A COMPARATIVE STUDY ON THREE UNIQUE GALACTOSYLATED CATIONIC LIPOSOMES WITH THEIR STERICALLY STABLIZED COUNTERPARTS, IN HEPG2 CELLS.

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A Comparative Study on Three Unique Galactosylated Cationic Liposomes with their Sterically Stablized Counterparts, in HepG2 Cells.

by

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Submitted in fulfilment of the academic requirements for the degree of Master of Science in the School of Life Science,

University of KwaZulu-Natal

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

Supervisor: Dr M Singh Signed _____ Date _____

Co-Supervisor: Professor M. Ariatti

Co-Supervisor: Dr B Masola

ABSTRACT

Receptor mediated endocytosis allows for the site specific delivery of exogenous DNA via appropriate ligand-receptor interactions. Various ligands have been used to target the asialoglycoprotein receptor (ASGP-R) present on the hepatocyte cell membrane viz. asialofeutin, asialoorosomucoid, lac-BSA, asialolactoferrin, asialo-transferrin, asialo-ceruloplasmin and galactose. The high affinity that the receptor displays for the galactose sugar moiety has led to the development of several new galacto-lipids for the incorporation into liposomes intended for hepatocyte targeting.

In this study, three cholesteryl derivatives displaying galactose units linked to the sterol skeleton by different spacer elements have been formulated into cationic liposomes with and without polyethylene glycol (PEG) accessories. The three galactosylated liposomal formulations were prepared using near equimolar amounts of MSO9 (N,N-dimethylaminopropylamidosuccinyl-cholesterylformylhydrazide) and DOPE (dioleoylphosphotidylethanolamine) together with the respective galactose derivative (at 10 mole % w/w) viz. Cholesteryl-3β-N-(4-aminophenyl-β-D-galactopyranosyl) carbamate; Cholesteryl (1-β-D-galactopyranosyl-1,2,3 triazol-4-yl) carbonate; and Cholesteryl-β-D-galactopyranoside. All liposomes displayed DNA binding, nuclease protective capabilities to plasmid DNA, low cytotoxicity (cell viability being within 60-101 %) and an increase in transfection activities, in the human hepatocellular carcinoma cell line HepG2, which expresses the ASGP-R abundantly. The results obtained correlate well with differences in the spacer element in the 3 galactosylated cholesterol derivatives under study and the presence and absence of 2 mole % DSPE-PEG_{2000} in the liposome formulations.

Overall, it was observed that the cationic liposome containing cholesteryl (1-β-D-galactopyranosyl-1,2,3 triazol-4-yl) carbonate (with and without PEGylated accessories), which was synthesised chemically using “click chemistry”, afforded the highest *in vitro* transfection activity, and may be optimised and studied further. The highest levels of transfection activity, *in vitro,* were attributed to the increased length of the spacer arm between the galactose moiety and the cholesteryl anchor of the

targeting component. Two formulations were then subjected to *in vivo* studies, using male Sprague Dawley rats which yielded little or no transgene expression.

PREFACE

The experimental work described in this dissertation was carried out in the Department of Biochemistry, School of Life Sciences, University of KwaZulu-Natal (Westville Campus), Durban from July 2010 to December 2012, under the supervision of Dr Moganavelli Singh and the co-supervision of Professor M. Ariatti and Dr B. Masola.

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

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DECLARATION – PLAGIARISM

I, ___Dhineshree Govender___, declare that

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

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

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ABBREVIATIONS

ATP	Adenosine-5-triphosphate
BCA	Bicinchoninic acid
Chol-T	3 β [N-(N', N'-dimethylaminopropane)-carbamoyl] cholesterol
CMV	Cytomegalovirus
DC-Chol	3 [N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DOGS	Dioctadecylamidoglycyl – spermine
DOPC	dioleolyphosphatidylcholine
DOPE	Dioleoylphosphatidylethanolamine
DOSPA	2,3 – dioleyoxy-2(6carboxylspermyl)-propylamide
DOTAP	N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulphate
DOTMA	N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
EDTA	Ethylenediamine tetra-acetic acid
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FR	Folate receptor
GAGs	Glycosaminoglycans
HBS	Hepes buffered saline
HCC	Hepatocellular Carcinoma
HEK 293	Human embryonic kidney cell line
HepG2	Human hepatocellular carcinoma cell line
HR	Hyaluronan receptors
MEM	Minimum essential medium

MS1	Cholesteryl-β-D-galactopyranoside
MSO9	N,N-dimethylaminopropylamidosuccinyl-cholesterylformylhydrazide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NN	Cholesteryl-3β-N-(4-aminophenyl-β-D-galactopyranosyl) carbamate
PBS	Phosphate buffered saline
PEG	Polyethylene Glycol
RES	reticuloendothelial system
RME	receptor mediated endocytosis
SDS	Sodium dodecyl sulphate
UV	Ultraviolet
WT	Cholesteryl (1-β-D-galactopyranosyl-1,2,3 triazol-4-yl) carbonate

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CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

1.1.GENE THERAPY

The concept of gene therapy emerged some 30 years ago and has enabled treatment of human disease at the genetic level. This therapeutic approach is superior to other systems that are based on merely treating the symptoms of genetic diseases (Motoyama *et al*., 2011 and Mountain, 2000).

Gene therapy involves the administration of therapeutic genes (DNA) to patients, with the goal of enhancing the expression of specific genes or inhibiting the production of specific target proteins (Kawakami *et al*., 2008). Since both the cell membrane and DNA are negatively charged they tend to repel one another. Therefore carrier systems (also called vectors) are required for the delivery of these therapeutic genes (Templeton *et al*., 2002) into the cell. Various systems, such as viral and non-viral vectors, have been engineered and extensively analysed. These carrier systems seem to excel in some areas but still possess downfalls, in terms of their gene delivery efficiency (reaching their specific target cell) and their resulting transfection efficiency. Although viral vectors have produced greater transfection efficiency, they have shown the potential, to be highly immunogenic, oncogenic (chromosomal integration) and to lack target specificity. Non-viral vectors however, are virtually non immunogenic, are safe to use, easy to produce and can be engineered to achieve organ/tissue specificity (Zhang *et al*., 2010).

Liposomal based gene delivery is the most frequently analysed synthetic (non-viral) vector, with approximately 45 open clinical trials being carried out worldwide (Simões *et al*., 2005). Liposomes are microscopic lipid bubbles which tend to form by self-assembly to yield a basic structure, of being vesicular. The bubble structure of a

liposome is a result of interactions that occur between the lipids in the solution, and the structure is completely dependent on the components in the solution (Karmali and Chaudhuri, 2007). Lipids contain a hydrophobic tail and a hydrophilic head group, and those that are used in the formation of liposomes, are connected by a backbone linker (usually glycerol). Liposomes form by separation of hydrophilic components from the hydrophobic components (Balazs and Godbey, 2011), resulting in the shielding of the hydrophobic components from the aqueous environment (Figure 1.1).

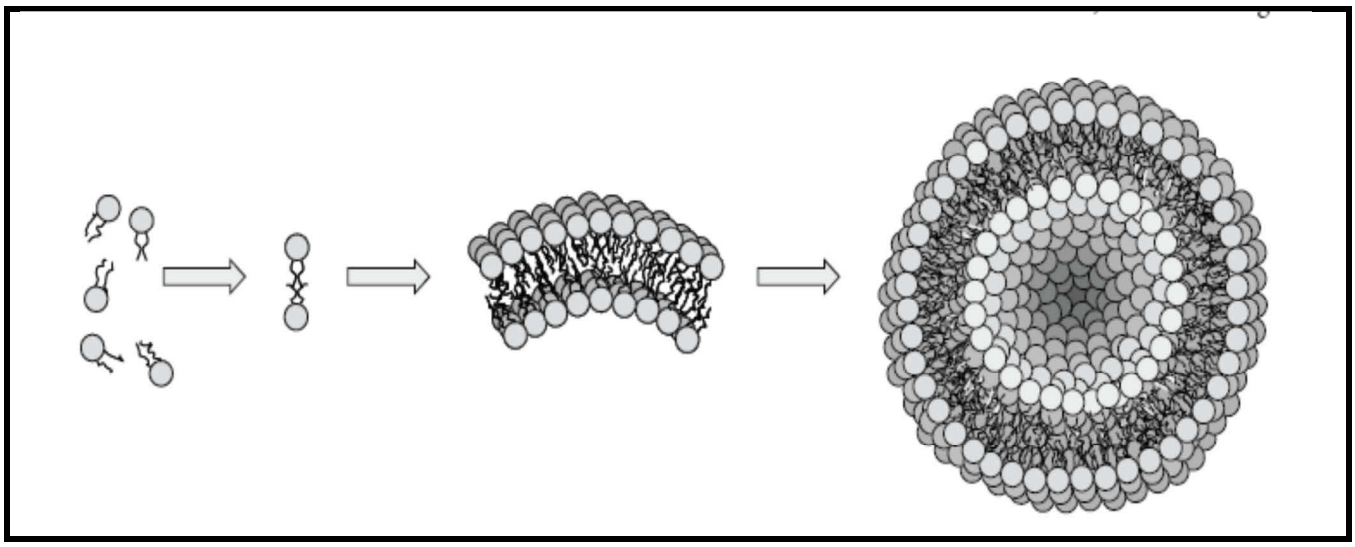


Figure 1.1: Basic formation of liposomes (Adapted from Balazs and Godbey, 2011).

Lipid vesicles such as liposomes, possess an abundant array of advantages such as the ability to be easily engineered and synthesized, they bind or envelop therapeutic drugs, confer protection to the genetic therapeutic drugs, have virtually no immunogenic response, and given their differential release mechanisms and low cost (Zhang *et al*., 2004) these vehicles are an ideal partner for effective therapeutic gene delivery. Cationic liposomes are usually engineered such that they possess an amine polar head group region which facilitates the binding of negatively charged molecules, such as DNA. These liposomes have been shown to bind and complex with almost all of the DNA molecules in a given solution (Zhdanov *et al*., 2002) thus gaining much attention in gene delivery.

Cationic liposomal formulations generally consist of a cationic lipid and, a neutral helper lipid. Some common cationic lipids that are often used in the synthesis of

liposomes include, dimethylaminoethane carbamoyl cholesterol (DC-Chol), *N*-[1-(2,3-dioleyoxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA), dioctadecylamidoglycylspermine (DOGS) and dioleoyl trimethylammonium propane (DOTAP) (Figure 1.2). These lipids form the hydrophobic domain of the liposome (Karmali and Chaudhuri, 2007) while the head groups form the hydrophilic surface.

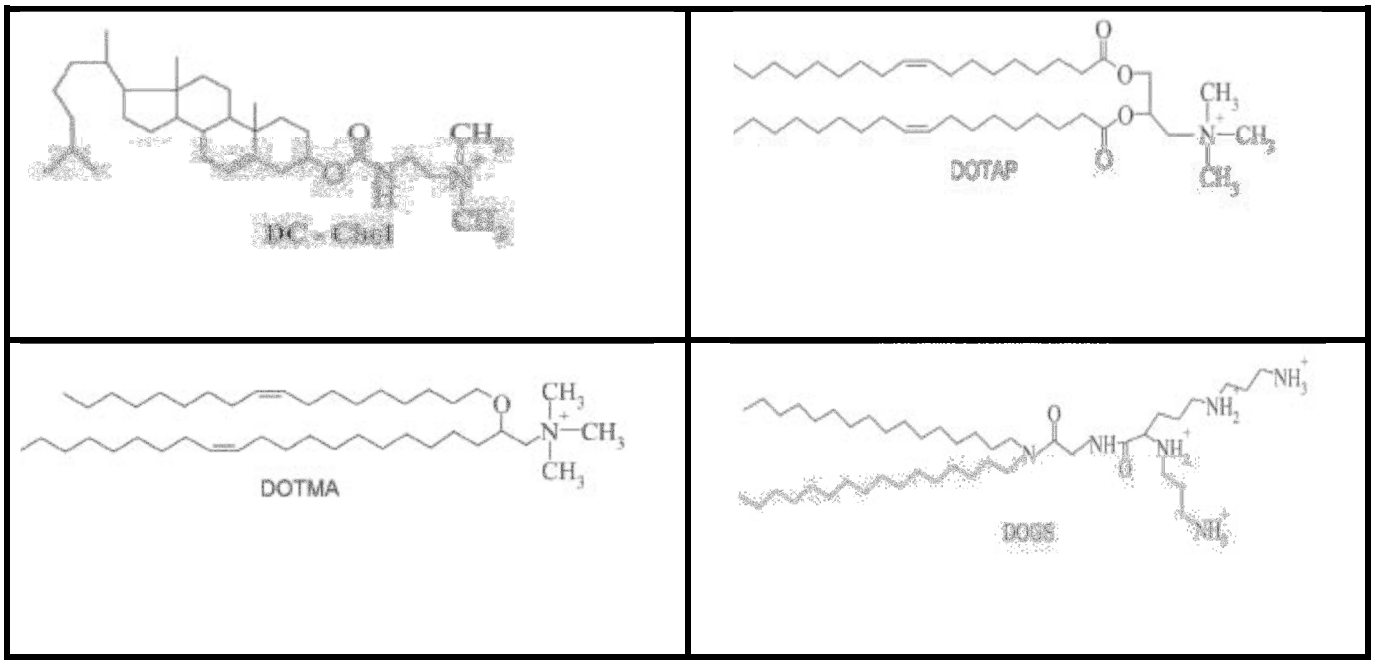


Figure 1.2: Common cationic lipids that have been extensively used to engineer effectively competent liposomes.

Lipids used for the formation of liposomes may contain up to three aliphatic chains. Previous research suggest that liposomes produced from a lipid containing one aliphatic chain form micelles and those formed from lipids containing three aliphatic chains transfect cells poorly (Karmali and Chaudhuri, 2007). Hence the hydrophobic domain of conventional liposomes usually contains two aliphatic linear chains and has shown to form transfection competent liposomes that possess a bilayer. The linker bond forms a bridge between the polar head group and the hydrocarbon anchor. The linker group controls the degree of transfection since it governs the conformational flexibility, biodegradability and stability of the liposome. Commonly used linkers are shown in Table 1.1.

Table 1.1: Various linker groups and advantages for their use in liposomal formulations (Adapted from Karmali and Chaudhuri, 2007).

Linker Group	Example	Advantages	Disadvantages
Carbamates	DC-CHOL	Chemically stable; Biodegradable	*None
Esters	DOTAP	Biodegradable; Less Toxic	Not chemically stable
Disulfides	DOPE	Chemically stable; Biodegradable	*None
Ethers	DOTMA	Chemically stable	Non-biodegradable
Amides	DOGS	Chemically stable; Biodegradable	*None

*No disadvantages documented thus far.

Helper lipids that are generally used in the formation of liposomes include; dioleolyphosphatidylcholine (DOPC) and dioleoylphosphotidylethanolamine (DOPE). These are neutral lipids that impart no charge to the liposome, but are responsible in increasing the stability of the cationic liposome/DNA complex (lipoplexes) (Kawakami *et al*., 2008). Several studies have shown that liposomes engineered to contain DOPE in their formulation, yielded higher transfection efficiency (Zhang *et al*., 2010).

When lipoplexes form, they exist as a lamellar packing of lipid and DNA. It is thought that DOPE, at a low pH, causes a conformational change in the packaging structure of the resulting lipoplexes. DOPE has been shown to convert this lamellar packaging into an inverted hexagonal packing structure. When in this conformation, the DNA is compacted further through electrostatic interactions (Chesnoy and Huang, 2000) reducing premature loss of the DNA before reaching the aimed destination. The hexagonal conformation also allows for the efficient release of complexed DNA from the endosomal membrane (Zuhorn *et al*., 2005). In addition, DOPE has the ability to reduce the energy required for DNA binding by facilitating the release of ions from the cationic head group (Zuidam and Barenholz, 1998).

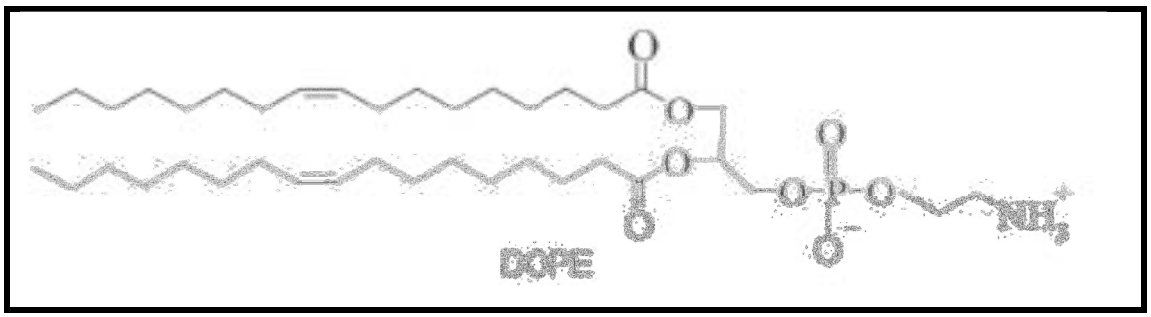


Figure 1.3: Structure of DOPE, a common neutral helper lipid used in liposomal formulations.

Tang and Hughes (1998) was the first to engineer a cationic lipid with a disulfide bond. The introduction of the disulfide linker was aimed to circumvent the poor release of the therapeutic DNA from the liposome into the cell cytoplasm. It has been shown that the incorporation of auxiliary lipids (such as DOPE and/or cholesterol) can render previously incompetent liposomes, competent (Mukherjee *et al*., 2005) and this characteristic enhances the probability of researchers to utilize such a lipid in existing and novel formulations.

1.2. THE USE OF POLYETHYLENE GLYCOL (PEG)

Liposomes are nanoparticles that have gained much attention in gene delivery since they have the potential to penetrate through capillaries and reach target sites due to their small size. However when injected intravenously, liposomes are treated as foreign material by the host cell. Generally when liposomes are administered into a host, the host reticuloendothelial system (RES) recognises the material as foreign, resulting in the rapid elimination of the delivery vehicle. The surface of cationic liposomes is also known to attract plasma proteins which reduce the efficiency of the liposomes. Liposomes were thus engineered to incorporate polyethylene glycol (PEG) in their formulation (Managit *et al*., 2005; Pathak *et al*., 2008).

PEG is a neutral and synthetic polymer. This molecule is known to be soluble in water and in some organic solvents such as ethanol, methylene chloride, chloroform and acetone. Solubility, in an aqueous solution, can be attributed to the ether oxygen

linkage that acts as a hydrogen bond acceptor (Gad, 2007). The incorporation of PEG into liposomes yields an attractive liposomal formulation that is more resistant to degradation by serum nucleases *in vivo.* PEG is non-toxic to the cells, available in numerous molecular weights, easy to use and is readily excreted by the kidney, thus making it a desirable additive in liposomal generation (Metselaar *et al*., 2003).

The U.S Food and Drug Administration (FDA) have approved the use of PEG in various applications including injectable pharmaceutical formulations, due to the non-toxic properties of PEG. *In vivo* studies have shown that when a PEGylated formulation is administered into the bloodstream it is rapidly expelled from the body. The clearance rate of PEG is considered to be inversely proportional to the molecular weight of the polymer, with PEG < 30 kDa being excreted via the kidney, and PEG molecules >20 kDa being excreted via the faeces (Gad, 2007). After its entry into the body, PEG is known to accumulate mainly in the liver, skin, muscle and bone. It is important to note that the accumulation of the polymer is independent of molecular weight.

The first research group to engineer PEGylated liposomes was Shimada and co-workers (1997). They designed a liposome with PEG coupled to a glycoprotein. The liposome differed from the 'norm' since the galactose sugar incorporated in the liposome was separated from the lipid interface by the PEGylated chains at different lengths. This particular system was engineered to evaluate the effect of the space of the PEG chains (PEG 10/20/40) and the anti-opsonic action of the PEGylated molecules. The main aim for varying spacer lengths was to improve the exposure of the ligand to its cognate receptor.

PEGylated liposomes are frequently referred to as "stealth liposomes' since they have been shown to increase the stability of the liposome, thus increasing the circulation time of the lipid structure, within the cell. Increase in stability is a result of the PEG molecules surrounding the surface of the liposome and forming a steric barrier which prevents (to a certain degree) the clearance of the lipoplexes by macrophages. The only disadvantage seen thus far with the use of PEG molecules is their lack of targeting to a specific organ. However it was also shown that the incorporation of PEG into liposomal formulation could inhibit the internalization of the lipoplexes via

endocytosis. Inhibition is dependent on both the percentage (in moles) of PEG molecules on the liposome and the type of functional group conjugated to the liposome (Shi *et al*., 2002).

PEG can be incorporated into the liposomal bilayer (Moghimi *et al*., 2001) or activated and anchored into reactive phospholipids groups of previously formed liposomes. It has been demonstrated that the use of 3-7 mole % of PEG, increases the circulation time of the PEGylated particle, provided that the final size of the particle is within a size range of 70-200 nm. Circulation of the PEGylated liposome may also increase when phospholipids and cholesterol are added component to the formulation. The mechanism, by which the PEG increases the circulation of a given liposome, is governed by the ability of the PEG, to prevent binding of the liposome with opsonins in the blood. PEG molecules contain a "flexible" chain that has been shown to occupy the periliposomal layer (the space adjacent to the surface of the liposome) which shields the liposome. This enables the cationic liposome to escape recognition by the cell as foreign material. Thus the liposome is not rapidly removed by the Kupffer cells present in the liver (Immordino *et al*., 2006).

With respect to the circulation half-lives of these stealth liposomes (PEGylated liposomes), it is estimated that in humans that liposomes remain in circulation in the blood for up to 45 hours following administration, and 15-24 hours in much smaller animals such as rodents. The incorporation of PEG into liposomes is considered as a great stepping stone towards the perfect gene delivery vehicle for *in vivo* applications. PEG has been shown to be non-toxic to animals, and to shield cationic liposomes from proteolytic enzymes (Hinds and Kin, 2002). The PEGylated molecules provide the gene delivery vehicle with a camouflage that almost avoids recognition by the RES and prevents aggregation of the lipoplex thus prolonging circulation time (Gad, 2007). PEG is capable of decreasing immunogenicity by blocking antibody sites. This in turn, increases solubility and allows for frequent dosing of the test formulation (Gad, 2007).

There exist various types of PEG, with differences noted in the type of configuration, conformation, as well as in the molecular weight. With respect to the conformation, the PEG molecule may exist as a linear, branched or multiple-branched molecule. A

branched configuration is more efficient than a PEG molecule that bears a linear conformation (Chirino *et al*., 2004).

1.3. NEW GENERATION OF LIPOSOMES: TARGETING TO A SPECIFIC ORGAN.

There are two types of targeting that are associated with gene delivery; passive and active targeting (Figure 1.4). In passive targeting, genes or drugs are targeted to their specific target organ based on the physicochemical properties as well as the size of their carrier or themselves (in terms of the targeting of drugs to specific cells). Formulations already approved for human treatment, is the anthracycline drug, doxorubicin. Pharmaceutically this drug in its PEGylated form, is known as, Doxil, and is used to treat cancer in patients diagnosed with AIDS-related Kaposi sarcoma, as well as in patients with multiple myeloma. The drug offers lower cardiotoxicity whilst maintaining an increase in its efficacy when compared with treatment using free doxorubicin (Ning *et al*., 2007).

Active targeting on the other hand employs the use of ligands that essentially will traffic the desired gene or drug to the specific target cell (Zhang *et al*., 2010). This approach allows for a considerable amount of control in the targeting of genes to a specific tissue or organ.

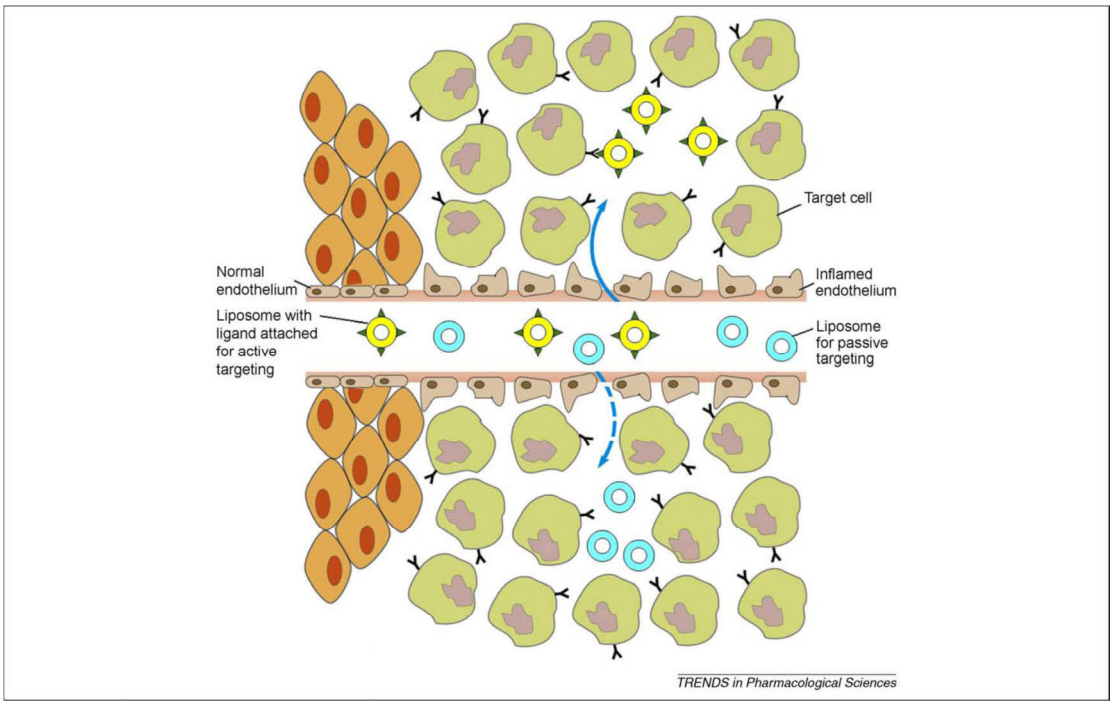


Figure 1.4: Active and passive targeting to cells for drug targeting using liposomes. (Adapted from Ghosh *et al*., 2008).

At regions of pathology where inflammation of the endothelium layer is predominant, different types of mediators (vascular endothelium growth factor and prostaglandins) have been shown to increase endothelial permeability. Underlying pathologies include cancer, rheumatoid arthritis and infection. Liposomes extravasate through the gaps between cells and enter the interstitial fluid (Ghosh *et al.,* 2008*).* Active targeting is achieved by conjugating ligands to the liposome that bind to a specific target cell receptor, leading to internalization or release of the drug. Table 1.2 provides some examples of active targeting. Passive targeting can be mediated by internalization or local high-concentration release of the drug (Zhang *et al*., 2010).

Table 1.2: Examples of active targeting drug delivery systems. aAGIP, amyloid growth inhibitory peptide; ASGP, asialoglycoprotein; mBAFF, mutant B cell activating factor belonging to the TNF family; SAP, sweet arrow peptide; TfR, transferrin receptor (Adapted from Malam *et al*., 2009).

Ligand	Receptor/ Target	Study Findings
Anti-CD74 antibody, LL1	CD74 receptor	Ligand covalently attached to liposomes; selective for malignant B lymphoma cells
TfR-targeting peptide HAIYPRH	TfR	Conjugation to the TfR-binding peptide significantly improves the anti-cancer potency and selectivity of the anti-cancer drug artemisinin
Folate	Folate receptor (FR)	FR is overexpressed on cancer cells Folate has been conjugated on liposomes loaded with doxorubicin for targeting of cancer and on NPs for targeted paclitaxel delivery
mBAFF	BAFF receptor	BAFF is the usual endogenous ligand for the BAFF receptor; mBAFF is a soluble BAFF mutant in which amino acids 217–224 are replaced by two glycine residues that can bind to BAFF receptors. PEGylated liposomes developed with mBAFF as a targeting ligand target certain B lymphoma cells in vitro.
Hyaluronic acid	Hyaluronan receptors (HR)	HT-29 cancer cells overexpress HR. Hyaluronic acid incorporated in chitosan NPs loaded with the anti-cancer drug 5-flurouracil exhibited higher cytotoxicity in vitro
Galactose	ASGP receptors	ASGP receptors are overexpressed on hepatoma cells. Dextran-based polymeric micelles were used to target liver cancer in vivo with superior results

With passive and active targeting, the delivery of transgenes and their subsequent expression is notably governed by the barriers that the delivery system encounters. Active targeting of genes is considered the most favourable choice in targeting since it has been observed to reduce serious adverse effects such as immune responses or cytotoxicity, and increase the transfection efficacy (Zhao *et al*., 2008).

The delivery of genetic material to hepatocytes presents great potential to science and disease therapy since the hepatocytes are cells that play a key role in the synthesis of various proteins that participate in biological processes both inside and outside of the

liver itself (Shigeta *et al*., 2007). Due to its relatively large size, the liver is a major site for lipid and protein production, allows accessibility to larger molecules, plays a role in metabolism, and is the site for the secretion of serum circulating polypeptides and numerous enzymes. The organ is also described as being partially "immuno-priviledged" since it bares tolerance to immunological reactions that occur when foreign material escapes the gastrointestinal tract and gains entry into the liver via the portal system (Pathak *et al*., 2008).

The liver is an organ consisting of different cell types. These include the highly important liver parenchymal cells (hepatocytes), liver macrophages (kupffer cells) and the sinusoidal endothelial cells. Hepatocytes make up 80 % of the total mass of the liver and hence serve as a potential target for gene delivery aimed at treating various liver associated diseases, such as Hereditary Hemochromatosis, Wilson's disease and Hepatocellular Carcinoma (HCC) (Pathak *et al*., 2008).

Nanomedicine is a promising approach to clinical practice, providing resolution to crucial limitations faced by the diagnoses, treatment and maintenance of human diseases. The various branches of nanomedicine include drug delivery (Yokoyama 2005), regeneration of tissues (Zhang and Webster, 2009) as well as imaging using Quantum dots (Jamieson *et al*., 2007). The use of liposomes as delivery vehicles is regarded as a 'mainstream' gene or drug delivery technology. Various ligands have been engineered for their bio-sensing and bio-signalling to liver hepatocytes. Hepatocytes express the asialoglycoprotein receptor (ASGP-R) on their sinusoidal surface. These receptors recognize and specifically bind asialoglycoproteins or galactosylated polymers and internalize these molecules. From all ligands researched for gene delivery, the galactose moiety has proven to be the ligand with the greatest potential for targeting to hepatocytes. Numerous attempts have been made by researchers, to use galactosylated liposomes to target liver parenchymal cells, both *in vitro* and *in vivo* (Kawakami *et al*., 2000, Kawakami *et al*., 2002, Higuchi *et al*., 2006).

1.3.1. The Asialoglycoprotein Receptor (ASGP-R)

Present on the surface of mammalian hepatocytes are the asialoglycoprotein receptors. These receptors are abundantly expressed on the surface of the liver parenchymal cells and thus have gained much interest in gene therapy. Besides being expressed in abundance, the ASGP-R contains approximately 1 - 5 x 10^5 binding sites/cell. The ASGP-R is randomly dispersed throughout the basolateral sinusoidal plasma membrane domain, in the direction facing the capillaries. Furthermore the receptors are thought to be found in either uncoated or coated pits and usually located with mannose-6-phosphate, poly-(Ig) and transferrin receptors (Pathak *et al*., 2008).

The morphology of the blood capillaries varies between different organs, with the liver containing discontinuous capillaries that exhibit inter-endothelial junctions with a large diameter of up to 150 nm. Thus the size of the DNA/cationic liposome complex should ideally be less than 150 nm in size, in order for the lipoplexes to bypass the capillaries (Takakura *et al*., 1998). The receptors recognize their specific substrates, which are glycoproteins that bear either a terminal galactose or an N-acetyl glucosamine. The proteins are internalized via clathrin coated pits and transported to the intracellular compartments. This natural process is adapted such that synthetic substances may be coupled with a desired ligand (lactose or galactose) and internalized into the cell by a process termed receptor-mediated endocytosis (RME). The attachment of a lactosylated or galactosylated moiety to the surface of a liposome was found to increase the uptake of the liposomal formulation by the liver cells (Wu *et al*., 2002).

1.3.2. Endocytosis

The success of a delivery vehicle to efficiently deliver nucleic acids to a specific site is dependent on the ability of the vehicle to firstly enter the cell; bypass digestion by serum nucleases, and to gain access to a specific target organ in an appropriate concentration. Generally, the nucleic acid adheres to a cell surface protein and is internalized into the cell. The nucleic acids are then released into the cell and are exposed to the internal barriers associated with gene delivery (Karmali and

Chaudhuri, 2007). The electrostatic repulsion that exists between naked DNA (negatively charged) and the phosphatidyl bilayer (negatively charged) lowers the possibility for the diffusion of the naked DNA into the cell, to occur. DNA may enter the cell through the process of endocytosis (Figure 1.5).

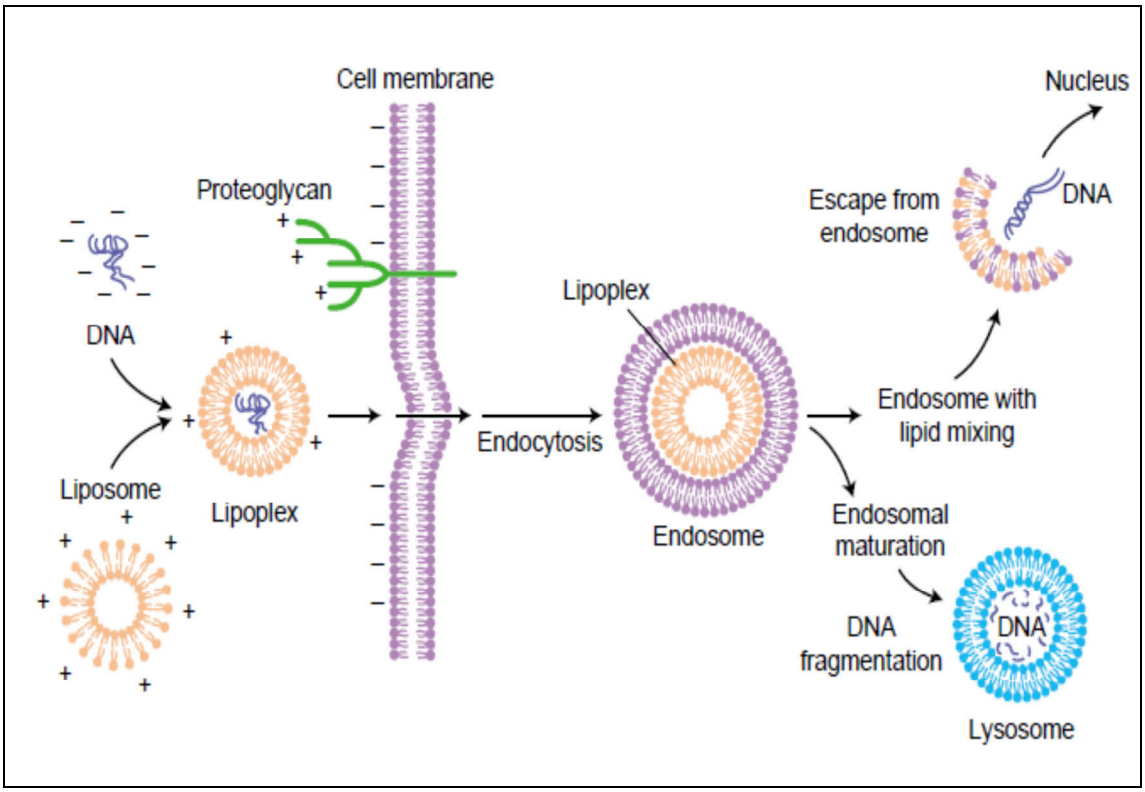


Figure 1.5: Endocytosis of cationic liposomes into the cell (Adapted from Parker *et al*., 2003).

The endolytic pathway is subdivided into two classes, pinocytosis (phenomenon called cell drinking) and phagocytosis (phenomenon called cell eating). The process of endocytosis involves several steps. It is believed that the spontaneous engulfment of DNA initiates the start of the endolytic pathway. This is followed by invagination of the plasma membrane (inward folding of the membrane) which results in the formation of a droplet of the extracellular membrane. The droplet is fully formed once it pinches off from the cell membrane which yields an endosome (an intracellular coated vesicle which envelopes the ingested material DNA). These endosomal vesicles are capable of increasing in size when they fuse together with other

endosomes. Endosomes may fuse with primary lysosomes that contain numerous hydrolytic enzymes resulting in the formation of a secondary lysosome. Due to the large content of hydrolytic enzymes in the lysosome, the formation of the secondary lysosome, results in the termination of the gene delivery process since the DNA is degraded by the enzymes. The endocytic membrane is re-cycled as it is known to return to the plasma membrane. It is only when a delivery vehicle can bypass the lysosomes, that the DNA can transverse the cytoplasm and reach the nucleus (Pathak *et al*., 2008; Elouahabi and Ruysschaert, 2005).

Cationic liposomes face numerous barriers with the most potent barrier, *in vivo*, being digestion of the therapeutic DNA by serum nucleases present in the blood (Yang and Huang, 1997). The anionic proteins present in the serum are thought to have an inhibitory effect on transfection (Audouy *et al*., 2000). Once the lipoplex enters the cell, the negatively charged serum proteins are attracted to the cationic liposome/DNA complex and attach to the complex. This results in the aggregation of lipoplex which either precipitates out the complex or causes the complex to disintegrate, thus releasing the corrective DNA well before it can reach its target site. Hence serum proteins have led to the reduction or inefficiency of corrective DNA delivery (Li *et al*., 2011).

