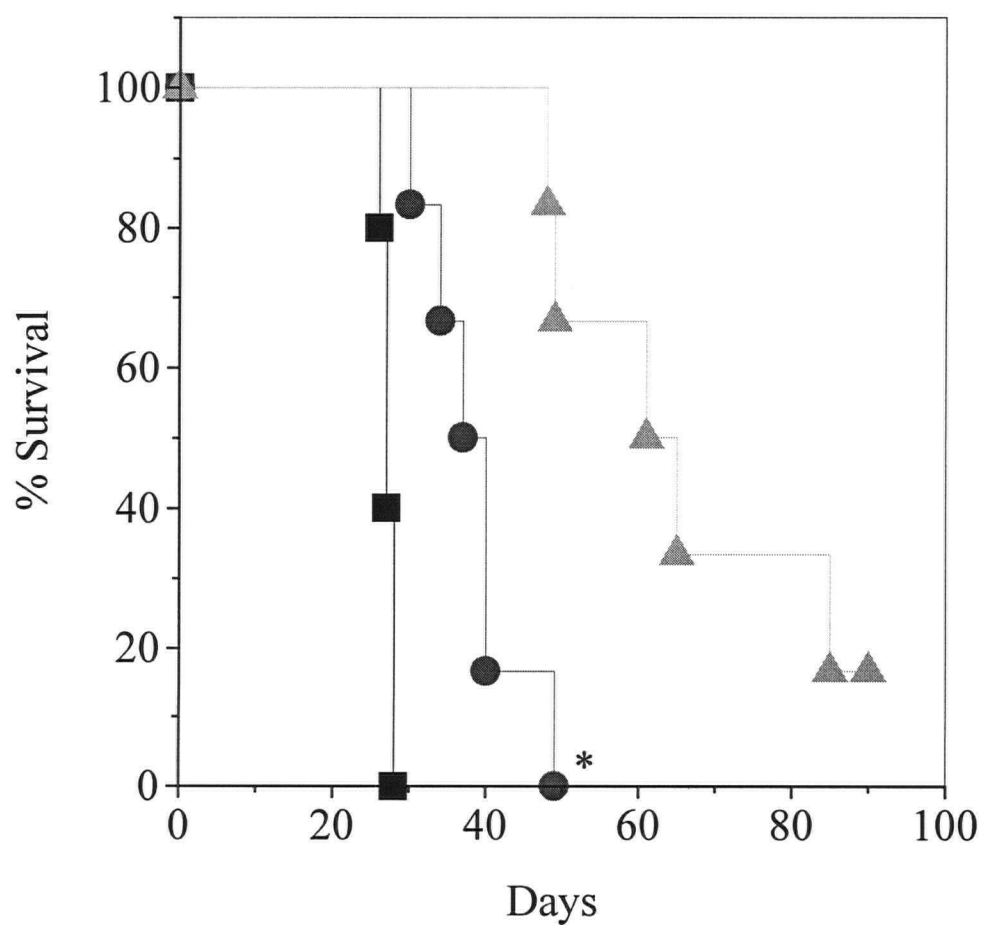
Table 5.2

Treatment of pfp/Rag-2 male mice bearing DoHH2 tumours

Pfp/Rag-2 mice were inoculated with 5×10^6 DoHH2 cells i.v. Animals were treated with None (saline only), ASO only (5 mg/kg injected i.p. QOD for 14 treatments beginning Day 4) or in combination with CPA (injected i.p. on Days 4, 8, 12)

Treatment	Dose mg/kg	Schedule	N	Median Survival	% 90 day survival	p-value[3]
None	NA[1]	NA	5	27	0	NA
ASO	5.0	QOD[2]	6	37	0	<0.01
ASO+CPA	5.0+35.0	QOD+4,8,12	6	61	17	<0.01

[1]NA: Not applicable
[2]QOD: Every other day treatment, animals were treated alternate days, including weekends.
[3]P-values were derived from using the log-rank test comparing animals treated with ASO alone vs control and ASO+CPA vs ASO alone.

combination, the median survival times were 72 and 84 days when treated with CPA at 15 mg/kg and ASO at 2.5 and 5 mg/kg, respectively. Both of these treatment groups exhibited long-term survival rates of 50% or more.

A similar pattern was also seen in animals treated at the 35 mg/kg dose of CPA. When animals were treated with CPA 35 mg/kg or ASO at 5 mg/kg/injection, they exhibited median survival times of 47 and 79 days, respectively, with no long-term survivors for CPA and 48% for ASO. When treated with both drugs the median survival times could not be determined because long-term survival exceeded 60% (range 61 to 100%, depending upon cohort), regardless of whether the ASO dose was 2.5 or 5 mg/kg/injection. CPA administered in combination with the controls RPOs and MMOs resulted in a median survival of 57 and 53 days, respectively, and this was not significantly different than the activity observed for CPA given alone (median survival of 47 days). It is worth noting that long-term survival increased significantly when CPA was combined with ASOs given by the less effective QD dosing schedule. As summarised in Table 5.1B, mice treated with ASO at 5 and 12.5 mg/kg/injection (QD) in combination with a 35 mg/kg dose of CPA exhibited long-term survival rates of 100 and 83%.

5.4 Discussion

These pre-clinical *in vitro* and *in vivo* studies have attempted to model the behaviour of human lymphoma using the DoHH2 cells implanted i.v. in SCID mice. This cell line carries the t(14;18) translocation associated with Bcl-2 overexpressing lymphomas (365). Chemotherapy alone in a moderate dose range had an impact on prolonging median survival but with no long-term survivors. Treatment with G3139 alone resulted also in some long-

term disease-free survivors. However, when ASO was combined with low dose CPA the majority of animals were long-term disease-free survivors with no histological or molecular evidence of persistence of lymphoma. It was noted that animals treated with ODNs, whether alone or in combination, exhibited splenomegaly, which correlated with dose. This effect did not appear to cause distress in the animals and no evidence of ASO-related toxicity was observed. This splenic hyperplasia may be specific to murine species, since it was not observed in primates or after clinical therapy with ASO (355).

Low dose chemotherapy with an ASO that is directed at bcl-2 has the potential to lower the amount of antineoplastic agent required to eliminate disease and therefore reduce the associated toxicity. It is important to note that downregulation of the specific antisense target protein need only be transient in order to minimise the growth advantage of the tumour cell such that the effect of chemotherapy is maximal. These data demonstrated that G3139 shows specific downregulation of Bcl-2 and enhances the activity of low doses of CPA in this model and in perforin knockout mice. When cured SCID/Rag-2 mice were challenged with DoHH2 cells, all animals exhibited symptoms of Bcl-2 expressing lymphoma and died within 35 days similar to control. This would indicate that ASO treatment did not stimulate a lasting immune response. Further, the studies in the pfp/Rag-2 mice strongly suggest that immunostimulation is not a factor in eradication of lymphoma cells *in vivo*. Rather, specific ASO/mRNA interactions leading to downregulation of Bcl-2 protein may, in fact, prime the tumour cells for death pathways, i.e. apoptosis, leading to chemosensitisation. This is significant because follicular lymphomas overexpress Bcl-2 as a survival advantage and, in many instances, this overexpression is the sole determinant keeping at least a percentage of

the malignant cells from going down the apoptotic pathway. If the apoptotic barrier is relieved, these cells appear sufficiently damaged for death.

These studies conclusively demonstrate that combination treatment of CPA and ASO renders SCID/Rag-2 mice curable of human lymphoma in a large fraction of the animals. The interaction between the two agents shows dose-response correlations (Tables 5.1A & 5.1B). For both doses of CPA, increasing the dose of ASO from 2.5 to 5 mg/kg resulted in longer median survivals and an overall increase in long-term survivors. A rather striking result was achieved when a completely ineffective dose of CPA (15 mg/kg - median survival 36 days and no long-term survivors) was combined with a modestly effective dose of ASO (2.5 mg/kg - median survival 61 days and 16% long-term survivors) to produce a 72 day median survival and 50% long-term survivors. These results suggest that chemotherapy at very modest doses could be made more effective with use of ASOs without increasing the toxicity to normal tissues. Such an increase in the efficacy of currently available chemotherapeutic agents could significantly alter the prognosis of a large number of moderately sensitive human tumours, resulting in improved clinical outcomes or increasing the potential for cure.

The model has direct relevance to the clinical situation faced in non-Hodgkin's lymphoma where patients typically present with what appears to be a chemotherapy-sensitive tumour at diagnosis which regresses only to recur within months to years post-treatment. Clinically, the prevalence of Bcl-2 overexpression in non-Hodgkin's lymphoma is high, consisting of 90% in follicular and mantle cell histologies and 50% of diffuse large cell disease. The DoHH2 cell line was derived from a follicular lymphoma carrying a t(14;18) which results in constitutive bcl-2 gene overexpression. The aggressive nature of the disease

in this model, however, is more suggestive of a transformation to a higher-grade histology, a common event in follicular lymphoma. Indeed, a recent re-exploration of the molecular and cytogenetic features of the cell line, using more sensitive detection techniques, has revealed a second translocation involving the c-myc oncogene with a resultant derivative chromosome 8 carrying t(8;14;18) (366). This clinical phenomenon of double translocation and constitutive overexpression of both bcl-2 and c-myc in a subset of patients with small non-cleaved cell (Burkitt-like) lymphoma, which represents a very aggressive form of the disease, has been recently described (371).

The data presented in this chapter addresses the potential role ASOs directed at the bcl-2 gene message in enhancing the therapeutic efficacy of a cytotoxic agent in non-Hodgkin's lymphoma. In a model of human melanoma implanted subcutaneously in SCID mice, B. Jansen *et al.* have shown chemosensitisation to dacarbazine (DTIC) with ASOs directed at bcl-2 resulting in reduced tumour volumes (348). This chapter utilises a similar strategy to increase the cure rate of a systemically distributed metastatic malignancy in a xenograft model. Moreover, the data suggest that improved clinical outcomes may be achieved with standard, or even lower, doses of anticancer drugs when combined with ASOs, potentially impacting overall clinical tolerance and costs of care. As a single agent, G3139 has entered clinical testing and promising initial results have been published - with a number of responses seen - in addition to downregulation of Bcl-2 protein in clinical samples from patients treated for non-Hodgkin's lymphoma (355). Based directly on these results Phase I/II clinical trials of chemotherapy in combination with G3139 in patients with non-Hodgkin's lymphoma have been initiated.

CHAPTER 6

A LIPID-BASED DELIVERY SYSTEM FOR ANTISENSE OLIGONUCLEOTIDES

DERIVED FROM A HYDROPHOBIC COMPLEX[*]

6.1 Introduction

Chapter 5 describes the utility of an ASO (G3139) directed against bcl-2 to enhance the therapeutic activity of a conventional chemotherapy agent (cyclophosphamide) against a B-cell lymphoma. Despite the promising results and its entry into Phase I/II clinical trials, ASO activity could be increased substantially if administered in an appropriately designed delivery system. The optimal vehicle for ASO would prevent nuclease degradation of the nucleotide backbone and enhance cellular permeability, thereby effecting delivery of intact molecules in a form that still facilitates downregulation of the target protein (372). In order to achieve therapeutic activity, ASO must first reach the target site and bypass the cell membrane in a form that retains its ability to complementary base-pair with the corresponding mRNA. Bypassing the cell membrane is a problematic venture since the very polar ASOs cannot passively diffuse through the cell membrane. Various methods of overcoming the obstacle to ASO delivery have been described, including direct 5' modification with cholesterol (373) and carrier technologies such as poly-L-lysine (374), transferrin-polylysine (375) and various forms of cationic lipids (94, 376, 377). This chapter focuses on the development of a novel method for generating a lipid-based delivery system for ASOs. These studies are based on the belief that lipids are a relatively safe and pharmaceutically viable technology for use in treatment of human diseases (378, 379).

The efficacy of lipid-based gene therapeutics is dependent upon initially protecting and then subsequently dissociating the lipids from the ASO molecule after reaching the target site (276, 380). The ASOs must be available to associate to the target mRNA in such a way that complementary base-pairing can occur. The presence of any associated lipid, in particular cationic lipids, may block the therapeutic potential of these molecules since even a single base mismatch can prevent specific association (381). Previous results have emphasised the importance of lipid dissociation for lipid-based gene transfer (272, 274, 276).

Results described in Chapter 2 and others have shown that solubilized cationic lipids can be added to plasmid DNA in order to form a hydrophobic complex (203, 313). This chapter demonstrates that the parameters relevant to the formation of this cationic lipid/DNA complex are also applicable to lipid/ASO formulations. In addition, a protocol was developed to convert a hydrophobic lipid/ASO complex into a lipid-ASO particle through addition of PEG-conjugated phosphatidylethanolamines. *In vitro* delivery studies were initiated using these novel lipid-antisense bcl-2 particles (LAPs) to the B-cell lymphoma cell line, DoHH2. The results suggest that the lipid-based delivery system promotes efficient association of fluorescein-labelled ASOs to this cell line; however, no significant decrease in Bcl-2 expression levels was observed. The LAPs were also assessed *in vivo* where the formulated ASO was at least as active as the free ASOs. It is suggested that the presence of PEG-PEs may be a detriment in the delivery of lipid-based ASO delivery systems not only by decreasing ASO to cell binding but also by modulating processing events associated with dissociation from the lipid carrier. Methods that facilitate loss of the PEG-lipids from the carrier following administration are considered in the discussion of these data.

6.2 Materials and Methods

6.2.1 Materials

DC-Chol and DOTAP were obtained from Sigma Chemical Co. (Oakville, ON) and Avanti Polar Lipids (Alabaster, AL), respectively. Polycationic lipids – Lipofectamine™ (3:1 w/w DOSPA:DOPE), Transfectam™ (DOGS) were purchased from Gibco BRL (Burlington, ON). All other lipids, egg-PG, DMPE-PEG$_{2000}$ and DSPE-PEG$_{2000}$, were obtained from Northern Lipids (Vancouver, B.C.).

All other materials used are described in Chapter 2, section *2.2.1*; Chapter 3, section *3.2.1*; Chapter 5, section *5.2.1*.

6.2.2 Methods

6.2.2.1 Bligh and Dyer extraction of ASOs

All monocationic and polycationic lipids were solubilized in chloroform and made up to a concentration of 1600 nmol/mL in a Bligh and Dyer monophase consisting of chloroform:methanol:water (1:2.1:1 v/v/v) (312). ASOs (2 - 40 µg) were resuspended in Bligh and Dyer monophase and combined in a 1:1 volume ratio with DODAC (0 - 640 nmol) to give a total volume of 1 mL. The monophase solution was subsequently partitioned into a two-phase system *via* the addition of 250 µL each of chloroform and water allowing for the ASO to partition into either an aqueous or organic phase. At this point the two-phase system was vortexed vigorously for 1 min and centrifuged at 600×g for 5 min at room temperature. The aqueous (~1 mL) and organic (~0.5 mL) layers were then separated and the amount of ASO in the aqueous layer was determined.

6.2.2.2 Quantification of ASO

The loss of the ASO from the aqueous phase (into the organic phase) was assessed by measuring the optical density at a wavelength of 250 nm using a Beckman UV spectrophotometer (DU-54) (Mississauga, ON). The amount of ASO retained in the aqueous phase following extraction was determined over a range of DODAC concentrations and was expressed as percent of total ASO initially present in the monophase system. Verification that the ASO was, in fact, recovered in the organic phase was accomplished using fluorescent bcl-2 ASO, assuming that it behaves in a similar manner to unlabelled ASO. Spectrofluorometric readings of the organic and aqueous layers were performed using a Luminescence Spectrometer 50B (Perkin-Elmer; St-Laurent, Quebec). All sample readings were recorded using an excitation wavelength of 493 nm and an emission wavelength of 517 nm and were expressed as arbitrary fluorescence units. Evaluation of the presence of ASO in the organic phase was further characterised by back extraction of the ASO into an aqueous phase generated from a 1 mL Bligh and Dye monophase partitioned by the addition of 250 µL each of chloroform and water. In order to dissociate the ASO/cationic lipid complex the anionic lipid 1600 nmol egg-PG was added. This system was vortexed for 1 min and centrifuged at 600×g for 20 min. Quantification of fluorescent ASO present in the aqueous phase was carried out under the conditions described above.

6.2.2.3 Effects of other lipids on the hydrophobic lipid/ASO complex

In order to assess the effects of the zwitterionic lipids DOPE and DOPC on the formation and/or dissociation of the DODAC/ASO complex, increasing amounts of these neutral lipids were then added to the system in one of two ways. In the first method, the

DODAC/ASO complex was formed prior to the addition of the other lipids. The neutral lipids were added into the organic phase following partitioning of the Bligh and Dyer monophase into two phases. The samples were mixed vigorously by vortexing and separated by centrifugation as previously described. The effect of these added lipids on the dissociation of the complex was evaluated by quantifying the ASO in the aqueous phase. Alternatively, the effects of the additional lipids on the formation of the complexes were evaluated by mixing them with ASO prior to the addition of DODAC. As a control, DOPE and DOPC were added to the ASO in the absence of DODAC to ensure that these lipids did not mediate the extraction of the ASO into the organic phase.

6.2.2.4 Formulation of lipid-ASO particles

DODAC and 100 µg ASO were made up in 1 mL Bligh and Dyer monophase at a charge ratio of 1.3:1 (+/-). The monophase was partitioned using 250 µL each of water and chloroform and vortexed vigorously for 1 min. The samples were centrifuged at 600×g for 20 min at room temperature. Spectrophotometric measurement of the aqueous layers following phase separation indicated that >95% of initial ASO had been extracted into the organic phase. The organic phase was dried down using a stream of nitrogen gas. Subsequently, an equimolar amount of DOPE and 2 - 30 mol % DSPE- or DMPE- PEG (in chloroform:methanol 1:1 v/v) was added to the film. The resultant lipid/ASO film was vacuum dried for at least 2 h. Rehydration of the LAPs was carried out using 500 µL of phosphate buffered saline (PBS, pH 7.2) and then incubated overnight at room temperature. It was not possible to hydrate the LAPs without forming large aggregates (>1000 nm) unless at least 2 mol % PEG-conjugated lipid was present in the film prior to addition of buffer.

The LAP solution was vortexed vigorously for at least 2 min to minimise adherence of the lipid-ASO particle film to the test tube. Particle sizing was assessed using a NICOMP model 270 submicron particle sizer. No further processing of the lipid-based structures was required.

6.2.2.5 Preparation of LUV/ASO complexes

Methods were previously described in Chapter 5, section *5.2.2.1*

6.2.2.6 FITC-labelled ASO Delivery to DoHH2 cells

DoHH2 cells (1 mL) at a concentration of 1×10^6 cells/mL were added to 48-well plates in RPMI without serum. LAPs (100 µL), prepared using FITC-labelled ASO, were added to each well. After a 4 h incubation at 37°C, cells were removed from 4 wells and combined. Cells were washed in PBS by centrifugation at 100×g for 5 min. After the last wash, the cells were kept in a pellet and placed, using a transfer pipet, onto a glass slide. The cells were viewed under a Leica Duralex fluorescent microscope and photographs were taken.

6.2.2.7 Western Blot

Methods were previously described in Chapter 5, section *5.2.2.3*.

6.2.2.8 Formation of encapsulated ASO

An encapsulated formulation of ASO was used as a positive control to compare against LAP formulations. Others have established that the EnASO formulation described

below has increased circulation longevity and efficacy in subcutaneous breast tumour models (D. Saxon, personal communication). Stock solution of lipids, DODAP, DSPC, Chol, PEG-CerC14 were made up in 100% ethanol and combined in proportion of 20:25:45:10 mol %. ASO were solubilized in 300 mM citrate buffer, pH 4.0. The ASO and lipid solutions were warmed briefly in a 65°C water bath. Lipids were then added dropwise to the ASO solution, resulting in an alteration from a clear and colourless to cloudy solution. No visible aggregates were observed. Five freeze-thaw cycles were performed by placing the solution in cryo-vials, which were then subjected to freezing in liquid N_2 and a 65°C water bath. The lipid-ASO solution was then extruded 10× through 3 stacked polycarbonate filters (Nucleopore, Pleasanton, CA or Poretics Corp, Mississauga, ON) of pore size 100 nm using an Extruder (Lipex Biomembranes Inc., Vancouver, BC). The extruded lipid-ASO solution was dialysed against 1 L 300 mM citrate, pH 4.0 for 3 h and 1 L HBS (150 mM NaCl, 20 mM HEPES), pH 7.5 to remove remaining ethanol and raise the pH. The EnASO was separated from free ASO through passage down a DEAE Biogel A agarose column (Bio-Rad Laboratories; Richmond, CA), equilibrated with HBS, pH 7.5. If necessary, the concentration of the EnASO solution was increased using a concentrator from Amicon Inc. (Beverly, MA).

6.2.2.9 Plasma elimination and biodistribution studies

SCID/Rag-2 mice (6 per group) were injected i.v. *via* the lateral tail vein with a total of 5 mg/kg dose of ASO and ~150,000 dpm of radioactivity. The ASO formulations injected were free ASO, EnASO, or LAPs containing 10% DSPE- or DMPE- PEG. At 1, 2, 4, and 24 h, 25 μL whole blood was collected *via* tail nicks, and placed into 5% EDTA-containing

microfuge tubes. Plasma was isolated by two centrifugations of whole blood at 500×g for 10 min each. Samples (200 μL) were collected after each centrifugation, pooled and assessed for radioactivity ([^{3}H]-ASO or [^{14}C]-lipid).

Animals were terminated by CO_2 asphyxiation and tissues were harvested at the 24 h time point. Distilled water was added to livers in a volume sufficient to generate a 25% (w/v) homogenate and homogenised with a Polytron tissue homogeniser (Kinematica, Switzerland). To aliquots of the liver homogenate (200 μL) or whole spleens and kidney, 500 μL Solvable™ was mixed and incubated at 50°C for at least 3 h. After cooling of the resultant mixture to room temperature, 50 μL of 200 mM EDTA, 200 μL 30% H_2O_2 and 25 μL 10 N HCl were added. Femurs were flushed with 3 mL PBS to recover bone marrow and incubated with 500 mL Solvable™. Pico-fluor scintillant was added to all samples and allowed to rest overnight prior to assessing radioactivity ([^{3}H]-ASO or [^{14}C]-lipid). At 24 h, there were significant levels of radioactivity in order to calculate ASO to lipid ratios.

6.2.2.10 Efficacy studies

Male SCID/Rag-2M mice used for these studies were obtained from a breeding colony and maintained in a pathogen-free environment at the BC Cancer Agency. Mice were used for experiments when they were 6 - 9 weeks old and weighed 22 g or more. Viable DoHH2 cells (5×10^6 cells in 200 μL) were injected i.v. *via* the tail vein of each animal and disease was allowed to establish for 4 days. Cohorts of at least 6 animals were then treated in the following groups: untreated control (injected with saline) (group 1), EnASO (group 2) or lipid-ASO particles containing 10% DSPE- (group 3) or DMPE- PEG (group 4). I.p. injections, containing a total ASO dose of 5 mg/kg, were administered Monday, Wednesday

and Friday for 14 treatments. Animals were assessed for illness by technicians blinded to the group's treatment and terminated at signs of illness including, but not limited to, paralysis in the hind region, scruffy coat, lethargy, weight loss of >20%, or if they survived past 90 days.

