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**The Comparative Effects of Acetylated and Deacetylated  
Galactose Derivatives in Liposomal Gene Delivery**

By

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Submitted in fulfilment of the academic requirements for the degree of  
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## Abstract

The use of cationic liposomes remains the most attractive non-viral approach in gene therapy as these gene carriers provide for ease and versatility in design. In targeted gene delivery, liposomes are coupled to ligands tailored to possess desired characteristics for improved cell-specificity. Carbohydrates have been established as useful targets for the asialoglycoprotein (ASGP) receptor in liver-directed delivery. The main purpose of this study was to comparatively evaluate physicochemical characteristics, DNA-binding interactions and *in vitro* transfection activities of hepatocyte-targeted liposomes bearing acetylated and deacetylated galactosides in ASGP receptor-mediated gene delivery. Furthermore, *in silico* studies were carried out to assess ligand-receptor interactions for both galactosides.

Novel targeted cationic liposomes conjugated with galactosyl ligands *viz.* cholest-5-en-3-yl 2-[4-( $\beta$ -D-galactopyranosyl-1-oxymethyl)-1*H*-1,2,3-triazol-1-yl]ethylcarbamate (Sc6) and cholest-5-en-3-yl 2-[4-(2,3,4,6-tetra-O-acetyl- $\beta$ -D-galactopyranosyl-1-oxymethyl)-1*H*-1,2,3-triazol-1-yl]ethylcarbamate (Sc9) were formulated with cytofectin 3 $\beta$ [N-(N',N'-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) and the neutral co-lipid dioleoylphosphatidyl ethanolamine (DOPE), using the thin film hydration–sonication method.

Characterisation of lipoplexes by cryo-transmission electron microscopy (cryo-TEM) showed unilamellar liposomes, and lipoplexes ranging between ~80 – 140 nm. DNA was fully liposome-bound at N:P ratios 2.5:1 – 3:1. Upon inclusion of polyethylene glycol 2000 -distearoylphosphatidyl ethanolamine (DSPE-PEG<sub>2000</sub>) in liposome formulations, vesicles were more compacted due to steric stabilisation. UnPEGylated lipoplexes achieved better condensation of DNA as determined in band shift and ethidium bromide displacement assays. Nuclease digestion assays revealed suitable protection of cargo DNA by some formulations, with the least protection afforded by the acetylated SM3 derivatives. Cytotoxicity studies in the HEK293 and HepG2 cell lines revealed good cell viabilities under transfection conditions for all liposomes. Transfection efficiency was assessed using the luciferase reporter gene assay. Higher transfection activities were observed in the ASGP receptor-positive HepG2 cell line than the ASGP receptor-negative HEK293 cells line for all lipoplexes. While the acetylated unPEGylated derivative (SM3)

demonstrated better transgene expression levels compared to other derivatives, this was not found to be significant. High transfection levels were attributed to favourable size and surface charge, as well as galactoside ligand accessibility to the receptor. In the presence of excess asialofetuin, a marked decrease in transfection efficiencies was observed for all targeted derivatives.

Docking scores further confirmed good binding affinity for the deacetylated Sc6 ligand and acetylated Sc9 ligand at -6.7 and -5.5 kCal/mol, respectively. The acetylated SM3 however, achieved avidity to the binding site through hydrogen bonding via the triazine linker. Overall transfection efficiency results were corroborated by outcomes from molecular studies as both galactoside ligand-conjugated liposomes presented similar binding affinities and transfection efficiency results. It is thus concluded that both these galactosides, with further optimization could present the potential for hepatocyte-specific delivery via ASGP receptor-mediated endocytosis.

## **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 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.

## **Declaration – Plagiarism**

I, **Seipati Rosemary Mokhosi**, declare that:

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## List of Abbreviations

AON	Antisense oligonucleotides
ASGP	Asialoglycoprotein
ATP	Adenosine triphosphate
B16 cells	Mouse melanoma cell line
BCA	Bicinchoninic acid
BGSC	Bis-guanidinium-spermidine-cholesterol
BSA	Bovine serum albumin
pDNA	Plasmid DNA
Chol	Cholesterol
Chol-T	3 $\beta$ [N-(N',N'-dimethylaminopropane)-carbamoyl] cholesterol
Chol-Q	3 $\beta$ [N-(N',N',N'-trimethylammonium propane)-carbamoyl] cholesterol iodide
Chol- $\beta$ -Gal	Cholesteryl- $\beta$ -D-galactopyranoside
Chol- $\beta$ -Glu	Cholesteryl- $\beta$ -D-glucopyranoside
CME	Clathrin-mediated endocytosis
CRD	Carbohydrate recognition domain
Cryo-TEM	Cryo-transmission electron microscopy
CvME	Caveolae-mediated endocytosis
DC-Chol	3 $\beta$ [N-(N'',N'-dimethylaminoethyl) carbamoyl) cholesterol)
DDAB	Dioctadecyldimethylammonium bromide
DLS	Dynamic light scattering
DM	Diabetes mellitus

DMRIE	1,2-dimyristyloxypropyl-3-dimethylhydroxyethylammonium bromide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DOGS	Diocetylamido-glycylspermine
DOPC	Dioleoylphosphatidylcholine
DOPE	Dioleoyl phosphatidylethanolamine
DOTAP	1,2-dioleoyloxy-3-[trimethylammonio]-propane
DOTMA	N-(1-(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride)
DOXIL	Doxorubivin Hcl liposome injection
DPPE	1,2-Bis(diphenylphosphino)ethane
DSPC	1,2-distearoyl- <i>sn</i> -glycero-3-phosphocholine
DSPE	Distearoylphosphatidylethanolamine
DSPE-PEG <sub>2000</sub>	1,2-distearoyl- <i>sn</i> -glycero-phosphoethanolamine- <i>N</i> - [carboxy(polyethylene glycol)2000]
DSTAP	1,2-distearoyl-3-[trimethylammonio]-propane
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMEM	Eagle's minimal essential medium
EPR	Enhanced permeation and retention
EtBr	Ethidium bromide
Fab	Fragment antigen binding
FBS	Foetal bovine serum

Fc	Fragment crystallizable
Gal	Galactose
Gal-C4-Chol	Cholesten-5-yloxy-N-(4-((1-imino-c- $\beta$ -D-thiogalactosylethyl)amino)butyl)formamide
GAlNAc	<i>N</i> -acetylgalactosamine
Glu195	Glutamate 195
Glu196	Glutamate 196
Glu277	Glutamate 277
Glu279	Glutamate 279
GUV	Giant unilamellar vesicles
H1299	Human non-small lung carcinoma cell line
HBS	Hepes-buffered saline
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HEK293	Human embryonic kidney 293 cell line
HeLa	Human cervical adenocarcinoma cell line
HEPES	2-[-(2-hydroxyethyl)-piperazinyl]-ethanesulphonic acid
HepG2	Human hepatocellular carcinoma cell line
HIV	Human immunodeficiency virus
LacNac	<i>N</i> -acetylactosamine
LDL	Low-density lipoprotein
LNC	Lipid nanocapsules

LUV	Large unilamellar vesicles
MAb	Monoclonal antibody
MEM	Minimum essential medium
miRNA	MicroRNA
MLV	Multilamellar vesicles
MPS	Mononuclear phagocyte system
mRNA	Messenger RNA
MTT	(3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide)
MW	Molecular weight
PAMAM	Poly(amido amine)
PBS	Phosphate buffered saline
PC	Phosphatidyl choline
PDI	Polydispersity index
pDNA	Plasmid deoxyribonucleic acid
PEG	Polyethylene glycol
PEI	Polyethylenimine
PLL	Poly(l-lysine)
RES	Retilo-endothelial system
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNase H	Ribonuclease H
RNAi	RNA interference
RLU	Relative light units



SAINT	<i>N</i> -methyl-4-(dioleyl)methylpyridinium
Sc6	Cholest-5-en-3-yl-2-[4-( $\beta$ -D-galactopyranosyl-1-oxymethyl)-1 <i>H</i> -1,2,3-triazol-1-yl]ethylcarbamate
Sc9	Cholest-5-en-3-yl-2-[4-(2,3,4,6-tetra-O-acetyl- $\beta$ -D-galacto-pyranosyl-1-oxymethyl)-1 <i>H</i> -1,2,3-triazol-1-yl]ethylcarbamate
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulphate
Ser163	Serine 163
Ser196	Serine 196
siRNA	Small interfering RNA
SUV	Small unilamellar vesicles
TEM	Transmission electron microscopy
Thr226	Threonine 226
TLC	Thin layer chromatography
T <sub>m</sub>	Transition temperature
UV	Ultraviolet
Val196	Valine 196
WHO	World Health Organisation
X-SCID	X-linked severe combined immunodeficiency

## CHAPTER ONE: INTRODUCTION

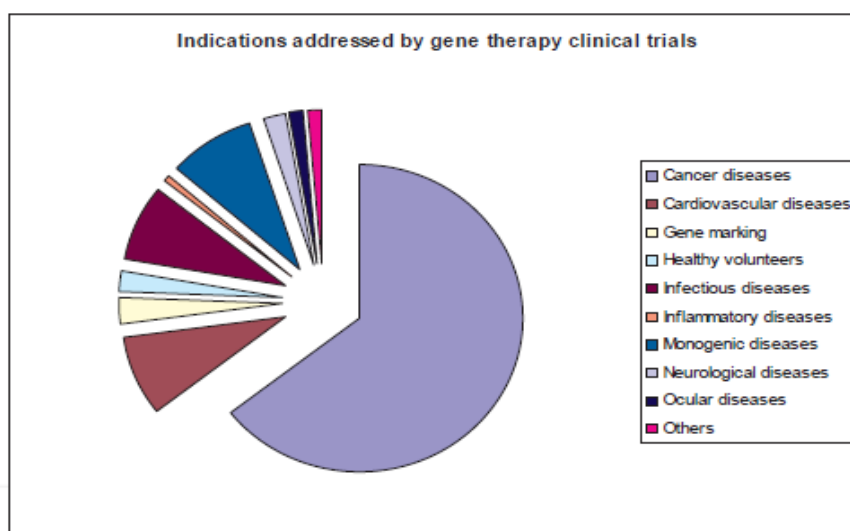
### 1.1 Gene therapy as an alternative therapeutic strategy

Despite great improvements since the 1950s, many of the global health challenges to-date remain unaddressed and often present major causatives of deaths worldwide. These are unique and multi-faceted challenges which range from lack of basic healthcare, lack of suitable methods of practice, and increasing economic pressures due to growing health demands. Limitations in conventional therapies such as surgery, radiation and chemotherapy (in the case of cancer), coupled with the emerging trend of multi-drug resistance in disease-causing pathogens and ineffective use of antibiotics have further exacerbated the problem (Websource:[www.globalissues.org](http://www.globalissues.org); Lee and Lee, 2012). According to the 2008 World Health Organisation (WHO) report, non-communicable diseases such as cardiovascular diseases, chronic lung diseases, diabetes and cancer result in over 36 million deaths worldwide each year. The majority of these deaths are found to occur in developing countries (Websource:[www.globalissues.org](http://www.globalissues.org)). One of the global responses in the past decades has been geared towards intensive research in new and alternative therapeutic strategies.

A promising approach was established in the past three decades, based on the novel concept of gene therapy. The idea of gene therapy originated in the 1960s; however its potential and feasibility as an alternative therapy in eradication of diseases was only realised in the 1990s (Abdallah *et al.*, 1995; Schmitz *et al.*, 2002; Lee and Lee, 2012). The underpinning principle entails delivery of genomic material into defective cells in order to correct or replace an abnormality, which in turn restores or presents the cells with a new functionality (Abdallah *et al.*, 1995; Martin *et al.*, 2003; Uddin, 2007; Lee and Lee, 2012). An effective and successful gene therapy requires that the therapeutic gene be delivered to a particular cell population efficiently. Thus many of the early gene therapy clinical trials reported utilised viral vectors (Li *et al.*, 2011; Lee and Lee, 2012, Gasc n *et al.*, 2013).

The initial focus was to restore diseases and disorders of a monogenic nature, but it has since expanded to include treatment and prevention of many other diseases. These include

acquired diseases such as AIDS, cardiovascular diseases as well as chronic diseases (Stephan *et al.*, 1996; Moghaddam *et al.*, 2011; Zhao *et al.*, 2012). It is important to note that more than 1400 clinical studies conducted for gene therapy, almost 70% have been in the area of cancer (Figure 1.1) (Choi *et al.*, 2001; El-Aneed, 2003; Uddin, 2007; Lee and Lee, 2012; Nayerossadat *et al.*, 2012).



**Figure 1.1** Statistical analysis of gene therapy trials and indications addressed (Gasc n *et al.*, 2013)

One of the earliest gene therapy clinical trials reported was conducted in 1989 using a retroviral vector. In 1990, severe combined immunodeficiency disorder (SCID) patients were treated using gene therapy (Lee and Lee, 2012; Nayerossadat *et al.*, 2012). However, when a patient enrolled in adenoviral-mediated gene transfer clinical trial for the human ornithine transcarbamylase cDNA died in 1999, it resulted in a major setback for gene therapy (Lee and Lee, 2012; Nayerossadat *et al.*, 2012). There was optimism when patients with X-linked severe combined immunodeficiency (X-SCID) were successfully **treated using retroviral gene vectors. Some of the patients; however, developed leukemia** due to viral integration, leading to activation of the LMO2 gene (Simões *et al.*, 2005; Karmali and Chaudhuri, 2007). While retroviral vectors are still the most commonly employed viral gene delivery vectors, low efficiency *in vivo*, immunogenic problems and their inability to transfect non-dividing cells has made them undesirable. Additionally, risk of insertion can lead to oncogene activation or inactivation of tumour-suppressor genes. Acute immunological responses have limited clinical application of many adenoviral vectors to a few tissues such as liver, and lung for cystic fibrosis treatment.

Severe side effects and fatalities have been reported in some cases (Simões *et al.*, 2015; Nayerossadat *et al.*, 2012; Lee and Lee, 2012). Various other viral delivery systems are currently being investigated and include those derived from the vaccinia virus, human cytomegalovirus and Epstein-Barr virus (Nayerossadat *et al.*, 2012). However, it remains an open question if the application of viral gene delivery systems will enjoy sustained popularity.

Regardless of past successes and failures in viral vectors, gene therapy still presents a powerful tool in a curative approach to diseases. This has inevitably presented an urgent expediency in development of non-viral delivery systems. It has also meant that researchers had to adopt a more responsive approach to the challenges encountered, for successful application of non-viral gene therapy. Perhaps the most important challenge in gene therapy lies with the actual delivery itself. This is dependent on two factors: choice of therapeutic gene to be introduced, and the delivery carrier to be used (Schmitz *et al.*, 2002; Simões *et al.*, 2005; De Laporte *et al.*, 2006; Pathak *et al.*, 2008; Bhattacharya and Bajaj, 2009; Elsabahy *et al.*, 2011; Li *et al.*, 2011; Lee and Lee, 2012; Nayerossadat *et al.*, 2012).

Traditionally, gene therapy has been nucleic acid-based in its approach where antisense and RNA interference have been reportedly used in cancer therapy. In use of antisense oligonucleotides (AONs), complementary hybridization of these 15-30 nucleotide fragments with the target messenger RNA results in its degradation by RNase H. This then results in direct blocking of the production of the disease-causing protein. RNAi mechanisms rely on the functionality of two small RNA molecules, which are microRNA (miRNA) and small interfering RNA (siRNA). Following delivery of these RNAi molecules into the cytoplasm, the target mRNA is incorporated into the RNA-induced silencing complex (RISC) in a sequence-specific manner, leading to cleavage of the target mRNA. In essence, both approaches are successful in that **there is translation inhibition** by downregulation or loss of functionality of the unwanted protein (Sonoke *et al.*, 2011; Shim *et al.*, 2013). Plasmid DNA (pDNA) has often been used in restoration of functionality of a defective protein by introducing the appropriate wild-type gene. While nucleic acid-based therapeutics present with real potential, limitations in application of these macromolecules *in vivo* include problems with selective delivery to target tissues, instability in serum and rapid clearance by the reticulo-endothelial system (RES) (Pathak

*et al.*, 2008; Podesta and Kostarelos, 2009; Elsabahy *et al.*, 2011; Maslov *et al.*, 2011; Sonoke *et al.*, 2011; Zhang *et al.*, 2012; Cheng *et al.*, 2013; Shim *et al.*, 2013).

Improvements in vector characteristics are particularly emphasised as they determine efficiency in delivery and expression, specificity to target cells and the immune response by the host. An ideal vector has to display minimal toxicity, protect the therapeutic cargo from degradation and evade opsonisation by the mononuclear phagocytic system (MPS) (Schmitz *et al.*, 2002; Gascon *et al.*, 2013). Additionally, it must show increased cell specificity, and result in prolonged and efficient transfection. In essence, the vector-cargo delivery system should be able to travel as safely as possible bypassing various extracellular barriers *en route* to the specific cell population as well as overcoming intracellular barriers for expression (Niidome and Huang, 2002; Schmitz *et al.*, 2002; Elsabahy *et al.*, 2011; Zhao *et al.*, 2012; Gascon *et al.*, 2013; Xiang and Zhang, 2013).

## 1.2 Gene delivery systems

Gene delivery systems are mainly categorised into: viral and non-viral systems (Safinya *et al.*, 2006; Uddin, 2007; Mintzer and Simanek, 2009; Nayerossadat *et al.*, 2012; Gascon *et al.*, 2013).

### 1.2.1 Viral gene delivery

Viral systems have over the years primarily made use of retroviruses, and adenoviruses. Clinical applications of adeno-associated viruses in gene therapy have also been reported. The key reason viral carriers were considered for gene transfer was due to their naturally infecting or transducing properties (Cristiano *et al.*, 1993; Karmali and Chaudhuri, 2006). Viral vectors thus gained research popularity due to higher transfection efficiency compared to non-viral delivery systems. However, the many disadvantages associated with their use have hindered their application *in vivo*. These include generation of immune responses to expressed viral proteins, integration of some viral vectors into the host chromosome as well as difficulties in engineering viral envelopes for targeted delivery to desired cells (Cristiano *et al.*, 1993; Aissaou *et al.*, 2004; El-Aneed, 2004; Nayerossadat *et al.*, 2012). Coupled to this, is the potential generation of replication

competent infectious viruses, the inability to administer certain viral vectors more than once, limitations in terms of cargo nucleic acid size and high costs in production of large amounts of high-titer viral stocks for use in clinic (Templeton, 2002; Wasungu and Hoekstra, 2006; Pathak *et al.*, 2008; Zhi *et al.*, 2010, Lee and Lee, 2012; Nayerossadat *et al.*, 2012).

While viral systems have dominated vector systems in the human clinical trials, there continues to be robust advancement in development and application of non-viral systems to provide for a safer alternative to viral delivery systems (Mintzer and Simanek, 2009; Nayerossadat *et al.*, 2012; Gasc n *et al.*, 2013; McCrudden and McCarthy, 2013).

### **1.2.2 Non-viral gene delivery**

The design of non-viral delivery systems requires an understanding of the various extracellular and intracellular challenges presented in their application *in vivo* (Pouton and Seymour, 2001; Khalil *et al.*, 2006). Most non-viral delivery systems have addressed the safety issue by making biocompatibility a key consideration. However, there are other equally important factors to consider. Perhaps the most obvious problem is that these carrier systems are in essence foreign to our bodies. Consequently, there is often rapid clearance of the introduced non-viral carrier system (Khalil *et al.*, 2006; Montier *et al.*, 2008). Thus there is a need to evade the immune response for minimal clearance by the system. Another fundamental issue relates to the biological, physical and chemical stability of the non-viral carrier and its cargo. The integrity of the carrier system should be maintained upon storage, as well as after administration, as this will translate into increased circulation time in the bloodstream (Pouton and Seymour, 2001; Yadav *et al.*, 2011; Laouini *et al.*, 2012). Another critically important aspect relates to intracellular delivery to the affected cells, tissues or organs, where adequate levels of expression of the delivered therapeutic genes are required (Pouton and Seymour, 2001; Templeton, 2002; Montier, *et al.*, 2008; Mintzer and Simanek, 2009; Nayerossadat *et al.*, 2012; Padeganeh *et al.*, 2012). Hence, the pursuit in many of the non-viral carrier designs is to address all the challenges mentioned.

While the task has remained daunting, several non-viral therapeutics has advanced to clinical trial stage. These include cationic lipid and polymer-mediated gene delivery

systems, which have been used to successfully target both genetic diseases and cancer (Table 1.1) (Liu and Song, 1998; Ropert, 1999; Mintzer and Simanek, 2009; Zhi *et al.*, 2010; Lee and Lee, 2012).

**Table 1.1** Gene therapy clinical trials conducted using non-viral carriers (adapted from Mintzer and Simanek, 2009)

<b>Disease</b>	<b>Major Carrier</b>	<b>Construct</b>	<b>Status</b>
<b>Melanoma</b>	DMRIE/DOPE	Allovectin-7	Phase III
<b>Melanoma and renal cell cancer</b>	DMRIE/DOPE	Allovectin-7	Phase II
<b>Solid tumours</b>	DOSPA/DOPE	Allovectin-7	Phase I
<b>Head and neck cancer</b>	DC-Chol/DOPE	tgDCC-E1A	Phase II
<b>Ovarian Cancer</b>	DC-Chol/DOPE	tgDCC-E1A	Phase I
<b>Glioblastoma multiform</b>	DC-Chol/DOPE	LIPO-HSV-1-tk	Phase II
<b>Cystic fibrosis</b>	DC-Chol/DOPE	LIP-HSV-1-tk	Phase I
	DOTAP		Phase I
<b>Cystic Fibrosis</b>	GL-67/DOPE	GL-67:DOPE-pCF1-CFTR	Phase I
<b>Bladder cancer</b>	<i>in vivo</i> jetPEI	BC-819	Phase IIb
<b>Ovarian Cancer</b>	PEG-PEI-Chol	EGEN-001	Phase I
<b>Cystic Fibrosis</b>	PEGylated 30mer PLL	DermaVir	Phase II
<b>HIV</b>	PEI mannose and dextrose	DermaVir	Phase II
<b>Solid tumours</b>	Cyclodextrin-based polymer	CALAA-01	Phase 1

Most literature characterises non-viral systems as either chemical or physical, based on the nature of their synthesis, and methods of introduction to the body (Table 1.2) (Kong *et al.*, 2012; Padeganeh *et al.*, 2012; Gascon *et al.*, 2013).

**Table 1.2** Non-viral delivery systems for gene therapy (adapted from Gascon *et al.*, 2013)

Category	System for gene delivery
<b>Physical methods</b>	Needle injection, ballistic DNA injection  Electroporation, Sonoporation,  Hydroporation
<b>Inorganic particle</b>	Calcium phosphate, Silica, Gold, Magnetic
<b>Synthetic or natural biodegradable particles</b>	Polymeric-based non-viral vectors  Cationic lipid-based non-viral vectors  Peptide-based non-viral vectors

Physical force can be applied in order to overcome the cell membrane barrier and facilitate gene transfer. Naked DNA can be directly injected into tissues of concern using a syringe. In particle bombardment, a microprojectile gene transfer or a gene gun is utilised and DNA-coated gold particles are propelled towards the cells at high pressure for final delivery into the nucleus. The latter method has been used in treatment of ovarian cancer and provides for precision in DNA doses administered (Niidome and Huang, 2002; Nayerossadat *et al.*, 2012; Lee and Lee, 2012). Naked DNA delivery can also be achieved by rendering the cell membrane porous and permeable thus allowing uptake of DNA. In electroporation, this is achieved by using electric pulses while in sonoporation, ultrasound is used. Efficiency in both methods is determined by the



intensity of the pulses, frequency and duration. Sonoporation provides a non-invasive and site-specific choice. Electroporation has been successfully implemented in cancer therapy (Lee and Lee, 2012). Hydrodynamic gene delivery has also been frequently used for gene delivery to the liver in rodents. This is carried out by rapid injection of large volumes of DNA solution via the tail vein. Due to fenestrae enlargement, plasma membrane defect associated with this kind of administration, requires the injection volume to be reduced in order to make it more applicable in humans. The most important limitation in clinical application of naked DNA is its susceptibility to nuclease degradation (Niidome and Huang, 2002; Mintzer and Simanek, 2009; Nayerossadat *et al.*, 2012; Gasc n *et al.*, 2013).

Recent use of inorganic particles has sparked huge interest in their application for gene therapy. This is attributed to their nanosize structure and ease in preparation. For instance, surface-coated silica nanoparticles present excellent characteristics such as biocompatibility, colloidal stability, reduced cytotoxicity and they have resulted in good transfection efficiency (Gasc n *et al.*, 2013). Recently, gold nanoparticles have been investigated for gene therapy. The ability to modify the surface of these gold nanoparticles for enhanced efficiency presents yet another advantage. However, the research challenge in the past three decades for all non-viral gene vector designers has been in matching transfection efficiencies to those displayed by their viral counterparts (Mintzer and Simanek, 2009; Gasc n *et al.*, 2013).

Relevant to this thesis, only the polymeric-based and cationic lipid-based non-viral vectors are discussed more extensively.

### ***1.2.2.1 Liposome-mediated gene delivery***

Liposomes are small spherical vesicles which are created from cholesterol and natural non-toxic phospholipids. They were first described by Alec Bangham and colleagues in the 1960s who noted spontaneous multilamellar vesicular arrangement of phospholipids in aqueous solution (Bangham *et al.*, 1965; Dua *et al.*, 2012; Laouini *et al.*, 2012; Akbazerdah *et al.*, 2013). These arrangements arise through repulsive forces which sort the amphiphilic molecules in such a way that the hydrophobic acyl-chains interact minimally with the surrounding aqueous medium. This is further driven by various

intermolecular forces such as electrostatic interactions, hydrogen bonding and van der Waals bonds. This results in a central aqueous and hydrophilic compartment, which is surrounded by one or more phospholipid layers (Montier *et al.*, 2008; Kulkarni *et al.*, 2011; Dua *et al.*, 2012; Laouini *et al.*, 2012; Allen and Cullis, 2013, Perche and Torchillin, 2013).

The potential of liposome-mediated gene transfer was elucidated by the 1970s, with several publications reporting liposomal carrier systems delivering exogenous genetic material, RNA and DNA into host cells (Ropert, 1999; Torchillin, 2005). This is particularly critical with the hydrophilic and anionic nature of nucleic acids, and therefore the unique ability of liposomes to bind the nucleic acids is a crucial benefit in gene delivery. This, and other considerations which are discussed in later sections, has meant that development of liposomes for delivery of therapeutic molecules continues to be of growing research interest (Ropert, 1999; Torchillin, 2005; Mintzer and Simanek, 2009; Kulkarni *et al.*, 2010). Early pioneering work proposed that these inherent properties of liposomes make them unique and versatile delivery vehicles for small therapeutic molecules. This is attributed to their biocompatibility, biodegradability, the protection conferred to their therapeutic cargo as well as the flexibility to trap both hydrophilic molecules in the central aqueous core, as well as hydrophobic molecules within the bilayer (Torchillin, 2005; Akbarzadeh *et al.*, 2013).

Structural parameters such as vesicle size and number of phospholipid bilayers are used to classify liposomes into: 1) unilamellar and 2) multilamellar vesicles. Further to this, there are small unilamellar vesicles (SUV), or large unilamellar vesicles (LUV), while the phospholipid charge makes them neutral, anionic or cationic (Dua *et al.*, 2012; Akbarzadeh *et al.*, 2013; Allen and Cullis, 2013; Patil and Jadhav, 2014). Over time, an important classification was developed which takes into account composition and application of the liposomes. Thus to-date most literature will cite conventional, stealth or long-circulating, ligand-targeted, cationic and immuno-liposomes (Torchillin, 2005; Patil and Yadhav, 2014). This type of classification also provides an overview of the development and evolution of liposomes over time.

#### 1.2.2.1.1 Conventional liposomes

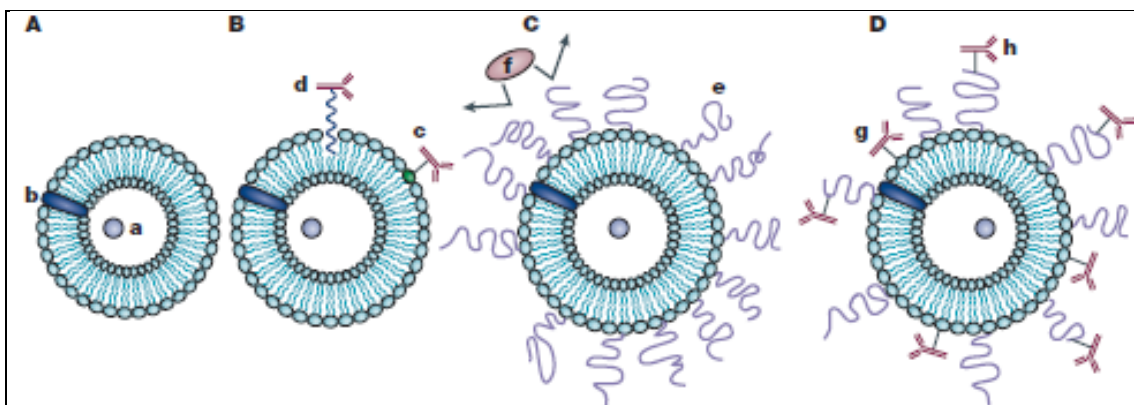
From early studies, some of the major problems identified with liposomal delivery systems included successful retention of the therapeutic molecules, rapid clearance by the MPS, and successful intracellular delivery of the therapeutic molecules. Initial attempts to tackle these issues were based on modifying the physicochemical properties of these conventional or „classical“ liposomes to impact on their behaviour and fate *in vivo* (Allen and Cullis, 2013). For example, by changing the lipid membrane components, the bilayer fluidity may be influenced. Incorporating cholesterol was found to „tighten“ or increase the packing of phospholipids in the lipid bilayer which would result in fluid phase switch to solid phase bilayer. Early studies have demonstrated that using liposomes made from saturated phosphatidylcholine (PC) or sphingomyelin presented more stability in the blood than liposomes prepared from PC with unsaturated fatty acyl chains. Overall, this resulted in better stability and reduced leakage of the entrapped molecules (Immordino *et al.*, 2006; Allen and Cullis, 2013).

Another fundamental challenge in use of conventional liposomes is related to their rapid uptake and clearance by the MPS (Allen and Cullis, 2013). This not only results in reduced distribution in other tissues of the body, but toxic accumulation in some of the MPS organs. By modulating liposome size and charge, it has been observed that this influences their uptake by the MPS, particularly in the liver and spleen. Larger liposomes were found to have a shorter blood circulation time than smaller ones. These findings suggested that opsonisation by the phagocytes depends on the size of the liposomes, such that SUVs had a longer half-life in blood than the LUVs. Positively charged liposomes have also been found to be more toxic, and were observed to be removed quickly from the system. Equally, negatively charged liposomes were reported to activate the complement system via a different pathway for clearance (Immordino *et al.*, 2006). While conventional liposomes provided a benchmark, their inherent inability to address some of these critical issues sustainably led to more research advancements in liposomal development.