Numerous strategies have been developed to overcome this barrier. These include the manipulation of existing liposomal formulations such as the amino acids and alkaline cationic lipids (Obata *et al*., 2008) and the addition of helper lipids such as PEGylated lipids (Ross and Hui, 1999). An increase in the positive charge ratio of the cationic liposome/DNA complex (1.0-4.0) has also shown increased transfection in 20% serum (Yang and Huang, 1997). However in the case of excessive positive charge, the cationic liposome is directed almost exclusively to the lung. Hence targeting to another organ, such as the liver, would render this approach unfavourable (Kawakami *et al*., 2008). Li and co-workers (2011) have shown that the fate of lipoplexes *in vivo* is dependent on three crucial aspects: the headgroup of the liposome, the stability if the lipoplex and the degree of cellular uptake. An increase in the lipid content during lipoplex formulation yields a lipoplex size of approximately 200 nm.

1.4. RECEPTOR MEDIATED ENDOCYTOSIS AND THE FATE OF LIPOSOMES *IN VIVO*

In vivo studies often face barriers that dampen their effective delivery to the target cell. These include enzymatic degradation of the nucleic acids in the blood; the lipoplex interaction with the blood components causing aggregation (mentioned above), as well as the non-specific uptake of the lipoplex by other cells (Kawakami *et al*., 2008). It has been shown that the administration of naked therapeutic DNA (intravenous route) induces virtually no gene expression (Kawabata *et al*., 1995). Hence advances have been made regarding a carrier system for the plasmid DNA.

Since the first successful *in vivo* transgene expression which was reported by Zhu *et al* (1993) using the DOTMA/DOPE system, considerable strides have been made in gene delivery. Cationic liposomes possess the ability to condense DNA which helps prevent the premature detachment of the corrective DNA prior to the DNA reaching its target cell, allowing for a greater amount of the corrective DNA reaching the target cell. Intravenous injection of traditional lipoplexes, have shown to accumulate predominantly in the lung since the lungs are the first to trap these complexes, creating a problem when the target organ is not the lung.

Many researchers have shown that target organ specificity may be achieved via receptor mediated endocytosis. Liposomes can be grafted such that the polar head group region bears a ligand that is specific for a receptor that is located, in abundance, on the target cell. For example, liver cells overexpressing the asialoglycoprotein receptor (ASGP-R) are specific for the sugar galactose. Hence tagging a cationic liposome with galactose moiety would essentially target the liposome to hepatocytes, achieving liver-targeting drug delivery (as shown below).

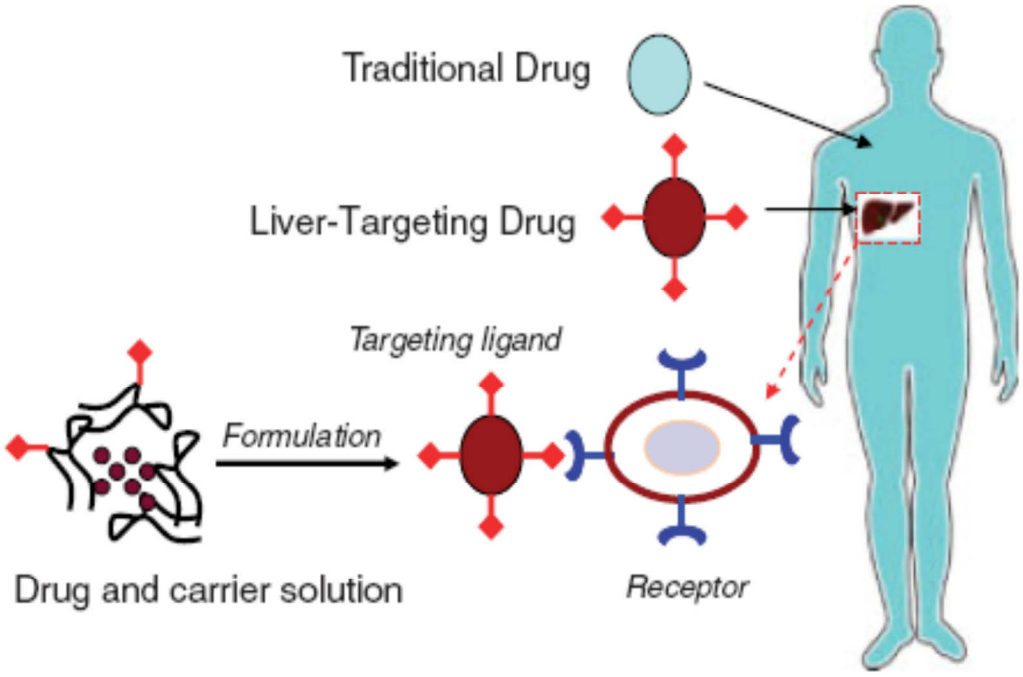


Figure 1.6: The generation of a cationic liposome grafted such that it possesses a ligand (galactose) that binds to the ASGP receptor present on the surface of hepatocytes (Adapted from Zhang *et al*., 2010).

Galactosylated cationic liposomes, such as Gal-C4-Chol (Kawakami *et al*., 2000; Fumoto *et al*., 2003) showed successful *in vivo* gene delivery specifically to hepatocytes. The ability to travel through the vascular walls of the cell presents a problem for delivery vehicles. In the liver, the sinusoidal wall does not possess a basement membrane but contains a fenestrated endothelium which is approximately 100-150 nm in diameter. Thus for efficient gene delivery to hepatocytes, the delivery vehicle should ultimately be smaller than 100-150 nm in size to travel through the fenestrae and gain access to the space of Disse (or perisinusoidal space) which lead directly to the target hepatocytes (Pathak *et al*., 2008). The studies of Shimada and co-workers (1997), using Gal-PEG10-lip showed that when the liposome was administered *in vivo*, the formulation was rapidly cleared from the system with a half-life ($t_{1/2}$) of 30 minutes. They compared this with a control (liposome without Gal-PEG-10-lip) and established that the control liposome had a half-life ($t_{1/2}$) of 12 hours. The group further showed that the rapid clearance was due to an enhanced uptake of the Gal-PEG-10-lip liposome (>90 % of the dose administered) by kupffer cells present in the liver. The percentage of the liposomal formulation that accumulated in

the spleen was only 1 % of the total volume injected into the animal. Interestingly, injecting Gal-PEG-Lip liposomes following the administration of N-acetylgalactosamine, showed a reduction in the rate of clearance compared to the control levels (initial rate of plasma clearance). The group also investigated the effect of PEG-DSPE (monomethoxypoly(ethyleneglycol)) incorporated into the Gal-PEG10-Lip and found that the PEG-DSPE only partially reversed the rate of plasma clearance of the liposome in the liver and spleen. They concluded that Gal-liposomes are recognized by the kupffer cells and removed from the cell.

Murao and co-workers (2002), evaluated the targeting efficiency of galactosylated liposomes to hepatocytes, based on a varying amount of lipid. The group synthesized various liposomal formulations, which consisted of 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol (CHOL), and cholesten-5-yloxy-N-(4((1-imino-2-D-thiogalactosylethyl)amino)butyl)formamide. The liposomal formulations were analysed *in vivo*, through intravenous injection to mice. They proved that liposomal formulations containing galactose were solely taken up by the liver hepatocytes, whereas those formulations without the galactose sugar were found to be distributed amongst the hepatocytes and nonparenchymal cells. However, when the molar ratio of DSPC was increased (to 90 %), in both the liposomal formulations, the uptake of the liposomes into the nonparenchymal cells increased dramatically. Since the results were constant for liposomes containing and those lacking the sugar moiety, it is possible that the uptake and internalization of the liposomes were based on a mechanism other than internalisation by the asialoglycoprotein receptor.

As mentioned previously, the major limitation of the use of cationic liposomes in gene delivery is the poor targeting potential. Liposomes have to be targeted to their specific target organ. Once the lipoplex is inside the cell, efficient endosomal release of the pDNA into the cell cytosol is crucial. Once released, the DNA is further subjected to cytosolic nucleases that may degrade existing DNA. Trafficking of adequate DNA into the nucleus is important since the DNA must gain access to the transcriptional machinery (Karmali and Chaudhuri, 2007). Targeted gene delivery was one of the most crucial barriers that have now been overcome using cationic liposomes. However, when targeting a lipoplex to a specific organ the size of the lipoplex has to

be within a specific size range due to the structural differences of the blood capillary walls in various tissues and organs.

An ideal nanosystem ensures that the lipoplex arrives and acts only on the selected targeted cells. The carrier must be biocompatible and biodegradable. This means that the liposome, which forms part of the lipoplex, should, upon entering the cell, metabolize or degrade into non-toxic components. Furthermore, these components should be rapidly expelled from the cell upon degradation.

1.5. HYPOTHESIS

RME allows for the site specific delivery of foreign genes via receptors. Various ligands have been used to target the receptors present on the surface of the hepatocytes (asialofeutin, asialoorosomucoid, lac-BSA, asialolactoferrin, asialoceruloplasmin, and galactose). However the ligand showing the greatest interest for gene delivery thus far (both *in vitro* and *in vivo*), is the galactose moiety. Thus three different galactosylated cationic liposomal formulations were subjected to *in vitro* analyses in an attempt to compare the three formulations, to determine, which formulation yields the best transfection efficiency. The best liposomal formulation *in vitro* will determine the direction in which future studies need to be focussed, in order to produce a viable gene delivery vector for clinical studies.

1.6. OUTLINE OF THESIS

In this project, a comparative study of three distinct, galactosylated cationic liposomal formulations in gene delivery was carried out. The three galactosylated liposomal formulations in this study were targeted specifically to the liver hepatocytes via the ASGP-R to establish which of the three liposomes was the most efficient in hepatocyte transfection. Of the three liposomes being tested, two were synthesized and analysed at the UKZN-Westville, Biochemistry department and the other at the Chemistry department, University of the Witwatersrand. All three liposomes were previously synthesized and analysed separately but never compared to one another

under similar conditions despite exhibiting high transfection activities *in vitro* (Narainpersad *et al*., 2012; Singh *et al*., 2007).

Both PEGylated and non PEGylated galactosylated cationic liposomes were investigated, *in vitro*, for cytotoxicity and luciferase gene expression, in the human hepatocellular carcinoma cell line, HepG2, and the human embryonic kidney cell line, HEK293 (control ASGP-R negative cell line). The three unique galactose targeted liposomes are: Cholesteryl-3β-N – (4-aminophenyl-β-D-galactopyranosyl) carbamate; Cholest-5-en-3-yl [1-(β-D-galactopyranosyl)-1*H*-1,2,3-triazol-4-yl]-methylcarbamate; Cholesteryl-β-D-galactopyranoside. Untargeted, PEGylated and non PEGylated liposomes were also be prepared and investigated for comparison. All cationic lipids were analysed by NMR, and liposomes and lipoplexes were characterised by electron microscopy. Liposome:DNA interactions were characterised by band shift and nuclease digestion assays. The best formulation was subjected to *in vivo* analyses.

CHAPTER TWO

PREPARATION AND CHARACTERISATION OF LIPOSOMES

2.1. INTRODUCTION

Liposomes are formed when amphiphilic molecules, are exposed to an aqueous environment. This is a spontaneous reaction in which the hydrophilic molecules protect the hydrophobic molecules, from the aqueous layer (Karmali and Chaudhuri, 2007).

Liposomes have the ability to entrap drugs within their aqueous core or to bind them on the surface of their structure. The process is greatly dependent on the method used to synthesize these nano-sized vesicles. Often when liposomes are prepared, conditions are metastable. This means that the state of free enthalpy and the environment in which the liposome are synthesized are not in equilibrium. Liposomes synthesized in a metastable state, have the capability to change their morphological conformation producing a heterogeneous mixture of size, lamellarity, and shape, with time (Lautenschlager, 2006). A variety of techniques have been employed for the preparation of liposomes. These include extrusion of lipid suspensions through filters, ethanol injection, detergent depletion, ether injection, thin lipid film hydration and reverse phase evaporation (Lasic, 1997; Campbell, 1995; Torchilin, 2003; Deamer and Bangham, 1976; Gao and Huang, 1991; Szoka and Papahadjopoulos, 1978).

Lipid amphiphiles are chemically designed to embody three unique structural domains; the hydrophobic anchor, the linker and/or spacer element and the hydrophilic headgroup (Figure 2.1). The nature of the cationic lipid used as the hydrophobic anchor, can either consist of a fused ring system such as that seen in steroids (cholesterol) or lipids containing long hydrocarbon chains engineered in the past, that proved less effective (example DOTMA and DOTAP).

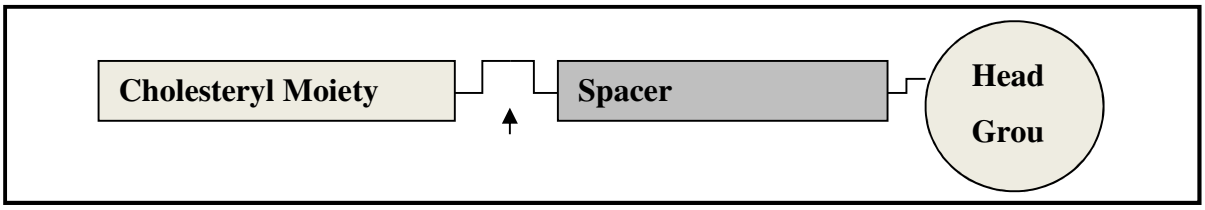


Figure 2.1: Molecular arrangement of a typical lipid amphiphile found in liposome preparations.

DC-Chol was the first steroid-based lipid to be used in liposomal formulations and to date is the most commonly utilized cationic lipid. It has been shown that this particular steroid-based lipid confers lower toxicity and greater transfection efficiency. Cationic cholesterol lipids proved superior over previously used glycerol-based amphipiles (Biswas *et al*., 2009). The major advantage of using cholesterol is that the 3β-OH of the lipid accommodates for structural modification at the linker element and hydrophilic head group.

The linker element attaches the cholesteryl moiety to the cationic head group. Commonly used linker elements are esters, ethers and amides. The linker group governs the biodegradability, chemical stability and transfection efficiency of the cationic lipid. The linker bridge is sensitive to numerous biological stimuli within the cell. This sensitivity allows for the release of DNA from the lipoplex (liposome:DNA complex) at specifically defined end points (Biswas *et al*., 2009). Helper lipids generally form a component of the cationic liposomal formulation. Co-lipids such as DOPE, cholesterol and DOPC assist in low cell toxicity and effective gene transfer (Mukherjee *et al*., 2005). The positively charged hydrophilic head group allows for the interaction and optimal binding of the negatively charged corrective DNA. During the spontaneous formation of the lipoplex, the cationic lipid acts as a catalyst and aids in the binding of the DNA to the liposome and its subsequent compaction. An imperative property of the amphiphile is its geometry. When suspended in an aqueous environment, the cationic lipids can adopt various structural phases. These phases include the lamellar, cubic, micellar and inverted hexagonal phase (Wasungu and Hoekstra, 2006).

This chapter describes and discusses the preparation and characterization of eight unique cationic liposomes. All contain the cationic cholesterol derivative, N,N-dimethylaminopropylamido- succinylcholesterylformylhydrazide (MSO9) and the neutral co-lipid dioleoylphosphatidylethanolamine (DOPE). The three targeted liposomes have been strategically formulated to incorporate a galacto glycolipid (10 mole %). Liposomes were prepared with and without dioleolphosphatidylethanolamine-polyethylenegylcol$_{2000}$ (DOPE-PEG$_{2000}$) at 2 mole %.

2.2. MATERIALS AND METHODS

2.2.1. Materials

Dioleoylphosphatidylethanolamine was purchased from the Sigma Chemical Company, St Louis, MS, USA. Silca gel 60 F_{254} chromatography plates and the 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulphonic acid (HEPES) were purchased from Merck, (Damstadt, Germany). Dioleoylphosphatidylethanolamine polyethylene glycol$_{2000}$ (DOPE-PEG$_{2000)}$ was purchased from Avanti Polar Lipids, Alabaster, USA. MS04 was synthesised previously in the Biochemistry laboratory. All other chemicals were of analytical grade.

2.2.2. Methods

2.2.2.1. Synthesis of the cholesterol derivative, N,N-dimethylaminopropylamidosuccinylcholesterylformylhydrazide (MSO9) from Cholesterylformylhydrazide (MSO4*)*

MSO4 (223 mg, 0.5 mmole) was treated with succinic anhydride (55 mg, 0.5 mmole) in dimethylformamide:pyridine (5 ml), and incubated at room temperature overnight. The solvent was then removed using a rotary evaporator, under reduced pressure. Thereafter, absolute ethanol was added to the flask, yielding the product, cholesterylformylhydrazide hemisuccinate (MSO8) as white crystals. The yield of the product was 188 mg (0.34 mmol, 68 %). MSO8 (188 mg, 0.34 mmol), together with

140 mg dicyclohexylcarbodiimide (DCC, 0.68 mmol) and 78 mg N-hydroxysuccinimide (0.68 mmol) were dissolved in 3 ml of DMF. This was then warmed at ± 50 ^{o}C. The reaction was monitored by TLC, using a silca gel 60F_{254} TLC plate in 9:1 ($^{v}/_{v}$) choloform:methanol. The reaction was allowed to proceed overnight whereupon dicyclohexylurea crystals formed. The crystals were then removed by filtration, followed by evaporations of the filtrate under vacuum. Chloroform was then added to the product followed by water to extract any excess N-hydroxysuccinimide. The chloroform layer was then evaporated, and petroleum ether was thereafter added to remove any excess DCC. The product (MSO9) was crystallized from ethanol.

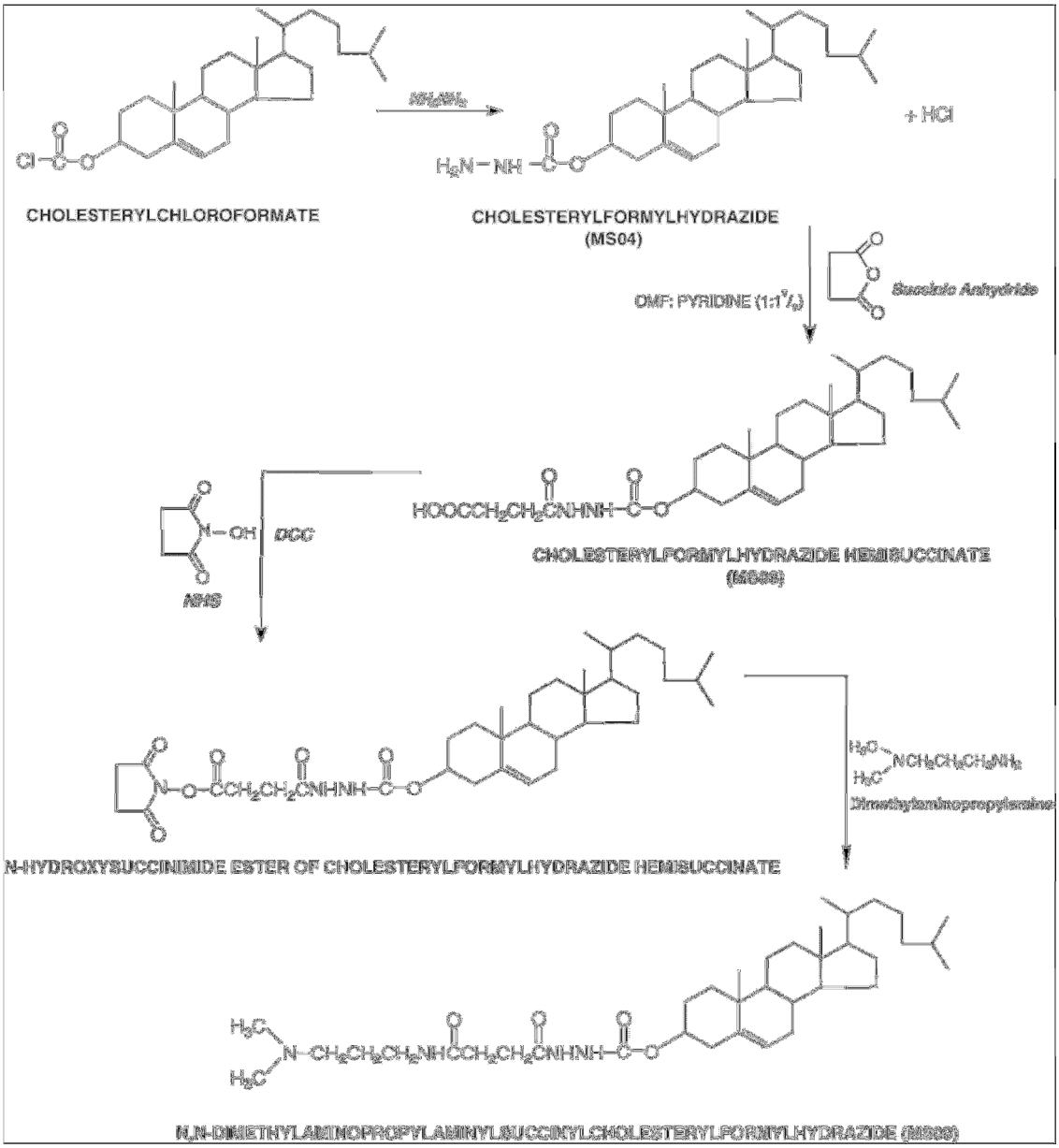


Figure 2.2: Chemical Synthesis of MS09 (Adapted from Singh and Ariatti 2006).

2.2.2.2. Cholesterol 3β-N-(4-aminophenyl-β-D-galactopyranosyl) carbamate

The synthesis of, cholesterol 3β-N-(4-aminophenyl-β-D-galactopyranosyl) carbamate was adapted from Narainpersad et al., (2012). Cholesteryl chloroformate (33 mg, 74 µmoles) and 4-aminophenyl-β-D-galactopyranoside (p-NH_2-Ø-Gal) (20 mg, 74 µmoles) were dissolved in DMF (2 ml). Thereafter triethylamine (10 µl) was added and the reaction mixture was set aside at room temperature overnight. Completion of the reaction was confirmed by TLC as described below.

The reaction mixture was tested against the starting galactoside, p-NH_2-Ø-Gal, on silica gel 60 F_{254} plates, in a chloroform:methanol (4:1 $^{v}/_{v}$) solvent system. DMF, the solvent component of the reaction product, was evaporated via rotary evaporation using a Buchii Rotavapor-R. Water was added to the residue to extract any remaining unreacted starting galactoside. The mixture was incubated at 4 ^{o}C for 2 hours and the crude product isolated by filtration. This was extracted further with ether (overnight) and the product was finally isolated by centrifugation in a MSE bench top centrifuge (3000 rpm, 5 minutes). A white powdery product was obtained.

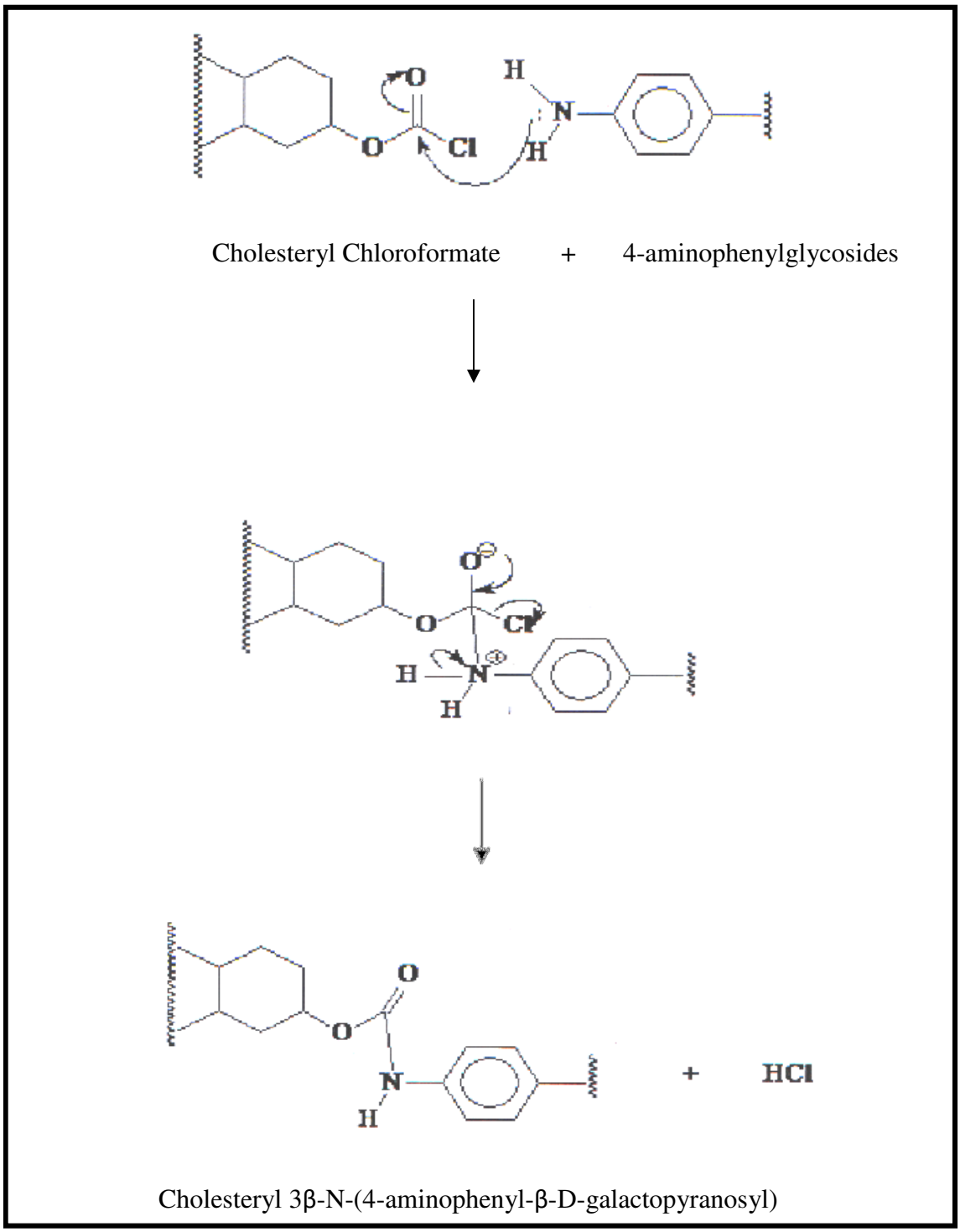


Figure 2.3: Chemical Synthesis of Cholesteryl 3β-N-(4-aminophenyl-β-D-galactopyranosyl).

2.2.2.3. Cholest-5-en-3-yl [1-(β-D-galactopyranosyl)-1*H*-1,2,3-triazol-4-yl]-methylcarbamate (WT)

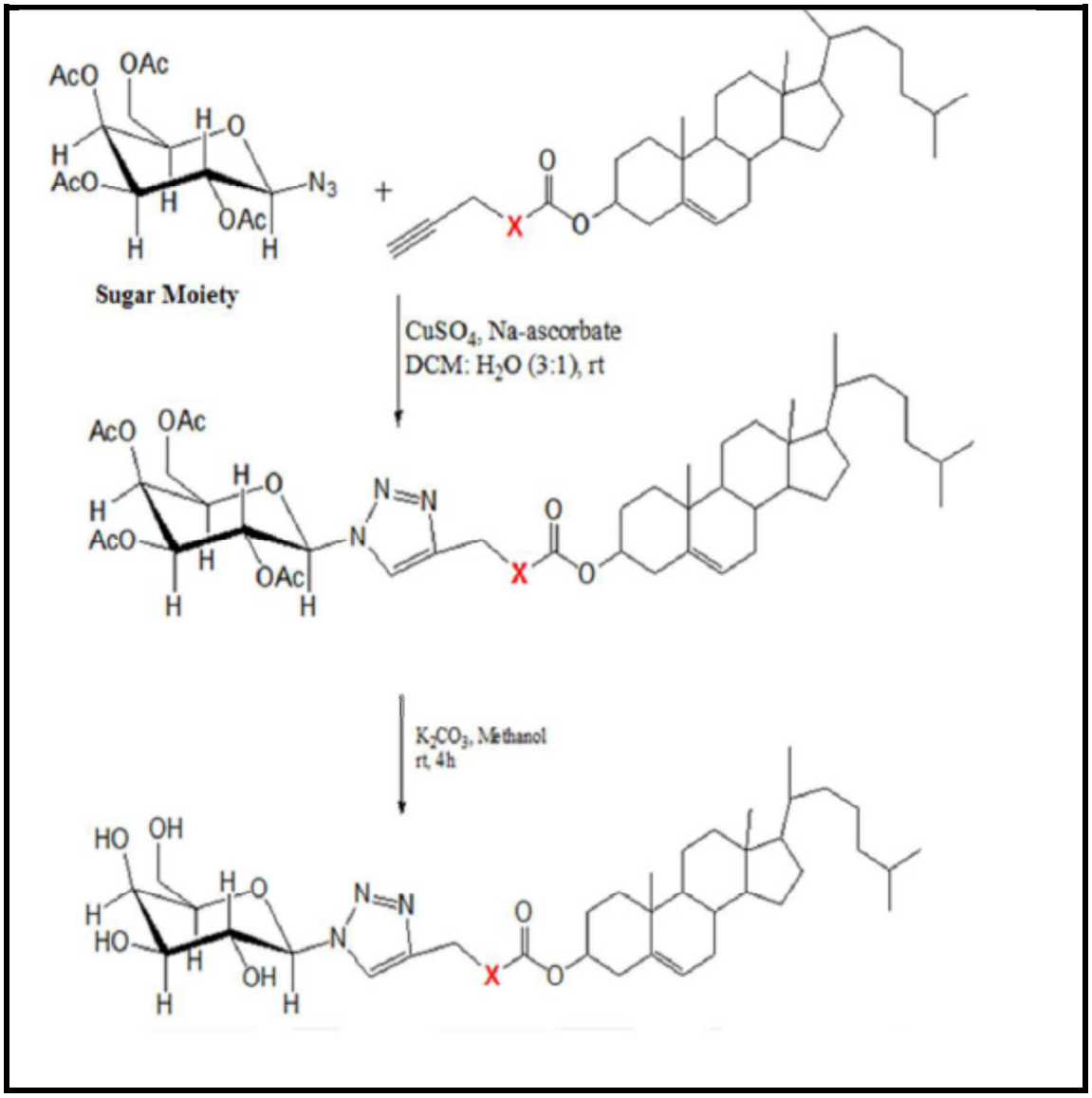


Figure 2.4: Schematic representation of the synthesis of WT. The conjugation of the acetylated sugar moiety to the cholesterol derivative is achieved through click chemistry.

2.2.2.4. Preparation of Cationic Liposomal Formulations

All of the eight cationic liposome formulations (both PEGylated and non PEGylated) were prepared by the method of Gao and Huang (1991). The specific quantities of each component of each liposome are as follows:

Table 2.2: The composition of each liposomal formulation.

Cationic Liposome Formulation	MSO9 (µmol)	DOPE (µmol)	GAL (µmol)	PEG_{2000} (µmol)
Control 1	2	2	-	-
Control 2	2	1.8	-	0.2
MS 1	2	1.6	0.4	-
MS 2	2	1.4	0.4	0.2
NN 1	2	1.6	0.4	-
NN 2	2	1.4	0.4	0.2
WT 1	2	1.6	0.4	-
WT 2	2	1.4	0.4	0.2

Non-PEGylated

Control 1= Untargeted

MS1= Cholesteryl-β-D-Galactopyranoside

NN1= Cholesteryl-3β-N-(4-aminophenyl-β-D-galactopyranosyl) carbamate

WT1= Cholest-5-en-3-yl [1-(β-D-galactopyranosyl)-1H-1,2,3-triazol-4- yl]methylcarbamate

PEGylated

Control 2= Untargeted

MS2= Cholesteryl-β-D-Galactopyranoside

NN2= Cholesteryl-3β-N-(4-aminophenyl-β-D-galactopyranosyl) carbamate

WT2= Cholest-5-en-3-yl [1-(β-D-galactopyranosyl)-1H-1,2,3-triazol-4- yl]methylcarbamate

The appropriate reagents were dissolved in 1 ml dry chloroform. The chloroform was evaporated and the sample was deposited on the inner wall of the test tube by rotary evaporation, *in vacuo* at 21 ^{o}C, using a Büchii Rotavapor-R. Thereafter the sample was subjected to a further drying step in a drying pistol for approximately 1.5 hours. Sterile HBS (20 mM HEPES, 150 mM NaCl, pH 7.5, 1 ml) buffer was used to

rehydrate the sample. The samples were incubated overnight at 4 ^{o}C, and thereafter, vortexed and sonicated for 5 minutes on a Transonic bath-type sonicator to generate unilamellar liposomes. Samples were then stored at 4 ^{o}C. Thereafter all liposomal formulations were vortexed for 30 seconds and sonicated for 30 seconds, prior to use and storage.

All formulations (excluding C1 and C2) contained a constant molar quantity of the galacto component (10 mole %). PEGylated liposomes contained DSPE-PEG_{2000} (2 mole %).

2.2.2.5. Nuclear Magnetic Resonance (NMR) Spectrometry

Samples of the cholesteryl galacto derivatives (4 mg) were dissolved in deuteropyridine (C_5P_5N) and subjected to ^{1}H and ^{13}C NMR one dimensional analysis on a Bruker Advance 400 spectrometer.

2.2.2.6. Characterization by Transmission Electron Microscopy (TEM)

For TEM, liposome suspensions were diluted 1:4 in HEPES Buffer Solution (HBS). Approximately 1 µl of the diluted liposomal formulation was pipetted onto a carbon coated copper grid. This was followed by the addition of 1 µl of uranyl acetate (saturated) which was smeared across the grid using the tip of the pipette. After 2-3 minutes the excess liquid was removed using filter paper. The grid was thereafter quickly dipped into liquid nitrogen using a spring loaded Leica CPC system. The grids were transferred to a Gatan cryotransfer system. An appropriate amount of liquid nitrogen was poured over the grid to cover it and cool the system. The grids were viewed using a Jeol 1010 Megaview Soft TEM system, operating at 80 kV.

2.3. RESULTS AND DISCUSSION

2.3.1. Synthesis of the Cholesteryl Derivative, MSO9

The synthesis of MS09 was followed using Thin Layer Chromatography (TLC) on silica $60F_{254}$ plates developed in $CHCl_3$:MeOH (4:1 $^{v}/_{v}$). The schematic representations of the TCL plates are shown below.

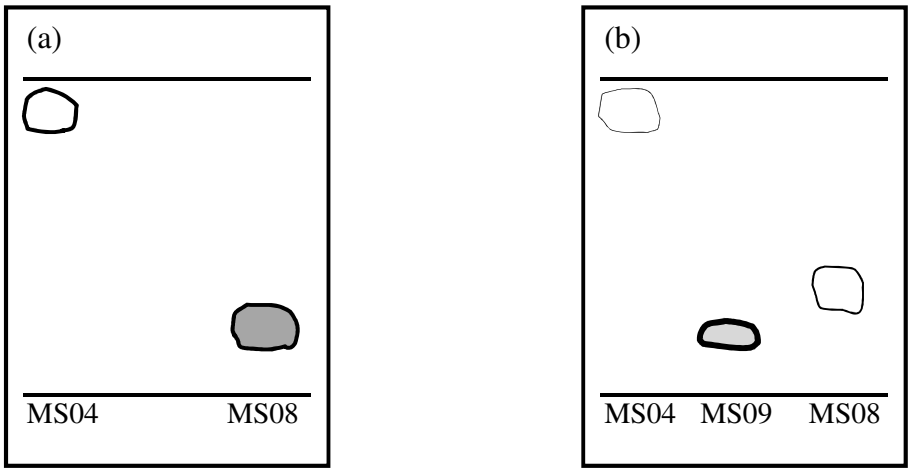


Figure 2.5: TLC representing two crucial steps of MS09 synthesis. (a) Products of the reaction between MS04 and succinic anhydride (b) Final step of the synthesis of MS09.

The animal cell membrane is constituted largely by the lipid cholesterol and due to its biocompatibility, the lipid has been frequently employed as the hydrophobic segment of cationic cytofectin. The use of cholesterol and cholesterol derivatives, as additive to conventional liposomal formulations, have been shown to rapidly reduce the release of bioactive compounds that is encapsulated (Tran *et al*., 2009). Cholesterol is composed of a hydroxyl group (polar) and a steroidal ring system (non-polar), with an isopentyl chain. Embedded within the liposomal bilayer of cationic liposomes are apolar cholesteryl skeletons which represent the hydrophobic anchors of the liposome. The anchor is interconnected to the targeting moiety via a linker (Singh *et al*., 2001;

Reynier *et al*., 2004), which may be an amide (Hasegawa et al., 2002), ether (Ghosh et al., 2000), ester (Lee *et al*., 2004) or carbamoyl entity (Gao and Huang, 1991).

The synthesis of the cholesterol derivative, MS09 was successfully achieved in 4 steps (Figure 2.2). The first step involving addition of previously synthesized MS04, succinic anhydride. As shown in Figure 2.5 (a), the product yielded was MS08. However, the reaction mixture contained a small proportion of unreacted MS04. MS04 displays an amino functionality that is succinylated in the formation of MS08. The hydrazide product MS04 is relatively non-polar and displays a high R_f value in CHCl:MeOH (4:1 v/v).

The next step involved the coupling of the N,N-dimethylaminopropylamine and hemisuccinate (MS08) to form a chemically stable amide linkage. This was achieved by N-hydroxysuccinimide activation of the MS08 carboxyl group. The TLC plate in Figure 2.5 (b) shows the three components in the final step of the synthesis. The desired product, MS09 was synthesized in high yield, with only a small fraction of the starting compound MS04 in the reaction mixture.

As mentioned before the cationic cholesteryl derivative synthesized in this study has the classic structure of a cytofectin, with a cationic head group, spacer arm (of varying lengths), linker bond and the hydrophobic lipid anchor. MSO9 is a cholesterol derivative that has been shown to be effective and efficient in attaining sustainable transfection efficiency in different cell lines (Singh and Ariatti, 2006). The lipid boasts a dimethylamino cationic head group, attached to a fused ring system. This cationic lipid has been synthesized previously by Singh and Ariatti (2006) and has promoted remarkably high transgene expression, in the HepG2 cell line. The lipid showed minimal toxicity to the cell lines, and it is due to this characteristic, that MS09 was adopted for the liposomal formulations in this study.