6.2.2.11 Statistical analysis

All data values for downregulation studies as assessed by densitometry are presented as the mean ± SEM. Statistical analysis was completed as described in Chapter 3, section *3.2.2.10.*

ANOVA (analysis of variance) was performed on the plasma elimination studies after administration of free ASO, EnASO and LAPs. Comparisons were made for various common time points using the Post Hoc comparison of Means, Scheffé test. Differences were considered significant if $p < 0.05$ and p-values are reported.

Statistical analysis for efficacy studies was completed as described in section *5.2.2.7.*

6.3 Results

6.3.1 Characterisation of cationic lipid binding to ASO

Chapter 2 describes the parameters for the preparation of a hydrophobic cationic lipid/plasmid DNA complex and the recovery of this complex in high yield (>95%) into the organic phase of an aqueous/organic partitioning system [Chapter 2, (203, 313)]. In samples containing 40 nmoles of cationic lipid and 10 µg of DNA where the charge ratio of lipid to nucleotide phosphate (+/-) was 1:1, >95% of DNA present could be extracted into the organic phase (313). This work prompted an investigation into the possibility that the same

interaction might be observed between cationic lipid and an ASO targeting the bcl-2 gene product.

Evaluation of the formation and extraction of the cationic lipid-ASO complex in the organic phase was completed using FITC-labelled ASO. Figure 6.1A shows that with increasing amounts of a monovalent cationic lipid (DODAC) added the ASO concentration in the aqueous phase (■) decreases with a corresponding increase in the organic phase (●). In a second experiment, 1600 nmol egg-PG, (Figure 6.1B) was used to dissociate the hydrophobic cationic lipid-FITC-labelled-ASO complexes present in the organic phase. The ASO released from the complex was subsequently re-extracted into an aqueous phase and the results suggest that >90% of the bcl-2 ASO in the organic phase could be recovered following addition of the anionic lipid. Similar results were observed by measuring unlabelled ASO recovery in the aqueous phase using spectrophotometry (OD_{260}). Further experiments were carried out under the assumption that unlabelled bcl-2 ASO behaves in a manner equivalent to that of the fluorescent probe-labelled bcl-2 ASO.

DODAC/ASO complex formation was determined over a broad range of ASO concentrations (2 - 40 μg) and the results are shown in Figure 6.2. The results demonstrate that the extraction of ASO out of the aqueous phase is concentration-dependent. Panel A shows that for 10, 20, and 40 μg of ASO >95% of the ASO was extracted from the aqueous phase when 40, 80, and 160 nmol of DODAC was added, respectively. When the charge ratio (+/-) of the cationic DODAC to anionic ASO was calculated, these data suggested that efficient extraction of ASO into the organic phase was achieved when a charge ratio (+/-) of 1:1 or greater was obtained (Figure 6.2B). This relationship between charge ratio and complex formation was no longer valid when the amount of ASO present in the assay was

Figure 6.1

Formation of the hydrophobic DODAC/ASO complex

A: Recovery of 10 μg of FITC-labelled ASO from aqueous (■) and organic (●) fractions following Bligh and Dyer extractions upon increasing amounts of DODAC.

B: Recovery of 10 μg of ASO from the organic fraction over increasing amounts of DODAC following Bligh and Dyer extractions and back-extraction into an aqueous phase using 1600 nmol egg-PG to dissociate the lipid-ASO complex.

Data are expressed as arbitrary fluorescence units and represent values obtained using an excitation wavelength of 493 nm and an emission wavelength of 517 nm.
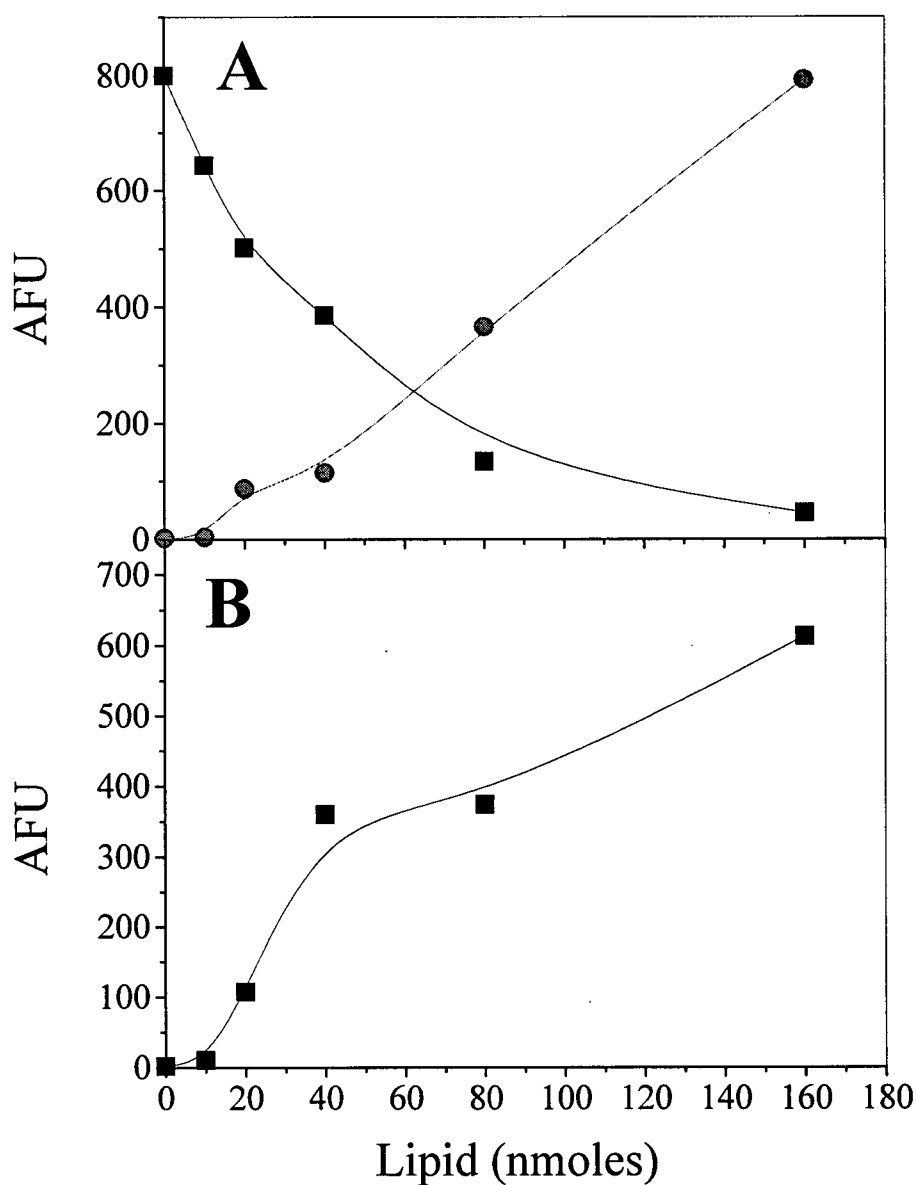
Figure 6.2

Recovery of various initial amounts of ASO following Bligh and Dyer extraction

Effect of increasing amounts of DODAC on ASO recovery (%) in the aqueous phase. Initial ASO amounts were: 2 μg (■), 5 μg (●), 8 μg (▲), 10 μg (▼), 20 μg (◆), and 40 μg (+). Data are represented as increasing concentrations of DODAC (**A**) or calculated charge ratios (**B**). All data points were averaged from three separate experiments and expressed ± SEM.
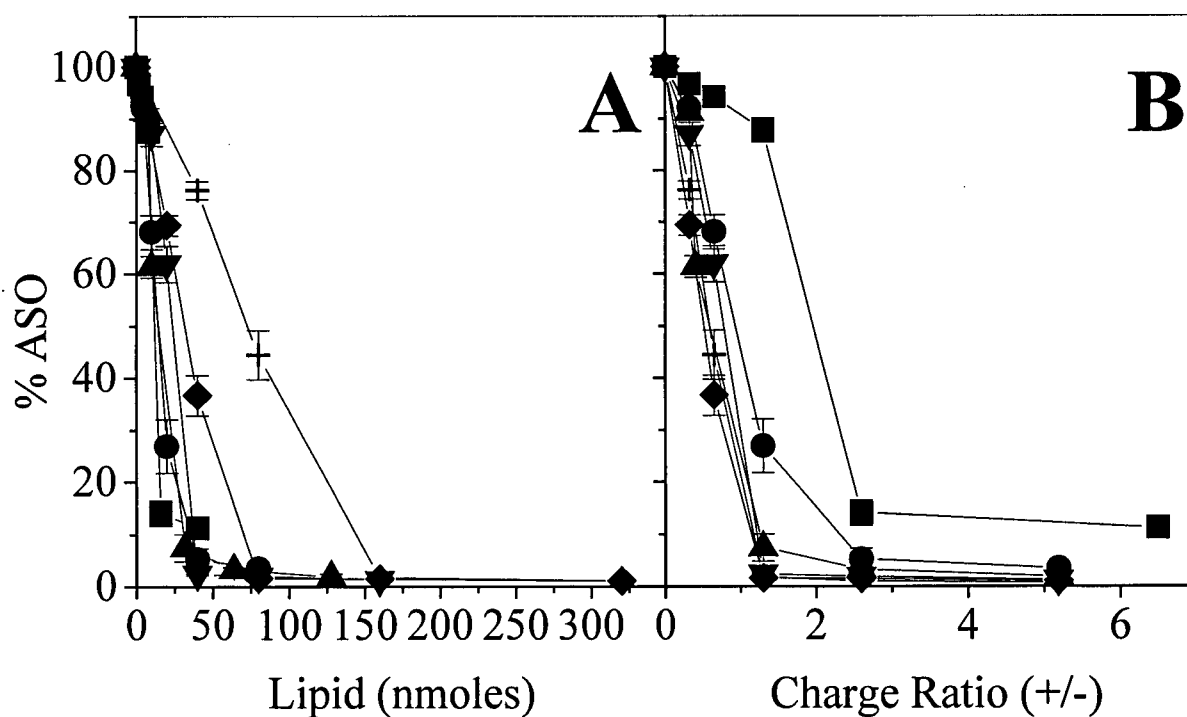
below 10 μg as illustrated in Figure 6.2A. These results suggest that a minimum concentration of lipid (40 nmol per assay) was necessary to effect the formation of the hydrophobic complex when the ASO concentration was below 10 μg.

With the parameters of DODAC/ASO complex formation defined, a series of experiments were performed in which the chemical attributes of the cationic lipid species were varied. Two classes of cationic lipids were employed: monocationic (DDAB, DC-Chol, DOTAP, Figure 6.3A) and polycationic (DOGS and DOSPA, Figure 6.3C). Formation of the lipid-ASO complex, as measured by a decrease in the proportion of ASO (10 μg per assay) isolated in the aqueous phase, appeared to be dependent on achieving charge neutralisation for the lipids DDAB and DOTAP; this was similar to the data obtained with DODAC. Figure 6.3A shows that for 10 μg of ASO, >95% of the ASO was extracted from the aqueous phase when 40 nmol of DODAC, DDAB or DOTAP were added [corresponding to a charge ratio (+/-) of 1:1 (Figure 6.3B)]. However, the monovalent cationic lipid DC-Chol was required in a higher amount, at a charge ratio (+/-) of 5.2, to efficiently extract ASO into the organic phase.

The multivalent cationic lipids DOGS (Transfectam™) and DOPSA (in Lipofectamine™) were unable to mediate efficient extraction of the ASO into the organic phase and form a hydrophobic complex. DOGS consists of four positive charges on each headgroup per two acyl chains whereas DOSPA consists of five positive. In both cases, >95% of ASO was retained in the aqueous phase even when the cationic lipid was increased to a charge ratio (+/-) as high as 5.2. Although charge neutralisation may result as a consequence of electrostatic interactions between the polyvalent positive headgroups with the ASO phosphate groups, the hydrophobic nature of the complex is conferred by the amount of

Figure 6.3

Recovery of ASO by cationic lipids following Bligh and Dyer extraction

Effect of increasing amounts of monocationic lipids (**A, B**) or polycationic lipids (**C, D**) on ASO recovery (%, 10 μg initial) in the aqueous phase. Monocationic lipids used are DDAB (■), DOTAP (●), DODAC (▲), and DC-Chol (▼). Polycationic lipids used are Lipofectamine (■) and Transfectam (●). Data are represented as increasing amount of cationic lipid added (**A, C**) or calculated charge ratios (**B, D**).
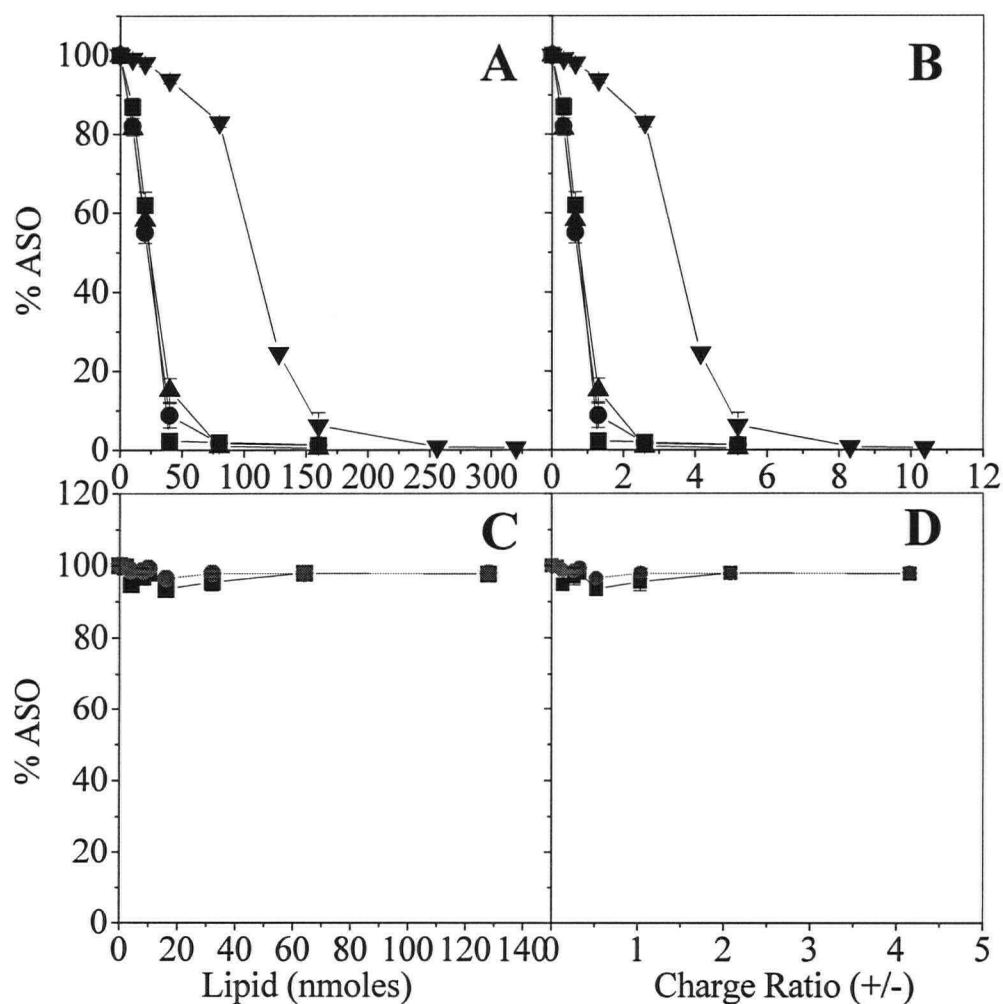
acyl chains present. The ratio of hydrophobic acyl chains (2) to positive charges (4 or 5) for the multivalent cationic lipid was insufficient to render the ASO-lipid complex hydrophobic.

6.3.2 Effect of additional phospholipids on hydrophobic complex

Other lipids (i.e. DOPE), in addition to cationic lipids, will play a critical role in enhancing the effectiveness of ASOs and will contribute to defining the physical and chemical characteristics of the delivery system. For this reason, it is important to assess hydrophobic complex formation and stability in the presence of other lipids. The presence of secondary lipids can affect lipid/ASO complex formation in two ways. Additional lipids present at the interface might displace cationic lipids and effectively reduce the amount of DODAC at the interface; alternatively, the added lipid might interact directly with the cationic lipid, preventing complex formation.

Studies evaluating the effect of added lipids on cationic lipid/ASO complex formation and/or destabilisation were based on a lipid titration where the secondary lipid amount was increased in a system containing 10 µg of ASO and 40 nmol of DODAC (Figure 6.4). The second lipid, DOPE or DOPC, was added either before or after complex formation. Three points are evident from these studies.

First, complex formation was inhibited in a concentration-dependent fashion when DOPE was added prior to complex formation (Figure 6.4A).

Second, as shown in Figure 6.4B, the addition of increasing amounts of DOPE destabilised the cationic lipid/ASO complex when the zwitterionic lipid was added after complex formation. This effect was evident as ASO retention in the aqueous phase with increasing concentration of DOPE. These results are consistent with previous studies using

Figure 6.4

Effect of lipids on the formation and dissociation of the hydrophobic lipid/ASO complex

DOPE (■) and DOPC (●) were added to assess the recovery of 10 μg of ASOs from the aqueous phase following Bligh and Dyer extractions. The neutral lipids were added before (to assess effect on formation) (**A**) or after (to assess effect on dissociation) (**B**) formation of the hydrophobic complexes using 40 nmoles of DODAC.
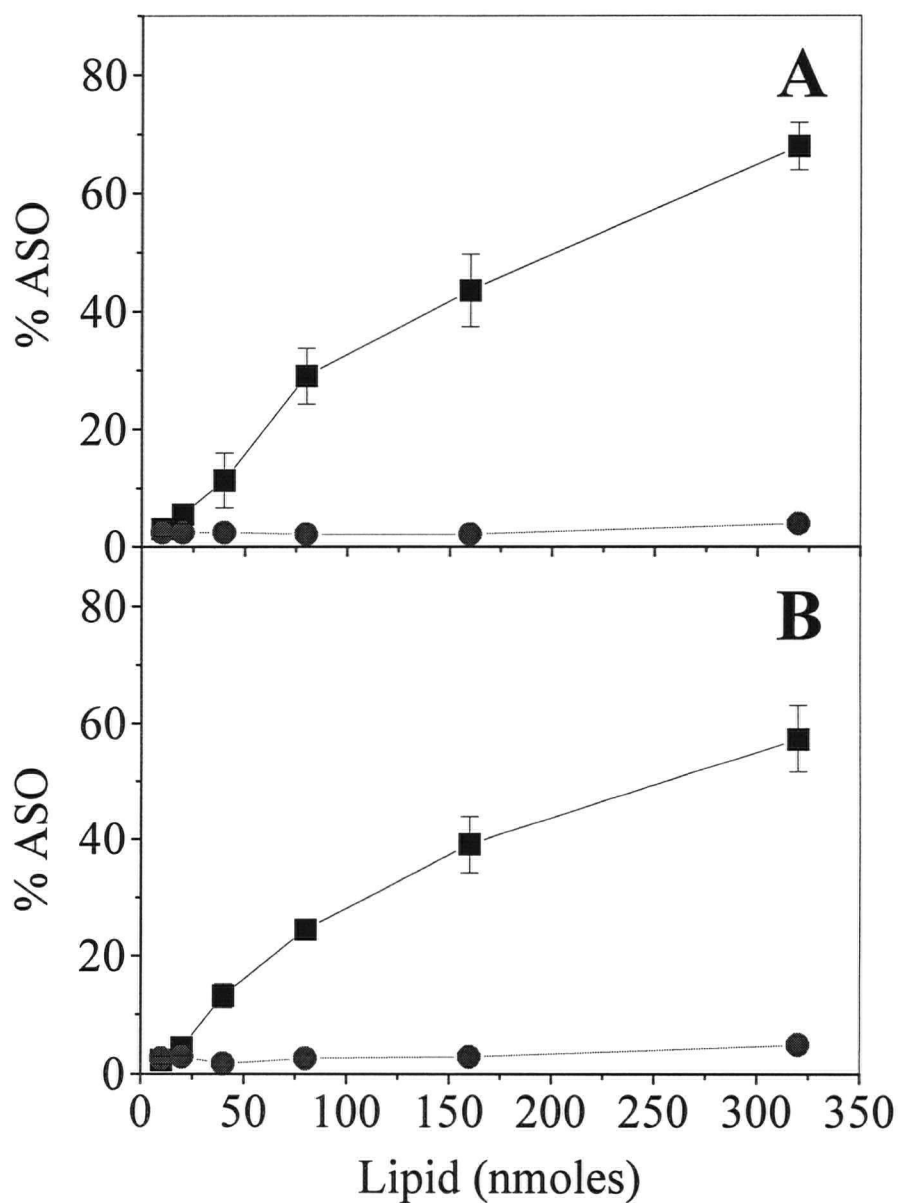
plasmid DNA [Chapter 2 & 3, (110, 313)], where it was postulated that destabilisation may be a consequence of direct DOPE/DODAC interactions.

Third, the addition of DOPC had no impact on formation or stability of the DODAC/ASO complex even at levels approaching 10-fold molar excess relative to DODAC.

6.3.3 Formation of lipid-ASO particles

The interest in the characterisation of cationic lipid/ASO complexes lies in the implication of this research for the preparation of well-defined lipid-ASO particles for use in gene therapy. Two approaches may be considered using a hydrophobic cationic lipid/ASO complex as an intermediate: First, particles could be prepared from solutions where cationic lipids, secondary lipids, and ASO are mixed in the presence of detergent, a procedure that is analogous to that which is described for plasmid DNA [Chapter 3, (110, 318, 319)]; second, solvent-based approaches could be considered where hydrophobic cationic lipid/ASO complexes are mixed with selected lipids prior to solvent removal and subsequent hydration to achieve particle formation. This chapter details the formation of novel lipid-ASO particles based on the latter approach.

An important goal of these studies was to determine whether lipid-ASO particles could be generated using the hydrophobic complex as an intermediate and subsequently to determine whether these particles were useful as delivery systems for ASOs. In order to ensure >95% recovery of the ASO in the organic phase, LAPs were generated with DODAC added at an excess charge ratio (+/-) of 1.3:1 and no other lipids were added at the time of phase partitioning. Upon formation of the hydrophobic lipid/ASO complex and its extraction into the organic phase, the upper aqueous phase was removed. However, the aqueous phase

was checked periodically by spectrophotometric measurement and ASO concentration in the aqueous phase never exceeded 5% of the total ASO added. The organic phase was dried down using a stream of nitrogen gas. Subsequently, an equimolar amount of DOPE and 2 - 30 mol % DSPE- or DMPE-PEG (in chloroform:methanol 1:1 v/v) was added to the film and vacuum dried for at least 2 h. Rehydration of the lipid-ASO particle was carried out using PBS and then incubated overnight at room temperature.

