TARGETED GENE TRANSFER TO MAMMALIAN SYSTEMS USING LIPOSOME CONSTRUCTS CONTAINING CHOLESTEROL COMPONENTS WITH OR WITHOUT BIOTINYLATED MOLECULAR ACCESSORIES

by

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DECLARATION

The work described in this thesis was carried out in the Department of Biochemistry, Faculty of Science and Agriculture, University of KwaZulu-Natal, Westville Campus, from January 2000 to December 2004, under the supervision of Professor Mario Ariatti.

These studies represent original research and has not been submitted in any form to another University. Where use is made of the work of others, it has been duly acknowledged in the text.

Moganavelli Singh

I

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I wish to express my sincere thanks and appreciation to the following people:

This thesis is dedicated to my
family:
my mother, late father,
my husband and children,
Nalendran and Teshnee.

ABSTRACT

The development of low toxicity cationic liposome-based gene delivery systems with the design capacity for systemic application *in vivo* is gaining momentum. In this study, three untargeted and six ligand-directed cationic liposome-based non-viral delivery systems have been optimized for gene transfer *in vitro*. Thus three novel cationic cholesterol derivatives have been prepared and formulated into unilamellar liposomes with the helper lipid dioleoylphosphatidylethanolamine (DOPE). Their interaction with the luciferase expression plasmid pGL3 affords lipoplexes which have been characterized by three separate assays viz. band shift, serum nuclease digestion, ethidium displacement and by transmission electron microscopy. All derivatives feature a dihydrazide linker in the spacer separating the cationic head groups and the cholesteryl moieties. Toxicities and transfection activities were determined *in vitro* in four transformed cell lines. N,N-Dimethylaminopropylaminylsuccinylcholesteryl-formylhydrazide (MS09), with a tertiary amino head group and a twelve atom 15 Å spacer, afforded weakly cytotoxic lipoplexes at the optimal liposome:DNA +/- transfection ratio (0.7) and exceeded Lipofectin™-mediated transgene activity in the three human transformed cell lines HeLa (cervix), HepG2 (hepatocyte) and SNO (oesophagus). Glycylcholesterylformylhydrazide (MS10) and β-alanylcholesteryl-formylhydrazide (MS11)-based lipoplexes also proved to be effective mediators of transfection but did not attain the levels reached by MS09, which was selected for incorporation into ligand-directed transfecting complexes.

Consequently, MS09 was formulated into two separate liposome preparations containing DOPE and either biotinylcholesterylformylhydrazide (MSB1) or aminohexanoylbiotinylcholesterylformylhydrazide (MSB2). The biotinyl appendage was used to secure a streptavidin(bio^3-transferrin) conjugate to the liposome while the cationic component (MS09) interacted with the pGL3 plasmid DNA. The resulting ternary complexes, which displayed good stability in 10% foetal bovine serum (FBS), achieved high transfection activity in HeLa cells which express the transferrin receptor. In the presence of free iron-loaded transferrin (400 μg/ ml), activity was reduced by 60-

70% to levels obtained by untargeted biotinylated lipoplexes therefore strongly supporting the notion that complexes entered the cells largely by transferrin receptor-mediation. Moreover, the presence of 10% FCS in transfection mixtures was virtually without effect on transfection activity.

Furthermore, four cholesteryl glycosides were prepared for inclusion into a cationic liposome-based system intended for gene targeting to the asialoglycoprotein receptor expressed in HepG2 cells. Hence cholesteryl-α-D-galactopyranoside (MSαGAL), cholesteryl-β-D-galactopyranoside (MSβGAL), cholesteryl-α-D-glucopyranoside (MSαGLU) and cholesteryl-β-D-glucopyranoside (MSβGLU) were separately formulated with MS09, DOPE and pGL3 plasmid DNA into lipoplexes. Competition assays with asialofetuin (600 µg/ ml), a cognate ligand for the asialoglycoprotein receptor, reduced transfection activity by >90% in all cases to levels of transfection achieved in HeLa cells, which are asialoglycoprotein receptor-negative. Although all four lipoplexes promoted high levels of luciferase gene expression, interestingly, the MSβGAL system achieved a four fold greater activity than its MSαGAL counterpart and an eight fold increase over the MSαGLU and MSβGLU lipoplexes. Not only does this demonstrate an anomeric preference by the asialoglycoprotein receptor for the MSβGAL lipoplexes but an epimeric preference is also apparent with respect to the C-4 position of the hexose, the position at which D-galactose and D-glucose differ. The transfection activity displayed by MS09 lipoplexes in the cell systems investigated in this study was high, but showed significant improvement by the addition of the targeting components discussed. High gene expression levels coupled to low toxicity *in vitro* render these novel liposome formulations promising candidates for possible future *in vivo* studies.

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LIST OF ABBREVIATIONS

Å	Angstrom
AAV	Adeno-associated virus
AFM	Atomic force microscopy
AIDS	Acquired Immunodeficiency Syndrome
AOM	Asialoorosomucoid
ASGP	Asialoglycoprotein
ASGP-R	Asialoglycoprotein receptor
ASF	Asialofetuin
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CD	Circular dichroism
Chol-T	3β[N-(N',N'-dimethylaminopropane)-carbamoyl]cholesterol
CLSM	Confocal laser scanning microscopy
Cpm	Counts per minute
CTAB	Cetyltrimethylammonium bromide
DC-CHOL	3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol
DCCI	Dicyclohexylcarbodiimide
DMF	Dimethylformamide
DMRIE	Dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DOGS	Dioctadecylamidoglycyl-spermine
DOPC	dioleoylphosphatidylcholine
DOPE	Dioleoyl-phosphatidylethanolamine
DOSPA	2,3-dioleyoxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate
DOSPER	1,3-dioleoyloxy-2(6-carboxyspermyl)-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
DPPES	Dipalmitoyl-phosphatidylethanolamido spermine
DSPE	Distearoylphoshatidylethanolamine
DTAB	Dodecyltrimethylammonium bromide
EDTA	Ethylenediamine tetra-acetic acid
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FTIR	Fourier transform infrared
GalNAc	N-acetylgalactosamine
GAP-DLRIE	(+)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis-(dodecyloxy)-1-propanaminium bromide
HEPES	2-[4-(2-hydroxyethyl)-piperazinyl]-ethanesulfonic acid
HIV-1	Human immunodeficiency virus type 1
HSV	Herpes Simplex Virus
HVJ	Haemagglutinating virus of Japan
IR	Infrared
LDL	Low density lipoprotein
LUV	Large unilamellar vesicle
MLV	Multilamellar vesicle
MSB1	Biotinylcholesterylformylhydrazide
MSB2	Aminohexanoylbiotinylcholesterylformylhydrazide
MSαGAL	Cholesteryl-α-D-galactopyranoside
MSβGAL	Cholesteryl-β-D-galactopyranoside
MSαGLU	Cholesteryl-α-D-glucopyranoside
MSβGLU	Cholesteryl-β-D-glucopyranoside
MS01	N-tritylglycylcholesterylformylhydrazide
MS02	N-tityl-β-alanylcholesterylformylhydrazide
MS04	Cholesterylformylhydrazide
MS05	N-trityl-β-alanine-NHS ester
MS06	N-trityl-β-alanine
MS07	N-tritylglycine NHS ester

MS08	Cholesterylformylhydrazide hemisuccinate
MS09	N,N-dimethylaminopropylaminylsuccinylcholesterylformylhydrazide
MS10	Glycylcholesterylformylhydrazide
MS11	β-alanylcholesterylformylhydrazide
NHS	N-hydroxysuccinimide
NMR	Nuclear magnetic resonance
NPC	Nuclear pore complex
PBS	Phosphate buffered saline
PEI	Polyethyleneimine
PLL	Poly-L-lysine
PEG	poly(ethylene glycol)
PG	phosphatidylglycerol
PS	Phosphatidylserine
PVP	Polyvinyl pyrrolidone
RLU	Relative light units
RNA	Ribonucleic acid
Rpm	Revolutions per minute
SAXS	Small angle X-ray scattering
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulphate
SUV	Small unilamellar vesicle
TEM	Transmission electron microscopy
Tf	Transferrin
TfR	Transferrin receptor
TLC	Thin layer chromatography
Tris	Tris(hydroxymethyl)aminomethane
TTAB	Tetradecyltrimethylammonium bromide

CHAPTER ONE

INTRODUCTION

1.1. GENE THERAPY

Malignant transformations leading to an unregulated growth of cells is almost always caused by genetic aberrations and often is associated with epigenetic events. Hence in the last few decades gene transfer has emerged as an important technique to shed some light on this potentially fatal process. Gene therapy was originally envisaged as a treatment for inherited monogenic disorders such as cystic fibrosis that could be treated by the restoration of the function of a single gene. In 1990 the first gene therapy trial for the treatment of adenosine deaminase (ADA) deficiency using retroviral vectors was undertaken (Blaese *et al.*, 1990). It has since been extended to include numerous diseases or disorders viz. arthritis (Kang *et.al.,* 1997; Jorgensen *et al.*, 2002), cardiovascular diseases (Ylä-Herttuala and Martin, 2000), cancer, Parkinson's disease (Rubanyi, 2001) and the acquired immunodeficiency syndrome (AIDS) (Haynes, 1996; Weber, 1996).

The concept of gene therapy appears simple, i.e. the delivery of a good copy gene or DNA that will ultimately correct the defects of a disease arising from a defective gene. However execution of this procedure is far from being simple. The Gene Therapy Advisory Committee of the United Kingdom defines gene therapy as "the deliberate introduction of genetic material into human somatic cells for therapeutic, prophylactic or diagnostic purposes". Gene transfer could entail the transfer of new genetic material or the manipulation of an existing gene. Gene therapy can also be used to destroy unwanted cells such as cancer cells. Genes can be introduced directly into the target cells in the body or the target cells can be modified outside the body *ex vivo* and then reimplanted. Major hurdles to successful gene therapy include delivery of the gene, vector design, gene regulation and avoidance of immune responses (Romano *et al.*, 1998). Hence basic research is still required to answer these pressing issues.

Genetic material is usually delivered by specific vectors. One hurdle is that most existing vectors are far from being ideal. An ideal vector can be described as one that will transfer the gene of interest to a specific target cell, bringing about regulated and sustained expression of the gene product without being toxic to the cells. Most vectors can meet several of these requirements but not necessarily all (Hodgson, 1995). The choice of a vector will largely depend on the therapeutic needs of the target disease. Among the strategies utilized for introducing genes are viral and non-viral methods that possess unique characteristics.

1.2   VIRAL GENE DELIVERY SYSTEMS

Viral vectors have been successfully utilized for the delivery of genetic material as they contain all the features necessary for efficient gene transfer such as cell adhesion, membrane translocation, efficient transcription, and translation (Cristiano, 1998). Although viral methods seem to offer high efficiencies in gene delivery and stable integration of exogenous genes into host chromosomes in the case of retroviruses, they may possess long term risks such as possible recombination with endogenous viruses, insertional mutagenesis, immunogenecity and oncogenic effects (Joo and Kim, 2002). The recent report on the occurrence of leukemia in children treated with viral gene therapy (retroviral based system) against severe combined immunodeficiency (SCID), demonstrated the toxicity problems associated with viral gene therapy (Kaiser, 2003). Another limitation of viruses is their relatively small packaging capacity of therapeutic DNA (Luo and Saltzman, 2000). Viruses commonly used in gene therapy include retroviruses, adenoviruses, lentiviruses, poxviruses, Sendai virus (or haemagglutinating virus of Japan), vaccinia virus (VV), and herpes viruses that carry the therapeutic gene in their capsid, thus protecting it from enzyme digestion.

Retroviruses are RNA viruses that undergo reverse transcription after infecting the cell, hence producing double stranded DNA. Retroviral vectors are modified retroviruses in which the genes required for replication have been removed and replaced with a

therapeutic or marker gene. Once they have been "packaged" in a protein coat, they operate in their normal manner to infect a cell. They were the first viral system adapted for gene therapy. They infect only actively dividing cells, leaving quiescent cells unaffected (Miller *et al.*, 1990). This aids in the selection of neoplastic cells over normal cells. Retroviruses have a large cloning capacity of about 10 kb (Trono, 2000). The DNA integrates stably, but in a random fashion into the host genome. Hence using this method the DNA is permanently integrated into the host genome, thus raising long-term safety concerns including possible mutagenesis. Recently an oncoretroviral vector was utilized in gene transfer to keratinocytes which revealed the absolute need for proliferation in order to achieve efficient gene transfer. The virus itself is difficult to produce at high titres. These problems represent a clear limitation of retroviral systems (Serrano *et al.*, 2003).

Adenoviruses are DNA viruses that infect the eukaryotic cell. They lose their protein coat and transfer DNA to the nucleus where it is transcribed. DNA is not integrated into the host genome. The average size of the genome is about 36 kb (Zhang Wei-Wei, 1999) and the vector can accommodate large DNA inserts of approximately 7-8 kb. Adenoviral vectors are capable of infecting non-dividing cells and hence will not be able to discriminate between neoplastic and normal cells. There are more than 40 different human serotypes, which are classified into 6 subgroups (A-F) based on their properties. Serotype 2 (Ad2) and 5 (Ad5) are the most studied adenoviruses in gene therapy. Concerns about the use of adenoviral vectors include pro-inflammatory properties, cytotoxic properties and possible immune responses to the vector capsid protein and transfected cells in some tissues (Amalfitano and Parks, 2002) and the lack of cell-specific targeting. Adenoviral vectors have been used in delivery of tumour-suppressor genes, antisense oncogenes, suicide / toxin genes, and have shown anticancer immunity in different animal models (Addison *et al.*, 1995). They can be produced in high titre (about 10^{11} /ml).

Herpesvirus vectors are usually derived from the strains of the Herpes Simplex virus type 1 (HSV-1). Herpes Simplex Virus (HSV), one of the human herpesviruses is a

natural pathogen causing recurrent oropharyngeal cold sores. They are double stranded DNA viruses that have the ability to remain latent in tissues and to be reactivated at the original site of infection. HSV-1 replicates within the infected cells and causes cell lysis and infection of surrounding cells. They can incorporate large amounts of exogenous DNA and can be grown to high titres. Vectors based on the herpes virus seem to be attractive possibilities for CNS gene therapy (Parks and Bramson, 1999). The disadvantages of these viruses include cytotoxicity that was reported especially *in vitro*, limited duration of transgene expression, and marked inflammatory response in the host.

Lentiviruses belong to the family of retroviruses. Lentiviral vectors eg. The human immunodeficiency virus type 1 (HIV-1), can replicate in non-mitotic cells similar to the adenoviral vector (Trono, 2000). Recent studies have shown that recombinant vectors derived from HIV-1 lentivirus can transduce a variety of non-dividing human cells, including neurons, liver, muscle, and keratinocytes (Kuhn *et al.*, 2002 ; Serrano *et al.*, 2003). Gene expression from a lentiviral vector has been shown to be stable for up to 14 months (Amado and Chen, 1999). Lentiviral vectors have a total insert capacity of 10 kb (Romano *et al.*, 1998). They seem to be a popular choice for genetic modification of retinal cells. HIV vector-mediated gene therapy is often utilized in treatment of recessive forms of inherited retinal degeneration (Miyoshi *et al.*, 1997). Despite this, lentiviral vectors seem to be very poor in delivering genes *in vivo* into liver and muscle (Trono, 2000).

Poxviruses are large, enveloped viruses containing a double stranded DNA genome. They are predominantly engineered for gene therapy to stimulate the immune system. They have a large packaging capacity of up to 25 kb. These viruses have been used extensively in humans for vaccine research and clinical vaccine application (Paoletti, 1996). Vaccinia virus vectors belong to the family of poxviruses. They have a long history in medicine as they were the first vectors used for vaccinating humans against smallpox. They are double-stranded DNA viruses with a genome of approximately 200 kb. Vaccinia is a promising vector since the virus infects nearly all mammalian cell

types (Smutzer, 2000). However the main disadvantage of poxviruses is their high immunogenicity and their use is limited to therapy requiring transient expression (Paoletti, 1996).

Adeno-associated viruses (AAV) have been used more often in pre-clinical / clinical trials over the past few years. Adeno-associated viruses are non-enveloped parvoviruses with a 4.7 kb single-stranded DNA genome (Srivastava *et al.*, 1983). They do not seem to be associated with any human disease since no pathology was found to be associated with infection with AAV and it is widely believed AAV-2 (human) can replicate efficiently in human cells when a helper virus is applied. This is because AAV is not a fully functioning virus and cannot replicate in host cells without co-infection with a helper virus such as, adenovirus (Atchison *et al.*, 2003), vaccinia virus and herpes simplex virus (Schlehofer *et al.*, 1986 ; Smith-Africa and Bartlett, 2001). The mechanisms of AAV integration are still poorly understood. They can target a wide range of cells derived from different tissues as well as non-proliferating cells and hence have a lower immunogenic property. They have the ability to establish latent infection by viral genome integration into the cell genome. High titers of this virus are difficult to obtain in the pure form (Romano *et al.*, 1998). However AAV have a relatively small transgene capacity of less than 5 kb, low transfection efficiency in non-dividing cells, and the possibility of humoral responses occurring (Kootstra and Verma, 2003).

Lastly, Sendai virus or haemagglutinating virus of Japan (HVJ) is a member of the paramyxoviruses. It has been incorporated in several different gene delivery systems, eg. the Sendai virus has been combined with liposomes to produce a novel gene delivery system, called HVJ-liposomes. These viruses are being assessed for gene therapy of liver diseases (Nakanishi, *et al.*, 1999). A glycoprotein of the vesicular stomatitis virus envelope has also been developed for gene therapy (Schuster *et al.*, 1999).

1.3 NON-VIRAL GENE DELIVERY SYSTEMS

Non-viral gene delivery systems are techniques designed to introduce a coding DNA sequence without the means of a virus. There has been an increase over the years in the development and application of non-viral vectors in gene therapy. Non-viral gene therapy has a significant clinical potential, although its therapeutic use has been limited thus far by extracellular and intracellular barriers. Non-viral vectors can now hope to rival the viral vectors in respect of transfection efficiency. Most non-viral gene therapy research has focused on the delivery of plasmids which is an attractive approach for gene transfer applications due to flexibility. Plasmids may be engineered to incorporate large DNA segments (Ahearn and Malone, 1999).

Non-viral vectors can be introduced either by physical or chemical methods. Physical transfection of genes can be accomplished by electroporation, microinjection, or the use of ballistic particles (gene gun method). Chemical transfection introduces DNA by calcium phosphate, lipids and / or protein complexes. Some of the current popular methods of non-viral gene transfer viz. gene gun method, electroporation, direct gene (DNA) transfer, cationic polymers and lipid based delivery systems will be discussed.

1.3.1   Gene Gun Method

This method is also referred to as the ballistic method, the particle acceleration method, or the micro-projectile method. It involves the penetration of the physical barrier of mammalian cells eg. the cell membrane and core membrane, by coated gold particles. A compressed shock wave of helium gas is created that accelerates the DNA-coated gold particles of varying sizes (1-3 µm) to a high speed. This force is sufficient to penetrate substantial physical barriers such as plant cell walls, cell membranes, and the stratum corneum of the mammalian epidermis (Yang et al., 1999).

The use of high velocity microprojectiles was initially developed for the delivery of nucleic acids into plant (onion) cells and was later extended to microbes and animals (Klein *et al.*, 1992). The main limitation of this process is the requirement of accessible tissue surface for application. Hence this method has thus far been largely used in gene transfer to the skin due to its physical accessibility. This method allows DNA to penetrate directly into the cytoplasm or nuclei and to bypass the endosome / lysosome, thereby avoiding enzyme degradation (Nishikawa and Huang, 2001).

A major application of the gene gun method is in genetic vaccination, and may be employed in combination with intramuscular injection of DNA or conventional protein antigens to achieve an optimal immunization effect (Yang *et al.*, 1999). Gene gun transfection was applied to the skin tissue of mice (Larson *et al.*, 1998), pigs (Macklin *et al.*, 1998), rhesus monkey (Fuller *et al.*, 1996), and horses (Lunn *et al.*, 1999), where humoral and cellular immune responses were elicited. These responses were seen to protect the animals from infection by viruses, bacteria, and parasites.

1.3.2   Electroporation and Nucleofection

Electroporation is a commonly applied technique to introduce DNA and other macromolecules such as antibodies and restriction nucleases into cells (Meaking *et al.*, 1995). This approach was pioneered in the 1960's by Coster, and has been used in microbiology and eukaryotic cell culture laboratories around the world (Vienken, 1978). Electroporation involves the application of brief but intense high voltage electric pulses to cells resulting in the formation of nanometer-sized pores in the cell membrane. The expression vector (DNA), or other molecules can enter the cytoplasm directly via the pores or as a result of redistribution of the membrane components following closure of the pores.

Electroporation depends on the field intensity, pulse duration, number of pulses and the direction of the field incident to the cell membrane (Meaking *et al.*, 1995). This is a

reproducible and a relatively simple technique, but seems to be restricted to *in vitro* gene delivery. The usage of high voltages can cause a fair amount of cell death, hence electronic parameters need to be optimized for each cell type. Electroporation was described in the delivery of mRNA encoding tumour antigens into human dendritic cells, suggesting an application in future dendritic cell-based tumour vaccines (Van Tendeloo *et al.*, 2001). This technique has also been applied to the skin (Titomirov *et al.*, 1991), the liver (Heller *et al.*, 1996) and muscle (Aihara and Miyazaki, 1998). From the tissues examined, skeletal muscle required mild electrical field strength of 100-400 V/cm while the skin and liver required shorter and more intense electrical pulses (Aihara and Miyazaki, 1998). The possibility that electroporation could give rise to DNA damage was investigated and was found to be partly dependent on the duration for which the current is applied (Meaking *et al.*, 1995).

Nucleofection is a novel transfection technology and a further development of electroporation. It is cell type-specific and depends on less harmful electric pulses and specific solutions optimized for specific cell types. Nucleofection transports DNA directly into the nucleus leading to rapid expression of the delivered gene within as low as forty minutes. The use of nucleofection has been reported recently in the delivery of small interfering RNA molecules (siRNA) into primary human cells (Chun *et al.*, 2002).

1.3.3   Direct / "Naked" DNA Delivery

The use of "naked" DNA is probably the simplist non-viral gene delivery system available. It was originally thought that "naked" / purified DNA could not enter eukaryotic cells due its large size and high negative surface charge. However it was first reported in 1990 that murine skeletal muscle could be transfected by injection of "naked" DNA (Wolff *et al.*, 1990). This muscle transfection ability has led to "naked" DNA being administered intramuscularly for vaccination purposes, where DNA encoding an antigen is administered with the aim of developing a protective immune response to the transgene antigenic product (Smith *et al.*, 1998).

Besides muscle, "naked" DNA has been applied to the liver (Zhang *et al.*, 1997), solid tumours, e.g. melanomas (Yang and Huang, 1996; Vile and Hart, 1993), skin epidermis, hair follicles (Yu *et al.*,1999) and urological organs (Yoo *et al.*, 1999). Although expression was achieved in direct cutaneous injection in patients suffering from lamellar ichthyosis, a genetic skin condition caused by the loss of expression of the enzyme transglutaminase, the pattern of expression was not uniform and failed to correct the histological and functional abnormality of the disease (Choate *et al.*, 1997).

Although direct transfer of DNA is not very efficient and gene expression levels are relatively low, immunization effects have been observed, making it sufficient for vaccination purposes. The transferred plasmid is usually maintained extrachromosomally in a circular form making expression variable. Immune responses produced have been shown to protect animals from a variety of live infectious agents, leading to a new class of therapeutic agents called "DNA vaccines" (Danko and Wolff, 1994). The mechanism for naked DNA-mediated gene transfer is not clearly understood as yet, but recent studies suggest that naked DNA is taken up by the parenchymal cells *in vivo* by an active, receptor-mediated process (Budker *et al.*, 1996). Application of "naked" DNA to the site of pathology and away from the degradative elements such as plasma is thus a viable strategy for gene therapy. However, it is ineffective if DNA dosing to anatomically inaccessible sites e.g. solid tumours in organs, is required (Brown *et al.*, 2001).

To improve DNA delivery across cellular barriers and to increase its stability of DNA against enzymatic degradation, DNA can be associated with or formulated with viral or non-viral delivery systems. The gene gun method and electroporation were developed to enhance gene transfer via "naked" DNA. These physical approaches also allow the DNA to penetrate the cell membrane and bypass the endosome / lysosome, avoiding enzymatic degradation. Intramuscular injection of plasmid DNA followed by electroporation produced impressive levels of gene expression in mice (Li and Huang, 2000). Recently, a single injection of a keratinocyte growth factor DNA encoded on a plasmid coupled with electroporation was found to improve and accelerate wound

closure in a delayed wound-healing mouse model (Marti *et al.*, 2004). The most common method to increase transfection efficiency of "naked" DNA involves the use of liposomes (Eck, 1999).

1.3.4   Cationic Polymers

Polymer based gene delivery systems are actively studied with greater interest focused on cationic polymers, although some neutral polymers eg. Polyvinyl pyrrolidone (PVP), have been utilized to improve gene transfer by direct intramuscular injection (Mumper *et al.*, 1996). Polymers bearing cationic groups are protonated at physiological pH and have been used most frequently. They have been widely accepted due to their ability to condense DNA and interact with cells often better than lipids. The complexes formed between cationic polymers and DNA are referred to as "polyplexes" (Felgner *et al.*, 1997 ; Ogris *et al.*, 1999). Cationic polymers complex with DNA and protect it against the action of nucleases. The cellular uptake of the plasmid DNA in "polyplexes" occurs by non-specific adsorptive endocytosis, similar to that of cationic liposomes (Nishikawa and Huang, 2001).

The cationic polymers produced to date are relatively poor in carrying DNA molecules across the membrane (Mansouri *et al.*, 2004). The most commonly used cationic polymers are polyethyleneimine (PEI) (Boussif *et al.*, 1995 ; Kichler *et al.*, 1999) (Figure 1.1) and poly-L-lysine (PLL) (Wu and Wu, 1987 ; Perales *et al.*, 1994) (Figure 1.2). Several novel cationic polymers have been synthesized, viz. gelatin (Leong *et al.*, 1998), polybrene (Mumper *et al.*, 1996), chitosan (Corsi *et al.*, 2003), poly (L-histidine)-graft-poly-L-lysine (Benns *et al.*, 2000) and oligoaminosiloxanes, e.g. Poly (alkylamino-siloxane) (Kichler *et al.*, 2003).
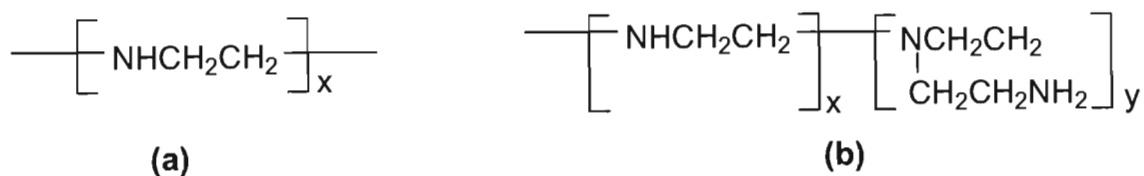
Figure 1.1 : Structure of (a) linear and (b) branched polyethyleneimines.
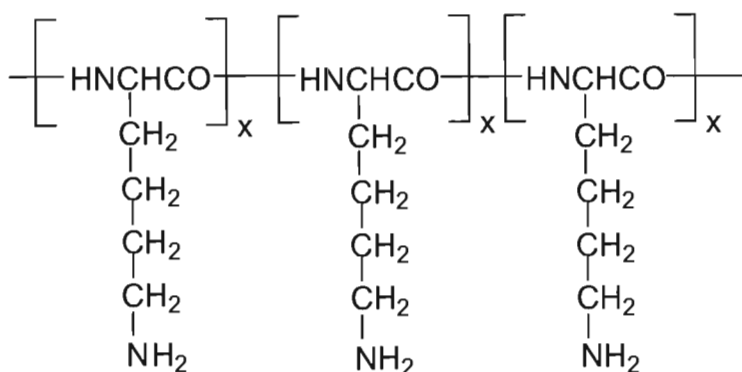
Figure 1.2 : Structure of poly-L-lysine

Polyethyleneimines exist as both branched and linear polymers (Figure 1.1). PEI is a polymer with one of the highest densities of amine functions. It has shown efficient gene transfer without the need for endosomolytic or lysosomotropic agents, such as chloroquine, to enhance receptor-mediated endocytosis (Brown *et al.*, 2001). Endocytosed PEI, whether administered with or without DNA, undergoes nuclear localization in the form of ordered structures (Godbey *et al.*, 1999). The transfection efficiency of PEI, depends largely on its molecular weight and isoform (branched or linear) (Fischer *et al.*, 1999). The addition of targeting ligands have enhanced activity in some cell lines, although some toxicity to cells has been reported (Boussif *et al.*, 1995).

Poly-L-lysine was the first polycation utilized for gene delivery and was conjugated to asialoorosomucoid for hepatocellular gene targeting (Wu and Wu, 1987). It has also been complexed to ligands such as transferrin (Cotton et al., 1990), and folate (Mislick et al., 1995). Gene transfer of poly-L-lysine polymers without the use of receptor-mediated strategies is poor unless endosomolytic or lysosomotropic agents are added (Brown et al., 2001). Poly-L-lysine polymers incorporating poly-L-histidine were also used to deliver genes to mammalian cells (Benns et al., 2000). Glycosylated and α-mannosylated polylysine were synthesized for gene transfer into normal human and cystic fibrosis tracheal gland serous cells, with the glycosylated polylysines ("glycofectins") showing greater efficiency than the α-mannosylated derivatives (Allo et al., 2000).

Chitosan [(1→4)2-amino-2-deoxy-β-D-glucan)] is obtained by alkaline deacetylation of chitin, a polysaccharide found in the exoskeleton of crustaceans and insects. It is a copolymer of N-acetyl-D-glucosamine and D-glucosamine. Chitosan is a non-toxic biodegradable polycationic polymer with low immunogenicity. DNA-loaded chitosan microparticles were also found to be stable during storage. Chitosan was shown to be able to enhance the transport of drugs across the cell membrane and has been used in the delivery for nasal, ocular and oral drug delivery to prolong contact time and improve drug absorption (Mansouri et al., 2004). Ligand modified chitosan for targeting of DNA to cell specific receptors has resulted in increased transfection efficiency (Sato et al., 2001).

Oligoaminosiloxanes are also able to condense DNA and deliver it to cells by adsorptive endocytosis. Unlike poly-L-lysine, the oligoaminosiloxanes are only partially protonated at physiological pH. These polymers display a pH- and substitution-dependent structure and are hence thought to be useful in controlled release applications (Kichler et al., 2003). The potential for cationic polymers used for gene therapy to adversely interact with host genes exists, and hence presents itself as a concern when using these methods to treat human diseases (Godbey et al., 1999).

1.3.5   Lipid based Gene Delivery Systems

Although gene expression can be achieved by direct intra-tissue injection of naked DNA, gene transfer via other routes of administration e.g. intravenous injection will require the use of a delivery vector or vehicle. Some of the more popular lipid based systems developed in the past include liposomes and non-liposomal cationic lipids or polymers.

1.3.5.1   Liposomes

The most widely used non-viral transfection system for gene therapy applications are lipid based artificial macromolecular complexes. These complexes generally involve liposomes formed from diverse synthetic and often novel and amphiphilic lipids in combination with helper lipids (eg. phosphatidylethanolamine), and sometimes natural and synthetic polycations (polylysine, polyethyleneimine) or hydrophobic oligo- and polycations (Zhdanov *et al.*, 2002).

Liposomes were first described by Bangham *et al.*, in 1965. It was found that when specific extracted lipids from egg yolks were placed in water, the lipids naturally organized themselves into microscopic spheres under appropriate conditions. The membranes enclosing these spheres resemble in appearance and function the biological membranes that enclose cells and constitute their boundary. Due to this similarity liposomes were initially used in studies as model membrane systems. They have also been used to investigate permeability parameters of various molecules and ions across the lipid bilayer. Later, however, the carrier potential of liposomes was realised. Various peptides and proteins such as insulin, immunoglobulins, and enzymes have been incorporated into liposomes and delivered to their target cells (Mukherjee *et al.*, 1978).

Liposomes have been described as assemblages of phospholipids and other lipids sustaining a bimolecular configuration and not requiring mechanical support for their

stability (Gregoriadis, 1976). Liposomes are vesicles consisting of one or more concentric lipid bilayers alternating with aqueous compartments, within which is entrapped a variety of lipid-soluble or water-soluble substances respectively. Liposomes can be characterized by their size as: small unilamellar vesicles (SUVs) that are up to 200 nm in size, large unilamellar vesicles (LUVs) , that are greater than 500 nm in size, and large multilamellar liposomes (MLVs) that are very much larger than 500 nm. However it has been reported that complexation of DNA with multilamellar liposomes of diameter of 300-700 nm mediated a two fold higher gene expression than those prepared with small unilamellar vesicles of about 50-100 nm (Felgner *et al.*, 1994).

Among the more conventional transfection methods, liposomes have become increasingly more popular and acceptable as a convenient and reproducible vehicle for DNA-mediated transfection. There are generally four major classes of liposome types viz. conventional liposomes (those that are anionic or neutral), cationic, 'stealth' or sterically stabilized liposomes (carry polymer coatings to obtain prolonged circulation times), and targeted liposomes (Storm and Crommelin, 1998). Most of the early studies on liposomes used anionic and neutral liposomes.

Transfection with anionic liposomes generally requires that the DNA be entrapped in the aqueous space of the liposomes. Improvements in liposome technology now permit the encapsulation of nucleic acids in liposomes. Liposomes encapsulating RNA and DNA can be introduced and genetic material expressed in mammalian cells (Fraley and Papahadjopoulos, 1981) and in plant cells (Fraley *et al.*, 1982 ; Gad *et al.*, 1990; Rosenberg *et al.*, 1988). This method however has not been widely used for gene transfer probably because encapsulation efficiency is variable and the actual process very time consuming. Neutral and anionic liposomes however are less toxic and more compatible with biological fluids and therefore may be suitable for systemic gene delivery (Li and Ma, 2001).

Steric stabilization of liposomes not only increases the colloidal stability of the lipid dispersion but it also promotes its biological stability. Stabilisation can be achieved by grafting or by absorption of a polymer to the liposomal surface. The so called grafting method is more commonly used and is achieved by incorporation of poly(ethylene glycol)-phospholipids. It was shown that poly(ethylene glycol) (PEG) grafting to liposomes hinders protein interaction with the liposome surface, whereas liposome-grafted dextran (a more rigid polymer) in similar quantities does not affect protein-liposome interaction (Torchilin *et al.*, 1994). Polymer stabilized liposomes can be thought of as structural mimics of red blood cells in which a relatively thick glycocalyx layer shields the lipid bilayer membrane (Chisti, 1999).

These sterically stabilised liposomes help prolong the circulation time of the liposome from minutes to days (about one hundred times longer than conventional liposomes) (Lasic and Papahadjopoulos, 1995), and also increases their efficiency in drug delivery. This is due to the apparent repulsive hydrophilic barrier around the liposomes provided by the covalently attached PEG, which prevents liposomes from cell adhesion and from being opsonised by proteins (Du *et al.*, 1997). Grafting of PEG into lipids or liposomes leads to the dehydration of the lipid headgroup region and a decrease in bilayer effects and enhanced lateral packing of the phospholipid acyl chains. All these factors contribute to steric stabilization (Barenholz, 2001). A typical PEG-liposome resembles a lipid bilayer with the grafted PEG moieties protruding from the surface and the hydrophobic tails of these molecules remaining inserted in the lipid bilayer (Kuhl *et al.*, 1998) (Figure 1.3). These liposome types will play an important role especially in *in vivo* gene transfer.

(a)

(b) DSPE-PEG
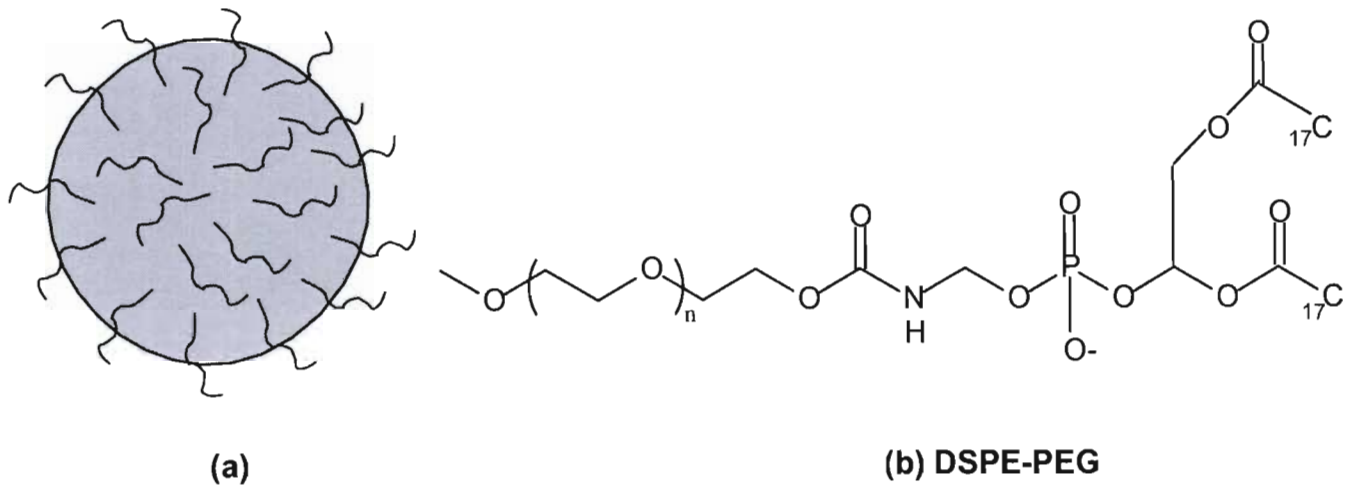
Figure 1.3 : Schematic representation of a sterically stabilised liposome with PEG (a). The common PEG-lipid DSPE(distearoylphoshatidylethanolamine)-PEG where n ranges from 17 to 114 in PEG molecular structure (b).

Another method employed to enhance the stability and circulation time of liposomes *in vivo* is via the use of pH sensitive liposomes. These pH sensitive liposomes help to mediate the introduction of highly hydrophobic molecules or macromolecules into the cytoplasm and generally contain phosphatidylethanolamine and titratable stabilizing amphiphiles. They are stable at pH 7.4 but undergo destabilization and acquire fusogenic properties under acidic conditions, leading to the release of their aqueous contents in the endocytotic pathway (Simões *et al.*, 2004).

1.3.5.2   Cationic Liposomes and Cationic Lipids

Cationic liposomes, on the other hand, do not require the DNA to be entrapped. DNA-liposome complexes can be easily prepared by simply mixing the liposome and DNA solutions and incubating the mixtures for a short while (Farhood *et al.*, 1992). Cationic liposomes tend to have favourable interactions with DNA which is bound to the external

16

layer of the liposome membrane due to the strong interactions between the cationic head groups of the lipid and the DNA (Behr *et al.*, 1989). Theoretically, there is no strict size limitation on DNA and almost 100% of the DNA can be recovered in a complexed form (Li and Ma, 2001). There is an extensive lipid rearrangement during complex formation, with resulting complexes bearing little resemblance to the starting or original liposome. Cationic liposome/DNA complexes have been simply referred to as 'lipoplexes' (Felgner *et al.*, 1997). 'Lipopolyplexes' can also be produced and are defined as being composed of vectors that have a polycation-condensed DNA coated with lipid bilayers The resulting net charge of the liposome-DNA complex (lipoplex) (positive charge) facilitates fusion with the negatively charged cell membrane (Li and Ma, 2001).

In addition to DNA, cationic liposomes can be used to deliver RNA (Malone *et al.*, 1989), proteins (Debs *et al.*, 1990), and antisense oligonucleotides (Jääskeläinen *et al.*, 2002). Among the non-viral vectors for gene transfer, cationic liposomes have intrinsic properties which make them attractive as vehicles for gene delivery. They are synthetic, and hence manufacturable to drug standards, biodegradable and seem to have the added advantage of low toxicity, higher efficiency, non-immunogenicity, easiness of use, and the design capability for cell-specific targeting. Furthermore cationic liposomes can interact with DNA to promote its transfection into both replicating and non-replicating cells (Birchall *et al.*, 1999). An understanding of the phospholipid-cationic lipid interactions is essential for rational development of the potential of cationic liposomes for gene /drug delivery.

Since the pioneering work of Felgner and coworkers in 1987 (Felgner *et al.*, 1987), who used a monocationic lipid, N-[1-(2,3-dioleyloxy) propyl]- N,N,N- trimethylammonium chloride (DOTMA), (Figure 1.4), in combination with a neutral zwitterionic phospholipid, dioleoyl-phosphatidylethanolamine (DOPE), several other related compounds or formulations have been reported and have proven to be valuable tools in gene delivery (Behr,1994). Many of these cationic lipid formulations are now available commercially.

All cationic lipids have four functional domains viz. a positively charged head group(s), a spacer of varying length, a linker bond and a hydrophobic anchor (Figure 1.5)
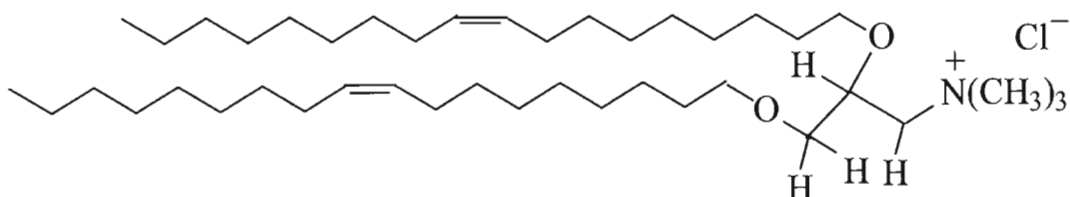
Figure 1.4 : Structure of DOTMA
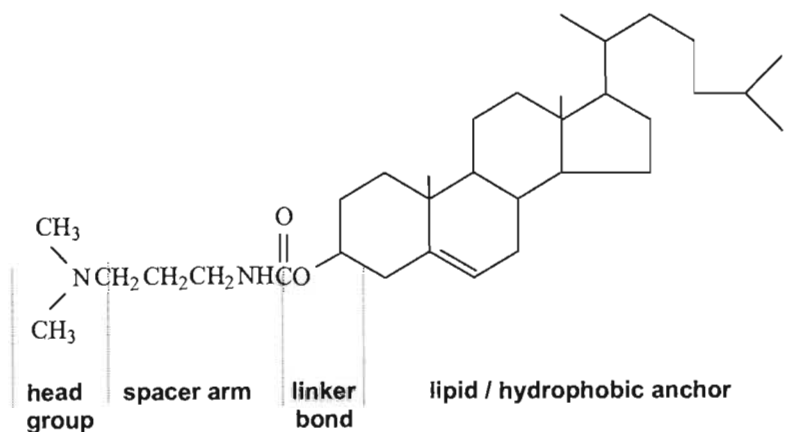
Figure 1.5 : An example of a cationic cholesterol derivative (CHOL -T) defining the four components of a typical cationic lipid.

The lipid anchor is usually a fatty acid chain or a cholesterol ring . The linker bond determines the lipid's stability and biodegradability. The length of the spacer arms differs in various lipid preparations and may be as critical as the nature of the head groups for transfection activities. The hydrophobic component ensures that the cationic

lipids assemble into lipid bilayer vesicles on dispersion in aqueous media, shielding the hydrophobic portion of the molecule and exposing the head group to the aqueous medium. The head group is the DNA binding moiety of the liposome (Brown *et al.*, 2001).

Based on the differences in the hydrophobic anchor, cationic lipids can be grouped into three different categories: cholesterol-anchored lipids eg. 3β[N-(N',N'-dimethylaminopropane)-carbamoyl]cholesterol (Chol-T) (Figure1.5) (Singh *et al.*, 2001b), and 3β[N,N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-CHOL), (Figure 1.6), (Gao and Huang, 1991); double chain hydrocarbon-anchored lipids eg. N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulphate, (DOTAP) (Figure1.7), dimyristyloxypropyl-3-dimethyl-hydroxyethylammonium bromide (DMRIE), (Harrison *et al.*, 1995), 1,3-dioleoyloxy-2 (6-carboxyspermyl) - propylamide (DOSPER), (Figure 1.8) (Buchberger *et al.*,1996), (+)-N-(3-aminopropyl)-N,N-dimethyl-2,3-bis-(dodecyloxy)-1-propanaminium bromide (GAP-DLRIE), (Figure 1.9), (Wheeler *et al.*, 1996); and 2,3-dioleyoxy-N-[2(sperminecarboxamido) ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), (Harrison *et al.*, 1995); and single chain hydrocarbon anchored lipids, eg. dodecyltrimethylammonium bromide (DTAB), cetyltrimethylammonium bromide (CTAB), and tetradecyltrimethylammonium bromide (TTAB) (Figure1.10) (Li and Ma, 2001).

Single chain hydrocarbon anchored lipids are commonly known as detergents and generally too toxic to be used alone in transfection and are therefore mixed with DOPE, a neutral helper lipid, to decrease toxicity (Pinnaduwage *et al.*, 1989). Single chain cationic lipids are generally less efficient than double chain cationic lipids and are hence not suitable for *in vivo* gene transfer. The lipids currently under investigation and ones used in liposome formulations in this thesis are cholesterol anchor-based cationic lipids.
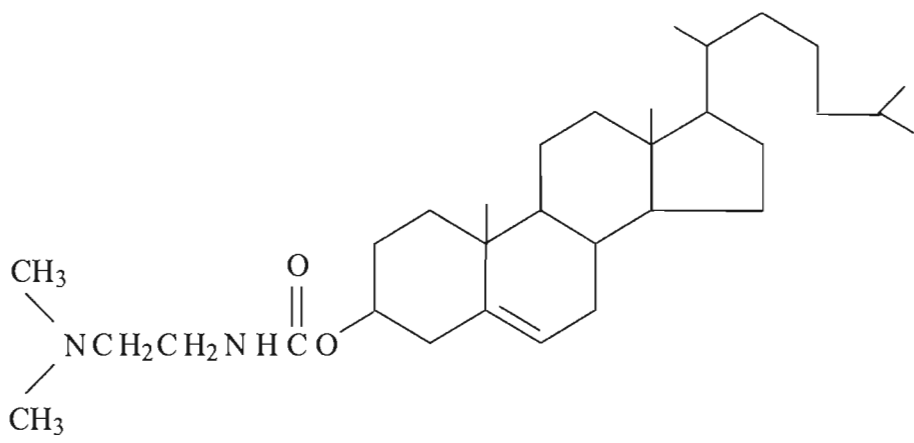
Figure 1.6 : Structure of DC-Chol
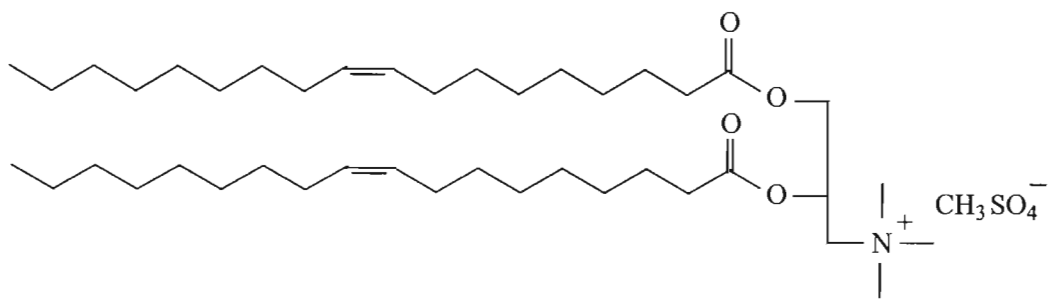
Figure 1.7 : Structure of DOTAP
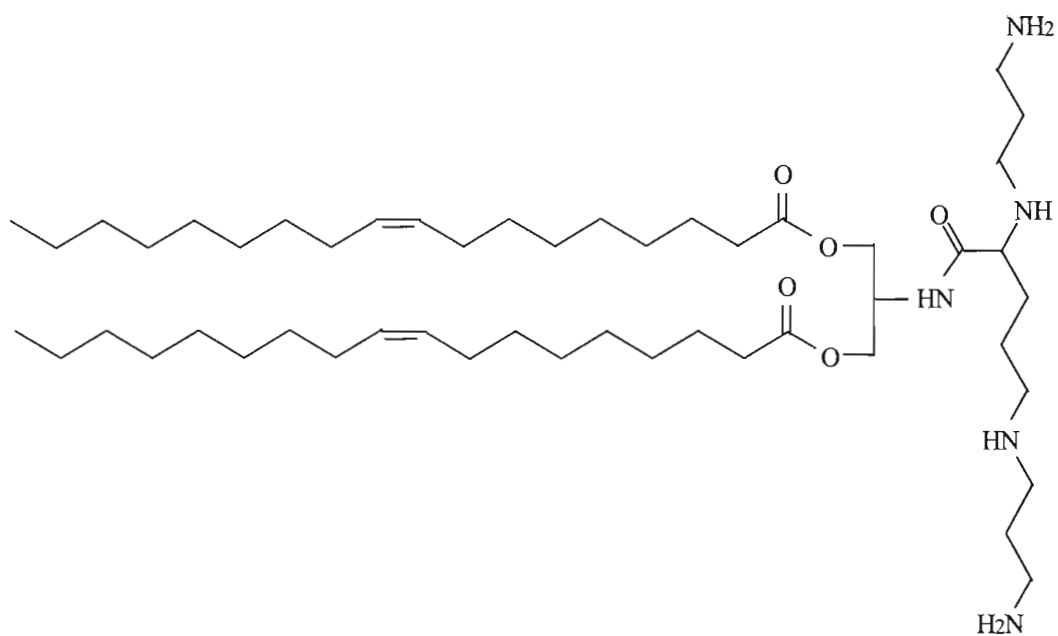
Figure 1.8 : Structure of DOSPER
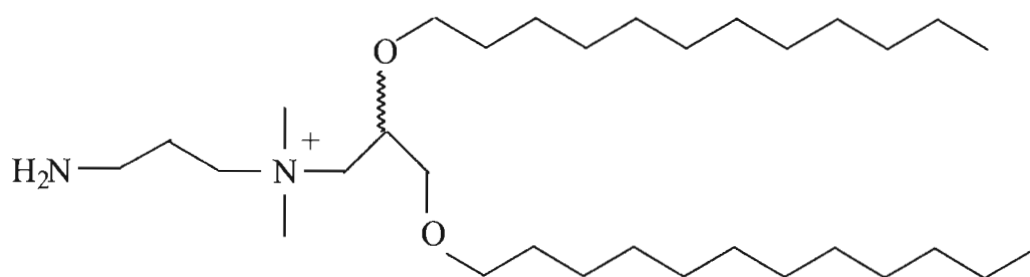
Figure 1.9 : Structure of GAP-DLRIE
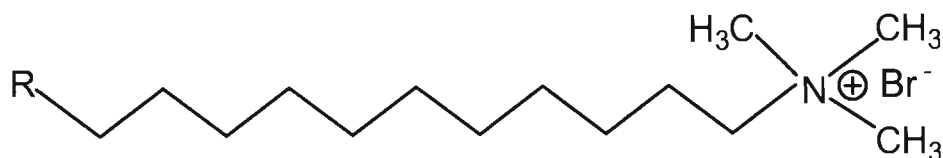
DTAB : R = H
TTAB : R = CH_3CH_2
CTAB : R = $CH_3(CH_2)_3$

Figure 1.10 : Structure of single chain hydrocarbon-anchored lipids

Two lipopolyamines, dioctadecylamidoglycyl-spermine (DOGS), (Figure 1.11), and dipalmitoyl-phosphatidylethanolamido spermine (DPPES), (Figure 1.11), were also synthesised for delivery of DNA into eukaryotic cells (Behr *et al.*, 1989). In these polyamines, the headgroups interact strongly with the minor groove of DNA. A nucleosome type structure is formed in which the DNA molecule is packaged. DOGS has a higher cationic charge number in its headgroup than DOTMA, hence favouring a stronger interaction with the negative DNA, resulting in a compact DNA-DOGS complex.

DOTMA, on the other hand, forms liposomes in aqueous solutions which aggregate spontaneously with negatively charged DNA molecules (Fortunati *et al.*, 1996). The combination of equimolar quantities of DOPE and DOTMA was widely used in liposome formulations for lipofection (Felgner *et al.*, 1994). Liposomes containing DOTMA have been formed to facilitate transfection of cultured mammalian cells with recombinant DNA vectors to a greater extent than conventional transfection techniques ((Felgner *et al.*, 1997).
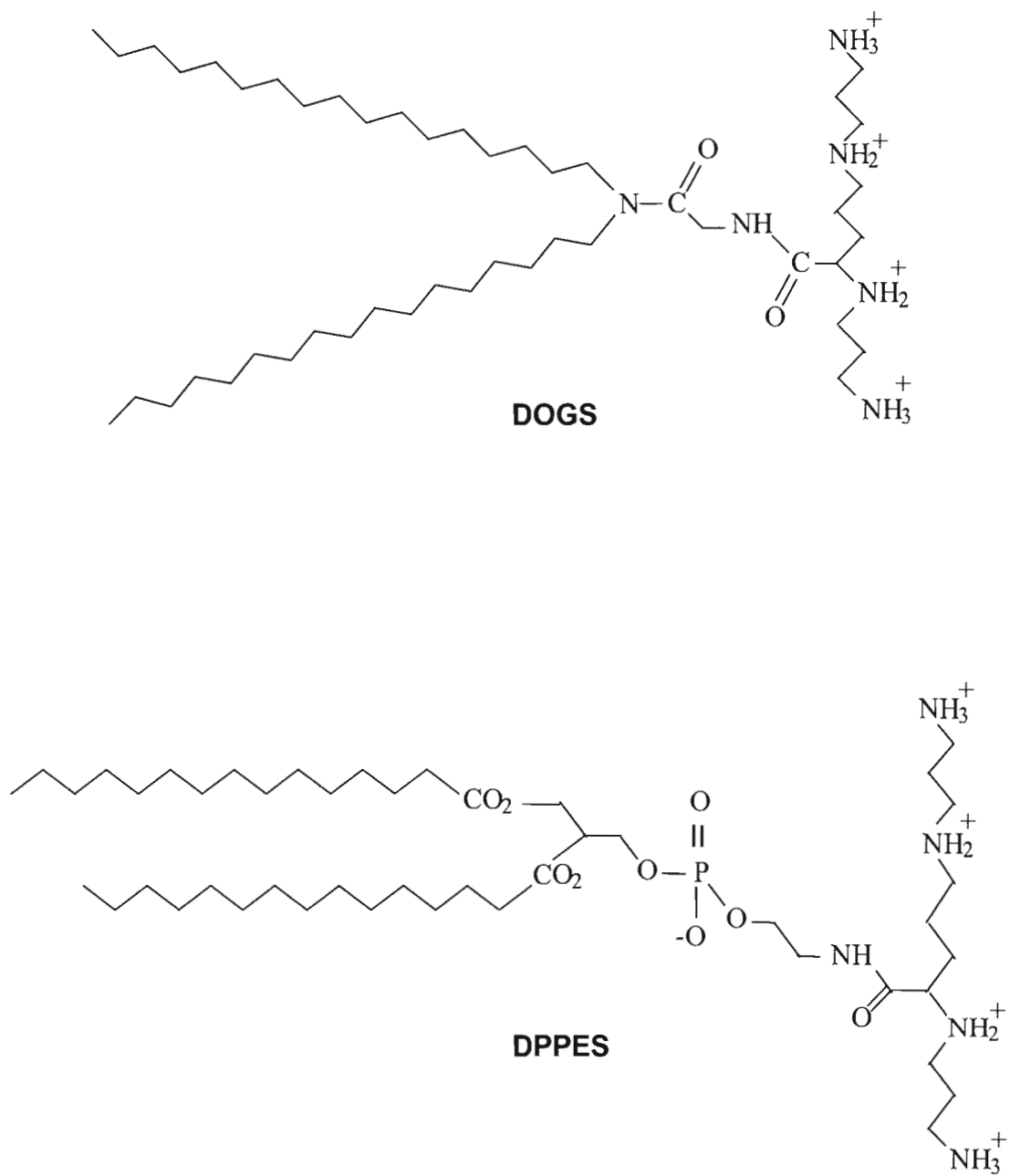
Figure 1.11: Structure of lipopolyamines DOGS and DPPES

Since their introduction in the late 1980's, cationic liposomes such as DOTAP were quickly and widely adopted as convenient, reliable and flexible tools for gene transfer studies in mammalian cells. DOTAP has been used with varying degrees of success in more than fifty different cell types, to introduce DNA, RNA, oligonucleotides and peptides into cells. However it was found to have a deleterious effect on erythrocytes (Uchegbu *et al.*, 1998) and macrophages (Filion and Phillips, 1998). DOTAP differs from DOTMA in that its two oleic acid moieties are in ester linkage to the propyl moiety whereas in DOTMA the two oleyl chains are in ether linkage to the propyl backbone. The ester bonds are inherent to natural lipids and DOTAP offers high transfection efficiency and lower toxicity. This is probably due to the fact that cellular lipases or esterases easily degrade this artificial lipid (Buchberger *et al.*, 1996). It has been found to exert a potent destabilizing effect on the lipid bilayer physical properties which may be important in drug delivery (Campbell *et al.*, 2001). DOTAP-based liposomal carriers were found to be more effective in the delivery of antisense oligonucleotides than cationic polymer-based systems (Jääskeläinen *et al.*, 2002).