2.3.2. Synthesis of Cholesteryl Derived Ligand [Cholesteryl-3β-N-(4-aminophenyl-β-D-galactopyranosyl) carbamate]

The product was synthesized as outlined in section 2.2.2.2, and completion of the reaction was confirmed visually using TLC.

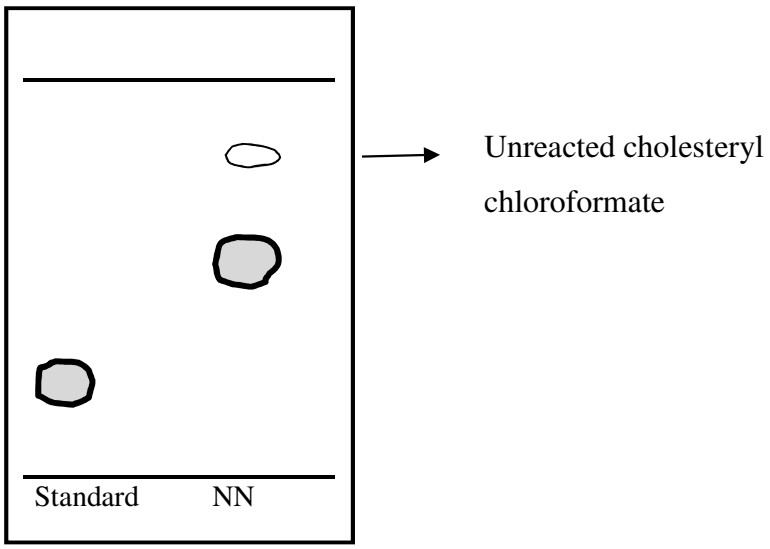
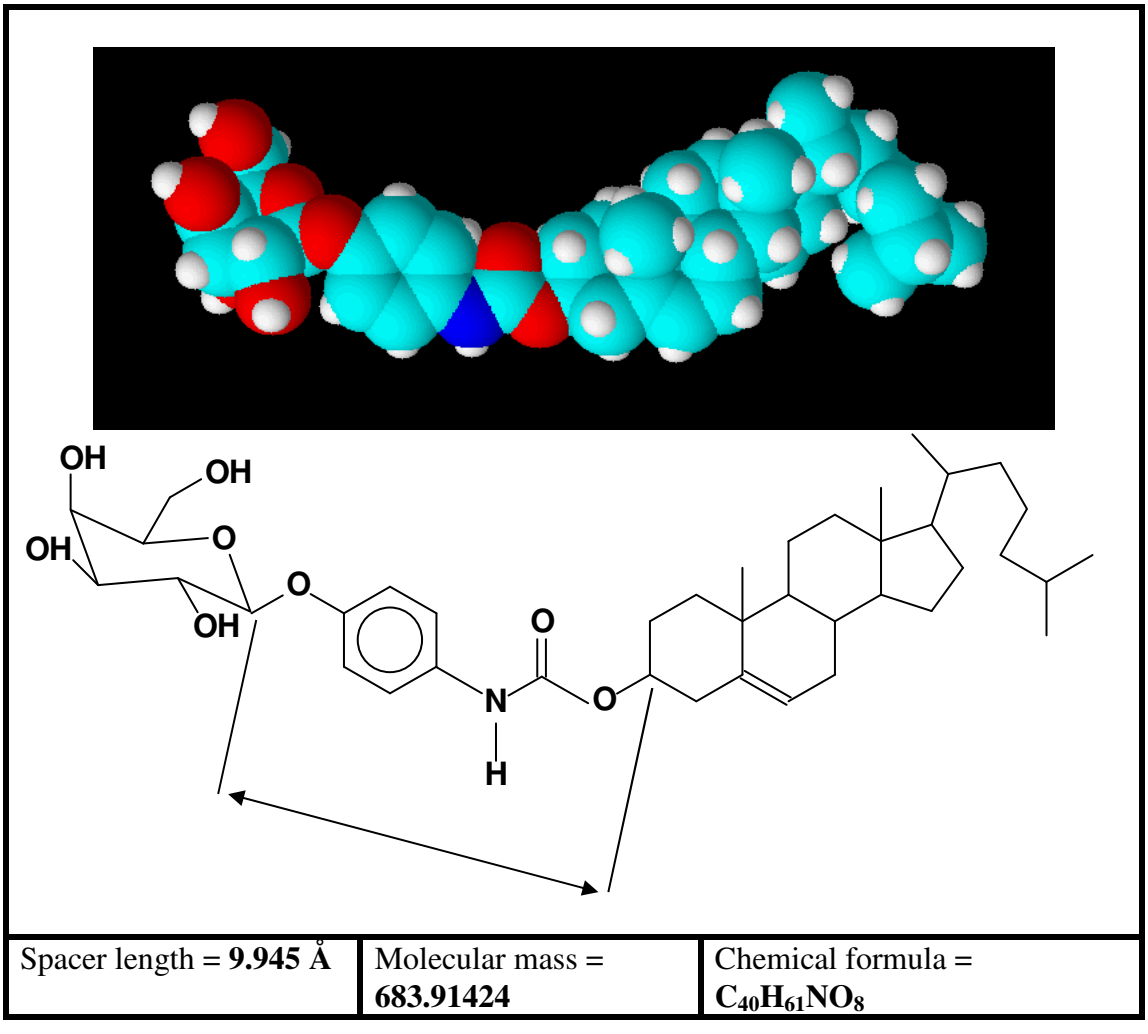


Figure 2.6: Thin Layer Chromatography of cholesteryl-3β-N-(4-aminophenyl-β-D-galactopyranosyl) carbamate in a 4:1 ($^{v}/_{v}$) chloroform:methanol (Standard = p-NH_2-ø-Gal; NN = Product).

Cholesteryl-3β-N-(4-aminophenyl-β-D-galactopyranosyl) carbamate (NN) was synthesized from cholesteryl chloroformate and p-aminophenyl-β-D-galactopyranoside. The product was visualised against the standard p-NH_2-ø-Gal (Figure 2.6). The synthesis of NN, resulted in a high yield of the product in pure form. The TLC plate was sprayed with 10 % ($^{v}/_{v}$) sulphuric acid to dehydrate the reaction products and enhance visualization of the products (brown/purple spots).

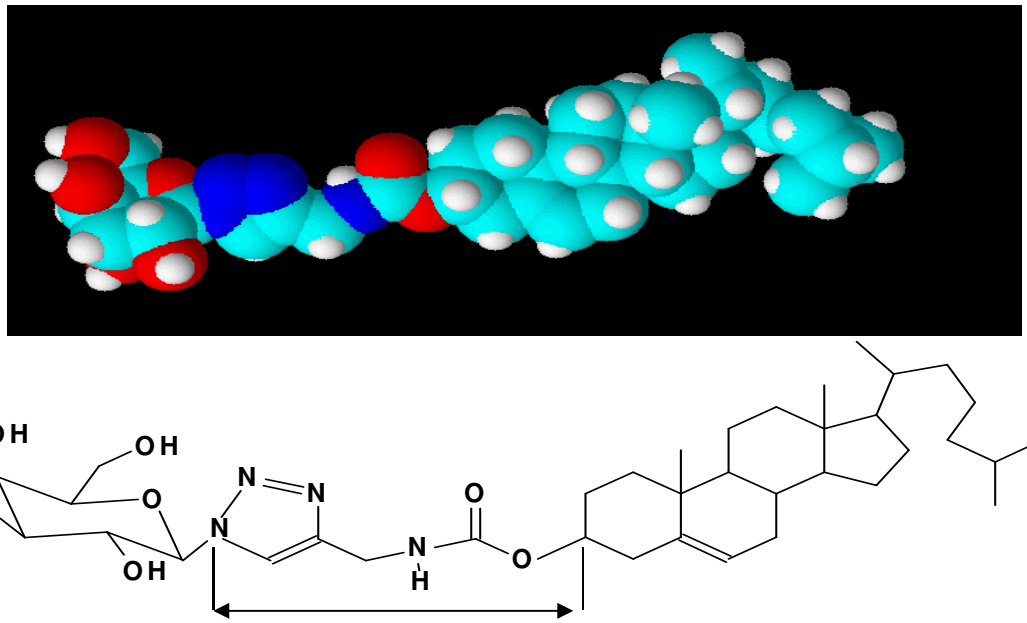
2.3.3. Preparation of Cationic Liposomes

All liposomes were prepared in accordance with Table 2.2 above. Figures 2.7-2.9 illustrate the chemical structure and the key properties of each cholesteryl galacto derivative.



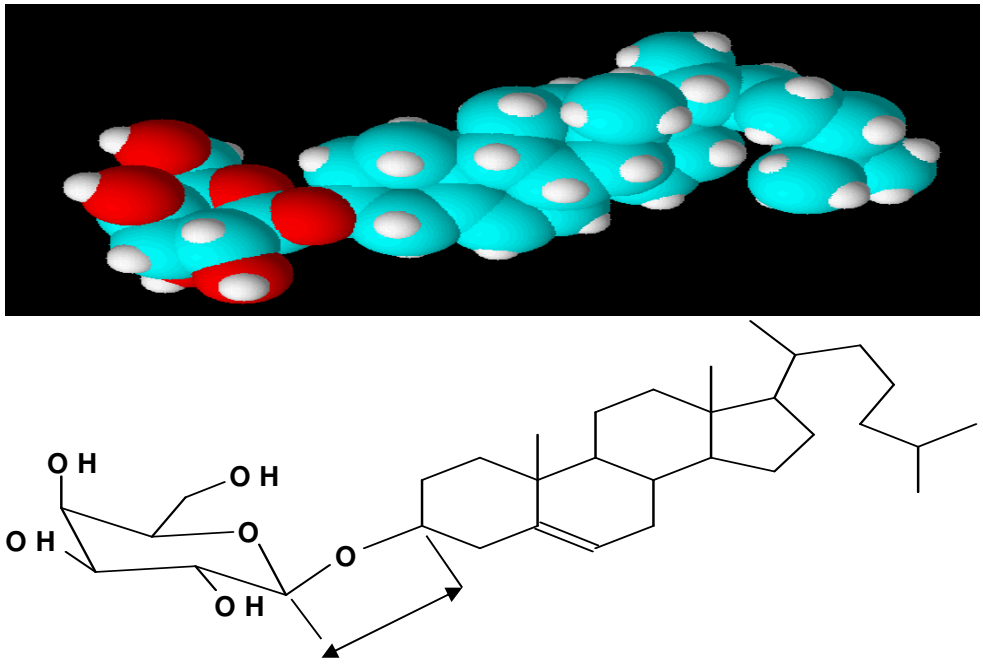
Spacer length = **9.945 Å**	Molecular mass = **683.91424**	Chemical formula = **$C_{40}H_{61}NO_8$**

Figure 2.7: Cholesteryl-3β-N-(4-aminophenyl-β-D-galactopyranosyl) carbamate.



Spacer length = **10.007 Å**	Molecular mass = **672.8949**	Chemical formula = **$C_{37}H_{60}N_4O_7$**

Figure 2.8: Cholest-5-en-3-yl [1-(β-D-galactopyranosyl)-1H-1,2,3-triazol-4-yl]methylcarbamate.



Spacer length = **2.42 Å**	Molecular mass = **548.79414**	Chemical formula = **$C_{33}H_{56}O_6$**

Figure 2.9: Cholesteryl-β-D-galactopyranoside.

Cationic liposomal formulations were prepared from near equimolar ratios of MS09 and DOPE. The two crucial factors that govern the linkage element, that adjoins the anchor to the targeting moiety, are the chemistry and length of the linker element. These two factors accommodate the cohesive charge–charge interactions in lipoplexes thus influencing their resulting degree of transfection obtained (Singh and Ariatti, 2006 and Reynier *et al*., 2004). Figures 2.7-2.9 exhibit the chemical structure and formula of each galacto derivative, together with the measured spacer length. Cholest-5-en-3-yl [1-(β-D-galactopyranosyl)-1H-1,2,3-triazol-4- yl]methylcarbamate displays the greatest spacer length (10.007 Å) between the galactose sugar and the cholesterol derivative. This is marginally greater than that in cholesteryl-3β-N-(4-aminophenyl-β-D-galactopyranosyl) carbamate (Figure 2.7), with a spacer length of 9.945 Å. The greatest difference evaluated was the spacer length of cholesteryl-β-D-galactopyranoside (2.42 Å). Singh and Ariatti (2006) demonstrated the vast increase in transgene expression with the use of a longer spacer.

When liposomes are engineered to contain equimolar quantities of DOPE and the cationic lipid, higher transfection efficiency *in vitro* can be achieved compared to the use of a different helper lipid such as DOPC. We can attribute this to the unique ability of DOPE, to transition the bilayer into an inverted hexagonal configuration, under specific conditions, such as acidic pH. This transition facilitates endosomal membrane destabilization and subsequent release of the liposome. *In vivo* studies have shown that the inclusion of cholesterol, instead of DOPE, as a co-lipid significantly increased transgene expression, since lipoplexes formed demonstrated vastly improved stability.

2.3.4. Characterization of liposomes and lipoplexes by TEM

2.3.4.1. TEM micrographs of the eight unique liposomal formulations

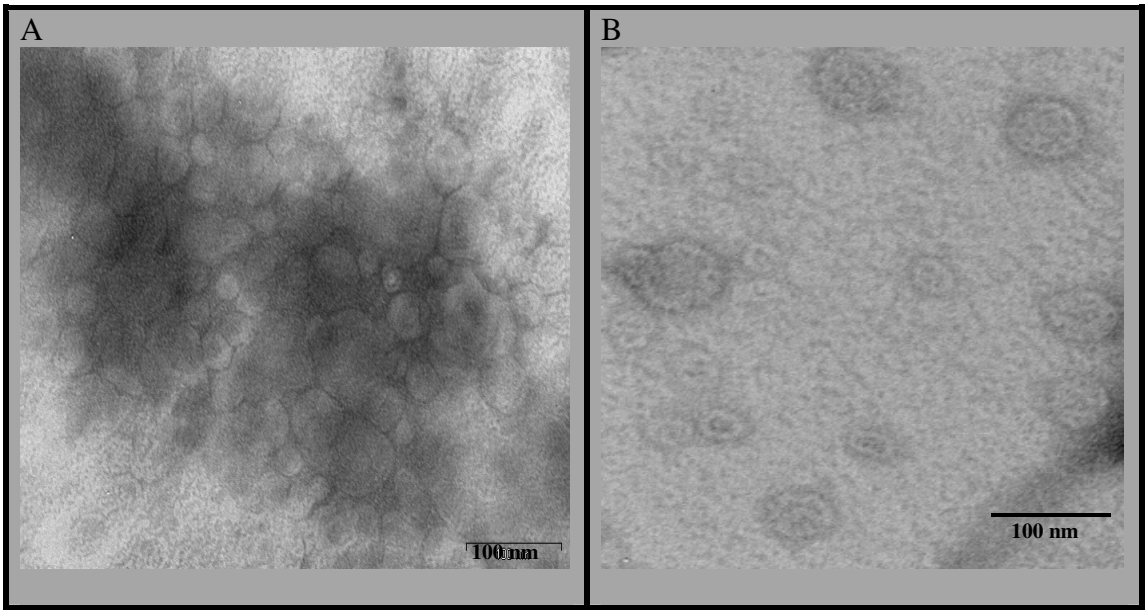


Figure 2.10: Ultrastructure images of (A) untargeted cationic liposomes, C1 and (B), untargeted PEGylated cationic liposomes, C2. Bar =100 nm.

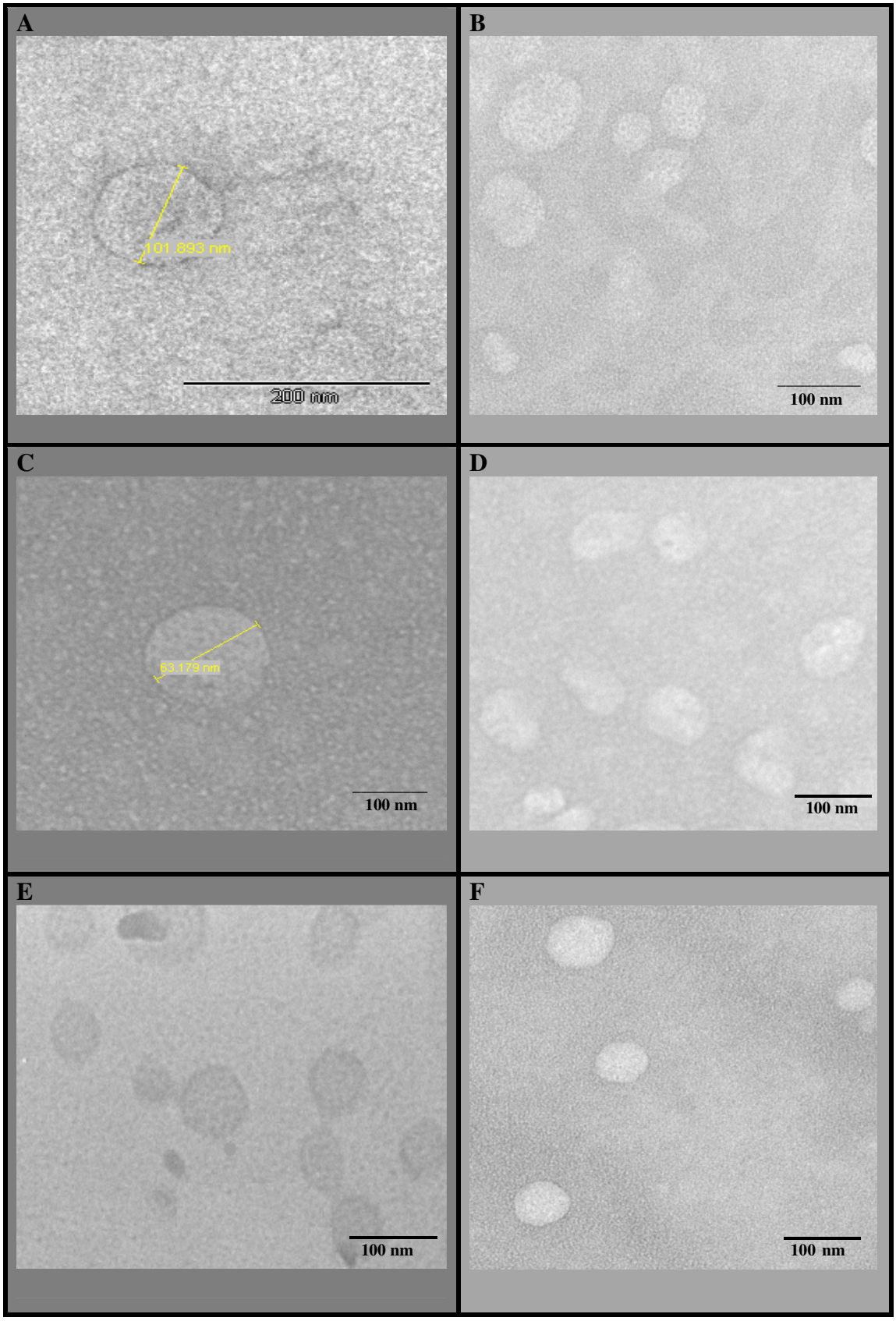


Figure 2.11: The morphology of targeted cationic liposomes and the PEGylated counterparts (A) MS1 (B) MS2 (C) NN1 (D) NN2 (E) WT1 (F) WT2. Bar = 100 nm except in A (Bar =200 nm).

The morphology of the liposomes was analysed using TEM. There are many tools and techniques that have been developed to determine particle size distribution. There are many techniques that have been developed to determine particle size and distribution. These include the zeta potential and TEM. However, visual characterization of liposomes is considered the most important technique (LeDoux, 2008). By visualizing a sample by electron microscopy, the true size distribution and shapes of the liposome can be determined (Betageri *et al*., 1993**;** Frederik *et al*., 1996).

Liposomes were prepared from the cytofectin MSO9 and DOPE at near equimolar ratios, at a concentration of 4 μmol lipid per millilitre of HBS. From the diluted samples, visualized by TEM (Figure 2.10-2.11) it can clearly be concluded that all liposomal formulations, consisted of unilamellar liposomes. Unilamellar liposomes can be formed when multilamellar liposomes are sonicated. Small unilamellar liposomes range between 10-100 nm in size while the large unilamellar liposomes range between 150-250 nm (Karmali and Chaudhuri, 2007). These can be stable at 4 ^{o}C for several months. Multivalent cationic lipids have a probability of forming micelles which yield unstable liposomes. All TEM ultrastructures obtained show that the liposomal formulations yielded liposomes that were unilamellar and mostly spherical in shape (Figure 2.9 A-H). The estimated size range for the unilamellar liposomes was between 60-120 nm.

CHAPTER THREE

PREPARATION AND CHARACTERIZATION OF GALACTOSYLATED CATIONIC LIPOSOME – DNA COMPLEXES OR LIPOPLEXES

3.1. INTRODUCTION

Liposome:DNA complexes are self-assembled nanosystems that form as a result of electrostatic interactions that occur between the positively charged lipid and the anionic DNA (Felgner *et al*., 1997). The formation of the cationic liposome:DNA complexes, occurs in two unique steps. The first step is an endothermic process involving the rapid attraction and subsequent binding, between the DNA and the liposomal surface. The second step is a gradual endothermic reaction that has been suggested to be involved in the fusion of the two entities and the resulting structural rearrangements (Zhdanov *et al*., 2002). During lipoplex formation, the electrostatic interactions that occur between the cationic lipid and the anionic DNA, results in the collapse of the DNA structure, a process known as condensation. The DNA is tightly compacted or condensed to the lipid which allows the lipid to shield and protect the DNA from nucleases (Wasungu and Hoekstra, 2006).

During the preparation of lipoplexes, the order in which the liposome preparation and DNA solution are introduced to each other is crucial. When the negatively charged plasmid DNA is added to a specific volume of liposome, the resulting lipoplex formed will exhibit a gradual increase in size. However, when the cationic lipid is added to the plasmid DNA, the lipoplexes generated following incubation, are roughly similar in size and the preparations seem more stable. The lipoplexes continue to form until the cationic lipid (positive charge) exceeds the nucleic acids (negative charge) available in solution (Kennedy *et al*., 2000). The morphology of the lipoplex, size of the lipoplex, the ratio of cationic lipid to nucleic acid and liposome composition govern the transfection efficiency that can be achieved in a cell line (Boffi *et al*., 2002; Obata *et al*., 2008). Positively charged liposomes that complex with DNA, have been shown to yield heterogeneous lipoplexes that vary in their size, shape and

composition. Many morphological structures have been reported, which include the commonly seen string of beads (Gershon *et al*., 1993), spaghetti and meat ball, inverted hexagonal phase, sliding columnar phase, map-pin and the multilamellar structures (Ma *et al.,* 2007). Various factors that affect the success of the liposomal formulations and their resulting gene transfer efficiency are outlined below in Table 3.1. The nature of the initial lipid, size of the plasmid DNA, and the inclusion of helper co-lipids such as DOPE, modulate lipoplex stability and transfection efficiency (Wasungu and Hoekstra, 2006). In the present study we have taken all of the below mentioned factors listed below into consideration, prior to the initiation of liposomal preparations.

Table 3.1: Factors governing the biological activity of lipoplexes, *in vitro* and *in vivo* (Simões *et al*., 2005).

Related Properties	**Affecting Factors**
Mode of lipoplex formation - morphology and structure	Nature of cationic and helper lipid, stoichiometry of cationic lipid and DNA, nature of the medium (ionic strength, pH and temperature), DNA structure
Control of physicochemical properties - size - net charge	Mode of lipoplex preparation (type of liposomes, DNA structure, order of addition and rate of mixing, lipid and DNA concentration), role of adjuvants (polycations, surfactants, cryoprotectants)
In vitro and *in vivo* performance - stability in the presence of serum - resistance to DNA nuclease degradation -pharmacokinetics/ biodistribution -passive versus active targeting -surpass the endothelial barrier -transfection efficacy -cytotoxicity	Route of administration, nonspecific interaction with serum components, interaction with blood cells, net charge of the lipoplexes, nature of the colipid (cholesterol versus DOPE) Interaction with serum components, opsonisation, prolonged circulation time (inclusion of poly[ethyleneglycol]) Size, use of ligands and antibodies Unknown Level and duration of gene expression Type and concentration of lipid, type of cell
Lipoplex–cell interaction - mode of cellular internalisation -escape from endosomes -DNA dissociation from the complex -trafficking of DNA into the nucleus	Size, liposome composition, net charge and topology of the complexes, presence of ligands Nature of helper lipid, use of endosome disrupting agents Net charge of the complex, nature and valency of the cationic lipid. Cellular factors unknown Degree of DNA condensation/compaction, protection from nucleases, size of the plasmid, targeting to the nucleus.

In 1998, using x-ray diffraction, Koltover and co-workers showed that during lipoplex formation, there exists a major structural rearrangement that yields three different structural configurations (complexes contained DOTAP as the cationic lipid). Figure 3.1 shows the structure of the lamellar, the inverted hexagonal and the hexagonal phases of cationic liposome-DNA complexes.

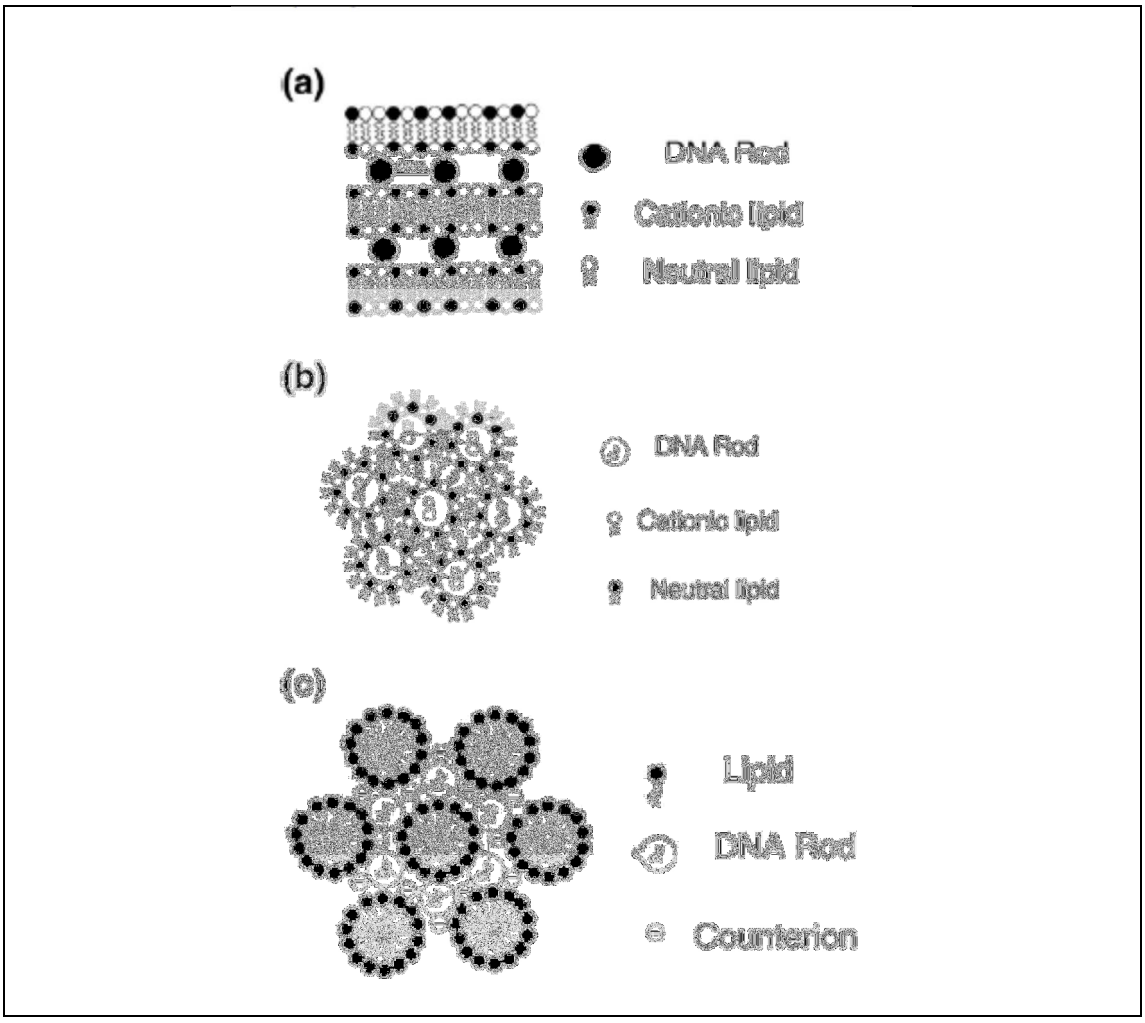


Figure 3.1: Schematic representation of the three different structural phases (a) the lamellar structural phase (L^C_α); (b) the inverted hexagonal structural phase (H^C_{II}); and the hexagonal structural phase (H_I) of a lipoplex (Ewert *et al*., 2006).

The lamellar phase exists such that the DNA rods are sandwiched between the lipid bilayers. This phase differs drastically from the inverted hexagonal and the intercalated hexagonal structural phase. As displayed in Figure 3.1 (b) in the inverted hexagonal phase, the DNA rods are coated with the lipid monolayer and are typically

arranged on a hexagonal lattice. In contrast, in the hexagonal structural phase the DNA rods are coated by three honeycombs of lipid micelles that are also arranged on a hexagonal lattice. It has been shown that the lamellar structural phase is present during liposome:DNA condensation and its subsequent transportation to the cell membrane (Hulst *et al*., 2004). However, immediately after contact with the cell membrane, the lipoplex transitions into the inverted hexagonal structure (Zabner *et al*., 1995).

The number of amine groups (+) on the cationic lipid in relation to the number of phosphate groups (-) on the DNA gives rise to the charge ratio. It has been suggested that a charge ratio of 1:1 lipid/DNA (neutral charge) must be avoided since the neutral charge generates large aggregates that are >1 µm (Xu *et al*., 1999) and would be of little value *in vivo*. In contrast lipoplexes prepared with a net positive charge ratio yield large multilamellar vesicles that possess a diameter of approximately 300-700 nm. These lipoplexes have been shown to be more successful in transfecting cells than small unilamellar vesicles (SUV) which are approximately 50-200 nm in size (Simões *et al*., 2005) *in vitro*. The size of the lipoplex is pivotal since the complexes must be < 150 nm in order to extravasate from the capillaries and reach their specified target cell. The final size of the lipoplex is proportional to the mass of the nucleic acid compacted by the vector (Simões *et al*., 2005; Uddin, 2007).

For our own investigations prepared cationic liposomes were mixed with plasmid DNA (pDNA). The resulting lipoplexes were further characterized according to their morphology and degree of protection conferred against serum nuclease digestion. Agarose gel electrophoresis was employed to examine the electrostatic interactions between the cationic liposome (positively charged) and the anionic DNA, which results in the binding and subsequent compaction of the nucleic acid in the lipoplex. The degree of protection conferred to the DNA, against serum nucleases, was assessed using the nuclease protection assay. The presence and the morphology of the lipoplexes were verified using transmission electron microscopy (TEM).

3.2. MATERIALS AND METHODS

3.2.1. Materials

2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulphonic acid (HEPES), Tris-HCl and EDTA were purchased from Merck, Damstadt, Germany. FBS was purchased from Highveld Biological, Lyndhurst, South Africa. The pCMV-Luc plasmid was purchased from Plasmid Factory, Bielfeld, Germany. The molecular biological grade agarose was purchased from Bio-Rad Laboratories, California, USA. Lysozyme was purchased from Sigma Chemical Company, St Louis, MS, USA. All other reagents were of analytical grade.

3.2.2. Methods

3.2.2.1. Gel Retardation Assay

The gel retardation assays allow one to establish the ratio at which the plasmid DNA is completely bound to the cationic liposome.

A 1 % agarose gel was prepared by dissolving 0.2 g of agarose in 18 Mohm water. The solution was brought to boil and then placed on the bench top to cool to temperature of 75 ^{o}C (the temperature was determined using a thermometer). Once the solution had cooled to the desired temperature, 2 ml of 10 x electrophoresis buffer and ethidium bromide (final concentration 1 µg/ µl) was introduced into the agarose gel mixture. The gel was allowed to set for a minimum of 60 minutes.

A standard amount of pCMV-Luc DNA (0.5 µg) was added to an increasing amount of cationic liposome (0,2,4,6,8,10,12,14 µg), in separate micro-centrifuge tubes. The samples were made up to a final volume of 12 µl with HBS (20 mM HEPES, 150 mM NaCl, pH 7.5) and incubated for 30 minutes at room temperature. Each sample was vortexed and centrifuged for 30 seconds in an Eppendorf microcentrifuge (12 000 rpm), prior to incubation. Thereafter, 4 µl of the gel loading buffer (50 % glycerol;

0.05 % bromophenol blue and 0.05 % xylene cyanol) was added to all tubes. The samples were then subjected to electrophoresis in a Bio-Rad mini-sub electrophoresis tank containing electrophoresis buffer (36 mM Tris-HCl, 30 mM sodium phosphate, 10 mM EDTA at a pH of 7.5), for 60 minutes at 50 volts. Following electrophoresis, the gel was viewed under UV transillumination (300 nm) and images were captured using a Vacutec Syngene G: Box gel documentation system.

3.2.2.2. Nuclease Protection Assay

Lipoplexes were prepared, incubated with serum and analysed using the nuclease protection assay to determine the degree of protection conferred by the lipid to the DNA. Varying amounts of cationic liposome (as illustrated in Table 3.2 below) were added to a constant amount of pCMV-Luc DNA (0.5 μg). The volume in each sample tube was made up to 10 μl with HBS, vortexed and centrifuged for 30 seconds and then incubated for 30 minutes at room temperature. Foetal bovine serum (FBS) was thereafter added to the complexes to a final concentration of 10 % ($^{v}/_{v}$). Two controls were used in this assay, a negative control using only pCMV-Luc plasmid and a positive control containing pCMV-Luc DNA and FBS. The tubes were then incubated for 4 hours, in a 37 ˚C water bath. Following incubation, ethylenediaminetetraacetic acid (EDTA) was added to all tubes, except the negative control (pCMV-Luc and liposome only), to a final concentration of 10 mM. This step was carried out as to stop the nuclease reaction. Sodium dodecyl sulphate was thereafter added to all tubes except the negative control, to obtain a final concentration of 0.5 % ($^{w}/_{v}$). Tubes were then incubated in a 55 ˚C water bath, for 20 minutes. Thereafter, 4 μl of the gel loading buffer was added to all tubes and the samples were subjected to agarose gel electrophoresis (as outlined in section 3.2.2.1 above), for 2 hours at 50V. Gels were then viewed and the images were captured using Vacutec Syngene G: Box gel documentation system as in 3.2.2.1.

Table 3.2: Liposome:DNA ratios analysed using nuclease protection assays.

Liposome	Liposome Amount (µg)			DNA (µg)
C1	1	2	3	0.5
C2	2	3	4	0.5
MS1	1	2	3	0.5
MS2	3	4	5	0.5
NN1	3	4	5	0.5
NN2	2	3	4	0.5
WT1	4	5	6	0.5
WT2	2	3	4	0.5

3.2.2.3. Transformation and isolation of plasmid DNA (pCMV-Luc)

3.2.2.3.1. Transformation

Approximately 100 µl of JM109 cell suspension and 10 µl of the pCMV-Luc control vector solution (1 µg) was pipetted into a microcentrifuge tube, and placed on ice for 30 minutes. Thereafter the tube was incubated in a 42 ˚C water bath for 90 seconds, followed by further incubation on ice for 2 minutes (transformed cells). The contents of the microcentrifuge tubes were then added to the LB broth containing ampillicin, and incubated on a 37 ^{o}C shaker for 48 hours.

3.2.2.3.2. DNA isolation

The broth containing cells were decanted into 4 centrifuge tubes (50 ml) and were centrifuged at 12 000 rpm for 15 minutes at 4˚C. The supernatant was then removed and discarded. Cells were re-suspended in 6 ml of freshly prepared lysis buffer (25 mM Tris-HCl (pH 7.5), 10 mM EDTA, 15 % sucrose, 25 mg/ml lysozyme). The tubes were thereafter placed in ice water for 20 minutes. This was followed by the addition of 12 ml of 0.1 N NaOH, and 1 % SDS (freshly prepared). The tubes were mixed

carefully by gentle inversion several times (not vortexed). Approximately 7.5 ml of potassium acetate (5 M) solution, pH 4.8 was added to each tube and mixed carefully by inversion. The tubes were thereafter incubated in ice water for 10 minutes. The tubes were then centrifuged at 12 000 rpm for 15 min and the supernatant was transferred to a fresh tube, avoiding the white precipitate. Approximately, 50 µl of RNase A (1mg/ml stock) was added to the supernatant in each tube. The tubes were then incubated for 20 min at 37 ˚C. One volume of TE buffer (10 mM Tris-HCl and 1 mM EDTA) saturated phenol-chloroform-isoamyl alcohol (24:1 $^{v}/_{v}$) was added to each tube and the tubes were centrifuged at 12000 rpm for 10 minutes. The upper aqueous phase was saved and the above step was repeated. One volume of chloroform-isoamyl alcohol (24:1 $^{v}/_{v}$) was added to each tube, which were then vortexed for 1 min and centrifuged at 12000 rpm for 20 minutes. The supernatant was then removed and the resultant pellet washed with 70% ethanol by centrifugation at 12000 rpm for 5 min. The pellet was thereafter dried under vacuum. The pellet was then dissolved in water and the DNA concentration and purity was analysed using a ThermoScientific Nanodrop 2000c spectrophotometer (Wilmington, USA) and agarose gel electrophoresis respectively.