The formulation strategy using equimolar DODAC and DOPE was similar to that when generating lipid-DNA particles [Chapter 3 and (110)]. However, it was not possible to hydrate the film prepared using the hydrophobic lipid/ASO complexes therefore, at least 2 mol % PEG-PE was added prior to film formation. In the absence of PEG-PE the majority of the lipid complex remained bound to the glass surface, even under conditions where the sample was heated and vortexed vigorously. As long as a minimum of 2 mol % PEG-PE was present in the formulation, all particles generated following hydration were <200 nm. There was no further processing of the LAPs required prior to their use in the *in vitro* studies described below.

LAPs were generated containing up to 30% PEG-PE, although it was not determined whether all the PEG-PE lipid was incorporated into the particle or whether excess PEG-lipid resulted in generation of micellar structures that were free of ASOs. The presence of PEG-PE was clearly of benefit in assisting hydration of the dried lipid film and it was anticipated that PEG-PE incorporation would result in several important properties that would also be of value for *in vivo* applications. The utility of PEG-conjugated PEs for lipid-based delivery of drugs, nucleic acid-based, or conventional cytotoxic agents, is well described (160, 382, 383). These advantages include reducing aggregate formation, prevention of serum protein

binding, increasing circulation longevity, and decreasing cell-binding attributes, among others. The latter characteristic is of particular interest when considering the following *in vitro* studies that assess LAPs for delivery of fluorescein-labelled ASO and their ability to downregulate Bcl-2 protein.

6.3.4 Delivery and activity of LAPs

The extent of delivery of cell-associated FITC-ASO mediated by LAPs was assessed qualitatively using fluorescence microscopy and compared to a positive control consisting of DODAC:DOPE liposomes complexed with ASO (Figure 6.5). For all treatment groups 2.5 μM FITC-ASO was added to DoHH2 cells for 4 h, washed and immediately viewed. Formulations were also evaluated for ASO and/or lipid toxicity by measuring trypan blue exclusion. It was necessary to use a carrier system, in the form of DODAC:DOPE liposomes (LAAs) or as LAPs, with the ASO in order to observe levels of fluorescence above background. Free ASO did not accumulate in cells under the conditions used here. When LAAs were added to DoHH2 cells *in vitro* for 4 h there were significant amounts of fluorescence accumulated in a few cells, approximately 5× greater than any of the LAPs studied. However, only ~60% of the observed cells were viable. These data suggests that in the absence of PEG-conjugated lipids there is significant cationic lipid-associated toxicity to cells *in vitro*. Cell-associated fluorescence was visualised for all observed cells when using LAPs and treated cells appeared viable as observed using microscopic evaluation of trypan blue treated cells. While the degree of fluorescence observed per cell was significantly decreased, when compared to LAAs, the number of cells with cell-associated FITC-ASO was increased over 5-fold. The fluorescence of FITC-ASO was compared within LAPs prepared

Figure 6.5

Delivery of FITC-ASO to DoHH2 cells

Fluorescent and the corresponding phase contrast micrographs of 2.5 µM FITC-ASO delivery mediated by lipid-based delivery systems. Vehicles and controls were added to DoHH2 cells *via* a 4 h pulse in media without serum. DoHH2 cells were incubated with PBS only (**DoHH2**), **Free ASOs**, or **LAAs**. **LAPs** added contained DODAC:DOPE at a 1:1 lipid ratio with **10**, **20**, or **30%** DSPE-PEG.
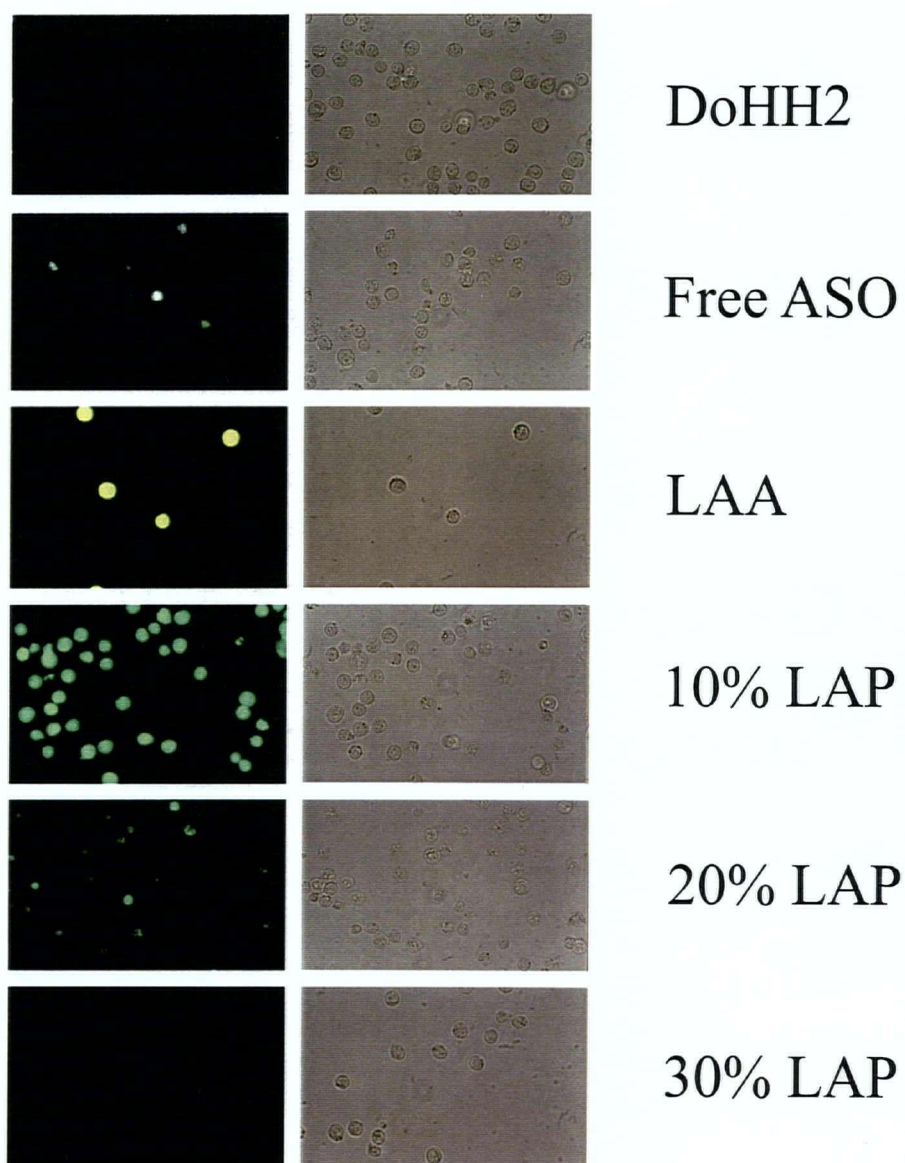
with high amounts of PEG-PE (10, 20, 30 mol %) in order to assess the extent of PEG-mediated ASO binding inhibition. Increasing the mole % of PEG-PE incorporated into the LAP decreased the amount of FITC-ASO associated per DoHH2 cell. However, the number of cells with some level of fluorescence is not different when comparing cells treated with LAPs containing 10 or 30% PEG-PE.

Chapter 5 demonstrated that G3139 ASO as LAAs, added to cells *in vitro*, specifically downregulates Bcl-2 protein when compared with RPO and MMO sequences. Suppression of Bcl-2 due to ASO addition was optimal at 72 h post-treatment, where LAA mediated complete elimination of Bcl-2 protein as demonstrated by Western analysis [Chapter 5 and (384)]. The cellular toxicity associated with LAA treatment was significant as there was at least 40% loss of cell viability when using trypan blue exclusion. Figure 6.6 confirms these results and the ability of LAPs to effect downregulation of Bcl-2 protein was evaluated under comparable conditions. LAPs formulated with either DSPE- or DMPE-PEG at 2 - 15% and were added in the presence of serum over 72 h or *via* a 4 h pulse without serum. Figure 6.6 shows representative results for DoHH2 cells incubated with the various formulations over 72 h in the presence of serum. The reason for using two different PEG-modified lipids is described in more detail later in this chapter. Briefly, DMPE-PEG (C14) is known to exchange out of membrane surfaces at a rate that is significantly fast than that observed for DSPE-PEG (C16). PEG-lipid exchange is dependent on the presence of other membranes, whether they are in the form of liposomes or cells. Regardless of the incubation time or the presence or absence of serum, the LAPs formulations containing DSPE- or DMPE- PEG did not decrease Bcl-2 expression in DoHH2 cells to a level equivalent to that seen with the LAAs. It was anticipated that reductions in Bcl-2 downregulation would

Figure 6.6

Western blot analysis of Bcl-2 expression after treatment with LAPs

Analysis of Bcl-2 expression after treatment with ASO alone or delivered by lipid-based delivery systems as determined by Western blot (**A, B**) and densitometry (**C, D**). DoHH2 cells were incubated with PBS only (**DoHH2, 1**), **Free ASOs** (**2**), or liposome-ASO aggregates (**LAAs, 3**) for 72 h in serum. Cell lysates were extracted 72 h post-treatment. Also, LAPs were added containing DODAC:DOPE at a 1:1 lipid ratio with **2** (**4**), **5** (**5**), **10** (**6**), or **15** (**7**)% of PEG-conjugated to **DSPE** (Panel **A**) or **DMPE** (Panel **B**).

Densitometry analysis was assessed from three separate experiments and reported as mean ± SEM.

*$p<0.0001$ when comparing LAAs to DoHH2

184
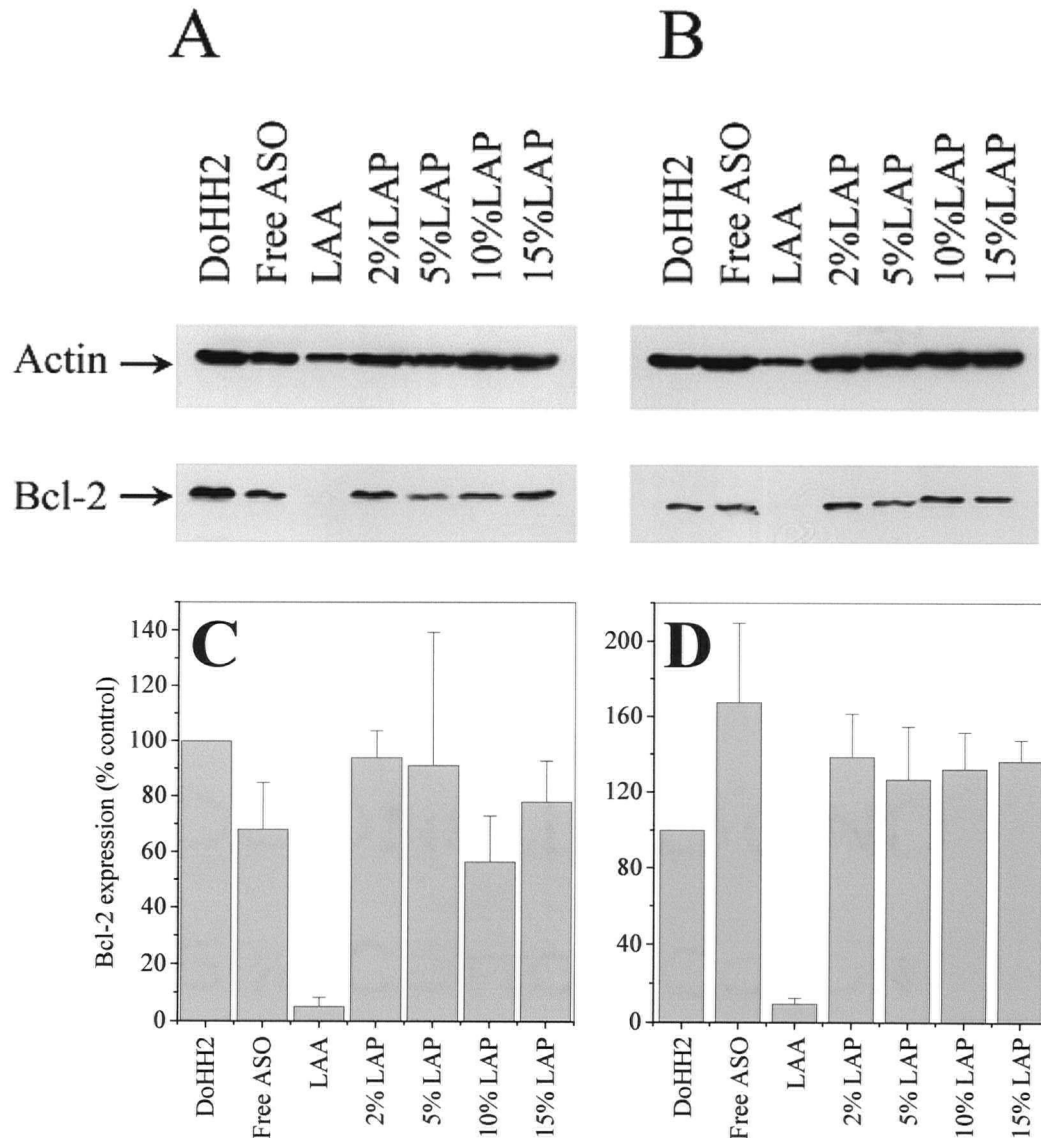
correlate with decreased ASO delivery. However, even under conditions where fluorescent ASO delivery was still observed (see 10% DSPE-PEG LAP formulation data shown in Figure 6.5) little to no downregulation of Bcl-2 was noted when compared to LAAs.

6.3.5 In vivo *studies*

Although the *in vitro* data described above would suggest that LAP formulations are not promising, there are several reasons for taking the formulations forward to studies *in vivo*: First, a lipid-based delivery system that is not capable of binding *in vitro* will also be resistant to serum protein and cell binding *in vivo*. Second, the rationale for including specific amounts of PEG-containing lipid was based on previous reports that suggested that the presence of PEG increases circulation lifetimes of lipid-based gene transfer vehicles (385). Third, recent reports suggest that therapeutic activity eliminated due to incorporation of PEG-modified lipids can be reinstated by facilitating loss of attached PEG-moieties (170). As such, the results obtained in Figure 6.5 demonstrating a decrease in cell-associated ASOs by formulating within LAPs were actually viewed as encouraging and the following *in vivo* studies were initiated.

The circulation longevity of lipid-based ASO formulations, generated either by encapsulation or through the Bligh and Dyer intermediate, were assessed. EnASO, prepared with DODAP:DSPC:Chol:PEG-CerC14 (20:25:45:10), was generated as described in the Methods, section *6.2.2.8*. This formulation has demonstrated increased circulation longevity, protection against nucleases and increased delivery of ASO to tumour site leading to a therapeutic benefit (386). LAPs were formed such that a charge ratio of 1.3:1 (+/-) of cationic lipid:anionic phosphate groups was used. A lipid ratio (DODAC:DOPE) of 1:1 with

10 mol % of DSPE- or DMPE- PEG was employed in the preparation of LAPs for these studies. After i.v. administration *via* the lateral tail vein of SCID/Rag-2M male mice, plasma samples were recovered over 24 h and radioactivity for ASO or total lipid was determined. The plasma elimination profiles of the various formulations of ASOs (A) and lipid (B) are shown in Figure 6.7. The plasma levels of ASOs, when given in free form, were close to detection limits and <1% of the total administered free ASO was recovered after 1 h. LAPs composed of DODAC:DOPE:PEG-PE (45:45:10) did not result in increased ASO levels in the circulation, despite the presence of PEG-conjugated lipids (DSPE-PEG or DMPE-PEG). Only the EnASO formulation facilitated a decrease in the elimination of the ASO from the plasma compartment, where ~25% of the total ASO administered could be detected by radioactivity at 1 h after administration. At 24 h post-injection, levels of ASO within EnASO detected were not significantly different from free ASO or LAP formulations.

Lipid elimination profiles for all formulations assessed were comparable to those observed for ASO, an initial indication that there was little release of ASO from the lipid formulations tested. This suggestion is confirmed for the EnASO formulation as the ASO to lipid ratio (Figure 6.7C) remained at a constant level over 24 h. In contrast, LAPs whether composed of DMPE- or DSPE- PEG appeared to undergo a significant increase in the ASO to lipid ratio over 24 h. Increases in the ASO to lipid ratio could be due to:

First, rapid elimination of a population of LAPs with low ASO to lipid ratios relative to those with a high ASO to lipid ratio. This would result in LAPs with high ASO to lipid ratios remaining in the plasma.

Figure 6.7

Plasma elimination of LAPs

SCID/Rag-2M mice were injected with a total of 5 mg/kg ASO i.v. via the tail vein. Radiolabeled ASO (**A**), lipid (**B**) or calculated ASO to lipid ratios (**C**) were assessed in plasma at 1, 2, 4, 24 h post-injection. Formulations injected were free ASO (■), encapsulated ASO (●), lipid-ASO with 10% DSPE-PEG (▲) or 10% DMPE-PEG (▼). *$p<0.05$ when comparing groups given EnASO to LAPs
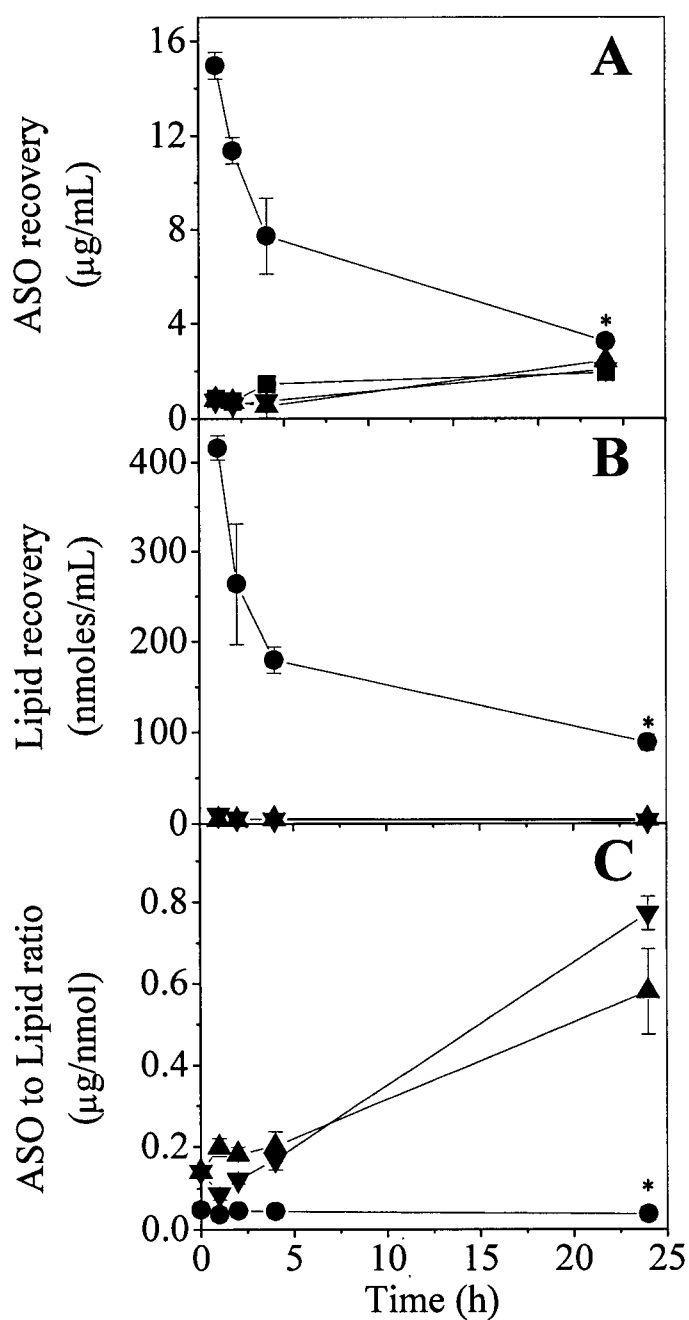
Alternatively, the LAPs that have localised in various tissues may be processed in a manner that allows the ASOs to be released as free drug. The release of ASO would suggest that the tissues could serve as a depot for ASOs.

At 24 h post-injection, mice were killed, tissues were excised and radioactivity was assessed in various organs. There are two important points to bear in mind when considering the results shown in Figure 6.8. First, ASO recovery in all tissues examined is greater when administered within a lipid carrier (A). Second, the amount of lipid recovery in all tissues was much greater for the EnASO formulation compared to animals given LAPs (B). These results and the calculated ASO to lipid ratios (C) are consistent with the concept that ASO are released following localisation in the tissues. Results observed following administration of EnASO show that the ASO and lipid recoveries in plasma are similar in all tissues indicative of a strong lipid-ASO association. In contrast, LAP containing DMPE-PEG, a lipid well known for its ability to readily exchange out of membranes (170, 343), resulted in high levels of ASO recovery in tissues relative to lipid recovery. The DMPE-LAPs treated animals exhibited the highest ASO to lipid ratios and this increase may be attributed to release of free ASO from other sites after LAP accumulation. It should be noted that for all formulations administered there were no significant levels of ASO or lipid recovered in the bone marrow. This is an important observation as the model for systemic B-cell lymphoma exhibits disease mainly in the spleen and bone marrow.

