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Galactosylated Liposomes with Proton Sponge Capacity: A Novel Hepatocyte-Specific Gene Transfer System

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Galactosylated Liposomes with Proton Sponge Capacity: A Novel Hepatocyte-Specific Gene Transfer System

by

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As the candidate's supervisor I have approved this dissertation for submission.

Supervisor: Dr M. Singh signed: _____ date:

Co-supervisor: Prof M. Ariatti signed: _____ date:

ABSTRACT

Hepatocyte-directed liposomal gene delivery systems have received much attention in view of the present lack of suitable treatment alternatives for several liver-associated disorders. While targeting of liposomes to the asialoglycoprotein receptor (ASGP-R), nearly-exclusive to hepatocytes, is a well-documented means of achieving cell-specificity, several intra- and extracellular barriers reduce the efficacy of liposomal gene transfer. These include the aggregation and opsonisation of lipoplexes by serum components; and endo/lysosomal degradation of internalised DNA. This study has attempted to address the individual concerns by modifying hepatotropic liposomes with a steric stabilising, polyethylene glycol (PEG) shroud, and an endosomal escape-inducing proton sponge moiety.

Novel galactosylated (SH02) and imidazolylated (SH04) cholesterol derivatives were successfully synthesised with the aim of conferring the respective functions of ASGP-R-specificity and proton sponge capability upon cationic liposome formulations. The individual derivatives afforded stable, unilamellar vesicles (< 200 nm, Z-average diameter) when incorporated at 10 % on a molar basis with the cytofectin, 3β[*N*-(*N'*,*N'*-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) and co-lipid, dioleoylphosphatidylethanolamine (DOPE). Modification of these formulations with 1,2-distearoyl-*sn*-glycero-phosphoethanolamine-*N*-[carboxy(polyethylene glycol)2000] (DSPE-PEG_{2000}), at 5 mol %, gave smaller vesicles (< 110 nm, Z-average diameter) and moderately reduced the instability associated with the combination of both SH02 and SH04 in a single formulation.

Individual preparations formed electrostatic complexes with pCMV-*luc* plasmid DNA, as demonstrated by gel retardation assays and electron microscopy. Furthermore, the liposomes afforded some protection to the DNA cargo against serum nuclease attack during a 4 hour-long exposure to foetal calf serum at 37 ˚C. However, the DNA-binding and protecting capabilities of the liposomes were reduced upon addition of the PEG coating.

Growth inhibition assays showed that lipoplexes derived from individual formulations were well tolerated by human hepatocyte-derived, HepG2, and embryonic kidney, HEK293, cell lines. Expression of the luciferase transgene mediated by non-pegylated formulations

containing SH02 was significantly higher in hepatocytes than in the ASGP-R-negative, kidney cells. Furthermore, receptor-mediated internalisation of non-pegylated, galactosylated carriers by hepatocytes was demonstrated by the gross inhibition of transfection in the presence of excess asialofetuin, a natural ligand to the ASGP-R. Liposome acid titration profiles highlighted the endosomal pH-buffering capacity afforded by SH04. However, the imidazolylated lipid enhanced the transfection activity of the non-sterically stabilised Chol-T/DOPE system, but not that of its targeted counterpart, and only with respect to HEK293 cells. Finally, pegylation reduced the transfection capability of liposomes by at least three orders of magnitude in both cell lines. The results suggest that further optimisation of liposome composition is necessary in order to achieve a liposomal system that simultaneously embodies hepatocyte-targeting, proton sponge and long-circulating properties.

PREFACE

The experimental work described in this dissertation was carried out in the Discipline of Biochemistry, School of Life Sciences, University of KwaZulu-Natal, Durban from February 2011 to May 2012, under the supervision of Dr Moganavelli Singh, and co-supervision of Prof Mario Ariatti.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

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DECLARATION – PLAGIARISM

I, Saffiya Habib, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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TABLE OF CONTENTS

PAGE

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF TABLES

LIST OF ABBREVIATIONS

AF	asialofetuin
AOM	asialoorosomucoid
APOBEC	apolipoprotein B mRNA editing enzyme, catalytic peptide-like
ASGP	asialoglycoprotein
ASGP-R	asialoglycoprotein receptor
ASGPs	asialoglycoproteins
BCA	bicinchoninic acid
Bcl-2	B-cell lymphoma 2
BGTC	bis(guanidinium)-tren-cholesterol
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
Cer	ceramide
CHE-LA	(5-Cholestan-3β-yl)-1-[2-(lactobionyl amido) ethylamido] formate
ChIm	cholesterol-(3-imidazol-1-yl-propyl) carbamate
Chol	cholesterol
Cholβgal	cholesteryl-β-D-galactopyranoside
Chol-T	3β[*N*-(*N′*, *N′*-dimethylaminopropane)-carbamoyl] cholesterol
CHS-ED-LA	(5-Cholesten-3β-yl)-4-oxo-4-[2-(lactobionyl amido) ethylamido] butanoate
CRD	carbohydrate recognition domain
CTLA4	Cytotoxic T-lymphocyte Antigen 4

LIST OF ABBREVIATIONS (continued)

DC-Chol	3β[*N*-(*N′*, *N′*-dimethylaminoethane)-carbamoyl] cholesterol
DCCI	*N*, *N′*-dicyclohexylcarbodiimide
DCU	dicyclohexylurea
DLS	dynamic light scattering
DMF	dimethylformamide
DMG	dimyristoylglycerol
DMPC	1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
DMRIE	(±)-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DOPC	dioleoylphosphatidylcholine
DOPE	dioleoylphosphatidylethanolamine
DOTAP	1,2-dioleoyl-3-(trimethylammonium) propane
DOTMA	*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride
DSPE	distearoylphosphatidylethanolamine
DSPE-PEG_{2000}	1,2-distearoyl-*sn*-glycero-phosphoethanolamine-*N*-[carboxy(polyethylene glycol)2000]
EDOPC	*O*-ethyldioleoylphosphocholine
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FRET	fluorescence resonance energy transfer

Gal-C4-Chol	cholesten-5-yloxy-N-(4-((1-imino-c-β-D-thiogalactosyl-ethyl)amino)butyl)formamide
Gal*N*Ac	*N*-acetylgalactosamine
GPr	galactose particle receptor
HBS	HEPES buffered saline
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
hCT (9-32)	human calcitonin C-terminal fragment 9-32
HEK293	human embryonic kidney 293
HEPES	2-[-(2-hydroxyethyl)-piperazinyl]-ethanesulphonic acid
HepG2	hepatoma G2
HPLC	High Performance Liquid Chromatography
HSPC	hydrogenated Soy L-α-phosphatidylcholine
IgA	immunoglobulin A
IgM	immunoglobulin M
IR	infrared
IUPAC	International Union of Pure and Applied Chemistry
KC	Kupffer cells
LDH	lactate dehydrogenase
LDL	low density lipoprotein
LSEC	liver sinusoidal epithelial cells

LIST OF ABBREVIATIONS (continued)

LUV	large unilamellar vesicles
MEM	minimal essential medium
MLV	large multilamellar vesicles
mp	melting point
MS09	*N*,*N*-dimethylaminopropylamidosuccinylcholesterylformylhydrazide
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H- tetrazolium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVL	multivesicular liposomes
NHS	*N*-hydroxysuccinimide
NMR	nuclear magnetic resonance
PAMAM	polyamidoamine
PBS	phosphate-buffered saline
PdI	polydispersity index
PE	phosphatidylethanolamine
PEG	polyethylene glycol
PEI	polyethylenimine
PLL	poly-L-lysine
PPI	polypropylenimine
PVP	polyvinylpyrrolidone

R_f	retention factor
RLU	relative light units
RME	receptor-mediated endocytosis
RNA	ribonucleic acid
rpm	revolutions per minute
SAINT	Synthetic Amphiphiles Interdisciplinary
SAINT-2	*N*-methyl-4-(dioleyl)methylpyridinium
SDS	sodium dodecylsulphate
SH01	cholesterylformylhydrazide
SH02	lactobionylcholesterylformylhydrazide
SH04	urocanylcholesterylformylhydrazide
SH05	*N*-tritylurocanylcholesterylformylhydrazide
SPC	soy phospholipids
Sper-Chol	spermine cholesterol
SUV	small unilamellar vesicles
TEM	transmission electron microscopy
TLC	thin layer chromatography
$TPPS_4$	meso-tetra-(4-sulphonatophenyl)-porphine
Tris-HCl	tris(hydroxymethyl)-aminomethane hydrochloride
UGT1A1	UDP-glucuronosyltransferase 1 family, polypeptide A1

LIST OF ABBREVIATIONS (continued)

UV	ultraviolet
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide

CHAPTER ONE

INTRODUCTION

1.1 Gene therapy: An overview

The central dogma of molecular biology dictates that hereditary information encoded by the DNA of a cell governs all aspects of its structure and function (Tzeng *et al*., 1996). Therefore, aberrations at the genetic level, both inherited and acquired, may result in cellular abnormalities that manifest as disease. In 1963, Joshua Lederberg predicted that the manipulation of nucleotide sequences in human chromosomes and integration of functional genes, would eventually present the opportunity to correct such disorders (Edelstein *et al*., 2004). True to this vision, advances largely in the field of recombinant DNA technology, have established the framework for a revolutionary approach to the treatment of disease – gene therapy (Cotrim and Baum, 2008).

In accordance with the therapeutic requirements of the target disorder, gene therapy may seek either to genetically modify cells with a new or restored function, or temporarily interfere with a cellular function (Sangro *et al*., 2003). While the stable introduction of a functional segment of DNA encoding a corrective phenotype into diseased cells classically defines gene therapy, it is worthy of note that current research has also led to the evaluation of antisense oligonucleotides (Rayburn and Zhang, 2008), RNA molecules (Caplen, 2004) and ribozymes (Mulhbacher *et al.*, 2010) as potential gene medicines. Furthermore, genes may be targeted to both somatic and germ-line cells. However, due to ethical issues associated with alteration of the latter cell type, most gene therapy protocols focus on the manipulation of somatic cells (Sharma *et al*., 2004).

Gene therapy is often described as a "*broad platform*" technology, having shown potential for the treatment of a variety of disorders (Mountain, 2000). In theory, gene-based approaches present an attractive alternative to conventional treatment, largely because gene therapy aims to treat the cause of disease rather than the symptoms (Mountain, 2000). Furthermore, these novel strategies may enhance patients' compliance by delivering treatment specifically to the site of disease, thereby avoiding potentially toxic systemic effects. Other advantageous

features include the ability to effect sustained expression of therapeutic proteins that are ordinarily short-lived in the body; and provide more affordable treatment modalities on a long-term basis (Sharma *et al*., 2004).

Since the first clinical trial performed by Rosenberg and colleagues in 1989, the field of gene therapy has experienced success and failure in almost equal measure (Cotrim and Baum, 2008). However, it is the promise of a permanent cure for disease that remains the primary motivation for the multitude of gene transfer approaches investigated.

1.2 The liver: An attractive target organ for gene therapy

Thus far, the introduction of genes into cells of several major organs, including the bone marrow, brain, lungs and liver has been attempted, albeit with varying success (Greenwell and McCulley, 2007). However, the liver is considered as one of the more attractive targets for the application of gene therapy (Grove and Wu, 1998).

The liver is the largest human internal organ, weighing up to 1.3 kg in an adult. This organ mediates a variety of metabolic functions that include detoxification, lipid and carbohydrate metabolism, protein synthesis and bile secretion (Fox, 1999). As a consequence of its central metabolic role, defects of the liver are often associated with the onset of disease (Ghosh *et al*., 2000). Unfortunately, conventional medicine has failed to provide stable treatment for several diseases of this organ (Brunetti-Pierri and Lee, 2005).

For example, chronic viral hepatitis B and C affects millions globally. If untreated, HBV infection may culminate in cirrhosis and hepatocellular carcinoma (HCC). Therefore, it is a major concern that more than 70 % of patients do not respond to standard treatment using interferon alpha. Furthermore, the liver is a primary location for the incidence of tumour metastases, with HCC recognised as the second most common form of cancer (Prieto *et al*., 2003). While liver transplantation is a treatment option in this instance, approximately 15 % of patients succumb while awaiting a donor organ (Brunetti-Pierri and Lee, 2005); and even in the event of a successful procedure, the need for life-long immunosuppression raises concerns of susceptibility to further illness (Alt and Caselmann, 1995).

Therefore, in recent years, medical research has shown great interest in the prospect of applying gene-based strategies to the treatment of liver diseases. In fact, the objectives of gene transfer to the liver (Table 1.1) have expanded with the growing list of liver-associated ailments that are considered amenable to gene therapy (Table 1.2).

Table 1.1: Goals of liver-directed gene therapy (adapted from Chowdhury, 2010; Ghosh *et al*., 2000).

Purpose of nucleic acid transfer	**Examples**
Replacement of a missing gene product	UGT1A1, LDL receptor, coagulation factors
Inhibition of gene expression	Viral genes, oncogenes, mutant α_1-antitrypsin (AAT-Z)
Overexpression or ectopic expression of a gene product	APOBEC in the liver, Bcl-2, CTLA4
Expression of pharmacological gene product	Insulin, growth hormones, vaccines, prodrugs
Site-directed gene repair	Correction of point mutation or single nucleotide deletions

Table 1.2: Partial list of disorders amenable to treatment by liver-directed gene therapy (adapted from Ghosh *et al*., 2000).

Inherited diseases of liver metabolism	**Acquired hepatic disorders**	**Inherited systemic disorders**
• Crigler-Najjar syndrome type 1 • Familial hypercholesterolemia • Phenylketonuria • Tyrosinemia • Wilson's disease • Oxaluria • α_1- Antitrypsin deficiency • Mucopolysaccharidosis VII	• Hepatitis B and C • Liver tumours • Allograft or xenograft rejection	• Hemophilia A and B • Oxalosis

The most appropriate strategy for the correction of inborn errors of liver metabolism entails replacing the defective gene with its functional counterpart (Ghosh *et al*., 2000). According to Alt and Caselmann (1995) the lack of safe, reliable techniques for site-specific integration of exogenous DNA into the human genome, has resulted in most gene therapy protocols attempting the transfer of therapeutic genes without replacing the aberrant form. Nonetheless, the potential for gene transfer to effect site-directed integration and repair of genes *in vivo* may be realised in the near future. With respect to genetic treatment of liver metastases, several strategies have been explored. These include the transfer of tumour suppressor and cytokine genes, tumour cell vaccination and the selective destruction of malignant cells by prodrug activation induced via the introduction of suicide genes (Xia *et al*., 2004). In other instances, treatment may require the downregulation or inhibition of gene expression. To this end, liver-directed gene therapy has developed strategies based on the use of antisense oligonucleotides, and RNA interference (Ghosh *et al*., 2000).

A further prospect of gene transfer to the liver exploits the physical location of the liver relative to the circulatory pathway and its role as a secretory organ, in order to systemically introduce therapeutic proteins (Xia *et al*., 2004). For example, nucleic acid-carrier constructs may be engineered to permit the overexpression of endogenous proteins, such as insulin and growth hormones that are not ordinarily expressed by the liver, to treat disorders affecting other organs. Similarly, the liver may serve as the production site of therapeutic foreign proteins, termed biodrugs, which include vaccines, single chain antibodies and immunomodulatory substances (Chowdhury, 2010; Ghosh *et al*., 2000).

1.2.1 Hepatocytes as targets for gene delivery

The liver comprises three major cell types: hepatocytes, Kupffer cells (KC) and liver sinusoidal epithelial cells (LSEC). However it is the hepatocytes, liver parenchymal cells, which account for approximately 80 % of the liver by volume (Stolz, 2011) and mediate most of its metabolic activity, that are often the targets of gene therapy directed to the liver (Wu *et al*., 2002).

Hepatocytes are polyhedral in shape with either one or two distinct nuclei, and range between 12 and 25 μm in diameter (Kamimura and Liu, 2008). They contain an abundance of organelles which correlates with their role in more than 5000 identified metabolic pathways (Pathak *et al*., 2008). Hepatocytes are arranged as hepatic plates which are organised as liver lobules (Figure 1.1). The hepatic plates are separated within these functional units by large capillary spaces, the sinusoids. The plate structure of the liver affords individual hepatocytes direct contact with the blood (Fox, 1999), which serves as a transport system for the delivery of gene carriers to the parenchymal cells and, if necessary, the distribution of therapeutic gene products to other tissues (Wu and Wu, 1998).

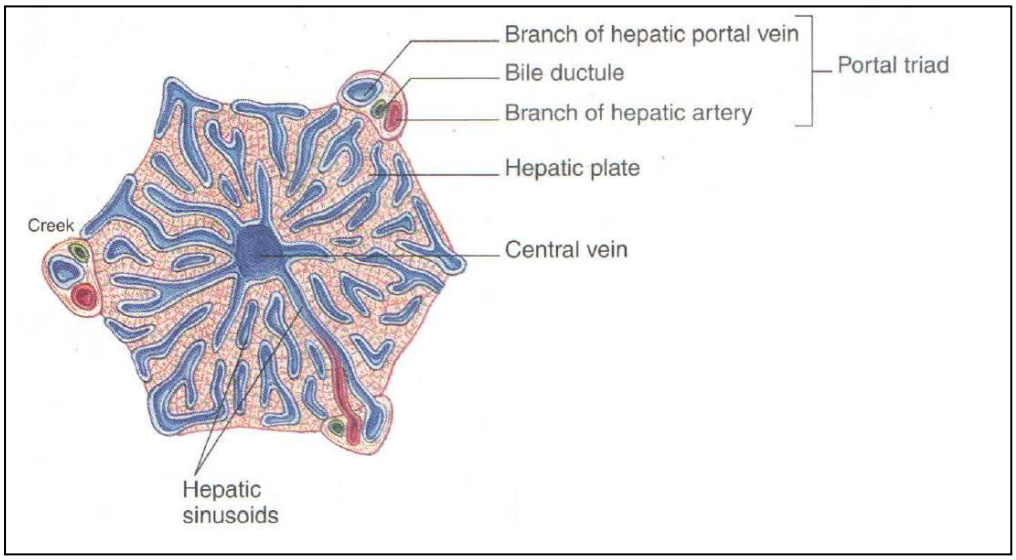


Figure 1.1: Cross section of a liver lobule (Fox, 1999).

Various pathological processes may cause injury to hepatocytes, which then produce inflammatory mediators that may result in the onset of disease (Wu *et al*., 2002). In addition, genetic defects associated with hepatocytes have been implicated in the deficiency of α_1-antitrypsin and lipoprotein receptors, among other disorders (Pathak *et al*., 2008). This major correlation with disease, in conjunction with their active metabolism, slow cell turnover and rich blood supply, renders hepatocytes important targets for the delivery of therapeutic genes (Pathak *et al*., 2008). Conversely, strategies for liver-directed gene therapy seek to avoid LSEC, which form a physical barrier between hepatocytes and the administered nucleic acid constructs, permitting entry only to relatively small particles; and KC which are macrophages

of the liver that have been implicated in the clearance of gene transfer agents from circulation (Brunetti-Pierri and Lee, 2005).

1.3 Vectors for hepatocyte-directed gene transfer

Nucleic acids are not amenable to simple diffusion across biological membranes as these are relatively large, anionic molecules (Ledley, 1996). Therefore the success of a gene-based approach is largely dependent on the development of an effective vehicle, termed a vector, to carry corrective genetic material into cells (Li and Huang, 1999). Such a carrier should be simple to construct, accommodate relatively large amounts of nucleic acids, and amenable to cost-effective, large scale production (Dani, 1999; Pfeifer and Verma, 2001). The vector must guard its cargo against enzymatic degradation, and deliver it in a cell-specific manner, without eliciting any harmful toxic or immunological response (Gao *et al*., 2007). The perfect vector, however, remains elusive. In an attempt to direct therapeutic genes into hepatocytes, a plethora of gene transfer modalities, as summarised in Table 1.3, has been investigated.

Table 1.3: Strategies for gene transfer into hepatocytes (adapted from Hart, 2010).

Mode of gene transfer				Examples	Advantages	Disadvantages
Viral methods				• Retrovirus • Lentivirus • Adenovirus • Adeno-associated virus • Herpes simplex virus	• extensively studied and well-characterised system • high transfection efficiency • potential for sustained gene expression with integrating vectors	• risk of insertional mutagenesis • activation of oncogene(s) • reversion of replication-defective virus to wild-type • immunogenicity
Non-viral methods	**Physical methods**			• Electroporation • Gene gun • Hydrodynamic injection • Ultrasound	• potential to administer nucleic acid directly to target cells • most protocols are relatively simple to perform	• tissue damage • limited gene expression • may require surgical procedure to expose hepatocytes
	Chemical methods	**Polymers**	Natural	• Chitosan	• biocompatibility • biodegradability • low toxicity	• poor transfection efficiency
			Synthetic	• Polyethylenimine • Poly-L-lysine	• chemical flexibility • can be conjugated to functional, hepatocyte-specific ligands • amenable to systemic administration	• immunogenicity • poor transfection efficiency
		Lipid-based methods	Liposomes	• Conventional liposomes • Cationic liposomes • Stealth liposomes • Targeted liposomes	• simple preparation methods • wide variety of formulations • relatively inexpensive reagents • amenable to chemical modification • amenable to systemic administration • easily targeted to hepatocytes	• transient gene expression • low transfection efficiency (compared to viral vectors) • may be associated with toxicity and • immunogenicity

1.3.1 Viral Methods

Viruses are natural gene transfer agents as their replication cycle is dependent on the successful transfer of viral genetic material into the nucleus of the host cell (Dani, 1999). The earliest attempts at gene therapy entailed the construction of replication-defective viral vectors, by replacing regions of the viral genome which are responsible for pathogenicity and the synthesis of progeny virions with therapeutic genes, while retaining the ability of the viral particle to penetrate the cell and transfer nucleic acids (Pfeifer and Verma, 2001; Rubyani, 2001). Attenuated viral systems applied to hepatocyte-directed gene transfer, either *in vitro* or *in vivo*, include those based on the retrovirus, lentivirus (Dariel *et al*., 2009), adenovirus (Suzuki *et al*., 2003), adeno-associated virus, baculovirus (Hofmann *et al*., 1995) and herpes simplex virus (Xia *et al*., 2004). Unfortunately, the potential for insertional mutagenesis, immunogenicity and reversion to an active viral particle following recombination with a wild-type virus, among other concerns, has tainted the early success achieved using such carriers (Alt and Caselmann, 1995).

Recently, however, Kozlowski and coworkers (2007) demonstrated that the transduction of hepatocytes by an adeno-associated viral construct, bearing a functional insulin transgene under the control of a liver specific promoter, restored blood-sugar levels to near-normality in diabetic mice. In addition, researchers have explored the possibility of preparing hybrid viral particles, which are chimaeras of two viruses, as a means of enhancing gene transfer to hepatocytes (Müller *et al*., 2005). Nonetheless, current work has emphasised the difficulty of maintaining long-term expression of the transgene while avoiding the cellular immune response to several virus-based carriers (Puppi *et al*., 2004; Seiler *et al*., 2007).

1.3.2 Non-viral methods

Although viral vectors remain the most widely employed gene transfer system in clinical trials to date (Herzoq *et al*., 2010), the associated safety concerns have encouraged research into alternate strategies. However, the design of a non-viral method with transfection potential to rival that of the viral vectors is a major challenge (Nishikawa and Huang, 2001).

1.3.2.1 Physical methods

Apart from being associated with vectors, DNA may be introduced into mammalian cells via the application of physical force (Fouillard, 1996). All physical methods encourage gene transfer by bringing DNA within close proximity of the plasma membrane and/or facilitating the passage of DNA into cells via transient microdisruption of the membrane (Wells, 2004). Physical methods such as electroporation (Matsuno *et al*., 2003), hydrodynamic injection (Liu *et al*., 1999) and ultrasound (Guo *et al.*, 2006) have been employed to introduce genes into hepatocytes. However, such gene transfer modalities are limited by the accessibility of target tissue, the requirement of surgery to expose liver parenchymal cells, tissue damage due to the physical force applied and their overall lack of feasibility for use in humans (Kamimura and Liu, 2008).

1.3.2.2 Chemical methods

Chemical methods of gene delivery, by broad definition, entail the construction of complexes with therapeutic nucleic acids (Dani, 1999). While such strategies encompass a variety of carriers, polymer and lipid-based systems, modified with hepatocyte-specific homing devices (refer to section 1.4), have been widely investigated for application to liver-directed gene therapy (Pathak *et al*., 2008).

Several natural and synthetic polymers have been explored as potential gene carriers (Dang and Leong, 2006; Kundu and Sharma, 2008). The most promising thereof, as illustrated in Figure 1.2, bear functional groups that are protonated at physiological pH. Therefore such polymers possess the cationic property required to electrostatically bind and condense nucleic acids into microspheric particles, known as polyplexes (Dang and Leong, 2006).

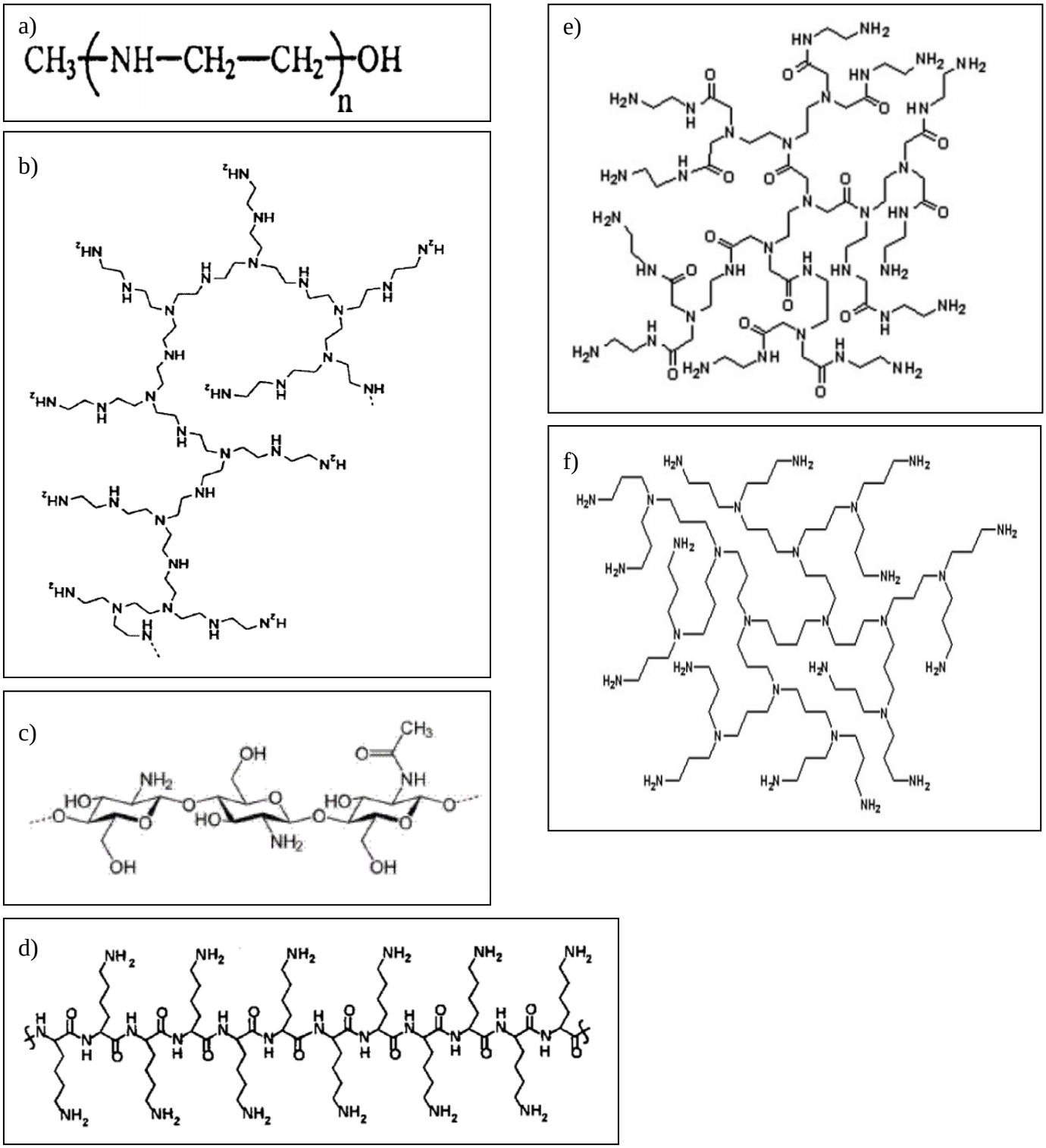


Figure 1.2: Cationic polymeric nucleic acid carriers, a) linear polyethylenimine (PEI); b) branched PEI (Lungwitz *et al*., 2005); c) chitosan (Martinez-Huitle *et al*., 2009); d) poly-L-lysine (PLL) (Tang and Szoka, 1997); e) polyamidoamine (PAMAM) dendrimer; and f) polypropylenimine (PPI) dendrimer (Pathak *et al*., 2009).

While the primary amino functions of polymers such as PEI, PLL and dendrimers facilitate effective binding of nucleic acids, these groups are believed to contribute, by and large, to the cytotoxicity associated with these carriers (Pathak *et al*., 2009). In addition, recent studies have presented evidence that short-term gene expression afforded by PEI and PLL, arguably the most widely documented cationic, polymeric carriers, may be attributed to their induction of apoptosis in several human cell lines (Hunter, 2006). Of particular interest to the present discussion are reports which demonstrate that PEI and PLL have induced toxic effects in hepatocytes (Bandyopadhyay *et al*., 1998). It is therefore evident that the performance of such carriers is yet to be optimised (Lutz, 2006). Consequently, chemical manipulation has been explored as a means of attenuating undesired effects (Pathak *et al*., 2009).

In spite of difficulties associated with many chemical methods, lipid-based vehicles, the liposomes, have maintained interest in this area (Lasic and Templeton, 1996). In fact, liposomes have been given greater recognition than any other non-viral gene transfer system in clinical trials (Tu *et al*., 2010). Therefore the application of this vector to the field of hepatocyte-specific gene transfer merits further discussion.

1.3.2.2.1 Liposomes

Liposomes are defined by Schuber and colleagues (1998) as "*spherical structures consisting of single or multiple concentric bilayers resulting from the self-assembly of amphiphilic molecules, such as phospholipids, in an aqueous medium*," (Figure 1.3). The scientific community was first introduced to liposomes by Bangham and coworkers (1965), who observed that lipids extracted from egg yolk naturally organised into micro-spheres upon introduction to water. The membranes encompassing these spheres closely resembled biological membranes; therefore, liposomes were initially studied as model membrane systems (Schuber *et al*., 1998).

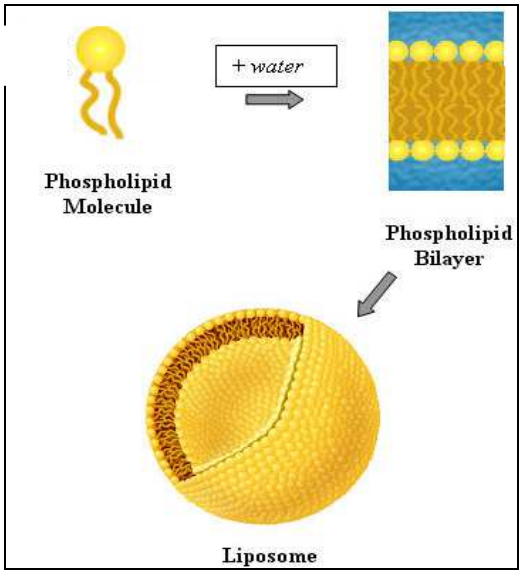


Figure 1.3: Formation of conventional liposomes from the spontaneous arrangement of phospholipids (http://www.nanolifenutra.com/liposome_technology.html).

Studies initially conducted by Hoffman and colleagues (1978), highlighted the nucleic acid carrying potential of liposomes. This group encapsulated DNA of high molecular weight within liposomes consisting of egg lecithin, which rendered the DNA resistant to the degradative action of DNase. Such liposomes, prepared from neutral and anionic lipids that are either naturally occurring or synthetic, are termed conventional liposomes (Lasic, 1997). However early attempts at hepatocyte-directed gene transfer using conventional liposomes demonstrated poor transfection efficiency, mainly due to massive accumulation of the administered liposomes in the lung. This phenomenon and the technical difficulties associated with DNA encapsulation limited the use of conventional liposomes in gene transfer applications (Ledley, 1996; Li and Huang, 1999). However, it was the advent of the cationic liposome, in conjunction with targeting strategies, which potentiated more feasible liposome-mediated gene transfer systems.

1.3.2.2.1.1 Cationic liposomes

Cationic liposomes, as the name suggests, possess a net positive charge on the outer surface of the bilayer, due to the incorporation of cationic lipids, termed cytofectins. Nucleic acids may therefore be electrostatically bound to the surface of such a liposome, following

incubation of the nucleic acids with liposomes. As such condensed nanostructures, known as lipoplexes, are formed (Lasic, 1997). Often, lipoplexes are constructed to bear a net positive charge as this encourages cellular uptake by way of their affinity for anionic biological surfaces (Felgner *et al*., 1994). While several mechanisms for the cellular entry of lipoplexes have been proposed (Figure 1.4), according to Zhdanov and colleagues (2002), the lipoplexes predominantly enter via endocytosis or direct membrane fusion, following adherence to the plasma membrane.

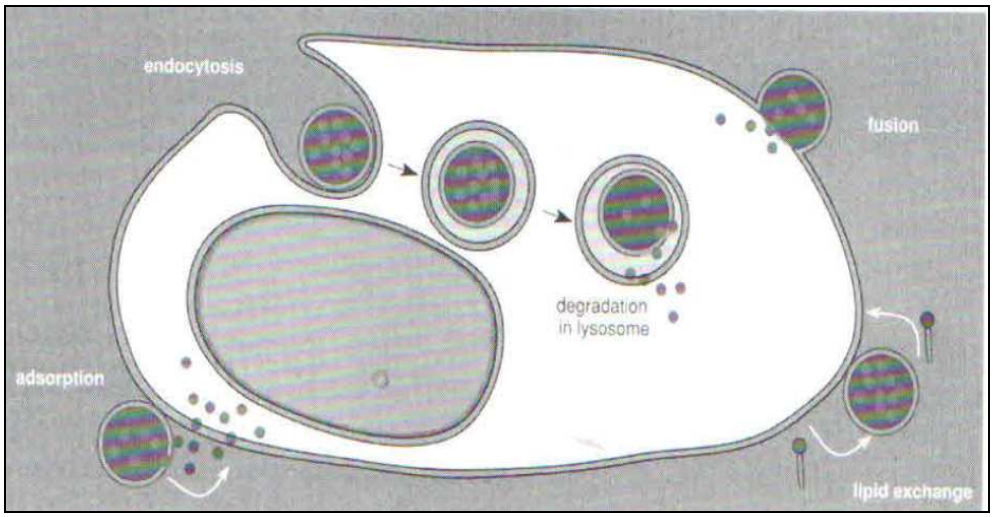


Figure 1.4: Possible liposome-cell interactions (Lasic, 1997).

Positively charged lipids, except for sphingosine and a few lipids in primitive organisms, do not exist in nature. Therefore, the positive charge was initially conferred upon liposomes by incorporating cationic detergents into the bilayer. However, the toxicity of such formulations severely limited their use (Lasic, 1997). In 1987, as a result of studies by Felgner and coworkers, the concept of the cationic lipid as an agent of transfection was made practical. This group successfully demonstrated the transfection of cultured cells using a liposome formulation prepared from equimolar quantities of the synthetic cationic lipid DOTMA (*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride) and neutral lipid DOPE (L-α-dioleoylphosphatidylethanolamine) (Felgner *et al*., 1987). The lack of tedious nucleic acid-encapsulation procedures, as well as the chemical flexibility, targeting potential and low toxicity of such a system; have contributed to the popularity of the cationic liposome as a non-viral vector (Lasic, 1997).

In the years to follow, much progress in the field of cationic liposome-mediated gene transfer has occurred in parallel with advances in cytofectin design (de Lima *et al*., 2001). A typical cationic amphiphile for use in transfection studies, as represented in Figure 1.5, consists of a hydrocarbon anchor for stable insertion into the liposomal bilayer; a hydrophilic headgroup that is protonated at physiological pH in order to bind and condense nucleic acids; a linker bond and spacer between the aforementioned components.

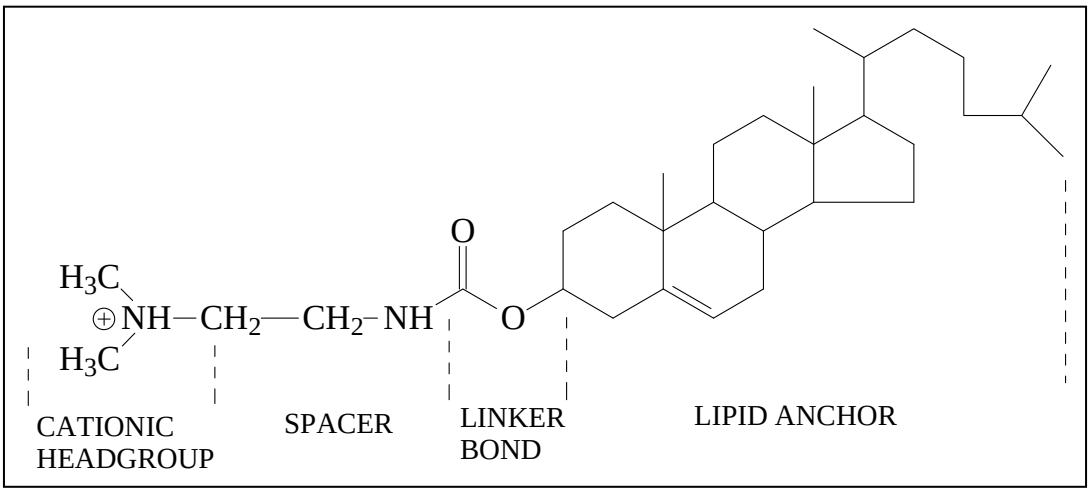


Figure 1.5: The four functional domains of a cytofectin, illustrated using 3β[*N*-(*N′*,*N′*-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol) as an example (Lasic and Templeton, 1996).

Studies have demonstrated that the nature of the respective functional domains contribute to critical features of the vector such as nucleic acid-binding capacity, stability, biodegradability and toxicity; all of which ultimately influence its transfection capabilities (Karmali and Chaudhury, 2007; Rao, 2010). Therefore, in an attempt to optimise cationic liposome-mediated transfection, libraries of novel cationic lipids, of which a few examples are provided in Figure 1.6, have been synthesised from varying combinations of the four domains (Cao *et al*., 2006). In fact, Sherman and colleagues (1998) have reported that several cationic lipids are undergoing safety and efficacy evaluation for use in clinical trials.

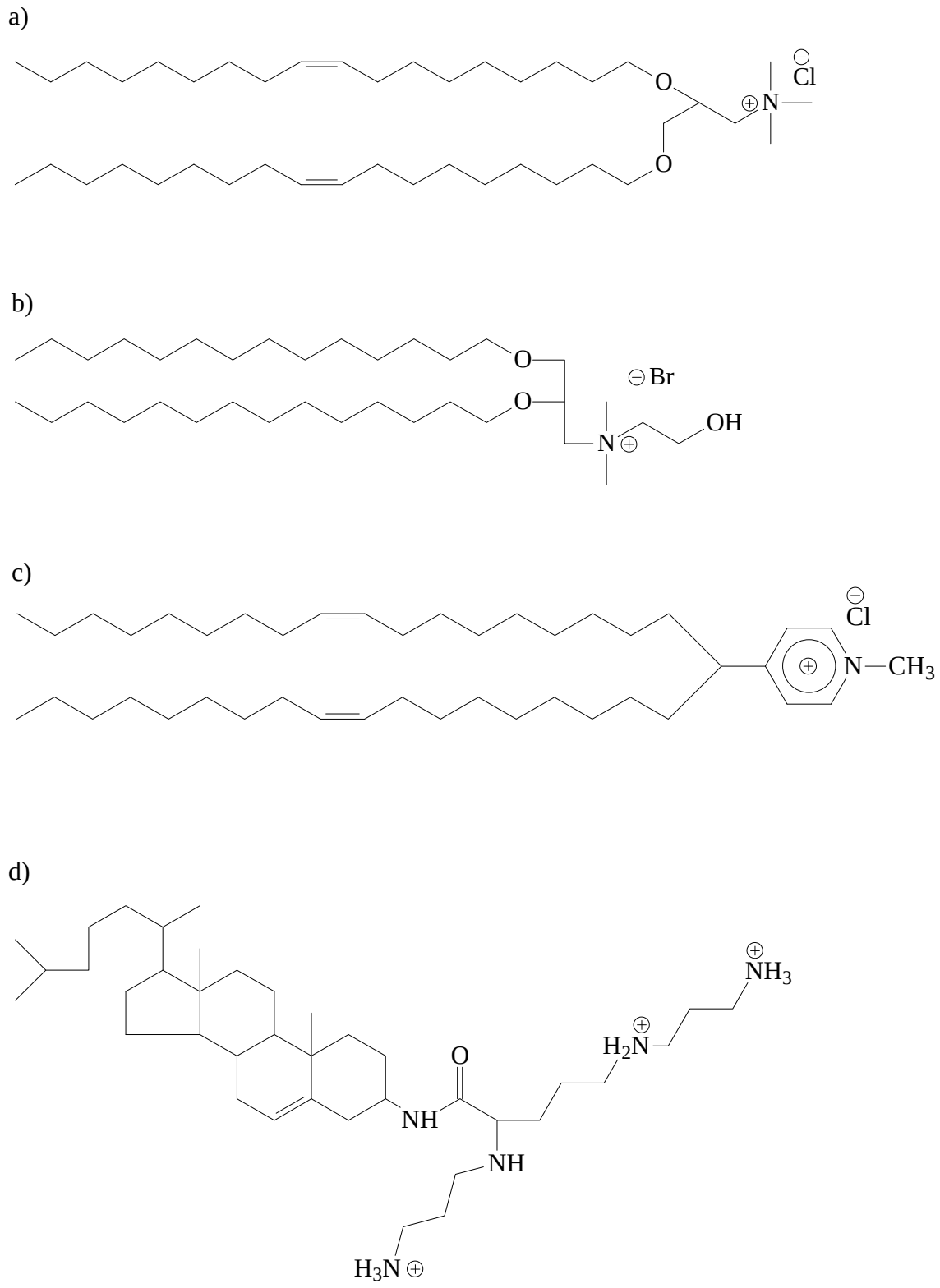


Figure 1.6: Cationic lipids for use in gene transfer, a) *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) (de Lima *et al*., 2001); b) (±)-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide (DMRIE) (de Lima *et al*., 2001); c) *N*-methyl-4-(dioleyl)methylpyridinium (SAINT-2) (Hoekstra *et al*., 2007); and d) Spermine cholesterol (Sper-Chol) (Lasic and Templeton, 1996).

Despite continuing reports of promising transfection studies achieved with cationic liposome technology; according to Wu and coworkers (2002), liposomal gene transfer to the liver remains significantly more challenging than to other organs, such as the lungs. Several factors, presented in Figure 1.7, which hamper the successful transfer of genes to hepatocytes using cationic liposomes, both *in vitro* and *in vivo*, have been identified. While the genetic modification of rat hepatocytes has been achieved by cationic liposome-mediated transfection *ex vivo*, current research seeks to avoid surgical procedures, as these are associated with significant mortality or adverse effects on the patient in the long term (Rangarajan *et al*., 1997). Therefore, in order to achieve the eventual application of systemically administered cationic liposomes to routine treatment of liver disease, researchers have attempted to address these concerns by exploring numerous strategies, and combinations thereof. The discussion to follow focuses on strategies aimed at adapting cationic liposomal systems towards hepatocyte-directed gene transfer, with emphasis on overcoming the problems of poor cell-specificity, lipoplex aggregation, recognition by the immune system and damage due to endosomal processing.

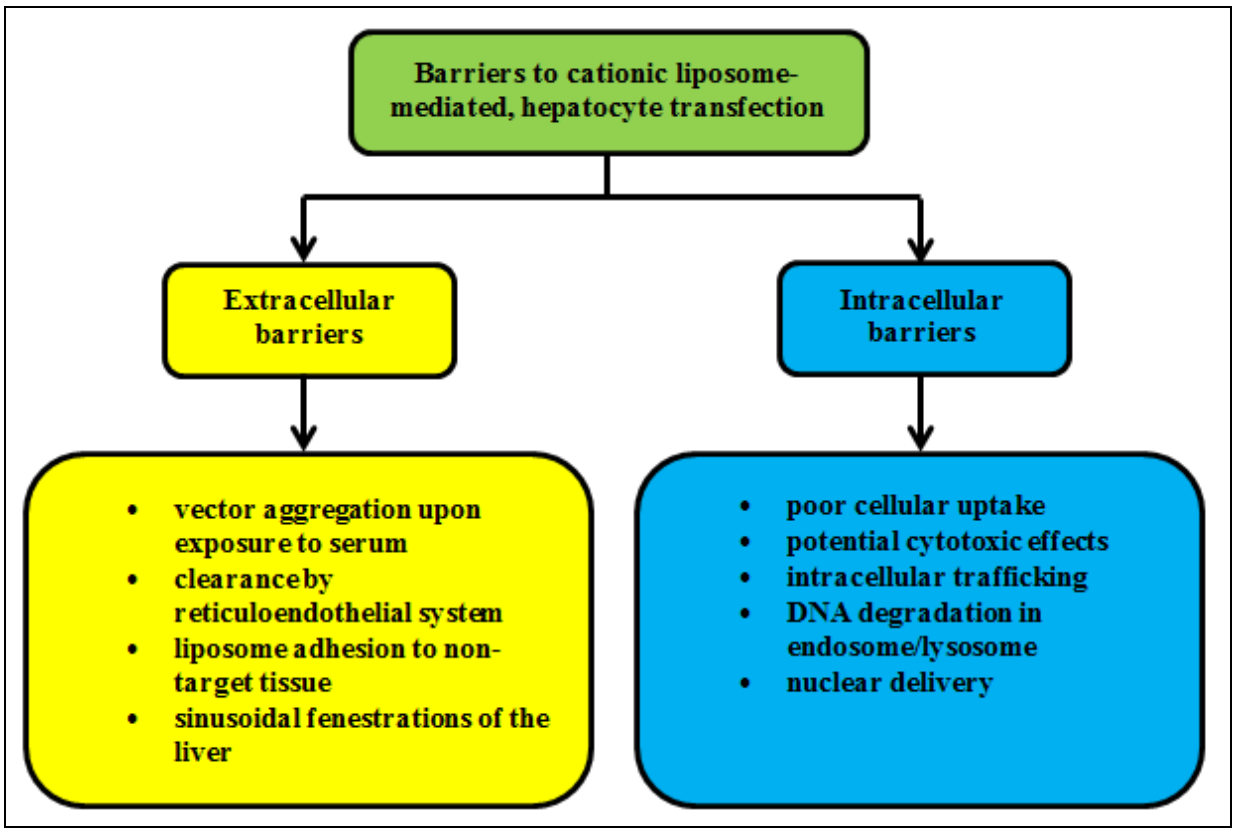


Figure 1.7: Biological barriers to hepatocyte-directed, cationic liposome-mediated gene transfer (adapted from Pathak *et al*., 2009; Wiethoff and Middaugh, 2003; Wu *et al*., 2002).

1.4 Cell-specific gene transfer

A key feature of any vector is its ability to deliver the therapeutic gene to specific diseased cells (Nishikawa and Huang, 2001). To this end various hepatocyte-targeting strategies have been investigated. Passive targeting approaches seek to raise the local vector concentration for enhanced diffusion into hepatocytes, while minimising non-specific interactions with, and/or adverse effects to other cells and organs. This may be achieved by modifying the size, charge or mode of administration of the vector. Alternatively, active targeting approaches exploit receptor-ligand interactions (Li *et al*., 2010). In fact, the use of receptor-mediated endocytosis is considered among the more promising strategies (Hashida *et al*., 2001). Moreover, Li and coworkers (2010) suggest that both active and passive approaches may be used to complement each other in a gene delivery strategy.

1.4.1 Receptor-mediated endocytosis (RME)

RME is a natural process by which cells internalise a variety of molecules such as serum transport proteins, hormones, growth regulators, antibodies and toxins prior to intracellular processing (Pastan and Willingham, 1985). According to Stahl and Schwartz (1986) this process occurs in almost all eukaryotic cells, with the exception of mature erythrocytes. The mechanisms of RME were first outlined by Goldstein and Brown (1977) as a consequence of studies concerning the uptake of low density lipoprotein (LDL) by cultured human fibroblasts.

Each class of molecules which follows this avenue of entry, is recognised by and bound to a specific cell surface protein, termed a receptor (Figure 1.8a). The receptor-ligand complexes migrate laterally through the plasma membrane and, guided by β–arrestin proteins, cluster in pits coated with the triskelion protein clathrin (Phillips, 1995; Sorkin, 2004). Studies have shown that in some instances the clustering of receptors is induced by binding of the cognate ligand, with reference to epidermal growth factor (EGF) and insulin. Conversely, receptors for other biomolecules, such as LDL and asialoglycoproteins (ASGPs), are concentrated in coated pits irrespective of ligand binding (Basu, 1984). In addition, the membrane adaptor

protein complex, which is a major component of the clathrin coat, mediates concentration of receptors within coated pits by binding to endocytosis motifs present in their cytosolic tails (Benmerah *et al*., 1998). The coated pits are structures that are continually formed, irrespective of receptor-ligand binding, and therefore contain a variety of both receptor-ligand complexes and unoccupied receptors (Pastan and Willingham, 1985). These coated pits progressively invaginate, and enter the cell as coated vesicles (Basu, 1984).

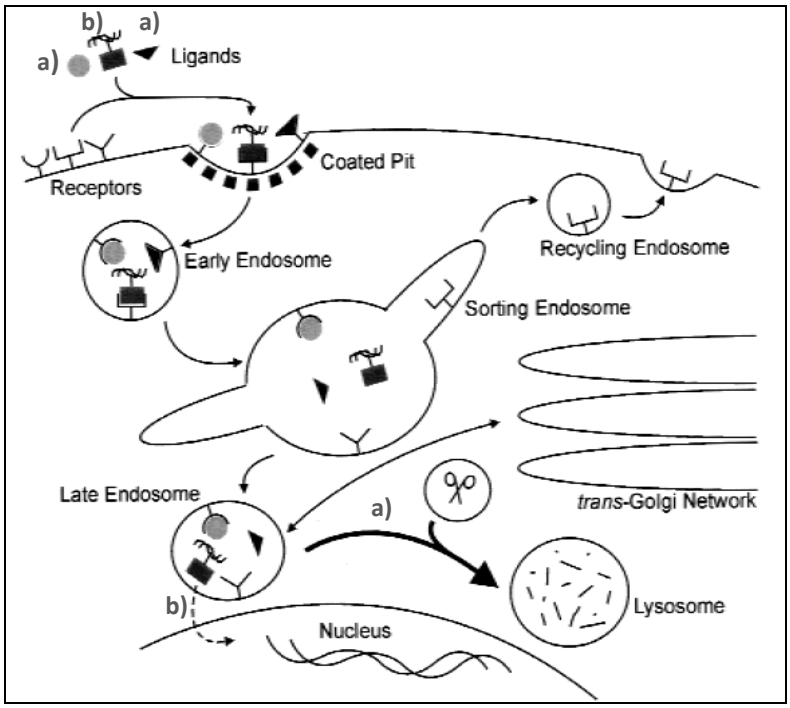


Figure 1.8: Receptor-mediated endocytosis of a) biomolecules and b) targeted vector constructs (adapted from Varga *et al*., 2000).

Within the cytosol, an ATP-dependent enzyme mediates removal of the clathrin coat (Wilemann *et al*., 1985) which is recycled to the plasma membrane. The uncoated vesicles subsequently fuse to afford the early endosome, with an internal pH of approximately 6.5 (Goldstein *et al*., 1985; Ropert, 1999). The endosomal compartment is a network of membranous tubules, the structure of which may differ according to cell type, where internalised molecules are organised before being transported to their appropriate destinations. Initially, the dissociation of ligand molecules from their respective receptors occurs within the acidic environment of the endosome, induced by the action of ATP-driven proton pumps (Stahl and Schwartz, 1986).

The subsequent stages of the endocytic pathway depend on the nature of the receptor and cognate ligand. As listed in Table 1.4, four possible routes have been identified. However, the ensuing discussion is focused on the classical pathway that entails conservation of the receptor, and degradation of the ligand. According to this route, receptors migrate to a pole of the endosome that pinches off as a recycling vesicle and, as such, are returned to the plasma membrane (Goldstein *et al*., 1985). The endosome experiences a further drop in pH, and is referred to as the late endosome (pH 5.5 – 6.0) (Ropert, 1999). This compartment then fuses with vesicles containing hydrolytic enzymes, resulting in maturation of late endosomes into lysosomes (with pH values less than 5.0), the end-point of the endocytic pathway. The enzymes, which are active within the acidic environment, facilitate catabolism of the internalised molecules (Varga *et al*., 2000). The breakdown products then diffuse out of the lysosome, and are available either for use or removal by the cell (Stahl and Schwartz, 1986).

As represented in Figure 1.8b, vector targeting approaches modelled on the process of RME are based on the premise that, having modified a vector with a ligand to a receptor known to undergo RME, the ligand binds to this receptor and permits entry of the carrier into cells bearing such receptors (Grove and Wu, 1998).

Table 1.4: Fate of receptor and cognate ligand following delivery to the endosome (adapted from Goldstein *et al*., 1985).

Route	**Description**	**Example(s)**
1	Receptor is recycled, while ligand is degraded.	LDL, ASGPs, insulin
2	Both receptor and ligand are recycled.	Transferrin
3	Both receptor and ligand are degraded.	EGF
4	Both receptor and ligand are transported.	IgA, IgM

1.4.2 The asialoglycoprotein receptor (ASGP-R)

The ASGP-R was first discovered in the mid-1960s by Ashwell and Morell during their studies on the metabolism of serum glycoproteins (Wiegel and Yik, 2002), and is a well-characterised endocytic transport receptor (Spiess, 1990). This receptor is of great significance to the field of hepatocyte-directed gene delivery, as it is expressed nearly exclusively and at high density ($1 – 5 \times 10^5$ receptors per cell) on the sinusoidal face of the plasma membrane of liver parenchymal cells (Pathak *et al*., 2008).

The hepatic lectin is largely recognised for its binding and internalisation of asialoglycoproteins and neoglycoproteins bearing terminal *N*-linked D-galactose or D-*N*-acetylgalactosamine (Gal*N*Ac) moieties (Stokmaier *et al*., 2009; Wiegel and Yik, 2002). However, other biological roles of this receptor include the clearance of cellular fibronectin, lipoproteins, serum IgA (Park *et al*., 2006) and apoptotic cells (Tozawa *et al*., 2001).

It is important to note that there is evidence for the existence of ASGP-R homologues on extrahepatic tissue such as the intestinal epithelium, thyroid and kidney (Park *et al*., 1998; Seow *et al*., 2002). However such receptors are believed to mediate functions different from that of this hepatic lectin (Wiegel and Yik, 2002), recognise different motifs on their cognate ligands (Stefanich *et al*., 2008) and are not as abundantly expressed (Park *et al*., 1998).

The human ASGP-R is a hetero-oligomeric protein made up of two homologous subunits (designated H1 and H2), of 46 and 50 kDa respectively (Becker *et al*., 1995). These subunits are expressed in a molar ratio of approximately 5:1 yielding the active integral membrane protein (Pathak *et al*., 2008). Four functional domains of this receptor, illustrated in Figure 1.9a, have been designated as follows: a cytosolic, N-terminal domain, a single transmembrane domain, stalk segment and a C-terminal carbohydrate recognition domain (CRD) (Massarelli *et al*., 2010).