The use of DOTMA is restricted due to its toxicity to treated cells, and also the cost of the reagent. DOSPER, on the other hand, is a polycationic amphiphile with ester bonds similar to DOTAP, which can be biodegraded by the target cells. DOSPER, with fatty acids connected to the 1 and 3 positions and the polyamine component attached to the 2 position of the propane backbone, also forms liposomes (Buchberger *et al.*, 1996). DMRIE is a polycationic lipid and has been used complexed to DOPE for *in vivo* gene delivery in mice and in clinical trials to deliver genes into cutaneous tumours in late stage melanoma patients (Nabel *et al.*, 1993).

GAP-DLRIE was found to enhance plasmid DNA delivery and expression in mouse lung cells (Wheeler *et al.*, 1996). DC-CHOL contains a cationic lipid component which is a tertiary amine conjugated to a cholesterol group via a short spacer arm and a carbamoyl bond. It is stable at $4^{\circ}C$ and has been used in clinical trials for cancer gene therapy. Farhood *et al.* (1992) synthesised four different cationic cholesterol derivatives containing tertiary or quaternary amino head groups, with and without succinyl spacer

arms. They found that the tertiary amine derivatives showed greater transfection activities and were less toxic to the cells than the quaternary ammonium derivatives.

Most cationic lipid reagents are formulated as liposomes containing two lipid species viz. a cationic amphiphile and a neutral lipid such as DOPE which is important in facilitating membrane fusion during cationic lipid mediated DNA delivery. The term "cytofectins" describes this class of positively charged lipids. Cationic lipids can have monocationic head groups eg. DOTMA, DOTAP, DC-CHOL, or polycationic head groups eg. DOSPA and DOGS (Felgner et al., 1994). Multivalent cationic lipids are thought to condense DNA more strongly than monovalent cationic lipids in vitro.

Successful gene transfer is a combination of delivery and expression of the foreign gene in the target cell (Gao and Huang, 1993). The cationic lipids discussed usually provide non-specific targeting to the negatively charged (sialic acid bearing) components of the cell surface. Inclusion of lipids such as DOPE that have a low transition temperature are thought to be important for the activity of these cationic lipids (Cotten and Wagner, 1993). DOPE is a common helper lipid used in the formation of cationic liposomes, except in DOGS-based liposomes. In most cases an equimolar mixture of cationic lipid and DOPE ensures optimal transfection efficiency. The liquid-crystalline nature of the DOPE / lipid bilayer promotes efficient binding and penetration of plasma proteins into the liposomal structure (Felgner et al., 1994).

Although the use of cationic liposomes for in vitro gene transfer has become common-place, whole organism studies are increasing in number. An early study has shown that injected DNA complexed with a cationic liposome (lipofectin i.e. DOTMA:DOPE) mediated transfection in the mouse (Brigham et al., 1989). Cationic liposome-DNA complexes were also expressed after intra-arterial administration (Nabel et al., 1990) and aerosol inhalation (Stribling et al., 1992). Some liposomal formulations have undergone clinical evaluation as vectors for gene therapy in cancer (Nabel et al., 1993), and cystic fibrosis (Mclachlan et al., 1996).

1.4   CATIONIC LIPOSOME-DNA INTERACTION

Several techniques have been employed to study the organization of DNA in lipoplexes. The cationic liposome DOTAP:DOPE has been commonly utilized in these studies. DNA / lipid association is extremely rapid and occurs within one minute after mixing (Gershon *et al.*, 1993). The DNA / lipid complex will continue to change over time in a maturation process. The lipoplex structure is determined by a number of factors, including the structure or type of cationic lipid used, the charge ratio between the cationic lipid and the DNA, the concentration of lipid and DNA used, time allowed for lipoplex formation, the temperature at which lipoplexes are preformed, the nature of the solvent or buffer system employed during sonication or extrusion of liposomes and the helper lipid utilized (Bennett *et al.*, 1996). Depending on the pKa of the cationic lipid, the pH may also affect the interaction of cationic lipids with DNA and the structure of the resulting complex. Incubation of cationic liposomes with DNA usually leads to a heterogeneous population of particles (Zabner *et al.*, 1995).

Studies have shown that lipoplexes generated from SUV's are two phase systems over most lipid / DNA charge ratios, but are single phase systems for LUVs and MLVs over a broad range of mixing ratios (Concalves *et al.*, 2004). Small angle X-ray scattering (SAXS) has shown the coexistence of lamellae and hexagonal lipid-DNA domains in lipoplexes. Atomic force microscopy (AFM) has revealed that even in lipoplexes with an excess cationic charge, there still exist regions of DNA not covered by cationic lipid. Circular dichroism (CD) was employed to investigate secondary and tertiary DNA structures in complexes, and it was found that a highly condensed form of DNA is generated known as Ψ⁻DNA, a form which is easily promoted by cationic liposomes (Simberg *et al.*, 2001).

The absolute structure of the DNA within lipoplexes is under discussion. It was previously assumed using CD data, that DNA is converted from its B form to the C form in complexes (Zuidam *et al.*, 1999 ; Simberg *et al.*, 2001), but recent investigations using vibrational spectroscopy have revealed that the DNA maintains its B form

conformation, but tends to become dehydrated at the liquid interface. This results in the apolar region of the lipid bilayer becoming fluidized upon the binding of DNA (Choosakoonkriang *et al.*, 2001). Furthermore, supercoiled plasmid DNA seems to undergo a significant loss of rotational strength upon interaction with a cationic lipid. The use of Fourier transform infrared (FTIR) and Raman spectroscopy have shown that these changes occur within the B type conformation of DNA and also within short stretches of DNA. It appears that lipid / DNA interactions extend far beyond direct electrostatic coupling between the lipid headgroup and the phosphate backbone, to alteration in base stacking interactions as well as hydrogen bonding (Braun *et al.*, 2003).

Almost all aspects of the structure of lipid / DNA complexes and of gene transfer mechanisms are connected to electrostatic interactions and the important role of the charge i.e bioelectrochemistry, since it is generally accepted that cationic liposomes interact with DNA through charge attraction. Electrostatic interaction and hydrophobic bonding are proposed as driving forces for complex formation. Hydrophobic forces between lipid side chains predominate but upon mixing with DNA, electrostatic interactions draw the positive surface of the cationic liposomes together with the negative phosphate backbone of the plasmid (Brown *et al.*, 2001).

The formation of the liposome-DNA complex involves two processes viz. a fast exothermic process attributed to the electrostatic binding of DNA to the liposomal surface and a slower endothermic process caused by the fusion of the two components and their rearrangement into a new structure. These two processes result in an homogeneous and physically stable suspension being formed (Zhdanov *et al.*, 2002). The thermodynamic driving force for lipid / DNA association is the entropy increase from the release of counterions and bound water associated with the plasmid DNA and the lipid surface. A similar result was seen for DNA that occured in the linear form (Kennedy *et al.*, 2000). Several models have been proposed for the structure of cationic liposome-DNA complexes. These will be discussed further in chapter two.

1.5 MECHANISM OF LIPOFECTION

Despite the enormous amount of work using liposomes as drug and gene carriers, the actual molecular mechanism of the liposome-cell interactions (lipofection) has not been fully elucidated. Lipofection involves formation of the DNA-liposome complex, the entry of the complex into the cell, escape of the DNA from the endosomal compartments and finally entry into the nucleus (Kennedy et al., 2000). Liposome uptake by different cells has a varying dependence on liposome composition, eg small mole percentages of negatively charged phospholipids viz. phosphatidylserine (PS), phosphatidylglycerol (PG) etc., in the liposome enhanced uptake twenty fold when compared to uncharged liposomes in CV 1 cells (Straubinger et al., 1983). The molecules on the cell surface that are responsible for interaction with lipoplexes are also not well defined. However proteoglycans on the cell membrane appear to play an important role, and cells deficient in proteoglycan synthesis are more difficult to transfect (Li and Huang, 2000).

Various methods have been postulated over the years (Figure 1.12), but recently two pathways have emerged as the main route of entry of liposome-DNA complexes viz. direct fusion with the cellular membrane (Felgner et al., 1987), and endocytosis with subsequent destruction of an endosome within the cell (Zabner et al., 1995 ; Leventis and Silvius, 1990). It has been suggested that the hydrophobic moiety of the cationic lipid is involved in cell membrane interaction, membrane passage, and escape from the endosome (Wetzer et al., 2001). Adhesion of cationic liposome complexes onto the negatively charged outer membrane of the cell occurs through electrostatic interactions (Hui et al., 1996). It was also proposed that DNA delivery and release from cationic liposome complexes occurs via anionic lipid exchange with the cytoplasmic surface of the cell membrane. (Xu and Szoka, 1996).
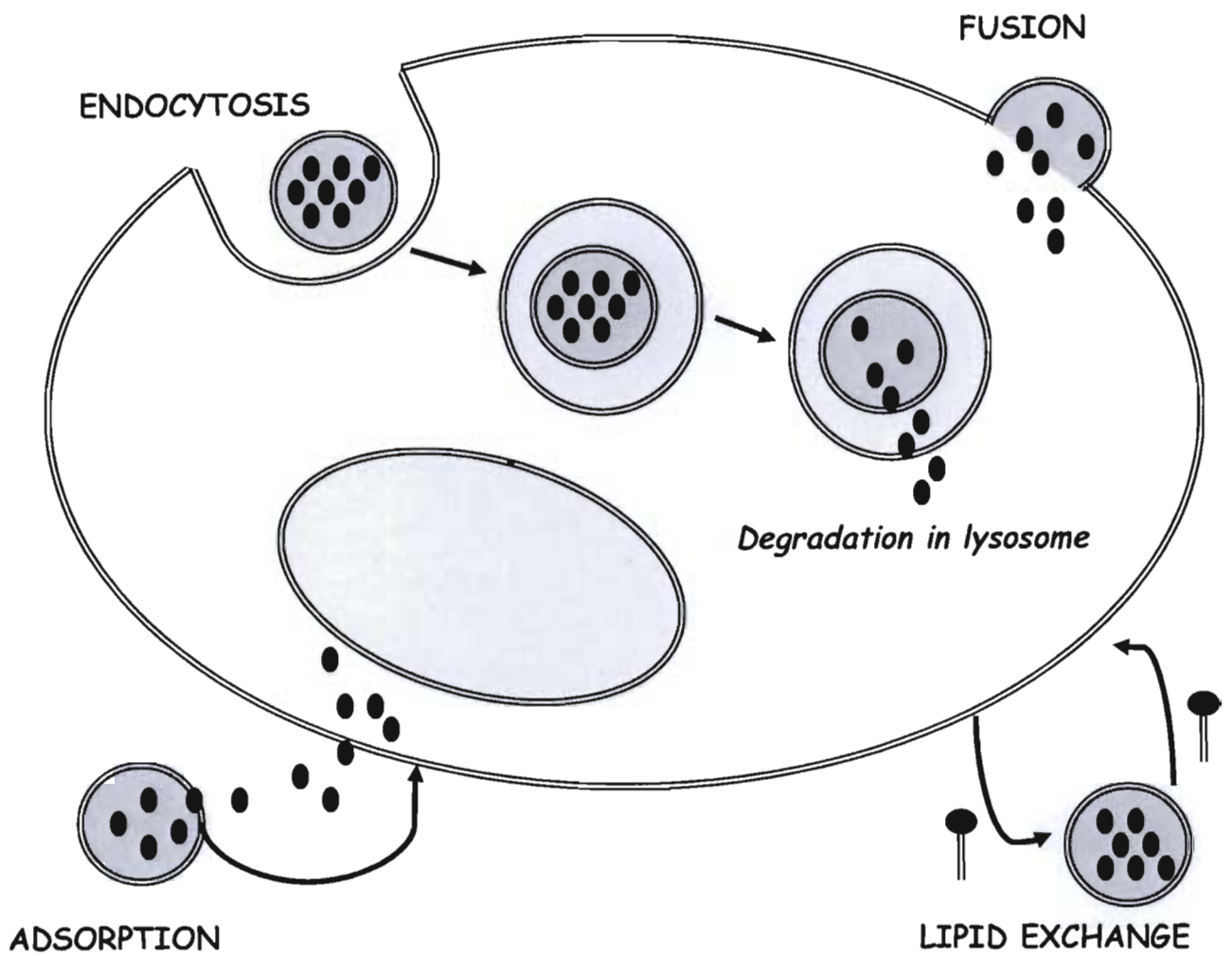
FIGURE 1.12 : Four different interactions of liposomes and cells : fusion, endocytosis, adsorption, and lipid exchange (adapted from Lasic,1997). The methods of fusion and endocytosis are the main postulated mechanisms of liposome delivery.

In an early study by Papahadjopoulos *et al.*, 1973, it was suggested that the liposome membrane fuses with the plasma membrane. It was proposed that fusion depended on the phospholipid composition of the liposome and also on the phase in which it exists eg. a liquid crystalline phase produces greater fusion than a gel phase. This mechanism postulates that the liposome membrane fuses with the plasma membrane of two adjacent cells, hence providing a bridge between the two cells. However lipid mixing assays did not reveal much correlation between fusion events of lipoplexes and cellular membranes and their transfection efficiency (Zhou and Huang, 1994).

Although fusion of liposomes with cell membranes is a proposed mechanism of DNA delivery by cationic liposomes it is more likely that the liposome-DNA complex enters the cell by endocytosis (Pinnaduwage *et al.*, 1989). It was found that most complexes are internalized by endocytosis and only 2% of the cells are transfected via direct complex membrane fusion (Zhou and Huang, 1994). Plasmid DNA, like all endocytosed molecules, travels after internalization in the cell via an endosomal compartment where it is degraded. However for a transfected gene to be expressed, the intact DNA molecule must cross either the endosomal or lysosomal membrane into the cytosol and finally into the nucleus (Wattiaux *et al.*, 2000). Once in the cell, liposomes then encounter a low pH compartment (Straubinger *et al.*, 1983). It is generally accepted that the liposomes and bound DNA enter the lysosomes.

Evidence for endocytosis is based on application of inhibitors eg. wortmannin, chloroquine, monensin and NH_4Cl (Simões et.al., 1999b; Zuhorn *et al.*, 2002). The use of inhibitors of endocytosis indicated that fusion occurred primarily at the plasma membrane. These inhibitors also inhibited transfection, further indicating that endocytosis is the primary route of gene delivery, despite fusion at the plasma membrane. However the precise effects of inhibitors are difficult to interpret as both increases and reductions in transfection efficiency have been observed (Zuhorn *et al.*, 2002). Electron microscopic studies of cells incubated with liposomes containing gold have revealed that the liposomes are taken up via endocytosis in coated pits (Straubinger *et al.*, 1983). This was supported by confocal laser scanning microscopy

(CLSM) and atomic force microscopy (AFM). These techniques also revealed that the cationic liposome:DNA complexes were transferred to lysosomes by a microtubule-mediated pathway and that membrane fusion between cationic liposomes and the endosome membrane leads to the displacement of DNA from the cationic liposome (Nakanishi, 2003).

Noguchi *et al.* (1998), provided evidence that cationic liposome:oligonucleotide complexes fused with the endosome and at low pH the oligonucleotide was released into the cytoplasm. In addition microtubules were found to be involved in the intracellular dynamics of gene transfection (Nakanishi and Noguchi, 2001). The delivery mechanism of the liposomes involve the destabilisation of the liposome bilayer in the late endosome at acidic (pH 5.0 to 6.3), and destabilisation and fusion of the vesicle membrane within the endosome. The intracellular fate of the delivered DNA is still poorly understood (Legendre and Szoka, 1992). Furthermore the plasmid DNA must dissociate from the cationic liposome complex prior to entry into the nucleus of the cell, since cationic lipid:DNA complexes microinjected directly into the nucleus exhibit a low transfection efficiency (Zabner et.al., 1995; Alino *et al.*, 2000). This has led to the development of pH-sensitive liposomes that could destabilize and undergo membrane fusion at mildly acidic pH and also sufficiently destabilize the endosomes to deliver their macromolecules into the cytoplasm. Unfortunately one of the drawbacks of pH-sensitive liposomes is their rapid removal from circulation *in vivo* (Torchilin *et al.*, 1993).

Various approaches such as the use of fusogenic peptides have been employed to facilitate release of DNA into the cytoplasm. These are unusually water soluble at physiological pH and are converted to an amphipathic alpha helix at low pH. Upon association with the lipid bilayers they trigger pH-dependent fusion of small liposomes. When co-delivered with a lipoplex, they disrupt the endosome and improve cytoplasmic delivery of DNA (Simões et.al., 1998; Li and Ma, 2001). In the case of the polycation PEI, a proton sponge mechanism has been proposed in which the PEI buffers the acidification by sequestration of protons and counterions, resulting in osmotic swelling and subsequent rupturing of the endosomes (Boussif *et al.*, 1995).

It was recently shown that cholesterol depletion results in the inhibition of clathrin-mediated endocytosis, whereas non-clathrin mediated endocytosis is much less affected. Decrease in clathrin-mediated endocytosis is caused by the disability of the coated pits to invaginate and detach from the plasma membrane. A clathrin independent pathway, of which caveolae form a part, may also be a delivery route. It is a pathway commonly utilised by viruses. Caveolae generally cluster to form vesicles called caveosomes. The drugs, filipin III and chlorpromazine have been used to distinguish between clathrin-mediated and caveolae-mediated internalization of lipoplexes. Filipin III is a sterol-binding pentaene macrolide antibiotic, that inserts into lipid membranes containing cholesterol. It inhibits the caveolae invagination by formation of cholesterol precipitates, leaving the coated pits unaffected. Chlorpromazine on the other hand, is a cationic amphiphilic drug that causes localization and accumulation of clathrin in late endosomes, thereby inhibiting coated pit endocytosis.

Only a fraction of lipoplexes are internalized via caveolae, and eventually lipoplexes accumulate into the lysosomal compartment, which is the end of an endocytotic rather than a caveolae-mediated internalization. Hence clathrin-mediated endocytosis is the major pathway for lipoplex internalization (Simões et.al., 1999b; Zuhorn *et al.*, 2002). Clathrin is involved in two crucial transport steps viz. receptor-mediated and fluid phase endocytosis for plasma membrane to early endosomes; and transport from the trans-Golgi network (TGN) to the endosomes (Marsh and McMahon, 1999).

1.5.1 Receptor-Mediated Endocytosis

Liposomes targeted to receptors on the cell surface usually follow the receptor-mediated endocytotic pathway. Receptor-mediated endocytosis is an effective method for gene delivery into target cells. It is a natural process that cells utilise for the uptake of proteins or peptides such as asialoglycoproteins, transferrin, epidermal growth factor, insulin, low density lipoproteins, diptheria toxin and also vitamins such as folic acid, and

viruses. One advantage of the receptor-mediated endocytosis of DNA is the ability to target DNA to specific cells by coupling the DNA-binding vector to cell specific ligands, and thus generating a cell specific DNA-delivery conjugate (Cotten *et al.*, 1990).

The first step in receptor-mediated endocytosis involves the binding of the ligand to its cognate cell surface receptor (Figure 1.13). Subsequently, the receptors undergo a conformational change which leads to the clustering of the receptor-ligand complexes in coated pits located on the plasma membrane (Wagner *et al.*, 1994). These pits are lined on the intracellular surface by a clathrin triskelion structure comprising three polypeptide heavy chains (190 kDaltons) and three polypeptide light chains of approximately 25 kDaltons each. These triskelions oligomerise *in vivo* and *in vitro* to produce polygonal arrays generally seen under electron microscopy. In cultured cells, the assembly of a clathrin coated vesicle during endocytosis, takes about one minute and several hundred to a thousand or more can form every minute (Marsh and McMahon, 1999). Receptor internalization is actively coupled to the formation of coated pits. The formation of clathrin is the driving force behind membrane invagination (Mousavi *et al.*, 2004).

Movement of the clathrin molecules results in deepening of the pits and the clathrin-associated membrane being "pinched off" (scission) as a clathrin coated vesicle (CCV). This invagination process requires the presence of G-proteins and ATP. Upon the removal of the clathrin coat, the vesicles fuse with and become part of the acidic endosomal compartments. The endosome is converted into a CURL (compartment of uncoupling of receptor and ligand) vesicle. The ligands and the receptors have different fates. Receptors can either be destroyed in a degradative pathway, or be brought back to the cell surface in a recycling pathway (Cotten *et al.*, 1993). The endosome combines with lysosomes and the ingested material is degraded by the lysozymes. Degradation products may be released into the extracellular fluid by the process of exocytosis.

Receptor-mediated endocytotic pathways differ according to the type of receptor-ligand pair. Low endosomal pH may or may not trigger dissociation of the receptor and the ligand. In the case of transferrin, the iron loaded transferrin releases the iron at low

endosomal pH, while the apotransferrin remains bound to the receptor. Other ligands and receptors also dissociate under these conditions (Cotten *et al.*, 1990). Endocytotic vesicles formed are usually transported to the lysosome, where the contents are degraded. The hydrolysis of the vector is a prerequisite for the endocytosed DNA to quickly reach the lysosomes. Such a hydrolysis would take place in the endosomal compartment that contains peptidases (Berg *et al.*, 1995). The asialoorosomucoid (AOM) endocytotic pathway is ultimately degradative, with the delivery of the ligand to the lysosomes, early in the internalisation process (Tolleshaug *et al.*, 1979). Hence effective delivery of DNA into cells via receptor-mediated endocytosis depends upon the escape of DNA from the endosome during transport (Lyerly, 1993). It has been proposed that pore formation in the endosome may be a possible mechanism of escape for the complexes or of free DNA into the cytoplasm (Simões *et al.*, 1999a). The DNA must finally enter the nucleus to ensure gene expression.

It is presumed that lipoplexes bind non-specifically to cellular receptors that become clustered in coated regions and are internalized. Internalisation of ligand-receptor complexes are generally rapid (about one minute), but internalization of lipoplexes is a relatively slow process. This could be due to possible restraints placed on lipoplexes to enter coated vesicles. Hence the rate limiting step includes the rate of association of the receptors and the lipoplexes, and the extent to which these receptors can non-specifically associate with the lipoplexes (Zuhorn *et al.*, 2002).
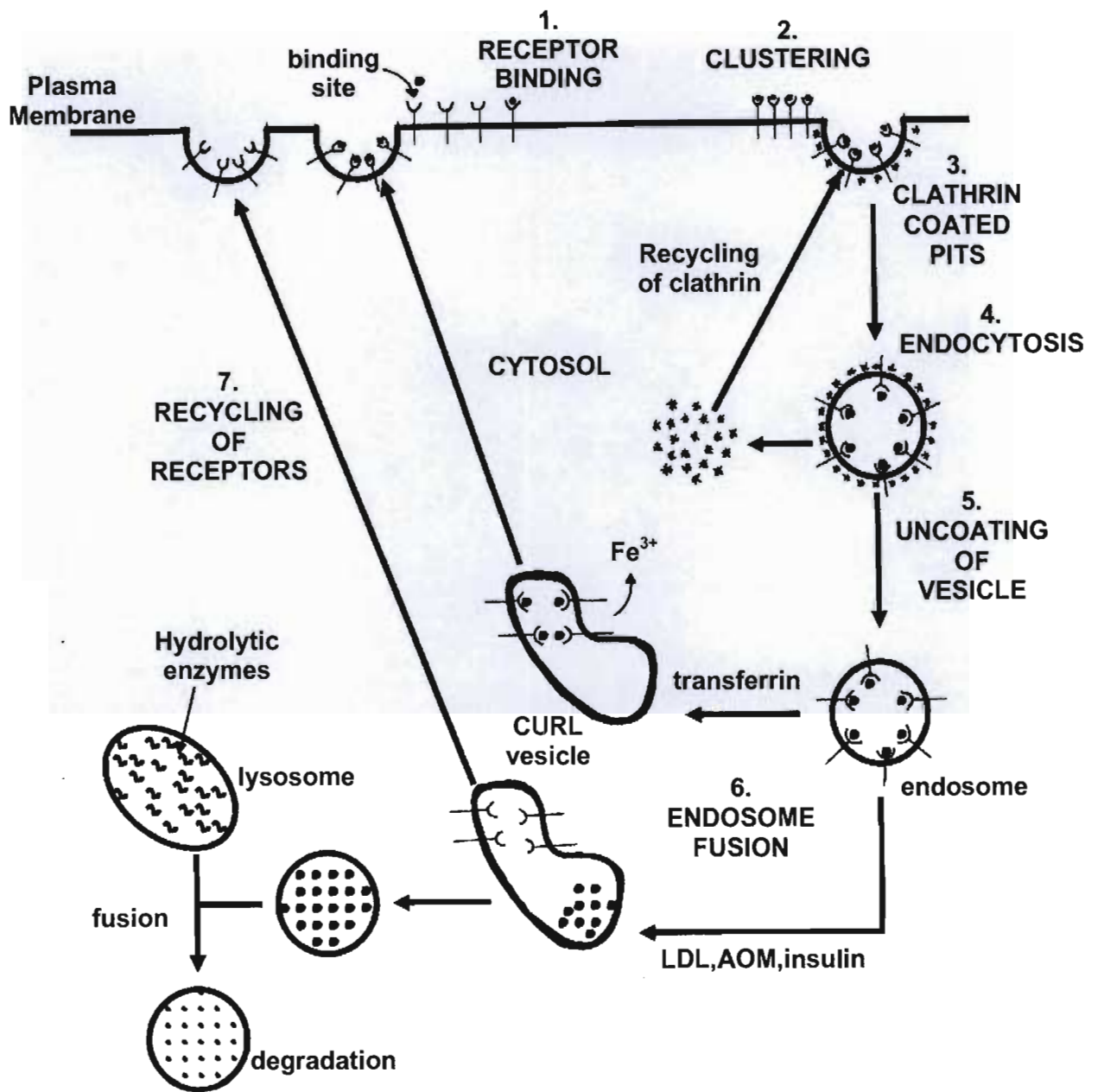
Figure 1.13 : Receptor-mediated endocytosis of hormones , growth factors, AOM , transport proteins and low density lipoproteins (LDL). In the case of transferrin the iron is released, and both the ligand and the receptor are recycled to the cell surface. In the case of insulin , LDL and AOM, the ligand is delivered to the lysosome while the receptors are recycled to the cell surface.

1.5.2 Nuclear Uptake

Ultimately, the DNA must be delivered to the nucleus for transcription of the transgene to take place. The mechanisms of DNA nuclear translocation are not fully understood but appear to depend on the type of delivery vehicle employed (Weithoff and Middaugh, 2003). The efficiency with which the DNA is imported into the nucleus also depends on its size and conformation. Linear plasmid DNA seems to be translocated more efficiently into the nucleus than other conformations of plasmid DNA (Li and Ma, 2001). The nuclear membrane is a barrier that prevents the uptake of most macromolecules greater than 70 kDaltons into the nucleus, unless they are able to interact with the nuclear pore active transport system. The actual pore size in the nuclear membrane is about 55 Å in diameter (Li and Ma, 2001). Poor access of plasmid DNA into the nucleus represents a major barrier to the success of non-viral gene therapy (Pouton and Seymour, 2001).

Three possible routes for DNA transport into the nucleus have been proposed. The DNA can pass into the nucleus through the nuclear pores, it can become physically associated with chromatin during mitosis when the nuclear envelope breaks down, or it could traverse the nuclear envelope. The latter mechanism (or "route") seems to be the most widely accepted, but there exists little experimental support for this proposed route of entry (Weithoff and Midduagh, 2003). It is assumed that the crossing of large molecules across the nuclear membrane is facilitated by a nuclear pore complex (NPC), which is a supramolecular aggregate of about 124 megaDaltons. The NPC generally binds proteins that carry consensus basic amino acid sequences called nuclear localization signals (NLS) (Li and Ma, 2001).

1.6   TARGETING OF LIPOSOMES

Of all the colloidal drug / gene carrier systems proposed for site-specific delivery, liposomes have attracted the most attention (Lasic, 1998).   Liposomal gene delivery has often been regarded as being non-specific as liposomes do not posses specific receptors for attachment to and subsequent entry into target cells. Antibodies and several low molecular weight ligands have been used to target vectors to cell surface receptors. Adding a targeting ligand to the cationic lipid / DNA complexes via a covalent or non-covalent linkage can improve transfection efficiency.   A targeting system may contain the liposome / DNA complex together with a ligand or an antibody conjugate, as was used by Trubetskoy et.al. (1992) (Figure 1.14).   More specifically a monoclonal antibody against an epitope expressed on the lung endothelial cells was used. The antibody was covalently linked to the positively charged poly-L-lysine which formed part of a ternary complex containing cationic liposomes and DNA (Figure 1.14).
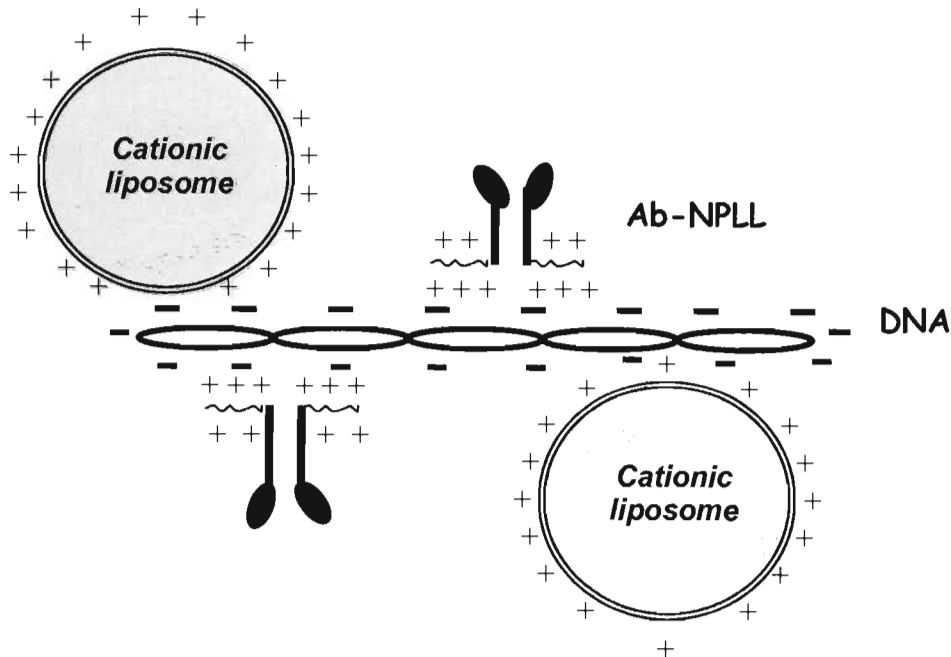
Figure 1.14:   Ternary complex between antibody(Ab), N-poly-L-lysine (NPLL), DNA and cationic liposomes .The DNA acts as an ionic bridge between the two cationic liposomes and modified antibodies.

Monoclonal antibodies have been the most widely used molecules for targeting liposomes as they have a high affinity and specificity for cell surface antigens against which they have been raised. Antibody-targeted liposomes are referred to as immunoliposomes, and have been used for selective drug delivery to cancer cells by targeting to tumour-associated antigens (Lopes de Menezes *et al.*, 1998).

Hence the antibody or ligand can be targeted to a specific tissue, depending on the ability of the antibody or ligand to facilitate cell specific docking (Lee and Low, 1994). Protein conjugates are recognised by cell surface receptors and antibodies by histocompatability antigens (Mack *et al.*,1994). By careful selection of the appropriate ligand-receptor interaction for liposome targeting, one may be able to enhance liposomal uptake by target cells. Targeted delivery systems would be of advantage in drug delivery and gene transfer as they would permit directed incorporation of selected macromolecules into a specific type of cell (Rosenberg *et al.*, 1987).

The process of targeted delivery can be roughly divided into two phases viz. a transport phase where liposomes travel from the site of administration (in vivo) to the target cells, and the effector phase that includes the specific binding of liposomes to the target cells and the subsequent delivery of the bound genes or drugs (Mastrobattista *et al.*, 1999).

Selective localization of genes or drugs can be obtained using either "passive" or "active" targeting. "Passive" targeting refers to the natural localisation patterns of liposomes when introduced into the body. It is a process by which the physical properties of the liposomes together with the microanatomy of the target tissue determine gene or drug selective localization (Barenholz, 2001). Liposomes injected intravenously localise mostly in mononuclear phagocytes of the reticuloendothelial system in the liver, spleen, bone marrow and in monocytes. Hence liposomes can be targeted to these cells passively (Poste *et al.*, 1984). "Active" targeting on the other hand, requires in addition to reaching the diseased site in specific cells, tissues or organs, a homing device, such as receptor ligands, antibodies, etc. to be attached to the liposome surface so that the liposome may recognize specific 'diseased" cells and bind

to them (Barenholz, 2001). Once the DNA-protein-liposome complex reaches the target cell, it is internalised . The targeting of liposomes may be carried out *in vivo* and *in vitro*.

Targeting to specific cell types *in vitro* poses fewer challenges than the more complex task of directing vectors to target tissues *in vivo*, although problems may arise in employing cell culture techniques. A mitotic cell population which has the capability to expand is a prerequisite (Hodgson, 1995). *In vitro* targeting provides the convenience of working with well characterised cells in culture and liposomes can be added directly to the desired target cells. These methods can hopefully be utilised eventually to develop methods to be used *in vivo*.

Various naturally occurring ligands to cell surface receptors such as hormones, vitamins (folate), growth factors, transferrin and asialoorosomucoid have been employed. They offer some advantages over antibodies such as lack of immunogenicity and low preparation costs (Ishii *et al.*, 1989). In a non-liposomal approach, asialoorosomucoid-poly-L-lysine-DNA conjugates were successfully delivered into asialoglycoprotein receptor-positive HepG2 cells (Wu and Wu, 1988). More recently a polycationic lipid (DOGS)-asialoorosomucoid-DNA ternary complex was effectively delivered to the same human hepatocyte derived cell line (Mack *et al.*, 1994). Furthermore, cationised asialoorosomucoid, used in a ternary complex with novel cationic liposome formulations and DNA showed promising results in gene delivery to HepG2 cells *in vitro* (Singh *et al.*, 2001b; Singh and Ariatti, 2003). Targeting of 'lipoplexes' (Tros de Ilarduya and Düzgüneş, 2000), and 'polyplexes' (Ogris *et al.*, 1999) to the transferrin receptor has also been attempted.

Liposomes to which the epidermal growth factor (EGF) was covalently coupled were targeted to hepatocytes expressing the EGF receptor. Since many carcinoma cells show a high expression of EGF receptors, EGF-targeted liposomes would be useful for tumour targeting (Ishii *et al.*, 1989). The folate targeted liposomes were found to bind specifically to cells that overexpress folate receptor. The folate receptor has been identified as an important surface marker in ovarian carcinomas (Lee and Low, 1995).

Folate receptor targeting has been found to enhance intracellular delivery of attached proteins, liposomes, viruses, antisense oligonucleotides, gene therapy vectors, polymeric drug carriers, imaging compounds, and neutron activation agents (Low and Antony, 2004). Targeting of cationic liposomes to specific target cells will be discussed in greater detail in chapters 3 and 4.

1.7   LIPOSOMES AS DRUG CARRIERS

Liposomes have steadily gained interest as potential carriers of drugs for certain diseases, especially for the chemotherapy of neoplastic diseases (Poste *et al.*, 1984). The amphiphilic nature of liposomes, with the hydrophobic bilayer and the hydrophilic inner core, enable solubilisation or encapsulation of both hydrophobic and hydrophilic drugs.   They are clinically the most developed of all drug carriers. Association of a drug with liposomes markedly alters the drug's distribution in the body and its pharmacology, which may be exploited to achieve targeted therapies (Chisti, 1999). Liposomes offer several attractive features as carriers for drugs and antigens:

Solubilisation : Liposomes may solubilise lipophilic drugs that would otherwise be difficult to administer intravenously.

Protection : Liposome-encapsulated drugs are inaccessible to metabolizing enzymes and most body components such as erythrocytes or tissues at the injection site are not directly exposed to the full dose of the drug. Liposomes can also protect the patient against possible side effects of the encapsulated drug.

Duration of action : Liposomes can prolong drug action by slowly releasing the drug in the body.

Directing potential : Targeting options change the distribution of the drug over the body.

Internalisation : Liposomes are generally endocytosed or phagocytosed by cells, opening up opportunities to use "liposome-dependent drugs'. Lipid based structures (non-viral) can also be utilized in this way.

Amplification : Liposomes can be used as adjuvants in vaccine formulations.

(Storm and Crommelin, 1998).

The main applications of liposomes in drug delivery technology is in passive tumour targeting, as vaccine adjuvants, passive targeting to lung endothelium, targeting to regional lymph nodes, and targeting to cell surface ligands in various organs or sites of pathology (Uchegbu, 1999). Most of the interest in liposomes as drug delivery vehicles stems from the potential to target these liposomes to specific cell types or organs by the incorporation of ligands into the surface membrane that could selectively bind to cell surface determinants on the desired target cells (Poste *et al.*, 1984). This will reduce the possibility of harmful side effects due to the minimized distribution of the drug to non-targeted tissues. Furthermore they are able to deliver high concentrations of the desired drug. Folate receptor-targeting is being exploited for the delivery of drugs for the treatment of cancer and some inflammatory diseases (Low and Antony, 2004; Lu *et al.*, 2004) such as rheumatoid arthritis where activated macrophages show an elevated level of folate receptor expression (Paulos *et al.*, 2004).

Cationic liposomes themselves can act as antimicrobial agents causing death of bacteria and fungi at concentrations that barely affect mammalian cells in culture. In addition, they are thought to be excellent solubilisers for hydrophobic drugs. Amphotericin B was effectively solubilised by the cationic liposome synthesized from dioctadecyldimethylammonium bromide (DODAB) (Carmona-Ribeiro, 2000). Liposomal amphotericin B (Ambisome) was the first liposomal preparation to be licensed for clinical use in the treatment of systemic fungal infections. Anticancer drugs that usually produce toxic side effects have been encapsulated within liposomes for tumour targeting. The use of liposomes in drug delivery was originally hampered by the fact that they were rapidly cleared from the circulation and mostly taken up by liver and spleen macrophages (reticuloendothelial system). The production of liposome surface ligands and "stealth" liposomes have reduced the uptake of liposomes by macrophages and increased their circulation time (Uchegbu, 1999).

1.8   OUTLINE OF THESIS

Currently viral based gene delivery systems are the most widely used in clinical gene therapy approaches. Patient safety will be of paramount importance and this is where viral systems may be disadvantaged, since they have the problem of immune responses of the host against viral proteins. Non-viral methods of gene delivery are beginning to emerge as more attractive alternatives since they are considered much safer. However, poor delivery and lack of sustained gene expression are generally the drawbacks associated with non-viral vectors. This thesis attempts to address the issue of delivery of genes *in vitro* using novel cationic liposome systems that show promise and that may be considered for further development.

The aim of this research is to prepare new and untested cholesteryl derivatives for incorporation into liposomes and to determine their transfection capabilities in a variety of cell lines.   Thus three cationic, two biotinyl and four glycosyl derivatives were synthesized for evaluation in various phases of this project.

Chapter two focuses on the the synthesis and analyses of three cationic cholesterol derivatives, their formulation into liposomes, and a study of their effectiveness as gene transfer vectors in four cell lines viz. human cervical carcinoma (HeLa) cells, mouse fibroblast (NIH-3T3) cells, human esophageal cancer (SNO) cells and hepatocellular carcinoma (HepG2) cells. Results obtained were compared with a commercially available cationic liposome formulation. The final results obtained were encouraging and the most efficient liposome preparation was utilized in the development of the targeting systems that are discussed in chapters three and four.

Chapter three deals with the targeting of biotinylated cationic liposomes to the transferrin receptor on HeLa cells using iron-loaded transferrin bound to a biotin-streptavidin complex.

In chapter four the targeting of lipoplexes to the asialoglycoprotein receptor is explored. Thus α and β anomers of both galactopyranosyl cholesterol and glucopyranosyl cholesterol were prepared and separately incorporated into cationic liposomes. Glycosylated lipoplexes were then evaluated for receptor-specific uptake into HepG2 cells *in vitro*.

Various aspects of the liposome formulations were studied. These include aspects of lipoplex formation and characterisation, liposome toxicity to cells, and finally transfection activity. The gene expression levels of the cationic liposome mediated transfections were assessed using the luciferase reporter gene assay in a transient expression system. The targeting systems designed and examined *in* vitro during the course of this study are of potential interest as transferrin receptor expression is elevated in certain cancers, while the asialoglycoprotein receptor is exclusively expressed in liver hepatocytes.

CHAPTER TWO

PREPARATION OF NOVEL CATIONIC LIPOSOME FORMULATIONS AND THEIR EFFICIENCY AS LIPOSOMAL GENE DELIVERY VEHICLES *IN VITRO*

2.1   INTRODUCTION

Most of the cationic compounds intended for liposome preparations are either those that possess cholesterol as an anchor lipid eg. DC-CHOL and CHOL-T, or those that possess double acyl chains of varying lengths and variable hydrocarbon saturation, as anchors eg. DOTMA and DOSPER (refer to chapter one).   The selection of the appropriate lipid anchor is of great importance as it was found that headgroups attached to cholesterol anchors were generally more active than those linked to diacyl chains (Marshall *et al.*, 1999). The liposomes discussed in this chapter are all cholesterol anchor based.

This chapter focuses on the synthesis of three different and untested cationic cholesterol derivatives N,N-dimethylaminopropylaminylsuccinylcholesterylformyl-hydrazide (MS09), glycylcholesterylformylhydrazide (MS10), β-alanylcholesteryl-formylhydrazide (MS11) that were formulated to produce three unique cationic liposomes. All cationic cholesterol derivatives were analysed and formulated in a 1:1 ratio with dioleoylphosphatidylethanolamine (DOPE) as the helper lipid. Liposome characterization was achieved using transmission electron microscopy (TEM). Lipoplex formation was analysed using gel retardation studies, nuclease digestion experiments, dye displacement assays and TEM. All three liposomes were assessed for cytotoxicity and transfection efficiency on four different cell lines, viz. HeLa, NIH-3T3, SNO, and HepG2 cell lines. The pGL3 plasmid DNA was complexed to each cationic liposome in different ratios respectively and introduced to the cells. Transfections were assayed using the Luciferase assay system.

2.1.1   Liposome Composition

The composition of lipid bilayers can be manipulated to influence the physicochemical characteristics of the liposomes, eg. surface charge, sensitivity to pH change, and bilayer rigidity (Gregoriadis, 1995). Most cationic liposomes are composed of a neutral lipid and a cationic lipid. The most frequently used "helper" lipids in cationic liposome formulations are the neutral lipids, dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE) and cholesterol (Figure 2.1). The role of "helper" lipids was shown to be critical for gene delivery (Campbell *et al.*, 2001).

Cholesterol is often added to improve mechanical stability of the bilayer and to decrease leakage of any encapsulated material (Lasic, 1997). It was also found to be associated with increased stability of cationic lipid:DNA complexes in serum, better than DOPE (Crook *et al.*, 1998). Furthermore Sakurai et.al. (2001) observed that complexes were stabilized against fusion with red blood cells when administered intravenously. Both helper lipids DOPE and cholesterol can significantly increase the resistance of the formulated complexes to polyanion mediated disassembly (Wiethoff *et al.*, 2001). Hence addition of 'helper' lipids such as DOPE to cationic liposomes tends to destabilize the cationic liposome (Farhood *et al.*, 1995), and can further increase their transfection efficiency (Smutzer, 2000). It is proposed that in those cases where DOPE improves transfection, it may do so by acting as an emulsifier, facilitating the dispersion of the lipid so that more of it becomes capable of interacting with the plasmid DNA (Rakhmanova *et al.*, 2000). The use of DOPE as the colipid (50 mol %) with cationic lipid resulted in a 2-5 fold increase in the transfection activity compared to formulations containing the same mole percentage of dioleoylphosphatidylcholine (DOPC) or those without any neutral lipid (Felgner *et al.*, 1994).

The use of PE in liposome preparations facilitates membrane fusion unlike the use of phosphatidylcholine (PC) that was found to inhibit fusion (Düzgüneş and Nir, 1999). PE and PC are both zwitterionic phospholipids. Phosphatidylethanolamine (PE) has a minimally hydrated and small head group that occupies a lower volume compared to

the respective hydrocarbon chains, exhibiting a cone shape which favours strong intermolecular interactions between the amine and phosphate groups of the polar headgroups (Simões *et al.*, 2004). PE is unable to form stable bilayer vesicles at neutral pH in physiological saline, and it undergoes a phase transition to an inverted bilayer hexagonal (H_{II}) phase (Farhood *et al.*, 1995), above the phase transition temperature (T_H), which for DOPE is 10°C (Simões *et al.*, 2004). This phase transition is an important factor in cellular delivery of nucleic acids and promotes fusion between liposome:plasmid DNA complexes and cell membranes such as the endosomal membrane. This also assists in the improved transfer of internalised cationic liposome:DNA complexes from the endocytotic cellular compartments into the cell cytoplasm (Farhood *et al.*, 1995).

PC, however, forms stable bilayer vesicles in physiological saline. For DNA transfection PC is generally less efficient than PE because of the reduced tendency to undergo the structural transition important for DNA delivery. PC liposomes are generally more suitable for entrapping and delivering therapeutic agents (Campbell *et al.*, 2001). Nevertheless, high levels of stable transfection were achieved in human cervical carcinoma cells (HeLa), using unilamellar liposomes prepared from lipids, phosphatidylcholine (PC), phosphatidylglycerol (PG), and cholesterol together with a cationic cholesterol derivative, N,N-dimethylaminopropylaminylsuccinylcholesterol. (Singh *et al.*, 2001a).

Dioleoylphosphatidylcholine (DOPC)

Dioleoylphosphatidylethanolamine (DOPE)

Cholesterol
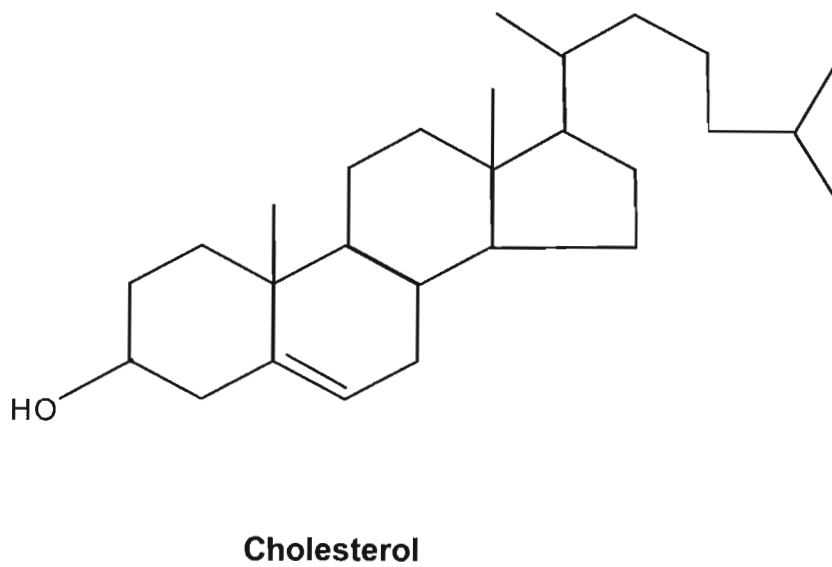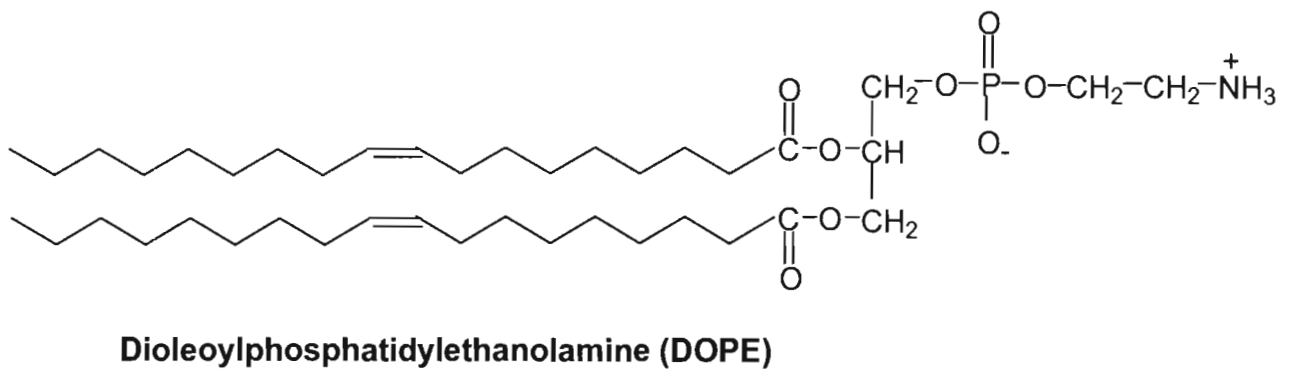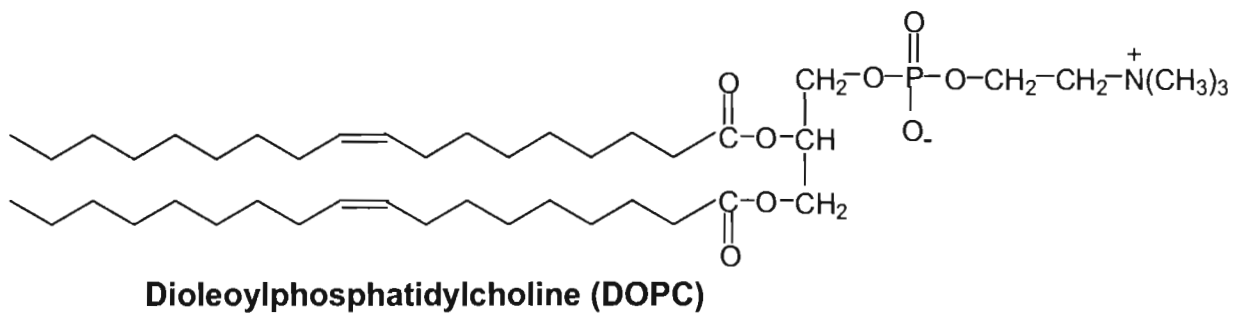
Figure 2.1 : Neutral lipids commonly used as 'helper' lipids in gene transfection.

2.1.2 Liposome Preparation

There are various methods that have been employed over the years in the preparation of liposomes. The common step in all these methods entails evaporating the organic solvent in which the lipids are generally dissolved and / or stored. Cholesterol and charged lipids are often added to the composition of liposomes. Phospholipids are characterised by a phase transition temperature (Tc) that permits membrane transition from a so-called crystal liquid to a gel state. In the gel state, liposomal membranes become fluid as a result of phase transition. Tc increases with increasing acyl chain length and is greater for saturated phospholipids.

Cationic liposomes used for gene delivery generally contain the phospholipid, dioleoylphosphatidylethanolamine (DOPE) as the neutral lipid, and a cationic lipid as is the case in this study. The dried lipids are then dispersed in an aqueous buffer solution. It is predicted that the furthest distance between two opposing lipids in the liposome bilayer is about 6 nm, depending on ionic activity of the aqueous solvent (Smith *et al.*, 1993). The preparation methods for liposomes differ in the way the lipids are dispersed. Some of these methods can be classified as follows :

    i)   The original hand shaken preparation producing multilamellar vesicles (Bangham *et al.*, 1965).

    ii)   Small unilamellar vesicles prepared by sonication (Papahadjopoulos and Miller, 1967 ; Chapman, 1984).

    iii) The ethanol injection technique (Batzri and Korn, 1973 ; Campbell, 1995).

    iv) Gel filtration of micelles (Brunner *et al.*, 1976).

    v)   Ether infusion technique that produces large unilamellar vesicles (Daemer and Bangham, 1976).

    vi) Reverse phase evaporation technique (Szoka and Papahadjopoulos, 1978).

    vii) Thin lipid film hydration (Gao and Huang, 1991).

The latter method was adapted for the preparation of the cationic liposomes used in this study.

2.1.3 Preparation of Liposome:DNA Transfection Complexes

Emerging from the extensive search for versatile DNA carriers, are complexes formed between DNA and cationic liposomes, that promise to be viable alternatives to the predominantly viral vectors. However little is known about the exact structure and properties of such complexes. In this chapter the preparation of three different complexes of this type and an evaluation of their transfection capabilities in various cell lines is reported. Complexes were prepared with cationic liposomes N,N-dimethylaminopropylaminylsuccinylcholesterylformylhydrazide (MS09), glycyl-cholesterylformylhydrazide (MS10), β-alanylcholesterylformylhydrazide (MS11), and pGL3 DNA respectively. The liposome:DNA complexes were subjected to gel retardation assays to determine optimum binding ratios and their ultrastructure was visualized by transmission electron microscopy.

The exact mechanism of cationic liposome-DNA interaction , and the structure of such complexes are still not fully understood. The DNA binds to the surface of cationic liposomes, but size and shapes are maintained (Felgner and Ringold, 1989). Complete complexation is achieved by simply mixing and incubating the cationic liposomes and DNA (Felgner *et al.*, 1987). Liposomes are dynamic in nature, and are able to interact or associate in different conformations depending upon size, charge, and conditions of formation (Smith *et al.*, 1993). When forming a complex with DNA, a profound structural rearrangement of the liposome structure occurs (Birchall *et al.*, 1999).

The positively charged lipid complex reacts with the negatively charged phosphate groups of the DNA resulting in formation of the liposome:DNA complex. It is unlikely that all the negative and positive charges are involved in ionic interactions because both the supercoiled DNA and the liposomes are bulky enough to produce steric hindrance preventing close contact to each other (Farhood *et al.*, 1992). Short alkyl chains of liposomes and complex polar headgroups with quaternary and primary amino groups, could impart physical properties to the DNA:liposome complex, resulting in maximum DNA:liposome interaction, and lipid:DNA packing (Wheeler *et al.*, 1996). It is proposed

that the ability of a cationic lipid or liposome to rapidly wrap and condense plasmid DNA, in conjunction with the facilitation of DNA release from a lipoplex by DOPE, allows for efficient lipoplex-mediated gene transfer (Zuhorn et al., 2002).

The use of fluorescence, gel electrophoresis, and electron microscopy techniques have helped to elucidate the structural features of the resulting complexes. There are currently three models of DNA:liposome interactions which either situate the DNA on the external or internal surface of the liposome. These models generally apply to unilamellar liposomes but could be extended to multilamellar liposomes as well. The internal model essentially describes the entrapment of DNA within the liposome. The other two models viz. the electrostatic model and the coated electrostatic model involve cationic liposome:DNA interaction and will be discussed further, since all studies are based on cationic liposome:DNA binding.

The electrostatic model is based on the probability that electrostatic forces underlie the successful interaction between cationic lipids and DNA, and also assist the entry of the complex into the cell. This type of model has been proposed by Felgner and Ringold in 1989 for the commercially available liposome formulation, Lipofectin™ [DOTMA:DOPE (1:1)]. Their model proposed that the plasmid DNA interacts with the cationic liposome such that one plasmid molecule is trapped in the interior of a complex of four liposomes (Figure 2.2).