### 1.2.2.1.2 Long-circulating liposomes

An important advancement in liposome-mediated gene delivery to deal with the issue of longevity, and stability was the development of stealth technology. Long-circulating liposomes otherwise known as „stealth“ liposomes, represents one of the important cornerstones in the success of liposomal gene delivery (Torchillin, 2005; Allen and Cullis, 2013).

The necessity to evade the MPS is paramount for efficient delivery. By safely creating a physical barrier on the liposomal surface, it was envisioned that there would be prevention of access and binding of blood plasma opsonins. This would inevitably inhibit interactions of MPS macrophages with such liposomes. Use of hydrophilic polymers was found to increase the hydrophilicity of the liposomal surface. This in turn leads to steric stabilization and increases their longevity in circulation (Allen and Cullis, 2013). This concept has been extensively studied and was based on previous work reported by Abuchowski and colleagues (1977) where PEG was attached to proteins leading to increased circulation half-life (Abuchowski *et al.*, 1977). The underlying principle functions on the assumption that the polymer's flexible chain which occupies the immediate space of the liposomal surface results in exclusion of other macromolecules from this space. In fact long circulating liposomes have been found to possess increased bioavailability (Figure 1.2 C) (Torchillin, 2005; Podesta and Kostarelos, 2009; Zhang *et al.*, 2012).

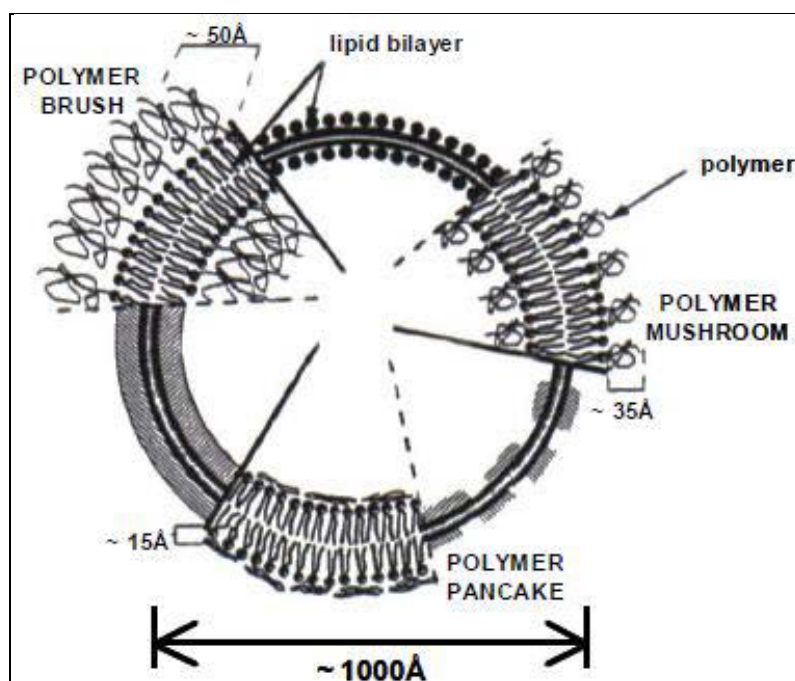


**Figure 1.2** Developmental milestones in design of liposomes: A. Early conventional liposomes with: a) entrapped therapeutic molecule in aqueous core, b) entrapped therapeutic molecule in the bilayer. B. Targeted immunoliposomes with antibody that is: c) covalently coupled to phospholipid bilayer and d) modified with hydrophobic moiety and anchored onto liposomal membrane. C. Long-circulating liposomes incorporating e)

hydrophilic polymer such as PEG, and f) long-circulating immunoliposomes comprising both protective polymer PEG and antibody attached to liposomal surface. D. Long-circulating liposomes with targeting moiety grafted onto g) liposomal surface or h) attached to the distal end of protective polymeric chain (adapted from Torchillin, 2005)

Early attempts to increase longevity in circulation were achieved by mimicking the erythrocyte membrane by modifying the surface with gangliosides and sialic acid derivatives, such as GM1. GM1 was used in combination with cholesterol to construct the first long circulating liposomes. Subsequent use of polyethylene glycol (PEG) followed and gained much popularity. PEG is a linear polyether diol comprising the monomeric repeat unit [-CH<sub>2</sub>-CH<sub>2</sub>-O]-, commonly trusted as polymeric steric stabilizer due to its low immunogenicity and toxicity (Podesta and Kostarelos, 2009; Allen and Cullis, 2013). Surface modification of liposomes with PEG can be achieved in several ways: by physically adsorbing the polymer onto the surface of the vesicles, by incorporating the PEG-lipid conjugate during liposome preparation, or by covalently attaching reactive groups onto the surface of preformed liposomes. Particles with size diameter less than 100 nm allow for easy modification with PEG (Torchillin, 2005; Yadav et al., 2011; Kong et al., 2012).

Depending on the molecular weight and grafting density, various configurations have been proposed which include the mushroom, brush and pancake regimes. These in turn affect surface coverage by the polymer and distance between graft sites. A brush-like PEG conformation is characteristic of high PEG grafting, while mushroom conformation is observed at low grafting density. The brush conformation has been reported to result in more efficient reduction of serum interactions (Figure 1.3) (Immordino et al., 2006; Zhang et al., 2012; Nag and Awasthi, 2013; Perche and Torchillin, 2013).



**Figure 1.3** Schematic representation of different conformational regimes assumed by PEGpolymer grafted onto liposomal surface (Immordino *et al.*, 2006)

Apart from the simplicity with which it can be conjugated, PEG is more soluble in aqueous and organic media and hence there is little impact on mechanism of action of a therapeutic molecule. The most widely used method at present is to anchor the polymer in the liposomal membrane via a cross-linked lipid such as distearoylphosphatidylethanolamine (Immordino *et al.*, 2006; Yadav *et al.*, 2011). Additionally, incorporation of PEG into the lipid bilayer offers a platform on which to add targeting moieties. Monoclonal antibodies, Fab fragments and peptides have all been successfully used for active targeting of long-circulating liposomes. Use of PEG as a linker for specific ligands has also been explored and found to lead to longevity and effective targeting (Martin *et al.*, 2003; Podesta and Kostarelos, 2009). The most significant properties of PEGylated vesicles are their strongly reduced MPS uptake and prolonged blood circulation. This means improved distribution in perfused tissues. Moreover, the PEG chains on the liposome surface prevent vesicle aggregation, improving stability of formulations (Immordino *et al.*, 2006; Zhang *et al.*, 2012).

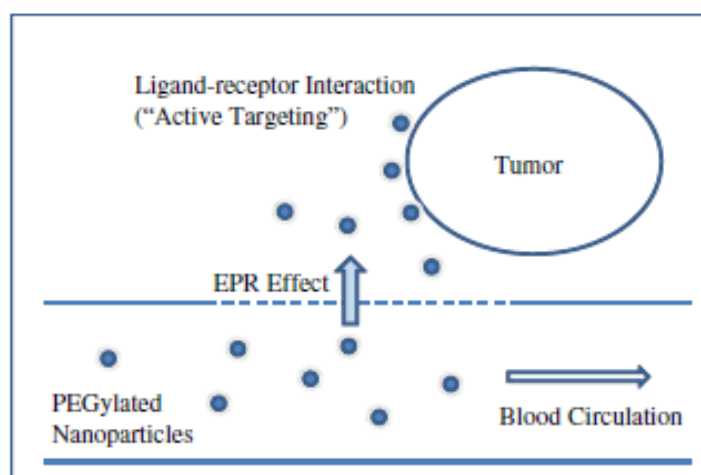
New improvements in PEG liposomes include detachable PEG conjugates constructed by mild thiolysis of the dithiobenzylurethane linkage between PEG and an amino-containing

substrate. Currently, there are some PEG liposomal formulations that are approved for clinical use for Kaposi sarcoma and ovarian cancer (Torchillin, 2005; Garg and Kokkoli, 2011; Levine *et al.*, 2013). The superiority of PEG-coated liposomes has revolutionised liposomal drug and gene delivery systems. In fact, incorporation of PEG in liposomal formulations has become the gold standard for many biomedical applications, and there is continued research interest in use of PEG liposomes (Torchillin, 2005; Kong *et al.*, 2012; Zhang *et al.*, 2012).

#### 1.2.2.1.3 Targeted liposomes

One of the crucial requirements to a successful and efficient gene therapy is delivery of therapeutic molecules to the target cells, i.e. cell-specific delivery. For this approach to be fully exploited, it is imperative to understand the significant steps involved in the gene transfer process and how to best overcome the challenges presented (Mintzer and Simanek, 2009; Gascon *et al.*, 2013). Essentially, the encapsulated therapeutic molecule has to be presented to the cell surface. At this step, targeting moieties can be coupled to the liposomal system for recognition by the target cell types. After binding to the cell membrane for the target cell-specific internalisation, the therapeutic molecules are directed towards the nuclear region for expression (Smith *et al.*, 1997; Mintzer and Simanek, 2009).

Long-circulating liposomes have also been able to achieve an indirect way to target certain tissues and organs. „Passive targeting“ is a phenomenon which results from the reduced uptake by MPS following PEG incorporation, thus allowing passive accumulation inside other tissues or organs (Figure 1.4) (Bee and Park, 2011; Zhang *et al.*, 2012).



**Figure 1.4** Targeted delivery of nanoparticles to tumours. Active targeting can be achieved by ligand-receptor interactions and passive targeting of PEGylated carriers by means of EPR effect (Bae and Park, 2011)

The disrupted and discontinuous endothelial lining in the tumor vasculature during angiogenesis facilitates extravasations of liposomal formulations into the interstitial space. There is preferential accumulation of liposomes in the tumour area due to the lack of efficient lymphatic drainage of the tumour. In a process known as the enhanced permeation and retention effect or EPR, there is improved therapy for the cancerous organ as there is slow release of the therapeutic over time. It must be remembered that liposomal formulations do not extravasate from the bloodstream into normal tissues that have tight junctions between capillary endothelial cells (Torchillin, 2005; Immordimo *et al.*, 2006; Bae and Park, 2011; Zhang *et al.*, 2012).

Wide use of targeted liposomes has become validated over the years and targeting moieties commonly include peptides, carbohydrates, growth factors, monoclonal antibodies (MAb) and receptor ligands. These are commonly attached to the vector, and some of these ligands have led to enhanced delivery efficiency (Gust and Zenke, 2002; Buñuales *et al.*, 2011; Fumoto *et al.*, 2013; Perche and Torchillin, 2013). Endocytosis has been proposed as the main route for uptake of most non-viral systems including DNA-liposome complexes, and not the direct fusion with the plasma membrane. Once bound, the complex is internalised into endosomes followed by release and escape into the cytoplasm. However, events leading up to the separation of the DNA-liposome complex for subsequent expression remain elusive (Immordimo *et al.*, 2006; Mintzer and Simanek, 2009).



The most significant advantage for targeting is the dramatic increase in therapeutic molecule delivered to the target. **Increasing** the number of ligand molecules exposed on the liposome surface, can also result in improved ligand avidity and uptake (Perche and Torchillin, 2013). In below subsections, two of the most commonly employed targeted liposomes are discussed viz. immunoliposomes and ligand-conjugated liposomes.

#### *a) Immunoliposomes*

In the last few years, there has been increased interest in antibody-based therapeutics, commonly known as immunoliposomes. These make use of immune-system components namely, fragment antigen binding (Fab) which is responsible for antigen recognition, and fragment crystallizable (Fc) which play a role in biological activity. Immunoliposome application has been explored for the treatment of cancer since the early 1980s. This is because of their ease in preparation, as well as high specificity. Immunoliposomes also presents a “bystander killing” effect, meaning that they can diffuse into neighbouring tumour cells. Because of their intrinsically short half-life, the stealth technique is commonly employed with immunoliposomes in order to increase their circulation time (El-Aneed, 2003; Martin *et al.*, 2003; Immordino *et al.*, 2006; Perche and Torchillin, 2013).

Various studies have shown improved transfection efficiency in tumours by use of antibody-attached long-circulating liposomes in tumours. In a study on rats with aggressive brain tumours, it was observed that their life-spans increased by 100% following administration of the epidermal growth factor (EGF) receptor antisense mRNA by immunoliposomes. While immunogenicity remains a concerning variable, it was found that use of Fab subunits instead of whole antibodies reduced risk. Post-insertion of a monoclonal antibody has been applied to commercially available doxorubicin-loaded liposomes (DOXIL) (El-Aneed, 2003; Immordino *et al.*, 2006; Perche and Torchillin, 2013).

#### *b) Ligand-conjugated liposomes*

Targeting has become an absolute essential in many gene delivery system designs. Apart from the most obvious benefit of cell-specificity, El-Aneed *et al.* (2003) also reported ligand-conjugated liposomes to be less immunogenic than immunoliposomes. This

approach entails incorporation of targeting ligands to bind to cell-surface receptors endogenously expressed by specific cells (Dan, 2002; Shim *et al.*, 2013).

Liver-directed gene delivery may be achieved by targeting the ASGP receptor (ASGP-R), which is exclusively expressed on liver parenchymal cells or hepatocytes (Kawakami, 1998; Singh *et al.*, 2001; Nishikawa *et al.*, 2003; Kawakami *et al.*, 2007; Fan and Wu, 2013). The incorporation of natural ligands for this receptor to the vector can lead to selective and specific recognition by the ASGP receptor. Studies have shown increased delivery to hepatocytes following asialofetuin-conjugated nanoparticles versus the non-targeted nanoparticles. Transferrin is another targeting ligand useful in liver cancer. Transferrin is overexpressed in these hepatocytes and PEI conjugated with transferrin was found to lead to enhanced transfection *in vivo* (Kawakami, 1998; Gust and Zenke, 2002; Arangoa *et al.*, 2003; Nishikawa *et al.*, 2003; McCrudden and McCarthy, 2013).

The epidermal growth factor receptor (EGFR) is a receptor found to be upregulated in a number of solid tumours of the breast, prostate and ovaries. Findings by Buñuales *et al.* (2011) demonstrated that EGF-lipoplexes prepared in their study had resulted in nanoparticles which were able to transfect different cancer cell lines more efficiently compared to a non-targeted system. By acquiring information about a certain receptor, and incorporating its targeting ligand into cationic non-viral systems, enhanced tumour-specific accumulation of the therapeutic nucleic acid is possible (Schaffer and Lauffenburger, 2002; Buñuales *et al.*, 2011; McCrudden and McCarthy, 2013).

### **1.2.2.2 Polymer-mediated gene delivery**

Cationic polymer vectors are synthetic gene delivery vectors are being extensively explored due to their versatile nature, as well as ease in design and manufacturing. Commonly used cationic polymers include poly(l-lysine) (PLL), polyethylenimine (PEI), chitosan and polyamidoamine dendrimers (PAMAM) (De Laporte *et al.*, 2006; McCrudden and McCarthy, 2013). The molecular weight of some of these synthetic compounds can be modified and also allow for ligand conjugation for improved delivery (El-Aneed, 2004; Mintzer and Simanek, 2009; Tros de Ilarduya *et al.*, 2010).

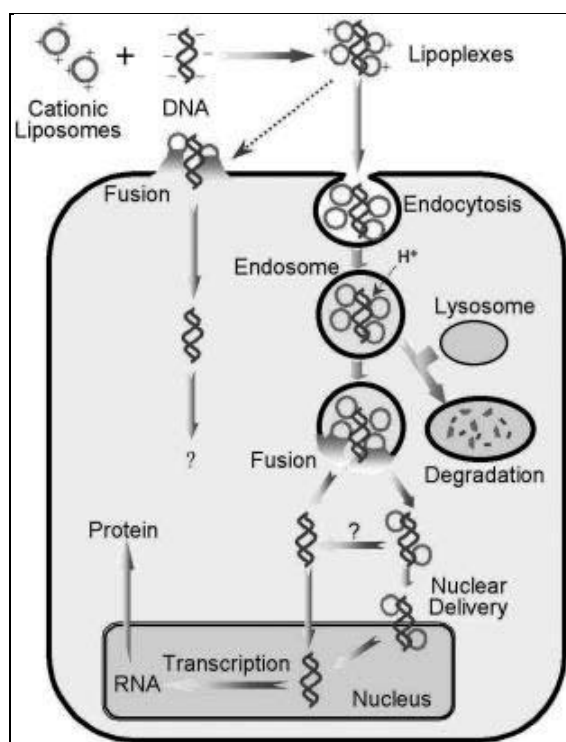
Polymers condense DNA into small particles (polyplexes) which are even smaller than cationic lipopolyplexes. This has been a major contributing factor to their wide use as delivery vectors, as smaller sizes are favoured for efficient transfection, particularly *in vivo*. In fact, polyplex formulations using cationic polymers have possibly been able to compete with viral vectors (Mintzer and Simanek, 2009; Nayerossadat *et al.*, 2011). PLL represent the first generation cationic polymers and their biodegradable nature is an added advantage to their *in vivo* applications. However, PLL polyplexes are rapidly cleared from circulation and are found to be mostly inefficient in transfection due to their inability to destabilise the endosomal compartments. Incorporation of PEG has led to increased circulation time, while targeting has enhanced cell-specificity. Additionally, co-transfection with endosome-lytic agents that create the proton sponge effect has resulted in improved transfection activity (El-Aneed, 2004; Mintzer and Simanek, 2009; Nayerossadat *et al.*, 2011; Zhang *et al.*, 2012).

PEI and PAMAM represent the second generation cationic polymers and are an improvement from first generation PLL polymers. This is attributed to their buffering capacity of the endosomal compartment. This induces osmotic swelling and rupture, also known as proton sponge effect – a widely accepted hypothesis over the years. Characteristics such as molecular weight, composition of complexes, size and charge density/zeta potential influence interactions of PEI and DNA, cytotoxicity and efficiency profile of PEI polyplexes are important points of consideration. Increase in length and branching results in better condensation of DNA and increased protection against degradation by nucleases. This in turn leads to improved uptake. Studies have shown that high molecular weight PEI (25kDa) was more efficient in DNA condensation, with polyplex sizes that range between 20 – 40 nm (El-Aneed, 2004; Mintzer and Simanek, 2009; Tros de Ilarduya *et al.*, 2010; Fant *et al.*, 2010; Zhang *et al.*, 2012). The highly branched PEIs condense DNA to a greater degree and achieve higher transfection efficiency. Low molecular weight and moderately branched PEI was found to exhibit low toxicity and fairly efficient delivery compared to high MW PEI. PEG conjugation which is employed for steric stability to PEI, and targeting have resulted in PEI polyplexes displaying reduced toxicity and improved transfection efficiency. PEI is possibly the most prominent polymer used for gene delivery both *in vitro* and *in vivo* (El-Aneed, 2004; Mintzer and Simanek, 2009; Tros de Ilarduya *et al.*, 2010; Zhang *et al.*, 2012; Gasc n *et al.*, 2013).

### 1.3 Cationic liposomes for gene delivery

In 1987, Felgner and colleagues reported use of the first cationic lipid-based liposomes in delivery of nucleic acids. To-date cationic liposomes represent the most well-characterised non-viral candidates in gene therapy (Felgner *et al.*, 1987; Pisani *et al.*, 2011; Zhao *et al.*, 2014). The most important difference between cationic lipids and natural phospholipids relates to charge. The natural phospholipids are either neutral (zwitterionic) or negatively charged. Thus cationic lipids, by virtue of their positive surface charge, will effortlessly and spontaneously condense the negatively charged phosphate backbones of a nucleic acid (such as DNA) to give rise to lipoplexes. Lipoplexes are highly structured self-assembling nanostructures which provide 100% loading efficiency for the DNA, and also offer protection for their cargo from degradation by nucleases. Other associated advantages include biocompatibility, biodegradability, ease with which they can be designed and manipulated; and their ability to transport large pieces of DNA (Gonçalves *et al.*, 2004; Khalil *et al.*, 2006; Zuhorn *et al.*, 2007; Tros de Ilarduya *et al.*, 2010).

The model determining formation of lipoplex formation has been studied extensively using atomic force microscopy and cryo-electron microscopy (Ma *et al.*, 2007; Wang *et al.*, 2012). The two-step mechanism is initiated when the positively charged amine headgroups bind via electrostatic interactions to phosphate backbones of DNA. During this step, there is collapse of the DNA structure resulting in condensation or compaction where DNA is shielded by the lipids. The second step involves lipid-mixing interactions which lead to fusion and rearrangements of the liposomes. Further lipid mixing results in cationic lipids which are wrapped around the DNA plasmids. Cationic lipids confer the positive charge of lipoplexes to the negatively charged cell membrane by non-specific electrostatic interactions. Endocytosis, as opposed to fusion has been established to be the main route of internalisation for non-viral gene delivery (Figure 1.5) (Khalil *et al.*, 2006; Ma *et al.*, 2007; Wasungu and Hoekstra, 2010).



**Figure 1.5** Fate of cationic liposome-DNA complexes in cells **viz.** initial internalisation, followed by uptake, intracellular trafficking and nuclear delivery (Khalil *et al.*, 2006)

It entails uptake of macromolecules and particles via invagination processes for internalisation into the cytoplasm for nuclear expression. It is believed that endocytosis leads to better transport towards the nucleus, thus avoiding to some degree cytosolic nucleases (Zuhorn *et al.*, 2007). A number of endocytic pathways are involved in lipoplex internalization and include phagocytosis, macropinocytosis, clathrin-mediated and non-clathrin-mediated endocytosis. Receptor-mediated endocytosis often employs the use of ligands which bind to cell surface receptors which are concentrated for internalisation (Khalil *et al.*, 2006; Zuhorn *et al.*, 2007).

The polymorphic properties of cationic liposomes are particularly highlighted in one of the most important rate-limiting steps where DNA is released from compartments such as endosomes into the cytoplasm for transport into the nucleus. As the pH of the endosome drops from pH 7 to 5.5, some DNA escapes from the early endosome into the cytoplasm. **Some of the lipoplexes also escape and dissociate to release DNA (Balasz and Godbey, 2011).** Several mechanisms have been postulated for the release of DNA from the endosome, with the most prominent being facilitated by the intrinsic membrane destabilisation activity of cationic lipids. Electrostatic interactions between the cationic

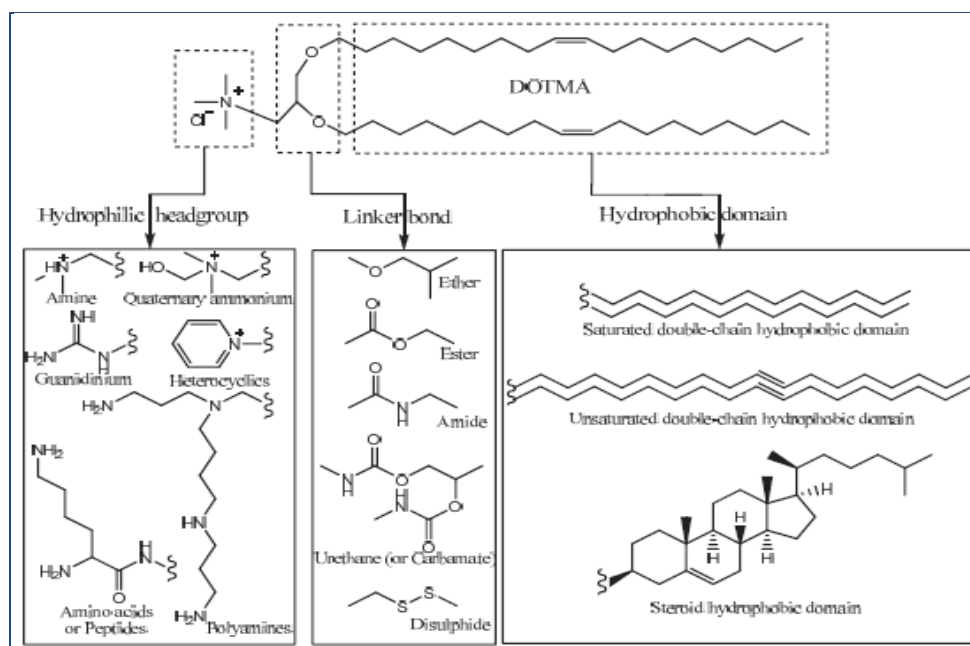
lipid and the endosome membrane are thought to induce the “flip-flop” movement of anionic lipids from the cytoplasm-facing monolayer of the endosome membrane. This will lead to the formation of a neutral ion pair with the cationic lipid and subsequent dissociation of the DNA and its release into the cytosol. Incorporation of neutral lipids such as DOPE and cholesterol has been confirmed to lead to improved transfection efficiency (Zuhorn *et al.*, 2007; Balasz and Godbey, 2011). In this mechanism, there is a bilayer “flip-flop” of the negatively charged phospholipids from the cytoplasmic leaflet to the luminal leaflet of the endosome which subsequently results in neutral ion pairs being formed. The weakening electrostatic interactions between the liposome and DNA, ultimately lead to separation and release of the DNA from the complex. Another mechanism that has been proposed is the proton sponge hypothesis. This is achieved by endosome buffering which results in osmotic swelling and endosome disruption. The released DNA is believed to then enter the nucleus for expression (Aissaoui *et al.*, 2002; Karmali and Chaudhuri, 2007; Zuhorn *et al.*, 2007; Montier *et al.*, 2008; Bhattacharya and Bajaj, 2009; Tros de Ilarduya *et al.*, 2010; Zhao *et al.*, 2013).

The successful delivery and expression efficiency of cationic liposomes also requires full understanding of the structure of the cationic lipids and all the governing factors that impact on the transfection mechanism. This takes into account the chemical basis of cationic lipids, and the resultant complexes formed with nucleic acids such as DNA (Karmali and Chaudhuri, 2007). Clinical use of cationic liposomes has been limited by their instability, rapid clearance, toxicity upon repeated administration, as well as the potential immuno-stimulation and complement activation. These disadvantages most often result in low delivery and expression efficiency which is a major drawback for liposomes (Stephan *et al.*, 1996; Simões *et al.*, 2005; Karmali and Chaudhuri, 2007; Mintzer and Simanek, 2009; Tros de Ilarduya *et al.*, 2010). Despite the above-mentioned challenges, cationic liposomes have in the past 3 decades remained the most explored non-viral delivery vector (Bhattacharya and Bajaj, 2009; Tros de Ilarduya *et al.*, 2010).

### **1.3.1 Structural features of cationic lipids**

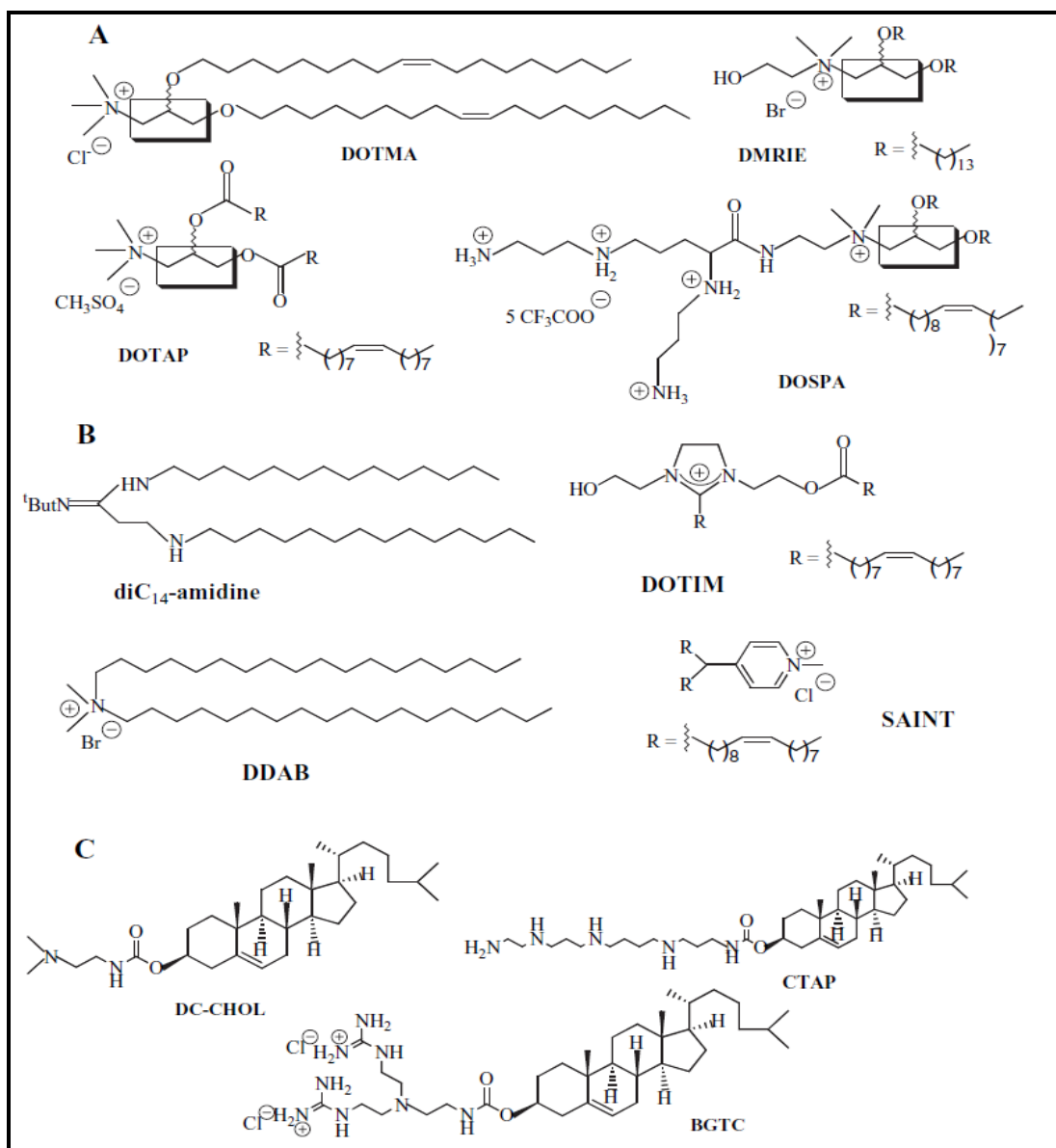
Cationic lipids are amphiphiles which commonly comprise three functional domains: 1) a polar hydrophilic headgroup 2) a linker bond, and 3) a hydrophobic domain. Since the

successful *in vitro* transfection achieved by Felgner and colleagues (1987) using DOTMA (N-(1-(2,3-dioleoyloxy)propyl)- N,N,N-trimethyl ammonium chloride), many cationic lipids have emerged. Most cationic lipid structures are based on the common foundation features presented by these early liposomes (Figure 1.6) (Felgner *et al.*, 1987; Martin *et al.*, 2003; Martin *et al.*, 2005; Karmali and Chaudhuri, 2007).