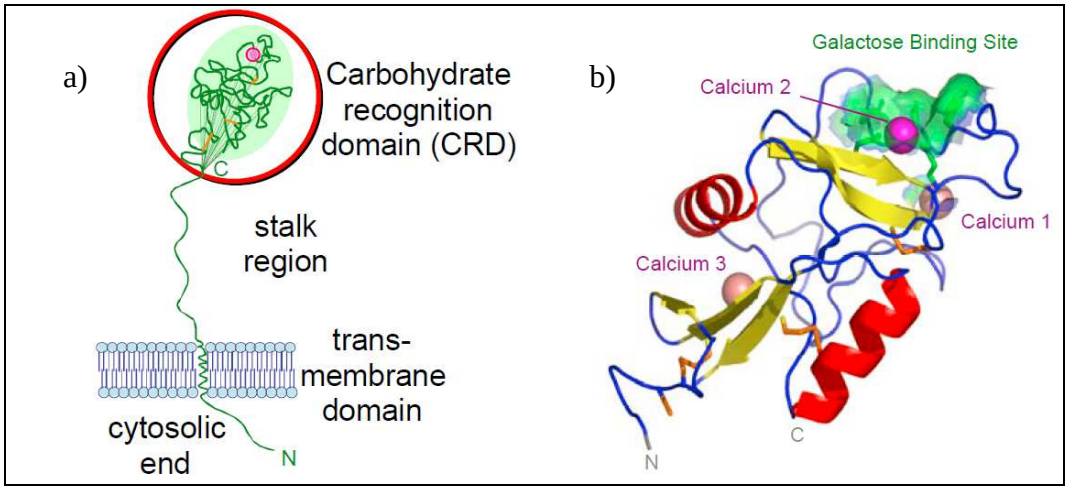


Figure 1.9: Schematic representations of a) the functional domains and b) H1-CRD of the asialoglycoprotein receptor (Khorev, 2007).

Characterised as a C-type mammalian lectin, the ASGP-R displays a requirement for calcium ions, at optimal concentrations of between 0.1 and 2.0 mM, for carbohydrate binding (Khorev, 2007; Massarelli *et al*., 2010; Zelensky and Gready, 2005). While both subunits possess a CRD, it is the CRD of H1 that associates with ligand molecules. The H2-subunit has been implicated in directing the assembly of the functional receptor and its basolateral sorting. Therefore, simultaneous expression of both subunits is essential to the proper functioning of the ASGP-R (Fuhrer *et al*., 1994; Khorev, 2007).

Meier and colleagues (2000) have presented an X-ray crystal structure of the CRD of the H1 subunit. According to this model, three calcium ions form an essential component, as presented in Figure 1.9b: one Ca^{2+} ion is implicated in the process of ligand binding, while the others contribute to the structural integrity of the protein. The terminal monosaccharides of ligand molecules are believed to co-ordinate directly with a Ca^{2+} ion in the galactose binding region, displacing two water molecules that co-ordinate this cation in the absence of a cognate ligand. In addition, hydrogen bonding of the hydroxyl groups at positions 3 and 4 of galacto-entities with carboxyl and amide side-chains of aspartate and glutamine residues, at this site, contributes to the strong ligand-binding interaction, such that the the dissociation constant has been calculated to within the nanomolar range (Meier *et al*., 2000; Schwartz *et al*., 1981).

Studies by Schwartz and coworkers (1982), using a human hepatoma cell line have demonstrated that an unoccupied ASGP-R binds to its cognate ligand within approximately 8.7 minutes, is internalised and returned to the plasma membrane with mean times of 2.2 and 4.2 minutes respectively. This high rate of internalisation and receptor recycling present further advantages for the hepatocyte-specific entry of vector constructs (Hashida *et al*., 2001).

1.4.3 Targeted gene transfer to hepatocytes

The first demonstration of the potential for hepatocyte-specific gene transfer via ASGP-R-mediated endocytosis emerged in the 1980s. The pioneering study by Wu and Wu entailed the covalent attachment of asialoorosomucoid, a natural ligand to the ASGP-R, to poly-L-lysine, for the introduction of the chloramphenicol acetyltransferase (CAT) reporter gene into liver parenchymal cells *in vitro* and *in vivo* (Grove and Wu, 1998; Wu and Wu, 1998). However the sophisticated procedure involved in the preparation of this carrier (Ren *et al*., 2001) and its reproducibility (Kawakami *et al*., 1998) were problematic. Therefore subsequent attempts at directing genes to hepatocytes by receptor-mediation employed more promising non-viral gene transfer agents, notably the cationic liposomes.

1.4.3.1 Liposome modification with ASGP-R-specific ligands

1.4.3.1.1 Asialoglycoproteins (ASGPs)

The asialoglycoproteins are a class of endogenous glycoproteins from which the terminal sialic acid residue has been enzymatically removed. Asialoorosomucoid (AOM), asialofetuin (AF), asialolactoferrin, asialotransferrin and asialoceruloplasmin have been appended to liposomal carriers in order to direct hepatocellular recognition (Pathak *et al*., 2008).

Singh and colleagues (2010) made use of the high affinity streptavidin-biotin interaction to attach AOM to a cationic liposome. This multicomponent lipoplex, illustrated in Figure 1.10,

not only mediated high transfection activity in cultured hepatocytes, but also showed favourable size, charge and cytotoxicity profiles for potential *in vivo* application.

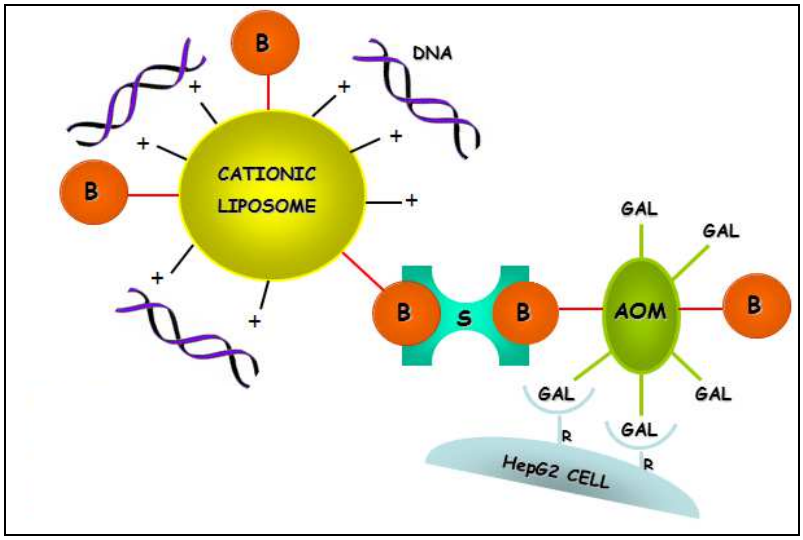


Figure 1.10: Schematic representation of the hepatocyte-specific modular complex formed between plasmid DNA, biotinylated liposomes, streptavidin and dibiotinylated AOM. B: biotin, S: streptavidin, AOM: asialoorosomucoid, GAL: galactose residue, R: asialoglycoprotein receptor (Singh *et al*., 2010).

In a deviation from the use of unmodified ASGPs as targeting ligands, Singh and Ariatti (2003) designed a hepatotropic transfecting complex assembled from the spontaneous electrostatic interactions between activated cationic liposomes, pRSVL plasmid DNA and a carbodiimide-cationised derivative of AOM. In transfection studies employing the human hepatoma cell line, HepG2, the ternary complex demonstrated luciferase activity four times higher than complexes assembled from non-cationised AOM.

The inclusion of asialofetuin as a targeting component of liposomal gene transfer systems is relatively prominent in the literature. Early work by Hara *et al*. (1995) entailed the application of both detergent removal and freeze thaw procedures to simultaneously permit the encapsulation of pSV2CAT DNA within, and its binding to the membranes of AF-labelled cationic liposomes. Arangoa and coworkers (2003) attempted to develop this strategy by designing a serum-tolerant hepatocyte-directed lipoplex. The reporter plasmid was condensed via a cationic peptide, protamine, and subsequently complexed with AF-modified DOTAP/Chol liposomes. The ASGP-R-affinity of AF, combined with the nuclear localisation

potential of protamine, afforded successful introduction of reporter genes into liver parenchymal cells following systemic administration in mice, without inducing organ damage. More recently, efforts by Tros de Ilarduya (2010) produced high levels of transfection in HepG2 cells, having improved the serum tolerance of AF-modified DOTAP/Chol liposomes. Furthermore, studies by Dasi *et al*. (2001) adds credence to the feasibility of AF-modified liposomes as gene transfer agents to cells of hepatic origin, as such a vector facilitated the introduction and sustained expression of the medically significant α_1-antitrypsin gene in an animal model.

1.4.3.1.2 Galactose

While ASGPs are proven to facilitate effective targeting of liver parenchymal cells, their application as components of non-viral vectors is limited by the high cost and tedious procedures required for their purification (Hwang *et al*., 2001). Due to the fact that the terminal galactose and Gal*N*Ac residues of ASGPs mediate their recognition by binding to the ASGP-R; these monosaccharides and other galactose-terminated compounds, such as lactose (Watanabe *et al*., 2007), lac-BSA (Pathak *et al*., 2008) and lactobionic acid (Yu *et al*., 2007), were investigated as alternative hepatocyte-targeting moieties. In recent years, structure-affinity studies involving the human ASGP-R have established that Gal*N*Ac binds with 50 fold greater affinity than galactose (Khorev, 2007; Westerlind *et al*., 2004). However, it is the galactose moiety that has received the most attention as a hepatocyte-specific homing device for gene transfer applications (Pathak *et al*., 2008).

Several liposomal gene transfer approaches employ galacto-entities that are displayed from the surface of the liposome, by way of appending the monosaccharide to a membrane-compatible lipid that permits its stable anchorage to the bilayer. Cholβgal (Figure 1.11a), composed of a β-D-galactose residue attached via a glycosidic bond to a cholesterol anchor, is among the simplest glycolipids (Singh *et al*., 2007). However, in the interest of ensuring optimal interaction between the liposome-appended targeting moiety and the ASGP-R, glycolipid designs have developed to include lipids bearing both cyclic and open galacto-headgroups (Mukthavaram *et al*., 2009), multifunctional lipids (Kawakami *et al*., 2000), variation in the length of the spacer between the lipophilic anchor and monosaccharide

(Kawakami *et al*., 1998), as well as the number of galactose residues tethered to a single lipid (Jiang *et al*., 2008; Tang *et al*., 2007). In fact, several synthetic glycolipids (Figure 1.11) have yielded encouraging results *in vitro* and *in vivo*.

As an example, Kawakami and coworkers (1998) evaluated the efficacy of liposomes prepared from DC-Chol, DOPE and a newly synthesised cationic glycolipid Gal-C4-Chol (Figure 1.11b). This vector system exhibited markedly higher transfection activity (as measured by reporter gene expression and [^{32}P] DNA uptake) in HepG2 cells, than cationic liposomes formulated without the glycolipid. Furthermore, gene expression using the targeted formulation was markedly reduced after exposing the cells to 20 mM galactose; which affirmed ASGP-R- mediated vector internalisation by the liver parenchymal cells. In a related study, Gal-C4-Chol/DOTMA/Chol liposomes achieved hepatocyte-specific introduction of the luciferase reporter gene following intraportal administration in mice (Kawakami *et al*., 2000).

Importantly, several groups have demonstrated that although the inclusion of a prominently displayed hepatocyte-targeting moiety is essential, physical features such as galactose density, lipid composition, size, stability, and charge ratio of the lipoplexes must be optimised in order to develop effective liver-directed, cationic liposomal gene transfer systems (Fumoto *et al*., 2004; Kawakami *et al*., 2000; Managit *et al*., 2005).

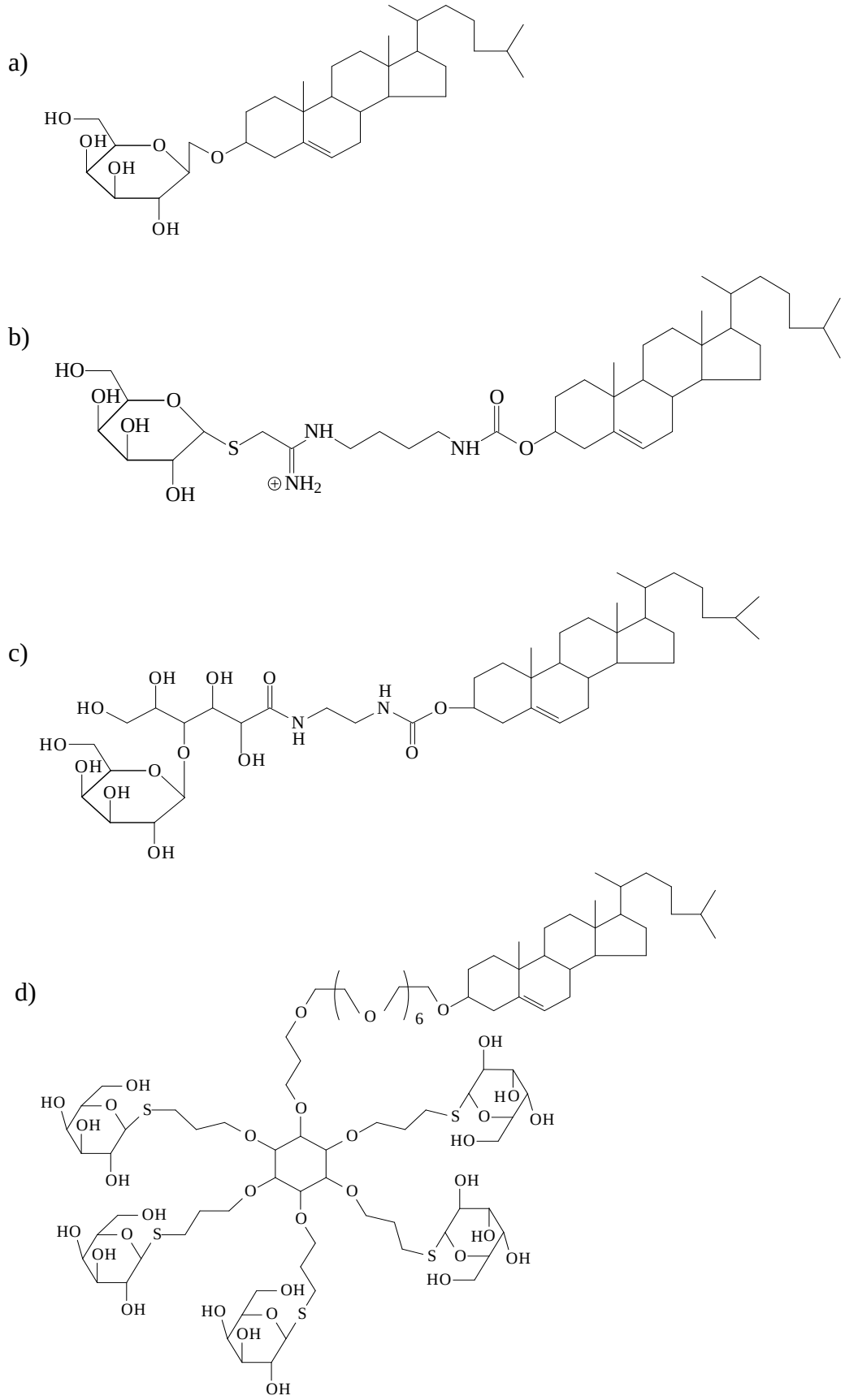


Figure 1.11: Examples of glycolipids used in hepatotropic liposome formulations, a) cholesteryl-β-D-galactopyranoside (Cholβgal) (Singh *et al*., 2007); b) cholesten-5-yloxy-*N*-(-

4-((1-imino-c-β-D-thiogalactosyl-ethyl) amino) butyl) formamide (Gal-C4-Chol) (Shigeta *et al*., 2007); c) (5-cholestan-3β-yl)-1-[2-(lactobionyl amido) ethylamido] formate (CHE-LA) (Yu *et al*., 2007); and d) penta-antennary thiogalactoside L-II (Jiang *et al*., 2008).

1.5 Steric stabilisation of liposomes

One of the prerequisites for successful gene transfer is that the vector should remain stable in circulation until it reaches the target tissue. However, upon exposure to the bloodstream, the transfecting complexes may be opsonised by serum proteins, causing aggregation. This phenomenon is prominent with cationic lipoplexes as their net positive surface charge encourages association with proteins bearing negative charges such as albumin and lipoproteins (Wu *et al*., 2002). Large aggregates tend to both accumulate in the lung, and undergo rapid clearance by the reticuloendothelial system. This reduces the effective vector dose and circulation time; and impacts adversely on liposome-mediated gene transfer, even in the presence of a targeting ligand (Pathak *et al*., 2009).

In response to this challenge, the steric stabilisation of liposomes was proposed. To this end, "stealth" liposomes were designed by modifying the surface of the vector with inert, hydrophilic polymers such as polyethylene glycol (PEG), poly[*N*-(2-hydroxypropyl)methacrylamide)], poly-*N*-vinylpyrrolidones and polyvinyl alcohol (Karanth and Murthy, 2007). However the linear polyether diol, PEG, is the most widely employed liposomal steric stabilising agent (Immordino *et al*., 2006).

1.5.1 Polyethylene glycol (PEG)

Several features of PEG render it suitable for combination with liposomal systems. Firstly, the polymer is easily synthesised by low-cost methods which afford high product yield and purity (Rejman *et al*., 2004b). Furthermore, PEG is known for its excellent biodistribution characteristics, low toxicity and weak immunogenicity (Zalipsky, 1995). Importantly, the presence of PEG chains provides a hydrated "cloak" over the liposomal bilayer that is analogous to the glycocalyx that encompasses erythrocytes (Immordino *et al*., 2006). The

flexible polymer extends approximately 3 – 5 nm from the bilayer, depending on its length (Song *et al*., 2002), occupying the space in immediate proximity to the liposome surface, and serves to exclude other macromolecules. This hinders the access and binding of plasma opsonins to the liposome surface, as represented in Figure 1.12 (Immordino *et al*., 2006). In addition, PEG chains are believed to exert a stabilising effect on the structure of the bilayer itself, although a mechanism for this phenomenon has not, as yet, been proposed (Varga *et al*., 2010).

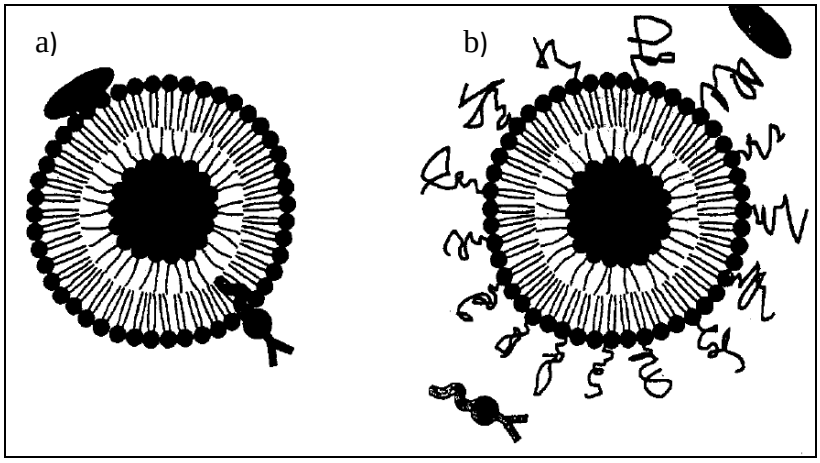


Figure 1.12: Interaction of opsonins with a) non-pegylated and b) pegylated liposomes (Schuber *et al*., 1998).

Liposomal systems may be modified with a PEG shroud in one of two ways, as shown in Figure 1.13. The pre-pegylation strategy entails the incorporation of PEG-lipid conjugates as components of the liposome formulation, prior to its association with nucleic acids. Conversely, the polymer coat may be introduced after the assembly of liposome-DNA complexes from non-pegylated liposomes, by incubating lipoplexes with pegylated lipids (Peeters *et al*., 2007). However, it is the former approach that is commonly employed (Immordino *et al*., 2006).

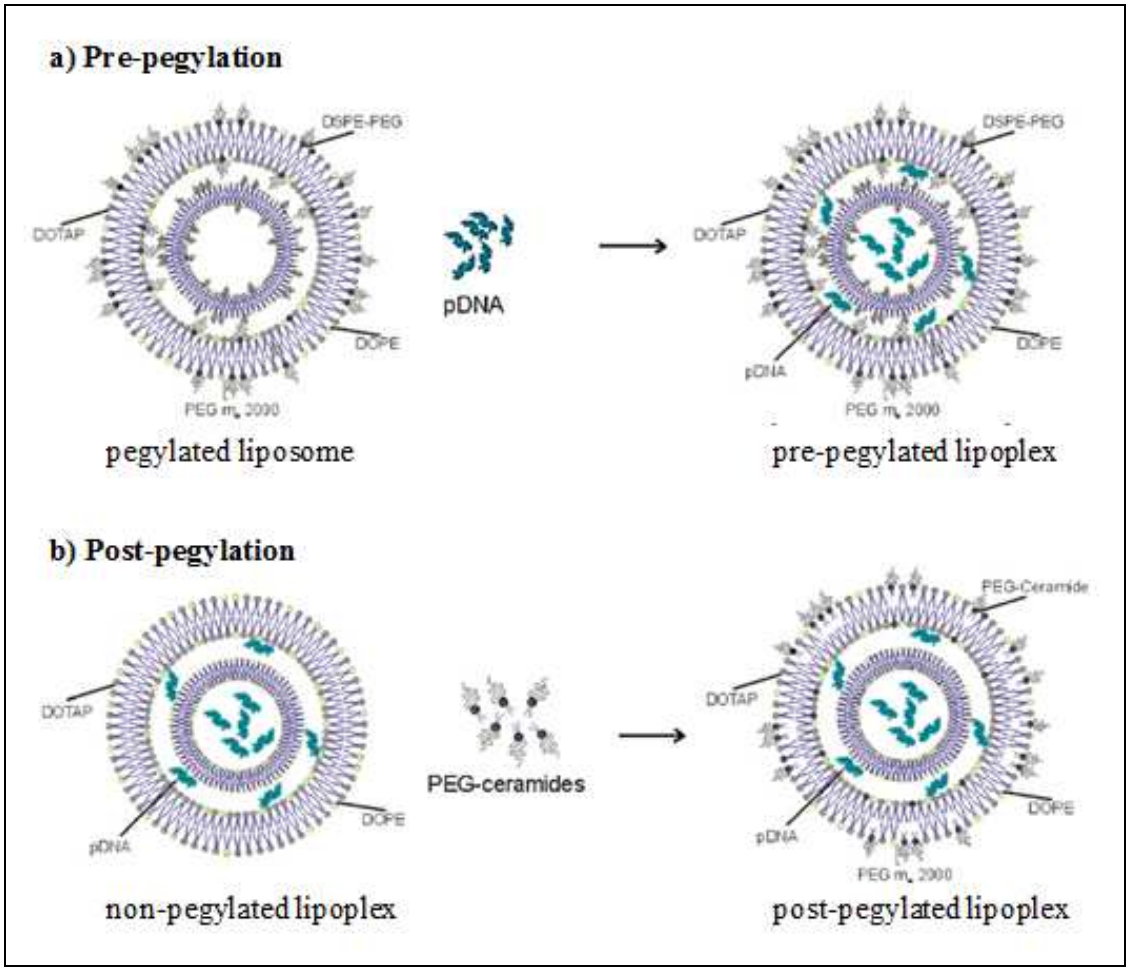


Figure 1.13: Representation of a) pre- and b) post-pegylation of liposomal carriers (adapted from Peeters *et al*., 2007).

The PEG coat assumes different conformations around liposomes (Figure 1.14) depending on the molecular weight of the polymer and the distance between the sites of attachment of neighbouring polymer chains (Čeh *et al*., 1997). When liposomes are pegylated at low density and with short PEG molecules, the polymer assumes a pancake-like structure. As the degree of grafting is increased, PEG exists as isolated grafts which typifies the "mushroom" conformational regime. It is only at high grafting densities that the PEG chains are able to interact sufficiently to encompass the vector as a shroud or "brush" (Ishida and Kiwada, 2008; Needham *et al*., 1997).

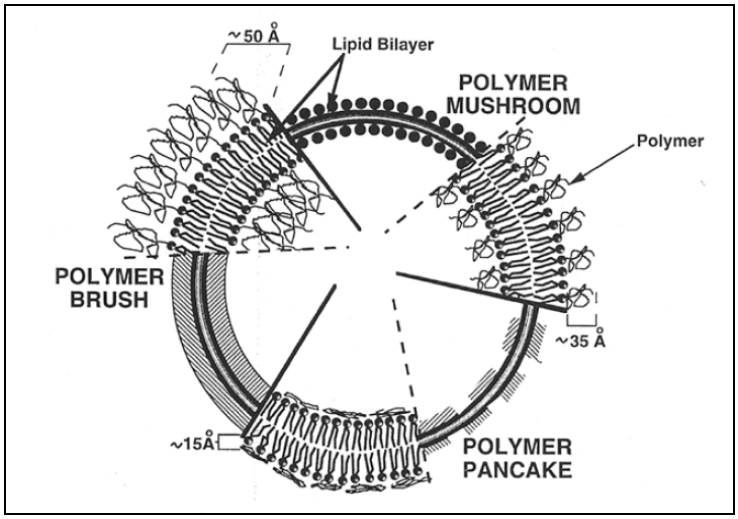


Figure 1.14: The different conformational regimes of PEG grafted to a bilayer. The length, in angstroms, reflects the approximate width of the polymer coating (Čeh *et al*., 1997).

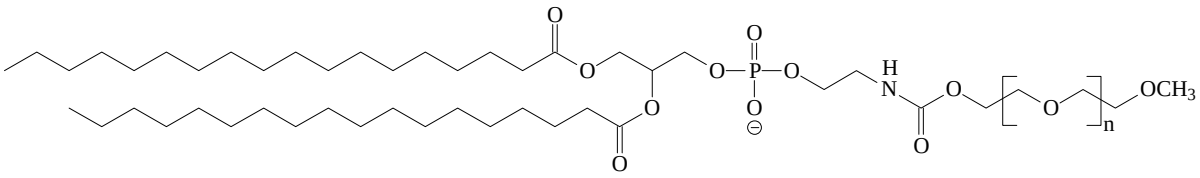
1.5.2 PEG-lipids

Although PEG is, by and large, grafted to phosphatidylethanolamines (Figure 1.15a) for liposome modification, the polymer has also been coupled to diacylglycerides (Figure 1.15b), ceramides (Figure 1.15c), cholesterol and phosphatidic acid (Carrion *et al*., 2001; Zalipsky, 1995).

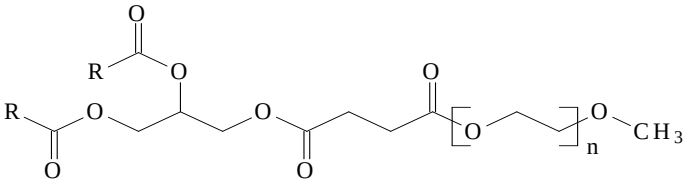
Pegylation of liposomal drug carriers has attained recognition as a viable treatment option (Ishida and Kiwada, 2008). However, the successful application of stealth technology to liposomal gene transfer is more complicated. This is because pegylation of liposomes has been associated with a drop in transfection activity, in correlation with the degree of pegylation. It has been proposed that the external corona of polyethylene glycol hinders liposome-cell bilayer interactions, which are important for successful vector internalisation and endosomal escape (Remaut *et al*., 2007; Singh *et al*., 2011). In response, the design of PEG-lipids has grown more elaborate to include functionalities which induce detachment of the polymer shroud, once it has fulfilled its role, thus permitting the necessary interactions between bilayers of the endosome and liposome. For example, PEG chains have been conjugated to their respective hydrophobic moieties via linkages, such as the ortho ester (Guo *et al*., 2003; Masson *et al*., 2004) and vinyl ether bonds (Shin *et al*., 2003), which are

sensitive to the acidic environment prevalent within the endosome. In addition, Zalipsky and colleagues (1999) introduced a disulphide-containing dithiobenzylurethane spacer between $mPEG_{2000}$ and DSPE, to permit depegylation under the thiolytic conditions of the endosomal compartment (Heyes *et al*., 2006). As an alternative, the use of exchangeable PEG-lipids, which are released from the liposome following interaction with biological membranes, was investigated (Rejman *et al*., 2004b).

a)

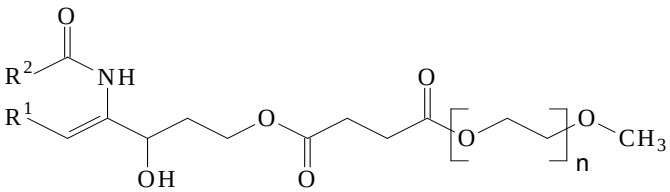


b)



$R = C_{13}H_{27}$

c)



$R^1 = C_{13}H_{27}$

$R^2 = C_7H_{15}$

Figure 1.15: PEG-lipids, a) DSPE-PEG (Immordino *et al*., 2006); b) PEG-DMG; and c) PEG-$CerC_8$ (Ambegia *et al*., 2005). DSPE: distearoylphosphatidylethanolamine, DMG: dimyristoylglycerol, Cer: ceramide.

1.5.3 Pegylated, hepatocyte-directed, liposomal gene transfer systems

Early research demonstrated that the incorporation of 10 % DSPE-PEG, on a molar basis, into galactose-modified N-glutaryl-phosphatidylethanolamine liposomes, enhanced the P:NP ratio, which is a measure of liposome accumulation in hepatocytes (P) relative to non-parenchymal liver cells (NP), by a factor of approximately 6.5 (Nag and Ghosh, 1999). However, other studies have reported that the hydrophilic PEG chains may shield the ligand displayed on the liposome surface and, as such, reduce target cell uptake, in comparison with its non-pegylated counterpart (Narainpersad, 2009). This effect may be modulated by optimising the degree of pegylation for respective liposome formulations, or by functionalisation of the PEG chains.

In exploring the latter option, Shimada and coworkers (1997) covalently attached a galactose residue to the distal ends of diacylglyceride-grafted PEG chains (containing between 10 and 40 oxyethylene residues). However, intrahepatic distribution studies showed that liposomes formulated with these bifunctional lipids failed to escape the macrophages of the liver. It was suggested that the orientation of the galacto-entity afforded by this arrangement was recognised by the galactose particle receptor (GPr) on the Kupffer cells, rather than the ASGP-R. Letrou-Bonneval and colleagues (2008) refined this concept to merit hepatocyte-specificity by displaying galactose residues at the cytosolic termini of polyethylene glycol (F108). In this study, the derivatised steric stabiliser (F108-GAL) was mixed with cationic liposomes prepared from bis(guanidinium)-tren-cholesterol (BGTC) and DOPE, and plasmid DNA to afford a multimodular gene transfer system. Transfection of isolated rat hepatocytes with BGTC/DOPE/DNA complexes, at a charge ratio of 2, resulted in a dramatic increase in luciferase gene expression as the F108-GAL/DNA ratio was increased. It was suggested that the length of the steric stabiliser may contribute both towards the formation of these supramolecular assemblies and the accessibility of the ligand for the RME pathway.

1.6 Preventing lipoplex degradation during endocytic trafficking

Once internalised by the ASGP-R, both the vector and its associated DNA are bound for degradation within the lysosome. In fact, the efficacy of liposome-mediated gene transfer, both targeted and non-targeted, may be limited by the low levels of therapeutic nucleic acids that reach the nucleus in intact form (Wu *et al*., 2002). Consequently, design of liposomal vector constructs was based on the premise that the expression of exogenous genes in target cells may be enhanced provided that lysosomal digestion of the vector is prevented. Attempts at preserving the integrity of internalised lipoplexes, until the required dissociation and nuclear translocation of the nucleic acid cargo, have been directed at both the endosome and lysosome (Wiethoff and Middaugh, 2003). Although numerous endosome- and lysosome-disrupting devices have collectively perpetuated more effective modes of non-viral gene transfer (Ciftci and Levy, 2001; Varkouhi *et al*., 2010; Wagner, 1998), the following discussion is limited to techniques that have been investigated with respect to cationic liposomal systems and, more specifically, those directed at the liver parenchymal cells.

1.6.1 Lysosomotropic agents

Lysosomotropic agents are defined as substances that selectively accumulate within the lysosomal compartment regardless of their chemical structure or the manner in which they are taken up (Ciftci and Levy, 2001). Several such chemicals (Table 1.5) have been applied to lipid-based gene transfer in order to minimise lysosomal hydrolysis of the vector by inactivating enzymes of this compartment; inhibiting fusion of the lysosome with the vector-containing endosome; or liberating the vector from the lysosome following the fusion event, by inducing osmotic rupture (Ciftci and Levy, 2001).

The weak base chloroquine, which interestingly also displays endosomolytic activity, is the most widely investigated member of this class. It has been suggested that the mechanism by which chloroquine facilitates early escape of vectors from the endosomal/lysosomal pathway may be concentration dependent (El-Sayed *et al*., 2009). Studies conducted by Ciftci and Levy (2001) revealed that the chloroquine-mediated transfection enhancement of Lipofectin-

pSV-gal complexes in cultured human fibroblasts was cell-specific and, in some instances, hampered by cytotoxic effects. Moreover, evidence that chloroquine, as a component of vector constructs, induces systemic toxicity following its introduction to the liver militates against its application to gene transfer directed at this organ (Zhang *et al*., 2003).

Table 1.5: Examples of lysosomotropic agents employed in gene transfer applications (adapted from Ciftci and Levy, 2001).

Agent	**Mechanism of lysosome disruption**
Chloroquine	• Inhibits activity of lysosomal enzymes by raising the pH to sub-optimal levels. • Alters osmotic balance of lysosome, inducing lysis.
Polyvinylpyrrolidone (PVP)	• Prevents fusion of lysosome with early endosome.
Sucrose	• Induces osmotic swelling and lysis.

1.6.2 Endosome-destabilising devices

Several agents, both natural and synthetic, have demonstrated the ability to disrupt endosomal membranes (Table 1.6). However, the toxic or immunogenic properties inherent to many, has precluded their application to liposomal gene transfer. Instead, an understanding of the mechanisms by which these agents effect escape from the endosomal compartment, prior to its maturation to the lysosome, has presented potential strategies to inhibit damage to the vector (Varkouhi *et al*., 2010).

Table 1.6: Endosomal escape agents (adapted from Midoux *et al.*, 2009; Varkouhi *et al.*, 2010).

Category	Examples	Source	Mechanism of endosomal escape
Proteins and peptides	Haemagglutinin	viral	endosomal membrane fusion
	Poly-L-histidine	synthetic	proton sponge effect
	Listeriolysin O	bacterial	pore formation in endosomal membrane
	Saporin	plant	not yet elucidated
	Mellitin	insect	pore formation in endosomal membrane
	hCT(9-32)	human	not yet elucidated
Chemicals	Chloroquine	synthetic	proton sponge effect
	Methylamine	synthetic	proton sponge effect
	Ammonium chloride	synthetic	proton sponge effect
Photosensitisers	Dendrimer pthalocyanine	synthetic	photochemical disruption of endosomal membrane
	$TPPS_4$	synthetic	photochemical disruption of endosomal membrane
Cationic polymers	Polyethylenimine	synthetic	proton sponge effect
	Polyamidoamines	synthetic	proton sponge effect
Imidazole-containing lipids	Gal-His-C4-Chol	synthetic	proton sponge effect
	ChIm	synthetic	proton sponge effect

1.6.2.1 Fusion with endosomal membrane

1.6.2.1.1 Fusogenic lipids

The use of the fusogenic lipid DOPE (Figure 1.16) as a helper lipid in cationic liposome formulations is well documented. DOPE induces endosome destabilisation by promoting the transition of lipid bilayers from the lamellar to the inverted hexagonal phase, which leads to fusion of the liposomal bilayer and that of the endosome at temperatures above 10 ˚C (Midoux *et al*., 2009; Wasungu and Hoekstra, 2006). However, several groups have demonstrated that the combination of other endosomal escape strategies with DOPE-containing liposomal systems has improved transfection levels (Midoux *et al*., 2009).

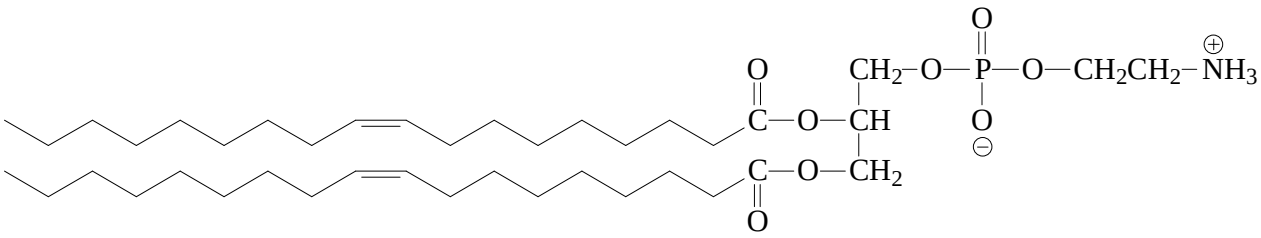


Figure 1.16: Chemical structure of DOPE (Lasic, 1997).

1.6.2.1.2 Fusogenic peptides

It has been long recognised that several pathogens evade destruction within host cells by escaping the endosomal compartment, as they express amphipathic fusogenic peptides (Cho *et al*., 2003; Varkouhi *et al*., 2010). Such peptides, which exist as random coils under neutral conditions, assume α-helical conformation when subjected to the low pH of the endosome (van Rossenberg *et al*., 2002), in response to protonation of acidic amino acid residues. Consequently hydrophobic regions of the peptide are exposed, which interact with the endosomal bilayer, inducing destabilisation (Plank *et al*., 1998). Therefore, researchers have attempted incorporating the fusogenic segments of proteins derived from biological agents as components of cationic liposomes (Wagner, 1999). In fact, van Rossenberg and coworkers (2002) have demonstrated the potential for the conjugation of hepatotropic ligands to a

fusogenic segment of a viral envelope protein with endosome-specific, bilayer-destabilising activity.

However, current research in this regard favours the use of synthetic peptides, notably analogues of their native counterparts. This is because peptide synthesis offers greater control over features that influence endosome-destabilising ability, such as peptide length, protein conformation, hydrophobicity and importantly, immunogenicity; by manipulating the amino acid sequence (Cho *et al*., 2003; Tu and Kim, 2008; Wagner, 1999).

1.6.2.2 Proton sponge effect

In the 1980s, following elucidation of the mechanisms governing RME, several studies demonstrated that weak bases inhibit this process by disrupting endosome function (Wilemann *et al*., 1985). This observation was later explained by Behr (1997), who hypothesised that the transfection efficiency of weakly basic polycations, in the absence of lysosomotropic agents, was due to their ability to circumvent degradation during endocytic trafficking by inducing rupture of the endosomal compartment.

Several polymeric carriers (Table 1.7) possess amino functions that exhibit buffering capacity within the endosomal range. When, as a consequence of endocytic uptake, such vectors reach the acidic environment of the endosomal compartment, their amino functions resist the proton pump-mediated change in pH by sequestering protons. Protonation of the vector results in an accumulation of positive charge within the endosome. This is accompanied by an influx of chloride ions, via membrane channels, in an attempt to maintain electroneutrality of the biological system. However, the accumulation of anions encourages excessive diffusion of water molecules, and causes swelling of the endosome. Once the osmotic pressure within the endosome rises beyond levels tolerable by the endosomal bilayer, it ruptures, releasing the vector to the cytosol. This phenomenon, summarised in Figure 1.17, is known as the proton sponge effect (Putnam *et al*., 2001).

Table 1.7: Non-viral vectors that exhibit intrinsic proton sponge capacity.

Gene carrier	**Reference**
Polyethylenimine	Akinc *et al*., 2005
Lipopolyamines	Behr, 1997
Polyamidoamine dendrimers	Pichon *et al*., 2010
Histidylated oligolysines	Pichon *et al*., 2000

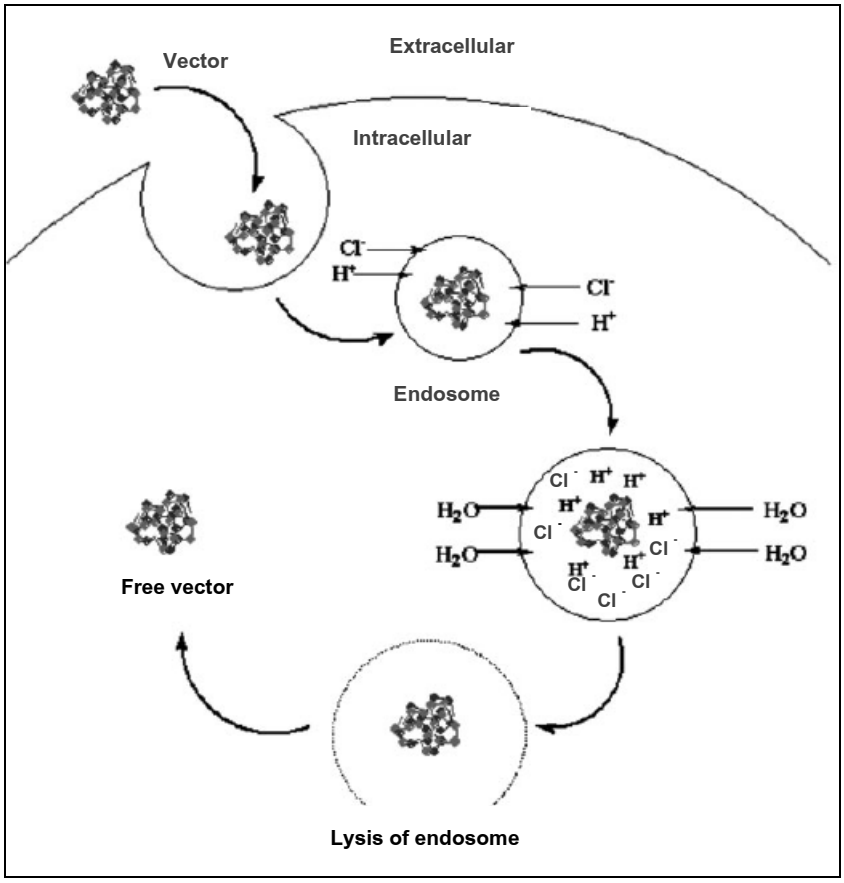


Figure 1.17: The proton sponge mechanism (adapted from Pathak *et al*., 2009).

While the use of certain polymers as gene transfer agents is often limited by cytotoxic effects, their proton sponge capacity has been profitably harnessed in combination with liposome-based strategies. As an illustration, Bandyopadhyay and colleagues (1998) have demonstrated the efficacy of second generation vectors, lipopolyplexes, in modulating PEI-induced toxicity in liver parenchymal cells. A targeted cationic liposome, formulated using DOTAP, DOPC

and galactocerebroside, was used to encapsulate PEI/DNA complexes. This vector construct exhibited an approximate two-fold increase in exogenous gene expression in cultured human hepatocytes when compared to the transfection activity of the polyplex alone. However, in recent years, liposomes have been endowed with proton sponge capability by way of incorporating pH-sensitive lipids in their formulations (Midoux *et al*., 2009). The discussion to follow pays attention to this emerging trend.

1.6.2.2.1 Imidazole-modified lipids

The amphoteric heterocycle, imidazole, has pKa of approximately 6.0 (Figure 1.18), which falls within the acidity range of the endosomal lumen. This aromatic compound is therefore able to induce rupture of the endosome via the proton sponge mechanism. Consequently imidazole and imidazole-containing compounds, notably the amino acid histidine, have received much attention in lipid design as a means of introducing endosome-destabilising functions into liposome formulations (Midoux *et al*., 2009).

Among the earliest attempts in this regard, is the design of a pH-responsive liposomal system; following the synthesis of three novel lipids, having tethered the imidazole ring to either cholesterol (Figure 1.19a), 1,2-dioleoyldeoxyglycerol or 1,2-dipalmitoyldeoxyglycerol as hydrophobic skeletons. Importantly, imidazole-conferred proton sponge capability was confirmed by a marked reduction in the *in vitro* transfection activity of such liposomes in the presence of bafilomycin A1, which inhibits endosomal acidification (Budker *et al*., 1996; Midoux *et al*., 2009).

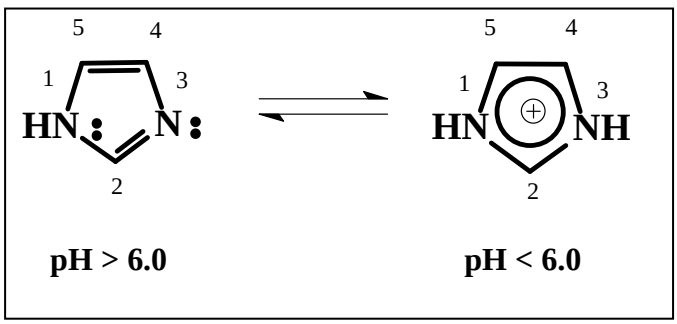


Figure 1.18: pH-dependent protonation of N_3 of imidazole ($C_3H_4N_2$) (adapted from Palleros, 2010).

In view of wide-spread interest in improving the efficacy of cationic liposomal gene transfer, the imidazole moiety has been applied to the preparation of a new class of helper lipids. These lipids were constructed from a lipophosphoramidate skeleton, with a headgroup composed of either a histidine methyl ester (Figure 1.19b) or histidine residue. *In vitro* experiments revealed that the novel co-lipids enhanced the transfection capacity of lipophosphoramide cytofectins, by a factor of up to 350, when combined in equimolar ratios. Furthermore, these liposomal systems achieved levels of reporter gene expression superior to those designed using DOPE (Mével *et al*., 2008).

Alternatively, chemical elaboration has presented the possibility of integrating the proton sponge-inducing moiety within the cytofectin. As an example, Kumar and colleagues (2003) appended a single histidine residue to the headgroup of twin-chain cationic amphiphiles (Figure 1.19c). The combination of such lipids with twice the molar quantity of cholesterol demonstrated a hundred-fold greater transfection activity in the HepG2 cell line when compared with two commercially available cationic liposome formulations. Furthermore, the fluorescence resonance energy transfer (FRET) technique was used to show that the histidine residue of the cationic lipids mediates membrane fusion within the pH range of 5 – 7 which, in addition to the proton sponge effect, contributes to endosome destabilisation and vector release. In a related study, a series of membrane-compatible amphiphiles was synthesised by the covalent grafting of multiple histidine residues to the hydrophilic end of a cationic cholesterol derivative. While it was concluded that there is no linear relationship between the number of histidine residues in the headgroup and the transfection potential of this class of lipids; the introduction of liposomes prepared from mono- and tri-histidylated derivatives, in several cell lines, produced remarkably high levels of reporter gene expression in the presence of up to 50 % serum (Karmali *et al*., 2006).

More recently, Shigeta and coworkers (2007) incorporated a histidine residue into an existing galactosylated cationic cholesterol derivative (Gal-C4-Chol), to afford a multifunctional lipid, Gal-His-C4-Chol (Figure 1.19d); which displayed integrated properties of hepatocyte-specificity and endosomal pH-sensitivity. Liposomes formulated using the histidylated amphiphile, mediated significantly higher luciferase activity, in human hepatoma cells, than those formulated from the parent lipid, at all lipoplex charge ratios investigated.

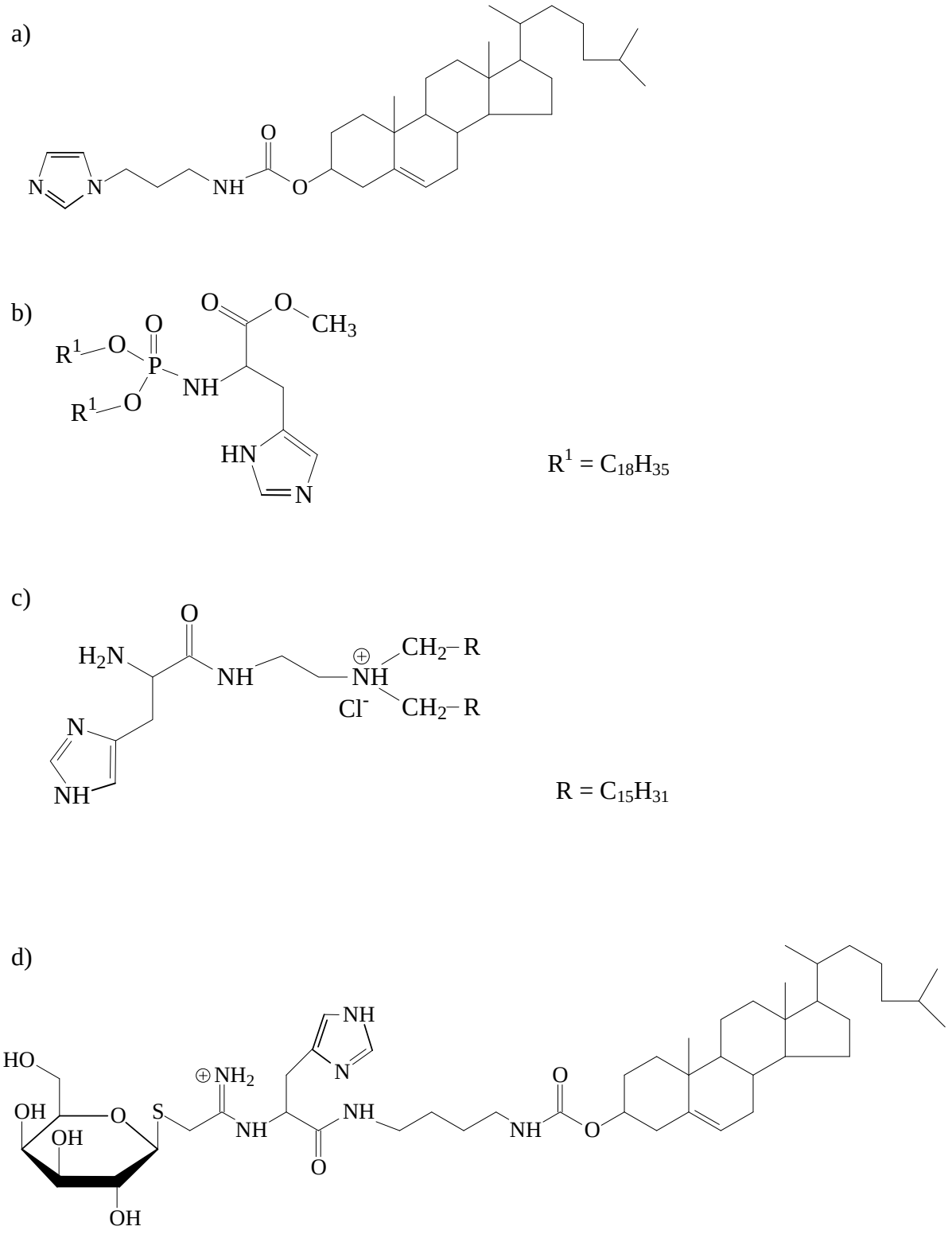


Figure 1.19: Endosomal pH-sensitive lipids with imidazole groups, a) Cholesterol-(3-imidazol-1-yl-propyl) carbamate (ChIm) (Midoux *et al*., 2009); b) A neutral imidazole-lipophosphoramidate (Mével *et al*., 2008); c) L-histidine-(*N*,*N*-di-n-hexadecylamine)ethylamide) (Kumar *et al*., 2003); and d) Gal-His-C4-Chol (Shigeta *et al*., 2007).

1.7 Outline of research

Galactosylated liposomes that are directed towards the ASGP-R, nearly-exclusive to hepatocytes, represent a well-documented, liver-specific gene transfer modality. Despite numerous advances in this regard, a feasible targeting strategy alone is not sufficient to merit the eventual application of such methods to the routine treatment of liver disease. In fact, several issues that influence the successful transfer of liposome-associated therapeutic genes to hepatocytes remain to be addressed (Wu *et al*., 2002). These include adverse serum-lipoplex interactions which are responsible for vector aggregation and rapid clearance by the reticuloendothelial system; and damage to the internalised vector within the acidic endosomal compartment and subsequently, enzyme-rich lysosome. The aforementioned effects have been attenuated with the advent of stealth liposomes that possess an external corona of steric-stabilising polyethylene glycol chains. In addition, liposomes have been modified with a variety of agents that possess proton sponge capability in order to facilitate early escape of vector constructs from the endosome. However, a hepatotropic liposomal system with propensity to evade both, recognition by the reticuloendothelial system; and damage within the endosome has not hitherto been designed.

Therefore, this study was aimed at investigating the combined effect of stealth, proton sponge and hepatocyte-targeting functions in a cationic liposome formulation. To this end, the study entailed the synthesis of two novel cholesterol derivatives for incorporation into pegylated and non-pegylated cationic liposomes. These lipids were designed to incorporate either an imidazole ring, to serve as a proton sponge; or a galactose moiety to afford hepatocyte-specificity. At the outset, the ability of each preparation to bind and protect DNA was assessed. In addition, liposomes and lipoplexes were characterised by transmission electron microscopy and dynamic light scattering (DLS)-based methods. Finally the cytotoxicity, transfection activity and hepatotropic potential of individual liposome preparations were evaluated using cultured human cell lines. In each case, the properties of the novel liposomal carriers were compared with liposomes prepared having omitted either the galactose- and/or imidazole-bearing lipid.

CHAPTER TWO

SYNTHESIS OF NOVEL CHOLESTEROL DERIVATIVES

2.1 Introduction

Membrane-compatible amphiphiles are the fundamental constituents of liposomes. Therefore, the development of liposomal systems, both for drug and gene delivery applications, is largely dependent on advances in lipid design and synthesis. This chapter details the synthesis of two novel cholesterol derivatives, based on the principles of chemical peptide synthesis, for the formulation of hepatotropic liposomes with proton sponge capability.

2.1.1 Cholesterol as a component of liposomal systems

Cholesterol (Figure 2.1) is a natural component of mammalian membranes, and constitutes approximately 30 % by weight. Although unable to form bilayers in isolation, this membrane active sterol influences membrane organisation and modulates its fluidity (Barenholz, 2002; Horton *et al*., 1996). Therefore, in early studies, cholesterol was employed to control the permeability of drug-loaded liposomes (Kirby *et al*., 1980); and as a helper lipid upon the advent of the cationic liposome (Battacharya and Haldar, 1996). Later, the planar ring system was exploited as a hydrophobic anchor, for the attachment of functional groups that confer useful properties upon liposomal carriers. A notable example thereof is presented by the work of Gao and Huang (1991; 1993). This group designed the cationic lipid DC-Chol, having appended a tertiary amino group to cholesterol via an ethyl spacer and carbamoyl bond; and initiated the development of a new class of cytofectins based on the cholesterol ring system. To date, several similar lipids in combination with neutral lipids have afforded stable cationic liposomes with more favourable cytotoxicity profiles and transfection activity than dialkyl-anchored cytofectins (Gao and Hui, 2001; Kisoon *et al*., 2002; Singh and Ariatti, 2006). Progress made in this regard led to the investigation of ionisable entities such as basic amino acids (Li *et al*., 2011), various heterocyclic (Gao and Hui, 2001) and guanidinium groups (Vigneron *et al*., 1996), as cationic substituents on the sterol ring. In addition, a study by

Bajaj and coworkers (2007) showed that cholesterol-based gemini lipids, which comprise two lipids separated by a spacer, are more potent cytofectins than their monomeric counterparts.