According to this model the cationic liposomes approach opposite sides of the anionic DNA strand and fuse with each other. The final arrangement of the lipoplex involves the coating of the DNA strand with a single lipid bilayer. It is assumed that these liposomes have an average diameter of 250 nm and would hence each contain 2500 lipid molecules, half (1250) of which are positively charged (DOTMA). For a standard 2500 base pair plasmid, approximately 5000 negative charges will be available. Hence to totally neutralize the positive charges on the cationic liposome (1250), requires four liposomes . Although four liposomes may interact with one plasmid, very little of the evenly distributed lipid charges would be utilised, and the size of the plasmid could bring

50

the four liposomes into close proximity, so that they would repel each other and may dissociate from the DNA. This model fails to consider the possibilities of charge localization, sharing of charges between inner and outer lipid layers, and the structural differences between linear and supercoiled plasmid DNA (Smith *et al.*, 1993).
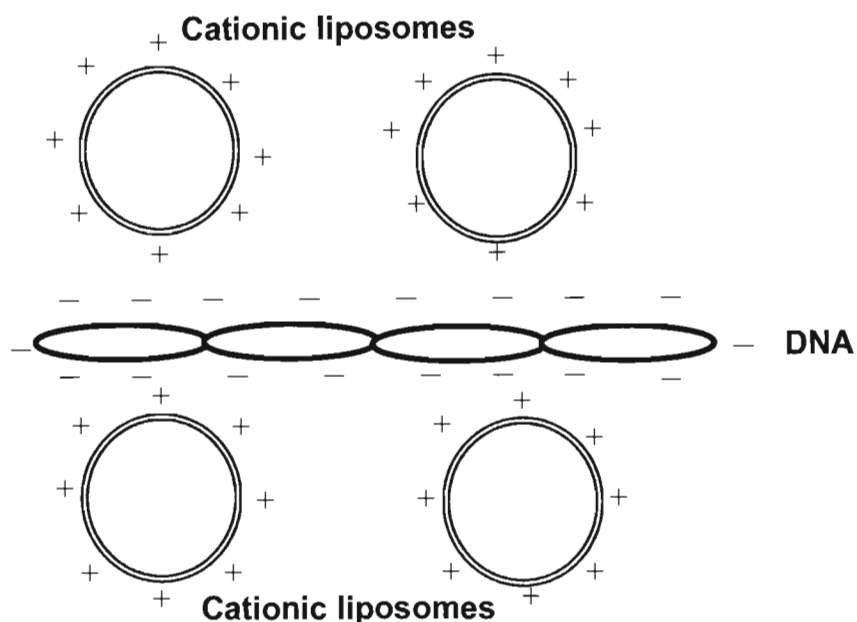
Figure 2.2 : The electrostatic model of lipid-DNA complexes proposed by Felgner and Ringold, 1989.

Boles *et al.*, 1990, proposed that the supercoiled plasmid, assumes a branched plectonemic form with a superhelix axis length of about 41% of the total DNA length (Figure 2.3). The four branches of the DNA protrude from the corners of a central core branch, and are each of equal length. This structure may be conducive to DNA-liposome complex formation, consisting of several liposomes clustered like grapes around compacted, branched DNA molecules. Such structures have been visualized by transmission electron microscopy in studies of the ultrastructure of lipoplexes.

These structures are common for DNA at both high and low levels of supercoiling. It is assumed that for a 2500 base pair plasmid, there exists a structure with four branches

at an average of 70 nm in length and 5 nm in diameter and a central "trunk" of similar length and diameter from which they protrude.
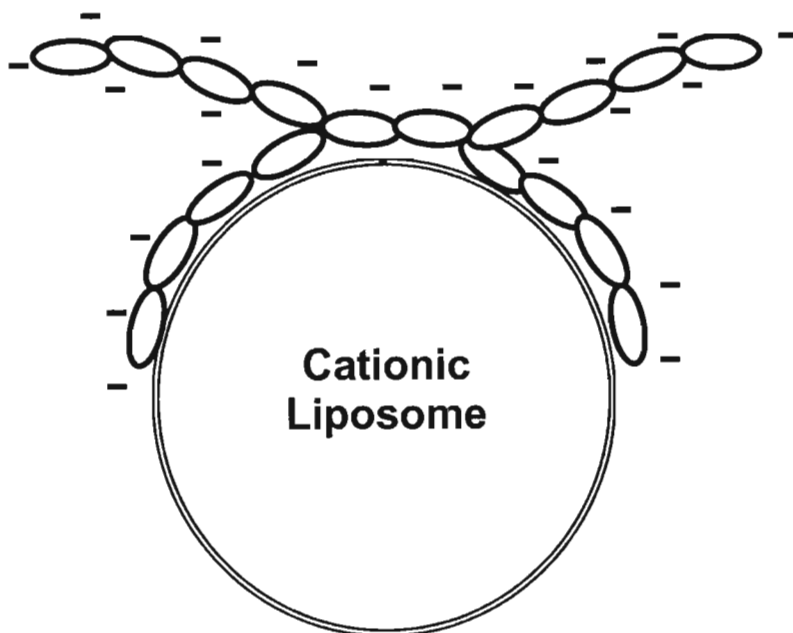
Figure 2.3 : Model depicting interaction between the supercoiled plasmid DNA and the cationic liposome as proposed by Boles *et al.*, 1990.

In the coated electrostatic model, DNA is coated by continuous lipid bilayers due to the DNA being entrapped between lipid bilayers during liposome fusion that draws bilayers together. An electron microscopy study by Gershon *et al.*, 1993, suggested that cationic liposomes are attached like beads on a string, gradually covering the DNA chain until at a specific lipid to DNA ratio, a complete lipid coating is reached, composed of a multilayer of liposomes. At a critical liposome density two processes occur, viz. DNA induced membrane fusion, and liposome induced DNA collapse. The lipid coating seems to protect the DNA and this should enhance the uptake by recipient cells.

Using freeze fracture electron microscopy, it was shown that DNA:liposome structures formed often resembled tubular "spaghetti-like" structures. The diameter of these tubular structures is approximately 7 nm (Sternberg *et al.*, 1994). The ratio of DNA to

cationic liposomes determines the net surface charge on the complex. This will alter the interaction of the complex with other ligands, such as asialoorosomucoid and transferrin and further alter its ability to bind and enter cells *in vivo* (Zhu *et al.*, 1993).

A mixed model can also be proposed where the DNA is bound both on the inside and on the outside of the liposomes. In the case of cationic liposomes it is assumed that the DNA will prefer those regions of the liposome bilayer that contain the greatest positive charge or it will draw these charges together. Since DNA is added after formation of cationic liposomes, the continuous lipid bilayer must become transiently discontinuous to allow entry of the DNA. This could occur by membrane fusion via the formation of an inverted cylinder or inverted micellar intermediate. DNA seems to be capable of acting as a fusogenic agent in the presence of cationic liposomes (Smith *et al.*, 1993).

2.1.4.   Tissue Culture and Transfection

Transfection in eukaryotic cells is a powerful tool in cell biology. A major focus is on the improvement of methods for the introduction of DNA into appropriate cells, and also on the quality of DNA used which is also significant in transfection efficiency. Gene transfer methods include *in vitro* and *in vivo* gene delivery. Using *in vitro* gene transfer, appropriate cell lines can be targeted. Transfected cells can be selected and expression of the transgene monitored. A limited degree of success has been achieved using intra-peritoneal, intra-arterial, intra-hepatic, intra-muscular, and intra-tracheal routes using physical or viral methods (Lyerly, 1993 ; Li and Ma, 2001).

The aim of this study was to develop synthetic gene transfer systems based on cationic liposome mediated transfection, that could be subsequently utilized for targeting to a specific cell type. Liposome mediated transfection is regarded as being a reliable and reproducible technique for gene transfer (Stamatatos *et al.*, 1988). Conventional cationic liposomes as prepared in this chapter have no cell-specific targeting

properties, and may be elaborated by addition of a targeting component for targeted *in vivo* gene transfer applications. The pGL3 plasmid DNA is used to detect transient expression of the luciferase gene (*luc*+) in mammalian cells grown in culture, since these cells lack endogenous luciferase activity. The intensity of luciferase activity will be a measure of the success with which gene transfer has been effected.

The use of vectors in transfection allows considerable flexibility in regulating the expression of cloned genes. Various plasmid vectors have been constructed and contain characteristics which render them amenable for gene cloning. A common feature of all plasmid based vectors is the presence of a prokaryotic replication origin (replicon), and a selectable marker gene to permit the recombinant DNA molecule to be amplified in *E.coli*. Most vectors have in addition a eukaryotic replication origin or a eukaryotic selectable marker. This latter feature is required for stable expression. The eukaryotic replication origin is usually derived from viruses such as bovine papilloma virus (BPV), Simian virus 40 (SV40), or Epstein Barr virus (EBV) (Murray, 1993).

Transfection of eukaryotic cells with reporter gene constructs have been widely used. The reporter gene encodes a reporter enzyme that is used in detection of gene expression. The most popular reporter enzymes are chloramphenicol acteyltransferase (CAT), β-galactosidase (β-Gal), and luciferase, which accumulate in the cytoplasm during experimental gene expression. The detection of luciferase activity is thought to be more sensitive than assaying for the chloramphenicol acetyltransferase expression. In addition the green fluorescent protein has been used in many gene expression studies (Blum *et al.*, 2004).

The pGL3 DNA vector, incorporating the firefly luciferase gene, *luc*+, was used in this study as the expression vector. The luciferase that was isolated from the North American firefly *Photinus pyralis* is one of the most extensively studied enzymes that catalyse light production in bioluminescent organisms. It has a molecular weight of approximately 62 000 daltons (Wood *et al.*, 1985), and it requires luciferin, ATP, and

oxygen as substrates. The structure of firefly luciferin (Figure 2.4) has been determined and the chemical synthesis of the carboxylic acid has been reported (Bowie, 1978).
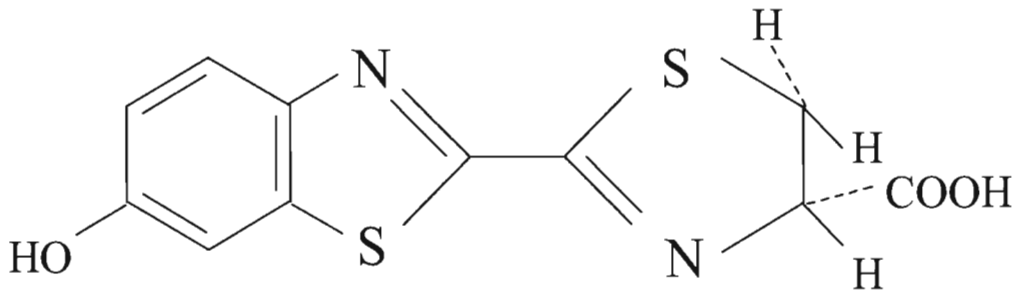
Figure 2.4 :   Structure of firefly D-(-)-Luciferin

The luciferase gene is a single copy gene in firefly *P. pyralis*. It is composed of seven exons separated by six very short introns ranging from 48 to 58 bases in length. The nucleotide sequence of the six introns was also determined from analysis of cDNA and genomic clones (De Wet *et al.*, 1987), (Figure 2.5). The structure and restriction map of the pGL3 plasmid DNA can be seen in Figure 2.6.
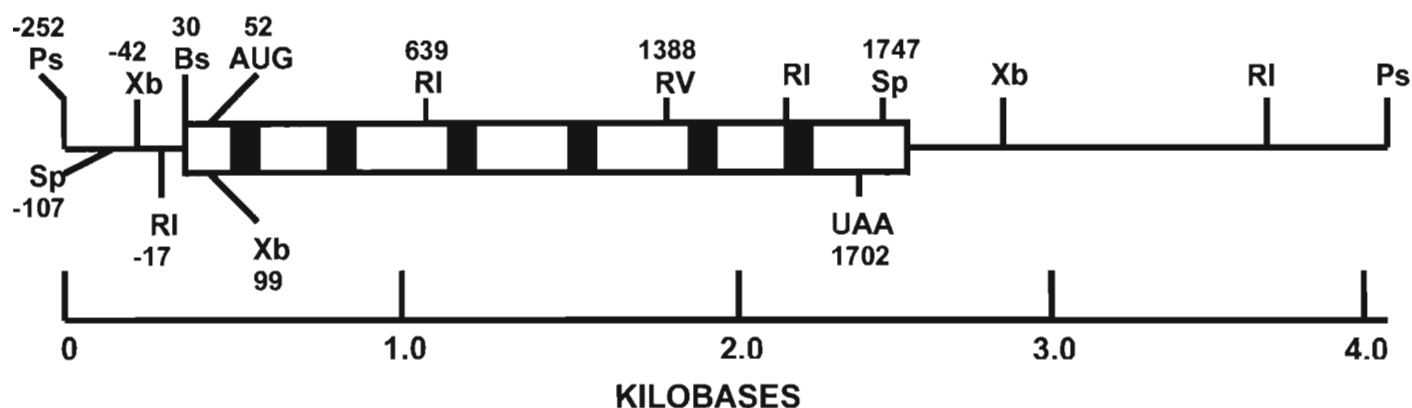
INTRON 1 (58 bp)	TTGCTTTT	GTGAGTATTTCTGTCTGATTTCTTTCGAGTTAACGAAATGTTCTTATGTTTCTTTAG				ACAGATGC
	177	10 20 30 40 50				178

INTRON 1 (58 bp) TTGCTTTT   GTGAGTATTTCTGTCTGATTTCTTTCGAGTTAACGAAATGTTCTTATGTTTCTTTAG   ACAGATGC
177   10   20   30   40   50   178

INTRON 2 (51 bp) TAATGAAC   GTAAGCACCCTCGCCATCAGACCAAAGGAATGACGTATTTAATTTTTAAG   GTGAATTG
385   10   20   30   40   50   386

INTRON 3 (48bp)   CATGCAAG   GTATGTCGTATAACAAGAGATTAAGTAATGTTGCTACACACATTGTAG   AGATCCTA
719   10   20   30   40   720

INTRON 4 (49bp)   GCAAAACG   GTGAGTTAAGCGCATTGCTAGTATTTCAAGGCTCTAAAACGGCGCGTAG   CTTCCATC
1040   10   20   30   40   1041

INTRON 5 (43bp)   GATATCAG   GTAATGAAGATTTTTACATGCACACACGCTACAATACCTGTAG   GTGGCCCC
1395   10   20   30   40   1396

INTRON 5 (47bp)   CGTCGCCA   GTAAATGAATTCGTTTTACGTTACTCGTACTACAATTCTTTTCATAG   GTCAAGTA
1561   10   20   30   40   1562

Figure 2.5 : Structure of the *P. pyralis* luciferase gene. The luciferase gene exons appear as open boxes and the introns as shaded boxes . Restriction sites are abbreviated as *Ps = Pst 1* , *Sp = Ssp 1* , *Xb = Xba 1* , *R1=EcoR 1* , *Bs=Bsm 1* , and *RV=EcoR V*. The nucleotide sequence of the six introns are indicated above.

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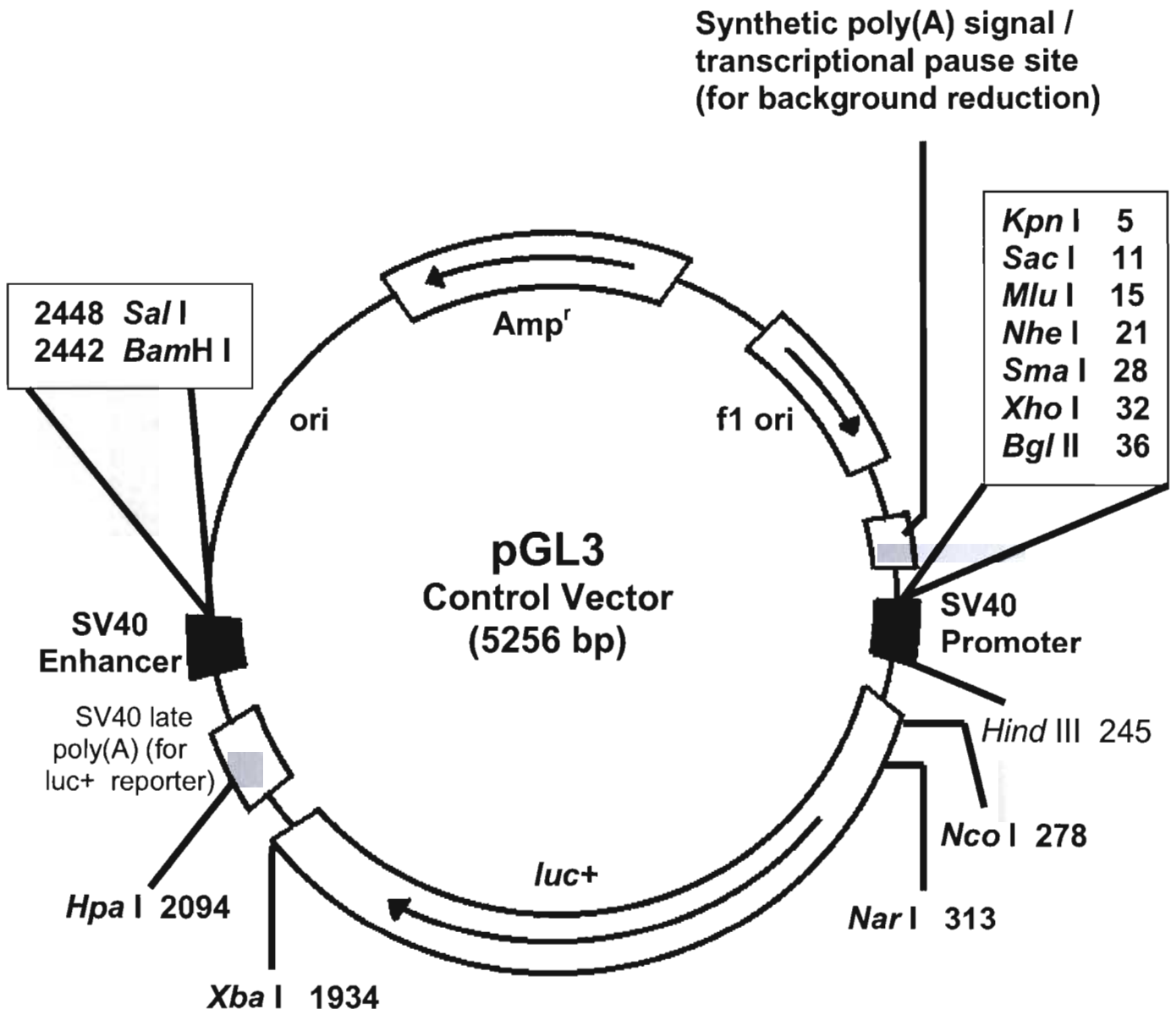
Figure 2.6 : Construction of the pGL3-control vector circle map (Promega).

Many expression vectors are derivatives of pSV2, an SV40 early-region promoter vector (Subramani and Southern, 1983), and contain the SV40 small T antigen intron, and an SV40 polyadenylation signal. The pGL3 control vector contains SV40 promoter and enhancer sequences resulting in strong expression of *luc+* in many types of mammalian cells. All pGL3 vectors contain a high copy number prokaryotic origin of replication for maintenance in *E. coli*, an ampicillin-resistance gene (Amp^r) for selection, and a filamentous phage origin of replication (f1 ori) for single stranded DNA (ssDNA) production. Restriction sites for insertion of DNA fragments are located upstream and downstream of the luciferase gene (*luc+*). Two of the upstream sites (*Xho* 1 and *Bgl* II) yield cohesive ends compatible with the downstream sites (*Sal* I and *Bam*H I) respectively, allowing for interchange of DNA inserts (Promega Technical Manual).

The luciferase gene is one of the most versatile reporter genes. Monitoring promoter activity using firefly luciferase has been described for cultured animal cells (Gould and Subramani, 1988 ; Brasier *et al.*, 1988), bacteria (Wolk *et al.*, 1991), insects and yeasts (Aflalo, 1990). Besides firefly luciferase, various other luciferases of eukaryotic and prokaryotic origin have been employed using different substrates and reaction conditions.

2.2.   MATERIALS AND METHODS

2.2.1   MATERIALS FOR CHEMICAL SYNTHESIS

Cholesterylchloroformate, N-hydroxysuccinimide (NHS), dimethylaminopropylamine, N-trityl glycine, β-alanine, trityl chloride, dicyclohexylcarbodiimide (DCCI) and succinic anhydride were obtained from Sigma-Aldrich (St.Louis, MO, USA).   Hydrazine, chloroform, methanol, pyridine, dimethylformamide (DMF), ethanol, ethyl acetate, sulphuric acid, diethylamine, ether, propan-2-ol, and Silica gel $60F_{254}$ chromatography plates were all purchased from Merck (Darmstadt, Germany). All other reagents used were of analytical grade. Infrared (IR) spectra were recorded with a Nicolet Impact 420 spectrophotometer using the KBr disc technique (0.5% dilution). NMR spectra were obtained on a Gemini 300 instrument. Electrospray mass spectra were recorded on a Waters APIQ-TOF Ultima instrument (ES-TOF).

Solvent system A = chloroform: methanol (9:1 $^v/_v$)

Solvent system B = chloroform: methanol (9.5:0.5 $^v/_v$)

2.2.2   SYNTHESIS OF CATIONIC CHOLESTEROL DERIVATIVE : N,N-DIMETHYLAMINOPROPYLAMINYLSUCCINYLCHOLESTERYL-FORMYLHYDRAZIDE (MS09)

The synthesis of N,N-dimethylaminopropylaminylsuccinylcholesterylformylhydrazide (MS09),   was achieved in four steps, the initial step being the preparation of cholestrylformylhydrazide. (Figure 2.7).

2.2.2.1   Preparation of Cholesterylformylhydrazide (MS04)

Cholesterylchloroformate (1.13 g, 2.5 mmoles) was dissolved in 5 ml chloroform and the resultant solution was chilled to 0°C. Hydrazine (240 mg, 7.5 mmoles) was added to about 3 ml chloroform and sufficient methanol (approximately 0.6 ml) was introduced to

ensure complete dissolution. This solution was also chilled to 0°C. The chilled chloroformate solution was then added dropwise, with continual stirring, to the solution of hydrazine. The resultant milky solution was kept at room temperature overnight. The reaction was monitored by thin layer chromatography (TLC) using silica gel 60 F_{254} chromatography plates, and solvent system A. Cholesterol containing compounds were visualised using a 33% sulphuric acid spray. The reaction mixture was concentrated *in vacuo* at 37°C to produce a crystalline mass. The product was recrystallised from methanol (100 ml) and chloroform (25 ml). The resultant crystals were filtered under vacuum and dried overnight under vacuum using a Xerotec Freeze Drier. Product produced a single spot by TLC (R_f 0.79, solvent A) with a mp of 225-227 °C and a yield of 917 mg (83%).

IR (film) : 3416 (b, N-H), 2929 (st, C-H), 1731 (m, C=O), 1495 (m, C=C). (Appendix 2C).

^{1}H NMR (300 MHz, DMSO d^6) : δ 0.66 (s, 3H, C-CH_3), 0.86 (d, 6H, CH-CH_3), 0.91 (d, 3H, CH-CH_3), 0.99 (s, 3H, C-CH_3), 3.88 (bs, 2H, NH_2), 4.38 (m, 1H, Chol-$H_{3\alpha}$), 5.33 (d, 1H, Chol-H_6), 7.93 (s, 1H, NH) ppm. (Appendix 1C).

MS,$^m/_z$, ES-TOF : 445.4358 [M + H$^+$], 467.3932[M + Na$^+$].

2.2.2.2 Preparation of Cholesterylformylhydrazide Hemisuccinate (MS08)

Cholesterylformylhydrazide (89 mg, 0.2 mmoles) and succinic anhydride (20 mg, 0.2 mmoles) were dissolved in 2 ml DMF:pyridine (1:1 $^v/_v$). The reaction mixture was maintained at room temperature overnight. The reaction was monitored by TLC in solvent system A. The reaction mixture was evaporated to dryness under reduced pressure in a Büchi Rotavapor-R, to produce a thin white film on the walls of the flask. Ethanol (3 ml) was added to the flask and the product crystallized out as fine white crystals. The crystalline suspension was heated to induce the crystals to redissolve in the ethanol. Thereafter they were left to recrystallise at 4°C in the refrigerator. Crystals were filtered and dried overnight using a Büchi TO-50 pistol drier. The product gave a single spot by TLC (R_f 0.21, solvent A) with a mp of 195-196 °C and a yield of 58 mg (53%).

IR (film) : 3452 (b, N-H), 2934 (m, C-H), 1675 (m, C=O), 1567 (w, C=C). (Appendix 2G).

^{1}H NMR (300 MHz, DMSO d^6) : δ 0.64 (s, 3H, C-CH_3), 0.83 (d, 6H, CH-CH_3), 0.89 (d, 3H, CH-CH_3), 0.96 (s, 3H, C-CH_3), 4.3 (m, 1H, Chol-H$_{3\alpha}$), 5.34 (d, 1H, Chol-H$_6$) ppm. (Appendix 1D).

MS, $^m/_z$, ES-TOF: 545.09 [M + H$^+$] , 567.63 [M + Na$^+$].

2.2.2.3 Preparation of N-hydroxysuccinimide Cholesterylformylhydrazide Hemisuccinate

Cholesterylformylhydrazide hemisuccinate (82 mg, 0.15 mmoles), DCCI (62 mg, 0.3 mmoles) and N-hydroxysuccinimide (NHS) (35 mg, 0.3 mmoles) were dissolved in DMF (1ml). The reaction mixture was maintained at room temperature and the product monitored by TLC as described above. Complete reaction was detected after 48 hours. The dicyclohexylurea crystals (53 mg) were filtered off under vacuum and washed with DMF. The filtrate was reduced under vacuum in a rotary evaporator to a thin white film on the walls of the flask. The product was extracted using a CHCl$_3$: H$_2$O (1:1) mixture. The water layer was removed as it contained the water soluble NHS. The chloroform layer containing the product was treated with anhydrous Na$_2$SO$_4$ to absorb any excess water. This was kept overnight at room temperature.

The chloroform extract was carefully removed and evaporated to dryness in a rotary evaporator. To the dried product was added petroleum ether (60-80°C, 10 ml), to dissolve any carbodiimide present. This was kept overnight at room temperature. The ether was then carefully removed using a Pasteur pipette and the product was dried using a rotary evaporator. The final product was recrystallised from ethanol and dried under vacuum in the Xerotec Freeze Drier. The product produced a single spot by TLC (R$_f$ 0.93, solvent A) and a yield of 53 mg (55%).

2.2.2.4 Preparation of N,N-dimethylaminopropylaminylsuccinyl-cholesterylformylhydrazide (MS09)

To the N-hydroxysuccinimide ester of cholesterylformylhydrazide hemisuccinate (53 mg, 0.083 mmoles) was added dimethylaminopropylamine (36 mg, 0.35 mmoles), in H_2O:pyridine (3:7 $^V/_V$; 0.5 ml). To obtain a homogeneous solution, however, H_2O:DMF (1:1 $^V/_V$; 1 ml) was added. The reaction mixture was maintained at room temperature and monitored by TLC in solvent system B, until the reaction had reached completion. The reaction mixture was applied to four 10 x 20 cm silica gel 60 F_{254} TLC plates which were developed in solvent system B. The product band was scraped off the plate and placed in a mixture of $CHCl_3$: CH_3OH (1:1 $^V/_V$) to extract the product. The mixture was filtered using a small glass funnel, to remove the silica gel residue. The filtrate was evaporated to dryness in the rotary evaporator and dried in a Xerotec Freeze Drier. The product produced a single spot by TLC (R_f 0.16, solvent A) and gave a mp of 155-156 °C with a yield of 20 mg (38%).

IR (film) : 3441 (b, N-H), 2945 (m, C-H), 1634 (m, C=O), 1557 (w, C=C). (Appendix 2H).

^{1}H NMR (300 MHz, $CDCl_3$) : δ 0.65 (s, 3H, C-CH_3), 0.84 (d, 6H, CH-CH_3), 0.88 (d, 3H, CH-CH_3), 0.98 (s, 3H, C-CH_3), 2.26 (s, 6H, N-CH_3), 3.28 (q, -CH_2 –CH_2 –NH -), 4.48 (m, 1H, Chol-$H_{3\alpha}$), 5.35 (bs, 1H, Chol-H_6) ppm. (Appendix 1E).

MS, $^m/_z$, ES-TOF: 629.82 [M + H$^+$].
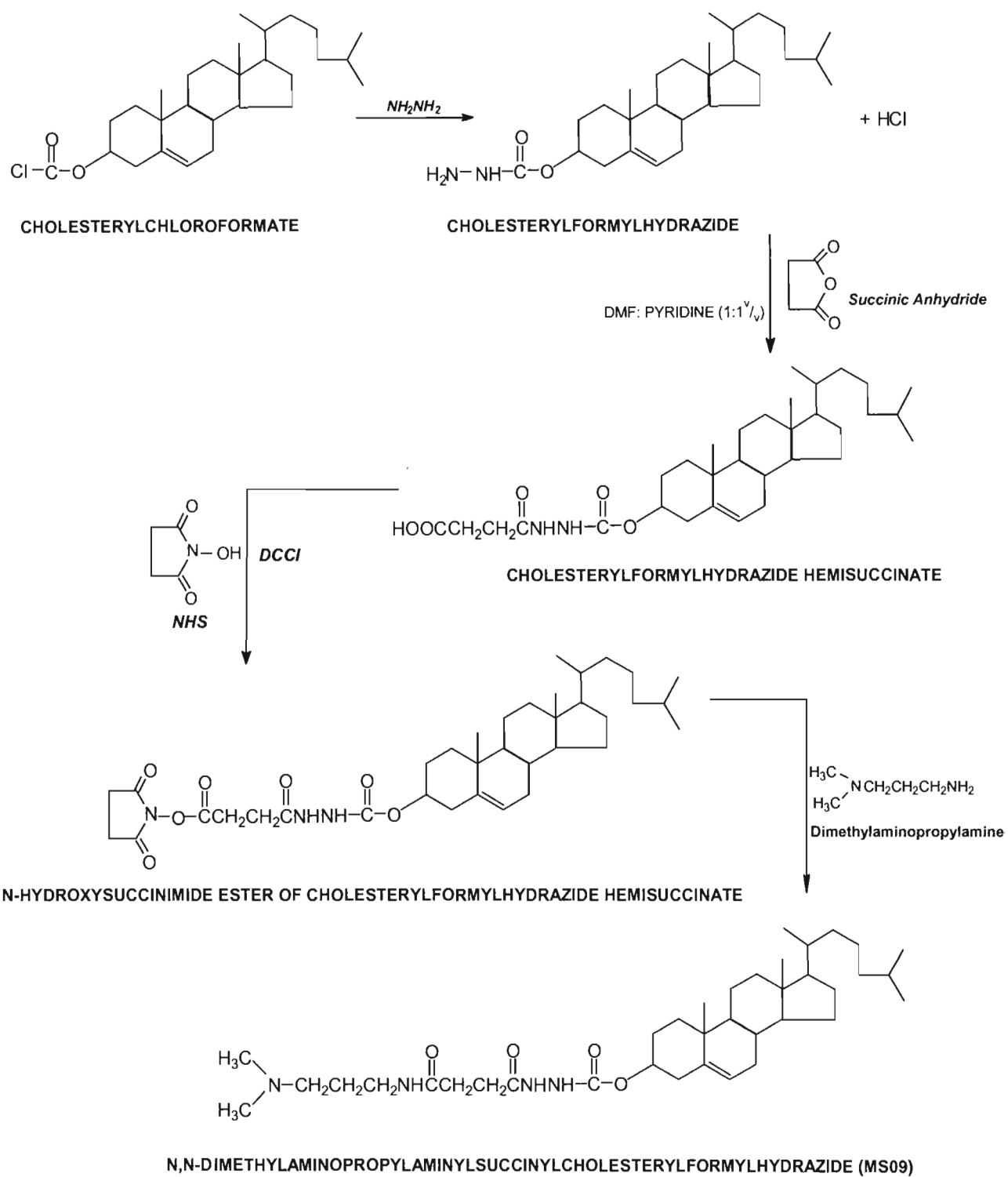
Figure 2.7 : Scheme for the synthesis of N,N-dimethylaminopropylaminylsuccinyl-cholesterylformylhydrazide (MS09)

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2.2.3 SYNTHESIS OF CATIONIC CHOLESTEROL DERIVATIVE : GLYCYLCHOLESTERYLFORMYLHYDRAZIDE

This synthesis was achieved in three steps (Figure 2.8).

2.2.3.1 Preparation of N-tritylglycine NHS ester (MS07)

N-tritylglycine (635 mg, 2 mmoles) was dissolved in DMF (2 ml). To this was added NHS (230 mg, 2 mmoles). Thereafter a solution of DCCI (433 mg. 2.1 mmoles) in DMF (2 ml) was added dropwise to the above reaction mixture. The mixture was then left in the dark for 24 hours. The reaction was monitored by TLC using the silica gel $60F_{254}$ chromatography plates and solvent system A. The plates were sprayed with a 2% ($^W/_w$) $HClO_4$ solution for the detection of the trityl group. After 24 hours, the reaction mixture was filtered under vacuum. The dicyclohexylurea crystals on the filter paper were discarded and the filtrate containing the product was evaporated to dryness using the rotary evaporator. This removed the DMF from the filtrate. The product was crystallized from ethanol. After crystallization most of the ethanol was removed using a Pasteur pipette. prior to rotary evaporation of the product to remove any further traces of ethanol. The resultant crystals were finally dried under vacuum in the Xerotec Freeze Drier overnight. The product was subjected to TLC in solvent system A. . NHS and NHS containing compounds were detected by viewing the TLC plate under UV_{254} illumination. The trityl group was detected by using a 2% ($^W/_w$) $HClO_4$ spray, and on a separate TLC plate the final product (active ester) was viewed using an active ester spray [14% ($^W/_v$) hydroxylamine hydrochloride, 3.5 M NaOH (20:8.5 $^V/_v$), which was sprayed first followed by a spray of $FeCl_3$ in 1.2 M HCl (5% $^W/_v$)]. The single spot on the chromatograms was UV_{254} absorbing, trityl positive, and found to be an active ester confirming its structure . R_f 0.93, mp 126-128 °C , yield 630 mg (76%).
IR (film) : 3436 (b, N-H), 2929 (w, C-H), 1731 (s, C=O), 1060 (m, C-O). (Appendix 2F).

2.2.3.2   Preparation of N-tritylglycylcholesterylformylhydrazide (MS01)

Cholesterylformylhydrazide (44.2 mg, 0.1 mmoles) and N-tritylglycine NHS ester (41.4 mg, 0.1 mmoles) were dissolved in a mixture of DMF : pyridine (1:1 $^V/_v$) , in a total volume of 800 µl. The reaction mixture was kept at room temperature overnight. The reaction was monitored by TLC in solvent system B. The product was extracted using a $CHCl_3$: H_2O (1:1 $^V/_v$) mixture since the NHS dissolves in water and will be eliminated in the H_2O layer. The $CHCl_3$ extract was evaporated under reduced pressure and any excess pyridine co-evaporated with ethanol. The final product was crystallized from ethanol. The product was monitored using TLC and spraying with 33% sulphuric acid. Product produced a single spot by TLC (R_f 0.96, solvent B) with a mp of 108-110 °C and a yield of 63.7 mg (86%).

IR (film) : 3436 (b, N-H), 2929 (st, C-H), 1449 (m, C=C), 1654 (m, C=O), 1378 (w, C-N), 1510 (v, aromatic rings). (Appendix 2A).

^{1}H NMR (300 MHz, $CDCl_3$) : δ 0.68 (s, 3H, C-CH_3), 0.86 (d, 6H, CH-CH_3), 0.89 (d, 3H, CH-CH_3), 0.99 (s, 3H, C-CH_3), 3.1 (bs, 1H, NH), 4.54 (m, 1H, Chol-H$_{3\alpha}$), 5.37 (d, 1H, Chol-H$_6$), 7.2-7.4 (15H, trityl group) ppm. (Appendix 1A).

MS, $^m/_z$, ES-TOF: 744.55 [M + H$^+$] , 767.21 [M + Na$^+$].

2.2.3.3   Preparation of Glycylcholesterylformylhydrazide (MS10)

To N-tritylglycylcholesterylformylhydrazide (30 mg, 0.04 mmoles) was added 300 µl $CHCl_3$ to dissolve the crystals. To this was added 600 µl glacial acetic acid and 60 µl H_2O. The reaction mixture was maintained at room temperature for 3 hours. The product was monitored by TLC in solvent system B. TLC plates were first sprayed with ninhydrin to detect the presence of the amino groups and then with 33% H_2SO_4 to detect the cholesteryl moiety. The reaction mixture was evaporated to dryness under reduced pressure and sufficient petroleum ether (100-120°C) was added to the sample

to remove any tritanol present in the reaction mixture. This mixture was then kept at room temperature overnight.

The ether was finally removed with a Pasteur pipette and the mixture evaporated to dryness in a Rotavapor-R rotary evaporator, to produce a powder.   The product gave a single spot by TLC (R_f 0.10, solvent B) with a mp of 203-204°C and a yield of 19.1 mg (96%).

IR (film) : 3441 (b, N-H), 2934 (m, C-H), 1649 (m, C=O), 1260 (w, C-N), 1432 (m, C=C). (Appendix 2I).

^{1}H NMR (300 MHz, CDCl$_3$) : δ 0.69 (s, 3H, C-CH_3), 0.84 (d, 6H, CH-CH_3), 0.89 (d, 3H, CH-CH_3), 0.99 (s, 3H, C-CH_3), 3.49 (2H, NH$_2$), 4.53 (m, 1H, Chol-H$_{3\alpha}$), 5.36 (bs, 1H, Chol-H$_6$) ppm. (Appendix 1F).

MS, $^m/_z$, ES-TOF : 502,4764 [M + H$^+$], 524.4638 [M + Na$^+$].
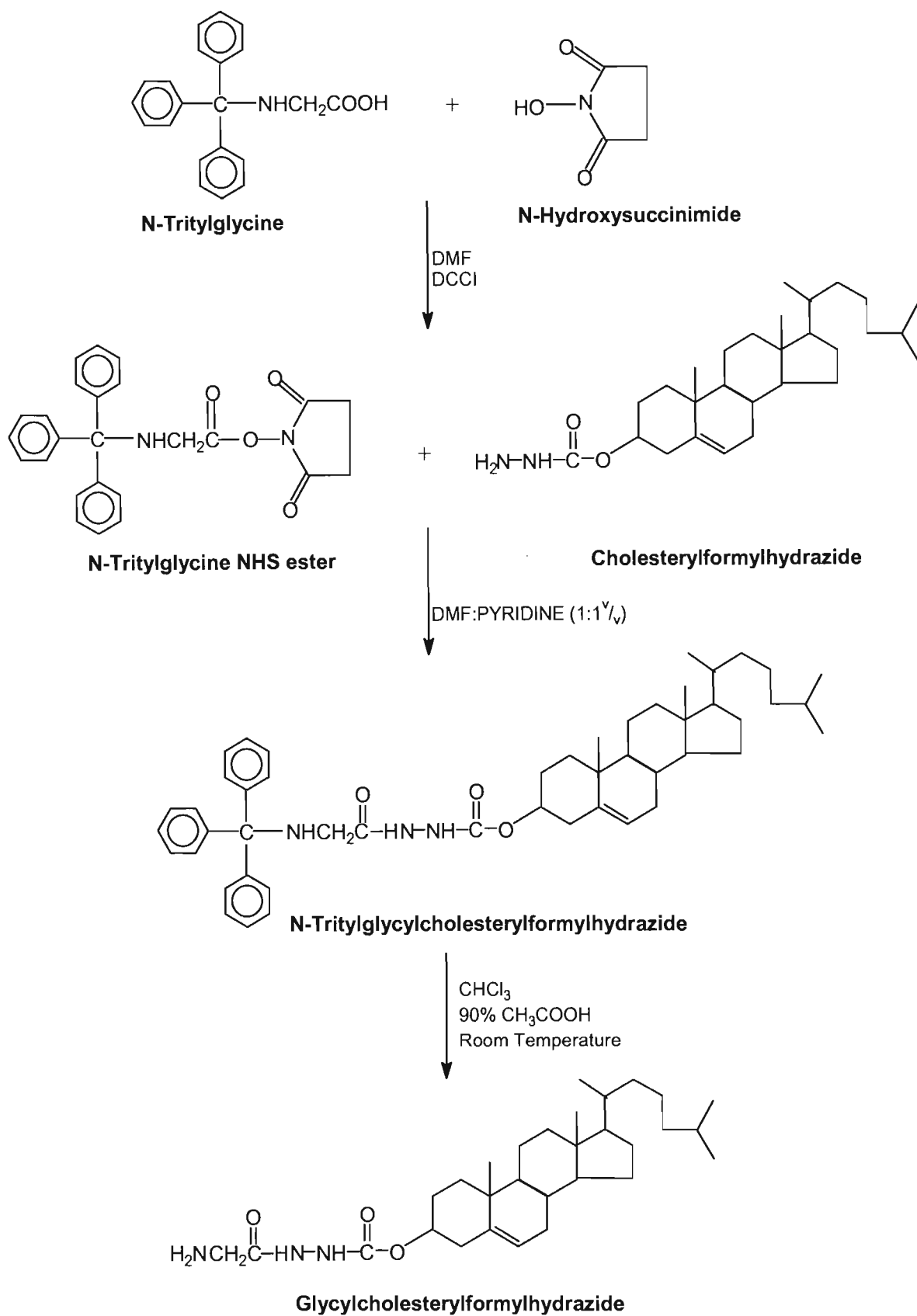
Figure 2.8 : Scheme for the synthesis of glycylcholesterylformylhydrazide (MS10).

2.2.4 SYNTHESIS OF CATIONIC CHOLESTEROL DERIVATIVE : β-ALANYLCHOLESTERYLFORMYLHYDRAZIDE

The preparation of β-alanylcholesterylformylhydrazide was carried out in four steps (Figure 2.9).

2.2.4.1 Preparation of N-trityl-β-alanine (MS06)

β-alanine was N-tritylated by a method described by Zervas and Theodoropoulos (1956) for the N-tritylation of amino acids. To a solution of β-alanine (1780 mg, 20 mmoles) in diethylamine (12 ml, 120 mmoles), H_2O (12 ml) and propan-2-ol (32 ml) was added powdered trityl chloride (7.2 g, 26 mmoles) in 10 equal aliquots with continuous stirring for one hour. The resulting milky solution was kept overnight at room temperature. Thereafter H_2O (80 ml) was added to the overnight solution. The resulting solution was extracted twice with $CHCl_3$ (2 x 100 ml), and the extract washed with H_2O (2 x 20 ml). The extract was concentrated under vacuum using a rotary evaporator. The final traces of $CHCl_3$ were removed by co-evaporation with 96% ethanol. The crude product was dissolved in ether (40 ml) together with a few drops of diethylamine. The sample was cooled to allow the diethylammonium salt of the N-trityl-β-alanine to crystallize.

The free N-trityl-β-alanine was obtained by dissolving the diethylammonium salt in H_2O (50 ml) and KOH (128 ml, 0.05 M). The liberated diethylamine was removed by rotary evaporation for about 10 minutes. The solution was then filtered using a buchner funnel. The precipitate was discarded and the supernatant was retained and kept in the cold. The product was obtained by acidification of the supernatant with acetic acid to a pH of about 5.0. The product was filtered using a buchner funnel and dried under vacuum using the Xerotec Vacuum Freeze Drier. The product was monitored by TLC in solvent system A, using both a ninhydrin spray for amino group detection and H_2SO_4 for cholesterol group detection. Product produced a single spot by TLC (R_f 0.51, solvent A) with a mp of 184-185°C (Ariatti and Hawtrey, 1975; 184-185°C) and a yield of

1.96 mg (32%). IR (film) : 3446 (b, N-H), 2924 (w, C-H), 1705 (s, COOH), 1449 (m, C=C), 1255 (m, C-N). (Appendix 2E).

2.2.4.2   Preparation of the NHS ester of N-trityl-β-alanine (MS05)

N-Trityl-β-alanine (662 mg, 2 mmoles) was dissolved in DMF (2 ml). The sample was warmed gently to completely dissolve the N-Trityl-β-alanine. To this was added NHS (230 mg, 2 mmoles). A solution of DCCI (433 mg, 2.1 mmoles) in DMF (2 ml) was added dropwise to the above mixture. The reaction mixture was maintained overnight in the dark and at room temperature. Thereafter the reaction was monitored by TLC in solvent system B. The UV absorbing NHS was viewed under UV_{254}, the trityl group was viewed using a 2% ($^{w}/_{w}$) $HClO_4$ spray, and the final product (active ester) was viewed using an active ester spray [14% ($^{w}/_{v}$) hydroxylamine hydrochloride, 3.5 M NaOH (20:8.5 $^{v}/_{v}$), which was sprayed first followed by a spray of $FeCl_3$ in 1.2 M HCl (5% $^{w}/_{v}$)].

The reaction mixture was then filtered under vacuum. The dicyclohexylurea was discarded. The supernatant containing the desired product was dried under reduced pressure using a rotary evaporator to remove any DMF residue. The product was dissolved with heating, using propan-2-ol and a mixture of $CHCl_3$: CH_3OH (1:1 $^{v}/_{v}$). The reaction mixture was then allowed to cool for the product to crystallize out. Some of the solvent was removed under reduced pressure and the product was maintained at 4°C for complete crystallization to take place. The product was then filtered under vacuum and crystals dried in a Xerotec Freeze Drier overnight. Product produced a single spot by TLC (R_f 0.92, solvent B) with a mp of 178-180 °C (Ariatti and Hawtrey, 1975 ; 178-179 °C) and a yield of 221.8 mg (26%).

IR (film) : 3452 (b, N-H), 2924 (w, C-H), 1736 (m, C=O), 1639 (m, aromatic rings). (Appendix 2D).

2.2.4.3 Preparation of N-trityl-β-alanylcholesterylformylhydrazide (MS02)

Cholesterylformylhydrazide (44.2 mg, 0.1 mmoles) and N-trityl-β-alanine NHS ester (42.8 mg, 0.1 mmoles), were dissolved in a mixture of DMF: pyridine (1:1 $^V/_v$) in a total volume of 800 µl and the resulting solution kept at room temperature overnight. The reaction was monitored by TLC in solvent system B. A mixture of $CHCl_3$: H_2O (1:1 $^V/_v$) was added to the reaction mixture to extract the product. The free NHS was eliminated in the water layer and the chloroform extract was evaporated under reduced pressure. The excess pyridine was co-evaporated with ethanol. The product was crystallized from methanol and dried under reduced pressure. The product afforded a single spot by TLC (R_f 0.97, solvent B) with a mp of 110-112 °C and a yield of 54.9 mg (72%).

IR (film) : 3426 (b, N-H), 2929 (st, C-H), 1649 (m, C=O), 1460 (m, C=C), 1383 (w, C-N), 1523 (v, aromatic rings). (Appendix 2B).

^{1}H NMR (300 MHz,$CDCl_3$) : δ 0.65 (s, 3H, C-CH_3), 0.86 (d, 6H, CH-CH_3), 0.89 (d, 3H, CH-CH_3), 0.98 (s, 3H, C-CH_3), 4.45 (m, 1H, Chol-$H_{3\alpha}$), 5.34 (bs, 1H, Chol-H_6) 7.18-7.52 (15H, trityl group) ppm. (Appendix 1B).

MS, $^m/_z$, ES-TOF : 758.5532 [M + H$^+$], 781.55 [M + Na$^+$].

2.2.4.4 Preparation of β-alanylcholesterylformylhydrazide (MS11)

N-trityl-β-alanylcholesterylformylhydrazide (49.9 mg, 0.066 mmoles) was dissolved in $CHCl_3$ (500 µl). Approximately 100 µl of 90% acetic acid was added to the reaction mixture, which was then incubated at 37°C and the product was monitored by TLC in solvent system B over a five hour period until the reaction had reached completion. The reaction mixture was thereafter evaporated to dryness to form a white film on the flask. Sufficient petroleum ether (100-120°C) was added to fully immerse the product which was kept at room temperature overnight. The ether was removed with a Pasteur pipette and the product dried in a rotary evaporator to produce a white powder. The product

was then recrystallised from ethanol and dried in the Xerotec Freeze Drier. The product affords a single spot by TLC (R_f 0.08, solvent B) and a yield of 33.5 mg (98%).

IR (film) : 3431 (b, N-H), 2934 (st, C-H), 1664 (m, C=O), 1250 (m, C-N), 1408 (m, C=C). (Appendix 2J).

MS, $^m/_z$, ES-TOF : 516.5375 [M + H$^+$], 538.5215 [M + Na$^+$]
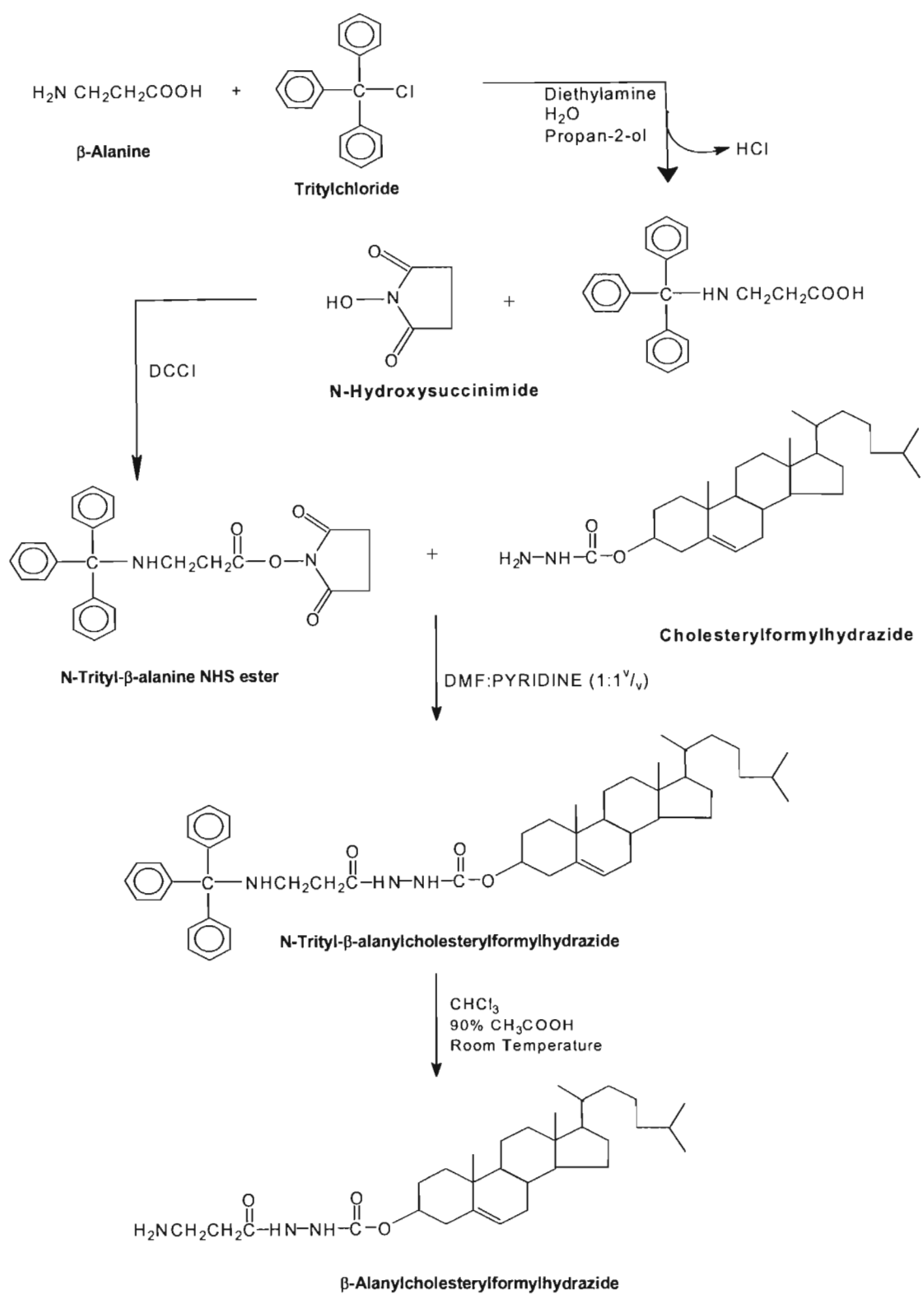
Figure 2.9 : Scheme for the synthesis of β-alanylcholesterylformylhydrazide (MS11).

2.2.5 PREPARATION AND CHARACTERISATION OF MS09, MS10 AND MS11 CONTAINING LIPOSOMES

2.2.5.1 Materials

DOPE was purchased from Sigma-Aldrich (St.Louis, MO, USA). 2-[4-(2-hydroxyethyl)-piperazinyl]-ethanesulfonic acid (HEPES), glutaraldehyde, osmium tetroxide, propylene oxide, lead citrate, sodium chloride and chloroform were obtained from Merck (Darmstadt, Germany). The components of Spurr's Resin (GRL 4206, DER 736, NSA , S-1), were purchased from TAAB Laboratories, United Kingdom. Copper grids for electron microscopy were obtained from Capital Labs (KwaZulu-Natal, South Africa). Bovine serum albumin (BSA) was purchased from Roche Diagnostics (Mannheim, Germany). All other reagents were of analytical grade.

2.2.5.2 Formulation of Cationic Liposomes

The preparation of the cationic liposomes was adapted from the method employed by Gao and Huang (1991). Liposome components (MS09, MS10 and MS11) were mixed in a 1:1 molar ratio respectively with DOPE as the 'helper' lipid, as set out in Table 2.1

Table 2.1 : The lipid components of cationic liposomes MS09 , MS10 and MS11.

LIPOSOME COMPONENTS	MOLAR RATIOS FOR RESPECTIVE LIPIDS (µmoles)			
	DOPE	MS09	MS10	MS11
Liposome MS09	2	2	-	-
Liposome MS10	2	-	2	-
Liposome MS11	2	-	-	2

The total reaction mixture of each formulation contained 4 μmoles of lipid. The liposome components of each cationic liposome preparation were dissolved in $CHCl_3$ and the resultant mixture evaporated to a thin film on the inside of a quickfit tube using a Büchii Rotavapor-R rotary evaporator. The thin film of sample was then rehydrated in 1 ml sterile HEPES buffer overnight (20 mM HEPES, 150 mM NaCl, pH7.5). The suspension was then vortexed and sonicated for five minutes at 20°C in a Transonic bath type sonicator and then placed at 4°C overnight.

2.2.5.3 Characterisation of Cationic Liposomes using Transmission Electron Microscopy

To one drop of each liposome suspension was added two drops of 5% bovine serum albumin (BSA) made up in 0.1 M Tris-HCl buffer (pH 7.2). BSA was added for the complexation of the liposome suspension thereby ensuring that a gel is produced. This was mixed and one drop of 25% glutaraldehyde was added. The resultant lemon coloured gels (after approximately 20 minutes) were then diced with a blade and transferred to a vial containing osmium tetroxide. The vials were capped and kept in the dark for 24 hours. Thereafter the samples were dehydrated for 15 minutes each in 70%, 95% and in absolute ethanol. The gels were then placed successively in propylene oxide for 20 minutes, and then in propylene oxide:Spurr's resin (1:1 $^V/_V$) for another 20 minutes. The samples were finally placed in pure Spurr's resin for 45 minutes. Thereafter the samples were embedded in beem capsules *in vacuo* at 60°C for 48 hours.

The resultant blocks were sectioned using a Reichert-Jung ultracut microtome. Sections were picked up onto C-200 copper grids. The grids were stained with uranyl acetate for 10 minutes, washed with distilled water, stained with lead citrate for a further 10 minutes, and washed again with distilled water. The stained sections were viewed using a Jeol 1010 Transmission Electron Microscope at 60 kV. Liposomes were photographed at a 2 second exposure on a fine grain release positive film.

2.2.6 LIPOPLEX FORMATION OF MS09, MS10 AND MS11 LIPOSOMES

2.2.6.1 Materials

The pGL3 control vector was purchased from Promega Corporation (Madison, USA), and amplified according to the manufacturers protocol. pBR322 plasmid DNA was supplied by Roche Diagnostics (Mannheim, Germany). Agarose was obtained from Bio-rad Laboratories (Richmond, CA, USA). Tris-HCl, NaH_2PO_4, HEPES, sodium chloride (NaCl), ethylene diamine tetra-acetic acid (EDTA), sodium dodecyl sulphate (SDS), glycerol, bromophenol blue, xylene cyanol, cholesterol hemisuccinate, $CHCl_3$, Whatman paper no.1 (1.3 cm), uranyl acetate and ethidium bromide were purchased from Merck, Darmstadt, Germany. Fetal bovine serum was obtained from Delta Bioproducts, Johannesburg, South Africa. All other reagent were of analytical grade. Formvar coated grids were prepared by the Electron Microscope Unit, University of KwaZulu Natal (Pietermaritzburg Campus). [^{3}H]pRSVL (specific activity : 2.1 x 10^6 cpm / µg) DNA was supplied by Professor Hawtrey, University of Stellenbosch.

The interactions between the various cationic liposomes and plasmid DNA were determined by gel retardation assays, dye displacement assays, transmission electron microscopy and nuclease digestion studies. In addition cationic cholesterol derivative MS09 was subjected to a DNA -binding assay.

2.2.6.2 Gel Retardation Assays

Varying ratios of different cationic liposome : DNA complexes were set up as in Table 2.2 (MSO9 liposomes), Table 2.3 (MS10 liposomes), and Table 2.4 (MS11 liposomes) respectively. All cationic liposome:DNA complexes were incubated for 30 minutes at room temperature for lipoplex formation. At the end of the incubation, 3 µl of gel loading buffer / stop solution (50% glycerol, 0.5% bromophenol blue, 0.5% xylene cyanol in a 2 x gel buffer) was added to all eight samples. The samples were then loaded onto a

1% agarose gel and subjected to agarose gel electrophoresis for approximately 90 minutes, at 50 V and in a 1 x electrophoresis / gel buffer (0.036 M Tris-HCl, 0.03 M NaH$_2$PO$_4$, 0.01 M EDTA, pH 7.5). The gel was stained with ethidium bromide (1 μg /ml) for 30 minutes and viewed under transillumination at 300 nm using a UVP gel documentation system. Images were obtained and photographed at 320 millisecond exposure.