Efficacy studies were initiated based on the observations that suggested the ASO distribution was changed as a consequence of lipid-based carrier association. Figure 6.9 and Table 6.1 provide a summary of the data obtained following treatment of DoHH2-bearing mice with free ASO, EnASO, and LAPs. Similar to previous results, SCID/Rag-2M mice

Figure 6.8

Biodistribution of LAPs after i.v. injection

SCID/Rag-2M mice were injected with a total of 5 mg/kg ASO i.v. *via* the tail vein. Radiolabeled ASO (**A**), total lipid (**B**) or the calculated ASO to lipid ratios (**C**) were assessed in liver, spleen, lung, kidney and bone marrow. Formulations injected were free ASO (■), encapsulated ASO (▨), lipid-ASO particles with 10% DSPE-PEG (■) or 10% DMPE-PEG (☐).
(*Recovery of lipid and ASO was assessed in one femur.)
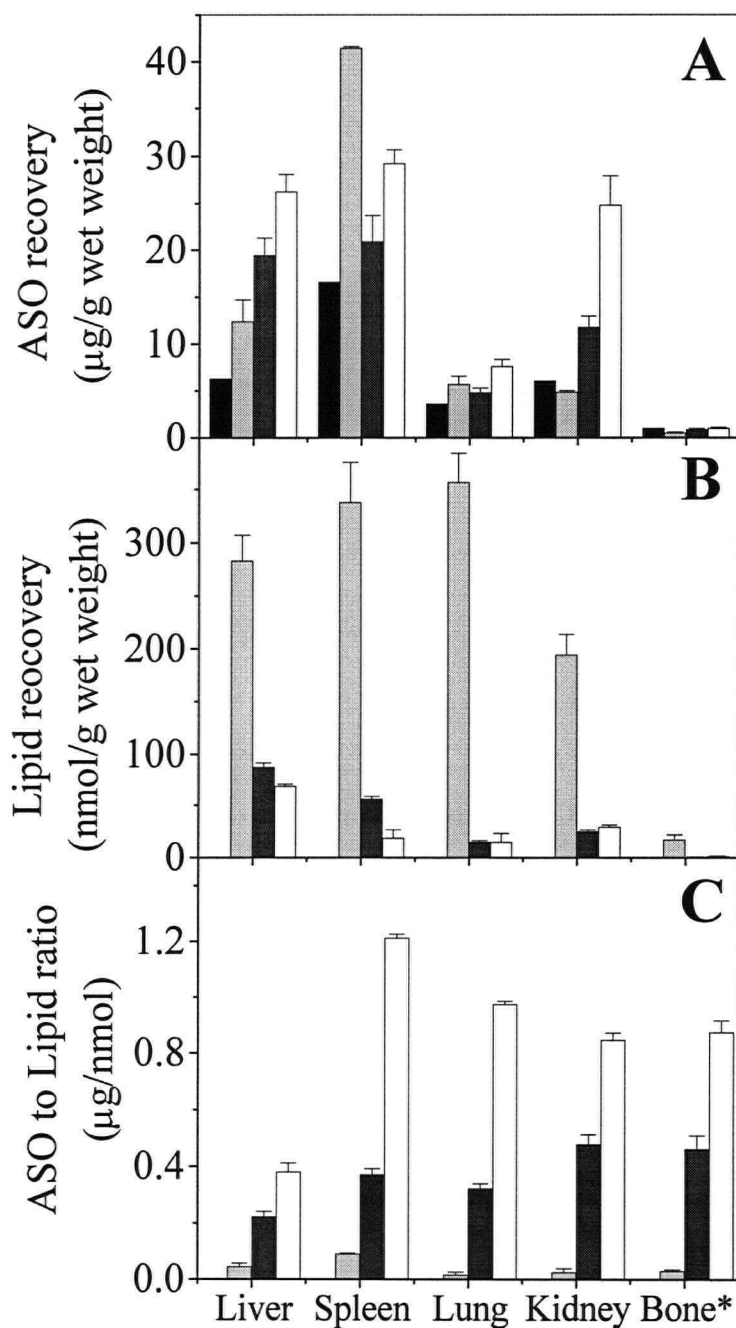
Figure 6.9

Survival curves of SCID/Rag-2M mice bearing B-cell lymphoma treated with LAPs

SCID/Rag-2M male mice were injected with 5×10^6 DoHH2 cells i.v. at Day 0. On Day 4 treatments began with a total of 5 mg/kg AS injected MWF for 14 treatments. Formulations used were none (■), free ASO (●), EnASO (▲), lipid-ASO particles with 10% DSPE-PEG (▼) and 10% DMPE-PEG (◆).
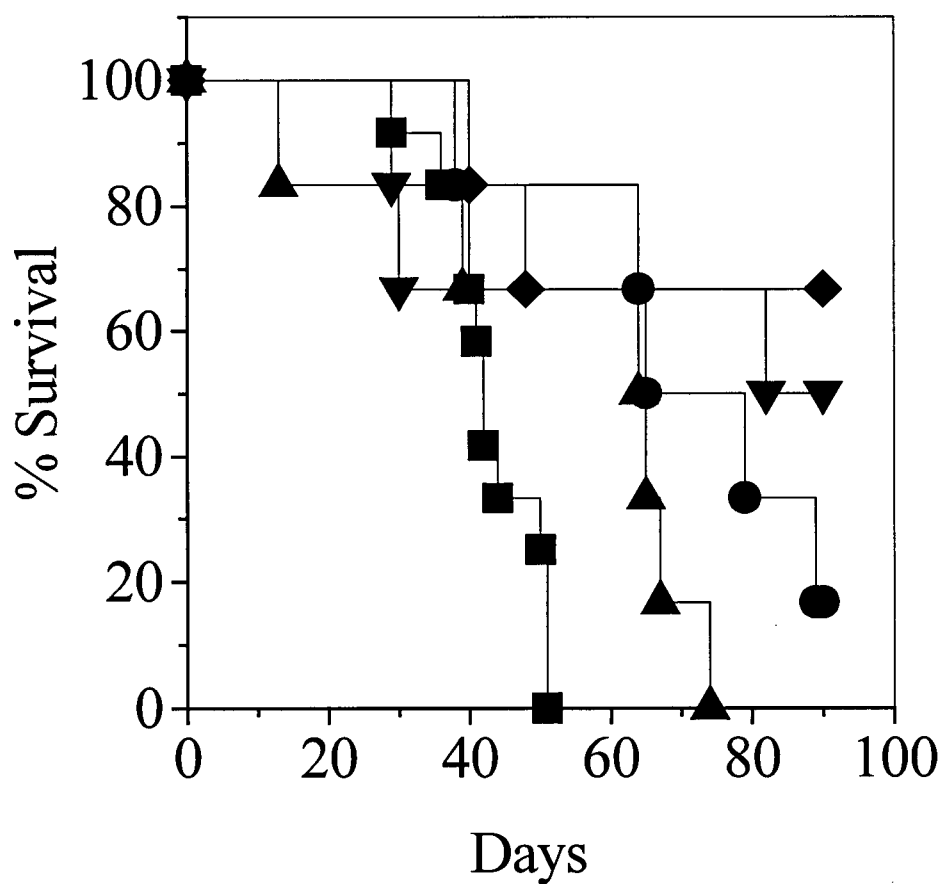
Table 6.1

Effect of lipid-based ASO formulations on SCID/Rag-2 male bearing DoHH2 tumours

SCID/Rag-2 mice were inoculated with 5×10^6 DoHH2 cells i.v. Animals were treated with none (saline only), free ASO, encapsulated ASO and LAPs containing 10% DSPE- or DMPE- PEG. All formulations were injected i.p. MWF for 14 treatments.

Treatment	Dose mg/kg	Schedule	N	Median Survival	% 90 day survival	p-value[3]
None	NA[1]	NA	12	42	0	NA
Free ASO	5.0	MWF	6	72	17	<0.01
EnASO	5.0	MWF	6	65	0	NS[4]
DSPE	5.0	MWF	6	87	50	NS
DMPE	5.0	MWF	6	NO[2]	67	ND[5]

[1]NA: Not applicable
[2]NO:Not obtained, if greater than 50% of the animals survived past 90 days then median survival could not be obtained.
[3]P-values were derived from using the log-rank test comparing Free ASO vs control or each lipid formulation vs Free ASO.
[4]NS: Not significant, $p>0.05$ when compared to control animals.
[5]ND: Not determined, there were <3 uncensored samples and estimated parameters were not reliable.

bearing the B-cell lymphoma, DoHH2 tumours, had a median survival of 42 days while treatments using free ASO extended median survival to 72 days [Chapter 5 and (384)]. No significant increases in median survival times were observed for any of the lipid-based ASO carriers when compared to survival curves for free ASO. However, LAP formulations showed a slight, but not significant, increase in median and 90-day survival. The DMPE-containing LAPs provides greater efficacy than DSPE-containing formulations where long-term survival was 67% and 50%, respectively. Notably, despite the results showing increases in ASO circulation longevity, the median survival time for animals treated with EnASO was no better than free ASO. The EnASO-treated animals had a decreased median survival (65 days) and no 90-day survivors compared to free ASO (72 day and 17% survivors).

6.4 Discussion

It was demonstrated in Chapter 2, and by others, that a cationic lipid/plasmid DNA complex can be generated using the Bligh and Dyer extraction procedure (203, 313). These previous studies suggest that the cationic lipid/DNA complex forms at the aqueous/organic interface and that DNA/lipid binding is dependent on multivalent interactions at this interface. The formation of the hydrophobic cationic lipid/DNA complex was dependent on charge neutralisation. The results described in this chapter are similar using ASOs and indicate that many of the same factors apply in the formation of lipid/ASO complexes.

Three of the monocationic lipids used in this study - DODAC, DDAB, and DOTAP, could extract >95% of the ASO into the organic phase provided that charge neutrality is achieved. Surprisingly, DC-Chol, which also exhibits a single charge per hydrophobic unit, must be added to ASO in quantities far surpassing charge neutralisation (+/- of 5.2) in order

to effect the same level of extraction. DC-Chol is a derivatized cholesterol and its sterol rings may restrict the accessibility of the cationic headgroup at the interface where the binding reactions occur. As a result, cationic lipid-anionic phosphate ion pairing may not be sufficient to achieve charge neutralisation and subsequent extraction into the organic phase.

In contrast to the results with plasmid DNA [see Chapter 2 and (203, 313)], the polyvalent cationic lipids DOGS and DOSPA were unable to mediate extraction of ASOs into the organic phase. DOGS and DOSPA are transfection reagents that consist of a polyvalent cationic lipid with four and five positive head groups, respectively. Each headgroup on the polyvalent lipid has the potential to neutralise more than one negative phosphate group of the ASO. Therefore, the hydrophobic nature of the spermidine is reduced since each polycationic headgroup (4 or 5 positive charges) is associated with only two acyl chains, whereas monocationic lipids have two acyl chains per positive charge. For example, in the DODAC system, an 18 mer ASO consists of 18 negative phosphate groups that can interact with 18 cationic lipids resulting in a total of 36 bound acyl chains. In contrast, when using the polycationic lipid DOGS, 4 negative phosphates are neutralised by one molecule of lipid that contains to two acyl chains. Therefore, there will be approximately 9 acyl chains present on one 18 mer ASO. The data shown suggests that this is not sufficient to confer hydrophobicity to the complex, preventing its extraction into the organic phase.

It is believed that pharmaceutically useful formulations for NAs may be dependent on a number of specific properties (i.e. small size, serum-stable, long circulation lifetimes); however, it has yet to be determined what specific attributes are important to maximise the effectiveness of lipid-based ASO complexes. It can be suggested that lipid carriers containing PEG-modified lipids will be biologically superior. Poly(ethylene glycol)-

conjugated phospholipids have become a vital component in protection of drugs and other small molecules against serum protein absorption (387), extending circulation lifetimes *in vivo* (155), and increasing delivery to disease outside the blood compartment (157). These properties are attributed to the steric hindrance and a hydrophilic barrier associated with the attached PEG polymer (163). This barrier is believed to prevent opsonization with serum proteins, which in turn have the propensity to promote the elimination of lipid-based carrier systems.

Although PEG-conjugated lipids are typically associated with beneficial attributes for liposome-based delivery systems, PEG-PE incorporation could also reduce ASO activity *in vitro* since it has been established that surface grafted-PEG can inhibit cell binding and other surface driven binding reactions (343). Results shown in this chapter demonstrate that there is significant cell-associated ASO delivery to DoHH2 cells *in vitro* even when the particles are formulated using 10 mol % PEG-PE (Figure 6.5). These LAPs were also eliminated rapidly (<1 h) and did not exhibit increased circulation longevity consistent with formulation that have sterically stabilised surfaces (Figure 6.7). These data indicate that PEG does not have the propensity to eliminate surface-mediated binding in these LAP formulations. As such, the presence and concentration of the PEG-lipids to modulate surface charge characteristics are important parameters that have not been well characterised in this report. Binding to cells *in vitro* and rapid elimination of i.v. injected LAPs may be a consequence of insufficient shielding of surface charge and this could be the result of three reasons: (i) the methodology used to formulate LAPs does not incorporate a sufficient concentration of PEG-lipid on the surface; (ii) PEG-lipids may be rapidly exchanging out LAPs, forming micelles

in solution or exchanging with membranes upon injection; and (iii) the PEG polymer configuration may not be appropriate for optimal shielding properties.

While PEG-lipids can contribute to effecting LAP attributes at an extracellular level, these lipids may also have the ability to effect intracellular processing following cell localisation. Western blot analysis demonstrates that Bcl-2 protein downregulation is not achieved with any of the formulations tested (Figure 6.6). These data are consistent with previous results that show inhibition of gene expression engendered by incorporation of PEG-lipids, even under conditions where cell delivery is still evident (388). Therefore, decreases in cell binding can not explain the levels of reduced transfection (for plasmid DNA) or downregulation of protein (for ASOs). If one assumes that reductions in cell binding can not account for the lack of protein downregulation, then the influence of PEG-PE on intracellular ASO processing events must be considered.

Another factor that may be of great influence in governing of ASO activity is the propensity for bound lipids (cationic, phospholipid or PEG lipids) to detach from the therapeutic molecule (276). For comparative purposes, this discussion will focus on the properties of the EnASO formulation to the LAPs prepared with DMPE-PEG. The plasma elimination and biodistribution studies establish that encapsulated ASO exhibit stable, long-circulating characteristics with lipids stably associated with the ASO over the 24 h time-course. While one would have predicted a similar plasma elimination profile for LAPs, results shown in Figure 6.8 indicate remarkable differences. In fact, despite incorporation of 10% PEG-PE, ASO circulation longevity was not enhanced over that of free ASOs. The LAPs-injected mice exhibited increasing ASO to lipid ratios in the plasma over the 24 h time course, in contrast to the EnASO formulation (Figure 6.8C).

One possible explanation for this result is the "depot" effect initially described by G. Storm (389). The results in this chapter indicate that ASO within LAPs is eliminated rapidly from the circulation and distributed throughout various tissues. Subsequently, the LAPs may be processed in a manner that causes free ASO to be re-released into the circulation. It is important to remember that the ASO used here is active in its free form [Chapter 5 and (384)]. Although it is not known what mediates the delivery of free ASOs *in vivo*, it is active in the absence of a lipid-based carrier system. The plasma elimination profiles shown in Figure 6.7 clearly indicate the plasma concentration is increasing as a function of time. This observation is consistent with a depot model where tissue localised ASO is released in to the plasma compartment in free form. It is not known whether the released ASO is bound by serum proteins, but the biological benefit of the "depot" would be to reduce the ASO clearance and retain bioavailability. Regardless of the mechanism, the efficacy studies in mice bearing DoHH2 tumours confirm this mechanism of an increased activity of LAPs over free ASOs. For example, the survival at 90 days was increased from 17% to 67% when comparing animals treated with free ASO and DMPE-containing LAPs.

As suggested above, the presence of PEG-PE may prevent any one of a number of steps required to achieve ASO activity. This reduction in ASO activity may be a result of lack of lipid dissociation, prevention of endosomal release, lack of nuclear transport, or hindering complementary base-pairing with the target mRNA. As such, work in generating a "transformable" lipid-based carrier system may be advantageous if PEG-PE incorporation is considered to be detrimental to intracellular processing of ASOs. Transformable systems are those which have the capability to release PEG-PE, either by transferring to another lipid membrane (170, 343, 390) or by having degradable linkage between the PEG polymer and

the phospholipid anchor (169). Regardless of the mechanism, it may be a fundamental requirement for all lipids associated with the ASO delivery system to be removed to permit ASO specific downregulation of protein levels.

An important point to bear in mind when considering these results is the nature of the disease being treated. Chapter 5 describes, in detail, the model of systemic B-cell lymphoma, a disease distributed mainly in the bone marrow and spleen. As such, an effective formulation strategy will likely mediate ASO delivery specifically to these sites. It is well known that lipid-based formulations accumulate passively in disease sites, particularly tumours, as a result of increased microvasculature (157, 391). However, in a model of systemic disease where tumour cells seed in the bone marrow, increasing circulation longevity of an encapsulated formulation may not be effective in delivering ASO to the target site. Further, the ASO within a lipid carrier that may succeed in reaching the disease site may not destabilise sufficiently in order to release its associated drug. For systemic disease, such as B-cell lymphoma, the difficulty is directing ASO to tumour cells to the exclusion of a normal cell. As such, the inclusion of a targeting moiety with LAPs may be warranted.

CHAPTER 7

CONCLUSIONS

7.1 Summary of results

At the onset of this thesis, I believed that a systematic approach to the generation of a lipid-based delivery system for NAs would be a viable alternative to the aggregation events induced by liposomes added to NA. This is based on the well known observation that the propensity for aggregation events using liposomal-based delivery systems limit their application as a systemic pharmaceutical. Although the findings in this thesis did not significantly alter the activity of NAs that utilise cationic lipids as delivery agents, those interactions that govern the formation and release of a lipid-NA particle were discerned by assessing basic binding characteristics of a hydrophobic cationic lipid/NA intermediate.

The underlying theme of these studies was the binding reaction between the cationic lipid and the NA. The Bligh and Dyer extraction used throughout this thesis demonstrated that a hydrophobic cationic lipid/NA complex could be recovered upon partitioning of a monophase into an aqueous and organic phase (Chapter 2). Hydrophobicity is conferred to the DNA through neutralisation of the anionic phosphate charges on the DNA as well as by the acyl chains of the bound cationic. In this two-phase system the electrostatic interactions occur at the level of the interfacial region between the aqueous and organic phase, where each component – lipid or DNA – in the absence of the other, exists solubilized within its own medium.

While binding characteristics were readily assessed using the Bligh and Dyer extraction procedure, it is also possible to generate a hydrophobic intermediate using a

detergent to solubilize the cationic lipid. In this way, cationic lipids in monomeric or micellar form were capable of binding to DNA as described in Chapter 3. Upon removal of the detergent, lipid-DNA particles formed spontaneously. LDPs have distinct characteristics when compared to LDAs. For example: the average size is smaller and distribution is much narrower for LDPs; the extent of TO-PRO-1 binding is increased for LDP formulations; and serum nuclease degradation is diminished. While I could not demonstrate that LDPs were superior in activity, I did determine that LDPs were valuable in assessing the effects of other phospholipids on formation and dissociation of cationic lipid and DNA. Lipids containing the PE headgroup mediated dissociation and prevented formation of the hydrophobic lipid/DNA complex in addition to providing less protection of DNA against serum degradation. In contrast, while results showed that LDPs with PE were not as stable as those containing PC-lipids these formulations were by far better at transfecting B16/BL6 tumours *in vitro* and *in vivo*. It is important to bear in mind that the formulation that demonstrated the greatest propensity for destabilisation translated to the highest level of activity. These results expanded the utility of PE lipids to induction of destabilisation events and release DNA from the carrier in addition to the previously described mechanism of mediating fusion.

While protection of the NA and ensuing release is one barrier that must be overcome leading to transfection activity, another barrier - access to the target cell - requires the close association of the lipid-NA complex to the cell membrane. In order to model this reaction a polystyrene latex bead was used as a model cell membrane; the bead provides a suitable model because it exhibits an overall negative surface charge (Chapter 4). Using the microelectrophoresis apparatus data demonstrated that electrostatic interactions could bring about close association as shown by net zero electrophoretic mobility of the anionic beads.

Further, the presence of the partial charges on the PE-lipids altered LDPs binding to the anionic beads.

While DNA is a good model to discern multivalent binding characteristics and can be utilised to demonstrate reporter gene activity, the studies completed with plasmid DNA did not lead to a therapeutically relevant carrier system (Chapter 2, 3, 4). As such, I initiated studies characterising the therapeutic activity of an ASO against bcl-2. Chapter 5 showed that there was a good therapeutic response when G3139 ASO was administered alone and in combination with a low dose of CPA in a systemic model of B-cell lymphoma. Further, there was little evidence that this ASO activity was mediated *via* stimulation of a natural killer cell response.

As this thesis is focused on generating a viable lipid-based carrier system for NA, I incorporated the above therapeutic molecule with lipids. Similar to the LDPs study, LAPs were generated using a hydrophobic lipid/ASO complex as an intermediate (Chapter 6). In contrast to the DNA studies, multivalent cations such as DOGS and DOSPA could not confer hydrophobicity to the ASO. In addition, the use of poly(ethylene glycol) polymer conjugated to a lipid anchor was added to effect hydration of the LAP and foreseeably incorporate biological benefits – such as circulation longevity. Despite the use of a systematic approach to the design of LAP, initial results measuring Bcl-2 downregulation did not demonstrate a potential for therapeutic activity. Interestingly, efficacy studies in SCID/Rag-2 mice bearing a B-cell lymphoma showed that LAPs were at least as active as free ASO and formulations containing a DMPE-PEG were likely more active. These results suggest that LAPs may not be effecting increases in a therapeutic response by increasing circulation longevity, rather efficacy may be the result of re-release of bioavailable ASO into the circulation.

7.2 Significance of results

The hydrophobic intermediate formed by the Bligh and Dyer extraction procedure led to characterisation of the cationic lipid-NA binding reaction and demonstrated the effects of other phospholipids on this binding. I believe that the hydrophobic cationic lipid/NA complex can be beneficial for use in evaluating the extent of NA binding by newly synthesised cationic lipids and to systematically assess the effect of co-lipids or transfection enhancing molecules. The hydrophobic lipid/DNA complex can be used to demonstrate whether a formulation will encapsulate a NA and prevent destabilisation of the lipid-based delivery system prior to administration. It is hoped that this methodology will also be more efficient - binding all NAs without waste of therapeutic compound.

While assessing the effect of other phospholipids on cationic lipid binding it was determined that PE-lipids were unique in their capacity to destabilise cationic lipid binding. The mechanism by which this occurs may be through PE headgroup interactions with the cationic lipid headgroup. This mechanism used to induce NA release is in addition to previous observations that show PE-induced H_{II} phase intermediates in the process of membrane fusion. Maximising intracellular delivery may be an irrelevant endeavour if the NA is not capable of expressing its activity due to the presence of bound cationic lipids. One major finding in this thesis is that PE-lipids are capable of interacting with cationic lipids through electrostatic interactions or other effects to obstruct NA binding.

While adding lipids to NAs through a hydrophobic intermediate seemed to be a viable alternative to liposomes in generating homogenous carriers for NA, the resultant LDPs and LAPs were not any more effective at mediating transfection or downregulation when compared to LDAs or LAAs, particularly in systems *in vitro*. This is because design features

of LDPs and LAPs prevent binding interactions (i.e. to cells and serum proteins) in the anticipation of increasing circulation longevity. While the complexity of an *in vivo* system makes assessment of activity more difficult, it may be that LDPs and LAPs are readily amenable to mediating a therapeutic response within a complex biological milieu.

7.3 Future directions

There are a number of polymorphic changes that a lipid-based carrier undergoes when complexed with a NA. As such investigators attempt to divulge structure-function relationships in order to predict the composition of an effective carrier. One way in which this could be enhanced is through the use of molecular modelling. This is of particular interest when considering the results obtained in Chapter 2 and 3. Deciphering the orientation of the PE-lipid and the cationic lipid, particularly at the headgroup region, would allow further understanding of the steric interactions involved. While it is known that PE induces destabilisation events, molecular modelling could define whether this is a function of electrostatic interactions or H-bonding between lipids. Further, it may be that the presence of DNA may limit PE accessibility through steric repulsion induced by its secondary and tertiary structure; these parameters, although difficult to assess, will give us more insight to the structural features that exhibit attributes for efficient transfection.