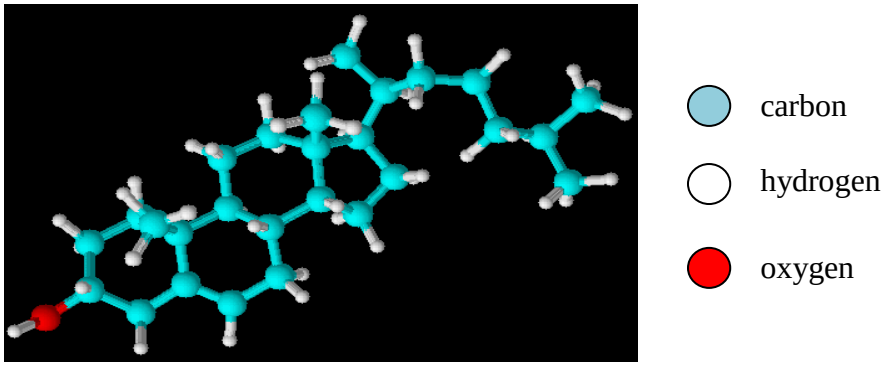


Figure 2.1: A ball and stick model of cholesterol.

In recent years, receptor-mediation, as a means of targeting liposomes to desired cell types, has become a prominent theme in the literature. To this end, cell-specific ligands have been displayed from the liposomal bilayer, having attached such entities to the cholesterol ring. These include galactose (Kawakami *et al*., 1998; Singh *et al*., 2007), mannose (Kawakami *et al*., 2002) and folate (Sunamoto and Ushio, 2006) to direct liposomes to receptors expressed at high density by hepatocytes, macrophages and tumour cells respectively.

Furthermore, the use of cholesterol as a scaffold for steric stabilisers (Boomer *et al*., 2009) and endosome-disrupting agents in liposomal systems (Midoux *et al*., 2009) has been reported. Other studies have employed cholesterol derivatives constructed with functional groups that induce destabilisation and release of liposomal contents under conditions prevalent at the target site (Davis and Szoka, 1998).

It is therefore evident that liposomes may be conveniently engineered to fulfil specific roles, and possibly overcome many of the challenges associated with liposomal gene transfer, through the use of appropriately designed cholesterol-based amphiphiles.

2.1.2 Carbodiimide coupling reagents

Chemical processes which facilitate the linkage of the carboxyl- and amino-termini of individual compounds via the amide bond have received great attention since the work of Emil Fischer in the early 1900s, largely due to their application in the preparation of synthetic peptides (Bodanszky *et al*., 1976). Methods developed to forge amide bonds, which do not form spontaneously except at elevated temperatures, are based on enhancing the electrophilicity of the carbonyl carbon of the acid, rendering it susceptible to nucleophilic attack by the amino group (Bodanszky, 1988). Among them, the use of carbodiimide coupling reagents, first proposed in 1955 by Sheehan and Hess, remain well-documented (Han and Kim, 2004).

The carbodiimides belong to a large class of compounds, known as the heterocumulenes. These possess unsaturated groups based on the allene structure (Williams and Ibrahim, 1981). Carbodiimide-mediated coupling is initiated by the highly electrophilic central carbon atom of the diimide group, which reacts preferentially with carboxyl groups, forming active *O*-acylisourea intermediates. As shown in Figure 2.2, route A, the electron withdrawing capacity of the N=C moiety contributed by the coupling agent, allows for direct nucleophilic attack by the amine-bearing compound which results in the formation of the amide linkage. In the process the carbodiimide, that has strong dehydrating ability, is converted to a urea byproduct. Alternatively (Figure 2.2, route B), the reaction may proceed via a symmetrical anhydride that forms due to interactions between the active intermediate and an unreacted carboxyl group. The anhydride subsequently acylates the amine (Bodanszky *et al*., 1976). However, the latter pathway is prevalent only in instances where the carboxylic acid is in excess relative to the coupling reagent (Williams and Ibrahim, 1981).

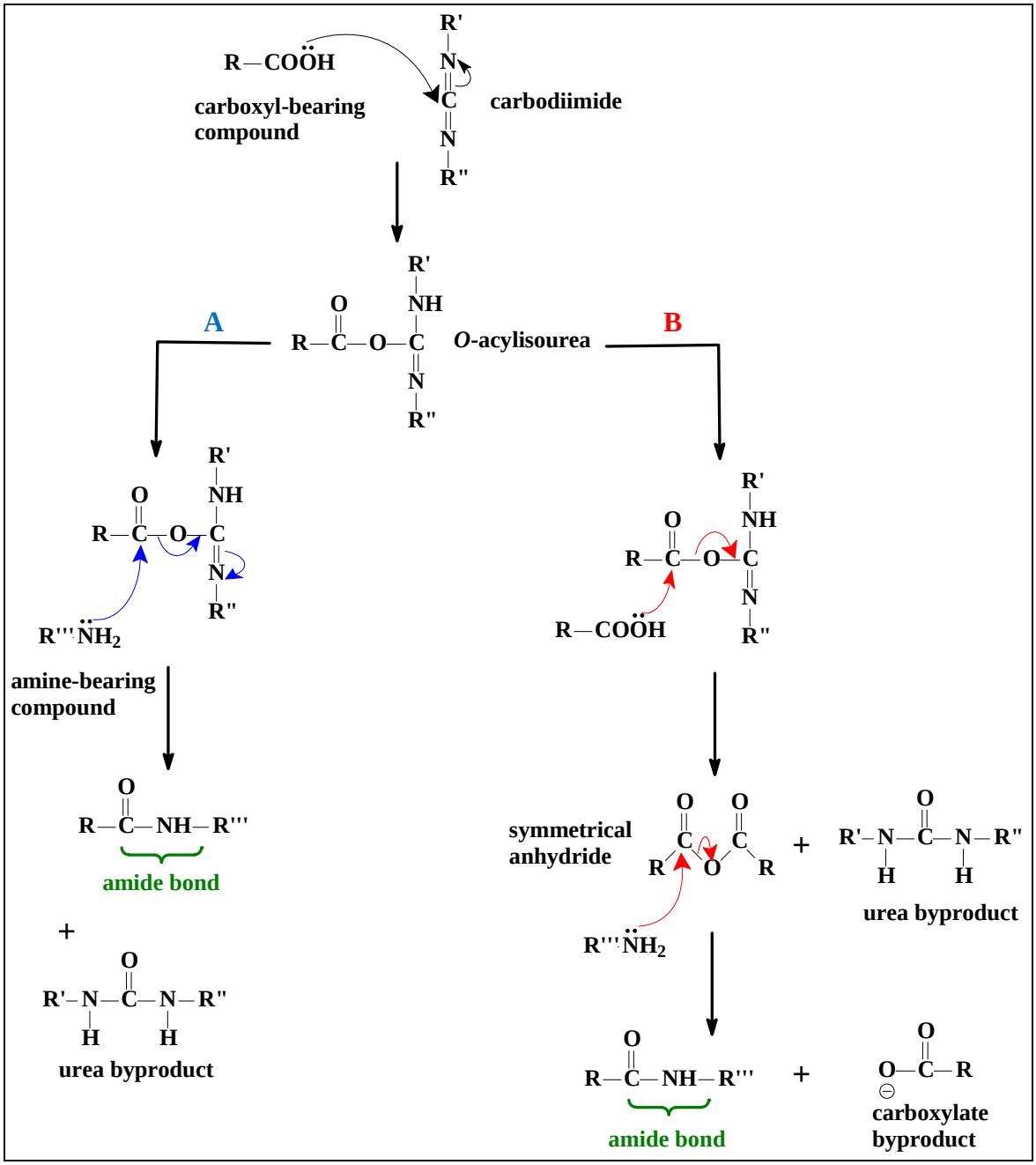


Figure 2.2: Carbodiimide-mediated coupling reactions. Synthesis of the amide bond may proceed either via the formation of A) an *O*-acylisourea intermediate alone, or B) a subsequent anhydride intermediate, (adapted from Bailey, 1990).

In recent years, carbodiimide-mediated coupling has extended beyond chemical peptide synthesis, having been employed in the modification of other biomolecules. These include lipids (Karmali *et al.*, 2006; Kumar *et al.*, 2003; Wang *et al.*, 2006) and polymers (Du *et al.*, 2011; Kim *et al.*, 2003; Lin *et al.*, 2011, Roy *et al.*, 2003) that have been incorporated in the design of non-viral gene carriers. Although several carbodiimides have been synthesised and

modified, *N*, *N′*-dicyclohexylcarbodiimide (DCCI), represented in Figure 2.3, is arguably the most popular (Han and Kim, 2004), and is the coupling agent of choice in this study. DCCI rapidly facilitates concurrent activation and coupling when introduced into a mixture of the carboxyl- and amine-bearing compounds. Other synthetic routes entail the use of DCCI in the generation of active carboxylic acid esters, to which the amino component is added at a later stage. In general, DCCI-mediated couplings rapidly give high yields especially when used in conjunction with strategies which limit undesired reactions with other functional groups on the starting material. Finally, the urea byproduct of DCCI reactions is insoluble in most organic solvents, and is easily eliminated by filtration following product formation (Bodanszky, 1988).

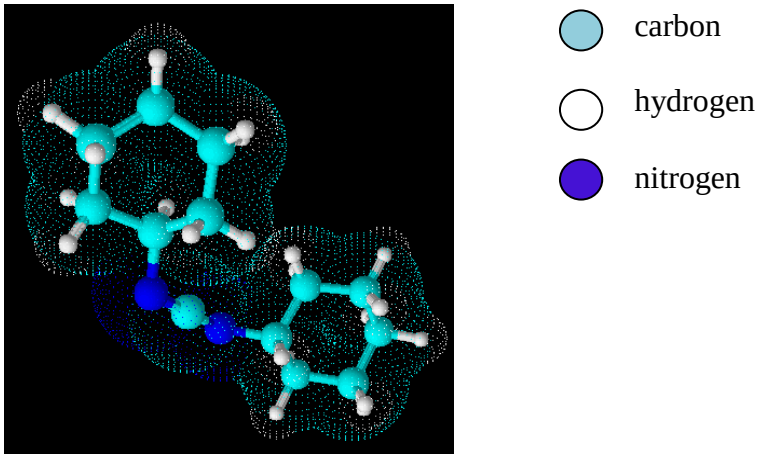


Figure 2.3: A ball and stick model of DCCI.

2.2 Materials and methods

2.2.1 Materials

Cholesterylchloroformate, hydrazine monohydrate (60 % hydrazine), lactobionic acid (97 %) and *N*-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich, St. Louis, USA. Urocanic acid (99 %), tritylchloride and *N*, *N′*-dicyclohexylcarbodiimide (DCCI) were obtained from Sigma-Aldrich, Germany. Triethylamine and silica gel $60F_{254}$ chromatography plates were supplied by Merck, Darmstadt, Germany. All other reagents were of analytical grade.

2.2.2 Methods

2.2.2.1 Synthesis of cholesterylformylhydrazide (SH01)

Cholesterylformylhydrazide was prepared according to the method published by Singh and Ariatti (2006).

Briefly, a solution of hydrazine (240 mg, 7.5 mmol) in $CHCl_3$:MeOH (3:0.6 ml) was added dropwise, with stirring, to an ice-cold solution of cholesterylchloroformate (1.13 g, 2.5 mmol) in $CHCl_3$ (5 ml). The reaction was maintained at room temperature for 2.5 hours, during which it was periodically monitored by TLC on silica gel $60F_{254}$ plates developed in $CHCl_3$:MeOH (95:5, v/v). Cholesterol derivatives appeared as purple spots after the plate was treated with 10 % H_2SO_4 and heated to approximately 60 ˚C. The crude product was deposited as a white crystalline mass after rotary evaporation (Rotavapor-R, Büchi) of the solvent. This was recrystallised from $CHCl_3$:MeOH (approximately 4:1, v/v). Liquors were collected and subjected to further recrystallisation. Finally 927 mg of product (83.4 % yield) was obtained. R_f (retention factor) = 0.56. mp (melting point), (first crop): 211 – 213 ˚C. mp (second crop): 217 – 219 ˚C.

2.2.2.2 Synthesis of lactobionylcholesterylformylhydrazide (SH02)

Lactobionic acid (107 mg, 0.3 mmol), which was dried *in vacuo*, and SH01 (111 mg, 0.25 mmol) were dissolved, with heating, in pyridine (2 ml). Thereafter, a solution of DCCI (85 mg, 0.4 mmol) in pyridine (0.5 ml) was introduced. Product formation was monitored by TLC on silica gel $60F_{254}$ plates in $CHCl_3$:MeOH:H_2O (6:4:1, v/v/v) as recommended by Wang *et al*. (2006), R_f = 0.39. Components of the reaction mixture were observed after spraying the plate with 10 % H_2SO_4, followed by heating. After 6 days at room temperature, the solution was filtered to remove dicyclohexylurea (DCU) crystals and concentrated to a foam *in vacuo*. Residual pyridine and DCCI were eliminated by co-evaporation with toluene and extraction with petroleum ether (60 – 80 ˚C) respectively. Thereafter, the crude product was evaporated to dryness. Water was added to the residue, in order to extract unreacted

lactobionic acid, whereupon a gel was formed. The gel was centrifuged at 3000 rpm (Eppendorf 5702R centrifuge, Merck, Darmstadt, Germany) for 5 minutes. After discarding the supernatant, a slurry remained. This was dried *in vacuo*, with $CHCl_3$:MeOH (approximately 1:1, v/v) and $CHCl_3$:EtOH:MeOH (undefined ratios) respectively, to an off-white powder (102 mg, 52 % yield). Approximately 20 mg of product was purified on a silica gel 60 column (7.95 cm^3), eluted with $CHCl_3$:MeOH (4:1, v/v), $CHCl_3$:MeOH (6:4, v/v) and $CHCl_3$:MeOH:H_2O (6:4:1, v/v/v) respectively. Fractions of approximately 3 ml each were collected and analysed by TLC using the corresponding solvent system. Finally, fractions exhibiting greatest product purity were combined and evaporated to dryness, to afford 17 mg of SH02. mp: 188.5 ˚C (decomp.).

2.2.2.3 Synthesis of urocanylcholesterylformylhydrazide (SH04)

The coupling of urocanic acid to the cholesterol anchor molecule, SH01, was achieved in several steps.

2.2.2.3.1 Preparation of the diethylammonium salt of *N*-tritylurocanic acid

This protocol is a modified version of that which was employed by Cloninger and Frey (1998) for the synthesis of the dibutylammonium salt of *N*-tritylurocanic acid.
Urocanic acid (276 mg, 2 mmol) was partially dissolved in dry dimethylformamide (DMF) (7 ml). Upon introduction of triethylamine (1.09 g, 10.8 mmol) and tritylchloride (1.22 g, 4.4 mmol) a homogeneous solution was obtained. This was stirred at room temperature for 24 hours. High Performance Liquid Chromatography (HPLC)-grade methanol (7 ml) was added, and the solution stirred for an additional 24 hours, after which it was dried to a brown oil under vacuum. The oil was diluted with ethylacetate, until the solution separated into two layers. Following *in vacuo* concentration of the organic layer, diethylamine (146 mg, 2 mmol) was added, and the solution stored overnight at 4 ˚C. The product was obtained as crystals, which were repeatedly washed with ethylacetate and hexane. The washings were collected and dried under vacuum, followed by the addition of hexane, in order to increase product yield. Eventually 954 mg (quantitative yield) of the diethylammonium salt of *N*-tritylurocanic acid was recovered.

2.2.2.3.2 Preparation of *N*-tritylurocanic acid

The diethylammonium salt of *N*-tritylurocanic acid (408.3 mg, 0.9 mmol) was dissolved, with heating, in ethanol (15 ml), and mixed with distilled water (15 ml). A pellet of NaOH (52.1 mg, 1.3 mmol) was introduced to displace diethylamine, which was subsequently expelled under mild vacuum. The solution was acidified to a pH of approximately 5, by the dropwise addition of glacial acetic acid. Thereafter, excess NaCl was added to induce precipitation of the free acid. The product, which was recovered as a white powder (270 mg, 79 % yield) after vacuum filtration, was rinsed with distilled water and dried for approximately 12 hours in a drying pistol. mp: 218 – 220 ˚C. TLC: R_f = 0.1 (silica gel $60F_{254}$ plates in $CHCl_3$:MeOH, 95:5, v/v).

2.2.2.3.3 Preparation of the *N*-hydroxysuccinimide ester of *N*-tritylurocanic acid

N-tritylurocanic acid (250 mg, 0.66 mmol), DCCI (135.8 mg, 0.66 mmol) and NHS (75.7 mg, 0.66 mmol) were dissolved in dry DMF (3.5 ml) and maintained at room temperature. TLC was performed on silica gel $60F_{254}$ plates in $CHCl_3$:MeOH (95:5, v/v), in order to monitor product formation (R_f = 0.84). The active ester was observed as a purple spot after spraying the plate with a mixture (2.3:1, v/v) of 14 % (w/v) hydroxylamine hydrochloride and 3.5 N NaOH; and subsequently, $FeCl_3$ in 1.2 N NaOH (5 % w/v). After 5 days, the solution was filtered to remove DCU crystals, and concentrated *in vacuo*. The residue was then dissolved in warm isopropanol, and stored at 4 ˚C overnight, whereupon the product was deposited as a film. The supernatant was discarded and the film dried under vacuum, first using a rotary evaporator and subsequently a drying pistol, to afford 261 mg of product as white, amorphous solids (83 % yield). mp: 99 – 103 ˚C.

2.2.2.3.4 Preparation of *N*-tritylurocanylcholesterylformylhydrazide (SH05)

SH01 (46.7 mg, 0.1 mmol) and the *N*-hydroxysuccinimide ester of *N*-tritylurocanic acid (50 mg, 0.1 mmol) were each dissolved in 0.5 ml $CHCl_3$. The solutions were combined and stored in the dark, at room temperature. The reaction was monitored by TLC on silica gel $60F_{254}$ plates developed in $CHCl_3$:MeOH (98:2, v/v) and, after 7 days, product was recovered

via preparative TLC. Briefly, the reaction mixture was applied to four 10 cm × 20 cm TLC plates which were developed in the above-mentioned solvent system. The TLC plates were viewed under ultraviolet light, so as to locate the position of the product. Silica gel at this position was scraped off, the product extracted into EtOH with heating, and the eluent evaporated to dryness under vacuum. This afforded 45.9 mg (54 % yield) of product as a white powder, with mp of 124 – 127 ˚C, and R_f of 0.39.

2.2.2.3.5 Detritylation of SH05

SH05 (approximately 40 mg) was dissolved in 500 µl each of $CHCl_3$ and AcOH, and maintained at 37 ˚C for 8.5 hours. Samples were periodically applied to silica gel $60F_{254}$ plates which were developed in $CHCl_3$:MeOH (95:5, v/v), in order to monitor the removal of the trityl group, whereupon the solution was evaporated to dryness. The released tritanol was extracted twice into 5 ml petroleum ether (60 – 80 ˚C), and the crude product deposited as a white powder after rotary evaporation. This was dissolved, with heating, in a mixture of $CHCl_3$ and MeOH (undefined ratios); and purified on two 10 × 20 cm silica gel $60F_{254}$ TLC plates, which were developed in $CHCl_3$:MeOH (85:15, v/v), to afford 16 mg (57 % yield) of SH04. mp: 232 – 235 ˚C; 227 – 228 ˚C (decomp.). R_f = 0.41.

2.2.2.4 Spectral analyses

^1H and ^{13}C NMR spectra were obtained on a Varian Gemini 300 instrument (Varian Inc., Palo Alto, CA.) at 300 MHz and 75 MHz respectively. ^1H chemical shifts were recorded relative to C_5H_5N (8.57 ppm) or $CHCl_3$ (7.24 ppm) and ^{13}C chemical shifts relative to C_5H_5N (135.5 ppm). Abbreviations for signal multiplicities are as follows: s (singlet), d (doublet), t (triplet), m (multiplet). Chemical shifts are reported for ^1H spectra as: shifts (multiplicity, integration, coupling constant, coupling assignment). Mass spectra were obtained on a Bruker ESI-(Q)TOF instrument operating in positive mode. Infrared (IR) spectra were obtained on a Nicolet Impact 420 spectrophotometer using a KBr disc technique (0.5 % dilution).

NMR and mass spectra are given in Appendices 1 and 2, respectively. Note that naming of both cholesterol derivatives and intermediates in their synthesis, according to IUPAC

nomenclature, is listed in Appendix 4. This was performed using ChemAxon software accessed via http://www.chemicalize.org.

2.3 Results and discussion

2.3.1 Spectral analyses

a) Lactobionylcholesterylformylhydrazide (SH02)
^{1}H NMR (C_5D_5N): cholesteryl moiety, δ = 0.64 (s, 3H, 18-H, C-C$\underline{H}_3$), 0.88 (d, 6H, J = 6.5 Hz, 26-H, 27-H, CH(C$\underline{H}_3$)$_2$), 0.92 (d, 3H, 6.5 Hz, CH-C$\underline{H}_3$); lactobionyl 4.10 – 5.50 (m, 13H, -C$\underline{H}$-). ESI-TOF *m/z*: 807.4625 ($C_{40}H_{68}N_2NaO_{13}$: 807.4614, $(M + Na)^+$).

b) *N*-hydroxysuccinimide ester of *N*-tritylurocanic acid
^{1}H NMR ($CDCl_3$): succinimide δ = 2.84 (bs, 4H, -C$\underline{H}_2$-C$\underline{H}_2$-); urocanyl 6.69 (d, 1H, J = 15 Hz, 2-H), 7.26 (s, 1H, 5´-H), 7.73 (d, 1H, J = 15 Hz, 3-H), 7.52 (s, 1H, 2´-H); trityl 7.12 (m, 6H, ortho), 7.36 (m, 9H, meta, para).

c) *N*-tritylurocanylcholesterylformylhydrazide (SH05)
^{1}H NMR ($CDCl_3$): cholesteryl moiety, δ = 0.67 (s, 3H, 18-H, C-C$\underline{H}_3$), 0.86 (d, 6H, J = 6.5 Hz, 26-H, 27-H, CH(C$\underline{H}_3$)$_2$), 0.91 (d, 3H, J = 6.5 Hz, CH-C$\underline{H}_3$), 4.56 (m, 1H, 3_α-H), 5.36 (m, 1H, 6-H); urocanyl 6.62 (d, 1H, J = 15 Hz, 2-H), 7.00 (s, 1H, 5´-H), 7.46 (s, 1H, 2´-H), 7.53 (d, 1H, J = 15 Hz, 3-H); trityl 7.12 (m, 6H, ortho), 7.35 (m, 9H, meta, para). ESI-TOF *m/z*: 807.5140 ($C_{53}H_{66}N_4O_3$: 807.5162), 565.4071 $(M – Tr + H)^+$.

d) Urocanylcholesterylformylhydrazide (SH04)
^{1}H NMR ($CDCl_3$): cholesteryl moiety, δ = 0.68 (s, 3H, 18-H, C-C$\underline{H}_3$), 0.87 (d, 6H, J = 6.4 Hz, 26-H, 27-H, CH(C$\underline{H}_3$)$_2$), 0.92 (d, 3H, J = 6.2 Hz, 21-H, CH-C$\underline{H}_3$), 4.54 (m, 1H, 3_α-H), 5.38 (m, 1H, 6-H); urocanyl 6.46 (d, 1H, J = 15 Hz, 2-H), 7.22 (s, 1H, 5´-H), 7.53 (d, 1H, J = 15 Hz, 3-H), 7.64 (s, 1H, 2´-H). ESI-TOF *m/z*: 565.4092 ($C_{34}H_{53}N_4O_3$: 565.4112, $(M + H)^+$).

2.3.2 Cholesterylformylhydrazide (SH01)

The hydrazide, SH01 (Figures 2.4a and 2.5a), was prepared as a synthon in the design of novel, bifunctional membrane-compatible lipids. The molecule bears the cholesterol ring system that has been widely documented as a suitable lipid anchor. Furthermore, the primary amino group, known for its strong nucleophilic properties and general high reactivity, provided a convenient means of appending functional groups, such as the ASGP-R- targeting and endosome-destabilising moieties, which were essential to the design of the liposomal vector system investigated (Bruice, 2007; Singh and Ariatti, 2006).

The synthesis of SH01, outlined in Figure 2.6, was achieved using a three-fold molar excess of hydrazine. This was intended to prevent the formation of higher homologues from the attachment of a cholesterylchloroformate molecule to each of the reactive amines of hydrazine. As a base, the excess hydrazine may react with hydrochloric acid that is generated as a byproduct, to yield mono and/or dihydrochloride salts of hydrazine which, like the product, appear as white crystalline solids. In order to confirm that the recrystallisation procedure had successfully eliminated such contaminants, the melting point of the product was determined. Hydrazine monohydrochloride and dihydrochloride melt at approximately 89 ˚C (Stratton and Wilson, 1932) and 198 ˚C (Abdel-Monem *et al*., 1975) respectively. However, the product melted at a higher temperature. Furthermore, observations using a light microscope revealed no changes, with respect to texture and optical properties, before the melting point was attained. It was noted, however, that crystals of the first and second crops of SH01, differed in appearance, birefringence, and melting point. This is not uncommon, as some impurities from the liquor may adhere to the crystals of the second crop, and alter its physical properties.

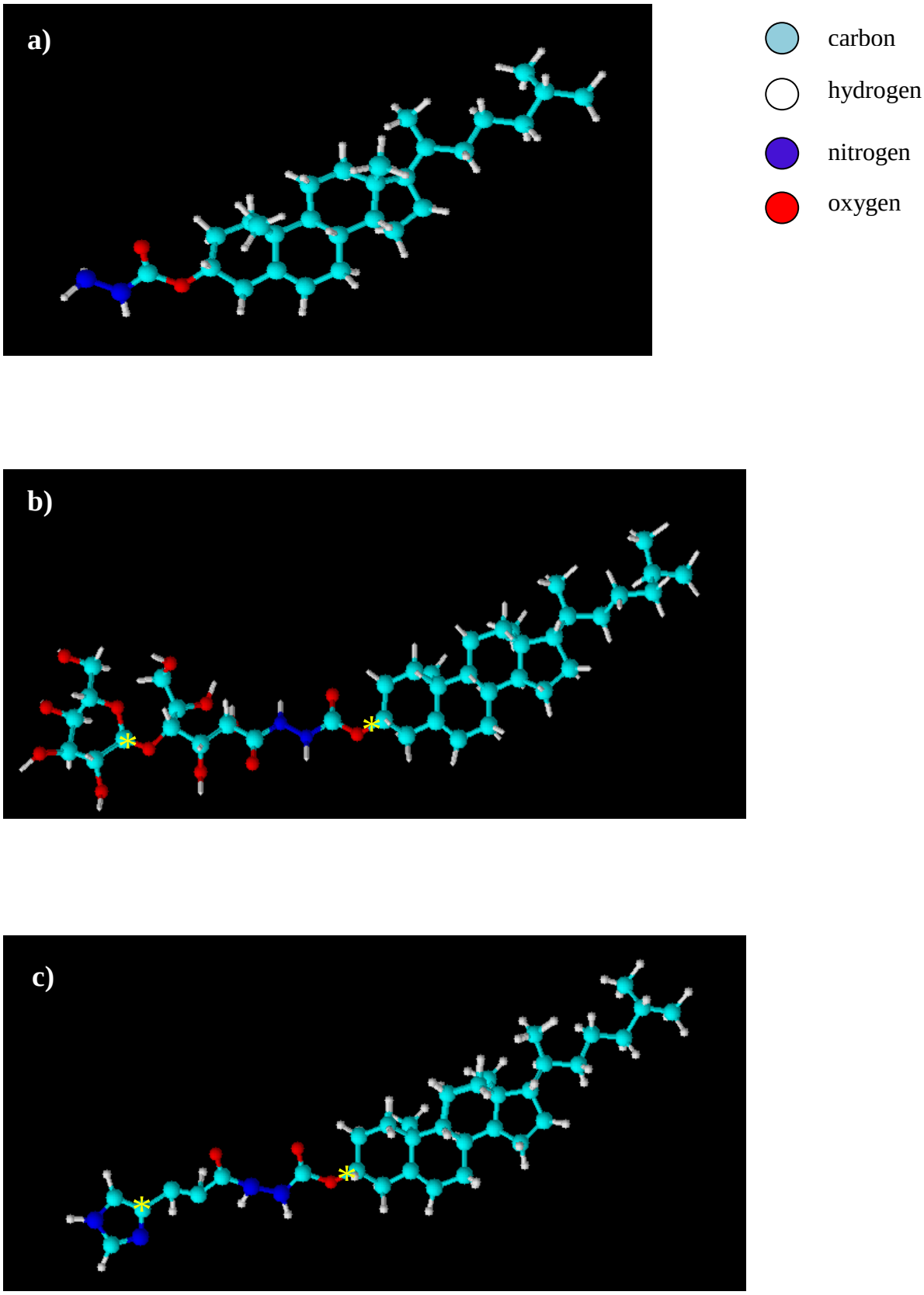


Figure 2.4: Ball and stick models of a) SH01, b) SH02 and c) SH04. Models were generated using ACD/3D Viewer software. The spacer lengths reported were measured as internuclear distances between the carbon atoms indicated with an asterisk.

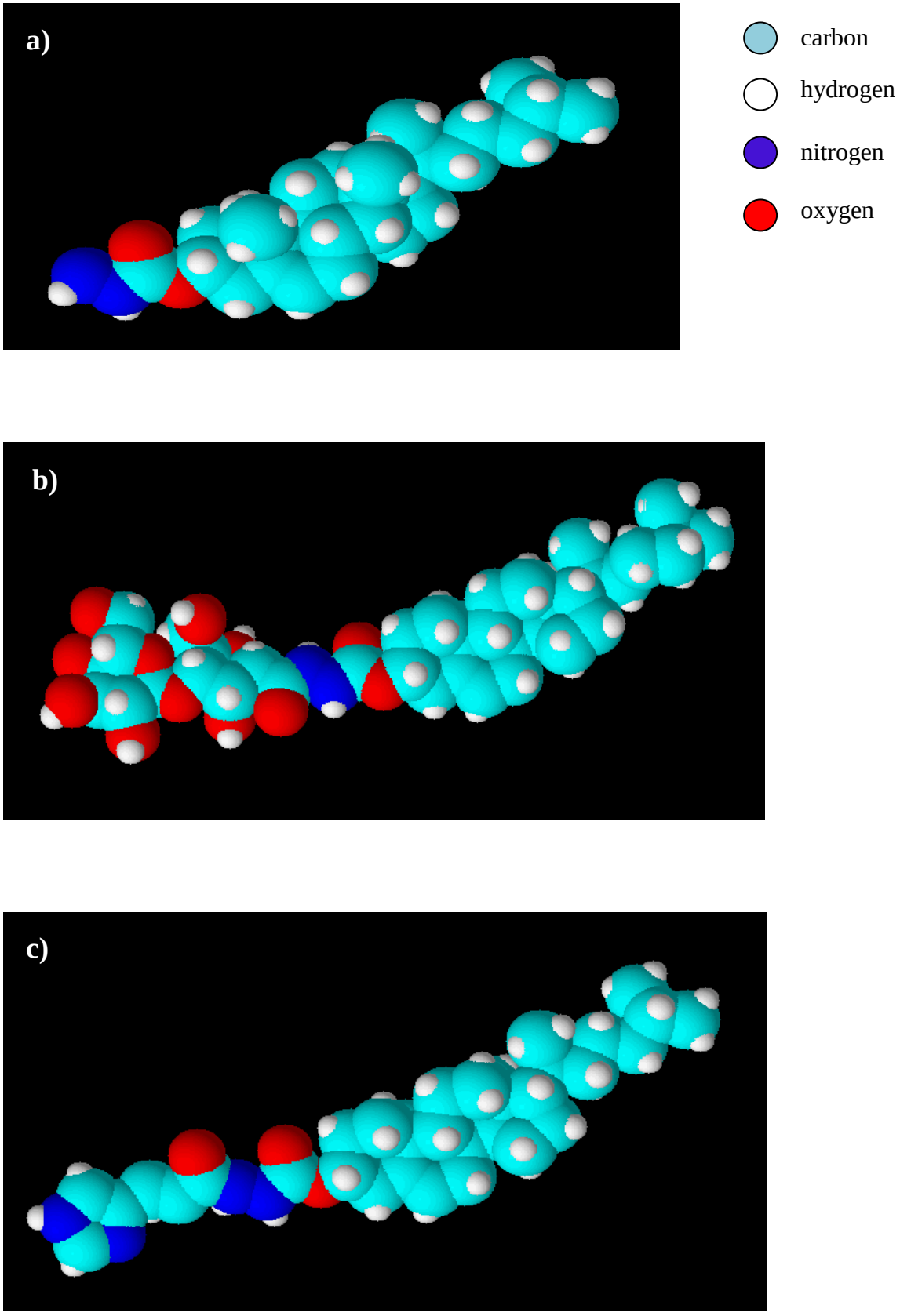


Figure 2.5: Spacefilling models of a) SH01, b) SH02 and c) SH04. Models were generated using ACD/3D Viewer software.

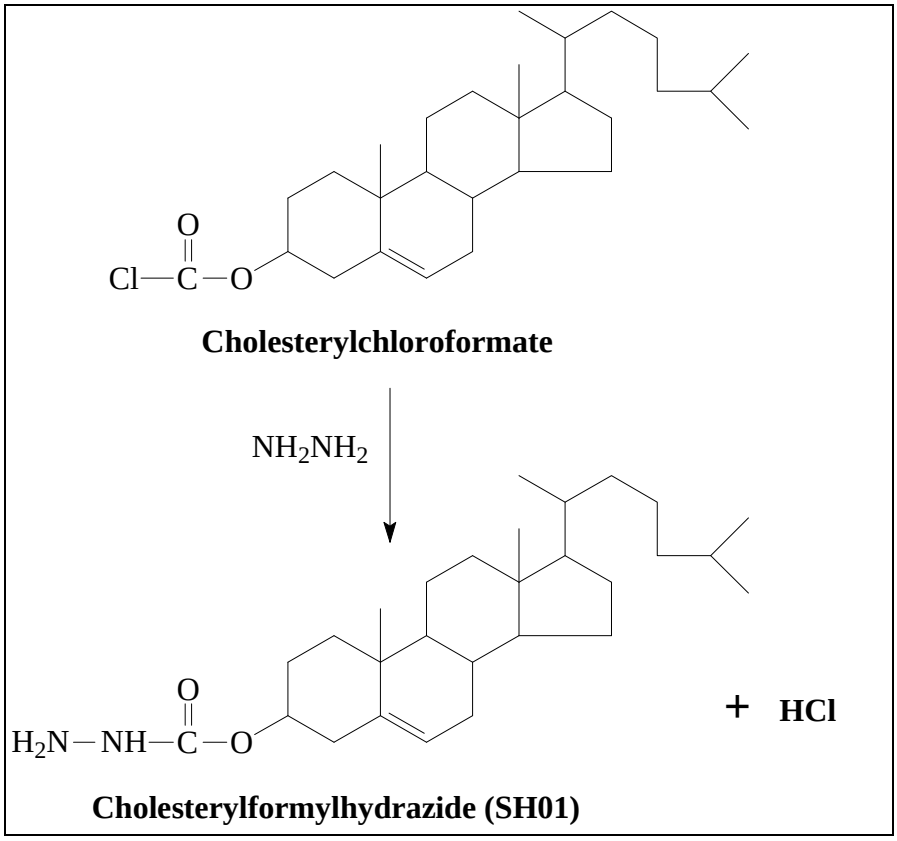


Figure 2.6: Synthesis of cholesterylformylhydrazide (SH01) from cholesterylchloroformate and hydrazine.

2.3.3 Lactobionylcholesterylformylhydrazide (SH02)

Lactobionic acid is the oxidised form of lactose. The molecule bears the galactose residue as the β-anomer, which is the configuration preferred by the ASGP-R (Singh *et al*., 2007). Due to its biocompatibility, lactobionic acid has been used as the galactosyl-bearing entity in several non-viral, hepatotropic gene carriers (Ghiamkazemi *et al*., 2010; Kim *et al*., 2005; Lin *et al*., 2011; Takahashi *et al*., 2009). In this study three strategies (Figure 2.7) for the synthesis of a novel galactosylated lipid, by the conjugation of lactobionic acid to cholesterylformylhydrazide (SH01), were explored.

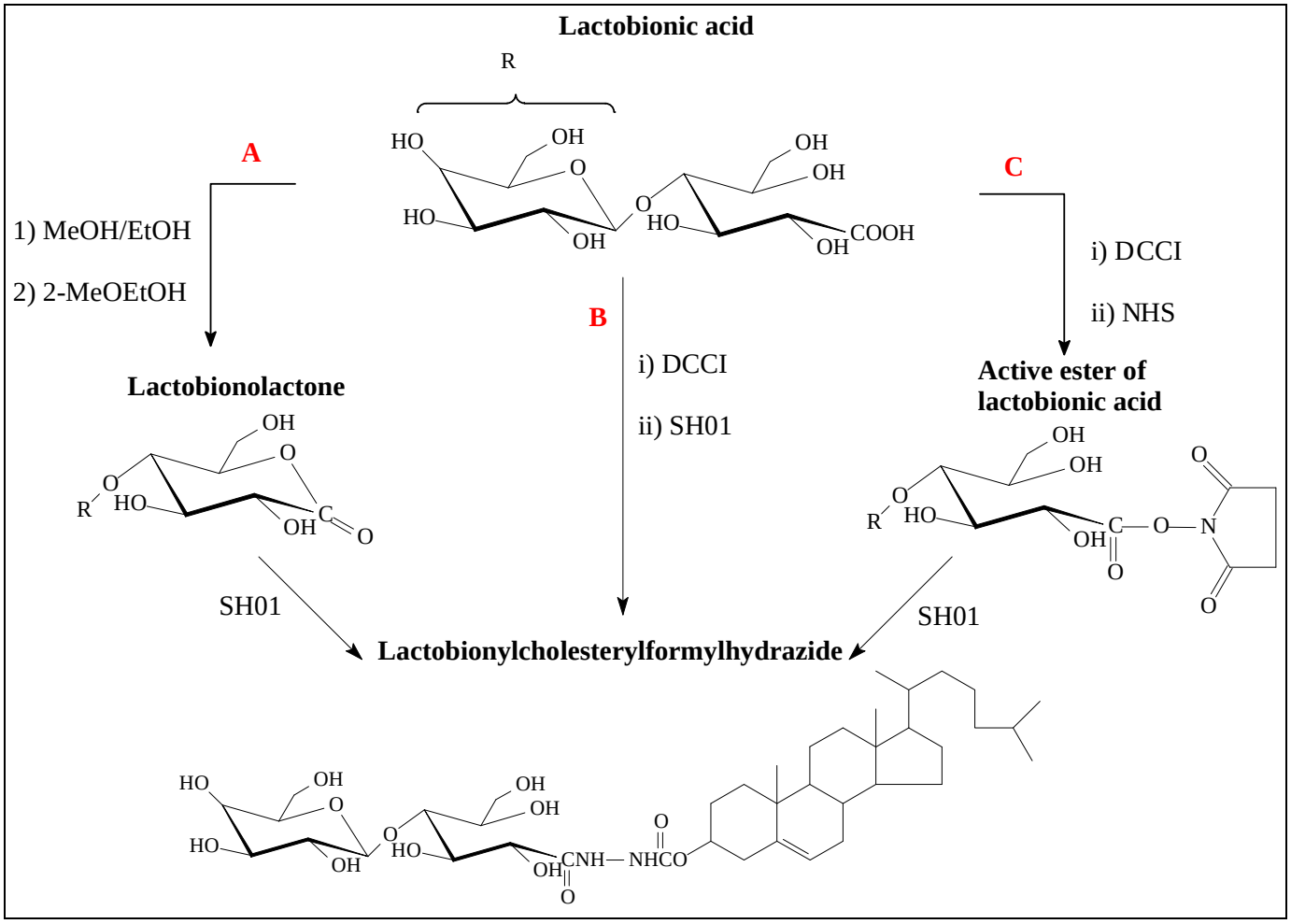


Figure 2.7: Possible strategies for the synthesis of lactobionylcholesterylformylhydrazide (SH02) from lactobionic acid and SH01. Synthetic routes investigated include A) dehydration of lactobionic acid to a lactone prior to reaction with SH01; B) direct coupling of lactobionic acid to SH01; and C) activation of the carboxyl group of lactobionic acid prior to reaction with SH01. Only route B proved successful.

It has been demonstrated that compounds, including lipids, which possess primary amino functions exhibit high reactivity towards lactones of aldobionic acids. This has served as the framework for the successful synthesis of several glycolipids displaying lactobionic acid headgroups (Grassert *et al*., 1997; Wang *et al*., 2006; Williams *et al*., 1979; Yu *et al*., 2007; Zhang *et al*., 1996). Therefore the conversion of lactobionic acid to lactobionolactone, prior to reaction with the primary amine-bearing SH01 was attempted (Figure 2.7, route A). Dehydration of lactobionic acid to its corresponding lactone was achieved by two methods: repeated evaporation of lactobionic acid from methanol and ethanol (designated Lactone 1) as recommended by Kim *et al*. (1996), Wang *et al*. (2006), and Yu *et al*. (2007); and, repeated

evaporation from 2-methoxyethanol and toluene (designated Lactone 3) according to Grassert *et al*. (1997) and Zhang *et al*. (1996). IR spectroscopy is diagnostic in terms of differentiating between the carbonyl moieties of the carboxylic acid and its corresponding lactone: stretching vibrations of the carbonyl group of the acid occur at a wavenumber of approximately 1650 cm^{-1}, while that of the lactone occurs near 1740 cm^{-1} (Wang *et al*., 2010). The IR spectra of Lactones 1 and 3 (refer to Appendix 3), displayed distinctive absorption bands at 1729.09 cm^{-1} and 1733.06 cm^{-1} respectively, which verified lactone formation via both methods.

The intramolecular ring closure of lactobionic acid due to dehydration in organic solvents may produce two different lactone species, each with either a five- or six-membered ring structure. The six-membered ring structure (lactobiono-1,5-lactone), which is the more reactive lactone species, forms rapidly at approximately 25 ˚C. In contrast, its five-membered counterpart, lactobiono-1,4-lactone, forms slowly, yet preferentially at higher temperatures (in excess of 50 ˚C), and demonstrates greater stability (Levene and Sobotka, 1927; Wang *et al*., 2010; Williams *et al*., 1979; Wolfrom, 1961). In this instance lactone preparation was effected at room temperature. Therefore, it is reasonable to assume that lactobiono-1,5-lactone is the major component of both Lactones 1 and 3.

Reactions were attempted with SH01 and a slight excess of the respective lactones, in a mixture of pyridine and DMF at room temperature. However, in spite of the presence of the reactive amine group, TLC revealed that Lactone 3 and SH01 did not react. Furthermore, the reaction of Lactone 1 with SH01 was extremely slow, displaying mere traces of product after 7 days; and attempts to encourage product formation by applying heat were unsuccessful.

The second synthetic route investigated (Figure 2.7, route C) entailed the activation of the weakly reactive carboxyl group of lactobionic acid with NHS. *N*-hydroxysuccinimide esters are generally very reactive towards compounds with primary amino groups, such as SH01 (Bailey, 1990; Bodanszky, 1988). Such strategies were recently documented by Huang *et al*. (2010) and Takahashi *et al*. (2009) for the galactosylation of polyamidoamine dendrimers. To this end, equimolar quantities of lactobionic acid, NHS and DCCI were dissolved in dry DMF, incubated at -15 ˚C for 90 minutes, stirred on ice, and set aside at room temperature. Despite the use of low temperature to discourage intramolecular ring closure of lactobionic acid molecules; and a minimal volume of solvent so as to promote interactions between the

carboxyl group of lactobionic acid and the hydroxyl group of NHS, TLC revealed that esterification did not occur.

Eventually, the direct coupling of lactobionic acid to cholesterylformylhydrazide, as outlined in Figure 2.8, produced the amphiphilic lipid, SH02 (Figures 2.4b and 2.5b), with a spacer arm of 12.4 Å between the galactose residue and cholesterol scaffold. Several authors have emphasised that the length of the spacer arm is an important feature of glycolipid design, as it influences both the display of the ligand on the liposome surface and its recognition by the ASGP-R. For example, Kawakami and coworkers (1998) conducted a comparative study with a series of galactosylated cholesterol derivatives. Competitive inhibition studies showed that cationic liposomes formulated with glycolipids designed with longer spacers were more effectively internalised by HepG2 cells via the ASGP-R. Conversely, Westerlind and coworkers (2004) reported that galactosides borne on short spacer elements (< 4 Å) were selectively bound to the galactose/fucose recognising GPr, an ASGP-R analogue that is exclusive to the liver macrophages (Rensen *et al*., 2001).

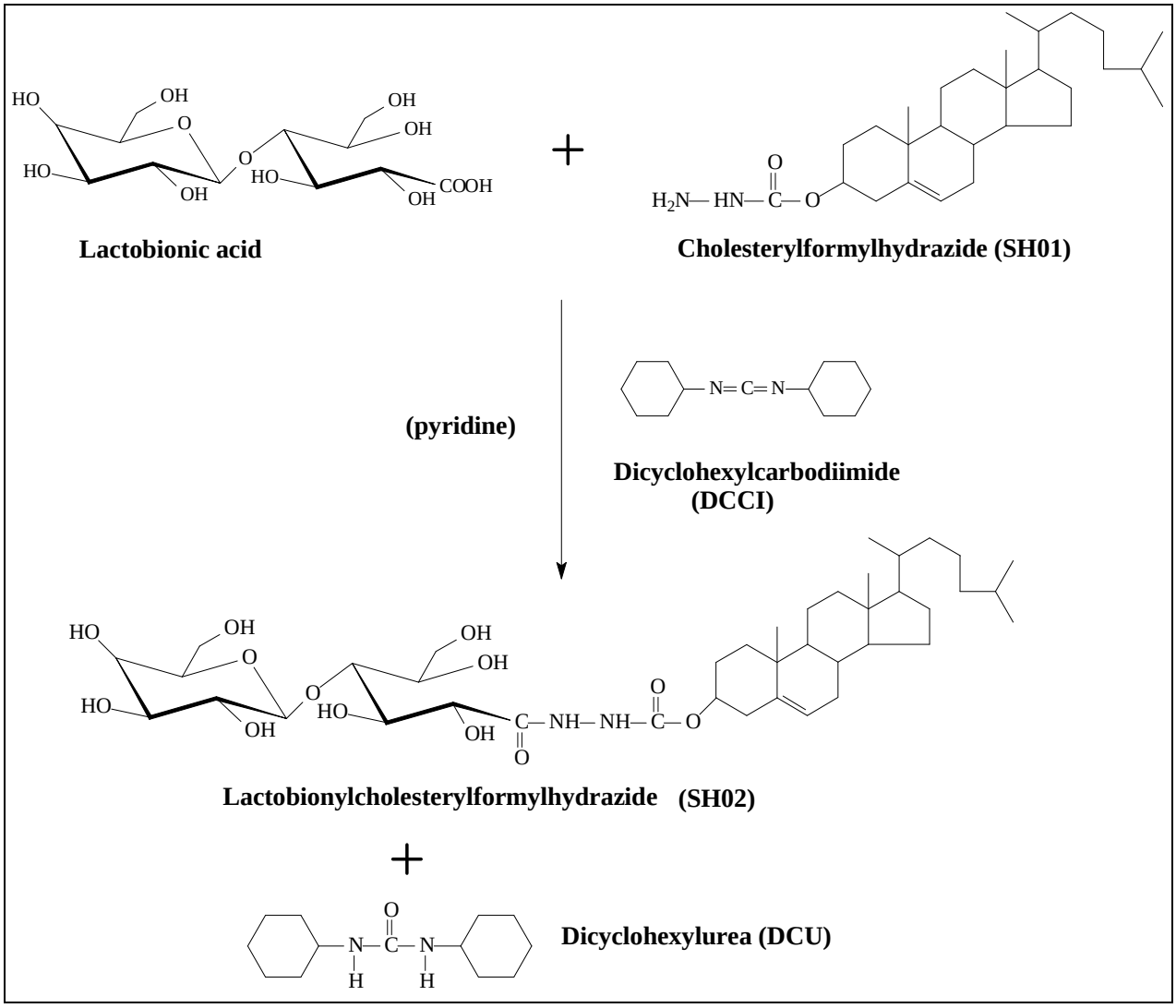


Figure 2.8: Synthesis of lactobionylcholesterylformylhydrazide (SH02) by the direct coupling of lactobionic acid to SH01.

It was initially believed that the synthesis of SH02 occurred by carbodiimide-mediated activation of the lactobionic acid carboxyl group, followed by nucleophilic attack of the SH01- amino group on the electron deficient carbonyl carbon atom of the *O*-acylisourea intermediate (Figure 2.9, route A). However, observations as per TLC (Figure 2.10) presented evidence for an alternative and/or additional mechanism. The dehydrating effect of the sulphuric acid applied, generates a colour characteristic of the class of compounds adhering to the plate: cholesterol and cholesterol derivatives are visualised as purple spots; while carbohydrate groups appear brown; with colour intensity indicative of concentration effects.

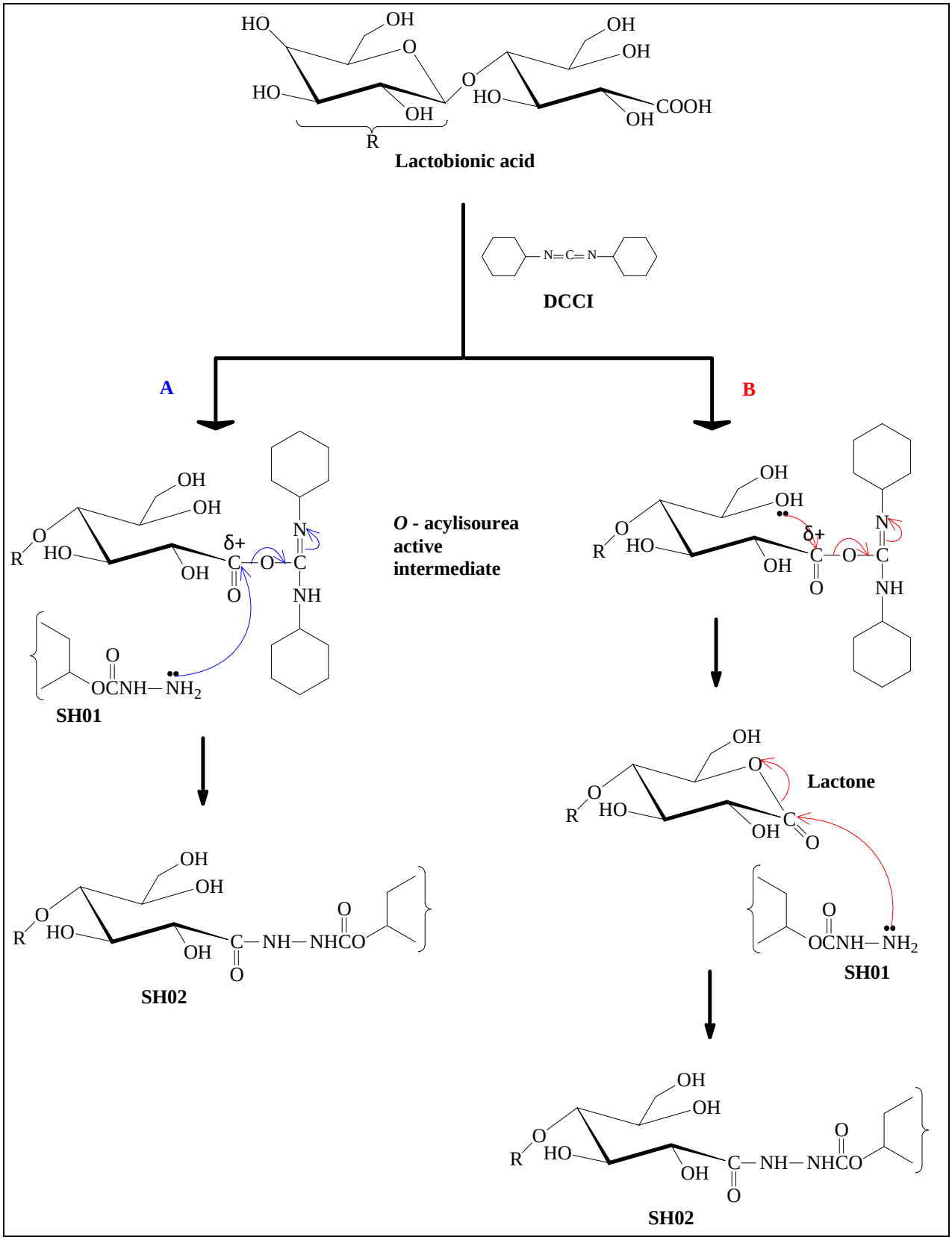
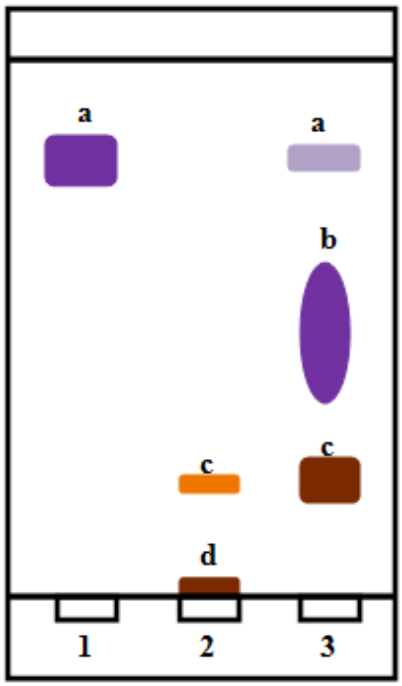


Figure 2.9: Possible mechanistic pathways for the reaction between lactobionic acid, DCCI and SH01 in the synthesis of SH02, A) SH01 carries out nucleophilic attack on the carbodiimide-activated lactobionic acid molecule; and B) DCCI facilitates dehydration of lactobionic acid to the lactone, which then undergoes nucleophilic attack by SH01.



Lane 1: SH01 in $CHCl_3$
Lane 2: Lactobionic acid dried *in vacuo*
Lane 3: Reaction mixture (lactobionic acid, DCCI and SH01 in pyridine)
a: SH01
b: Product (SH02)*
c: Lactone
d: Lactobionic acid

*Note that the product was purified by column chromatography before use.

Figure 2.10: Representation of the direct coupling of lactobionic acid to SH01, after 5 days at room temperature, as monitored on a silica gel $60F_{254}$ plate developed in $CHCl_3$:MeOH:H_2O (6:4:1, v/v/v).

While unreacted starting materials were evident during the course of the reaction, a carbohydrate, less hydrophilic than lactobionic acid, was observed in relatively high yield. The R_f value of this compound corresponded to that of lactobionolactone which was prepared, according to published methods, as part of an earlier attempt to synthesise SH02 (Figure 2.7, route A). While it is important to acknowledge the possible instability of the lactobionic acid stock utilised, and its *in vacuo* drying (Williams *et al*., 1979) prior to incorporation within the reaction mixture as contributors to the lactone detected; small amounts of the lactone species relative to the acid were observed having analysed the aforementioned samples by TLC (results not shown). In addition, the presence of low levels of lactone in both lactobionic acid samples analysed may be supported by information presented by Baminger and colleagues (2001), who reported that an aqueous solution of lactobionic acid at equilibrium at room temperature contains 84 % acid and 16 % lactobiono-1,5-lactone; especially in view of the fact that the lactobionic acid samples were dissolved in distilled water prior to application on the silica gel plate.