Table 2.2 : MS09 cationic liposome:DNA complexes. Incubation mixtures (10 μl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained cationic liposome (0 - 6 μg) and pGL3 DNA (0.5 μg).

LIPOPLEX COMPONENTS	1	2	3	4	5	6	7	8
pGL3 DNA (μg)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Liposome MS09 (μg)	-	0.5	1	2	3	4	5	6

Table 2.3 : MS10 cationic liposome:DNA complexes. Incubation mixtures (15 μl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained cationic liposome (0 - 7 μg) and pGL3 DNA (1 μg).

LIPOPLEX COMPONENTS	1	2	3	4	5	6	7	8
pGL3 DNA (μg)	1	1	1	1	1	1	1	1
Liposome MS10 (μg)	-	1	2	3	4	5	6	7

Table 2.4 : MS11 cationic liposome:DNA complexes. Incubation mixtures (15 μl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained cationic liposome (0 - 7 μg) and pGL3 DNA (1 μg).

LIPOPLEX COMPONENTS	1	2	3	4	5	6	7	8
pGL3 DNA (μg)	1	1	1	1	1	1	1	1
Liposome MS11 (μg)	-	1	2	3	4	5	6	7

2.2.6.3   Dye Displacement Assays

This assay was adapted from that of Tros de Ilarduya *et al.*, 2002. Ethidium bromide (1 µg) was added to 500 µl of 20 mM HEPES,150 mM NaCl, pH 7.5. This mixture was used to provide the baseline reading (0%) for fluorescence. Thereafter 6 µg of pBR322 DNA was added to the above solution and the reading taken was assumed to represent 100% fluorescence. Aliquots of cationic liposome (10 µg for MS09 ; 5 µg for MS10and MS11), were added, stepwise to the solution until 90 µg of MS09 liposome and 70 µg of MS10 and MS11 liposomes had been added. The solutions were mixed thoroughly after each addition and fluorescence was measured in a Shimadzu RF-551 spectrofluorometric detector, at an excitation wavelength of 520 nm and an emission wavelength of 600 nm, and at high sensitivity. Results were plotted relative to 100% fluorescence.

2.2.6.4   Binding of Radiolabelled DNA to Lipid Impregnated Whatman No.1 Paper Discs

Lipids (0.3 µmoles) were dissolved in $CHCl_3$ (25 µl) and applied to Whatman no.1 (1.3 cm diameter) paper discs. After evaporation of the solvent, discs were suspended in 200 µl buffer containing 0.25 M Tris-HCl (pH 7.6), 0.05 M NaCl, 2.5 mM EDTA, and pRSVL DNA (0.03 µg , 6.4×10^4 cpm). Discs were incubated overnight at 18°C and then washed with 3 x 5 ml incubation buffer, air dried and then kept at 70°C for 15 minutes. A control sample of $CHCl_3$ (25 µl) was also included. Radioactivity was measured by liquid scintillation.

2.2.6.5 Transmission Electron Microscopy of Lipoplexes (MS09, MS10, MS11)

To one drop of the respective lipoplex suspension on parafilm was added 0.5% uranyl acetate. This was mixed and allowed to stand for three minutes. The matt surface of formvar coated grids were brought into contact with the lipoplex - uranyl acetate mixture for 3 minutes. Thereafter discs were air dried overnight and viewed in a Jeol 1010 transmission electron microscope at 60 kV.

2.2.6.6 Nuclease Digestion Assays

Liposome:DNA complexes were set up as in Table 2.5 (MS09), Table 2.6 (MS10) and Table 2.7 (MS11).

Table 2.5 : MS09 cationic liposome:DNA complexes. Incubation mixtures (20 µl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained cationic liposome (0 - 14 µg) and pGL3 DNA (1 µg).

LIPOPLEX COMPONENTS	1	2	3	4	5	6	7	8
pGL3 DNA (µg)	1	1	1	1	1	1	1	1
Liposome MS09 (µg)	-	-	4	6	8	10	12	14

Table 2.6 : MS10 cationic liposome:DNA complexes. Incubation mixtures (15 µl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained cationic liposome (0 - 7 µg) and pGL3 DNA (1 µg).

LIPOPLEX COMPONENTS	1	2	3	4	5	6	7	8
pGL3 DNA (µg)	1	1	1	1	1	1	1	1
Liposome MS10 (µg)	-	-	2	3	4	5	6	7

Table 2.7: MS11 cationic liposome:DNA complexes. Incubation mixtures (15 μl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained cationic liposome (0 - 7 μg) and pGL3 DNA (1 μg).

LIPOPLEX COMPONENTS	1	2	3	4	5	6	7	8
pGL3 DNA (μg)	1	1	1	1	1	1	1	1
Liposome MS11 (μg)	-	-	2	3	4	5	6	7

Samples were made up to a total volume of 20 μl (MS09), or 15 μl (MS10 and MS11), with 20 mM HEPES, 150 mM NaCl, pH 7.5. Lipoplexes in tubes 3 to 8 were incubated at room temperature for 30 minutes. Thereafter fetal calf serum was added to lipoplexes in tubes 3 to 8 and to tube 2 containing only DNA, to a final serum concentration of 10% ($^v/_v$). Sample 2 to 8 were then incubated at 37°C for four hours. Therafter EDTA was added to a final concentration of 10 mM and sodium dodecyl sulphate (SDS) to a final concentration of 0.5% ($^w/_v$). The reaction mixtures were incubated further at 55°C for 20 minutes.

At the end of the above incubation 3 μl of gel loading buffer was added to all eight samples. Samples were loaded onto a 1% agarose gel and subjected to agarose gel electrophoresis for 90 minutes at 50 V in a 1 x electrophoresis buffer, as described for the gel retardation assays (2.2.6.2). The gel was stained with ethidium bromide (1 μg /ml) for 30 minutes and viewed under 300 nm transillumination using a UVP gel documentation system. Images were obtained and photographed at 320 millisecond exposure.

2.2.7   CELL CULTURE AND TRANSFECTION STUDIES

2.2.7.1   Materials

Cell lines (HeLa, NIH-3T3, SNO, HepG2) were obtained from Highveld Biologicals (Pty) Ltd (Kelvin, South Africa). Trypsin-EDTA, and penicillin-streptomycin mixtures were purchased from Whittaker, M.A. Bioproducts (Maryland, USA). Fetal bovine serum was supplied by Delta Bioproducts (Johannesburg, South Africa). Minimum essential medium (MEM) with Earle's salts was obtained from Gibco-BRL, Life Technologies Ltd, (Inchinnan, Scotland). The Luciferase assay kit, and the pGL3 control vector (prior to amplification) were purchased from the Promega Corporation (Madison, USA). All tissue culture plasticware was obtained from Bibby-Sterilin (Staffordshire, England). The Bicinchoninic acid (BCA) Protein Assay Kit was supplied by Sigma-Aldrich (St.Louis, MO, USA). Dimethylsulfoxide (DMSO), crystal violet, formaldehyde were supplied by Merck (Darmstadt, Germany). All other reagents were of analytical grade.

2.2.7.2   Growth and Maintenance of Cell Lines

Preparation of Culture Medium :

MEM powdered medium intended for 1 litre of medium was dissolved in 900 ml of 18 Mohm water. To this solution was added 10 mmoles sodium hydrogen carbonate, 20 mmoles HEPES, and 20 ml of antibiotic (5000 units penicillin G and 5000 µg streptomycin sulphate / ml). The pH was adjusted with sodium hydroxide to pH 7.2 - 7.3. The medium was then made up to 1 litre with 18 Mohm water. The culture medium was filter-sterilised with the aid of a Cole-Palmer Masterflex (model 7017-12) peristaltic pump, through a Sterivex-GS, 0.22 µm bell filter unit, into autoclaved 250 ml Schott bottles (200 ml aliquots). Before the medium could be added to the cells for growth, fetal bovine serum (FBS) was added to the medium to a final concentration of 10% ($^V/_V$).

Propagation of HeLa, SNO, NIH-3T3 and HepG2 Cells:

All cells were received from Highveld Biologicals in 2 x 25 cm^2 flasks in MEM, and were immediately incubated at 37°C overnight. Thereafter the medium from the cells was decanted, and the cells washed with 5 ml phosphate buffered saline (PBS) (150 mM sodium chloride, 2.7 mM potassium chloride, 1 mM potassium dihydrogen phosphate, 6 mM di-sodium hydrogen phosphate, pH 7.5). Excess PBS was removed and 1 ml trypsin-EDTA mixture (0.25% $^w/_v$ trypsin , 0.1% $^w/_v$ EDTA) was added to the cells for trypsinisation. Trypsinisation of the cells was observed under a Nikon TMS inverted microscope (incubation times were 3 minutes for HeLa, 3T3 and SNO, but 5-10 minutes for HepG2 at 37°C). The trypsin solution was then removed and the flasks lightly tapped against the base of the palm in order to dislodge the trypsinised cells. Thereafter approximately 1 ml of medium (MEM + 10 % FCS + antibiotics), was added to the cells. The cells were split in ratios of 1:2 (HepG2 cells) or 1:3 into 2 or 3 x 25 cm^2 flasks, each containing 5 ml medium. The cells were incubated at 37°C and medium was changed at the required intervals. Once cells had reached confluency they were once again trypsinised and split appropriately as required. Cells were routinely frozen and stored in a -80°C biofreezer.

Cryopreservation and Reconstitution of HeLa, SNO, NIH-3T3 and HepG2 Cells:

Confluent cells were washed with PBS and trypsinised. The cells were then pelleted by centrifugation at 1000 rpm for 3 minutes in a MSE bench top centrifuge. The cells were then resuspended in 0.9 ml medium and 0.1 ml DMSO. This cell suspension was dispensed into a cryogenic ampoule and sealed. The cells were frozen to about -70°C using a cold probe, at a drop rate of 1°C / minute, from room temperature before being transferred to the -80°C biofreezer for storage.

The cells were reconstituted when required by removing ampoules from the low temperature storage and immediately placing them in a 37°C water bath. Directly after

thawing, the ampoules were wiped with ethanol, and the cells were pelleted by centrifugation (1000 rpm for 3 minutes). The supernatant was discarded into a waste bottle, and the cells were resuspended in 1 ml fresh complete medium. The medium was changed after 24 hours, and the cells were monitored with frequent medium changes until they reached confluency. All cell lines were photographed on FP4 film using a Nikon camera attached to the inverted microscope, with sensitivity set at 200 and an exposure time of 2 seconds.

2.2.7.3   Growth Inhibition Assays

Cells were trypsinised and seeded into a 24 well plate at varying seeding densities. For studies involving liposome MS09 the seeding density was 2.0×10^4 cells/well for HeLa cells, 1.8×10^4 cells/well for SNO cells, 2.1×10^4 cells/well for NIH-3T3 cells and 1.8×10^4 cells/well for HepG2 cells. For liposome MS10 the seeding density was 2.6×10^4 cells/well for HeLa cells, 2.2×10^4 cells/well for SNO cells, 2.3×10^4 cells/well for NIH-3T3 cells and 2.0×10^4 cells/well for HepG2 cells, and for liposome MS11 the seeding density was 2.75×10^4 cells/well for HeLa cells, 2.0×10^4 cells/well for SNO cells, 2.2×10^4 cells/well for NIH-3T3 cells and 2.1×10^4 cells/well for HepG2 cells. Wells in row 1 received no cells and were used to obtain a blank (0) reading. Cells were incubated for 24 to 36 hours and allowed to attach to the wells and grow to semi-confluency. The liposome:DNA [pGL3 DNA (0.5 μg or 1 μg)] complexes were set up as outlined in Tables 2.8, 2.9, 2.10. The reaction mixtures were all made up to a constant volume with 20 mM HEPES, 150 mM sodium chloride, pH 7.5, and incubated at room temperature for 30 minutes.

Table 2.8 : MS09 cationic liposome:DNA complexes. Incubation mixtures (10 µl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained cationic liposome (0–7 µg) and pGL3 DNA (0.5 µg).

LIPOPLEX COMPONENTS	1	2	3	4	5	6
pGL3 DNA (µg)	-	0	0.5	0.5	0.5	0.5
Liposome MS09 (µg)	-	0	3	5	6	7

Table 2.9: MS10 cationic liposome:DNA complexes. Incubation mixtures (15 µl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained cationic liposome (0–7 µg) and pGL3 DNA (1 µg).

LIPOPLEX COMPONENTS	1	2	3	4	5	6
pGL3 DNA (µg)	-	0	1	1	1	1
Liposome MS10 (µg)	-	0	4	5	6	7

Table 2.10: MS11 cationic liposome:DNA complexes. Incubation mixtures (15 µl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained cationic liposome (0–7 µg) and pGL3 DNA (1 µg).

LIPOPLEX COMPONENTS	1	2	3	4	5	6
pGL3 DNA (µg)		0	1	1	1	1
Liposome MS11 (µg)	-	0	4	5	6	7

The cells were prepared by first removing the growth medium and replacing it with 0.5 ml of serum free medium. The reaction complexes were then added to the wells containing the cells. The assays were carried out in quadruplicate. The cells were then incubated at 37°C for 4 hours. Thereafter, the medium was replaced with complete

medium (MEM + 10% FCS + antibiotics). The cells were incubated again at 37°C for a further 48 hours. The cells were then quantified by the method of Schellekens and Stitz, 1980, which suggests that the viable cells will adhere to the flask surface and can be quantified by staining as described. The cells were washed twice with PBS and stained with 200 μl crystal violet solution (0.5% $^W/v$ crystal violet, 0.8% $^W/v$ sodium chloride, 5% $^V/v$ formaldehyde, 50% $^V/v$ ethanol) for 20 minutes. The stain was then removed and the cells washed extensively with water. The multiwell plate was then dried for 24 hours, and the stain extracted with 2-methoxyethanol (0.5 ml) over a period of 36 hours, with gentle rocking (20 rev / minute) on a Stuart Scientific STR 6 platform shaker. Absorbance values for the samples were then read in a Novospec spectrophotometer, at a wavelength of 550 nm.

2.2.7.4   Transfection Studies
(a)   Liposome MS09 : pGL3 DNA complexes
(b)   Liposome MS10 : pGL3 DNA complexes
(c)   Liposome MS11 : pGL3 DNA complexes
(d)   Lipofectin™ : pGL3 DNA complexes

Transfection of all lipoplexes was conducted using four cell lines viz. HeLa, NIH-3T3, SNO and HepG2 cells. Lipofectin™, a commercially available liposome preparation was utilized as a comparator. All cells were trypsinised and seeded at varying densities in 24 well plates. The cells were then allowed time to attach to the wells and to grow to semi-confluency. The transfection complexes (a – d) were then set up in quadruplicate for all cell lines as described in Tables 2.11, 2.12, 2.13 and 2.14. The transfection efficiencies of the three cationic liposome:DNA complexes (a – c) in the presence of 10% serum were also evaluated.

(a)   Liposome MS09 : pGL3 DNA complexes

Transfection complexes were added to 24-well plates containing cells at a seeding density of 2.25×10^4 cells/well for HeLa cells, 1.8×10^4 cells/well for SNO cells, 2×10^4 for NIH-3T3 cells/well and 1.9×10^4 cells/well for HepG2 cells. For transfection in the presence of 10% FCS the seeding densities were 2.35×10^4 cells/well for HeLa cells, 2.23×10^4 cells/well for SNO cells, 2.15×10^4 for NIH-3T3 cells/well and 2.1×10^4 cells/well for HepG2 cells.

Table 2.11 : MS09 transfection complexes as added to 24-well plates. Control 1 contained only cells, control 2 contained cells and pGL3 DNA (0.5 µg) only. Test samples 1 to 4 contained varying amounts of liposome MS09 (3, 5, 6 and 7 µg respectively). pGL3 DNA was kept constant at 0.5 µg. All complexes were made up in a final volume of 10 µl with 20 mM HEPES, 150 mM sodium chloride (pH 7.5).

Reaction Components	Control 1	Control 2	Test 1	Test 2	Test 3	Test 4
HeLa / SNO / 3T3 / HepG2 Cells	+	+	+	+	+	+
Liposome MS09 (µg)	-	-	3	5	6	7
pGL3 DNA (µg)	-	0.5	0.5	0.5	0.5	0.5

(b)   Liposome MS10 : pGL3 DNA complexes

Transfection complexes were added to 24-well plates containing cells at a seeding density of 2.6×10^4 cells/well for HeLa cells, 2.2×10^4 cells/well for SNO cells, 2.3×10^4 cells/well for NIH-3T3 cells and 2×10^4 cells/well for HepG2 cells. For transfection in the presence of 10% FCS the seeding densities were 2.25×10^4 cells/well for HeLa cells, 2.4×10^4 cells/well for SNO cells, 2.1×10^4 cells/well for NIH-3T3 cells and 1.8×10^4 cells/well for HepG2 cells.

Table 2.12 : MS10 transfection complexes as added to 24-well plates. Control 1 contained only cells, control 2 contained cells and pGL3 DNA (1 µg) only. Test samples 1 to 4 contained varying amounts of liposome MS10 (4,5,6 and 7 µg respectively). pGL3 DNA was kept constant at 1 µg. All complexes were made up in a final volume of 20 µl with 20 mM HEPES, 150 mM sodium chloride (pH 7.5).

Reaction Components	Control 1	Control 2	Test 1	Test 2	Test 3	Test 4
HeLa / SNO / 3T3 / HepG2 Cells	+	+	+	+	+	+
Liposome MS10 (µg)	-	-	4	5	6	7
pGL3 DNA (µg)	-	1	1	1	1	1

(c)   Liposome MS11 : pGL3 DNA complexes

Transfection complexes were added to 24-well plates containing cells at a seeding density of 2.75×10^4 cells/well for HeLa cells, 2×10^4 cells/well for SNO cells, 2.2×10^4 cells/well for NIH-3T3 cells and 2×10^4 cells/well for HepG2 cells. For transfection in the presence of 10% FCS the seeding densities were 2.1×10^4 cells/well for HeLa cells, 2.3×10^4 cells/well for SNO cells, 2×10^4 for cells/well NIH-3T3 cells and 1.8×10^4 cells/well for HepG2 cells.

Table 2.13 : MS11 transfection complexes as added to 24-well plates. Control 1 contained only cells, control 2 contained cells and pGL3 DNA (1µg) only. Test samples 1 to 4 contained varying amounts of liposome MS10 (4,5,6 and 7 µg respectively). pGL3 DNA was kept constant at 1 µg. All complexes were made up in a final volume of 20 µl with 20 mM HEPES, 150 mM sodium chloride (pH 7.5).

Reaction Components	Control 1	Control 2	Test 1	Test 2	Test 3	Test 4
HeLa / SNO / 3T3 / HepG2 Cells	+	+	+	+	+	+
Liposome MS11 (µg)	-	-	4	5	6	7
pGL3 DNA (µg)	-	1	1	1	1	1

(d) Lipofectin™ : pGL3 DNA complexes

Transfection complexes were added to 24-well plates containing cells at a seeding density of 2.25×10^4 cells/well for HeLa cells, 1.8×10^4 cells/well for SNO cells, 2×10^4 cells/well for NIH-3T3 cells and 1.9×10^4 cells/well for HepG2 cells.

Table 2.14 : Lipofectin™ transfection complexes as added to 24-well plates. Control 1 contained only cells, control 2 contained cells and pGL3 DNA (1 µg) only. Test samples 1 to 4 contained varying amounts of lipofectin (4, 5, 6 and 7 µg respectively). pGL3 DNA was kept constant at 1 µg. Lipoplexes were incubated in serum free medium in a final volume of 100 µl.

Reaction Components	Control 1	Control 2	Test 1	Test 2	Test 3	Test 4
HeLa / SNO / 3T3 / HepG2 Cells	+	+	+	+	+	+
Lipofectin™ (µg)	-	-	4	6	8	10
pGL3 DNA (µg)	-	1	1	1	1	1

Lipofectin™:DNA complexes were prepared according to manufacturer's protocol in quadruplicate. Lipofectin™ reagent (4, 6, 8, 10 µg), and pGL3 DNA (1 µg) were diluted separately in 50 µl serum free medium. Both solutions were allowed to stand for 30 minutes before they were combined. The two solutions were then mixed and incubated for a further 15 minutes at room temperature, for lipoplex formation. The lipoplexes were then added to the cells and transfection conducted as described below.

Typically liposome:DNA complexes (a – c) were incubated at room temperature for 30 minutes prior to addition to cells. The cells were prepared by removal of the growth medium, washing with 0.5 ml PBS and replenishing with 0.5 ml serum free medium (MEM + antibiotics). For the evaluation of transfection efficiency in the presence of 10% serum the medium utilized was complete medium (MEM + antibiotics + 10% FCS). Thereafter the transfection complexes (lipoplexes) were added to the cells. The cells were then incubated at 37°C for 4 hours. After 4 hours the medium was removed and

replaced with complete growth medium (MEM + antibiotics + 10% FCS). The cells were incubated for a further 48 hours at 37°C after which they were assayed for luciferase activity using the luciferase assay system (Promega).

2.2.7.5   Luciferase Assay

The luciferase assay was carried out using the Promega Luciferase Assay system. The luciferase assay reagent (20 mM tricine, 1.1 mM magnesium carbonate hydroxide, pentahydrate, 2.7 mM magnesium sulphate, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 µM coenzyme A , 470 µM luciferin, 530 µM ATP), was prepared by adding 10 ml of the given luciferase assay buffer to one vial of lyophilised luciferase assay substrate. The cell culture lysis reagent (5x) [25 mM Tris-phoshate, pH 7.8 ; 2 mM dithiothreiotol ; 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetra-acetic acid, 10% ($^V/_V$) glycerol, 1%($^V/_V$) Triton X-100], was diluted with distilled water to produce a 1 x stock. Both reagents were equilibrated to room temperature.

The cells were prepared by first removing the growth medium and washing twice with PBS (2 x 0.5 ml) being careful not to dislodge any of the attached cells. A minimal amount of 1 x cell culture lysis reagent (80 µl) was added to cover the cells. The multiwell plate was then placed on a platform rocker for 15 minutes. Thereafter the attached cells were dislodged from the wells and the cell solution was transferred to a microcentrifuge tube. The cells were spun briefly (5 seconds) in an Eppendorf microcentrifuge at 12 000 x g to pellet the large debris. The cell free extracts (supernatants) were retained to be assayed for luciferase activity. To 20 µl of cell free extract at room temperature was added 100 µl of luciferase assay reagent. The reaction mixture was immediately mixed and placed in a luminometer (Lumac Biocounter 1500). The light produced was measured for a period of 10 seconds. The protein determination of the cell free extracts was performed using the BCA assay (Sigma) with BSA as the standard protein (Smith et al., 1985).

2.3   RESULTS AND DISCUSSION

2.3.1   PREPARATION OF CATIONIC CHOLESTEROL DERIVATIVES

Three novel cationic cholesterol derivatives were synthesized for formulation into cationic liposomes (Figures 2.7, 2.8, 2.9). Each of these derivatives has a cholesterol anchor in common, but they differ in the lengths of their relatively polar spacer arms and the cationic head groups. MS09 has the longest spacer arm and a dimethylammonium head group. Of the two amino acid-containing derivatives, MS11 (the β-alanyl derivative) has just one extra carbon in its spacer arm compared to MS10 (the glycyl derivative). Both compounds have simple primary amino head groups. The head groups become protonated at physiological pH and thus behave as cationic headgroups, potentiating transfection. These compounds were all used in the formulation of three novel cationic liposomes which were assessed for their transfection efficiency in four different cell lines *in vitro*. As expected, the transfection efficiencies of the three liposome formulations were significantly different (2.3.4.3). This will be discussed later in this chapter.

It is assumed that derivatives that possess headgroups with a primary amine or a bulky tertiary amine produce lower transfection efficiencies than derivatives with a secondary amine headgroup (Hasegawa *et al.*, 2002). Furthermore the use of hydrophobic components ensure that cationic lipids assemble into lipid bilayer vesicles on dispersion in aqueous media, shielding the hydrophobic portion of the molecule and exposing the amine group/s to the aqueous medium. These amine groups are the DNA-binding moieties and upon electrostatic interaction with the DNA molecule, condense the larger liposomal structures into smaller transportable units or lipoplexes (Brown *et al.*, 2001). This can be seen in section 2.3.3.4.

Structure-activity relationship studies have also shown that the number of amine groups per molecule and the distance between the headgroup and the hydrophobic units affects gene delivery (Remy *et al.*, 1994 ; Wheeler *et al.*, 1996a). Finally the addition of

a cationic lipid into a liposomal formulation that has a good surface hydration will lead to increased transfection activity (Bennett *et al.*, 1996). The nature of a cationic lipid can be related to its aqueous stability, hydrophilic-hydrophobic balance, solubilizing power, affinity for membrane lipids and proteins, and biodegradability (Lasic, 1997).

2.3.2 PREPARATION AND CHARACTERISATION OF MS09, MS10 AND MS11 CONTAINING LIPOSOMES

2.3.2.1 Formulation of Cationic Liposomes

Cationic liposomes were successfully formulated using the method described. This was revealed using transmission electron microscopy. Liposomes were easily prepared using equimolar amounts of cationic lipid and DOPE. DOPE is often used as a helper lipid because most cationic surfactants form micelles but not liposomes. The addition of a phospholipid such as DOPE that has a strong tendency to adopt an inverted hexaganol structure (H_{11} phase) was found to be very important. DOPE assists in the formation of liposomes with a significantly increased transfection efficiency, while it tends to reduce cytotoxicity of the cationic surfactants (Sternberg *et al.*, 1994). The neutral lipid DOPE also plays a role in assisting fusion or membrane destabilisation in the cationic lipid-mediated DNA delivery mechanism. DOPE on its own is inactive as a transfection reagent (Gao and Huang, 1991), probably due to its inability to interact with DNA.

It is likely that the cationic lipid reacts with DOPE in a 1:1 ratio to form a heterodimer in the liposomal bilayer. This heterodimer may be formed due to interaction between the negatively charged phosphate on DOPE and the ammonium group on the cationic lipid. Upon formation of the heterodimer, the polar head group will be rendered more hydrophobic due to charge neutralisation after formation of the ion pair. Compounds which contain the hydroxyl group will remain in contact with the aqueous phase, maintaining the integrity of the bilayer structure, whereas those lacking the hydroxyl

group may become buried within the aliphatic region, disrupting the bilayer structure (Felgner *et al.*, 1994).

2.3.2.2 Characterisation of Liposomes by Transmission Electron Microscopy

Transmission electron microscopy revealed the unilamellar nature and the sizes of the different cationic liposome preparations. Liposome MS09 sizes varied from 80 nm to 150 nm, liposome MS10 from 100 nm to 200 nm, and liposome MS11 from 50 nm to 200 nm (Figures 2.10 to 2.12). A liposome size of 150 nm or less seems to favour the process of endocytosis (Bertling *et al.*, 1991).

It was shown using cryoelectron microscopy that cationic liposomes can exist in a variety of shapes such as tubular, dumbbell, pear, oval or elongated oval shapes, in addition to spherical shapes. Liposomes sometimes also appear invaginated , lenslike or flat (Lasic, 1997). In our preparations predominantly spherical or oval shaped liposomes were observed. Liposome diameter and size dispersity are important parameters in drug delivery applications (Campbell *et al.*, 2001).
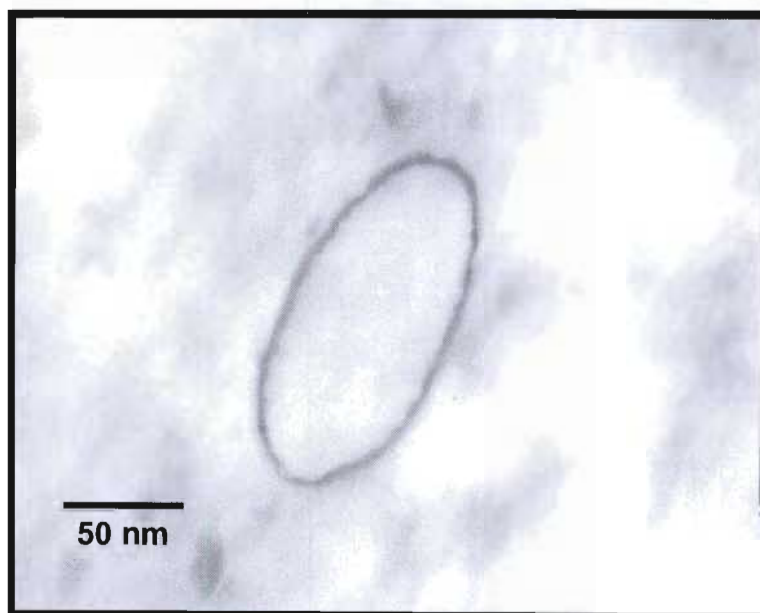
Figure 2.10 : TEM of liposome MS09. Liposome size ranged from 80 nm to 150 nm.
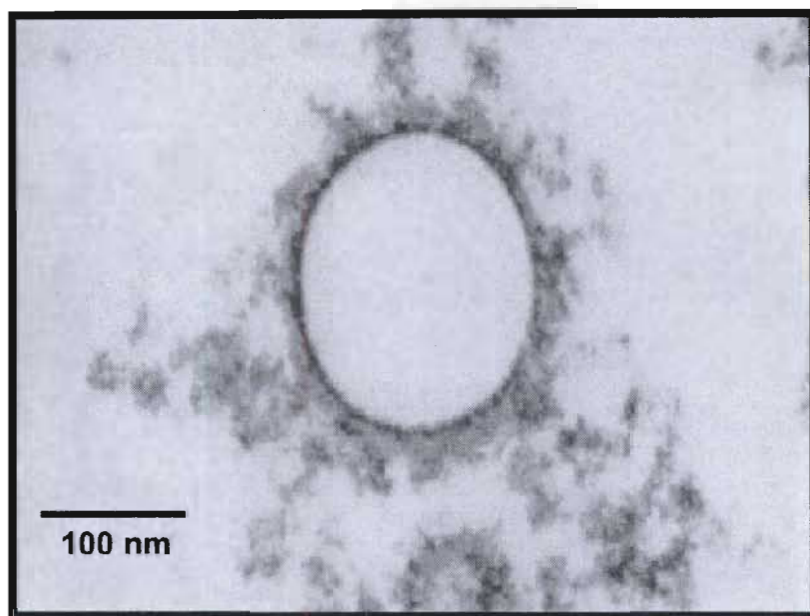
Figure 2.11 : TEM of liposome MS10. Liposome size ranged from 100 nm to 200 nm.
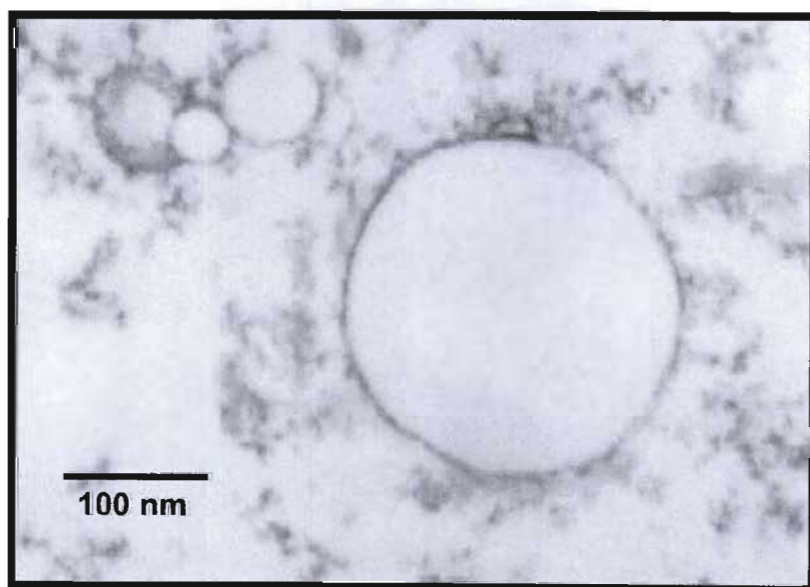
Figure 2.12 : TEM of liposome MS11. Liposome size ranged from 50 nm to 200 nm.

2.3.3   LIPOPLEX FORMATION OF MS09, MS10 AND MS11 LIPOSOMES

Lipoplexes (liposome:DNA complexes) are usually produced within 20 minutes of incubation at room temperature. They were demonstrated using gel retardation assays, dye displacement assays, nuclease digestion assays and transmission electron microscopy. The cationic cholesterol derivative, MS09 was further characterized for its ability to bind DNA using a filter paper binding assay.

2.3.3.1   Gel Retardation Assays

Gel retardation analyses were carried out on all three lipoplexes (MS09, MS10 and MS11), each yielding a different pattern of binding with a specific optimum binding ratio (Figures 2.13 and Figure 2.14).
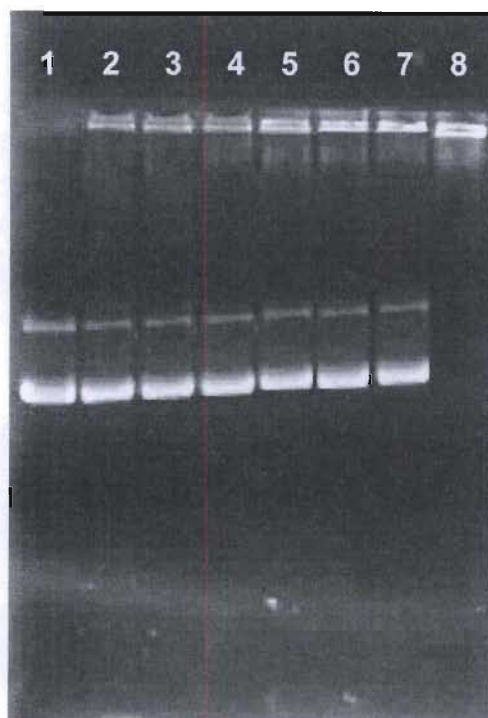
Figure 2.13 : Gel retardation study of MS09 cationic liposome:DNA complexes. Incubation mixtures (10 µl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained varying amounts of cationic liposome in lanes 1-8 (0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 µg), while the pGL3 DNA was kept constant at 0.5 µg.
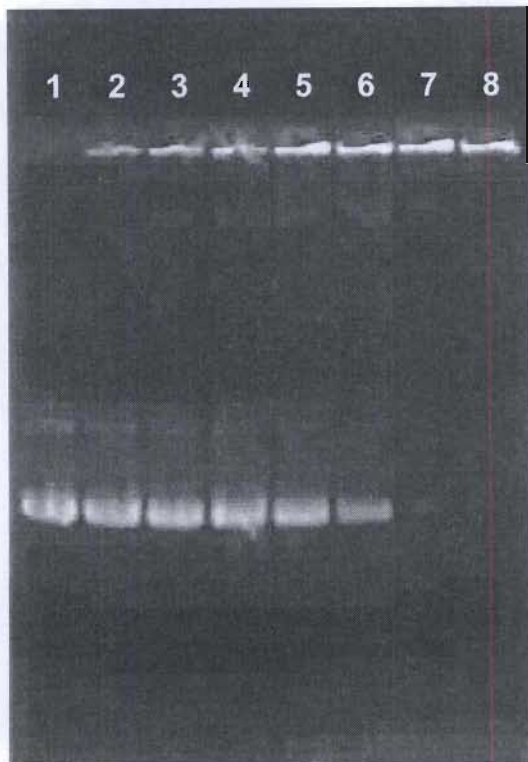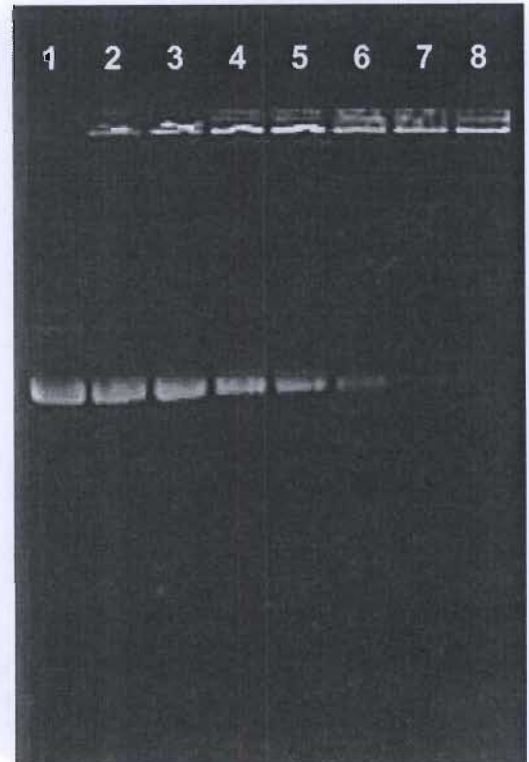
Figure 2.14 : Gel retardation study of (a) MS10 cationic liposome:DNA complexes, and, (b) MS11 cationic liposome:DNA complexes.
Incubation mixtures (15 µl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained varying amounts of cationic liposome in lanes 1-8 (0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 µg), while the pGL3 DNA was kept constant at 1.0 µg.

The use of agarose gel electrophoresis serves to demonstrate the formation of complexes between DNA and cationic liposomes. In the absence of liposomes, the DNA would migrate into the gel, but in the presence of increasing liposome concentrations the DNA is bound to the cationic liposome and retarded and hence retained in the wells. It is accepted that uncomplexed DNA will migrate into the gel and the liposome:DNA complexes will not (Bertling *et al.*, 1991).

It can be seen that the plasmid DNA (pGL3) produced the expected two bands (lane 1), viz. the supercoiled form of DNA (bottom band) and the nicked circular form (top band) which is more clearly visible in Figure 2.13. As the respective cationic liposome concentration increased, more DNA was bound by the cationic liposome and hence less DNA entered the gel. This can be seen by the ethidium bromide staining intensity. DNA that did not migrate into the gel can also be seen stained in the wells. Complete retardation of the DNA in the case of each liposome was obtained at different liposome:DNA binding ratios viz. 12:1 ($^{w}/_{w}$) for MS09, 6:1 ($^{w}/_{w}$) for MS10 and 6:1 ($^{w}/_{w}$) for MS11. These ratios will help to determine the optimum ratios for transfection studies.

Complete retardation suggests that the negative charges of the DNA are completely titrated by the cationic liposome's positive charges. This sometimes produces large electroneutral complexes that do not migrate into the agarose gel matrix. These complexes can be seen, once stained with ethidium bromide, to be present in the wells. Precipitation of the electroneutral complexes may also occur in the sample wells. Occasionally precipitated complexes may float out of the wells during electrophoresis, or during the staining / washing process, and are hence not seen under UV light in the wells even after staining with ethidium bromide. In these cases no DNA will be detectable in the wells. Liposomes MS10 and MS11 bound at a lower liposome:DNA ratio (6:1) and a +/- charge ratio of 0.8, whilst the MS09 bound at a higher liposome:DNA ratio (12:1)and a +/- charge ratio of 1.3.

2.3.3.2 Dye Displacement Assays

These assays showed that as the amount of liposome added to the DNA increased, the fluorescence emitted by the ethidium bromide was quenched due to the binding of cationic liposome to the plasmid DNA, hence displacing the ethidium bromide. This gradual decrease in fluorescence was observed with all three liposome formulations (Figure 2.15, Figure 2.16 and Figure 2.17).
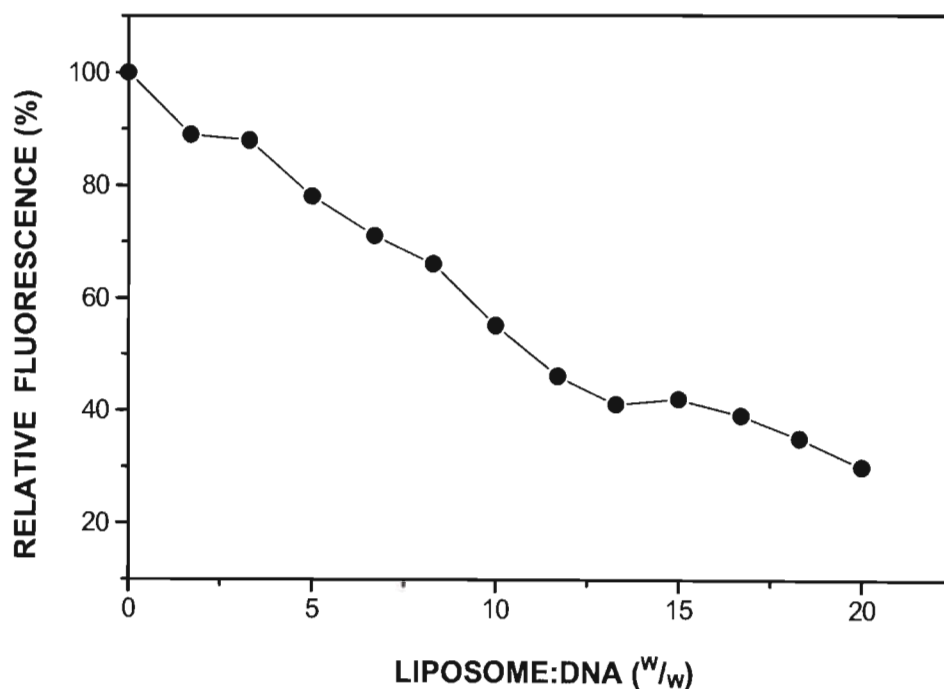
Figure 2.15 : Ethidium bromide intercalation assay for cationic liposome MS09 at varying liposome : DNA ($^w/_w$) binding ratios.
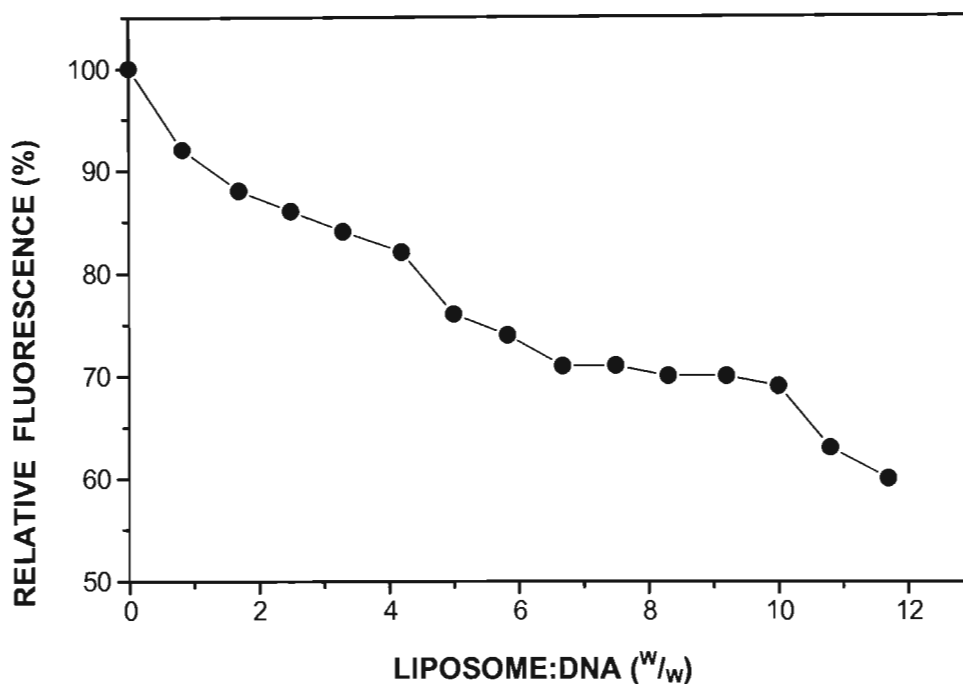
Figure 2.16 : Ethidium bromide intercalation assay for cationic liposome MS10 at varying liposome : DNA ($^w/_w$) binding ratios.
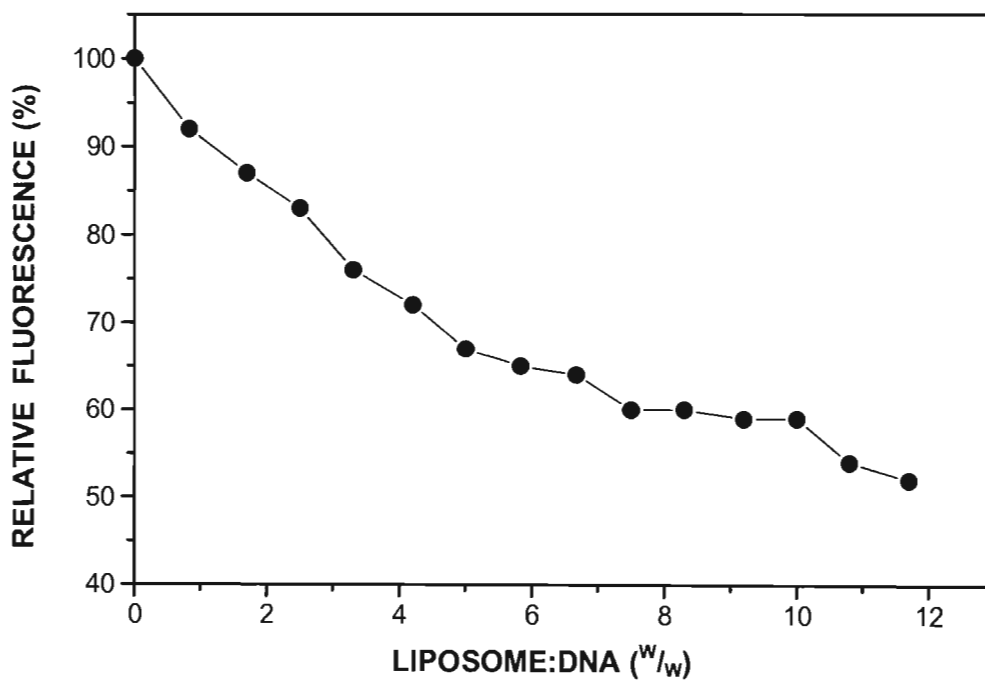
Figure 2.17 : Ethidium bromide intercalation assay for cationic liposome MS11 at varying liposome:DNA ($^w/_w$) binding ratios.

Ethidium bromide is an aromatic planar cationic fluorophore whose fluorescence increases upon intercalation between base pairs of the double-stranded DNA (Even-Chen and Barenholz, 2000). This was observed upon the initial addition of pBR322 DNA to the ethidium bromide solution in buffer which resulted in a marked enhancement of its fluorescence emission at 600 nm. This increase in fluorescence can be explained by the steric protection from molecular oxygen, inducing dequenching that is proportional to the level of fluorophore intercalation (Even-Chen and Barenholz, 2000). This was taken as being the 100% fluorescence reading. Upon the stepwise addition of the respective cationic liposome preparation, a steady decrease in fluorescence was observed. This was due to the formation of the cationic liposome:DNA complex that causes displacement of the intercalated ethidium bromide from the DNA, hence reducing the emitted fluorescence. The decrease in fluorescence occurs since the ethidium bromide will now be fully accessible to quenching by molecular oxygen. This change of fluorescence can be used to assess the level of condensation or encapsulation of DNA (Even-Chen and Barenholz, 2000). The displacement of ethidium bromide from DNA is generally a rapid process and is usually completed within 30 seconds (Xu and Szoka, 1996). Most cationic liposome preparations (DOTAP, DOTAP/DOPE, DOTAP/DOPC) were seen to displace ethidium bromide in a similar fashion (Xu et al., 1999).

The fluorescence decreased steadily until a point was attained which seemed to correlate with total retardation in gel retardation assays. The maximum displacement of ethidium bromide was obtained for liposome MS09 (approximately 65%) at the total retardation ratio of 12:1. Liposomes MS10 and MS11 both achieved maximum displacement of ethidium bromide at a liposome:DNA ratio of 6:1 (^{w}/$_w$), corresponding to the full retardation ratio obtained in band shift assays. The reduction in fluorescence at these points was, however, much less marked than that achieved by MS09 (29% and 36% respectively). This may be indicative of tighter packing of DNA by the MS09 liposomes and could account for the large differences in transfection efficiencies observed between MS09 lipoplexes and MS10 and MS11 lipoplexes (section 2.3.3.4).

Further increase in liposome:DNA ratios in the dye displacement assay resulted in the onset of turbidity and may account for the sharp decline in fluorescence observed at high ratios.

2.3.3.3 Binding of Radiolabelled DNA to Lipid impregnated Whatman No.1 Paper Discs

The results obtained in this assay showed that cationic cholesterol derivative MS09 binds very strongly to plasmid DNA (pRSVL DNA) (Table 2.15). This assay was conducted on cationic cholesterol derivative MS09 since it produced the highest transgene expression among the three cationic cholesterol derivatives in transfection assays.

Table 2.15 : Filter paper binding assay of cationic cholesterol derivative , MS09, compared against a control (CHCl$_3$) and standards (cholesterol and cholesteryl hemisuccinate).

COMPOUND	cpm / µmole*
Cholesterol	596*
Cholesteryl hemisuccinate	958*
Cationic Derivative (MS09)	6708*
CHCl$_3$ control (25 µl)	270

This strong affinity of cationic cholesterol derivative MS09 for DNA also correlates with its high fluorescence quenching due perhaps to tight binding of DNA.

2.3.3.4   Transmission Electron Microscopy of Lipoplexes

The enhancement of transfection efficiencies using non-viral methods such as liposomes requires a full understanding of the supramolecular structures of the cationic liposome:DNA complex and their interactions with the cell membrane (Safinya, 2001). This procedure was utilized to determine the size and ultrastructural morphology of the various lipoplexes formed at two different ratios. Each lipoplex was monitored at the ratio that produced complete retardation and at a higher ratio (Figures 2.18-2.20).

In the case of MS09 liposomes, the diameter of the lipoplex ranged from 200 nm at the lower liposome:DNA ratio of 12:1 (+/- charge ratio of 1.3) to 300 nm at a higher liposome:DNA ratio of 14:1 (+/- charge ratio of 1.7). The MS10 liposomes, gave lipoplex diameters ranging from 250 nm for a liposome:DNA ratio of 6:1 (+/- charge ratio of 0.8) to 350 nm for a liposome:DNA ratio of 7:1 (+/- charge ratio of 1). MS11 liposomes gave lipoplexes with diameters ranging in size from 200 nm for a liposome:DNA ratio of 6:1 (+/- charge ratio of 0.8) to 350 nm for the liposome:DNA ratio of 7:1 (+/- charge ratio of 1).
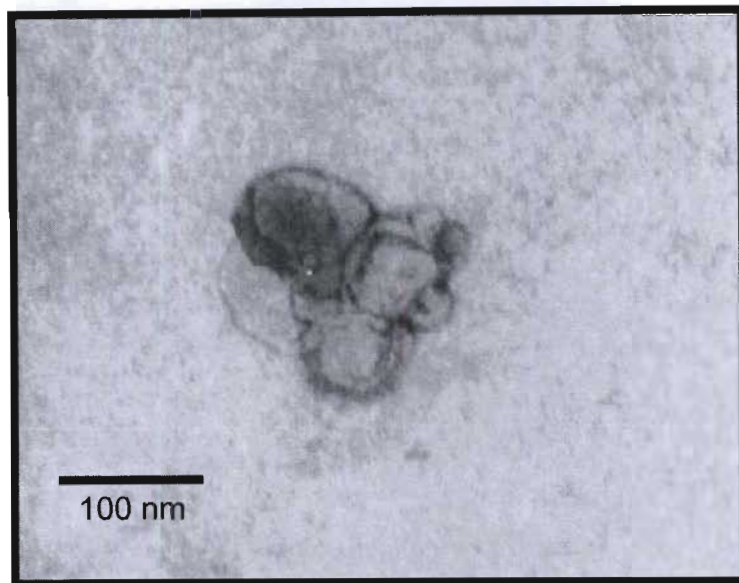
[a]
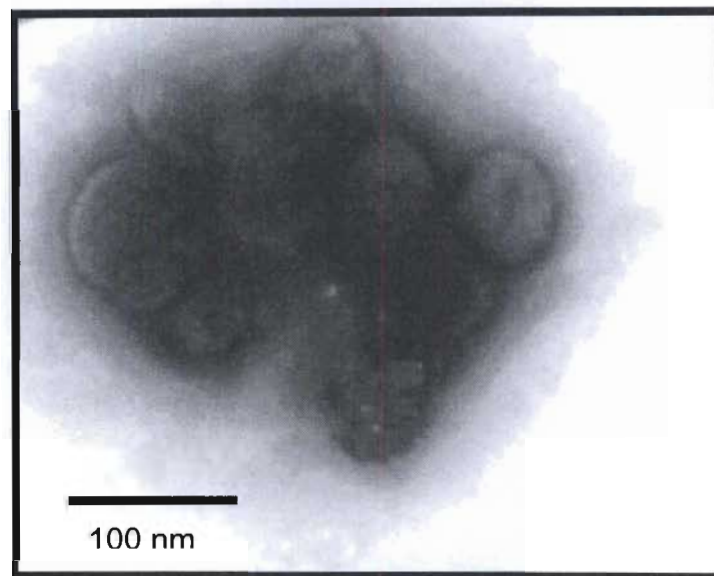
[b]

Figure 2.18 : TEM of MS09 lipoplexes at two different liposome:DNA ratios:
(a) 12:1 (+/- charge ratio of 1.3), and,
(b) 14: 1 (+/- charge ratio of 1.7)
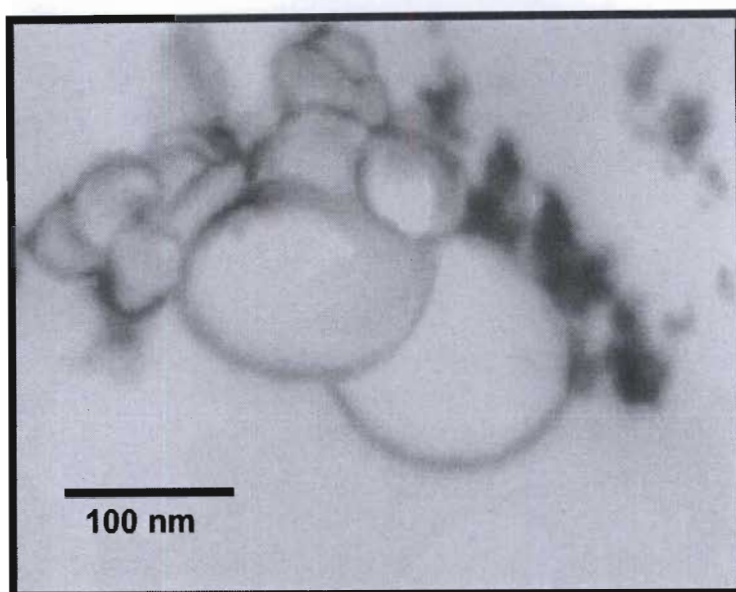
[a]
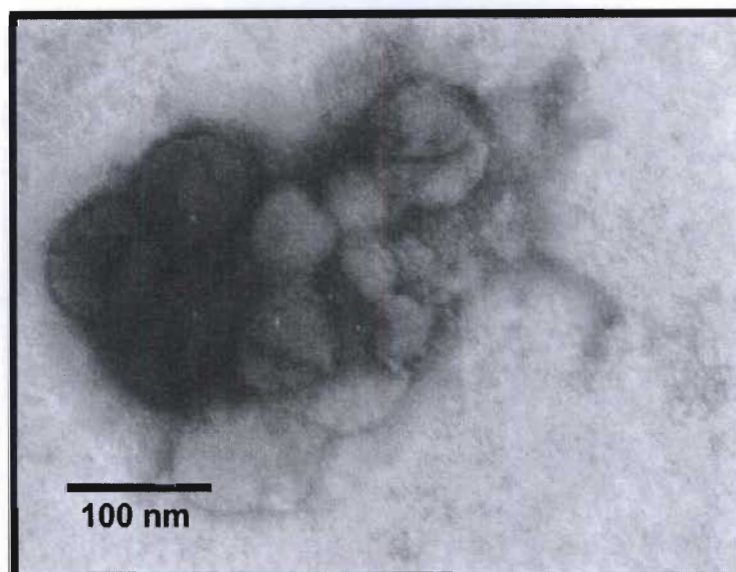
[b]

Figure 2.19 : TEM of MS10 lipoplexes at two different liposome:DNA ratios:
(a) 6:1   (+/- charge ratio of 0.8), and,
(b) 7: 1 (+/- charge ratio of 1).
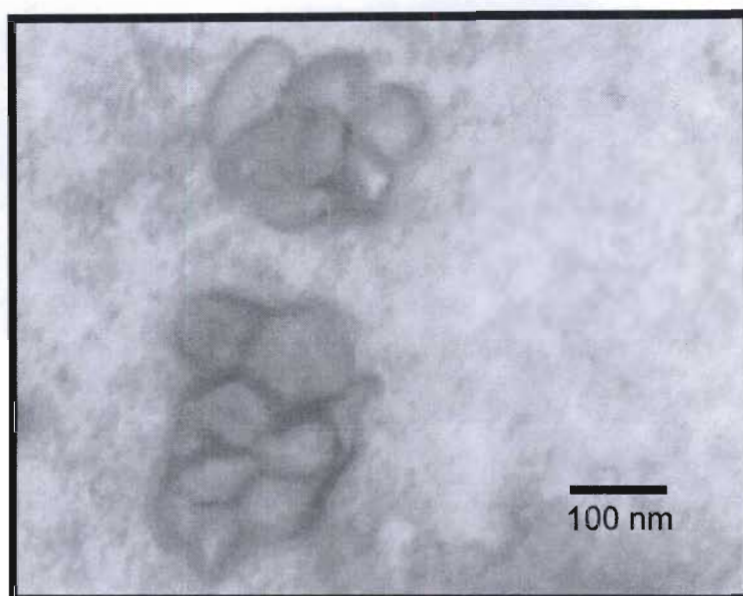
[a]
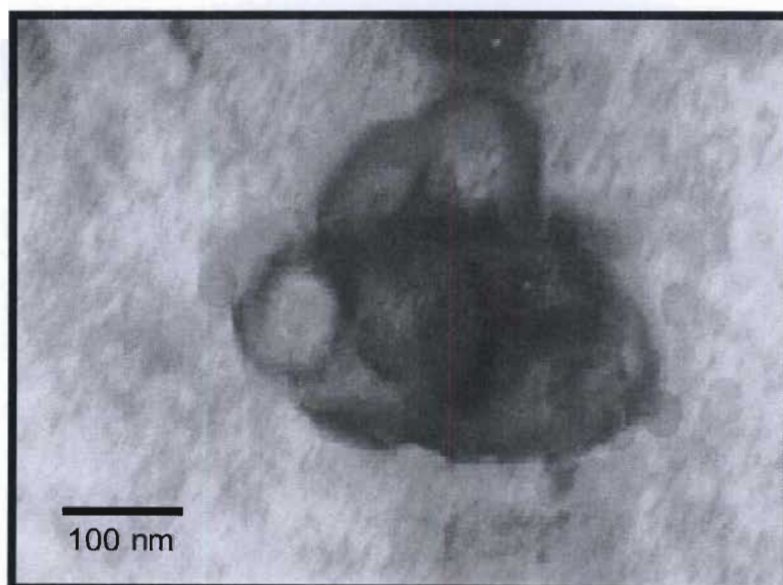
[b]

Figure 2.20 : TEM of MS11 lipoplexes at two different liposome:DNA ratios:
(a) 6:1 (+/- charge ratio of 0.8), and,
(b) 7: 1 (+/- charge ratio of 1).