Molecular modelling can also be used to determine whether the effects mediated by PE-lipids can lead to alternative methods for destabilisation of NA delivery systems. The interactions among NA, cationic lipids, DOPE, anionic lipids in membrane can lead to myriad of possible structures after association leading to heterogeneity of activity in any given sample. The packing parameters of these molecules in solution could be carefully

assessed in order to optimise strategies that would effect the greatest stability within circulation and release after intracellular uptake. Modelling can be used to anticipate the relevance of a molecule to effect good protection of DNA and subsequently be release from lipid carrier. This can be deduced by observing the relative "fit" of a molecule between the cationic lipid and NA. Complemented by a technique like the Bligh and Dyer extraction procedure, molecular modelling studies can lead to a wider examination of the orientation of lipids with NA and how these data can be interpreted in a functional way. The systematic generation of LDP and LAPs can then be used to correlate activity within *in vitro* and *in vivo* models testing efficacy. These will lead closer to deciphering structure-function relationships, a major goal in lipid-based delivery systems.

7.4 Advances in lipid-based carriers and gene therapy

The key step in lipid-based NA carrier design is knowing what factors are required to: (i) bind DNA in a form that prevents nuclease degradation and (ii) trigger release upon access to the cell's transcription machinery. Further studies may be needed to generate lipids that are designed to specifically hinder only nuclease recognition. Therefore, the entire NA molecule does not require encapsulation or cationic lipid association. Similar to chimeric ASOs, one may be able to design lipids that attach to specific regions on the plasmid that bind complement, serum proteins, or fits into the enzymatic site of a nuclease. Therefore, charge neutralisation of the entire molecule will not be of great importance if charges are buried through condensation or complexation reactions. The key is to design lipids capable of this level of control where lipids associate strongly to confer pharmacokinetic effects and

then release after achieving the target site. Based on the observations throughout this thesis, I believe that lipids can be designed for this sophistication of triggered release.

The first component in gene therapy that needs to be optimised is the design of NA. It is an unrepudiated fact that a NA must be effective in a diseased cell regardless of other environmental factors. As such, NAs must be designed to have a specific mechanism of action with a defined clinical performance. Based on these arguments I suggest that ASOs will be of more value than plasmid expression vectors. This is due to its simplicity in physical characteristics (smaller and single-stranded) and definitive mode of action (Watson-Crick interactions). It is this art of molecular design that needs to be perfected before synthetic carriers are a feasible option for gene therapy using NAs.

I believe that the promise of gene therapy will be fulfilled because of a NA's specificity toward a target. For example, the problems with conventional drug therapy are due to indiscriminate distribution of the drug to non-target tissue, leading to toxic side effects. Therefore, the benefits of gene therapy are such that individual gene expression can contribute to a specific attack on the disease. It is, however, important to note that this specificity is also a drawback in its ability to effect therapy. If only a single gene is affected then it must have the following characteristics: (i) be of direct therapeutic relevance; (ii) be a key player in causing disease and its activity must not be compensated through redundant pathways; (iii) be exclusively expressed on every tumour cell and not essential in normal cells; (iv) be expressed in cells that are accessible by synthetic gene carrier systems; and (v) its expression can be readily and externally controlled. If all of these conditions are fulfilled then a nucleic acid drug has the potential to be an effective agent in combating disease.

The likelihood that all patients, at some level, will require modulation of one specific protein through gene therapy and that this will be capable of influencing tumours of similar grades and proliferative status, is improbable, if not impossible. As such I believe that successful gene therapy regimes will involve unique, personalised protocol which caters to the needs of each individual patient. This would include a careful consideration of the patient's disease status and related genetic variations. Subsequently, the specific nucleic acid drug(s) required and the schedule of administration would be cautiously controlled in order to modulate expression of a specific protein and elicit a therapeutic response. This may require multiple treatment modalities where transfection enhancing agents or cytotoxic drugs would be used in combination with nucleic acid drugs. From a patient perspective, any treatment that has the potential to decrease toxic side effects with ease of use and generate maximal effects for their particular disease should be vigorously pursued.

REFERENCES

1. Mountain, A. (2000) Gene therapy: the first decade. *Trends Biotechnol.* 18:119-28.

2. Gomez-Navarro, J., Curiel, D.T. and Douglas, J.T. (1999) Gene therapy for cancer. *Eur. J. Cancer.* 35:2039-57.

3. Anderson, W.F. (1998) Human gene therapy. *Nature.* 392:25-30.

4. Hersh, E.M. and Stopeck, A.T. (1997) Advances in the biological therapy and gene therapy of malignant disease. *Clin. Cancer Res.* 3:2623-9.

5. Lesoon-Wood, L.A., Kim, W.H., Kleinman, H.K., Weintraub, B.D. and Mixson, A.J. (1995) Systemic gene therapy with p53 reduces growth and metastases of a malignant human breast cancer in nude mice. *Hum. Gene Ther.* 6:395-405.

6. Dedieu, J.F., Vigne, E., Torrent, C., Jullien, C., Mahfouz, I., Caillaud, J.M., Aubailly, N., Orsini, C., Guillaume, J.M., Opolon, P., Delaere, P., Perricaudet, M. and Yeh, P. (1997) Long-term gene delivery into the livers of immunocompetent mice with E1/E4-defective adenoviruses. *J. Virol.* 71:4626-37.

7. Stopeck, A.T., Hersh, E.M., Akporiaye, E.T., Harris, D.T., Grogan, T., Unger, E., Warneke, J., Schluter, S.F. and Stahl, S. (1997) Phase I study of direct gene transfer of an allogeneic histocompatibility antigen, HLA-B7, in patients with metastatic melanoma. *J. Clin. Oncol.* 15:341-9.

8. Link, C.J., Seregina, T., Traynor, A. and Burt, R.K. (2000) Cellular suicide therapy of malignant disease. *Oncologist.* 5:68-74.

9. Bestor, T.H. (2000) Gene silencing as a threat to the success of gene therapy. *J. Clin. Invest.* 105:409-11.

10. Verma, I.M. and Somia, N. (1997) Gene therapy -- promises, problems and prospects. *Nature.* 389:239-42.

11. Tiberghien, P. (1994) Use of suicide genes in gene therapy. *J. Leukoc. Biol.* 56:203-9.

12. National Center for Biotechnology Information. (2000) dbSNP Map Information. http://www.ncbi.nlm.nih.gov/SNP/get_html.cgi.

13. National Center for Biotechnology Information. (2000) Human Genome Sequencing. http://www.ncbi.nlm.nih.gov/genome/seq/.

14. Tripathy, S.K., Black, H.B., Goldwasser, E. and Leiden, J.M. (1996) Immune responses to transgene-encoded proteins limit the stability of gene expression after injection of replication-defective adenovirus vectors. *Nat. Med.* 2:545-50.

15. Setoguchi, Y., Jaffe, H.A., Chu, C.S. and Crystal, R.G. (1994) Intraperitoneal *in vivo* gene therapy to deliver α1-antitrypsin to the systemic circulation. *Am. J. Respir. Cell Mol. Biol.* 10:369-77.

16. Yang, Y., Nunes, F.A., Berencsi, K., Furth, E.E., Gonczol, E. and Wilson, J.M. (1994) Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc. Natl. Acad. Sci. USA.* 91:4407-4411.

17. Yang, Y., Li, Q., Erte, H.C.J. and Wilson, J.M. (1995) Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J. Virol.* 69:2004-15.

18. Dean, D.A., Dean, B.S., Muller, S. and Smith, L.C. (1999) Sequence requirements for plasmid nuclear import. *Exp. Cell Res.* 253:713-22.

19. Prazeres, D.M., Ferreira, G.N., Monteiro, G.A., Cooney, C.L. and Cabral, J.M. (1999) Large-scale production of pharmaceutical-grade plasmid DNA for gene therapy: problems and bottlenecks. *Trends Biotechnol.* 17:169-74.

20. Middaugh, C.R., Evans, R.K., Montgomery, D.L. and Casimiro, D.R. (1998) Analysis of plasmid DNA from a pharmaceutical perspective. *J. Pharm. Sci.* 87:130-46.

21. Calos, M.P. (1996) The potential of extrachromosomal replicating vectors for gene therapy. *Trends Genet.* 12:463-6.

22. Nabel, E.G., Gordon, D., Yang, Z.Y., Xu, L., San, H., Plautz, G.E., Wu, B.Y., Gao, X., Huang, L. and Nabel, G.J. (1992) Gene transfer in vivo with DNA-liposome complexes: lack of autoimmunity and gonadal localization. *Hum. Gene Ther.* 3:649-56.

23. Stewart, M.J., Plautz, G.E., Buono, L.D., Yang, Z.Y., Xu, L., Gao, X., Huang, L., Nabel, E.G. and Nabel, G.J. (1992) Gene transfer in vivo with DNA-liposome complexes: safety and acute toxicity in mice. *Hum. Gene Ther.* 3:267-75.

24. Sverdrup, F., Sheahan, L. and Khan, S. (1999) Development of human papillomavirus plasmid capable of episomal replication in human cell lines. *Gene Ther.* 6:1317-21.

25. Nichols, W.W., Ledwith, B.J., Manam, S.V. and Troilo, P.J. (1995) Potential DNA vaccine integration into host cell genome. *Ann. NY Acad. Sci.* 772:30-9.

26. Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M. and Danielsen, M. (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA.* 84:7413-17.

27. Jacobs, D. (1995) Long-term preservation of DNA in agarose gels using 70% ethanol. *Biotechniques.* 19:892-4.

28. Clark, A.R., Boam, D.S. and Docherty, K. (1989) A new series of CAT expression vectors. *Nucleic Acids Res.* 17:10130.

29. Herman, G.E., O'Brien, W.E. and Beaudet, A.L. (1986) An *E. coli* β-galactosidase cassette suitable for study of eukaryotic expression. *Nucleic Acids Res.* 14:7130.

30. Nordeen, S. (1988) Luciferase reporter gene vectors for analysis of promoters and enhancers. *Biotechniques.* 6:454-8.

31. Zolotukhin, S., Potter, M., Hauswirth, W.W., Guy, J. and Muzyczka, N. (1996) A "humanized" green fluorescent protein cDNA adapted for high-level expression in mammalian cells. *J. Virol.* 70:4656-54.

32. Heinzel, S.S., Krysan, P.J., Tran, C.T. and Calos, M.P. (1991) Autonomous DNA replication in human cells is affected by the size and the source of the DNA. *Mol. Cell Biol.* 11:2263-72.

33. Krysan, P.J., Haase, S.B. and Calos, M.P. (1989) Isolation of human sequences that replicate autonomously in human cells. *Mol. Cell. Biol.* 9:1026-33.

34. Nickoloff, J.A. and Reynolds, R.J. (1992) Electroporation-mediated gene transfer efficiency is reduced by linear plasmid carrier DNAs. *Anal. Biochem.* 205:237-43.

35. Cooper, M.J. (1995) Replicating plasmid vectors for cancer gene therapy. *In* Artificial self-assembling systems for gene delivery. P.L. Felgner, *et al.*, ed. American Chemical Society, Washington: 32-46.

36. Alter, D.C., Ramanujam, P., Yamaguchi, J. and Subramanian, K.N. (1989) Simple screening for the presence of antibiotic resistant CAT gene plasmids in bacteria. *Biotechniques.* 7:247-8.

37. Belt, P.B., Groeneveld, H., Teubel, W.J., van de Putte, P. and Backendorf, C. (1989) Construction and properties of an Epstein-Barr-virus-derived cDNA expression vector for human cells. *Gene.* 84:407-17.

38. Zouzias, D., Bertelsen, A.H. and Rush, M.G. (1981) DNA mediated gene transfer using simian virus 40 or polyoma virus morphological transformation as selective markers. *Arch. Virol.* 70:291-5.

39. Jenab, S. and Johnson, E.M. (1989) A dual-circular plasmid structure dependent on DNA replication generated in monkey COS7 cells and cell extracts. *Biochem. Biophys. Res. Commun.* 160:53-9.

40. Ledley, T.A.S. and Ledley, F.D. (1994) A multicompartment, numerical model of cellular events in the pharmacokinetics of gene therapies. *Hum. Gene Ther.* 5:679-91.

41. Vassilev, L. and Johnson, E.M. (1988) Evaluation of autonomous plasmid replication in transfected mammalian cells. *Nucleic Acids Res.* 16:7742.

42. Haase, S.B. and Calos, M.P. (1991) Replication control of autonomously replicating human sequences. *Nucleic Acids Res.* 19:5053-8.

43. Yates, J.L., Warren, N. and Sugden, B. (1985) Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. *Nature.* 313:812-5.

44. Radler, J.O., Koltover, I., Salditt, T. and Safinya, C.R. (1997) Structure of DNA-cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes. *Science.* 275:810-4.

45. Dean, N.M., McKay, R., Miraglia, L., Geiger, T., Muller, M., Fabbro, D. and Bennett, C.F. (1996) Antisense oligonucleotides as inhibitors of signal transduction: development from research tools to therapeutic agents. *Biochem. Soc. Trans.* 24:623-9.

46. Szczylik, C., Skorski, T., Nicolaides, N.C., Manzella, L., Malaguarnera, L., Venturelli, D., Gewirtz, A.M. and Calabretta, B. (1991) Selective inhibition of leukemia cell proliferation by BCR-ABL antisense oligodeoxynucleotides. *Science.* 253:562-5.

47. Ho, P.T. and Parkinson, R. (1997) Antisense oligonucleotides as therapeutics for malignant diseases. *Semin. Oncol.* 24:187-202.

48. Zamecnik, P.C. and Stephenson, M.L. (1978) Inhibition of Rous sarcoma virus replication and cell transformation by an oligodeoxynucleotide. *Proc. Nat. Acad. Sci. USA.* 75:280-4.

49. Stephenson, M.L. and Zamecnik, P.C. (1978) Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. *Proc. Nat. Acad. Sci. USA.* 75:285-8.

50. Curcio, L.D., Bouffard, D.Y. and Scanlon, K.J. (1997) Oligonucleotides as modulators of cancer gene expression. *Pharmacol. Ther.* 74:317-32.

51. Crooke, S.T. (1992) Therapeutic applications of oligonucleotides. *Biotechnology.* 10:882-6.

52. Flanagan, W.M. and Wagner, R.W. (1997) Potent and selective gene inhibition using antisense oligodeoxynucleotides. *Mol. Cell. Biochem.* 172:213-25.

53. Crooke, S.T., Lemonidis, K.M., Neilson, L., Griffey, R., Lesnik, E.A. and Monia, B.P. (1995) Kinetic characteristics of Escherichia coli RNase H1: cleavage of various antisense oligonucleotide-RNA duplexes. *Biochem. J.* 312:599-608.

54. Bennett, C.F. and Crooke, S.T. (1994) Regulation of endothelial cell adhesion molecule expression with antisense oligonucleotides. *Adv. Pharmacol.* 28:1-43.

55. Mizuno, T., Chou, M.Y. and Inouye, M. (1984) A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). *Proc. Natl. Acad. Sci. U S A.* 81:1966-70.

56. Marcusson, E.G., Yacyshyn, B.R., Shanahan, W.R., Jr. and Dean, N.M. (1999) Preclinical and clinical pharmacology of antisense oligonucleotides. *Mol. Biotechnol.* 12:1-11.

57. Warzocha, K. (1999) Antisense strategy in hematological malignancies. *Cytokines Cell. Mol. Ther.* 5:15-23.

58. Agrawal, S. (1996) Antisense oligonucleotides: towards clinical trials. *Trends Biotechnol.* 14:376-87.

59. Ma, L. and Calvo, F. (1996) Recent status of the antisense oligonucleotide approaches in oncology. *Fundam. Clin. Pharmacol.* 10:97-115.

60. Calogero, A., Hospers, G.A.P. and Mulder, N.H. (1997) Synthetic oligonucleotides: Useful molecules? A review. *Pharm. World Sci.* 19:264-8.

61. Deshmukh, R.R., Cole, D.L. and Sanghvi, Y.S. (2000) Purification of antisense oligonucleotides. *Methods Enzymol.* 313:203-26.

62. Zhao, Q., J., T., Iadarola, P.L., Jiang, Z. and Agrawal, S. (1996) Effect of different chemically modified oligodeoxynucleotides on immune stimulation. *Biochem. Pharmacol.* 51:173-82.

63. Scanlon, K.J., Ohta, Y., Ishida, H., Kijima, H., Ohkawa, T., Kaminski, A., Tsai, J., Horng, G. and Kashani-Sabet, M. (1995) Oligonucleotide-mediated modulation of mammalian gene expression. *FASEB J.* 9:1288-96.

64. Iverson, P.L., Copple, B.L. and Tweary, H.K. (1995) Characterization and toxicology of phosphorothioate oligonucleotides in the mouse, rat, monkey and man. *Toxicol. Lett.* 82-83:425-30.

65. Maier, M., Bleicher, K., Kalthoff, H. and Bayer, E. (1995) Enzymatic degradation of various antisense oligonucleotides: monitoring and fragment identification by MECC and ES-MS. *Biomed. Pept. Proteins Nucleic Acids.* 1:235-42.

66. Eckstein, F. (1985) Nucleoside phosphorothioates. *Annu. Rev. Biochem.* 54:367-402.

67. Uhlmann, E., Ryte, A. and Peyman, A. (1997) Studies on the mechanism of stabilization of partially phosphorothioated oligonucleotides against nucleolytic degradation. *Antisense Nucleic Acid Drug Dev.* 7:345-50.

68. Crooke, S.T. (1995) The medicinal chemistry of oligonucleotides. *In* Therapeutic applications of oligonucleotides. S.T. Crooke, ed. Springer-Verlang, Germany: 85-108.

69. Klimuk, S.A. (1999) Liposome encapsulation enhances the anti-inflammatory activity of an ICAM-1 antisense oligonucleotide. Ph.D. University of British Columbia, Vancouver.

70. Zon, G. (1995) Antisense phosphorothioate oligodeoxynucleotides: introductory concepts and possible molecular mechanisms of toxicity. *Toxicol. Lett.* 82-83:419-24.

71. Brazolot-Millan, C.L., Weeratna, R., Krieg, A.M., Siegrist, C.A. and Davis, H.L. (1998) CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. *Proc. Natl. Acad. Sci. USA.* 95:15553-8.

72. Chu, R.S., Targoni, O.S., Krieg, A.M., Lehmann, P.V. and Harding, C.V. (1997) CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J. Exp. Med.* 186:1623-31.

73. Wooldridge, J.E., Ballas, Z. and Krieg, A.M. (1997) Immunostimulatory oligodeoxynucleotides containing CpG motifs enhance the efficacy of monoclonal antibody therapy of lymphoma. *Blood.* 89:2994-8.

74. Weiner, G.J., Liu, H.M., Wooldridge, J.E., Dahle, C.E. and Krieg, A.M. (1997) Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. *Proc. Natl. Acad. Sci. USA.* 94:10833-7.

75. Ryte, A., Morelli, S., Mazzei, M., Alama, A., Franco, P., Canti, G.F. and Nicolin, A. (1993) Oligonucleotide degradation contributes to resistance to antisense compounds. *Anticancer Drugs.* 4:197-200.

76. Stein, C.A. and Cheng, Y.-C. (1993) Antisense oligonucleotides as therapeutic agents -- is the bullet really magical? *Science.* 261:1004-11.

77. Miller, P.S., Yano, J., Yano, E., Carroll, C., Jayaraman, K. and Ts'o, P.O. (1979) Nonionic nucleic acid analogues. Synthesis and characterization of dideoxyribonucleoside methylphosphonates. *Biochemistry.* 18:5134-43.

78. Tari, A.M., Tucker, S.D., Deisseroth, A. and Lopez-Berestein, G. (1994) Liposomal delivery of methylphosphonate antisense oligodeoxynucleotides in chronic myelogeneous leukemia. *Blood.* 84:601-7.

79. Saison-Behmoaras, T.E., Duroux, I., Nguyen, T.T., Asseline, U. and Helene, C. (1997) Antisense properties of end-modified oligonucleotides targeted to Ha-ras oncogene. *Antisense Nucleic Acid Drug Dev.* 7:361-8.

80. Ledley, F.D. (1995) Nonviral gene therapy: The promise of genes as pharmaceutical products. *Hum. Gene Ther.* 6:1129-44.

81. Cotten, M., Wagner, E., Zatloukal, K., Phillips, S., Curiel, D.T. and Birnstiel, M.L. (1992) High-efficiency receptor-mediated delivery of small and large (48 kilobase gene

constructs using the endosome-disruption activity of defective or chemically inactivated adenovirus particles. *Proc. Natl. Acad. Sci. U S A.* 89:6094-8.

82. Wagner, E., Plank, C., Zatloukal, K., Cotten, M. and Birnstiel, M.L. (1992) Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. *Proc. Natl. Acad. Sci. USA.* 89:7934-8.

83. Gottschalk, S., Sparrow, J.T., Hauer, J., Mims, M.P., Leland, F.E., Woo, S.L. and Smith, L.C. (1996) A novel DNA-peptide complex for efficient gene transfer and expression in mammalian cells. *Gene Ther.* 3:48-57.

84. Caplen, N.J., Alton, E.W., Middleton, P.G., Dorin, J.R., Stevenson, B.J., Gao, X., Durham, S.R., Jeffery, P.K., Hodson, M.E., Coutelle, C. and et al. (1995) Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis [published erratum appears in Nat. Med. 1995 Mar;1(3):272]. *Nat. Med.* 1:39-46.

85. Plank, C., Mechtler, K., Szoka Jr., F.C. and Wagner, E. (1996) Activation of the complement system by synthetic DNA complexes: A potential barrier for intravenous gene delivery. *Hum. Gene Ther.* 7:1437-46.

86. Gottschalk, S., Cristiano, R.J., Smith, L.C. and Woo, S.L. (1994) Folate receptor mediated DNA delivery into tumor cells: protosomal disruption results in enhanced gene expression. *Gene Ther.* 1:185-91.

87. Schuber, F., Kichler, A., Boeckler, C. and Frisch, B. (1998) Liposomes: from membrane models to gene therapy. *Pure Appl. Chem.* 70:89-96.

88. Tanford, C. (1978) The hydrophobic effect and the organization of living matter. *Science.* 200:1012-8.

89. Sen, A. and Hui, S.W. (1988) Direct measurement of headgroup hydration of polar lipids in inverted micelles. *Chem. Phys. Lipid.* 49:179-84.

90. Dill, K. (1986) The chain conformations in membranes and micelles. *Adv. Colloid Interface Sci.* 26:99-110.

91. Bangham, A.D., Standish, M.M. and Watkins, J.C. (1965) Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.* 13:238-52.

92. Lohri, A., Reuter, J., Gudat, F. and Herrmann, R. (1997) Topoisomerase IIa mRNA and tumour cell proliferation in non-Hodgkin's lymphoma. *J. Clin. Pathol.* 50:22-6.