**Figure 1.6** The cationic lipid DOTMA showing the different structural components viz. hydrophilic headgroup, linker bond, and hydrophobic domain (Zhao *et al.*, 2013)

It is unarguable that a relationship exists between transfection efficiency, cytotoxicity and the nature of the components of the cationic lipids. For example, certain aspects of the linker can be modified to impact on how well it is tolerated in circulation (Smith *et al.*, 1997; Karmali and Chaudhuri, 2007). The most common classification of cationic lipids is founded on the nature of these parameters, depending on whether they are glycerol-based, non-glycerol-based and cholesterol-based cationic lipids (Figure 1.7) (Simões *et al.*, 2005; Martin *et al.*, 2003; Martin *et al.*, 2005; Karmali and Chaudhuri, 2007). Hence, it is important to discuss these parameters in separate subsections below.



**Figure 1.7** Structures of various transfection lipids classified as: A) glycerol-based, B) non glycerol-based and C) cholesterol-based cationic lipids (Karmali and Chaudhuri, 2007)

a) *Headgroup domain*

The most commonly employed hydrophilic headgroup comprises a tertiary amine or a quaternary ammonium group as seen in DOTMA and DOTAP. Felgner and colleagues later synthesised a number of 2,3-dialkyloxy quaternary hydroxyl-containing compounds and these resulted in better transfection than DOTMA. Several cationic lipids that delocalise the positive charge in the heterocyclic headgroups such as pyridinium, imidazole and piperazine (SAINT) conjugated to cholesterol have resulted in higher transfection efficiency and lower cytotoxicity. Multivalency is introduced by the



incorporation of polyamines and spermines. The first multivalent lipopolyamine, DOGS (dioctadecylamido-glycylspermine) was introduced in 1989 by Behr and colleagues (Behr *et al.*, 1989). These have been observed to compact DNA far more efficiently resulting in better transfection than the monovalent quaternary amine counterparts (DOTMA, DC-Chol and DMRIE). However, toxicity due to the micelles formed presents a challenge. This can be circumvented by addition of pyridine and guadinine for better positive charge spread (BGSC) (Aissaoui *et al.*, 2002; Martin *et al.*, 2003; Karmali and Chaudhuri, 2007; Montier *et al.*, 2008; Bhattacharya and Bajaj, 2009; Zhao *et al.*, 2013).

#### *b) Linker bond*

Linker groups act as bridges between the cationic headgroup and the hydrophobic domain. The commonly used linkers include ether, ester, amide or carbamate groups. Linkers influence conformational flexibility, stability, biodegradability and toxicity; and ultimately impact on transfection efficiency. Although the ester-linked DOTMA and similar liposomes exhibited high transfection efficiency, they were found to be very stable but toxic. Biodegradability and reduced toxicity was reported with use of ether bonds such as in DOTAP, and led to increased transfection efficiency of cholesterol-based cationic liposomes compared to those cationic lipids containing ester linkers. Structural analogues of DOTMA, by substitution of the ester linkage with ether linkers, DDAB (dimethyldioctadecyl ammonium bromide) and DOTAP (1,2-dioleoyloxy-3-[trimethylammonio]- propane) are commercially available. Carbamate linkers are chemically more stable and biodegradable. The first clinical trial using a liposome-based delivery system included the carbamate-linked DC-Chol cytofectin as it displayed better transfection efficiency, stability and reduced toxicity. Another important attribute in carbamate-linked structures is based on the theory that when the pH drops in the early endosomes, it triggers a disconnection of hydrophobic and hydrophilic portions of the lipoplex thus resulting in DNA release. Therefore transfection is found to be higher with the use of carbamate groups. Linkers consisting of redox-sensitive disulphide bonds have been investigated for stimuli-triggered to favour early release of DNA (Aissaoui *et al.*, 2002; Karmali and Chaudhuri, 2007; Montier *et al.*, 2008; Bhattacharya and Bajaj, 2009; Zhao *et al.*, 2013).

Structural parameters such as length, hydrocarbon chain position and nature of chemical bonds that make up the linker have also been reported to influence transfection efficiency,

biodegradability and stability of the cationic lipids. The length of the linker, such as the incorporation of oxyethylene units between cholesterol moiety and the polar headgroup has been reported to increase hydration level, and lead to decreased transfection efficiency (Karmali and Chaudhuri, 2007). The added benefit is that side chains for enhanced targeting, uptake and intracellular trafficking can be added into the linker region (Aissaoui *et al.*, 2002; Karmali and Chaudhuri, 2007; Montier *et al.*, 2008; Bhattacharya and Bajaj, 2009; Zhao *et al.*, 2013).

### *c) Hydrophobic domain*

Hydrophobic domains of cationic lipids are derived from either simple aliphatic hydrocarbon chains or steroids. The aliphatic chains can be linear and saturated, or linear and mono-unsaturated. The common variations include branched, acetylenic or cis-mono-unsaturated alkyl chains. The majority of cationic lipids synthesised thus far comprise double-chain hydrocarbons. Length, asymmetry, branching and saturation of the chains have been correlated to transfection efficiency (Aissaoui *et al.*, 2002; Karmali and Chaudhuri, 2007; Montier *et al.*, 2008; Bhattacharya and Bajaj, 2009; Zhao *et al.*, 2013).

Many studies have reported an inverse relationship between transfection efficiency and the length of the chain of cationic lipids. It is stated that shorter lengths, branched and unsaturated chains lead to decreased phase transition temperature and increased membrane fluidity. This favours higher intermembrane transfer rate and lipid mixing leads to potential disruption of endosomal membrane thus facilitating DNA release (Zhi *et al.*, 2010). The shorter chains are believed to confer good membrane fluidity and good lipid bilayer mixing. Unsaturated chains are found to be the best choice for transfection, with C18:1 oleyl being optimal (Aissaoui *et al.*, 2002; Karmali and Chaudhuri, 2007; Montier *et al.*, 2008; Bhattacharya and Bajaj, 2009; Zhao *et al.*, 2013).

The natural properties of cholesterol have ensured that it is the most commonly employed alternative to aliphatic chains. These attributes include rigidity, biodegradability and fusion activity. These essentially lead to better protection of the liposome-nucleic acid complex from nucleases, enhanced stability and reduced toxicity. Additionally, factors such as phase transition temperature and membrane fluidity which play a major role in DNA dissociation and release from the lipoplex in endosome are favourably influenced. Other steroid compounds include vitamin D, antibiotics, cholestane and lithocholic acid

(Zhi *et al.*, 2010). There have been recent developments in the use of fluorinated lipoplexes for enhanced transfection. This is due to the favourable biological and physicochemical characteristics with which they are attributed (Aissaoui *et al.*, 2002; Karmali and Chaudhuri, 2007; Montier *et al.*, 2008; Bhattacharya and Bajaj, 2009; Zhao *et al.*, 2013).

### **1.3.2 Factors governing lipoplex-mediated transfection efficiency**

There are many factors that govern liposome-mediated gene delivery. The mode of lipoplex formation also has a bearing on the physicochemical properties of the lipoplexes. Lipoplex size, charge density and stability are among the most important parameters that ultimately influence transfection efficiency. Additionally, there are indirect aspects such as protection of the nucleic acids from nucleases, use of targeting ligands for specificity, and inclusion of helper lipids for avoidance of DNA from lysosomal degradation (Simões *et al.*, 2005; Wasungu and Hoekstra, 2006; Wang *et al.*, 2012).

These aspects are perhaps best discussed in the context of the processes they are involved in, particularly if they are rate-limiting steps during transfection. It is interesting to note the coincidence of the site of impact by these factors and the cellular barriers presented during non-viral gene delivery.

#### **1.3.2.1 Lipoplex formulation**

Formation of lipoplexes is a highly dynamic process which essentially could determine the morphology and ultimately the transfection potential of lipoplexes. Formulations for delivery of nucleic acids include SUVs, giant unilamellar vesicles (GUV) and (multilamellar vesicles) MLVs. SUVs condense DNA to give spaghetti-like shaped lipoplexes which have been found to exhibit higher *in vitro* transfection efficiency. This is attributed to the extreme curvature with small radii, which means that lipoplexes attach to cells more easily. The residual positive charges on their surfaces lead to enhanced interaction with the cell for internalisation and transfection (Smith *et al.*, 1997; Templeton, 2002; Ma *et al.*, 2007).

Another important trait to take into account relates to stability during formulation. It has been found that coexistence of polydispersed size distributions may affect stability. Also, poor mixing of cationic lipids and helper lipids will often result in poorly stabilised complexes, which effectively leads to low transfection efficiencies (Wasungu and Hoekstra, 2007). PEG is often introduced to provide steric stabilisation to the liposomes; however, this often leads to masking of the positive charges. Thus, PEG serves a dual function in promoting stability and as a result protection of DNA cargo from serum nuclease degradation. Adversely, the “PEG dilemma” arises and leads to poor interaction of the lipoplex with target cells (Tros de Ilarduya *et al.*, 2010; Kibria *et al.*, 2011). This has undoubtedly led to hindered application of PEG liposomes as gene carriers. The introduction of ligands for recognition by the specific receptors of the target cells is employed to circumvent this PEG effect (Xiang *et al.*, 2012). Additionally, there have been investigations into cleavable PEG cationic liposomes, where the PEG is released when exposed to the acidic environment of the endosomal compartment (Templeton, 2002; Wasungu and Hoekstra, 2006).

### **1.3.2.2 Binding and internalisation**

As mentioned, the cationic lipoplex surface charge is inherently exposed to the negatively charged serum components. These result in interference of their interactions with the cell surface. Targeting by attachment of sugar residues was found to provide specificity in delivery using receptors. DC-Chol/DOPE based lipoplexes exhibited improved transfection efficiency when they were sugar-linked. This was attributed to enhanced cellular attachment and internalisation. In this case, it was assumed that targeting led to increased gene delivery to the nucleus (Wasungu and Hoekstra, 2006).

Lipoplex size has been determined to be a major parameter for lipoplex-mediated transfection efficiency. Size is largely influenced by factors such as cationic lipid ratio, types of liposomes formed, and exposure to serum components. For example, large and stable lipoplexes of 700 nm have been shown to resist transfection inhibition by serum, while lipoplexes less than 250 nm in diameter showed efficient transfection in the absence of serum (Ma *et al.*, 2007). However, while smaller-sized lipoplexes are

generally expected to be more efficiently internalized via endocytosis, *in vitro* studies have reported that larger lipoplexes often exhibit improved transfection activities (Ross and Hui, 1999; Zuhorn *et al.*, 2007). This can simply be due to enhanced sedimentation of the larger particles for maximum contact with the cells. Another contributing factor could be that once larger lipoplexes are taken up, the large intracellular vesicles are more prone to disruption and release of DNA into the cytoplasm. Contrasting results however, show that *in vivo* transfection with larger complexes is not feasible as the capillary network present size constraints in the range of 40 – 80 nm; while *in vitro* the optimal size has been proposed to be between 200 – 400 nm (Khalil *et al.*, 2006; Wasungu and Hoekstra, 2006; Ma *et al.*, 2007; Zuhorn *et al.*, 2007).

Studies with DOTAP lipoplexes have also confirmed that size is perhaps far more critical to transfection efficiency than lipoplex charge or the zeta potential. It is believed that size is an important trigger of uptake via a distinct transfection-efficient pathway. However, it must be stated that aspects that pertain to particle shape, cell type and even culture conditions have also been reported to be involved in selection of uptake pathways of non-viral gene complexes (Ma *et al.*, 2007; Xiang *et al.*, 2012). In a study on the inhibitory effects by non-phagocytic B16 cells, the internalisation mechanism was found to be size-dependent. Particles of about 250 nm were found to be endocytosed exclusively via clathrin-coated pits while those of 500 nm were internalised by means of caveolae (Wasungu and Hoekstra, 2006). Internalization pathways have a huge impact on the fate of the lipoplexes, which will be discussed in later sections. Various intracellular compartments convey certain properties that ultimately influence how efficiently DNA is trafficked into the cells until expression (Wasungu and Hoekstra, 2006; Zuhorn *et al.*, 2007; Wang *et al.*, 2012).

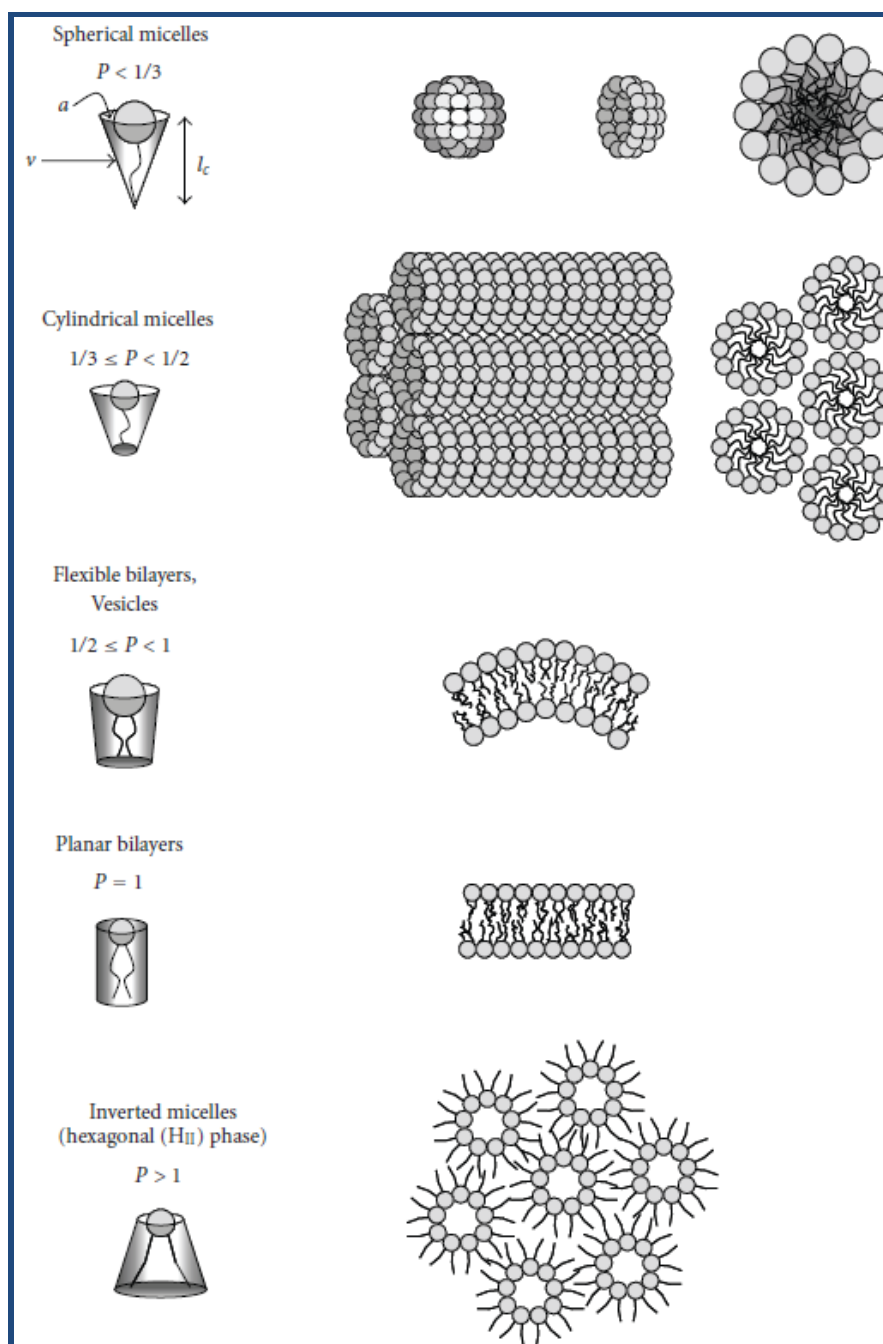
While the DNA:lipid ratio will determine the overall charge density; other properties such as complex size and shape, lipid formulation and encapsulation efficiency of nucleic acids by liposomes play a role in the colloidal properties of the complexes (Templeton, 2002). Preparations of cationic liposomes require that there be a slight excess of positive charge, which has been seen to result in higher transfection efficiency. In untargeted liposomes, this promotes the interaction between the liposome and the negatively charged cell surface proteoglycans. Neutrally charged lipoplexes tend to result in aggregated assemblies which can impact on transfection negatively. These large and unstable

lipoplexes often result in poor transfection efficiency (Zuhorn *et al.*, 2007; Montier *et al.*, 2008; Bhattacharya and Bajaj, 2009).

### ***1.3.2.3 Release of DNA from endosomes***

Lysosomal degradation has to be avoided if DNA has to successfully reach and be transcribed in the nucleus. This is achieved through escape from the acidic environment of the endosomal compartment. The hexagonal phase transition of the cationic lipid membrane is thus seen as an important process in this regard. Cationic lipids adopt various structural phases such as micellar, lamellar, cubic and inverted hexagonal phase. This is predicted by the packing parameter,  $P$ , where  $P = v/al_c$  which is defined as the ratio of the hydrocarbon volume,  $v$  and the product of effective head group area,  $a$ , and the critical length of the lipid tail,  $l_c$  (Figure 1.8) (Karmali and Chaudhuri, 2007; Ma *et al.*, 2007; Montier *et al.*, 2008; Bhattacharya and Bajaj, 2009; Tros de Ilarduya *et al.*, 2010; Balasz and Godbey, 2011; Zhao *et al.*, 2013).

When  $P$  is greater than  $l_c$ , and the area occupied by hydrocarbon chains is larger than of the headgroup, the cationic lipid assumes the inverted hexagonal phase. The inverted hexagonal and micellar cubic phases lead to the destabilisation of the bilayer structure and subsequent release of the lipoplex into the cytoplasm. This phase conversion is also found to be instrumental in DNA dissociation from the lipoplex where cubic phase adopting cationic lipid systems have been observed to express the highest transfection efficiency, even more than the hexagonal H<sub>II</sub> phase (Figure 1.8) (Safinya *et al.*, 2006; Wasungu and Hoekstra, 2006; Ma *et al.*, 2007).



**Figure 1.8** Schematic diagram showing the organization and phase structure of self-assembled cationic lipids as determined by the packing parameter,  $P$  (Balasz and Godbey, 2011)

Helper lipids such as DOPE are commonly used in most liposome formulations as they promote conformational change from a lamellar bilayer into an inverted hexagonal structure following a drop of pH in endosome. This results in the destabilisation of the endosomal membrane, leading to release of lipoplex. The dissociation of lipoplex into the respective liposome and nucleic acid cargo leads to improved lipoplex-mediated transfection (Hui *et al.*, 1996; Safinya *et al.*, 2006; Karmali and Chaudhuri, 2007; Ma *et*

*al.*, 2007; Montier *et al.*, 2008; Bhattacharya and Bajaj, 2009; Tros de Ilarduya *et al.*, 2010; Balasz and Godbey, 2011; Zhao *et al.*, 2013).

One cannot address PEGylated cationic liposomes in isolation without discussing the PEG effect on the phase conversion of these liposomes. Tight intermembrane interactions are required between the lipoplex surface and the endosomal membrane. While steric stabilisation of the lamellar phase afforded by PEG is crucial for longer circulation, release from the endosome is compromised, as it would have to be dissociated. This poses a major problem, which may be circumvented by use of cleavable pH-sensitive PEG analogues. Targeting moieties also have to be designed in a manner that they do not interfere with the tight intermembrane interactions necessary between the endosomal membrane and the lipoplex surface. Improved targeting due to the sugar-linked lipoplexes has been reported. However, poor delivery **due to poor** escape from the endosomal compartment can result in lower transfection efficiencies (Aissaoui *et al.*, 2002; Wasungu and Hoekstra, 2006; Ma *et al.*, 2007; Tros de Ilarduya *et al.*, 2010).

#### **1.3.2.4 Trafficking into nucleus**

Nuclear entry is a major rate-limiting step in lipoplex-mediated transfection. It has been hypothesised to take place in one of two ways viz. 1) by passive entry during cell division when the nuclear membrane temporarily disintegrates, or 2) by active transport through the nuclear pores. Naked DNA is prone to degradation by cytosolic nucleases during its trafficking to the nucleus. Therefore transit time to the nucleus has to be as short as possible. Once reached, a critical factor relates to the limited size of the nuclear pores. The passive limit is about 70 kDa molecular mass or 10 nm diameter and hence even if condensed in a lipoplex, nuclear entry is impossible (Khalil *et al.*, 2006). During active transport, the nuclear pore size is stretched to about 30 nm. Mitosis is believed to play an important role in transfection. DNA enters the nuclear compartment during mitosis when the integrity of the nuclear membrane is transiently compromised. However, this is the case for *in vitro* transfection of dividing cells, and does not translate to *in vivo* transfection which usually targets differentiated non-dividing cells. Thus molecules of large sizes can penetrate into the nucleus during interphase through the nuclear pores



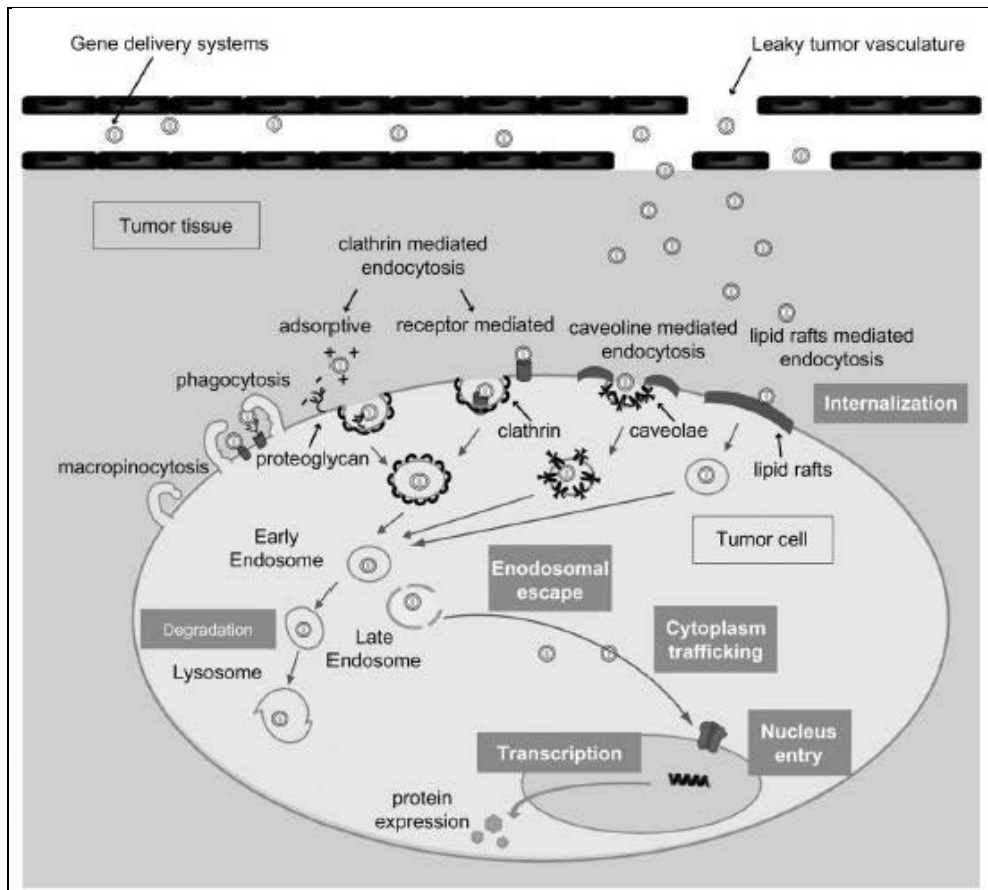
(Karmali and Chaudhuri, 2007; Montier *et al.*, 2008; Bhattacharya and Bajaj, 2009; Tros de Ilarduya *et al.*, 2010; Balasz and Godbey, 2011; Zhao *et al.*, 2013).

## **1.4 Uptake pathways for non-viral gene delivery**

Early studies had proposed that fusion with the cell membrane is responsible for non-viral delivery. However, various studies have indisputably established that endocytosis is the major path of entry for non-viral delivery vectors. This was demonstrated by Payne and colleague following studies on intracellular trafficking of PEI- and Lipofectamine-DNA complexes (Payne *et al.*, 2007; Mintzer and Simanek, 2009; Xiang *et al.*, 2012; McCrudden and McCarthy, 2013). There is also a need to develop a comprehensive understanding of non-endocytic pathways as they form a significant part of non-viral gene delivery.

### **1.4.1 Endocytic uptake pathways**

Endocytosis is classified into two main categories such as phagocytosis and pinocytosis. Pinocytic pathways are characterised as follows: clathrin-coated endocytosis, caveolae, macropinocytosis and clathrin/caveolae independent endocytosis (Figure 1.9.) (Khalil *et al.*, 2006; Morille *et al.*, 2008; Resina *et al.*, 2009; Ziello *et al.*, 2010; Xiang *et al.*, 2012; Gasc n *et al.*, 2013; Xiang and Zhang, 2013).



**Figure 1.9** Schematic representation of the various internalisation pathways and hurdles encountered for intracellular trafficking (Morille *et al.*, 2008)

Phagocytosis is generally restricted to specialised cells such as macrophages, neutrophils and monocytes with the main function to remove pathogens and other unwanted particles from the system. While this process does not contribute significantly to gene delivery pathways, it cannot be completely ignored for its potential to influence internalization (Khalil *et al.*, 2006; Mintzer and Simanek, 2009). Macropinocytosis is currently being explored for a possible role in gene delivery and transfection pathways. This is due to advantageous properties displayed by this pathway, which include efficient uptake of fluid-phase macromolecules and ease of escape from the inherently leaky uncoated and larger sized (up to 0.2  $\mu\text{m}$  in diameter) macropinosomes. Most importantly, internalised particles can avoid lysosomal degradation as there is no fusion with lysosomes. However, much needs to be done in order to target this pathway for gene delivery systems (Khalil *et al.*, 2006; Mintzer and Simanek, 2009; Resina *et al.*, 2009; Ziello *et al.*, 2010; Xiang and Zhang, 2013).

#### 1.4.1.1 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME) is possibly the best-characterised pathway for endocytosis. It is constitutively used in biological processes which require uptake of nutrients, growth factors and pathogens. This is seen in low-density lipoprotein (LDL) internalisation by binding to the LDL receptors on the liver surface (Khalil *et al.*, 2006). Internalisation of particles in this pathway seems to be size-dependent, with size ranges of 150 – 200 nm. This is attributed to the limiting size of the clathrin coated pits which have a radius of approximately 100 nm. This is followed by clustering of the internalised complexes in coated pits on the plasma membrane. The coated pits then invaginate and pinch off from the plasma membrane to form intracellular clathrin-coated vesicles. The internalised complexes are then depolymerised in the early endosomes. There is a rapid drop in pH from neutral to pH 5.9 – 6.0 in the lumen of these early endosomes, with a further reduction to pH 5 during progression from late endosomes to lysosomes (Khalil *et al.*, 2006; Morille *et al.*, 2008; Ziello *et al.*, 2010; Xiang *et al.*, 2012; Gascón *et al.*, 2013; Xiang and Zhang, 2013).

A common misconception has resulted in the clathrin-mediated endocytosis and receptor-mediated endocytosis being deemed to be one and the same. As shown in Figure 1.9, clathrin-mediated endocytosis can occur in an adsorptive or receptor-mediated manner. Additionally, it has also been established that most of the pinocytic pathways, including CME involve receptor-ligand interactions. Commonly, in gene delivery, the clathrin-mediated pathway is targeted by using certain ligands, which can specifically recognize certain receptors on the cell surface. This results in an increase in the internalization of the particles and offers the possibility of targeting specific cells that substantially over-express certain receptors (Khalil *et al.*, 2006; Xiang and Zhang, 2013). Use of receptor-mediated endocytosis has been widely explored over the years, particularly for its role in liver-directed gene therapy. The ASGP receptor-mediated endocytosis has been exploited by coupling appropriate motifs, glycoproteins, lactose or galactose to delivery systems. While there are several ligands that have been investigated for cell selective transgene expression both *in vivo* and *in vitro*, galactose is possibly the most extensively studied ligand for ASGP receptor recognition (Pathak *et al.*, 2008; Stokmaier, 2010; Motoyama *et al.*, 2010).

### **1.4.1.2 Caveolae-mediated endocytosis**

Caveolae are small, hydrophobic membrane flask-shaped microdomains that are rich in cholesterol and sphingolipids. Classically, CvME occur in the wall lining of endothelial cells. Caveolae are found in many different cell types, with diameter in the 50 – 100 nm size range (Ziello *et al.*, 2010; Xiang *et al.*, 2012).

Caveolae-mediated endocytosis (CvME) is dynamin-dependent and receptor-mediated, and its role in DNA delivery was reported in the internalisation of cationic PEI polyplexes. This was observed to take place by both clathrin and caveolae-mediated mechanisms. An intriguing finding using inhibitor studies showed the caveolae-mediated route to have led to productive transfection. Larger particle sizes in the order of 500 nm are preferentially taken up via this route, while smaller particles may use clathrin-mediated endocytosis (Zuhorn *et al.*, 2007; Xiang *et al.*, 2012). The high transfection efficiency could be attributed to lack of pH drop in this pathway. Studies have shown caveolae-internalised pathogens to be transported in the cytoplasm to other organelles thus avoiding lysosomal degradation. While it remains unlikely that they contribute significantly to constitutive endocytosis, caveolae-mediated endocytosis is still a promising strategy for gene delivery especially if the internalization can be increased, possibly through the use of specific receptors for caveolae (Khalil *et al.*, 2006; Morille *et al.*, 2008; Ziello *et al.*, 2010; Xiang *et al.*, 2012; Gasc n *et al.*, 2013; Xiang and Zhang, 2013).

## **1.5 Strategies in liver-directed gene delivery**

### **1.5.1 Overview**

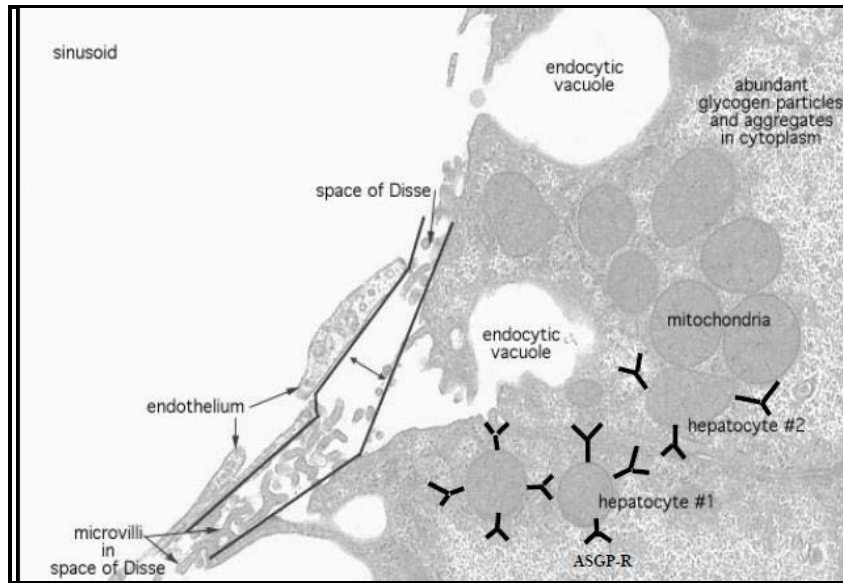
The liver is the largest visceral organ in the body. It plays an integral and central role in many metabolic pathways such as in synthesis of proteins, posttranslational modifications as well as lipid synthesis. It is also associated with many genetically-based diseases such as haemophilia, lipoprotein receptor deficiency, cancer and life-threatening Hepatitis B. For this reason, the liver is considered one of the most important targets for gene therapy (Pathak *et al.*, 2008; Loduca *et al.*, 2009; Daniels *et al.*, 2010; Mishra *et al.*, 2013).