Given the relative abundance of the lactone present in the reaction mixture, the dehydrating potential of DCCI, and the close proximity of the C_5 hydroxyl group of lactobionic acid to the electrophilic carbon of the reactive intermediate, it appeared that the reaction proceeded largely via a lactone intermediate (Figure 2.9, route B). In order to explore this possibility, lactobionic acid (dried *in vacuo*) and DCCI (1:1 molar ratio) were reacted in pyridine at room temperature. Analysis on TLC (Figure 2.11) revealed two compounds, one of which corresponded to lactobionic acid (R_f = 0.05), and a more dominant species with migration characteristic of the lactone (R_f = 0.23). The IR spectrum of the product of the lactobionic acid/DCCI reaction (designated Lactone 2) was characterised by an absorption band at 1737.97 cm^{-1}, and the absence of a signal in the region of 1650 cm^{-1}. This demonstrated the ability of DCCI to effect intramolecular ring closure of lactobionic acid to yield the lactone, during the synthesis of SH02.

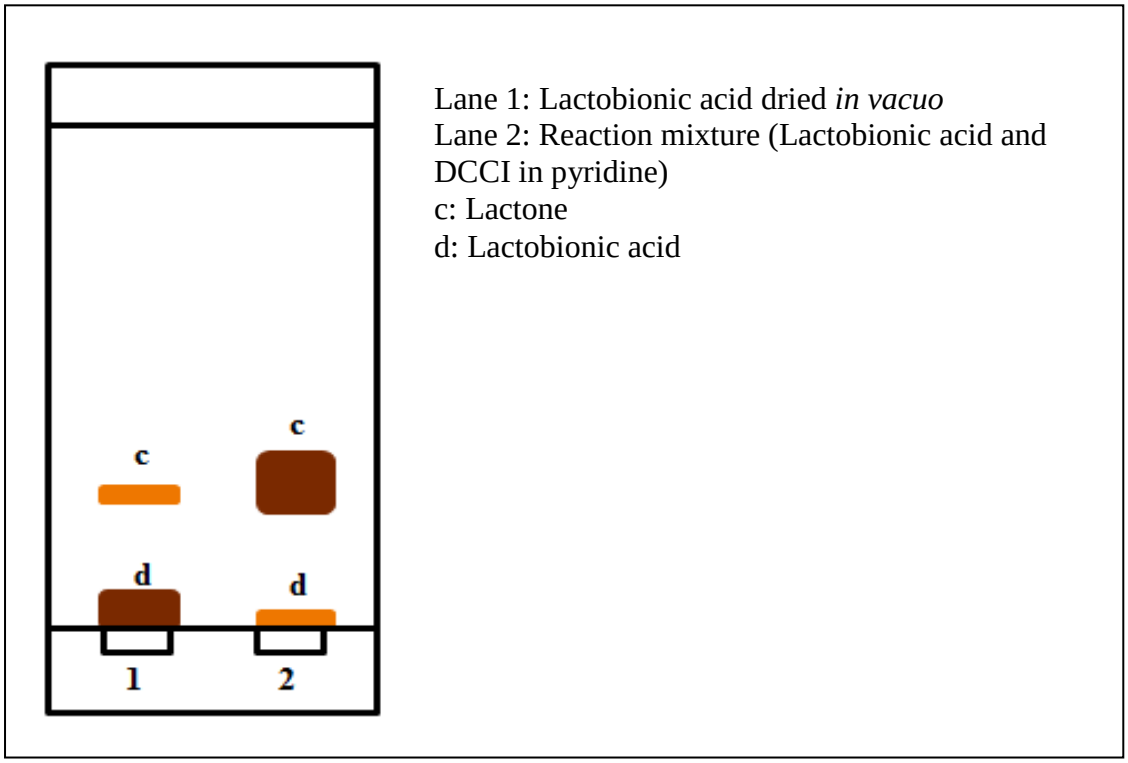


Figure 2.11: Representation of the reaction between lactobionic acid and DCCI, after 4 days at room temperature, as visualised on a silica gel $60F_{254}$ plate developed in $CHCl_3$:MeOH:H_2O (6:4:1, v/v/v).

It is noteworthy that SH02 did not melt but decomposed, as evidenced by a change in the birefingent crystals from colourless to dark brown, due to charring of the sugar residues. The fact that no additional change occurred even when SH02 was subjected to temperature in excess of 230 ˚C, which corresponds to the melting point of DCU, confirms that DCU was not present as a contaminant. This was further corroborated by the absence of a peak corresponding to the molecular weight of DCU in the mass spectrum of SH02. A disadvantage associated with DCCI coupling protocols is the possibility of *O* to *N* transacylation within some of the *O*-acylisourea intermediate species, resulting in stable *N*-acylisourea, due to competition between the amine-bearing compound and the nucleophilic center of *O*-acylisourea for the acyl group (Bodanszky, 1988; Bodanszky *et al*., 1976). However the mass spectrum confirmed that SH02 was free of *N*-acylisourea molecules, as a peak corresponding to a molecular weight of this species was not evident (refer to Appendix 2).

2.3.4 Urocanylcholesterylformylhydrazide (SH04)

It has been reported that urocanic acid-modification improved the *in vitro* transfection potential of cationic polymers, chitosan (Kim *et al*., 2003; Wang *et al*., 2008) and polyphosphazene (Yang *et al*., 2008; Yang *et al*., 2010). As an intermediate of amino acid metabolism in humans (Hawkinson, 1977), urocanic acid is non-toxic and enhanced the biocompatibility of the carriers. Furthermore, the molecule bears the imidazole heterocycle that, with a pKa of 6.9, can facilitate early release of the vector from the endosome via the proton sponge mechanism as highlighted by bafilomycin A1 inhibition experiments (Kim *et al*., 2003; Yang *et al*., 2010). Therefore, in an attempt to design a membrane-compatible lipid capable of inducing the proton sponge effect, urocanic acid was linked to the cholesterol anchor-molecule, SH01. The synthetic route adopted for the synthesis of this pH-sensitive lipid is outlined in Figure 2.12.

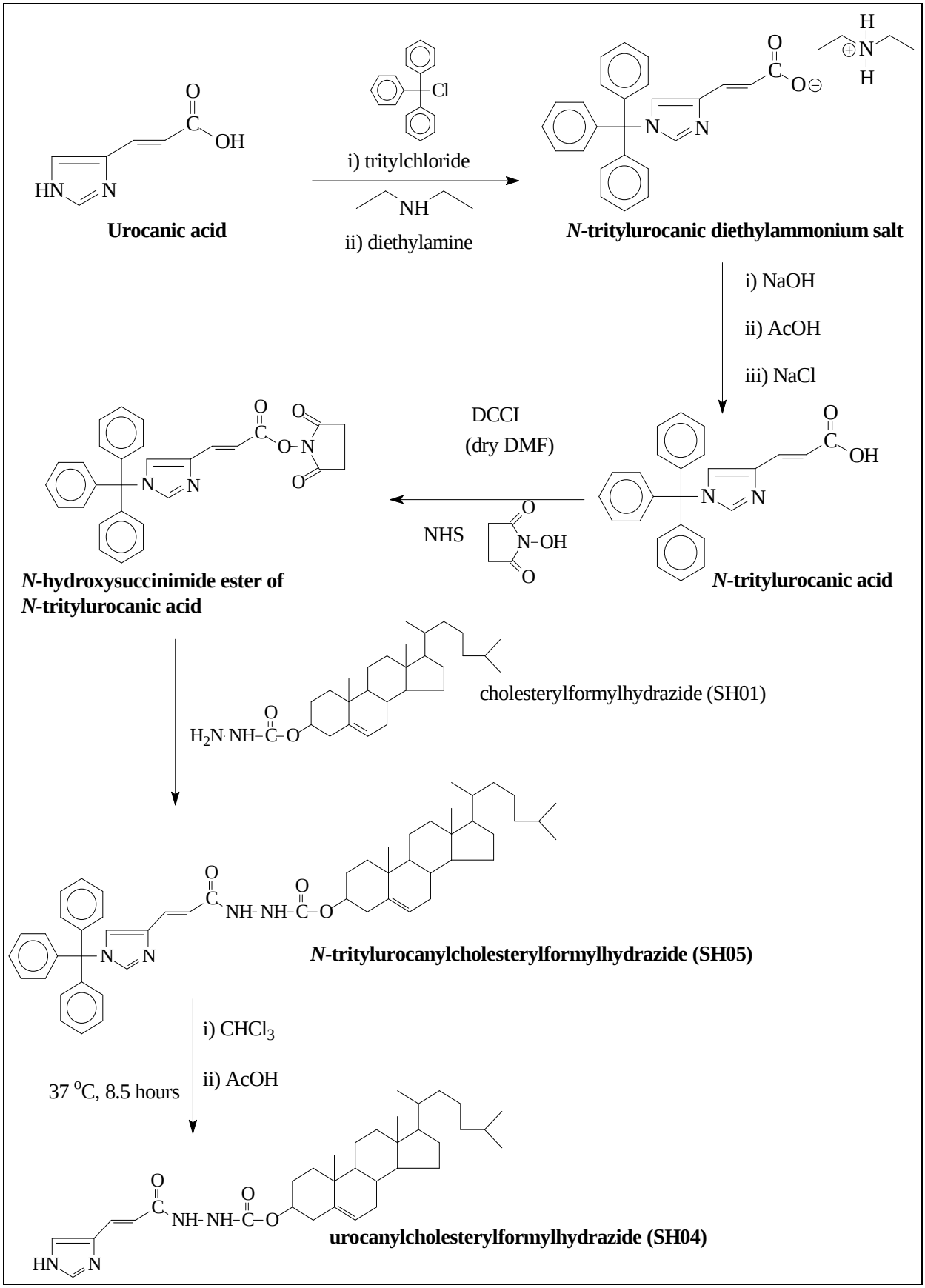


Figure 2.12: Scheme outlining the synthesis of urocanylcholesterylformylhydrazide (SH04) from urocanic acid and SH01.

An urocanic acid molecule contains both amino and carboxyl functionalities, from which amide bonds may be derived via DCCI coupling reactions. Therefore, the introduction of a coupling reagent to a mixture of urocanic acid and SH01 may generate homopolymers and SH01-linked higher homologues of urocanic acid, in addition to the desired novel lipid. This would impact negatively on the yield of the desired product; and presents difficulties with respect to product purification. In an attempt to prevent the formation of amide bonds between urocanic acid molecules, its amino group was rendered inert by appending a trityl group. The trityl moiety is well documented as a protecting group for amines. It is relatively simple to introduce and can be removed under conditions which preserve the integrity of the newly synthesised molecule (Divakaran, 2008).

The reaction of urocanic acid with tritylchloride generates hydrochloric acid as a byproduct, which is likely to react with the basic nitrogen atom of the imidazole ring, to yield a hydrochloride salt. This was prevented by performing the reaction in the presence of a 5.4 molar excess of the organic base, triethylamine that was introduced into the reaction mixture prior to tritylchloride. Triethylamine served to sequester hydrochloric acid, forming its hydrochloride salt, which is insoluble in DMF. Solubilisation of triethylamine hydrochloride was achieved by the addition of methanol. In fact, methanol serves a dual purpose in this reaction as it also eliminates unreacted tritylchloride as methyl ethers. For the purposes of product recovery the tritylated urocanic acid was obtained in high yield as a diethylammonium salt. The unreactive nature of this compound necessitated its conversion to the free acid. However, due to the low reactivity of the carboxyl functionality, and with reference to the preparation of urocanic acid-modified carriers reported in the literature (Bailey, 1990; Yang *et al*., 2010), this group required activation to merit its participation in subsequent synthesis. For this reason NHS was coupled to the free acid, to afford an active ester.

Apart from the procedure for product visualisation outlined in 2.2.2.3.3, two other methods also confirmed the formation of the active ester via TLC, both during its synthesis and after product recovery. The phenolic ring system of the trityl group and heterocyclic ring structures of urocanic acid and NHS are able to absorb ultraviolet (UV) light. Therefore, UV illumination of the fluorescent silica gel plate enabled visualisation of the product and unreacted starting material as dark spots. An alternative method entailed spraying the plate

with sulphuric acid, whereupon the product assumed an intense yellow colour (Figure 2.13). This is because the acid releases the trityl group as a stable carbocation, which is yellow in colour. In other TLC analyses, the active ester was detected as a dark purple spot on the plate after applying basic hydroxylamine and ferric chloride. Excess hydroxylamine in alkaline medium is known to convert *N*-hydroxysuccinimide and its esters to their corresponding hydroxamic acids. In the presence of Fe(III) cations, hydroxamic acid forms a ferric hydroxamate complex that is purple in colour (Maguire and Dudley, 1977; Ong and Brady, 1972).

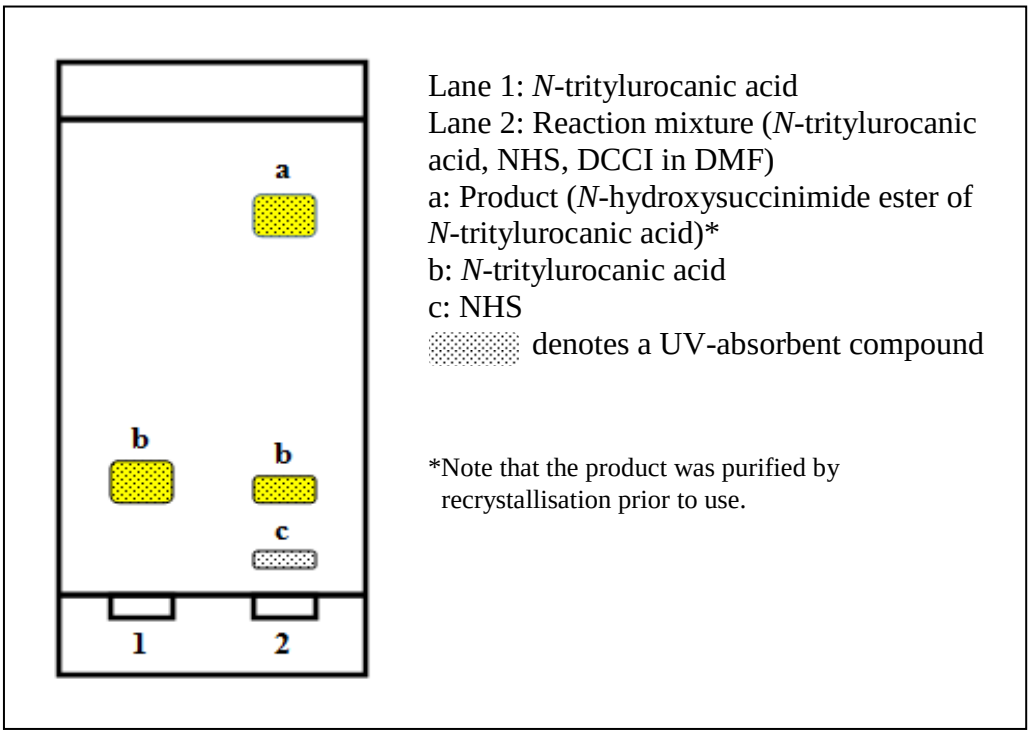
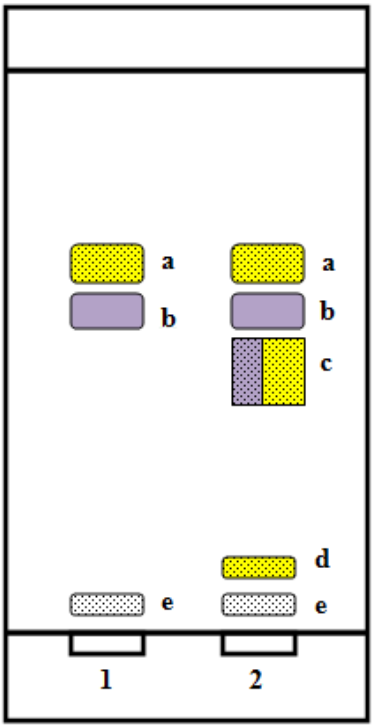


Figure 2.13: Formation of the active ester of *N*-tritylurocanic acid after 24 hours of reaction time, as observed on silica gel $60F_{254}$ plates developed in $CHCl_3$:MeOH (95:5, v/v). The plate was viewed under ultraviolet light and thereafter, sprayed with 10 % H_2SO_4 and heated.

The *N*-hydroxysuccinimide ester of *N*-tritylurocanic acid prepared exactly as outlined in 2.2.2.3.3 was used as a precursor to SH04 in this study. At a later stage however, a second batch of the active ester was prepared, and ethanol was employed as the solvent for recrystallisation. In this instance, the product was obtained in high yield (80.8 %) as white, birefringent crystals, which melted at a higher temperature (127-130 ˚C). A comparison on

TLC revealed that the R_f values of the amorphous and crystalline products were identical. It appears, therefore, that ethanol is a more suitable solvent for the recovery of the active ester of *N*-tritylurocanic acid in crystalline form.

The next reaction entailed the formation of the amide linkage between the activated, *N*-protected urocanic acid and the cholesterol anchor molecules (SH01). Progress of this reaction was evidenced by the release of UV-absorbent NHS by TLC (Figure 2.14). Eventually the product, SH05, was identified on the chromatogram due to its UV-absorbent, trityl-positive and cholesterol-positive properties. Finally, the trityl group was removed under mildly acidic conditions to afford the imidazolylated lipid, SH04, with a 9.771 Å spacer between the cholesterol and imidazole rings (Figures 2.4c and 2.5c).



Lane 1: *N*-hydroxysuccinimide ester of *N*-tritylurocanic acid, SH01 and NHS, each applied successively.
Lane 2: Reaction mixture (*N*-hydroxysuccinimide ester of *N*-tritylurocanic acid and SH01 in $CHCl_3$)
a: *N*-hydroxysuccinimide ester of *N*-tritylurocanic acid
b: SH01
c: Product (SH05)*
d: *N*-tritylurocanic acid
e: NHS
denotes a UV-absorbent compound

*Note that the product was purified by preparative TLC before use.

Figure 2.14: Coupling of carboxyl activated *N*-tritylurocanic acid to SH01 as observed on silica gel $60F_{254}$ TLC plates in $CHCl_3$:MeOH (98:2, v/v). Reaction components were visualised under UV illumination, and after applying 10 % H_2SO_4.

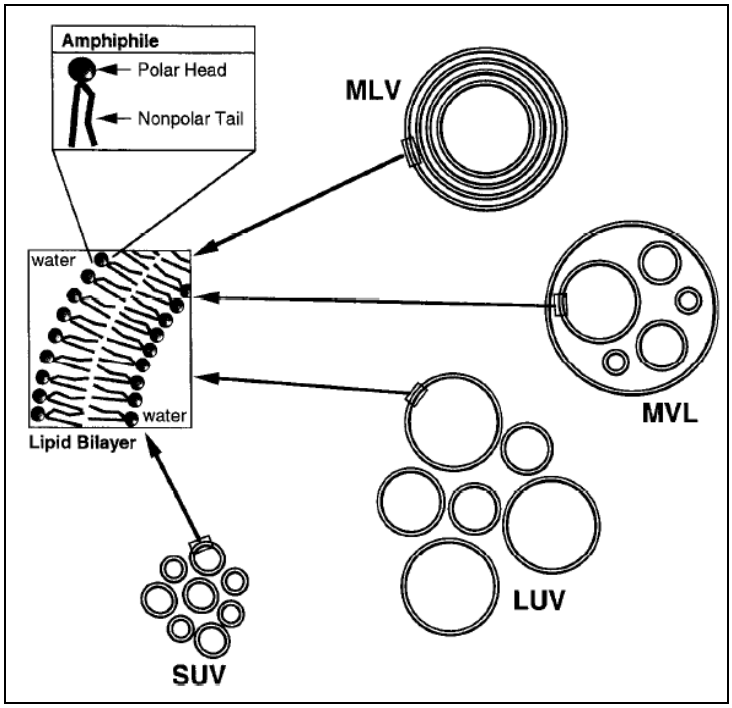
In summary, two novel cholesterol derivatives, lactobionylcholesterylformylhydrazide (SH02) and urocanylcholesterylformylhydrazide (SH04) were successfully synthesised in reactions mediated by the coupling reagent dicyclohexylcarbodiimide. Cholesterylformylhydrazide (SH01) was prepared from cholesterylchloroformate and hydrazine as a precursor to both SH02 and SH04. A direct coupling reaction between SH01 and lactobionic acid gave the glycolipid, SH02. Observations made as per TLC suggested that this reaction proceeded largely via a lactone intermediate. The synthesis of the imidazolylated lipid, SH04, from urocanic acid and SH01 was achieved in several steps: urocanic acid was *N*-tritylated, recovered as a diethylammonium salt, and converted to the free acid; the amide bond was formed between the *N*-protected urocanic acid and SH01, and the trityl group was removed. NMR and high resolution mass spectrometry were used to validate the structures of both cholesterol derivatives.

CHAPTER THREE

LIPOSOME PREPARATION AND CHARACTERISATION

3.1 Introduction

Liposomes are classified on the basis of size and lamellarity (the number of lipid rings) as illustrated in Figure 3.1. It is possible to discern small (up to 200 nm), large (an upper limit of 1 μm) and giant (between 1 μm and 50 μm) sized vesicles; as well as unilamellar, oligolamellar (between 2 and 10 concentric lipid rings) and multilamellar structures (Lasic and Templeton, 1996).



SUV: small unilamellar vesicles

LUV: large unilamellar vesicles

MLV: large multilamellar vesicles

MVL: multivesicular liposomes

Figure 3.1: Liposome characterisation according to size and lamellarity (Lasic and Templeton, 1996).

Studies have shown that each class of vesicles is best suited for a specific application. For example, large vesicles with multiple concentric bilayers possess high internal volume and have shown promise for the effective encapsulation of macromolecules (Riaz, 1996); while small unilamellar vesicles are preferred as gene carriers (Lasic, 1997). Although lipid suspensions often consist of a heterogeneous mixture of vesicles (Zhu and Szostak, 2009),

significant control over the type of vesicles formed and their stability may be exerted at the level of liposome preparation. Moreover, the preparation method is a key determinant of the economic feasibility of the liposomal carrier (Hatziantonioy and Dementzos, 2008).

In view of the importance of the liposome preparation method to the design of liposomal systems, a variety of techniques have been developed (Figure 3.2). All processes are designed to permit the introduction of lipids into an aqueous environment, which is the fundamental requirement for the formation of liposomes, but differ in the manner in which the lipids are dispersed (Lasch *et al*., 2003).

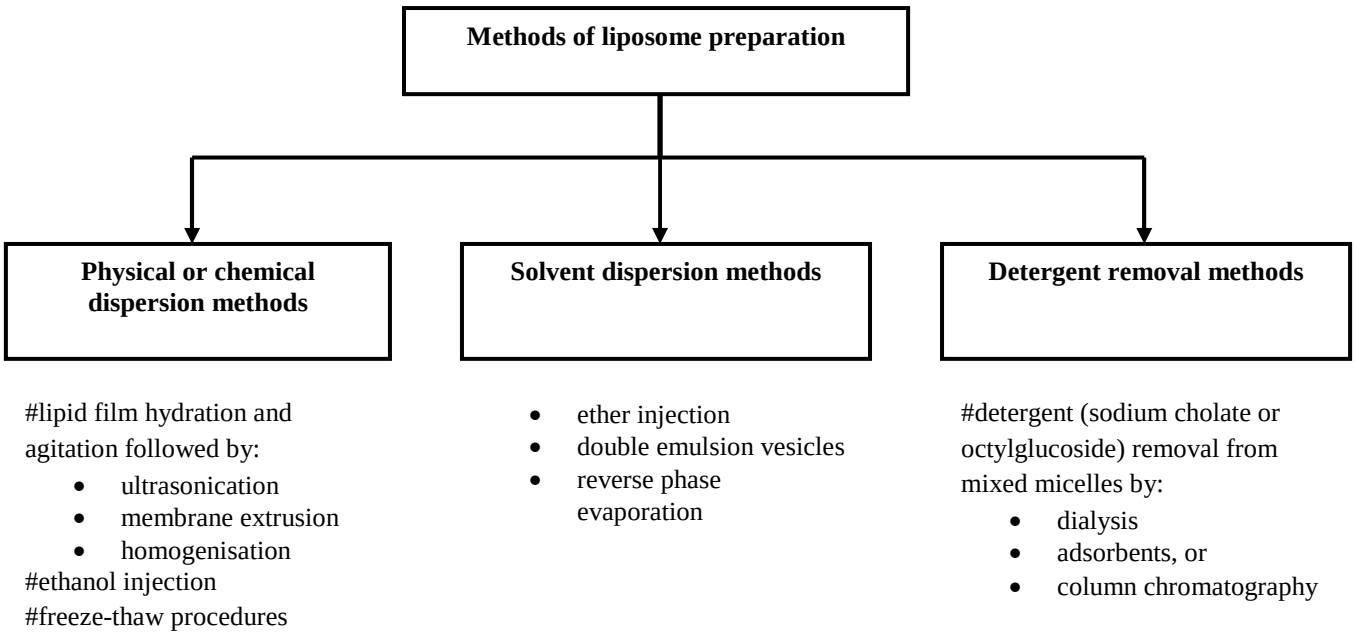


Figure 3.2: Flow diagram outlining the different methods employed in liposome preparation (adapted from Patel, 2006).

The conventional method reported by Bangham and colleagues (1965), is among the oldest and most widely used means of preparing liposomes (Riaz, 1996; Uhumwangho and Okor, 2005). According to this method, lipid mixtures in organic solvents are dried to a thin film that is subsequently hydrated in a buffered aqueous medium (Singh and Ariatti, 2008). Although it is generally accepted that dry lipid films spontaneously form large multilamellar structures upon hydration, Lasch and coworkers (2003) comment that this is incorrect. Instead, the lipid film swells and grows into multiple thin lipid tubules, whilst remaining

attached to the film support, as shown in Figure 3.3. It is only with mechanical agitation that the tubules are fragmented. The fragments self-seal to form MLV due to exposure of non-polar regions of the tubules to the hydrophilic suspension medium (Lasic, 1997). The size and lamellarity of the vesicles may be reduced in several ways (Uhumwangho and Okor, 2005). Therefore, the process of lipid film hydration has been relatively easily adapted for the successful preparation of many documented liposomal gene carriers (Almofti *et al*., 2003a; Balram *et al*., 2009; Mignet *et al*., 2008; Roux *et al*., 2004; Singh and Ariatti, 2008).

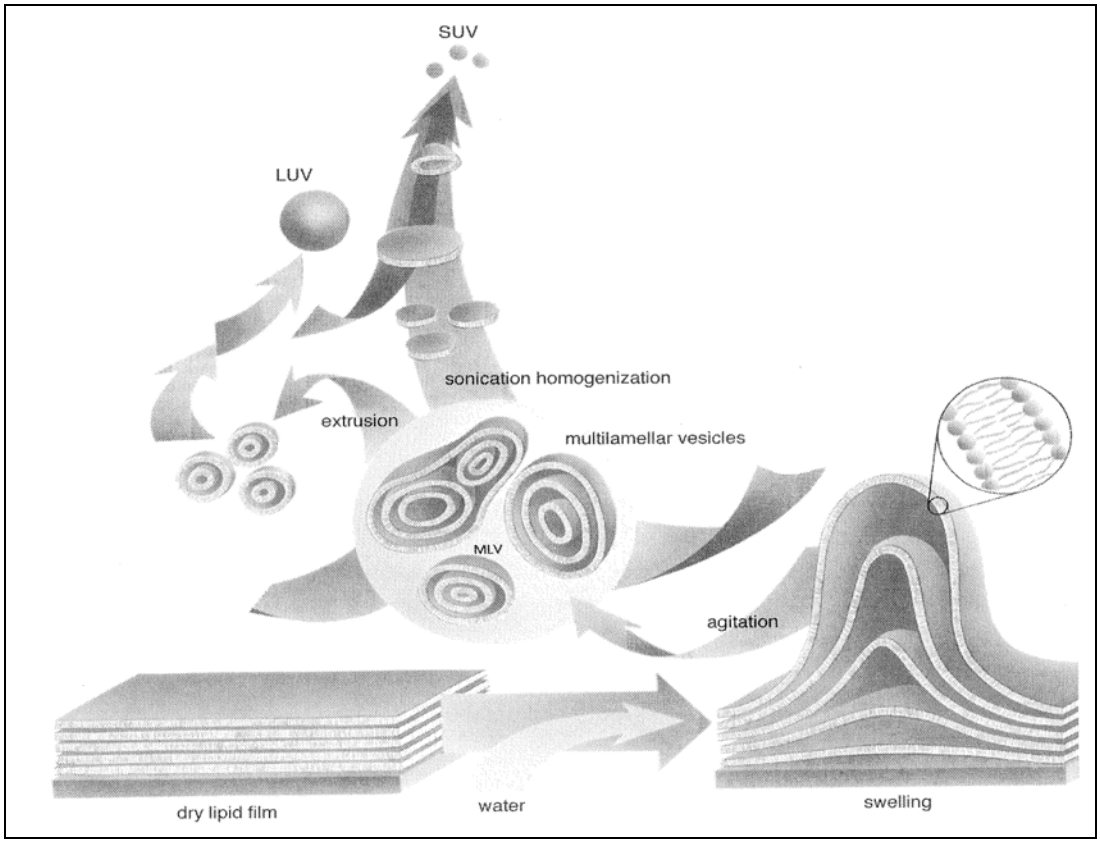


Figure 3.3: Schematic representation of the formation of liposomes from the hydration of a dry lipid film (adapted from Lasic, 1997).

Suspensions of MLV may be forced through filters with extremely small pore diameters (0.8 – 1 μm). Repeated extrusions through the filters removes successive lipid membranes until a relatively homogeneous population of small vesicles with a single bilayer remain (Uhumwangho and Okor, 2005). In this regard, high-pressure devices such as the French pressure cell may be useful (Hamilton *et al*., 1980). Alternately, the use of high frequency sound waves, sonication, provides the energy necessary to disrupt MLV forming SUV, that

are evenly dispersed in the suspension (Lasic, 1997). Bath-type sonicators are favoured over sonicator probes, because contamination of the sample may be avoided; and the temperature at which samples are processed can be controlled (Vemuri and Rhodes, 1995). Accordingly, lipid film hydration with vesicle dispersion by bath-sonication was the method of choice for the preparation of cationic liposomes in this study.

3.2 Materials and Methods

3.2.1 Materials

The cationic lipid 3β[*N*-(*N′, N′*-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) was previously prepared in the Laboratory of Bioorganic Synthesis, Department of Biochemistry, University of KwaZulu-Natal (UKZN, Westville); according to the protocol of Gao and Huang (1991). Lactobionylcholesterylformylhydrazide (SH02) and urocanylcholesterylformylhydrazide (SH04) were prepared as described in Chapter Two. L-α-phosphatidylethanolamine dioleoyl (DOPE), and 1,2-distearoyl-*sn*-glycero-phosphoethanolamine-*N*-[carboxy(polyethylene glycol)2000] (ammonium salt) (DSPE-PEG_{2000}), were supplied by Sigma, St. Louis, MO, USA, and Avanti Polar Lipids, Albaster, USA, respectively. 2-[-(2-hydroxyethyl)-piperazinyl]-ethanesulphonic acid (HEPES) was from Merck, Darmstadt, Germany.

3.2.2 Methods

3.2.2.1 Liposome preparation

The individual lipids were dissolved in appropriate organic solvents (refer to Table 3.1) and combined in the quantities shown in Table 3.2. Lipid mixtures were concentrated *in vacuo* (Büchi Rotavapor-R) to a thin film, deposited on the inner walls of a quick-fit tube. Residual solvent was removed by drying the lipid film under high vacuum (Büchi-TO pistol drier), for 30 minutes. The film was rehydrated in 1 ml sterile HBS (HEPES buffered saline containing 20 mM HEPES, 150 mM NaCl, pH 7.5) at 4 ˚C, overnight. The suspension was agitated by

vortexing for 30 seconds, and then sonicated (Elma, Transsonic 460/H bath sonicator) at room temperature for 5 minutes. Liposome preparations were stored at 4 ˚C for the duration of the study, and subjected to brief sonication (30 seconds) prior to use.

Table 3.1: Organic solvents used for lipid solubilisation during liposome preparation.

Lipid	Organic solvent
Chol-T	Chloroform
DOPE	Chloroform
SH02	Pyridine
SH04	chloroform:methanol, 1:1 (v/v)
DSPE-PEG_{2000}	Chloroform

Table 3.2: Composition of pegylated and non-pegylated cationic liposomes.

Liposome preparation		Lipids (µmol)					Total lipid (µmol)
		Chol-T	DOPE	SH02	SH04	DSPE-PEG_{2000}	
Non-pegylated liposomes	**1**	2	2	-	-	-	4
	2	1.8	1.8	0.4	-	-	4
	3	1.8	1.8	-	0.4	-	4
	4	1.6	1.6	0.4	0.4	-	4
Pegylated liposomes	**5**	2	1.8	-	-	0.2	4
	6	1.8	1.6	0.4	-	0.2	4
	7	1.8	1.6	-	0.4	0.2	4
	8	1.6	1.4	0.4	0.4	0.2	4

3.2.2.2 Characterisation of liposomes

3.2.2.2.1 Cryo-transmission electron microscopy (cryo-TEM)

Suspensions of non-pegylated and pegylated liposomes were diluted 1:4 (v/v) and 1:6 (v/v) in HBS, respectively. Aliquots (1 µl) were placed on formvar-coated copper grids and stained with a solution of uranyl acetate (2 % w/v, 1 µl) for approximately 60 seconds. Excess liquid was removed using filter paper, and samples were flash-frozen in liquid propane (- 170 ˚C). Preparations were viewed with a JEOL JEM.1010 transmission electron microscope (JEOL Ltd., Tokyo, Japan) operating at an accelerating voltage of 100 kV. Images were captured using a MegaView III digital camera and iTEM Universal Imaging Platform software (Tokyo, Japan).

3.2.2.2.2 Size measurements

Liposome suspensions were diluted 1:19 (v/v) in sterile HBS. Samples (1 ml) were transferred to disposable polystyrene sizing cuvettes. Vesicle size and size distribution patterns were obtained using a Malvern Zetasizer Nano-ZS instrument (Malvern Instruments Ltd., Worcestershire, UK.) operating at 25 ˚C. In all instances, the viscosity and refractive index of the dispersant was assumed to be 0.8872 and 1.330 respectively. Data was recorded using Zetasizer Software, version 6.30, which generated plots of size distribution by intensity. Particle size and size distribution were expressed as the intensity-weighted mean hydrodynamic size (Z-average diameter) and polydispersity index (PdI) respectively. Three measurements were performed per sample.

3.2.2.2.3 Statistical analysis

Statistical analyses were performed using ANOVA (one-way analysis of variance), followed by Tukey's Multiple Comparison Test to compare between groups (GraphPad Prism version 5.04, GraphPad Software Inc., USA). *P* values less than 0.05 were considered significant.

3.2.2.3 Buffering capacity of liposomes

Large scale preparations of liposomes 1 (Chol-T/DOPE) and 3 (Chol-T/DOPE/SH04) were hydrated in glass-distilled, deionised water (10 ml). The aqueous suspensions and 10 ml distilled water, which served as a blank, were each transferred into flat-bottomed flasks with a 20 mm internal diameter. The flasks were rested on a polystyrene sheet throughout the experiment to minimise temperature fluctuations. The blank and test samples were adjusted to pH 10 with 0.35 N NaOH. Titrations were conducted at room temperature by the stepwise addition of 0.01 N HCl (20 μl) until 400 μl of the acid was added. Approximately 30 seconds was allowed for equilibration before the introduction of a subsequent aliquot, and pH was recorded using a standard pH probe (Metrohm 620 pH meter).

3.3 Results and discussion

3.3.1 Liposome preparation

Pegylated and non-pegylated cationic liposomes were successfully prepared by the lipid film hydration method. The discussion to follow details the rationale for the formulation of these liposomes.

Chol-T (Figure 3.4) served as the cytofectin in all eight formulations. Several features of this monocationic cholesterol derivative contribute to its suitability for use in the gene transfer system under investigation. Firstly, amphiphiles with tertiary amino headgroups, such as Chol-T, are favourable agents of transfection, due to their reduced toxicity (Lv *et al*., 2006). Secondly, the dimethylamino headgroup of the molecule is borne at the end of a 6.183 Å propyl spacer arm. It has been demonstrated that a spacer region extending between 3 and 6 atoms in length, like that of Chol-T, encourages transfection activity (Gao and Hui, 2001); possibly because it promotes accessibility of the cationic headgroup with the anionic phosphate moieties of the DNA backbone (Singh and Ariatti, 2006). In addition, the substituents on the sterol ring are tethered via a carbamoyl bond that is chemically stable but easily biodegradable following the release of DNA (Cao *et al*., 2006; Tseng and Huang,

1998). Therefore, cytofectins designed with carbamoyl linker bonds are generally less toxic both *in vitro* and *in vivo* (Gao and Hui, 2001).

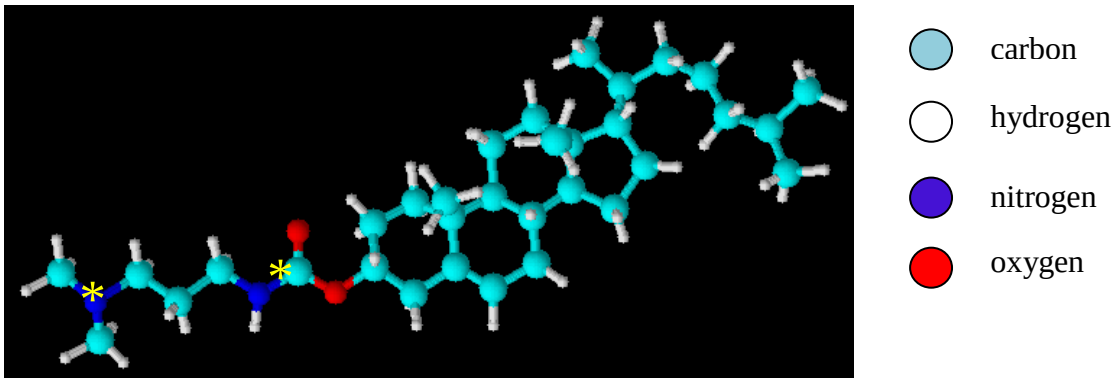


Figure 3.4: A ball and stick model of Chol-T. The spacer length reported was measured as the internuclear distance between the atoms indicated with an asterisk.

Liposomes were formulated from Chol-T and DOPE in either equimolar (as for the non-pegylated liposomes) or near-equimolar (with respect to pegylated formulations) quantities, in keeping with evidence that these lipids, in the aforementioned proportions, form stable unilamellar liposomes with favourable DNA-binding and protecting character (Kisoon *et al*., 2002; Singh *et al*., 2011). DOPE is the most commonly employed helper lipid (Rao, 2010), and is particularly effective for *in vitro* gene transfer (Hirsch-Lerner *et al*., 2005). According to Felgner and coworkers (1994), the transfection-enhancing effect of DOPE arises from the formation of heterodimers in the liposomal bilayer, as a consequence of ion pairs which form between the ammonium headgroup of the cytofectin and the phosphate moiety of DOPE. Apart from reducing the toxicity of cationic lipids, studies have shown that DOPE also assists in the processes of lipid dehydration and counterion release that are necessary for the assembly of liposomal carriers with DNA (Hirsch-Lerner *et al*., 2005). In the current study, several lipid derivatives were introduced into the Chol-T/DOPE formulation with a view to designing liposomes displaying properties of hepatocyte-specificity, endosomal pH-sensitivity and/or steric stability; while liposome 1, which consisted of Chol-T and DOPE alone, served as a control.

The galactose density on the surface of liposomes is known to influence their intrahepatic distribution patterns, and is therefore an important parameter in the design of hepatotropic liposomes. It has been demonstrated that liposomes formulated with galactosides at 50 %, on

a molar basis, were internalised largely by Kupffer cells (Yu *et al*., 2007). This was attributed to binding of the sugar moieties to the ASGP-R analogue expressed by these cells (Rensen *et al*., 2001). In contrast, liposomes containing up to 5 % of the targeting component were selectively taken up by liver parenchymal cells via the ASGP-R (Yu *et al*., 2007). Furthermore, a comparative study by Managit and coworkers (2005) showed that liposomes containing a cationic, galactosylated cholesterol derivative at concentrations within a range of 5 – 9 % (mol/mol) were successfully recognised by the ASGP-R. More recently, a neutral, lactobionic acid-modified cholesterol derivative (CHS-ED-LA), similar to SH02, afforded high levels of receptor-mediated liposome accumulation in hepatocytes, when incorporated at 10 % (mol/mol) (Wang *et al*., 2006). Therefore, the novel glycolipid SH02 was used to formulate the targeted liposomes 2, 4, 6, and 8, at 10 % on a molar basis.

While several lipids with proton sponge capability have shown promise in liposomal systems, only three other cholesterol derivatives bearing the imidazole moiety have been previously reported (Midoux *et al*., 2009). The first, ChIm, synthesised by Budker and coworkers (1996), consists of an imidazole ring attached to the cholesterol anchor via a propyl spacer arm and carbamoyl bond. Although ChIm successfully formed liposomes when combined with DOPE, such a system does not display permanent cationic charges at physiological pH. Therefore, acidic conditions were required for lipoplex assembly. Later, Singh and coworkers (2004) designed a cholesterol-based, histidylated cationic lipid. This lipid proved effective as a cytofectin, and exhibited sensitivity to endosomal acidification when formulated with DOPE, at a molar ratio of 2:1. Most recently, Shigeta and colleagues (2007) reported the synthesis and application of Gal-His-C4-Chol. This is a pH-sensitive cytofectin with a hepatotropic ligand. Therefore, its incorporation in a cationic liposomal system at 25 % on a molar basis was intended to permit optimal demonstration of all three properties. It was apparent that none of these lipids presented an appropriate system upon which the molar composition of SH04 in cationic liposomes could be modelled. Due to the fact that optimisation of liposome composition falls beyond the scope of this study, SH04 was set at the same molar concentration as SH02, i.e. 10 % in the relevant formulations. Nonetheless, the optimisation of the molar composition of SH04 in liposome formulations could present opportunity for further study.

Liposomes 5 to 8 were modified with a steric stabilising shield, using the pegylated lipid DSPE-PEG_{2000}. Polyethylene glycol that is carbamate-linked to a phospholipid anchor, presents a well-documented means of stably grafting the polymer to the liposomal bilayer (Webb *et al*., 1998). However, pegylated lipids may be prone to the formation of curved micelles (Figure 3.5), instead of lipid vesicles. This imposes a limitation on the amount at which pegylated lipids may be incorporated within a bilayer (Discher, 2003). It has been noted that, increasing the length of a PEG chain attached to a hydrocarbon skeleton increases the ratio of the polar region of the lipid relative to its apolar component; and this disturbs the organisation of the bilayer (Photos *et al*., 2003). Although longer PEG chains and higher grafting densities would theoretically confer greater steric protection, according to Hristova and coworkers (1995), there exists a critical quantity of pegylated lipids that can be assembled into a liposomal bilayer beyond which transition from vesicular to micellar structures occur. This amount is specific to the composition of the bilayer and the nature of the PEG-lipid conjugate. These complexities, taken into consideration, have led to the use of PEG of molecular weight 2000, as opposed to larger polymer chains, in several long-circulating liposomal systems (Kim *et al*., 2003; Managit *et al*., 2003; Narainpersad *et al*., 2012; Santel *et al*., 2006; Shi and Pardridge, 2000).

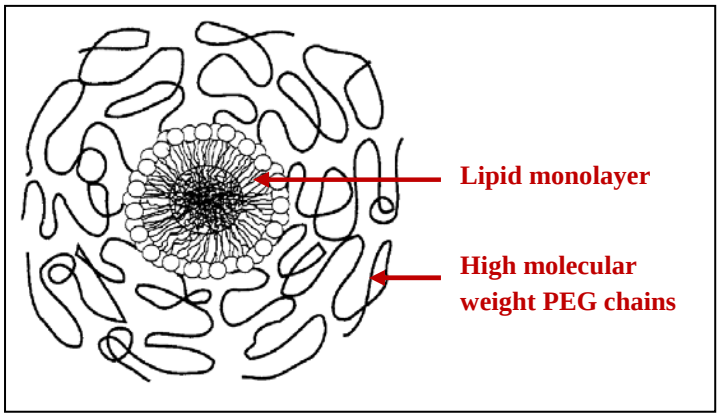


Figure 3.5: A representation of a micelle formed by introducing a combination of phospholipids and a high concentration of pegylated lipids into an aqueous environment (adapted from Hristova *et al*., 1995).

PEG_{2000}-lipid derivatives may be stably incorporated in liposomal bilayers at concentrations of up to10 % (mol/mol) (Markus, 2001). However, a dense polymer shroud is likely to shield essential functional groups on the liposome surface, thereby reducing transfection capability (Kichler, 2004; Romberg *et al*., 2008). For this reason liposomal gene transfer systems are

often modified with PEG_{2000} at relatively low densities (Needham *et al*., 1997; Singh *et al*., 2011; Song *et al*., 2002). In an attempt to minimise the adumbrating effect of PEG, in this study, liposomes were formulated with DSPE-PEG_{2000} at 5 % on a molar basis.

3.3.2 Characterisation of liposomes

The accurate characterisation of liposomes is important as it provides useful information regarding the efficacy of the preparation method (Hupfeld, 2009), and can be used to explain the biodistribution properties of the carrier (Gabizon *et al*., 1990) and assess its suitability towards its intended application. In this study liposomes were characterised using both cryo-TEM and a DLS device, in keeping with reports which suggest that a combination of techniques allows for a more precise characterisation of nanoparticles (Kim *et al*., 2003; Murdock *et al*., 2007; Ruozi *et al*., 2007; Škalka *et al*., 1998). While TEM provides useful information regarding liposome morphology and can be calibrated to measure vesicle diameter, it cannot take into account the relative dispersion of vesicles and is restricted to the field of view. It has been reported that even an average of 500 random measurements is limiting (Murdock *et al*., 2007). DLS, however, provides the average hydrodynamic diameter and size distribution patterns of nanoparticles by analysing fluctuations in the intensity of light scattered by particles in suspension because of Brownian motion, over a period of time. DLS-based methods are highly sensitive, and provide data that is representative of the entire preparation (Kaszuba *et al*., 2008). As such, DLS has emerged as an effective tool for determining the size of non-viral vectors (Kawakami *et al*., 1998; Kawakami *et al*., 2000; Managit *et al*., 2005; Mével *et al*., 2007; Yang *et al*., 2008).

The micrographs in Figures 3.6 and 3.7 show that all formulations, with the exception of liposome 4, afforded small, unilamellar vesicles. The vesicles of non-pegylated liposomes 1, 2 and 3 (Figure 3.6a, b and c, respectively) were predominantly spherical in shape, not unlike those prepared by Kisoon and colleagues (2002) from equimolar quantities of Chol-T and DOPE. Liposome 4, however, consisted of deformable structures and aggregates of varying size (Figure 3.6d). Nonetheless, the non-aggregated lipid bodies in this preparation were observed to possess a single bilayer.

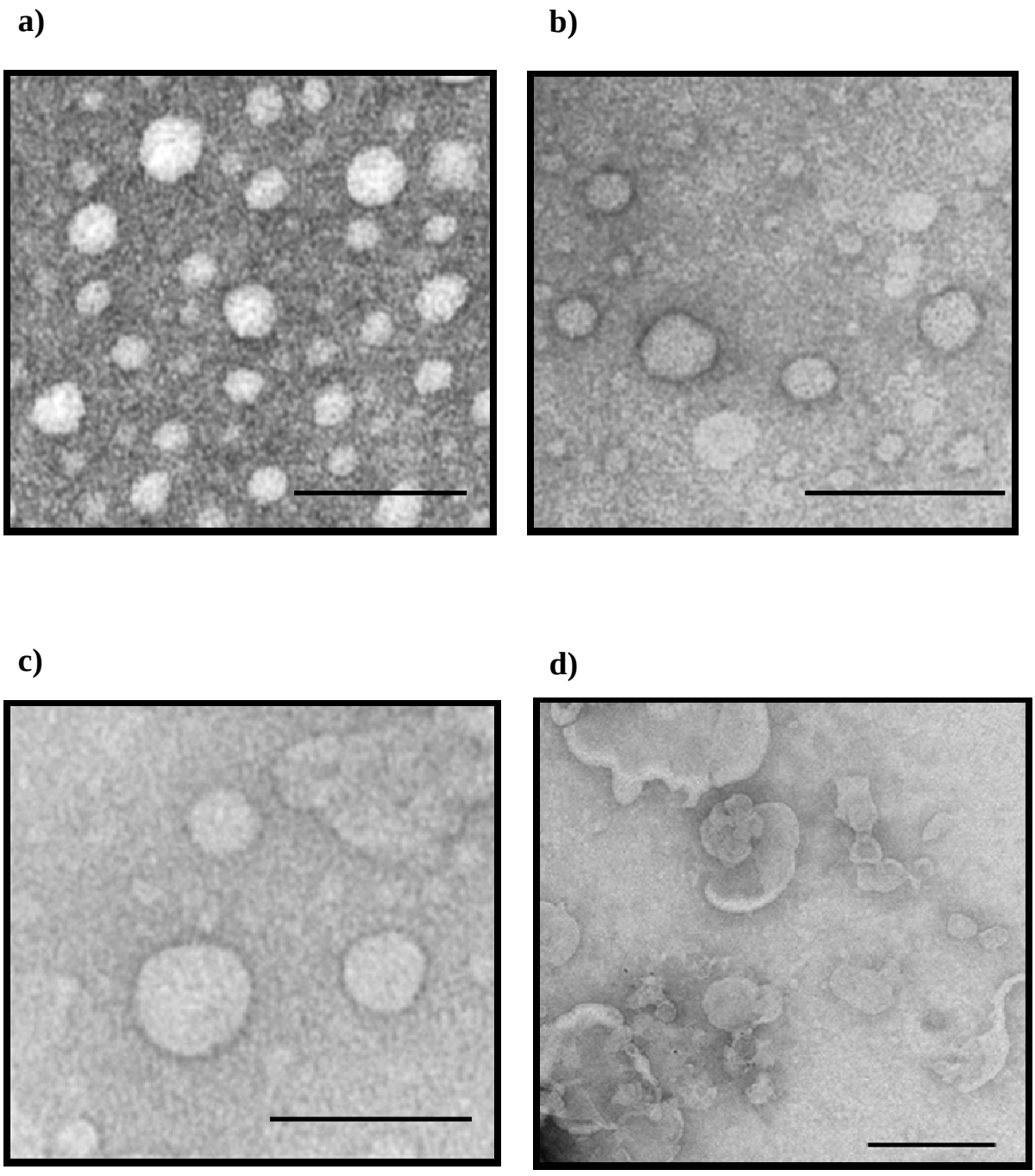


Figure 3.6: Transmission electron micrographs of non-pegylated cationic liposomes a) 1; b) 2; c) 3; and d) 4. (Refer to Table 3.2 for composition of liposomes). Liposome suspensions were stained with uranyl acetate and cryopreserved using liquid propane prior to viewing. Scale bar = 100 nm (a, and c); 200 nm (b); 500 nm (d).

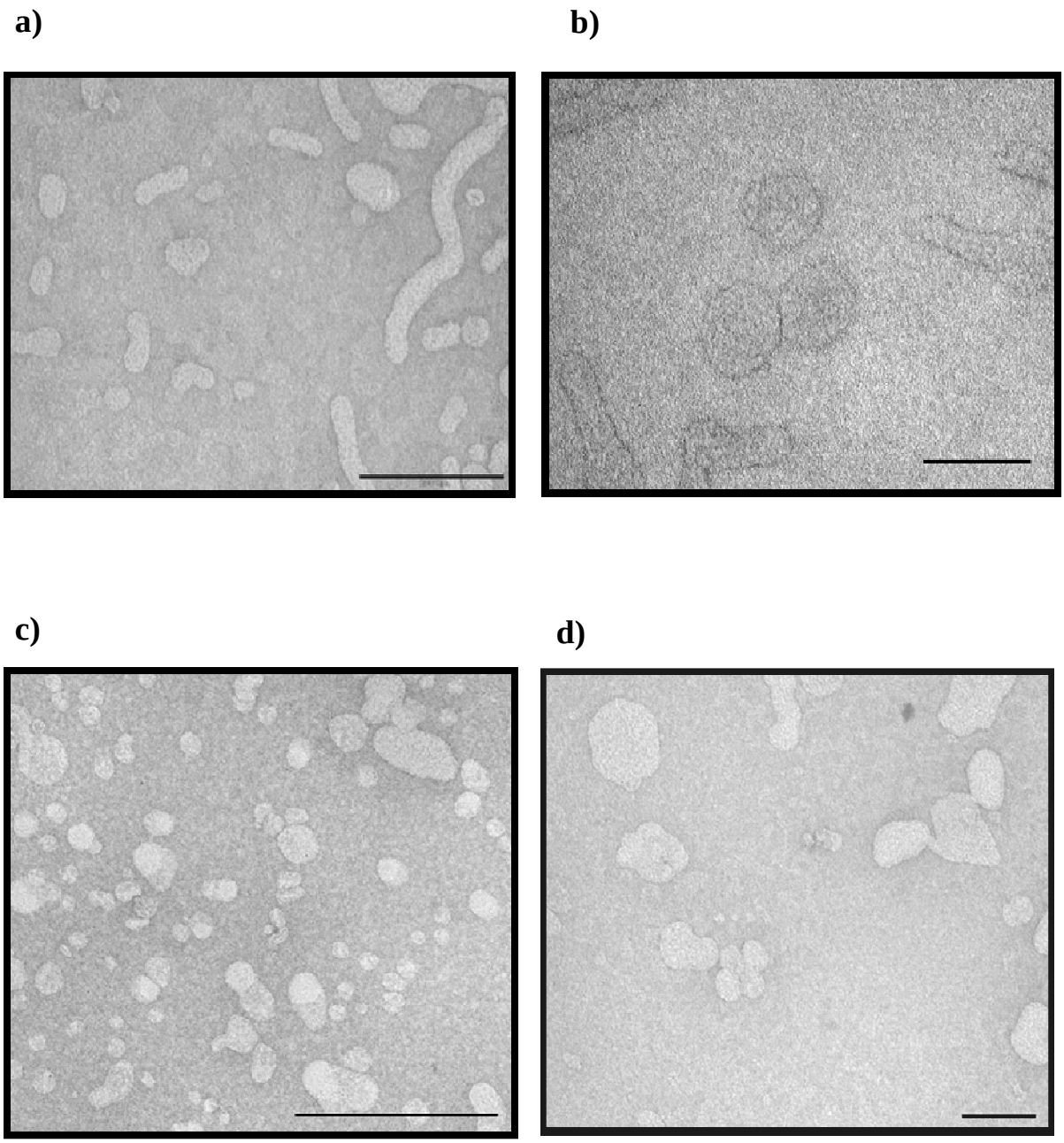


Figure 3.7: Transmission electron micrographs of pegylated cationic liposomes a) 5; b) 6; c) 7; and d) 8. (Refer to Table 3.2 for composition of liposomes). Liposome suspensions were stained with uranyl acetate and cryopreserved using liquid propane prior to viewing. Scale bar = 200 nm (a, and d); 100 nm (b); 500 nm (c).

The pegylated liposomes displayed considerable heterogeneity with regard to vesicle shape (Figure 3.7a-d). Elongated-oval, tubular, club- and bean-shaped structures appeared to be almost as prevalent as spherical vesicles in these preparations. This is not uncommon as

similar non-conventional lipid structures have been observed upon cryo-electron microscopy of other cationic liposomes. Moreover, the presence and relative abundance of such unusually shaped lipid vesicles in a liposome suspension may occur as a result of the cumulative effect of the structural phases preferred by the individual lipids; the lipid composition and the method employed for liposome preparation (Lasic, 1997).

The vesicle size and size distribution of the liposome preparations is summarised in Table 3.3. The PdI of each sample was noted in order to assess the uniformity of the vesicle size distribution. The PdI ranges between 0 and 1, with these extreme values corresponding to entirely monodisperse and polydisperse samples respectively (Colas *et al*., 2007). According to Gramdorf and coworkers (2008) PdI values between 0.1 and 0.2 reflect narrow particle size distributions; while those in excess of 0.25 indicate particle sizes distributed over a broad range.