3.2.2.4. Transmission Electron Microscopy (TEM)

Characterization of the cationic liposome:DNA complexes was carried out in accordance with the protocol for liposomes outlined in Chapter 2, Section 2.2.2.5.

3.3. RESULTS AND DISCUSSION

3.3.1. Gel Retardation Assay

Lipoplex formation between the cationic liposome and the DNA was analysed by varying the cationic lipid to pCMV-Luc DNA ratio, using the gel retardation assay. The assay determined the ability of the cationic liposome to completely bind to the negatively charged backbone of the plasmid DNA.

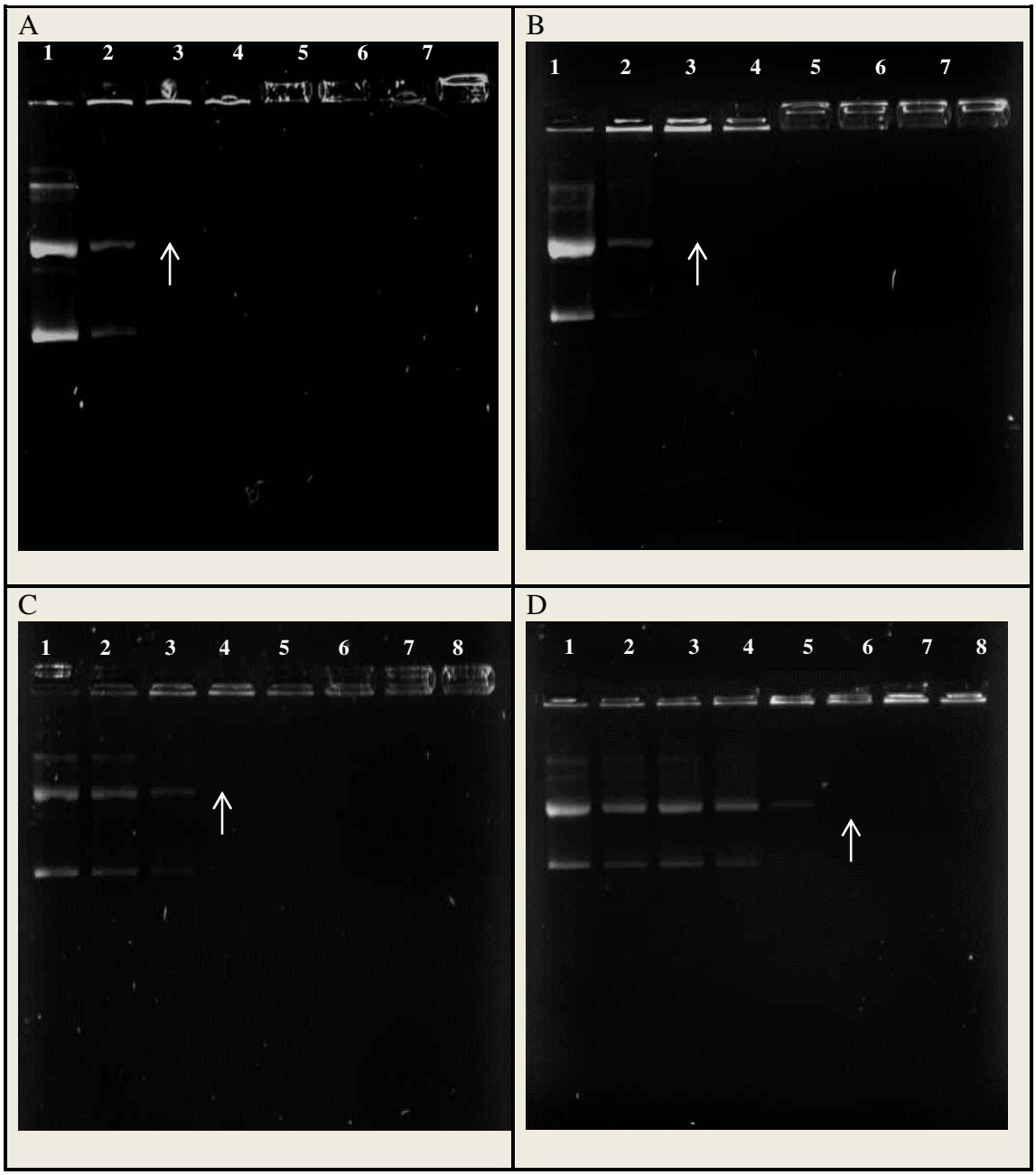


Figure 3.2: Gel retardation assay. Reaction mixtures (14 µl) consisted of pCMV-Luc DNA (0.5 µg) and an increasing amounts of the non-PEGylated cationic liposome, in HBS. DNA: cationic liposome ratio ($^{w}/_{w}$): Lane 1-8 (1:0, 1:2, 1:4, 1:6, 1:8, 1:10, 1:12, 1:14). (A) C1; (B) MS1; (C) NN1; (D) WT1.

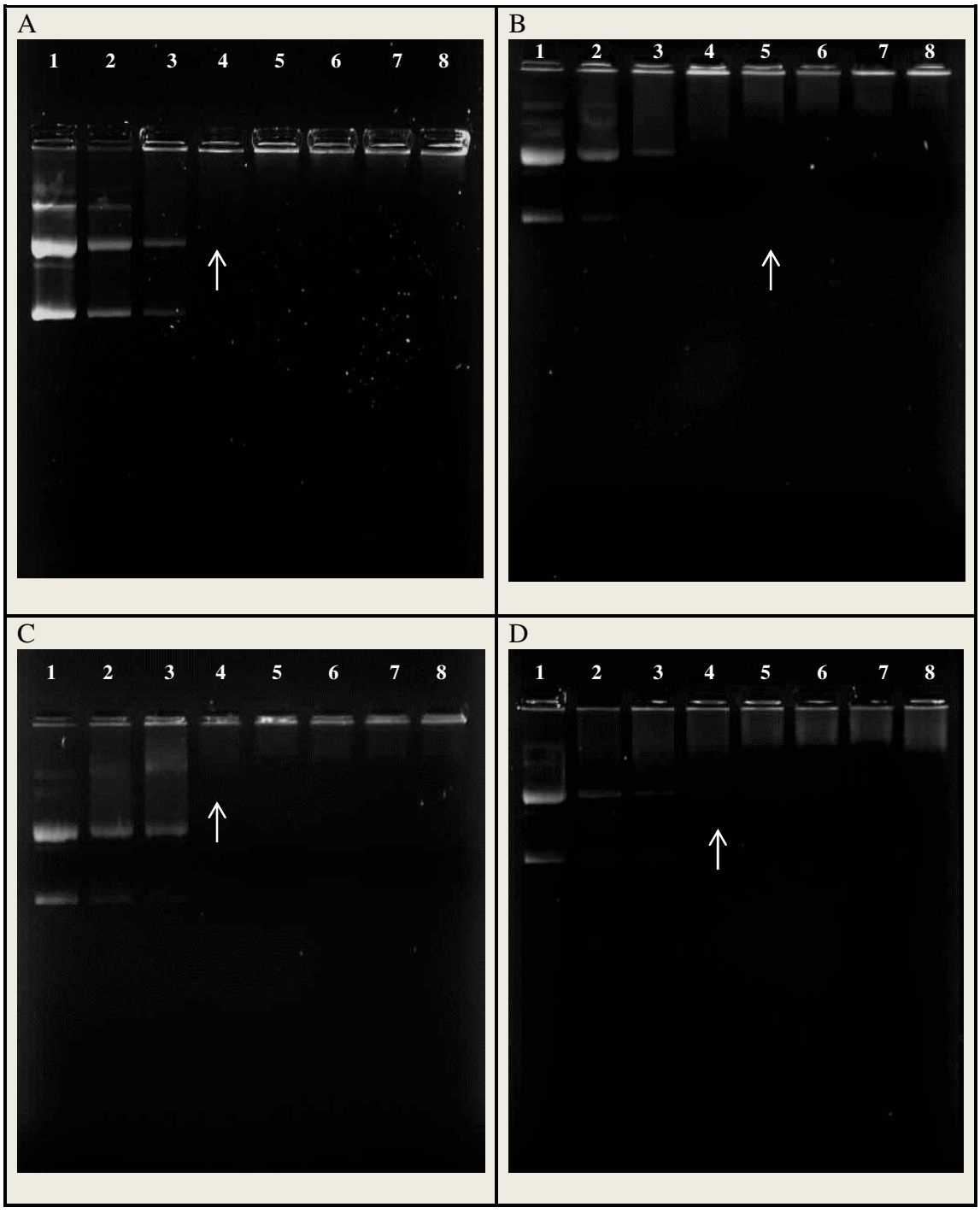


Figure 3.3: Gel retardation assay. Reaction mixtures (14 µl) consisted of pCMV-Luc DNA (0.5 µg) and an increasing amounts of the 2 % PEGylated cationic liposome. DNA: cationic liposome ratio ($^{w}/_{w}$): Lane 1-8 (1:0, 1:2, 1:4, 1:6, 1:8, 1:10, 1:12, 1:14). (A) C2; (B) MS2; (C) NN2; (D) WT2.

Table 3.3: Liposomal formulation and optimal binding ratio, as established using gel retardation assay.

Liposomal Formulation	Mass (mg)				Retardation		Charge ratio* (DNA:Liposome) (-:+)
	MSO9	DOPE	GAL	PEG_{2000}	Amount of Liposome (µg)	DNA: Liposome Ratio (w/w)	
C1	1.26	1.5	-	-	2	1:4	1:1.0
C2	1.26	1.5	-	0.56	3	1:6	1:1.2
MS1	1.26	1.19	0.22	-	2	1:4	1:1.0
MS2	1.26	1.0	0.22	0.56	4	1:8	1:1.8
NN1	1.26	1.19	0.26	-	4	1:8	1:2.1
NN2	1.26	1.0	0.26	0.56	3	1:6	1:1.4
WT1	1.26	1.19	0.28	-	5	1:10	1:2.6
WT2	1.26	1.0	0.28	0.56	3	1:6	1:1.4

*End point ratios. In calculating the nitrogen:phosphorous (N/P, positive:negative) ratio, MS09 is assumed to carry one positive charge at physiological pH and each nucleotide in DNA is assumed to have a molecular weight of 350 and to carry one negative charge.

The gel retardation assay is a mobility shift assay performed using agarose gel. As mentioned before this assay is used to investigate the ability of the cationic charge present on the liposomal surface, to neutralize the anionic charge of the phosphate backbone, producing electroneutral complexes that are unable to migrate during electrophoresis (Huang *et al*., 1998). Gene delivery by a lipid based vector can only be achieved when DNA is complexed to the vector. The above results (Figure 3.2 and 3.3) show that the cationic lipid completely condensed the anionic DNA. In each of the images above (Figure 3.1 A, B, C, D and Figure 3.2 A, B, C, D), lane 1 represents a negative control (DNA only). The negatively charged DNA migrates easily towards the cathode during electrophoresis exhibiting the three conformations of undigested DNA, viz. supercoiled, closed circular and linearized DNA. The supercoiled DNA migrates the fastest towards the anode. This is sequentially followed by the migration

of closed circular and linearized DNA. Upon the addition of an increasing amount of cationic liposome across lanes 2-8, the amount of DNA that migrates freely through the agarose gel decreases. Thus the amount of migrating DNA is suggested to be inversely proportional to an increase in the cationic liposome. The migration of the pCMV-Luc DNA will continue to decrease until the negatively charged DNA is completely bound to the cationic liposome. This is known as the optimal binding ratio and it is the point at which the complex is electroneutral. Lipoplexes were assembled at or close to electroneutrality to minimize ligand-receptor interactions whilst minimizing non-specific electrostatic interactions with the negatively charged plasma membrane. This was evident in competition experiments (Figure 4.11) where transfection levels dropped by more than 95% in the presence of the competing ligand, asialofetuin.

The optimal binding ratios obtained for each of the liposomal formulations are documented in Table 3.3 above. Non-PEGylated liposomal formulations NN1 and WT1 required a larger amount of lipid than their PEGylated counterparts, to optimally bind the negatively charged DNA (binding ratios for NN1 and WT1 are1.8 and 1.10 respectively, with the counterparts NN2 and WT2 both binding the DNA at a ratio of 1:6 DNA:cationic liposome). PEGylated MS2 required twice the amount compared to its non-PEGylated counterpart to optimally bind the anionic DNA.

It has been suggested that electrostatic interactions that occur during lipoplex formation, impact on the organization of the solvent present around the complex. This leads to the restriction on the number of sites exposed for pDNA binding (Ferrari *et al*., 2001). Thus this shielding of binding sites could provide justification for some complexes displaying a high binding ratio whilst others display a low cationic liposome:DNA binding ratio. Furthermore, targeting ligands possess lipids that form part of the lipid bilayer, and head groups that protrude from the bilayer of the liposome (structural representations shown in Chapter 2.3.3). It is possible that these protruding groups may also block or shield other binding sites on the liposomal surface. Therefore, sites may be available for DNA binding but due to the restriction and blockage caused by the structural arrangements, a large amount of lipid will be required to bind the DNA. This contrasts with the length of the spacer in liposomal preparations NN and WT which is longer when compared to liposomal formulation

MS. Thus MS requires a smaller amount of cationic liposome to bind the pCMV-Luc DNA while WT1 and NN1 both require more lipid to completely bind the DNA.

Optimal binding ratios obtained using the gel retardation assay, have been considered in assembling lipoplexes for cytotoxicity and transfection studies, in both the HEK293 and HepG2 cell lines (Chapter 4).

3.3.2. Nuclease Protection Assay

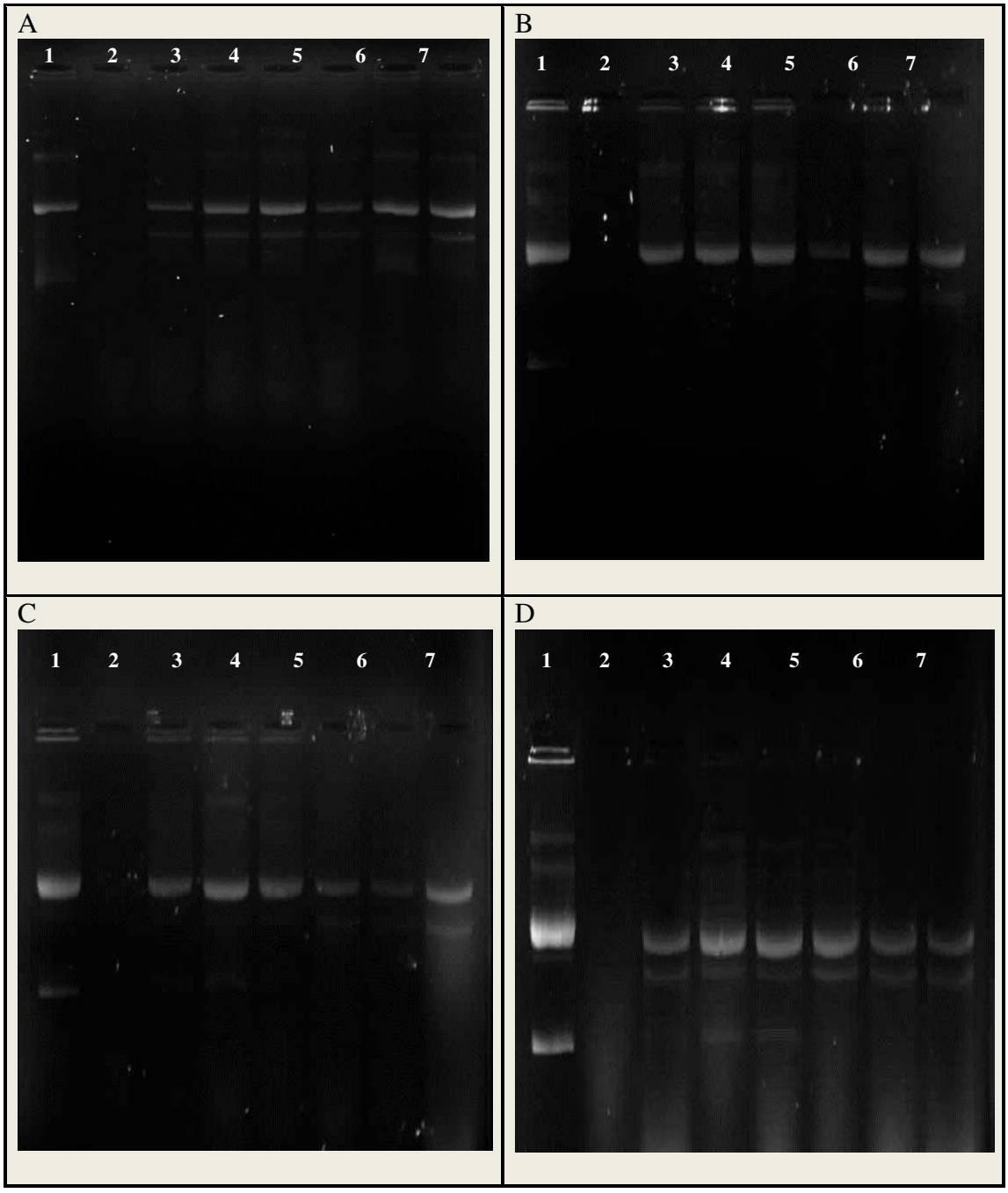


Figure 3.4: Nuclease protection assay of cationic liposome/DNA complexes. Plasmid pCMV-Luc DNA (0.5 μg) completely bound to the liposomal formulations, were incubated in the presence of 10 % serum. Lane 1- pCMV-Luc DNA, lane 2- pCMV-Luc DNA in the presence of serum.

A) Lane 3-5 C1, Lane 6- 8 Pegylated C2
B) Lane 3-5 MS1, Lane 6-8 Pegylated MS2
C) Lane 3-5 NN1, Lane 6-8 Pegylated NN2
D) Lane 3-5 WT1, Lane 6-8 Pegylated WT2

In the present study we evaluated the role played by cationic liposomes in protecting the negatively charged DNA. As mentioned before, when the liposome bound DNA complex enters the bloodstream, the complex is subjected to nucleases present in the serum. The ability of a liposome to protect the DNA is undoubtedly the most crucial feature affecting its biological activity. To assess the complex's degree of resistance to degradation by serum nucleases, lipoplexes were prepared at specific ratios (as outlined in Table 3.2 above) and incubated with serum. The assay mimics an *in vivo* system thus determines the capability of the lipoplex to prevent degradation by serum nucleases. The serum:lipoplex mixture was then subjected to agarose gel electrophoresis (Figure 3.4).

As indicated previously, a negative and positive control, were employed for this assay. The negative control using only pCMV-Luc DNA is represented in lane 1 of Figure 3.3 (A-D) and demonstrates the migration of undigested DNA. The positive control containing pCMV-Luc and 10 % FBS is represented in lane 2 of Figure 3.4 (A-D) which displays the fate of naked DNA in the presence of 10 % serum. Both controls are used to establish the extent of protection offered to the DNA by the cationic liposome. From the results above (Figure 3.4) lanes 3-8 in each image indicate the migration of the lipoplex bound DNA following incubation with 10 % serum. When lanes 3-8 are compared to lane 1 and lane 2 (negative and positive control) we can conclude that all cationic liposomal formulations (at, below and above electroneutrality) displayed a high degree of protection to the plasmid DNA, when subjected to 10 % serum. This is consistent with earlier findings (Kawakami *et al*., 2007) that show that complexes prepared to afford a neutral charge are capable of DNA protection. It is important to note, however, that the lipoplex-bound DNA is largely in the relaxed closed circular form after incubation with serum indicating some nicking of the plasmid. Similarly complexes prepared with an excess positive charge are capable of fully condensing DNA to form a complex that is highly resistant to DNase 1. In contrast, complexes that possess a net negative charge offer little or no protection to the DNA.

The protection of plasmid DNA by the liposome could be attributed to the stability of the complexes or to the electrostatic forces between the positively charged liposomes and the negatively charged DNA. This leads to the formation of highly organised

supramolecular structures where DNA is condensed or compacted and thus largely protected against nuclease degradation (Singh *et al*., 2006).

The results obtained provide clear evidence that all cationic liposomes and PEGylated cationic liposomes form lipoplexes with DNA, and at or near optimal binding ratios, these lipoplexes afford considerable serum nuclease protection. Taking this into consideration, lipoplexes were subjected to *in vitro* transfection and later *in vivo* studies (Chapters 4 and 5).

3.3.3. TEM micrographs of the lipoplexes

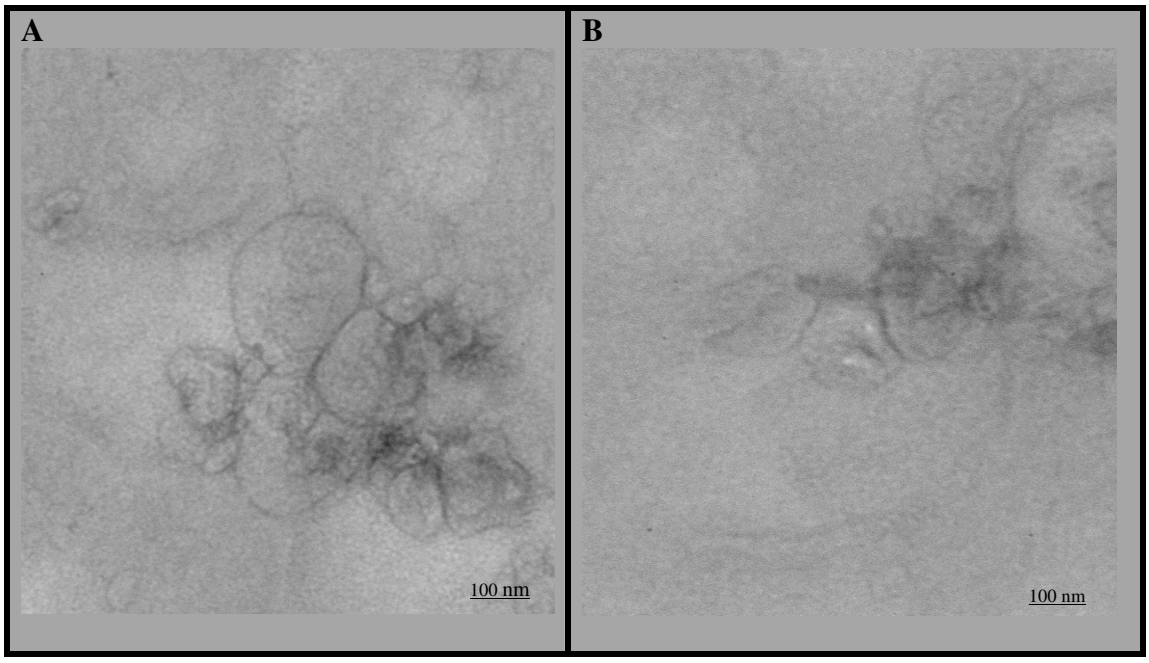


Figure 3.5: Transmission electron micrographs illustrating the morphology of (A) untargeted cationic lipoplexes, C1 and (B) Untargeted PEGylated cationic lipoplexes, C2. Bar =100 nm.

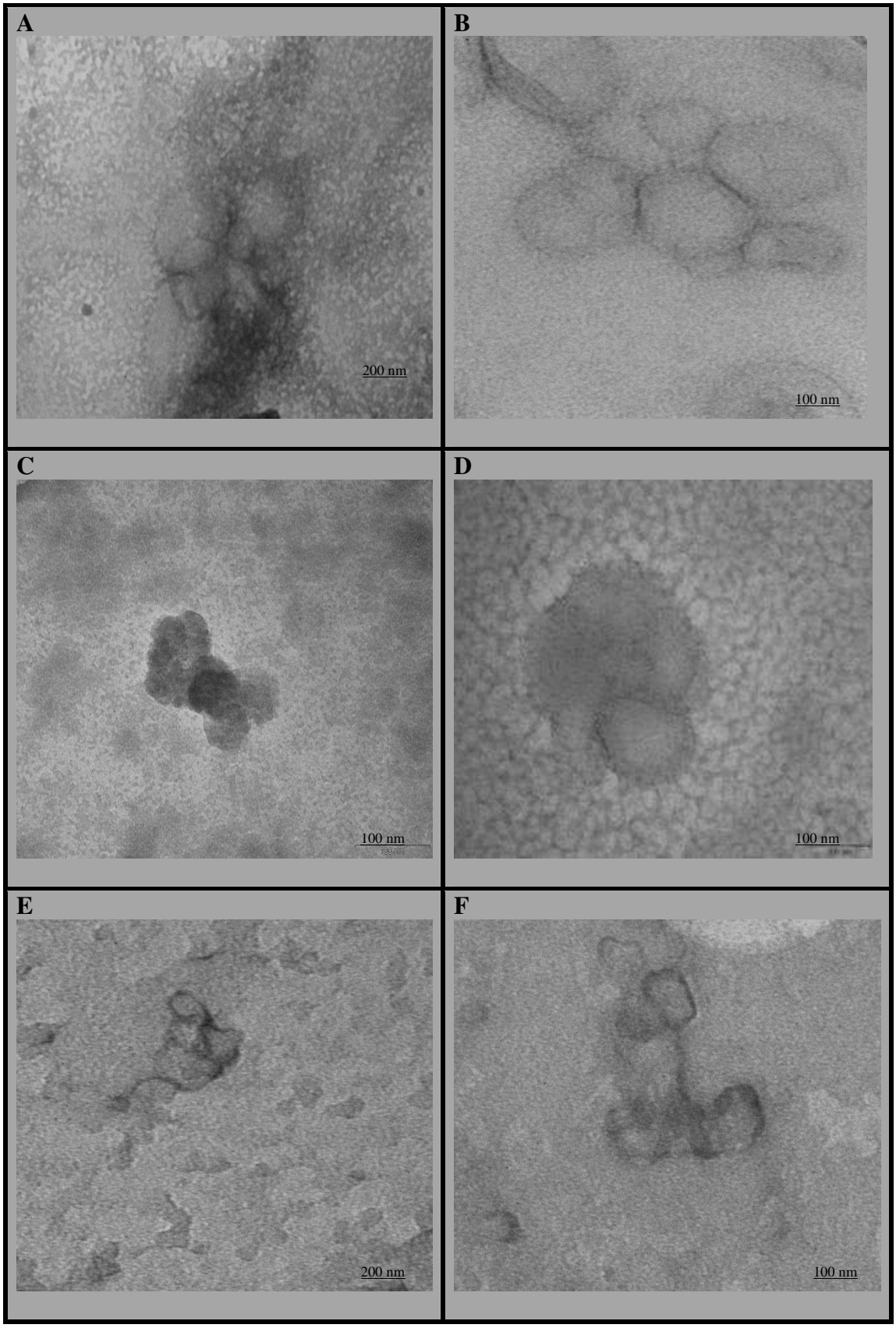


Figure 3.6: The morphology of targeted cationic lipoplexes and the PEGylated counterparts (A) MS1; (B) MS2; (C) NN1; (D) NN2; (E) WT1; (F) WT2.

The size of the lipoplex is relatively heterogeneous when DNA is completely compacted with the cationic liposome, with an estimated diameter of 100-450 nm per lipoplex. Complexes prepared with a lipid-DNA charge ratio of approximately 1:1, yielded complexes between 350-1200 nm in diameter. These complexes present a better colloidal stability when compared with those demonstrating a net positive charge. Multilamellar liposomes form lipoplexes which have been shown to physically exhibit "swiss roll" type of appearance. The resulting lipoplex is practically unusable for systematic gene delivery due to its large size and its inability to disentangle at the cell surface and bind to receptors adequately (Gustafsson *et al*., 1995).

From the results obtained (Figure 3.5-3.6) in cryoelectron microscopy studies, it can be seen that the unilamellar liposomes completely condensed the negatively charged DNA, to yield lipoplexes in a size range that show distinct potential for target cell entry by endocytosis.

CHAPTER FOUR

TISSUE CULTURE AND *IN VITRO* TRANSFECTION STUDIES

4.1. INTRODUCTION

Transfection is a term that has been associated with the delivery of nucleic acids and their analogues to cells, *in vitro* and *in vivo*. Therapeutic applications, specializing in cancer treatment, have exploited the delivery of genes, as plasmid DNA (Min *et al*., 2010). The main aim of gene therapy is to engineer a vector that can successfully transport exogenous DNA to a target cell and ensure high levels of transgene expression, *in vivo*. Prior to *in vivo* studies, formulations are evaluated *in vitro* to determine their safety and efficiency in gene delivery. The transfection efficiency of a vector is an approximate measure of the amount of genetic material successfully transferred and its subsequent transcription within a given cell (Rahau *et al*., 2012). The efficiency of liposomes to optimally transfect cells *in vitro* is dependent on the chemical and physical properties of the liposome. The chemical structure of the lipid, the percentage of helper lipid incorporated in the formulation and the charge ratio between the lipid and the DNA are factors that affect transfection efficiency of a given formulation. Physical properties such as the size and the structure of the lipoplex also play a role in efficient transfection. Other factors governing successful transfection include the type and quantity of lipid used, the type of cell line and the cell density employed during the assay (Xin *et al*., 2012).

A major challenge in systemic targeting is the inability of a gene delivery vector to circulate in the blood for a sufficient period of time, resulting in lower levels of cell toxicity and higher levels of exogenous gene delivery to targeted cells (Morille *et al*., 2009). To avoid non-specific interactions, selective ligands have been incorporated into gene delivery systems. This generates vectors that are internalized via receptor mediated endocytosis (active targeting). The aim of this study was to synthesize and compare of three unique cationic liposomal formulations. Cell specific targeting was achieved by incorporating galactose (10 mole %) into the lipid bilayer of each of the three formulations tested. Target specificity was determined using the human

hepatocellular carcinoma (HepG2) and the human embryonic kidney (HEK293) cell lines. The HepG2 cells (established in 1979 from hepatoma tissue) mimics the liver parenchymal cells since they synthesize and secrete several serum proteins. The cell line originally isolated from a liver biopsy of hepatocellular and hepatoblastoma carcinomas (Aden *et al*., 1979), displays a unique characteristic exhibiting one nucleus and 48-54 chromosomes/ cell (Wilkening *et al*., 2003). This enables the cell line to be an ideal candidate for the analysis of cytotoxicity and pharmaceutical studies, targeted to the liver (*in vitro*). The cell line expresses the asialoglycoprotein receptor and is known for its rapid internalization rate and high ligand affinity. The receptor is known to exhibit high affinity for the galactose located at the non-reducing termini of the asialoglycoprotein heteroglycan structures, thus galactose molecules may be grafted on to the surface of liposomes to promote their cellular uptake by ASGP-R mediation (Figure 4.1).

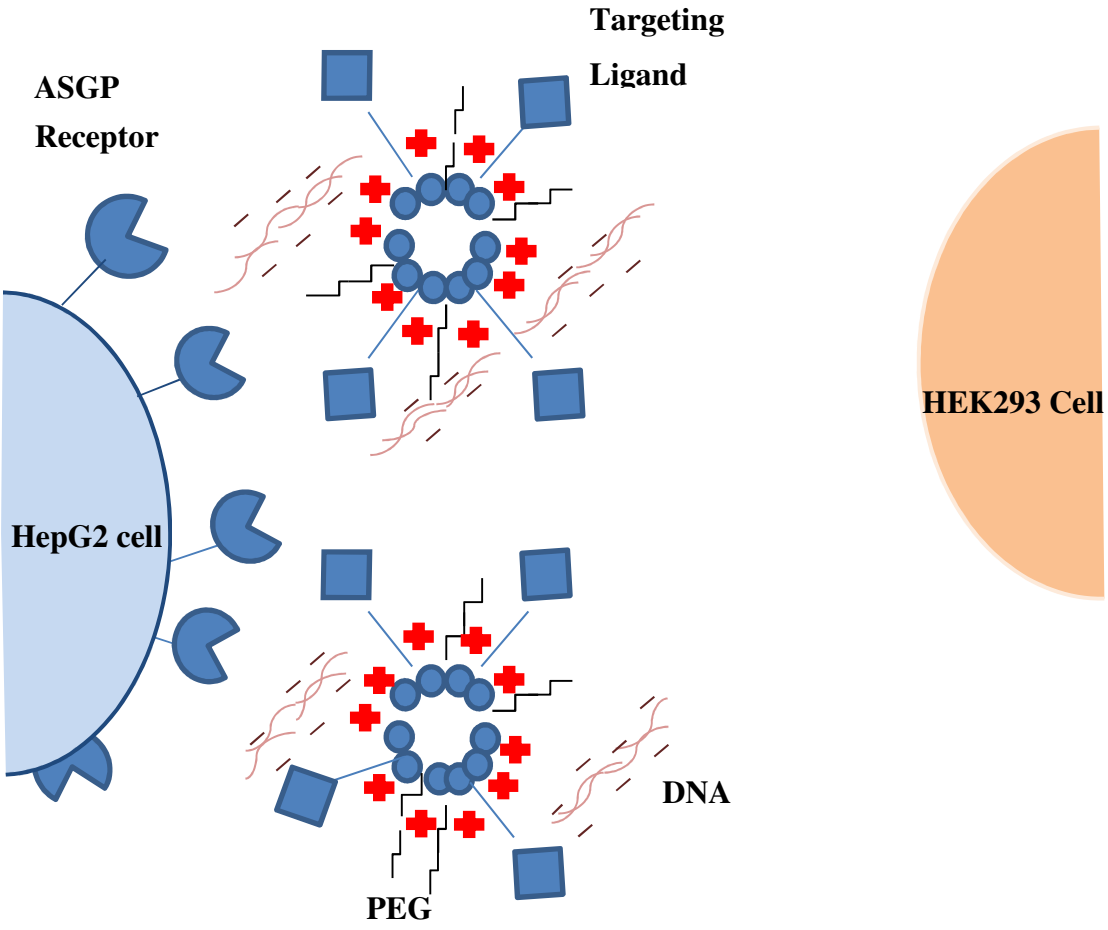


Figure 4.1: Schematic representation outlining the interactions between the HepG2 cell line and the galactosylated targeting system. The galactose moiety is recognized and internalized by the ASGP-R present on the HepG2 cell. The HEK293 cell line is ASGP-R negative (Diagram not drawn to scale).

DSPE-PEG_{2000}, (2 mole %) was employed in the preparation of the liposomes. PEGylation of complexes has been shown to increase the lipoplex circulation period sufficiently, as to allow for the complexes to reach their target organ (Braet and Wisse, 2002). Liposomes are frequently studied as alternatives to toxic viral vectors. Due to the low levels of exogenous gene expression *in vivo*, an increased dosage of the liposomal formulation is required to increase transgene expression. An increase in liposomal dose consequently increases cytotoxicity (Dass and Choong, 2006). This can be attributed to the hydrophobic and polar domains of cationic lipids (Hongtao *et al*., 2006). Thus this study has employed the use of lipids, such as cholesterol and DOPE (Kawakami *et al*., 1998) that have been known to exhibit low levels of cell toxicity *in vitro*. Various protocols (indirect methods using chromogenic indicators) have been developed to determine the effect of the lipoplex on cell toxicity. The most common protocol is the MTT cell proliferation assay, which is based on the conversion of a water soluble salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to an insoluble formazan. The formazan is solubilized and the optical density of the extract is determined at 570 nm.

The cytomegalovirus (CMV) promoter has been extensively used to detect the expression of exogenous proteins *in vitro*. The pCMV-Luc plasmid DNA expresses the luciferase gene under the control of a CMV promoter sequence. This plasmid was used in all studies conducted. The cationic: pCMV-Luc DNA complexes were prepared at different ratios of lipid to a constant amount of pCMV-Luc and subjected to analyses in the HepG2 and HEK293 cell lines. The pCMV-Luc reporter plasmid has been utilized in this study, to assess transgene expression in two cell lines, which typically do not otherwise display luciferase activity. The pCMV-Luc plasmid DNA encodes the firefly luciferase gene. This ATP-dependent gene was initially isolated from the North American firefly, *Photinus pyralis*.

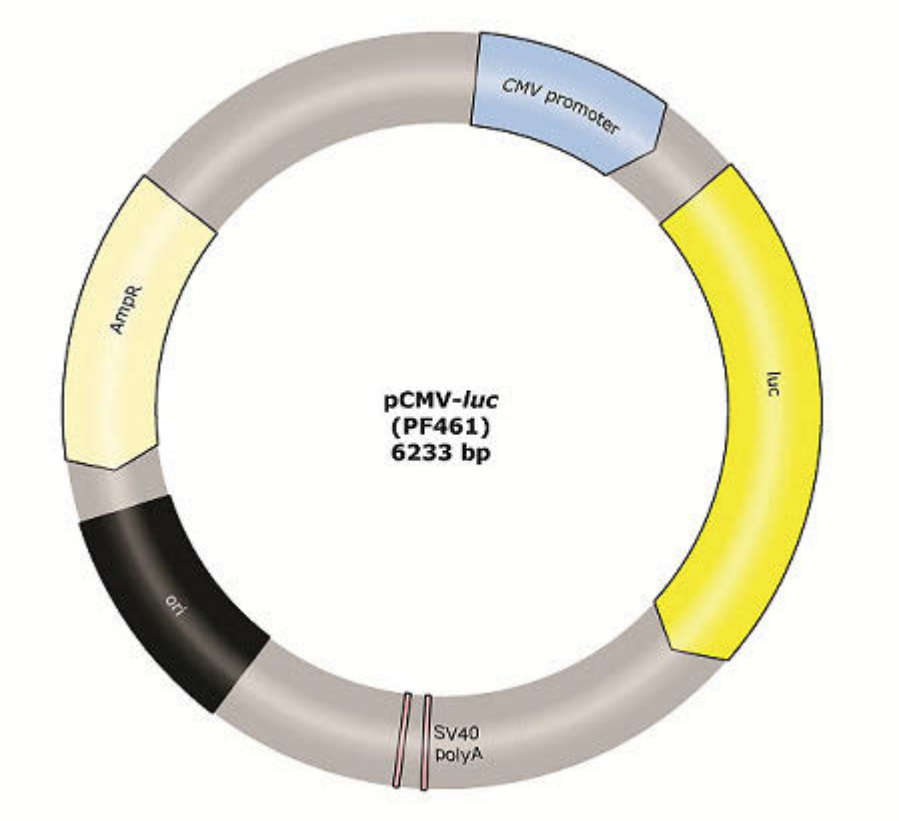


Figure 4.2: Basic schematic representation of the pCMV-Luc DNA control vector that expresses the luciferase gene (*luc*) (Plasmid Factory, Bielefeld, Germany).