Liver cancer has been established as one of the most malignant cancers. Statistics have shown that it is fifth most frequent cancer in the world. However, this value rises to a third in developing countries among men after lung and stomach cancer. Poor prognosis may also contribute to the high mortality associated with this cancer and makes it the third leading cause of cancer deaths globally (Ananthkrishnan *et al.*, 2006; Wang *et al.*, 2009).

Hepatocellular carcinoma (HCC) is the most common primary liver cancer, often associated with Hepatitis B virus (HBV) and Hepatitis C (HCV) infections. Other risk factors include diabetes mellitus (DM), aflatoxin exposure, excessive alcohol intake, and tobacco use. Statistics have also shown that the highest HCC incidents are in China, Taiwan and sub-Saharan Africa. Interestingly, these regions are found to be endemic for HBV infections, aflatoxin exposure, alcohol intake and tobacco use (Ananthkrishnan *et al.*, 2006; Datta, 2008; Wang *et al.*, 2009). In a report by Hall *et al.* (2011), it was projected that Africa will have an estimated 23.9 million people living with DM by 2030. This undoubtedly bears significant implications for the currently high HCC prevalence. Coupled with high rates of communicable diseases such as HIV, malaria and tuberculosis in the region, morbidity and mortality will be consequentially impacted (Hall *et al.*, 2011). Development of new therapeutic modalities is hence very important for the future therapy of liver associated diseases.

### **1.5.2 Asialoglycoprotein (ASGP) receptor-mediated delivery for hepatocyte-specific therapy**

One of the major challenges faced by many therapeutics in systemic gene delivery to the liver relates to traversing **the** vascular walls of hepatocytes (Kawakami *et al.*, 2001; Higuchi *et al.*, 2006). Figure 1.10 provides a diagrammatic representation of hepatocytes showing the sinusoidal wall of the liver.

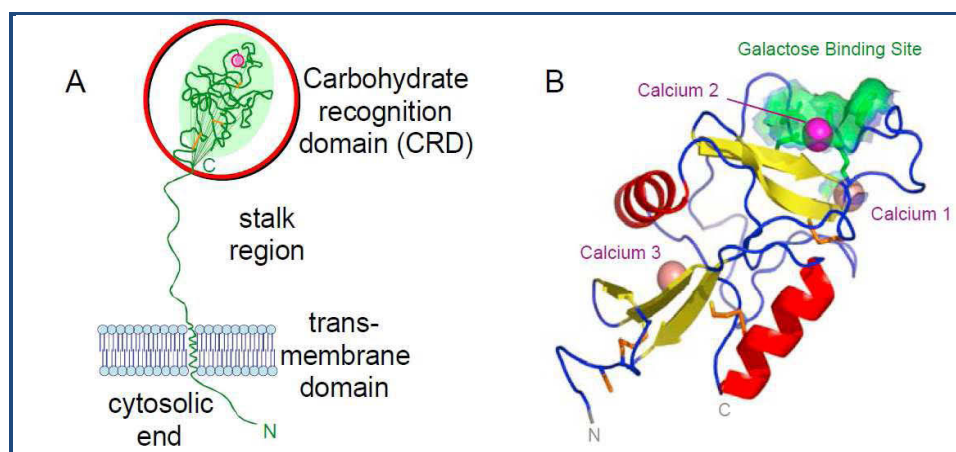


**Figure 1.10** Diagrammatic representation of hepatocytes (Pathak *et al.*, 2008)

It is known to lack a basement membrane and rather possesses a fenestrated endothelium of 100 nm diameter pore size. Therefore, for efficient internalization, vector-cargo complexes should be small enough to pass through the fenestrae and enter into the space of Disse, which is in direct contact with hepatocytes (Higuchi *et al.*, 2006; Pathak *et al.*, 2008; Mishra *et al.*, 2013). Additionally, while many of these complexes are able to deliver genes to the liver, they often do not efficiently target the parenchymal hepatocyte (Ganem, 1999). One of the reasons relates to the three additional Gal/GalNac C-type lectin receptors viz. the Kupffer cell receptor, the macrophage galactose lectin and the scavenger C-type lectin. This may lead to the misdirection of vectors (Khorev *et al.*, 2010; Sonoke *et al.*, 2011).

The ASGP receptor is a high-capacity membrane-bound carbohydrate-binding protein located abundantly on parenchymal hepatocytes. ASGP receptors are calcium-dependent (C-type) lectins randomly located on the sinusoidal (basolateral) plasma membrane domain facing the capillaries. These receptors present in excess about  $1 \times 10^5$  -  $5 \times 10^5$  binding sites per cell (Lee and Lee, 1987; Cristiano *et al.*, 1993; Kawakami *et al.*, 1998; Kawakami *et al.*, 2001; Singh and Ariatti, 2003; Stokmaier *et al.*, 2009; Xiao *et al.*, 2013). One of the important roles of the ASGP receptor is to maintain serum glycoprotein levels by processing and clearing desialylated glycopeptides and liposaccharides that contain terminal end galactose and N-acetylgalactosamine (GalNac) residues (Stokmaier

*et al.*, 2009; D'Souza *et al.*, 2010; Christie *et al.*, 2014). This activity of the ASGP receptor was initially described by Ashwell and colleagues who discovered the removal of diasialyated glycoproteins from the circulation and their subsequent degradation in the liver (Ashwell and Kawasaki, 1978; Ashwell and Harford, 1982; Bernades *et al.*, 2010; Stokmaier, 2010). The X-ray crystal structure of the human ASGP receptor has been determined as shown in Figure 1.11 (Khorev, 2007; Khorev *et al.*, 2008).



**Figure 1.11** X-ray structure of the ASGP receptor showing the active binding site located in the carbohydrate recognition domain (Khorev, 2007)

It comprises 2 single-spanning membrane proteins, designated the H1 and H2 subunits in the ratio of 5:1. Both of these subunits are required for full functionality of the receptor. The carbohydrate recognition domain (CRD) is found on the major H1 subunit. The major subunit, H1, exclusively contains the signal for endocytosis by mediation of the  $\text{Ca}^{++}$ -dependent recognition of D-galactose and N-acetyl-D-galactose (D-GalNac) resulting in displacement of two water molecules which co-ordinate this action in the absence of a cognate ligand. H2 contributes an important element to basolateral sorting of the hetero-oligomeric receptor molecules and thus, is more involved in the functional configuration (Khorev *et al.*, 2008; Stokmaier *et al.*, 2009; Bernardes *et al.*, 2010).

Evaluations were carried out on affinity of D-GalNac derivatives to the rat hepatocyte ASGP receptor. The highest affinity reported was for the tri-galactosides, followed by bi-galactosides and lastly, mono-galactosides. This phenomenon has been described as the “cluster effect”. And thus, modifications of liposomes by incorporating tri-galactoside ligands have resulted in increased recognition and avidity by these ligands for this

receptor (Kawakami, 2000; Singh and Ariatti, 2003; Khorev, 2007; Stokmaier *et al.*, 2009). Although triantennary ligands have displayed a higher affinity than the mono- and diantennary counterparts, some of the challenges they present include complexity in structure, and in upscaling to industrial production as well as possible antigenicity. The reduced complexity with which monoantennary ligands can be synthesised still makes them the preferred choice in ASGP receptor-mediated delivery (Singh and Ariatti, 2003; Bernades *et al.*, 2010; Stokmaier *et al.*, 2009). Optimisation of these small monoantennary ligands by addition of small-molecule analogues can provide the potential to increase the relatively weak binding kinetics (Mamidyala *et al.*, 2011; Maslov *et al.*, 2011).

Acetylation, PEGylation, and glycosylation have been reported to be effective strategies towards improvement of transfection efficiency in dendrimers (Fant *et al.*, 2010; Hu *et al.*, 2011; Maslov *et al.*, 2011). Glycosylation and PEGylation have been extensively explored in lipoplex formulations. **However, currently there is limited data available pertaining to modifications of mono-antennary ligands by acetylation, and how that impacts on their binding affinity to the ASGP receptor.** Nevertheless, the exclusive expression of ASGP receptors by parenchymal hepatocytes coupled with high specificity and capacity for internalisation of cognate ligands make the ASGP receptor an attractive target in development of hepatocyte-specific therapeutics. It is imperative to investigate ways by which these can be modified for improved binding affinity to the receptor, which will essentially have an impact on transfection efficiency (Stokmaier *et al.*, 2009; Khorev *et al.*, 2010).

## **1.6 Outline of thesis**

An introduction to gene therapy as an alternative therapeutic strategy for treatment of many diseases is presented in Chapter One. While many advances have been made, it is imperative that more robust efforts be focused on non-viral gene delivery systems. Cationic liposomes remain vectors of choice due to their many important attributes; however, it is pertinent that key mechanisms and challenges presented in gene delivery be addressed. In targeted delivery, the use of ligands becomes even more useful in order to



enhance cell-specificity. The effects of acetylation on galactose-derived (galactoside) ligands were investigated for *in vitro* and *in silico*.

Thus the primary aims and objectives of this study as presented in Chapter Two were to:

- a) formulate cationic liposomes that exhibit dual-functionality in respect of targeting and long-circulation for liver-targeted delivery. Comparative analysis has thus been carried out for feasibility of targeting, where galactoside ligands viz. Sc6 and Sc9 have been formulated into liposomes and attached for recognition via the ASGP receptor. Additionally, effects of PEG have been investigated with some liposomes incorporating PEG in their formulations.
- b) carry out *in vitro* investigations to characterise morphological and physicochemical properties of the liposomes, the DNA-binding interactions, serum nuclease protection and cytotoxicity assays.
- c) evaluate the *in vitro* transfection activity via the ASGP receptor-mediated endocytosis in hepatocellular carcinoma cell line (HepG2). This has been achieved by comparing transgene expression in the ASGP receptor-negative HEK293 and receptor-positive HepG2 cell lines. Furthermore, receptor-mediated internalisation was verified by introducing a competitive inhibitor, asialofetuin and comparing the gene expression levels against the unsaturated receptor levels.
- d) assess *in silico* behaviour of binding affinities of galactose ligands by performing molecular modelling and docking studies using the Autodock Vina software programme.

Findings from the study are presented in Chapter Three. Discussions have centred around the various aspects critical for successful gene delivery as per the aims and objectives. Ultimately, transfection efficiencies for both ligand-derived lipoplexes were evaluated and comparative analysis was drawn between the derivatives. Additionally, calculated binding affinities of the ligands in question were discussed in terms of their avidity for the ASGP receptor. Chapter 4 provides overall conclusions drawn from the findings of the study including future considerations and recommendations.

## CHAPTER TWO: MATERIALS AND METHODS

### 2.1 Materials

The cationic lipid 3 $\beta$ [*N*-(*N*' , *N*' -dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) was previously prepared in the Department of Biochemistry, University of KwaZulu-Natal (UKZN, Westville) according to the protocol of Gao and Huang (1991). Galactosides *viz.* 1) cholest-5-en-3-yl 2-[4-( $\beta$ -D-galactopyranosyl-1-oxymethyl)-1*H*-1,2,3-triazol-1-yl]ethylcarbamate (Sc6) and 2) cholest-5-en-3-yl 2-[4-(2,3,4,6-tetra-O-acetyl- $\beta$ -D-galactopyranosyl-1-oxymethyl)-1*H*-1,2,3-triazol-1-yl]ethylcarbamate (Sc9) were synthesised in the Department of Chemistry of the University of Witswatersrand (South Africa). L- $\alpha$ -phosphatidylethanolamine dioleoyl (DOPE) was supplied by Sigma, St. Louis, MO, USA. 1,2-distearoyl-*sn*-glycerophosphoethanolamine-*N*-[carboxy(polyethylene glycol) 2000] (ammonium salt) (DSPE-PEG<sub>2000</sub>) was obtained from Avanti Polar Lipids, Alabaster, USA.

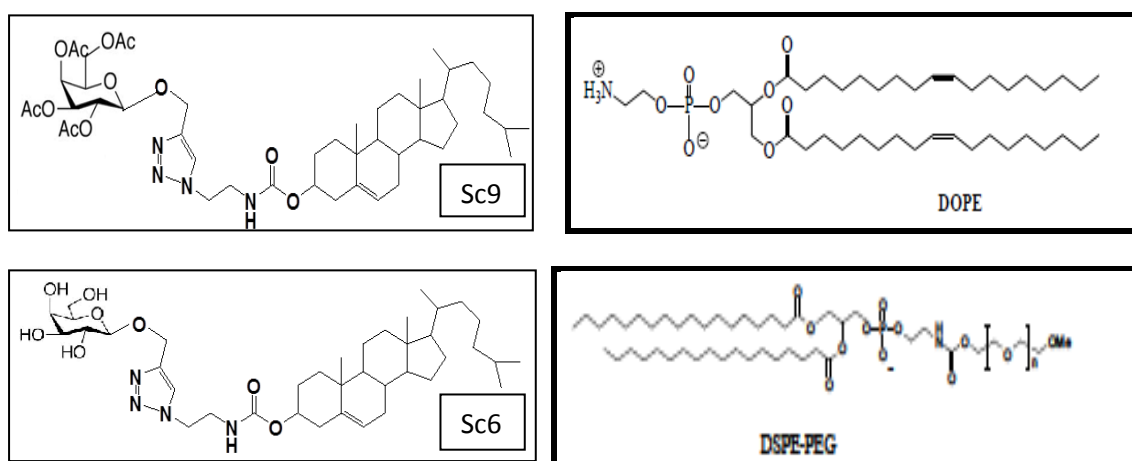
2-[4-(2-hydroxyethyl)-1-piperazinyl] ethane sulphonic acid (HEPES) and the MTT salt (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide) were purchased from Merck, Damstadt, Germany. Ultrapure DNA grade agarose was purchased from Bio-Rad Laboratories, Richmond, USA. pCMV-*luc* DNA was purchased from Plasmid Factory, Bielefeld, Germany. HEK293 cells were supplied by the University of the Witwatersrand Medical School (South Africa) and HepG2 cells were obtained from Highveld Biologicals (Pty) Ltd. (Lyndhurst, South Africa). 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) were obtained from Sigma-Aldrich Inc., St, Louis, MO, USA. Phosphate-buffered saline (PBS) tablets and trypsin-versene were purchased from Calbiochem, Canada and Lonza Biowhittaker, Walkersville, MD respectively.

The cell culture lysis reagent (1 X) (25 mM Tris-phosphate, pH 7.8; 2 mM dithiothreitol; 2 mM 1,2-diaminocyclohexane-*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  $\mu$ M coenzyme A; 470  $\mu$ M luciferin; 350  $\mu$ M ATP) were

purchased from Promega Corporation, Madison, WI, USA. Penicillin/streptomycin mixture (10 000 U/ml penicillin, 10 000 µg/ml streptomycin) and fetal bovine serum (FBS) were purchased from Gibco Invitrogen™, New Zealand. All tissue culture plastic consumables were purchased from Corning Incorporated, New York, USA. All other reagents were of analytical grade.

## 2.2 Formulation of liposomes

Six liposomal formulations were prepared using components as shown in Figure 2.1. Preparations was done in 1 ml of sterile HBS (20 mM HEPES, 150 mM NaCl, pH 7.5) at a total lipid concentration of 4 mM as per Table 3.



**Figure 2.1** Structures of components of liposomal formulations used in this study

**Table 2.1** Components of the liposomal formulations (µmole/ml)

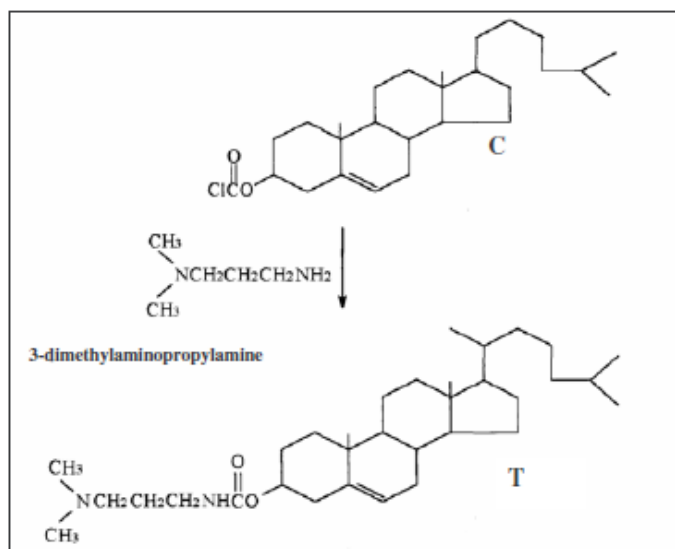
	<b>Chol-T</b>	<b>DOPE</b>	<b>Sc6</b>	<b>Sc9</b>	<b>DSPE-PEG<sub>2000</sub></b>
<b>SM1</b>	2	2	-	-	-
<b>SM1(+PEG)</b>	2	1.8	-	-	0.2
<b>SM2</b>	2	1.6	0.4	-	-
<b>SM2(+PEG)</b>	2	1.6	0.4	-	0.2
<b>SM3</b>	2	1.6	-	0.4	-
<b>SM3(+PEG)</b>	2	1.6	-	0.4	0.2

Untargeted and unPEGylated control (SM1) comprised of equimolar quantities of Chol-T and DOPE. The PEGylated control contained DSPE-PEG<sub>2000</sub> at 0.2  $\mu$ mole/ml equivalent to 5% mole ratio. Targeted liposomes contained varying amounts of Chol-T, DOPE and respective galactosides *viz.* Sc6 (SM2 and its PEGylated derivative) and Sc9 (SM3 and its PEGylated derivative). The PEGylated formulations all contained DSPE-PEG<sub>2000</sub> at 5% mole ratios.

The following mixtures: *viz.* SM1; SM1(+PEG); SM2 and SM2(+PEG) were dissolved in 1 ml of dry chloroform, while SM3 and SM3(+PEG) mixtures were dissolved in 1 ml of chloroform:methanol (50:50 v/v) mixture. The lipid components were deposited as a thin film in a test tube by rotary evaporation of the solvent *in vacuo* at 25 °C. Thereafter, the film was hydrated at 4 °C for 24 h and the resulting mixture was vortexed. The suspension was then sonicated in an Elma Transsonic bath type sonicator (T 460/H) for 5 min to obtain unilamellarity as determined by transmission electron microscopy (TEM). Liposomes were routinely stored at 4 °C until use.

### **2.2.1 Preparation of cationic cholesterol derivative 3 \_ [N-(N', N'-dimethyl-aminopropane)-carbamoyl] cholesterol (Chol-T)**

The synthesis steps of the Chol-T were carried out as follows: 3-dimethylaminopropylamine was added to a solution of cholesteryl chloroformate in dichloromethane. This synthesis reaction proceeded for an 1 hour at room temperature (Figure 2.2) and monitored by TLC (results not shown). The solvent (dichloromethane and excess 3-dimethylaminopropylamine) was subsequently removed by rotary evaporation in a Büchii Rotavapor-R. The residue resulting from the evaporation was dissolved in absolute ethanol and allowed to crystallise overnight at -4 °C. The product was then recrystallised, filtered under a stream of dry nitrogen gas and further dried by rotary evaporation to yield whitish coloured crystals.



**Figure 2.2** Synthesis reaction scheme of cationic cholesterol derivative Chol-T (T) from starting material cholesteryl chloroformate (C)

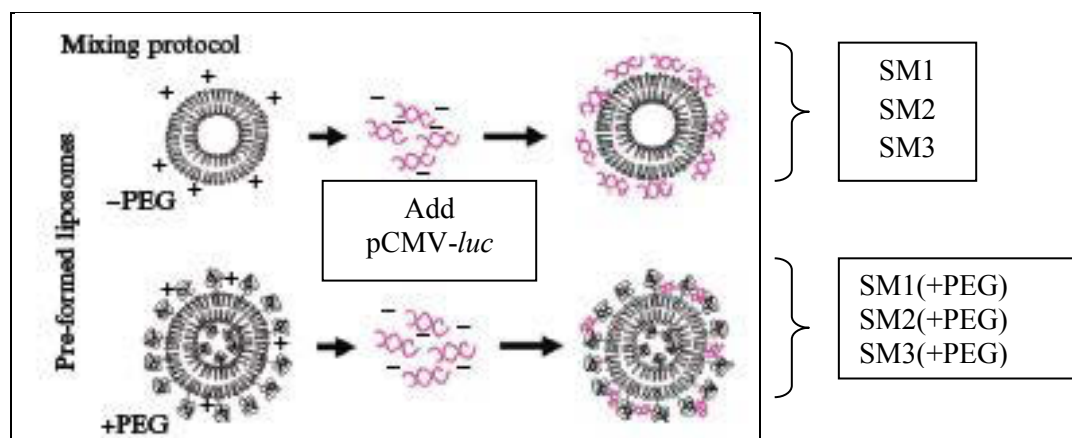
## 2.3 Formulation of lipoplexes

### 2.3.1 Plasmid amplification and isolation

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). These were maintained in a shaking incubator at 37 °C for 36 hours, in order to select transformants. The pCMV-*luc* DNA was then isolated according to the protocol outlined in the Promega Technical Manual no. 033 for pGL3 Luciferase Reporter Vectors. Purity and amount of plasmid DNA isolated was determined using a ThermoScientific NanoDrop 2000c spectrophotometer (Wilmington, USA). The isolated 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. For verification of the plasmid, the isolate (0.5 µg) and pCMV-*luc* stock (0.5 µg), each in 10 µl HEPES buffered saline (HBS) were mixed with gel loading buffer (2 µl) and subjected to electrophoresis for 60 minutes at 50 V on 1 % agarose gel in a buffer comprising 36 mM Tris–HCl, 30 mM sodium phosphate and 10 mM EDTA (pH 7.5).

### 2.3.2 Formation of lipoplexes

Lipoplex formation was achieved by the addition of the isolated pCMV-*luc* DNA (0.5 µg) to increasing amounts of PEGylated and unPEGylated liposome suspensions resulting in different liposome (+) / DNA (-) ratios (Figure 2.3).



**Figure 2.3** Diagrammatic representation showing formulation of the various lipoplexes (adapted from Podesta and Kostarelos, 2009)

The added pCMV-*luc* DNA and liposomal suspension volume was brought to a final volume of 10 µl with HBS. The liposome (+) / pDNA (-) charge ratios ranged from 0.5/ 1 to 6/1. Lipoplexes were then incubated at room temperature for 20 min prior to use. Lipoplexes were used within one hour of preparation time.

### 2.4 Gel retardation assay

Lipoplexes were prepared as described above to achieve (+) liposome / (-) DNA charge ratios in the range 0.5:1 to 6:1. After 20 mins of incubation, 2 µl of the gel loading buffer (0.05% bromophenol blue, 40% sucrose) was added to the complexes. The samples were then subjected to electrophoresis (50 V) on a 1% agarose gel containing ethidium bromide (EtBr) (1.5 µg/ml) in a 1x electrophoresis buffer comprising 36 mM Tris-HCl, 30 mM sodium phosphate and 10 mM EDTA (pH 7.5) for 90 min. Viewing of the gels was carried out in a Vacutec Syngene G:box gel documentation system (Syngene, Cambridge, UK) under UV transillumination at 300 nm, and images were captured using GeneSnap software.

## 2.5 Serum nuclease digestion assay

The complexes containing 0.5 µg pCMV-*luc* DNA at sub-optimal, optimal and super-optimal retardation ratios as determined from the gel retardation assay (section 2.4), were incubated in HBS for 20 min at room temperature. Foetal bovine serum (FBS) was added to a final concentration of 10% (v/v) and samples were incubated for 4 h at 37 °C. Thereafter, EDTA and SDS were added to final concentrations of 10 mM and 0.5% (w/v), respectively and complexes incubated for a further 20 min at 55 °C. Thereafter, approximately 2 µl of the gel loading buffer (0.05% bromophenol blue, 40% sucrose) was added to the suspension. Electrophoresis was carried out at 50 V on a 1% agarose gel containing ethidium bromide (1.5 µg/ml) in a buffer comprising 36 mM Tris-HCl, 30 mM sodium phosphate and 10 mM EDTA (pH 7.5) for 90 min. Gels were viewed as described in section 2.4.

## 2.6 Ethidium bromide intercalation assay

Approximately, 2 µl of ethidium bromide (EtBr) solution (100 µg/ml) was added to 100 µl of HBS in wells of a black 96-well microtitre plate. Fluorescence was measured at an emission wavelength of 580 nm and an excitation wavelength of 525 nm in a Glomax multi-detector system (Promega Biosystems, Sunnyvale, USA). This initial reading was set as 0% relative fluorescence. The next reading was taken after addition of 2.4 µl (1.2 µg) of pCMV-*luc* DNA to the sample and set as 100% relative fluorescence. Thereafter, cationic liposome suspensions (1 µl) were added stepwise to the pDNA-EtBr solution. The fluorescence intensity was recorded after every liposomal addition, until a plateau in the readings was observed. Results were presented as relative fluorescence versus liposome concentration.

## 2.7 Cryo-transmission electron microscopy

Liposome and lipoplex suspensions were diluted 1:5 with sterile HBS. Approximately 2 µl, of liposome/lipoplex suspension was then deposited on a carbon coated 400-mesh copper grid. To this was added 2 µl of 1% (w/v) uranyl acetate and the grid was allowed to dry for 2 min. Excess suspension was removed using filter paper and the grid was

placed under liquid nitrogen. Grids were then immediately transferred into a GATAN cryo-holder maintained at  $-170\text{ }^{\circ}\text{C}$ , and thereafter introduced into the microscope for observation at  $-150\text{ }^{\circ}\text{C}$ . Images were obtained under cryogenic conditions at 100 kV using a JEOL JEM1010 electron microscope (Tokyo, Japan). The micrographs were generated by a MegaView III camera and SIS i-TEM software facilitated measurements of liposomes on calibrated images.

## **2.8 Size and zeta potential measurements**

Size distribution patterns and zeta potentials were measured by dynamic light scattering (DLS) using a Malvern Zetasizer Nano-ZS instrument (Malvern Instruments Ltd., Worcestershire, UK.) operating at  $25\text{ }^{\circ}\text{C}$ . Liposome suspensions were prepared by addition of  $10\text{ }\mu\text{l}$  of liposome suspension to  $990\text{ }\mu\text{l}$  HBS. Lipoplexes were formed by complexing  $10\text{ }\mu\text{l}$  of liposome suspension with corresponding amount of DNA to obtain optimal end-point ratios. The final volume was made up to 1 ml. The 1 ml suspensions were added to the appropriate cells and thereafter, readings were taken. A DTS0012 polystyrene disposable sizing cuvette for the Z-average diameter measurements and a DTS-1061 electrocell for the zeta potential measurements were used. All measurements were done in triplicate.

## **2.9 Cell culture and maintenance**

HEK293 and HepG2 cells were maintained in  $25\text{ cm}^2$  flasks containing EMEM and placed in a Steri-cult  $\text{CO}_2$  incubator HEPA Class 100 (Thermo-Electron Corporation, Waltham, Massachusetts, USA) at  $37\text{ }^{\circ}\text{C}$  under 5%  $\text{CO}_2$ . Cell culture medium was supplemented with 10% (v/v) FBS, penicillin (100 units/ml), streptomycin (100  $\mu\text{g}/\text{ml}$ ), and L-glutamine (4 mM) (Gibco BRL Life Technologies). Cells were split 1:3 every 4–5 days or when required.



## **2.10 MTT cell viability assay**

Both cell lines were seeded at a density of  $2.5 \times 10^4$  cells per well in a 48-well microtitre plates in complete medium (EMEM, 10% FBS, streptomycin (100 µg/ml), and penicillin (100 U/ml)) and incubated at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub> for 24 h. Thereafter, the medium was discarded and fresh complete medium (0.3 ml) was added to the wells. The lipoplexes were prepared in triplicate at the sub-optimal, optimal and super-optimal ratios as in section 2.5, and added to the cells. Cells not treated with the lipoplexes were set up as controls (100%). Incubation of the cells was carried out for a further 48 h at 37 °C. Thereafter the spent medium was removed, and cells rinsed with PBS, followed by addition of 0.2 ml of EMEM and 0.2 ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide solution (MTT). The cells were incubated for an additional 4 h at 37 °C. Thereafter, the MTT-containing medium was removed from the wells, and 0.2 ml of dimethylsulphoxide (DMSO) was added to dissolve the formazan crystals produced by the cells. The absorbance was measured at 570 nm using a Mindray MR-96A micro plate reader (Vacutec, Hamburg, Germany). Cell viability was determined as the percentage fluorescence relative to the untreated control (100%). The results were calculated and expressed as the mean  $\pm$  standard deviation. Experiments were performed in triplicate.

## **2.11 Transgene expression and Protein Determination Assays**

### **2.11.1 Transfection assay in HEK293 and HepG2 cell lines**

HEK293 and HepG2 cells were seeded in a 48-well plate at a density of  $2.5 \times 10^4$  cells per well in 0.3 ml complete medium. Thereafter, the medium was discarded, and wells were rinsed with PBS, being careful not to dislodge the cells. The lipoplexes were prepared as in section 2.5 and added to a set of triplicate wells for each ratio. Two controls, one containing only cells and the other containing cells with naked DNA were also set up. Following lipoplex addition, the plate was incubated for a further 48 h at 37 °C. Thereafter, the medium was removed and cells washed twice with PBS, being careful not to dislodge the cells. This was then followed by the determination of luciferase gene expression using the Luciferase Reporter Gene assay system (Promega).

### **2.11.2 Competitive inhibition assay in the HepG2 cell line**

HepG2 cells were seeded in a 48-well plate at a density of  $2.5 \times 10^4$  cells per well in 0.3 ml complete medium. Thereafter the medium was removed and cells replenished with fresh medium. The lipoplexes were prepared to sub-optimal, optimal and super-optimal ratios as in section 2.5. Free asialofetuin (final concentration 200  $\mu$ M) was introduced to the cells 20 min prior to the addition of the lipoplexes. After lipoplex addition, the plate was incubated for a further 48 h at 37 °C. This was followed by the Luciferase assay for gene expression.