Table 3.3: Vesicle size and polydispersity indices of pegylated and non-pegylated cationic liposomes.

Liposome preparation		**Lipid content**	**Z-average diameter[a] (nm)**	**PdI[a]**
Non-pegylated liposomes	1	Chol-T/DOPE	207.70 ± 1.59	0.222 ± 0.006
	2	Chol-T/DOPE/SH02	170.37 ± 1.26	0.198 ± 0.011
	3	Chol-T/DOPE/SH04	168.37 ± 2.92	0.192 ± 0.016
	4	Chol-T/DOPE/SH02/SH04	765.40 ± 58.58$^{###}$	0.535 ± 0.042$^{###}$
Pegylated liposomes	5	Chol-T/DOPE/DSPE-PEG_{2000}	121.53 ± 2.04**	0.246 ± 0.005
	6	Chol-T/DOPE/SH02/DSPE-PEG_{2000}	109.10 ± 0.76*	0.209 ± 0.005
	7	Chol-T/DOPE/SH04/DSPE-PEG_{2000}	100.32 ± 1.59*	0.221 ± 0.005
	8	Chol-T/DOPE/SH02/SH04/DSPE-PEG_{2000}	140.80 ± 1.65***	0.254 ± 0.003***

[a] Each value represents the mean ± SD (n = 3).

*P < 0.05, **P < 0.01, ***P < 0.001 *vs*. the respective non-pegylated counterpart.

$^{###}P$ < 0.001, non-pegylated liposomes *vs*. liposome 1. P > 0.05, pegylated liposomes *vs*. liposome 5.

The plots of size distribution by intensity (Appendix 6), showed a single peak, indicating a monomodal size distribution pattern, with respect to liposomes 1 and 4. Although the remaining liposomes gave two peaks, a dominant population of small vesicles was apparent in each case. Furthermore, the second population of vesicles in liposomes 2, 3, 5, 6, and 7, which were between 2 and 5 μm in diameter, accounted for less than 1 – 4 % of the individual preparations by intensity. Due to the fact that large particles scatter light to a greater extent, DLS-based size determination is sensitive to minute quantities of large particles in a sample (Mattison *et al*., 2003). In a review of liposome preparation methods Riaz (1996) commented that even sonicated suspensions of small unilamellar vesicles may contain traces of large multilamellar vesicles; and this may account for the large particles detected in the above-mentioned preparations. It is worth mentioning, at this point, that size distributions by volume and number may also be generated by the Zetasizer Software system used. However, the primary distribution profile generated is intensity-weighted; and distributions by volume and number are derived hereof using mathematical theories. Consequently, volume- and number-based distributions are less accurate than distributions by intensity, with the greatest error associated with number distributions. Moreover, for samples in which the larger particles that are known for high intensity light scattering account for a small percentage by intensity, as noted for the above-mentioned liposomes, such particles are often negligible by volume. Therefore, these may not be represented when the data is converted to volume-weighted distributions. For these reasons, only the intensity-weighted size distribution profiles were presented in this study.

In general, the pegylated liposomes were approximately half the size of their non-pegylated counterparts. These observations are supported by studies which show that DSPE-PEG_{2000}, at concentrations of up to 7 % (mol/mol), decreases vesicle size due to steric repulsion by the PEG chains grafted to neighbouring vesicles (Liu *et al*., 2003; Yoshida *et al*., 1999). It has also been shown that while non-pegylated nanoparticles, including liposomes, often aggregate over extended periods of time, pegylation maintains particle size (Zhao *et al*., 2007). However, the PdI values of the pegylated liposomes 5, 6 and 7 were not significantly different from that of their non-pegylated counterparts 1, 2 and 3, respectively. Conversely, it was expected that the pegylated vesicles would be distributed over a narrower range due to steric stability. Nonetheless, these findings may be attributed to the non-conventionally shaped vesicular bodies observed by electron microscopy in pegylated preparations alone;

taking into account that DLS assesses particle size according to the equivalent sphere principle, which assumes a spherical shape for individual particles (Gaumet *et al*., 2008). Therefore, a sample containing particles which are both non-spherical and heterogeneously shaped is likely to be characterised by an elevated PdI value.

It was found that the introduction of either SH02 or SH04, at the given molar ratio, did not significantly alter the size and size distribution patterns of the Chol-T/DOPE liposomes. Similar observations were made when comparing the pegylated liposomes 6 and 7, with the Chol-T/DOPE/DSPE-PEG_{2000} formulation. However, liposome 4, which incorporated both SH02 and SH04, gave larger, more heterogeneously sized vesicles than liposome 1. Although the Z-average vesicle diameter and PdI of its pegylated counterpart, liposome 8, was not significantly different from that of liposome 5, both liposomes 4 and 8 were not as stable as the remaining 6 preparations with extended storage. The discussion to follow pays attention to the characteristics of these liposome preparations.

Liposome 4 (Chol-T/DOPE/SH02/SH04) was characterised by the highest Z-average diameter (765.40 nm) and PdI value (0.535) across the eight liposomes prepared. This may be ascribed to the observation that liposome 4 partially aggregated with extended storage time, even though all liposome suspensions were routinely sonicated for the duration of the study. The results obtained are supported further by the visual detail provided by the electron micrographs of this liposome suspension (Figure 3.6d). According to the size distribution profile of liposome 4, the single population of vesicles, most prevalent by intensity, was centered around 473.0 ± 69.6 nm. Due to the fact that the cumulants algorithm which gives the Z-average diameter is based on the equivalent sphere principle, in suspensions, such as liposome 4, which display high heterogeneity in size, this value may be inclined towards the larger particles (Crawford *et al*., 2011; Gaumet *et al*., 2008; Mattison *et al*., 2003). According to Gaumet and coworkers (2008), this holds true even in instances where a small number of aggregates are present, relative to the other particles in suspension. Therefore, given the instability of liposome 4, the visible lipid precipitation and the consequent size heterogeneity, it is understandable that the Z-average diameter obtained was excessively large, despite the prevalence of smaller sized vesicles.

Liposome 8 was also visibly unstable, and large particles in the suspension accounted, on average, for 8.1 % by intensity. The characteristics of liposomes 4 and 8 indicate that combination of both novel cholesterol derivatives, with Chol-T and DOPE, at the molar ratios employed in this study, does not support the formation of stable cationic liposomes. However, a comparison of the DLS-data and electron micrographs of these liposomes, suggests that the addition of DSPE-PEG_{2000}, at low molar concentration, moderately reduced the instability associated with the incorporation of both SH02 and SH04. Unlike its non-pegylated counterpart, the hydrated lipids of liposome 8 that settled out of suspension after long-term storage were redispersed with vortexing and sonication. Furthermore, liposome 8 did afford small unilamellar vesicles which were distributed over a narrower size range than liposome 4. This may be attributed to the effect of the phospholipid, DSPE, in stabilising the lamellar phase of the lipid bilayer (Shi *et al*., 2002), and/or the inter-vesicle repulsion and bilayer-stabilising ability of the PEG chains (Varga *et al*., 2010).

3.3.3 Buffering capacity of liposomes

Gene carriers with proton sponge capability are characterised by the ability to resist pH change within the endosomal range (Midoux *et al*., 2009). A comparison of the acid titration profiles of liposomes 1 and 3 (Figure 3.8) revealed that liposome 3 was able to resist change in pH within the range of 5.1 – 5.8. This confirms that the introduction of SH04 into the Chol-T/DOPE formulation, at 10 % on a molar basis, afforded the liposome buffering capacity within mildly acidic conditions, as is prevalent in the endosome. The results are supported by similar titration curves that have been reported for both liposomal and non-liposomal imidazolylated carriers. In each instance, the buffering capacity of the vector was attributed to protonation of the imidazole ring (Benns *et al*., 2000; Shigeta *et al*., 2007).

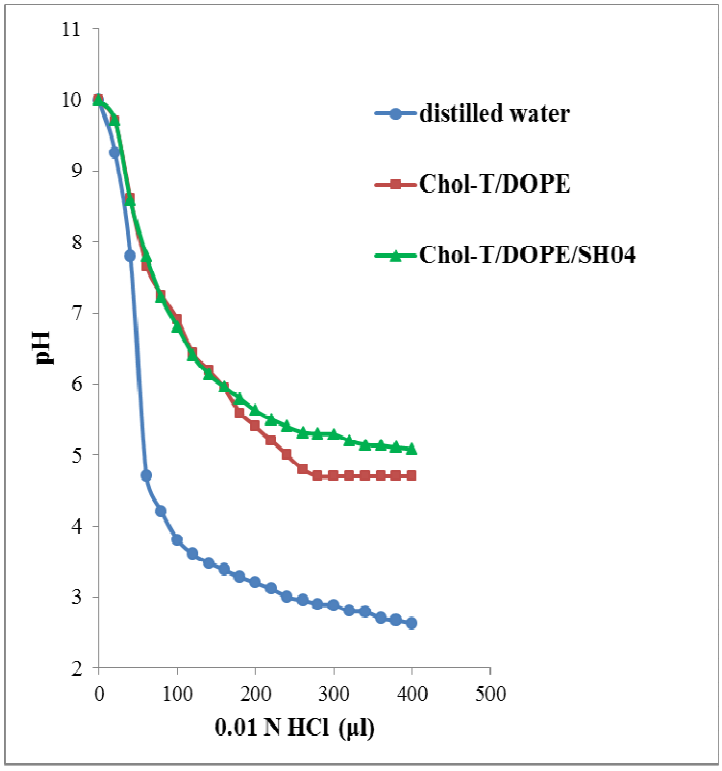


Figure 3.8: Titration profiles of liposomes 1 and 3. Aqueous suspensions of liposomes (10 ml) were adjusted to pH 10 using 0.35 N NaOH before titration with acid.

In summary, a series of pegylated and non-pegylated cationic liposomes, incorporating the newly synthesised cholesterol derivatives, SH02 and SH04, each at 10 mol %, was successfully prepared using the lipid film hydration method. In each formulation, the cytofectin, Chol-T, and co-lipid, DOPE, were combined in either equimolar or near-equimolar quantities. Steric stabilisation of liposomes was achieved by the introduction of DSPE-PEG_{2000} directly into lipid mixtures. Liposomes formulated with either SH02 or SH04, remained stable for the duration of the study, while those containing both novel compounds showed visible instability with extended storage time. However, pegylation at 5 mol % reduced this effect. Characterisation of liposomes by cryo-TEM and DLS revealed that all liposome preparations, with the exception of liposome 4 (Chol-T/DOPE/SH02/SH04) afforded small unilamellar vesicles. In general, pegylated liposomes were nearly half the size of their non-pegylated equivalents, but showed greater heterogeneity with regard to vesicle shape. Finally, an acid titration experiment demonstrated that the incorporation of SH04 at 10 mol % into the Chol-T/DOPE formulation afforded buffering capacity within the endosomal range.

CHAPTER FOUR

LIPOSOME-DNA INTERACTIONS

4.1 Introduction

The nature of the association between cationic liposomes and DNA influences the size, stability, and transfection efficiency of the resulting lipoplexes (Huebner *et al*., 1999; Kennedy *et al*., 2000; Šmisterová *et al*., 2001). Consequently, an understanding of liposome-DNA interactions is essential in order to optimise vector performance (Dan, 1998). This chapter outlines the characterisation of interactions between pegylated and non-pegylated cationic liposomes with plasmid DNA, based on the DNA-binding affinity and DNA-protecting capabilities of the carriers, according to the gel retardation and nuclease digestion assays respectively. In addition, transfecting complexes were characterised by cryo-TEM and DLS-based methods.

4.1.1 Lipoplex assembly

The primary event in the association of cationic liposomes with DNA is the nearly-spontaneous formation of ion pairs between the positively charged headgroups of the cytofectin and negatively charged phosphate moieties of DNA (Wasungu and Hoekstra, 2006). Pozharski and MacDonald (2003) investigated the binding free energy of lipoplex formation by monitoring the dissociation of EDOPC/DNA complexes at increasing ionic strength. It was found that although the interaction between an isolated pair of oppositely charged entities within the lipoplex is relatively weak, high affinity binding of DNA to cationic liposomes is the cumulative effect of many such interactions acting simultaneously. Furthermore, ion pair formation is accompanied by the release of counterions, initially associated with the liposomal bilayer and DNA molecules (Figure 4.1), and partial dehydration of both components. It is these processes which largely account for the entropy gain that renders lipoplex formation thermodynamically favourable (Bruinsma, 1998; Pozharski and MacDonald, 2003).

Although several models have been proposed, it is agreed that lipoplex assembly proceeds via a multistep mechanism (Gershon *et al*., 1993; Huebner *et al*., 1999; Kennedy *et al*., 2000; Wasungu and Hoekstra, 2006). An early model of lipoplex assembly was proposed by Gershon and colleagues (1993) following electron microscopy, lipid mixing and fluorescence quenching experiments with DOTMA/PE/DNA complexes. This group postulated that cationic liposomes initially bind to DNA molecules, and form clusters along the nucleic acid. However, more recent studies have presented images of DNA-coated vesicles, and show that it is the cationic bilayer which forms the scaffold for the attachment of DNA molecules (Huebner *et al*., 1999). Nonetheless, it is widely conceded that at a critical liposome density, DNA-induced membrane fusion and liposome-induced DNA collapse result in condensed structures of DNA that are encompassed by fused lipid bilayers (Ma *et al*., 2007). Further research has provided insight into these key processes.

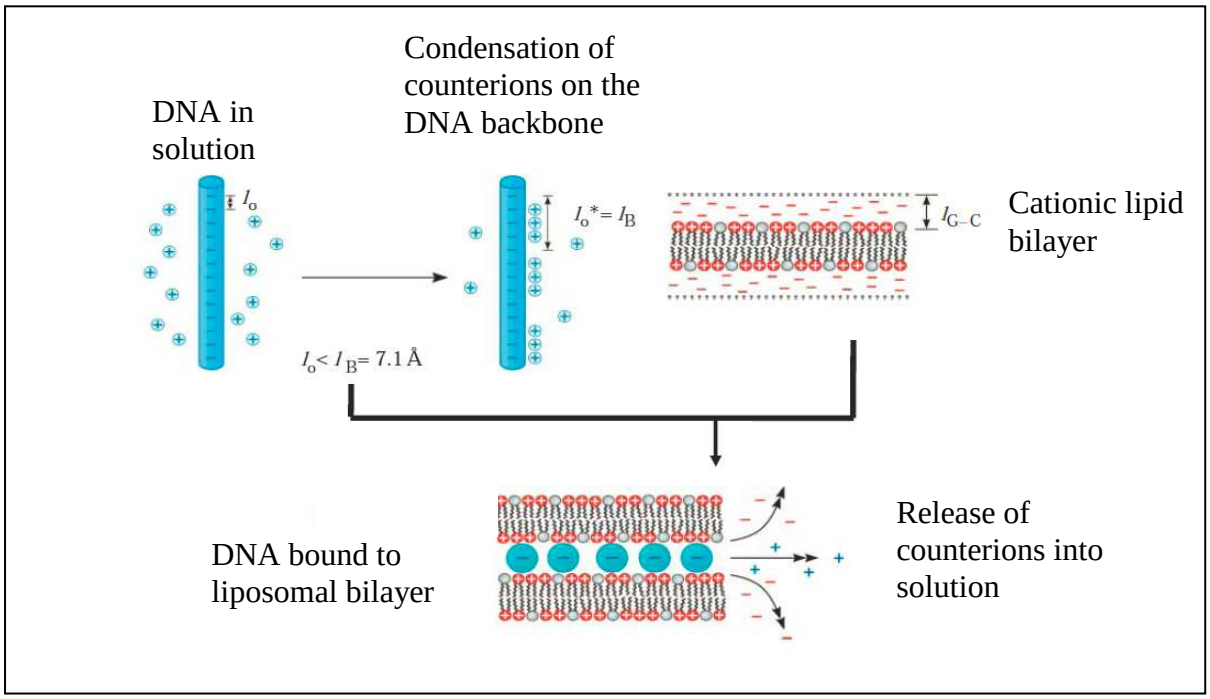


Figure 4.1: Lipoplex formation is driven by the release of counterions associated with the polyanionic DNA and cationic liposomal bilayer (adapted from Safinya *et al*., 2006).

It is only the outer surface of the liposome that is available for binding DNA. Therefore, the electrostatic attachment of DNA, which increases the hydrophobicity of the cytofectin headgroup, induces an uneven packing pressure upon the bilayer. This in turn causes deformation of cationic vesicles, to the extent that membrane destabilisation occurs (Huebner

et al., 1999). Several destabilised vesicles may fuse due to lipid mixing. Alternatively, the stress imposed by the binding of DNA may induce rupture of liposomes, exposing hydrophobic regions of the membrane, which merge with neighbouring vesicles (Wasungu and Hoekstra, 2006). Huebner and coworkers (1999) have extended this hypothesis by suggesting that the bilayers of ruptured vesicles "roll over" adjacent vesicles due to the inherent flexibility of the lipid membranes. Both mechanisms of DNA-induced lipid reorganisation are represented in Figure 4.2.

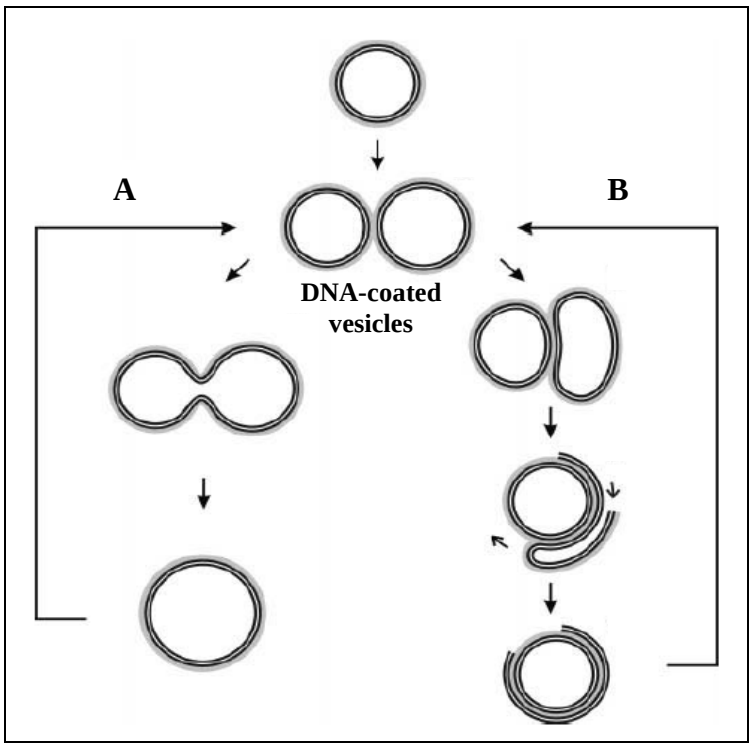


Figure 4.2: Reorganisation of cationic liposomal bilayers upon binding DNA, as proposed by Huebner and colleagues (1999). Scheme A shows the fusion of DNA-coated vesicles, while scheme B outlines DNA-induced membrane rupture and resealing. The black and grey regions represent lipid bilayers and DNA respectively.

Investigations that have contributed towards current models of liposome-DNA associations have also focused on the conformation of DNA within transfecting complexes. Braun and coworkers (2003) have presented evidence that liposome-associated DNA exists in a variant B-form, having employed several spectroscopic techniques and a computational-modelling system (Figure 4.3). This group proposed that the observed alterations in base-stacking interactions of DNA within lipoplexes are a consequence of interactions between the cytofectin headgroups and DNA bases. In addition, DNA bound to adjacent lipid bilayers

within individual lipoplexes align in an orderly fashion, due to long-range correlation forces between the DNA molecules themselves (Battersby *et al*., 1998; Huebner *et al*., 1999).

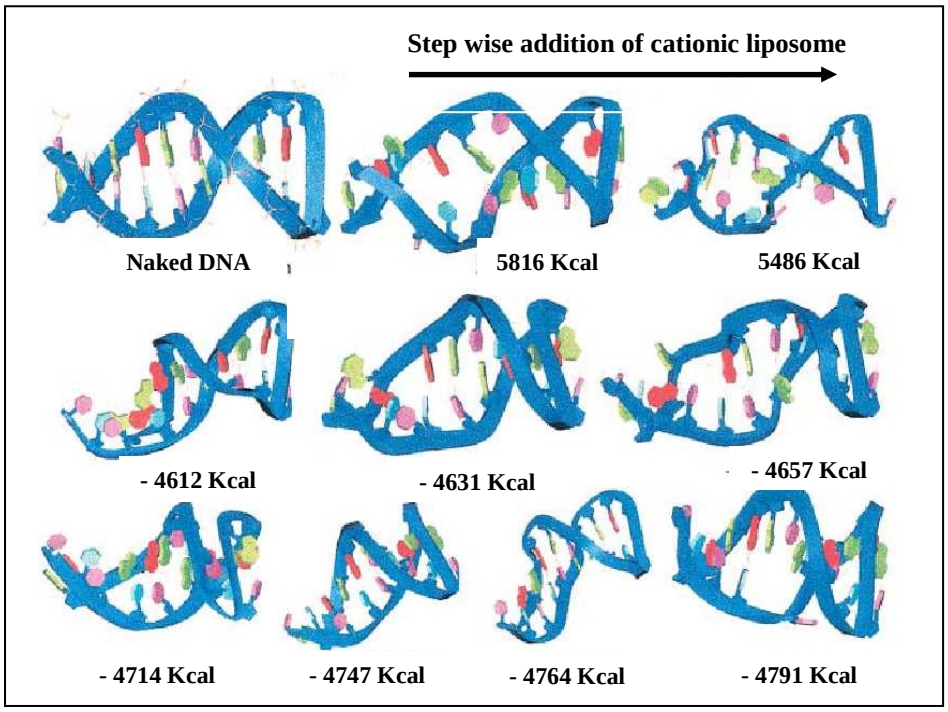


Figure 4.3: A dynamic simulation of the conformation of double stranded DNA at different stages in its interaction with cationic liposomes, in order of increasing favourable free energy (adapted from Braun *et al*., 2003).

4.1.2 Lipoplex morphology

The rearrangement of lipid and DNA components, in the formation of lipoplexes, may be influenced by several factors. These include composition of the cationic liposome, the lipid:DNA charge ratio, presence and concentration of divalent cations in the incubation medium, and the order and rate of addition of nucleic acids and liposomes (Huebner *et al*., 1999; Kennedy *et al*., 2000; Mozafari *et al*., 2005). As a consequence, liposome/DNA complexes, as investigated in individual studies have displayed varying structural detail. However, a consensus has not been reached with regard to which lipoplex structures are most physiologically relevant (Karmali and Chaudhuri, 2007). This is primarily because studies aimed at exploring the structure-function relationships of lipoplexes employ widely differing

liposome formulations and cell lines. Therefore, comparisons between such studies are not feasible (Šmisterová *et al*., 2001). Nonetheless, three main lipoplex configurations (Figure 4.4a-c) have been proposed using information obtained through a wide range of techniques, which include conventional electron microscopy, small angle X-ray scattering and atomic force microscopy (Huebner *et al*., 1999).

Dan (1998) used a mathematical model to show that the liposomes are least distorted when in a lamellar arrangement with DNA (Figure 4.4a); and are more stable than cylindrical lipoplex structures (Figure 4.4c), in which lipid bilayers surround DNA molecules. However, the hexagonal or "honeycomb" complex (Figure 4.4b), that consists of closely packed units of DNA that are coated by lipid monolayers, is believed to be the most stable among the three structures. Moreover, the structure of lipoplexes, which have exhibited promising transfection capabilities, changes at different stages of the gene transfer process; as reported by Hulst and colleagues (2004) in a study of modified SAINT lipid/DOPE/DNA systems. For example, lamellar or "sandwich" lipoplex structures were observed upon condensation of DNA by the liposomes and during transport to the cells. However, the transfecting complex adopted a less stable inverted hexagonal form, when in contact with the cell membranes and, as such, permitted cellular entry. Other poorly characterised lipoplex structures are believed to exist as intermediates in the transition from lamellar to inverted hexagonal configuration (Ma *et al*., 2007).

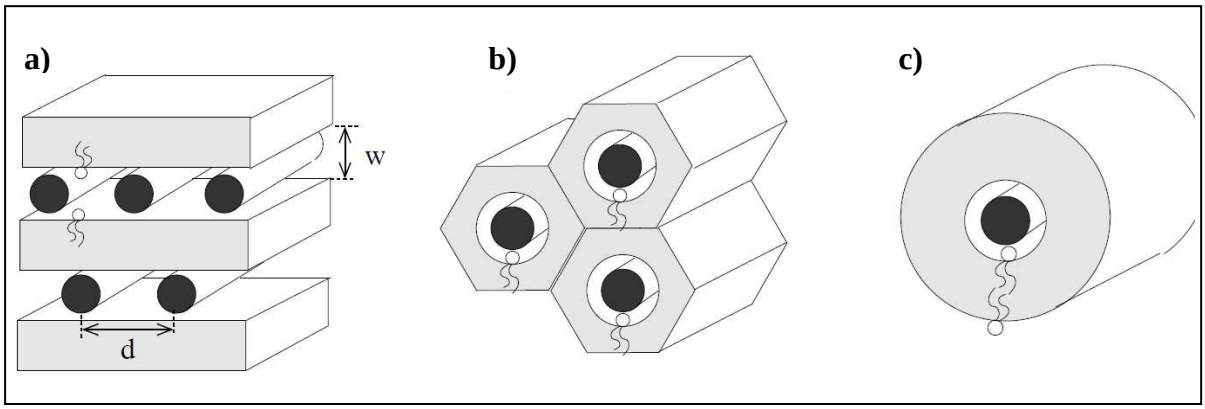


Figure 4.4: Three main lipoplex geometries, a) lamellar or sandwich structure, b) hexagonal or honeycomb complex, and c) the bilayer-coated complex. The grey regions represent the non-polar lipid core, and the solid circles, cross-sections of DNA. In a) d is the DNA-DNA distance; and w is the width of the water gap between adjacent bilayers (May and Ben-Shaul, 2004).

4.2 Materials and Methods

4.2.1 Materials

pCMV-*luc* plasmid DNA (1 μg/μl stock) was obtained from Plasmid Factory, Bielefeld, Germany. (Plasmid amplification and isolation is outlined in Appendix 5). Ultrapure™ agarose and heat-inactivated newborn calf serum were purchased from Gibco Invitrogen, USA. Tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) and ethylenediamine-tetraacetic acid (EDTA) (disodium salt, dihydrate) were from Merck, Darmstadt, Germany and Calbiochem, Germany respectively. Promega, Madison, USA supplied the ethidium bromide solution (10 mg/ml stock). All other reagents were of analytical grade.

4.2.2 Methods

4.2.2.1 Gel retardation assays

4.2.2.1.1 Preparation of 1 % agarose gel

Agarose powder (0.2 g) was suspended in 18 Mohm water (18 ml) and heated to boiling point. After cooling to approximately 75 ˚C, 2 ml 10 × electrophoresis buffer (0.36 M Tris-HCl, 0.3 M NaH_2PO_4, 0.1 M EDTA, pH 7.5) and 25 μg ethidium bromide were added. The mixture was then poured into a gel-casting tray fitted with an eight-well comb, and allowed to set for 30 – 45 minutes at room temperature.

4.2.2.1.2 Preparation of lipoplexes

Seven lipoplexes were assembled per liposome, at Chol-T:DNA (w/w) ratios ranging from 0.25:1 to 1.75:1, in increments of 0.25 with respect to non-pegylated preparations; and 1:1 to 4:1, in increments of 0.5 for pegylated carriers.

Varying amounts of liposome was added to pCMV-*luc* DNA (0.5 μg) in HBS (11 μl), so as to correspond to the aforementioned Chol-T:DNA (w/w) ratios. The mixtures were incubated

for 30 minutes at room temperature to permit the formation and maturation of electrostatic complexes.

4.2.2.1.3 Electrophoresis

Lipoplexes were mixed with 3 μl gel loading buffer (containing 40 % sucrose and 0.5 % bromophenol blue) and loaded onto 1 % agarose gel. Electrophoresis was carried out for 90 minutes in a Mini-Sub^{R} apparatus (Bio-Rad, Richmond, CA) containing 1 × electrophoresis buffer (36 mM Tris-HCl, 30 mM NaH_2PO_4, 10 mM EDTA, pH 7.5), using a Bio-Rad PowerPac^{TM} Basic unit operating at 50 V. The gel was viewed under ultraviolet transillumination using a Vacutec SynGene G:Box (Cambridge, UK) gel documentation system. Images were captured with GeneSnap software following exposure times of 1 – 2 seconds.

4.2.2.2 Characterisation of lipoplexes

Pegylated and non-pegylated lipoplexes were assembled with pCMV-*luc* DNA at Chol-T:DNA (w/w) ratios which exhibited optimum binding, and optimum transfection in the target cells. Lipoplexes prepared at these ratios alone were characterised by cryo-TEM and DLS.

4.2.2.2.1 Cryo-TEM of lipoplexes

Complexes prepared with pegylated and non-pegylated lipoplexes were diluted 1:9 (v/v) and 1:6 (v/v) in HBS respectively. Samples (1 μl) were cryopreserved and viewed according to the protocol outlined in 3.2.2.2.1.

4.2.2.2.2 Measurement of particle size

Lipoplexes were diluted 1:100 (v/v) in HBS to a total volume of 1 ml. The size and size distribution were obtained using the Zetasizer Nano ZS (Malvern Instruments Ltd.,

Worcestershire, UK). Measurements were read at 25 ˚C. In all instances, the refractive index and viscosity of the dispersant were assumed to be 1.330 and 0.8872 respectively.

4.2.2.2.3 Statistical analysis

Data was analysed by one-way ANOVA, followed by Tukey's Multiple Comparison Test. *P* values below 0.05 were considered significant.

4.2.2.3 Nuclease digestion assays

Lipoplexes, corresponding to the optimal, sub- and super-optimal DNA-binding ratios (as determined by the gel retardation assays), were incubated with serum (10 % by volume) for 4 hours at 37 ˚C. As a control, 0.5 μg naked pCMV-*luc* DNA was subjected to the same treatment. EDTA was added to a final concentration of 10 mM, in order to halt nuclease activity. Thereafter, complexes were disassembled by the addition of SDS to a final concentration of 0.5 % (w/v). Reaction mixtures were maintained at 55 ˚C for 20 minutes. Finally, 3 μl gel loading buffer was added; and electrophoresis was conducted for 120 minutes as previously described (4.2.2.1.3).

4.3 Results and discussion

4.3.1 Gel retardation assays

The gel retardation assays, represented in Figures 4.5 and 4.6, demonstrate the association of cationic liposomes with plasmid DNA. This electrophoretic assay is based on the premise that the migration of DNA that is entirely bound by cationic liposomes is retarded in an electric field because the large, electroneutral complexes fail to enter the matrix of agarose gel (Kisoon *et al*., 2002).

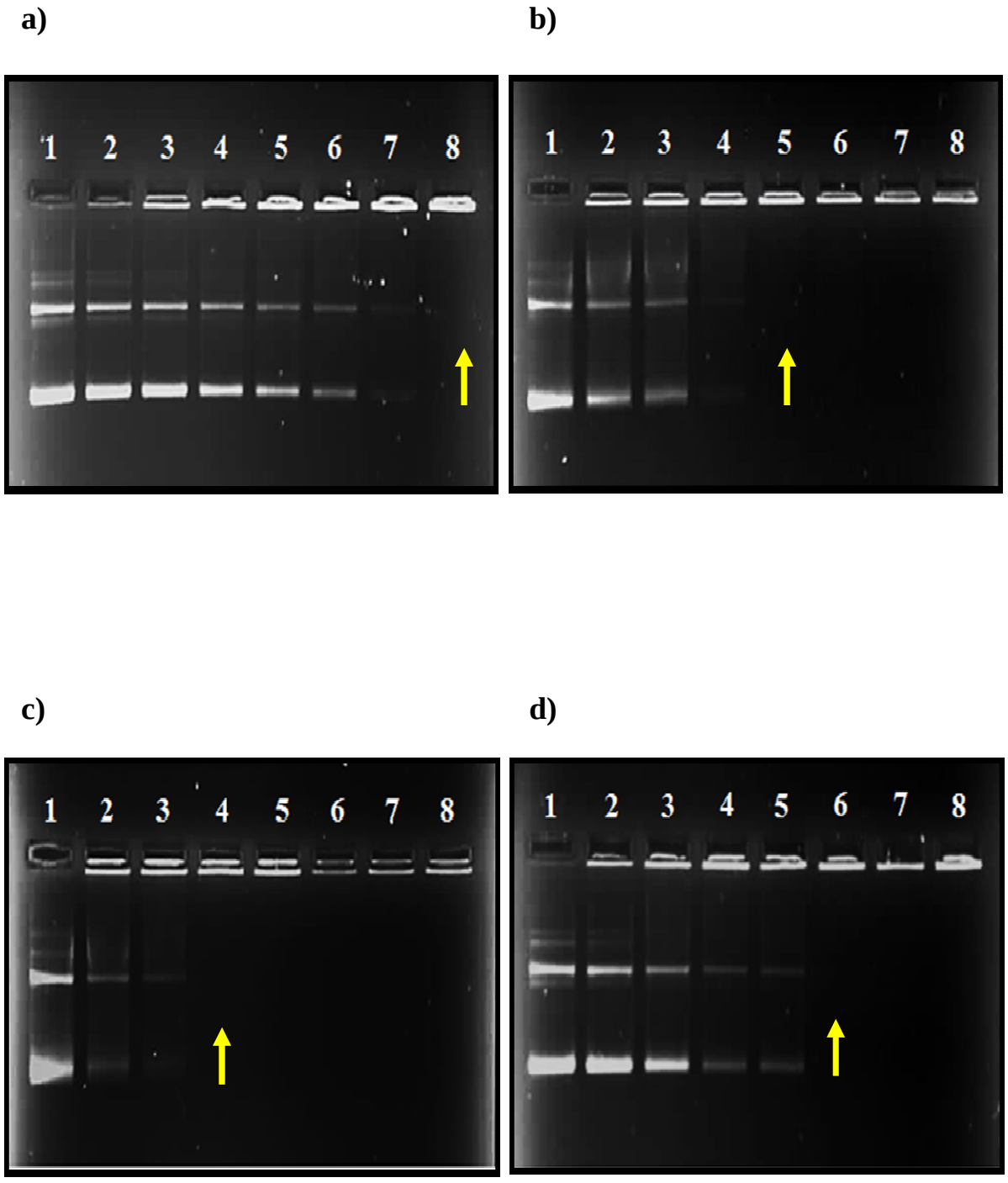


Figure 4.5: Gel retardation study of the binding interactions between plasmid DNA and non-pegylated liposomes, a) 1; b) 2; c) 3; and d) 4. Incubation mixtures (11 µl) contained HBS, pCMV-*luc* DNA (0.5 µg) and varying amounts of Chol-T (0, 0.125, 0.25, 0.375, 0.5, 0.625, 0.75 and 0.875 µg) per liposome in lanes 1-8 respectively. In each instance, an arrow indicates the point at which DNA was entirely liposome-bound.

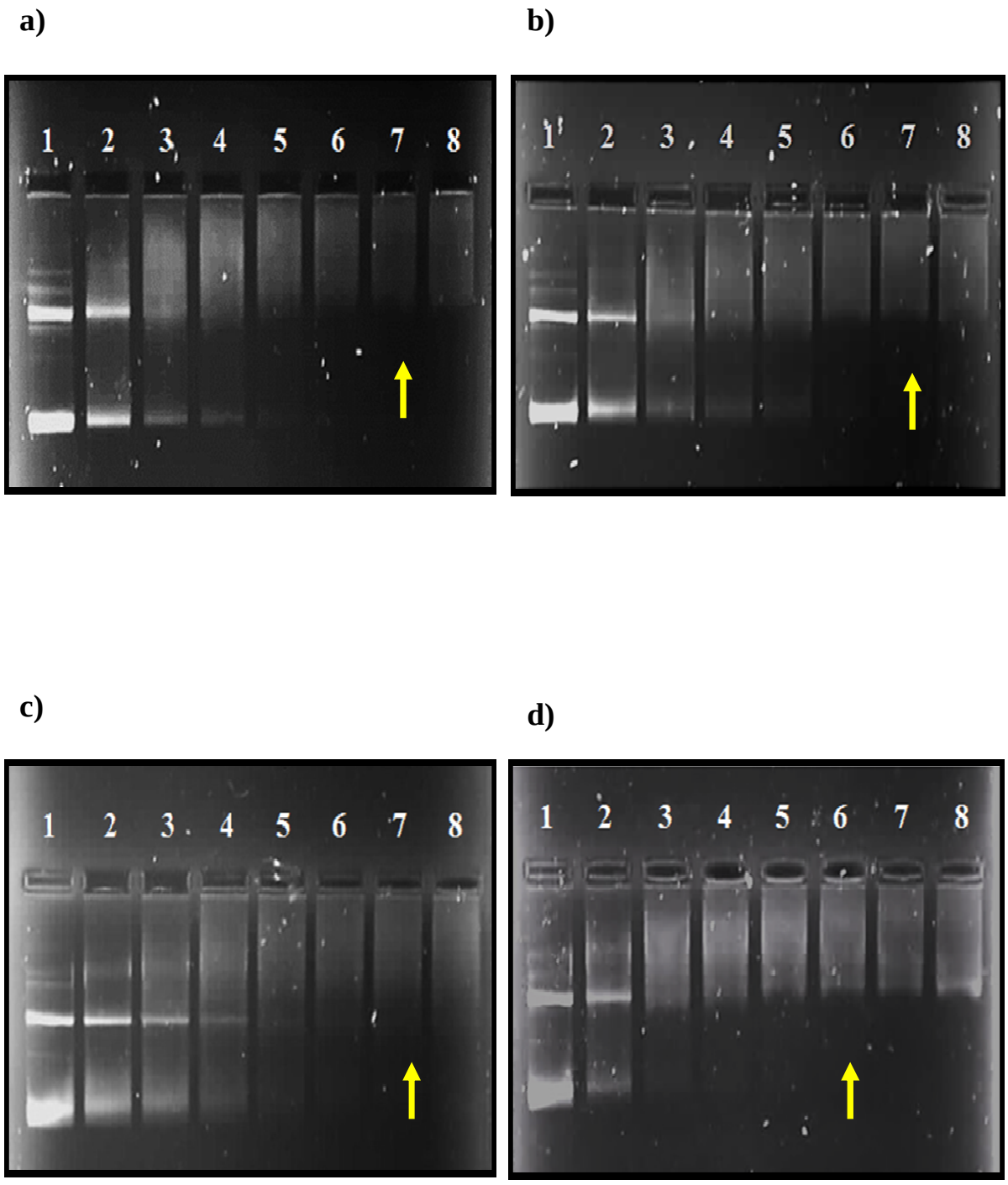


Figure 4.6: Gel retardation study of the binding interactions between plasmid DNA and pegylated liposomes, a) 5; b) 6; c) 7; and d) 8. Incubation mixtures (11 µl) contained HBS, pCMV-*luc* DNA (0.5 µg) and varying amounts of Chol-T (0, 0.5, 0.75, 1.0, 1.25., 1.5, 1.75 and 2.0 µg) per liposome in lanes 1-8 respectively. In each instance, the point at which the liposomes best bound DNA is highlighted by an arrow.

In lane 1 of each gel, two forms, namely the superhelical and closed circular forms of the uncomplexed plasmid were prominent. As the amount of liposome relative to DNA was increased across lanes 2 to 8, more DNA became liposome-associated. This was observed as a gradual decrease in the intensity of DNA bands that had migrated into the gel, accompanied by an increase in DNA retained in the wells, in the form of lipoplexes. However, in some instances, as in Figures 4.5c and 4.6a-d, further addition of liposome did not visibly correlate with intensified DNA accumulation in the wells. This is due to the formation of complexes near neutrality, which may float out of the wells and into the electrophoresis buffer. Eventually, optimal binding of DNA by the liposomes was achieved when DNA failed to migrate away from the well. In this way, the minimum amount of each liposome preparation required to effectively bind a fixed amount of DNA (0.5 μg) was established. This was also expressed as a function of the amount of Chol-T present in the respective quantity of liposome, relative to DNA, on a weight basis (Table 4.1); because it is the cytofectin that confers the positive charge necessary to bind DNA. The lipoplexes assembled at the optimal, sub- and super-optimal DNA-binding ratios were explored further with respect to their nuclease-resistance, cytotoxicity and transfection potential.

Table 4.1: Optimum DNA-binding ratios for liposome preparations.

Liposome	**Chol-T:DNA (w/w) ratio**	**Liposome:DNA (w/w) ratio**	**Cytofectin:DNA (+/-) charge ratio**
1	**1.75:1**	4.2:1	1.1:1
2	**1.0:1**	2.8:1	1:1.5
3	**0.75:1**	2.0:1	1.0:2
4	**1.25:1**	3.9:1	1:1.2
5	**3.5:1**	10.0:1	2.3:1
6	**3.5:1**	11.3:1	2.3:1
7	**3.5:1**	11.0:1	2.3:1
8	**3.0:1**	10.8:1	2.0:1

The non-pegylated liposomes 1 to 4 demonstrated complete binding of DNA (Figure 4.5a-d). Liposomes 2 to 4, which were formulated with the newly-synthesised cholesterol derivatives, bound DNA with higher affinity than the Chol-T/DOPE formulation, as evidenced by the lower cytofectin:DNA ratios at which the migration of DNA was entirely retarded (Table 4.1). In addition, liposomes which contained either SH02 or SH04 (liposomes 2 and 3, respectively) showed greater DNA binding capability than liposome 4, which was formulated with both novel lipids. These observations are in keeping with studies conducted by Ferrari and colleagues (2002) which show that the DNA-binding characteristics of the individual preparations is primarily a consequence of the lipid composition; possibly due to its bearing on the distribution of the cytofectin within the inner and outer leaflets of the bilayer, and the exposure of the cationic headgroup to the external environment. In support of this argument, it has been shown that the tendency of some lipids to self-associate in the bilayers of small unilamellar vesicles promotes an asymmetric distribution of other lipids (Boggs, 1987). Furthermore, studies conducted by Pysher and Hayes (2005) suggest that the fluidity of the liposomal membrane and the mobility of the cationic lipid often influence a non-uniform charge distribution on the surface of liposomes. It is likely, therefore, that the introduction of SH02 and/or SH04 induced greater prevalence of cytofectin molecules within the outer leaflet of the lipid bilayer, thereby enhancing the DNA-binding ability of the Chol-T/DOPE formulation. Similarly, the exposure of the cytofectin appears to have been more pronounced in liposomes which contained either SH02 or SH04, than when both lipids were incorporated in a single formulation.

While the gel retardation assays of the non-pegylated liposomes gave clearly discernable end-points, the same did not hold true for their pegylated counterparts. Instead, these liposomes (preparations 5 to 8) demonstrated near-complete binding of DNA. From Figure 4.6a-d, it is apparent that there exists a threshold beyond which further binding of plasmid to the pegylated liposomes did not occur. Therefore, the point at which distinct bands of DNA were no longer visible on the gel was accepted as the ratio at which these preparations best bound DNA. In all instances, the superior DNA-binding affinity of the non-pegylated carriers over their pegylated counterparts was emphasised by the markedly lower amounts of liposome or Chol-T necessary to achieve full retardation of DNA (Table 4.1). Other authors have also reported a reduction in the DNA-binding capabilities of cationic liposomes upon pegylation (Singh *et al*., 2011; Zhang *et al*., 2010; Zhong *et al*., 2005). This has been attributed to partial

shielding of the cationic headgroups by the polymer chains, which impedes their interaction with DNA (Templeton, 2002). Nonetheless, recent studies have shown that liposomes formulated with cholesteryl cytofectins and DSPE-PEG_{2000} at 5 % on a molar basis achieved complete retardation of plasmid DNA (Singh *et al*., 2011; Zhang *et al*., 2010), albeit at higher ratios than their non-pegylated equivalents (Zhang *et al*., 2010). Although the sterically stabilised liposomes in this study were modified with the same pegylated lipid conjugate and at the same concentration, these carriers were unable to fully bind DNA. However, it is important to note that the overall composition of the pegylated liposomes in the aforementioned published works differs from those formulated in the current study. This presents the possibility that, in addition to the charge-adumbrating effect of the PEG chains, the incorporation of DSPE-PEG_{2000} in liposomes 5 to 8 may have promoted a higher density of Chol-T molecules on the inner surface of the liposomal bilayer, resulting in a lower effective positive charge presented on the external surface.

It is generally accepted that, upon complete retardation, the positive charges on the liposomal bilayer are entirely titrated by the negative charges on DNA molecules, resulting in electroneutral complexes (Kisoon *et al*., 2002). However, according to the charge ratios estimated, at optimum binding, only lipoplexes assembled from preparations 1 and 4 appear near neutrality. Complexes resulting from liposomes 2 and 3 bear net negative charges, while those of the remaining formulations are positive. This may be attributed to the assumptions upon which the calculation of the cytofectin:DNA charge ratios listed in Table 4.1 were based. In each case it was assumed that Chol-T is fully protonated at physiological pH; each negative charge on the DNA backbone is associated with a positive charge on the liposome surface; and that the average molecular weight of a nucleotide, bearing a single negative charge, is 330 (Singh and Ariatti, 2006).

Inaccuracies associated with the aforementioned assumptions are highlighted by Muñoz-Ùbeda and colleagues (2011) who recently showed that fewer negative charges are available for binding to cationic headgroups on plasmid DNA, that is predominantly supercoiled, as compared with linearised DNA; and Pysher and Hayes (2005) who report the uneven distribution of charged entities in a bilayer. Furthermore, according to the charge ratios calculated, the pegylated lipoplexes bear a higher apparent net positive charge than the non-pegylated lipoplexes at the optimum DNA-binding ratio. However, this is merely a

consequence of the larger amounts of the cytofectin that are required to bind the same quantity of DNA, when incorporated in pegylated liposomes than non-pegylated liposomes; and does not reflect the net charge on the surface of the carrier. In fact, Silvander (2002) showed, using a laser light scattering device, that the introduction of pegylated phospholipids at 5 mol % effectively reduces the surface charge of liposomes. It is therefore suggested that more accurate measurements of lipoplex surface charge or zeta potential be obtained in the event of the current study being extended. Although the Zetasizer, capable of determining zeta potential of both liposomes and lipoplexes, was made available during the latter stages of this study, due to lack of the appropriate measurement cells this property of the vectors was not investigated.

4.3.2 Characterisation of lipoplexes

In addition to the gel retardation assay, the formation of liposome-DNA complexes was confirmed by electron microscopy. Lipoplexes formed at the ratios which demonstrated optimum binding of DNA and optimum transfection in HepG2 cells were characterised. Other ratios were not explored. Although the characterisation of the latter lipoplexes was carried out following the gene transfer experiments outlined in Chapter Five, the data was included in this chapter in order to compare the ultrastructural detail of all lipoplexes investigated, in an orderly fashion.

Cryo-TEM and DLS together showed that lipoplexes derived from each of the eight liposome preparations differed in size and shape. Documented transmission electron micrographs of a variety of cationic liposome-DNA complexes have also revealed variable morphology (Safinya *et al*., 2006). Furthermore, published works suggest that the lipoplex morphologies observed are a consequence of both the lipid composition of the carrier, which influences its assembly with DNA; and the technique employed to prepare and view individual lipoplexes (Huebner *et al*., 1999; Karmali and Chaudhuri, 2007). As an example, several lipoplexes in this study (Figures 4.7d; 4.8a, c, d; and 4.10a, b) appeared as clusters of vesicles, however, the number, size, shape and arrangements of these smaller units within aggregates differed across the individual liposome formulations, and Chol-T:DNA ratios. Cationic lipoplexes containing cholesterol-based cytofectins are reported to have assumed comparable structures

(Singh *et al*., 2006; Singh *et al*., 2007; Singh and Ariatti, 2008). Other complexes observed include those of a globular nature, in which membranes of vesicles appear to have fused (Figures 4.7a; 4.8b; and 4.9d). This is similar to the general lipoplex ultrastructure described by Gonçalves and coworkers (2004). In this regard, atomic force microscopy studies carried out by Oberle and colleagues (2000) have shown that DNA within a lipoplex may be encased by up to five bilayers, depending on the number of fusion events that take place during its assembly. Interestingly, liposome 3, at the optimum DNA-binding ratio (Figure 4.7c), gave multilamellar structures. Battersby and coworkers (1998) described similar structures, visualised by cryo-electron microscopy of DMPC/DC-Chol lipoplexes, as fingerprint-like patterns. These were attributed to the ordered arrangement of parallel DNA helices between successive lipid bilayers.

It is worthy of note that complexes formed from a single liposome preparation at the respective Chol-T:DNA ratios exhibited different morphological traits. For example, at the optimum DNA-binding ratio, Chol-T/DOPE/DNA complexes appeared as aggregates of several fused vesicles that were flattened at the points of membrane contact (Figure 4.7a); while at the ratio which demonstrated optimum transfection, lipoplexes resembled large vesicles onto which smaller lipid bodies were attached (Figure 4.9a). These observations are in agreement with a report by Huebner and coworkers (1999). This group proposed that the process of lipid and DNA reorganisation involved in lipoplex assembly varies at different lipid:DNA ratios for a single liposome suspension, in order to explain the noticeable differences in the structure of DMPC/DC-Chol/DNA complexes at varying lipid:DNA mixing ratios. In addition, studies have shown that plasmid size influences the size and arrangement of liposome/DNA complexes (Madeira *et al*., 2007; Oberle *et al*., 2000). For this reason a single type of plasmid, i.e. pCMV-*luc*, was used for the assembly of lipoplexes throughout this study.

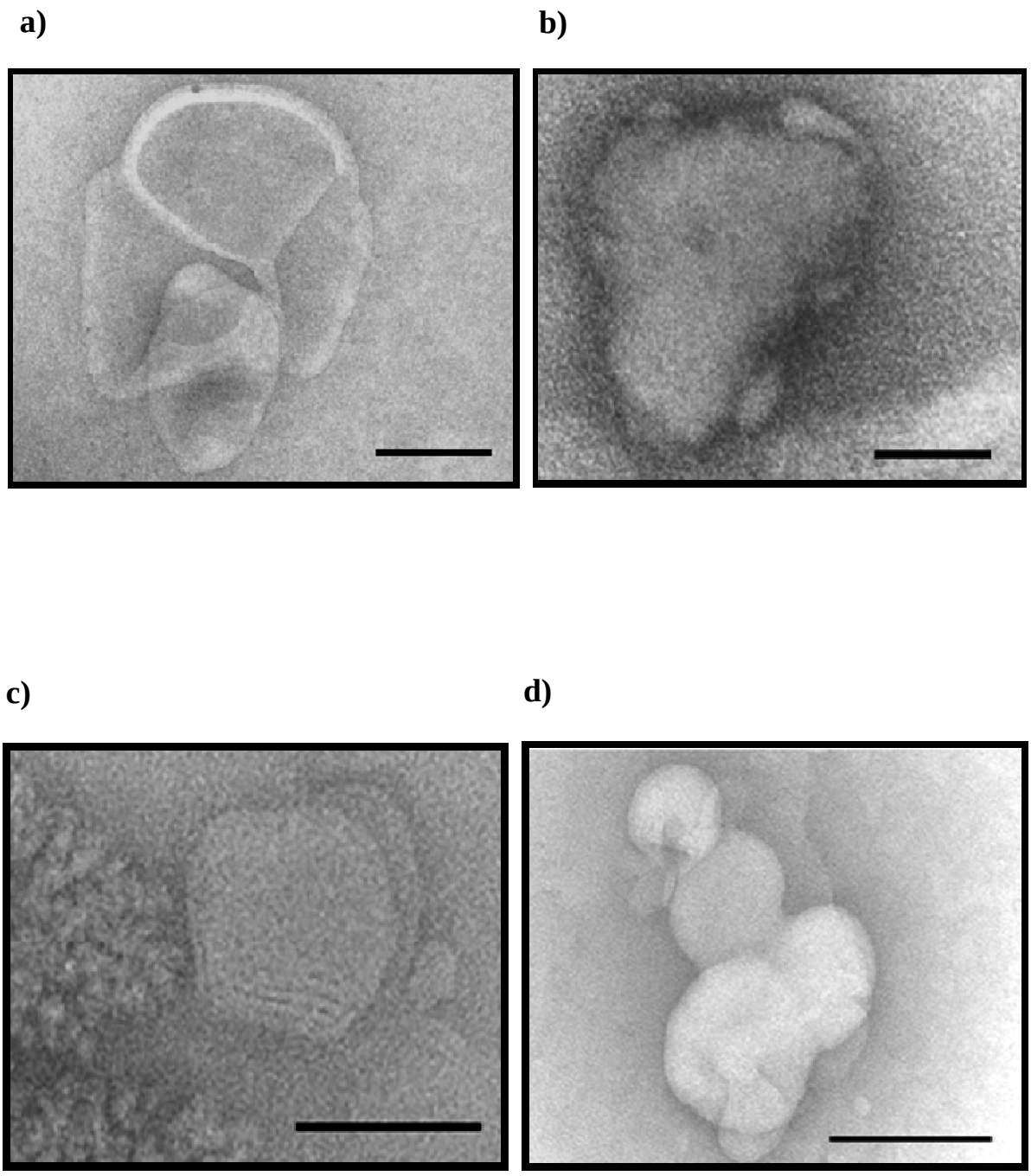


Figure 4.7: Transmission electron micrographs of lipoplexes assembled from pCMV-*luc* DNA and non-pegylated liposomes, a) 1; b) 2; c) 3; and d) 4, at the respective optimum DNA- binding ratios. Chol-T:DNA (w/w) ratios were as follows: 1.75:1 (a); 1:1 (b); 0.75:1 (c); and 1.25:1 (d). Lipoplex suspensions were stained with uranyl acetate and cryopreserved using liquid propane prior to viewing. Scale bar = 100 nm (b, and c); 200 nm (a, and d).