The degree of cytotoxicity and transfection were measured and compared between the two cell lines, for all liposomal formulations, at different lipid: plasmid DNA ratios. The formulations displaying the highest transfection activity were subjected to the competition assay in an attempt to confirm that the galactosylated lipoplexes were actually internalized by ASGP-R mediation. The formulation showing the greatest potential in the transfection studies was then subjected to *in vivo* studies.

4.2. MATERIALS AND METHODS

4.2.1. Materials

HepG2 cells and foetal bovine serum (FBS) were obtained from Highveld Biological (PTY) LTD., Lyndhurst, South Africa. HEK293 cells were obtained from University of Witwatersrand, Johannesburg, SA. Minimum Essential Medium (MEM), Trysin-versene and penicillin/streptomycin mixtures were purchased from Lonza BioWhittaker, Walkersville, USA. All tissue culture plastic consumables were purchased from Corning Incorporated, New York, USA. The Luciferase Assay kit was purchased from the Promega Corporation, Madison, USA. The Bicinchoninic acid (BCA) used in the BSA protein assay was purchased from the Sigma-Aldrich Co., St. Louis, USA. 2-[4-(2-hydroxyethyl)-1-piperazinzyl] ethanesulphonic acid (HEPES), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Phosphate buffered saline (PBS) tablets were purchased from Merck, Darmstadt, Germany. All other reagents were of analytical grade.

4.2.2. Methods

4.2.2.1. Preparation of tissue culture medium

To Minimum Essential Medium (MEM) was added penicillin G and streptomycin to a final concentration of 100 u/µl and 100 µg/µl respectively. Finally the FBS was added (10 % by volume).

4.2.2.2. Cell maintenance and culture

All media preparation and other cell culture work performed for this study, was executed in a class II Biohazard laminar flow hood. The cell lines (HepG2 and HEK293) were frequently inspected for contamination and the growth patterns monitored. Cells were subcultured or frozen when they reached a state of semi-

confluency. Cells were cultured in 25 cm^2 flasks and routinely spilt 1:3 every 4-5 days.

4.2.2.3. Reconstitution of the cell lines, HepG2 and HEK293

Cells are cryopreserved as per standard procedures, in cryogenic vials and stored temporarily in a - 80 ˚C Biofreezer. Upon reconstitution of the appropriate cell line (HepG2 and/or HEK293), the previously cyropreserved vial containing cells are removed from the Biofreezer and thawed in a 37 ˚C water bath. Once the medium containing cells had completely thawed, the cryogenic vial was wiped thoroughly with 70 % ethanol and placed into the laminar flow hood. The vial was thereafter opened aseptically, and decanted into a 15 ml centrifuge tube. The tube was then centrifuged at 3000 rpm for 30 seconds. Under sterile conditions, the centrifuge tube was opened and the supernatant (old medium) was discarded. The remaining pellet was resuspended with 1 ml of freshly prepared complete medium (MEM, antibiotics and 10 % FBS). The tube was then vortexed for a few seconds to ensure that the pelleted cells were completely resuspended in the medium. Cells were thereafter transferred to 25 cm^2 flask containing 4 ml of complete medium and incubated at 37 ˚C to allow cells to attach to the bottom of the flask. Cells were monitored on a daily basis and the medium changed every 2 days and replaced with fresh complete medium. This was carried out until cells reached the state of confluency/ semi-confluency.

4.2.2.4. Propagation of Cells (HepG2 and HEK293 cell lines)

When the cells reached confluency/ semi-confluency, they were trypsinized to be used in cell assays or spilt into 2 flasks (25 cm^2). The process of trypsinization firstly involves, discarding the medium that is present in the flask, and washing the attached cells with 5 ml PBS (phosphate buffered saline) (150 mM NaCl, 2.7 mM KCl, 1 mM KH_2PO_4, 6 mM Na_2HPO_4; pH 7.5). The excess PBS was then discarded into the waste bottle. Thereafter, 1 ml of trypsin-versene was added to the flask containing the cells, and cells were observed under a Nikon TMS inverted light microscope for rounding off (indication of cells being trypsinized). Once the cells had rounded off, 2 ml of

complete medium was added to the flask. Essentially, it is necessary that 20 % of FBS is added to the flask since this terminates the action of the trypsin (prolonged exposure to trypsin could result in cell death). The flask was then tapped against the palm of the hand to dislodge the cells. The cells were then resupended using a 2 ml pipette and thereafter either divided into sterile culture flasks containing 5 ml of complete medium or plated in multi-welled plates, for culture assays. Cells were continuously monitored with regular mediun change. Once the cells had reached the state of confluency, they were trypsinized and spilt once again, and used for assays or cryopreserved.

4.2.2.5. Cryopreservation of Cells

The cryopreservation protocol is similar to that of the propagation of cells. The cells were trypsinized as described in section 4.2.2.4, above. Upon the addition of trypsin, the cells are dislodged, resuspended and transferred to a 15 ml centrifuge tube. The tubes were then centrifuged at 3000 rpm for 1 minute and the supernatant was discarded. Complete medium (0.9 ml) and dimethylsulfoxide (DMSO) (0.1 ml) was then added to the pelleted cells. The tube was then gently vortexed to resuspend the cells, in the complete medium containing 10 % DMSO. The suspension was then immediately aliquoted into cryogenic vials, labelled and placed in a cryogenic container in the biofreezer for slow freezing (1 ˚C/minute).

4.2.2.6. Growth Inhibition Studies

Upon confluency, the cells were trypsinized (section 4.2.2.4) and seeded into a 48 well plate (cell density = 1.2 x 10^4 cells/well). The plate was sealed with para-film, appropriately labelled and incubated in a 37 ˚C incubator for 24 hours at 37 ˚C, to allow the cells to attach to the base of each well. Following this incubation, the lipoplexes (cationic liposome-DNA complexes) were prepared as per Table 3.2. The volume of each lipoplex suspension was then brought up to a final volume of 10 µl with HBS. Lipoplexes were then briefly vortexed and centrifuged (Eppendorf

microcentrifuge, 3 000 rpm) and incubated for 30 minutes at room temperature. Assays were conducted in triplicate.

Thereafter the old medium was removed and replenished with 0.25 ml of serum free medium containing antibiotics (penicillin G and streptomycin). The lipoplexes were then added to the appropriate wells and the plate was incubated for 4 hours at 37 ˚C. Thereafter the serum free medium was removed and replaced with 0.25 ml complete medium and the plate was incubated for 48 hours at 37 ˚C. Following the incubation period, the medium was removed and 0.2 ml of complete medium and 0.2 ml of the MTT solution (5 mg / ml PBS) was added to each well and the plate was incubated at 37 ˚C for 4 hours to allow for the production of formazan crystals. Thereafter the MTT solution and medium in each well was removed and discarded. The formazan crstals were then dissolved by the addition of 0.2 ml of dimethylsulfoxide (DMSO) to each well and incubated for 5 minutes. The DMSO extract (0.1 ml) was removed from each well and transferred to a 96 well plate. The samples were read in a Mindray 96A microplate reader (Vacutec) using DMSO as the blank. The 100 % viability value was obtained from the OD 570 value measured for cells, without complexes. Test samples were measured relative to untreated cells. Viability = $\frac{\text{Test } OD_{570} - \text{Blank } OD_{570}}{\text{Untreated } OD_{570} - \text{Blank } OD_{570}}$

4.2.2.7. Transfection

4.2.2.7.1. Luciferase Assay

Confluent cells were trypsinized and seeded into a 48 well plate, at a seeding density of, 1.9 x 10^4 cells/well. Cells were incubated overnight, at 37 ˚C, to allow the cells to attach to the base of each well. Once attachment was confirmed by microscopic observation, the appropriate lipoplexes were prepared as per Table 2.2 and allowed to incubate at room temperature for 30 minutes. The medium present in each well in the plate, was removed and replaced with serum free medium (0.25 ml). Following the 30 minute incubation period, the complexes were added to their appropriate wells. Assays were conducted in triplicate. Each test plate contained two controls, one

containing only the cells, and the other containing cells plus naked pCMV-Luc DNA (1 µg). The plate was then incubated for 4 hours in a 37 ˚C incubator. Thereafter the medium was removed and replaced with 0.25 ml complete medium (MEM + 10 % foetal bovine serum) and cells incubated for a further 48 hours, at 37 ˚C.

The luciferase assay was carried out using the Promega Luciferase Assay kit. The 5x lysis reagent (25 mM trisphosphate, pH 7.8, 2 mM dithiothreiotol, 2 mM 1,2-diaminocyclohexane-N, N, N'N'-tetraacetic acid, 10 % ($^{v}/_{v}$) glycerol, 1 % ($^{v}/_{v}$) Triton X-100) was allowed to thaw at room temperature. This was then diluted with distilled water to obtain a 1x stock.

The medium from the wells were then removed and the cells were washed twice with PBS (approximately 0.1 ml). A total volume of 80 µl of 1x cell lysis reagent was added to each well. The plate was placed on a mechanical platform shaker (Scientific STR 6, Surrey, UK), for 15 minutes at 30 rev / min. The cells were finally dislodged from the bottom of each well, by scrapping the base of each well with either a pasture pipette or the tip of a micropipette. The resulting cell solution was dispensed into microcentrifuge tubes and centrifuged at 12 000 rpm for 30 seconds. The clear supernatant, containing the cell free extract, was subjected to luciferase activity analyses. This involved the addition 100 µl of luciferase assay reagent to 20 µl of the cell free extract. This was immediately mixed and placed in the Lumac Biocounter 1500 luminometer (Landgraaf, Netherlands). The cell free extract was also subjected to the bicinchoninic acid (BCA) assay to determine protein content.

4.2.2.7.2. Competition Assay

Confluent cells were trypsinized and seeded into two 48 well plates (2 x 10^4 cells/well and 2.5 x 10^4 cells/well) and incubated at 37 ˚C, for 24 hours to allow the cells to adhere to the base of each well. The appropriate lipoplexes were prepared as per Table 3.2 and allowed to incubate at room temperature for 30 minutes. The assay was carried out in triplicate. The medium from each well was removed and replaced with serum free medium (0.25 ml). Thereafter 25 µl of asialofectuin (Stock of 10 µg/ µl) was added to the appropriate wells and incubated at 37 ˚C, for 20 minutes. The

complexes were then added to each well and the plate was incubated for 4 hours, at 37 ˚C. Thereafter the medium was removed and replaced with 0.25 ml complete medium (MEM + 10 % foetal bovine serum) and cells incubated for a further 48 hours at 37 ˚C. The luciferase assay was conducted as described in Section 4.2.2.7.1 above.

4.2.2.7.3. Statistical Analysis

A comparison between each of the positive controls and the respective test groups was analysed using the unpaired student t-test. A p-value of < 0.05 was considered as significant in this study.

4.3. RESULTS AND DISCUSSION

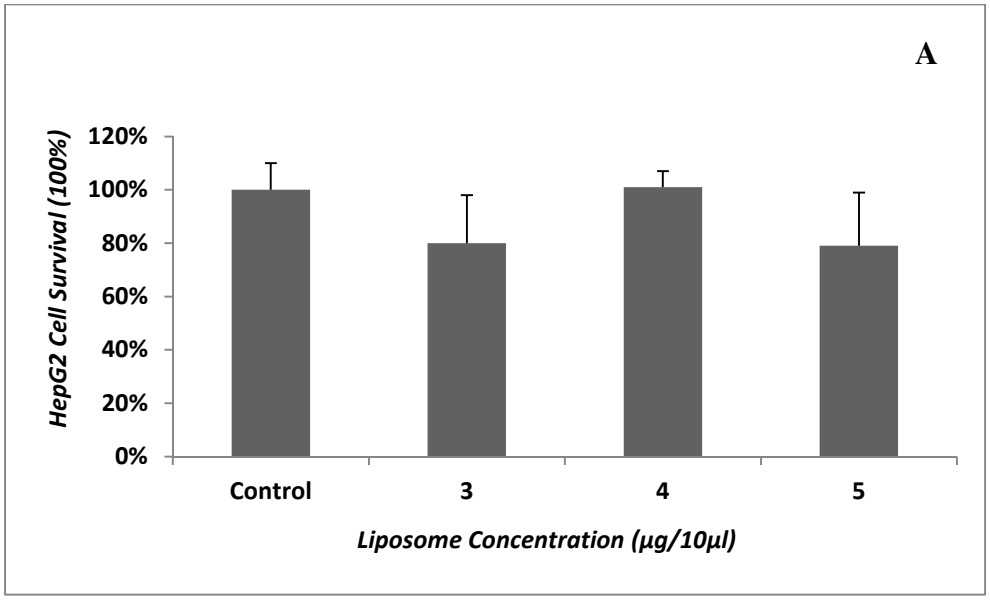
4.3.1. Growth Inhibition Assay

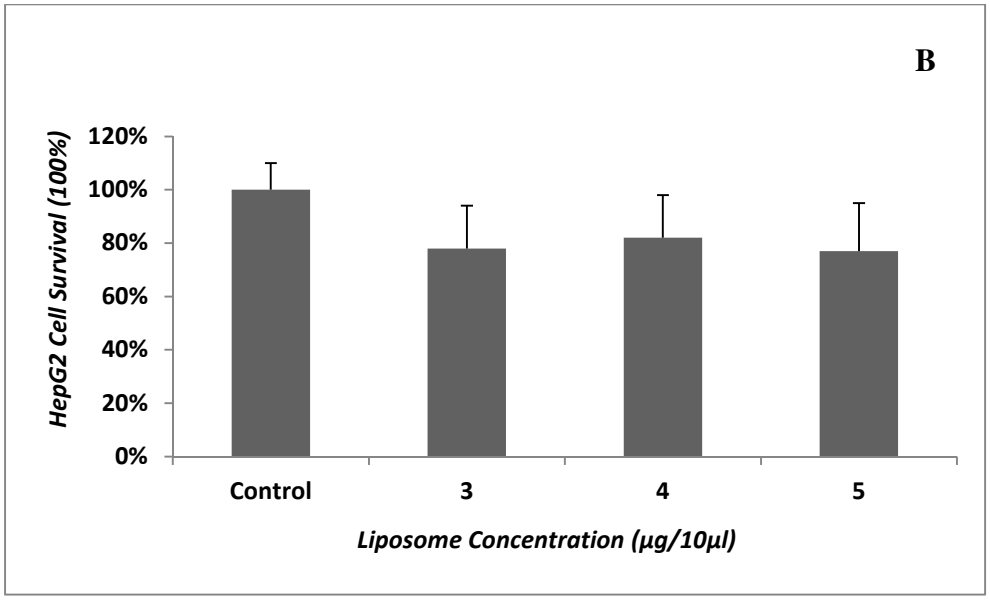
Cytotoxicity induced or caused by lipoplexes is an obstacle in gene therapy and cytotoxicity studies are used to determine the safety of the liposomal formulations *in vitro* and potentially *in vivo*. The MTT Assay is a simple method used to determine cell numbers spectrophotometrically. This assay was used to evaluate the effects of the lipoplex on cell survival. The assay was carried out using the ASGP-receptor negative cell line, HEK293 and the ASGP-receptor positive cell line, HepG2. The results obtained are shown below in, Figures 4.3-4.6.

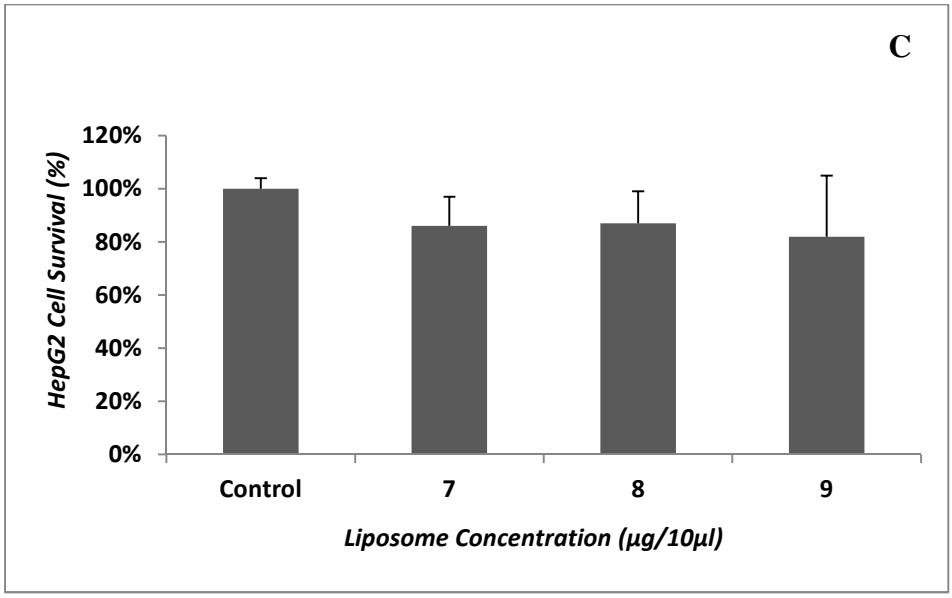
4.3.1.1. HepG2 and HEK293 Cell line

From the results obtained, all cationic liposome:DNA complexes analysed were well tolerated over the entire lipid concentration range (3-11 µg/10 µl). The study findings are in agreement with those of Percot *et al*. (2004), who showed that galactosylated cationic liposomes, containing DOPE, displayed low toxicity in the HepG2 cell line. Nguyen and co-workers (2007) also showed that there existed a distinct synergy between the plasmid DNA and the cationic lipid, which undoubtedly resulted in the cell toxicity. It was found that free naked DNA did not induce cell death, however when the cationic lipid was subjected to cell toxicity assays (Hela cell line), the lipid

slightly induced cytotoxicity. Cell death was further increased when the cationic lipid was complexed to the pDNA.







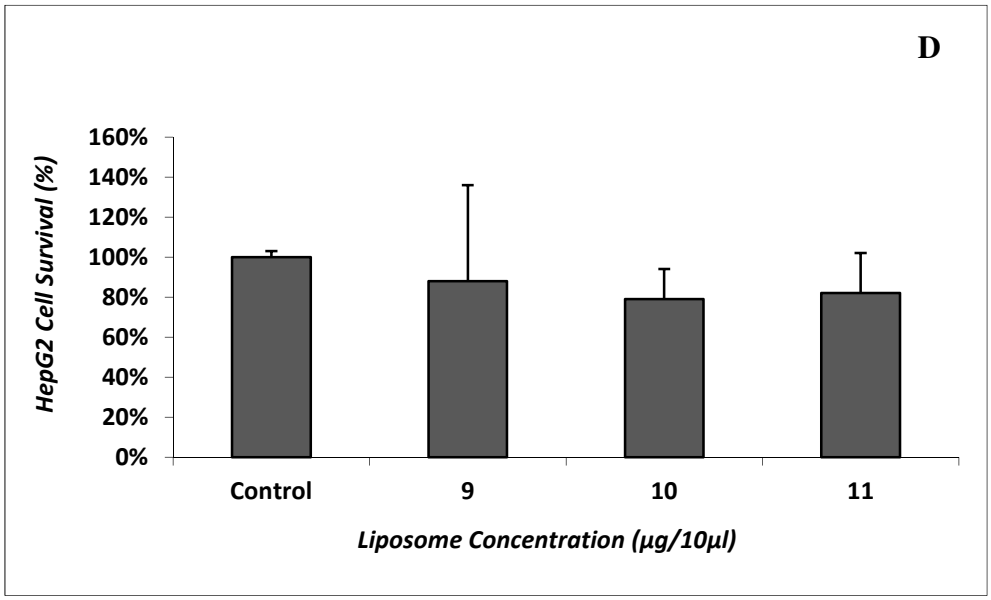
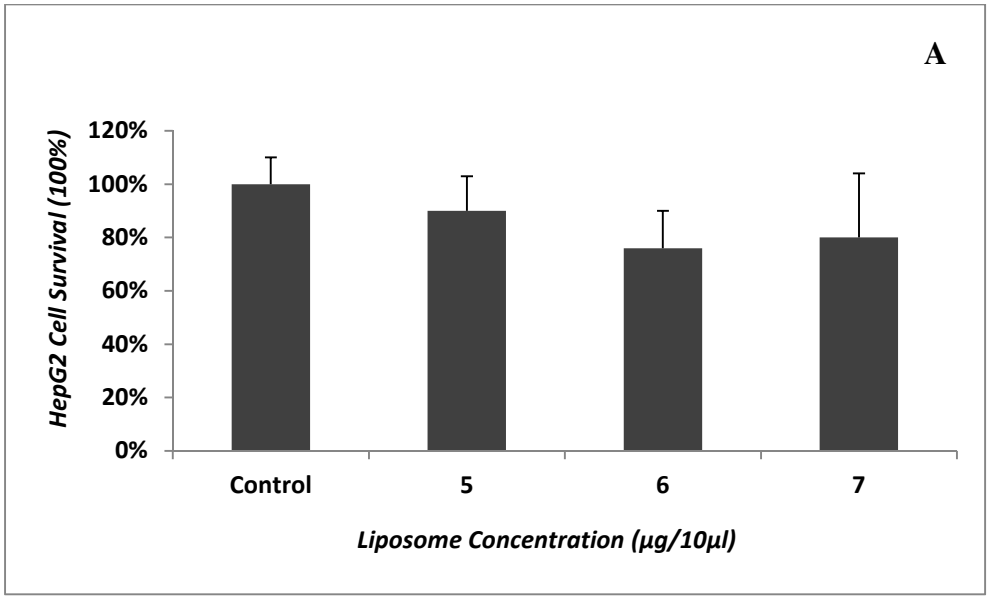
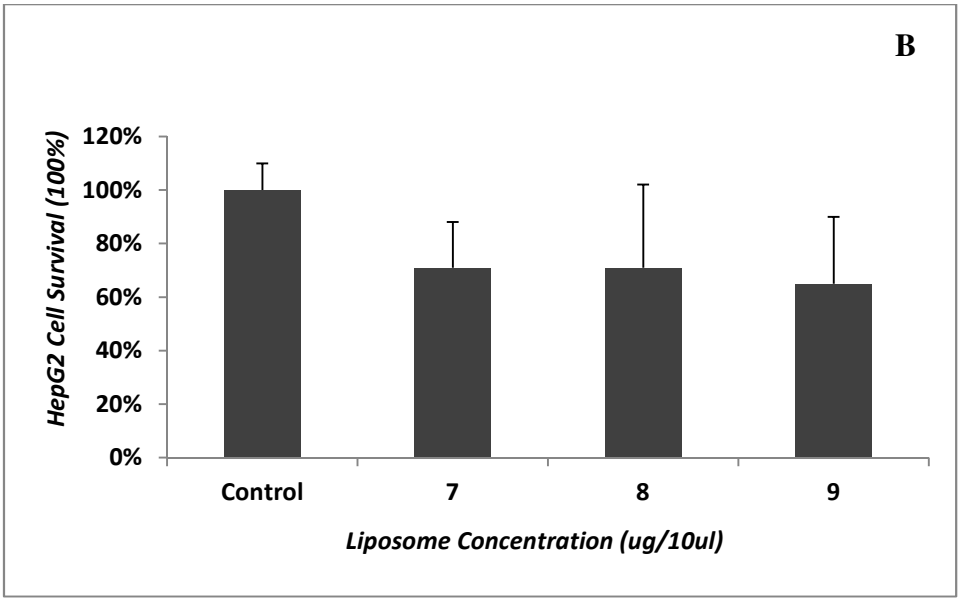
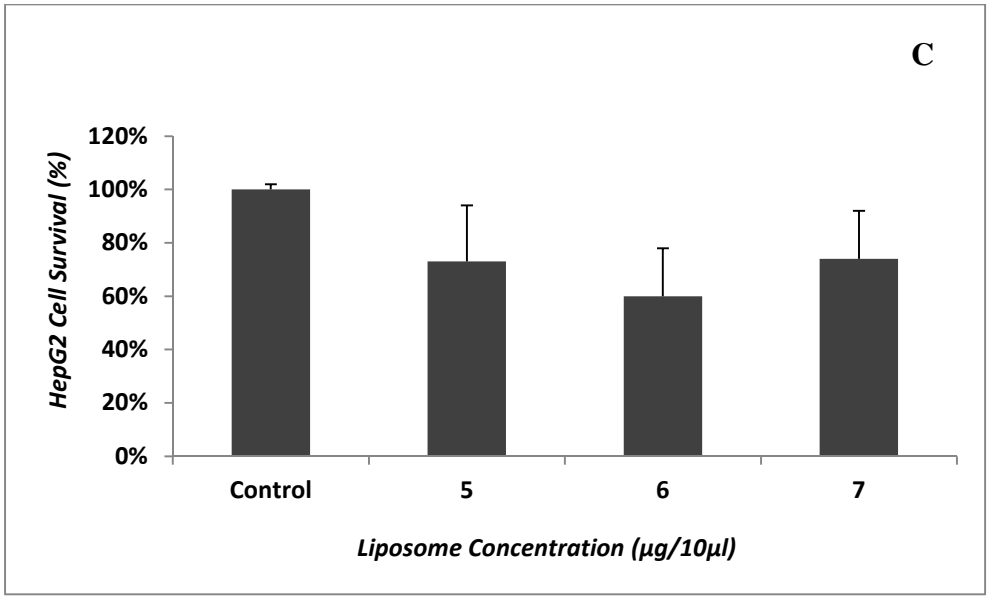


Figure 4.3: MTT cytotoxicity assay in HepG2 cells. Assay was conducted in a 48 well plate with 1 µg pCMV-Luc DNA/well and an increasing amount of the liposomal formulation, as indicated in each case above. (A) untargeted liposomal formulation C1; non-PEGylated formulations (B) MS1 (C) NN1 (D) WT1. Results are represented as means ± S.D (n=3).


A
HepG2 Cell Survival (100%)
120%
100%
80%
60%
40%
20%
0%
Control
5
6
7
Liposome Concentration (µg/10µl)


B
HepG2 Cell Survival (100%)
120%
100%
80%
60%
40%
20%
0%
Control
7
8
9
Liposome Concentration (ug/10ul)



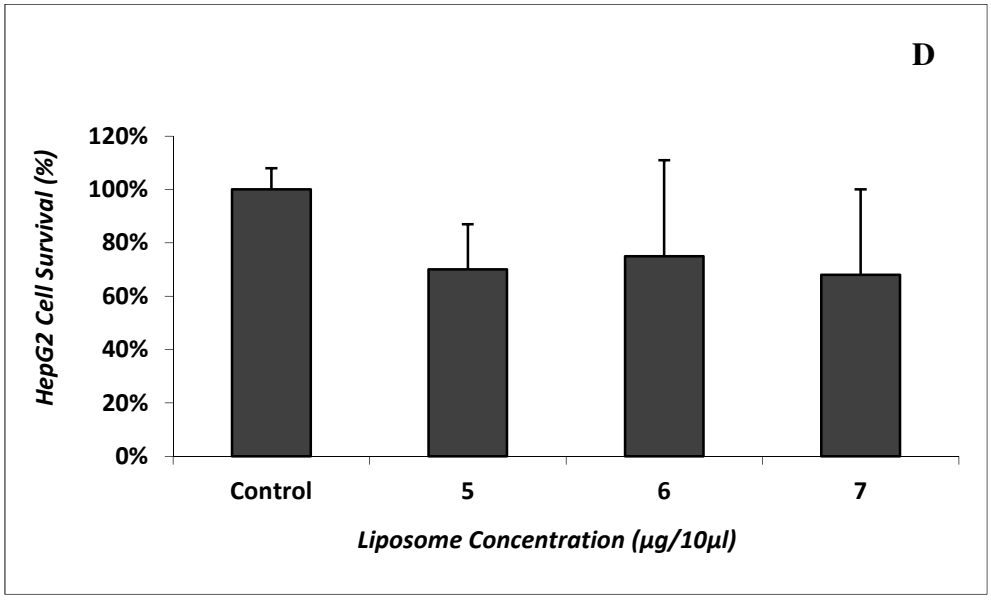
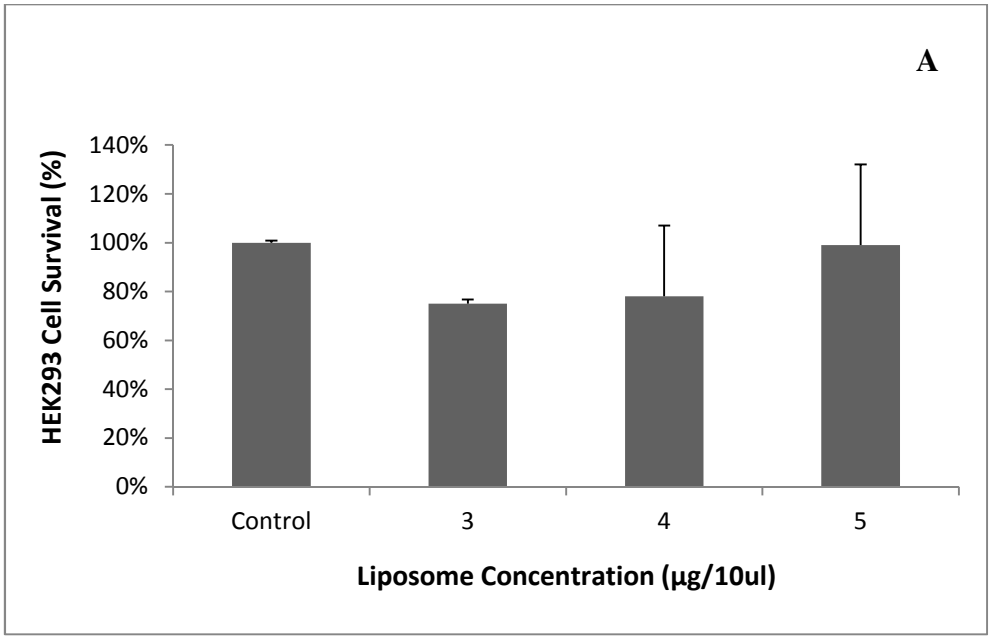
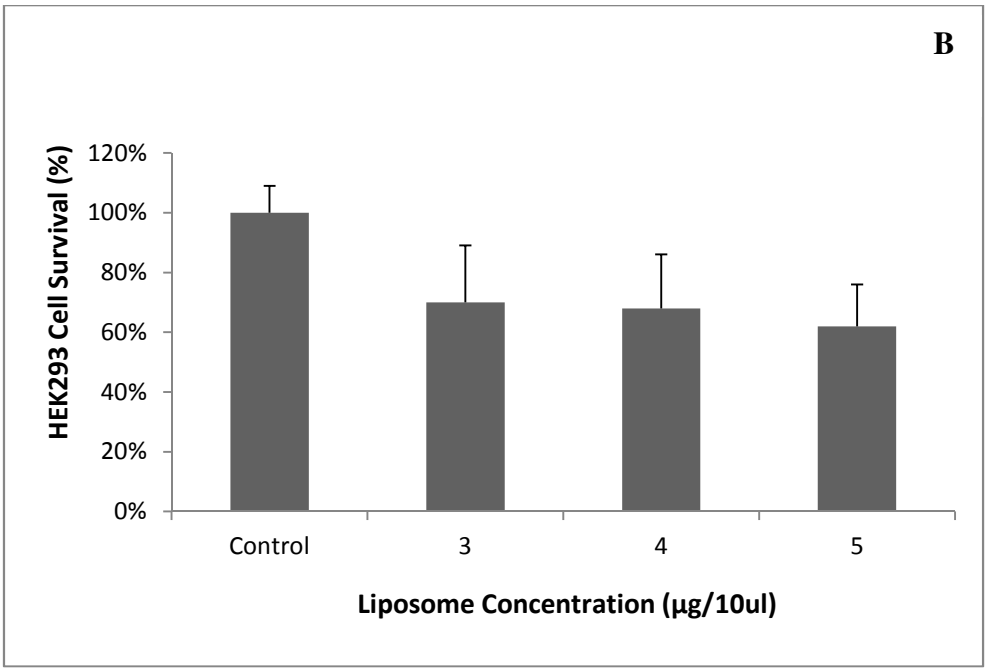
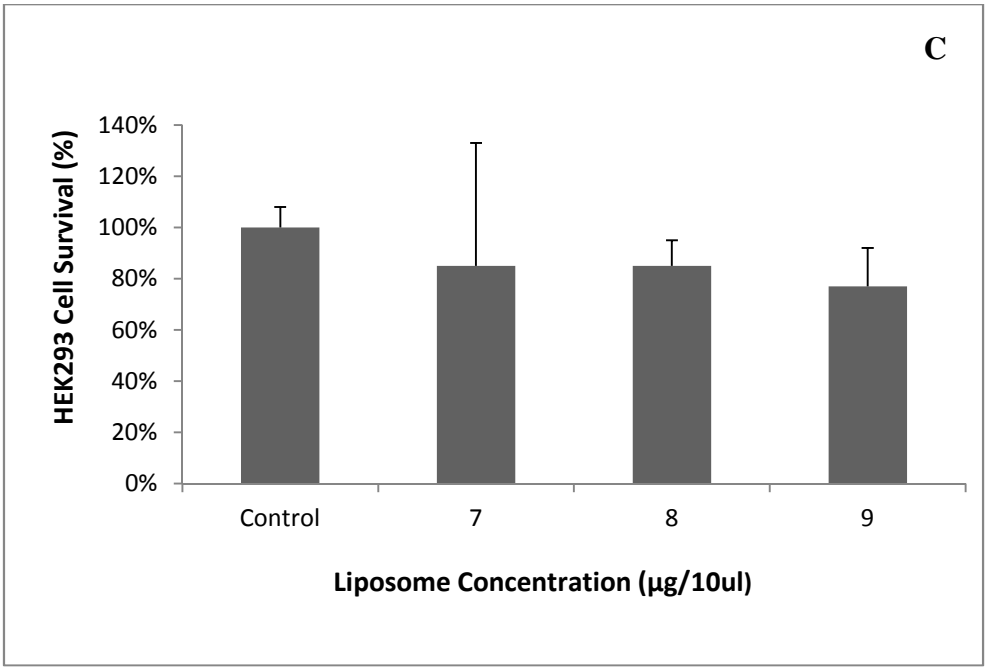


Figure 4.4: MTT cytotoxicity assay in HepG2 cells. Assay was conducted in a 48 well plate with 1 µg pCMV-Luc DNA/well and an increasing amount of the liposomal formulation, as indicated in each above. 2 mole % PEGylated (A) untargeted C2; (B) MS2; (C) NN2; (D) WT2. Results are presented as means ± S.D (n=3).