TEM studies of the various lipoplexes at different liposome:DNA ratios showed a variety of aggregates or complexes. Most of the lipoplexes appeared as groups or clusters of spherical, oblong or flattened liposomes. The ultrastructure of the lipoplexes seemed to depend on the liposome:DNA ratio. At low liposome:DNA (+/-) charge ratios the lipoplexes were more tightly packed and were smaller in size compared to lipoplexes at higher liposome:DNA (+/-) charge ratios, which seem to be larger and more loosely clustered. These types of aggregates or clusters have been described previously (Moodley et al., 2002) and were also referred to as resembling a cluster of blackberries (Cao et al., 2000). The lipoplex shape has also been characterized as resembling 'beans" or "map-pins" immediately after liposome:DNA mixing. It was further noted that after thirty minutes, aggregation takes place and any definitive shape is lost (Sun et al., 2004). The lipoplexes examined in this study were all freshly prepared to maintain their ultrastructural shape and no further maturation of the lipoplexes was allowed before fixing.

Gershon et al., 1993, reported that at a DNA:lipid (DOTMA) weight ratio of 1:1 approximately half of the DNA molecules are bound to the liposomes and complexes appeared spherical even at the lower ratios. However at higher liposome concentrations all the DNA is covered by liposomes and smooth rod-like structures were visualised. Similar structures were also noted later using freeze fracture EM (Sternberg et al., 1994), and small angle X-ray scattering (SAXS) (Rädler et al., 1997 ; 1998). Furthermore it has been suggested that a hexagonal arrangement of lipid:DNA complexes can help to promote transfection (Koltover et al., 1998). Hence it can be said that the ultimate lipoplex shape is an important factor in determining transfection efficiency. This ultimate lipoplex shape depends on the size of the plasmid DNA, the type of liposome, and the mechanisms and conditions utilized for the lipoplex formation (Sun et al., 2004).

As yet no concensus has been reached as to the exact structure or ultrastructure of the so called "active" cationic liposome:DNA complex. The interaction between DNA and cationic liposome is probably a process of self-assembly that is triggered by DNA-

mediated fusion of liposomes and could involve some lipid rearrangement. The negatively charged plasmid DNA seems to act as a fusogenic agent, drawing together the positively charged liposomes and forming semi-fused liposomes or aggregates. It was found that lipoplex structures at the optimal ratio produce forms different from those at sub-optimal ratios (Sternberg et al., 1994).

Investigators have also utilized cryo-TEM to visualize lipoplexes. Results have suggested that the DNA molecules are usually entrapped between the liposomal membranes in clusters, and that with increasing amounts of DNA, free or loosely bound plasmids were seen (Li and Ma, 2001). In some instances much larger aggregates were produced that were made up of distinct globular units generally of a constant size (Rädler et al., 1998). Depending on the pKa of a lipid, the pH can also affect the interaction of cationic liposomes with DNA and the structures of the lipoplexes (Li and Ma, 2001).

The 200 nm diameter lipoplex seems to predominate when complexes are prepared at net positive charge ratios (Zabner et al., 1995). There does not seem to be consensus on the exact lipoplex size required for efficient transfection amongst different researchers. It has been observed that liposome:DNA complexes with diameters in the 0.4 to 1.4 μm (Nakanishi, 2003) or 200 to 450 nm range (Templeton, 2003) were effective for gene transfection. For in vivo studies it is assumed that the larger the lipoplexes, the longer the circulation time in the blood (Templeton, 2003). However it has been suggested that large aggregates do not interfere with transfection and that since they cannot enter the cells by endocytosis, other cell entry mechanisms may be in place for the uptake of these larger complexes (Lasic, 1997).

The transfection efficiency and the cell association and uptake of lipoplexes by CHO cells were seen to increase with increasing lipoplex size (Ross and Hui, 1999). The effect of lipoplex size on transfection efficiency can be seen in the transfection studies presented in 2.3.4.3. The identification of the lipoplex that is most effective at delivering a gene could prove an extremely important factor in the improvement of gene transfer in

future studies. . It was shown that an increase in lipoplex size resulted in a decrease in transfection (Kawaura *et al.*, 1998).

It was observed that the lipoplex formation kinetics, and final lipoplex molar masses, geometric sizes, and density were all primarily determined by the DNA:cationic liposome charge ratio, with DNA concentration playing a very small role. Observation of charge ratios close to 1 showed that primary lipoplexes were unstable and subsequently aggregated to form DNA complexes, reaching a final stable state. For ratios above and below 1 the initial primary lipoplexes were stable and did not undergo any form of aggregation (Lai and van Zanten, 2002). It is assumed that if the lipoplex has an excess positive charge relative to the number of negatively charged phosphates of the plasmid DNA i.e. a net positive charge ratio (+ / -), the net surface charge (zeta potential) of the lipoplex will be positive. This is thought to facilitate the interaction with the anionic proteoglycans on the cell surface, and the lipoplex will be taken up by endocytosis (Zabner *et al.*, 1995). It is further suggested that the diameter of the lipoplexes reaches a maximum when the surface potential is neutral. *In vivo* studies have shown that biodistribution of complexes after intravenous injection resulted in accumulation of positively charged complexes in the lung with little or no accumulation of negatively charged complexes (Ishiwata *et al.*, 2000).

Furthermore it is suggested that a hexagonal arrangement of lipid:DNA complexes helps to promote transfection (Koltover *et al.*, 1998).

2.3.3.5 Nuclease Protection Assays

These studies were conducted to ascertain the protection afforded by the cationic liposomes to the bound DNA. All three lipoplexes (MS09:DNA, MS10:DNA and MS11:DNA) afforded good protection of the DNA from digestion by serum nucleases, as seen in Figures 2.21 and 2.22 respectively.
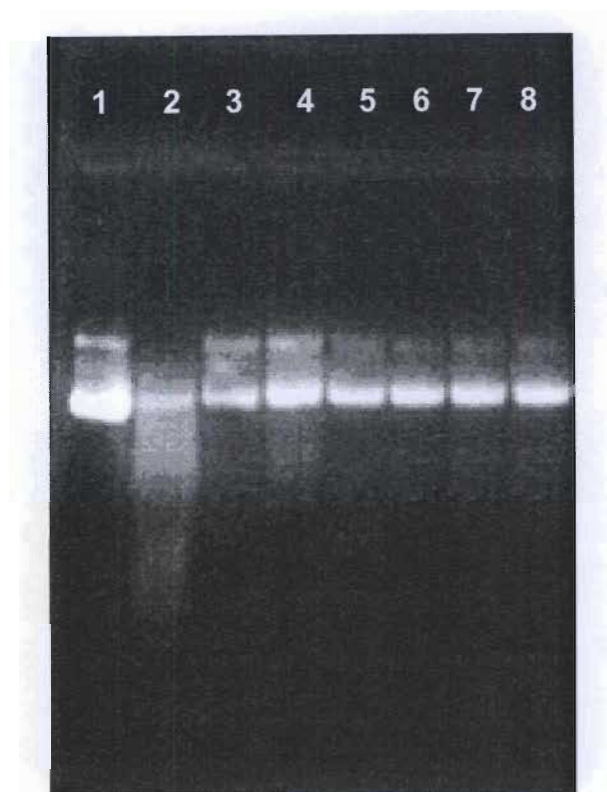
Figure 2.21 : Nuclease protection assay of cationic liposome MS09 and pBR322 DNA.
Lane 1 : undigested plasmid pBR322 DNA (1 µg)
Lane 2 : unprotected plasmid DNA (1 µg) digested by serum nucleases
Lanes 2-8 : Varying amounts of cationic liposomes (0, 4, 6, 8, 10, 12, 14 µg) with pBR322 DNA (1 µg) and serum (10%).
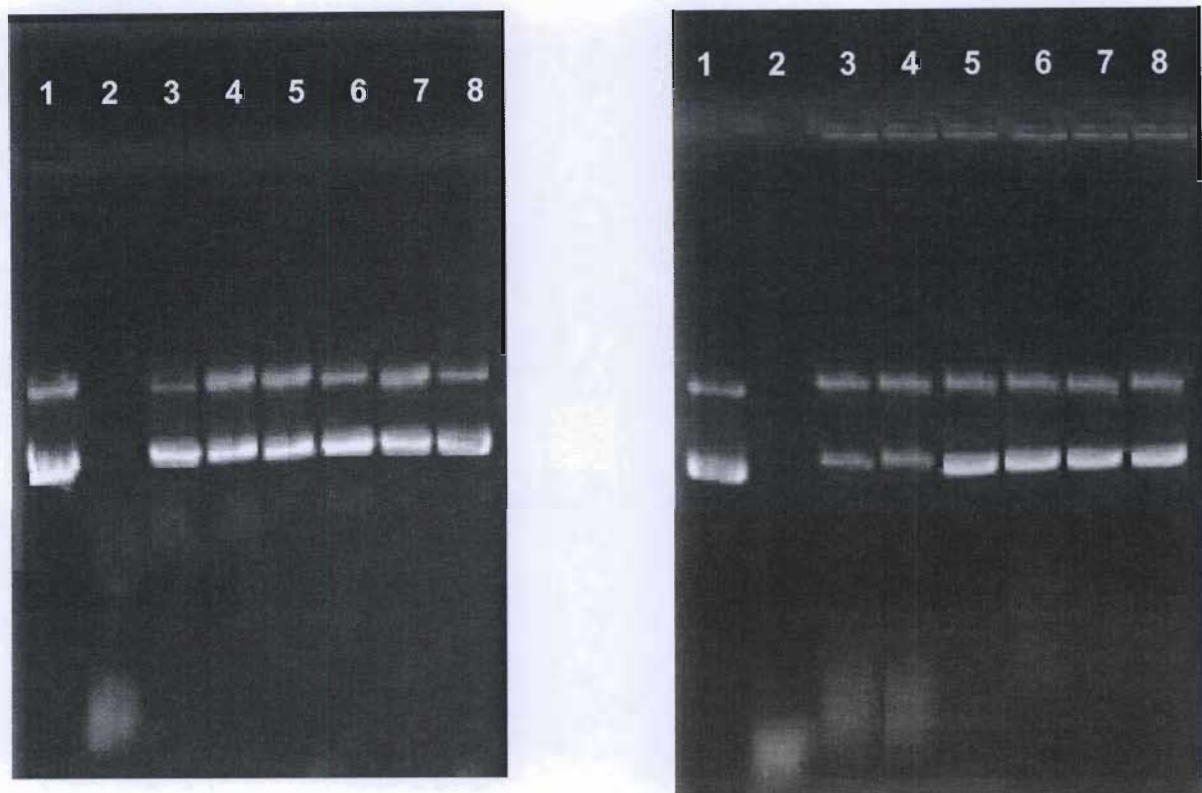
Figure 2.22 : Nuclease protection assay of :
(a) cationic liposome MS10 and pBR322 DNA, and,
(b) cationic liposome MS11 and pBR322 DNA
Lane 1 : undigested plasmid pBR322 DNA (1 µg)
Lane 2 : unprotected plasmid DNA (1 µg) digested by serum nucleases
Lanes 2-8 : Varying amounts of cationic liposomes (0, 2, 3, 4, 5, 6, 7 µg) with pBR322 DNA (1 µg) and serum (10%).

The addition of sodium dodecyl sulphate helps to liberate the bound and liposome protected DNA from the lipoplex so that it can be visualized on the agarose gel. The reaction between the positively charged cationic liposomes and the negatively charged DNA molecules via electrostatic forces results in the formation of highly organized supramolecular structures where the DNA molecules are condensed and protected against nuclease degradation (Pitard, 2002). This can be clearly seen in Figures 2.21, 2.22.

The naked DNA (lane 2) is not bound to liposomes and is extensively degraded by the presence of serum nucleases, whereas the DNA bound in lipoplexes is protected and not degraded, especially at the higher liposome:DNA ratios. The actual extent to which the DNA is protected by the liposomes will depend on the type of cationic liposome used. If the liposome:DNA association is not a strong or tight one, the nucleases may access and degrade the DNA, or, alternatively, destabilize the liposome:DNA complex (Yang and Huang, 1997).

The inhibitory effect of serum on the transfection efficiency of cationic liposome:DNA complexes (lipoplexes) is a major obstacle to the application of a gene delivery vector both *in vivo* and *in vitro*. (Yang and Huang, 1997; Ross and Hui, 1999).

2.3.4 CELL CULTURE AND TRANSFECTION STUDIES

2.3.4.1 Growth and Maintenance of Cell Lines

The four different cell lines viz. HeLa (Human cervical carcinoma cells), NIH-3T3 (mouse fibroblast cells), HepG2 (hepatocellular carcinoma cells) and SNO (human esophageal cancer cells) (Figures 2.23, 2.24, 2.25, 2.26), were successfully propagated in MEM + 10% heat inactivated fetal bovine serum + antibiotics. Trypsinised cells have a typical rounded appearance and can be seen for the HepG2 cells in Figure 2.27. Cell growth, especially for HepG2 cells, at times was relatively slow, with cells only reaching confluency after 4 to 6 days. The HeLa, HepG2 and SNO cells are epithelial in morphology while the NIH-3T3 cells are fibroblasts. All cell lines were photographed using a Nikon camera attached to the inverted microscope at a 2 second exposure with a film speed of 200 ASA.
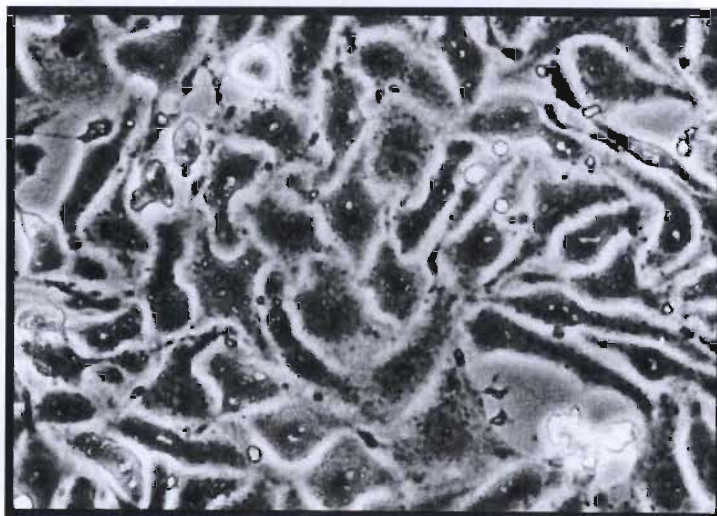
Figure 2.23 : HeLa cells at semi-confluency.
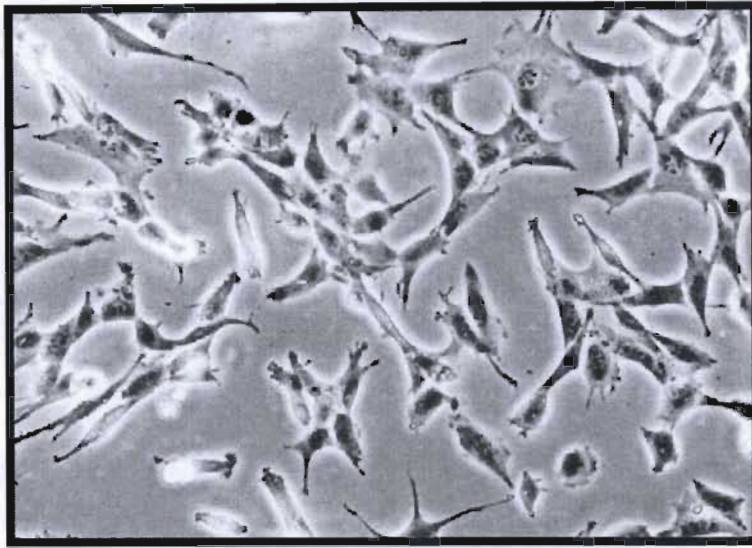
Figure 2.24 :   NIH-3T3 cells at semi-confluency.
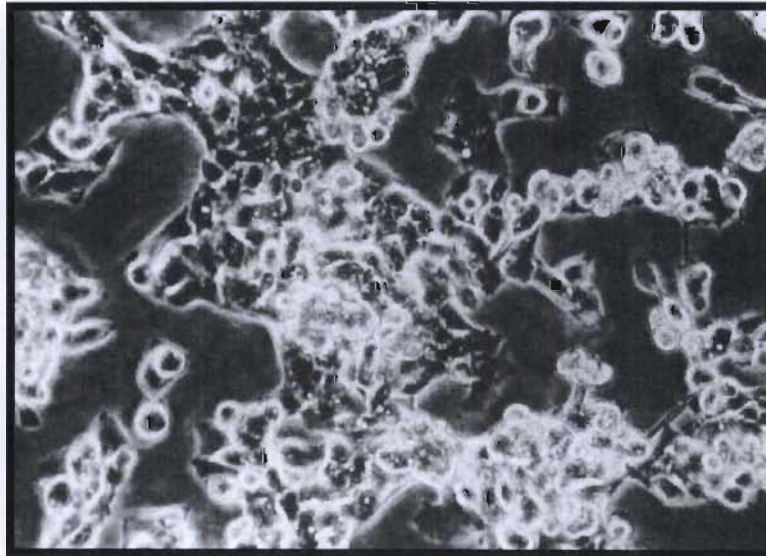
Figure 2.25 :   HepG2 cells at semi-confluency.
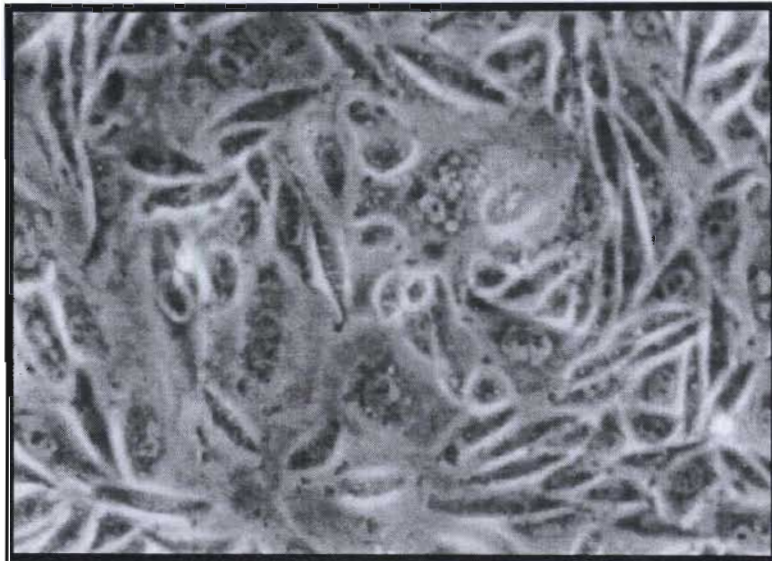
Figure 2.26 :   SNO cells at confluency.
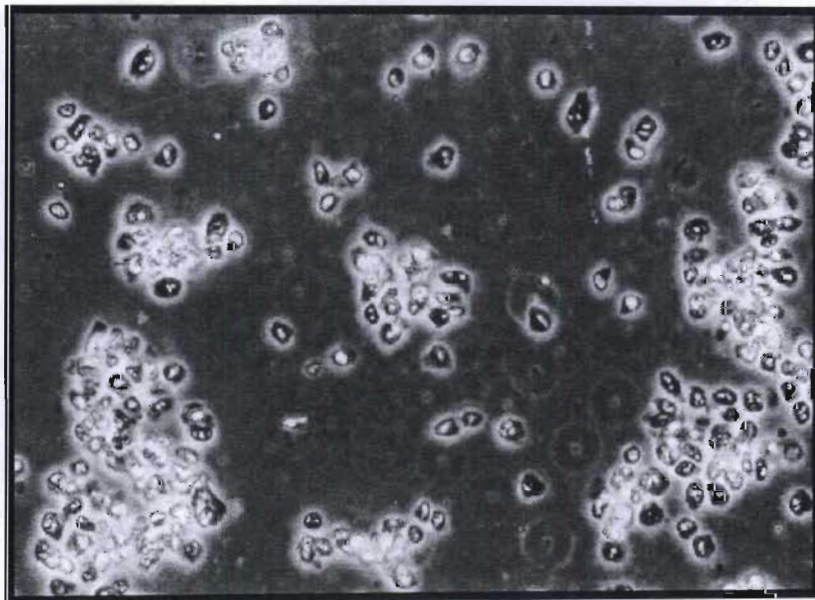
Figure 2.27 :   Typical rounded appearance of HepG2 cells after trysinisation.
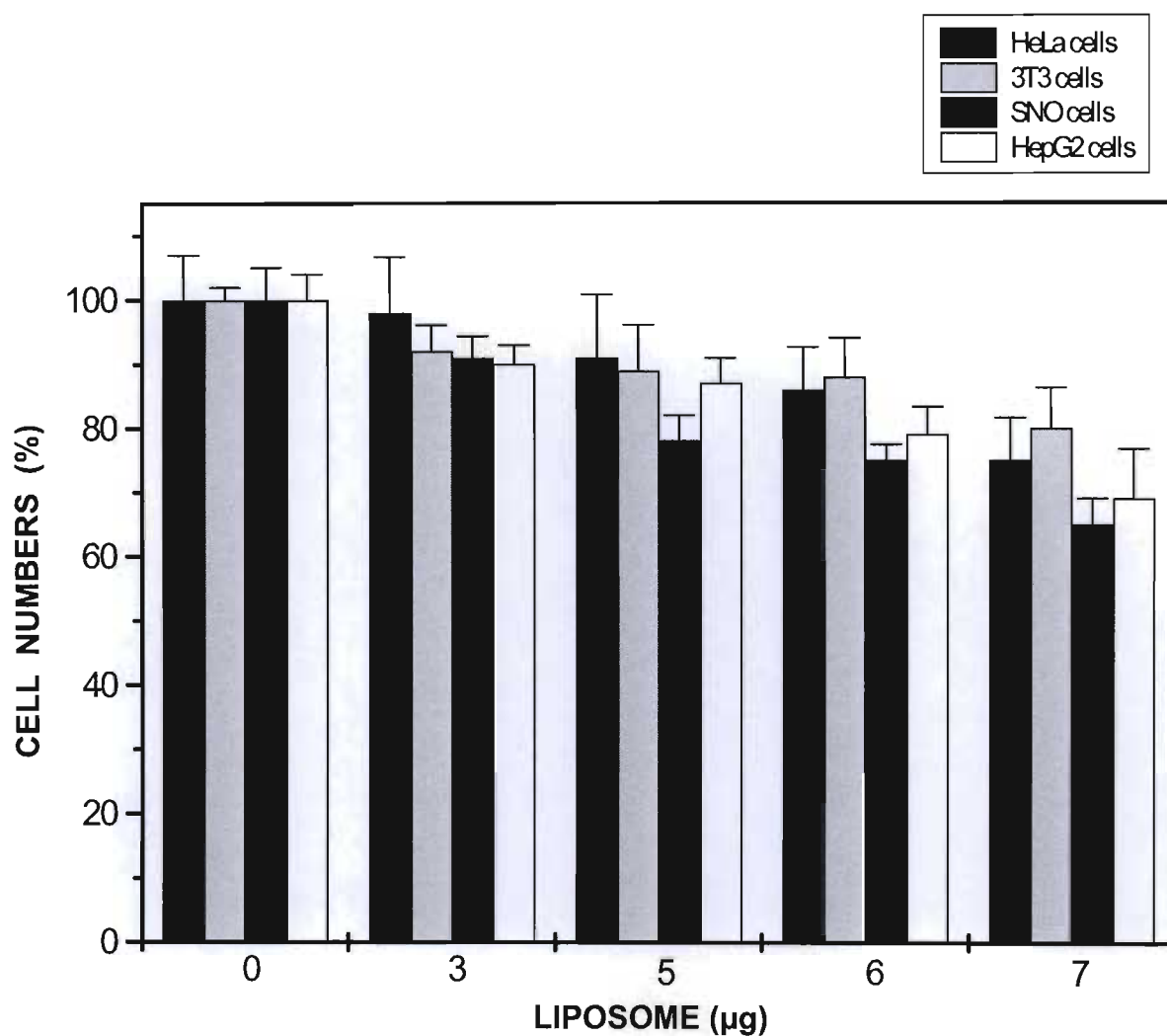
Figure 2.28 : Growth inhibition studies of liposome MS09:DNA complexes in four cell lines *in vitro*. Liposome MS09 was varied (0, 3, 5, 6 , 7 µg) while the DNA (0.5 µg) was kept constant in a total volume of 0.5 ml MEM. A control sample (no liposome), containing only cells was assumed to have 100% survival. Data are presented as means ± S.D (n = 4).
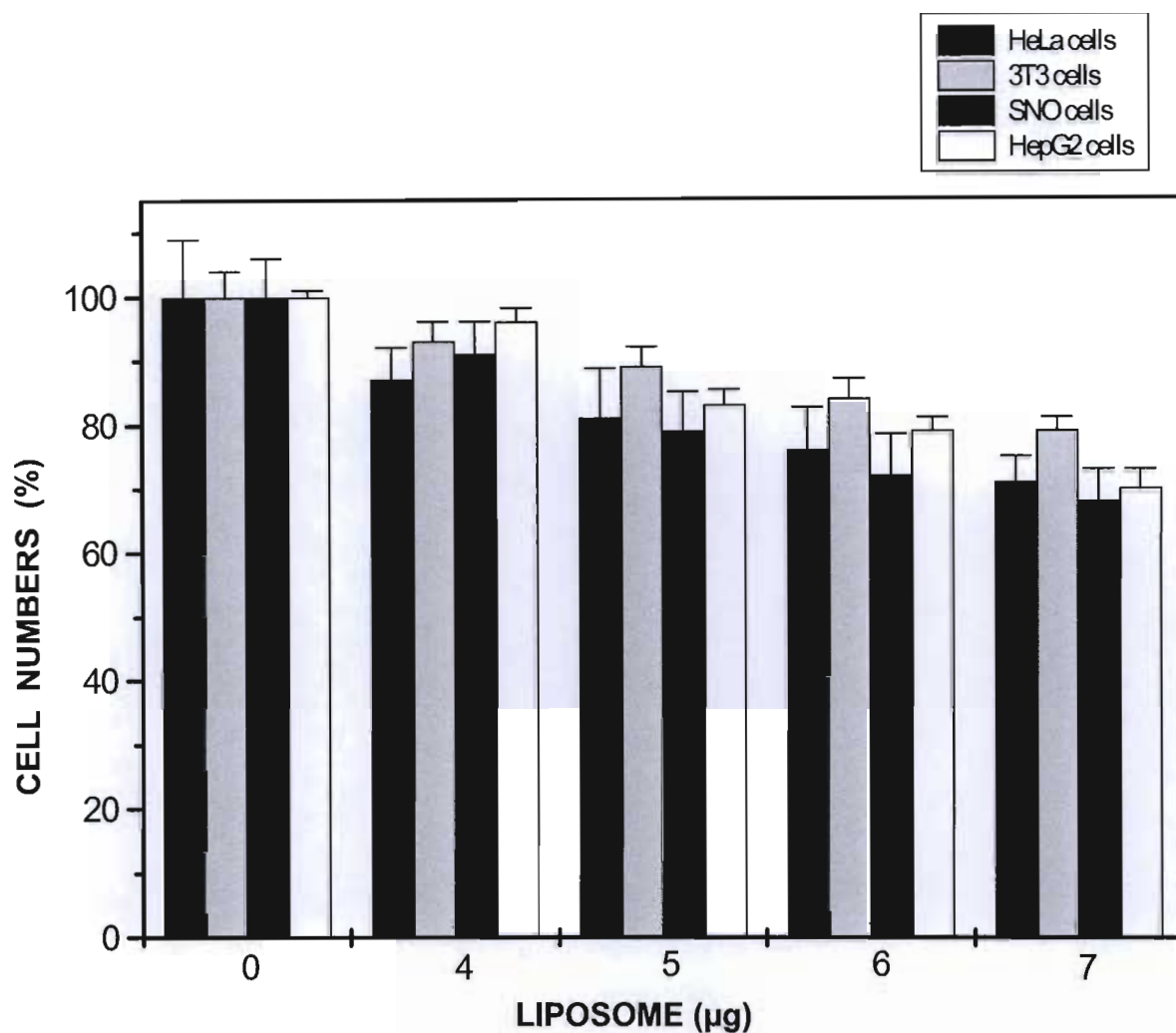
Figure 2.29 : Growth inhibition studies of liposome MS10:DNA complexes in four cell lines *in vitro*. Liposome MS10 was varied (0, 4, 5, 6 , 7 µg) while the DNA (1 µg) was kept constant in a total volume of 0.5 ml MEM. A control sample (no liposome) , containing only cells was assumed to have 100% survival. Data are presented as means ± S.D (n = 4).
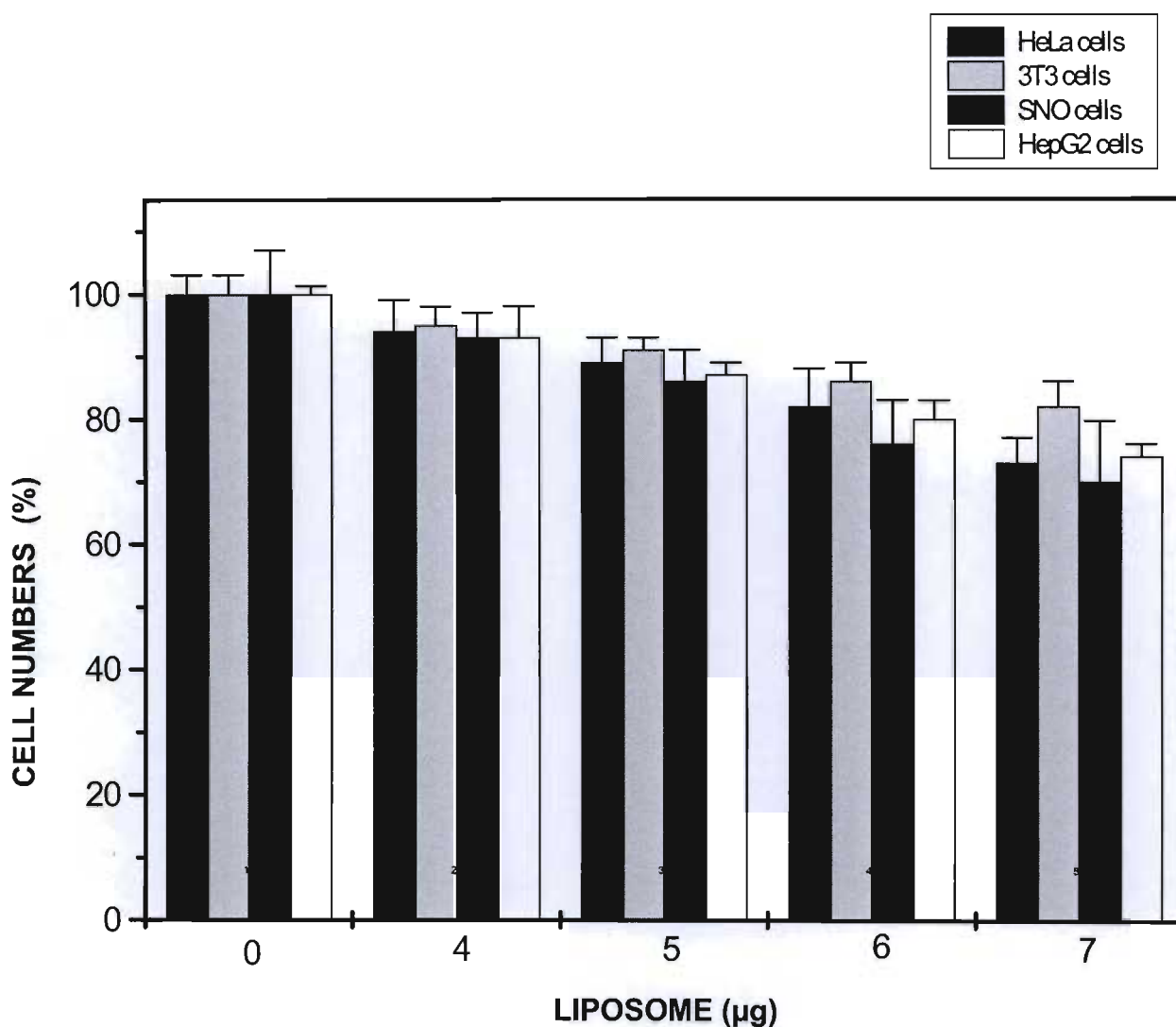
Figure 2.30 : Growth inhibition studies of liposome MS11:DNA complexes in four cell lines *in vitro*. Liposome M11 was varied (0, 4, 5, 6, 7 μg) while the DNA (1 μg) was kept constant in a total volume of 0.5 ml MEM. A control sample (no liposome), containing only cells was assumed to have 100% survival. Data are presented as means ± S.D (n = 4).

2.3.4.3 Transfection Studies

The three different cationic liposome formulations were tested for their *in vitro* transfection activity in four different cell lines viz. HeLa, NIH-3T3, SNO and HepG2 cells. Transfection activity was also compared to the cationic liposome formulation, Lipofectin™. Which is one of the first commercial lipofection agents and is an equimolar mixture of DOTMA and DOPE. Lipofectin™ was found to successfully transfect DNA into many tissue types but performed poorly in mouse fetal brain cells (Sakurai *et al.*, 2001). Significant transfection activity was observed for all three liposome formulations (MS09 – Figure 2.31 and Figure 2.32; MS10 – Figure 2.33 and Figure 2.34; and MS11- Figure 2.35 and 2.36), with different liposome formulations showing optimal transfection activity at specific liposome:DNA ratios. Beyond the optimal liposome:DNA ratio (+/- charge ratio of 1.3 FOR MS09 and 0.8 for MS10 and MS11 lipoplexes) there is a noticeable drop in transfection activity especially for liposome MSO9. This could be due to the formation of larger lipoplexes, as discussed in section 2.3.3.4, at higher liposome:DNA ratios, that do not easily facilitate endocytosis. For optimal transfection, complex aggregation as well as condensation are important considerations, and reinforce the thought that sizes of the lipoplexes are critical for gene transfection. Optimal transfection is thought to be a result of a delicate balance between the toxicity of a lipid and the level of expression (Lasic, 1997).

Transfections with no liposome or DNA (control 1) and with naked DNA (control 2) were the controls employed against which transfection was measured. Transfection activity in these control cells showed negligible luminescence, as can be seen in Figures 2.31-2.37). Liposome MS09 showed the highest transfection activity and performed much better in some cell lines than Lipofectin™ (Figure 2.37). Transfection activity was greatest at liposome:DNA (+/-) charge ratio of 1.3 for MSO9, and 0.8 to 1 for MS10 and MS11. Optimal transfection was obtained at a liposome:DNA ratio of 8:1(^{W}/$_W$) for Lipofectin™. It is suggested that the DNA:liposome mixing ratio significantly affects the intracellular trafficking of plasmid DNA complexed to cationic liposomes (Sakurai *et al.*, 2000). The highest transfection activity for MS09 was observed for the HeLa cell line

which was 3 fold greater (3 87 x 10^6 LU / mg protein) than that of Lipofectin^{TM} (1.10 x 10^6 LU / mg protein) at its optimal liposome:DNA ratio. This is also extremely promising, considering that only 0.5 µg pGL3 DNA was used in transfections with MS09 liposomes, while 1 µg pGL3 was utilized for transfection with Lipofectin^{TM}. Lipofectin^{TM} only performed slightly better (1.6 x 10^6 LU / mg) in the NIH-3T3 cell line when compared to MS09 (1.5 x 10^6 LU / mg protein). MS09 liposomes in SNO (2.0 x 10^6 LU / mg) and HepG2 (1.3 x 10^6 LU / mg) cells also performed slightly better than Lipofectin^{TM} (1.1 x 10^6 LU / mg for SNO cells and 1.0 x 10^6 LU / mg for HepG2 cells). Although liposomes MS10 and MS 11 showed significant transfection levels, they were not as high as those achieved using Lipofectin^{TM} or liposome MS09. It has been suggested that HeLa cells may be more easily transfected than fibroblasts (eg. 3T3 cells) due the the observation that after translocation of the DNA into the nucleus, it disappears rapidly in fibroblasts, but remains for a longer period in HeLa cells (Coonrod *et al.*, 1997 ; Zhdanov *et al.*, 2002).

Several factors are thought to influence the success of cationic liposome-mediated DNA transfer, viz: (Dass and Burton, 1999).

(i)   cell type and whether culture is primary or a subculture of a primary

(ii)   stage of cell in the growth cycle and cell seeding densities

(iii)   DNA:liposome ratios

(iv)   any chemical treatment of liposomes prior to addition of DNA

(v)   type and concentration of salts / biomolecules in liposome:DNA mixing medium and constitution of cell culture medium

(vi)   size of lipoplexes and time allowed for lipoplexes to mature before addition to cells

(vii)   dilution of liposome:DNA complex suspension

(viii) incubation time of lipoplexes with cells, and

(ix)   the lipid component making up the liposomal vesicles.

(a)

(b)
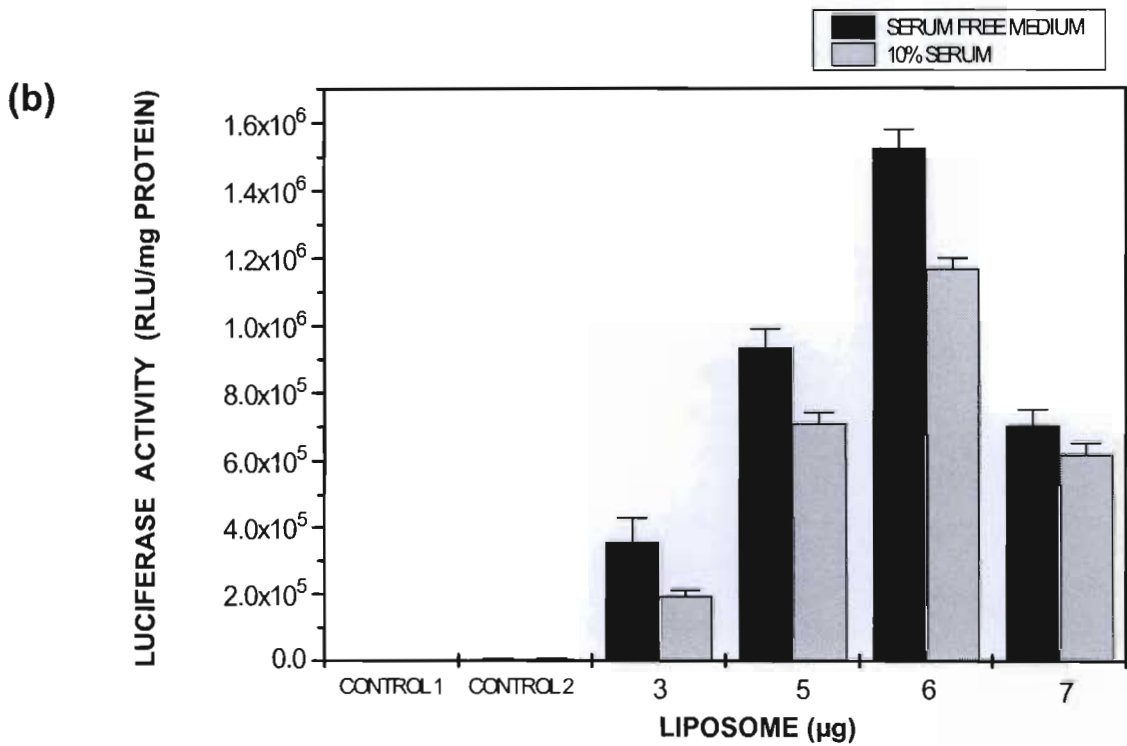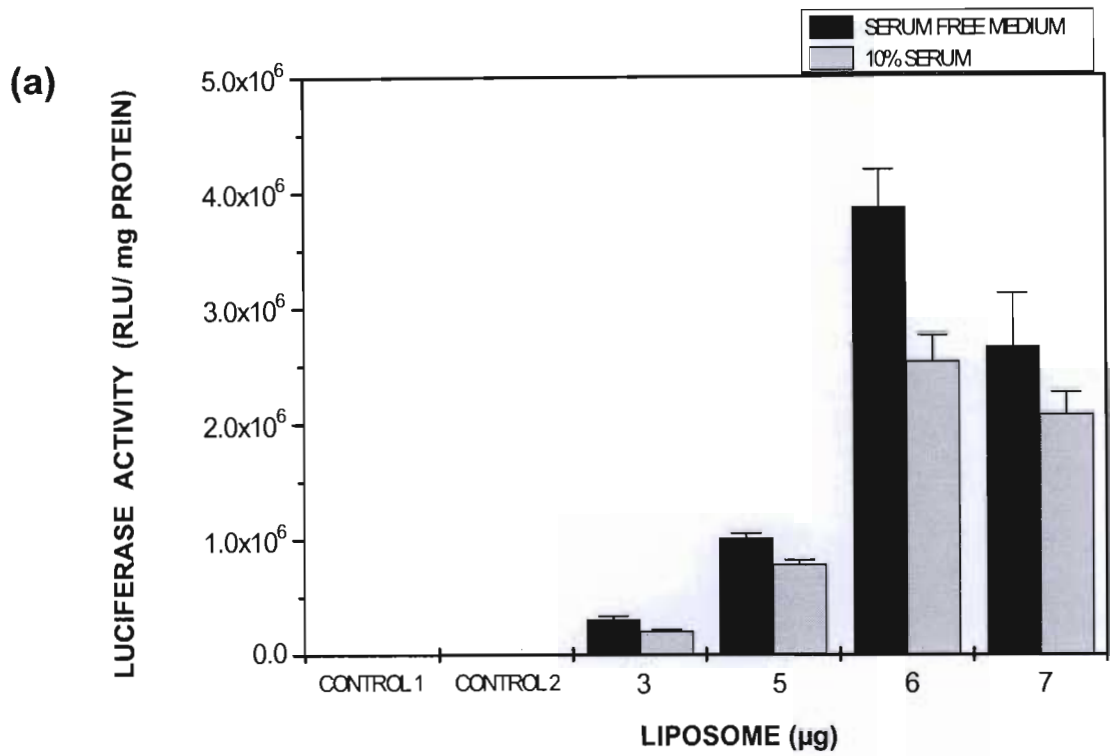
Figure 2.31 : Transfection studies of liposome MS09:DNA complexes (with and without incubation in 10% serum) in (a) HeLa cells and (b) NIH 3T3 cells *in vitro* . Liposome MS09 was varied (0, 3, 5 ,6 ,7 µg) while the DNA (0.5 µg) was kept constant in a total volume of 0.5 ml MEM. Control 1 contains only cells and control 2 contains naked DNA and no liposome. Data are presented as means ± S.D (n = 4).
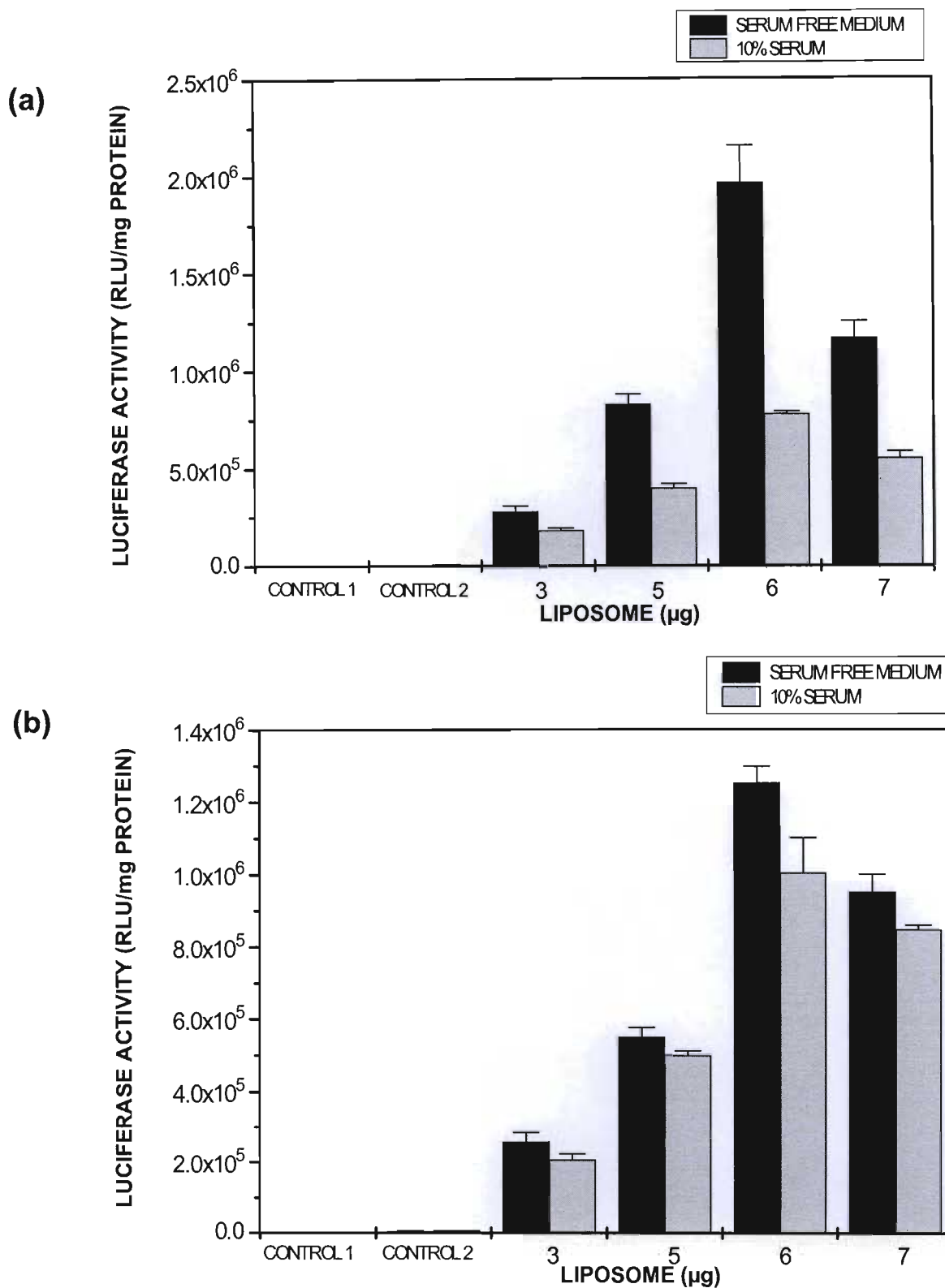
Figure 2.32 : Transfection studies of liposome MS09:DNA complexes (with and without incubation in 10% serum) in (a) SNO cells and (b) HepG2 cells *in vitro*. Liposome MS09 was varied (0, 3, 5, 6, 7 µg) while the DNA (0.5 µg) was kept constant in a total volume of 0.5 ml MEM. Control 1 contains only cells and control 2 contains naked DNA and no liposome. Data are presented as means ± S.D (n = 4).
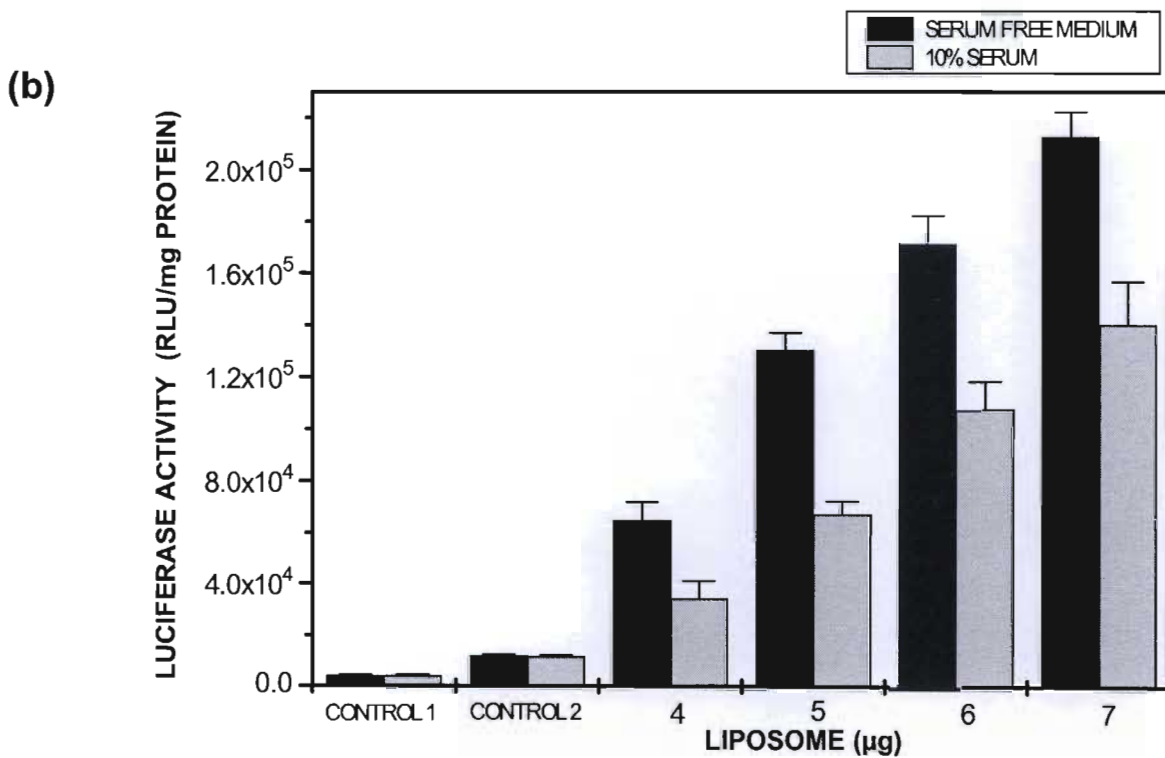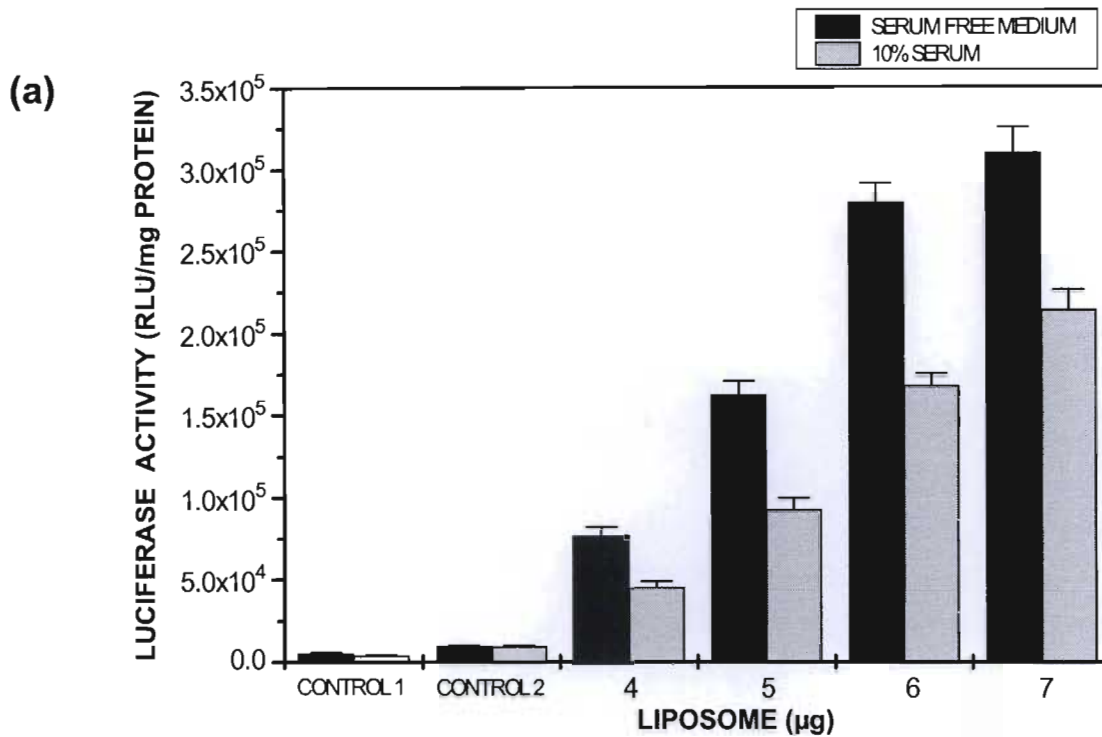
Figure 2.33 : Transfection studies of liposome MS10:DNA complexes (with and without Incubation in 10% serum) in (a) HeLa cells and (b) 3T3 cells *in vitro*. Liposome MS10 was varied (0, 4, 5, 6, 7 µg) while the DNA (1.0 µg) was kept constant in a total volume of 0.5 ml MEM. Control 1 contains only cells and control 2 contains naked DNA and no liposome. Data are presented as means ± S.D (n = 4).