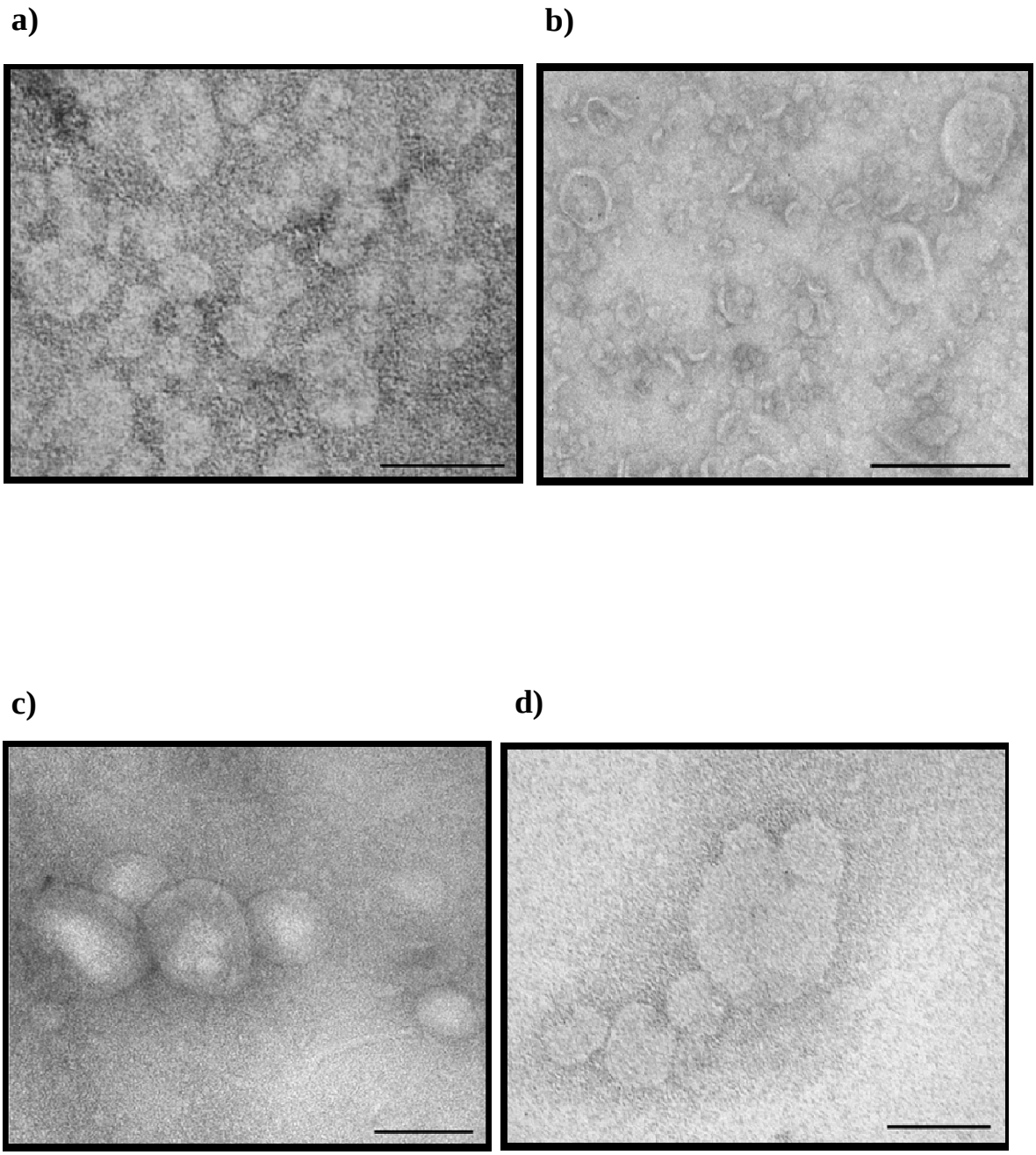


Figure 4.8: Transmission electron micrographs of lipoplexes assembled from pCMV-*luc* DNA and pegylated liposomes, a) 5; b) 6; c) 7; and d) 8, at the respective optimum DNA-binding ratios. Chol-T:DNA (w/w) ratios were as follows: 3.5:1 (a, b and c); and 3:1 (d). Lipoplex suspensions were stained with uranyl acetate and cryopreserved using liquid propane prior to viewing. Scale bar = 100 nm (a, c and d); 500 nm (b).

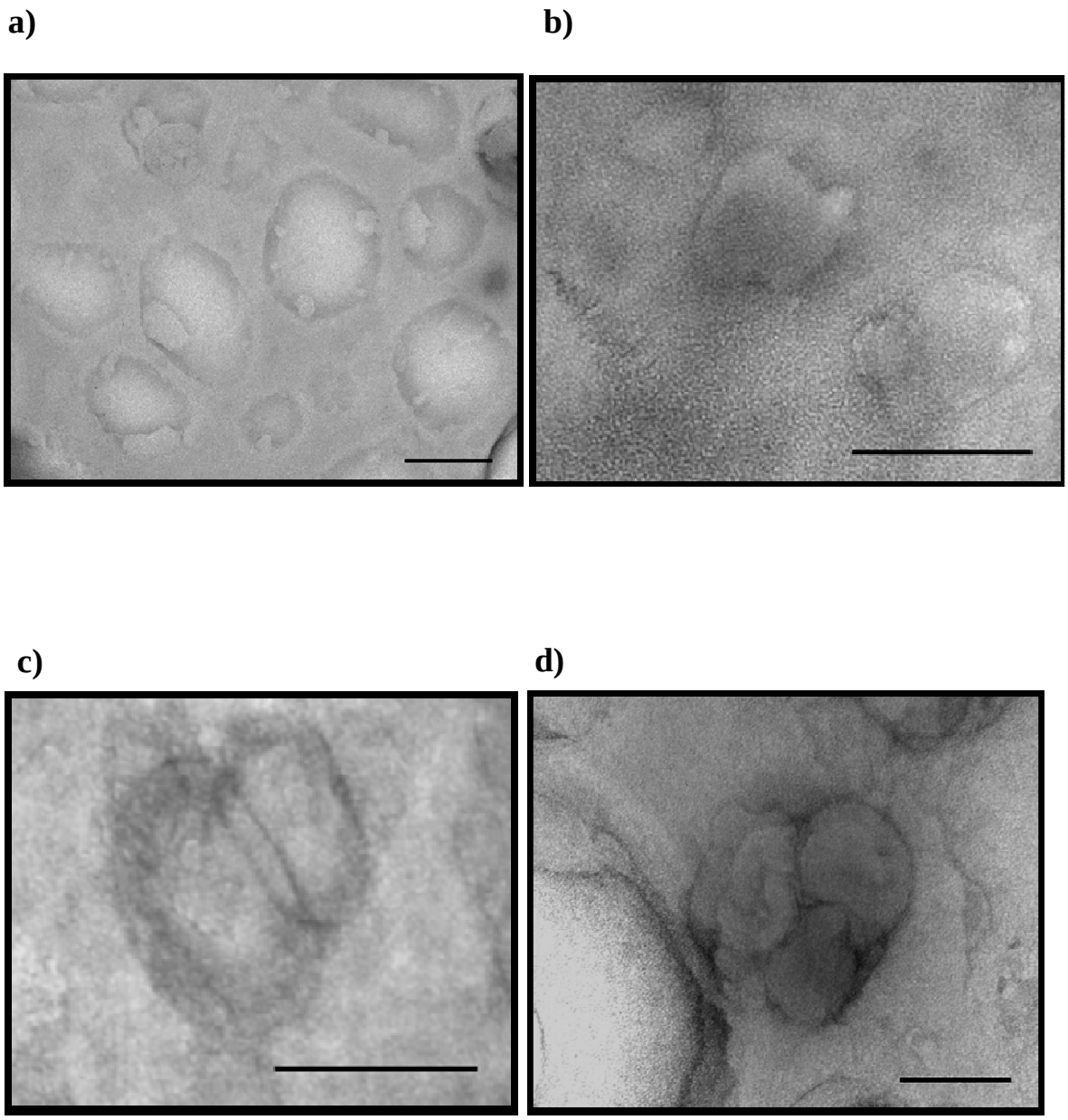


Figure 4.9: Transmission electron micrographs of lipoplexes assembled from pCMV-*luc* DNA and non-pegylated liposomes, a) 1; b) 2; c) 3; and d) 4, at Chol-T:DNA ratios which optimally transfected HepG2 cells. Chol-T:DNA (w/w) ratios were as follows: 2:1 (a); 0.75:1 (b); 1:1 (c); and 1.5:1 (d). Lipoplex suspensions were stained with uranyl acetate and cryopreserved using liquid propane prior to viewing. Scale bar = 200 nm (a); 100 nm (b, c and d).

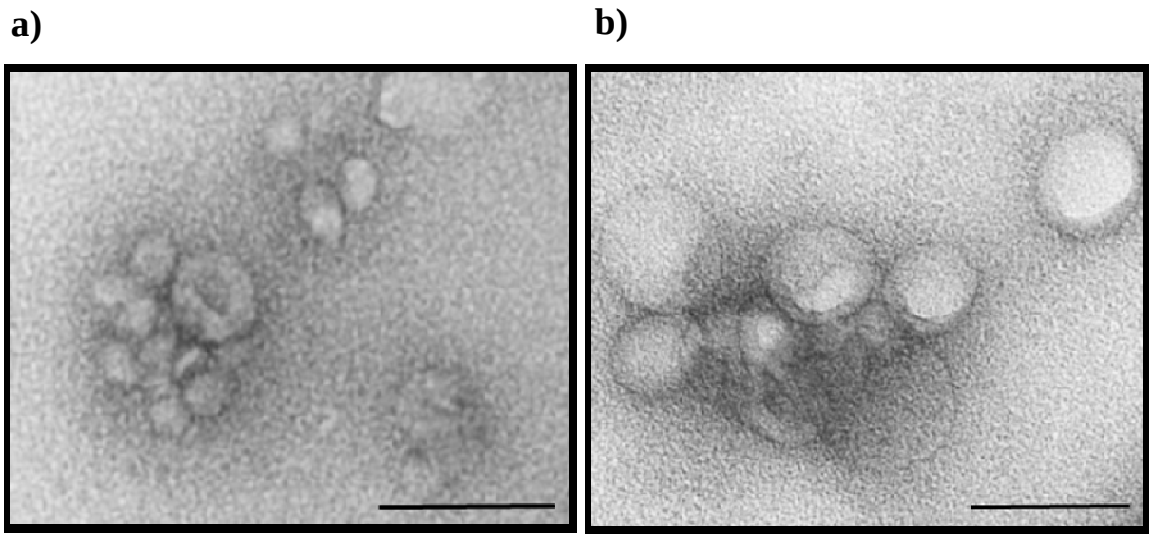


Figure 4.10: Transmission electron micrographs of lipoplexes assembled from pCMV-*luc* DNA and pegylated liposomes, a) 7; and b) 8, at Chol-T:DNA ratios which optimally transfected HepG2 cells. Chol-T:DNA (w/w) ratios were as follows: 3:1 (a); and 2.5:1 (b). Lipoplex suspensions were stained with uranyl acetate and cryopreserved using liquid propane prior to viewing. Note that liposomes 5 and 6 showed the highest transfection activity in HepG2 cells at the optimum DNA-binding ratio. Scale bar = 100 nm (a, and b).

Size is an important parameter governing the efficacy of liposomal gene carriers (Ma *et al*., 2007; Ross and Hui, 1999; Wasungu and Hoekstra, 2006). Although the vectors designed in this study are limited to *in vitro* assessment, the ultimate goal of any gene transfer strategy is to provide a platform for successful human gene therapy in the future. Studies in animal models have emphasised that small lipoplexes are better suited for *in vivo* gene transfer to liver parenchymal cells (Pathak *et al*., 2009). Firstly, in order to reach the hepatocytes, systemically administered lipoplexes must extravasate through the sinusoidal fenestrae of the liver. These serve as dynamic filters and range between 50 and 300 nm in diameter (Horn *et al*., 1987). In addition, the sinusoidal epithelial cells have a high acid DNase content that is potentially damaging to inadequately protected regions of the DNA cargo (Wattiaux *et al*., 2000). Secondly, large lipoplexes are often entrapped within the capillaries of the lung, a phenomenon termed the "*first-passage effect*" (Hashida *et al*., 2001; Kawakami *et al*., 2000). Finally, as a passive targeting strategy, small lipoplexes experience delayed recognition by the reticuloendothelial system (Hashida *et al*., 2001; Plank *et al*., 1996). As an example, Higuchi and coworkers (2006) investigated the effect of particle size on the intrahepatic distribution patterns of galactosylated lipoplexes. While hepatic transfection levels increased with increasing lipoplex size within the range of 141 nm to 253 nm, hepatocyte-specific

transfection was favoured by lipoplexes of 141 nm, with larger complexes displaying a tendency to accumulate in non-parenchymal cells.

Complexes assembled from each of the non-pegylated liposomes, at ratios which gave optimal binding and optimal transfection in HepG2 cells were characterised by Z-average diameters in the micrometre range (Table 4.2), which demonstrates the formation of large aggregates. Furthermore, individual lipoplex suspensions were highly polydisperse and, in most instances, PdI values either near or equal to 1.0 were obtained. This is not unusual as Rao (2010) commented in a recent review of cationic lipid-mediated gene transfer, that a single lipoplex suspension often contains complexes that differ widely in size. This is especially prevalent in instances where the carriers have not been sterically stabilised (Zhang *et al*., 2010). However, the high heterogeneity and predisposition of the non-pegylated lipoplexes towards aggregate formation, at the Chol-T:DNA ratios investigated, clearly renders them unsuitable for *in vivo* application. Nonetheless, these carriers showed appreciable transfection activity in human cell lines (refer to Chapter Five). This is because the size requirements of *in vitro* and *in vivo* transfection differ widely (Liu and Song, 1998; Rao, 2010). It has been observed that larger lipoplexes sediment to the bottom of tissue culture plates, and promote transfection by permitting close contact between the vector particles and cultured cells (Wasungu and Hoekstra, 2006). In this regard, size distribution profiles of all non-pegylated lipoplexes showed the presence of smaller, non-aggregated complexes in lipoplex suspensions. In most instances these were within the range of 200 – 400 nm which gave useful transfection activity in documented *in vitro* studies (Sternberg *et al*., 1998). In order to highlight this feature, the diameter of complexes most abundant by intensity was also summarised in Table 4.2, even though it is the Z-average and PdI values which typify any particle size characterisation by DLS.

Table 4.2: Sizes and size distributions of lipoplexes derived from non-pegylated cationic liposomes.

Liposome preparation	**Ratio (Chol-T:DNA, w/w)**	**Z-average diameter[a] (nm)**	**PdI[a]**	**Diameter[a,f] (nm) of particles most abundant by intensity**
1	1.75:1[b]	4083.7 ± 1465.6	1.000 ± 0	151.8 ± 41.58
	2.0:1[c,d]	2096.7 ± 169.9	1.000 ± 0	262.1 ± 101.6
2	1.0:1[b]	2693.3 ± 458.9	1.000 ± 0	325.8 ± 153.8
	0.75:1[c,e]	3380.0 ± 1771.5	1.000 ± 0	250.6 ± 129.3
3	0.75:1[b]	2396.7 ± 1078.1	0.909 ± 0.086	352.4 ± 236.4
	1.0:1[c,d]	1220.3 ± 250.8	0.800 ± 0.020	442.1 ± 150.4
4	1.25:1[b]	986.2 ± 65.0*	0.531 ± 0.238***	497.2 ± 148.3
	1.5:1[c,d]	2674.7 ± 531.5	0.810 ± 0.124	874.7 ± 87.5[##]

[a]Each value represents the mean ± SD (n = 3).

[b]Ratios which gave optimum binding of DNA.

[c]Ratios which gave optimum transfection in HepG2 cells

[d]These represent super-optimal DNA-binding ratios for the respective liposome formulations.

[e]These represent sub-optimal DNA-binding ratios for the respective liposome formulations.

[f]Note that the diameter reported represents the size around which the population of complexes in the sample which gave the highest % intensity was centered and does not reflect that all particles within that population are of this size. These were obtained from peak analysis of the intensity distribution data (Kaszuba *et al*., 2008).

*P < 0.05, ***P < 0.001 *vs.* liposome 1 (Chol-T/DOPE) at the respective optimum DNA-binding ratios.

[##]P < 0.01 *vs.* liposome 1 at the respective DNA-binding ratios which gave optimum transfection in HepG2 cells.

At the optimum DNA-binding ratio lipoplexes often display a tendency to aggregate due to the lack of inter-particle repulsion by the electroneutral complexes. Consequently, complexes formed at the sub- and super-optimal DNA-binding ratios, which bear a net surface charge, are expected to yield smaller Z-average diameters (Almofti *et al*., 2003b). This was observed only with respect to complexes derived from liposomes 1 and 3. In the case of liposome 2, the optimal (1:1) and sub-optimal (0.75:1) DNA-binding ratios were compared. At sub-optimal DNA-binding ratios, DNA is incompletely condensed, and bears a net negative charge. It has been reported that at such ratios, DNA strands may protrude from individual complexes (Ma *et al*., 2007). Due to the fact that DLS assesses particle size according to the equivalent sphere principle, surface protrusions on the particle are likely to give a higher apparent hydrodynamic size than at the ratio at which DNA was maximally compacted. This could possibly account for the larger hydrodynamic size obtained at the ratio of 0.75:1. A seemingly anomalous result was also obtained with respect to liposome 4. However, it was observed whilst performing size measurements on lipoplexes formed at optimal DNA-binding (1.25:1) that the count rate dropped upon successive runs. This indicates the formation of aggregates that were too large to remain in suspension, possibly as a consequence of the instability of the liposome formulation itself, resulting in fewer particles that were smaller in size being subjected to the beam of light. Consequently, suspensions of these complexes were characterised by a lower Z-average and PdI value than those assembled at the higher ratio (1.5:1).

Kearns and coworkers (2010) also used a Malvern Zetasizer Nano instrument and obtained similar Z-average and PdI values for non-pegylated lipoplexes derived from liposomes containing cholesteryl cytofectins at the optimum DNA-binding ratio. However, unlike the results obtained in this study, lipoplexes prepared at other ratios did not exhibit a tendency to aggregate and were more evenly distributed, due to repulsion afforded by neighbouring charged complexes. Other authors (Rädler *et al*., 1998; Sakurai *et al*., 2000; Zhang *et al*., 2010) have also reported similar trends with regard to the size of non-pegylated lipoplexes as a function of the lipid:DNA mixing ratio, which in effect determines the net surface charge (Ma *et al*., 2007). However, these groups varied the lipid:DNA ratios of the lipoplexes in increments much larger than that which was employed in this study. (In the current study, lipid:DNA ratios were increased in small increments so as to define the optimum DNA-binding ratios more accurately in gel retardation assays). Consequently, the lipoplexes

assembled at the sub- and super-optimal ratios, by the aforementioned groups, carried surface charges sufficient to afford the inter-particle repulsion necessary to maintain small, uniformly distributed sizes. Therefore, the sizes of lipoplexes assembled from preparations 1 to 4 at the sub- and super-optimal DNA-binding ratios suggest that these are not highly charged particles.

While the addition of DNA to the non-pegylated liposomes afforded particles that were many-fold larger in size, and distributed over a broader size range than the liposomal vesicles themselves; the same did not hold true for their pegylated counterparts (Table 4.3). The results show that pegylation at 5 mol % effectively overcame the aggregation tendencies inherent to the lipoplexes in the absence of the steric stabiliser. A comparative study conducted by Zhang and coworkers (2010) established similar findings upon incorporating DSPE-PEG_{2000} at the same molar concentration in a DC-Chol/DOPE formulation. Although the sizes of the pegylated lipoplexes and their distribution patterns appear suitable for *in vivo* gene transfer, the sterically hindered DNA-binding evidenced by gel retardation does not augur well for application in this regard.

Finally, it is important to note that the lipoplex size and shape detailed in this chapter does not necessarily reflect their morphological characteristics *in vivo*. This is because the adsorption of negatively charged serum proteins to the cationic bilayer is known to induce changes in size and shape of lipoplexes; and is one of the limitations associated with the use of several cationic liposome formulations (Sun *et al*., 2009, Wu *et al*., 2002). In an attempt to obtain a representation of lipoplexes as they might appear *in vivo*, Hwang and coworkers (2001) incubated the complexes in a medium containing 10 % serum prior to electron microscopy.

Table 4.3: Sizes and size distributions of lipoplexes derived from pegylated cationic liposomes.

Liposome preparation	**Ratio (Chol-T:DNA, w/w)**	**Z-average diameter[a] (nm)**	**PdI[a]**	**Diameter[a,e] (nm) of particles most abundant by intensity**
5	3.5:1[b,c]	93.1 ± 0.6	0.181 ± 0.015	110.7 ± 3.2
6	3.5:1[b,c]	106.6 ± 1.5***	0.227 ± 0.016	115.5 ± 4.7
7	3.5:1[b]	107.0 ± 1.5***	0.255 ± 0.021*	118.9 ± 5.4
	3.0:1[c,d]	109.4 ± 1.5[###]	0.245 ± 0.003	122.7 ± 5.8
8	3.0:1[b]	108.7 ± 1.9***	0.246 ± 0.048	119.8 ± 13.8
	2.5:1[c,d]	152.7 ± 1.2[###]	0.263 ± 0.016[#]	170.2 ± 6.1[###]

[a]Each value represents the mean ± SD (n = 3).

[b]Ratios which gave optimum binding of DNA.

[c]Ratios which gave optimum transfection in HepG2 cells.

[d]Note that these represent sub-optimal DNA-binding ratios for the respective liposome formulations.

[e]Note that the diameter reported represents the size around which the population of complexes in the sample which gave the highest % intensity was centered and does not reflect that all particles within that population are of this size. These were obtained from peak analysis of the intensity distribution data (Kaszuba *et al*., 2008).

*P < 0.05, ***P < 0.001 *vs.* liposome 5 (Chol-T/DOPE/DSPE-PEG_{2000}) at the optimum DNA-binding ratios.

[#]P < 0.05, [###]P < 0.001 *vs.* liposome 5 at the DNA-binding ratios which gave optimum transfection in HepG2 cells.

4.3.3 Nuclease digestion assays

The efficiency of lipid-based gene carriers is often severely limited in the presence of serum (Esposito *et al*., 2006). Therefore, Hwang and coworkers (2001) have suggested that the interaction of lipoplexes with serum may be a key point in predicting the *in vivo* efficiency of cationic liposomal carriers. To this end, the DNA-protecting capability of each liposome was assessed after a 4 hour long exposure to serum at body temperature. A detergent treatment released DNA from the complexes and its integrity was assessed on agarose gel as a measure of the protection provided by the carrier (Singh and Ariatti, 2006).

The results presented in Figures 4.11 and 4.12 show that all preparations afforded partial protection to their DNA cargo, under conditions which entirely destroyed the naked plasmid (lane 2 of Figures 4.11 and 4.12). A possible explanation for these observations is provided by work reviewed by Rao (2010). The reorganisation of liposomes and DNA involved in lipoplex assembly may produce structures in which DNA is partially exposed, even if fully bound to the lipid bilayers. Consequently, these regions of DNA are more vulnerable to serum nuclease attack. The partial DNA-protecting characteristics may be further attributed to the destabilisation of lipoplexes by the adsorption of serum proteins, with particular reference to the non-pegylated carriers (Schätzlein, 2003). This concept is supported by Sun and coworkers (2009) who demonstrated, using infrared spectroscopy and X-ray diffraction, that the hexagonal structure native to DOTAP/Chol/DNA complexes may be broken in the presence of serum. According to Esposito and colleagues (2006), the rate of lipoplex destabilisation in serum is largely influenced by the lipid composition. To this end, it has been suggested that lipids which confer greater membrane rigidity may contribute towards serum-resistance. However, such lipids may prevent the structural alteration of lipoplexes that is necessary for dissociation of the DNA cargo after cellular uptake (Rao, 2010). These concerns reflect the importance of optimising liposome formulations in order to achieve a compromise between liposome stability and transfection activity.

a) **b)**

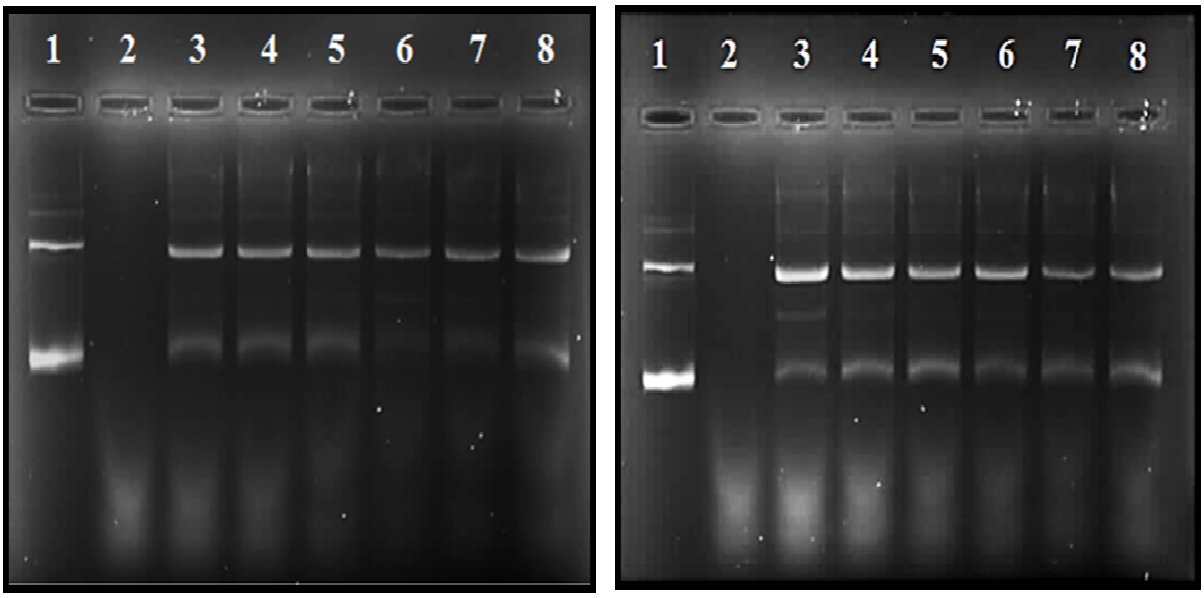


Figure 4.11: Nuclease digestion assay of non-pegylated liposomes. Liposome/DNA complexes were incubated with 10 % serum, for 4 hours at 37 ˚C, as described in 4.2.2.3. Lane 1: undigested pCMV-*luc* DNA (0.5 μg); lane 2: plasmid digestion (0.5 μg) in the absence of liposomes. Lipoplexes were assembled from 0.5 μg DNA and a) lanes 3-5: liposome 1 (containing 0.75, 0.875 and 1.0 μg Chol-T respectively); lanes 6-8: liposome 2 (containing 0.375, 0.5 and 0.625 μg Chol-T respectively); b) lanes 3-5: liposome 3 (containing 0.25, 0.375 and 0.5 μg Chol-T respectively; lanes 6-8: liposome 4 (containing 0.5, 0.625 and 0.75 μg Chol-T respectively).

a) **b)**

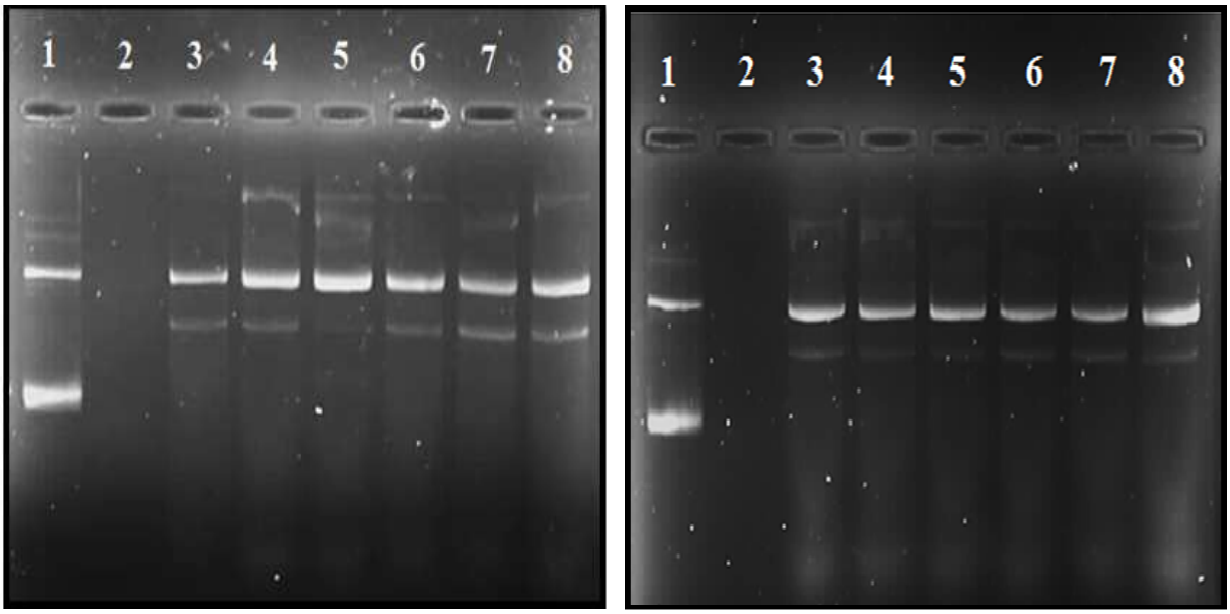


Figure 4.12: Nuclease digestion assay of pegylated liposomes. Liposome/DNA complexes were incubated with 10 % serum, for 4 hours at 37 ˚C, as described in 4.2.2.3. Lane 1: undigested pCMV-*luc* DNA (0.5 µg); lane 2: plasmid digestion (0.5 µg) in the absence of liposomes. Lipoplexes were assembled from 0.5 µg DNA and a) lanes 3-5: liposome 5 (containing 1.5, 1.75 and 2.0 µg Chol-T respectively); lanes 6-8: liposome 6 (containing 1.5, 1.75 and 2.0 µg Chol-T respectively); b) lanes 3-5: liposome 7 (containing 1.5, 1.75 and 2.0 µg Chol-T respectively); lanes 6-8: liposome 8 (containing 1.25, 1.5 and 1.75 µg Chol-T respectively).

Although PEG chains are known to hinder the binding of serum proteins to the cationic liposomal surface, it was observed that DNA complexed to each of the pegylated liposomes did sustain damage to approximately the same extent. Furthermore, pegylated liposome-associated DNA was characterised by the absence of the supercoiled plasmid, after exposure to serum. This was accompanied by both an increase in the intensity of the circular species, and the presence of a relatively small amount of linear DNA of lower molecular weight, in comparison with the untreated control. This suggests that the supercoiled plasmid was not entirely fragmented, but also nicked by nucleases to its relaxed form. In contrast, plasmid bound to the non-pegylated carriers retained some of its supercoiled character, with evidence of nuclease-mediated circularisation in the case of liposome 3. These results agree with reports by Zhang and Anchordoquy (2004) who used fluorometric imaging to show that plasmid complexed to relatively stable non-pegylated cationic liposomes lost approximately 70 % of its superhelical structure upon brief exposure to serum. In addition, the severity of the effect was exacerbated with increasing serum concentration. Consequently, this group has proposed that the analysis of lipoplex-serum interactions should be performed using higher serum concentrations, which more closely resemble *in vivo* conditions. In this study, the failure of the pegylated liposomes in protecting the most abundant form of plasmid DNA may be attributed to the incomplete condensation of DNA (Zhao and Lee, 2004) as a result of weaker interactions with DNA, which were emphasised by gel retardation (4.3.1). The results are further supported by a comparative study conducted by Remaut and coworkers (2005) which showed, by fluorescence correlation spectroscopy, that pegylation reduced the nucleic acid protecting capacity of DOTAP/DOPE liposomes. Consequently, this group suggested that a post-pegylation strategy may be advantageous as it would permit the effective complexation of DNA to the non-pegylated liposomes prior to surface modification with the steric stabiliser.

In summary, the formation of electrostatic complexes between cationic liposomes and pCMV-*luc* plasmid DNA was demonstrated by gel retardation assays and cryo-TEM. The gel retardation assays also permitted assessment of the DNA-binding capabilities of the liposomes. It was found, among the non-pegylated liposomes, that the introduction of SH02 and/or SH04 improved the DNA-binding ability of the Chol-T/DOPE formulation. However, modification of liposomes with PEG was associated with a reduction in DNA-binding affinity. Analysis of lipoplex size by DLS showed that non-pegylated liposomes formed

polydisperse suspensions of large aggregates (approximately 1 – 4 μm) when complexed with DNA. In contrast, sterically stabilised lipoplexes gave Z-average diameters of less than 200 nm, with more uniform size distribution patterns. Futhermore, cryo-TEM highlighted the fact that lipoplex morphology differs with liposome composition and the lipid:DNA mixing ratio. Lastly, nuclease digestion assays showed that individual liposome formulations partially protected DNA against nuclease catalysed degradation upon exposure to 10 % foetal calf serum at 37 ˚C for 4 hours. While the non-pegylated liposomes imparted some protection to the supercoiled form of the plasmid, under these conditions, their pegylated equivalents were unable to do so.

CHAPTER FIVE

IN VITRO CYTOTOXICITY TESTING AND TRANSFECTION STUDIES

5.1 Introduction

The ultimate goal in vector development is to facilitate the introduction of nucleic acids into the desired cell type without inducing toxic or immunological effects (Basarkar and Singh, 2007). To this end, the *in vitro* gene transfer capabilities of the pegylated and non-pegylated liposome formulations were studied using the luciferase assay for transient gene expression. In each instance, the effect of the carriers on cell growth was assessed by means of the MTT assay.

5.1.1 Cytotoxicity of cationic liposomes

Studies have shown that some cationic lipids and cationic polymers may induce toxic effects both *in vitro* and *in vivo*. As an example, Zhang and coworkers (2005) reported that the systemic administration of liposomes, formulated with several well-known cytofectins, was associated with harmful physiological responses in animal models. These include acute inflammation, hepatic, haematological and serological toxicity. Furthermore, at the cellular level, abnormalities such as cell shrinkage, vacuolisation of cytoplasm and reduction in the number of cell division cycles have been documented (Lappalainen *et al*., 1994). These adverse effects have primarily been attributed to structural features of the cytofectin and the net positive charge of the nucleic acid constructs. It has been put forward that the cationic lipoplexes may associate with anionic biological molecules and induce aggregation, or thrombosis. Cationic lipids are also believed to exert a cytotoxic effect by creating transmembrane pores which increase the permeability of the plasma membrane (Lasic, 1997); while more recent studies suggest that the cytofectin headgroup may interact with cellular proteins such as protein kinase C, and trigger an immune response (Lv *et al*., 2006). Moreover, factors such as lipoplex size, the lipid dose, and the biological activity of the cell

types involved, are believed to influence the overall toxic effect elicited (Lasic, 1997; Ma *et al*., 2007).

Research in recent years has provided greater insight into the relationship between cationic lipids, the behaviour of liposome/DNA complexes, and toxicity (Filion and Phillips, 1998; Lv *et al*., 2006; Zhang *et al*., 2005). This has promoted advances in cytofectin design, the use of co-lipids, and optimisation of liposome formulations, that have collectively enhanced the biocompatibility of cationic liposomal carriers. Nonetheless, it is imperative that the safety of any newly designed vector be investigated.

5.1.1.1 *In vitro* cytotoxicity tests

Significant differences exist between *in vitro* and *in vivo* scenarios with respect to cytotoxicity and cell growth. Nonetheless, *in vitro* assays are known to provide invaluable information about the activity of several agents at the cellular level. Therefore, as an initial step in safety assessment, the effect of lipoplexes on the proliferation of cells in culture is often considered (Lappalainen *et al*., 1994).

In early *in vitro* cytotoxicity studies, living cells were identified either by their ability to replicate DNA, which was demonstrated by the incorporation of radioactive nucleotides, or by negative staining in the presence of the dye trypan blue (Lappalainen *et al*., 1994). However, understanding the different parameters associated with both cell growth and death has provided the framework for the development of safer, more sensitive methods of quantifying viable cell density. To date, colorimetric or luminescent-based assays are commonly employed. For example, the lactate dehydrogenase (LDH) assay uses the integrity of the plasma membrane as an indicator of cell viability by quantifying the activity of the enzyme LDH released by damaged cells (Weyermann *et al*., 2005). Alternately, the number of living cells may be determined by measuring the absorbance of a dye, such as neutral red or crystal violet, which selectively accumulates within a specific organelle of uninjured cells (Chiba *et al*., 1998). In addition, the cellular ATP content may be correlated with cell survival in bioluminescent assays (Weyermann *et al*., 2005). The MTT assay, which is the cell

proliferation assay chosen to assess the cytotoxicity of lipoplexes in this study, relates the metabolic activity of mitochondria to cell viability.

5.1.1.2 The MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow, water-soluble tetrazolium dye. When introduced into the growth medium, MTT traverses the plasma membrane of living cells and enters the mitochondria (Kwak *et al.*, 2002). Within the mitochondria, succinate dehydrogenase enzymes cleave the tetrazolium ring and, as such, reduce MTT to purple, insoluble MTT formazan (Figure 5.1), which accumulates at this site (Fotakis and Timbrell, 2006). A purple coloured solution is obtained after dissolving the formazan crystals in an organic solvent; and its absorbance is measured spectrophotometrically at a wavelength specific to the solvent employed. Studies have shown that dimethylsulphoxide (DMSO) is the most appropriate solvent as it does not induce precipitation of either cell- or medium-derived proteins, and provides a formazan solution of stable optical density (Twentyman and Luscombe, 1987).

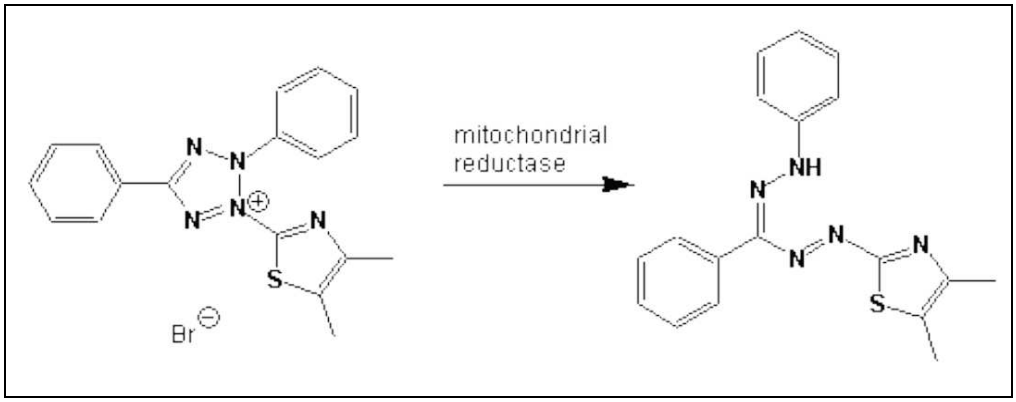


Figure 5.1: Reduction of MTT to MTT-formazan by mitochondrial succinate dehydrogenases (http://www.mnstate.edu/provost/MTT_Proliferation_Protocol.pdf).

The MTT assay is based on the premise that changes in viable cell density, translate into directly proportional changes in the level of functional dehydrogenase enzymes responsible for MTT reduction. Therefore, a linear relationship exists between absorbance of the formazan solution and the number of viable cells. This allows changes in cell growth to be

quantified, and the growth or death rate of cells to be measured. (http://www.atcc.org/CulturesandProducts/CellBiology/KitsPanels/MTTCellProliferationAssay).

The MTT assay is relatively simple to perform, requires equipment that is generally available in most laboratories and displays high sensitivity. Therefore use of the MTT assay remains well supported by the literature, even though modified tetrazolium salts, such as MTS and XTT, have been designed and are now commercially available as kits (Lappalainen *et al*., 1994).

5.1.2 Gene expression assays

Reporter genes, which manifest their expression in an easily detectable and quantifiable manner, have provided an effective platform for the study of gene expression both *in vitro* and *in vivo* (Alam and Cook, 2003). According to Bronstein and coworkers (1996) a gene must fulfil three criteria in order to be used as a marker of gene expression. Firstly, the protein product encoded by the gene must be absent in non-transformed cells. Secondly, the quantity of reporter protein expressed must be proportional to the level of mRNA transcribed. Thirdly, a simple and sensitive assay system must be available to identify and quantify the protein product.

Over the years, several genes have been adapted as markers of gene expression. These include genes which encode antibiotic resistance, enzyme activity and bioluminescence (Alam and Cook, 2003). Among them, genes that encode luciferases, enzymes that catalyse reactions that emit visible light, have received widespread attention in the study of gene expression (Baldwin, 1996; Greer and Szalay, 2002).

5.1.2.1 The luciferase assay

The North American firefly *Photinus pyralis* represents the most extensively studied natural bioluminescent system (Fraga, 2008). In 1985, DeLuca and coworkers successfully cloned

cDNA of the *luc* gene which encodes the luciferase expressed by *P. pyralis*. This provided an alternative source of the enzyme, and paved the way for its use as a reporter for gene expression (Baldwin, 1996). Early studies estimated that, depending on the instrument used to measure light production, the firefly luciferase system was 30 – 1000 fold more sensitive than the chloramphenicol acetyltransferase (CAT) reporter system, which was commonly employed at the time (De Wet *et al*., 1987). In addition, luciferase assays are favoured because they are rapid, versatile, inexpensive and safe, as radioactive material is not required (Bronstein *et al*., 1996; De Wet *et al*., 1987).

The *luc* gene consists of seven exons separated by 6 introns, which are shorter than 60 base pairs in length. This gene codes for a protein, which has a molecular weight of 62 kDa, and consists of 550 amino acid residues (De Wet *et al*., 1987; Fraga, 2008). The ribbon diagram of firefly luciferase, shown in Figure 5.2, illustrates that these amino acids are arranged as a large N-terminal domain (amino acids 1 – 436) that is connected to a smaller C-terminal domain (amino acids 440 – 550) via a hinge peptide (Branchini, 2010). The enzyme catalyses the ATP-dependent oxidative decarboxylation of the substrate, a benzothiazole, D-luciferin. This results in the emission of yellow-green light of maximum intensity at 562 nm at pH 7.5 – 8.5. The light emitted decays to approximately 10 % of its initial maximum within a minute, and is followed by low levels of light emission that decays at a markedly slower rate (De Wet *et al*., 1987; Greer and Szalay, 2002). A proposed mechanism for the reaction, as presented in a review by Baldwin (1996), is shown in Figure 5.3.

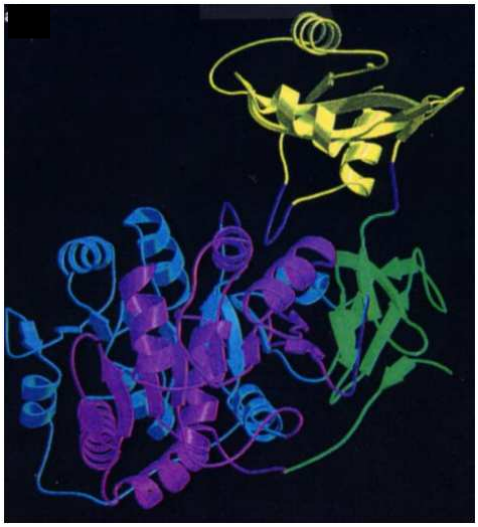


Figure 5.2: Ribbon diagram of the luciferase expressed by *P. pyralis* (Branchini, 2010).

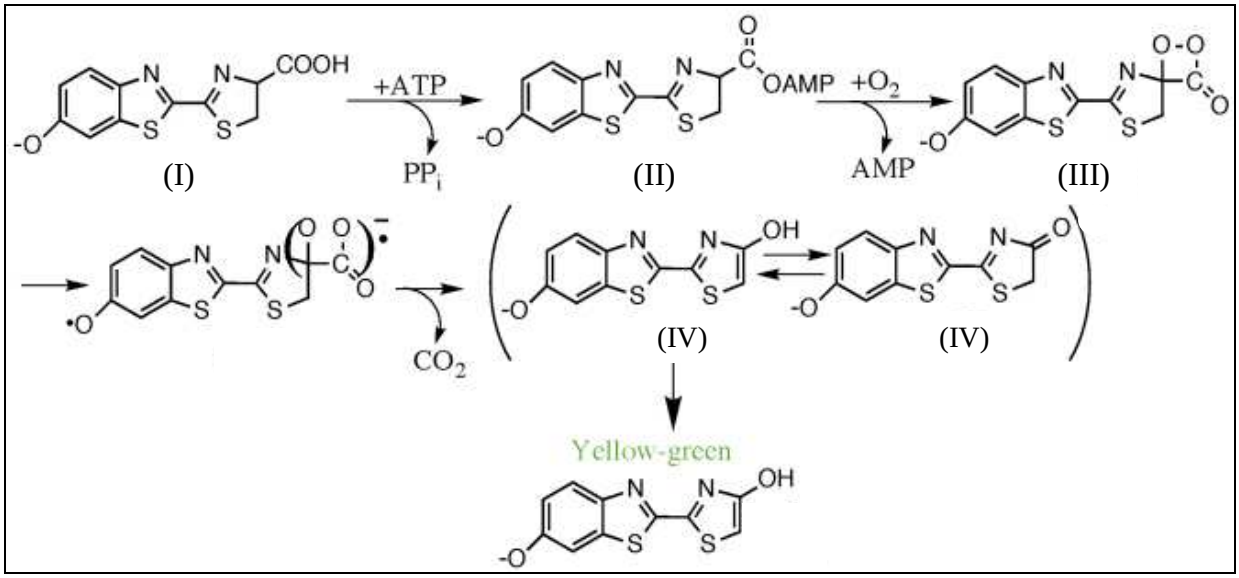


Figure 5.3: Proposed mechanism of the reaction catalysed by firefly luciferase. The Roman numerals correspond to the following compounds: (I) firefly luciferin, (II) adenylated luciferin, (III) luciferin dioxetenone, and (IV) oxyluciferin (adapted from Baldwin, 1996).

The *luc* gene has been incorporated into reporter plasmid vectors which are constructed with regulatory sequences necessary for its expression in transformed cells. The map of the pCMV-*luc* expression vector employed in this study is shown in Figure 5.4. Expression of the luciferase gene is driven by a strong promoter derived from the cytomegalovirus. In addition, the plasmid bears an antibiotic resistance gene that enables selection of transformants following plasmid amplification in bacterial cells.

The luciferase assay is based on the premise that when cells which have been successfully transfected with the *luc* gene are lysed, and excess substrate (D-luciferin) is added to the lysate, a flash of light is generated (De Wet *et al*., 1987). The intensity of the light emitted is easily quantified in a luminometer, and is taken as measure of the level of gene expression, because it is directly proportional to the quantity of the enzyme present in the cell lysate (Jouzani and Goldenkova, 2005).

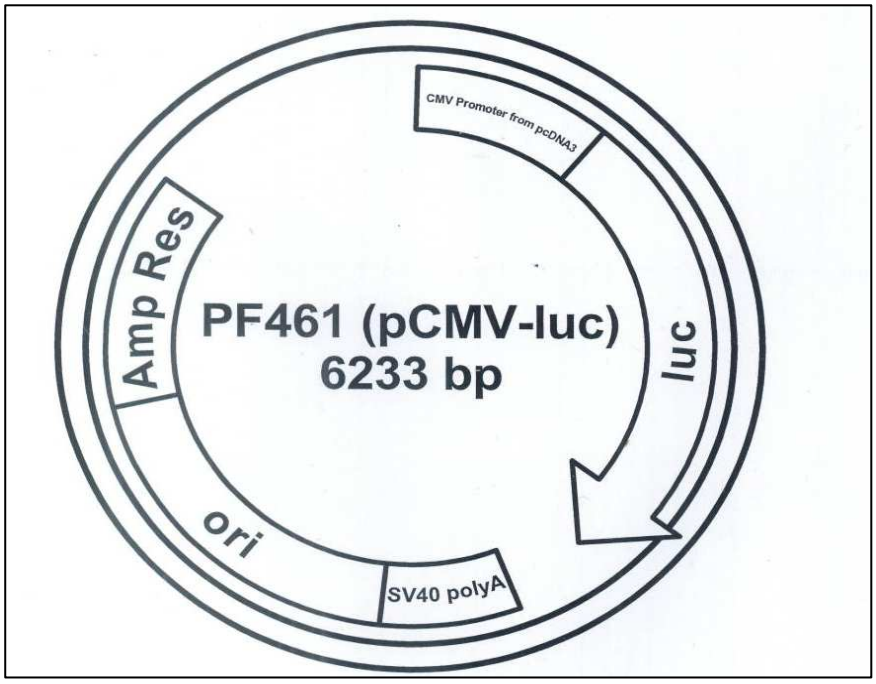


Figure 5.4: pCMV-*luc* vector circle map (http://www.plasmidfactory.com).

5.2 Materials and methods

5.2.1 Materials

5.2.1.1 Cell maintenance and cytotoxicity testing

HepG2 (hepatoma G2) cells were obtained from Highveld Biologicals (Pty) Ltd., Lyndhurst, South Africa. HEK293 (human embryonic kidney 293) cells were supplied by the University of Witwatersrand, Medical School, South Africa. The following was purchased from Gibco Invitrogen™, New Zealand: minimal essential medium (MEM) with Glutamax™, Earle's salts and 25 mM HEPES (pH 7.5); Penicillin/Streptomycin mixture (10 000 U/ml penicillin, 10 000 μg/ml streptomycin) and heat-inactivated, newborn calf serum. Phosphate-buffered saline (PBS) tablets and trypsin-versene were from Calbiochem, Canada and Lonza Biowhittaker, Walkersville, MD respectively. The MTT salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Merck, Darmstadt, Germany. All sterile plasticware for tissue culture was from Corning Inc., Corning, NY, USA. All other reagents were of analytical grade.

5.2.1.2 Gene transfer experiments

The following was obtained from Sigma-Aldrich Inc., USA: asialofetuin (Type I, from foetal calf serum), bicinchoninic acid (BCA) solution, copper (II) sulphate solution and the protein standard, bovine serum albumin (BSA) (1 mg BSA/ml in 0.15 M NaCl). The 5 × cell culture lysis reagent (25 mM Tris-phosphate, pH 7.8; 2 mM dithiothreitol; 2 mM 1,2-diaminocyclohexane-*N*-*N*-*N*′-*N*′-tetra-acetic acid; 10 % (v/v) glycerol; 1 % (v/v) Triton X-100) and luciferase assay reagent (20 mM tricine; 1.1 mM magnesium carbonate hydroxide, pentahydrate; 2.7 mM magnesium sulphate; 0.1 mM EDTA; 33.3 mM dithiothreitol; 270 μM coenzyme A; 470 μM luciferin; 350 μM ATP) were purchased from Promega Corporation, Madison, WI, USA.

5.2.2 Methods

5.2.2.1 Cell growth and maintenance

5.2.2.1.1 Reconstitution

Cells in cryovials were maintained in a water bath at 37 ˚C for 5 minutes. Cells were transferred into centrifuge tubes, and recovered as a pellet after centrifugation at 3000 rpm (Eppendorf 5702R centrifuge, Merck, Darmstadt, Germany) for 60 seconds. After resuspension in 5 ml complete medium (MEM, serum (10 %, v/v), 100 U/ml penicillin and 100 μg/ml streptomycin), cells were introduced into 25 cm^2 tissue culture flasks and incubated at 37 ˚C in a humidified, 5 % CO_2 incubator.

5.2.2.1.2 Change of medium

Medium was replaced within 24 hours of reconstitution, so as to eliminate residual DMSO. The spent medium was discarded and cells were rinsed with PBS (10 mM phosphate buffer at pH 7.4, 140 mM NaCl, 3 mM KCl, 2 ml). After adding complete medium (5 ml), cells were returned to the incubator (37 ˚C). Cell growth was monitored on a daily basis using an

Olympus inverted fluorescence microscope. Medium was replaced approximately every 24 – 48 hours, in accordance with such observations.

5.2.2.1.3 Trypsinisation

Growth medium bathing cells was removed. Cells were rinsed with PBS and incubated with trypsin-versene (1 ml) for approximately 60 seconds at 37 ˚C. Enzyme activity was halted by the addition of complete medium (2 ml) and cells were gently dislodged from the surface of the culture vessel. Cells were split into convenient ratios, introduced into flasks with complete medium to a final volume of 5 ml, and maintained at 37 ˚C.

5.2.2.1.4 Cryopreservation

Cells were trypsinised as described in 5.2.2.1.3 and centrifuged at 1200 rpm for 2 minutes. The pellet was resuspended in complete medium (1 ml) containing DMSO (10 %, v/v), transferred to cryovials and cooled at a rate of -1 ˚C/minute in a Nalgene^{TM} Cryo 1 ˚C freezing container with isopropanol until a temperature of – 80 ˚C was attained. The cells were stored temporarily in a biofreezer (- 80 ˚C).

5.2.2.2 Growth inhibition assays

Cells were trypsinised and seeded into 48 well plates (2×10^4 cells/well). These were grown to semi-confluency after incubation (37 ˚C, 24 hours) in complete medium (0.25 ml/well). Lipoplexes (10 μl in HBS) were assembled from pCMV-*luc* DNA (0.5 μg) and varying amounts of cationic liposomes (as shown in Table 5.1), 30 minutes prior to use. Complexes (10 μl/well) were introduced to cells in serum-free medium (0.25 ml/well), and incubated for 4 hours at 37 ˚C. After replacing the minimal medium with complete medium, cells were maintained at 37 ˚C for a further 48 hours. Growth medium was again removed and cells were incubated (37 ˚C) for 4 hours with 0.2 ml each of MTT solution (5 mg/ml in PBS) and complete medium per well. This was replaced with DMSO (0.2 ml/well) in order to permeate

cells and solubilise formazan crystals. Absorbances were read at 540 nm using a Mindray microplate reader, MR-96A. The percentage cell viability was calculated as follows:

% cell survival = [$A_{540\ nm}$ treated cells] / [$A_{540\ nm}$ untreated cells] × 100

Table 5.1: Cytofectin and corresponding lipid concentrations of lipoplexes, as introduced per well.

Liposome preparation	Chol-T:DNA ratio (w/w)	Cytofectin concentration (µg Chol-T/10 µl)	Lipid concentration (µg /10 µl)
1 (Chol-T/DOPE)	1.5:1[b]	0.750	1.833
	1.75:1[a]	0.875	2.139
	2.0:1[c]	1.000	2.445
2 (Chol-T/DOPE/SH02)	0.75:1[b]	0.375	1.044
	1.0:1[a]	0.500	1.392
	1.25:1[c]	0.625	1.739
3 (Chol-T/DOPE/SH04)	0.5:1[b]	0.250	0.672
	0.75:1[a]	0.375	1.008
	1.0:1[c]	0.500	1.344
4 (Chol-T/DOPE/SH02/ SH04)	1.0:1[b]	0.500	1.550
	1.25:1[a]	0.625	1.937
	1.5:1[c]	0.750	2.325
5 (Chol-T/DOPE/ DSPE-PEG_{2000})	3.0:1[b]	1.500	4.280
	3.5:1[a]	1.750	4.994
	4.0:1[c]	2.000	5.707
6 (Chol-T/DOPE/SH02/ DSPE-PEG_{2000})	3.0:1[b]	1.500	4.856
	3.5:1[a]	1.750	5.666
	4.0:1[c]	2.000	6.475
7 (Chol-T/DOPE/SH04/ DSPE-PEG_{2000})	3.0:1[b]	1.500	4.714
	3.5:1[a]	1.750	5.499
	4.0:1[c]	2.000	6.285
8 (Chol-T/DOPE/SH02/ SH04/DSPE-PEG_{2000})	2.5:1[b]	1.250	4.513
	3.0:1[a]	1.500	5.416
	3.5:1[c]	1.750	6.318

Note that the optimal, sub- and super-optimal DNA-binding ratios of individual liposomes are indicated by the superscripts a, b and c, respectively.

5.2.2.3 Gene transfer experiments

5.2.2.3.1 Transfection protocol

Cells were seeded into 48 well plates at a density of 2×10^4 cells/well and incubated (37 ˚C, 24 hours) in complete medium (0.25 ml/well) to allow cells to attach and grow to semi-confluence. Lipoplexes (10 μl in HBS) were prepared, 30 minutes prior to transfection, from pCMV-*luc* DNA (0.5 μg) and varying amounts of cationic liposomes (refer to Table 5.1). The growth medium was replaced with serum-free medium, and complexes (10 μl/well) were introduced. After incubation for 4 hours at 37 ˚C, the spent medium was replaced with complete medium. The plates were maintained at 37 ˚C for an additional 48 hours, whereupon medium was removed and cells were assayed for expression of the luciferase transgene (refer to 5.2.2.3.3).

5.2.2.3.2 Competition assay

This assay was conducted with HepG2 cells as per the transfection protocol described above, except that cells were incubated (37 ˚C, 20 minutes) with asialofetuin (250 μg/well), in the absence of serum, prior to the introduction of targeted lipoplexes in order to permit saturation of the ASGP-Rs.