A
HEK293 Cell Survival (%)
140%
120%
100%
80%
60%
40%
20%
0%
Control
3
4
5
Liposome Concentration (µg/10ul)


B
HEK293 Cell Survival (%)
120%
100%
80%
60%
40%
20%
0%
Control
3
4
5
Liposome Concentration (µg/10ul)



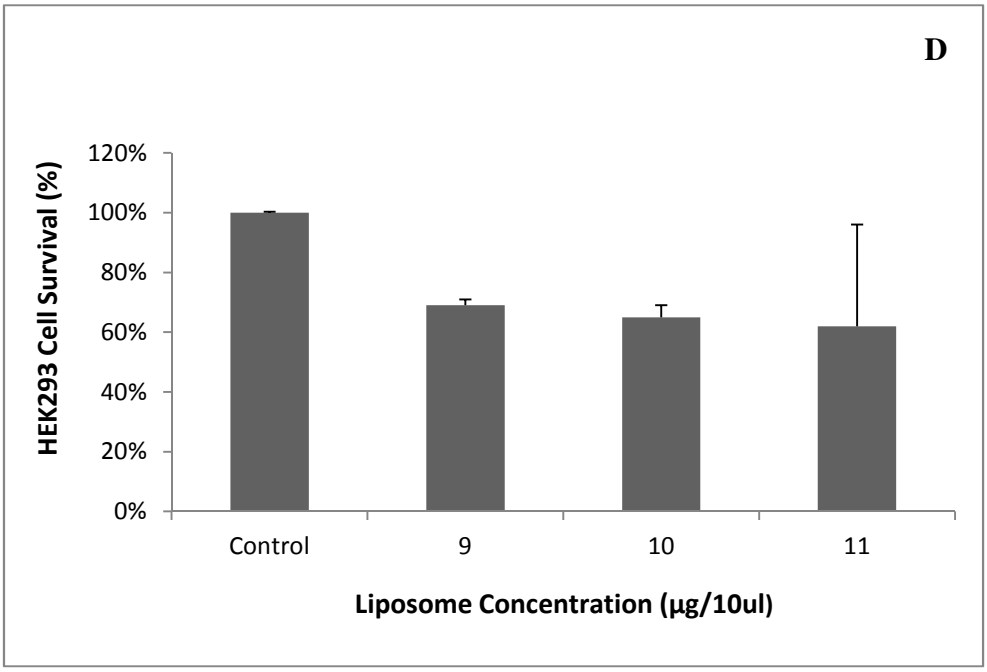
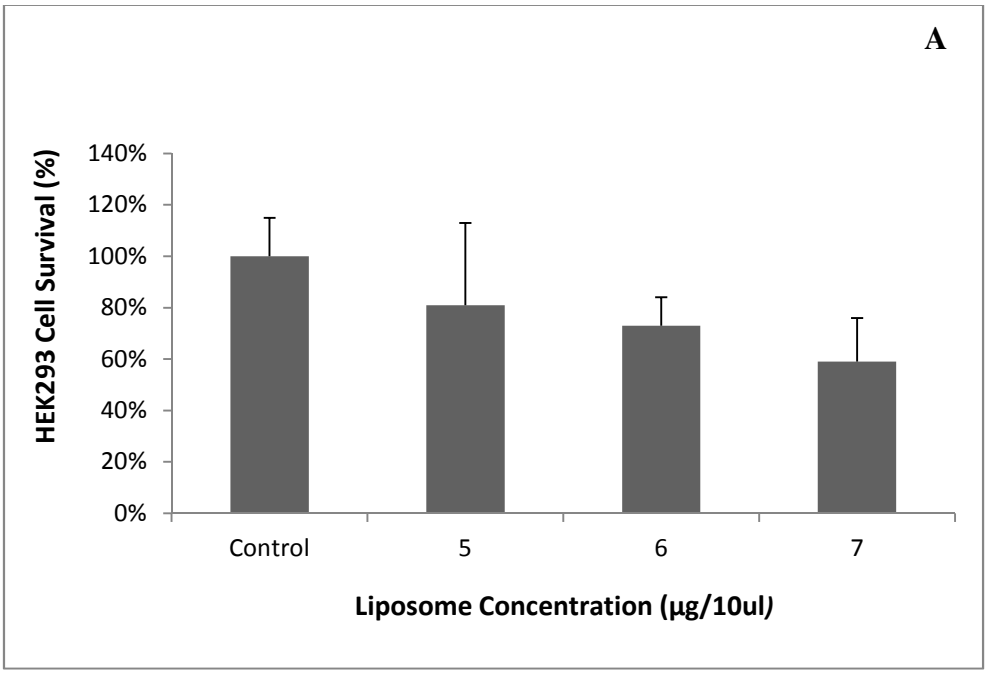
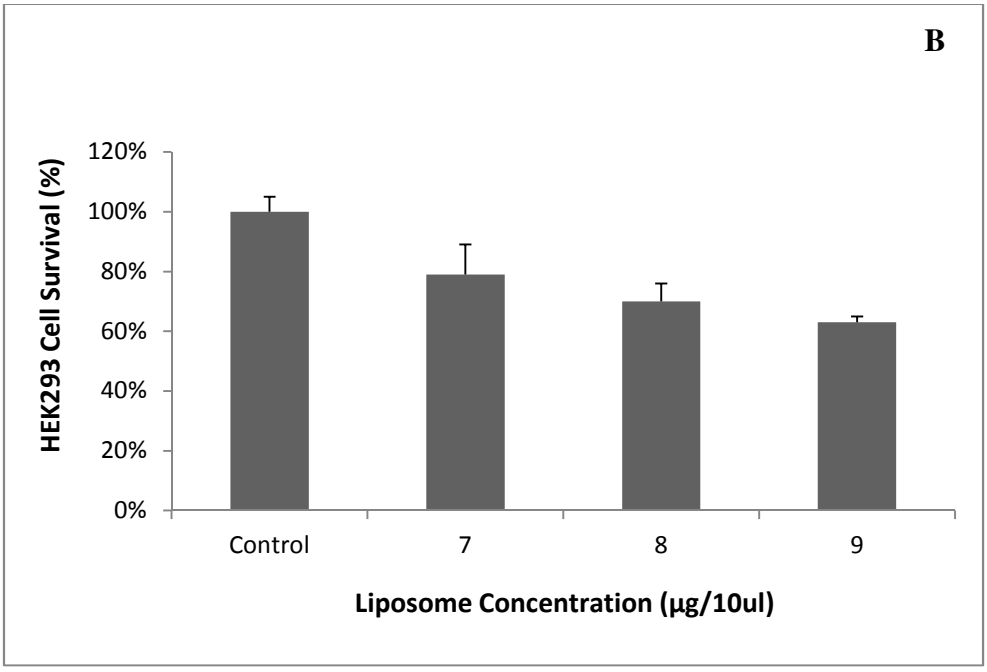
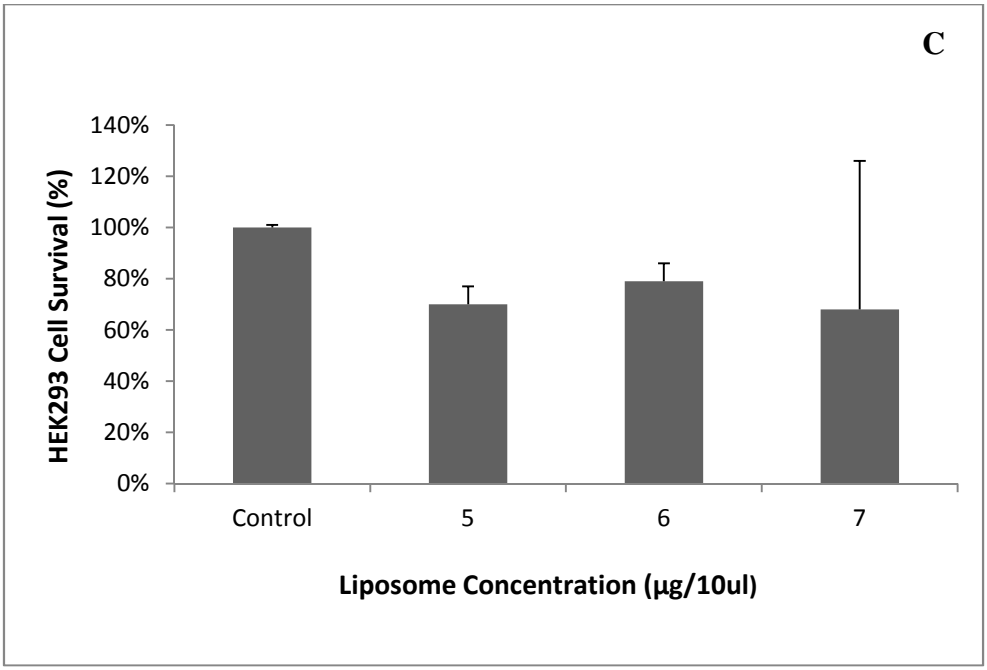


Figure 4.5: MTT cytotoxicity assay in HEK293 cells. Assay was conducted in a 48 well plate with 1 µg pCMV-Luc DNA/well and an increasing amount of the liposomal formulation, as indicated in each above. (A) Untargeted liposomal formulation, C1; non-PEGylated targeted formulations (B) MS1; (C) NN1; (D) WT1. Results are presented as means ± S.D (n=3).


A
HEK293 Cell Survival (%)
140%
120%
100%
80%
60%
40%
20%
0%
Control
5
6
7
Liposome Concentration (µg/10ul)


B
HEK293 Cell Survival (%)
120%
100%
80%
60%
40%
20%
0%
Control
7
8
9
Liposome Concentration (µg/10ul)



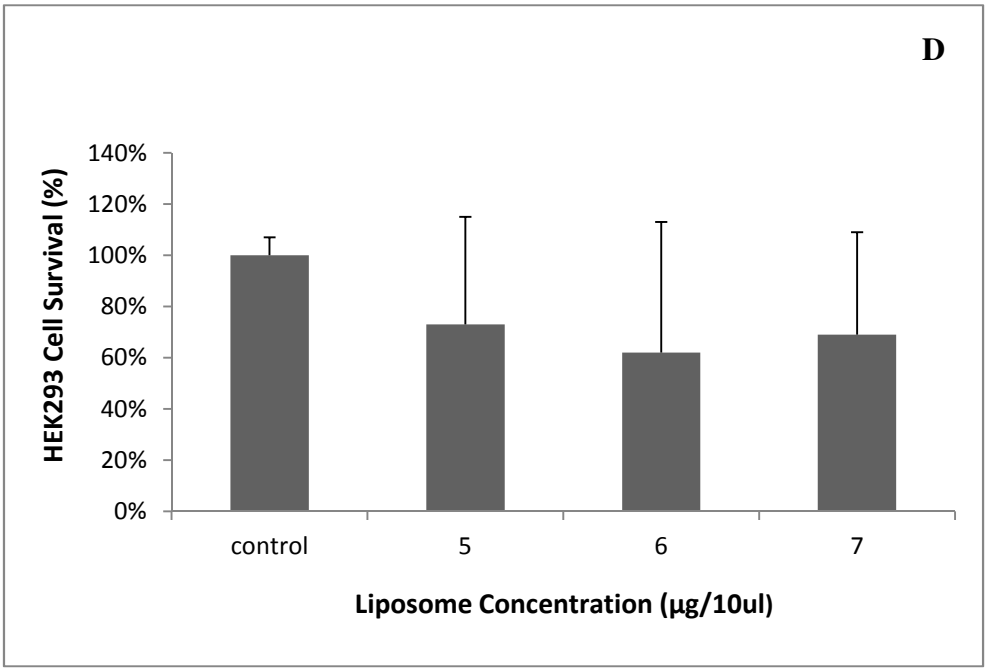


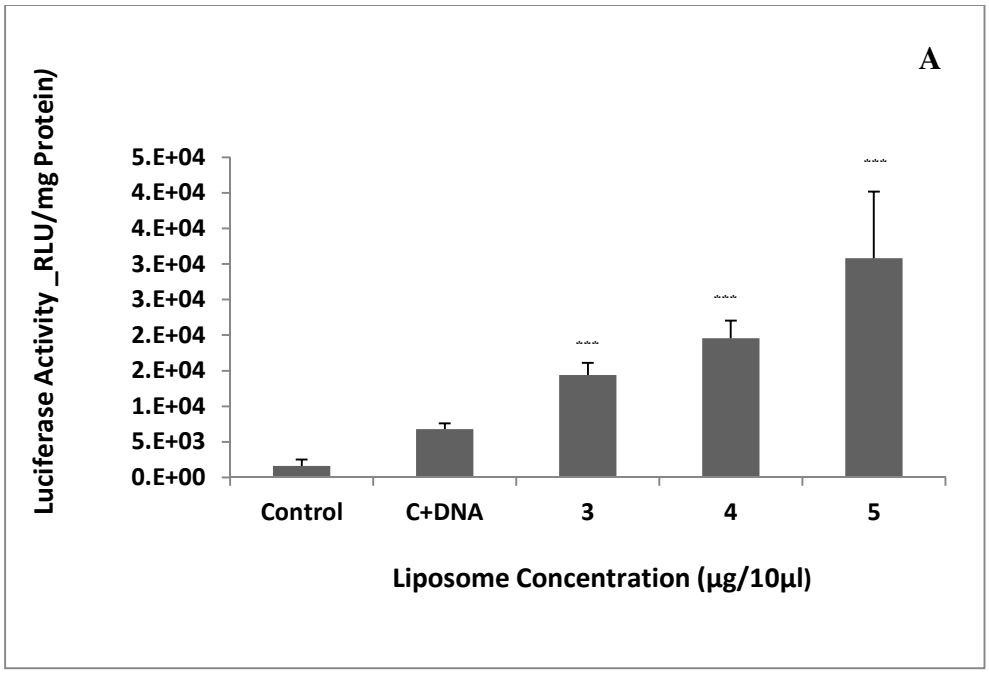
Figure 4.6: MTT cytotoxicity assay in HEK293 cells. Assay was conducted in a 48 well plate with 1 µg pCMV-Luc DNA/well and an increasing amount of the liposomal formulation, as indicated in each above. 2 mole % PEGylated (A) untargeted C2; (B) MS2; (C) NN2; (D) WT2. Results are presented as means ± S.D (n=3).

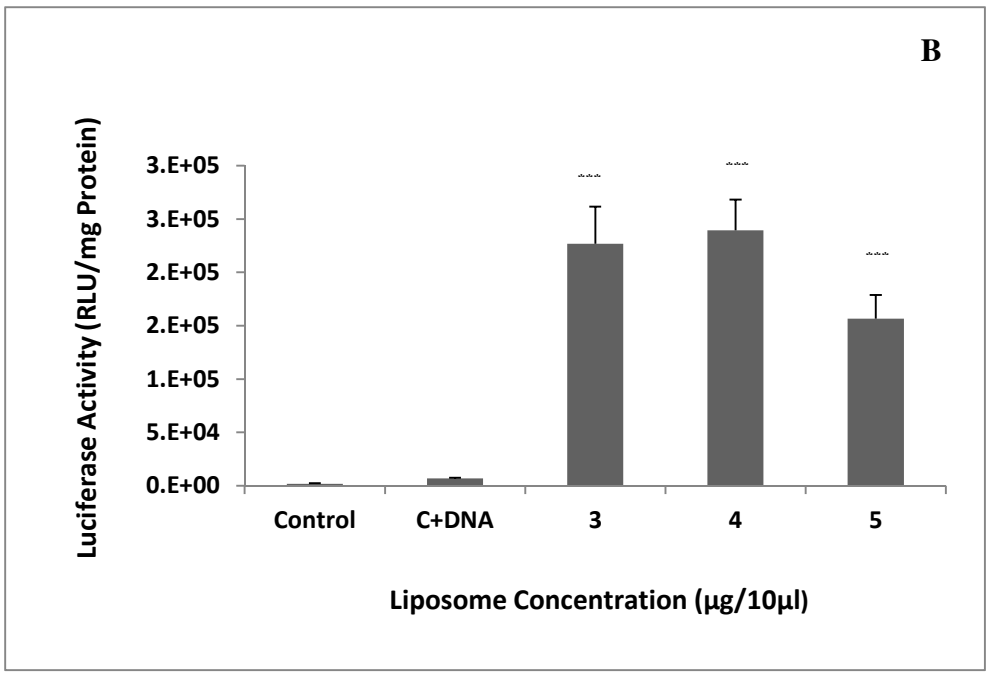
There are various factors affecting toxicity of lipoplexes, and these include the nature of the cationic lipid, the zeta potential, time of incubation, type of cell line and cell density (Dass *et al*., 2004). Is has been suggested that cell toxicity caused by cationic lipids is due to an increase in cell membrane permeability as well as the creation of transmembrane pores (Singh *et al*., 2006). Multivalent cationic compounds (such as 2,3-dioleoyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propaniminium trifluoroacetate) are known to induce toxicity since they form aggregates with intracellular organelles, resulting in cell death. Amino acid-based cationic lipids, in contrast, easily dissociate and are metabolized within the cell and thus are less toxic (Obata *et al.,* 2008).

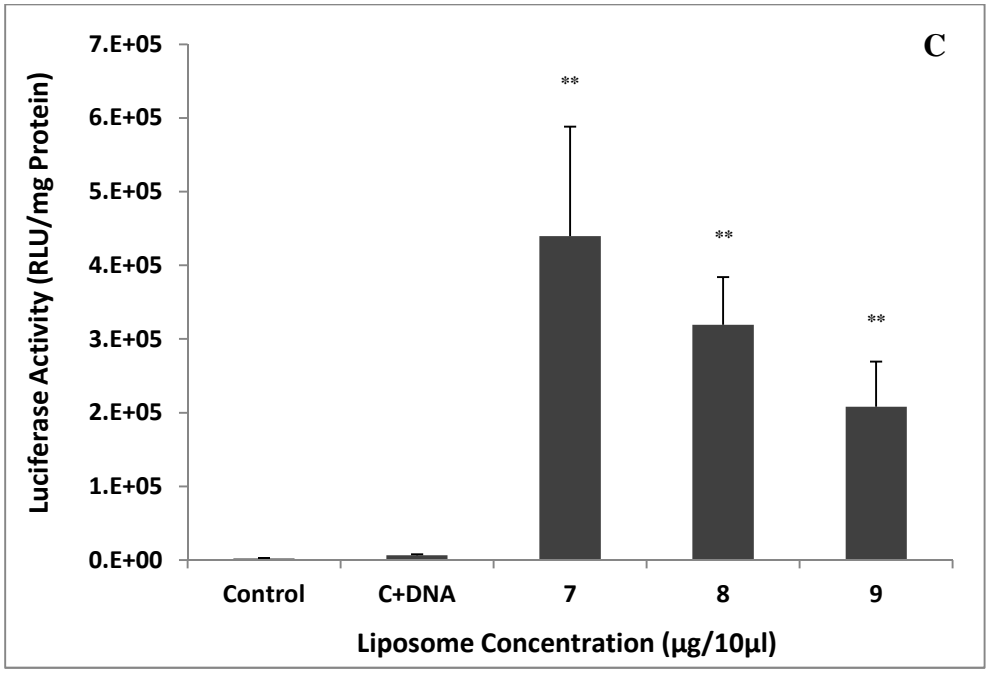
Maximum growth inhibition of untargeted non-PEGylated and PEGylated cationic liposomes in the HepG2 cell line was 12-27 % and 26-40 %, respectively (Figures 4.3-4.6). The untargeted formulations, C1 and C2, showed minimal toxicity with the untargeted, non-PEGylated complex, displaying a slight increase (1%) in cell survival. The receptor negative cell line displayed a similar degree of cell death with the respective liposomal formulations, with the untargeted PEGylated and non-PEGylated cationic liposomes exhibiting 15-38 % and 21-38 % cell death, respectively. In both the cell lines, the targeted PEGylated liposomal formulation showed a greater degree of cytotoxicity than their non-PEGylated counterparts. A recent study (He *et al*., 2009) on nanoparticles using the Chang cell line, established that an increase in the molecular weight of PEG is closely related to an increased in cell toxicity. Thus the findings in this study, with respect to increase toxicity in the PEGylated liposomes, can possibly be attributed to the presence of the PEG component in the formulation.

4.3.2. Luciferase Assay

In the presence of ATP and Mg^{2+}, the firefly luciferase produces light from the subtrate luciferin. The ATP dependent reaction is achieved by the oxidative decarboxylation of the beetle luciferin, resulting in the emission of light at a wavelength of 562 nm. Transient gene expression was measured with assemblies containing 3-11 µg/10 µl liposome, using the luciferase assay, in the HepG2 and HEK293 cell line.


A
Luciferase Activity _RLU/mg Protein)
5.E+04
4.E+04
4.E+04
3.E+04
3.E+04
2.E+04
2.E+04
1.E+04
5.E+03
0.E+00
Control
C+DNA
3
4
5
Liposome Concentration (µg/10µl)


B
Luciferase Activity (RLU/mg Protein)
3.E+05
3.E+05
2.E+05
2.E+05
1.E+05
5.E+04
0.E+00
Control
C+DNA
3
4
5
Liposome Concentration (µg/10µl)



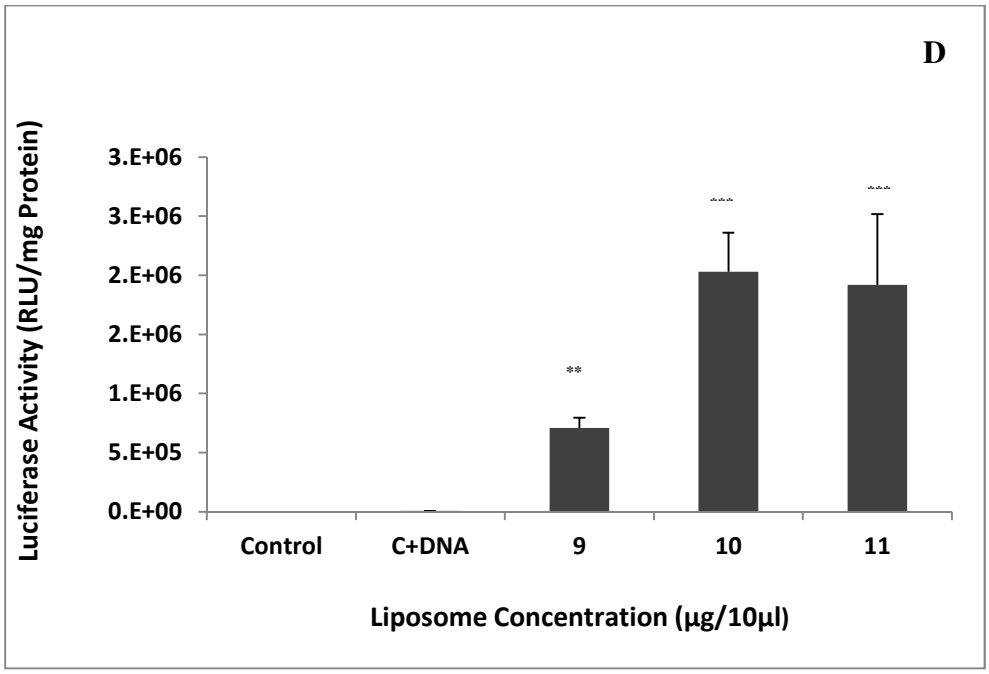
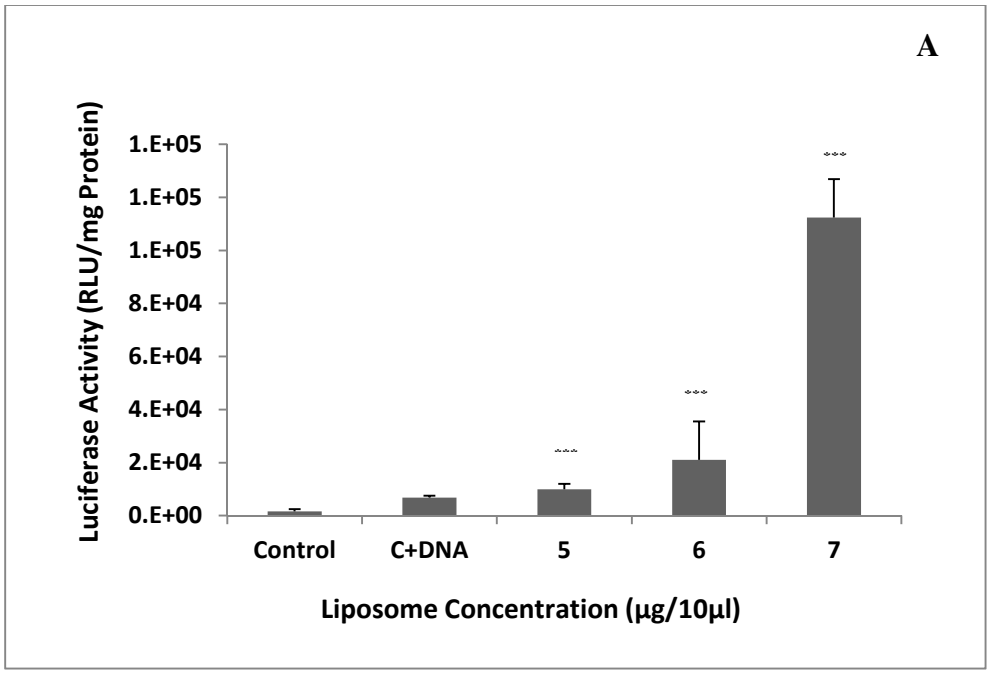
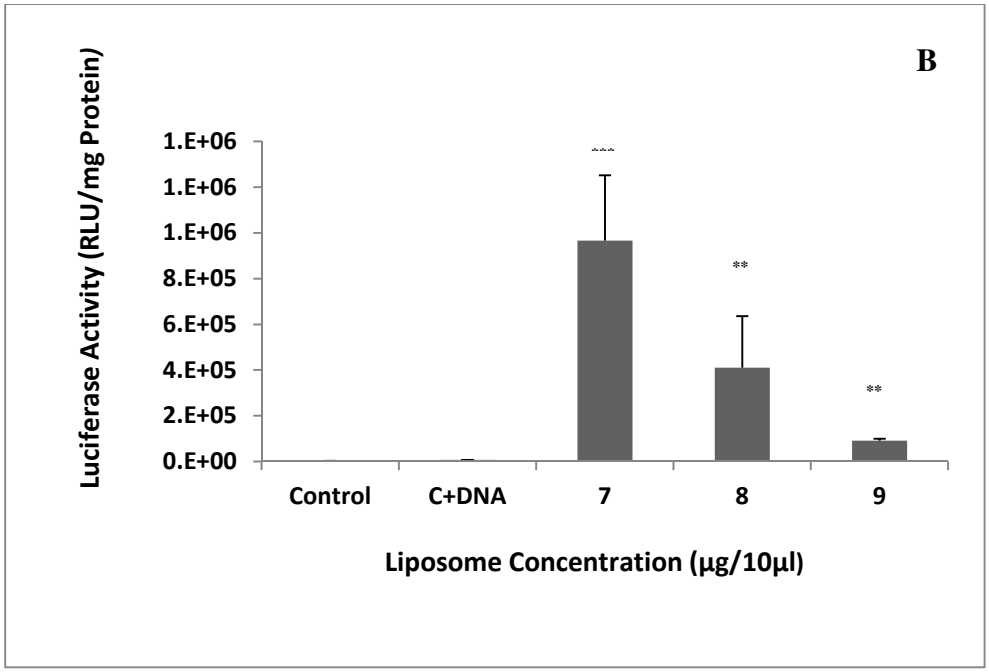
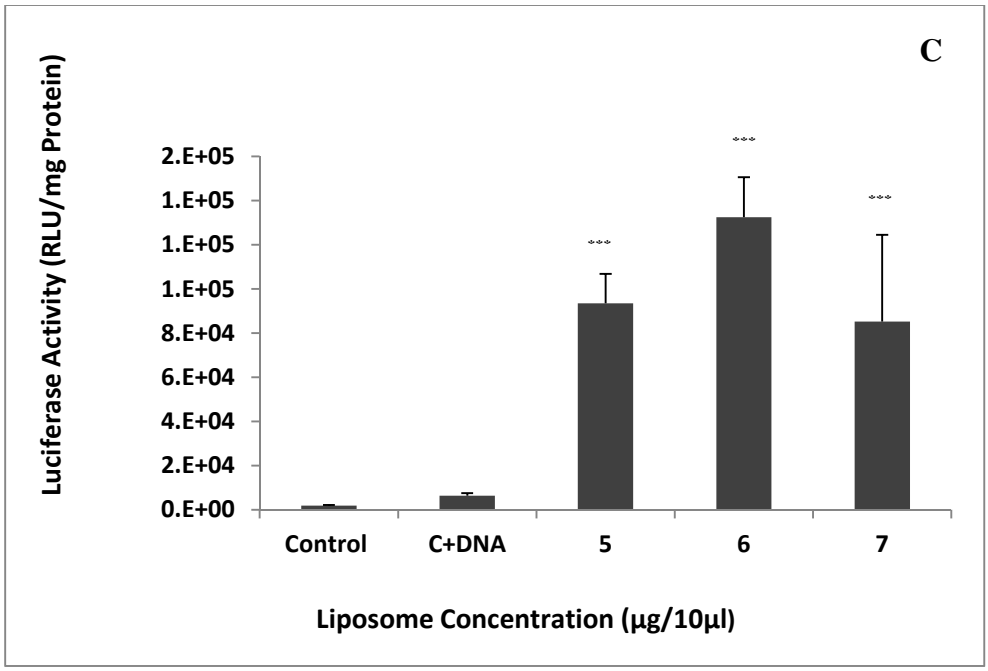


Figure 4.7: Luciferase transfection assay in the ASGP-R positive cell line, HepG2. Assay was conducted in a 48 well plate with 1 µg pCMV-Luc DNA/well and an increasing amount of liposome (as indicated above). (A) untargeted liposomal forumaltion, C1; non-PEGylated targeted formulations (B) MS1; (C) NN 1; (D) WT1. Results are presented as means ± S.D (n=3). Significant statistical difference of transfection between the lipoplexes and the control are reported as *p<0.05; **p<0.01; ***<0.001.


A
Luciferase Activity (RLU/mg Protein)
1.E+05
1.E+05
1.E+05
8.E+04
6.E+04
4.E+04
2.E+04
0.E+00
***
***
***
Control
C+DNA
5
6
7
Liposome Concentration (µg/10µl)


B
Luciferase Activity (RLU/mg Protein)
1.E+06
1.E+06
1.E+06
8.E+05
6.E+05
4.E+05
2.E+05
0.E+00
***
**
**
Control
C+DNA
7
8
9
Liposome Concentration (µg/10µl)



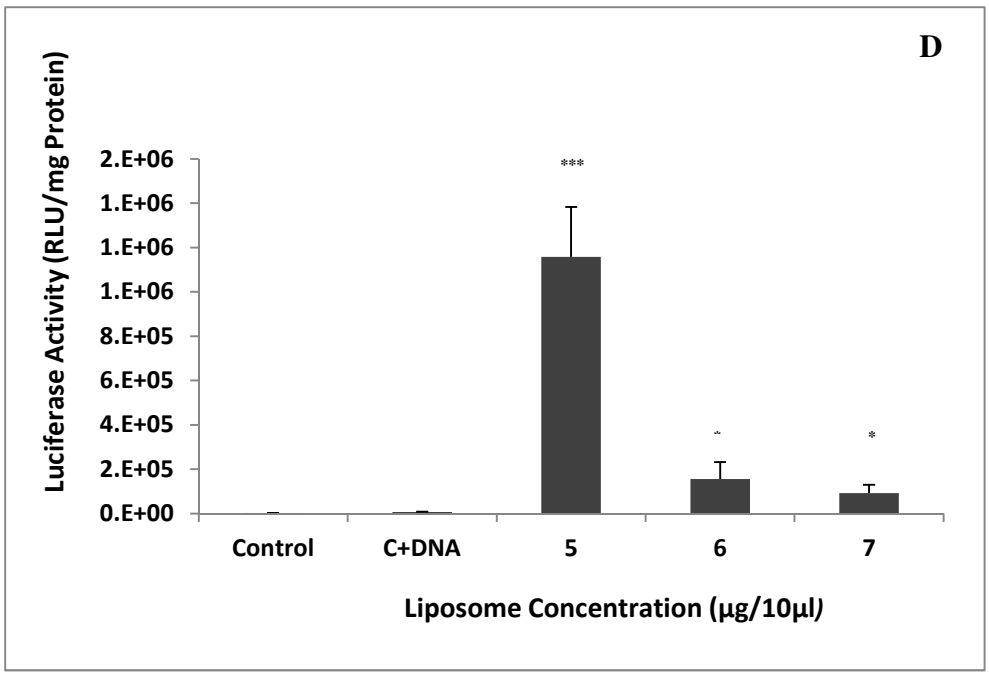
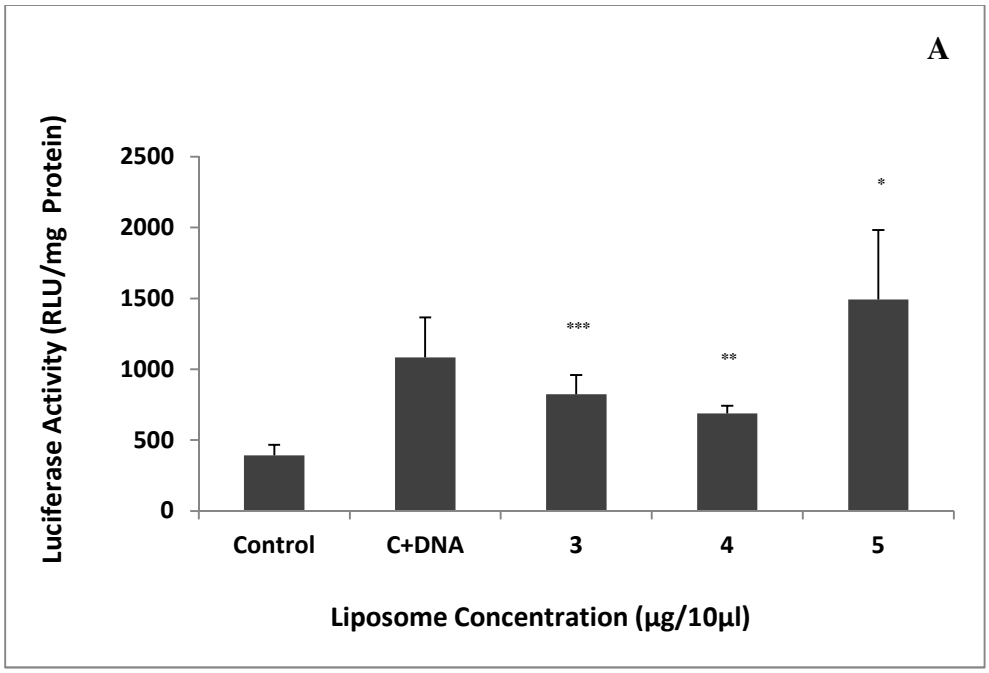
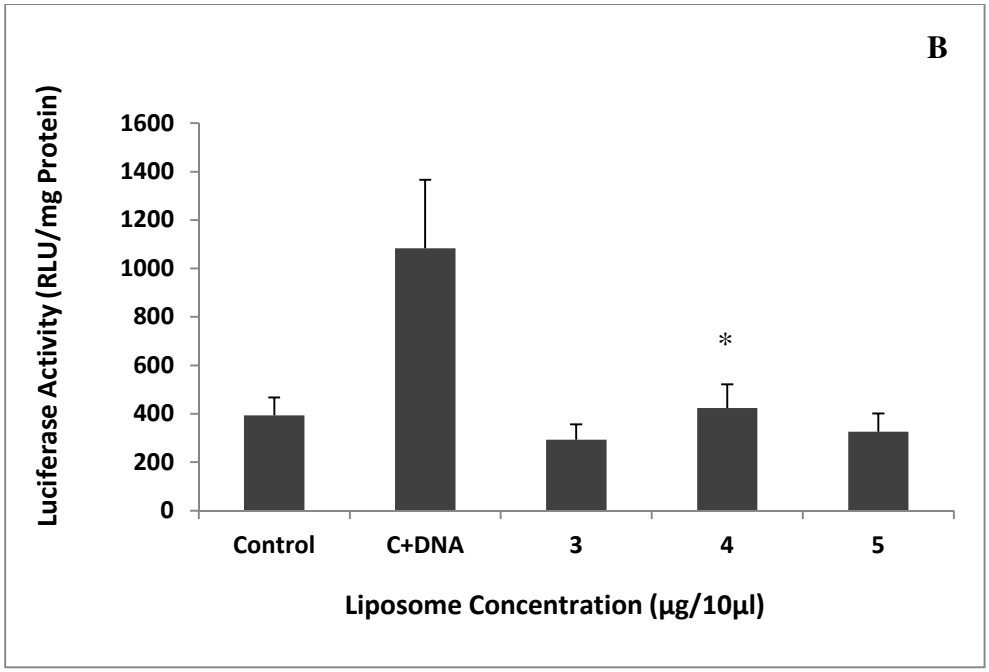
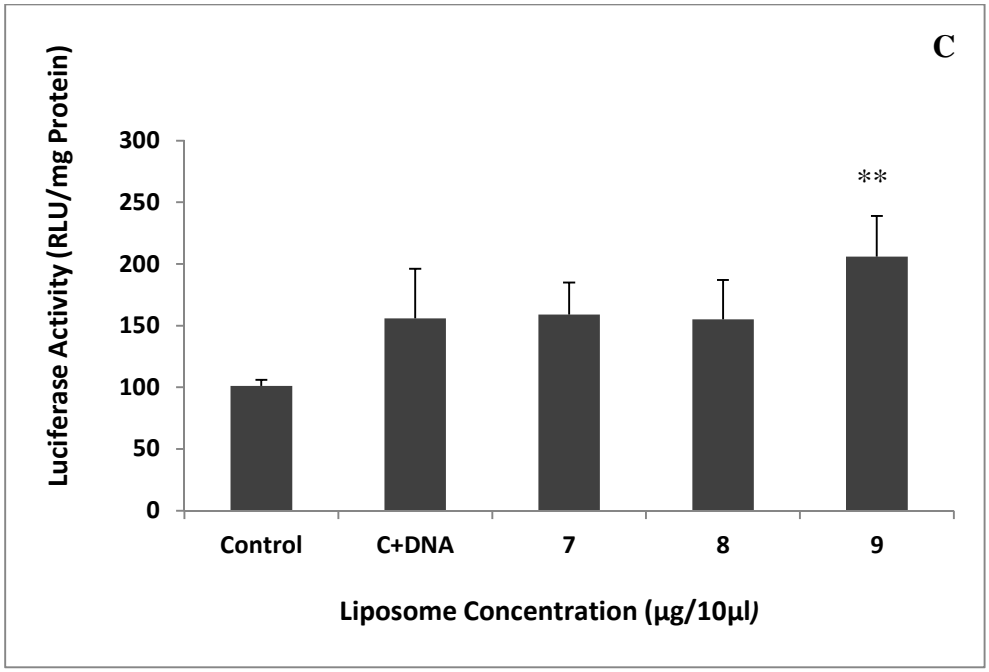


Figure 4.8: Luciferase transfection assay in the ASGP-R positive cell line, HepG2. Assay was conducted in a 48 well plate with 1 µg pCMV-Luc DNA/well and an increasing amount of liposome (as indicated above). 2 mole % PEGylated (A) untargeted C2; (B) MS2; (C) NN2; (D) WT2. Results are presented as means ± S.D (n=3). Significant statistical difference of transfection between the lipoplexes and the control are reported as *p<0.05; **p<0.01; ***<0.00


A
Luciferase Activity (RLU/mg Protein)
2500
2000
1500
1000
500
0
***
**
*
Control
C+DNA
3
4
5
Liposome Concentration (µg/10µl)


B
Luciferase Activity (RLU/mg Protein)
1600
1400
1200
1000
800
600
400
200
0
*
Control
C+DNA
3
4
5
Liposome Concentration (µg/10µl)