### **2.11.3 The luciferase assay**

The Luciferase Assay was carried out as per protocol set out by Promega. Briefly, after washing of cells with PBS, 100  $\mu$ l of freshly prepared of cell culture lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N,N'',N''-tetraacetic acid, 10 % v/v glycerol, 1 % v/v Triton X-100) (Promega) was dispensed into each well and the plate was gently rocked on a STR 6 (Stuart Scientific, Staffordshire, UK) platform rocker at 30 rpm for 15 minutes. Lysates were then transferred into microcentrifuge tubes and spun at 13 000 rpm in Eppendorf microcentrifuge for 30 secs. The level of luciferase gene expression was measured by adding 50  $\mu$ l of luciferase reagent (Promega) to 20  $\mu$ l of lysate or supernatant. Luminescence was measured using a Glomax Multi+ Detector system. Thereafter, protein content in the supernatant was determined using the BCA Protein Assay reagent (Sigma) with bovine serum albumin as the standard. The expression data are expressed as relative light units (RLU) per mg of total soluble cell protein. Two controls were employed i.e. C(-): untreated cells, C(+): cells with uncomplexed free DNA. All experiments were carried out in triplicate.

### **2.11.4 The bicinchoninic acid (BCA) assay**

Protein determination was carried out using the BCA assay. In order to construct a protein standard curve, standard BSA solutions (ranging from 0 to 30  $\mu$ g/50  $\mu$ l in increments of 5  $\mu$ g/50  $\mu$ l) were prepared in a final volume of 50  $\mu$ l 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 in a Mindray MR-96A microplate reader. The cell free extracts (50 µl) were mixed with BCA working reagent (1 ml) and treated in the same way as the standards. The soluble protein content of the extracts was obtained by extrapolation from the standard curve.

## **2.12 Statistical analysis**

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

## **2.13 Docking studies**

Docking studies were performed at Discipline of Pharmaceutical Chemistry, UKZN. Molecular flexible docking studies were performed using the AutoDock Vina and AutoDock graphical user interface (GUI). All non-standard amino acid residues were deleted. The crystal structure of the 3D ASGP receptor structure for carbohydrate-binding domain was downloaded from Protein Data Bank (Code IDV8, 2.3 Å resolution). All the crystallographic water molecules, other than the molecules forming coordinate bonds with Ca<sup>++</sup> ions within the binding sites, were removed. The bond orders and hydrogen atoms were assigned to define the correct ionization states. Optimized metal binding states were generated within pH 7.0± 4.0. An H-bond assignment was given to orient the water molecules to optimize H-bonding. Positional constraints were added to replace the oxygen atom of the Ca-coordinated water molecules as coordinate bonding of the ligand is essential for binding to ASGP receptor.

## CHAPTER THREE: RESULTS AND DISCUSSION

### 3.1 Physicochemical characterisation of liposomes

It is important to assess and analyse morphological characteristics of liposomes as they relate to functionality of the liposome-DNA complexes. Commonly investigated features include liposome size, surface charge, shape and lamellarity (Patil and Yadhav, 2014).

#### 3.1.1 Preparation of liposomes

##### 3.1.1.1 Components of liposomes viz. Chol-T, DOPE and PEG

All six liposomal formulations viz. SM1, SM2, SM3 and their PEGylated derivatives were visually observed to be stable for several months when stored at 4 °C. They comprised Chol-T and DOPE at varying molar ratios. Incorporating Chol-T in this study is a direct result of literature citations showing improved transfection by cholesterol-based lipids. Daniels *et al.* (2011) carried out a comparative analysis of use of Chol-T and Chol-Q as cationic cholesteryl moieties. Chol-T features a dimethylamino head group, and similar transfection agents including DC-Chol have been designed and reported to be efficient in transfection (Singh and Ariatti, 2006). DC-Chol represents the first cholesterol-based cationic lipids to be used in clinical trials owing to its low cytotoxicity (Battacharya and Bajaj, 2009). Chol-T was observed to result in the highest transfection efficiency, a threefold increase over Chol-Q, which has a trimethyl ammonium head group. The increase was attributed to promotion of endosomal escape of the DNA cargo. An important attribute of carbamate-linked lipids is the facilitated disconnection of hydrophobic and hydrophilic portions of the lipoplex as a result of the low pH in early endosomes. This in turn leads to DNA release (Balram *et al.*, 2009; Daniels *et al.*, 2011).

The hydrophobic cholesterol moiety not only enhances rigidity and thereby retention of the liposomal cargo, but also improves the stability of the liposomal bilayer. Additionally, there is protection of the liposomal membrane against dissociation by plasma proteins. These attributes are due to high gel-to-liquid crystalline phase  $T_m$  of about 55 °C – 58 °C which prevents coexistence of two phases at physiological temperature of 37 °C (Perche and Torchillin, 2013). Perhaps the most important reason relates to the ability of cholesterol to promote  $H_{II}$  phase organisation; a critical factor in endosomal escape. This

important trait is further exploited in the use of cholesterol in combination with a zwitterionic or neutral co-lipid, such as the fusogenic DOPE or DOPC. A study by Yang *et al.* (2013) investigating cholesterol content in DC-Chol/DOPE lipoplexes revealed that an increase in cholesterol content led to increased transfection activity. This was thought to be due to more stable liposomes with lower cytotoxicity profile and therefore higher transfection activity. It has been suggested that DOPE facilitates formation of lipid bilayers of cationic cholesteryl cytofectins. An important difference is that DOPC, unlike DOPE induces the lamellar phase in lipoplexes. DOPE however, was found to be more useful in its effect on transfection owing to its ability to induce inverted hexagonal phase. Study results showed that DOPE led to 70% better transfection than in complexes with same amount of DOPC. Another study demonstrated superior transfection results of DOPE when compared to the lamellar phase forming DPPE (Balram *et al.*, 2009; Ramezani *et al.*, 2009; Madeira *et al.*, 2011; Pisani *et al.*, 2011).

Investigations by Moghaddam and colleagues on lipoplex characteristics involving DOPE, DSPE, DOTAP and DSTAP revealed that the DSPE:DSTAP formulation was not possible. However, by introducing double bonds in the lipid tail through substitution of DSPE with DOPE or DSTAP with DOTAP in a DSPE:DSTAP lipid combination mixture, a less compact lipid system resulted. This system provided for better hydration and dispersion in water resulting in different structures such as micelles, inverted micelles and hexagonal or lamellar phases (Moghaddam *et al.*, 2011). By combining Chol-T and DOPE in a liposomal formula, it is expected that there would be increased transfection due to efficient endosomal escape of DNA. These results have been reported by Daniels *et al.*, (2011) who confirmed better transfection and additionally, reduced cytotoxicity of these liposomes.

Cationic liposomes, by virtue of their positive charge are prone to interactions with serum components which can lead to aggregated complexes. This may result in various morphological changes in size, charge and behaviour of lipoplexes. Charge neutralisation can result from the positively charged serum proteins coating the surface of the lipoplexes. This forms a corona, which creates a sticky adhesion resulting in vesicles aggregating. Additionally, the weakened repulsions due to neutralised surface charge can lead to the particles sticking together (Yang *et al.*, 2013). Aggregation and fusion not only impact on size, but also on the shelf life of liposomes. Electrostatic stabilisation can be

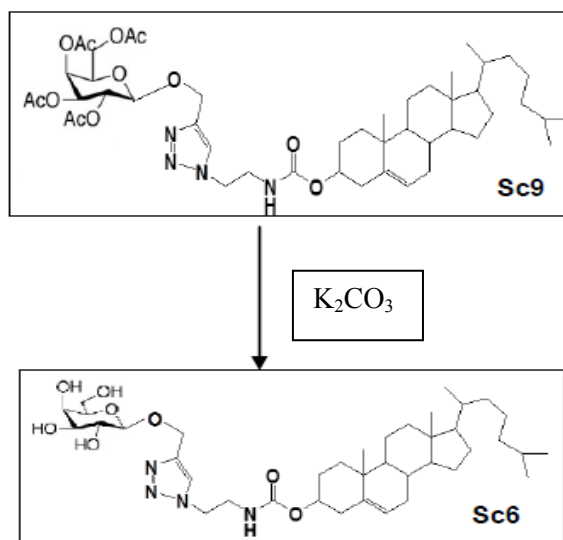
achieved by adjusting ionic strength or pH. This however, means that there is a degree of sensitivity related to changes in these parameters (Pannier *et al.*, 2008; Yadav *et al.*, 2011). PEG formulations have been associated with increased stability, increased DNA protection, reduced surface charge, and reduced cytotoxicity. PEG-liposomes are highly stable dispersions which display enhanced transfection potential (Pannier *et al.*, 2008; Tros de Illarduya *et al.*, 2010). For this reason, comparative analysis of PEG effects on liposomes was done, where PEG-modification was achieved by introducing 5 % molar ratio of DSPE-PEG<sub>2000</sub>.

Liposomes composed of cholesterol and phospholipids are impacted by the amount of grafted PEG and length/molecular weight of the polymer. Long-chained and higher molecular weight DSPE-PEG<sub>2000</sub> and DSPE-PEG<sub>5000</sub> have been reported to produce better results than shorter chained PEG<sub>750</sub> and PEG<sub>120</sub>. Managit *et al.*, 2003 demonstrated that PEG<sub>2000</sub> displayed a markedly higher longevity blood circulation than PEG<sub>350</sub> to PEG<sub>750</sub>. PEG<sub>2000</sub>-Gal liposomes were eliminated at a slower rate. PEG<sub>2000</sub> has been shown to be the best compromise in respect of molecular weight and was therefore chosen for this study. The quantity of PEG to be incorporated is another important factor to take into account. PEG concentrations of up to 6 mol % were found to shield surface charge moderately, but at higher concentrations of 15% mole ratio, PEG completely abolished the effect of the charged groups. This becomes critical for cationic lipid-mediated delivery, which relies on the positive charge. Surface modification with 5.7% mol PEG was shown to improve ODN loading where structural stability of the resulting lipoplexes comprising cationic lipid DOTPA, DOGS, DDAB; helper lipid DOPE and PEG were not lost, while size was affected due to aggregation (Campbell *et al.*, 2002; Immordimo *et al.*, 2006). Kibria *et al.*, (2011) have also reported on use of 5 mol% DSPE-PEG<sub>2000</sub> while Dadashzadeh *et al.* (2010) used 6.25 mol%. Hence in this study, we opted to use 5% mole ratio PEG which has been shown to reduce clearance while the cationic charge of the liposomes would still be retained (Perche and Torchillin, 2013).

### ***3.1.1.2 Targeting ligands***

Use of sugar-based cationic cholesterol derivatives has been extensively investigated for their ability to increase transfection in hepatocytes (Kawakami, 2000). The galactoside

cholesterol derivative ligand Sc9 was previously synthesized (Department of Chemistry, University of the Witwatersrand) by a copper-mediated „click“ reaction between the 2-propynylcarbamate derivative of cholesterol and *O*-tetraacetate galactose azide (Hean *et al.*, 2010). In an additional deacetylation step, the galactoside viz. Sc6, was formed (Figure 3.1).



**Figure 3.1** Click chemistry galactosides viz. Sc6 and Sc9

Click chemistry has emerged as an important tool when preparing structurally diverse glycoconjugates for biomedical purposes. This relates to its numerous advantages such as shorter synthetic sequences, higher cyclisation yields and ease in resolving anomeric mixtures thus allowing for preparation of well-defined analogues (Wilkinson *et al.*, 2009). Synthesis of the galactoside derivative, Sc9 using „click chemistry“ was found to be desirable owing to its simplicity, and it allows for easier scale-up preparation of the galactosides (Wilkinson *et al.*, 2009; Hean *et al.*, 2010). Additionally, Sc9 has been incorporated in liposomal formulations for improved efficiency to the liver. The lipoplexes were examined for their ability to deliver siRNA for inhibition of hepatitis B virus replication. They were reported to be useful vectors, displaying minimal cytotoxicity and immunostimulatory effects, even at high siRNA concentrations (Hean *et al.*, 2010).

The galactosides, Sc6 and Sc9 were successfully incorporated into the liposomal formulations SM2 and SM3 (and their PEGylated derivatives), respectively. Comparative analysis could thus be drawn on the effect of acetylation and deacetylation of the derived

liposomes, with particular focus on their respective affinity for the ASGP receptor and transfection efficiency for liver-targeted delivery.

### **3.1.1.3 Preparation method**

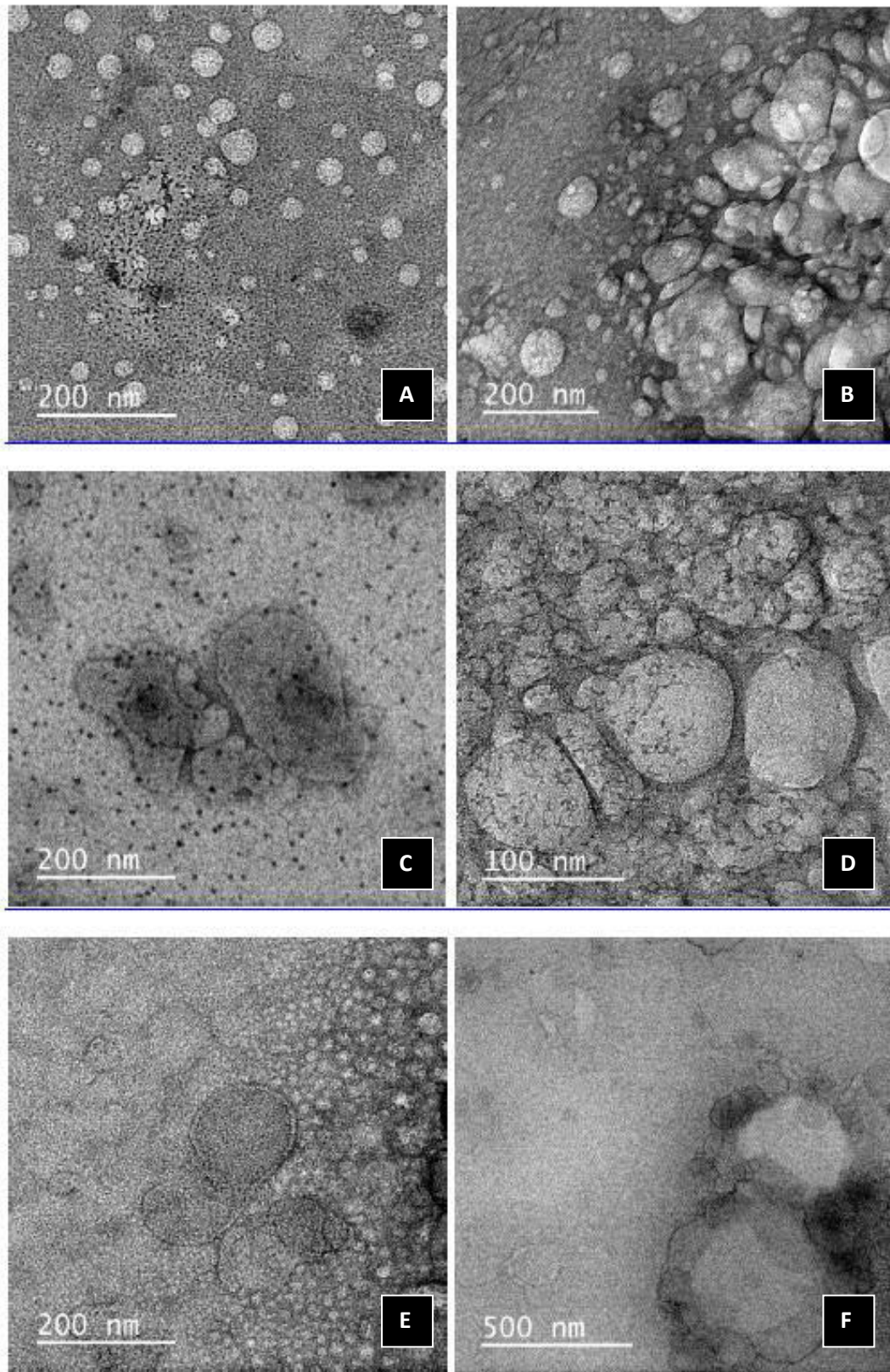
Formulation procedure and components impact the physical nature of lipoplexes (Ferrari *et al.*, 2002). Unilamellar liposomes can be produced by various methods including the classical thin film hydration (Levine *et al.*, 2013). Thin film hydration method was used to prepare the liposomal dispersion in this study. This technique requires that lipids be dissolved in organic solvents, usually chloroform or chloroform:methanol mixtures (Dua *et al.*, 2012). Following evaporation of the solvent, thin lipid films are hydrated by adding an aqueous medium such as HBS. Clear and stable MLV suspension were observed, which could be of heterogenous size and lamellarity (Patil and Jadhav, 2014).

Reduction in liposome size presents an important aspect in liposomal preparation, as it increases cellular uptake where the physiological barriers *in vivo* can be overcome (Malaekheh-Nikouei *et al.*, 2009). Whereas other methods are also known to reduce size such as French press and membrane extrusion, ultrasonic waves using a bath sonicator result in SUVs of satisfactory sizes. As expected, the suspension resulting from sonication was observed to be opalescent to clear and transparent. It is thus assumed that SUV of similar properties were produced; however, it is possible to have variations in size and size distributions between batches due to the fact that conditions of sonication are not always reproducible (Dua *et al.*, 2012; Laouini *et al.*, 2012; Akbarzadeh *et al.*, 2013; Patil and Jadhav, 2014).

### **3.1.2 Cryo-TEM images of liposomes**

Cryo-TEM allows for visualisation of liposomal morphology, lamellarity and importantly, it provides for quick estimation of size of individual liposomes (Smith *et al.*, 1997; Patil and Jadhav, 2014). All liposomal samples appeared to be unilamellar, and were observed to be predominantly spherical and distinct vesicles as observed in Figure 3.2. Studies on galactosylated cholesterol derivatised liposomes have shown similar morphological characteristics (Singh and Ariatti, 2006).





**Figure 3.2** Cryo-TEM images of liposomes showing distinct, spherical vesicles and clustered arrangements. A) SM1, B) SM1(+PEG), C) SM2, D) SM2(+PEG), E) SM3 and F) SM3(+PEG).

The vesicle sizes for the untargeted SM1 were reported to have a mean diameter of 110 nm. Similarly, Dorosamy *et al.* (2012) reported untargeted Chol-T:DOPE vesicle size diameters in the 80 – 100 nm range while inclusion of Chol- $\beta$ -Gal resulted in 30 – 80 nm size diameters. Addition of targeting moieties to the liposomal formations of SM2 and SM3 led to mean size diameters of 235 nm while the PEGylated derivatives measured had mean diameters ranging from 35 – 126 nm.

While cryo-TEM provided an estimation of sizes, these results cannot be conclusively taken to be representative of the liposomal populations in a sample. This is because most of the liposomes exist as a non-homogenous population. Sample preparations in TEM are generally complicated and can result in size and shape distortions. Cryo-TEM remains preferable to other microscopic techniques as it provides some morphological view; however, information gathered from this technique should ideally be used in conjunction with other techniques such as DLS (Laouini *et al.*, 2012). From these results, it could be concluded that SUVs were successfully prepared as discernible vesicles with mean sizes ranging between 35 nm and 235 nm.

### **3.1.3 Size and zeta potential measurements**

Dynamic light scattering techniques have been employed for quick estimation of size, measurements and distribution of liposomal formulations. Other colloidal properties are also assessed using this technique. Zeta potential ( $\zeta$ -potential) studies allow for the estimation in how the negatively charged nucleic acids interact with the positive charges of the cationic liposome (Tros de Ilarduya *et al.*, 2010; Patil and Jadhav, 2014).

All formulated liposomes were found to be below 150 nm in size (Table 3.1). This is a particularly desirable as smaller particles with size range of 50 – 300 nm are expected to display significantly longer circulation time due to reduced RES uptake and reduced clearance compared to larger particles (Bae and Park, 2011).

**Table 3.1** Size distribution and zeta potential measurements for the liposomes using DLS

	<b>Size diameter (nm)</b>	<b>PDI value</b>	<b>ζ-potential (mV)</b>
<b>SM1</b>	125.00±0.44	0.208	+13.53
<b>SM1 (+ PEG)</b>	88.07±0.76	0.225	-1.92
<b>SM2</b>	121.10±1.91	0.212	+2.36
<b>SM2 (+ PEG)</b>	81.57±0.33	0.202	-4.97
<b>SM3</b>	140.27±0.06	0.228	+14.27
<b>SM3 (+ PEG)</b>	129.57±2.35	0.309	-2.21

No significant size difference was noted between the non-targeted control SM1 and the targeted SM2. As expected, PEG introduction resulted in clear compaction of sizes to ~80 nm for derivatives SM1(+PEG) and deacetylated SM2(+PEG). Daniels *et al.*, (2011) also reported stable PEGylated liposomes which were found to be in the 40 – 100 nm size range. Reduction in particle size diameter is attributed to the steric stabilisation through strong inter-membrane repulsive forces. The reduced Van der Waals forces between particles signify a lower tendency towards aggregation or fusion thus preventing formation of larger vesicles.

Similar observations by Nie *et al.* (2012) confirmed that incorporation of 5 mol% PEG resulted in a narrower size distribution than the conventional unPEGylated derivatives. Interestingly, liposomal formulations SM3 and its PEGylated derivative which comprised acetyl groups presented larger mean diameters of 140.27 nm and 129.57 nm, respectively. This was found to be larger than the liposomes containing the conventional D-galactopyranosyl group. This might be explained to structural dimensions where the acetyl groups possibly occupy more space due to the protruding methyl groups. Thus a bulkier configuration is most likely with SM3 and its PEGylated derivative SM3(+PEG) due to –CH<sub>2</sub>COO-(acetoxy) groups as opposed to -OH (hydroxyl) groups.

The polydispersity index (PDI) provides a means to monitor the relative width of liposomal size distributions (Levine *et al.*, 2013). PDI values ranging between 0.1 and 0.2 are indicative of narrow particle size distributions (Li *et al.*, 2011). And if these values are in excess of 0.25, the particle sizes are said to be distributed over a broad range. All liposomes displayed PDI values which were less than 0.25 (Table 3.1). However, the acetylated SM3(+PEG) had PDI of 0.309 which suggests existence of particle sizes which fall out of the given range. Thus, for most of the liposomes, size distribution was mainly monodispersed and homogenous around the z-average diameter given. SUVs are however, inherently unstable and will therefore tend to fuse thereby forming larger vesicles therefore it is not uncommon to have heterogeneity where there might be a small population present made up of larger sized vesicles (Patil and Jadhav, 2014).

$\zeta$ -potential is an important parameter used to characterise the surface of charged colloidal systems. It represents the electric potential in the interfacial double layer of nanoparticles. Measurements are based on particle electrophoretic mobility, which is converted to  $\zeta$ -potential using Smoluchowski or Huckel theory (Salopek *et al.*, 1992; Laouini *et al.*, 2012; Honary and Zahir, 2013). The existence of positive charge is an important requirement in pDNA-cationic liposomes condensation (Ramezani *et al.*, 2009).

The untargeted control SM1 preparation exhibited an overall positive charge of +13.53 mV. The cholesteryl galactoside targeting moieties by galactoside cholesterol Sc6 (in SM2) and Sc9 (in SM3) had varying effects on the surface charge. SM2 liposome displayed a near neutral surface charge of +2.36 mV, while the acetylated SM3 liposome retained a positive charge of +14.27 mV. This could play an important role in how they interact with the negatively charged DNA, where SM3 would be expected to condense it more efficiently. The absolute value of surface charges of PEGylated derivatives viz. SM1(+PEG), SM2(+PEG) and SM3(+PEG) was reduced upon DSPE-PEG<sub>2000</sub> inclusion. Reduction resulted in near-neutral charges at -1.92, -4.97 and -2.21 mVs, respectively. This can be explained in the context of PEG shielding effect where the hydrophilic polymer acts by masking of the surface charge and lowering electrophoretic mobility of the particles (Campbell *et al.*, 2002; Nie *et al.*, 2012). The surface charge of the near-neutral SM2 was not significantly reduced upon PEG inclusion, which was consistent with results by Nie *et al.* (2012). While it is true that conventional liposomes with neutral charge are unstable and aggregate, this is not likely to occur in this case as steric-

repulsive propensity is conferred by the PEG polymer (Xiang and Zhang, 2013). In fact, neutrally charged PEGylated doxorubicin liposomes were shown to exhibit increased therapeutic activity, unaffected by their charge (Perche and Torchillin, 2013).

## **3.2 Liposome-DNA interactions**

### **3.2.1 Gel retardation assay**

The gel retardation assay is commonly used to study the pDNA binding affinity of cationic liposomes. The formation of lipoplexes is due to strong electrostatic interactions between the cationic charges from lipids and the anionic DNA. The principle is thus based on the electrophoretic migration of the negatively charged pDNA to the positive anode in an electric field unless it is retarded or fully bound by the liposome to form large complexes possessing an electroneutral charge (Li *et al.*, 2011). By determining the least amount of liposome required to bind all pDNA, optimal binding ratios can be obtained.

In lanes 1 of each gel (Figures 3.3 and 3.4), three conformations of uncomplexed pDNA are visible, namely the superhelical, closed circular and linear. From lanes 2 to 8, increasing amounts of liposomes were added resulting in DNA becoming liposome-bound. This is also observed as a reduction in band intensity because of reduced amounts of DNA migrating into the gel.

The untargeted control liposomes (SM1) were shown to fully bind the pDNA at a N:P ratio of 2.5:1. This end-point ratio possibly represents an N:P ratio where the liposome cationic charge is fully neutralised by the negatively charged phosphate groups of DNA. No further migration of free pDNA was observed in subsequent lanes, suggesting that the DNA was indeed fully-bound. The resulting lipoplexes could also be large and unable to diffuse through the agarose matrix as reported by Li *et al.*, (2011). The PEGylated derivative SM1(+PEG) was observed to bind at a higher end-point ratio of 3:1. Inclusion of the galactosides *viz.* Sc6 and Sc9 in SM2 and SM3, respectively did not alter the optimal N:P binding ratios when compared to the untargeted SM1. These were maintained at 2.5:1 for both liposomes (Figure 3.4).

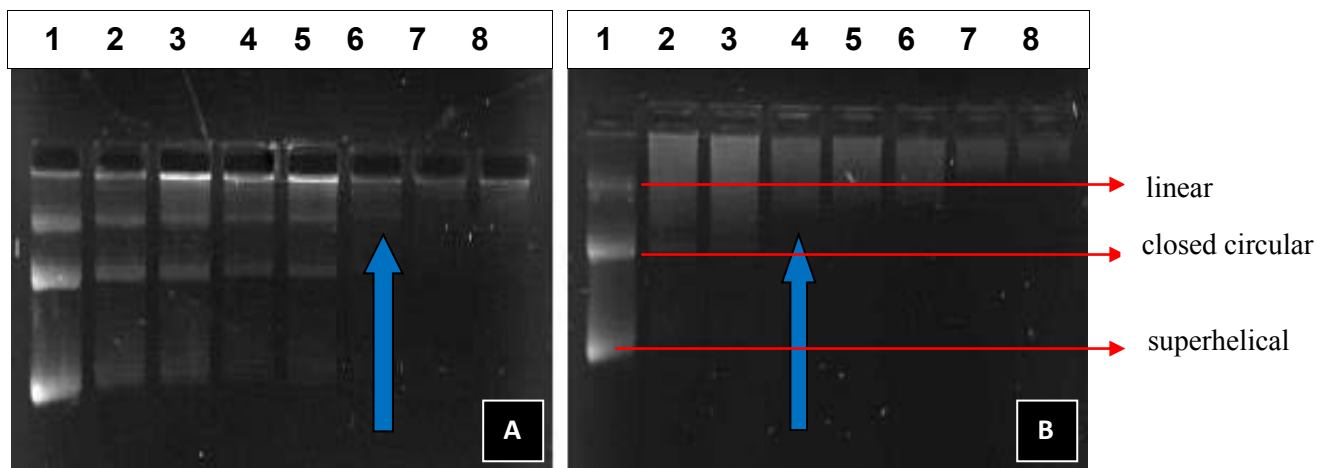


Figure 3.3 Gel retardation assays showing optimal binding ratios for A) SM1 where lanes 2 – 8 corresponded to N:P ratios 0.5:1, 1:1, 1.5:1, 2:1, 2.5:1, 3:1 and 3.5:1, respectively; and B) SM1(+PEG) where lanes 2 – 8 corresponded to N:P ratios 1:1, 2:1, 3:1, 4:1, 5:1, 6:1 and 7:1, respectively.

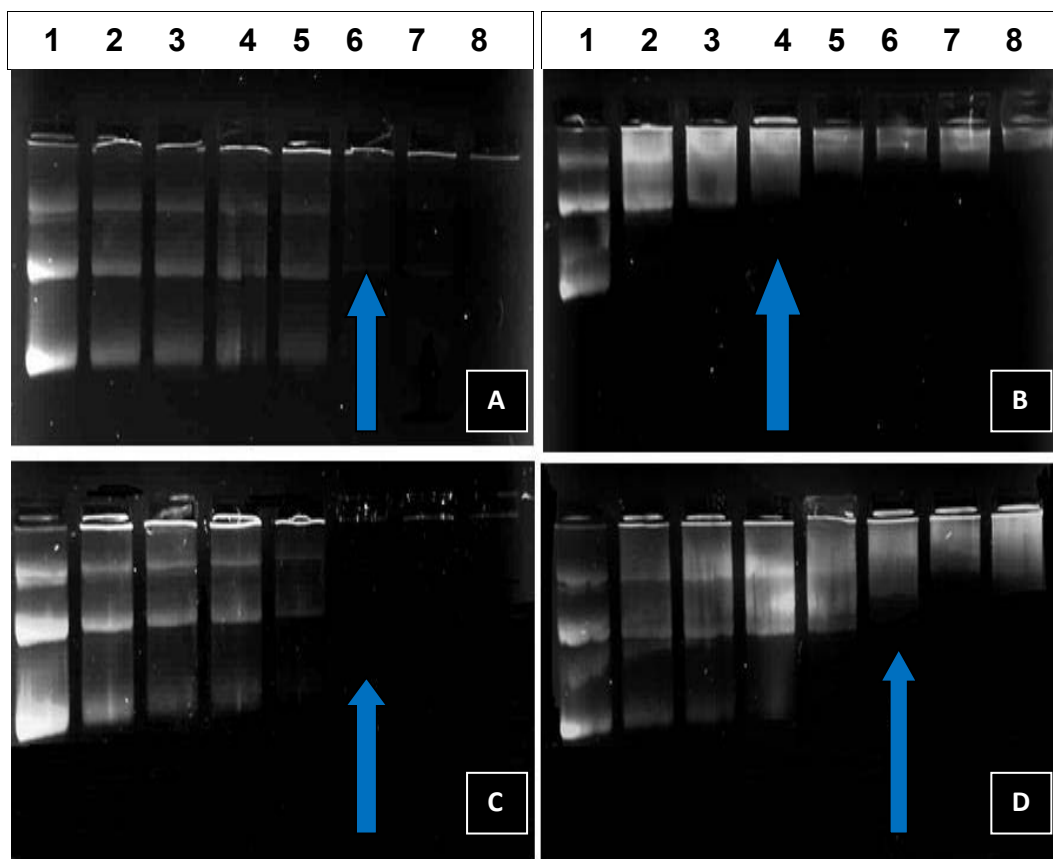


Figure 3.4 Gel retardation assays showing optimal binding for A) SM2 where lanes 2 – 8 corresponded to N:P ratios 0.5:1, 1:1, 1.5:1, 2:1, 2.5:1, 3:1 and 3.5:1, respectively; B) SM2(+PEG) where 2 – 8 corresponded to N:P ratios 1:1, 2:1, 3:1, 4:1, 5:1, 6:1 and 7:1, respectively; C) SM3 where lanes 2 – 8 corresponded to N:P ratios 0.5:1, 1:1, 1.5:1, 2:1, 2.5:1, 3:1 and 3.5:1, respectively; and D) SM3(+PEG) where lanes 2 – 8 corresponded to N:P ratios 0.5:1, 1:1, 1.5:1, 2:1, 2.5:1, 3:1 and 3.5:1, respectively.