5.2.2.3.3 Luciferase assay

Luciferase activity was determined using the Promega Luciferase Assay System. Briefly, cells were rinsed twice with PBS (0.25 ml/well) and 1 × lysis buffer (60 μl/well) was introduced. The plate was then agitated on a platform shaker (Stuart Scientific STR6, Surrey, UK) operating at 30 rev/minute for 15 minutes. Any cells adhering to the wells were scraped off, and cell free extracts were obtained after centrifugation (12 000 rpm, using an Epindorf 5415D microcentrifuge, Merck, Darmstadt, Germany; for 30 seconds, at room temperature). Luciferase reagent (50 μl) was introduced to lysates (20 μl), and the mixture vortexed briefly (10 seconds). Finally, light production was quantified using a luminometer (Lumac Biocounter M1500, Landgraaf, Netherlands) as relative light units (RLU) emitted for 10

seconds. Gene expression was reported as RLU per mg protein, the latter having been determined using the BCA assay.

5.2.2.3.4 The bicinchoninic acid (BCA) assay

In order to construct a protein standard curve, standard BSA solutions (ranging from 0 to 30 μg/50 μl in increments of 5 μg/50 μl) were prepared in a final volume of 50 μl with 18 Mohm water. These were mixed with 1 ml BCA working reagent (BCA solution:copper (II) sulfate solution, 50:1 v/v) and maintained at 37 ˚C for 30 minutes. Solutions were cooled to room temperature and absorbances read at 540 nm (Mindray microplate reader MR-96A). The cell free extracts (50 μl) were mixed with BCA working reagent (1 ml) and treated in the same way. The soluble protein content of the extracts was obtained by extrapolation from the standard curve.

5.2.2.4 Statistical analysis

Statistical analyses were performed using ANOVA (one-way analysis of variance), followed by Tukey's Multiple Comparison Test to compare between groups (GraphPad Prism version 5.04, GraphPad Software Inc., USA). *P* values less than 0.05 were regarded as significant.

5.3 Results and discussion

5.3.1 Cell growth and maintenance

The HepG2 cell line was chosen as an appropriate system to model hepatocytes *in vitro*, because it is derived from a human source and expresses the ASGP-R, to which the gene transfer system under investigation is directed, at high densities (Knowles *et al*., 1980; Schwartz *et al*., 1981). In several related studies this cell line has been employed to assess the efficacy of hepatotropic liposomes (Kawakami *et al*., 1998; Shigeta *et al*., 2007; Singh and Ariatti, 2003; Singh *et al*., 2010). As a receptor-negative control, a second human cell line

was employed i.e. HEK293. These transformed kidney cells have also been widely used in gene transfer experiments (Thomas and Smart, 2005). Both cell lines (Figures 5.5 and 5.6) were successfully propagated after reconstitution using the methods described in 5.2.2.1.

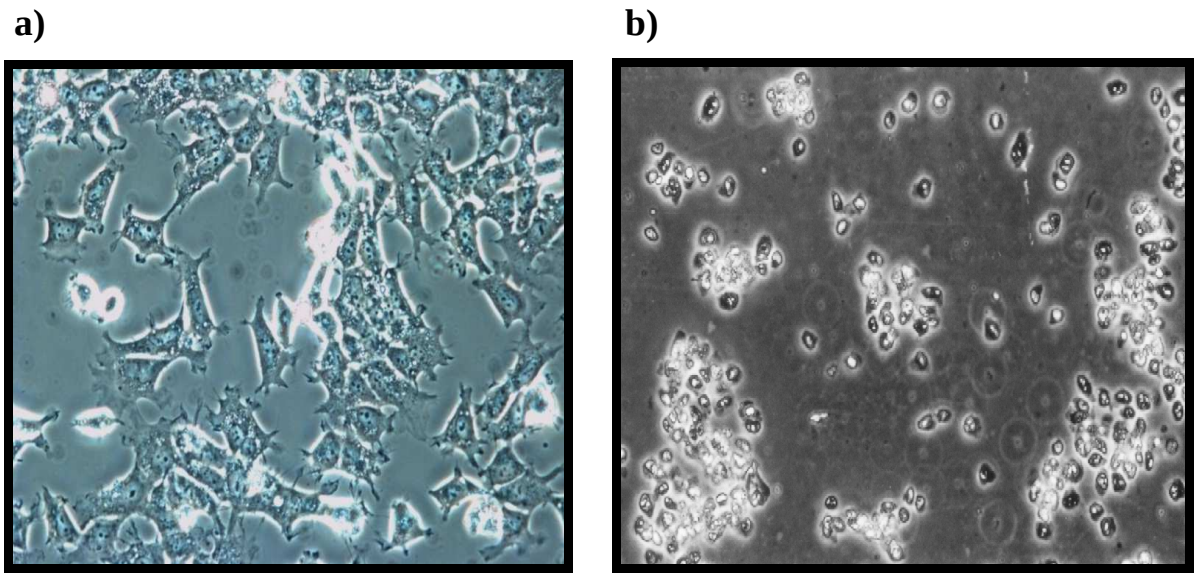


Figure 5.5: HepG2 cells, a) semi-confluent and b) trypsinised, as viewed under an inverted fluorescence microscope (10 ×).

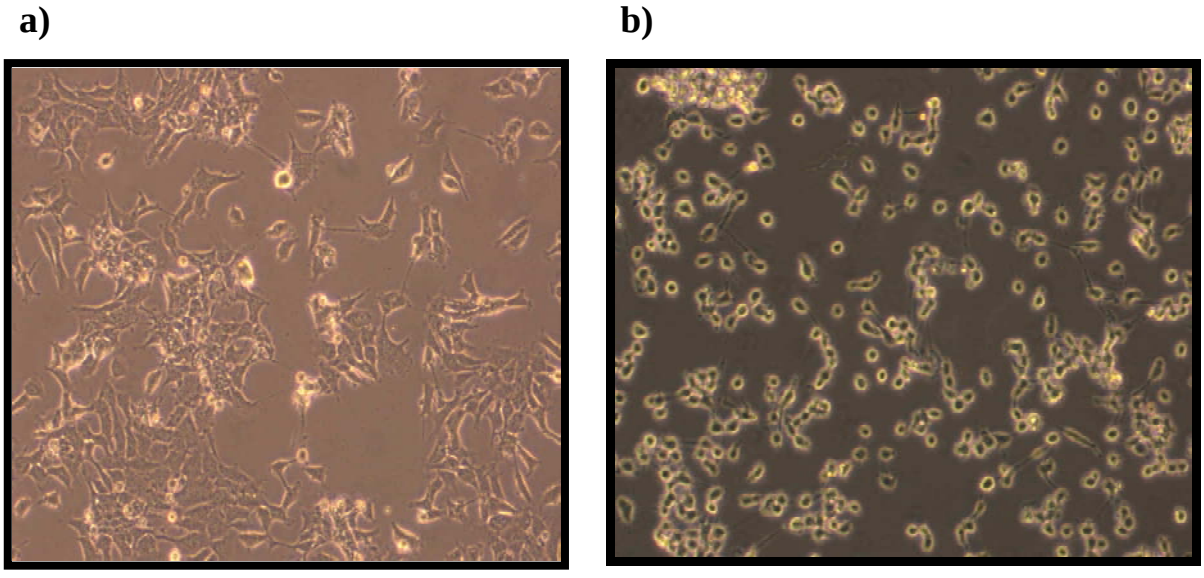


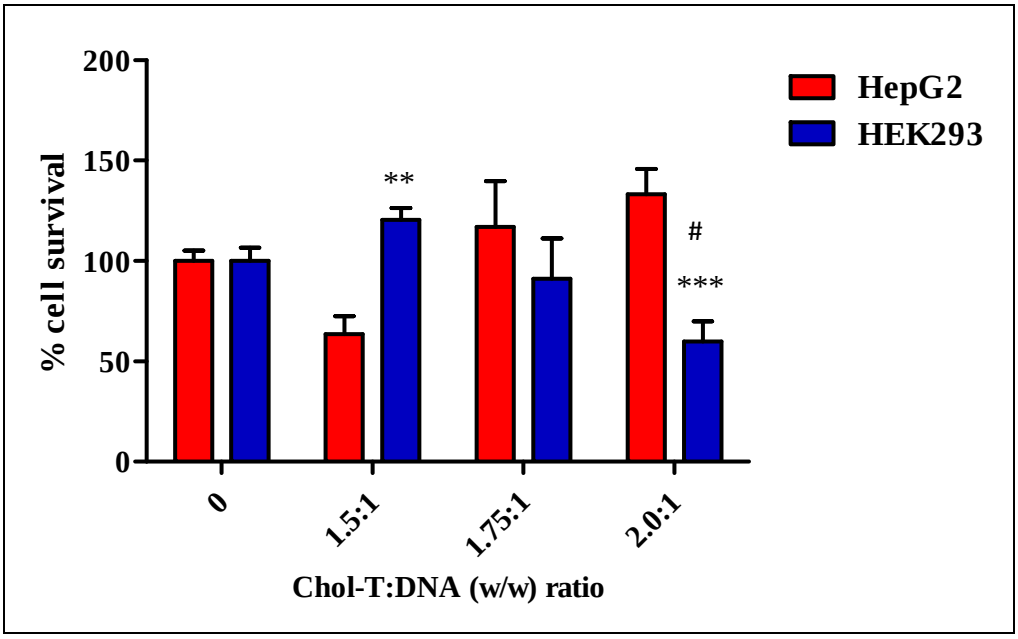
Figure 5.6: HEK293 cells, a) semi-confluent and b) trypsinised, as viewed under an inverted fluorescence microscope (10 ×).

5.3.2 Growth inhibition studies

The cytotoxicity of lipoplexes was assessed under the conditions adopted for transfection studies, as there often exists a correlation between the effect of vectors on cell growth and their gene transfer potential (Wiethoff and Middaugh, 2003). Throughout the study lipoplexes were assembled in HBS to reduce the possibility of the assays being influenced by osmotic effects (Singh *et al*., 2007). This buffer maintains osmolarity at approximately 290 mosmol/kg; and is optimal for cultured human cells as it corresponds to that of human plasma (Freshney, 2005).

In this study, cytotoxicity profiles of the liposomes formulated are represented, in Figures 5.7 and 5.8, as the percentage cell survival after exposure to lipoplexes assembled at different Chol-T:DNA ratios by weight. A prevalent trend in the literature is that cytotoxicity increases with increasing lipoplex charge ratio (Lv *et al*., 2006; Masotti *et al*., 2008). This is largely because a higher charge ratio correlates with the inclusion of higher levels of cationic amphiphiles, which is in turn associated with adverse effects on a molecular level (Lasic, 1997). However, Masotti and colleagues (2008) have reported that this pattern does not hold true for all cationic liposome formulations. This is possibly a consequence of helper lipids included in their composition, as these also influence cell growth; and the specific cell line employed in the investigation. Moreover, the lipid concentration to which cells are exposed, the duration of exposure and the cell density employed in *in vitro* growth inhibition studies are known to influence the end result (Pazner and Jansons, 1979). Therefore, in comparing the cellular response to each liposomal carrier, at the optimal, sub- and super-optimal DNA-binding ratios, due consideration was given to the cytofectin and total lipid concentration (Table 5.1) introduced per cell sample.

a)


200
150
100
50
0
% cell survival
**
#
***
HepG2
HEK293
0
1.5:1
1.75:1
2.0:1
Chol-T:DNA (w/w) ratio

b)

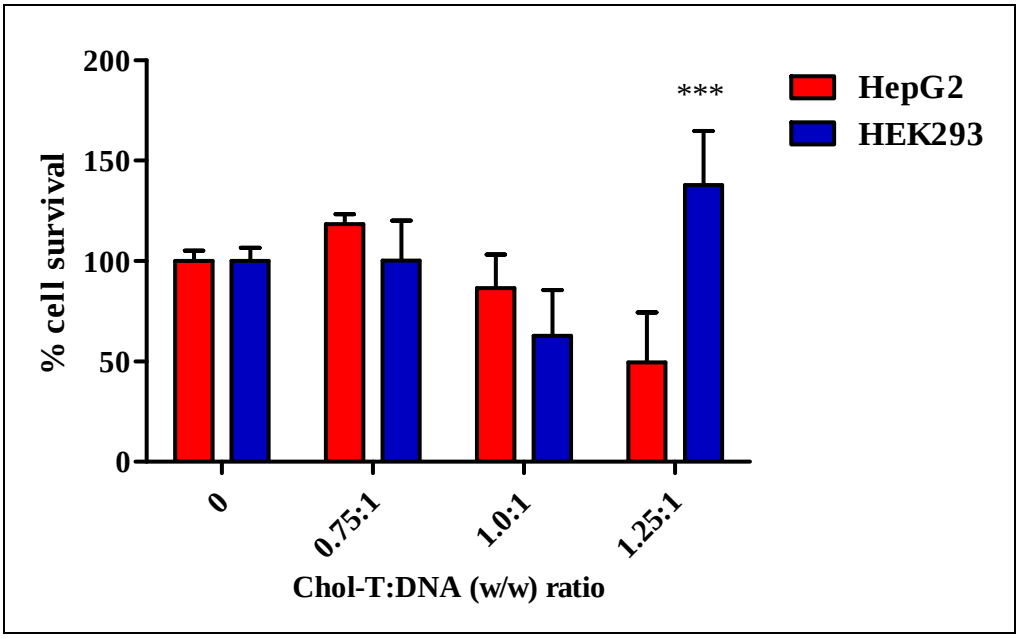
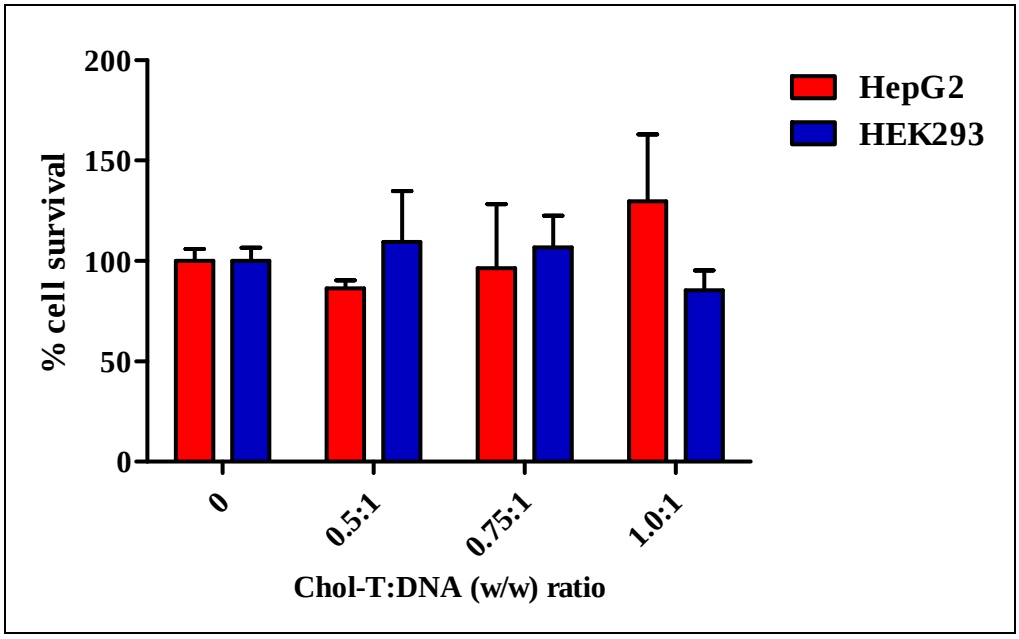

200
150
100
50
0
% cell survival
***
HepG2
HEK293
0
0.75:1
1.0:1
1.25:1
Chol-T:DNA (w/w) ratio

Fig. 5.7

c)



d)

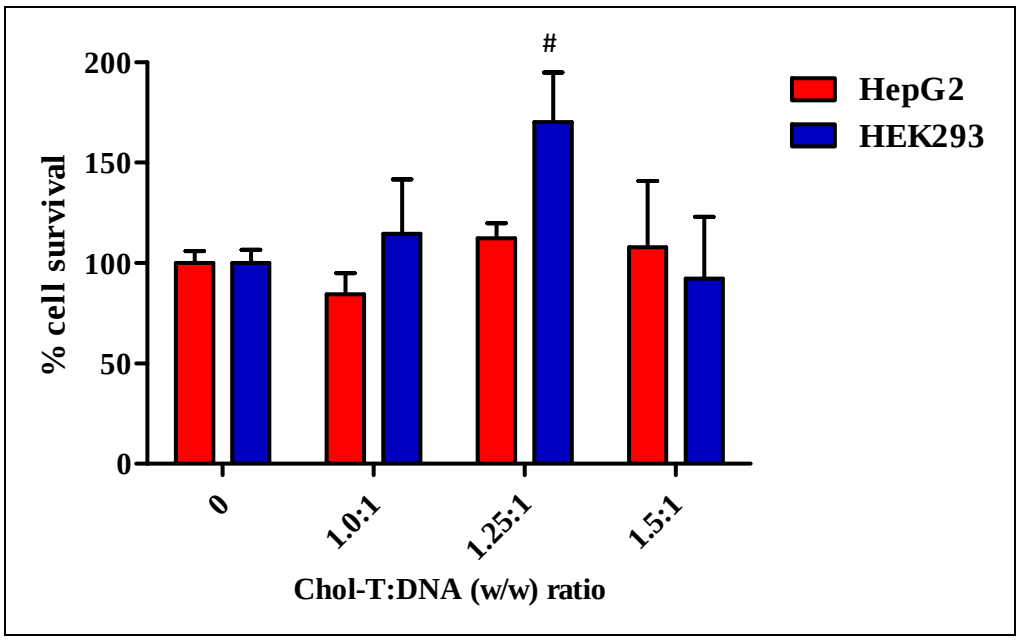
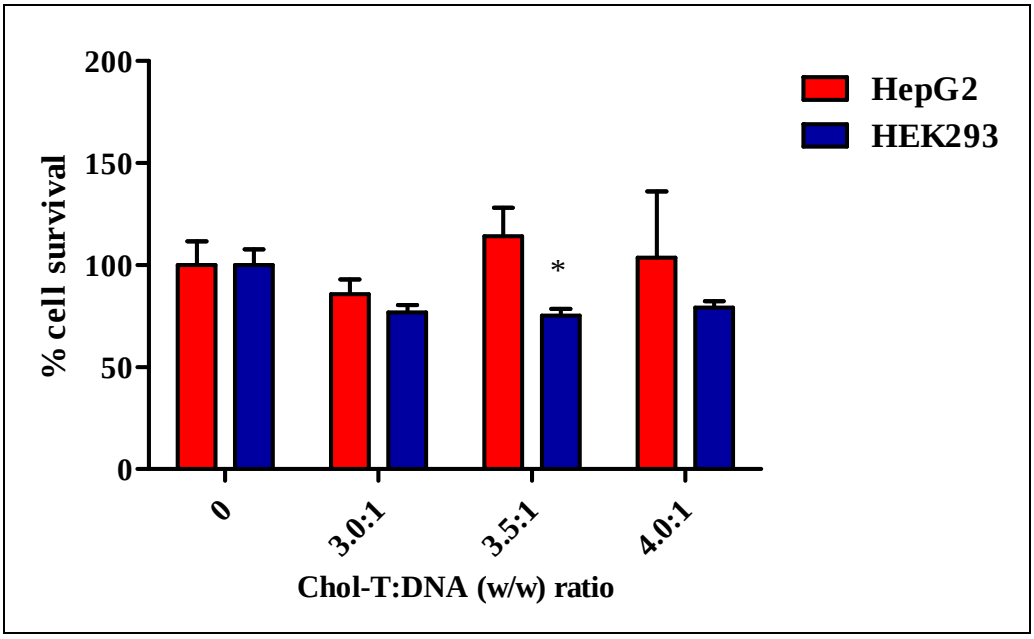


Figure 5.7: Growth inhibition studies of non-pegylated liposomes, a) 1; b) 2; c) 3; and d) 4. Cells in serum-free medium were subjected to 4 hour exposure to lipoplexes (10 µl in HBS) prepared from 0.5 µg pCMV-*luc* DNA and different amounts of liposome corresponding to optimal, sub- and super-optimal DNA-binding ratios. Each column represents the mean ± SD (n = 3). $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* HepG2; $^{\#}P < 0.05$ *vs.* the relevant control.

a)


200
150
100
50
0
% cell survival
HepG2
HEK293
*
0
3.0:1
3.5:1
4.0:1
Chol-T:DNA (w/w) ratio

b)

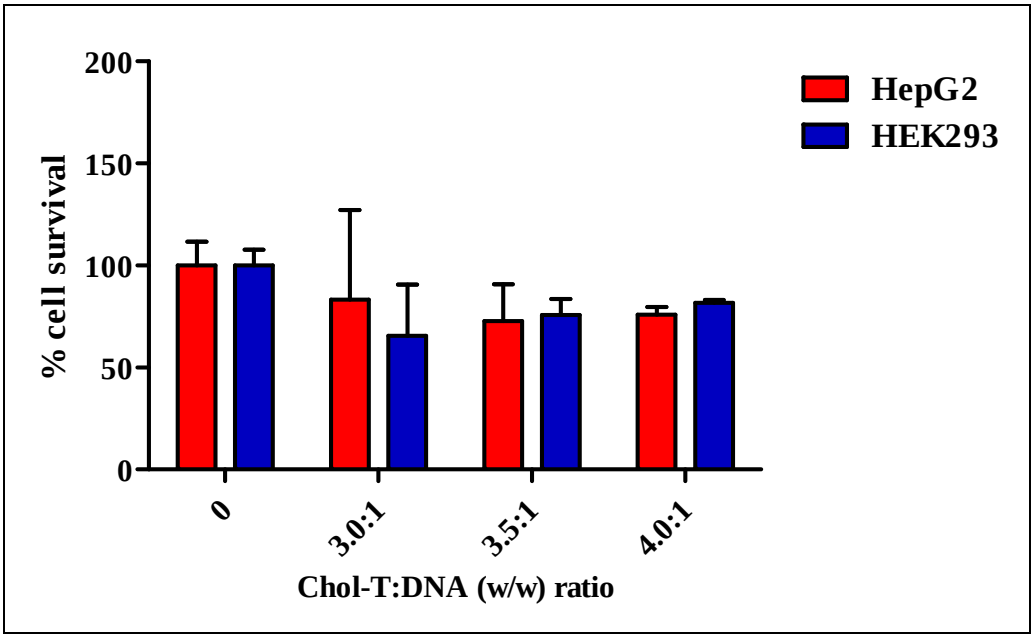
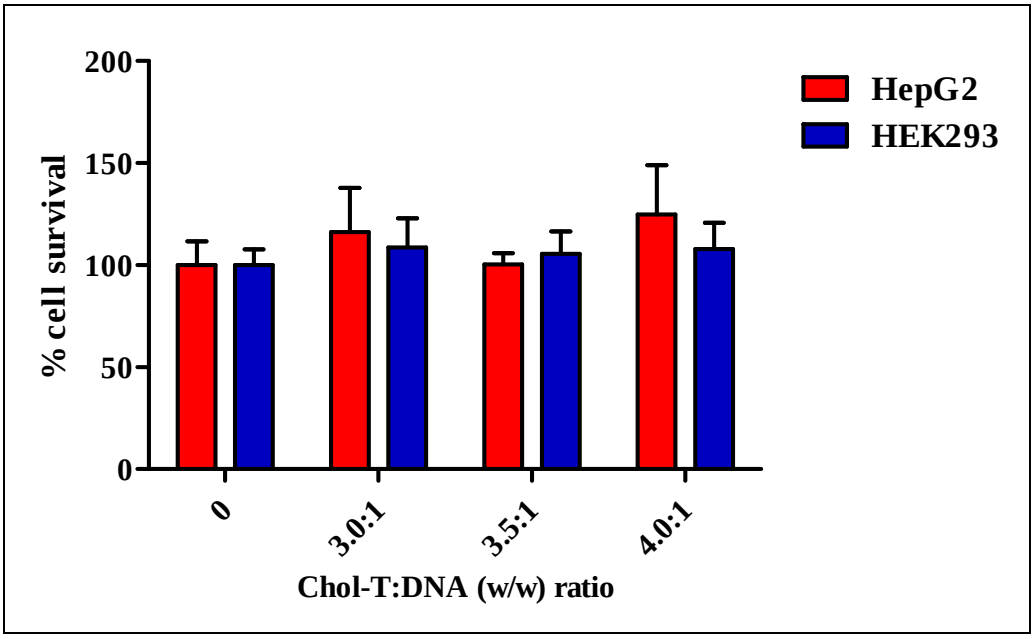

200
150
100
50
0
% cell survival
HepG2
HEK293
0
3.0:1
3.5:1
4.0:1
Chol-T:DNA (w/w) ratio

Fig. 5.8

c)



d)

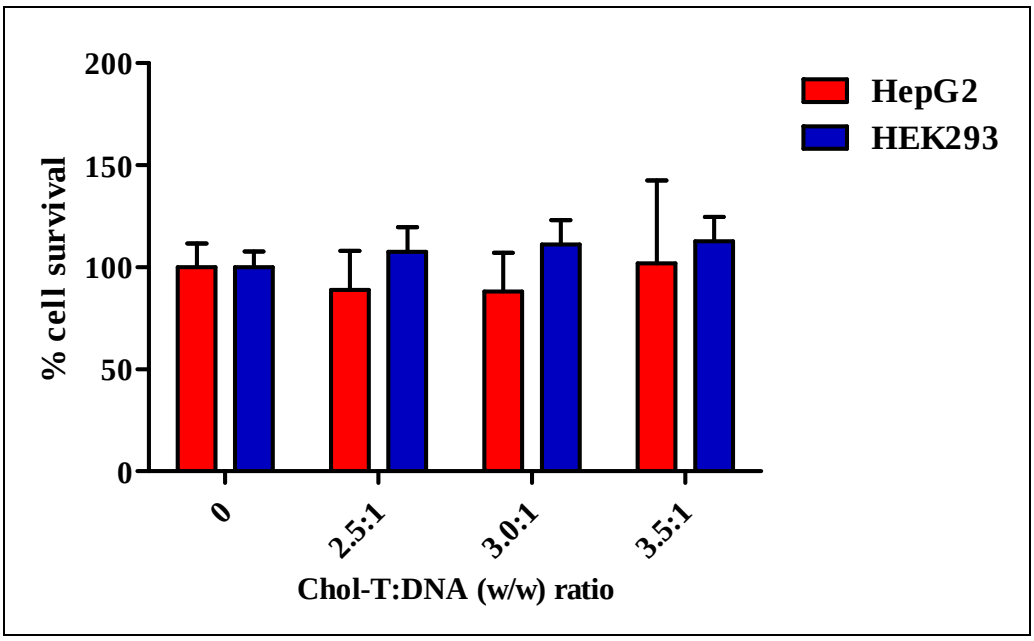


Figure 5.8: Growth inhibition studies of pegylated liposomes, a) 5; b) 6; c) 7; and (d) 8. Cells in serum-free medium were subjected to 4 hour exposure to lipoplexes (10 µl in HBS) prepared from 0.5 µg pCMV-*luc* DNA and different amounts of liposome corresponding to optimal, sub- and super-optimal DNA-binding ratios. Each column represents the mean ± SD (n = 3). *P < 0.05 *vs.* HepG2.

In general, pegylated and non-pegylated lipoplexes demonstrated favourable biocompatibility towards both HepG2 and HEK293 cells. However, cellular growth patterns in response to several liposome formulations differed noticeably in the two cell lines. For example, survival of hepatoma cells increased with increasing concentrations of liposome 1 within a range of 1.8 – 2.4 μg/well, having promoted growth by approximately 33 % at the super-optimal DNA-binding ratio. In contrast, the viability of the kidney cell line steadily decreased to 60 %, after an initial increase in cell numbers, in response to the same concentrations of the Chol-T/DOPE formulation (Figure 5.7a). Other authors have also reported that equimolar combination of Chol-T and DOPE correlates with cell survival rates in excess of 60 % (Balram *et al*., 2009; Singh and Ariatti, 2003; Singh *et al*., 2010). Furthermore, increasing the cytofectin:DNA ratio of lipoplexes assembled from pegylated preparations 5 – 8 in HepG2 cells was, in general, accompanied by a measurable change in cell numbers. However, in HEK293 cells, a similar response to the three different Chol-T:DNA ratios was observed with the exception of liposome 6 (Figure 5.8a, c, and d). In instances where similar effects on cell growth were observed between cell lines, such as the response to liposome 4, (Figure 5.7d), the growth inhibitory or growth stimulatory effect of individual lipoplexes often appeared more profound in one cell line than the other. Across the eight vector formulations, maximal growth inhibition was recorded at 50 % with respect to hepatocytes upon exposure to the Chol-T/DOPE/SH02 lipoplexes at the ratio of 1.25:1; and at 40 % in HEK293 cells in the presence of Chol-T/DOPE lipoplexes at a ratio of 2:1. These findings are not unusual as cell-specific responses to several cationic liposomal carriers have been documented (Lv *et al*., 2006; Ma *et al*., 2007; Romøren *et al*., 2004). According to Romøren and coworkers (2004) this may be attributed to differences in cellular accumulation of the vector, as a consequence of variation in cell surface characteristics; and its intracellular processing among cell lines.

A comparison of the cytotoxicity profiles of each liposome in a single cell line demonstrates that, in addition to lipid concentration, the type, properties and relative proportions of individual lipid components greatly influences the effect of transfecting complexes on cell proliferation. Similar statements were forwarded by Lv and coworkers (2006) in a review of cationic lipid-mediated toxicity. As an illustration, the introduction of the galactosylated lipid SH02 into the Chol-T/DOPE formulation, reversed the cell proliferation pattern that was observed upon exposure of HepG2 cells to liposome 1, with increasing Chol-T:DNA ratio. Figure 5.7b, shows that survival of hepatoma cells steadily decreased until maximal growth

inhibition of 50 % was attained at the super-optimal (1.25:1) DNA-binding ratio. Transfection studies support the fact that these galactose-modified lipoplexes, were effectively internalised by HepG2 cells, due to ASGP-R-mediated endocytosis (refer to 5.3.3). The cell-specific accumulation mediated by liposome 2, may account for its more profound cytotoxic effect, especially at higher liposome and cytofectin concentrations. As a further example, modification of the Chol-T/DOPE formulation with the imidazolylated lipid, SH04, at the same molar ratio as SH02, led to improved cell tolerance, having permitted cell survival in excess of 84 % at all ratios investigated (Figure 5.7c). Due to the stronger DNA-binding affinity of liposome 3 (Chol-T/DOPE/SH04), smaller amounts of liposome, corresponding to lower levels of cytofectin, were required to prepare lipoplexes from this formulation, than liposomes 1 and 2. In this regard, it has been reported that cationic liposome formulations that are able to complex DNA with minimal lipid content, are favourable gene transfer agents as these generally display minimal cytotoxicity (Muñoz-Úbeda *et al*., 2011).

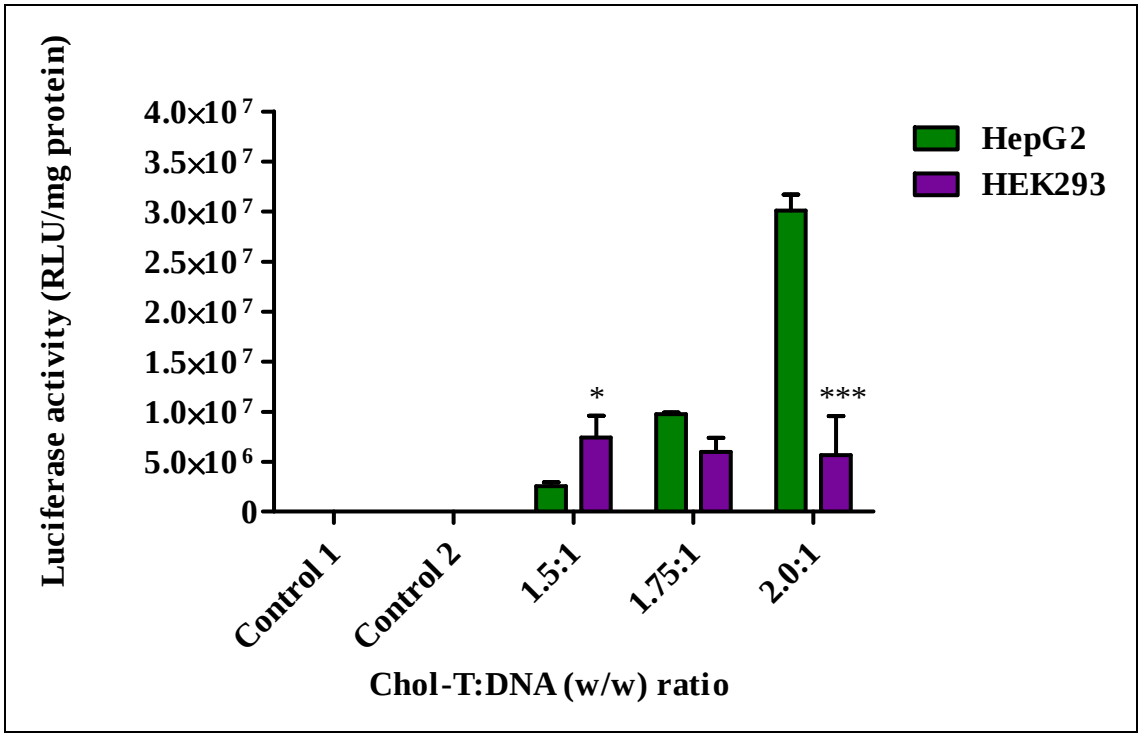
Among the non-pegylated carriers, lipoplexes prepared from liposomes 3 and 4, gave the most favourable cytotoxicity profiles in both cell lines, with cell survival in excess of 84 % at the different DNA-binding ratios investigated. In addition, their pegylated counterparts were best tolerated among the sterically stabilised preparations: liposome 8 resulted in a slight inhibition of growth, of approximately 10 %, when complexed with DNA at ratios of 2.5:1 and 3:1 in HepG2 cells alone (Figure 5.8d); while liposome 7 mediated a slight increase in the growth of both cell lines at all DNA-binding ratios investigated (Figure 5.8c). On the whole, pegylated lipoplexes prepared at the optimal, sub- and super-optimal DNA-binding ratios contained larger amounts of liposome and, accordingly, cytofectin, than their non-pegylated counterparts due to their weaker DNA-binding capacity. Nonetheless, the pegylated liposomes permitted cell survival in excess of 66 %, and often elicited growth promoting effects (Figures 5.8a-d). In fact, other authors have reported that modification of cationic vectors with PEG enhanced the biocompatibility of the carriers (Nagasaki *et al*., 2004; Narainpersad *et al*., 2012). Although this study was limited to *in vitro* assays, it is worthy of note that steric stabilisation of liposomes using DSPE-PEG_{2000} at maximal concentration of 10 %, was shown to minimise liposome-induced inflammatory toxicity in animal models, by preventing interactions between liposomes and cells of the immune system (Filion and Phillips, 1998; Sakurai *et al*., 2002; Zhang *et al*., 2005).

5.3.3 Gene expression assays

The gene expression assays included two controls (Figures 5.9 and 5.10). The first, which consisted of cells alone, was used to establish the effect of background luminescence. The second control consisted of cells to which naked plasmid DNA was introduced, in the same quantity as was used to prepare lipoplexes, and incubated under the same conditions. This control highlights the inability of plasmid DNA to facilitate gene expression in the absence of a carrier.

In the case of lipoplexes derived from the Chol-T/DOPE control (Figure 5.9a), an increase in the Chol-T:DNA ratio within the range of 1.5:1 and 2:1, was accompanied by significant elevations ($P < 0.001$) in gene expression in the hepatoma cells. Maximum luciferase activity in this cell line was achieved at the super-optimal DNA-binding ratio of 2:1. This may be attributed to both the growth stimulating effect of this lipoplex in HepG2 cells, and its net positive surface charge. It is generally accepted that positive surface charge is necessary for efficient transfection of cells in culture, due to enhanced affinity of the vector for association with anionic plasma membranes (Cao *et al*., 2000; Reimer *et al*., 1997). Nonetheless, the ratio at which transgenes are optimally delivered and expressed may vary among cell lines (Farhood *et al*., 1992; Vigneron *et al*., 1996). In keeping herewith, the highest transgene expression levels in HEK293 cells was achieved at the sub-optimal DNA-binding ratio (1.5:1), but was approximately four times lower than optimal levels in hepatocytes. Similar observations were made with respect to several of the other liposomes formulated in this study. This may be ascribed to differences in the cellular surface characteristics, which influence internalisation of the vector (Romøren *et al*., 2004). An additional reason for the cell-specific differences in gene expression afforded by individual carriers, relates to the longevity of the DNA cargo, following its escape from the endosome. Although the mechanism by which exogenous DNA translocates to the nucleus is poorly understood, it has been reported than the efficiency with which DNA enters the nucleus of different cell lines varies according to their ability to divide (Karmali and Chaudhuri, 2007). Furthermore, the DNA cargo may remain stable for a longer period of time in some cell lines, often leading to higher gene expression levels in such cells (Zhdanov *et al*., 2002).

a)


Luciferase activity (RLU/mg protein)
4.0×10 7
3.5×10 7
3.0×10 7
2.5×10 7
2.0×10 7
1.5×10 7
1.0×10 7
5.0×10 6
0
*
***
HepG2
HEK293
Control 1
Control 2
1.5:1
1.75:1
2.0:1
Chol-T:DNA (w/w) ratio

b)

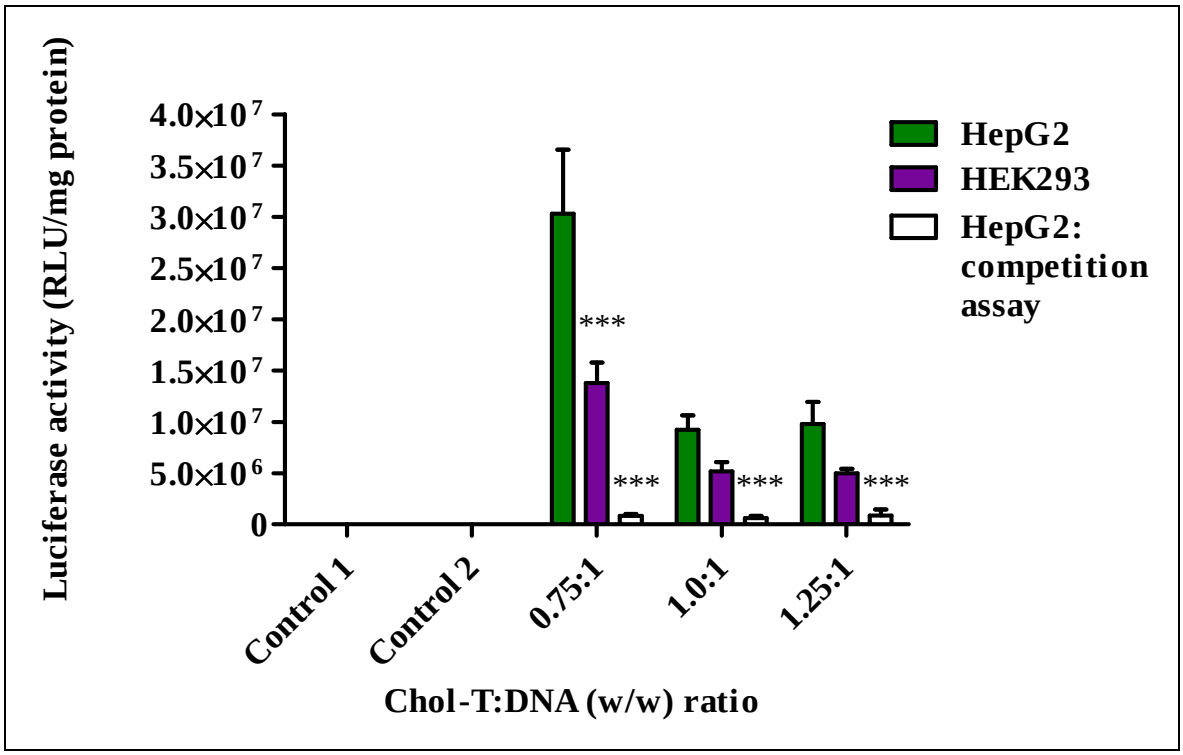

Luciferase activity (RLU/mg protein)
4.0×10 7
3.5×10 7
3.0×10 7
2.5×10 7
2.0×10 7
1.5×10 7
1.0×10 7
5.0×10 6
0
***
***
***
***
HepG2
HEK293
HepG2: competition assay
Control 1
Control 2
0.75:1
1.0:1
1.25:1
Chol-T:DNA (w/w) ratio

Fig. 5.9

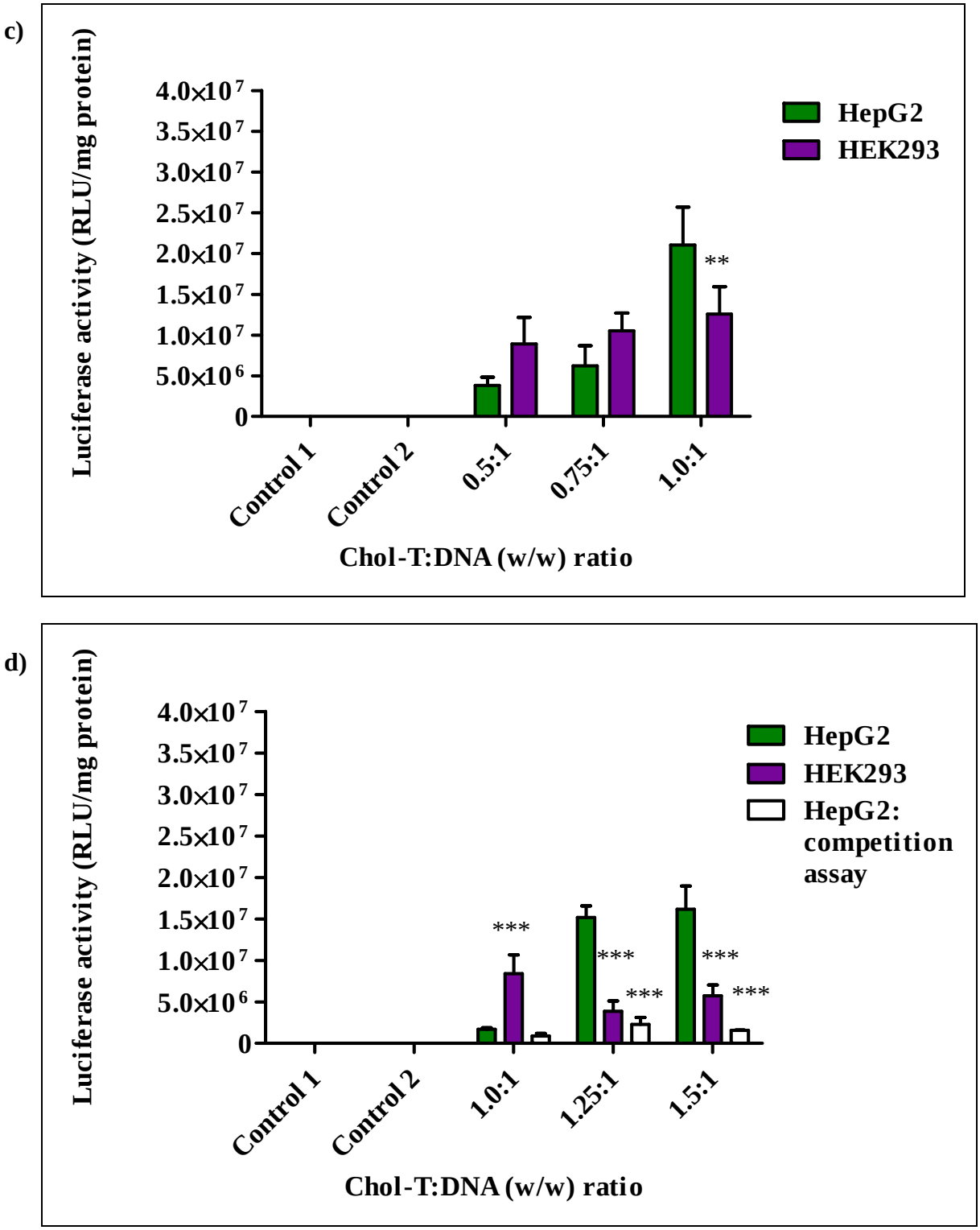
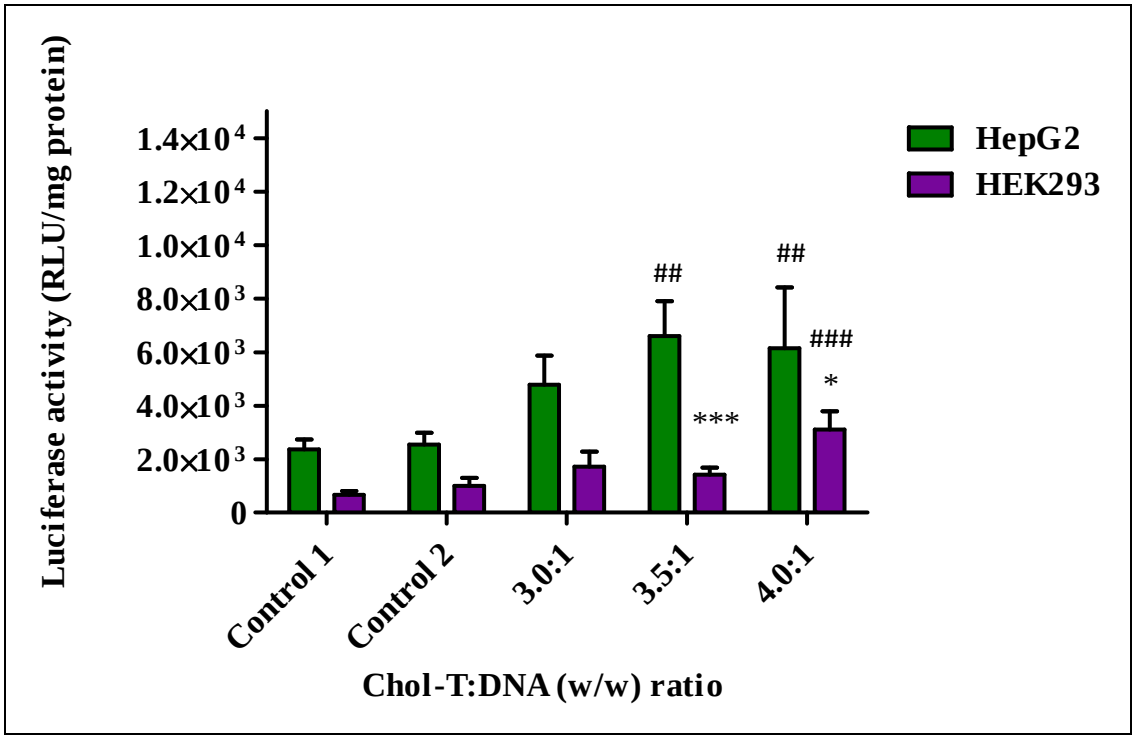


Figure 5.9: Transfection capabilities of non-pegylated cationic liposomes, a) 1; b) 2; c) 3; and d) 4, in the absence of serum. Cells were exposed to lipoplexes (10 µl in HBS) assembled from 0.5 µg pCMV-*luc* DNA and varying amounts of liposome corresponding to the optimal, sub- and super-optimal DNA-binding ratios for 4 hours at 37 ˚C. Control 1 consisted of cells alone, while control 2 contained cells and naked plasmid DNA. The controls gave readings below 3×10^3 RLU/mg protein in both cell lines. Each column represents the mean ± SD ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs.* HepG2.

a)


Luciferase activity (RLU/mg protein)
1.4×10⁴
1.2×10⁴
1.0×10⁴
8.0×10³
6.0×10³
4.0×10³
2.0×10³
0
HepG2
HEK293
##
##
###
*
***
Control 1
Control 2
3.0:1
3.5:1
4.0:1
Chol-T:DNA (w/w) ratio

b)

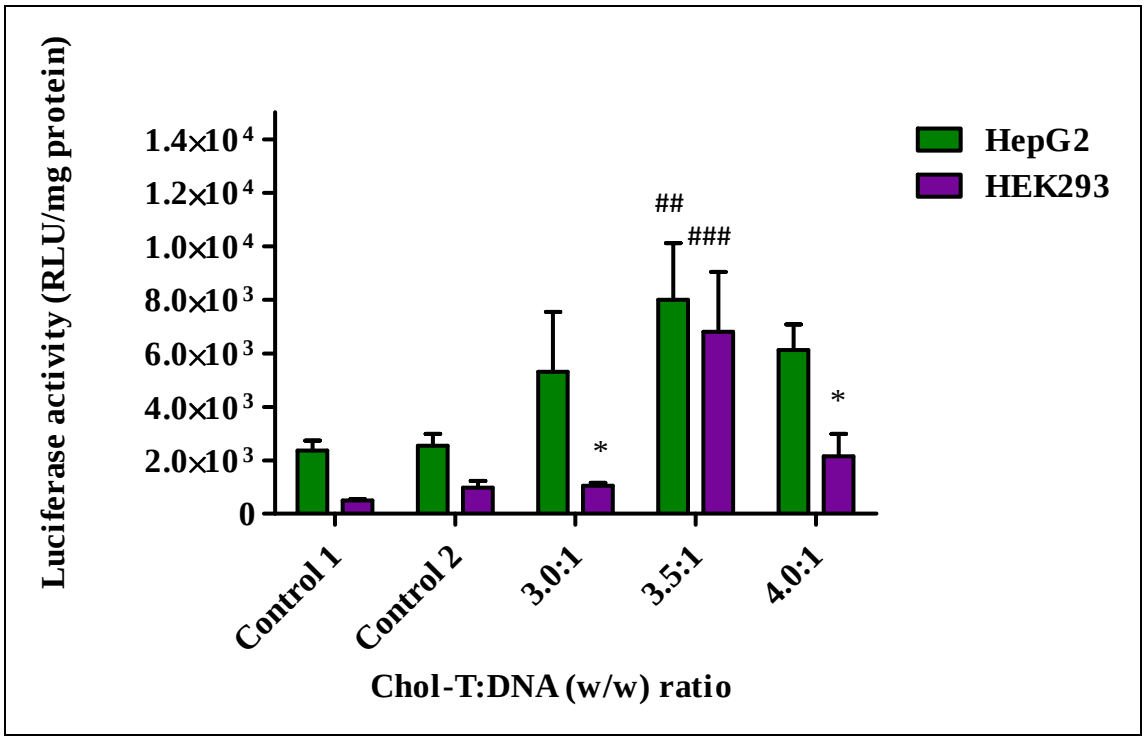
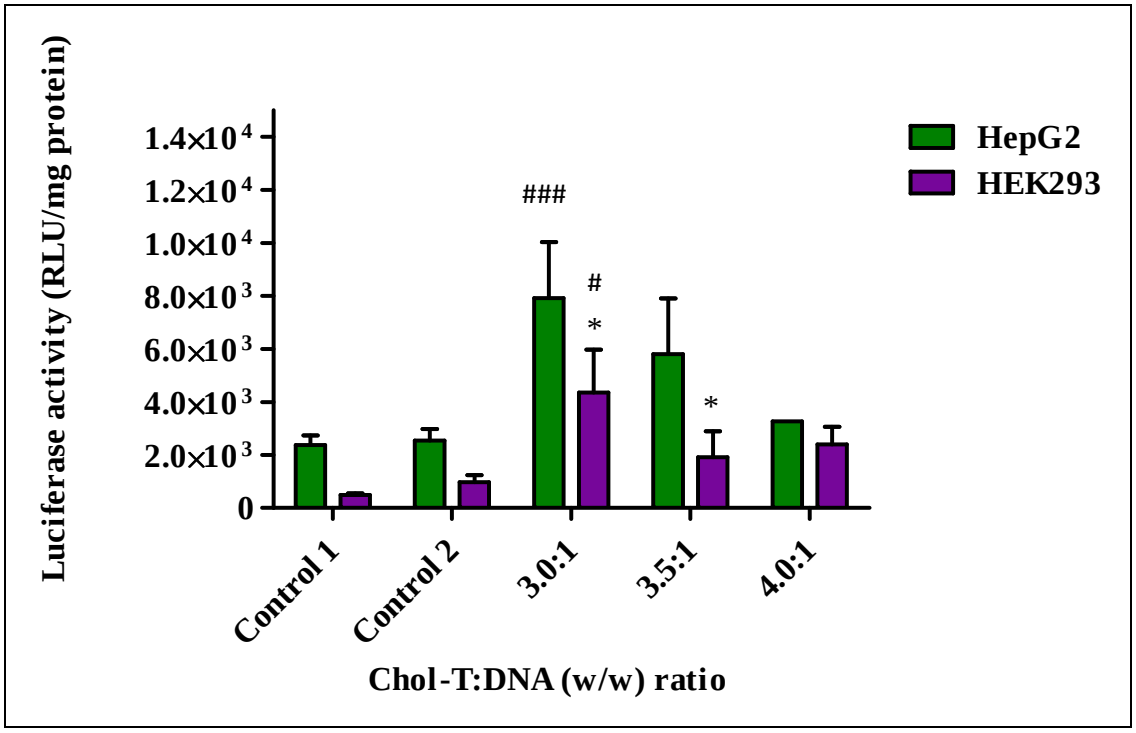

Luciferase activity (RLU/mg protein)
1.4×10⁴
1.2×10⁴
1.0×10⁴
8.0×10³
6.0×10³
4.0×10³
2.0×10³
0
HepG2
HEK293
##
###
*
*
Control 1
Control 2
3.0:1
3.5:1
4.0:1
Chol-T:DNA (w/w) ratio

Fig. 5.10

c)



d)

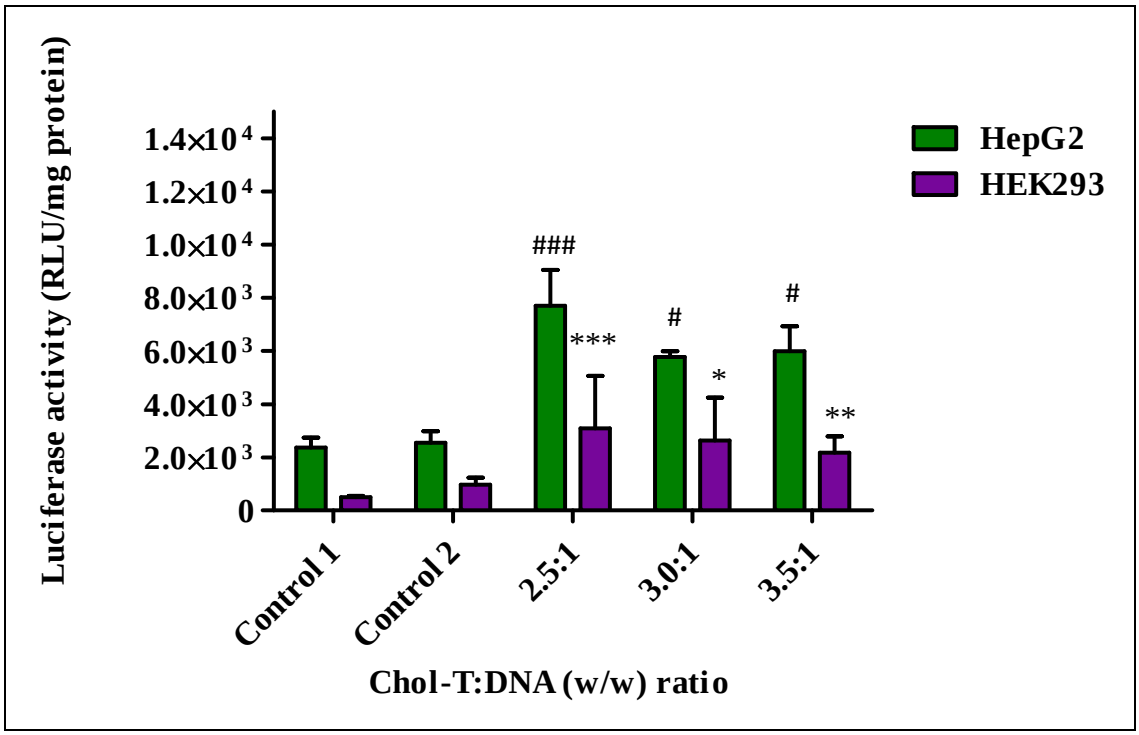


Figure 5.10: Transfection capabilities of pegylated cationic liposomes, a) 5; b) 6; c) 7; and d) 8, in the absence of serum. Cells were exposed to lipoplexes (10 μl in HBS) assembled from 0.5 μg pCMV-*luc* DNA and varying amounts of liposome corresponding to the optimal, sub- and super-optimal DNA-binding ratios for 4 hours at 37 ˚C. Control 1 consisted of cells alone, while control 2 contained cells and naked plasmid DNA. Each column represents the mean ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 *vs.* HepG2; $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001 *vs.* control 2 in the relevant cell line.