93. Lou, D. and Saltzman, W.M. (2000) Synthetic DNA delivery systems. *Nat. Biotechnol.* 18:33-7.

94. Capaccioli, S., Di Pasquale, G., Mini, E., Mazzei, T. and Quattrone, A. (1993) Cationic lipids improve antisense oligonucleotide uptake and prevent degradation in cultured

cells and in human serum [published erratum appears in Biochem. Biophys. Res. Commun. 1994 May 16;200(3):1769]. *Biochem. Biophys. Res. Commun.* 197:818-25.

95. Crook, K., McLachlan, G., Stevenson, B.J. and Porteous, D.J. (1996) Plasmid DNA molecules complexed with cationic liposomes are protected from degradation by nucleases and shearing by aerosolisation. *Gene Ther.* 3:834-9.

96. Allen, T.M., Mehra, T., Hansen, C. and Chin, Y.C. (1992) Stealth liposomes: an improved sustained release system for 1-β-D-arabinofuranosylcytosine. *Cancer Res.* 52:2431-39.

97. Rahman, A., Kessler, A., More, N., Sikic, B., Rowden, G., Woolley, P. and Schein, P.S. (1980) Liposomal protection of adriamycin-induced cardiotoxicity in mice. *Cancer Res.* 40:1532-7.

98. Lee, K.D., Hong, K. and Papahadjopoulos, D. (1992) Recognition of liposomes by cells: in vitro binding and endocytosis mediated by specific lipid headgroups and surface charge density. *Biochim. Biophys. Acta.* 1103:185-97.

99. Uchiyama, K., Nagayasu, A., Yamagiwa, Y., Nishida, T., Harashima, H. and Kiwada, H. (1995) Effects of the size and fluidity of liposomes on their accumulation in tumors: a presumption of their interaction with tumors. *Int. J. Pharm.* 121:195-203.

100. Poate, G., Bucana, C., Raz, A., Bugelski, P., Kirsh, R. and Fidler, I.J. (1982) Analysis of the fate of systemically administered liposomes and implications for their use in drug delivery. *Cancer Res.* 42:1412-22.

101. Lasic, D. (1996) Sterically stabilized liposomes. *Science Med.* 1:1.

102. Israelachvili, J.N. and Mitchell, D.J. (1975) A model for the packing of lipids in bilayer membranes. *Biochim. Biophys. Acta.* 389:1-19.

103. Markin, V.S., Kozlov, M.M. and Borovjagin, V.L. (1984) On the theory of membrane fusion. The stalk mechanism. *Gen. Physiol. Biophys.* 3:361-77.

104. Siegel, D.P. (1993) Energetics of intermediates in membrane fusion: comparison of stalk and inverted micellar intermediate mechanisms. *Biophys. J.* 65:2124-40.

105. Epand, R.M. (1998) Lipid polymorphism and protein-lipid interactions. *Biochim. Biophys. Acta.* 1376:353-68.

106. Tarahovsky, Y.S. and Invanitsky. (1998) Liposomes in gene therapy. Structural polymorphism of lipids and effectiveness of gene delivery. *Biochemistry (Moscow).* 63:607-18.

107. Cullis, P.R. and de Kruijff, B. (1979) Lipid polymorphism and the functional roles of lipids in biological membranes. *Biochim. Biophys. Acta.* 559:399-420.

108. Seddon, J.M. (1990) Structure of the inverted hexagonal (HII) phase, and non-lamellar phase transitions of lipids. *Biochim. Biophys. Acta.* 1031:1-69.

109. Liu, N.I. and Kay, R.L. (1977) Redetermination of the pressure dependence of the lipid bilayer phase transition. *Biochemistry.* 16:3484-6.

110. Wong, F.M.P., Zhang, Y., Harvie, P. and Bally, M.B. (2000) Phosphatidylethanolamine mediated destabilization of lipid-based plasmid delivery systems: DNA dissociation in regulating transgene expression. *Biochim. Biophys. Acta.* (submitted).

111. Carnie, S., Israelachvili, J.N. and Pailthorpe, B.A. (1979) Lipid packing and transbilayer asymmetries of mixed lipid vesicles. *Biochim. Biophys. Acta.* 554:340-57.

112. Lee, A.G. (1975) Functional properties of biological membranes: a physical-chemical approach. *Prog. Biophys. Mol. Biol.* 29:3-56.

113. Shalaev, E.Y. and Steponkus, P.L. (1999) Phase diagram of 1,2-dioleoylphosphatidylethanolamine (DOPE):water system at subzero temperatures and low water contents. *Biochim. Biophys. Acta.* 1419:229-47.

114. Boggs, J.M. (1980) Intermolecular hydrogen bonding between lipids: influence on organization and function of lipids in membranes. *Can. J. Biochem.* 58:755-70.

115. Papahadjopoulos, D., Jacobson, K., Nir, S. and Isac, T. (1973) Phase transitions in phospholipid vesicles. Fluorescence polarization and permeability measurements concerning the effect of temperature and cholesterol. *Biochim. Biophys. Acta.* 311:330-48.

116. Chapman, D. (1975) Phase transitions and fluidity characteristics of lipids and cell membranes. *Q. Rev. Biophys.* 8:185-235.

117. Cameron, D.G., Gudgin, E.F. and Mantsch, H.H. (1981) Dependence of acyl chain packing of phospholipids on the head group and acyl chain length. *Biochemistry.* 20:4496-500.

118. van Dijck, P., W., de Kruijff, B., Aarts, P.A., Verkleij, A.J. and de Gier, J. (1978) Phase transitions in phospholipid model membranes of different curvature. *Biochim. Biophys. Acta.* 506:183-91.

119. Finegold, L. and Singer, M.A. (1986) The metastability of saturated phosphatidylcholines depends on the acyl chain length. *Biochim. Biophys. Acta.* 855:417-20.

120. Dixon, G.S., Black, S.G., Butler, C.T. and Jain, A.K. (1982) A differential AC Calorimeter for biophysical studies. *Anal. Biochem.* 121:55-61.

121. Rudolph, A.S., Crowe, J.H. and Crowe, L.M. (1986) Effects of three stabilizing agents--proline, betaine, and trehalose--on membrane phospholipids. *Arch. Biochem. Biophys.* 245:134-43.

122. Johnston, D.S., Sanghera, S., Pons, M. and Chapman, D. (1980) Phospholipid polymers--synthesis and spectral characteristics. *Biochim. Biophys. Acta.* 602:57-69.

123. Phillips, M.C., Ladbrooke, B.D. and Chapman, D. (1970) Molecular interactions in mixed lecithin systems. *Biochim. Biophys. Acta.* 196:35-44.

124. Blume, A. (1979) A comparative study of the phase transitions of phospholipid bilayers and monolayers. *Biochim. Biophys. Acta.* 557:32-44.

125. Wilkinson, D.A. and Nagle, J.F. (1981) Dilatometry and calorimetry of saturated phosphatidylethanolamine dispersions. *Biochemistry.* 20:187-92.

126. Webb, M.S., Hui, S.W. and Steponkus, P.L. (1993) Dehydration-induced lamellar-to-hexagonal-II phase transitions in DOPE/DOPC mixtures. *Biochim. Biophys. Acta.* 1145:93-104.

127. Wong, F.M.P., Bally, M.B. and Brooks, D.E. (1999) Electrostatically mediated interactions between cationic lipid-DNA particles and an anionic surface. *Arch. Biochem. Biophys.* 366:31-9.

128. Hofland, H.E.J., Shephard, L. and Sullivan, S.M. (1996) Formation of stable cationic lipid/DNA complexes for gene transfer. *Proc. Natl. Acad. Sci. USA.* 93:7305-9.

129. Sternberg, B., Sorgi, F.L. and Huang, L. (1994) New structures in complex formation between DNA and cationic liposomes visualized by freeze-fracture electron microscopy. *FEBS Lett.* 356:361-6.

130. Gustafsson, J., Arvidson, G., Karlsson, G. and Almgren, M. (1995) Complexes between cationic liposomes and DNA visualized by cryo-TEM. *Biochim. Biophys. Acta.* 1235:305-12.

131. Lasic, D.D., Strey.H., Stuart, M.C.A., Podgornik, R. and Frederik, P.M. (1997) The structure of DNA-liposome complexes. *J. Am. Chem. Soc.* 119:832-3.

132. Mannino, R.J. and Gould-Fogerite, S. (1988) Liposome mediated gene transfer. *Biotechniques.* 6:682-90.

133. Kanamaru, T., Takagi, T., Takakura, Y. and Hashida, M. (1998) Biological effects and cellular uptake of c-*myc* antisense oligonucleotides and their cationic liposome complexes. *J. Drug Target.* 5:235-46.

134. Eastman, S.J., Lukason, M.J., Tousignant, J.D., Murray, H., Lane, M.D., St. George, J.A., Akita, G.Y., Cherry, M., Cheng, S.H. and Scheule, R.K. (1997) A concentrated

and stable aerosol formulation of cationic lipid:DNA complexes giving high-level gene expression in mouse lung. *Hum. Gene Ther.* 8:765-73.

135. Zou, Y., Zong, G., Ling, Y.H., Hao, M.M., Lozano, G., Hong, W.K. and Perez-Soler, R. (1998) Effective treatment of early endobronchial cancer with regional administration of liposome-p53 complexes. *J. Natl. Cancer Inst.* 90:1130-7.

136. Wasan, E., Harvie, P., Edwards, K., Karlsson, G. and Bally, M.B. (1999) A multi-step lipid mixing assay to model structural changes in cationic lipoplexes used for *in vitro* transfection. *Biochim. Biophys. Acta.* 1461:27-46.

137. Jaaskelainen, I., Monkkonen, J. and Urtti, A. (1994) Oligonucleotide-cationic liposome interactions. A physiochemical study. *Biochim. Biophys. Acta.* 1195:115-23.

138. Zabner, J., Fasbender, A.J., Moninger, T., Poellinger, K.A. and Welsh, M.J. (1995) Cellular and molecular barriers to gene transfer by a cationic lipid. *J. Biol. Chem.* 270:18997-19007.

139. Tomlinson, E. and Rolland, A.P. (1996) Controllable gene therapy: Pharmaceutics of non-viral gene delivery systems. *J. Controlled Release.* 39:357-72.

140. Escriou, V., Ciolina, C., Helbling-Leclerc, A., Wils, P. and Scherman, D. (1998) Cationic lipid-mediated gene transfer: Analysis of cellular uptake and nuclear import of plasmid DNA. *Cell Biol. Toxicol.* 14:95-104.

141. Bally, M.B., Harvie, P., Wong, F.M.P., Kong, S., Wasan, E.K. and Reimer, D.L. (1999) Biological barriers to cellular delivery of lipid-based DNA carriers. *Adv. Drug Del. Rev.* 38:291-315.

142. Aoki, H., Tottori, T., Sakurai, F., Fuji, K. and Miyajima, K. (1997) Effects of positive charge density on the liposomal surface on disposition kinetics of liposomes in rats. *Int. J. Pharm.* 156:163-74.

143. Chonn, A., Cullis, P.R. and Devine, D.V. (1991) The role of surface charge in the activation of the classical and alternative pathways of complement by liposomes. *J. Immunol.* 146:4234-41.

144. Gabizon, A. and Papahadjopoulos, D. (1992) The role of surface charge and hydrophilic groups on liposome clearance in vivo. *Biochim. Biophys. Acta.* 1103:94-100.

145. Li, S., Tseng, W.C., Stolz, D.B., Wu, S.P., Watkins, S.C. and Huang, L. (1999) Dynamic changes in the characteristics of cationic lipidic vectors after exposure to mouse serum: implications for intravenous lipofection. *Gene Ther.* 6:585-94.

146. Fox, B.A., Drury, M., Hu, H.-M., Cao, Z., Huntzicker, E.G., Qiem, W. and Urba, W.J. (1998) Lipofection indirectly increases expression of histocompatibility complex class I molecules on tumor cells. *Cancer Gene Ther.* 5:307-12.

147. Filion, M.C. and Phillips, N.C. (1997) Anti-inflammatory activity of cationic lipids. *Br. J. Pharmacol.* 122:551-7.

148. Filion, M.C. and Phillips, N.C. (1998) Major limitations in the use of cationic liposomes for DNA delivery. *Int. J. Pharm.* 162:159-70.

149. Filion, M.C. and Phillips, N.C. (1997) Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells. *Biochim. Biophys. Acta.* 1329:345-6.

150. Arnold, K., Aschoernig, O., Barthel, D. and Herold, W. (1990) Exclusion of poly(ethylene glycol) from liposome surfaces. *Biochim. Biophys. Acta.* 1022:303-310.

151. Allen, T.M., Hansen, C. and Rutledge, J. (1989) Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other tissues. *Biochim. Biophys. Acta.* 981:27-35.

152. Maruyama, K., Yuda, T., Okamoto, A., Kojima, S., Suginaka, A. and Iwatsuru, M. (1992) Prolonged circulation time in vivo of large unilamelar liposomes composed of distearoyl phosphatidylcholine and cholesterol containing amphipathic poly(ethylene glycol). *Biochim. Biophys. Acta.* 1128:44-9.

153. Malcolm, G.N. and Rowlinson, J.S. (1957) The thermodynamic properties of aqueous solutions of poly(ethylene glycol), polypropylene glycol and dioxane. *Trans. Faraday Soc.* 53:921-31.

154. Du, H., Chandaroy, P. and Hui.S.K. (1997) Grafted poly-(ethylene glycol) on lipid surfaces inhibits proteins adsportion and cell adhesion. *Biochim. Biophys. Acta.* 1326:236-48.

155. Allen, T.M., Hansen, C., Martin, F., Redemann, C. and Yau-Young, A. (1991) Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo. *Biochim. Biophys. Acta.* 1066:29-36.

156. Yoshioka, H. (1991) Surface modification of haemoglobin-containing liposomes with polyethylene glycol prevents liposome aggregation in blood plasma. *Biomaterials.* 12:861-4.

157. Gabizon, A. and Papahadjopoulos, D. (1988) Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. *Proc. Natl. Acad. Sci. USA.* 85:6949-53.

158. Hong, K., Zheng, W., Baker, A. and Papahadjopoulos, D. (1997) Stabilization of cationic liposome-plasmid DNA complexes by polyamines and poly(ethylene glycol)-phospholipid conjugates for efficient in vivo gene delivery. *FEBS Lett.* 400:233-7.

159. Meyer, O., Kirpotin, D., Hong, K., Sternberg, B., Park, J.W., Woodle, M.C. and Papahadjopoulos, D. (1998) Cationic liposomes coated with polyethylene glycol as carriers for oligonucleotides. *J. Biol Chem.* 273:15621-7.

160. Zelphati, O., Imbach, J., Signoret, N., Zon, G., Rayner, B. and Leserman, L. (1994) Antisense oligonucleotides in solution or encapsulated in immunoliposomes inhibit replication of HIV-1 by several different mechanisms. *Nucleic Acids Res.* 22:4307-4314.

161. MacDonald, R.I. (1985) Membrane fusion due to dehydration by polyethylene glycol, dextran, or sucrose. *Biochemistry.* 24:4058-66.

162. Burgess, S.W., McIntosh, T.J. and Lentz, B.R. (1992) Modulation of poly(ethylene glycol)-induced fusion by membrane hydration: importance of interbilayer separation. *Biochemistry.* 31:2653-61.

163. Needham, D., McIntosh, T.J. and Lasic, D.D. (1992) Repulsive interactions and mechanical stability of polymer-grafted lipid membranes. *Biochim. Biophys. Acta.* 1108:40-8.

164. Mori, A., Klibanov, A.L., Torchilin, V.P. and Huang, L. (1991) Inflence of the steric barrier activity of amphipathic poly(ethyleneglycol) and ganglioside GM1 on the circulation time of liposomes and on the target binding of immunoliposomes in vivo. *FEBS Lett.* 284:263-6.

165. Kenworthy, A.K., Hristova, K., Needham, D. and McIntosh, T.J. (1995) Range and magnitude of the steric pressure between bilayers containing phospholipids with covalently attached poly(ethylene glycol). *Biophys. J.* 68:1921-36.

166. Needham, D., McIntosh, T.J. and Lasic, D.D. (1992) Repulsive interactions and mechanical stability of polymer-grafted lipid membranes. *Biochim. Biophys. Acta.* 1108:40-8.

167. Kuhl, T.L., Majewski, J., Wong, J.Y., Steinberg, S., Leckband, D.E., Israelachvili, J.N. and Smith, G.S. (1998) A neutron reflectivity study of polymer-modified phospholipid monolayers at the solid-solution interface: polyethylene glycol-lipids on silane-modified substrates. *Biophys. J.* 75:2352-62.

168. Legendre, J. and Szoka Jr., F.C. (1992) Delivery of plasmid DNA into mammalian cell lines using pH-sensitive liposomes: comparison with cationic liposomes. *Pharm. Res.* 9:1235-42.

169. Kirpotin, D., Hong, K., Mullah, N. and Zalipsky, S. (1996) Liposomes with detachable polymer coating: destabilization and fusion of dioleoylphosphatidylethanolamine vesicles triggered by cleavage of surface-grafted poly(ethylene glycol). *FEBS Lett.* 388:115-8.

170. Adlakha-Hutcheon, G., Bally, M.B., Shew, C.R. and Madden, T.D. (1999) Controlled destabilization of a liposomal drug delivery system enhances mitoxantrone antitumor activity. *Nat. Biotechnol.* 17:775-9.

171. Gabizon, A., Horowitz, A.T., Goren, D., Tzemach, D., Mandelbaum-Shavit, F., Qazen, M.M. and Zalipsky, S. (1999) Targeting folate receptor with folate linked to extremities of poly(ethylene glycol)-grafted liposomes: *in vitro* studies. *Bioconjug. Chem.* 10:289-98.

172. Luo, D., Woodrow-Mumford, K., Belcheva, N. and Waltzman, W.M. (1999) Controlled DNA delivery systems. *Pharm. Res.* 16:1300-8.

173. Godbey, W.T., Wu, K.K., Hiraski, G.J. and Mikos, A.G. (1999) Improved packing of poly(ethylenimine)/DNA complexes increases transfeciton efficiency. *Gene Ther.* 6:1380-88.

174. Koiv, A., Palvimo, J. and Kinnunen, P.K.J. (1996) Evidence for ternary complex formation by histone H1, DNA, and liposomes. *Biochemistry.* 34:8018-27.

175. Haensler, J. and Szoka Jr., F.C. (1993) Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconjug. Chem.* 4:372-9.

176. Duiguid, J.G., Li, C., Shi, M., Logamn, M.J., Alila, H., Rolland, A., Tomlinson, E., Sparrow, J.T. and Smith, L.C. (1998) A physicochemical approach for predicting the effectiveness of peptide-based gene delivery systems for use in plasmid-based gene therapy. *Biophys. J.* 74:2802-14.

177. Caplen, N.J. (2000) Nucleic acid transfer using cationic lipids. *Methods Mol. Biol.* 133:1-19.

178. Brunette, E., Stribling, R. and Debs, R. (1991) Lipofection does not require the removal of serum. *Nucleic Acids Res.* 20:1151.

179. Quattrone, A., Papucci, L., Schiavone, N., Mini, E. and Capaccioli, S. (1994) Intracellular enhancement of intact antisense oligonucleotide steady-state levels by cationic lipids. *Anticancer Drug Des.* 9:549-53.

180. Reimer, D.L., Kong, S., Monch, M., Wyles, J., Tam, P., Wasan, E.K. and Bally, M.B. (1998) Liposomal lipid and plasmid DNA delivery to B16/BL6 tumors after intraperitoneal administration of cationic liposome DNA aggregates. *J. Pham. Exp. Ther.* 289:807-15.

181. Escriou, V., Ciolina, C., Lacroix, F., Byk, G., Scherman, D. and Wils, P. (1998) Cationic lipid-mediated gene transfer: effect of serum on cellular uptake and intracellular fate of lipopolyamine/DNA complexes. *Biochim. Biophys. Acta.* 1368:276-88.

182. Cudd, A. and Nicolau, C. (1985) Intracellular fate of liposome-encapsulated DNA in mouse liver. Analysis using electron microscope autoradiography and subcellular fractionation. *Biochim. Biophys. Acta.* 845:477-91.

183. Williams, S.A., Chang, L., Buzby, J.S., Suen, Y. and Cairo, M.S. (1996) Cationic lipids reduce time and dose of c-myc antisense oligodeoxynucleotides required to specifically inhibit Burkitt's lymphoma cell growth. *Leukemia.* 10:1980-9.

184. Jarnagin, W.R., Debs, R.J., Wang, S. and Bissell, D.M. (1992) Cationic lipid-mediated transfection of liver cells in primary culture. *Nucleic Acids Res.* 20:4205-11.

185. Keogh, M.-C., Chen, D., Lupu, F., Shaper, N., Schmitt, J.F., Kakkar, V.V. and Lemoine, N.R. (1997) High efficiency reporter gene transfection of vascular tissue *in vitro* and *in vivo* using a cationic lipid-DNA complex. *Gene Ther.* 4:162-71.

186. Mita, K., Zama, M. and Ichimura, S. (1977) Effect of charge density of cationic polyelectrolytes on complex formation with DNA. *Biopolymers.* 16:1993-2004.

187. Felgner, J.H., Kumar, R., Sridhar, C.N., Wheeler, C.J., Tsai, Y.J., Border, R., Ramsey, P., Martin, M. and Felgner, P.L. (1994) Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formations. *J. Biol. Chem.* 269:2550-61.

188. Zimmer, A., Atmaca-Abdel Aziz, S., Gilbert, M., Werner, D. and Noe, C.R. (1999) Synthesis of cholesterol modified cationic lipids for liposomal drug delivery of antisense oligonucleotides. *J. Pharm. Biopharm.* 47:175-8.

189. Egilmez, N.K., Iwanuma, Y. and Bankert, R.B. (1996) Evaluation and optimization of differant cationic liposome formulations for in vivo gene transfer. *Biochim. Biophys. Res. Commun.* 221:169-73.

190. Wheeler, C.J., Sukhu, L., Yang, G., Tsai, Y., Bustamente, C., Felgner, P., Norman, J. and Manthorpe, M. (1996) Converting an alcohol to an amine in a cationic lipid dramatically alters the co-lipid requirement, cellular transfection activity and the ultrastructure of DNA-cytofectin complexes. *Biochim. Biophys. Acta.* 1280:1-11.

191. Byk, G., Dubertret, C., Escriou, V., Frederic, M., Jaslin, G., Rangara, R., Pitard, R., Crouzet, J., Wils, P., Schwartz, B. and Sherman, D. (1998) Synthesis, activity, and structure-activity relationship studies of novel cationic lipids for DNA transfer. *J. Med. Chem.* 41:224-35.

192. Tang, F. and Hughes, J.A. (1998) Introduction of a disulfide bond into a cationic lipid enhances transgene expression of plasmid DNA. *Biochem. Biophys. Res. Commun.* 242:141-5.