**Table 3.2** Binding optimal N:P ratios and their corresponding N:P weight ratios

	<b>Optimal N:P charge ratio (+/-)</b>	<b>Optimal N:P weight ratio (w/w)</b>
<b>SM1</b>	2.5:1	4.41:1
<b>SM1(+PEG)</b>	3:1	5.84:1
<b>SM2</b>	2.5:1	4.39:1
<b>SM2(+PEG)</b>	3:1	6.45:1
<b>SM3</b>	2.5:1	4.49:1
<b>SM3(+PEG)</b>	2.5:1	5.47:1

However, upon PEG inclusion, there seemed to be increased optimal binding for the SM2(+PEG) derivative while the acetylated SM3(+PEG) maintained an optimal N:P binding ratio of 2.5:1. The corresponding optimal N:P weight ratios as observed in [Table 3.2](#), showed comparable ratios for the unPEGylated derivatives. Higher optimal N:P weight ratios were observed for the PEGylated derivatives with the highest weight ratio obtained for the SM2(+PEG) derivative.

It is interesting to note that the PEGylated derivatives viz. SM1(+PEG), SM2(+PEG) and SM3(+PEG) did not exhibit distinct obvious end-points. There was seemingly weaker binding avidity for DNA compared to their unPEGylated derivatives. Migration patterns of DNA with these liposomes exhibited what appeared to be streaking of the pDNA down the gel. This streaked or smeared DNA did not comprise either the circular or supercoiled conformations and rendered it difficult to determine the exact end-point ratios. The point at which distinct bands of DNA were no longer visible as distinct bands on the gel was accepted as the end-point ratio. Thus the PEGylated derivative as seen in (b) and (d) were assumed to bind the pDNA at a ratio of 3:1 for SM1(+PEG) and SM2(+PEG) and 2.5:1 for SM3(+PEG), respectively - albeit in an unconvincing manner. This general

characteristic of reduced DNA-binding abilities observed in PEGylated derivatives has been reported by other authors (Zhang *et al.*, 2010; Singh *et al.*, 2011)

Another possible explanation relates to the partial shielding of the cationic headgroups by the PEG polymer chains, which impedes their interaction with DNA (Templeton, 2002). It has been established that grafting densities of PEG will generally influence conformational disposition and configuration assumed on the liposomal surface. Grafted PEG<sub>2000</sub> will most likely result in the brush regime where the polymer projects by 50Å from liposomal surface. This can result in strong intermembrane repulsive forces leading to reduced DNA-liposome interactions. These effects may be compounded by the water shell resulting from the PEG polymer surrounding the liposomal surface (Immordino *et al.*, 2006; Daniels *et al.*, 2011; Gorle *et al.*, 2014). However, Daniels *et al.*, (2011) reported that liposomes comprising DSPE-PEG<sub>2000</sub> at 5 mol% had resulted in complete retardation of plasmid DNA.

Acetylation and PEGylation in SM3 and SM3(+PEG) derivatives surprisingly resulted in better associations with DNA. This is despite the assumption that the negatively charged acetyl groups would not allow for tight DNA-binding. However, it must be noted that the acetylated SM3 liposome had in fact retained positive charge measured at zeta potential of +14.27 mV far better than its SM2 counterpart at +2.36 mV. This would lead to better interactions with the negatively charged DNA. This is in contrast with reports showing acetylated and PEGylated dendrimers which displayed less condensed DNA when complexed with acetylated dendrimers when compared to the unmodified dendrimers (Fant *et al.*, 2010).

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.

### **3.2.2 Ethidium bromide intercalation assay**

The ethidium bromide intercalation assay is routinely carried out in order to monitor and evaluate the ability of liposomes to condense and compact the DNA using the monovalent



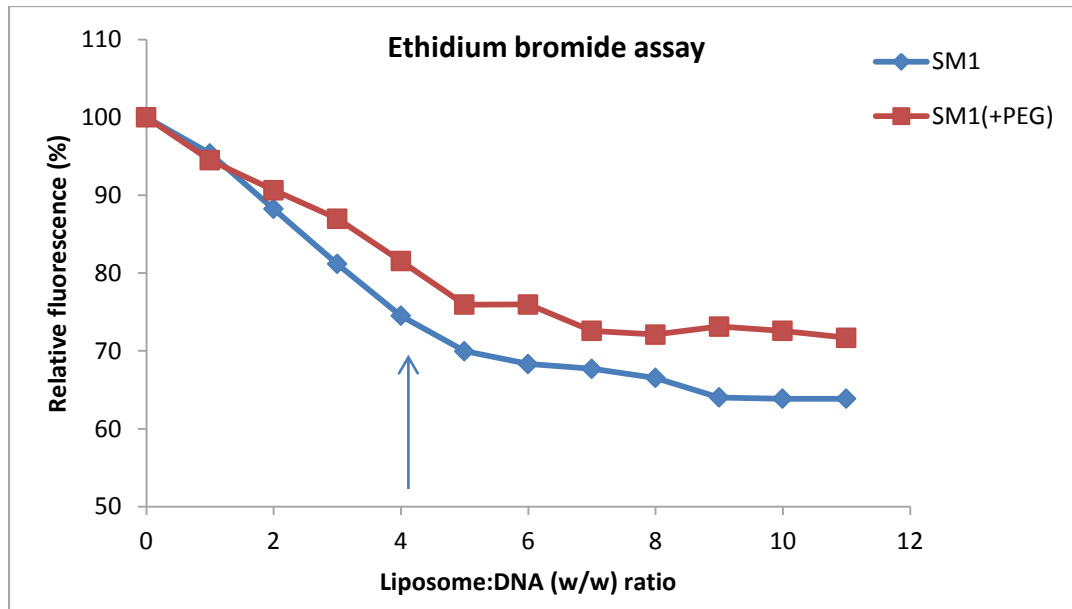
DNA-intercalating agent, ethidium bromide. The DNA-intercalated ethidium bromide dramatically enhances fluorescence, but it is quenched when it is displaced by higher affinity compounds that bind to DNA e.g. when adding liposomes to form complexes with the DNA (Tros de Ilarduya *et al.*, 2010).

Cationic liposome preparations are known to displace DNA-associated ethidium. By stepwise addition of cationic liposomes to the ethidium bromide-labeled DNA, condensation by liposomes results in stepwise displacement of the ethidium bromide and leads to reduced fluorescence (Pires *et al.*, 1999; Singh and Ariatti, 2006).

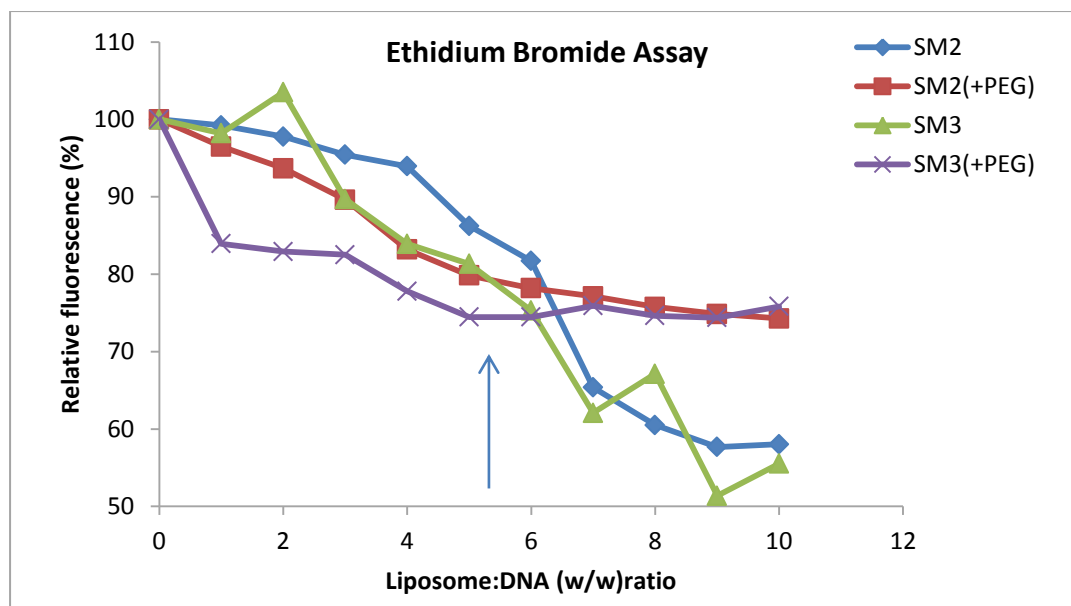
The control liposome SM1 and its PEGylated derivative, SM1(+PEG) (Figure 3.5) showed that upon stepwise addition of the respective liposomal formulation, a point of inflection was observed, which as at approximately 70% relative fluorescence. This point corresponded with the complete association of DNA with liposomes which also corresponded to the optimal N:P weight ratio of just above 4 as determined by gel retardation analysis. This is in confirmation of the results obtained in the gel retardation assay. The PEGylated SM1 derivative was observed to exhibit a reduced drop in fluorescence, which relates to looser associations with DNA compared to the unPEGylated derivative. This was in agreement with results obtained by Gorle *et al.* (2014) where ethidium displacement was greatest with untargeted unPEGylated liposomes than in the PEGylated-untargeted lipoplexes.

This trend is also seen in the targeted unPEGylated derivatives viz. SM2 and SM3 which showed far better condensation of DNA than their PEGylated derivatives (Figure 3.6). The targeted-PEGylated complexes also displayed a somewhat compromised quality of DNA condensation. This can be explained by the PEG shielding effect where the cationic charges of the liposomes lead to reduced interactions with the pDNA. PEGylation has been said to significantly reduce pDNA binding affinity of DC-Chol/DOPE liposomes (Chen *et al.*, 2013). In a study by Fant *et al.* (2010), availability for ethidium intercalation was observed to be significantly higher in the DNA-dendrimer complexes comprising acetylation and PEGylation modifications than in unmodified ones (Fant *et al.*, 2010). Additionally, low grafting density is essential so that the „mushroom“ regime assumed results in limited effect on the binding affinity of DNA to liposomes. At higher grafting density, the brush conformation can limit DNA interaction with the cationic headgroups

projected on the liposomal surface. In that instance, binding of DNA to the liposomal cationic headgroup can be found to be much looser and tenuous (Daniels *et al.*, 2011; Gorle *et al.*, 2014).



**Figure 3.5** Ethidium bromide interaction assays with stepwise addition of untargeted liposomes SM1 and its PEGylated SM1(+PEG) derivative. Arrow indicates point of inflection.



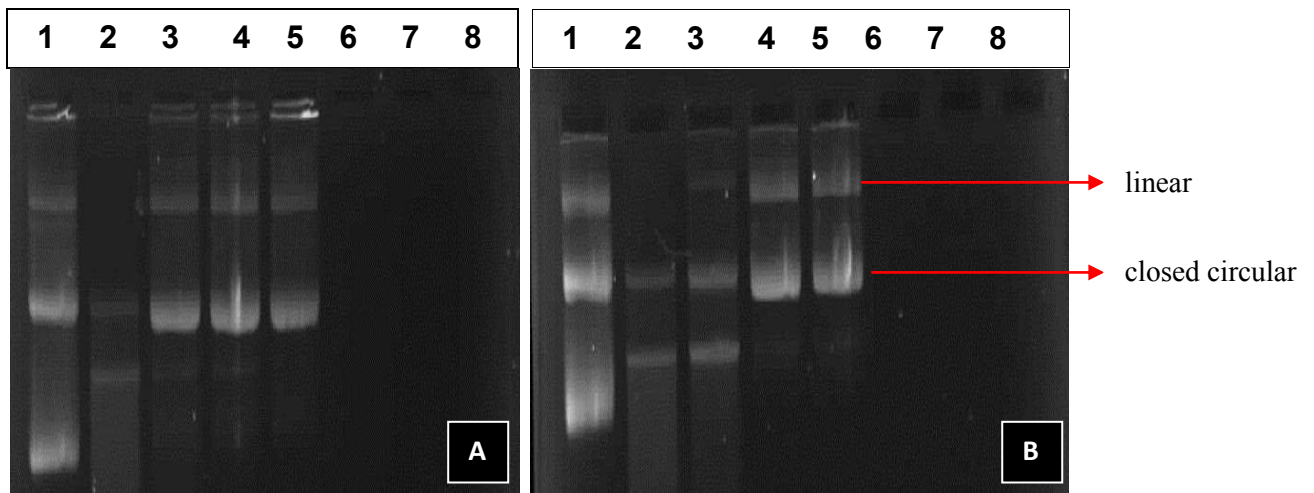
**Figure 3.6** Ethidium bromide interaction assays with stepwise addition of targeted liposomes SM2, SM3, and their PEGylated SM2(+PEG) and SM3(+PEG) derivatives. Arrow indicates point of inflection.

A sharper point of deflection was observed for the acetylated SM3 liposome which was observed at 40% displacement while a much steadier decline in fluorescence was observed for the SM2 liposome. For both these derivatives, the plateau points were reached at the correspondingly higher weight ratios. Other authors have reported a trend where targeting moieties such as  $\beta$ -D-galactopyranosyl resulted in higher N:P end-point ratios than untargeted derivatives (Dorasamy *et al.*, 2012; Gorle *et al.*, 2014). Once a plateau is reached, any further addition of liposome was observed to result in more modest reductions in fluorescence. This suggests that there was no further significant pDNA condensation beyond this point.

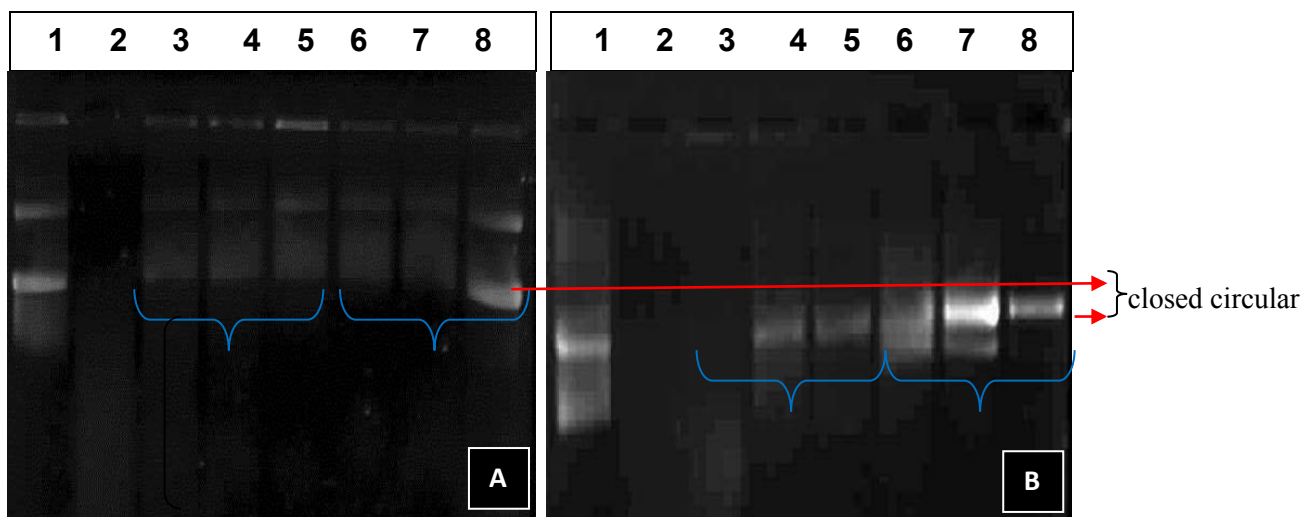
### 3.3 Serum nuclease digestion assay

Efficient gene delivery requires that the DNA cargo be protected from adverse effects of serum components. These include, but are not limited to, structural alterations of lipoplex structure which may lead to digestion by serum nucleases (Resina *et al.*, 2013). In this assay, the binding interaction of the DNA and liposome are investigated for their ability to provide protection to the bound DNA.

Lanes 1 in Figures 3.7 and 3.8 show the untreated DNA displaying the three DNA conformations, *viz.* linear, closed circular and superhelical. Lanes 2 represent the unbound naked DNA which is exposed to serum and is visibly digested by the nucleases. Lanes 3, 4 and 5 contain increasing ratios of liposome:DNA which conferred different degrees of protection. The untargeted control SM1 demonstrated good protection of the DNA in increasing ratios; however, there is evidence of nicking of the superhelical DNA (Figure 3.7). This is observed with the disappearance of this band, while the closed circular DNA is retained. PEGylated derivative SM1(+PEG) exhibited similar protection patterns and nicking of the superhelical DNA is evident in all ratios. The greatest susceptibility was observed in the PEGylated SM1(+PEG) liposome as the N:P ratio decreased to 2.5:1. This could be related to the unbound DNA molecules which are readily accessible for degradation.



**Figure 3.7** Serum nuclease digestion assays for liposomes: A) lanes 3-5: SM1 at N:P ratios of 2:1, 2.5:1, 3:1 and 3.5:1, respectively and B) lanes 3-5: SM1(+PEG) at N:P ratios of 2.5:1, 3:1 and 3.5:1, respectively.



**Figure 3.8** Serum nuclease digestion assays for liposomes: A) lanes 3-5: SM2 at N:P ratios of 2:1, 2.5:1, and 3:1, respectively; lanes 6-8: SM2(+PEG) at N:P ratios of 3:1, 3.5:1 and 4:1, respectively; and B) lanes 3-5: SM3 at N:P ratios of 2:1, 2.5:1 and 3:1, respectively; lanes 6-8: SM3 (+PEG) at N:P ratios of 2:1, 2.5:1 and 3:1, respectively.

The targeted SM2 liposome was also observed to provide partial protection of the DNA from nuclease degradation at all ratios as seen in Figure 3.8. However, the integrity of the protected pDNA had been preserved as seen by presence of both linear and closed circular conformations. A similar protection pattern was observed for PEGylated derivative SM2(+PEG). The acetylated SM3 liposome and its PEGylated derivative also resulted in compromised protection for the pDNA cargo. What was different with these derivatives however, was the evident nicking of the superhelical pDNA as its band is not seen on the gel. The least protection was afforded by the SM3 complex at the lowest N:P ratio of 2:1 as it resulted in complete degradation of the pDNA cargo. However, at optimal ratios, both SM3 and SM3(+PEG) resulted in increased band intensity of the closed circular form of DNA. This evidently suggested that nicking resulted in increased amounts of closed circular DNA being protected.

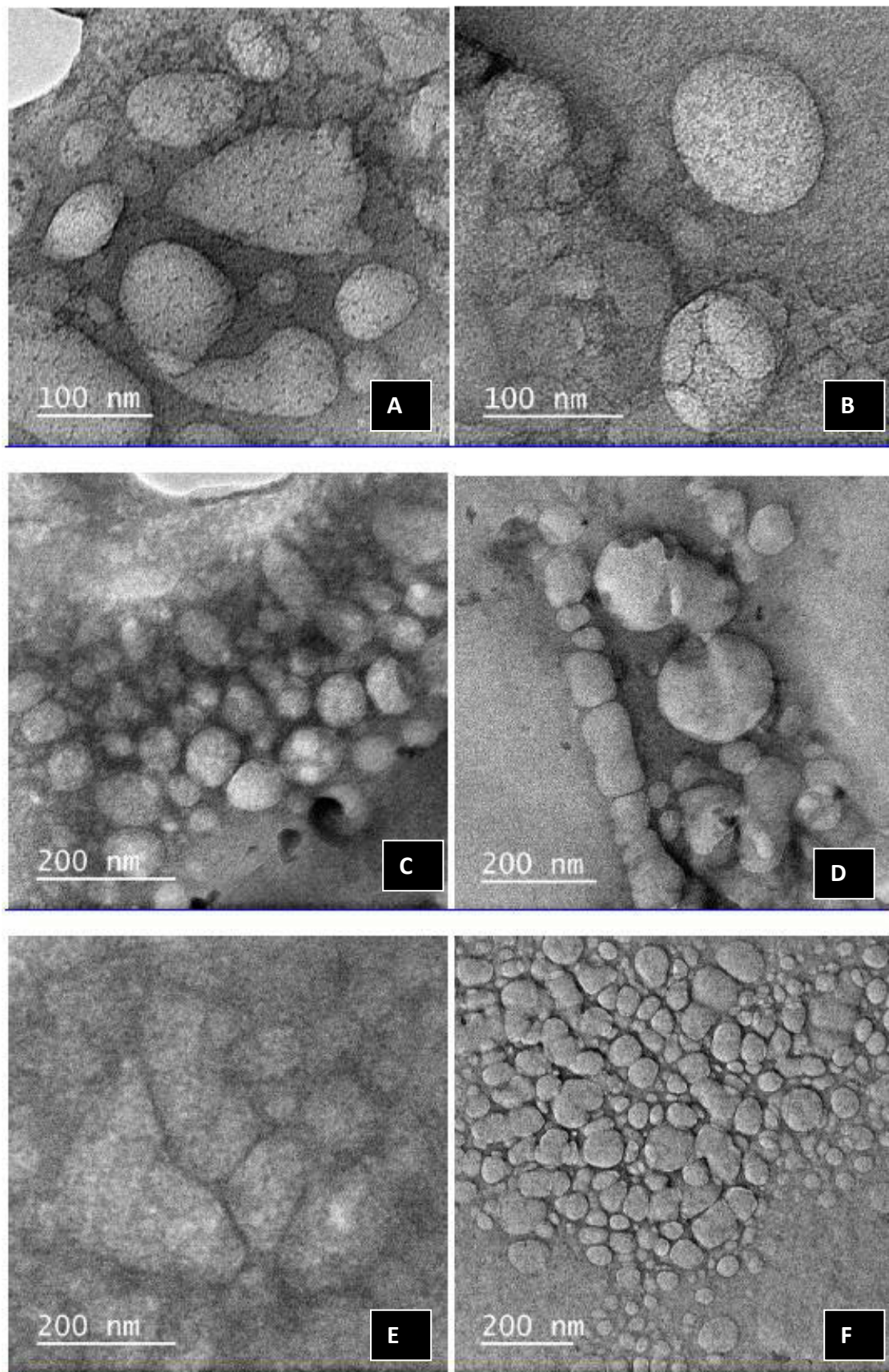
### 3.4 Assembly of lipoplexes

Significant changes take place with regards to the physico-chemical features of cationic liposomes upon complexation with negatively charged nucleic acids (Pires *et al.*, 1999; Madeira *et al.*, 2011; Paecharoenchai *et al.*, 2012).

### 3.4.1 Cryo-TEM of lipoplexes

Morphological characteristics of lipoplexes can be examined using cryo-TEM. Lipoplex structure has been linked to transfection efficiency, whereby size and shape of complexes can influence the internalisation pathway to be taken (Van Groll *et al.*, 2006; Madeira *et al.*, 2011). While cryo-TEM is a more reliable method of visualisation, it is limited due to low contrast which can result in some details not being observed. Additionally, the sample observed is not representative of the entire lipoplex population.

Lipoplexes prepared at liposome:DNA ratio corresponding to the minimum liposome suspension required to bind fully 0.5  $\mu\text{g}$  DNA were investigated. Micrographs showed varied arrangements seen as globular aggregates and predominantly cluster organisations of unilamellar vesicles (Figure 3.9). The distinct necklace-like structures observed in SM2(+PEG) (Figure 3.9 D) have been reported to be characteristic of linearised DNA complexes, while compact aggregates are commonly associated with circular DNA complexes (Van Groll *et al.*, 2006). An analysis of lipoplexes containing DOPE showed that DNA molecules are often sandwiched between the lamellae in exhibiting clustered multilamellar structures. Cationic lipoplexes containing cholesterol-based cytofectins have previously been reported to have exhibited compact aggregates (Ropert, 1999; Singh and Ariatti, 2006; Singh *et al.*, 2007).



**Figure 3.9** Cryo-TEM images of lipoplexes showing spherical vesicles arranged in linear as well as clustered aggregates. A) SM1, B) SM1(+PEG), C) SM2, D) SM2(+PEG), E) SM3 and F) SM3(+PEG)

The complex mean diameters of the untargeted control lipoplexes were found to range from 65 nm to 120 nm. The largest particle sizes were measured for targeted lipoplexes of SM2 and SM3 at ~ 250 nm. The PEGylated derivatives viz. SM1(+PEG) and SM2(+PEG) yielded lipoplexes which were marginally smaller, and consistent with what is expected for PEGylated formulations. Lipoplexes formed as a result of nucleic acids interacting with unilamellar vesicles of cationic lipids such as DOTAP combined with DOPE and cholesterol have been reported to result in multilamellar organisations, whereby the DNA is intercalated between the cationic lipid bilayers (Montier *et al.*, 2008).

### 3.4.2 Size and zeta potential measurements of lipoplexes

Size measurements of the complexes by DLS were given as a mean diameter (z-average). Generally, an increase in vesicle size is a result of reduced electrostatic interactions following association with pDNA. This in turn results in the tendency of lipoplexes to aggregate, thereby forming larger complexes (Mogghadam *et al.*, 2011). Complexation with DNA resulted in an increase in vesicle size for all liposomal formulations with a relatively homogenous size distribution (Table 3.3).

**Table 3.3** Size distribution and zeta potential measurements for the lipoplexes using DLS

	<b>Z-average (nm)</b>	<b>PDI value</b>	<b>ζ-potential (mV)</b>
<b>SM1</b>	1163.00±15.72	0.237	-25.93
<b>SM1(+PEG)</b>	99.76±1.56	0.241	-5.13
<b>SM2</b>	935.30±16.89	0.186	-28.00
<b>SM2(+PEG)</b>	102.77±0.75	0.264	-5.24
<b>SM3</b>	433.67±8.41	0.259	-15.37
<b>SM3(+PEG)</b>	126.87±1.29	0.247	-4.52



The untargeted and unPEGylated SM1 lipoplex displayed the largest vesicle size at over 1000 nm with a relatively narrow distribution of 0.237. Size diameters greater than 1000 nm of unPEGylated and untargeted lipoplexes of comparable composition were previously reported (Gorle *et al.*, 2014). These could also be explained in terms of stability properties, where the unPEGylated untargeted may aggregate and vesicle fusion processes could then result in larger complexes. The rigidity conferred by cholesterol ensures regular positioning of the positively charged cytofectin head groups on the lipidic surface. Thus upon interactions with DNA, there can be a formation of colloiddally stable and negatively charged complexes, with excess uncondensed DNA surrounding the lipoplexes (Turek *et al.*, 2000; Ferrari *et al.*, 2002).

Targeted liposomes gave rise to complexes with smaller size diameters compared to the untargeted SM1. Dorasamy *et al.* (2012) suggested that galactosylation may result in inhibition of vesicle fusion, thus affording smaller complex sizes compared to the non-targeted complex. SM2 measured an average of ~935 nm while SM3 with acetylated galactoside Sc9 displayed the lowest size diameter of ~434 nm. Although SM3 liposome had presented a larger diameter compared to SM1 and SM2, the resulting complexation with DNA led to the most compact lipoplex size. What is noteworthy, and may explain these results is that the acetylated SM3 liposomes displayed the most positive surface charge. This would have led perhaps to better liposome:DNA interaction. It is quite possible that the acetyl groups adjacent to the cationic head also contributed to the head group interactions with the DNA phosphodiester negative charges.

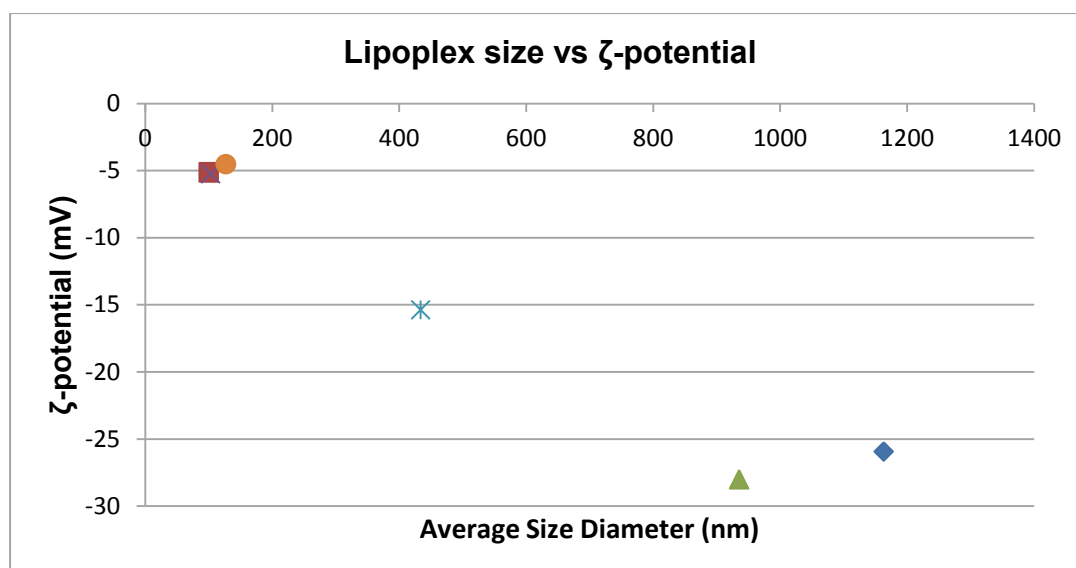
Significant size compaction for all three PEGylated derivatives was noted when associated with DNA. The greatest and most significant reduction was observed for the untargeted control SM1(+PEG) at size diameter of 99.76 nm. Both SM2(+PEG) and SM(+PEG) exhibited reduced size diameters of 102.77 nm and 126.87 nm, respectively. This can be related to PEG properties where steric hindrance is conferred and reduced aggregations discouraged vesicular fusion to form larger vesicles (Pannier *et al.*, 2008). Similar results were obtained by Chen *et al.* (2013) where incorporation of 6.25 mol% PEG led to reduced size and surface charge density of lipoplexes. PEG concentrations of 5 mol% have been previously reported to result in retention of positive cationic charge (Campbell *et al.*, 2002). This was however, not the case as PEG resulted in some masking

of the positive charges of the head group and DNA condensation led to net negative charge in these lipoplexes, attributable to uncondensed DNA.