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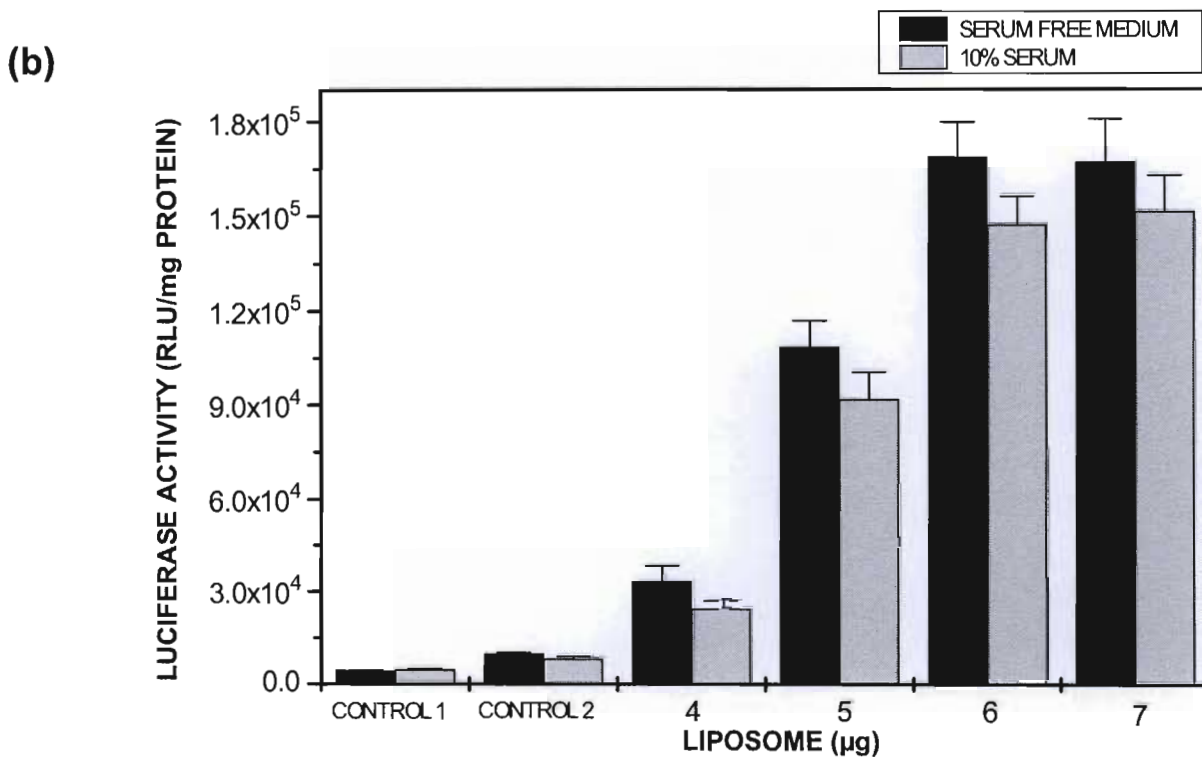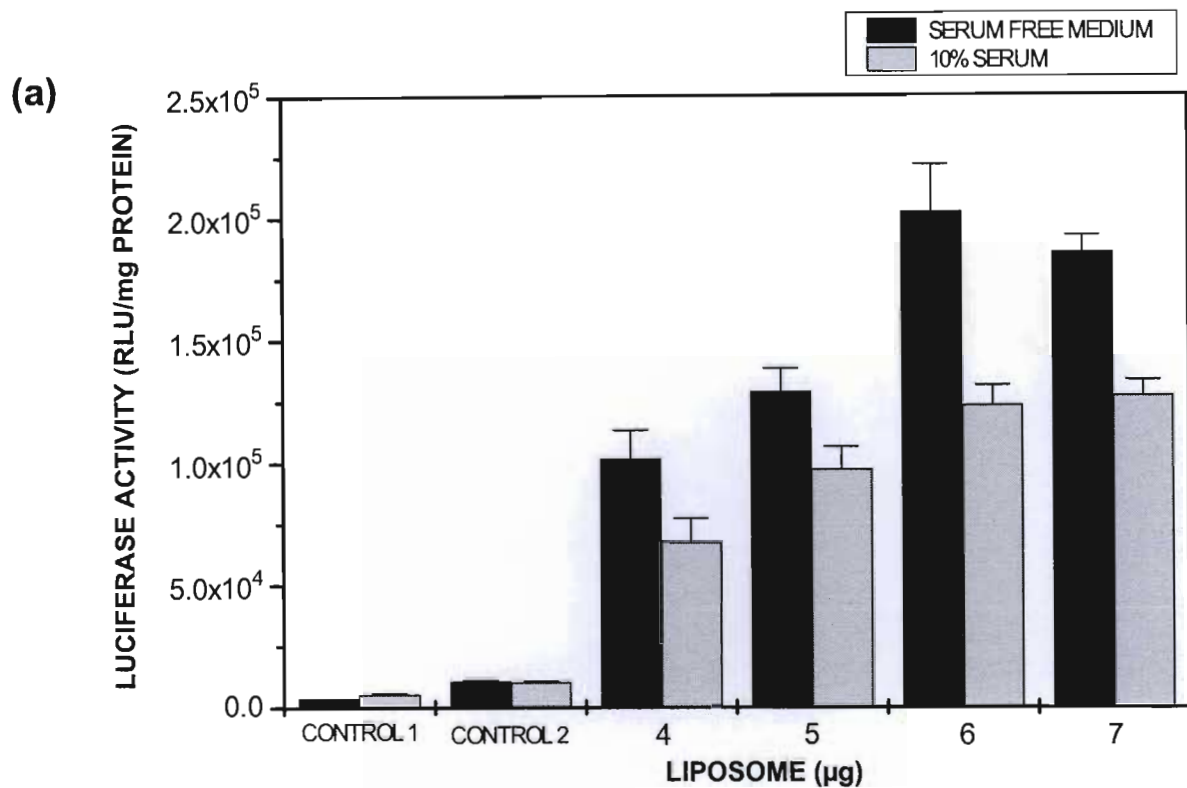
Figure 2.34 : Transfection studies of liposome MS10:DNA complexes (with and without Incubation in 10% serum) in (a) SNO cells and (b) HepG2 cells *in vitro*. Liposome MS10 was varied (0, 4, 5, 6, 7 µg) while the DNA (1.0 µg) was kept constant in a total volume of 0.5 ml MEM. Control 1 contains only cells and control 2 contains naked DNA and no liposome. Data are presented as means ± S.D (n = 4).
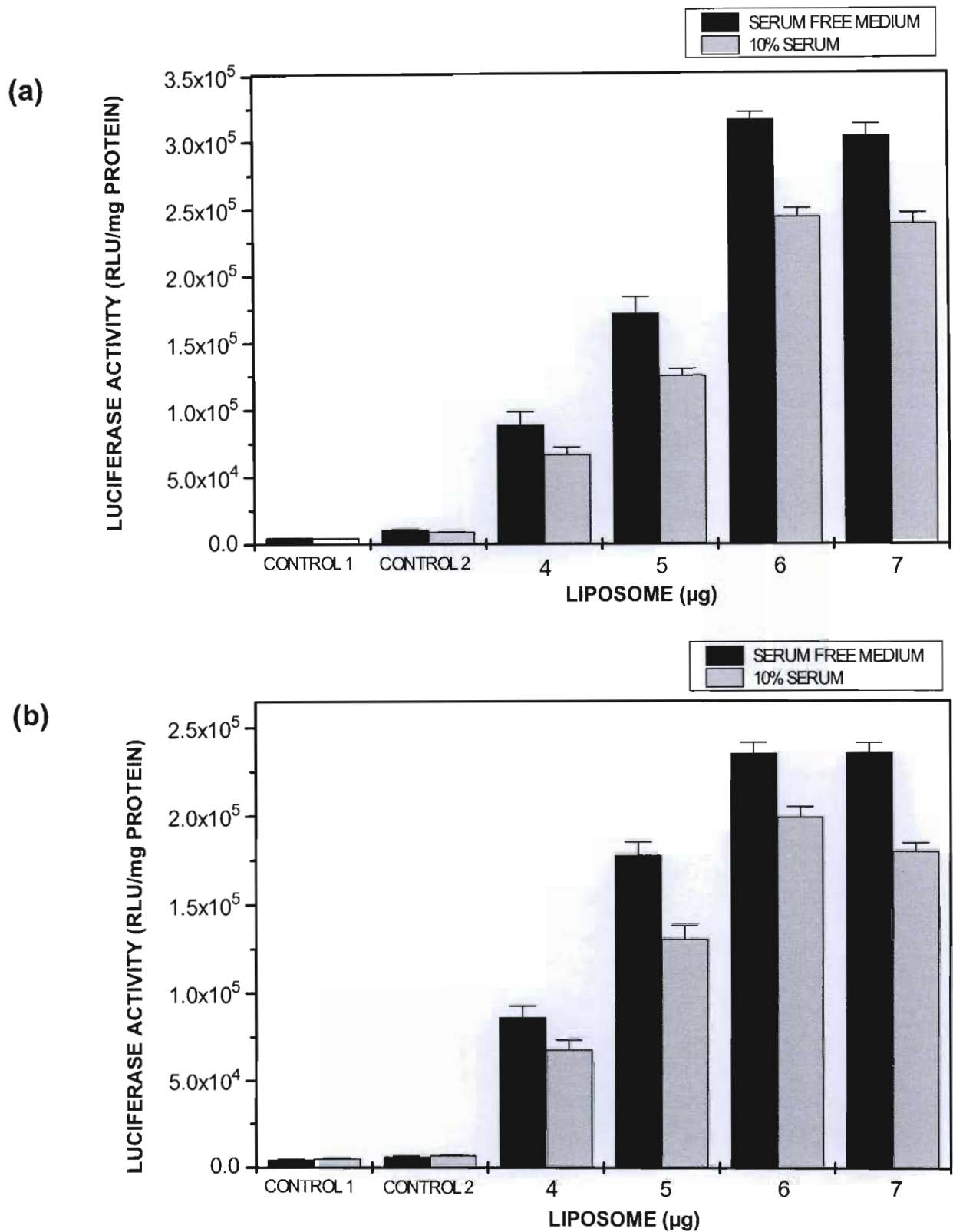
Figure 2.35 : Transfection studies of liposome MS11:DNA complexes (with and without Incubation in 10% serum) in (a) HeLa cells and (b) 3T3 cells *in vitro*. Liposome MS11 was varied (0, 4, 5, 6, 7 µg) while the DNA (1.0 µg) was kept constant in a total volume of 0.5 ml MEM. Control 1 contains only cells and control 2 contains naked DNA and no liposome. Data are presented as means ± S.D (n = 4).
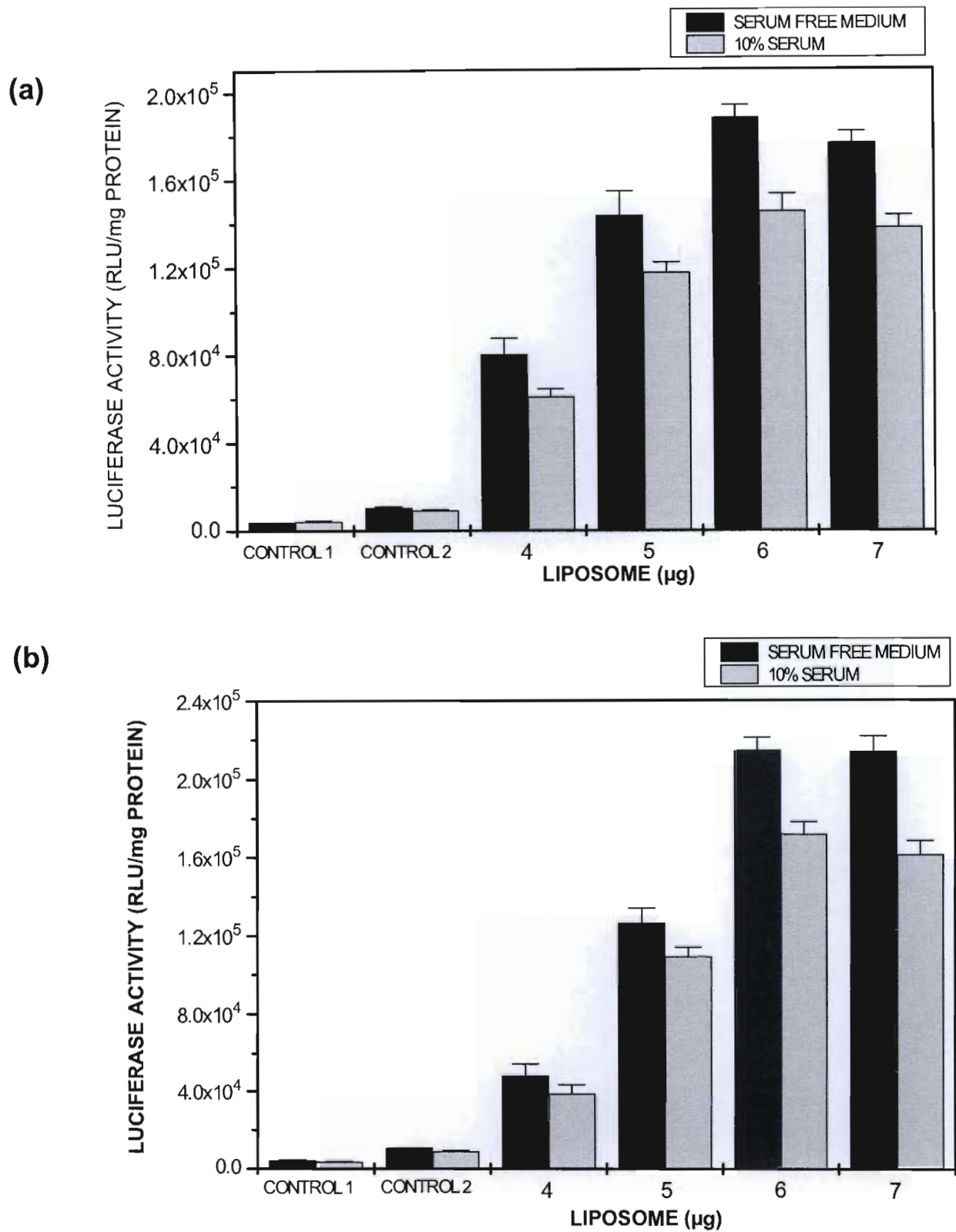
Figure 2.36 : Transfection studies of liposome MS11:DNA complexes (with and without Incubation in 10% serum) in (a) SNO cells and (b) HepG2 cells *in vitro*. Liposome MS10 was varied (0, 4, 5, 6, 7 µg) while the DNA (1.0 µg) was kept constant in a total volume of 0.5 ml MEM. Control 1 contains only cells and control 2 contains naked DNA and no liposome. Data are presented as means ± S.D (n = 4).

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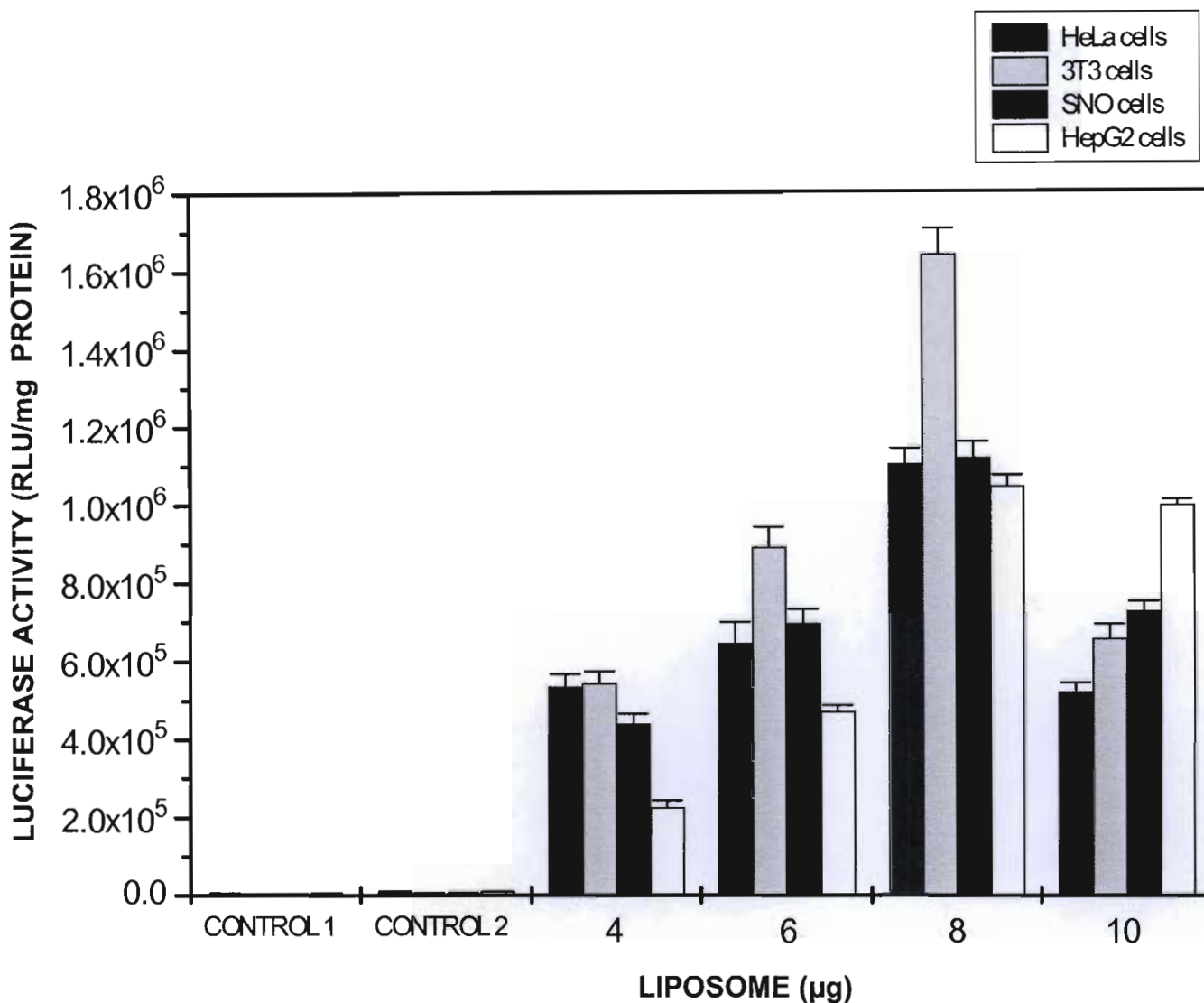
Figure 2.37 : Transfection studies of Lipofectin™:DNA complexes in four cell lines *in vitro*. Lipofectin™ was varied (0, 4, 6, 8, 10 µg) while the DNA (1.0 µg) was kept constant in a total volume of 0.5 ml MEM. Control 1 contains only cells and control 2 contains naked DNA and no liposome. Data are presented as means ± S.D (n = 4).

During the preparation of this thesis the synthesis of two cholesterol-based cationic lipids with a β-alanine [2-(N-β-alanyl)aminoethyl-cholesteryl ether], and a glycine [2-(N-glycyl)aminoethyl-cholesteryl ether] head group that are similar to head groups of our MS10 and MS11 derivatives was reported. These 2 cationic lipids showed large differences in their transfection efficiency, with the β-alanyl derivative being 10-24 fold more efficient in transfecting CHO, COS-1 and HepG2 cells than its glycyl analog. This was attributed to the extra methylene group of the β-alanine in its polar head group region (Singh and Chaudhuri, 2004). In our studies, however MS11 (β-alanyl derivative) showed only a slight increase in optimal gene expression over the MS10 (glycyl derivative) in HeLa cells (2% increase), NIH-3T3 (10% increase) and HepG2 (1.5% increase), but a somewhat higher increase in SNO cells (30%). Although spacer lengths in the β-alanyl and glycyl compounds reported by Singh and Chaudhuri are similar to those in MS11 and MS10 respectively, the spacer is attached to the cholesteryl moiety at its 3 position by an ether linkage rather than the carbamoyl linkage which is the case in the study reported here.

Transfections with liposomes MS09, MS10 and MS11 were also conducted in 10% serum to ascertain the effect of the serum components on transfection efficiency and to provide some insight into the future possibility of using these liposome formulations for *in vivo* experiments. From the results obtained it can be seen that the incubation of lipoplexes for the four hour period in 10% serum containing medium resulted in a decrease in transfection activity, with the HepG2 system being the least affected. This could be attributed to the protection afforded by the liposomes to the DNA molecules as seen in the nuclease digestion assays. It has been proposed that the efficiency of cationic liposome-mediated gene expression often decreases with addition of serum (Yang and Huang, 1997). Some investigators have even reported a total inhibition of transfection after incubation with serum for as little as two hours (Nchinda *et al.*, 2002).

The influence on transfection efficiency of lipoplexes by cationic liposome:DNA ratios, liposome type, incubation time in polyanion containing media, time of serum addition are all mediated through lipoplex size. Lipoplexes at a 2:1 charge ratio were seen to

grow in size in media containing polyanions. Size growth of the lipoplexes may be halted by the addition of serum to the incubation medium and large lipoplexes may help to overcome the inhibitory effects of the added serum (Ross and Hui, 1999). It has been reported that maximum gene expression occurs at intermediate stablilty, because stable complexes restrict DNA transcription and unstable complexes permit rapid degradation of the plasmid DNA (Luo and Saltzman, 2000).

Cationic liposome mediated transfections are often conducted in serum-free medium at least for the first few hours. A longer incubation time with the lipoplexes eg. four hours vs one hour results in a higher amount of internalized lipoplex and hence a higher degree of transfection (Zuhorn et al., 2002). This serum free medium which is removed after a few hours (4 hours in the experiments conducted in this study), does not affect or displace the liposome:DNA complex from the cell surface, since the DNA and liposome remain tightly complexed and bound to the cell surface. These phenomena were also observed using an inverted light microscope. The liposome:DNA complex therefore will be continuously internalised into the cell from the cell surface. Transfection of plasmid DNA is closely related to the problem of condensation of its molecules as the plasmid may be too large to effectively overcome the cellular membrane barrier. The formation of relatively small lipoplexes of 100 to 350 nm in diameter as seen in Section 2.3.3.4, is favourable for transfection. The transfected DNA is probably transported efficiently through the nuclear membrane during interphase, and it is assumed that the breakdown of the nuclear membrane during mitosis is crucial for the entry of the transfected DNA into the nucleus. Transfection efficiency hence depends upon cell division.

When using medium containing serum in the four hour incubation of lipoplexes, it is believed that polyvalent negatively charged molecules such as albumin, present in the serum may compete in charge interactions (Harrison et al., 1995). However the complete mechanism of serum inhibition of transfection is still unclear. Lipoplexes interact with blood components due to their strong positive charge. Binding of serum proteins on the lipoplexes is thought to be due to non-specific electrostatic interactions (Sakurai et al., 2001). The charged components of the serum may bind to the

metastable particles, and either facilitate aggregation or interfere with polynucleotide coating (Ahearn and Malone, 1999). The adsorption of negatively charged proteins neutralizes the cationic charge of the complex and also increases their size, which in turn will lead to a reduction in gene expression. If the size of the complex exceeds 5 μm in diameter during preparation or after interaction with serum components, it will not pass through capillaries and results in embolization of downstream tissues (Nishikawa and Huang, 2001). If however sufficient time is allowed for mature particles to form before addition of serum, there will be no decrease in transfection activity. Factors such as higher temperatures, concentration, higher liposome:DNA ratios, all increase the speed at which serum resistance is obtained (Yang and Huang, 1998). Fetal bovine serum was found to inhibit binding of cationic and anionic liposomes to rat liver parenchymal cells (Hoekstra and Scherphof, 1979).

A crucial step in the overall transfection process is the endosomal release of lipoplex and / or DNA into the cytosol. Hence, even if a substantial uptake of lipoplexes into cells occurs, failure of significant endosomal escape of plasmid prior to arrival of lipoplexes into lysosomes, where degradation occurs, will result in reduced transfection. This is an important factor to consider when comparing transfection efficiencies of different cell lines. Differences occur in efficiency of lipoplex internalization, endosomal escape and processing of DNA into the nucleus in different cells (Zuhorn et al., 2002). Although there is a correlation between delivery of DNA and gene expression, it is often found that DNA delivery into the nucleus of a particular cell is efficient but little or no gene expression is achieved (Templeton, 2003).

Both the quality of plasmid DNA and the transfection reagent used strongly influences the results of transfection experiments (Singer et al., 1999). The pGL3 plasmid DNA utilized in our study was amplified and prepared according to the manufacturer's protocol (Promega). It was a pure sample, free from contaminants, with a 260:280 ratio of not less than 1.8. Most of the plasmid was in the form of supercoiled DNA with less closed circular forms present. The use and choice of a suitable reporter gene expression system is also of great importance. The luciferase assay system was used

for the detection of transient gene expression in the cells. The increased sensitivity and ease of detection of the firefly luciferase has an appeal over the other reporter gene assays. Its increased sensitivity, especially over the CAT assay, allows transfection of fewer cells, using less material and a shorter turn-around time (Fulton and Van Ness, 1993).

The reaction catalysed by the firefly luciferase has the highest quantum efficiency known of any chemiluminescent reaction (Seliger and McElroy, 1960). Luciferase, is a monomeric protein (61 kD), that does not require post-translational processing for enzymatic activity, and can hence function as a genetic reporter immediately upon translation (de Wet *et al.*, 1985). The overall ATP-dependent reaction catalysed by the firefly luciferase is the oxidative decarboxylation of beetle luciferin, producing light emission (photon) at a wavelength of 562 nm. The principle of the luciferase reaction can be divided into a two stage process as set out below.

$$Mg^{2+}$$

1. Luciferase + Luciferin + ATP ——> Luciferase.luciferyl-AMP + ppi

2. Luciferase.luciferyl-AMP + O$_2$ ——> Luciferase + oxyluciferin + AMP + CO$_2$ +hν
(light)
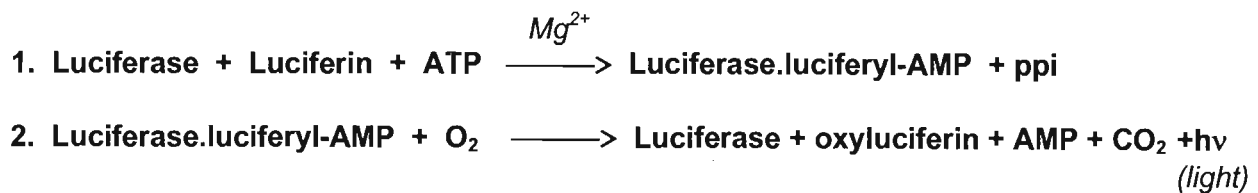
The first reaction involves the formation of luciferase.luciferyl-AMP. The second reaction involves the decarboxylation of the luciferase.luciferyl-AMP to produce oxyluciferin, CO$_2$, AMP and light (hν).

The coenzyme A (CoA) is also used as a substrate for luciferase. Oxidation occurs instead from luciferyl-CoA, as in the Promega assay system. The flash of light emitted by the luciferase assay rapidly decays after the enzyme and substrate are mixed. The system used in this assay produces a light intensity that is constant for up to several minutes. This light intensity, since it measures the rate of catalysis by the luciferase enzyme, is therefore dependent upon temperature. Optimum temperature for luciferase

activity is around room temperature (20 - 25°C). Hence the requirement for all the reagents to be equilibrated to room temperature before starting the assay procedure.

This overall process of transfection has many uses in medical research. It allows proteins whose functions are not well understood to be expressed in cultured cells (as seen above), so that their behaviour may be monitored. Liposome mediated transfection has also shown potential in predicting the optimal treatment for tumour cells by determining the tissue–specific expression of the target gene in prostate cancer cells (Suzuki *et al.*, 2001).

2.4   CONCLUSION

From the results obtained it can be concluded that the three novel cationic liposomes are capable of significant transfection activity. It is accepted that the transfection efficiency, mediated by mixtures of cationic lipids and neutral "helper lipids" varies widely and unpredictably (Safinya, 2001). The most important result to emerge was that cationic liposome MS09 showed extremely high transfection levels *in vitro* in all four cell lines, and performed better than Lipofectin^{TM} in the three human transformed epithelial cell lines, HeLa, HepG2 and SNO. Furthermore its relatively low toxicity to cells *in vitro* makes it a suitable formulation for further studies. In order to increase the efficiency of transfection by cationic liposomes one could add a targeting ligand or a built-in targeting moiety in the liposomal formulation for cell-specific delivery. For this reason the cationic cholesterol derivative MS09, which yielded the best results,   was utilised in studies described in Chapters 3 and 4, where it was formulated with targeting moieties to direct liposomes to the transferrin receptor (HeLa cells) and the asialoorosomucoid receptor (HepG2 cells) respectively. In both chapters, liposomes were prepared from MS09 and other novel cholesteryl derivatives as a prelude to the construction of larger assemblies intended for targeted delivery.

CHAPTER THREE

TARGETING OF CATIONIC BIOTINYLATED LIPOSOMES TO TRANSFERRIN RECEPTORS

3.1 TARGETING OF LIPOSOMES

The rate of entry of lipoplexes into cells varies with cell type and is generally a slow process (Zabner *et al.*, 1995). To speed up this process and to improve the cell specificity of gene expression by a liposome:DNA complex, the incorporation of a homing device into the complex is necessary (Remy *et. al.*, 1995; Nishikawa and Huang, 2001). The exploration of the potential of site-specific and targeted drug and gene delivery systems has gained enormous interest recently. The possibility of targeting genes or drugs to specific tissues and cells, as well as facilitating their uptake and cytoplasmic delivery has rendered liposomes as a versatile carrier system with numerous potential applications in medicine. Targeting provides an added advantage over non-targeted delivery systems in that it could prevent or minimize the side-effects of drugs or genes in healthy tissue in addition to enhancement of drug or gene uptake (Minko, 2004). It is possible that liposomes can bind to different cell surface receptors on different cells and that they could utilize more than one type of receptor on a particular cell (Düzgüneş and Nir, 1999).

A characteristic difference between normal and tumour cells which may be exploited is essential for targeting. This could be a structure expressed solely on tumour cells. However, there are very few known structures exclusively expressed on tumour cells. There could also be a difference in the number of an expressed structure. These structures, called tumour-associated antigens, are also often found in the organs from which the tumour originated. Antibodies are the most commonly used biomolecules in targeting against tumour-associated antigens. Other types of biomolecules suggested for targeting are hormones or ligands, which are directed towards normal cellular receptors that are overexpressed in certain tumour cells. Such biomolecules include the

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epidermal growth factor (EGF), asialoglycoprotein, folate, galactose, and transferrin that can be targeted to their respective receptors (Qian et al., 2002).

These naturally occurring ligands to cell surface receptors offer some advantages over antibodies, such as lack of immunogenicity, low toxicity, biodegradability and low preparation costs (Qian et al., 2002). The EGF-targeted liposomes may be useful in tumour targeting as many carcinoma cells show relatively high expression of this receptor (Kikuchi et al., 1996). The folate receptor has been identified as a marker in ovarian carcinomas. Folate-targeted liposomes have also been designed for this purpose and targeted to cells that overexpress the folate receptor (Lee and Low, 1994).

In this chapter we deal with the use of the transferrin ligand (Tf) in targeting to transferrin receptors on HeLa cells. Transferrin is a well studied ligand for tumour targeting due to the upregulation of transferrin receptors in numerous cancer cell types (Cotton et al., 1990; Wagner et al., 1994; Bellocq et al., 2003). It is also one of the most widely used ligands for synthetic targeting systems. The efficient cellular mechanism of transferrin uptake has been exploited for the delivery of anticancer drugs, proteins, and therapeutic genes into proliferating malignant cells that overexpress transferrin receptors (Singh, 1999). Transferrin can be utilized for targeting either in the form of drug conjugates, hybrid systems with macromolecules or as liposomal-coated systems. Various drugs such as doxorubicin and chlorambucil have already been conjugated to transferrin for delivery to certain cancer cells. The use of Tf-coupled liposomes for drug (eg doxorubicin) delivery has enhanced uptake by cells via receptor mediated endocytosis (Li and Qian, 2002). Other Tf conjugates such as Tf-polylysine (Cotton et al., 1990; Wagner et al., 1991; Curiel et al., 1992 ; Schoeman et al.,1995), Tf-PEI (Ogris et al., 1999; Kursa et al., 2003) and Tf-protamine enhanced liposomes (Tros de Ilarduya et al., 2002) have been shown to be efficient carriers for the introduction of genes into various cells such as human leukemic cells (K-562) and hematopoietic cells. Tf-PEI conjugates have been used to efficiently mediate topical gene transfer to the lungs after intratracheal nebulisation (Rudolf et al., 2002). The transferrin receptor also offers great

promise in the delivery of therapeutic agents across the blood-brain barrier to the brain (Li *et al.*, 2002).

For *in vivo* systems this process of targeted delivery, can be roughly divided into two phases viz. the transport phase in which the liposome travels from the site of administration to the target cells, and the effector phase that includes the specific binding of the liposome to the target cell receptor, and subsequent delivery of the bound gene or drug of interest (Mastrobattista *et al.*, 1999).

3.1.1   The Transferrin (Tf) ligand

Iron is transported in blood plasma and intestinal fluids bound to a carrier protein called transferrin (Tf). The iron requirement of any cell or tissue is particularly high during periods of cell proliferation and growth (Taylor and Morgan, 1990). Hence the Tf concentration in plasma varies in humans at different stages of the life cycle and in certain diseases. The serum transferrin concentration is about 2.5 mg/ml (35 μM) with 30% bound to iron (Li and Qian, 2002). The liver is the prime synthesizer of transferrin (Morgan, 1969), while a significant amount is also produced in the brain (Takeda, 2001).   However the levels of brain transferrin decrease with age and a dramatic decrease is observed in neurodegenerative diseases such as Alzheimer's disease or Parkinson's disease (Takeda, 2001).

Transferrins are 80 kDalton single-chain polypeptides of 670-700 amino acids (Li and Qian, 2002), and consist of two similarly sized homologous N and C lobes with a rninimum molecular weight in the range of 35-45 kDaltons (Baker *et al.*, 1968), which are further divided into two similarly sized domains (N1 and N2 in the N lobe and C1 and C2 in the C lobe). The C lobe has a carbohydrate chain attached with sialic acid residues which could affect the binding of metals to Tf. The most common Tf contains two biantennary complex-type glycans with a total of four sialic acid residues (Nagaoka and Maitani, 2001). The two iron binding sites are located within the interdomain cleft of

each lobe. These sites bind, with high affinity, one Fe^{3+} atom in combination with one CO_3^{2-} ion. Fe^{3+} binding is reversible and pH dependent. The amino acid tryptophan seems to be involved in the iron binding site (Mazurier et al., 1976). Tf can exist in three forms in the serum, viz. apotransferrin (no Fe^{3+}), monoferric Tf (one Fe^{3+}) and diferric Tf (two Fe^{3+}) (Mizutani et al., 1999).

The best known transferrins are serum transferrins that are found in blood, bile, amniotic fluid, cerebrospinal fluid, lymph, colostrum and milk; ovotransferrin found in egg white; lactoferrin found in mammalian milk, secretions (tears, saliva, mucus) and in white blood cells; and melanotransferrin (p97) that is found anchored to the membrane surface of melanocytes and other cells. All transferrins except lactoferrin are acidic proteins with the diferric species having an isoelectric point (pI) value of around 5.6-5.8 (Li and Qian, 2002). The transferrin ligands used in these studies are the serum transferrins.

3.1.2   The Transferrin Receptor (TfR)

There are two types of receptors on cells that are potentially able to mediate the uptake of Tf, viz. the specific Tf receptor which has a higher affinity for diferric Tf than the monoferric Tf, and, the asialoglycoprotein receptor which is believed to mediate the endocytosis and cellular acquisition of iron bound to asialotransferrin (Morgan, 1991). The reaction between Tf and TfR is reversible, pH dependent and influenced by the iron content of the Tf. At physiological pH, the receptor has a high affinity for iron-saturated Tf, especially diferric Tf, but a lower affinity for apotransferrin (Morgan, 1999). The TfR is a transmembrane homodimer made up of two subunits containing 760 amino acids each (Kuhn et al., 1984), joined by disulphide bonds at cysteine 89 and 98. The homodimer of TfR can bind up to two molecules of Tf. Human TfR is synthesized in the endoplasmic reticulum and post-translationally modified with both phosphates and fatty acyl groups (Qian et al., 2002). Oligosaccharides account for 5% of the receptor's 90 kDalton subunit molecular mass. Tf receptors on the outer face of the plasma

membrane bind iron loaded Tf with a very high affinity. The C-terminal domain of Tf appears to mediate binding to the receptor (Zak *et al.*, 1994).

Transferrin receptors are predominant or elevated in various forms of cancer cells, including squamous cell carcinomas such as oral tumours, and seem to correlate with the aggressive or proliferative ability of tumour cells. Hence, transferrin receptors may be useful as potential targets for drug / gene delivery (Joo and Kim, 2002). These rapidly dividing cells can express from 10 000-100 000 TfR molecules per cell (Inoue *et al.*, 1993). HeLa cells used in this study are amongst those cells that express the transferrin receptor (Derycke and De Witte, 2002). HeLa cells were found in some cases to express approximately 2×10^5 receptors /cell (Bridges and Smith, 1985). It was shown that cells in culture can also synthesise an increased number of Tf receptors when stimulated to proliferate (Iacopetta *et al.*, 1982). Rat reticulocytes were found to express an average of 85 000 Tf receptor sites per cell (Morgan, 1981), and hepatocytes about 18 600 receptors per cell (Bridle *et al.*, 2003). The Tf receptor has also shown potential for systemic p53 tumour suppressor gene therapy in a human breast cancer metastasis model (Xu *et al.*, 2002).

Transferrin receptors have been identified to be sometimes overexpressed in various cell types, viz. aortic endothelial cells (Voinea *et al.*, 2002), liver cells (hepatocytes) (Morgan, 1999), hepatic stellate cells, Kupffer cells, fibroblasts, neuronal cells (Bridle *et al.*, 2003), corneal endothelium cells (Tan *et al.*, 2001), adipocytes (Tros de Ilarduya *et al.*, 2002), in glioma cells where the number of receptors seems to increase with the severity of the tumour (Eavarone *et al.*, 2000) and in erythroblasts which have the highest number of Tf receptors (Vernet, 1999). Tf receptors can exist as cellular or serum Tf receptors. The serum Tf receptor is a truncated monomeric form of the cellular receptor and lacks its first 100 amino acids, and usually circulates as a Tf-TfR complex. Erythroblasts are the main source of serum TfR (sTfR) (Beguin, 2003). Serum transferrin receptors are unaffected in inflammatory diseases, infections, malignancies or cytolysis but are considered useful tools in diagnosis of different causes of anemia

(Vernet, 1999) and the bone marrow erythropoietic activity where there is either an 8 fold or a 20 fold decrease in sTfR levels (Beguin, 2003).

A second Tf receptor (TfR2) has been cloned and sequenced (Kawabata *et al.*, 1999). It has a similar function to TfR in Tf binding but its affinity for iron-loaded Tf is 25-fold lower. TfR2 is thought to be important in maintenance of iron haemostasis and is linked to the disorder of hemochromatosis (Li and Qian, 2002).

3.1.3   The Uptake of Transferrin

Cells possess endogenous pathways for internalization of macromolecules. The utilization of these pathways for the purpose of DNA delivery is advantageous. These cellular internalization pathways can be highly efficient, as seen in the internalization of Tf, which can be in the order of thousands of molecules per minute per cell. Hence, these pathways represent a potentially efficient physiological method for the transport of DNA across the cell membrane of eukaryotic cells (Curiel, 1996). The *in vitro* transfection efficiency of cationic liposomes can be increased when complexed to transferrin exploiting the mechanism of receptor-mediated endocytosis. Cells generally acquire iron from transferrin via receptor-mediated endocytosis of the transferrin-iron complex (Qian and Morgan, 1992).

Transferrin is first bound to the Tf receptor on the cell surface to form a Tf-receptor (TfR) complex which accumulates at one end of the cell (capping), and is then internalized by endocytosis (Hibbs, 2000). This process involving invagination of clathrin-coated pits and formation of endocytotic vesicles requires the short, 61 amino acid intracellular tail of the Tf receptor molecule (Miller *et al.*, 1991). Tf binding is a simple chemical event and not dependent on any metabolic energy. After endocytosis the iron is released from the Tf within the endosomes (pH 5.0-5.5) and enters the cytosol to be incorporated into heme, utilized as a cofactor for aconitase, cytochromes and RNA reductase, or stored as ferritin, while the apotransferrin and the TfR are recycled to the cell surface. Apotransferrin has a much lower affinity for Tf receptors at

physiological pH than at acidic pH, but was shown to mediate significant levels of gene expression at different lipid/DNA (+/-) ratios (Simões *et al.*, 1999).

The TfR seems to localize to three different populations of endosomal vesicle: those containing only Rab5, those containing Rab5 and Rab4, and those containing Rab4 and Rab11. These Rab endosomal proteins are small GTPases of the Ras superfamily (Jones *et al.*, 2003). Rab5 and Rab4 are localized in the early endosomes and regulate fusion between the endocytotic vesicles and the early endosomes, and recycling. Hence they are important in endosomal trafficking (McCaffrey *et al.*, 2001). Rab11 however has been shown to co-localise with internalized Tf in the pericentriolar recycling compartment of CHO and BHK cells (Ullrich *et al.*, 1996), but does not affect transferrin recycling in Hela cells (Wallace *et al.*, 2002). Tf-receptor complexes are recycled via exocytotic vesicles back to the cell membrane (Li and Qian, 2002), or, in the case of polarized cells, they can be transcytosed from the apical to the basolateral membrane (Jones *et al.*, 2003). The apotransferrin is released, due to its low affinity for its receptor at pH 7.4, into the medium, and the receptors bind more iron-transferrin to repeat the cycle (Qian and Morgan, 1992 ; Savigni and Morgan, 1998). Tf and its receptor seem to recycle from the cell surface to intracellular sites without degradation, with an average recycling time of 3-4 minutes in rat erythrocytes (Morgan and Peters, 1985). This was also observed in K562 cells with about 150 000 TfR per cell and a mean transit time of about 10 minutes. This leads to high turnover numbers of about 2×10^4 Tf molecules internalized per minute (Wagner *et al.*, 1994).

Tf undergoes conformational changes during both Fe^{3+} uptake and release, that are crucial for the selective recognition by the receptor. The two lobes of Tf open and close, by a mechanism which is not completely understood (Qian *et al.*, 2002). The transferrin molecule undergoes unfolding upon dissociation of the iron cations. However if one iron cation is removed the secondary structure of transferrin is not affected, but removal of the second iron cation results in simultaneous unfolding of the protein (Mazurier *et al.*, 1976). The rate limiting step for Tf-iron uptake by cells is the rate of Tf endocytosis which in turn is dependent, in part, on the number of recycling receptors in the cell (Qian and Morgan, 1992). Co-localization studies with transferrin, showed that it is

internalized via the clathrin-coated endocytotic pathway, and that the internalization via caveolae do not seem significant (Zuhorn *et al.*, 2002). Receptor-mediation, however only accounts for part of the Tf uptake, the remainder being derived from non-saturable adsorptive processes and fluid-phase endocytosis (Morgan, 1991). The endocytosis of Tf in the liver of rats was seen to increase during iron deficiency (Morgan *et al.*, 1986). Various binding studies on rat reticulocytes have shown that the forces involved in Tf binding are a combination of weak forces such as hydrophobic, hydrogen bonding or van der Waals forces. Binding involves a single bimolecular interaction between Tf and the binding sites. Two types of interaction between Tf and reticulocytes exist, viz. adsorption and association reactions (Baker and Morgan, 1969).

3.1.4 Streptavidin-Biotin Interaction

The use avidins offer an attractive approach to organ- or tissue-selective targeting. Avidin and streptavidin, the two biotin-binding proteins, can be targeted to specific tissues when modified with appropriate tissue markers. Their resistance to proteolytic enzymes supports long-term accumulation at the target tissue or organ, and their biotin binding sites permit the delivery of biotinylated molecules or carriers loaded with cytotoxic drugs or other bioactive substances (Chen *et al.*, 2000). The high affinity of avidin for biotin was first exploited in histochemical applications in the mid-1970s. This egg-white protein and its bacterial counterpart, streptavidin, produced by *Streptomyces avidinii*, have since been utilized for diverse applications (Wilchek and Bayer, 1988). Avidin is a highly cationic 66 kDalton glycoprotein with an isoelectric point of about 10.5. It is thought that avidin's positively charged residues and its oligosaccharide component (heterogeneous structures composed largely of mannose and *N*-acetylglucosamine) can interact non-specifically with negatively charged cell surfaces and nucleic acids, sometimes causing background problems in some histochemical applications (Bayer *et al.*, 1993). Therefore, instead of avidin, streptavidin has been frequently used due to the fact that it does not bind non-specifically.

Streptavidin is a non-glycosylated 52 800 Dalton protein with a near-neutral isoelectric point (pI 5-6), and exhibits less nonspecific binding than avidin. Since streptavidin is not a glycoprotein, its potential to bind to carbohydrate receptors is reduced. Both its pI value and its specificity reduce the chances of systemic clearance *in vivo* (Schnyder *et al.*, 2004). However, streptavidin contains the tripeptide sequence Arg–Tyr–Asp (RYD) that apparently mimics the Arg–Gly–Asp (RGD) binding sequence of fibronectin, a component of the extracellular matrix that specifically promotes cellular adhesion. This universal recognition sequence binds integrins and related cell-surface molecules. Background problems sometimes associated with streptavidin may be attributable to this tripeptide. (Alon *et al.*, 1993). Streptavidin is a small protein, which diffuses rapidly through leaky capillaries at sites of inflammation (Das *et al.*, 2002)..

Avidin and streptavidin each bind four biotins per molecule with high affinity and selectivity. Biotin has both high affinity for streptavidin and rapid whole body clearance (Das *et al.*, 2002). Amino acid residues are critical for stabilization of the tetrameric assembly and for the exceptionally tight binding of biotin. The binding of biotin to streptavidin involves a highly stabilized network of polar and hydrophobic interactions. Avidin, however, has a higher biotin affinity constant, probably due to the presence of additional hydrophobic and hydrophilic groups in its binding site (Livnah *et al.*, 1993). Dissociation of biotin from streptavidin is hence reported to be about 30 times faster than dissociation of biotin from avidin (Piran and Riordan, 1990). Their multiple binding sites permit a number of techniques in which avidin or streptavidin can be used to bridge two biotinylated reagents. This bridging method, which is commonly used to link a biotinylated probe to a biotinylated enzyme in enzyme-linked immunohistochemical applications, often eliminates the background problems that can occur when using direct avidin or streptavidin–enzyme conjugates.

Receptor-mediated endocytosis via the transferrin receptor using conjugates of streptavidin or avidin, biotinylated Tf and biotinylated polylysine and pRSVL DNA was described previously (Schoeman *et al.*, 1995 ; Strydom *et al.*, 1993). The addition of 2-4 PEG chains to the above conjugate seemed to increase luciferase activity 4-5 fold in a

HeLa cell system (Robinson *et al.*, 1997). The use of streptavidin as a linker between biotinylated polyethyleneglycol-phospholipid present in a liposome bilayer and an antibody raised against the rat Tf receptor in skeletal muscle was also recently investigated, and showed promising results for future targeting studies (Schnyder *et al.*, 2004).

3.2   OUTLINE OF CHAPTER

In this chapter we outline the synthesis of two biotinylated cholesterol derivatives, their formulation with cationic cholesterol derivative MSO9 (chapter 2) and DOPE to produce liposomes, and the formation of a ternary complex between these biotinylated liposomes, plasmid DNA (pGL3) and a streptavidin-biotin-transferrin conjugate. The transferrin ligand (targeting moiety) was first conjugated to three biotin molecules which were then bound to streptavidin to produce a streptavidin-biotin-transferrin (streptavidinbiotransferrin) complex. The streptavidin in the conjugate acts as a bridge between the transferrin and the biotin that is anchored by the cholesterol group in the liposomal lipid bilayer. The aim of these studies was to determine whether these novel liposomal formulations coupled to Tf are taken up by transferrin-specific receptors on HeLa cells *in vitro*.

3.3 MATERIALS AND METHODS

3.3.1 MATERIALS FOR CHEMICAL SYNTHESES

Cholesteryl formylhydrazide was prepared as described in section 2.2.2.1. Biotinamidocaproate-NHS, DCCI, NHS, apotransferrin, ferric citrate and streptavidin were purchased from Sigma-Aldrich (St.Louis, MO, USA). Dry pyridine, citrate, dimethylformamide (DMF), sulphuric acid, p- dimethlyaminocinnamaldehyde, NaCl, Tris-HCl and silica gel 60F$_{254}$ chromatography plates were obtained from Merck (Darmstadt, Germany). All other reagents were of analytical grade.

Solvent system A = chloroform: methanol (9:1 $^V/_v$).

3.3.2 PREPARATION OF BIOTINYLCHOLESTERYLFORMYLHYDRAZIDE (MSB1) (Figure 3.1)

Initially, the N-hydroxysuccinimide ester of biotin was prepared by an adaptation of the method described by Wilchek and Bayer, 1990. Briefly, biotin (122 mg, 0.5 mmole), DCCI (99 mg, 0.48 mmole) and NHS (60 mg, 0.52 mmole) were dissolved in 1.2 ml DMF. The resultant dicyclohexylurea crystals were removed by filtration and the DMF was evaporated to dryness in a Büchii Rotavapor-R. The residue was extracted three times with ether. The biotin-NHS was recrystallised from isopropanol. Mp 198°C.

Biotin-NHS (68 mg, 0.2 mmoles) and cholesterylformylhydrazide (88 mg, 0.2 mmoles) were dissolved with gentle heating in 4 ml dry pyridine. The reaction mixture was kept at room temperature in the dark for 48 hours and thereafter concentrated in a rotary evaporator. The product was then extracted with a CHCl$_3$: H$_2$O (1:1 $^V/_v$) mixture. The NHS was removed in the water layer and the product dissolved in the chloroform layer. The chloroform layer was evaporated under reduced pressure and the extract then dissolved in a CHCl$_3$: ethanol (1:1 $^V/_v$) mixture. The solvent mixture was evaporated under vacuum and the product was dissolved in hot ethanol. The resultant clear solution

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was kept at 4°C until crystal formation was complete. Excess ethanol was removed from the flask with a pasteur pipette and crystals dried in a rotary evaporator.

The product was purified further by preparative TLC on 10 x 20 cm silica gel 60F$_{254}$ chromatography plates developed in solvent system A. Plates were viewed under UV$_{254}$ light to locate the product bands, which were finally scraped off the plates and extracted in a CHCl$_3$: ethanol (1:1 $^v/_v$) mixture. The mixture was filtered to remove the silica gel and the filtrate evaporated to dryness in a rotary evaporator. The product was finally dissolved in ethanol (0.3 ml) and placed at 4°C overnight. The crystalline product was separated from the supernatant and dried by rotary evaporation. The product was monitored at each stage by TLC (silica gel 60F$_{254}$) in solvent system A. A developed TLC plate was sprayed with a biotin spray (0.2% p-dimethlyaminocinnamaldehyde in 2% sulphuric acid) to detect the biotin group that stained bright pink / red. The plate was then heated to produce the brown spots to indicate the presence of cholesterol. Product gave a single spot by TLC (R$_f$ 0.52, solvent A) with a mp of 192-193°C and a yield of 80.2 mg (60%).

IR (film) : 3288 (b, N-H), 2939 (st, C-H), 1695 (st, C=O), 1244 (m, C-N), 1465 (m, C=C), 1045 (m, C-S). (Appendix 2K).

^{1}H NMR (300 MHz, CDCl$_3$) : δ 0.65 (s, 3H, C-CH_3), 0,88 (d, 3H, J=6.5, C-CH_3), 0.84 (d, 6H, J=6.1, C-CH_3), 4.32 (m, 1H, biotin H), 4.48 (m, 2H, biotin H, Chol-H$_{3\alpha}$), 5.35 (bs, 1H, Chol-H$_6$). (Appendix 1G).

MS, $^m/_z$, ES-TOF : 693.6136 [M + Na$^+$], 694.6306 [M + H + Na$^+$].

3.3.3 PREPARATION OF AMINOHEXANOYLBIOTINYLCHOLESTERYL-FORMYLHYDRAZIDE (MSB2) (Figure 2)

Biotinamidocaproate-NHS (aminohexanoylbiotin-NHS) (11.5 mg, 0.025 mmoles) and cholesterylformylhydrazide (11.25 mg, 0.025 mmoles) were dissolved in 400 µl dry pyridine. The reaction mixture was kept at room temperature in the dark for 48 hours and thereafter evaporated to dryness in a rotary evaporator to produce a white film on the flask. Distilled water was added to completely cover the product. Free NHS was extracted into the water. The mixture was kept at 4°C overnight, and the water finally removed by filtration and product dried under vacuum. The product was monitored at each stage by TLC (silica gel 60F$_{254}$) in solvent system A. The TLC plate was sprayed with a biotin spray to detect the biotin group and then heated to indicate the presence of cholesterol. Product produced a single spot by TLC (R$_f$ 0.33, solvent A) with a mp of 194-195°C and a yield of 17.8 mg (91%).

IR (film) : 3446 (b, N-H), 2929 (st, C-H), 1669 (st, C=O), 1244 (w, C-N), 1460 (w, C=C), 1032 (m, C-S). (Appendix 2L).

MS, $^m/_z$, ES-TOF : 806.7360 [M + Na$^+$], 807.7563 [M + H + Na$^+$]
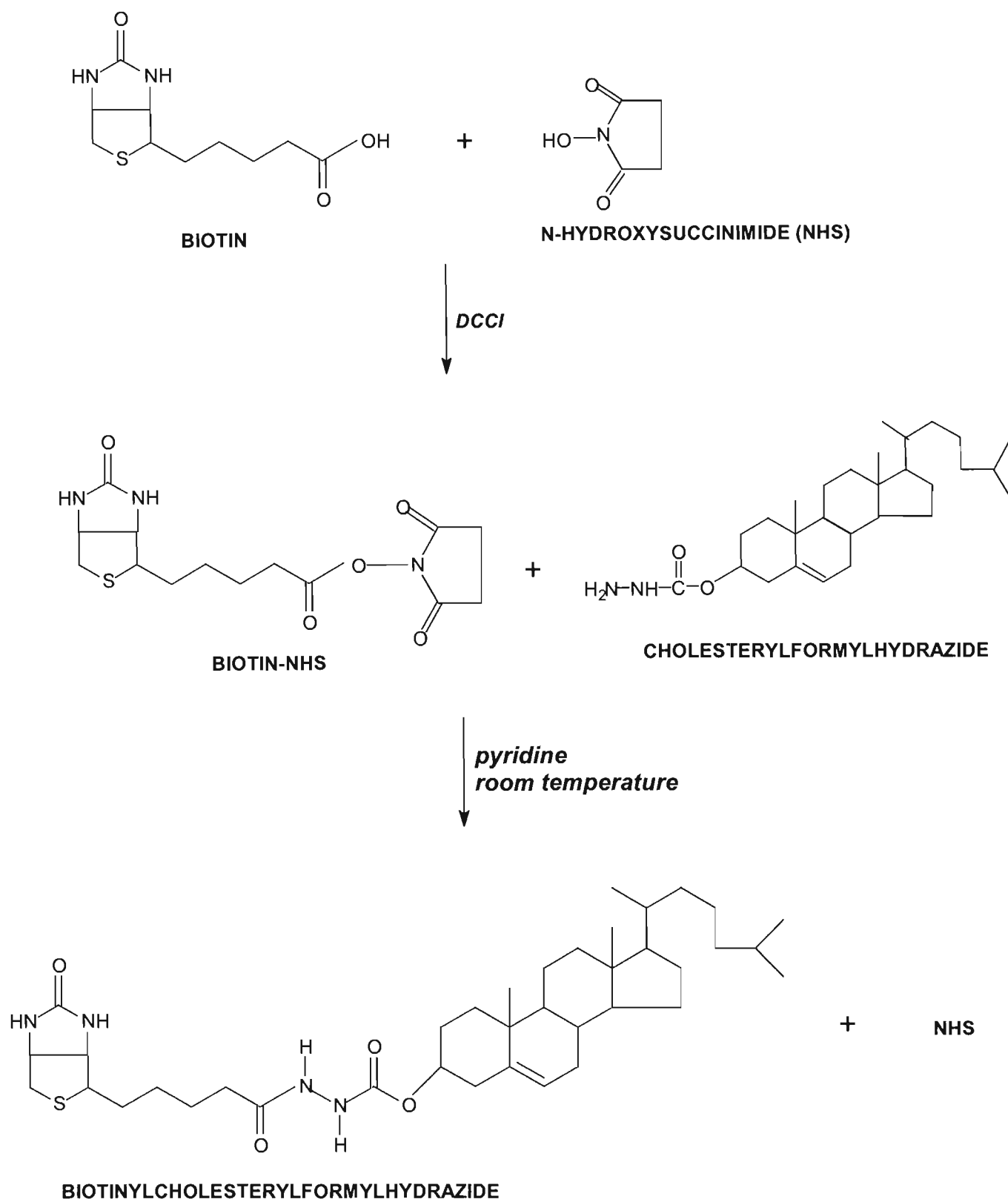
Figure 3.1 : Scheme for the synthesis of biotinylcholesterylformylhydrazide (MSB1).