193. Balasubramanian, R.P., Bennett, M.J., Aberle, A.M., Malone, J.G., Nantz, M.H. and Malone, R.W. (1996) Structural and functional analysis of cationic transfection lipids: the hydrophobic domain. *Gene Ther.* 3:163-72.

194. White, P.J., Fogarty, R.D., McKeanS.C., Venables, D.J., Werther, G.A. and WraightC.J. (1999) Oliognucleotide uptake in culture keratinocytes: Influences of confluence, cationic liposomes, and keratinocyte cell type. *J. Invest. Dermatol.* 112:699-705.

195. Reimer, D.L., Kong, S. and Bally, M.B. (1997) Analysis of cationic liposome-mediated interactions of plasmid DNA with murine and human melanoma cells *in vitro. J. Biol. Chem.* 272:19480-7.

196. Bennett, C.F., Mirejovsky, D., Crooke, R.M., Tsai, Y.J., Felgner, J., Sridhar, C.N., Wheeler, C.J. and Felgner, P.L. (1997) Structural requirements for cationic lipid mediated phosphorothioate oligonucleotides delivery to cells in culture. *J. Drug Target.* 5:149-162.

197. Barron, L., Meyer, K. and Szoka Jr., F. (1998) Effects of complement depletion on the pharmacokinetics and gene delivery mediated by cationic lipid-DNA complexes. *Hum. Gene Ther.* 9:315-23.

198. Schreier, H., Gagne, L., Bock, T., Erdos, G.W., Druzgala, P., Conary, J.T. and Muller, B.W. (1997) Physicochemical properties and in vitro toxicity of cationic liposome cDNA complexes. *Pharm. Acta Helv.* 72:215-23.

199. Gouy, G. (1910) Constitution of the electric charge at the surface of an electrolyte. *J. Phys.* 9:457-68.

200. Chapman, D.L. (1913) Theory of electrocapillarity. *Philos. Mag.* 25:475-81.

201. Grant, C.W.M. (1983) Lateral phase separation and the cell membrane. *In* Membrane Fluidity in Biology. R.C. Aloia, ed. Academic Press Inc., New York: 131-47.

202. Bloomfield, V.A. (1991) Condensation of DNA by multivalent cations: considerations on mechanism. *Biopolymers.* 31:1471-81.

203. Reimer, D.L., Zhang, Y., Kong, S., Wheeler, J.J., Graham, R.W. and Bally, M.B. (1995) Formation of novel hydrophobic complexes between cationic lipids and plasmid DNA. *Biochemistry.* 34:12877-83.

204. Wolff, J.A., Malone, R.W., Williams, P., Chong, W., Acsadi, G., Jani, A. and Felgner, P.L. (1990) Direct gene transfer into mouse muscle *in vivo. Science.* 247:1465-8.

205. Davis, H.L., Demeneix, B.A., Quantin, B., Coulombe, J. and Whalen, R.G. (1993) Plasmid DNA is superior to viral vectors for direct gene transfer into adult mouse skeletal muscle. *Hum. Gene Ther.* 4:733-40.

206. Manthorpe, M., Cornefert-Jensen, F., Hartikka, J., Felgner, J., Rundell, A., Margalith, M. and Dwarki, V. (1993) Gene therapy by intramuscular injection of plasmid DNA: studies on firefly luciferase gene expression in mice. *Hum. Gene Ther.* 4:419-31.

207. Zhang, G., Budker, V. and Wolff, J.A. (1999) High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum. Gene Ther.* 10:1735-7.

208. Liu, F., Song, Y.K. and Liu, D. (1999) Hydrdynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther.* 6:1258-66.

209. Hansen, S.H., Snadvig, K. and van Deurs, B. (1991) The preendosomal compartment comprises distinct coated and noncoated endocytic vesicle populations. *J. Cell Biol.* 113:731-41.

210. Stamatatos, L., Leventis, R., Zuckermann, M.J. and Silvius, J.R. (1988) Interactions of cationic lipid vesicles with negatively charged phospholipid vesicles and biological membranes. *Biochemistry.* 27:3917-25.

211. Behr, J., Demeneix, B., Loeffler, J. and Perez-Mutul, J. (1989) Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proc .Natl. Acad. Sci. USA.* 86:6982-6.

212. Zhou, X., Klibanov, A.L. and Huang, L. (1991) Lipophilic polylysines mediate efficient DNA transfection in mammalian cells. *Biochim. Biophys. Acta.* 1065:8-14.

213. van der Woude, I., Visser, H.W., ter Beest, M.B.A., Wagenaar, A., Ruiters, M.H.L., Engberts, J.B.F.N. and Hoekstra, D. (1995) Parameters influencing the introduction of plasmid DNA into cells by the use of synthetic amphiphiles as a carrier system. *Biochim. Biophys. Acta.* 1240:34-40.

214. Matsui, H., Johnson, L., Randell, S.H. and Boucher, R.C. (1997) Loss of binding and entry of liposome-DNA complexes decreases transfection efficiency in differentiated airway epithelial cells. *J. Biol. Chem.* 272:1117-26.

215. Hernandez-Caselles, T., Villalain, J. and Gomez-Fernandez, J.C. (1993) Influence of liposome charge and composition on their interaction with human blood serum proteins. *Mol. Cell. Biochem.* 120:119-126.

216. McLean, J.M., Fox, E.A., Baluk, P., Bolton, P.B., Haskell, A., Pearlman, R., Thurston, G., Umemoto, E.Y. and McDonald, D.M. (1997) Organ-specific endothelial cell uptake of cationic liposome-DNA complexes in mice. *Am. J. Physiol.* 273:H387-H404.

217. Aoki, H., Fuji, K. and Miyajima, K. (1997) Effects of blood on the uptake of charged liposomes by perfused rat liver: cationic glucosamine-modified liposomes interact with erythrocyte and escape phagocytosis by macrophages. *Int. J. Pharm.* 149:15-23.

218. Zhou, X. and Huang, L. (1994) DNA transfection mediated by cationic liposomes containing lipopolylysine: characterization and mechanism of action. *Biochim. Biophys. Acta.* 1189:195-203.

219. Gao, X. and Huang, L. (1991) A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochem. Biophys. Res. Commun.* 179:280-5.

220. Yagi, K., Noda, H., Kurono, M. and Ohishi, N. (1993) Efficient gene transfer with less cytotoxicity by means of cationic multilamellar liposomes. *Biochem. Biophys. Res. Commun.* 196:1042-5.

221. Ohki, S. (1991) Physico-chemical factors underlying membrane adhesion and fusion. *In* Cell and model membrane interaction. S. Ohki, ed. Plenum Press, New York: 267-84.

222. Israelachvili, J. and Wennerstrom, H. (1996) Role of hydration and water structure in biological and colloidal interactions. *Nature.* 379:219-25.

223. Bennett, M.J., Aberle, A.M., Balasubramaniam, R.P., Malone, J.G., Malone, R.W. and Nantz, M.H. (1997) Cationic lipid-mediated gene delivery to murine lung: correlation of lipid hydration with in vivo transfection activity. *J. Med. Chem.* :4069-78.

224. Lopez-Casillas, F., Wrana, J.L. and Massague, J. (1993) Betaglycan presents ligand to the TGF beta signaling receptor. *Cell.* 73:1435-44.

225. Wang, X.F., Lin, H.Y., Ng-Eaton, E., Downward, J., Lodish, H.F. and Weinberg, R.A. (1991) Expression cloning and characterization of the TGF-beta type III receptor. *Cell.* 67:797-805.

226. Berryman, D.E. and Bensadoun, A. (1995) Heparan sulfate proteoglycans are primarily responsible for the maintenance of enzyme activity, binding, and degradation of lipoprotein lipase in Chinese hamster ovary cells. *J. Biol. Chem.* 270:24525-31.

227. Chappell, D.A., Fry, G.L., Waknitz, M.A., Muhonen, L.E., Pladet, M.W., Iverius, P.H. and Strickland, D.K. (1993) Lipoprotein lipase induces catabolism of normal triglyceride-rich lipoproteins via the low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor in vitro. A process facilitated by cell-surface proteoglycans. *J. Biol. Chem.* 268:14168-75.

228. Yayon, A., Klagsbrun, M., Esko, J.D., Leder, P. and Ornitz, D.M. (1991) Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. *Cell.* 64:841-8.

229. Mislick, K.A. and Baldeschwieler, J.D. (1996) Evidence for the role of proteoglycans in cation-mediated gene transfer. *Proc. Natl. Acad. Sci. USA.* 93:12349-54.

230. Belting, M. and Peterson, P. (1999) Protective role for proteoglycan against cationic lipid cytotoxicity allowing optimal transfection efficiency in vitro. *Biochem. J.* 342:281-6.

231. Loke, S.L., Stein, C.A., Zhang, X.H., Mori, K., Nakanishi, M., Subasinghe, C., Cohen, J.S. and Neckers, L.M. (1989) Characterization of oligonucleotide transport into living cells. *Proc. Natl. Acad. Sci. USA.* 86:3474-8.

232. Fasbender, A., Zabner, J., Zeiher, B.G. and Welsh, M.J. (1997) A low rate of cell proliferation and reduced DNA uptake limit cationic lipid-mediated gene transgene to primary cultures of ciliated human airway epithelia. *Gene Ther.* 4:1173-80.

233. Schaffer, D.V. and Laffenburger, D.A. (1998) Optimization of cell surface binding enhances efficiency and specificity of molecular conjugate gene delivery. *J. Biol. Chem.* 273:28004-9.

234. Straubinger, R.M., Paphadjopoulos, D. and Hong, K. (1990) Endocytosis and intracellular fate of liposomes using pyranine as a probe. *Biochemistry.* 29:4929-39.

235. Wu, G.Y., Wilson, J.M., Shalaby, F., Grossman, M., Shafritz, D.A. and Wu, C.H. (1991) Receptor-mediated gene delivery in vivo. *J. Biol. Chem.* 266:14338-14342.

236. Friend, D.S., Papahadjopoulos, D. and Debs, R.J. (1996) Endocytosis and intracellular processing accompanying transfection mediated by cationic liposomes. *Biochim. Biophys. Acta.* 1278:41-50.

237. Stegmann, T. and Legendre, J.Y. (1997) Gene transfer mediated by cationic lipids: lack of a correlation between lipid mixing and transfection. *Biochim. Biophys. Acta.* 1325:71-9.

238. Lee, R.J. and Huang, L. (1996) Folated-targeted, anionic liposome-entrapped polylysine-condensed DNA for tumor cell-specific gene transfer. *J. Biol. Chem.* 271:8481-7.

239. Li, L.H. and Hui, S.W. (1997) The effect of lipid molecular packing stress on cationic liposome-induced rabbit erythrocyte fusion. *Biochim. Biophys. Acta.* 1323:105-16.

240. Bailey, A.L. and Cullis, P.R. (1997) Membrane fusion with cationic liposomes: Effects of target membrane lipid composition. *Biochemistry.* 36:1628-34.

241. Noguchi, A., Furuno, T., Kawaura, C. and Nkanishi, M. (1998) Membrane fusion plays an important role in gene transfection mediated by cationic liposomes. *FEBS Lett.* 433:169-73.

242. Kumenko, D.A. and Frolov, V.A. (1997) Quantitative evaluation of the kinetics of lipid probe redistribution between fusing cells. *Membr. Cell Biol.* 10:593-600.

243. Van Renswoude, J. and Hoekstra, D. (1981) Cell-induced leakage of liposome contents. *Biochemistry.* 20:540-6.

244. Spassova, M., Tsoneva, I., Petrov, A.G., Petkova, J.I. and Neumann, E. (1994) Dip patch clamp currents suggest electrodiffusive transport of the polyelectrolyte DNA through lipid bilayers. *Biophys. Chem.* 52:267-74.

245. Stubbs, C.D. and Slater, S.J. (1996) The effects of non-lamellar forming lipids on membrane protein-lipid interactions. *Chem. Phys. Lipids.* 81:185-95.

246. Scarlata, S. and Gruner, S.M. (1997) Role of phosphatidylethanolamine lipids in the stabilization of protein-lipid contacts. *Biophys. Chem.* 67:269-79.

247. Bazzi, M.D. and Nelsestuen, G.L. (1992) Interaction of annexin VI with membranes: highly restricted dissipation of clustered phospholipids in membranes containing phosphatidylethanolamine. *Biochemistry.* 34:10406-13.

248. McElhaney, R.N. (1989) The influence of membrane lipid composition and physical properties of membrane structure and function in Acholeplasma laidlawii. *Crit. Rev. Microbiol.* 17:1-32.

249. Epand, R.F., Martin, I., Ruysschaert, J.M. and Epand, R.M. (1994) Membrane orientation of the SIV fusion peptide determines its effect on bilayer stability and ability to promote membrane fusion. *Biochem. Biophys. Res. Commun.* 205:1938-43.

250. Farhood, H., Serbina, N. and Huang, L. (1995) The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochim. Biophys. Acta.* 1235:289-95.

251. Fasbender, A., Marshall, J., Moninger, T.O., Grunst, T., Cheng, S. and Welsh, M.J. (1997) Effect of co-lipids in enhancing cationic lipid-mediated gene transfer *in vitro* and *in vivo*. *Gene Ther.* 4:716-25.

252. Ellens, H., Bentz, J. and Szoka Jr., F.C. (1986) Fusion of phosphatidylethanolamine-containing liposomes and mechanism of the Lα-HII phase transition. *Biochemistry.* 25:4141-7.

253. Brown, P.M., Steers, J., Hui, S.W., Yeagle, P.L. and Silvius, J.R. (1986) Role of head group structure in the phase behavior of amino phospholipids. 2. Lamellar and nonlamellar phases of unsaturated phosphatidylethanolamine analogues. *Biochemistry.* 25:4259-67.

254. Allen, T.M., Hong, K. and Papahadjopoulos, D. (1990) Membrane contact, fusion, and hexagonal (HII) transitions in phosphatidylethanolamine liposomes. *Biochemistry.* 29:2976-85.

255. Hui, S.W., Langner, M., Zhao, Y., Ross, P., Hurley, E. and Chan, K. (1996) The role of helper lipids in cationic liposome-mediated gene transfer. *Biophys. J.* 71:590-599.

256. Gruner, S.M., Lenk, R.P., Janoff, A.S. and Ostro, M.J. (1985) Novel multilayered lipid vesicles: comparison of physical characteristics of multilamellar liposomes and stable plurilamellar vesicles. *Biochemistry.* 24:2833-42.

257. Lafleur, M., Bloom, M., Eikenberry, E.F., Gruner, S.M., Han, Y. and Cullis, P.R. (1996) Correlation between lipid plane curvature and lipid chain order. *Biophys. J.* 70:2747-57.

258. Janes, N. (1996) Curvature stress and polymorphism in membranes. *Chem. Phys. Lipids.* 81:133-50.

259. Chernomordik, L., Kozlov, M.M. and Zimmerberg, J. (1995) Lipids in biological membrane fusion. *J. Membr. Biol.* 146:1-14.

260. Boden, N., Jackson, P., Levine, Y.K. and Ward, A. (1976) Intramolecular disorder and its relation to mesophase structure in lipid/water mixtures. *Biochim. Biophys. Acta.* 419:395-403.

261. Tate, M.W. and Gruner, S.M. (1989) Temperature dependence of the structural dimensions of the inverted hexagonal (HII) phase of phosphatidylethanolamine-containing membranes. *Biochemistry.* 28:4245-53.

262. Epand, R.M., Fuller, N. and Epand, R.P. (1996) Role of the position of unsaturation on the phase behavior and intrinsic curvature of phosphatidylethanolamines. *Biophys. J.* 71:1806-10.

263. Kirk, G.L., Gruner, S.M. and Stein, D.L. (1984) A thermodynamic model of the lamellar to inverse hexagonal phase transition of lipid membrane-water systems. *Biochemistry.* 23:1093-102.

264. Leventis, R. and Silvius, J.R. (1990) Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles. *Biochim. Biophys. Acta.* 1023:124-32.

265. Hauser, H., Pascher, I., Pearson, R.H. and Sundell, S. (1981) Preferred conformation and molecular packing of phosphatidylethanolamine and phosphatidylcholine. *Biochim. Biophys. Acta.* 650:21-51.

266. McIntosh, T.J. (1996) Hydration properties of lamellar and non-lamellar phases of phosphatidylcholine and phosphatidylethanolamine. *Chem. Phys. Lipids.* 81:117-131.

267. McIntosh, T.J. (1980) Differences in hydrocarbon chain tilt between hydrated phosphatidylethanolamine and phosphatidylcholine bilayers. A molecular packing model. *Biophys. J.* 29:237-45.

268. Papahadjopoulos, D. and Watkins, J.C. (1967) Phospholipid model membranes II. Permeability properties of hydrated liquid crystals. *Biochim. Biophys. Acta.* 135:639-652.

269. Pink, D.A., McNeil, S., Quinn, B. and Zuckermann, M.J. (1998) A model of hydrogen bond formation in phosphatidylethanolamine bilayers. *Biochim. Biophys. Acta.* 1368:289-305.

270. Cullis, P.R., de Kruiff, B., Hope, M.J., Verkleij, A.J., Nayar, R., Farren, S.B., Tilcock, C., Madden, T.D. and Bally, M.B. (1983) Structural properties of lipids and their

functional roles in biological membranes. *In* Membrane Fluidity in Biology. R.C. Aloia, ed. Academic Press Inc., New York: 40-79.

271. Damodaran, K.V. and Merz, K.M. (1997) A comparison of DMPC- and DLPE-based lipid bilayers. *Biophys. J.* 66:1076-87.

272. Harvie, P., Wong, F.M.P. and Bally, M.B. (1998) Characterization of lipid DNA interactions I - Destabilization of bound lipids and DNA dissociation. *Biophys. J.* 75:1040-51.

273. Szoka Jr., F.C., Xu, Y. and Zelphati, O. (1996) How are nucleic acids released in cells from cationic lipid-nucleic acid complexes? *J. Liposome Res.* 6:567-87.

274. Xu, Y. and Szoka Jr., F.C. (1996) Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry.* 35:5616-23.

275. Wrobel, I. and Collins, D. (1995) Fusion of cationic liposomes with mammalian cells occurs after endocytosis. *Biochim. Biophys. Acta.* 1235:296-304.

276. Zelphati, O. and Szoka Jr., F.C. (1996) Mechanism of oligonucleotide release from cationic liposomes. *Proc. Natl. Acad. Sci. USA.* 93:11493-8.

277. Ouahabi, A.E., Thiry, M., Pector, V., Fuks, R., Ruysschaert, J.M. and Vandenbranden, M. (1997) The role of endosome destabilizing activity in the gene transfer process mediated by cationic lipids. *FEBS Lett.* 414:187-92.

278. Remy, J.S., Kichler, A., Mordvinov, V., Schuber, F. and Behr, J.P. (1995) Targeted gene transfer into hepatoma cells with lipopolyamine-condensed DNA particles presenting galactose ligands: a stage toward artificial viruses. *Proc. Natl. Acad. Sci. USA.* 92:1744-8.

279. Bhattacharya, S. and Mandal, S.S. (1998) Evidence of interlipid ion-pairing in anion-induced DNA release from cationic amphiphile-DNA complexes. Mechanistic implications in transfection. *Biochemistry.* 37:7764-77.

280. Wattiaux, R., Jadot, M., Warnier-Pirotte, M.-T. and Wattiaux-De Coninck, S. (1997) Cationic lipids destabilize lysosomal membrane in vitro. *FEBS Lett.* 417:199-202.

281. Shangguan, T., Pak, C.C., Ali, S., Janoff, A.S. and Meers, P. (1998) Cation-dependent fusogenicity of an N-acyl phosphatidylethanolamine. *Biochim. Biophys. Acta.* 1368:171-83.

282. Hara, T., Kuwasawa, H., Aramaki, Y., Takada, S., Koike, K., Ishidate, K., Kato, H. and Tsuchiya, S. (1996) Effects of fusogenic and DNA-binding amphiphilic compounds on the receptor-mediated gene transfer into hepatic cells by asialofetuin-labeled liposomes. *Biochim. Biophys. Acta.* 1278:51-8.

283. Jensen, T.G., Roses, A.D. and Jorgensen, A.L. (1994) Apolipoprotein E uptake and degradation via chloroquine-sensitive pathway in cultivated monkey cells overexpressing low density lipoprotein receptor. *Neurosci. Lett.* 180:193-6.

284. Capecchi, M.R. (1980) High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell.* 22:479-88.

285. Pollard, H., Remy, J.-S., Loussouarn, G., Demolombe, S., Behr, J.-P. and Escande, D. (1998) Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. *J. Biol. Chem.* 273:7507-11.

286. Lewis, J.G., Lin, K.-Y., Kothavale, A., Flanagan, W.M., Matteucci, M.D., DePrince, R.B., Mook, R.A., Hendren, R.W. and Wagner, R.W. (1996) A serum-resistant cytofectin for cellular delivery of antisense oligodeoxynucleotides and plasmid DNA. *Proc. Natl. Acad. Sci. USA.* 93:3176-81.

287. Nigg, E.A. (1997) Nucleocytoplasmic transport: signals, mechanisms and regulation. *Nature.* 386:779-87.

288. Mortimer, I., Tam, P., MacLachlan, I., Graham, R.W., Saravolac, E.G. and Joshi, P.B. (1999) Cationic lipid-mediated transfection of cells in culture requires mitotic activity. *Gene Ther.* 6:403-11.

289. Merrill, G.F. (1998) Cell synchronization. *Methods Cell Biol.* 57:229-49.

290. Peters, R. (1984) Nucleo-cytoplasmic flux and intracellular mobility in single hepatocytes measured by fluorescence microphotolysis. *EMBO J.* 3:1831-6.

291. Peters, R. (1986) Fluorescence microphotolysis to measure nucleocytoplasmic transport and intracellular mobility. *Biochim. Biophys. Acta.* 864:305-59.

292. Branden, L.J., Mohamed, A.J. and Smith, C.I. (1999) A peptide nucleic acid-nuclear localization signal fusion that mediates nuclear transport of DNA. *Nat. Biotechnol.* 17:784-7.

293. Zanta, M.A., Belguise-Valladier, P. and Behr, J.P. (1999) Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. *Proc. Natl. Acad. Sci. USA.* 96:91-6.

294. Subramanian, A., Ranganathan, P. and Diamond, S.L. (1999) Nuclear targeting peptide scaffolds for lipofection of nondividing mammalian cells. *Nat. Biotechnol.* 17:873-7.

295. De Lucia, F., Alilat, M., Sivolob, A. and Prunell, A. (1999) Nucleosome dynamics. III. Histone tail-dependent fluctuation of nucleosomes between open and closed DNA conformations. Implications for chromatin dynamics and the linking number paradox. A relaxation study of mononucleosomes on DNA minicircles. *J. Mol. Biol.* 285:1101-19.

296. Krylov, D., Leuba, S., van Holde, K. and Zlatanova, J. (1993) Histones H1 and H5 interact preferentially with crossovers of double-helical DNA. *Proc. Natl. Acad. Sci. USA*. 90:5052-6.