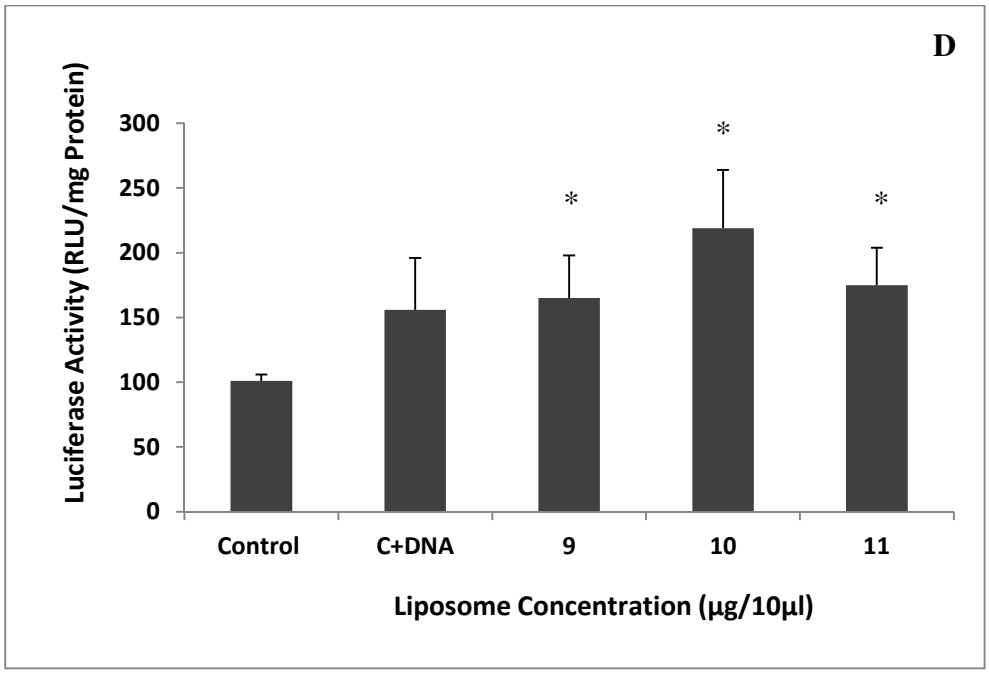
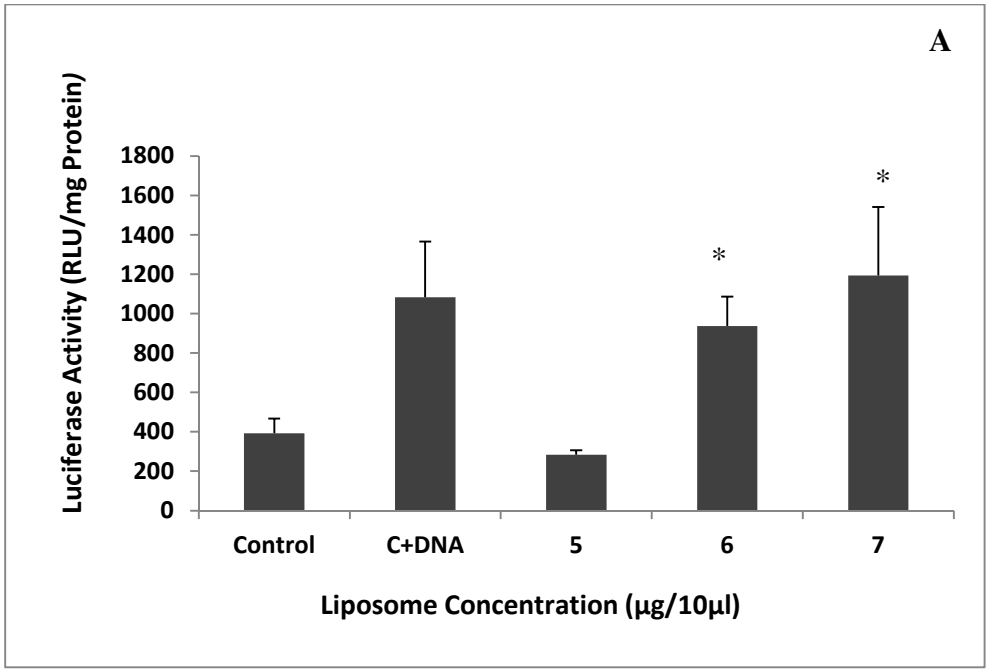
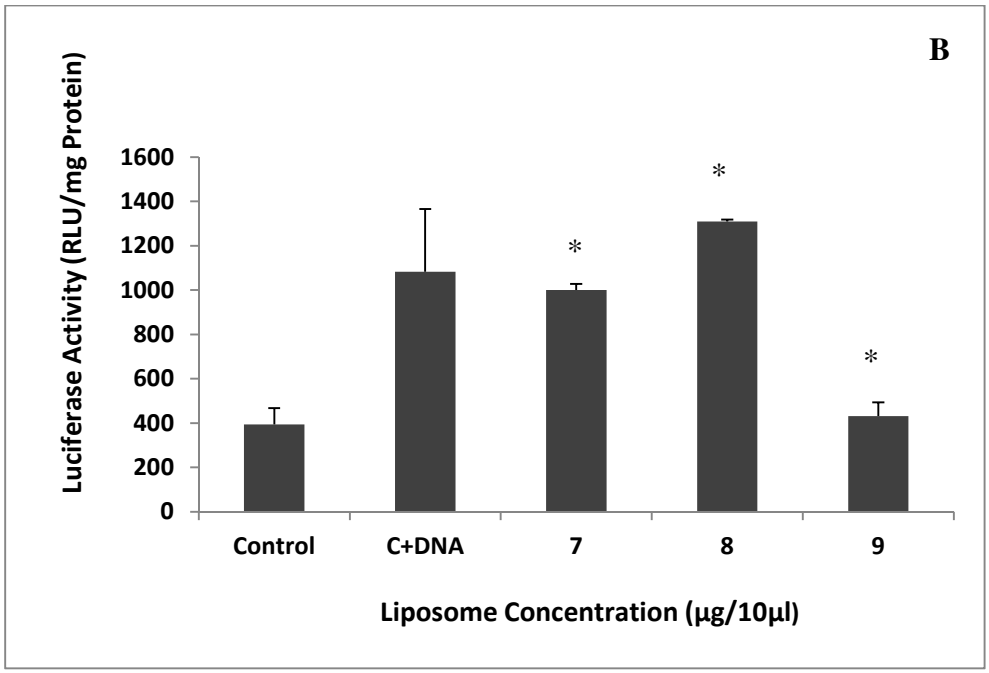
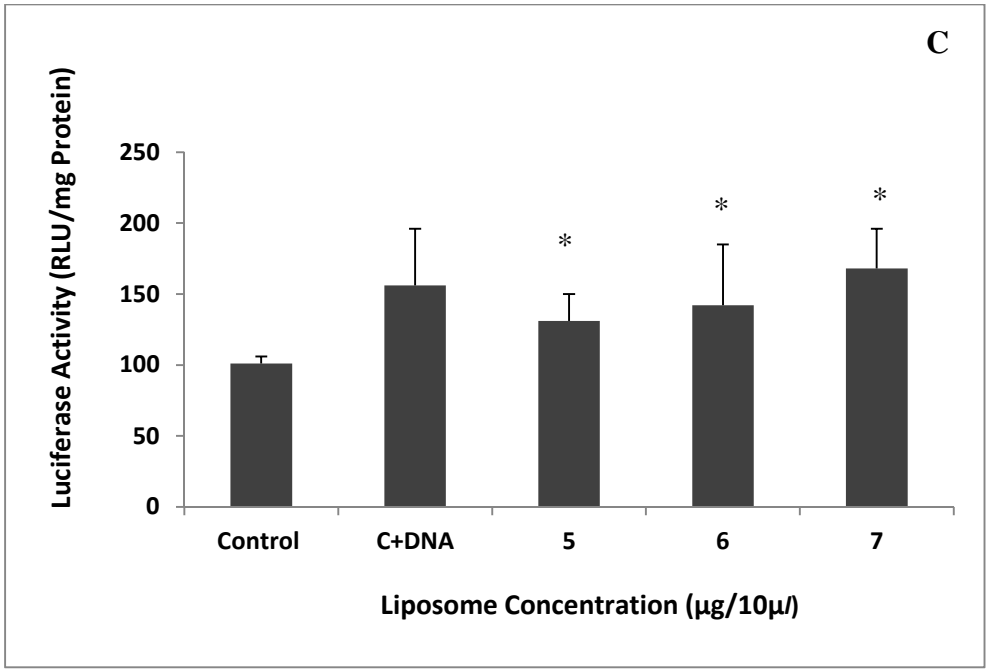


Figure 4.9: Luciferase transfection assay in the ASGP-R negative cell line, HEK293. Assay was conducted in a 48 well plate with 1 µg pCMV-Luc DNA/well and an increasing amount of liposome (as indicated above). (A) untargeted liposomal forumaltion, C1; non-PEGylated targeted formulations (B) MS1; (C) NN1; (D) WT1. Results are presented as means ± S.D (n=3). Significant statistical difference of transfection between the lipoplexes and the control are reported as *p<0.05; **p<0.01; ***<0.001.


A
Luciferase Activity (RLU/mg Protein)
1800
1600
1400
1200
1000
800
600
400
200
0
*
*
Control
C+DNA
5
6
7
Liposome Concentration (µg/10µl)


B
Luciferase Activity (RLU/mg Protein)
1600
1400
1200
1000
800
600
400
200
0
*
*
*
Control
C+DNA
7
8
9
Liposome Concentration (µg/10µl)



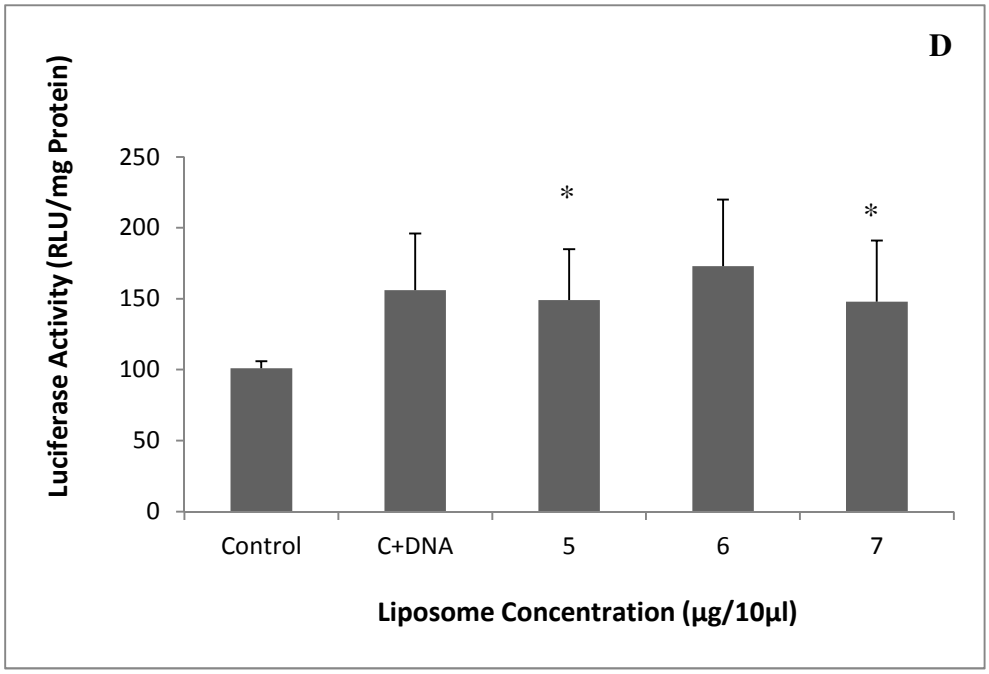


Figure 4.10: Luciferase transfection assay in the ASGP-R negative cell line, HEK293. Assay was conducted in a 48 well plate with 1 µg pCMV-Luc DNA/well and an increasing amount of liposome (as indicated above). 2 mole % PEGylated (A) untargeted C2; (B) MS2; (C) NN2; (D) WT2. Results are presented as means ± S.D (n=3). Significant statistical difference of transfection between the lipoplexes and the control are reported as *$p<0.05$.

Table 4.1: Summary of luciferase activity detected in each cell line for the respective liposomal formulations.

Lipoplex		Transfection (RLU/mg protein)	
Liposome formulation	**pCMV-Luc DNA: liposome (µg)**	**HepG2 Cell line**	**HEK293 Cell line**
C1	1:3	14413	824
	1:4	19565	689
	1:5	30800	1492
C2	1:5	10000	284
	1:6	21063	937
	1:7	112333	1193
MS1	1:3	226819	293
	1:4	239360	424
	1:5	156490	326
MS2	1:7	965666	1000
	1:8	409800	1310
	1:9	90467	431
NN1	1:7	439707	159
	1:8	319513	155
	1:9	208185	206
NN2	1:5	93460	131
	1:6	132531	142
	1:7	85293	168
WT1	1:9	707195	165
	1:10	2030200	219
	1:11	1919378	175
WT2	1:5	1157067	149
	1:6	155160	173
	1:7	91581	148

The galactosylated cationic liposomal formulations analysed in this study, showed varying degrees of transfection activity, when subjected to the luciferase assay (Figures 4.7-4.10). The transfection activities were measured in the galactose receptor positive cell line, HepG2 and the receptor negative cell line, HEK293. Results that were considered statistically significant, exhibited a p value below 0.05 (student t-test). Furthermore, each assay plate contained two controls, cells alone and cells with pDNA only. The two controls showed low luminescence levels in both of the cell lines. The control utilizing DNA and cells, showed a slight increase in luminescence when compared to the control, containing cells only ($p < 0.05$, both cell lines). This could be attributed to the fact that a small portion of naked DNA can gain access into the cell and be expressed.

When compared to the targeted liposomal formulations, untargeted non-PEGylated and PEGylated liposomes, exhibited the lowest transfection activity (Table 4.1). Furthermore, there existed a distinct difference in the transfection activity between the two cell lines utilized, using targeted liposomal formulations. Luciferase activity in the HepG2 cell line showed an greater increase when compared to the activity measured in the receptor negative cell line. This clearly indicates that liposomes containing galactose (10 mole %), was successful in specifically the targeting hepatocytes, *in vitro*. The highest transfection activity observed, in the HepG2 cells, was obtained with the WT1 liposomal formulation (2030200 RLU/ mg protein). This was followed by its PEGylated counterpart, WT2 (1157076 RLU/ mg protein). PEGylated liposomal formulations have been known to inhibit the transfer of DNA. It has been suggested that the presence of PEGylated chains restricts the close contact between the endosomal membrane and the lipids of the PEGylated liposomal complex. Therefore, DOPE is unable to destabilize the endosomal membrane and release the pDNA. As a result, the DNA is entrapped in the endosome while it fuses with the lysosome and is degraded. This prevents the effective delivery of the pDNA to the nucleus and thus can be attributed to the reduced transfection activity measure (Song *et al*., 2002).

The liposomal formulation containing the cytofectin WT was chemicaly synthesized through a process called “click chemistry”. The success of a liposome to target a specific cell is dependent on the biochemical and physiochemical properties of the formulation (Kawakami *et al*., 2001).

Attachment of the targeting ligand and the cholesteryl anchor (MSO9) has been achieved by two distinct linkages; the carbamoyl link (NN and WT) and the glycosidic linkage as observed in the MS formulations. The distance between C1' (anomeric carbon) of the pyranose ring and the C3 of cholesterol (considered the anchor point) have been assessed for all three formulations. The length of the spacer varies from 2.42 to 10.1 Å (Figures 2.7, 2.8 and 2.9). An important feature of the WT formulation is the increased spacer length (Figure 2.8) which exists between the galacto moiety and cholesteryl anchor thus displaying the sugar moeity more prominently therefore, allowing the nanosystem to gain access and bind the receptors comfortably. This is consistent with earlier findings (Kawakami *et al*., 1998; Singh and Ariatti, 2006) that an increase in the spacer length mediates higher transfection activity.

When results were compared to the binding ratios, it was noted that the formulations MS1, NN2 and WT1 displayed the highest luciferase activity at their optimum binding ratios. In contrast, formulations MS2, NN1 and WT2 performed best at their sub-optimum binding ratio. In the HEK293 cell line, it was observed that both the non-PEGylated and PEGylated formulations of MS and WT exhibited high transfection activity at their optimum binding ratios, whilst the NN formulation showed highest transfection at its supra optimum binding ratio. The possible explanation for the highest transfection activity achieved for each formualtion at varying ratios, could be associated with lipoplex size differences at different DNA:liposome ratios (Higuchi *et al*., 2006). All formulations were considered statistically significant from the control (cells only. The results obtained in this study clearly show that the transfection activity of the non-PEGylated complexes is directly proportional to an increase in C1-C3 spacer length. An increase in the length of spacer can increase the probability of the lipoplexes and their contents being taken up by the cell (Davis and Robinson, 2002).

4.3.3. Competition Assay

Specific targeting of lipoplexes to hepatocytes can be achieved via receptor mediated endocytosis (ligand-receptor interactions). The process aims to utilize the abundantly expressed asialoglycoprotein receptor which is unique to hepatocytes, in an attempt to internalize corrective DNA. The asialofectuin (AF) is a glycoprotein that possesses a

triantennary sugar chain with galactose terminals and is known to be the natural ligand which is selectively recognized by the ASGP-R (Arangoa *et al*., 2003).

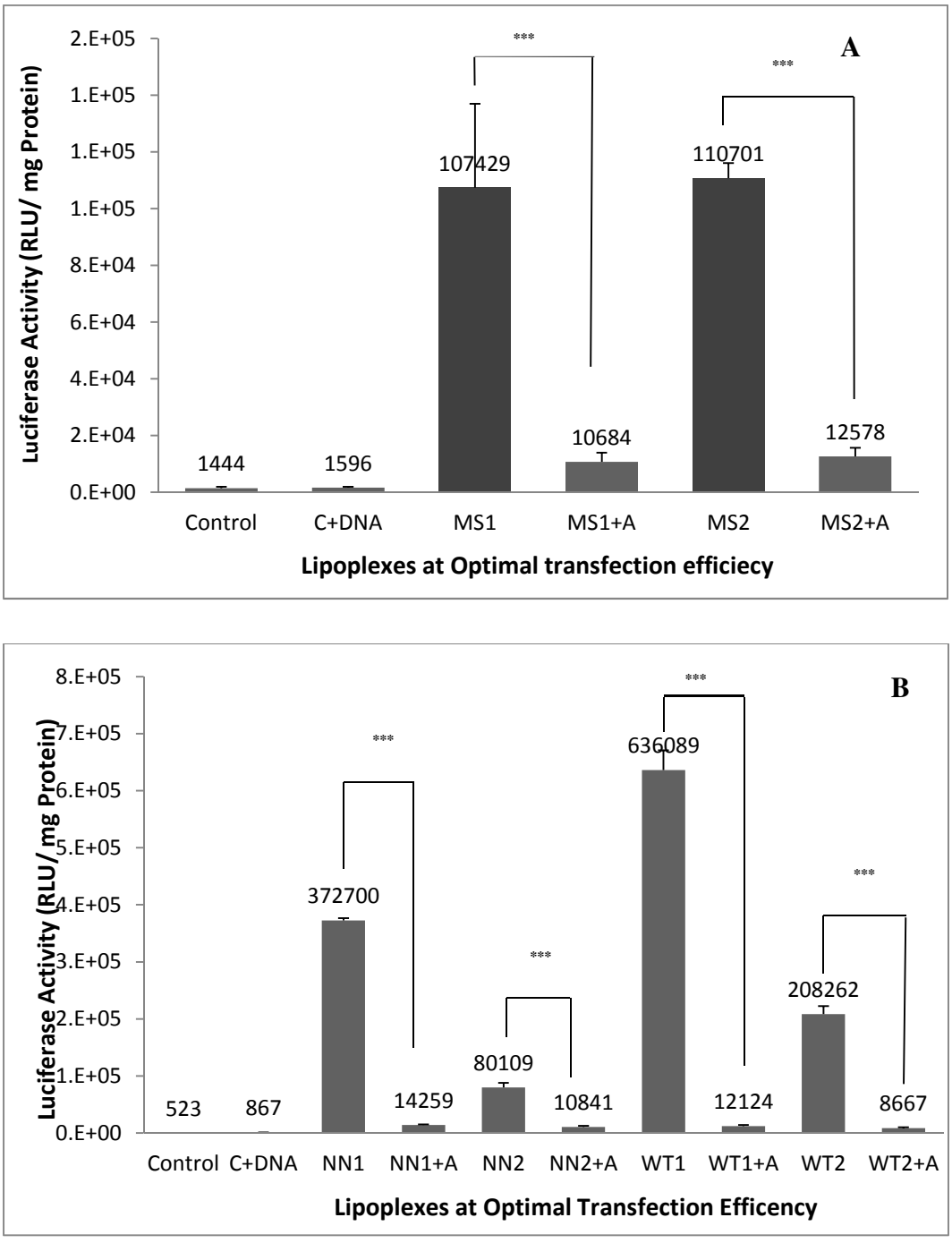


Figure 4.11: Competition assay with Control (cells only), C+DNA (cells plus naked pCMV DNA) and lipoplexes (A) MS1; MS2 and (B) NN1; NN2; WT1; WT2 without the ASGP-R natural ligand or lipoplexes with the natural ligand Asialofetuin in HepG2 cells. Assay was conducted in a 48 well plate with 1 µg pCMV-Luc DNA and an increasing amount of liposome (as indicated above). Asialofetuin concentration 25 µl per well (Stock of 10 µg/ µl). Results are represented as means ± S.D. (n=3). Significant statistical difference between transfection values in the presence or absence of competing ligand are reported as ***<0.001.

The competition assay utilizes free asialofetuin to assess and confirm the uptake of complexes by the ASGP-R. The free asialofetuin ligands in the test wells are bound and taken up by the receptors present on the HepG2 cell. This limits the percentage of ASGP-receptors available for the binding of the galactosylated liposomal formulations. The cells treated with the ligand asialofetuin prior to the addition of the galactosylated liposomal formulations, showed a marked and very significant ($p < 0.001$) decrease in transgene expression (Figure 4.11). This strongly suggests that lipoplexes are taken up specifically and almost exclusively by ASGP-R (Arangoa *et al* 2003).

CHAPTER FIVE

IN VIVO ANALYSIS OF A CATIONIC LIPOSOMAL FORMULATION SHOWN TO MEDIATE EFFICIENT TRANSFECTION *IN VITRO*

5.1. INTRODUCTION

For efficient delivery of a therapeutic gene it is necessary to develop a vector that has the ability to be used successfully both *in vitro* and *in vivo*. Thus once the three cationic galactosylated liposomal formulations and their PEGylated counterparts were analysed *in vitro,* the most efficient formulation/s were subjected to *in vivo* studies. Most vectors developed thus far are aimed at increasing exogenous gene expression but many are only evaluated *in vitro,* using cultured cells. This is particularly problematic, since several vectors/formulations that were previously shown to be efficient *in vitro* do not exhibit high exogenous gene expression *in vivo* (Fortes and Razquin, 2009).

Cationic liposomes are one of the most popular non-viral vectors used in gene therapy. These nanosystems exhibit desirable features such as, a net overall positive charge that allows for the binding to negatively charged DNA to the cell surface, for adequate internalization and potential transcription of the genes. Within the cell, the liposome also offers protection to the DNA from en-route DNases. The liposomal formulations that were subjected to *in vivo* analyses in this study displayed the following characteristics:

i. a cholesterol lipid anchor that has low toxicity and promotes high gene expression,
ii. DOPE as a co-lipid, for the initiation of endosomal fusion and enhancement of DNA release from the primary endosome to evade chemical degradation,
iii. the sugar moiety, galactose for specific targeting to the liver hepatocytes via receptor mediated endocytosis (ASGP-R)
iv. an increased spacer length in the spacer element resulted in increased extension of the galactopyranose sugar from the liposome surface to facilitate ASGP-receptor recognition

v. PEGylation to provide a steric barrier to the vector and cargo (DNA) which will inhibit opsonisation.

These ideal characteristics found in these formulations predict that they may have the ability and potential to achieve *in vivo* delivery. However, there are many parameters underlining successful delivery of therapeutic gene, hence making it unfeasible to expect a parallel relationship to exist between the chemical features of a delivery vector and the resulting transfection activity (Bhattacharya and Baja, 2009). With the synthetic vector facing various obstacles, achieving high levels of therapeutic gene transfection, *in vivo*, is a major challenge.

As mentioned previously, the complex must first gain access into the cell. In this study we have achieved cell specific targeting through the ASGP-receptor that internalizes the galactosylated vector carrying the DNA, facilitating expression of the transgene. The density of the galactose sugar on the surface of the liposome plays a crucial role in ASGP-R mediated uptake. The effect of galactose density was investigated by Managit and co-workers (2005). The group synthesized cholesten-5-yloxy-N-(4-(1-imino-2-D-thiogalactosylethyl)formamide (Gal-C4-Chol) and formulated it into liposomes at different molar ratios and tested the effect of galactose density on gene expression, *in vivo* via intravenous injection. The study showed that liposomes galactosylated with Gal-C4-Chol (3.5 %, 5 %, and 7.5 %), rapidly disappeared from the blood stream and accumulated in the liver. The approximate amount of the liposomal formulation to have gained access to the liver was calculated to be 80% of the initial dose injected into the animal. Liposomal formulations containing a lower percentage of the Gal-C4-Chol (1 % and 2.5 %) showed liver accumulation to a lower extent when compared to the liposomal formulation containing a higher percentage of the Gal-C4-Chol, and a relatively small difference to the control liposomes containing no Gal-C4-Chol. The most promising outcome of the above mentioned study was the establishment of the most promising formulation, in terms of the galactose density. The group clearly showed that liposomal formulations containing 5 % Gal-C4-Chol, were most efficient in transfection, since these liposomes were taken up rapidly by hepatocytes. In the present study, liposomes formulated with galactosylated cholesteryl derivatives at the 10 mole % level, promoted very efficient transfection of HepG2 cells *in vitro*. Moreover, lipoplex cell entry was shown, in a competition assay, to be largely by ASGP-R-mediation.

Following cell surface internalization, the next crucial step is the endosomal release of the cargo DNA from the lipoplex, to prevent lysosomal degradation. The incorporation of DOPE in the formulations, results in endosome disruptions accomplished by enhanced membrane fusion. This results in a flip-flop of the anionic endosomal monolayer and interaction with the cationic liposome. The DNA disassociates itself from the liposome due to the neutralization of liposomal charges by interaction between the collapsed membrane and the lipid (Abbott *et al*., 2005). Only if the DNA evades degradation by serum nucleases, will the genetic material reach its target cells and finally the nucleus where it will undergo transcription. The incorporation of 2 % DSPE-PEG_{2000} into the formulations tested affords protection to the lipoplexes from the serum nucleases. Key issues facing *in vivo* liposomal delivery are induced cytotoxicity, rapid blood clearance, and inability of targeting specific cells.

Administration of therapeutic exogenous genes can be accomplished via several different delivery routes. The abdominal cavity and the peripheral veins can be used as potential routes for the delivery of genetic material (Baumhofer *et al*., 1998 and Biewenga *et al*., 1995). Depending on the application, the administration routes employed in previous studies include; intravenous, intra-arterial, intratumoral, intramuscular, intraperitoneal, intrasplenic, intradermal, intratracheal, mucosal (nasal, rectal or vaginal), subcutaneous, subretinal and transdermal (Figure 5.1). Intravenous injection has been regarded as the “holy grail” for the delivery of therapeutic DNA (Templeton, 2001). Studies involving Glyco-poly-L-lysine, showed that liver targeting can be achieved *in vivo*, by using either intraperitoneal or intravenous administration (Yang *et al*., 2000). However, the intravenous route of administration results in higher transfection activity than the intraperitoneal administration and thus this method of gene delivery to the liver being more favourable was employed for this study.

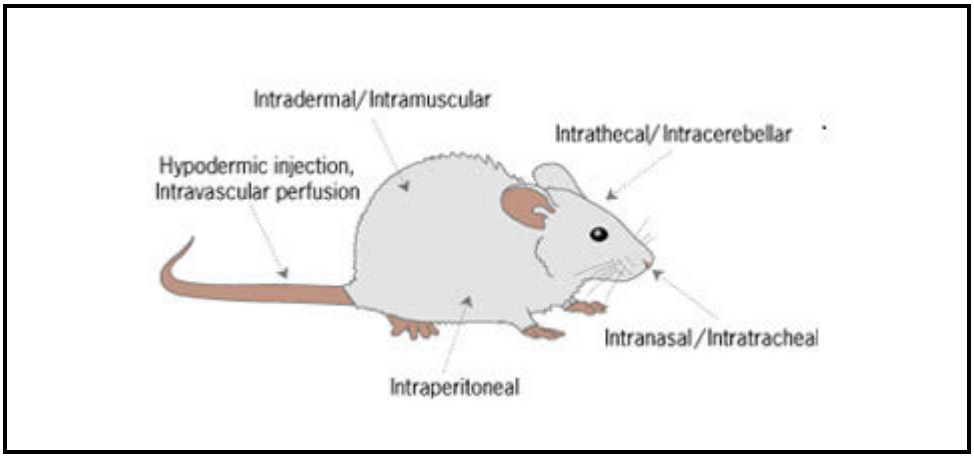


Figure 5.1: A representation of some of the routes of administration employed in gene delivery (http://www.initrogen.com/site/us/en/home/Reference/Ambion-Tech-Support/rnai-sirna/tech-notes/performing-rnai-experiments-in-animals.html).

Functional properties previously defined using *in vitro* analyses techniques, do not measure or predict the resulting stability of lipoplexes, the biodistribution and pharmacokinetics *in vivo,* of which all are key aspects for successful gene transfection. Seven crucial points have been highlighted for the optimization of therapeutic gene delivery and it has been suggested that these points be investigated in detail prior to initiation of animal studies. The seven key issues include (but are not limited to) the plasmid DNA design and its subsequent preparation method and resulting purity, the formulation of the delivery vector to be used, the route of gene/drug delivery, the dose to be administered, the administration schedule (incubation time) and the method used for the detection of exogenous gene expression or the transfection activity (Templeton, 2001).

In this case study three distinct, galactosylated cationic liposomal formulations were compared for their effectiveness in gene delivery, *in vitro*. As mentioned previously the three formulations were targeted specifically to the liver hepatocytes via the ASGP-R. Both pegylated and non pegylated galactosylated cationic liposomes were investigated, *in vitro*, in the human carcinoma cell lines, HepG2 and HEK293 (Chapter 3). The three unique galactose targeted liposomes prepared are: cholesteryl-3β-N – (4-aminophenyl-β-D-galactopyranosyl) carbamate; cholesteryl (1-β-D-galactopyranosyl-1,2,3triazol-4-yl)carbonate; cholesteryl-β-D-galactopyranoside. Untargeted, PEGylated and non PEGylated liposomes were also formulated and investigated for comparison.

From the results gained *in vitro*, the liposomal formulations WT1 and WT2 displayed the highest luciferase activity. The two formulations were therefore assessed for their ability to deliver corrective DNA to human cells, *in vivo* following injection via the tail vein into rats. The necessary organs were harvested using the method of Kawakami *et al*. (2000), with mild modifications (anesthesia of animals with Halothane). The *in vivo* experimentations were approved, by the University of KwaZulu-Natal Ethic Committee prior to initiation of the animal testing (approval number: 076/11/Animal-See Appendix 3).

5.2. METHODS AND MATERIALS

5.2.1. Materials

All chemicals utilized were of analytical grade. A total of 16 male Sprague Dawley rats (230-250 g) were obtained and harvested at the University of KwaZulu-Natal Biomedical Research unit (BRU).

5.2.2. Methods

5.2.2.1. Animal Maintenance

All animals were allowed free access to food and water. Cages housing the animals were kept clean, with the saw dust being changed once every two days. The rats were monitored twice a day, following the administration of test formulations.

5.2.2.2. Preparation of test formulations

Lipoplexes were prepared in accordance to Table 5.1 below, under aseptic conditions. The total volume of the formulations assessed, were brought up to 300 µl using the Sabex saline

solution. This study employed two controls, i.e. animals not treated with any of the formulations and animals treated with naked pCMV-Luc DNA.

Table 5.1: Preparation of different formulations, analysed *in vivo*.

Formulation Prepared	**Stock Liposomal Formulation** (µg/ µl)	**Liposome** (µl)	**pGL3** (µg)
Control 1	-	-	-
Control 2	20.00	-	20
C2 (MSO9:DOPE)	2.75	29.0	20
WT1 (MSO9:DOPE:GAL)	2.75	72.7	20
WT2 (MSO9:DOPE:GAL:PEG $_{2000}$)	3.08	39.0	20

5.2.2.3. *In vivo* Transfection

The method used in this study was adapted, from Kawakami *et al*., (2000). Animals were selected according to weight, sex and species (230-250 g, male, Sprague Dawley rats). A total of 4 rats per test group (outlined in Figure 5.2) were separated and labelled appropriately. Two controls were employed in this study. Control 1, were untreated animals that received no test formulation. The control 2 group were animals receiving only pCMV-Luc DNA. Control 2 and lipoplexes C1, WT1 and WT2 were prepared in accordance to Table 5.1 and incubated for 30 minutes. The rats (maximum of 2 animals at a time) were then placed in a heating chamber (chamber heated by a light bulb) for 10-15 minutes. This was conducted to allow for the dilation of the tail vein, to assist in a quick and proper administration of the test formulations. After the vein dilated and was visible to the naked eye, the animal was gently restrained by picking up the rat with the palm of the hand placed on the back of the animal. Upon placing of the hand on the animal, the upper thoracic and neck region was grasped between the thumb and index finger. The other hand was used to gently support the animal and hold the tail whilst the formulations were administered.

The tail vein was located, disinfected with hibitane and using a 23 gauge needle, the test formulations were carefully injected into the tail vein on the animal. The animals were taken to the autopsy room and were subjected to euthanasia, 5 hours after administration of the formulations. A cotton gauze was soaked with anaesthetic (halothane) and placed at the bottom of a bell jar. The gauze was then covered with a wire mesh to ensure that the animal did not come into contact with the anaesthetic. The animal was carefully placed into the jar and covered. After the animal's respiration stopped, the rat was removed from the jar and the pulse was checked to ensure that the animal was dead. The rat was then dissected and the required organs were harvested and placed (separately) in beakers containing ice cold Sabex saline solution. All tissues were placed on ice and transported to the laboratory for further analyses. Animal remains were incinerated by the BRU. Organs were washed with the Sabex solution, dried gently and weighed. Each organ was cut into tiny pieces and subjected to homogenization (Dounce homogenizer) following the addition of 5 µl/ mg rat tissue lysis buffer (2 mM EDTA, 0.1 M Tris-HCl, 0.1 % Triton X-100). The homogenate was then centrifuged at 4000 rpm for 10 minutes, at 4 ˚C (Figure 5.2).

The lysate (20 µl) was then added to the luciferase assay reagent (100 µl), (luciferase assay protocol, outlined in Chapter 4, section, 4.4.1). This was mixed immediately and placed in the Lumac Biocounter 1500 luminometer. The cell free extract was also subjected to the bicinchoninic acid (BSA) assay. Each sample was measured in triplicate.

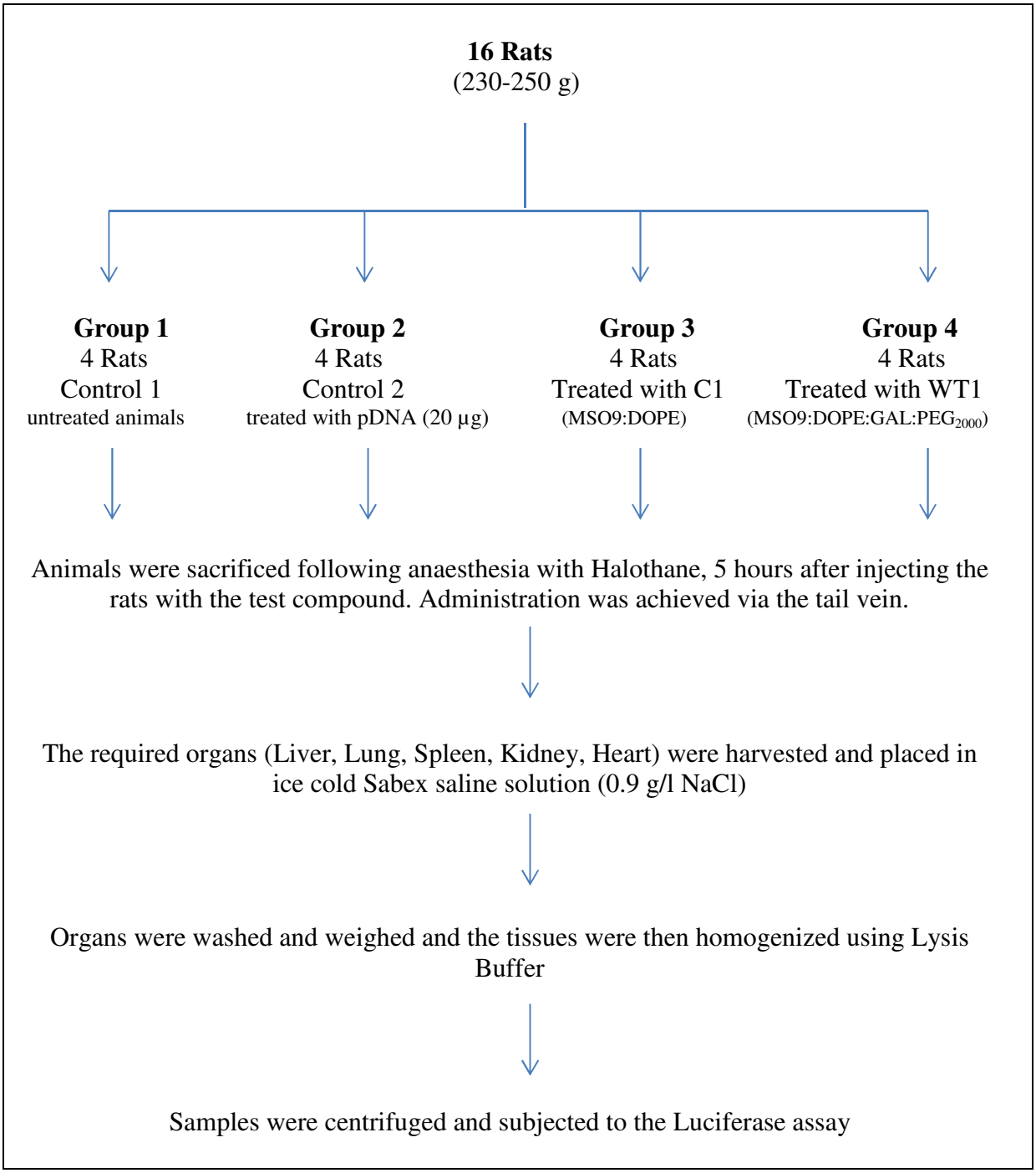


Figure 5.2: Flowchart indicating the precise steps undertaken for the *in vivo* analysis.