The galactosylated formulation, liposome 2, at the ratio of 0.75:1 afforded the best transgene activity across the eight carriers designed, in the hepatoma cell line. Although maximal luciferase activity of 3.02×10^7 RLU/mg protein reflected only a marginal improvement over that achieved by liposome 1 in HepG2 cells, the hepatotropic potential of this liposome formulation is of importance. Firstly, gene expression achieved by complexes assembled from liposome 2 in hepatocytes was approximately twice that attained in the ASGP-R-negative kidney cells, at all DNA-binding ratios explored. Secondly, transfection activity of this carrier in HepG2 cells was reduced by 93 – 97 % in the presence of excess asialofetuin, a natural ligand to the ASGP-R (Figure 5.9b). This competition assay confirmed that the principal route of entry of lipoplexes derived from liposome 2, in the liver parenchymal cells, is via ASGP-R-mediated endocytosis.

Studies with synthetic ligands to the ASGP-R, have demonstrated that the number and 3-dimensional orientation of galactosides influences the affinity with which such molecules are bound to the ASGP-R. According to Westerlind and coworkers (2004), the attachment of an additional monosaccharide ranging from a mono- to a tri-antennary ligand, enhances binding affinity 100 – 1000 fold; while further addition of sugar residues marginally increases binding affinity. This phenomenon, known as the "*glycoside cluster effect*," occurs due to the fact that a single ASGP-R possesses between three and six sugar binding sites (Khorev, 2007; Lee and Lee, 2000). According to Lee and coworkers (1983) the high affinity binding interaction between multivalent ligands and the receptor, is a consequence of the tendency of such ligands to simultaneously occupy the sugar-binding sites on individual receptors. Moreover, it has been proposed that multivalent ligands may induce the process of RME by encouraging clustering of receptor subunits (Khorev, 2007). Although SH02, which was used to glycosylate liposome 2, bears a single galacto-entity, lipoplexes derived hereof were successfully internalised by receptor-mediation. A possible explanation was offered by Murao and coworkers (2002) in a study of the targeting potential of galactosylated liposomes in an animal model. This group commented that the type and concentration of other lipids in the liposome formulation has a bearing on the mobility and distribution of the glycolipid. Consequently, it is possible for bilayer-anchored monogalactosides to form clusters on the surface of the vector. If the clustered sugar moieties are present in the correct orientation, relative to each other and the bilayer, these may behave as multivalent ligands and, as such, compensate for the relatively weak affinity displayed by a single galactose residue.

The fact that lipoplexes derived from liposome 2 did not mediate a significant increase ($P > 0.05$) in reporter gene expression in comparison with the Chol-T/DOPE control, may be associated with the size of the complexes (Audouy and Hoekstra, 2001). Although non-phagocytic eukaryotic cells may internalise particles of up to 1 μm, the upper size limit for endocytosis via clathrin-coated pits is approximately 200 nm (Rejman *et al*., 2004a). Moreover, studies by Rensen and colleagues (2001) have shown that in addition to the structure and orientation of the ligand on the liposome surface, the overall size of the particle influences its recognition and uptake via the ASGP-R. According to this group, smaller glycosylated liposomes are more effectively internalised by hepatocytes both *in vitro* and *in vivo*. Taking into consideration that lipoplexes derived from liposome 2 were distributed over a broad size range, and that these complexes entered hepatocytes predominately by ASGP-R-mediated endocytosis; it is reasonable to assume that, of the complexes introduced, only those which were sufficiently small to be processed by the ASGP-R entered hepatocytes. Therefore, cells were subjected to a lower effective liposome and DNA dose, despite the appropriate display of cognate ligands to the ASGP-R. In fact, this effective dose of lipoplexes assembled at the ratio of 0.75:1 may have been comparable to that of the Chol-T/DOPE lipoplexes at the ratio of 2:1, which were also entirely polydisperse with large hydrodynamic diameters, but entered hepatocytes via a non-specific pathway with different size requirements.

As shown in Figure 5.9c, liposome 3 (Chol-T/DOPE/SH04) optimally transfected both cell lines at the super-optimal (1:1) DNA-binding ratio. Although luciferase activity afforded by this formulation was significantly ($P < 0.01$) greater in HepG2 cells, liposome 3 failed to rival the transfection capability of the Chol-T/DOPE formulation in this cell line. In contrast, liposome 3 gave approximately twice the levels of transgene expression achieved by liposome 1 in the kidney cell line. Seeing that the acid titration profile of liposome 3 confirmed its pH-sensitivity within the endosomal range, this enhancement in transfection over the Chol-T/DOPE control may be a consequence of early escape of the vector from the endosome, due to the proton sponge capability of the imidazolylated lipid, SH04. It is important to note, however, that the concentration of SH04 was not optimised for liposome formulations with respect to either cell line. Therefore, a future *in vitro* investigation with Chol-T/DOPE liposomes formulated with different concentrations of SH04 is suggested.

It is known that the shape and size of lipoplexes has a bearing on the pathway by which carriers enter cells (Audouy and Hoekstra, 2001; Ma *et al*., 2007; Rejman *et al*., 2004a). However, different cell types are better suited towards different endocytic pathways (Elouahabi and Ruysschaert, 2005). These points, taken together, may account for the observable differences in the gene transfer capability of the same carrier in cell lines. It is therefore possible that the structural features of complexes assembled from liposome 1 at the Chol-T:DNA ratio of 2:1 were better suited than those of liposome 3 for internalisation by hepatocytes. Although lipoplexes derived from liposomes 1 and 3, at the ratios which gave the highest transfection in HepG2 cells, were characterised by high polydispersity and aggregation, a greater population or sub-population of complexes may have displayed features more appropriate for successful uptake by HepG2 cells, with respect to liposome 1. It is also likely that the surface charge of the Chol-T/DOPE complexes promoted greater contact with the hepatocyte membranes. Consequently, the lower luciferase activity afforded by complexes prepared with liposome 3, in hepatoma cells may be due to reduced internalisation of these lipoplexes.

According to the above discussion it is possible that the SH04-conferred pH-sensitivity of liposome 3 did permit early escape of the vector from the endosomal compartment in HepG2 cells even in the absence of a measurable increase in gene expression when compared with liposome 1. In order to explore this possibility, bafilomycin A1 inhibition assays in both cell lines would be most appropriate. Bafilomycin A1 is an antibiotic that inhibits the activity of the ATP-driven proton pumps that acidify the endosome during intracellular processing of endocytosed particles (Johnson *et al*., 1993). The presence of the antibiotic would render the endosomal lumen neutral, preventing protonation of weak bases, such as the imidazole ring (Midoux *et al*., 2009). Significant reduction in transfection activity of the vector in the presence of bafilomycin A1 would imply that its transfection capabilities are dependent on the process of endosomal acidification and, consequently, its induction of the proton sponge effect (Yang *et al*., 2010). Several authors have employed this inhibition assay as a means of validating the proton sponge capability of a variety of pH-sensitive carriers (Kichler *et al*., 2003; Midoux and Monsigny, 1999; Moreira *et al*., 2009; Shigeta *et al*., 2007; Yang *et al*., 2010).

Liposome 4 afforded the lowest levels of reporter gene expression across the four non-pegylated carriers in the HepG2 cell line, despite the incorporation of both SH02 and SH04. This is possibly due to the fact that this formulation and complexes derived therefrom were the least stable of the liposomes prepared. Nonetheless, the galactose moiety of SH02 was successfully recognised by the ASGP-R on hepatocytes, when incorporated into this formulation. This was demonstrated by the significant reduction ($P < 0.001$) in gene expression in the presence of excess asialofetuin (Figure 5.9d). However, unlike liposome 2, liposome 4 mediated higher levels of transgene expression in hepatoma cells in comparison with kidney cells, only at the optimal and super-optimal DNA-binding ratios. The significantly lower luciferase activity in hepatocytes mediated by complexes assembled at the ratio of 1:1 may be a consequence of the lipoplex ultrastructure and the processes involved in lipoplex assembly, both of which are dependent on the lipid:DNA mixing ratio (Huebner *et al*., 1999). It is likely that the rearrangement of lipid components induced by the addition of DNA at this ratio may have induced shielding of galactose residues, resulting in poor recognition by the ASGP-R. Alternately, the size and shape of complexes formed at the ratio of 1:1 may have been more appropriate for uptake by the kidney cells.

Liposome modification with 5 % PEG was accompanied by a dramatic reduction in reporter gene expression. In each case, the luciferase activity afforded by pegylated liposomes was at least three orders of magnitude lower than that of their non-pegylated equivalents in both cell lines. Although all pegylated liposomes achieved higher gene expression levels in hepatoma cells than in the kidney cell line, transfection activity of several lipoplexes was not significantly different from control 2 (Figure 5.10a-d).

Related studies have achieved useful transfection activity upon pegylation of cationic liposomes with DSPE-PEG_{2000} (Narainpersad *et al*., 2012; Singh *et al*., 2011). However authors have reported a reduction in gene expression in comparison with the performance of non-pegylated carriers *in vitro* (Narainpersad *et al*., 2012; Zhang *et al*., 2010). Furthermore, the effect is exacerbated with increased pegylation (Zhang *et al*., 2010). Several theories have been forwarded in order to explain these observations. Firstly, the polymer shroud masks a portion of cationic charges on the outer leaflet of the lipid bilayer, hinders its association with anionic cell surfaces and, as such, impedes endocytosis of the vector (Deshpande *et al*., 2004). In the case of ligand-modified vectors, PEG chains may adumbrate the targeting

moieties and inhibit their recognition by cell-specific receptors (Ulrich, 2002). Accordingly, transgene expression achieved by the galactosylated, pegylated liposomes, 6 and 8, in HepG2 cells was as low as that afforded by the non-targeted, pegylated carriers. Due to the poor levels of luciferase activity in comparison with control 2, these liposomes were not subjected to competition assays.

Song and coworkers (2002) have proposed that instead of inhibiting endocytosis of lipoplexes, the PEG chains prevent escape of lipoplexes from the endosome, depending on the nature of the hydrophobic skeleton and molecular weight of the PEG chains of the PEG-lipid conjugate. According to this group, the presence of PEG chains on the surface of the vector prevents close contact between the bilayers of the lipoplex and the endosome. This obstructs DOPE in inducing the inverted hexagonal phase that promotes destabilisation of the endosome. Consequently, such lipoplexes are often bound for lysosomal degradation, resulting in low levels of reporter plasmid reaching the nucleus in an intact form (Remaut *et al*., 2007). Furthermore, studies conducted by Shi and coworkers (2002) have shown that DSPE-PEG conjugates stabilise the lamellar phase of the bilayer and therefore discourage destabilisation of the endosomal membrane. In support of this observation, Rejman and colleagues (2004b) have reported that the bilayer stability afforded by DSPE as a lipid anchor of PEG moieties encumbers the process of lipid interchange between the bilayers of the lipoplex and the endosome and, as such, prevents release of the DNA cargo.

The poor transfection capability displayed by the pegylated liposomes in this study may be further attributed to the weak DNA-binding ability, and small size of the resulting liposome/DNA complexes. At all Chol-T:DNA ratios investigated pegylated lipoplexes were approximately 100 nm in size and more uniformly distributed than their non-pegylated counterparts. According to Kenworthy and coworkers (1995), PEG chains are known to contribute to the hydrodynamic radius of lipid-based carriers. Therefore, the pegylated lipoplexes are likely to be even smaller than the DLS measurements suggest. When introduced to cells in culture, these small lipoplexes might have not settled to the bottom of the wells during the four hour exposure time (Rao, 2010). Consequently, the carriers may not have been in contact with cell membranes long enough to permit internalisation. Although two of the pegylated liposomes (preparations 7 and 8) were formulated with the pH-sensitive lipid, SH04, that could potentially avert the inhibition of endosomal destabilisation by DSPE,

due to its proton sponge capability; this effect of SH04 could only be invoked if the carrier had been successfully internalised. Furthermore, the favourable cytotoxicity profiles of the pegylated lipoplexes could also be due to their poor cellular contact and internalisation. It is possible that with an extended incubation period (> 4 hours), pegylated lipoplexes may be deposited upon the layer of cells; and this may correlate with higher transfection levels.

However, if the above-mentioned transfection experiments are to be carried out, it is necessary that the corresponding growth inhibition assays be performed in parallel. In the event that extended exposure to the complexes improves transfection levels, competition assays should then be performed in order to establish whether or not the galactose residues of liposomes 6 and 8 are recognised by the ASGP-R in the presence of the polymer shield. In view of the importance of steric stabilisation to the clinical application of liposomal carriers, such investigations may present a useful addition to the current study. In addition, several modifications to the pegylated liposomal systems may be explored in order to improve transfection activity. Firstly, the cationic charge- and ligand-adumbrating effects of PEG may be reduced by modifying liposomes with a less dense polymer coating. In this regard, Singh and coworkers (2011) reported that cationic liposomes stabilised using DSPE-PEG_{2000} at 2 % on a molar basis, gave gene expression levels 15 % higher than those achieved at 5 % pegylation. Secondly, galactose moieties on sterically stabilised liposomes may be rendered more accessible to the ASGP-R, by increasing the length of the spacer element between the galactose residue and cholesterol anchor of the glycolipid (Rensen *et al*., 2001; Westerlind *et al*., 2004). Alternatively, the ligand may be covalently attached to the termini of bilayer-anchored PEG chains. Such a strategy, devised by Letrou-Bonneval and coworkers (2008) afforded encouraging levels of transgene expression in hepatocytes. Finally, the incorporation of linkages sensitive to reduction and acidity within the endosome, between the PEG moiety and its lipid anchor has shown potential in alleviating the inhibitory effect of PEG chains on the process of transfection. These novel PEG-lipid analogues permit loss of the hydrophilic polymer cloud such that it does not inhibit the inter-bilayer associations that are often critical to the transfection process (Guo *et al*., 2003; Masson *et al*., 2004; Shin *et al*., 2003; Zalipsky *et al*., 1999).

In summary, the non-pegylated liposomes were far superior to their pegylated counterparts with respect to *in vitro* transfection efficiency. However, the addition of DNA to the non-pegylated liposomes, at all Chol-T:DNA ratios explored, was associated with high polydispersity and the formation of large aggregates. Although large complexes (> 300 nm), with the exception of ASGP-R-targeted lipoplexes, are favourable for *in vitro* transfection (Narainpersad *et al*., 2012; Rensen *et al*., 2001), in most instances the Z-average diameters of lipoplex suspensions were well beyond the upper limit of 1 μm at which non-phagocytic cells may internalise particles. Nonetheless, it is important to note that the size limit for cellular uptake is often cell- and pathway specific (Elouahabi and Ruysschaert, 2005; Rejman *et al*., 2004a). This suggests that only a portion of the lipoplexes introduced successfully gained entry into cells in each case; resulting in a lower effective lipid and gene dose. Therefore, if the relative proportions of individual lipids and the lipid:DNA mixing ratios are optimised to merit more stable, uniformly distributed complexes, that are suitably sized for ASGP-R-mediated uptake; the hepatocyte-specificity of SH02 and proton sponge capacity of SH04 may be profitably harnessed in a hepatocyte-specific liposomal gene transfer strategy. However, the adaptation of such a system to display stealth properties, without compromising either the DNA-binding ability, the effect of functional lipids SH02 and SH04, or the process of vector internalisation, requires further research.

CONCLUSION

Glycosylated cationic liposomes have shown great potential as hepatocyte-specific gene transfer agents. However, further development of such systems is required before these attain the levels of efficiency necessary to merit clinical application. This study was aimed at investigating the *in vitro* applicability of cationic liposomes modified with hepatocyte-targeting, endosomal escape and stealth functionalities.

The successful synthesis of galactosylated (SH02) and pH-sensitive, imidazolylated (SH04) cholesterol derivatives by carbodiimide-mediated coupling was confirmed using ^{1}H NMR and high resolution mass spectrometry. These novel lipids were combined at 10 % on a molar basis with the cytofectin, Chol-T, and helper lipid, DOPE. Liposomes formulated with either SH02 or SH04 gave stable, small unilamellar vesicles. In contrast, less stable, large unilamellar vesicles resulted upon incorporation of both cholesterol derivatives in a single formulation. Nonetheless, all non-pegylated liposomes demonstrated effective binding of pCMV-*luc* plasmid DNA, to which each provided protection against serum nucleases. Moreover, SH02 and/or SH04-containing formulations bound DNA with higher affinity than the Chol-T/DOPE control.

Steric stabilisation of the aforementioned liposomes with 5 % (mol/mol) DSPE-PEG_{2000} encouraged the maintenance of smaller-sized vesicles, and moderately reduced the instability associated with the incorporation of both SH02 and SH04 in the same preparation. However, partial shielding of cytofectin headgroups by the polymer chains reduced the DNA-binding and protecting capabilities of the liposomes.

Lipoplexes derived from individual formulations, at the optimal, sub- and super-optimal DNA-binding ratios, were assessed in ASGP-R positive, HepG2 cells, and the receptor-negative, HEK293 cell line. On the whole, the cellular tolerance of lipoplexes was favourable. However, responses specific to liposome composition, lipid:DNA mixing ratio and cell type were noted; and reflect the importance of optimising vector design with respect to the target cells. Transfection levels, as measured by luciferase activity, of non-pegylated liposomes were at least three orders of magnitude greater than that of their pegylated equivalents in both cell lines. This dramatic decrease in reporter gene activity upon

pegylation occurred even having incorporated the pH-sensitive and/or ASGP-R-targeting components. It was suggested that the inferior transfection capability of pegylated liposomes was primarily as a consequence of poor cellular uptake, due to the small size of the pegylated lipoplexes, which discourages transfection *in vitro*; and the known inhibitory effect of PEG chains on lipoplex-cell and receptor-ligand interactions.

The highest transfection levels in HepG2 cells were obtained using the Chol-T/DOPE/SH02 formulation. Reduced internalisation in receptor negative cells, and competitive inhibition assays confirmed that non-pegylated, glycosylated liposomes were internalised by hepatocytes predominantly by ASGP-R-mediation. This confirms that the concentration of SH02 employed for liposome formulation in this study permitted suitable exposure of galacto-entities to the hepatic lectin. The incorporation of the pH-sensitive lipid, SH04, into the targeted formulation did not significantly alter recognition of the sugar moieties by the ASGP-R, nor did it further improve the transfection levels in hepatocytes. This can be ascribed to the instability associated with the Chol-T/DOPE/SH02/SH04 formulation and complexes derived therefrom.

In the current study the proton sponge capability afforded by SH04 at 10 % (mol/mol) could, at best, be inferred from acid titration experiments, and the enhancement of transfection mediated by the Chol-T/DOPE/SH04 formulation in HEK293 cells over the Chol-T/DOPE control. It is suggested that future investigations involving transfection in the presence of an inhibitor of endosomal acidification, such as bafilomycin A1, be performed in order to validate this feature of SH04-containing liposomes.

It appears that the design of a liposomal system that effectively displays both hepatocyte-targeting and proton sponge capabilities relies on the stable incorporation of both SH02 and SH04 within a single liposome formulation. To this end, optimisation of liposome formulation is essential. Furthermore, optimisation of lipoplex size, shape and surface charge characteristics by modulating the lipid:DNA mixing ratio may permit the internalisation of lipoplexes at higher levels. Although pegylation effectively overcame the aggregation and high heterogeneity associated with lipoplexes derived from non-pegylated liposomes in this study, the weaker interactions with DNA and poor transfection activity of pegylated liposomes remains to be addressed in order to provide hepatotropic, pH-sensitive liposomes

with long-circulating properties. In this regard, pegylation at lower densities, post-pegylation strategies, the use of transient PEG coatings and/or the display of ligands on PEG chains were proposed as useful alternatives to the pegylation strategy employed in this study.

Nonetheless, the novel lipids, SH02 and SH04, synthesised in this study have demonstrated the intended properties of ASGP-R-specificity and endosomal pH-sensitivity, respectively. Therefore, with further modulation of both liposome compositions and the physical properties of the transfecting complexes, these lipids may well provide a platform for the future design of clinically feasible hepatotropic liposomal gene transfer systems.

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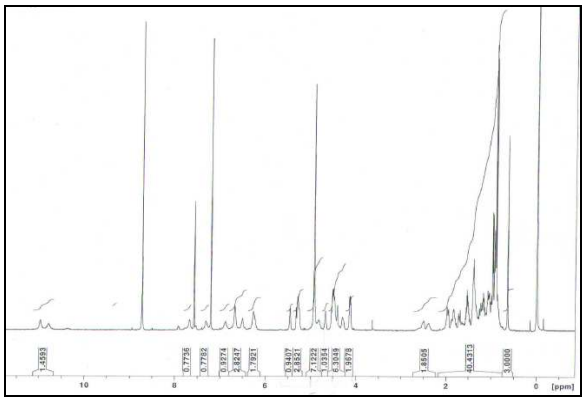
APPENDIX 1

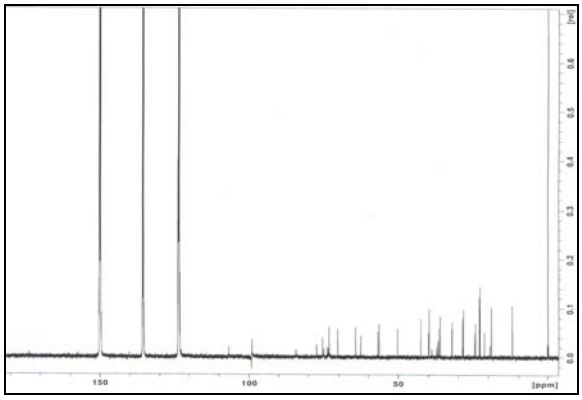
NMR SPECTRA

A



^{1}H NMR

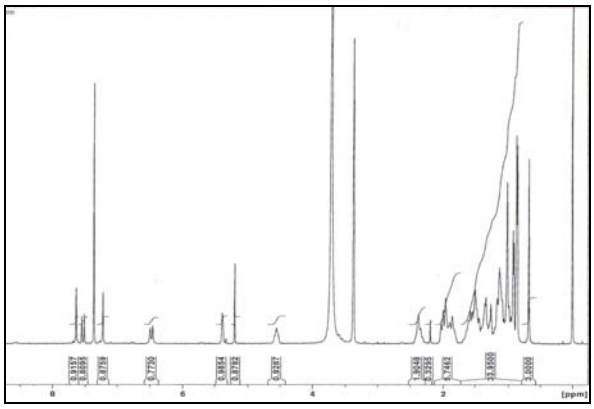
B



^{13}C NMR

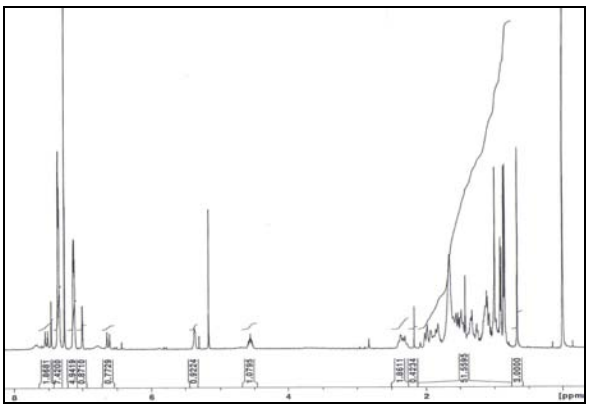
SH02: lactobionylcholesterylformylhydrazide

C



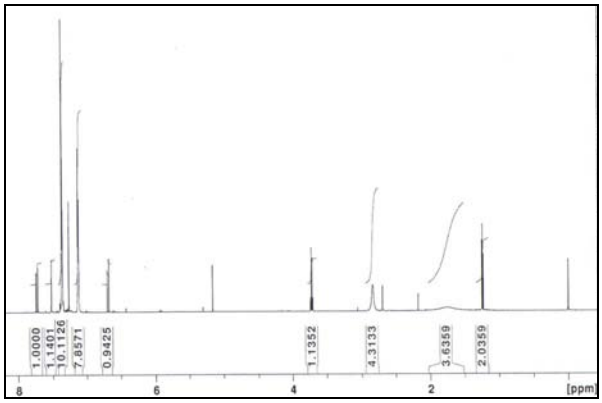
SH04: urocanylcholesterylformylhydrazide
^{1}H NMR

D



SH05: *N*-tritylurocanylcholesterylformylhydrazide
^{1}H NMR

E

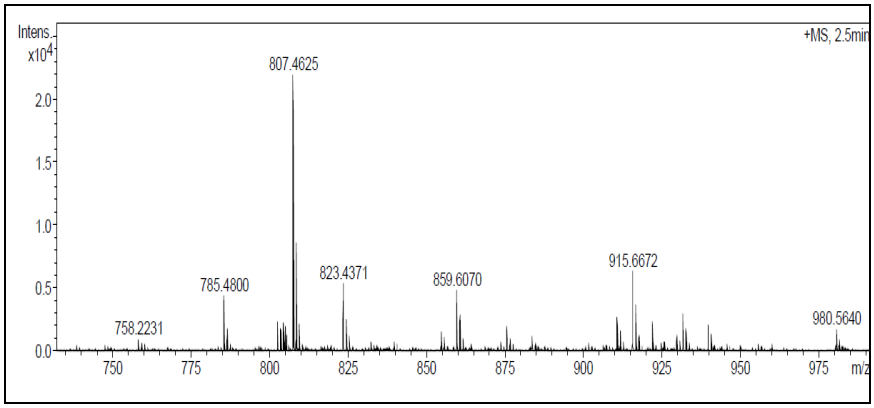


N-hydroxysuccinimide ester of *N*-tritylurocanic acid
^{1}H NMR

APPENDIX 2

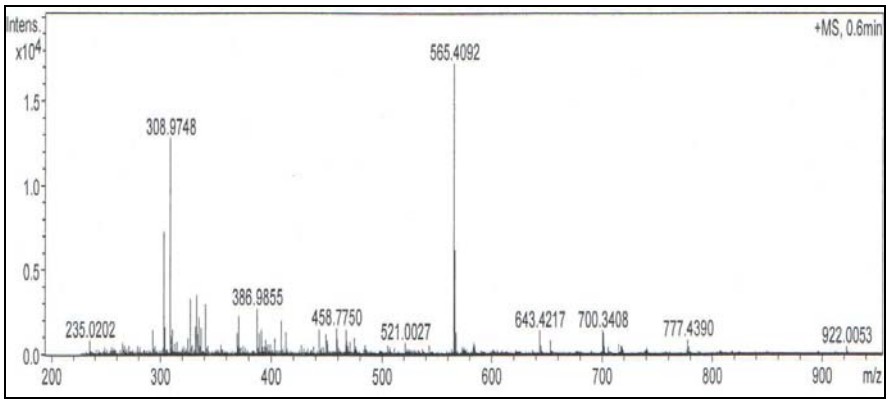
MASS SPECTRA

A



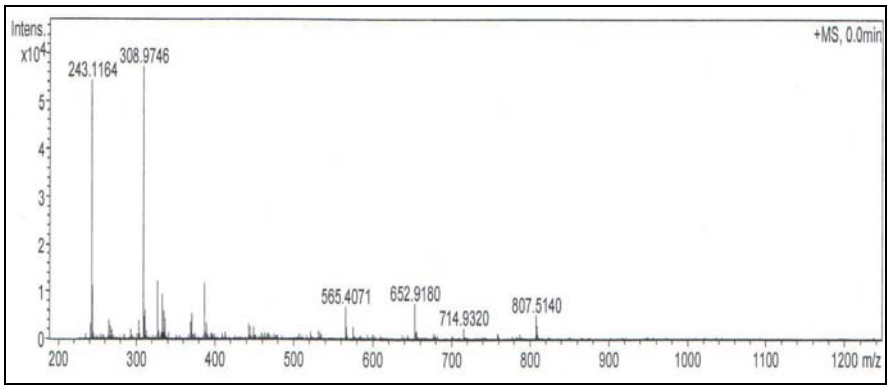
SH02: lactobionylcholesterylformylhydrazide

B



SH04: urocanylcholesterylformylhydrazide

C

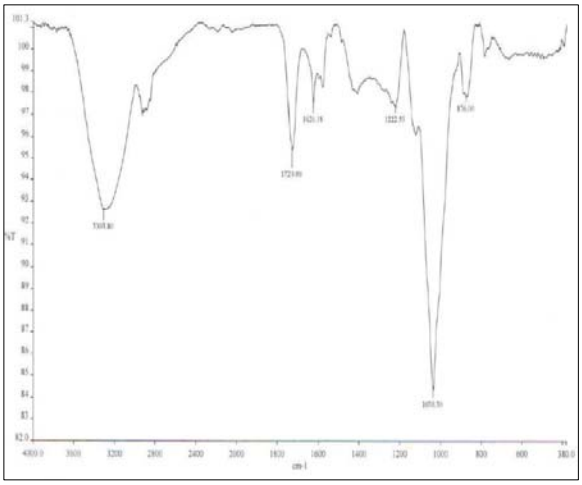


SH05: *N*-tritylurocanylcholesterylformylhydrazide

APPENDIX 3

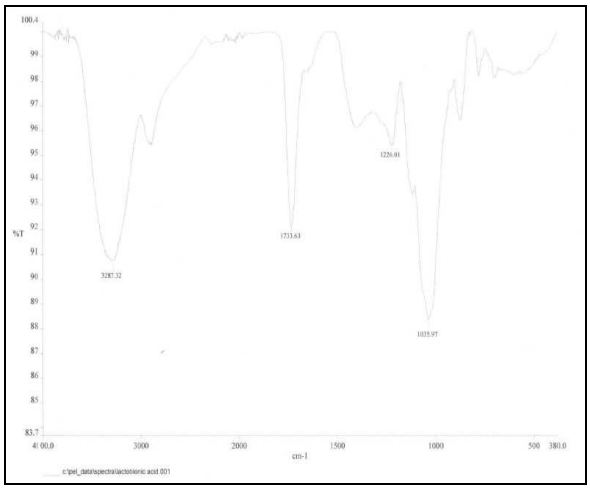
INFRARED (IR) SPECTRA

A



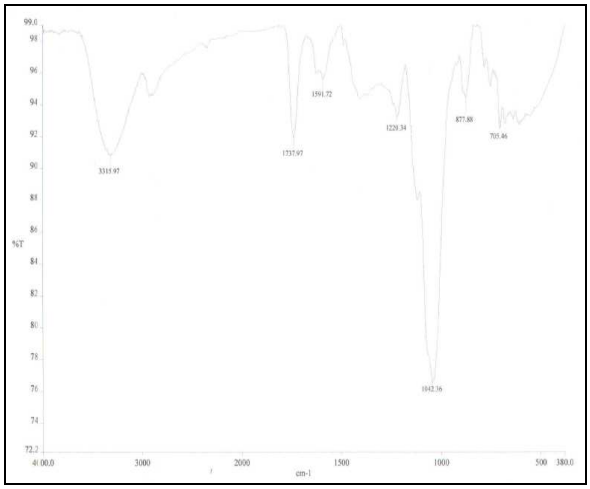
Lactobionic acid

B



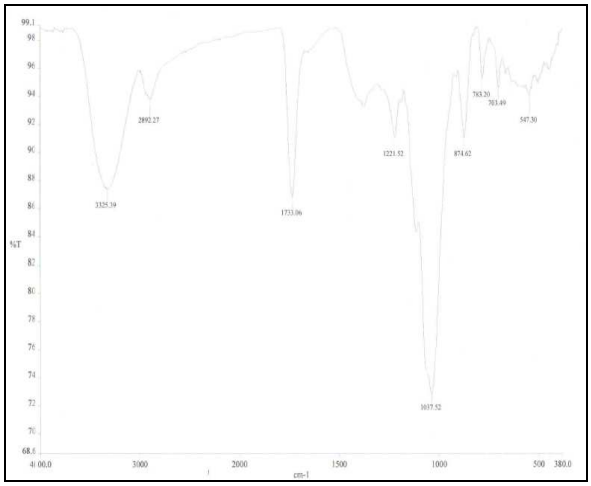
Lactone 1: formed by repeated evaporation of lactobionic acid from MeOH and EtOH.

C



Lactone 2: product of the lactobionic acid/DCCI reaction.

D



Lactone 3: formed by repeated evaporation of lactobionic acid from 2-MeOEtOH and toluene.

APPENDIX 4

IUPAC NAMES OF NOVEL CHOLESTEROL DERIVATIVES AND SYNTHESIS INTERMEDIATES

Compound	**IUPAC name**
Cholesterylformylhydrazide (SH01)	{[2,15-dimethyl-14-(6-methylheptan-2-yl)tetracyclo[8.7.0.0^{2,7}.0^{11,15}]heptadec-7-en-5-yl]oxy}formohydrazide
Lactobionylcholesterylformylhydrazide (SH02)	(2R,3R,5R)-N´-({[2,15-dimethyl-14-(6-methylheptan-2-yl)tetracyclo[8.7.0.0^{2,7}.0^{11,15}]heptadec-7-en-5-yl]oxy}carbonyl)-2,3,5,6-tetrahydroxy-4-{[(2S,3R,4S,5R,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy}hexanehydrazide
N-tritylurocanic acid	(2E)-3-[1-(triphenylmethyl)-1H-imidazol-4-yl]prop-2-enoic acid
N-hydroxysuccinimide ester of *N*-tritylurocanic acid	2,5-dioxopyrrolidin-1-yl (2E)-3-[1-(triphenylmethyl)-1H-imidazol-4-yl]prop-2-enoate
N-tritylurocanylcholesterylformyl hydrazide (SH05)	1-{[2,15-dimethyl-14-(6-methylheptan-2-yl)tetracyclo[8.7.0.0^{2,7}.0^{11,15}] heptadec-7-en-5-yl]oxy}-N´-(2E)-3-[1-(triphenylmethyl)-1H-imidazol-4-yl]prop-2-enoyl]formohydrazide
Urocanylcholesterylformylhydrazide (SH04)	(2E)-N´-({[2,15-dimethyl-14-(6-methylheptan-2-yl)tetracyclo[8.7.0.0^{2,7}.0^{11,15}]heptadec-7-en-5-yl]oxy}carbonyl)-3-(1H-imidazol-4-yl)prop-2-enehydrazide

APPENDIX 5

PLASMID AMPLIFICATION, ISOLATION AND VERIFICATION

Materials

Competent *Escherichia coli* JM109 cells were purchased from Promega, USA. pCMV-*luc* plasmid DNA (1 μg/μl stock) was obtained from Plasmid Factory, Bielefeld, Germany. Tryptone and yeast extract powder were purchased from Biolab Diagnostics, Midrand and Merck-biolab, Darmstadt, Germany respectively. Ampicillin (sodium salt) was supplied by Calbiochem, USA. Ribonuclease A (RNAse A) and chicken egg-white lysozyme were from EMB Biosciences, Madison.

Methods

Plasmid amplification

pCMV-*luc* DNA (1 μg) was added to competent cells (200 μl) and allowed to stand on ice for 30 minutes. The cells were subjected to heat-shock at 42 ˚C for 90 seconds and, thereafter, placed on ice for 2 minutes. Cells were then introduced into 250 ml sterile LB broth (containing 2.50 g tryptone, 1.25 g yeast extract powder, 1.25 g NaCl, and 10 μg/ml ampicillin), and maintained in a shaking incubator at 37 ˚C for 36 hours, in order to select transformants.

Plasmid isolation

pCMV-*luc* DNA was isolated according to the protocol outlined in the Promega Technical Manual no. 033 for pGL3 Luciferase Reporter Vectors.

APPENDIX 5 (continued)

Verification of DNA isolate

Spectrophotometry

The amount of plasmid DNA isolated was determined using a ThermoScientific NanoDrop 2000c spectrophotometer (Wilmington, USA). DNA was diluted to a concentration of 0.5 μg/μl with 18 Mohm water and stored, in aliquots of 200 μl, at – 20 ˚C.

Agarose gel electrophoresis

The plasmid isolate (0.5 μg) and pCMV-*luc* stock (0.5 μg), each in 10 μl HBS, were mixed with gel loading buffer (2 μl) and subjected to electrophoresis for 60 minutes at 50 V on 1 % agarose gel. For details of gel preparation and buffers used, refer to 4.2.2.1.

Results

Spectrophotometric analysis of isolated pCMV-*luc* DNA.

$Absorbance_{280nm}$	37.333
$Absorbance_{260nm}$	73.858
260/280 ratio	1.98
260/230 ratio	2.53
DNA concentration (μg/μl)	3.6929

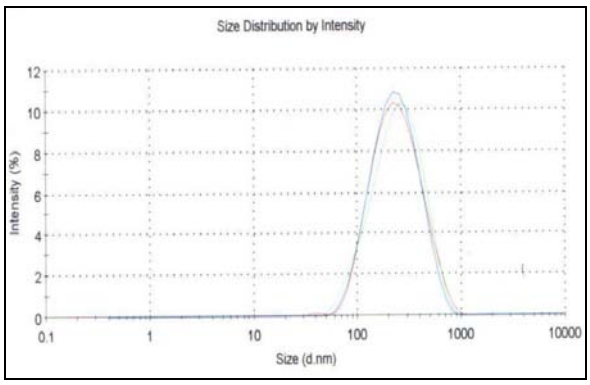
Plasmid DNA was successfully amplified and isolated in high yield. The integrity of the pCMV-*luc* isolate was assessed by electrophoresis on agarose gel, and all three forms, i.e. linear, circular and supercoiled forms, of plasmid DNA were observed. As expected, the supercoiled species was more abundant than the other two forms.

APPENDIX 6

SIZE DISTRIBUTION PROFILES (Size distribution by intensity)

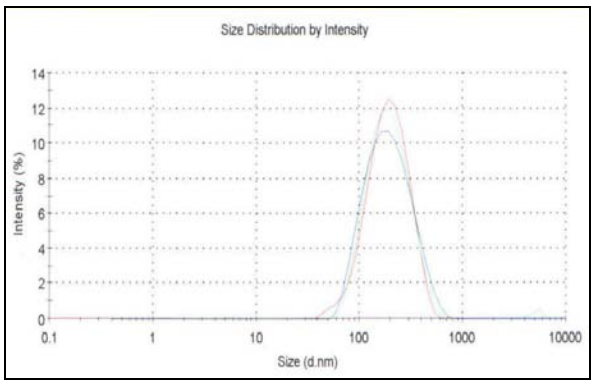
1. LIPOSOMES

A



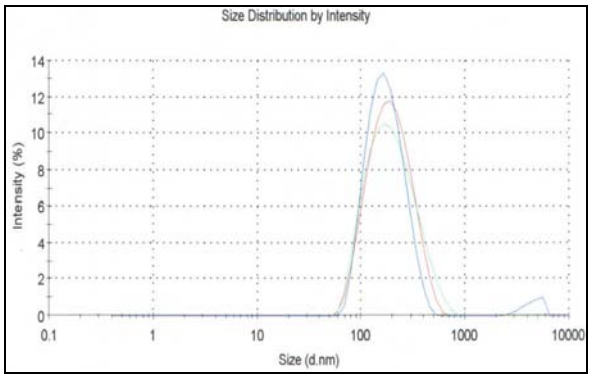
Liposome 1: Chol-T/DOPE

B



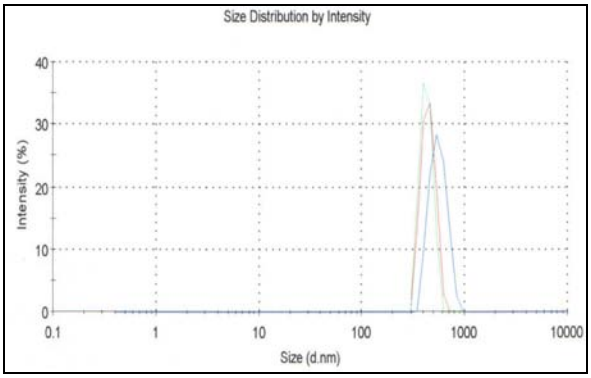
Liposome 2: Chol-T/DOPE/SH02

C



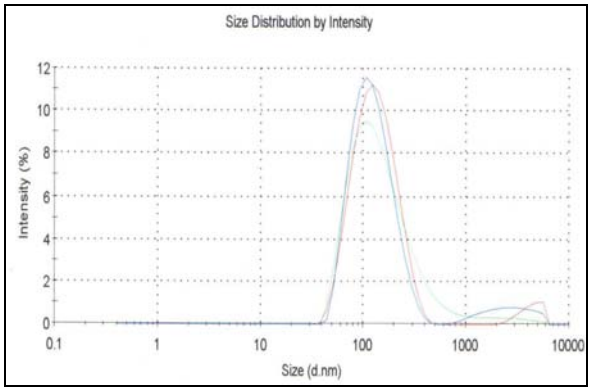
Liposome 3: Chol-T/DOPE/SH04

D



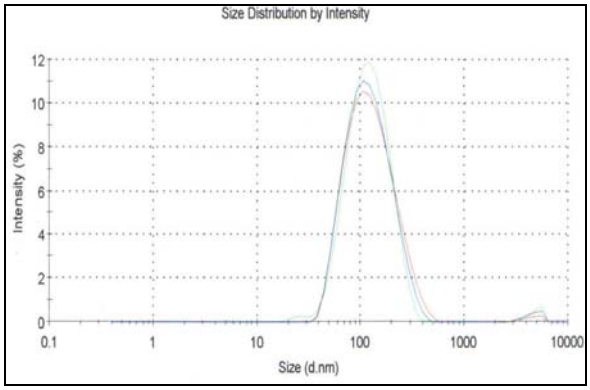
Liposome 4: Chol-T/DOPE/SH02/SH04

E



Liposome 5: Chol-T/DOPE/DSPE-PEG_{2000}

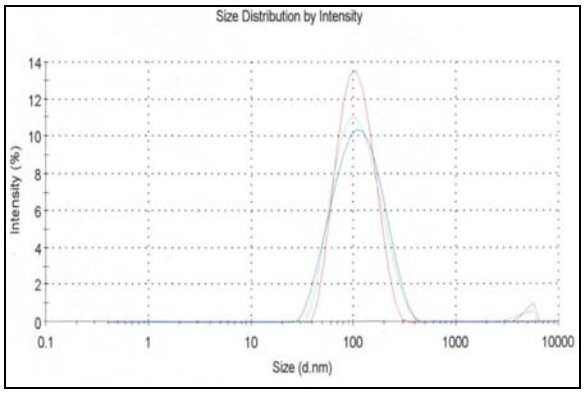
F



Liposome 6: Chol-T/DOPE/SH02/DSPE-PEG_{2000}

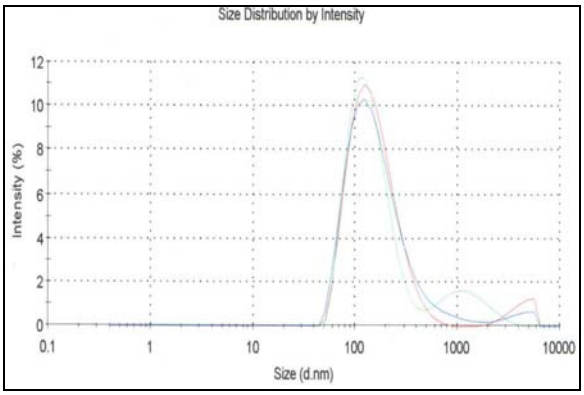
APPENDIX 6 (continued)

G



Liposome 7: Chol-T/DOPE/SH04/DSPE-PEG$_{2000}$

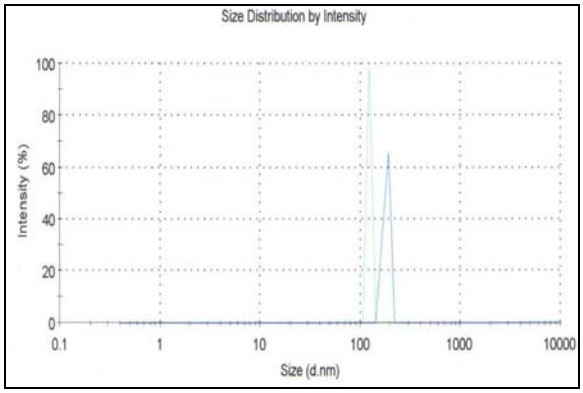
H



Liposome 8: Chol-T/DOPE/SH02/SH04/DSPE-PEG$_{2000}$

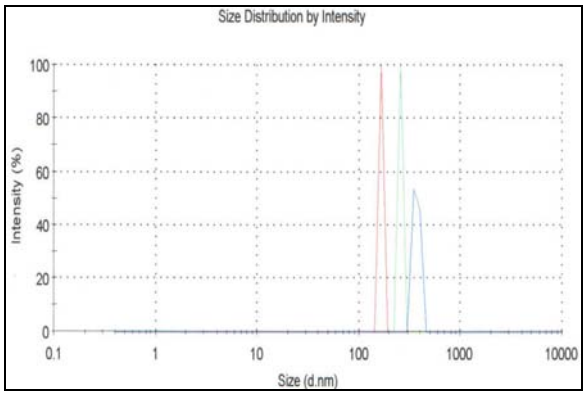
2. LIPOPLEXES

I



Chol-T:DNA (w/w) = 1.75:1

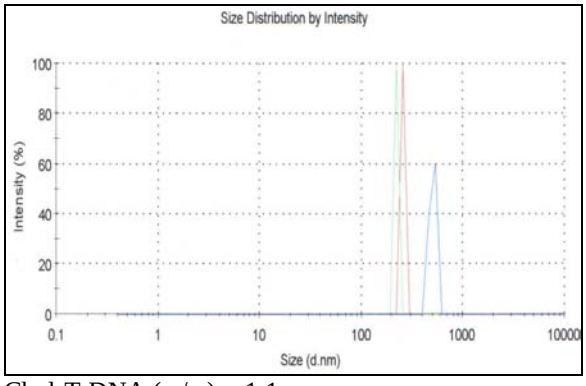
J



Chol-T:DNA (w/w) = 2.0:1

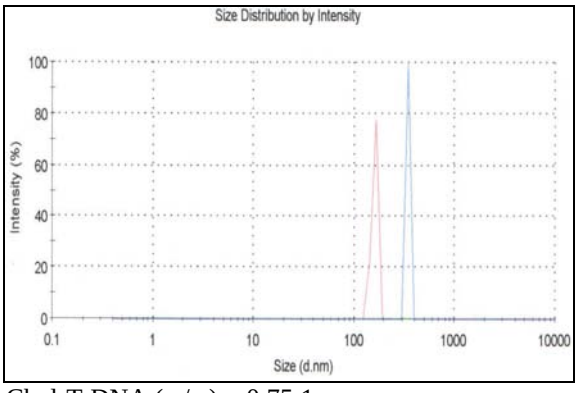
Liposome 1/DNA complexes

K



Chol-T:DNA (w/w) = 1:1

L

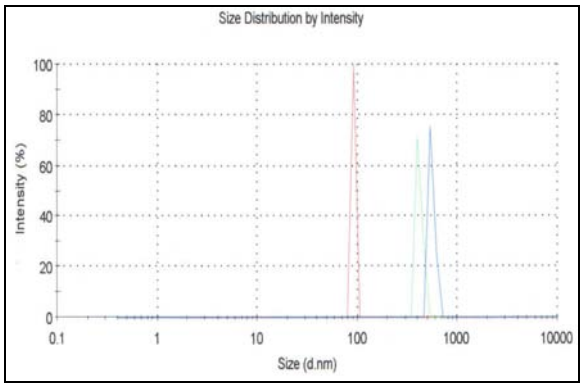


Chol-T:DNA (w/w) = 0.75:1

Liposome 2/DNA complexes

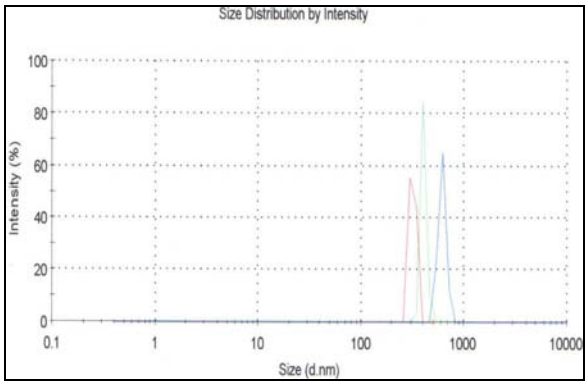
APPENDIX 6 (continued)

M



Chol-T:DNA (w/w) = 0.75:1

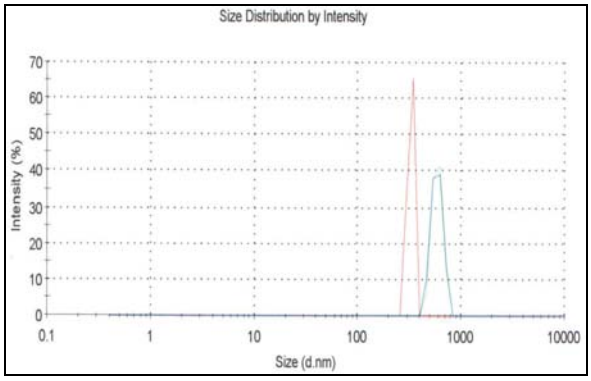
N



Chol-T:DNA (w/w) = 1:1

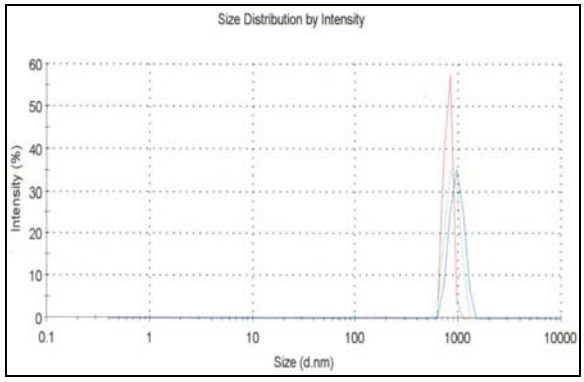
Liposome 3/DNA complexes

O



Chol-T:DNA (w/w) = 1.25:1

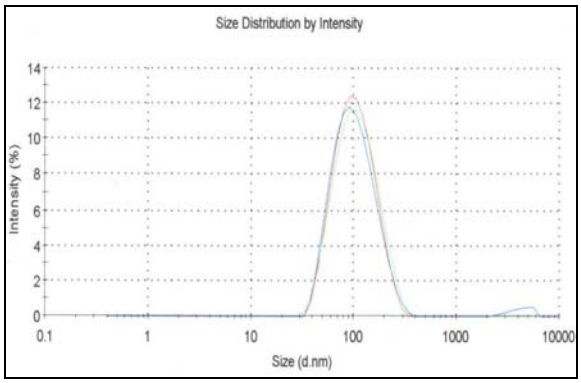
P



Chol-T:DNA (w/w) = 1.5:1

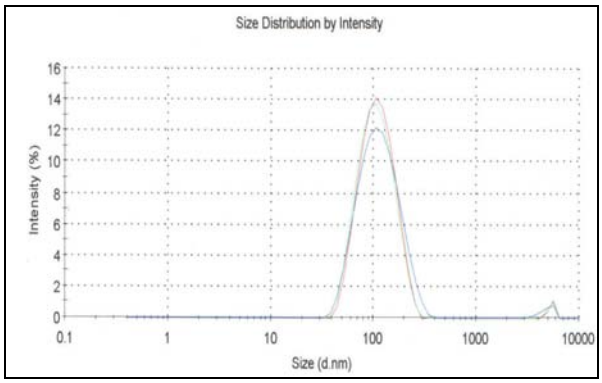
Liposome 4/DNA complexes

Q



Chol-T:DNA (w/w) = 3.5:1
Liposome 5/DNA complexes

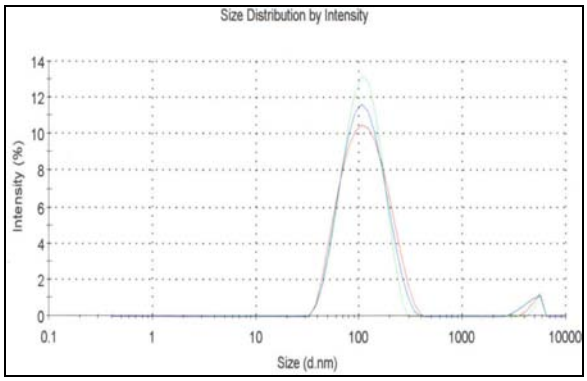
R



Chol-T:DNA (w/w) = 3.5:1
Liposome 6/DNA complexes

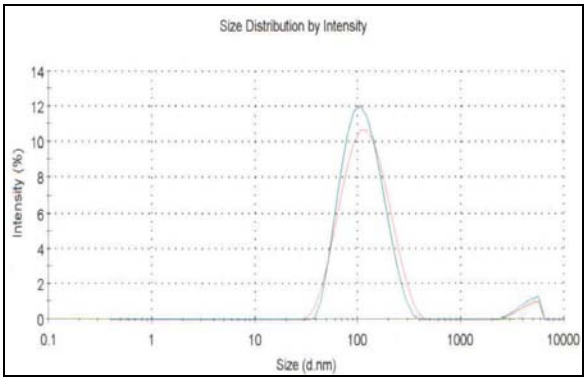
APPENDIX 6 (continued)

S



Chol-T:DNA (w/w) = 3.5:1

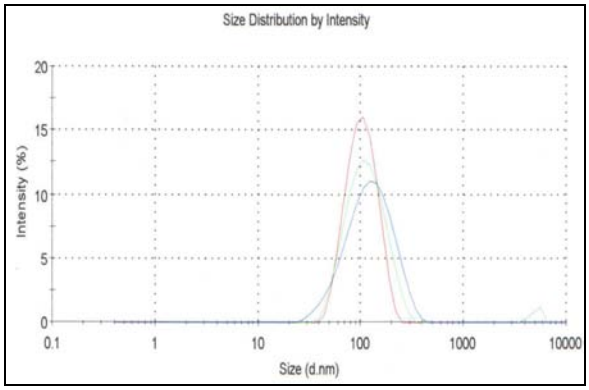
T



Chol-T:DNA (w/w) = 3.0:1

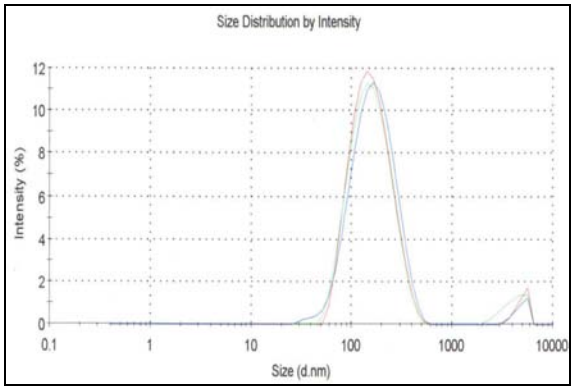
Liposome 7/DNA complexes

U



Chol-T:DNA (w/w) = 3.0:1

V



Chol-T:DNA (w/w) = 2.5:1

Liposome 8/DNA complexes

Note that each curve per size distribution profile represents a single measurement run by the Zetasizer.