297. Miller, N. and Whelan, J. (1997) Progress in transcriptionally targeted and regulatable vectors for genetic therapy. *Hum. Gene Ther.* 8:803-15.

298. Mummaneni, P., Walker, K.A., Bishop, P.L. and Turker, M.S. (1995) Epigenetic gene inactivation induced by a cis-acting methylation center. *J. Biol. Chem.* 270:788-92.

299. Bryans, M., Kass, S., Seivwright, C. and Adams, R.L. (1992) Vector methylation inhibits transcription from the SV40 early promoter. *FEBS Lett.* 309:97-102.

300. Thompson, J.P., Simkevich, C.P., Holness, M.A., Kang, A.H. and Raghow, R. (1991) In vitro methylation of the promoter and enhancer of Pro alpha 1(I) collagen gene leads to its transcriptional inactivation. *J. Biol. Chem.* 266:2549-56.

301. Ward, G.A., Stover, C.K., Moss, B. and Fuerst, T.R. (1995) Stringent chemical and thermal regulation of recombinant gene expression by vaccinia virus vectors in mammalian cells. *Proc. Natl. Acad. Sci. USA*. 92:6773-7.

302. Gao, X. and Huang, L. (1993) Cytoplasmic expression of a reporter gene by co-delivery of T7 RNA polymerase and T7 promoter sequence with cationic liposomes. *Nucleic Acids Res.* 21:2867-72.

303. Deng, H. and Wolff, J.A. (1994) Self-amplifying expression from the T7 promoter in 3T3 mouse fibroblasts. *Gene*. 143:245-9.

304. Boyd, A.C., Popp, F., Michaelis, U., Davidson, H., Davidson-Smith, H., Doherty, A., McLachlan, G., Porteous, D.J. and Seeber, S. (1999) Insertion of natural intron 6α-6β into a human cDNA-derived gene therapy vector for cystic fibrosis improves plasmid stability and permits facile RNA/DNA discrimination. *J. Gene Med.* 1:312-21.

305. Yang, E. and Korsmeyer, S.J. (1996) Molecular thanatopsis: a discourse on the BCL2 family and cell death. *Blood*. 88:386-401.

306. Reed, J.C. (1997) Double identity for proteins of the Bcl-2 family. *Nature*. 387:773-6.

307. Spiller, D.G., Biles, R.V., Broughton, C.M., Grzybowski, J., Ruddell, C.J., Tidd, D.M. and Clark, R.E. (1998) The influence of target protein half-life on the effectiveness of antisense oligonucleotide analog-mediated biologic response. *Antisense Nucleic Acid Drug Dev.* 8:281-93.

308. Mulligan, R.C. (1993) The basic science of gene therapy. *Science*. 260:926-31.

309. Gershon, H., Ghirlando, R., Guttman, S.B. and Minsky, A. (1993) Mode of formation and structural features of DNA-cationic liposome complexes used for transfection. *Biochemistry*. 32:7143-51.

310. Marx, K.A. and Ruben, G.C. (1983) Evidence for hydrated spermidine-calf thymus DNA toruses organized by circumerential DNA wrapping. *Nucleic Acids Res.* 11:1839-54.

311. Sambrook, J., Fritsch, E.F. and Maniatis, T., ed. (1989) Molecular Cloning: A Laboratory Manual. Vol. 2. Cold Spring Harbor Laboratory Press, Plainsview.

312. Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911-37.

313. Wong, F.M.P., Reimer, D.L. and Bally, M.B. (1996) Cationic lipid binding to DNA: characterization of complex formation. *Biochemistry.* 35:5776-63.

314. Wong, F.M.P., Macadam, S.A., Kim, A. and Oja, C. (2000) A lipid-based delivery system for antisense oligonucleotides derived from a hydrophobic complex. *Biochim. Biophys. Acta.* (submitted).

315. Sokolove, P.M., Brenza, J.M. and Shamoo, A.E. (1983) Ca^{2+}-cardiolopin interaction in a model system selectivity and apparent high affinity. *Biochim. Biophys. Acta.* 732:41-7.

316. Brenza, J.M., Neagle, C.E. and Sokolove, P.M. (1985) Interaction of Ca^{2+} with cardiolipin-containing liposomes and its inhibition by adriamycin. *Biochem. Pharmacol.* 34:4291-8.

317. Boggs, J.M. (1987) Lipid intermoleculer hydrogen bonding: influence on structural organization and membrane function. *Biochim. Biophys. Acta.* 906:353-404.

318. Zhang, Y., Reimer, D.L., Zhang, G., Lee, P.H. and Bally, M.B. (1997) Self-assembling DNA-lipid particles for gene transfer. *Pharm. Res.* 14:190-6.

319. Bally, M.B., Zhang, Y., Wong, F.M.P., Wasan, E.K. and Reimer, D.L. (1997) Lipid/DNA complexes as an intermediate in the preparation of particles for gene transfer: an alternative to cationic liposome/DNA aggregates. *Adv. Drug Del. Rev.* 24:275-90.

320. Crissman, H.A. and Hirons, G.T. (1994) Staining of DNA in live and fixed cells. *Methods Cell Biol.* 41:195-209.

321. Seed, B. and Sheen, J. (1988) A simple phase-extraction assay for chloramphenicol acetyltransferase activity. *Gene.* 67:271-7.

322. McElhaney, R.N. (1982) The use of differential scanning calorimetry and differential thermal analysis in studies of model and biological membranes. *Chem. Phys. Lipids.* 30:229-59.

323. Matubayasi, N., Shigematsu, T., Iehara, T., Kamaya, H. and Ueda, I. (1986) Miscibility of phosphatidylcholine binary mixtures in unilamellar vesicles: phase equilibria. *J. Membr. Biol.* 90:37-42.

324. Lewis, R.N.A.H., Mak, N. and McElhaney, R.N. (1987) A differential scanning calorimetric study of the thermatropic phase behavior of model membranes composed of phosphatidylcholines containing linear saturated fatty acyl chains. *Biochemistry.* 26:6118-26.

325. Zhang, Y., Lewis, R., Hodges, R. and McElhaney, R. (1995) Interaction of a peptide model of a hydrophobic transmembrane alpha-helical segment of a membrane protein with phosphatidylethanolamine bilayers: differential scanning calorimetric and Fourier transform infrared spectroscopic studies. *Biophys. J.* 63:847-57.

326. Hara, T., Liu, F., Liu, D. and Huang, L. (1997) Emulsion formulations as a vector for gene delivery *in vitro* and *in vivo*. *Adv. Drug Del. Rev.* 24:165-271.

327. Campbell, M.J. (1995) Lipofection reagents prepared by a simple ethanol injection technique. *Biotechniques.* 18:1027-32.

328. Slater, S.J., Ho, C., Taddeo, F.J., Kelly, M.B. and Stubbs, C.D. (1993) Contribution of hydrogen bonding to lipid-lipid interactions in membranes and the role of lipid order: effects of cholesterol, increased phospholipid unsaturation, and ethanol. *Biochemistry.* 32:3714-21.

329. Wimley, W. and Thompson, T. (1991) Phosphatidylethanolamine enhances the concentration-dependent exchange of phospholipids between bilayers. *Biochemistry.* 30:4200-4.

330. Smith, J.G., Walzem, R.L. and German, J.B. (1993) Liposomes as agents of DNA transfer. *Biochim. Biophys. Acta.* 1154:327-40.

331. Gao, X. and Huang, L. (1995) Cationic liposome-mediated gene transfer. *Gene Ther.* 2:710-22.

332. Felgner, P.L. and Ringold, G.M. (1989) Cationic liposome-mediated transfection. *Nature.* 337:387-388.

333. Duzgunes, N., Golstein, J.A., Friend, D.S. and Felgner, P.L. (1989) Fusion of liposomes containing a novel cationic lipid, N-[2,3-(Dioleyloxy)propyl]-N,N,N-trimethylammonium: Induction by multivalent anionic and asymmetric fusion with acidic phospholipid vesicles. *Biochemistry.* 28:9179-84.

334. Hope, M.J., Bally, M.B., Webb, M. and Cullis, P.R. (1985) Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. *Biochim. Biophys. Acta.* 812:55-65.

335. Seaman, G.V.F. (1975) Electrokinetic behavior of red cells. *In* The Red Blood Cell. D.M. Surgenor, ed. Academic Press, New York: 1135-229.

336. Liu, Y., Mounkes, L.C., Liggitt, H.D. and Debs, R.J. (1997) Factors influencing the efficacy of cationic liposome-mediated intravenous gene delivery. *Nat. Biotechnol.* 15:167-73.

337. Felgner, P.L. (1990) Particulate systems and polymers for *in vitro* and *in vivo* delivery of polynucleotides. *Adv. Drug Del. Rev.* 5:163-87.

338. Peitzsch, R.M., Eisenberg, M., Sharp, K.A. and McLaughlin, S. (1995) Calculations of the electrostatic potential adjacent to model phospholipid bilayers. *Biophys. J.* 68:729-38.

339. Sternberg, B. (1997) Morphology of cationic liposome/DNA complexes in relation to their chemical composition. *J. Liposome Res.* 6:515-33.

340. Bull, H.T. (1964) An Introduction to Physical Biochemistry. F.A. Davis Co., Philadelphia:

341. Marra, J. and Israelachvili, J.N. (1985) Direct measurements of forces between phosphatidylcholine and phosphatidylethanolamine bilayers in aqueous electrolyte solutions. *Biochemistry.* 24:4608-18.

342. Janzen, J., Song, X. and Brooks, D.E. (1996) Interfacial thickness of liposomes containing poly(ethylene glycol)-cholesterol from electrophoresis. *Biophys. J.* 70:313-20.

343. Holland, J.W., Hui, C., Cullis, P.R. and Madden, T.D. (1996) Poly(ethylene glycol)-lipid conjugates regulate the calcium-induced fusion of liposomes composed of phosphatidylethanolamine and phosphatidylserine. *Biochemistry.* 35:2618-24.

344. Cotter, F.E. (1997) Antisense therapy for lymphomas. *Hematol. Oncol.* 15:3-11.

345. Wintersberger, U. (1990) Ribonucleases H of retroviral and cellular origin. *Pharmacol. Ther.* 48:259-80.

346. Ballas, Z.K., Rasmussen, W.L. and Krieg, A.M. (1996) Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J. Immunol.* 157:1840-5.

347. Krieg, A.M., Yi , A.K., Matson, S., Waldschmidt, T.J., Bishop, G.A., Teasdale, R., Koretzky, G.A. and Klinman, D.M. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature.* 374:546-9.

348. Jansen, B., Schlagbauer-Wadl, H., Brown, B.D., Bryan, R.N., van Elsas, A., Muller, M., Wolff, K., Eichler, H. and Pehamberger, H. (1998) bcl-2 antisense therapy chemosensitizes human melanoma in SCID mice. *Nat. Med.* 4:232-4.

349. Monia, B.P., Johnston, J.F., Geiger, T., Muller, M. and Fabbro, D. (1996) Antitumor activity of a phosphorothioate antisense oligodeoxynucleotide. *Nat. Med.* 2:668-75.

350. Hijiya, N., Zhang, J., Ratajczak, M.Z., Kant, J.A., DeRiel, K., Herlyn, M., Zon, G. and Gewirtz, A.M. (1994) Biologic and therapeutic significance of MYB expression in human melanoma. *Proc. Natl. Acad. Sci. USA.* 91:4499-503.

351. Skorski, T., Nieborowska-Skorska, M., Nicolaides, N.C., Szczylik, C., Iversen, P., Iozzo, R.V., Zon, G. and Calabretta, B. (1994) Suppression of Philadelphia-1 leukemia cell growth in mice by BCR-ABL antisense oligonucleotide. *Proc. Natl. Acad. Sci. USA.* 91:4504-8.

352. Cotter, F.E., Johnson, P., Hall, P., Pocock, C., al Mahdi, N., Cowell, J.K. and Morgan, G. (1994) Antisense oligonucleotides suppress B-cell lymphoma growth in a SCID-hu mouse model. *Oncogene.* 9:3049-55.

353. Agrawal, S., Temsamani, J. and Tang, J.Y. (1991) Pharmacokinetics, biodistribution and stability of oligodeoxynucleotide phosphorothioates in mice. *Proc. Natl. Acad. Sci. USA.* 88:7595-9.

354. Raynaud, F.I., Orr, R.M., Goddard, P.M., Lacey, H.A., Lancashire, H., Judson, I.R., Beck, T., Bryan, B. and Cotter, F. (1997) Pharmacokinetics of G3139, a phosphorothioate oligodeoxynucleotide antisense to bcl-2, after intravenous administration or continuous subcutaneous infusion to mice. *J. Pharmacol. Exp. Ther.* 281:420-7.

355. Webb, A., Cunningham, D., Cotter, F., Clarke, P.A., di Stefano, F., Ross, P., Corbo, M. and Dziewanowska, Z. (1997) BCL-2 antisense therapy in patients with non-Hodgkin lymphoma. *Lancet.* 349:1137-41.

356. Reed, J.C., Miyashita, T., Takayama, S., Wang, H.G. and Sato, T. (1996) BCL-2 family proteins: regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. *J. Cell. Biochem.* 60:23-32.

357. Reed, J.C. (1997) Bcl-2 family proteins: regulators of apoptosis and chemoresistance in hematologic malignancies. *Semin. Hematol.* 34:9-19.

358. Reed, J.C. (1995) Bcl-2: prevention of apoptosis as a mechanism of drug resistance. *Hematol. Oncol. Clin. North Am.* 9:451-73.

359. Miyashita, T. and Reed, J.C. (1992) bcl-2 gene transfer increases relative resistance of S49.1 and WEHI7.2 lymphoid cells to cell death and DNA fragmentation induced by glucocorticoids and multiple chemotherapeutic drugs. *Cancer Res.* 52:5407-11.

360. Miyashita, T. and Reed, J.C. (1993) Bcl-2 oncoprotein blocks chemotherapy-induced apoptosis in a human leukemia cell line. *Blood.* 81:151-7.

361. Kamesaki, S., Kamesaki, H., Jorgensen, T.J., Tanizawa, A., Pommier, Y. and Cossman, J. (1993) bcl-2 protein inhibits etoposide-induced apoptosis through its effects on events subsequent to topoisomerase II-induced DNA strand breaks and their repair. *Cancer Res.* 53:4251-6.

362. Walton, M.I., Whysong, D., O'Connor, P.M., Hockenbery, D., Korsmeyer, S.J. and Kohn, K.W. (1993) Constitutive expression of human Bcl-2 modulates nitrogen mustard and camptothecin induced apoptosis. *Cancer Res.* 53:1853-61.

363. Reed, J.C., Kitada, S., Takayama, S. and Miyashita, T. (1994) Regulation of chemoresistance by the bcl-2 oncoprotein in non-Hodgkin's and lymphocytic leukemia cell lines. *Ann. Oncol.* 5 Suppl 1:61-5.

364. Keith, F.J., Bradbury, D.A., Zhu, Y.M. and Russell, N.H. (1995) Inhibition of bcl-2 with antisense oligonucleotides induces apoptosis and increases the sensitivity of AML blasts to Ara-C. *Leukemia.* 9:131-8.

365. Kluin-Nelemans, H.C., Limpens, J., Meerabux, J., Beverstock, G., Jansen, J.H., de Jong, D. and Kluin, P.M. (1991) A new non-Hodgkin's B-cell line (DoHH2) with a chromosomal translocation t(14;18)(q32;q21). *Leukemia.* 5:221-4.

366. Dyer, M.J., Lillington, D.M., Bastard, C., Tilly, H. and Lens, D. (1996) Concurrent activation of MYC and BCL2 in B cell non-Hodgkin lymphoma cell lines by translocation of both oncogenes to the same immunoglobulin heavy chain locus. *Leukemia.* 10:1198-208.

367. Kronenwett, R., Steidl, U., Kirsch, M., Sczakiel, G. and Haas, R. (1998) Oligodeoxyribonucleotide uptake in primary human hematopoietic cells is enhanced by cationic lipids and depends on the hematopoietic cell subset. *Blood.* 91:852-62.

368. Gascoyne, R.D., Adomat, S.A., Krajewski, S., Krajewska, M., Horsman, D.E., Tolcher, A.W., O'Reilly, S.E., Hoskins, P., Coldman, A.J., Reed, J.C. and Connors, J.M. (1997) Prognostic significance of Bcl-2 protein expression and Bcl-2 gene rearrangement in diffuse aggressive non-Hodgkin's lymphoma. *Blood.* 90:244-51.

369. Horsman, D.E., Gascoyne, R.D., Coupland, R.W., Coldman, A.J. and Adomat, S.A. (1995) Comparison of cytogenetic analysis, southern analysis, and polymerase chain reaction for the detection of t(14; 18) in follicular lymphoma. *Am. J. Clin. Pathol.* 103:472-8.

370. de Kroon, J.F., Kluin, P.M., Kluin-Nelemans, H.C., Willemze, R. and Falkenburg, J.H. (1994) Homing and antigenic characterization of a human non-Hodgkin's B lymphoma in severe combined immunodeficient (SCID) mice. *Leukemia.* 8:1385-91.

371. Macpherson, N., Lesack, D., Klasa, R., Horsman, D., Connors, J.M., Barnett, M. and Gascoyne, R. (1999) Small noncleaved, non-Burkitt's (Burkit-Like) lymphoma:

cytogenetics predict outcome and reflect clinical presentation. *J. Clin. Oncol.* 17:1558-67.

372. Haner, R. and Hall, J. (1997) The sequence-specific cleavage of RNA by artificial chemical ribonucleases. *Antisense Nucleic Acid Drug Dev.* 7:423-30.

373. Boutorin, A.S., Gus'kova, L.V., Ivanova, E.M., Kobetz, N.D., Zarytova, V.F., Ryte, A.S., Yurchenko, L.V. and Vlassov, V.V. (1989) Synthesis of alkylating oligonucleotide derivatives containing cholesterol or phenazinium residues at their 3'-terminus and their interaction with DNA within mammalian cells. *FEBS Lett.* 254:129-32.

374. Leonetti, J.P., Degols, G. and Lebleu, B. (1990) Biological activity of oligonucleotide-poly(L-lysine) conjugates: mechanism of cell uptake. *Bioconjug. Chem.* 1:149-53.

375. Citro, G., Perrotti, D., Cucco, C., D'Agnano, I., Sacchi, A., Zupi, G. and Calabretta, B. (1992) Inhibition of leukemia cell proliferation by receptor-mediated uptake of c-myb antisense oligodeoxynucleotides. *Proc. Natl. Acad. Sci. USA.* 89:7031-5.

376. Bennett, C.F., Chiang, M., Chan, H., Shoemaker, J.E. and Mirabelli, C.K. (1992) Cationic lipids enhance cellular uptake and activity of phsphorothioate antisense oligonucleotides. *Mol. Pharmacol.* 41:1023-33.

377. Zelphati, O. and Szoka Jr., F.C. (1996) Intracellular disrtibution and mechanism of delivery of oligonucleotides mediated by cationic lipids. *Pharm. Res.* 13:1367-1372.

378. Biffi, A., Sersale, G., Cassetti, A., Villa, A., Bordignon, C., Assael, B.M. and Conese, M. (1999) Restoration of bacterial killing activity of human respiratory cystic fibrosis cells through cationic vector-mediated cystic fibrosis transmembrane conductance regulator gene transfer. *Hum. Gene Ther.* 10:1923-30.

379. Waddill, W., Wright, W.J., Unger, E., Stopeck, A., Akporiaye, E., Harris, D., Grogan, T., Schluter, S., Hersh, E. and Stahl, S. (1997) Human gene therapy for melanoma: CT-guided interstitial injection. *Am. J. Roetgenol.* 169:63-7.

380. Marcusson, E.G., Bhat, B., Manoharan, M., Bennett, C.F. and Dean, N.M. (1998) Phosphorothioate oligodeoxyribonucleotides dissociate from cationic lipids before entering the nucleus. *Nucleic Acids Res.* 26:2016-23.

381. Flanagan, W.M., Wolf, J.J., Olson, P., Grant, D., Lin, K.Y., Wagner, R.W. and Matteucci, M.D. (1999) A cytosine analog that confers enhanced potency to antisense oligonucleotides. *Proc. Natl. Acad. Sci. USA.* 96:3513-8.

382. Wang, S., Lee, R.J., Cauchon, G., Gorenstein, D.G. and Low, P.S. (1995) Delivery of antisense oligodeoxyribonucleotides against the human epidermal growth factor receptor into cultured KB cells with liposomes conjugated to folate via polyethylene glycol. *Proc. Natl. Acad. Sci. USA.* 92:3318-22.

383. Ma, D.D.F. and Wei, A. (1996) Enhanced delivery of synthetic oligonucleotides to human leukaemic cells by liposomes and immunoliposomes. *Leukemia Res.* 20:925-30.

384. Klasa, R.J., Bally, M.B., Ng, R., Goldie, J.H., Gascoyne, R.D. and Wong, F.M.P. (2000) Eradication of human non-Hodgkin's lymphoma in SCID mice by bcl-2 antisense oligonucleotides combined with low dose cyclophosphamide. *Clin. Cancer Res.* 6:2492-500.

385. Ogris, M., Brunner, S., Schuller, S., Kircheis, R. and Wagner, E. (1999) PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther.* 6:595-605.

386. Semple, S.C., Klimuk, S.K., Harasym, T.O. and Hope, M.J. (2000) Lipid-based formulations of antisense oligonucleotides for systemic delivery applications. *Methods Enzymol.* 313:322-41.

387. Bradley, A.J., Devine, D.V., Ansell, S.M., Janzen, J. and Brooks, D.E. (1998) Inhibition of liposome-induced complement activation by incorporated poly(ethylene glycol)-lipids. *Arch. Biochem. Biophys.* 357:185-94.

388. Harvie, P., Wong, F.M.P. and Bally, M.B. (1999) Use of poly(ethylene glycol)-lipid conjugates to regulate the surface attributes and transfection activity of lipid-DNA particles. *J. Pharm. Sci. 89*:652663.

389. Storm, G., Steerenberg, P.A., Emmen, F., Waalkes, M.v. and Crommelin, D.J.A. (1988) Release of doxorubicin from peritoneal macrophages exposed *in vivo* to doxorubicin-containing liposomes. *Biochim. Biophys. Acta.* 965:136-145.

390. Rex, S., Zuckermann, M.J., Lafleur, M. and Silvius, J.R. (1998) Experimental and Monte Carlo simulation studies of the thermodynamics of polyethyleneglycol chains grafted to lipid bilayers. *Biophys. J.* 75:2900-14.

391. Richardson, V.J., Ryman, B.E., Jewkes, R.F., Jeyasingh, K., Tattersall, M.N.H., Newlands, E.S. and Kaye, S.B. (1979) Tissue distribution and tumour localization of 99m-technetium-labelled liposomes in cancer patients. *Br. J. Cancer.* 40:35-43.