AMINOHEXANOYLBIOTIN-NHS

+

CHOLESTERYLFORMYLHYDRAZIDE

pyridine
room temperature

AMINOHEXANOYLBIOTINYLCHOLESTERYLFORMYLHYDRAZIDE   +   **NHS**
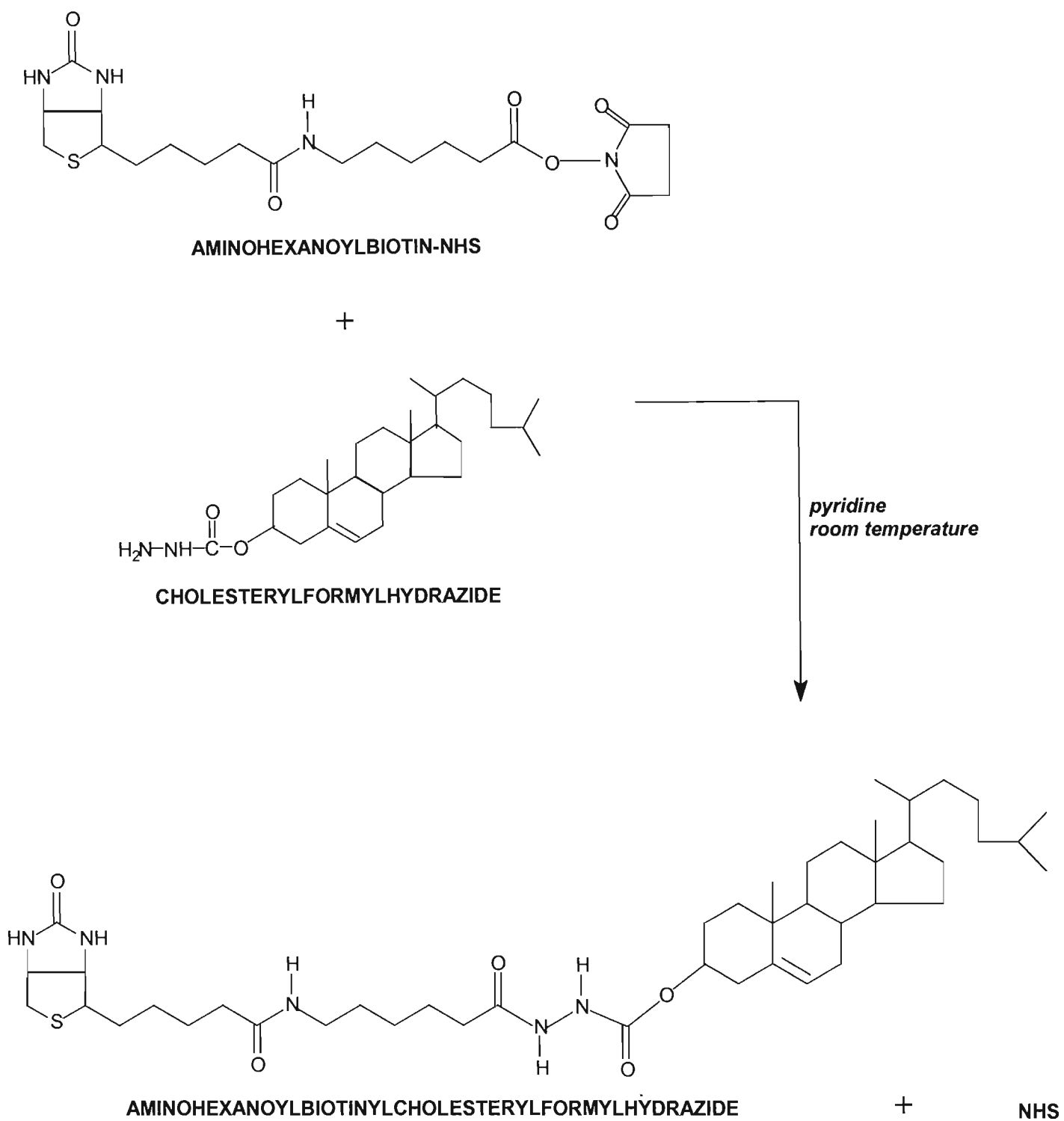
Figure 3.2 : Scheme for the synthesis of aminohexanoylbiotinyl-
cholesterylformylhydrazide (MSB2)

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3.3.4 Preparation of Iron (Fe^{3+}) loaded Transferrin

This procedure was adapted from that of Kursa *et al.*, 2003. Ferric citrate buffer (25 µl, 10 mM) in citrate buffer (200 mM adjusted to pH 7.8 with NaHCO$_3$), was added to transferrin (5 mg). This iron-loaded transferrin was made up in a total volume of 1 ml with 20 mM HEPES, 150 mM NaCl (pH 7.4), giving a final concentration of 5 mg / ml.

3.3.5 Preparation of Streptavidin(bio^3-transferrin)

This preparation was carried out as described by Schoeman *et al.*, 1995, at the University of Stellenbosch by Professor A.O. Hawtrey. Briefly, to streptavidin (3 mg, 0.05 µmole) was added 2 ml 0.2 M NaCl, 0.005 M Tris-HCl (pH 7.6). This was mixed gently. To bio^3-transferrin (0.55 ml, 4 mg, 0.05 µmole) was added 1.45 ml 0.2 M NaCl, 0.005 M Tris-HCl (pH 7.6). The streptavidin and bio^3-transferrin solutions were then mixed together quickly and checked for turbidity. This was kept at room temperature for one hour in the dark. Solutions were re-examined and were clear of any turbidity (Strydom *et al.*, 1993 ; Schoeman *et al.*, 1995).

Reaction mixtures were split into aliquots containing 7.14 µg/µl or 0.87 µg/µl streptavidin (bio^3-transferrin) and stored at -10°C.

3.3.6   PREPARATION AND CHARACTERISATION OF MSB1 and MSB2 CONTAINING LIPOSOMES

3.3.6.1   Materials

The synthesis of MS09 is described in chapter two section 2.2.2. DOPE was purchased from Sigma-Aldrich (St.Louis, MO, USA). HEPES, glutarylaldehyde, osmium tetroxide, propylene oxide, lead citrate, sodium chloride and chloroform were obtained from Merck (Darmstadt, Germany). The components of Spurr's Resin (GRL 4206, DER 736, NSA , S-1), were purchased from TAAB Laboratories (Berkshire, United Kingdom). Copper grids for electron microscopy were obtained from Capital Labs (KwaZulu-Natal, South Africa). Bovine serum albumin (BSA) was purchased from Roche Diagnostics (Mannheim, Germany). All other reagents were of analytical grade.

3.3.6.2   Formulation of Biotinylated Liposomes containing :
a) Biotinylcholesterylformylhydrazide (MSB1) and,
b) Aminohexanoylbiotinylcholesterylformylhydrazide (MSB2)

Components for liposome MSB1 and MSB2 were formulated in a 48:50:2 molar ratio of MS09:DOPE:MSB1/MSB2 with DOPE kept at a 2 μmol (Table 3.1).

Table 3.1 : Liposomal components of Biotinylated liposomes MSB1 and MSB2

LIPOSOME FORMULATION	MOLAR RATIOS FOR RESPECTIVE LIPIDS (μmoles)			
	DOPE	MS09	MSB1	MSB2
Liposome MSB1	2	1.92	0.08	-
Liposome MSB2	2	1.92	-	0.08

Liposome components were dissolved in 1 ml CHCl$_3$ and liposomes were prepared as described in chapter two, section 2.2.5.2.

3.3.6.3 Characterisation of Cationic Liposomes using Transmission Electron Microscopy:

Both liposome formulations were prepared for characterization by TEM as described in chapter two, section 2.2.5.3, viewed in a Jeol 1010 transmission electron microscope at 60 kV and photographed at a 2 second exposure on a fine grain release positive film.

3.3.7 LIPOPLEX AND TERNARY COMPLEX FORMATION OF MSB1 AND MSB2 LIPOSOMES WITH DNA AND TRANSFERRIN / STREPTAVIDIN (BIO³-TRANSFERRIN)

3.3.7.1 Materials

The pGL3 control vector was purchased from Promega Corporation (Madison,WI, USA), and amplified according to the manufacturer's protocol. pBR322 plasmid DNA was supplied by Roche Diagnostics (Mannheim, Germany). Transferrin was purchased from Sigma-Aldrich (St.Louis,Mo,USA). Agarose was obtained from Bio-Rad Laboratories (Richmond, CA, USA). Tris-HCl, NaH_2PO_4, HEPES, NaCl, EDTA, SDS, glycerol, bromophenol blue, xylene cyanol, , uranyl acetate and ethidium bromide were purchased from Merck (Darmstadt, Germany). Sypro red protein stain was purchased from Life Technologies (UK). Fetal calf serum was obtained from Delta Bioproducts (Johannesburg, South Africa). All other reagents were of analytical grade. Formvar-coated grids were prepared by the Electron Microscope Unit, University of KwaZulu-Natal (Pietermaritzburg Campus).

3.3.7.2 Gel Retardation Studies of Biotinylated Liposome:DNA Complexes

Varying ratios of different biotinylated liposome:DNA complexes were set up as indicated in Table 3.2 for MSB1 and Table 3.3 for MSB2 liposomes. All cationic

liposome:DNA complexes were incubated for 30 minutes at room temperature for completion of lipoplex formation. At the end of the incubation, 3 µl of gel loading buffer / stop solution (50% glycerol, 0.5% bromophenol blue, 0.5% xylene cyanol in a 2 x gel buffer) was added to all eight samples. The samples were then loaded onto a 1% agarose gel and subjected to agarose gel electrophoresis for approximately 90 minutes, at 50 V and in a 1 x electrophoresis / gel buffer. The gel was stained with ethidium bromide (1 µg /ml) for 30 minutes and viewed under transillumination at 300 nm using a UVP gel documentation system. Images were obtained and photographed at 320 millisecond exposure.

Table 3.2 : MSB1 liposome:DNA complexes. Incubation mixtures (10 µl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained cationic liposome (0 - 7 µg) and pGL3 DNA (1 µg).

LIPOPLEX COMPONENTS	1	2	3	4	5	6	7	8
pGL3 DNA (µg)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Liposome MSB1 (µg)	0	1	2	3	4	5	6	7

Table 3.3 : MSB2 liposome:DNA complexes. Incubation mixtures (10 µl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained cationic liposome (0 - 6 µg) and pGL3 DNA (1 µg).

LIPOPLEX COMPONENTS	1	2	3	4	5	6	7	8
pGL3 DNA (µg)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Liposome MSB2 (µg)	0	0.5	1	2	3	4	5	6

3.3.7.3   Gel Retardation Studies of Transferrin:DNA Complexes

Varying ratios of different transferrin:DNA mixtures were set up as shown in Table 3.4. Transferrin:DNA mixtures were incubated for 20 minutes at room temperature. At the end of the incubation, 3 µl of gel loading buffer / stop solution (50% glycerol, 0.5%

bromophenol blue, 0.5% xylene cyanol in a 2 x gel buffer) was added to all eight samples. The samples were then loaded onto a 1% agarose gel and subjected to agarose gel electrophoresis as indicated in 3.2.7.2. The gel was stained with ethidium bromide (1 µg /ml) for 30 minutes and viewed under transillumination at 300nm using a UVP gel documentation system. Images were obtained and photographed at 320 millisecond exposure.

Table 3.4 : Transferrin:DNA mixtures. Incubation mixtures (50 µl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained transferrin (0 - 50 µg) and pGL3 DNA (0.5 µg).

LIPOPLEX COMPONENTS	1	2	3	4	5	6	7	8
pGL3 DNA (µg)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Iron laoded transferrin (µg)	-	5	10	15	20	30	40	50

3.3.7.4 Gel Retardation Studies of Streptavidin (bio[3]-transferrin): Liposome:DNA Complex for MSB1 and MSB2 Liposomes

Varying ratios of different streptavidin (bio[3]-transferrin):liposome:DNA complexes were set up as indicated in Tables 3.5 and 3.6. The streptavidin (bio[3]-transferrin) complexes were first added to the liposomes and mixed prior to addition of DNA. After addition of the DNA the complexes were incubated for a further 30 minutes. At the end of the incubation, 3 µl of gel loading buffer / stop solution (50% glycerol, 0.5% bromophenol blue, 0.5% xylene cyanol in a 2 x gel buffer) was added to all eight samples. The samples were then loaded onto a 1% agarose gel and subjected to agarose gel electrophoresis as described in 3.2.7.2. The gel for samples in Table 3.5 was stained with ethidium bromide (1 µg /ml) for 30 minutes and gel for samples in Table 3.6 was stained for protein with a Sypro Red™ stain for 60 minutes, and viewed under

transillumination at 300 nm using a UVP gel documentation system. The images were obtained and photographed at 320 millisecond exposure. The latter samples were also stained for protein with a 0.5 % Coomassie Blue G250 solution for 20 minutes. Background staining was removed by destaining in a methanol : acetic acid : water (3:1:6 $^V/_V$) mixture for 3 to 4 hours before photographing the gels under white light at a 280 millisecond exposure.

Table 3.5 : Streptavidin (bio^3-transferrin):liposome:DNA complexes. Incubation mixtures (20 µl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained streptavidin (bio^3-transferrin) (0-12 µg) , liposome MSB1 or MSB2 (6 µg) and pGL3 DNA (1µg).

LIPOPLEX COMPONENTS	1	2	3	4	5	6	7	8
pGL3 DNA (µg)	1	1	1	1	1	1	1	1
Liposome MSB1 or MSB2 (µg)	-	2	3	4	5	6	7	8
streptavidin (bio^3-transferrin) (µg)	-	6	7	8	9	10	11	12

Table 3.6 : Streptavidin (bio^3-transferrin):liposome:DNA complexes. Incubation mixtures (20 µl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained streptavidin (bio^3-transferrin) (10 µg), liposome MSB1 or MSB2 (0-8 µg) and pGL3 DNA (1µg).

LIPOPLEX COMPONENTS	1	2	3	4	5	6	7	8
pGL3 DNA (µg)	1	1	1	1	1	1	1	1
Liposome MSB1 or MSB2 (µg)	-	2	3	4	5	6	7	8
streptavidin (bio^3-transferrin) (µg)	-	10	10	10	10	10	10	10

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3.3.7.5 Dye Displacement Assays

Dye displacement assays were carried out in four different ways to ascertain liposome:DNA binding and the effect of transferrin and streptavidin(bio[3]-transferrin) on binding of DNA.

(a) Addition of biotinylated liposomes to DNA-ethidium bromide solution

This assay was conducted in a manner similar to that described in Chapter 2, Section 2.2.6.3. Ethidium bromide (1 µg) was added to 500 µl of 20 mM HEPES,150 mM NaCl, pH 7.5. This mixture was used to provide the baseline reading (0%) for fluorescence. Thereafter 6 µg of pBR322 DNA was added to the above solution and the reading taken was assumed to represent 100% fluorescence. Aliquots of each biotinylated liposome, MSB1 and MSB2 (6 µg, 2.2 µl), were added, stepwise to the solution until 60 µg of each liposome had been added. The solutions were mixed thoroughly after each addition and fluorescence was measured in a Shimadzu RF-551 spectrofluorometric detector, at an excitation wavelength of 520 nm and an emission wavelength of 600 nm, and at high sensitivity. Results were plotted relative to 100% fluorescence.

(b) Addition of liposome:transferrin (1:2) complex to DNA

Ethidium bromide (1 µg) was added to 500 µl of 20 mM HEPES,150 mM NaCl, pH 7.5. This mixture was used to provide the baseline reading (0%) for fluorescence. Thereafter 6 µg of pBR322 DNA was added to the above solution and the reading taken was assumed to represent 100% fluorescence. Aliquots (3 µl) of each liposome:transferrin complex (3.9 µg lipid and 7.8 µg transferrin) were added stepwise to the solution until the total lipid was 39 µg and the total transferrin was 78 µg. The solutions were mixed thoroughly after each addition and fluorescence was measured as explained above (3.3.7.5a).

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(c) **Addition of streptavidin (bio³-transferrin) complex to liposome:DNA complex**

Ethidium bromide (1 µg) was added to 500 µl of 20 mM HEPES,150 mM NaCl, pH 7.5. This mixture was used to provide the baseline reading (0%) for fluorescence. Thereafter a biotinylated liposome:DNA lipoplex (6:1 $^w/_w$) , 36 µg lipid and 6 µg pBR322 DNA) was added to the above solution and the fluorescence reading recorded and reported as arbitary fluorescence units. Aliquots (2 µl, 1.74 µg) of the streptavidin (bio³-transferrin) complex were added stepwise to the solution until 26.1 µg of the streptavidin (bio³-transferrin) had been added. The solutions were mixed thoroughly after each addition and fluorescence was measured as explained in 3.3.7.5a.

(d) **Addition of transferrin to liposome:DNA complex**

Ethidium bromide (1 µg) was added to 500 µl of 20 mM HEPES,150 mM NaCl, pH 7.5. This mixture was used to provide the baseline reading (0%) for fluorescence. Thereafter a biotinylated liposome:DNA mixture (6:1 $^w/_w$) , 36 µg lipid and 6 µg pBR322 DNA) was added to the above solution and the fluorescence reading recorded and reported as arbitary fluorescence units. Aliquots (2 µl, 4 µg) of iron loaded transferrin were added stepwise to the solution until a total of 28 µg of transferrin had been added. The solutions were mixed thoroughly after each addition and fluorescence was measured as described in 3.3.7.5a.

3.3.7.6 Transmission Electron Microscopy of Lipoplexes (MSB1 and MSB2) and Liposome:DNA:streptavidin(bio³-transferrin) Ternary Complexes

Sample preparation was as described in Section 2.2.6.4. The lipoplexes and ternary complexes were viewed in a Jeol 1010 transmission electron microscope at 60 kV.

3.3.7.7 Nuclease Digestion Assays of Ternary Complexes

Liposome:DNA:streptavidinbiotransferrin complexes were set up as shown in Table 3.4.

Table 3.4 : Biotinylated liposome:DNA:streptavidinbiotransferrin complexes. Incubation mixtures (20 µl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained liposome MSB1 or MSB2 (0 - 8 µg), pGL3 DNA (1 µg) and streptavidinbiotransferrin (10 µg).

LIPOPLEX COMPONENTS	1	2	3	4	5	6	7	8
pGL3 DNA (µg)	1	1	1	1	1	1	1	1
Liposome MSB1 /MSB2 (µg)	-	-	3	4	5	6	7	8
Streptavidinbiotransferrin (µg)	-	-	10	10	10	10	10	10

Samples were made up to a total volume of 20 µl with 20 mM HEPES, 150 mM NaCl, pH 7.5. The streptavidin (bio^3-transferrin) complexes were first added to the liposomes and mixed prior to addition of DNA. After addition of the DNA, the complexes were incubated for 30 minutes. The procedure followed was as described in chapter two , section 2.2.6.6.

3.3.8 CELL CULTURE AND TRANSFECTION STUDIES

3.3.8.1 Materials

HeLa cells were obtained from Highveld Biologicals (Pty) Ltd (Kelvin, South Africa). Trypsin-EDTA, and penicillin-streptomycin mixtures were purchased from Whittaker, M.A. Bioproducts (Maryland, USA). Fetal calf serum was supplied by Delta Bioproducts, (Johannesburg, South Africa). Minimum essential medium (MEM) with Earle's salts was obtained from Gibco-BRL, Life Technologies Ltd. (Inchinnan, Scotland). The Luciferase assay kit , and the pGL3 control vector (prior to amplification) were purchased from the Promega Corporation (Madison,WI, USA). All tissue culture plasticware was obtained from Bibby-Sterilin (Staffordshire, England). The Bicinchoninic acid (BCA) protein assay kit was supplied by Sigma-Aldrich (St.Louis, MO, USA). Coomassie blue G250, dimethylsulfoxide (DMSO), crystal violet and formaldehyde were supplied by Merck (Darmstadt, Germany). All other reagents were of analytical grade.

3.3.8.2 Growth and Maintenance of HeLa cells

The HeLa cell lines were propagated in MEM with 10% FCS and antibiotics, and maintained as described in chapter two, section 2.2.7.2.

3.3.8.3 Growth Inhibition Assays

HeLa cells were trypsinised and seeded into a 24 well plate at a seeding density of 2.2×10^4 cells/well for both liposome MSB1:DNA complexes and liposome MSB2:DNA complexes. The HeLa cell seeding density was 2.1×10^4 cells/well for both streptavidin (bio^3-transferrin):liposome:DNA complexes. Wells in row 1 received no cells and were used to obtain a blank (0) reading, and wells in row 2 received only cells and no lipoplexes and was used to give the 100% reading. Cells were incubated for 24 to 36

hours and allowed to attach to the wells and grow to semi-confluency. The biotinylated liposome:DNA complexes and the streptavidin (bio^3-transferrin):liposome:DNA [pGL3 DNA, 1 μg)] complexes were set up as outlined in Tables 3.5 and 3.6 for both liposome MSB1 and MSB2. The lipoplexes were incubated for 30 minutes prior to addition to the cells. To make up the ternary complex, the streptavidin (bio^3-transferrin) complexes were first added to the liposomes and mixed prior to addition of DNA. After addition of the DNA the complexes were incubated for 30 minutes. The reaction mixtures were all made up to a constant volume with 20 mM HEPES, 150 mM sodium chloride, pH 7.5.

Table 3.5 : MSB1 or MSB2:DNA complexes as added to 24-well plates. pGL3 DNA was kept constant at 1 μg. Incubation mixtures (10 μl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained biotinylated liposomes (0–8 μg) and pGL3 DNA (1 μg).

Reaction Components	1	2	3	4	5	6
Liposome MSB1 or MSB2 (μg)	-	-	4	5	6	8
pGL3 DNA (μg)	-	-	1	1	1	1

Table 3.6 : Streptavidin(bio^3-transferrin):liposome:DNA complexes with liposomes MSB1 and MSB2. Incubation mixtures (20 μl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained streptavidin (bio^3-transferrin) (10 μg), biotinylated liposome (0–8 μg) and pGL3 DNA (1 μg).

LIPOPLEX COMPONENTS	1	2	3	4	5	6
pGL3 DNA (μg)	-	-	1	1	1	1
Liposome MS09 (μg)	-	-	4	5	6	8
Streptavidin(bio^3-transferrin) (μg)	-	-	10	10	10	10

3.3.8.4 Transfection studies with untargeted biotinylated liposomes
    (a)   Liposome MSB1: pGL3 DNA complexes
    (b)   Liposome MSB2 : pGL3 DNA complexes

Transfection by lipoplexes (a) and (b) were conducted on the HeLa cell line in quadruplicate in the absence of FCS and in the presence of 10% FCS. A competition assay using iron loaded transferrin was also conducted. HeLa cells were trypsinised and seeded into a 24 well plate at a seeding density of 2.8×10^4 cells/well for both liposome MSB1 and liposome MSB2 for studies conducted in the absence of FCS. For studies conducted in the presence of 10% FCS the seeding densities were 2.7×10^4 cells/well for both liposome MSB1 and liposome MSB2. For the competition assay the seeding densities were 2.5×10^4 cells/well for both liposome MSB1 and liposome MSB2. The cells were allowed time to attach to the wells and to grow to semi-confluency. The transfection complexes for (a) and (b) were set up as described in Table 3.7.

Table 3.7 : MSB1 or MSB2 transfection complexes as added to 24-well plates. Control 1 contained only cells, control 2 contained cells and pGL3 DNA (1 μg) only. Test samples 1 to 4 contained varying amounts of liposome MSB1 or MSB2 (4, 5, 6 and 8 μg respectively). pGL3 DNA was kept constant at 1 μg. All complexes were made up to a final volume of 10 μl with 20 mM HEPES, 150 mM sodium chloride (pH 7.5).

Reaction Components	Control 1	Control 2	Test 1	Test 2	Test 3	Test 4
HeLa Cells	+	+	+	+	+	+
Liposome MSB1 or MSB2 (μg)	-	-	4	5	6	8
pGL3 DNA (μg)	-	1	1	1	1	1

The liposome:DNA complexes were incubated at room temperature for 30 minutes prior to addition to cells. The cells were prepared by removal of growth medium, washing with 0.5 ml PBS and replenishment with 0.5 ml serum free medium (MEM + antibiotics). For the evaluation of transfection efficiency in the presence of 10% serum the medium utilized was complete medium (MEM + antibiotics + 10% FBS).

A competition assay utilizing 200 µg free iron loaded transferrin per well was also conducted. The iron loaded transferrin was added to the cells 10 minutes prior to the addition of the untargeted biotinylated lipoplexes. After the addition of the transfection complexes (lipoplexes) to the cells, the cells were incubated at 37°C for 4 hours, after which the medium was removed and replaced with complete growth medium (MEM + antibiotics + 10% FCS). The cells were incubated for a further 48 hours at 37°C after which they were assayed for luciferase activity using the luciferase assay system (Promega) as described in chapter two section 2.2.7.5. The protein determination of the cell free extracts was performed using the BCA assay (Sigma) with BSA as the standard protein.

3.3.8.5 Transfection Studies with Streptavidin(bio³-transferrin) Targeted Biotinylated Liposomes :

(a) streptavidin(bio³-transferrin):liposome MSB1:pGL3 DNA complexes

(b) streptavidin(bio³-transferrin):liposome MSB2:pGL3 DNA complexes

Transfection by ternary complexes (a) and (b) were conducted on the HeLa cell line in quadruplicate in the absence of FCS and in the presence of 10% FCS. A competition assay using excess iron loaded transferrin over streptavidin(bio³-transferrin) was also conducted. HeLa cells were trypsinised and seeded into a 24 well plate at a seeding density of 2.6×10^4 cells/well for both liposomes MSB1 and liposomes MSB2 for studies conducted in the absence of FCS. For studies conducted in the presence of 10% FCS the seeding density was 2.7×10^4 cells/well for both liposome MSB1 and liposome MSB2. For the competition assay the seeding density was 2.9×10^4 cells/well for both liposome MSB1 and liposome MSB2. The cells were allowed time to attach to the wells and to grow to semi-confluency. The transfection complexes for (a) and (b) were set up as described in Tables 3.8.

Table 3.8 : MSB1 or MSB2 ternary transfection complexes as added to 24-well plates. Control 1 contained only cells, control 2 contained cells and pGL3 DNA (1 µg) only, and control 3 contained the optimal liposome:DNA (6:1 $^w/_w$) ratio for untargeted transfection. Test samples 1 to 3 contained varying amounts of liposome MSB1 or MSB2 (5, 6 and 8 µg respectively). pGL3 DNA was kept constant at 1 µg. All complexes were made up in a final volume of 20 µl with 20 mM HEPES , 150 mM sodium chloride (pH 7.5).

Reaction Components	Control 1	Control 2	Control 3	Test 1	Test 2	Test 3
HeLa Cells	+	+	+	+	+	+
Liposome MSB1 or MSB2 (µg)	-	-	6	5	6	8
pGL3 DNA (µg)	-	1	1	1	1	1
streptavidin(bio^3-transferrin) (µg)	-	-	-	10	10	10

The streptavidin(bio^3-transferrin):liposome:DNA complexes were incubated at room temperature for 30 minutes prior to addition to cells. The cells were prepared as described in section 3.2.8.4. For the evaluation of transfection efficiency in the presence of 10% serum the medium utilized was complete medium (MEM + antibiotics + 10% FCS).

For the competition assay 200 µg free iron-loaded transferrin was utilized per well. The iron loaded transferrin was added to the cells 10 minutes prior to the addition of the targeted biotinylated complexes [streptavidin(bio^3-transferrin):liposome:DNA]. After the addition of the ternary transfection complexes to the wells, the cells were then incubated at 37°C for 4 hours. The medium was then removed and replaced with complete growth medium (MEM + antibiotics + 10% FCS). The cells were incubated for a further 48 hours at 37°C and thereafter were assayed for luciferase activity using the luciferase assay system (Promega) as described in chapter two, section 2.2.7.5. The soluble protein in the cell free extracts was determined using the BCA assay (Sigma) with BSA as the standard protein.

3.4   RESULTS AND DISCUSSION

3.4.1   CHEMICAL SYNTHESES AND PROTEIN MODIFICATIONS

3.4.1.1   Preparation of Biotinylated Cholesterol Derivatives

Two novel biotinylated cholesterol derivatives were successfully synthesized (Figures 3.1 and 3.2), by the condensation of N-hydroxysuccinimide activated biotinyl components and cholesterylformylhydrazide. Both derivatives have a common cholesterol anchor, but differ in the lengths of their spacer arms, with MSB2 having the longer spacer arm. Both compounds were used in the formulation of two novel biotinylated liposomes together with MS09 (chapter two) and DOPE, which were assessed for their transfection efficiency in the HeLa cell line with and without Tf as the targeting moiety. The transfection efficiencies of these two liposome formulations were not significantly different. They will be discussed later in this chapter.

3.4.1.2   Preparation of iron (Fe^{3+}) loaded transferrin

It is accepted that transferrin binds strongly to the Tf receptor only if it is iron (Fe^{3+}) loaded. For this reason the Tf (apotransferrin) was Fe^{3+} loaded and utilized in all studies conducted with Tf. Tf binds Fe^{3+} avidly with a dissociation constant of approximately 10^{22} M^{-1}. The Fe^{3+} iron complexes to the Tf only in the presence of an anion which is usually a carbonate, which serves as a bridging ligand between the Fe^{3+} and the Tf, with exclusion of water molecules from the two coordination sites (Aisen and Listowsky, 1980). Hence sodium carbonate was used during the preparation .

3.4.1.3   Preparation of Streptavidin(bio^3-transferrin)

The streptavidin biotransferrin complex has three aminohexanoylbiotin moieties attached to Tf. The biotin content of Tf is generally kept low to prevent any cross-linking of complexes and precipitation under normal buffer incubation conditions (Hawtrey and Ariatti, 1999), and to preserve the structural form with as little alteration to the protein as possible (Schoeman *et al.*, 1995). This TfR targeted complex was linked via the streptavidin component to the biotinylated liposome (Figure 3.5).

3.4.2   PREPARATION AND CHARACTERISATION OF MSB1 and MSB2 CONTAINING LIPOSOMES

The two biotinylated liposomes were successfully formulated using the method described (section 2.2.5.2). This was confirmed using transmission electron microscopy. Liposomes were easily prepared using the cationic cholesterol derivative , MSO9, the respective biotinylated cholesterol derivative (MSB1 or MSB2) and the neutral helper lipid, DOPE at the chosen ration of components. Liposomal suspensions were stable at 4°C over several weeks and components did not precipitate out of solution. The ratio utilized for the formulation therefore was a favourable combination and other ratios were not explored. Hence the liposomes formulated contained a cationic component for interaction with the plasmid DNA molecule, a biotinylated cholesterol derivative, intended for interaction with streptavidin in the streptavidinbiotransferrin complex, and a helper lipid, DOPE, that assists in the fusogenic processes and plays a role in increasing transfection efficiency.

3.4.2.1 Characterisation of Liposomes by Transmission Electron Microscopy

Transmission electron microscopy revealed the unilamellar nature and the sizes of the two biotinylated liposome formulations. Liposome MSB1 presented vesicles in the 50 nm to 200 nm size range, while MSB2 liposomes were from 100 nm to 300 nm in diameter (Figures 3.3 and 3.4). It is proposed that the liposome diameters or hydrodynamic radii and their population dispersity bear a direct relationship to liposome clearance rates in blood and can exert marked effects on the antitumour activity of therapeutic agents (Campbell *et al.*, 2001).

The size of liposomes and the rigidity of the liposomal membrane are also important factors for liposome removal by the reticuloendothelial system (RES). Small liposomes with less fluid membranes have longer half lives in the blood stream and are not easily opsonised by complement factors in the blood when compared to larger liposomes. Liposome sizes of 100-200 nm are also considered to be favourable for tumour targeting (Oku *et al.*, 2000).
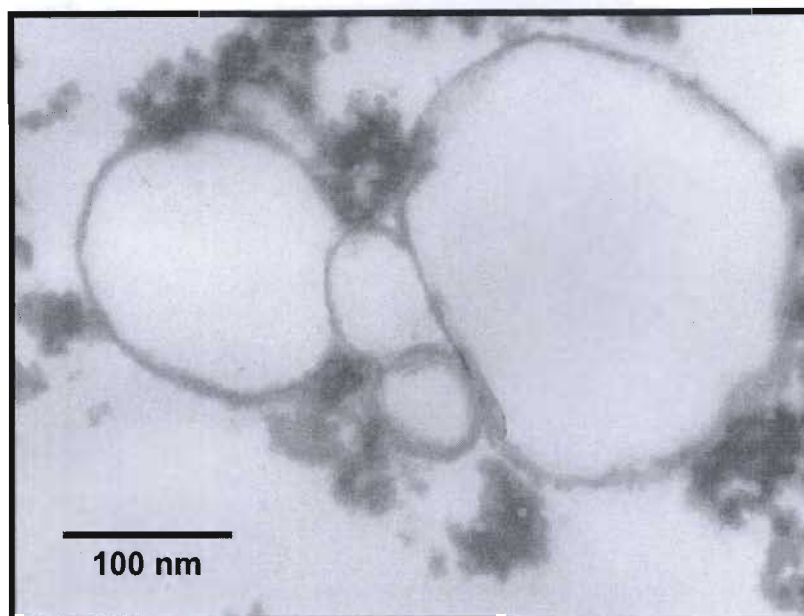
Figure 3.3: Representative TEM of liposome MSB1.
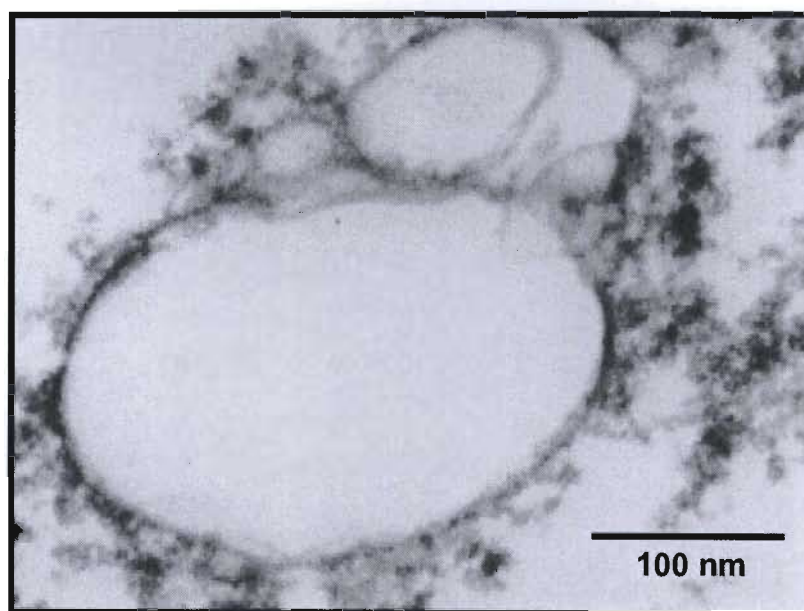Liposomes ranged in size from 50 nm to 200 nm.

Figure 3.4: Representative TEM of liposome MSB2.
Liposomes ranged in size from 100 nm to 300 nm.

3.4.3 LIPOPLEX AND TERNARY COMPLEX FORMATION OF MSB1 AND MSB2 LIPOSOMES WITH DNA, AND WITH TRANSFERRIN / STREPTAVIDIN (BIO^3-TRANSFERRIN)

Lipoplexes (liposome:DNA complexes) were generated by incubating liposomes and DNA at room temperature for 30 minutes although it is commonly held that 20 minutes under these conditions should suffice. The ternary complexes (streptavidinbiotransferrin:liposome:DNA complexes) were prepared by first adding the streptavidinbiotransferrin to the liposome followed by the DNA, allowing 15 minutes incubation after the addition of each component, thus providing sufficient time for the ternary complexes to form. All the complexes were monitored and characterized by gel retardation assays, dye displacement assays, nuclease digestion assays and transmission electron microscopy. The streptavidin-biotin interaction was therefore used to affix the transferrin moieties to the biotinylated liposomes (Figure 3.5).

It was reported previously, using ferritin molecules conjugated via streptavidin to biotinylated liposomes, that specific binding to the liposomes incorporating biotinylated lipids can be impaired by unfavourable electrostatic repulsion. The charge of the liposomes was adjusted by the addition of a cationic surfactant in the lipid bilayer (Velev, 1997). The liposomes produced in this study also contain a cationic cholesterol derivative (MS09) which produced very effective lipoplexes (chapter two). Hence the ternary complex formed brings together favourable interactions between the three components. The liposomes become coated with the Tf ligands for specific interaction with the cognate cell surface receptors thereby transporting the plasmid DNA that is bound to the cationic component of the liposome.
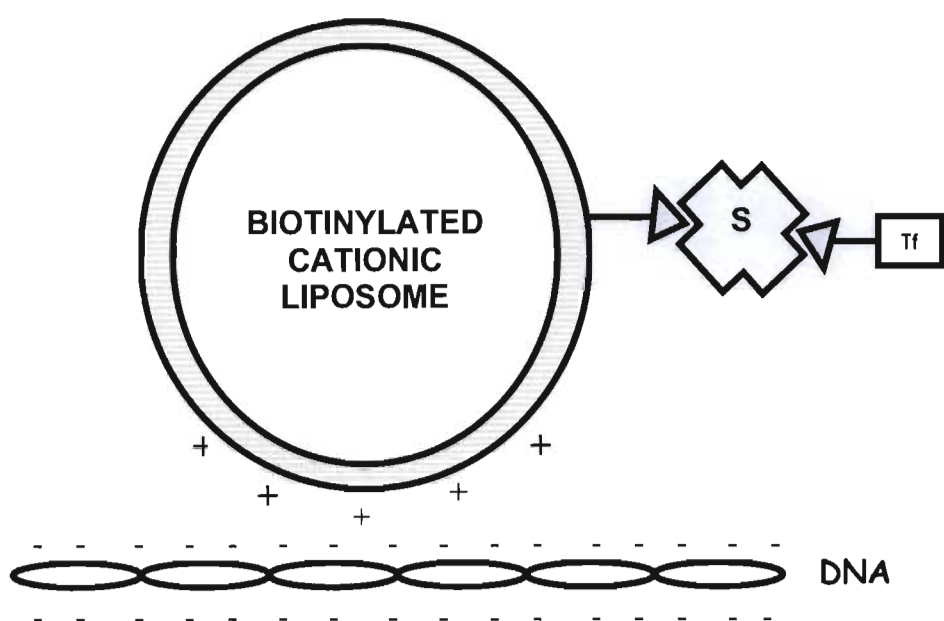
Figure 3.5 : Schematic representation of the ternary complex formed between streptavidinbiotransferrin, biotinylated liposome and DNA. Compound MS09 is the liposome cationic component for binding to plasmid DNA.

3.4.3.1 Gel Retardation Studies of Biotinylated Liposome:DNA Complexes

Agarose gel electrophoresis was once again utilized to demonstrate the formation of complexes between DNA and biotinylated liposomes.

Gel retardation studies were conducted on both lipoplexes (MSB1 and MSB2), each yielding a similar pattern of binding with a specific optimum binding ratio (Figures 3.6 and 3.7). From the results of the agarose gel electrophoresis, it can be seen that the plasmid DNA (pGL3) produced the expected two bands (lane 1), viz. the supercoiled form of DNA (bottom band) and the nicked circular form (top band). As the amount of cationic liposome increased, more DNA was electrostatically bound to the cationic liposome and hence less DNA entered the gel as was visualised by the ethidium bromide intercalation. DNA that was bound to the liposomes and does not migrate into the gel can also be seen as an intense fluorescence in the wells. Complete retardation of the DNA in the case of each liposome was obtained at the same biotinylated liposome:DNA binding ratios viz. 6:1(^{w}/$_{w}$). This complete retardation normally indicates that the negative charges of the DNA have been completely titrated by the cationic liposome's positive charges.

These ratios were then used in determining the optimal ratios for the formation of ternary complexes with streptavidinbiotransferrin and to ultimately identify the optimum ratios for transfection studies. By retaining these liposome:DNA ratios in ternary complexes, it was expected that ionic interaction between streptavidinbiotransferrin and the liposome component would be minimized thus favouring the biotin-streptavidin interaction as the docking force.

Figure 3.6 :   Gel retardation study of MSB1liposome:DNA complexes. Incubation mixtures (10 μl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained varying amounts of liposome in lanes 1-8 (0, 1.0, 2.0, 3.0, 4.0,5.0, 6.0, 7.0 μg), while the pGL3 DNA was kept constant at 0.5 μg per well.

Figure 3.7 : Gel retardation study of MSB2 liposome : DNA complexes. Incubation mixtures (10 µl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained varying amounts of cationic liposome in lanes 1-8 (0, 0.5,1.0, 2.0, 3.0, 4.0, 5.0, 6.0 µg), while the pGL3 DNA was kept constant at 0.5 µg per well.

3.4.3.2 Gel Retardation Studies of Transferrin:DNA Complexes

It is clear, from the results presented in Figure 3.8, that there is little if any binding of Tf to DNA (1 µg) over a wide protein range (0-100 µg) at pH 7.5. Hence ternary complexes, that are formed in this study, are held together largely by the biotin-streptavidin interaction (biotinylated liposomes and streptavidinbiotransferrin), and the ionic forces (liposome and DNA). It has been suggested, using DOTAP/DOPE liposomes, that Tf binds to these vesicles via negatively charged groups on this ligand, at physiological pH, to afford complexes which bind DNA through a charge-charge interaction (Tros de Illarduya and Düzgüneş, 2000).

In arriving at this conclusion it is assumed that any binding of Tf to DNA would produce larger complexes leading to a DNA band shift on agarose gel electrophoresis. However, the migration of the DNA exposed to large amounts of Tf was very similar to that of control DNA (Figure 3.8). This may be attributed, in part, to the fact that Tf molecules bear four negatively charged sialic acid residues (Nagaoka and Maitani, 2001), and hence is an acidic protein, with the diferric species having an isoelectric point (pI) in the range 5.6 - 5.8 (Li and Qian, 2002). It has previously been reported that Tf fails to interact with DNA as determined in a band shift assay (Huckett *et al.*, 1986). However, Joshee *et al.*, 2002, have suggested that Tf can interact with DNA to give a relatively stable complex. Based on evidence provided by electron microscopy, they have also proposed that a complex comprising DNA (1 µg) and Tf (20 µg) adopts an unusual toroidal ring structure (diameter : 160 nm) and a tail. Furthermore, the thickness of the DNA component was significantly greater than that of uncomplexed DNA.
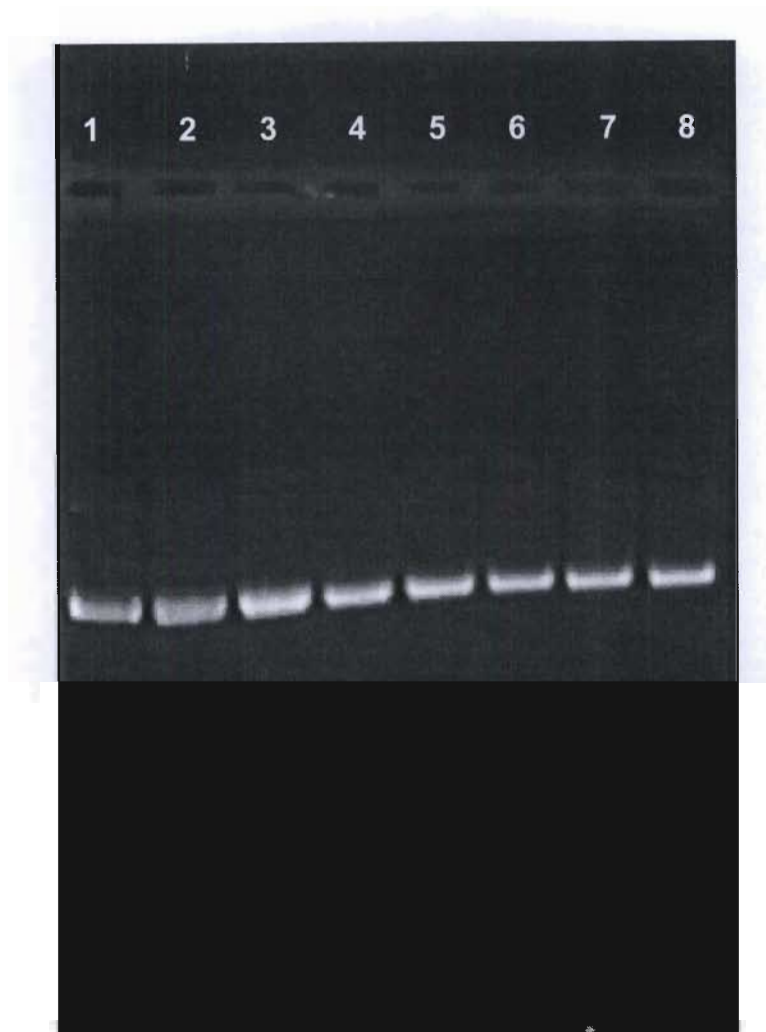
Figure 3.8 : Gel retardation study of transferrin:DNA complexes. Incubation mixtures (15 μl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained varying amounts of transferrin in lanes 1-8 (0, 10, 20, 40, 60, 70, 80, 100 μg), while the pGL3 DNA was kept constant at 1.0 μg.

3.4.3.3 Gel Retardation Studies of Streptavidin Biotransferrin: Liposome:DNA Complex for MSB1 and MSB2 Liposomes

Agarose gel electrophoresis was once again used to detect retardation patterns, this time with the streptavidinbiotransferrin:liposome:DNA complexes. At low biotransferrin:liposome ratios the biotransferrin may bind to the liposomes by charge-charge interactions which precludes binding of the DNA (lane 2, Figure 3.9; lanes 2 and 3, Figure 3.10). However, as the biotransferrin concentration increases it appears that the biotin-streptavidin interaction becomes the dominant cohesive force and DNA is able to successfully compete for binding to the cationic bilayer. It is clearly visible from the fluorescence observed after ethidium bromide staining that the complete retardation of the ternary complexes for both liposome formulations was achieved at a streptavidinbiotransferrin:liposome:DNA ratio of 10:6:1 (Figures 3.9 and 3.10). The determination of this ratio was critical as it established the optimal ratio of the ternary complex to be utilized for transfection purposes.

In separate binding studies the liposome amounts were varied from 0 to 8 µg while the streptavidinbiotransferrin was kept constant at 10 µg and the pGL3 DNA maintained at 1 µg. In this case the agarose gels were stained with a protein stain (Sypro Red^{TM}) and the fluorescence attributed to the protein was visualized under UV_{300} illumination. Thus it is noted in Figures 3.11 and 3.12 that the streptavidinbiotransferrin is liposome-bound and remains in or near the well. At low liposome concentration (lane 2, Figures 3.11 and 3.12), some migration of the protein conjugate towards the anode may be seen. A similar result was observed when gels were stained with Coomassie Blue to detect the protein (Figure 3.13). It may be inferred, therefore, that at least 3 or perhaps 4 µg of MSB1 or MSB2 liposomes are required to bind 10 µg of streptavidinbiotransferrin. These results serve to affirm the ratios of components that have been selected for the transfection studies.

Figure 3.9 : Gel retardation study of ternary complex of streptavidin-biotransferrin:MSB1 liposome : DNA complexes. Incubation mixtures (15 µl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained varying amounts of liposome in lanes 1-8 (0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 µg) and pGL3 DNA (1.0 µg). The streptavidinbiotransferrin was also varied (0, 6, 7, 8, 9, 10, 11, 12 µg).

Figure 3.10 : Gel retardation study of ternary complex of streptavidin-biotransferrin:MSB2 liposome : DNA complexes. Incubation mixtures (15 µl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained varying amounts of liposome in lanes 1-8 (0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 µg), and pGL3 DNA (1.0 µg). The streptavidinbiotransferrin was also varied (0, 6, 7, 8, 9, 10, 11, 12 µg).
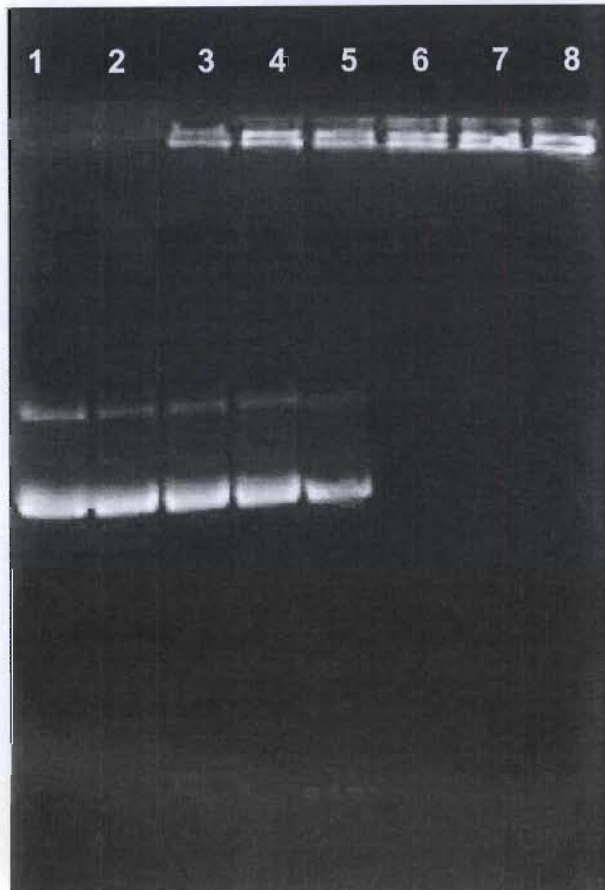
Figure 3.11 : Gel retardation study of ternary complex of streptavidin-biotransferrin:MSB1 liposome : DNA complexes. Incubation mixtures (15 μl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained varying amounts of liposome in lanes 1-8 (0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 μg), while the pGL3 DNA and streptavidinbiotransferrin was kept constant at 1.0 μg and 10 μg respectively. Gel was stained to detect protein using a Sypro Red[TM] fluorescent stain.
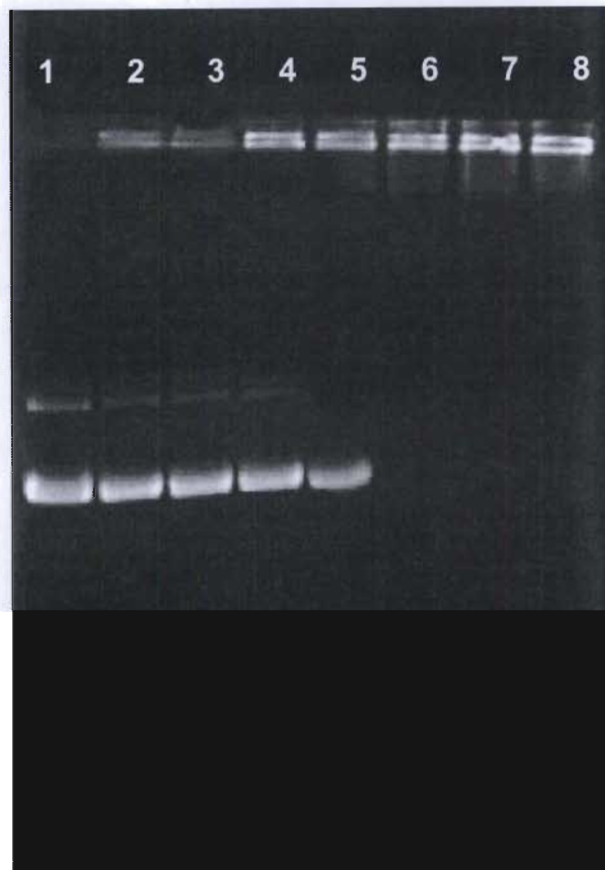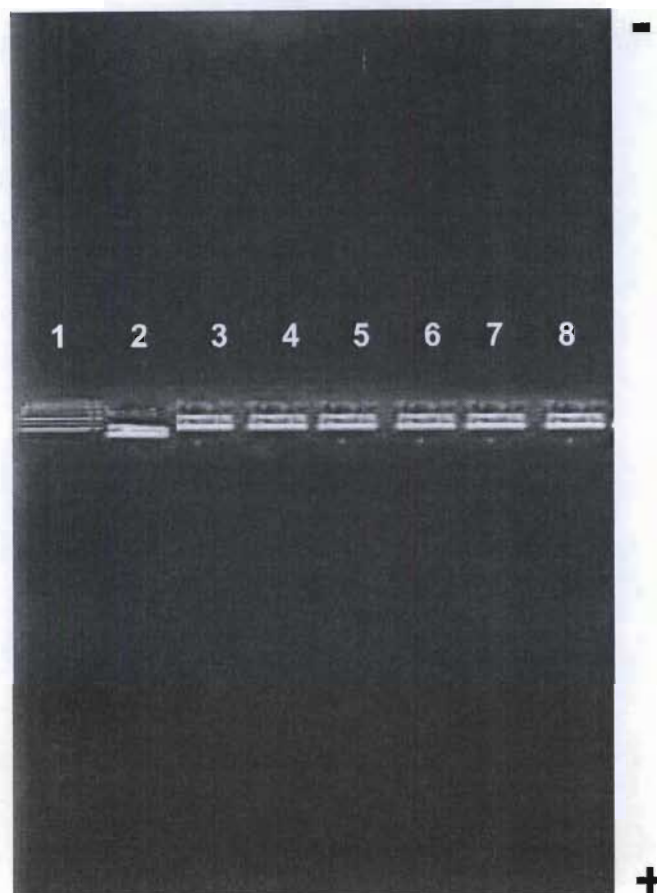
Figure 3.12 : Gel retardation study of ternary complex of streptavidin biotransferrin:MSB2 liposome : DNA complexes. Incubation mixtures (15 µl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained varying amounts of liposome in lanes 1-8 (0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 µg), while the pGL3 DNA and streptavidin biotransferrin was kept constant at 1.0 µg and 10 µg respectively. Gel was stained to detect protein using a Sypro Red^{TM} fluorescent stain.
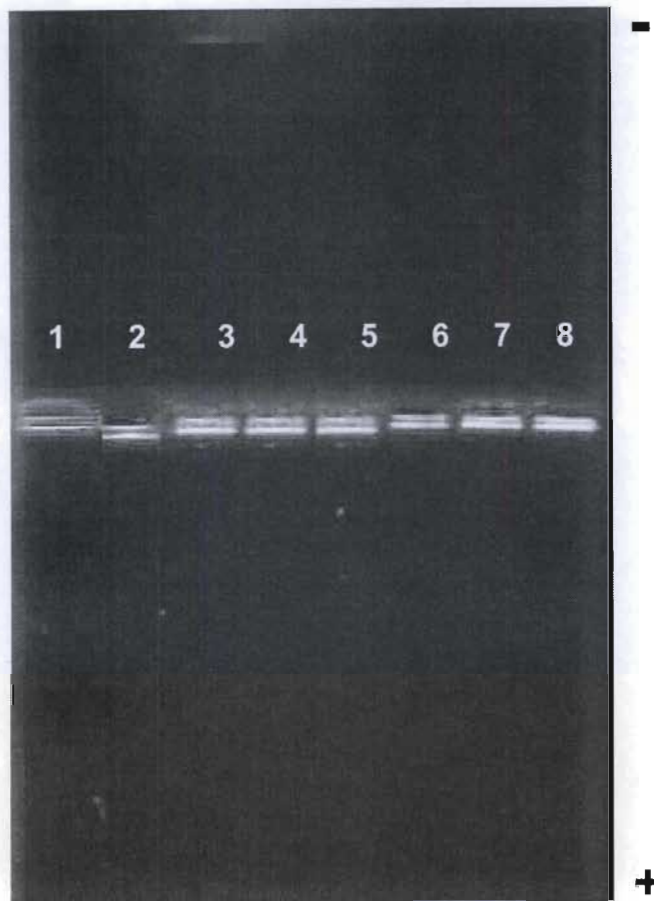
Figure 3.13 : Gel retardation study of ternary complex of streptavidinbiotransferrin: MSB1 liposome:DNA complexes. Incubation mixtures (15 μl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained varying amounts of liposome in lanes 1-8 (0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 μg), while the pGL3 DNA and streptavidinbiotransferrin was kept constant at 1.0 μg and 10 μg respectively. Gel was stained to detect protein with Coomassie Blue stain. MSB2 complexes produced the same result.
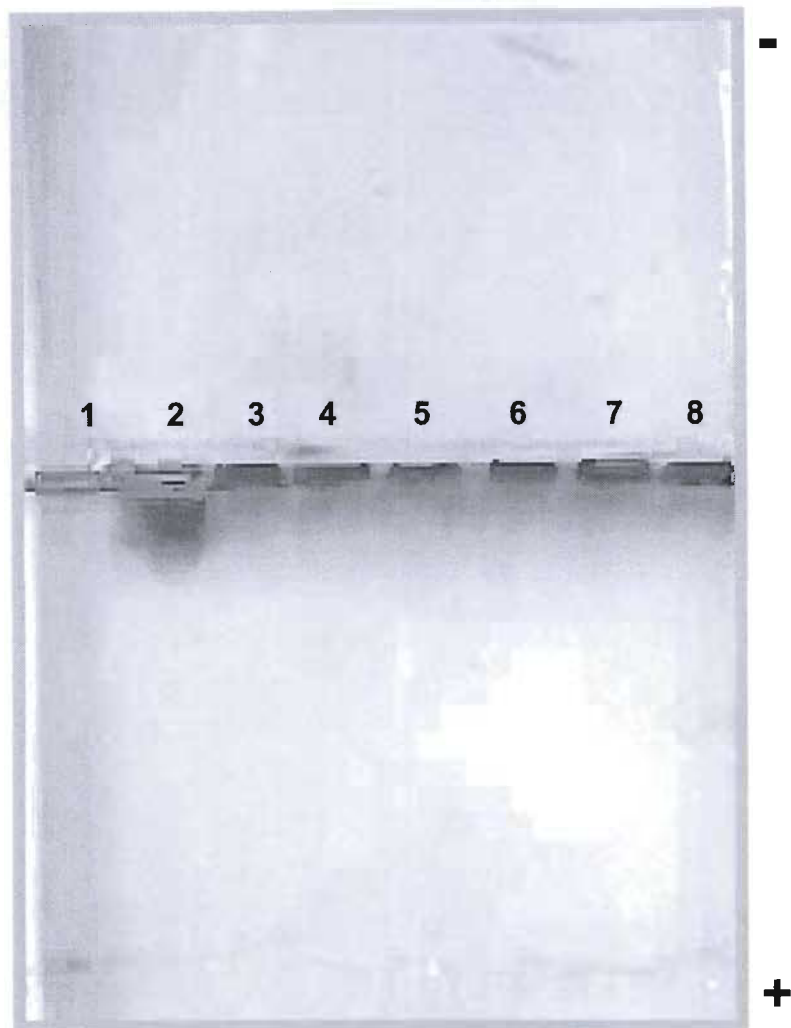
3.4.3.4   Dye Displacement Assays

In these assays the 100% fluorescence reading at 600 nm emission, was taken after the initial addition of pBR322 DNA to the ethidium bromide solution in buffer, which resulted in a higher fluorescence reading due to the intercalation of the ethidium into the DNA. The assays using the plain liposome:DNA complexes showed the general trend seen in Chapter 2, in that as the amount of liposome added to the DNA increased, the fluorescence emitted by the ethidium bromide decreased due to the binding of the cationic component of the biotinylated liposome to the plasmid DNA, hence displacing the ethidium bromide. This decrease in fluorescence was observed with both biotinylated liposome formulations (Figure 3.14). The fluorescence decreased steadily until a point was attained which seemed to correlate with total retardation seen for lipoplexes in the gel retardation assays. The maximum displacement of ethidium bromide as measured by fluorescence was obtained for liposome MSB1 and MSB2 at the total retardation ratio of liposome:DNA of 6:1($^{w}/_{w}$), and charge (+/-) ratio of 0.7. The reduction in fluorescence (45-50%) was less than that obtained for the MS09 liposomes (60%) in Chapter 2. This could be due to the added biotinylated cholesterol component that is also present in the liposomal bilayer which may be partially obstructing the DNA association with the MS09 component although MSB1 and MSB2 are present at a low percentage in the bilayer (2%).