5.3. RESULTS AND DISCUSSION

All animals used in the study showed no visible side effects or signs that the formulations were toxic. It is also noteworthy that no deaths were reported during the study prior to sacrifice. There were high expectations for high transfection in the WT1 and WT2 formulation since both formulations displayed the key characteristics (cholesterol lipid, DOPE as a co-lipid, Gal targeting ligand, and PEGylation) required for high transfection activity. However, after 5 attempts, both formulations showed no increased transfection activity *in vivo*, when compared to the controls. Results obtained from a representative experiment are shown below in Table 5.2.

Table 5.2: Transfection activity for the various organs, after *in vivo* gene expression. Results are measured against the two controls.

Organ	**Measured Luciferase Activity (RLU/ mg protein)**				
	Control 1	**Control 2**	**C1**	**WT1**	**WT2**
Lung	140	143	141	151	143
Liver	142	141	145	145	140
Kidney	136	133	132	140	130
Spleen	111	98	117	123	113
Heart	68	66	68	71	60

The method as outlined in Section 5.2.2.3 was the first attempt to analyse gene delivery *in vivo* on the UKZN Westville campus. The results portrayed above are obtained from this experimental attempt. Since no distinct increase in the transgene expression was noted, the current protocol was modified several times, with each attempt generating similar if not the same results. In the second attempt the homogeniser was switched from a Dounce to the Ultra Turrax. The Ultra Turrax allows for the increased and quick homogenization of tissue material. This rapid mechanical homogenizer was particularly effective in tissues such as the

heart and lung that were possibly not homogenized sufficiently in the first attempt (Dounce). In this attempt we assumed that the high speed homogenizer would shred the tissue sufficiently, releasing the soluble cellular material that may be taken for the luciferase assay. In the third attempt 3 freeze-thaw cycles were added to the method. Again, this was carried out to increase membrane disruption and allow the cellular contents to be released.

In the fourth attempt, the incubation time was altered from 5 hours to 24 hours. Animals were intravenously injected with the test formulations and were only dissected 24 hours later. It was reasoned that the added time would ensure hepatocyte accumulation of lipoplexes and promote higher transgene activity. In the final attempt the amount of pCMV-Luc DNA administered was increased. The DNA concentration was increased from 20 µg to 30 µg with the total volume being brought up to 400 µl. Although there was an increase in the total volume only 300 µl of the solution was injected into the animal due to weight/volume restrictions.

In all five attempts, the liposomal formulations show no significant transfection of exogenous DNA *in vivo*. The expression of pDNA *in vivo* is restricted by the cell's natural defence mechanisms, developed to prevent expression of toxic factors. Studies on the effects of the incubation period of lipoplexes on gene transfer revealed that lipoplexes that were prepared within an hour of incubation proved more effective in gene transfection than complexes prepared in 5 hour incubations (Zhang *et al*., 2003). It is possible that the lipoplexes prepared could have been rapidly cleared from the blood and excreted from the animal thus resulting in no significant expression of the luciferase gene.

Little or no gene expression *in vivo* could also be attributed to various factors including poor preparation techniques used during preparation of the pDNA, the sub-optimal promoter-enhancers present in the pCMV-Luc DNA, may not be optimal for the animal models chosen detection and measurement of transfection activity (gene expression). Furthermore, intravenously administered liposomes lose their ability to bind and carry the DNA in the presence of serum (> 10 % serum). The negatively charged plasma serum proteins bind to the cationic liposome: DNA complex and destabilizes and inactivates the lipoplex. This results in the aggregation of the lipoplex which either precipitates out the complex or causes the complex to disintegrate, thus releasing the corrective DNA well before it can reach its target

site. Hence serum proteins have led to the reduction or inefficiency of corrective DNA delivery (Li *et al*., 2011).

Initially it was postulated that PEGylation could reduce the probability of the liposome being taken up by the macrophages which in turn extends the circulation time and decreases initiation of inflammatory responses. However, it has also been shown that PEG in fact interferes with and inhibits the endosomal release of the DNA. The incorporation of 5 mole % PEG into the liposomal formulation has been shown to inhibit the disruption of the endosomal membrane and the subsequent release of the DNA (Song *et al*., 2002). This correlates with earlier findings by Mori et al., (1998) that established that the endosomal rupture was inhibited by PEGylated liposomes. Furthermore, materials are cleared from circulation, according to the size. Particles within 0-30 nm are cleared rapidly by renal excretion. Particles greater than 30 nm are cleared by the Kupffer cells and the spleen. The lack of activity *in vivo* may also be attributed to macrophage clearance.

The ability of the liposome to completely condense the anionic DNA to a relatively small size is another element affecting gene delivery. This is because particle size impacts on the efficiency of the internalization via endocytosis as well as the *in vivo* biodistribution (Sun *et al*., 2004). Interestingly it has also been shown that glycosaminoglycans (GAG) interact with the cationic liposome: DNA complex inhibiting gene delivery at the cell surface by destabilizing the complex causing the extracellular release of the therapeutic DNA (Ruponen *et al*., 2004). This could explain the results obtained since no gene expression was detected in any of the organs harvested.

The effects of serum (10 % $^{v}/_{v}$) on DNA liposome binding was measured on all of the liposomal formulations (Chapter 2), however results obtained from *in vitro* studies cannot always be correlated to success, *in vivo* (Bhattacharya and Baja, 2009) as can be seen in the current study. Further *in vivo* trials and optimizations of protocols *in vitro* and *in vivo* will also be needed in future studies if *in vitro* results are to be translated into an *in vivo* success.

CONCLUSION AND FUTURE DIRECTIONS

The perfect gene delivery vector must be a tool that boasts efficient and sustainable characteristics that enable the protection of the exogenous DNA from nucleases, target a specific cell population and have the potential to reach the nucleus in a deserved amount that will bring about change. Furthermore these characteristics must not only be exploited in *in vitro* studies but the vector should be capable of tolerating the extreme *in vivo* environment.

In this study, 3 different galactosylated liposomal formulations and their PEGylated counterparts, were synthesized and analysed, for their gene transfer efficacy, *in vitro*. After confirming their ability to bind and protect DNA, using the gel retardation and nuclease protection assay, liposomes were assessed using the cultured cell lines, HepG2 and HEK 293. Given the structural configurations and physico- chemical properties, the liposomes showed promising results, with reduced levels of cell toxicity and good transfection activity, in cultured cells. Furthermore, receptor-mediated endocytosis of lipoplexes via the ASGP-R was demonstrated using the competition assay in HepG2 cells. The liposomal formulations, WT1 and WT2, displayed the highest levels of targeted transfection activity *in vitro* that could be attributed to the increased length of the spacer arm in the targeting element. The two formulations were then subjected to *in vivo* studies, using rats. However, the sound results obtained in *in vitro* studies could not be correlated with the lack of positive results obtained in *in vivo* studies.

Thus it has been demonstrated *in vitro* that an increase in the spacer length in the galactosyl cholesteryl derivatives correlates with higher transfection activity. This was also dependent on the design of the liposomal formulation and the cell line used. Furthermore, although liposomes show enormous potential when subjected to *in vitro* analysis, a better understanding and further optimization is required to improve the reduced potential obtained in the current *in vivo* study. More detailed investigations are required that place emphasis on exposure (both short and long term) in animal, humans and their environment (Malam *et al*., 2009). The effect of the positively charged lipid on binding and compaction of the DNA and the stability of the complex in the cytosol, are key aspects that effect transfection and require further attention (Wasungu *et al*., 2006).

Gene delivery is indeed a multi-ordered process that requires a multi-functional vector to successfully enter the cell and surmount obstacles, within the cell. Thus future studies could be focused on vectors such as “artificial viruses” which can be synthesized to portray different ligands in a manner that mimics a viral system (Morille *et al*., 2008).

REFERENCES

Abbott NL, Jewell CM, Hays ME, Kondo Y, Lynn D M. Ferrocene-Containing Cationic Lipids: Influence of Redox State on Cell Transfection. J Am Chem Soc. 2005;27*:*11576–11577.

Aden CJ, Kim S-H. Accessible surface areas of nucleic acids and their relation to folding, conformational transition and protein recognition. In: Sarma RH, editor. Stereodynamics of Molecular Systems. New York (NY): Pergamon Press; 1979. pp. 331-350.

Arangoa MA, Duzgunes N, Tros IC. Increased receptor-mediated gene delivery to the liver by protamine-enhanced-asialofetuin-lipoplexes. Gene Ther. 2003;10:5–14.

Audouy S, Molema G, de Leij L, Hoekstra D. Serum as a modulator of lipoplex-mediated gene transfection: dependence of amphiphile, cell type and complex stability. J Gene Med. 2000; 2:465–476.

Balazs DA, Godbey WT. Liposomes used for gene delivery. J Drug Deliv. 2011; 10:1-12.

Baumhofer JM, Beinhauer BG, Wang JE, Brandmeier H, Geissler K, Losert U, Philip R, Aversa G, Rogy MA. Gene transfer with IL-4 and IL-13 improves survival in lethal endotoxemia in the mouse and ameliorates peritoneal macrophages immune competence. Eur J Immunol. 1998;28:610-615.

Betageri GV, Jenkins SA, Parsons DL. Liposome Drug Delivery Systems. Lancaster, PA: Technomic Publishing Company; 1993. Page:16–17.

Bhattacharya S, Baja A. Advances in gene delivery through molecular design of cationic lipids. Chem Commun. 2009;4632-4656.

Biewenga J, van der Ende MB, Krist LF, Borst A, Ghufron M, van Rooijen N. Macrophage depletion in the rat after intraperitoneal administration of liposome encapsulated clodronate:

depletion kinetics and accelerated repopulation of peritoneal and omental macrophages by administration of Freund's adjuvant. Cell Tissue Res. 1995;280:189-196.

Biswas J, Raja G-V, Bhattacharya S. Biological and material properties of various cholesterol based systems. J Indian Institute Sci. 2009;89:137-146.

Boffi F, A. Bonincontro, F. Bordi, E. Bultrini, C. Cametti, A. Congiu-Castellano, F. De Luca, G. Risuleo. Two-step mechanism in cationic lipoplex formation as observed by dynamic light scattering, dielectric relaxation and circular dichroism methods. Phys Chem. 2002;4:2708–2713.

Braet, F, Wisse, E, Structural and functional aspects of liver sinusoidal endothelial cell fenestrae: a review. Gene Ther. 2002;4:1-17.

Campbell, MJ. Lipofection reagents prepared by a simple ethanol injection technique. Biotechniques. 1995;18: 1027-1032.

Chesnoy S, Huang L. Structure and function of lipid-DNA complexes for gene delivery. Ann Rev Biophys Biomol Struc. 2000; 29:27-47.

Chirino AJ, Ary ML, Marshall SA. Minimising the immunogenicity of protein therapeutics. Drug Discov Today. 2004;9:82-90.

Dass CR, Choong PFM. Targeting of small molecule anticancer drugs to the tumour and its vascultare using cationic liposomes: lessons from gene therapy. Cancer Cell Int. 2006:6:1-9.

Dass CR. Lipoplex-mediated delivery of nucleic acids: factors affecting in vivo transfection. J Mol Med. 2004;82:579–591.

Davis BG, Robinson MA. Drug delivery systems based on sugar-macromolecule conjugates. Current Opin Drug Discov Develop. 2002;5:279 - 288.

Deamer D, Bangham AD. Large volume liposomes by ether vaporisation method. Biochim Biophys Acta. 1976;443: 629-624.

Elouahabi A, Ruysschaert JM. Formation and Intracellular Trafficking of Lipoplexes and Polyplexes. Mol Ther. 2005;11:336-347.

Ewert KK, Evans HM, Zidovska A, Bouxsein NF, Ahmad A, Safinya CR. A columnar phase of dendritic lipid-based cationic liposome- DNA complexes for gene delivery: hexagonally ordered cylindrical micelles embedded in a DNA honeycomb lattice. J American Chem Soc. 2006;128:3998–4006.

Felgner PL, Barenholz Y, Behr JP, Cheng SH, Cullis P, Huang L, Jessee JA, Seymour L, Szoka F, Thierry AR, Wagner E, Wu G. Nomenclature for synthetic gene delivery systems. Hum Gene Ther. 1997;8:511–512.

Ferrari ME, Rusalov D, Enas J, Wheeler CJ. Trends in lipoplex physical properties dependent on cationic lipid structure, vehicle and complexation procedure do not correlate with biological activity. Nucleic Acid Res. 2001;29:1539-1548.

Fortes P, Razquin N. *In Vivo* Evaluation of Regulatory Sequences by Analysis of Luciferase Expression. Promega Corporation. Retrieved from http://www.promega.com/resources/articles/pubhub/in-vivo-evaluation-of-regulatory-sequences-by-analysis-of-luciferase-expression

Frederik PM, Stuart MCA, Bomans PHH, Lasic DD. Cryo-electron microscopy of liposomes. In: Lasic DD, Barenholz Y, editors. Nonmedical Applications of Liposomes. Boca Raton, FL: CRC Press; 1996. Page: 309–322.

Fumoto S, Nakadori F, Kawakami S, Nishikawa M, Yamashita F, Hashida M. Analysis of hepatic disposition of galactosylated cationic liposome/plasmid DNA complexes in perfused rat liver. Pharm Res. 2003;20:1452 – 1459.

Gad S. Handbook of Pharmaceutical Biotechnology. John Wiley and Sons Inc. Hoboken, New Jersey; 2007. pp 965-968.

Gershon H, Ghirlando R, Guttman SB, Minsky A. Mode of formation and structural features of DNA-cationic liposome complexes used for transfection. Biochem. 1993;32:7143-7151.

Ghosh P, Han G, De M, Kim CK, Rotello VM. Gold nanoparticles in delivery applications. Adv Drug Deliv Rev. 2008;60: 1307-1315.

Ghosh YK, Visweswariah SS, Bhattacharya S. Nature of linkage between the cationic headgroup and cholesteryl skeleton controls gene transfection efficiency. FEBS Lett. 2000;473:341–344.

Goa X, Huang L. A novel cationic reagent for efficient transfection of mammalian cells. Biochem Biophys Res Commun. 1991;170:280-285.

Gustafsson J, Arvidson G, Karlsson G, Almgren M. Complexes between cationic liposomes and DNA visualized by cryo-TEM. Biochim Biophys Acta. 1995;1235:305–312.

Hasegawa S, Hirashima N, Nakanishi M. Comparative study of transfection efficiency of cationic cholesterols mediated by liposome-based gene delivery. Bioorg Med Chem Lett. 2002;12:1299 – 1302.

He L, Yang L, Duan Y, Deng L, Sun X, Gu X, Zhi-Rong Z. Cytotoxicity and hemocompatibility of a family of novel MeO-PEG-Poly (D,L-Lactic-co-glycolic acid)-PEG-OMe triblock copolymer nanoparticles. J Appl Polymer Sci. 2009;113:2933-2944.

Higuchi Y, Kawakami S, Fumoto S, Yamashita F, Hashida M. Effect of the particle size of galactosylated lipoplex on hepatocyte-selective gene transfection after intraportal administration. Biol Pharm Bul. 2006;29:1521-1523.

Hinds KD, Kim SW. Effects of PEG conjugation on insulin properties. Adv Drug Deliv Rev. 2002; 54:505-530.

Hongtao LV, Zhang S, Wang B, Cui S, Yanc J. Toxicity of cationic lipids and cationic polymers in gene delivery. J Controlled Release. 2006;114:100-109.

Huang CY, Uno T, Murphy JE, Lee S, Hamer JD, Escobedo JA, Cohen FE, Radhakrishna R, Dwarki V, Zuckermann RN. Lipitoids-novel cationic lipids for cellular delivery of plasmid DNA *in vitro*. Chem Biol. 1998;5:345-354.

Hulst R, Muizebelt I, Oosting P, Pol C, Wagenaar A, Šmisterová J, Bulten E, Driessen C, Hoekstra D, Engberts J. Sunfish amphiphiles: conceptually new carriers for DNA delivery. Eur J Org Chem. 2004;4:835–849.

Immordino MR, Dosio F, Cattel L. Stealth liposomes: review of the basic science, rationale and clinical applications, existing and potential. Int J Nanomed. 2006;1:297-315.
Jamieson T, Bakhshi R, Petrova D, Pocock R, Imani M, Seifalian AM. Biological applications of quantum dots. Biomaterials. 2007; 28:4717-4732.

Karmali PP, Chaudhuri A. Cationic liposomes as non-viral carriers of gene medicines: resolved issues, open questions, and future promises. Med Res Rev. 2007;27:696-722.

Kawabata K, Takakura Y, Hasida M. The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake. Pharm Tes. 1995;12:825-835.

Kawakami H, Hiraka K, Tamai M, Horiuchi A, Ogata A, Hatsugai T, Yamagichi A. Oyaizu K, Yuasa M. pH-sensitive liposome retaining Fe-porphyrin as SOD mimic for novel anticancer drug delivery system. Polymers Adv Technol. 2007; 18:82-87.

Kawakami S, Fumoto S, Nishikawa M, et al. *In vivo* gene delivery to the liver using galactosylated cationic liposomes. Pharm Res. 2000;17:306–13.

Kawakami S, Fumoto S, Nishikawa M, Yamashita F, Hashida M. *In vivo* Delivery to the Liver Using Novel Galactosylated Cationic Liposomes. J Pharm Res. 2002;17:306-313.

Kawakami S, Higuchi Y, Hashida M. Nonviral approaches for targeted delivery of plasmid DNA and oligonucleotides. J Pharm Sci. 2008; 97:726-745.

Kawakami S, Munakata C, Fumoto S, Yamashita F, Hashida M. Novel galactosylated liposomes for hepatocyte-selective targeting of lipophilic drugs. J Pharm Sci. 2001;90:150-113.

Kawakami S, Yamashita F, Nishikawa M, Takakura Y, Hashida M. Asialoglycoprotein receptor mediated gene transfer using novel galactosylated cationic liposomes. Biochem Biophy Res Commun. 1998;252:78–83.

Kennedy MT, Pozharski EV, Rakhmanova VA, MacDonald RC. Factors governing the assembly of cationic phospholipid-DNA complexes. J Biophys. 2000;78:1620-1633.

Koltover I, Salditt T, Radler JO, Safinya CR. An inverted hexagonal phase of cationic liposome-DNA complexes related to DNA release and delivery. Sci. 1998;281:78–81.

Lasic DD, Strey H, Stuart MCA, Podgornik R, Frederik PM. The Structure of DNA-Liposome Complexes. J Am Chem Soc.1997;119:832 - 833.

Lautenschlager H. Liposomes. Handbook of Cosmetic Science and Technology. Barel AO, Paye M and Maibach HI, editors. CRC Press Taylor & Francis Group, Boca Raton; 2006. pp. 155-163.

LeDoux J. Gene Therapy Protocols: Production and In Vivo Applications of Gene Transfer Vectors. Humana Press; 2008. Page 43-44.

Lee MC, Miller EA, Goldberg J, Orci L, Schekman R. Bi-directional protein transport between the ER and Golgi. Annu Rev Cell Dev Biol. 2004; 20:87–123.

Li L, Song H, Luo K, He B, Nie Y, Yang Y,Wu Y, Gu Z. Gene transfer efficacies of serum-resistant amino acids-based cationic lipids: Dependence on headgroup, lipoplex stability and cellular uptake. Int J Pharm. 2011;408:183-190.

Ma B, Zhang S, Jiang H, Zhao B, Lv H. Lipoplex morphologies and their influence on transfection efficiency in gene delivery. J Controlled Release. 2007;123:184-194.

Malam Y, Loizidou M, Seifalian AM. Liposomes and nanoparticles: Nanosized vehicles for drug delivery in cancer. Trends Pharm. 2009; 30:592-598.

Managit C, Kawakami S, Yamashita F, Hashida M. Effect of galactose density on asialoglycoprotein receptor-mediated uptake of galactosylated liposomes. J Pharm Sci. 2005;94:2266–2275.

Metselaar JM, Bruin P, De Boer LW, Vringer T, Snel C, Oussoren C, Wauben MH, Crommelin DJ, Storm G, Hennink WE. A novel family of L-amino acid-based biodegradable polymer-lipid conjugates for the development of long-circulating liposomes with effective drug-targeting capacity. Bioconj Chem. 2003;14:1156-1164.

Min JH, Kim SM, Bae H, Shin MS, Kim S, Park S, Lee H, Park RW, Kim IS, Kim K, Kwon IC, Jeong SY, Lee DS. Tumoral acidic pH-responsive MPEG-poly(beta-amino ester) polymeric micelles for cancer targeting therapy. J Controlled Release. 2010;144:259–266.

Moghimi SM, Hunter AC, Murray JC. Long-circulating and target-specific nanoparticles: Theory to practice. Pharmacol Rev. 2001; 53:283-318.

Mori A, Chonn A, Choi LS, Isreals A, Monck MA, Cullis PR. Stabilization and regulated fusion of liposomes containing a cationic lipid using amphipathic polyethylenegylcol derivatives. J Liposome Res. 1998;8:195-211.

Morille M, Passirani C, Vonarbourg A, Clavreul A, Benoit J-P. Progress in developing cationic vectors for non-viral systemic gene therapy against cancer. Biomaterials. 2008;29:3477-3496.

Morille M, Passirani C, Letrou-Bonneval E, Benoit JP, Pitard B. Galactosylated DNA lipid nanocapsules for efficient hepatocyte targeting. Int J Pharm. 2009;379:293–300.

Motoyama K, Nakashima Y, Aramaki Y, Hirayama F, Uekama K, Arima H. In Vitro Gene Delivery Mediated by Asialofetuin-Appended Cationic Liposomes Associated with γ-Cyclodextrin into Hepatocytes. J Drug Deliv. 2011;10:1-13.

Mountain A. Gene therapy: The first decade. TIBTECH. 2000; 18:119-128.

Mukherjee KM, Sen J, Chaudhuri A. Common co-lipids, in synergy, impart high gene transfer efficacy to a transfection-incompetent cationic lipids. FEBS Lett. 2005;579: 1291-1300.

Murao A, Nishikawa M, Managit C, Wong J, Kawakami S, Yamashita F, Hashida M.. Targeting efficiency of galactosylated liposomes to hepatocytes *in vivo*: Effect of lipid composition. Pharm Res. 2002;19:1808-14.

Narainpersad N, Singh M, Ariatti M. Novel Neo Glycolipid: Formulation into PEGylated cationic liposomes and targeting of DNA lipoplexes to the hepatocyte-derived cell line HepG2. Nucleosides, Nucleotides and Nucleic Acid. 2012;31:206-223.

Nguyen LT, Atobe K, Barichello JM, Ishida T, Kiwada H. Complex formation with plasmid DNA increases the cytotoxicity of cationic liposomes. Biol Pharm Bull. 2007;30:751–757.

Ning YM, He K, Dagher R, Sridhara R, Farrell AT, Justice R, Pazdur R. Liposomal doxorubicin in combination with bortezomib for relapse or refractory multiple myeloma. Oncol. 2007; 21: 1503-1508.

Obata Y, Suzuki D, Takeoka S. Evaluation of cationic assemblies constructed with amino acid based lipids for plasmid DNA delivery. Bioconjug. Chem. 2008;19:1055–1063.

Parker AL, Newman C, Briggs S, Seymour L, Sheridan PL. Lipoplex mediated transfection and endocytosis. Expert Rev Mol Med. 2003;5:1-6.

Pathak A, Suresh PV, Gupta KC. Nano-vectors for efficient liver specific gene transfer. Int J Nanomed. 2008;3:31-49.

Percot A, Briane D, Coudert R, Reynier P, Bouchemal N, Lievre N, Hantz E, Salzmann JL, Cao A. A Hyroxyehylated cholesterol-based cationic lipid for DNA delivery: effect of conditioning. Int J Pharm. 2004;278:143-163.

Rahau S, Shirazi RS, Kai K, Ewert KK, Bruno F, Silva B, Cecilia C L, Youli YL, Cyrus R, Safinya CR. Structural evolution of environmentally responsive cationic liposome-DNA complexes with a reducible lipid linker. J Am Chem Soc. 2012;28:10495-503.

Reynier P, Briane D, Coudert R, Fadda G, Bouchemal N, Bissieres P, Taillandier E, Cao A. Modifications in the head group and in the spacer of cholesterol-based cationic lipids promote transfection in melanoma B16-F10 cells and tumours. J Drug Target. 2004;12:25-38.

Ross PC, Hui SW. Polyethylene glycol enhances lipoplex-cell association and lipofection. Biochim Biophys Acta. 1999;1421:273-283.

Ruponen M, Honkakoski P, Tammi M, Urtti A. Cell-surface gylcosaminoglycans inhibit cation-mediate gene transfer. J Gene Med. 2004; 6:405-414.

Shi F, Wasungu L, Nomden A, Stuart MCA, Polushkin E, Engberts JBFN, Hoekstra D. Interference of poly(ethylene glycol)-lipid analogues with cationic-lipid-mediated delivery of oligonucleotides; role of lipid exchangeability and non-lamellar transitions. J Biochem. 2002; 366: 333-341.

Shimada K, Kamps JM, Regts J, Ikeda K, Shiozawa T, Hirota S, Scherphof GL. Biodistribution of liposomes containing synthetic galactose-terminated diacylglycerylpoly(ethyleneglycol)s. Biochim Biophys Acta- Biomembranes. 1997;1326:329–41.

Simões S, Filipe A, Faneca H, Mano M, Penacho N, Duzgunes N, Lima MP. Cationic liposomes for gene delivery. Expert Opin Drug Deliv. 2005;2:237-254.

Singh M, Ariatti M. A cationic cytofectin with long spacer mediates favourable transfection in transformed human epithelial cells. Int J Pharm. 2006;309:189-198.

Singh M, Kisoon N, Ariatti M. Receptor-mediated gene delivery to HepG2 cells by ternary assemblies containing cationic liposomes and cationized asialoorosomucoid. Drug Deliv. 2001;8:29-34.

Singh M, Hawtrey A, Ariatti M. Lipoplexes with biotinylated transferrin accessories: novel, targeted, serum-tolerant gene carriers. Int J Pharm. 2006;321:124–137.

Singh M, Rogers CB, Ariatti M. Targeting of glycosylated lipoplexes in HepG2 cells: Anomeric and C-4 epimeric preference of the asialoglycoprotein receptor. SA J Sci. 2007;103: 204-210.

Song LY, Ahkong QF, Rong Q, Wang S, Ansell S, Hope MJ, Mui B. Characterisation of the inhibitory effect of PEG-lipid conjugates on the intracellular delivery of plasmid and antisense DNA mediated by cationic lipid liposomes. Biochim Biophys Acta. 2002;1558:1-13.

Sun X, Zhang HW, Zhang ZR. Transfection efficiency of pORF lacZ plasmid lipopolyplex to hepatocytes and hepatoma cells. World J Gastroenterol. 2004;10:531–534.

Szoka Jr FC, Papahadjopoulos D. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc Nat Acad Sci. 1978;75: 4194 - 4198.

Tang F, Hughes JA. Introduction of a disulfide bond into cationic lipid enhances transgene expression of plasmid DNA. Biochem Biophy Res Commun. 1998; 242:141-145.

Templeton NS. Developments in liposomal gene delivery systems. Informa Health Care. 2001;1:567-570.

Templeton NS. Cationic Liposome-mediated gene delivery *in vivo*. Biosci Report. 2002;22:283-295.

Torchilin V. Liposomes: a practical approach. 2nd Edition. Oxford University Press, New York. 2003: Page 1-15.

Tran MA, Watts RJ, Robertson GP. Use of liposomes as drug delivery vehicles for treatment of melanoma. Pigment Cell Melanoma Res. 2009;22:388–399.

Uddin SN. Cationic lipids used in non-viral gene delivery systems. Biotech Mol Biol Rev. 2007;2:058-067.

Wasungu L, Hoekstra D. Cationic lipids, lipoplexes and intracellular delivery of genes. J Controlled Release. 2006;116:255-264.

Wilkening S, Stahl F, Bader A. Comparison of primary human hepatocytes and hepatoma cell line HepG2 with regard to their biotransformation properties. Drug Metabol Disposit. 2003;31:1035-1042.

Wu J, Hantz M, Zern MA. Targeting hepatocytes for drug and gene delivery: emerging novel approaches and applications. Frontiers Biosci. 2002;7:717-725.

www.initrogen.com/site/us/en/home/Reference/Ambion-Tech-Support/rnai-sirna/tech-notes/performing-rnai-experiments-in-animals.html

Xu Y, Hui SK, Frederik P, Szoka Jr FC. Physiochemical characterization and purification of cationic lipoplexes. Biophys J. 1999;77:341–353.

Yang JP, Huang L. Overcoming the inhibitory effect of serum on lipofection by increasing the charge ratio of cationic liposomes to DNA. Gene Ther. 1997;4:950-960.

Yang B, Fukuda N, Van-Hoek A, Matthay MA, Ma T, Verkman AS . Carbon dioxide permeability of aquaporin-1 measured in erythrocytes and lung of aquaporin-1 null mice and in reconstituted proteoliposomes. J Biol Chem. 2000;275:2686–2692.

Yokoyama M. Drug targeting with nano-sized carrier systems. J Artif Organs. 2005;8:77–84.

Takakura Y, Oka Y, Hashida M. Cellular uptake properties of oligonucleotides in LLC-PK1 renal epithelial cells. Antisense Nucl. Acid Drug Develop**.** 1998;8: 67-73.

Zabner J, Fasbender AJ, Moninger T, Poellinger KA, Welsh MJ. Cellular and molecular barriers to gene transfer by a cationic lipid. J Biol Chem. 1995; 270:18997–19007.

Zhang Y, Boado RJ, Pardridge WM. Marked enhancement in gene expression by targeting the human insulin receptor. J Gene Med. 2003;5:157–163.

Zhang H, Ma Y, Sun XL. Recent developments in carbohydrate-decorated targeted drug/gene delivery. Med Res Rev. 2010; 30: 270-289.

Zhang L, Webster TJ. Nanotechnology and nanomaterials: promises for improved tissue regeneration. Nano Today. 2009; 4:66-80.

Zhang S, Zhao Y, Zhao B, Wang B. Hybrids of Nonviral Vectors for Gene Delivery. Bioconj Chem. 2010;21:1003-1009.

Zhang SB, Xu YM, Wang B, Qiao WH, Liu DL, Li ZS. Cationic compounds used in lipoplexes and polyplexes for gene delivery. J Controlled Release. 2004;100:165-180.

Zhao Q, Chen J, Han M, Liang W, Tabata Y, Gao J. Combination of poly (ethylenimine) and chitosan induces high gene transfection and low cytotoxicity. J Biosci Bioeng. 2008;105:65–68.

Zhdanov RI, Podobed OV, Vlassov VV. Cationic lipid-DNA complexes-lipoplexes for gene transfer and therapy. Bioelectrochemistry. 2002;58:53-64.

Zhu N, Liggitt D, Liu Y, Debs R. Systemic gene expression after intravenous DNA delivery into adult mice. Sci. 1993;126:209-211.

Zuhorn IS, Bakowsky U, Polushkin E, Visser WH, Stuart MCA, Engberts JBFN, Hoekstra D. Nonbilayer phase of lipoplex-membrane mixture determines endosomal escape of genetic cargo and transfection efficiency. Mol Ther. 2005;11: 801-810.

Zuidam NJ, Barneholz Y. Electrostatic and structural properties of complexes involving plasmid DNA and cationic lipids commonly used for gene delivery. Biochim Biophys Acta. 1998;1368:115-128.

APPENDIX A

Spectral data of galactosylated moieties analysed in the present study.

Compound:
3β[N-(4-aminophenyl-β-D-galactopyranosyl) carbamoyl] cholesterol (NN)

IR: 3333 (m, OH, CONH); 2933, 2900, 2867 (m, CH, CH_2, CH_3); 1698 (m, urethane); 1603 (m, benzene ring); 1510 (s, benzene ring); 1414 (m, O-H); 1381 (w, $C(CH_3)_2$); 1214 (s, C-O); 1050 (s, C-OH); 833 (m, aromatic C-H) cm-1. ^{1}H NMR (600 MHz, C_5D_5N, TMS): sugar 5.54 (d, 1H, J = 6.7Hz, H-1"), 4.77 (dd, 1H,J = 9.3, 7.9 Hz, H-2"); aromatic 7.45 (d, 2H, J = 9 Hz, H-3', H-5'), 7.88 (d, 2H, J = =8.2 Hz, H-2', H-6') sterol 0.67 (s, 3H, CH_3, C-18), 0.91 (d, 6H, J = 6.8 Hz, CH_3, C-26, C-27), 0.99 (d, 3H, J = 4.8 Hz, CH-CH_3, C-21), 5.41 (dd, 1H, J = 3.2, 2.3 Hz, H-6), 4.89 (m, 1H, H-3$_\alpha$) ppm. ^{13}C NMR (150.9 MHz, C_5D_5N, TMS): sugar 103.56 (C-1"), 77.45 (C-5"), 75.37 (C-3"), 72.31 (C-2"), 70.28 (C-4"), 62.41 (C-6"); aromatic 154.44 (C-4'), 135.00 (C-1'), 117.75 (C-3', C-5'); sterol 140.21 (C-5), 122.78 (C-6), 56.81 (C-14), 56.36 (C-17), 50.28 (C-9), 42.52 (C-13), 39.96 (C-12), 39.76 (C-24), 37.29 (C-1), 32.07 (C-8), 28.65 (C-16), 28.53 (C-25), 24.52 (C-15), 24.18 (C-23), 22.97 (C-27), 22.72 (C-26), 21.30 (C-11), 19.39 (C-19), 18.97 (C-21), 12.03 (C-18) ppm.

Compound:
Cholest-5-en-3-yl [1-(β-D-galactopyranosyl)-1H-1,2,3-triazol-4-yl]methylcarbamate (WT)

IR: 3381, 3130, 2941, 2358, 1740, 1682, 1460 cm-1
^{1}H NMR (300 MHz, $CDCl_3$): 0.64 (s, 3H, CH_3), 0.83-0.85 (d, 6H, J = 6 Hz, CH_3), 0.90-0.92 (d, 3H, J = 6 Hz, CH_3), 0.98 (s, 3H, CH_3), 1.03-1.52 (m, 23H), 1.79-1.97 (m, 5H), 2.20-2.30 (m, 2H), 2.49-2.50 (t, 2H, J = 4.2 Hz), 3.45-3.54 (m, 3H), 3.67-3.70 (t, 1H, J = 4.2 Hz), 3.75 (d, 1H, J = 1.8 Hz), 3.97-4.01 (t, 1H, J = 6.9 Hz), 4.22 (s, 2H), 4.30-4.38 (m, 1H), 4.63-4.69 (m, 1H), 5.33-5.43 (m, 1H), 5.43-5.45 (d, 1H, J = 6.9 Hz), 7.99 (s, 1H) ppm; ^{13}C (75 MHz, $CDCl_3$): 11.4 (C-18), 18.3, 18.8 (C-21), 20.3 (C-19), 22.1(C-26), 22.4 (C-27), 23.0, 23.6 (C-23), 27.2, 27.5 (C-16), 27.6 (C-25), 31.1, 31.2, 35.0, 35.4, 35.8 (C-20), 36.4 (C-22), 38.1, 38.7 (C-24), 49.3 (C-9), 55.4, 55.9 (C-17), 60.1, 68.1, 69.1, 73.0, 73.4 (C-3), 78.7, 78.9, 79.0, 87.8, 121.1, 121.6 (C-6), 139.5 (C-5), 145.2, 155.5 ppm.

APPENDIX B

pCMV-Luc Plasmid Sequence:

cccgggaggtaccgagctcttACGCGTTGACATTGATTATTGACTAGTTATTAATAGTAATC
AATTACGGGGTCATTAGT

TCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGAC
CGCCCAACGACCCCCGCC

CATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGT
CAATGGGTGGACTATTTA

CGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGA
CGTCAATGACGGTAAATG

GCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCT
ACGTATTAGTCATCGCTA

TTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGG
GGATTTCCAAGTCTCCAC

CCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCG
TAACAACTCCGCCCCATT

GACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCTCTGGCTAA
CTAGAGAACCCACTGCTT

ACTGGCTTATCGAAATTAATACGACTCACTATAGGGAGACCCAAGCTTggcattccggtact
gttggtaaaatggaagac

gccaaaaacataaagaaaggcccggcgccattctatcctctagaggatggaaccgctggaga
gcaactgcataaggctat

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tcacgtacgcggaatact

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atcagggttattgtctca

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APPENDIX C

Animal Experimentation carried out in the present study was approved by the University of KwaZulu-Natal Animal Ethics Committee.

UNIVERSITY OF
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INYUVESI
YAKWAZULU-NATALI

Govan Mbeki Centre, Westville Campus,
University Road, Chiltern Hills, Westville, 3629, South Africa
Telephone 27 (031) 260-2275/35 Fax (031) 260-2384
Email: Animalethics@ukzn.ac.za

29 September 2011

Reference: 076/11/Animal

Miss D Govender
Biochemistry Department
Block F3 Room 12
Private Bag X 54001
WESTVILLE CAMPUS

Dear Miss Govender

Ethical Approval of Research Projects on Animals

I have pleasure in informing you that the Animal Ethics Sub-committee of the University Ethics Committee has granted ethical approval for 2011 on the following project:

Max. 50 characters including spaces: A comparative study on three unique galactosylated cationic liposomes with their sterically stablized counterparts, in HepG2 cells.

Yours sincerely

Professor Theresa HT Coetzer
Chairperson: Animal Ethics Sub-committee

Cc Registrar, Prof. J Meyerowitz
Research Office, Mr N Moodley
Head of School, Prof. B Pillay
Supervisor, Dr M Singh
Biomedical Resource Unit, Dr S Singh

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