PDI values for untargeted SM1 and its PEGylated derivative SM1(+PEG) were found to be within a range of 0.237 and 0.241 indicating homodispersion or homogeneity in size distributions. This was consistent with the targeted SM2, SM3 and their PEGylated derivatives, SM2(+PEG) and SM3(+PEG) which also displayed fairly narrow size distribution with PDI values ranging from 0.186 to 0.268.

Lipoplex structure, and its biological activity is influenced by surface charge on the liposomes prior to complexation (Ferrari *et al.*, 2002). In cationic liposomes, it is the charge density of the cationic moiety at the surface of liposomes that influences their  $\zeta$ -potential. It is also an important parameter which contributes to transfection efficiency (Takeuchi *et al.*, 1996; Nie *et al.*, 2011). Overall, reductions in  $\zeta$ -potential values were observed for all lipoplex formulations. In a study by Pires *et al.* (1999), complexation of DNA with DOTAP-containing liposomes led to decreased  $\zeta$ -potential values. Reduction in surface charge is suggested to be beneficial for DNA dissociation from the lipoplex following uptake by the cells (Mogghadam *et al.*, 2011). UnPEGylated derivatives *viz.* SM1, SM2 and SM3 resulted in relatively stable complexes. Before DNA complexation, the overall charge was +13.53, +2.36 and +14.27 mVs (Table 3.2), respectively and upon binding with DNA, these values were reduced to -25.93, -28.00 and -15.37 mVs (Table 3.3), respectively. An excess of positive or negative charge of  $>+30$  and  $<-30$  mV is believed to confer sufficient electrostatic repulsive forces to prevent aggregation (Salopek *et al.*, 1992; Nie *et al.*, 2011). SM1 and SM2 can thus assumed to be the most stable complexes when compared to SM3 as they measured  $\zeta$ -potentials of -25.93 and -28.00 mVs, respectively.

Figure 3.10 presents a distribution pattern of the lipoplexes where the PEGylated derivatives are observed in the near-neutral zone with size ranges below 200 nm. However, the unPEGylated derivatives suggest that an increase in average size diameter corresponds with a decrease in surface charge. It seems that a correlation could be drawn with the liposomes we have, where an increase in diameter resulted in decrease in zeta potential measurements, particularly when observing the unPEGylated derivatives.



**Figure 3.10** Size distribution of lipoplexes in relation to zeta potential

Marginal reduction in surface charge was observed for the stable complexes with PEGylated derivatives viz. SM1(+PEG), SM2(+PEG) and SM3(+PEG). Addition of DSPE-PEG<sub>2000</sub> in complexes has been reported to promote stabilisation in complexes (Hu *et al.*, 2010). The final surface charge remained in the -5 mV range, which places it within the neutral zone. SM2(+PEG) was found to exhibit the most negative surface charge at -5.24 mV. Reduction of neutral  $\zeta$ -potential value to a negative charge could be attributed to the phosphate groups in DSPE. The negative values obtained with the use of DSPE-PEG<sub>2000</sub> could be attributed to the presence of negative PEG dipoles that can form a mushroom/brush intermediate PEG conformation (Morille *et al.*, 2008; Kibria *et al.*, 2011). While extensive aggregation and fusion would be favoured in the case of neutral complexes, steric hindrance conferred by PEG results in their stability. There is general assumption that positively charged liposomes should maintain positive charge even after interaction with the negatively charged DNA in order to facilitate interaction with target cells. However, numerous literature reports have stated that *in vivo* application of positively charged lipoplexes does not always guarantee higher transfection. In fact, these are prone to rapid clearance from circulation due to binding with anionic plasma components (Wasungu and Hoekstra, 2006). Additionally, some authors have reported on negatively charged lipoplexes which have not been impeded in their transfection potential (Resina *et al.*, 2009).

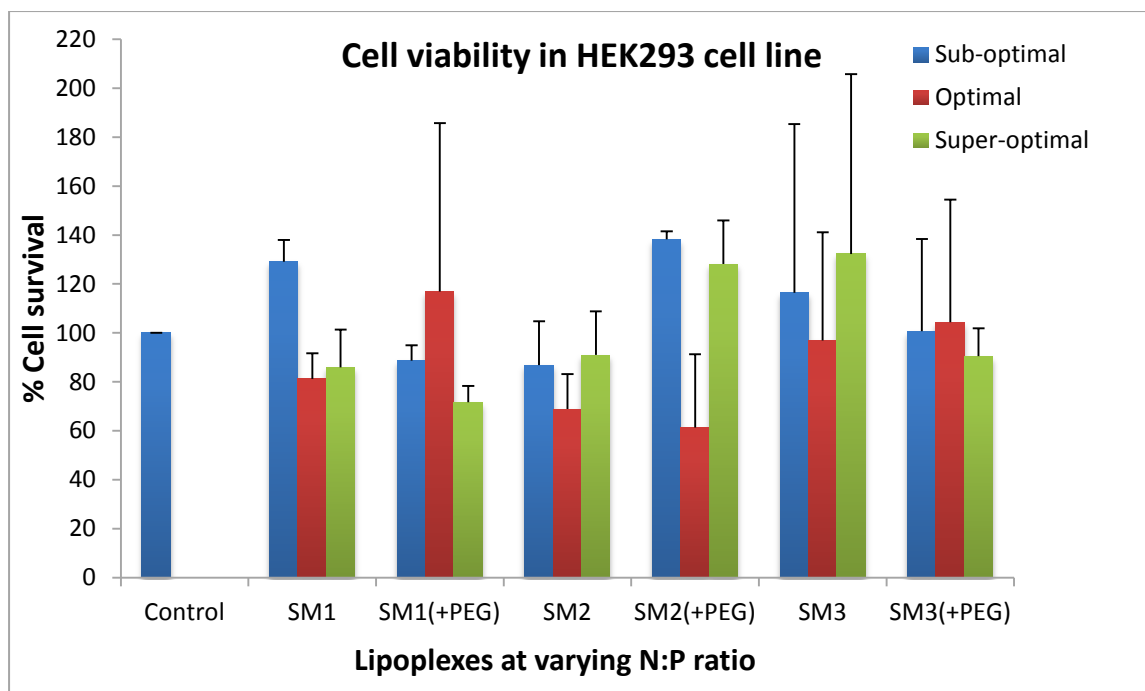
### 3.5 *In vitro* evaluation of lipoplexes on HEK293 and HepG2 cell lines

#### 3.5.1 Cytotoxicity assay

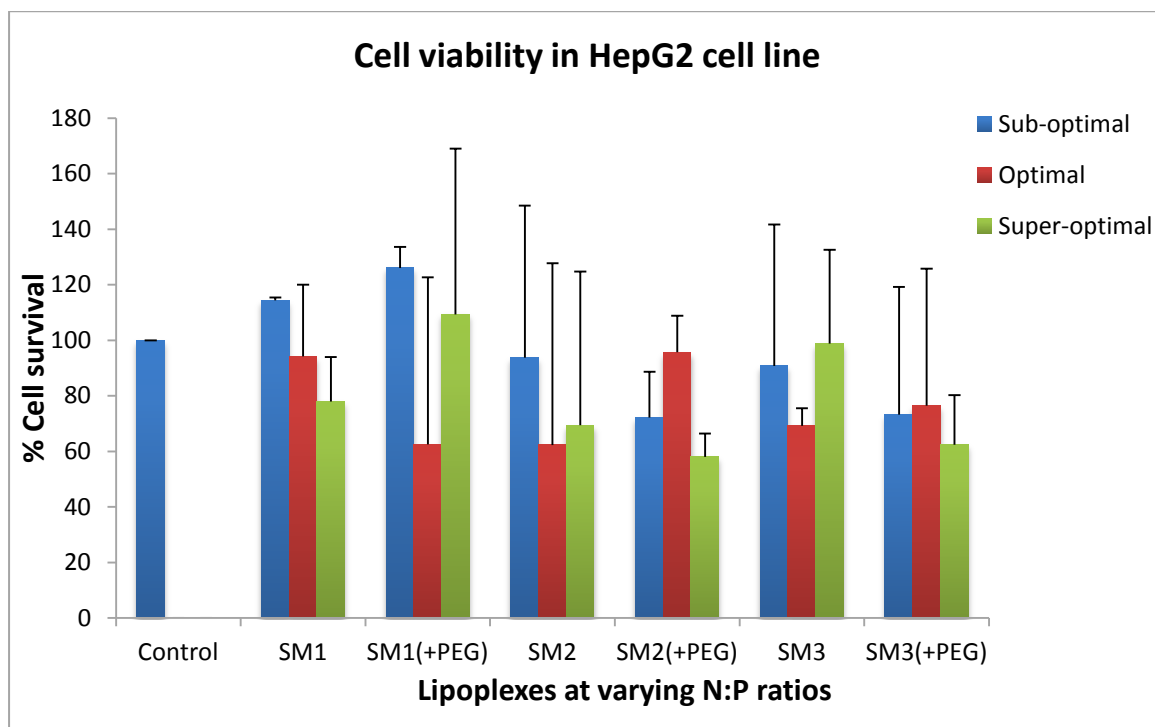
Early studies in non-viral gene delivery related transfection efficacy in terms of carrier binding affinity to nucleic acids or drugs, encapsulation efficiency, biodistribution following administration, and uptake by cells for expression. Cationic lipids particularly, being membrane active may interfere with membrane function and cell integrity which can ultimately lead to toxicity. Thus safety remains a critical factor in their application *in vivo* (Romøren *et al.*, 2004; Paecharoenchai *et al.*, 2012; Wang *et al.*, 2012).

Cytotoxicities of formulated lipoplexes were assessed at liposome:DNA sub-optimal, optimal and super-optimal ratios using the MTT assay. The biochemical reaction is based on metabolically active cells, which are able to reduce 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide solution to form purple, insoluble MTT formazan crystals. Viable cell density is thus related to functional mitochondrial enzymes responsible for MTT reduction (Rodrigues *et al.*, 2012). Figures 3.11 and 3.12 represent cytotoxicities of lipoplexes in HEK293 and HepG2 cell lines, respectively.

All liposomal formulations including the PEGylated derivatives presented an above average cell viability greater than 60% at optimal binding ratios in the HEK293 cell line. At these optimal ratios, the highest cell viabilities >80% were observed for the control liposome SM1, the targeted SM3 and their PEGylated derivatives, viz. SM1(+PEG) and SM3(+PEG). Neutral lipids have been associated with lower cytotoxicities due to reduced interactions with serum (Simões *et al.*, 2005). The degree of PEGylation has been observed to reduce cytotoxicity in DC-Chol/DOPE complexes where 5 mol% DSPE-PGE showed the least cytotoxic effect (Chen *et al.*, 2013). However, SM2 and its PEGylated derivative presented with lowest cell survival at 65% and 62%, respectively. The observed toxicities could be linked to compromised membrane integrity resulting from increased interactions with serum components (Singh and Ariatti, 2006). The acetylated SM3 and its PEGylated derivative resulted in the highest cell viabilities. This has been reported extensively in other systems where these modifications, viz. acetylated and PEGylation were found to result in reduced cytotoxicities of dendrimers (Fant *et al.*, 2010; Hu *et al.*, 2011)



**Figure 3.11** Cell viability studies of lipoplexes for the HEK293 cell line in varying amounts of liposome corresponding to the optimal, sub- and super-optimal DNA-binding ratios. Each column represents the mean  $\pm$  SD (n = 3). \* $P < 0.05$  vs control



**Figure 3.12** Cell viability studies of lipoplexes for the HepG2 cell line in varying amounts of liposome corresponding to the optimal, sub- and super-optimal DNA-binding ratios. Each column represents the mean  $\pm$  SD (n = 3). \* $P < 0.05$  vs control

Lower liposomal concentrations resulted in reduced cell growth inhibition for most of the liposomal formulations. Cell viabilities >100% were also observed in HEK293 cells and may arise through metabolised components of the liposomes promoting cell growth. Untargeted lipoplexes SM1 and its PEGylated derivative SM1(+PEG) resulted in above average cell viability of >65% in HepG2 cell line as shown in Figure 3.12. The targeted lipoplexes resulted in relatively greater cytotoxicities up to 38% when compared to the untreated control. The SM2(+PEG) lipoplexes were well-tolerated by the HepG2 cells with cell viability above 80% while the acetylated SM3 resulted in cell viabilities of ~70%. Hepatotoxic effects were reported for siRNA lipoplexes formulated with Sc9 ligands. These led to increased levels of circulating interleukin-6 and tumour necrosis factor following the fourth injection of these complexes in an animal study (Hean *et al.*, 2010). And thus, this remains an important factor to consider when it comes to *in vivo* application.

Earlier reports by Singh and Ariatti (2006) had demonstrated that cationic lipoplexes result in growth inhibitions of up to 31% for HepG2 cell lines. Upon investigations by Dorasamy *et al.*, (2011), galactosylated Chol-T siRNA lipoplexes were well-tolerated by the HepG2 cells at viabilities > 80%. At ratios below liposome:DNA optimal binding, there was increase in cell viabilities for all liposomal formulations. An increase in liposome:DNA ratios did not result in significantly lower cell viabilities thus suggesting that the liposomes do not present adversely cytotoxic effects for the HepG2 cells. Lipoplexes that comprise cholesterol and DOPE have been reported to display good cell compatibility (Yang *et al.*, 2013). Growth inhibitions of up to 18% were reported for Chol $\beta$ Gal and Chol $\alpha$ Gal lipoplexes comprising co-helper DOPE in Hela and HepG2 cell lines (Singh *et al.*, 2007).

### 3.5.2 Transfection assays

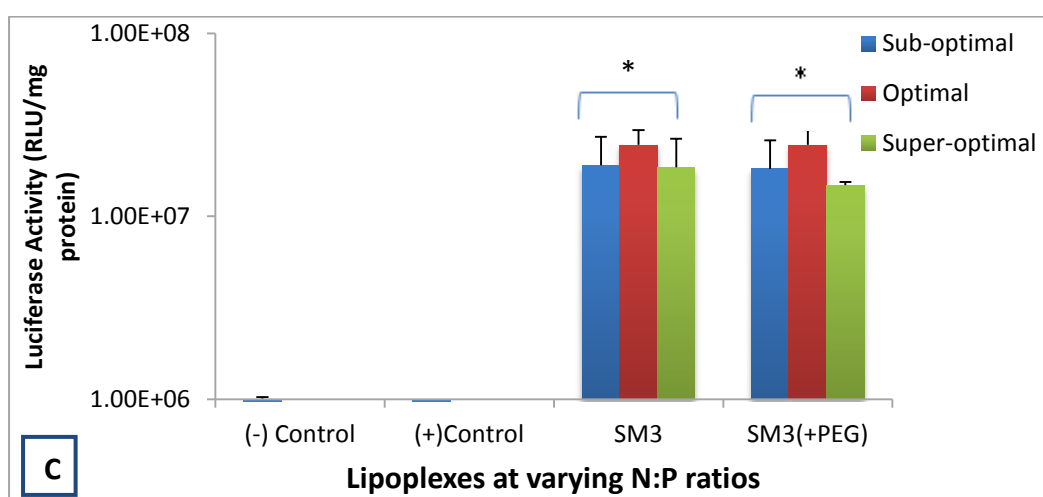
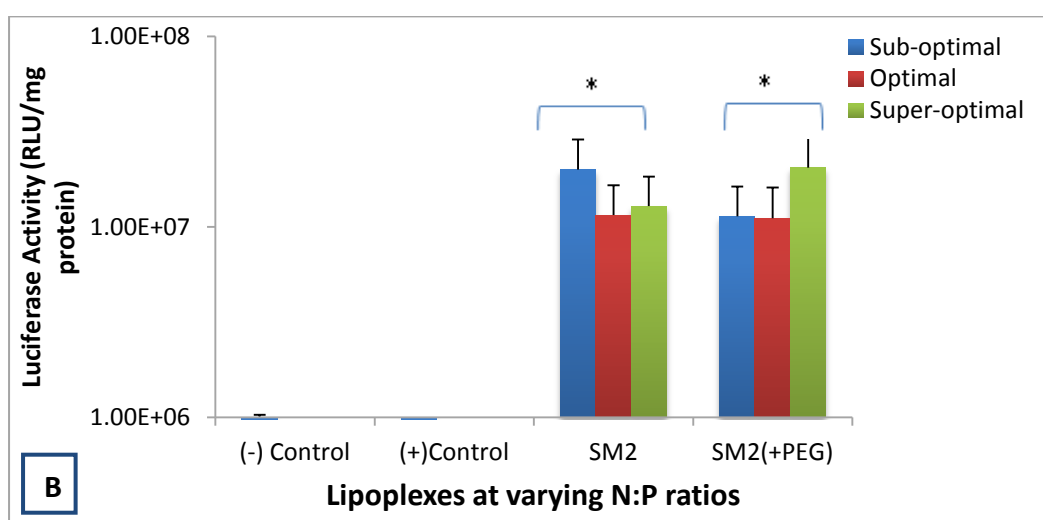
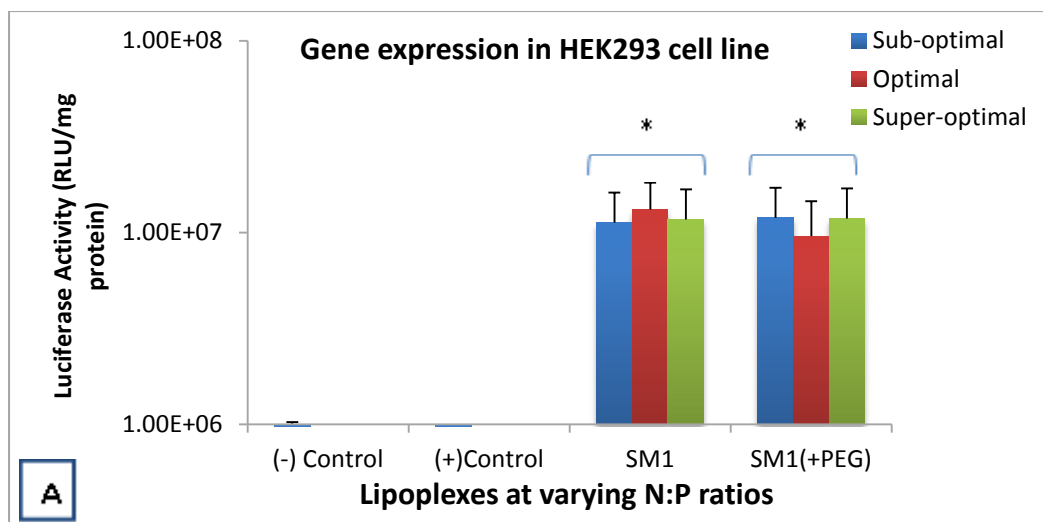
The luciferase assay employs the pCMV-*luc* reporter gene vector for expression of the luciferase gene. Cells which had been successfully transfected with the *luc* gene were lysed, and excess substrate (D-luciferin) was added to the lysate generating luminescence (De Wet *et al.*, 1987). The intensity of the light emitted was quantified and taken as a measure of gene expression level. This is directly proportional to the quantity of the

enzyme present in the cell lysate. Results were normalised by determining the soluble protein concentration in the lysate and results were expressed as relative light units (RLU)/ $\mu\text{g}$  protein.

An overall assessment of gene expression levels was conducted in the ASGP receptor-negative HEK293 cell line by lipoplexes viz. SM1, SM2 and SM3 and their PEGylated derivatives at N:P ratios below, above and at optimal points. The controls are classified as: (-)control, where there was no DNA added, and (+)control, where naked uncomplexed DNA was added to the cells (Figure 3.13).

The untargeted SM1 derivative was observed to result in comparably high transfection to that achieved by the other smaller-sized liposome derivatives. Size has been determined to influence choice of internalisation pathway and ultimately, the fate of the lipoplexes (Rejman *et al.*, 2004; Khalil *et al.*, 2006). SM1 presented with the highest complex size diameter of over 1000 nm, followed by the targeted SM2 at 935 nm. Cellular uptake for these complexes could have been resulted from phagocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis or macropinocytosis. It has been proposed that cationic complexes without the targeting moiety are internalised as a result of various endocytotic routes. PEI-DNA complexes were proposed to be internalised following binding of the complex with the transmembrane heparin sulphate proteoglycans. This leads into clustering into cholesterol-rich lipid rafts which pulls the complex into the cell via phagocytosis. Cationic lipoplexes were reported to interact with proteoglycans where lack of proteoglycans resulted in complexes being unable to transfect Raji cells *in vitro*. The clathrin-mediated endocytosis could only have been achieved following fragmentation of the complexes into smaller units (Rejman *et al.*, 2004; Khalil *et al.*, 2006; Ma *et al.*, 2007; Minzter and Simanek, 2009).

There is also preferential uptake via caveolae-mediated transfection for particles with sizes ranging  $>200$  nm to 1 000 nm in diameter. This would explain the comparatively high transfection efficiencies noted for all unPEGylated liposomes viz. SM1, SM2 and SM3. In this pathway, a crucial benefit is the ability for particles to avoid the lysosomal degradation



**Figure 3.13** Transfection capabilities of A) SM1 and SM1(+PEG), B) SM2 and SM2(+PEG) and C) SM3 and SM3(+PEG) lipoplexes in HEK293 cell line at varying amounts of liposome corresponding to the optimal, sub and super-optimal DNA-binding ratios. Each column represents the mean  $\pm$  SD ( $n = 3$ ).  $*P < 0.05$  vs naked DNA control



While it may not be an ideal situation to have particles as large as 935 nm or 434 nm in the *in vivo* setting, larger sizes have been reported to result in enhanced sedimentation thus allowing for maximum contact with the cells *in vitro*. Optimal sizes for high transfection *in vitro* have been suggested to lie between 200 nm – 400 nm. Macropinocytosis has been reported to lead to uptake of larger sized cationic lipoplexes for efficient transfection. Again, while these pathways are not necessarily mutually exclusive, it cannot be concluded that all of them result in productive transfection (Rejman *et al.*, 2004; Simões *et al.*, 2005; Ma *et al.*, 2007; De Garibay *et al.*, 2013).

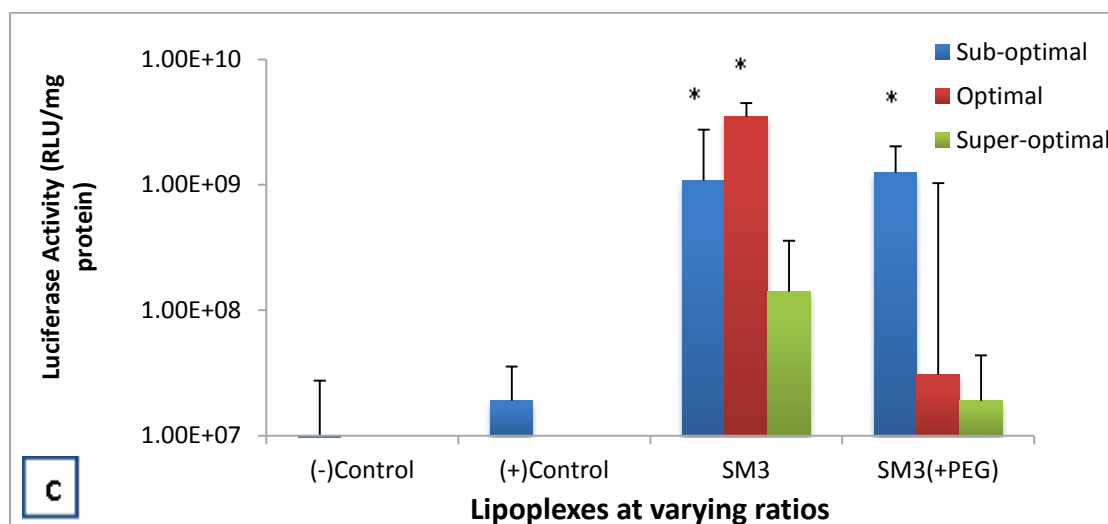
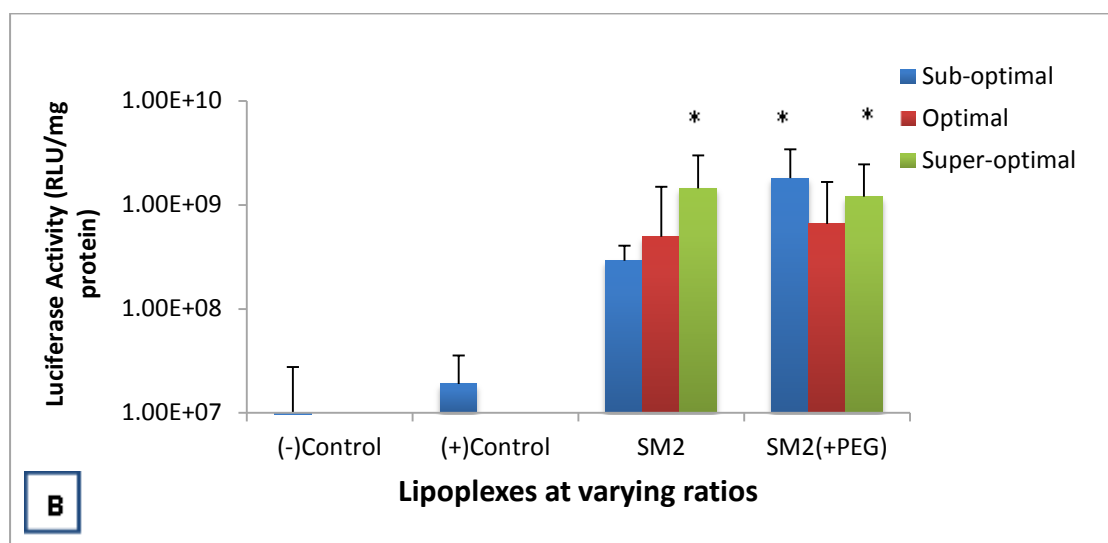
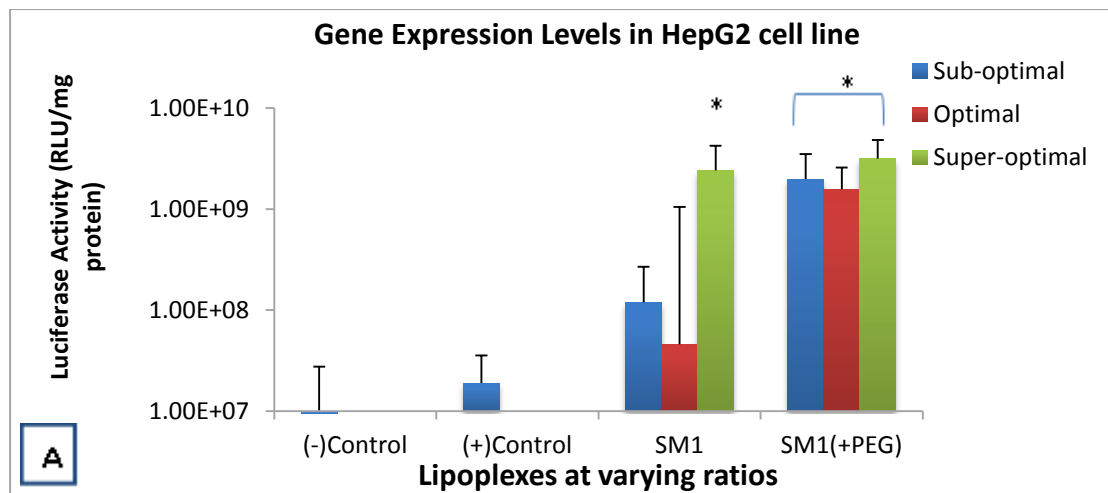
Another important aspect relates to serum effects. Interactions with serum components by larger lipoplexes have been reported to result in lower transfection due to decreased delivery and dissociation of the complexes. However, in a contrasting report, large cationic complexes of over 700 nm have been observed to result in enhanced transfection due to delayed dissociation of the lipid from DNA (Fumoto *et al.*, 2005; Ma *et al.*, 2007). For particles with size diameters less than 250 nm, the clathrin-mediated pathway would be the most likely internalisation pathway for complexes in this size range. This is the suggested internalisation pathway for the PEGylated SM1(+PEG), SM2(+PEG) and SM3(+PEG) derivatives as they presented size diameters less than 126 nm at optimal ratios (Simões *et al.*, 2005; Khalil *et al.*, 2006; Ma *et al.*, 2007; Minzter and Simanek, 2009).

Surface charge is an equally important determining factor in how the complexes interact with the target cell membrane (Zuhorn *et al.*, 2007). The unPEGylated derivatives of SM1, SM2 and SM3 presented with net negative charges of -22.93, -28.00 and -15.37 mVs, respectively. It would be expected that this would render the complexes incapable of interacting with the cell membrane. However, comparably high transfection efficiencies were noted for all these derivatives at optimal binding ratios. Negatively charged lipoplexes are often associated with lower stability due to aggregation. According to one report, a zeta potential with a range of  $\sim$ -5 mV to -15 mV is said to be ideal for nanoparticles designed for biological systems (Leary, 2011). The PEGylated derivatives presented stable complexes with near-neutral charges. While it has been suggested that a fall of net positive charge to neutrality can result in inhibited transfection, this was not the case for these liposomes. This relates to the steric hindrance afforded by PEGylation

which does not allow aggregation (Simões *et al.*, 2005; Ma *et al.*, 2007; Xiang and Zhang, 2013).

Overall results showed higher transfection levels by liposomes in HepG2 cells when compared to HEK293 cells (Figure 3.14). Cell type-dependent differences in transfection efficiencies relate to cell cycle and cell division frequency and the cells' endocytic capacity. Transfection efficiencies of F108-coated DNA lipid nanocapsules (LNC) in HeLa cells were reported to be slightly superior to those in H1299. It is also quite possible that the different optimal cell densities required to carry out the transfection assays is cell type-dependent. Lipofectamine 2000 optimal cell densities are reported to be higher in HEK293 cells for the  $\beta$ -galactosidase assay and lower in luciferase gene expression assay for the HeLa cells (Zuhorn *et al.*, 2007; Wang *et al.*, 2012).

The untargeted SM1 derivative showed the overall lowest transgene activity, with the exception of the super-optimal N:P ratio. This could be explained in terms of the complex large size diameter of over 1000 nm. In a study by Ross and Hui (1999), it was determined that an increase in size of larger lipoplexes results in greater cellular association and uptake when compared to smaller complexes. This is attributed to the larger surface area presented by the larger complexes making uptake more favourable. Once taken up by the cells, it is believed that larger lipoplexes result in large intracellular vesicles which can be disrupted more easily for release of DNA into cytoplasm (Ma *et al.*, 2007; Mintzer and Simanek, 2009; Malaekheh-Nikouei *et al.*, 2009). The lower transfection efficiency for the sub-optimal and optimal ratios could be attributed to the serum effects on large lipoplexes. Introduction of serum to large DC-Chol/DOPE lipoplexes results in adsorption of large amounts of pDNA onto their cationic surfaces. This has been found to confer the excess negative charge onto the lipoplex surface. This can lead to reduced interactions of the cationic lipids with the endosomal membrane and ultimately interfere with the membrane hexagonal phase transition for endosomal escape. Additionally, it can result in conversion of the supercoiled structure of the pDNA to the open-circular conformation which is transcribed less efficiently. Thus there is low transfection efficiency exhibited by these lipoplexes (Malaekheh-Nikouei *et al.*, 2009; Yang *et al.*, 2013).



**Figure 3.14** Transfection capabilities of A) SM1 and SM1(+PEG), B) SM2 and SM2(+PEG) and B) SM3 and SM3(+PEG) lipoplexes in HepG2 cell line at varying amounts of liposome corresponding to the optimal, sub and super-optimal DNA-binding ratios. Each column represents the mean  $\pm$  SD (n = 3)\* $P < 0.05$  vs naked DNA control

The PEGylated derivative SM1(+PEG) lipoplex however, presented a mean size range of 99 nm at optimal ratio, and a near neutral zeta potential (-5 mV) with higher transfections being noted. Small liposomes with diameters below 100 nm are reported to have relatively easy access to the transendothelially located hepatocytes, even without using any targeting ligand. This is related to size restrictions of 175 nm for traversing through the fenestrae into the Disse space (Hattori *et al.*, 2000; Bae and Park, 2011; Patil and Jadhav, 2014). However, the observed transfection results for the untargeted SM1(+PEG) were somewhat unexpected as they were comparable and even higher than those of targeted SM2, SM3 and their PEGylated derivatives viz. SM2(+PEG) and SM3(+PEG). However, Morille *et al.* (2008) also reported that galactosylated DNA LNCs were as efficient in transfecting primary hepatocytes as the untargeted DOTAP/DOPE lipoplexes. At this size range of ~120 nm, the main mode of internalisation could be as a result of adsorptive clathrin-mediated endocytosis. The near-neutral zeta potential (within the ~ -5 mV range) also advantageously played a significant role in terms of interactions with the cell membrane. Reduction in surface charge of cationic lipoplexes is assumed to be beneficial for DNA dissociation following cellular uptake for trafficking to the nucleus (Zuhorn *et al.*, 2007; Moghaddam *et al.*, 2011).

It has been suggested that size effects are probably more pronounced in targeted complexes when compared to the non-targeting counterparts (Minzter and Simanek, 2009). The targeted SM2 derivative presented a complex with negative zeta potential which was -25 mV, and a size diameter of ~935 nm at optimal N:P ratio. While it is believed that size ranges between 650 nm to 1500 nm are likely to be internalised via the caveolae-mediated pathway, and thereby avoid lysosomal degradation – this does not guarantee high transfection levels (Zuhorn *et al.*, 2007; Brgles *et al.*, 2012). Again, these parameters can be altered under serum-present conditions, resulting in lower transfection efficiency. For instance, larger complexes will tend to display a weakened ability to protect DNA following interactions with serum. Thus while they are able to escape from the endosomes efficiently, they often result in poor transfections. This was observed to be true particularly for the sub-optimal ratio in SM2 lipoplex. At this ratio, the liposomes did not afford optimal serum nuclease protection for the DNA cargo as there was some degradation of the DNA cargo (Yang *et al.*, 2013). Its PEGylated derivative viz. SM2(+PEG) was however, observed to display relatively better transfection efficiency. Inclusion of PEG led to improved transfection efficiency by enhancing stability and as a

result of desirable size reduction. Literature reports have indicated that galactosylated lipoplexes with a mean size of 141 nm result in higher transfection in parenchymal cells *in vivo*. While size could be the main contributing factor, stability and efficacy in serum is said to be critical for successful delivery (Singh *et al.*, 2007).

The highest gene expression levels were noted for the unPEGylated SM3 at optimal ratio. The acetylated SM3 lipoplexes led to sustained transfection owing to their favourable zeta potential (15 mV), and size diameter (434 nm). Size could be an important contributing factor where moderately sized vesicles of 400 nm were reported to result in higher transfection efficiency (Malaekah-Nikouei *et al.*, 2009). Another interesting aspect relates to the serum degradation assay results, which showed nicking of the supercoiled pDNA thus resulting in the prevalence of open circular pDNA. Van Groll (2006) reported that while linearised DNA presents a more stable DNA conformation, they exhibit lower transfection efficiency compared to circular complex. In fact, it was observed that when complex size diameter ranged from 100 to 500 nm using circular pDNA, there was accentuated transfection efficiency.

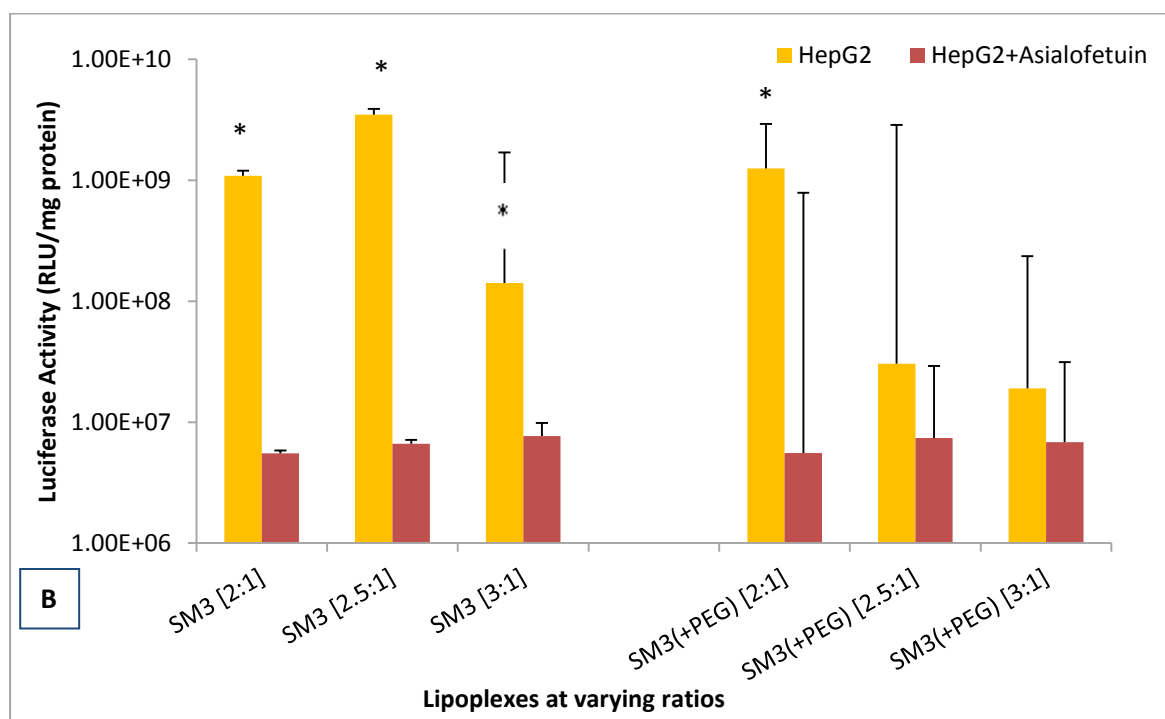
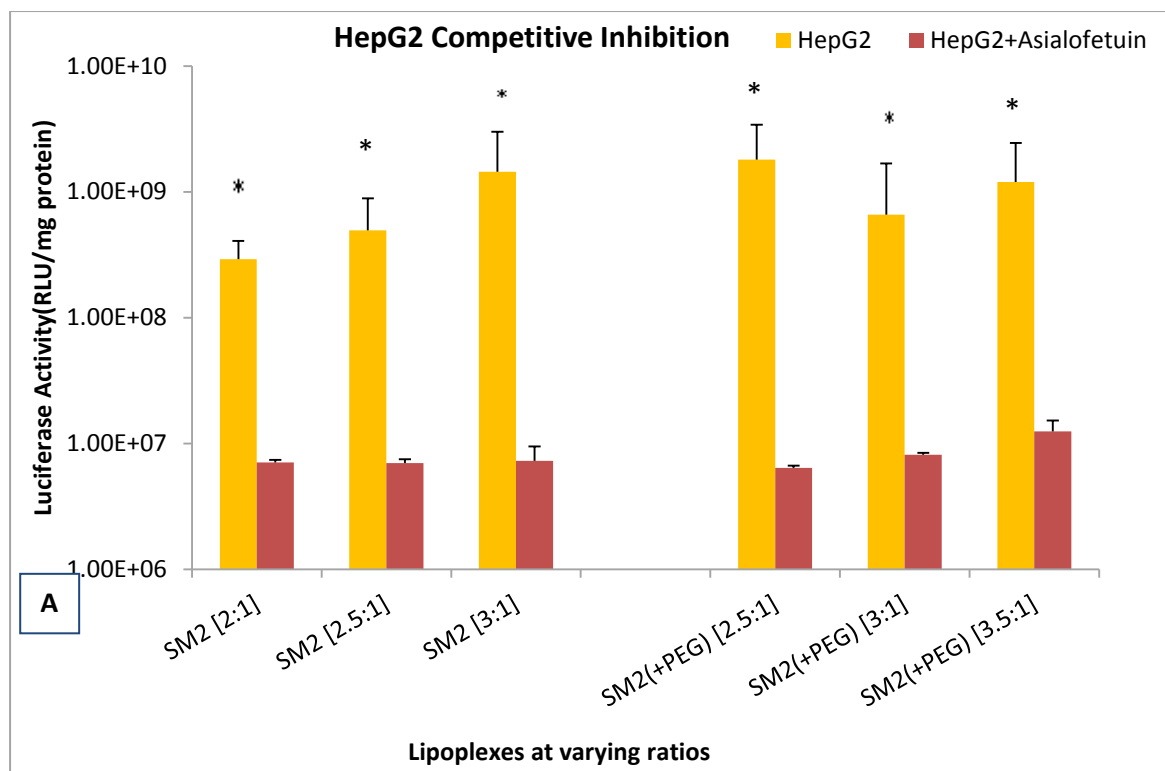
While targeting is important, it remains critical to contextualise it in terms of other contributing factors that impact on transfection efficiency. This was particularly evident in the case of the SM3(+PEG) lipoplexes with acetylation and PEGylation modifications. The SM3(+PEG) lipoplex exhibited the overall lowest transfection efficiency when compared to the other targeted lipoplexes. Transfection efficiency of acetylated and PEGylated dendrimers was also observed to be lower than that of the corresponding unmodified dendrimers (Fant *et al.*, 2010). Slightly better transfection efficiency levels were observed for the sub-optimal ratio; however, it was almost abolished at optimal and super-optimal N:P binding ratio. This was despite the enhanced stability properties where a 120 nm size diameter and near neutral surface charge were reported.

This can, perhaps, be explained in respect of the PEG dilemma. PEGylation has been reported to lead to reduced association with the target cell membrane, but in context of targeted delivery, it could result in masking of the galactose moiety. While it has been established that DSPE-PEG<sub>2000</sub> confers a mushroom/brush intermediate or mushroom spatial conformation for accessibility of the galactose moieties, it could still present an impediment. In a folded conformation, the galactose moiety might be hidden (Morille *et*

*al.*, 2010). Morille *et al* (2010) also reported similar results in the sterically stabilised DNA LNCs with DSPE-mPEG<sub>2000</sub> that displayed reduced transfection efficiencies in comparison to non-coated DNA LNCs in H1299 and HeLa cell lines. This remains the primary drawback of PEG and is attributed to the obstructed association with target cells. Additionally, PEG can be an impediment in endosomal escape due to the lower surface charge on lipoplexes, although this was not observed in the case for the SM2(+PEG) lipoplexes (Morille *et al.*, 2010). The 120 nm size often results in clathrin-mediated endocytosis, but is subject to rapid processing and clearing. This, coupled with an impeded dissociation of lipoplexes from the endosomes would have resulted in poor transfection efficiency. Size-dependent variations in transfection results have been cited in various literature reports (Mintzer and Simanek, 2009). And thus, the results of the unPEGylated SM3 derivatives can also explained by the possibility that the ligand could have been more readily accessible, thus resulting in better receptor recognition and increased transfection efficiency than its PEGylated counterpart.

To verify the ASGP receptor-mediated internalisation, a competitive inhibition study was carried out for targeted lipoplexes viz. SM2, SM2(+PEG), SM3 and SM3(+PEG) as seen in Figure 3.15. Asialofetuin is a natural ligand which shows strong affinity for the ASGP receptor. It has three asparagine-linked triantennary complex carbohydrates with terminal N-acetylglucosamine (LacNAc) residues.

Upon addition of the competitive inhibitor, asialofetuin, a marked decrease of up to 2 order of magnitude in transfection activity was noted for the SM2 and SM2(+PEG) lipoplexes. This undoubtedly showed that the ASGP receptor had been saturated by asialofetuin thereby preventing ASGP receptor-mediated internalisation and expression of the pCMV-*luc* gene. This was also observed in the SM3 and SM3(+PEG) derivatives where the most significant reduction was observed for the optimal ratio in SM3 and the sub-optimal ratio of SM3(+PEG). This was clear evidence of the ASGP receptor-mediated endocytosis; however, the effects were less pronounced for the low transfecting SM3(+PEG) lipoplexes at optimal and super-optimal ratios.



**Figure 3.15** Transfection capabilities of A) SM2 and SM2(+PEG); and B) SM3 and SM3(+PEG) lipoplexes in varying amounts of liposome corresponding to the optimal, sub- and super-optimal DNA-binding ratios for the HepG2 cell line in presence of competitive inhibitor. Each column represents the mean  $\pm$  SD (n = 3). \* $P < 0.05$  HepG2 vs HepG2+Asialofetuin

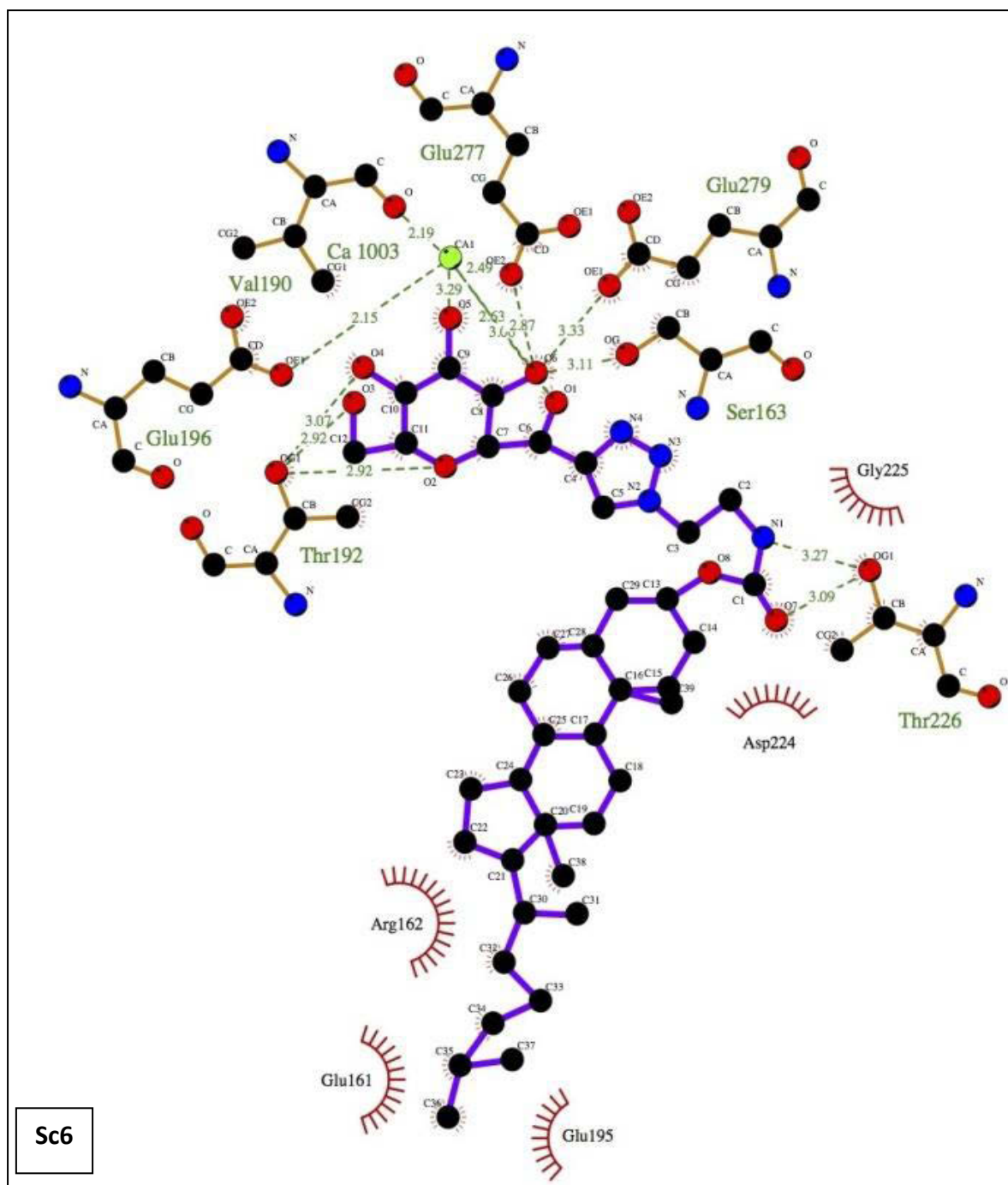
Overall, targeting of the ASGP receptor was found to present the main mode of internalisation of lipoplexes and eventual gene expression in the SM2, SM3 and their PEGylated derivatives. However, it must be stated that internalisation modes are not mutually exclusive and can thus occur simultaneously (Zuhorn *et al.*, 2007). The unPEGylated SM3 comprising of the acetylated Sc9 galactoside presented with superior transfection efficiencies in both the HEK293 and HepG2 cell lines.

### 3.6 *In silico* evaluation of ligand-receptor interactions

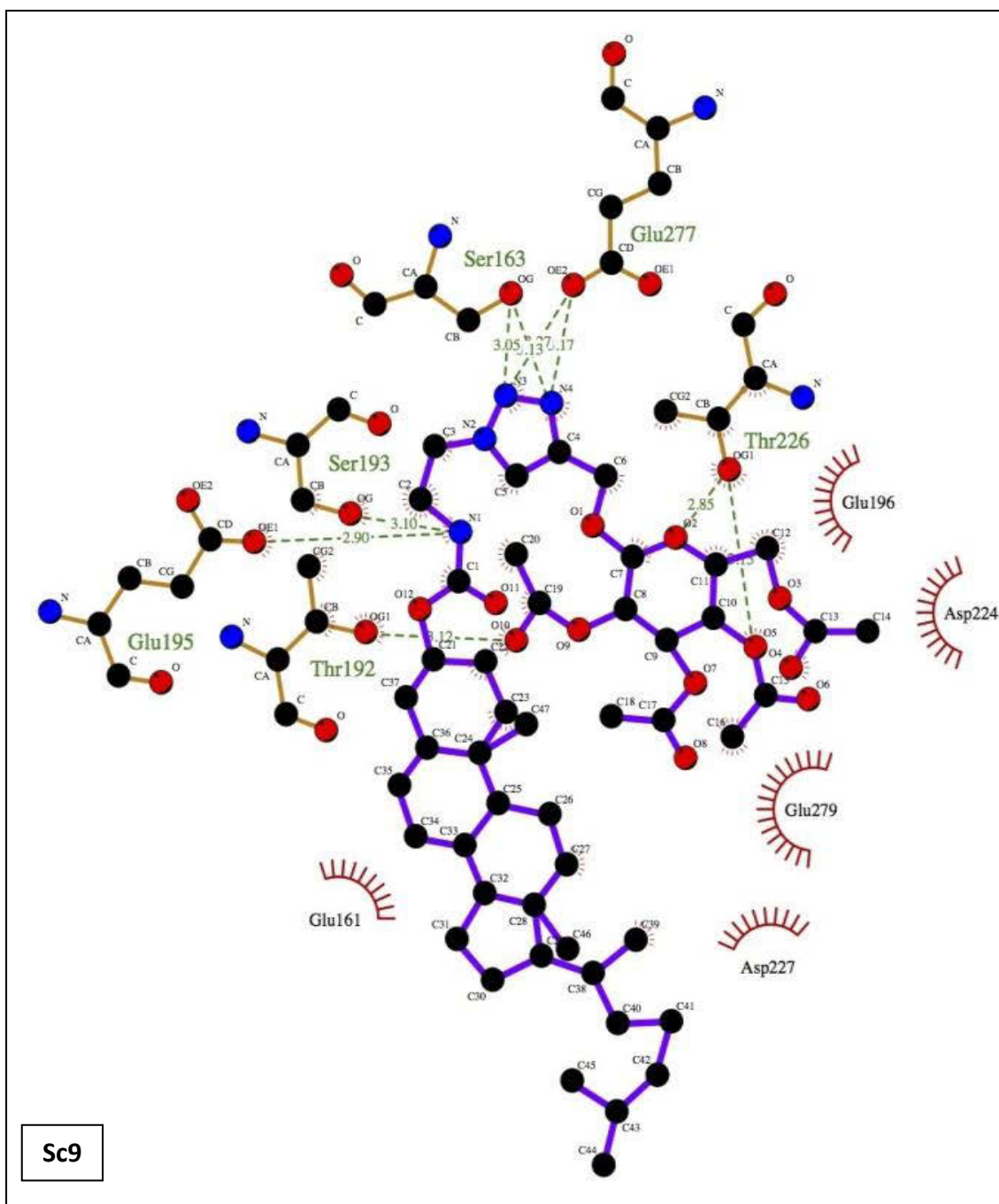
Docking analyses serve to predict the *in vivo* setting in respect of bound conformations and their binding affinity (D'Souza *et al.*, 2013). Figures 3.16 and 3.17 illustrate the docked pose by the Sc6 and Sc9 galactoside ligands to the ASGP receptor using Ligplot.

The conformation shown in Figure 3.16 demonstrates the best docked pose of the Sc6 galactoside in the receptor binding site. It takes into account the coordinate bonds, hydrophobic bonds, and hydrogen bonds, all critical in ligand-receptor interactions. In particular, hydrogen bonding was observed to be prevalent between the galactoside head group where the C<sub>4</sub>, C<sub>5</sub> and C<sub>5</sub> hydroxyl groups of the galactose sugar interacted extensively with Thr192. Stabilisation of the receptor by calcium ion (Ca1003) was observed to be quite significant where further interactions with two hydroxyl groups of the galactose molecule (C<sub>2</sub> and C<sub>3</sub>) were observed. The above are in line with GalNac receptor interactions, where the C<sub>3</sub>- and C<sub>4</sub>-hydroxyl groups are in coordination with the calcium ion (Stokmaier, 2010; Mamidyala *et al.*, 2012). It has been suggested that the hydroxyl group position plays an important role for the sugar moieties (D'Souza *et al.*, 2013). Simultaneously, Ca1003 is observed to form hydrogen bonds with amino acids, Val190 and Glu196. These interactions with calcium have been suggested to be important in ASGP receptor binding; preferentially with the 3- and 4- hydroxyl groups (D'Souza *et al.*, 2012). Amino acids viz. Glu277, Glu279 and Ser163 also formed strong hydrogen bonds with the hydroxyl group on C<sub>2</sub> of the galactose head. These extensive interactions clearly seem to result in steady and strong binding for the receptor. Hydrogen bonding of carboxyl and amide side-chains of aspartate and glutamine residues with C<sub>3</sub> and C<sub>4</sub> hydroxyl groups have been reported to contribute to the strong ligand-binding interactions (Meier *et al.*, 2000).





**Figure 3.16** Docking of Sc6 galactoside at the active binding site of ASGP receptor and its ligand interaction diagrams (Ligplot)



**Figure 3.17** Docking of Sc9 galactoside at the active binding site of ASGP receptor and its ligand interaction diagrams (Ligplot)

The acetylated Sc9 galactoside ligand comprised of the hydrophobic and bulkier acetoxy groups in place of the hydroxyl groups. Figure 3.17 shows the optimal docking model for this galactoside in the active site of the ASGP receptor. Their absence in this ligand was expected to result in compromised binding; however, contortion of the Sc9 ligand seemed to confer optimal docking conformation. Contrary to Sc6 docking disposition, extensive hydrogen bonds were observed to be with the triazine ring in the linker region of the ligand to the receptor amino acids viz. Ser163 and Glu277. The galactose head displayed minimal interactions and only involved Thr226 and Ser193. It must be noted that there were no interactions with the calcium ions in the active site – which is reported to be a critical component for the active binding to the receptor. This would have led perhaps to a lesser degree of affinity to the receptor when compared to the Sc6 ligand. It has been suggested that another critical factor that influences receptor-ligand binding relates to the stereochemical conformation of the ligands (Mamidyala *et al.*, 2012; D'Souza *et al.*, 2013).

Calculated binding affinities revealed negative docking scores for Sc6 and Sc9 galactoside ligands of  $-6.7$  kCal/mol and  $-5.4$  kCal/mol, respectively. These scores also corresponded with Glide scores reported by D'Souza *et al.* (2013) for the D-galactose polymers including the arabinogalactan, comprising  $\beta(1\rightarrow3)$ D-galactose monomers. These also ranged from  $-5.5$  kCal/mol to  $-9.79$  kCal/mol. However, there was a marginally higher binding affinity calculated for the Sc6 ligand to the receptor compared to that of the acetylated Sc9. This was somewhat expected, given the reasons stated earlier. Additionally, the lower binding affinity of Sc9 galactoside can be explained in context of findings by Mamidyala *et al.* (2012). By replacing the anomeric hydroxyl group of the galactose monomer with a  $\beta$ -4-methyl substituent, a 5-fold loss of affinity for the ASGP receptor was reported. Thus the absence of interactions with the anomeric hydroxyl groups by the Sc9 ligand led to decrease in its binding avidity for the receptor.

## CHAPTER FOUR: CONCLUSION

Cationic liposomes represent the most attractive non-viral gene carriers due to their biocompatibility, ease of manufacture and versatility in design. This is evident with a proportion of these vectors being used in various *in vivo* clinical trials, following the immunogenicity impasse presented by use of viral vectors. In contrast to their viral counterparts which have developed mechanisms to overcome cellular barriers, cationic liposomes and other non-viral vectors remain hindered by these obstacles presented *in vivo* (Schaffer and Lauffenburger, 2000; Mintzer and Simanek, 2009). Furthermore, the process of internalisation and transfection has continued to be elusive. This is owing to the many factors that influence the liposome/DNA complex, including size, lamellarity, charge ratio and density. However, because these properties also influence each other, their defined roles are often obscured (Brgles *et al.*, 2012).

Design and synthesis of novel cationic lipids for an efficient gene delivery still presents a formidable task, and is often the result of trial and error (Mintzer and Simanek, 2009; Brgles *et al.*, 2012). Composition thus plays an important role in design of liposomal formulations for gene delivery. Many cationic liposomal compositions entail the cationic lipid such as DOTAP/DOTMA, neutral lipid such as cholesterol and/or a co-lipid such as DOPE (Montier *et al.*, 2008). The deployment of PEG has become common practice in the design of stealth nano-therapeutics for sustained release of small molecules to nucleic acids. In fact, stealth liposomes have been employed in treatment of Kaposi sarcoma and various other cancers e.g. breast, ovarian and solid tumours (Garg and Kokkoli, 2010; Allen and Cullis, 2013). Coating with PEG polymers has been shown to circumvent toxicity by forming a stabilising interface between the liposome and its environment resulting in increased circulation (Elsabahy *et al.*, 2011). DOPE is proposed to improve transfection by promoting disruptions in the endosomal membrane (Aissaoui *et al.*, 2002; Montier *et al.*, 2008; Bhattacharya and Bajaj, 2009; Zhi *et al.*, 2010).

The targeting galactoside ligands viz. Sc6 and Sc9 were incorporated into liposomal formulations and comparatively evaluated for *in vitro-in silico* characteristics. Galactosylated cholesterol derivatives have been shown to exhibit lower cytotoxicity and improved transfection efficiency in HepG2 cells. The higher transfection potential was

attributed to affinity of cellular receptors for galactosylated ligands (Kawakami *et al.*, 2000; Kawakami *et al.*, 2007; Balazs and Godbey, 2011). Efficient gene delivery *in vivo* requires that complexed liposomes range between 50 nm and 200 nm (Ma *et al.*, 2007; Bae and Park, 2011; Patil and Jadhav, 2014). The unPEGylated liposomes displayed larger than desirable size diameters showing tendencies to aggregate. However, the sterically stabilised PEGylated liposomal formulations displayed more compact size diameters ranging from 80 – 130 nm and near-neutral surface charges. Both Sc6- and Sc9-galactoside-bearing liposomes exhibited a capacity to bind DNA and provide some protection against nuclease digestion in serum, albeit partial. All lipoplexes were well-tolerated by both the HEK293 and HepG2 cell lines as determined in cell viability studies. The acetylated SM3 lipoplex displayed the greatest transgene expression levels in both the HEK293 and HepG2 cell lines; however, it was not found to be a statistically significant effect. The mode of internalisation was unambiguously revealed to be via the ASGP receptor-mediation for the targeted liposomes bearing both galactoside ligands.

Overall, these results confirmed the unequivocal relevance and potential of targeting by galactosylation in ASGP receptor mediated delivery. However, modifications such as in acetylation and PEGylation did not attest much significant effect with respect to transfection *in vitro*. An equally important finding from the study pertained to the comparable transfection levels of the acetylated Sc9 and deacetylated Sc6 targeted lipoplexes in the HepG2 cell line, which were corroborated quite remarkably by the binding affinities calculated for both these ligands. This essentially reinforced the high correlation that clearly exists between *in vitro* and *in silico* studies.

It is regrettable that most of the liposomes which have reached the various stages in clinical trials are untargeted, with much fewer targeted formulations having progressed that far. Nevertheless, the field of ligand-targeting continues to grow and presents many possibilities (Allen and Cullis, 2013). Future attempts at exploiting ligands for receptor-mediated delivery should have a holistic approach. In performing molecular simulation studies, this could serve as a way to predict ligand receptor interactions and give some insight of the likely outcome *in vitro*. It is however, impossible to account more accurately for the properties that ultimately influence the disposition of ligands *in vitro* or *in vivo*. Despite this, the benefits of docking analyses cannot be undermined. Coupled with the invaluable *in vitro* experiments, optimisation of lipoplex formulations could be

achieved much more expediently this way. This in turn, will ultimately lead to a more focused approach toward *in vivo* applications. In the long term, it could then allow for the design and development of multitude virtual libraries comprising small molecule therapeutics modified with suitable ligands (natural or synthetic) for enhanced specificity and binding affinity geared for the ASGP receptor (Zuhorn *et al.*, 2007; D'Souza *et al.*, 2013).

## CHAPTER FIVE: REFERENCES

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