For the addition of the liposome:Tf complex to the DNA (Figure 3.15), there was a rapid decrease in the fluorescence which seemed to stabilize at a Tf:liposome:DNA ratio of about 12:6:1 (w/w/w/) for both liposome formulations. As the Tf:liposome complex was added 3 µl at a time, the fluorescence intensity decreased due to binding of the DNA by the cationic component of the liposome. The negatively charged transferrin is expected to bind to the cationic liposomes by electroststic interactions. Results presented in Figure 3.15 confirm, however, that the plasmid DNA is able to displace the transferrin, as the dye displacement ceases at a liposome:DNA ratio of 6:1($^{w}/_{w}$), the same value achieved in the absence of transferrin. In Figure 3.16, the lipoplex (biotinylated liposome:DNA , 6:1 $^{w}/_{w}$) was first added to the ethidium bromide solution prior to

addition of the streptavidinbiotransferrin complex. There was an initial gradual increase in fluorescence upon addition of the streptavidinbiotransferrin complex. This could be due to the binding of the streptavidinbiotransferrin complex to the biotinylated liposomes which would have coated the liposomes. This may have interfered with the binding of the DNA to the cationic component of the liposome, thereby partly releasing the DNA from the liposome.

(a)   Addition of biotinylated liposomes to DNA-ethidium bromide solution

Figure 3.14 : Ethidium bromide intercalation assay for liposomes MSB1 and MSB2 at varying liposome : DNA ($^{w}/_{w}$) binding ratios (section 3.3.7.5a).

(b)   Addition of liposome:transferrin (1:2) complex to DNA

Figure 3.15 :   Ethidium bromide intercalation assay for liposomes MSB1 and MSB2 at varying Tf:liposome:DNA (w/w/w) binding ratios. Incubation mixtures (500 µl) contained increasing amounts of a liposome:transferrin (1:2 $^w/_w$) solution (1.3 µg liposome and 2.6 µg Tf / µl HEPES buffered saline), as indicated in the figure, and 6 µg pBR322 plasmid DNA.

(c) **Addition of streptavidinbiotransferrin complex to liposome:DNA complex**

Figure 3.16 : Ethidium bromide intercalation assay for liposome MSB1 and MSB2 at varying streptavidinbiotransferrin:liposome : DNA ($^{w}/_{w}$) binding ratios. Incubation mixtures (500 µl) contained liposomes (36 µg), pBR322 DNA (6 µg) and increasing amounts of streptavidin(bio[3]transferrin) as indicated in the figure.

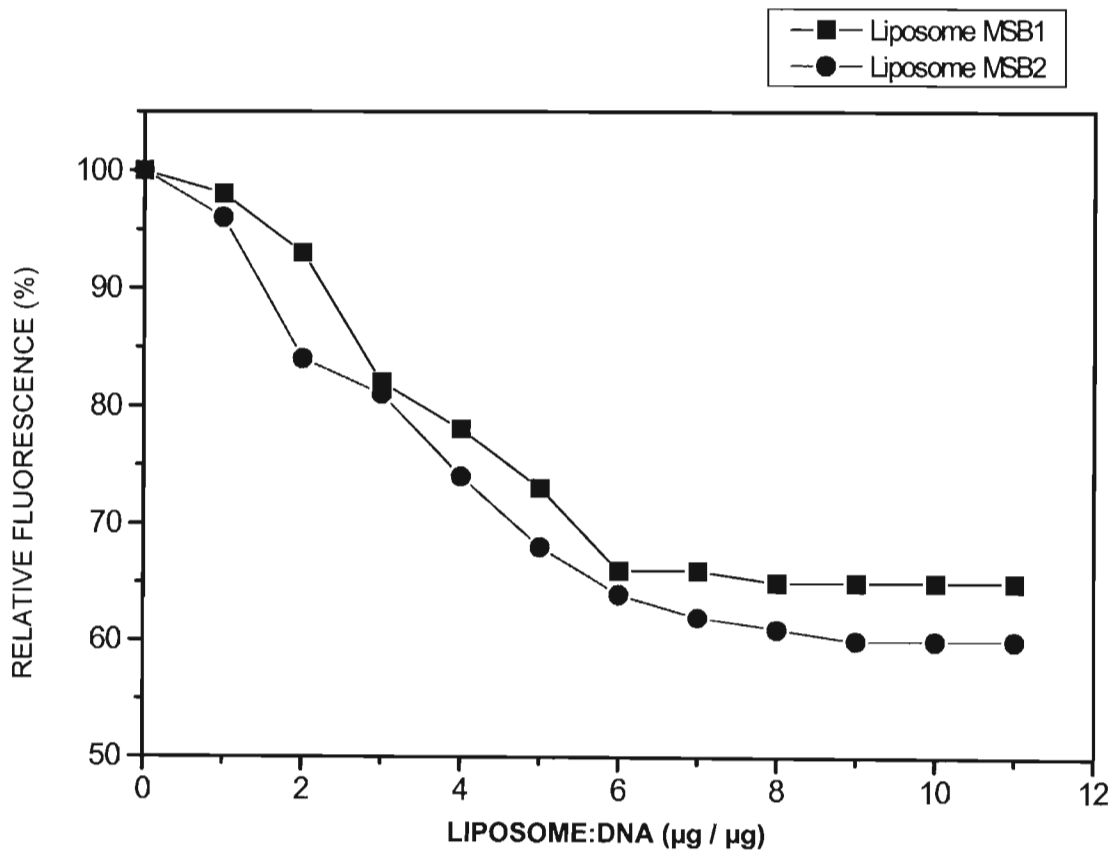**(d)** **Addition of transferrin to liposome:DNA complex**
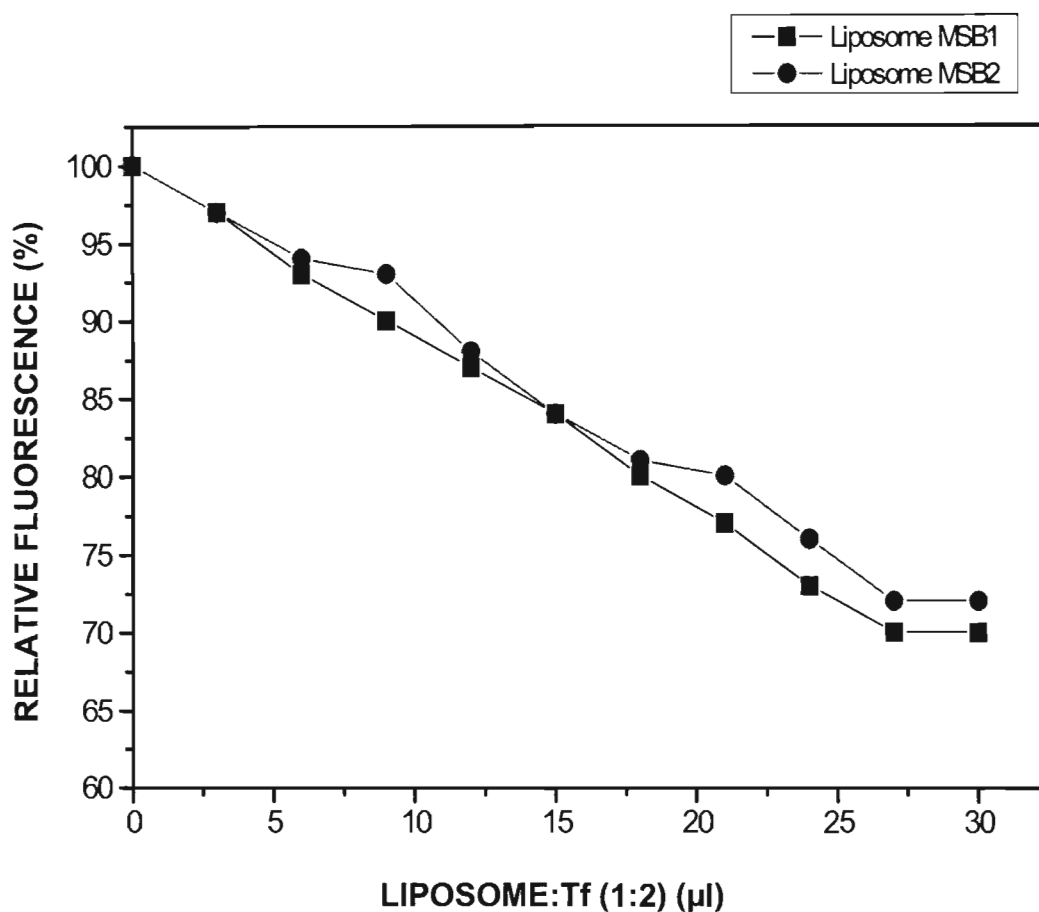

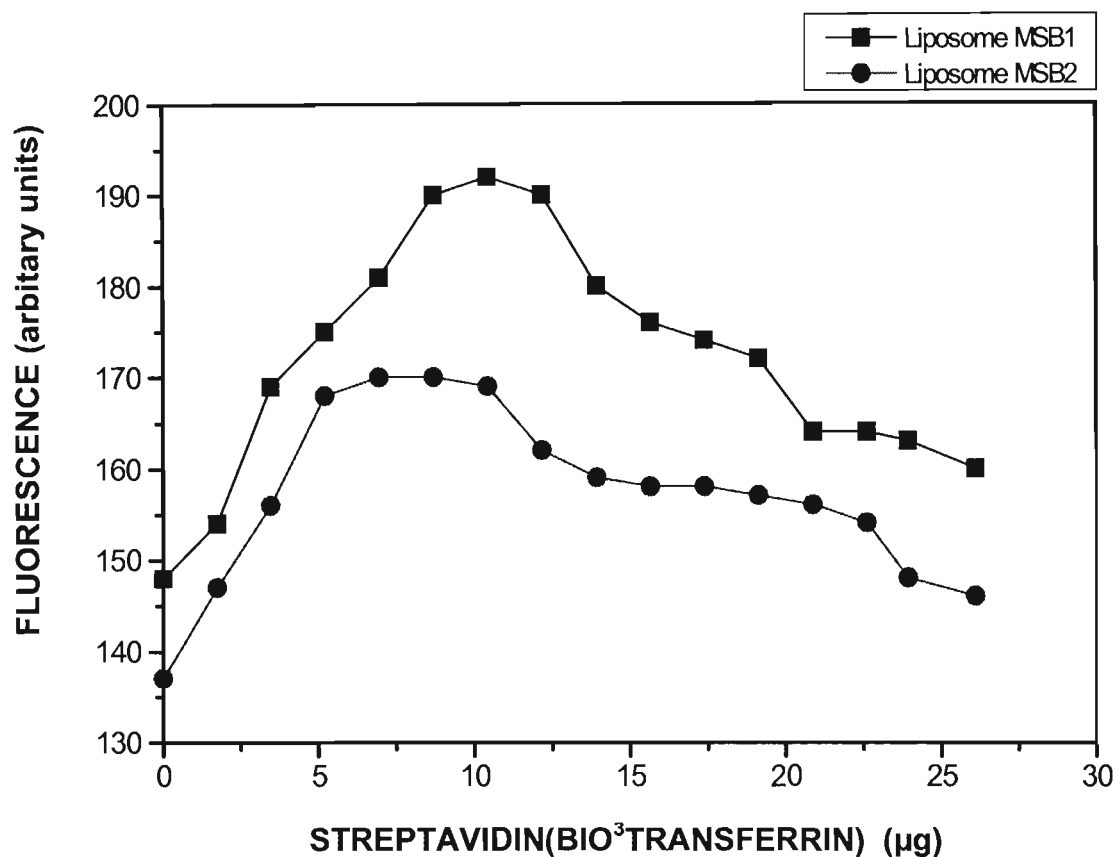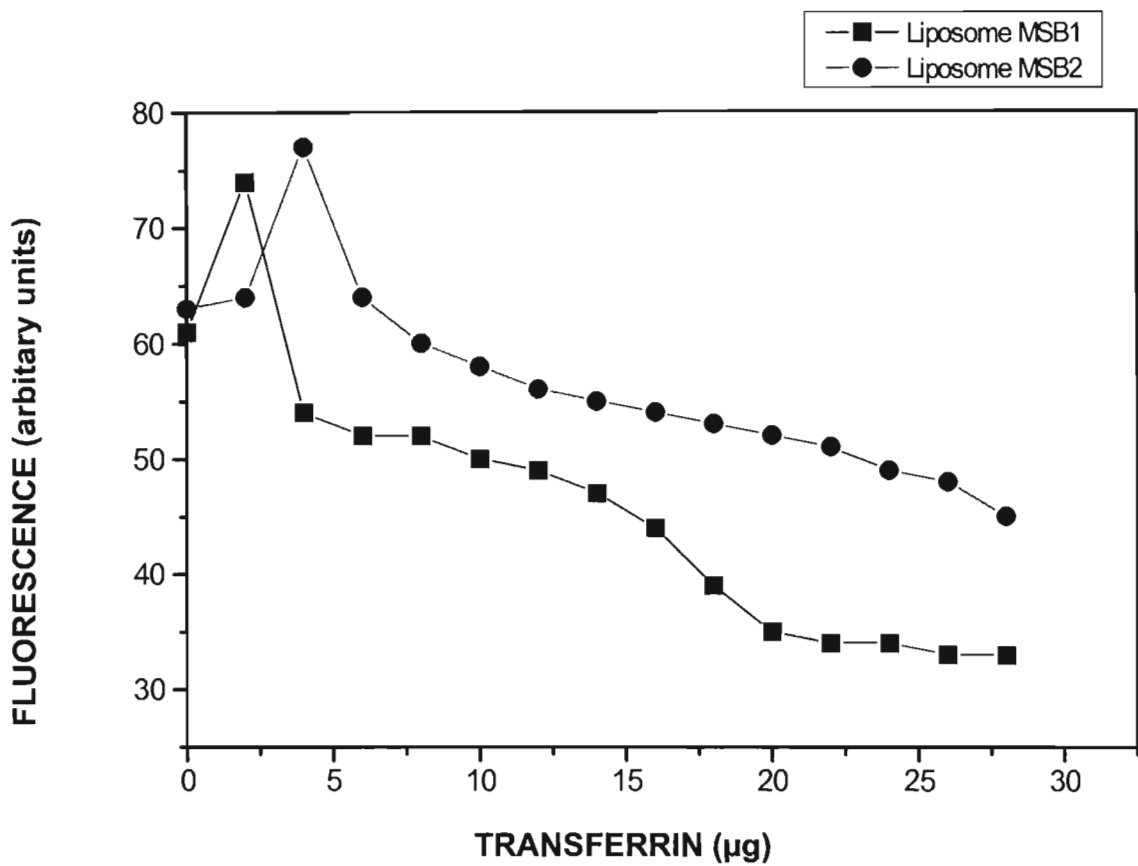**Figure 3.17 :** Ethidium Bromide intercalation assay for liposome MSB1 and MSB2 at varying transferrin:liposome:DNA ($^{w}/_{w}$) binding ratios. Incubation mixtures (500 µl) contained liposomes (36 µg) and pBR322 DNA 6 µg) in HEPES buffered saline and increasing amounts of iron loaded Tf as indicated.

The released DNA then intercalates the ethidium bromide resulting in enhanced fluorescence. The increase in fluorescence observed was more dramatic for liposome MSB1 than for MSB2, suggesting that the liposome MSB2 which has a longer spacer offered less hinderence to the DNA bound to the liposome than is the case with liposome MSB1 which has a much shorter spacer.

This increase in fluorescence was observed until about 6 µg of streptavidinbiotransferrin had been added, from which point there was little or no change in fluorescence (flattening in the curve) until about 11 µg of streptavidinbiotransferrin. This could be due to the fact that no further DNA was displaced from the liposomes. Thereafter there was a gradual quenching in the fluorescence which apparently marked the reassociation of DNA with the liposome complex. The plasmid DNA was able to bind to the cationic liposomal component which could have now become more readily accessible. It seems, therefore, plausible to suggest that the ionic interaction between the liposomes and streptavidinbiotransferrin assumes a lesser importance and that the modified transferrin remains liposome-bound largely through the biotin-streptavidin interaction. This is corroborated by the gel retardation studies which show a strong interaction of streptavidinbiotransferrin with liposomes that are also associated with a saturating amount of DNA (Figures 3.9 to 3.13).

When iron-loaded Tf (holotransferrin) was added to the liposome:DNA complex (Figure 3.17), there was an initial sudden increase followed by a sudden drop in the measured fluorescence. This could be a result of the binding of the transferrin to the liposome displacing some of the DNA in the process. The ethidium bromide then intercalates into the DNA thereby increasing fluorescence levels. This binding of Tf reaches a peak at about 4 µg after which fluorescence drops again very sharply to levels even below that of the initial reading, signalling the reattachment of the partially displaced DNA. The further drop in fluorescence is in agreement with the notion that the lipoplex contains more compacted DNA with increasing iron loaded transferrin. It is noteworthy that in this experiment liposomes and plasmid DNA were premixed at a ratio of 6:1 (^{w}/$_w$), which

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results in complete retardation of DNA in a band shift assay (Figures 3.6 and 3.7), and marks the saturation point for binding of the DNA to the liposomes in the dye displacement assay (Figure 3.14).

3.4.3.5 Transmission Electron Microscopy of liposome:DNA complexes (MSB1 and MSB2)

Lipoplexes were successfully assembled from MSB1- and MSB2-containing cationic liposomes and plasmid DNA. After mixing the lipidic and nucleic acid components, a 30 minute period at room temperature was allowed for maturation before samples were taken for TEM. The lipoplexes observed were very similar to those reported for the cationic liposomes not containing biotin appendages in chapter two. The size and ultrastrucuture of these lipoplexes were observed at their optimal transfection ratio of liposome:DNA (6:1 $^w/_w$) and charge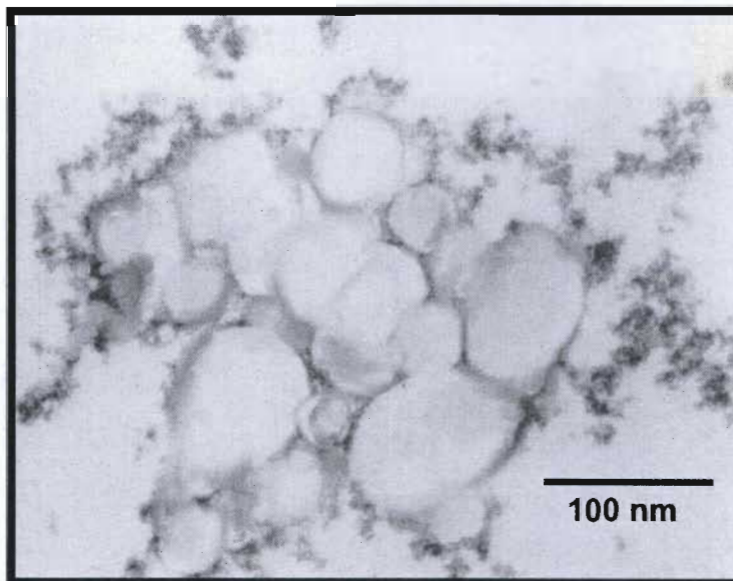 (+/-) ratio of 0.7. Lipoplex sizes ranged from 100 to 200 nm for MSB1 lipoplexes and 100 to 300 nm for MSB2 lipoplexes (Figure 3.18). Most of the lipoplexes appeared as aggregates or clusters of liposomes. Some of the lipoplexes appeared much more closely or tightly packed (especially the MSB1 lipoplexes) than others. These lipoplexes were randomly dispersed and were not encountered as groups of lipoplexes. Studies have shown that highly positively charged complexes, where DNA is completely condensed, or highly negatively charged complexes with an excess of DNA over the lipid, exhibit an homogeneous size distribution of between 100 nm and 450 nm in diameter. Neutral complexes however are characterized by a heterogeneous size distribution from about 300 nm to 1200 nm in diameter with a much lower colloidal stability (Pedroso de Lima et al., 2001).

The sizes of the lipoplexes are potentially important properties of synthetic gene delivery systems. DNA complexes can range from 50 to 1000 nm depending on DNA:carrier ratio, total concentrations, ionic strength of buffer, and kinetics of mixing, with the lower size limit not easily adjustable and hence intrinsic to that specific formulation (Schätzlein, 2003).
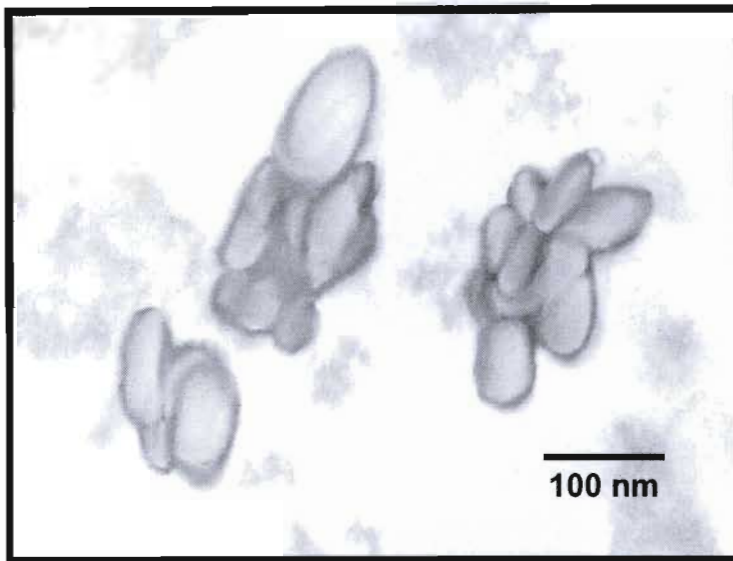
(a)

(b)

**Figure 3.18 :**   TEM of biotinylated liposome:DNA complexes at optimal ratio of 6:1 (µg / µg) and charge (+/-) ratio of 0.7. Lipoplex sizes ranged from (a) 100 - 200 nm for liposome MSB1:DNA, and, (b) 100 - 300 nm for liposome MSB2:DNA complexes

3.4.3.6 Transmission Electron Microscopy of MSB1 and MSB2 streptavidinbiotransferrin: liposome:DNA complexes

The ternary complexes formed (Figures 3.19 to 3.20) varied in size from 100 nm to 250 nm for complexes with MSB1 at a streptavidinbiotransferrin:liposome MSB1:DNA ratio of 10:6:1 (w/w/w), and 200 nm to 500 nm at a higher ratio of 10:7:1 (w/w/w). The complexes for liposome MSB2 varied in size from 150 nm to 300 nm at a ratio of 10:6:1(w/w/w) for the streptavidinbiotransferrin:liposome MSB2:DNA complexes , and from 200 nm to 500 nm at a higher ratio of 10:7:1. The ultrastructure of these complexes did not differ much from that of their corresponding lipoplexes (3.3.3.5) in that they formed clusters of varying sizes. Sizes reported for some Tf containing complexes using light scattering measurements ranged from 500 nm to 880 nm in diameter (Joshee *et al.*, 2002).

The DNA seemed to be condensed by the complex, which is important for efficient gene transfer. However there was one significant difference in these ternary complexes in that rod-like or elongated tubular forms were visible (Figure 3.19) at the higher charge ratio. These could be due to the fusion of the lipid membranes during formation of the ternary complex which completely covers the DNA, drawing the three components closer together. Such tubular or rod shaped structures have been observed previously for some lipoplexes (Gershon *et al.*, 1993; Chiruvolu *et al.*, 1994). These tubules were about 1 µm in diameter and up to 100 µm in length and most of them appeared entangled with each other (Chiruvolu *et al.*, 1994). However this was not the case for lipoplexes prepared in the present study and rods seemed to be considerably shorter and occurred singly. The sizes of tubular structures were approximately 0.25 µm in diameter and about 4 µm in length (Figure 3.19b). Rod like structures were also observed at the higher charge ratio viz.(+/-) ratio of 1.5. It was suggested that these unusual structures are formed above the critical ratio and are complexes where the DNA molecules are packed and completely encapsulated within a smooth lipid bilayer (Gershon *et al.*, 1993). The liposome:DNA charge ratio at which tubular formations were observed in the present study was 0.8 (+/-).
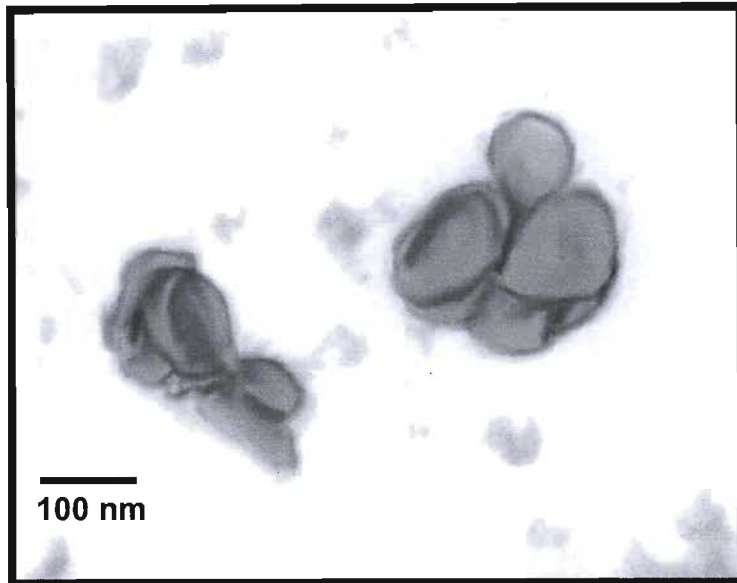
(a)
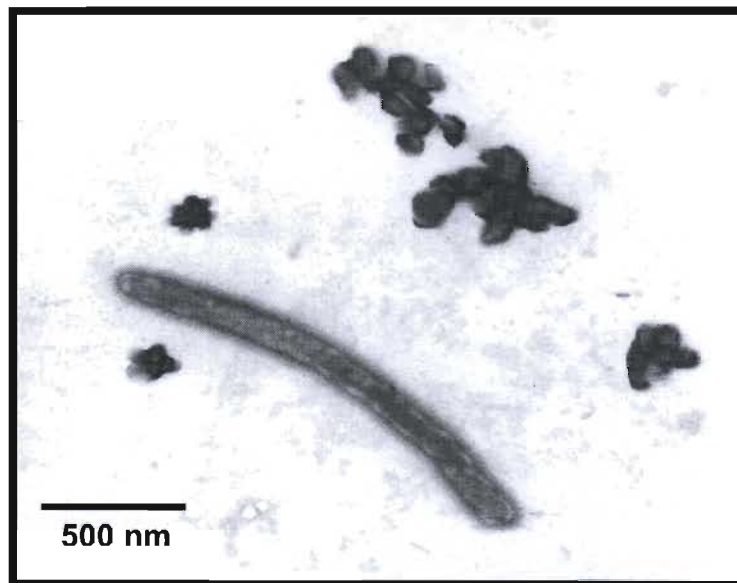
(b)

Figure 3.19 : TEM of streptavidinbiotransferrin: liposomeMSB1:DNA complexes at
(a) optimal transfection ratio of 10:6:1, and,
(b) at a higher ratio of 10:7:1.

(a)

(b)

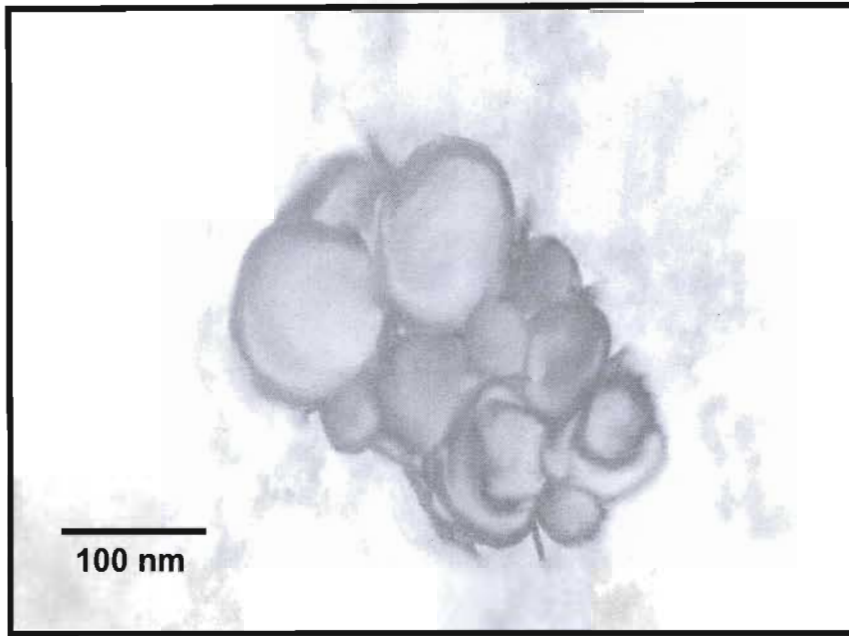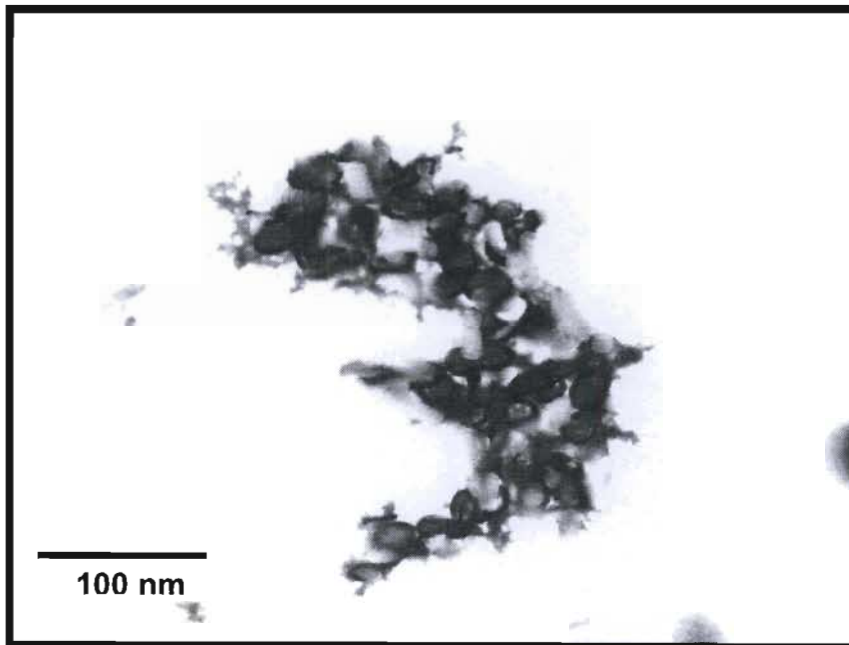**Figure 3.20 :** TEM of streptavidinbiotransferrin: liposomeMSB2:DNA complexes at
(a) optimal transfection ratio of 10:6:1, and,
(b) at a higher ratio of 10:7:1.

Furthermore some free liposomes were noted, unlike the preparations of plain lipoplexes, where it seemed that all the liposomes were complex-associated. Wagner *et al.*, 1991, have also shown that Tf-complexes produced toroidal-like structures.

It was found by some investigators that the inclusion of Tf in the formulation to produce a ternary complex, increased the size of the complex especially if the DNA is added last. If the Tf or the liposome was added last, the size of the complex was unchanged (Joshee *et al.*, 2002). Lipoplexes reported herein were prepared by the addition of the Tf component first followed by the DNA, and no prolonged maturation of the complexes (i.e > 30 minutes) was carried out. Transferrin (80 kDalton) itself, is approximately 2 orders of magnitude smaller than the average lipoplex size of 200 nm. These 200 nm complexes can be accommodated in clathrin-coated vesicles since it was observed under electron microscopy that coated vesicle sizes from 100-200 nm exist. Hence, it has been suggested that clathrin–mediated endocytosis of particles exceeding diameters of 200 nm is possible and that the process may involve a considerable degree of size flexibility of the coated vesicles (Zuhorn *et al.*, 2002). The attachment of Tf to liposomes tends to increase the hydrodynamic radius of the Tf:liposome:DNA complex (Joo and Kim, 2002). Ideally, for successful endocytosis, the size of the ligand must not exceed the internal diameter of the coated pit. Vance and Vance (1991) have reported that the coated pit diameter lies within the 100-500 nm range.

It has been suggested that ternary complexes of 400-500 nm in diameter are appropriate for transfection (Tokunaga *et al.*, 2004). The average size of the most effective ternary complex *in vitro* utilizing Lipofectin[TM] (Tf:liposome:DNA), as determined by light scattering was between 500-880 nm, and complex sizes ranged from 400-1400 nm (Joshee *et al.*, 2002). These complexes do not completely resemble the condensed lipoplexes seen by Gershon *et al.*, 1993. Hence the results obtained by Joshee *et al.*, 2002, suggest that uncondensed DNA can also yield high transfection efficiency in liposome-based gene transfer systems. This is unlike the molecular conjugate based vectors where small vector sizes (up to 17 nm) produced high transfection rates (Perales *et al.*, 1994).

3.4.3.7 Nuclease Digestion Assays of Ternary Complexes

These studies have shown that the ternary complexes effectively protected the plasmid DNA from attack by the serum nucleases. This could be due to the stability of these complexes or to their compaction by condensation. The ternary complexes as seen under electron microscopy are highly condensed supramolecular structures. This reinforces the notion that the DNA in these complexes is protected from nuclease degradation.

The naked DNA (lane2, Figures 3.21a and b) is uncomplexed (absence of liposomes and streptavidinbiotransferrin), and is completely degraded by the presence of serum nucleases whereas the DNA bound in the ternary complexes is protected and undegraded, at all streptavidin(biotransferrin):liposome:DNA ratios. The actual extent to which the DNA is protected by the liposomes will depend on the type of liposome utilized and the type of complex formed. If the ternary complex association is not a strong or tight one, the nuclease enzymes can easily access and degrade the DNA or alternatively destabilize the complex. It was observed by some investigators that ternary complexes prepared using streptavidin complexes seem to stabilize and protect the DNA from digestion by DNase enzymes (Xu et al., 1998). This could also account for the protection of the DNA revealed by agarose gel electrophoresis in the present study (Figures 3.21)

It was seen using DNA/Tf/PEI complexes that aggregates of the complexes were produced upon addition of serum, due to binding of serum proteins to the complexes (Ogris et al., 1999). However with our ternary complexes no aggregation or precipitation was observed upon addition of serum and there was no noticeable change in the turbidity of the liposomal solution. Hence it can be concluded that these ternary liposome complexes could be suitable vehicles for the safe transport of DNA into cells in vitro and could perhaps be useful in vivo. This observation seems to correlate with results obtained for transfection studies of the ternary complexes in the presence of 10% fetal bovine serum.

Liposome clearance *in vivo*, however, will depend upon the amount of serum proteins or opsonins (complements, immunoglobulins and fibronectins) that are bound to the liposomes (Oku *et al.*, 2000).

(a)   **(b)**
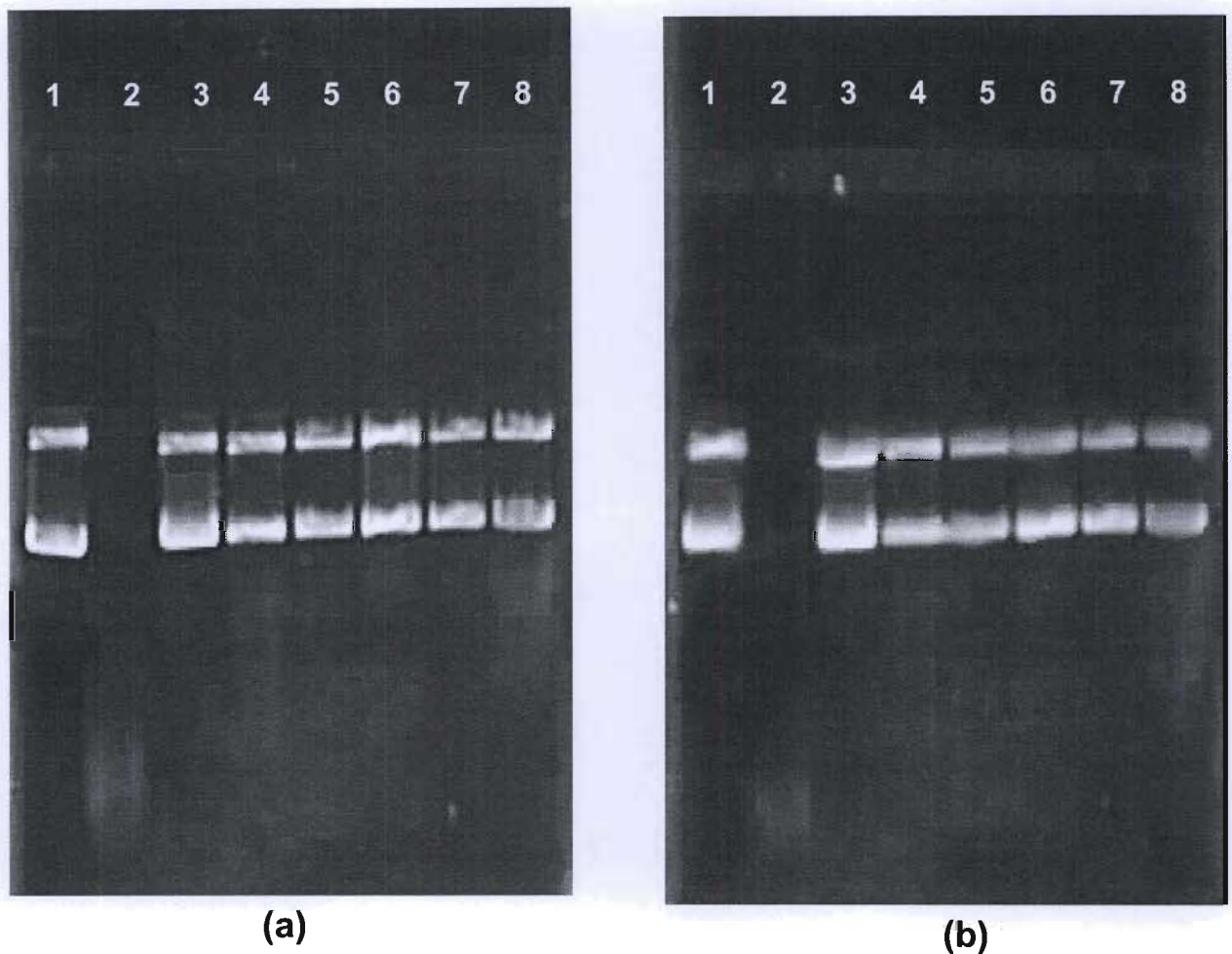
Figure 3.21 : Nuclease protection assay of ternary assemblies containing
(a) MSB1, pBR322 DNA and streptavidinbiotransferrin, and,
(b) MSB2, pBR322 DNA and streptavidinbiotransferrin.
Lane 1 : untreated marker plasmid pBR322 DNA (1 μg)
Lane 2 : unprotected plasmid DNA (1 μg) in the presence of 10% FCS
Lanes 3-8 : Varying amounts of cationic liposomes (2, 3, 4, 5, 6, 7 μg) with pBR322 DNA (1 μg) and streptavidinbiotransferrin (10 μg) and 10% FCS.

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3.4.4   CELL CULTURE AND TRANSFECTION STUDIES

3.4.4.1   Growth and Maintenance of HeLa cells

The HeLa cells (Figure 2.28) were successfully propagated as outlined in chapter two.

3.4.4.2   Growth Inhibition Assays

These studies were conducted on the untargeted biotinylated liposome:DNA complexes and on the ternary complexes for both liposome preparations (MSB1 and MSB2). As can be seen in Figures 3.22 and 3.23 there was a small but measurable difference in the growth inhibition levels for the plain lipoplexes and the ternary targeted complexes. The ternary complexes (streptavidinbiotransferrin:liposome:DNA) showed less cytotoxicity than the plain lipoplexes even at higher liposome ratios. This could be attributed to the presence of the transferrin component which is the only difference between the two types of complexes. It bears mentioning also that there were marked ultrastructural differences (TEM) between the targeted and untargeted assemblies (Figures 3.18 to 3.20).

Maximum cell death recorded was 32% for liposome MSB1:DNA (Figure 3.23) at a liposome:DNA ratio of 8:1, (+/-) charge ratio of 1.0, and 31% for liposome MSB2:DNA (Figure 3.22) at the same ratio.   The best survival rates observed were at the liposome:DNA ratio of 4:1, (+/-) charge ratio of 0.5, and were 91% and 92% respectively for liposome MSB1 and MSB2. In the case of the ternary complexes the maximum cell death recorded at the highest streptavidinbiotransferrin:liposome:DNA ratio of 10:8:1 was lower with 23% for both complexes (Figure 3.22 and 3.23). At the lower ternary complex ratio of 10:4:1 the survival rate was 96% and 95% for complexes with liposome MSB1 and MSB2 respectively. The extent of cytotoxicity varies with different liposome formulation and also with the type of complexes being formed with the liposomes. The seeding density of cells in the plate could also play a role in the estimation of toxicity

levels in the different complexes and formulations. The general trend that the level of toxicity increases with increasing amounts of liposome is also observed for these liposomes. These low levels of toxicity are very important as they could indicate the potential of these novel liposomes, lipoplexes, and ternary complexes to be utilized in future *in vivo* studies.
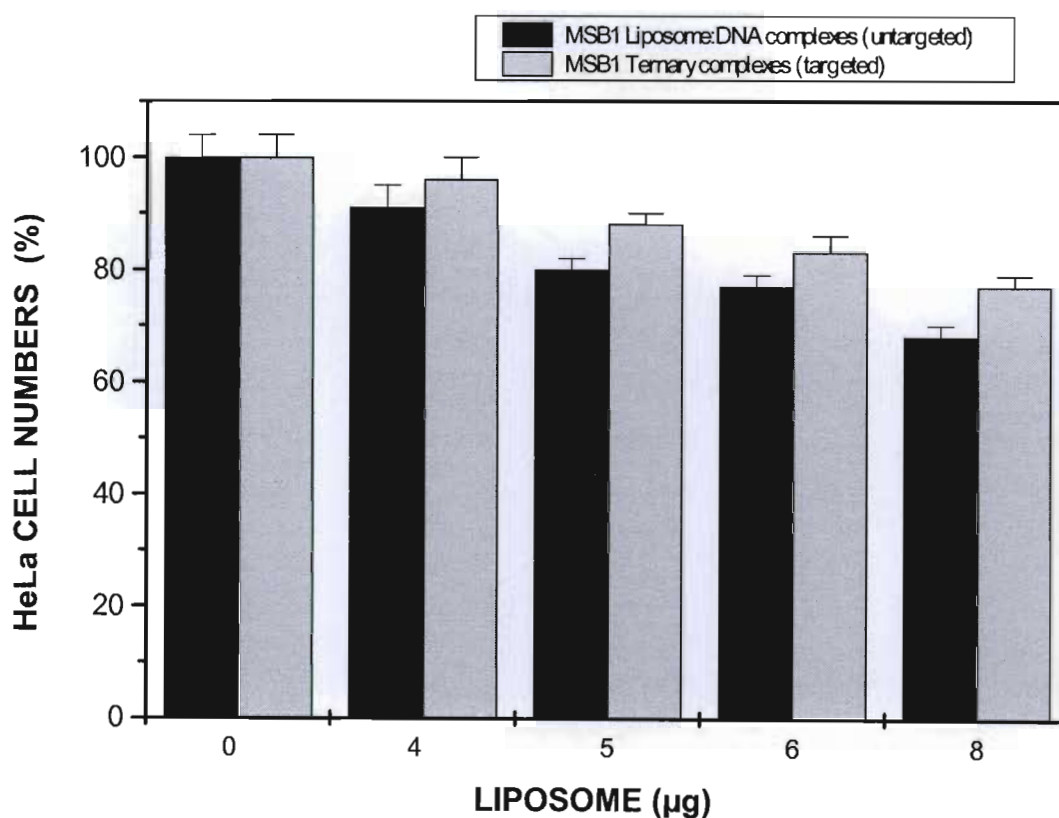
Figure 3.22 : Growth inhibition studies of liposome MSB1:DNA and ternary complexes (streptavidinbiotransferrin:liposome MSB1:DNA) to the HeLa cell line *in vitro*. The amount of liposome MSB1 was varied (0, 4, 5, 6 ,8 µg) while the DNA (1 µg) and streptavidinbiotransferrin (10 µg) was kept constant in a total volume of 0.5 ml MEM. A control sample (no liposome), containing only cells was assumed to have 100% survival. Data are presented as means ± S.D (n = 4).

Figure 3.23 : Growth inhibition studies of liposome MSB2:DNA and ternary complexes (streptavidinbiotransferrin:liposome MSB2:DNA) to the HeLa cell line *in vitro*. The amount of liposome MSB2 was varied (0, 4, 5, 6 ,8 µg) while the DNA (1 µg) and streptavidinbiotransferrin (10 µg) was kept constant in a total volume of 0.5 ml MEM. A control sample (no liposome), containing only cells was assumed to have 100% survival. Data are presented as means ± S.D (n = 4).
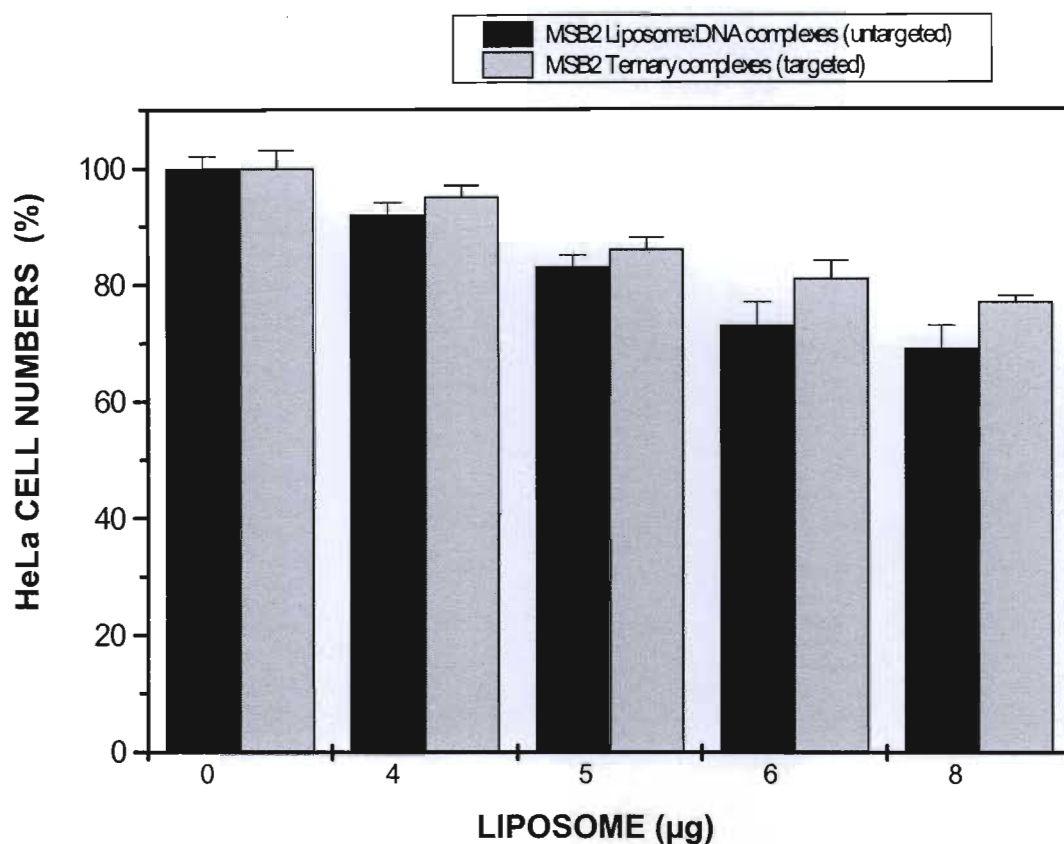
3.4.4.3 Transfection studies with untargeted and targeted biotinylated liposomes :

(a) liposome MSB1:pGL3 DNA complexes
(b) liposome MSB2:pGL3 DNA complexes
(c) streptavidin(biotransferrin):liposome MSB1:pGL3 DNA complexes
(d) streptavidin(biotransferrin):liposome MSB2:pGL3 DNA complexes

Tf-facilitated lipofection has been investigated by many researchers who have shown that it occurs with high efficiency. It was shown that the mixing of transferrin with a cationic liposome prior to addition of DNA, greatly enhanced the lipofection efficiency in Panc 1 cells. Hence it is proposed that the ligand-facilitated gene delivery depends on the order of mixing of these components. The most efficient method tested was the addition of the plasmid DNA last (Joshee *et al.*, 2002). Our ternary complexes were all prepared by the initial addition of the Tf component to the liposome, followed by a period of incubation, and then the addition of the DNA followed by a further incubation. This seemed to afford reasonable transfection activity. Some results have suggested that the addition of Tf to the liposome prior to the addition of DNA prevents DNA condensation by the liposome and helps to segregate DNA-Tf complexes from the liposome, in addition to facilitating the endocytosis step which will increase delivery of the DNA to the cells (Joshee *et al.*, 2002). It was found that a decrease in the net charge of a complex upon addition of DNA led to a decrease in the extent of binding, and cell association and fusion. However addition of Tf to the complexes resulted in a significant increase in cell binding and transfection. Tf was found to trigger internalization of complexes and to mediate fusion with the endosomal membrane (Girão da Cruz *et al.*, 2001).

Transferrin is regarded as a potent ligand that affords effective receptor-mediated gene transfer (Rudolf *et al.*, 2002). In this study the transfection efficiency probably depended on several factors viz. the biotinylated Tf containing three biotin moieties: the reaction between the biotinylated Tf and the streptavidin, the mole percentage of the biotinylated cholesterol derivative in the liposome formulation, and finally the interaction between the biotinylated liposome and the streptavidinbiotransferrin to produce a stable and efficient

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complex for receptor mediated transfection. The results obtained for the untargeted and targeted complexes can be seen in figures 3.24 to 3.27.

Figure 3.24 : Transfection studies of liposome MSB1:DNA complexes (with and without incubation in 10% serum and in the presence of 200 µg freeTf) in HeLa cells *in vitro*. Liposome MSB1 was varied (0, 4, 5 , 6 , 8 µg) while the DNA (1 µg) was kept constant in a total volume of 0.5 ml MEM. Control 1 contains only cells and control 2 contains naked DNA and no liposome. Data are presented as means ± S.D (n = 4).
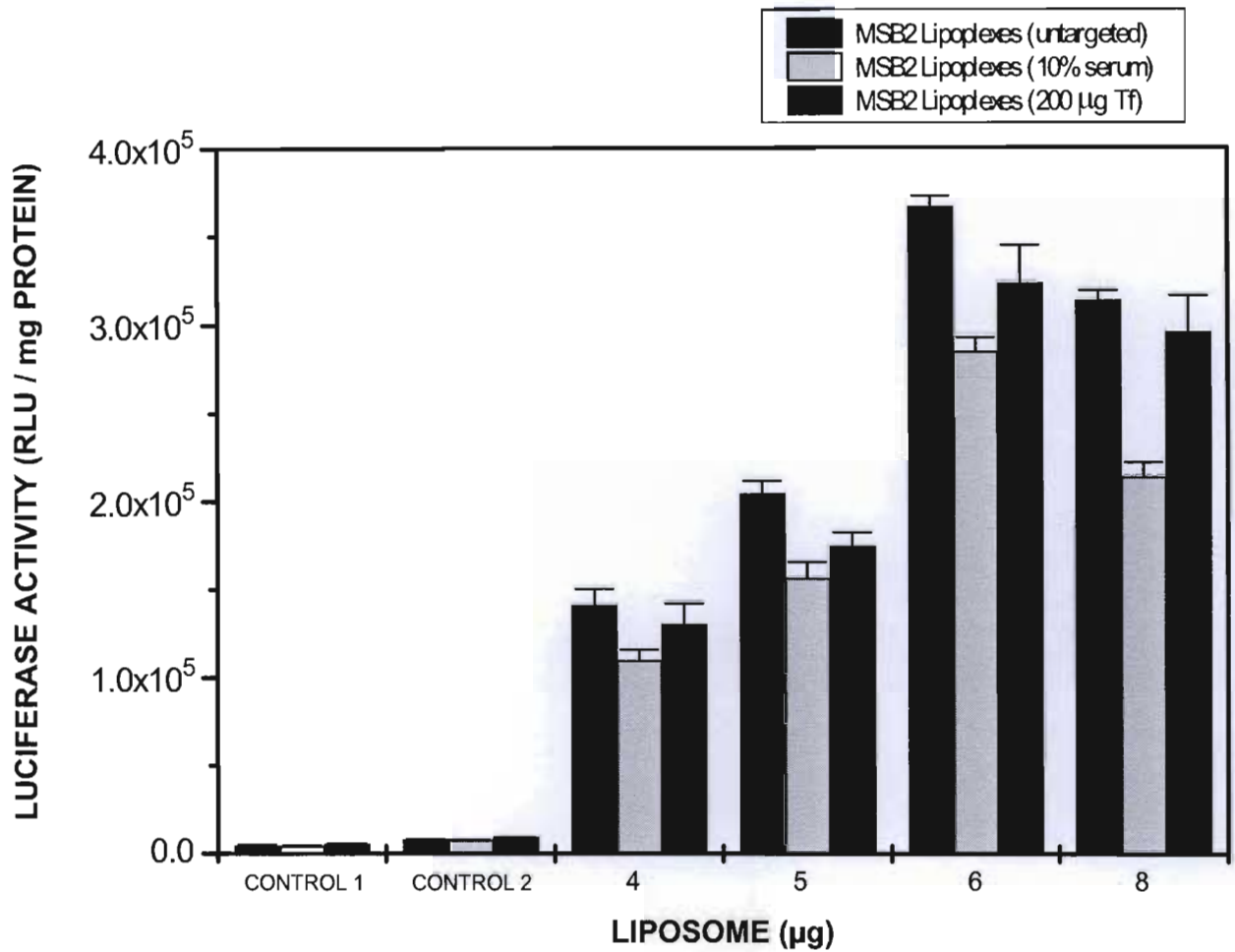
Figure 3.25 : Transfection studies of liposome MSB2:DNA complexes (with and without incubation in 10% serum and in the presence of 200 µg freeTf) in HeLa cells *in vitro*. Liposome MSB2 was varied (0, 4, 5 , 6 , 8 µg) while the DNA (1 µg) was kept constant in a total volume of 0.5 ml MEM. Control 1 contains only cells and control 2 contains naked DNA and no liposome. Data are presented as means ± S.D (n = 4).
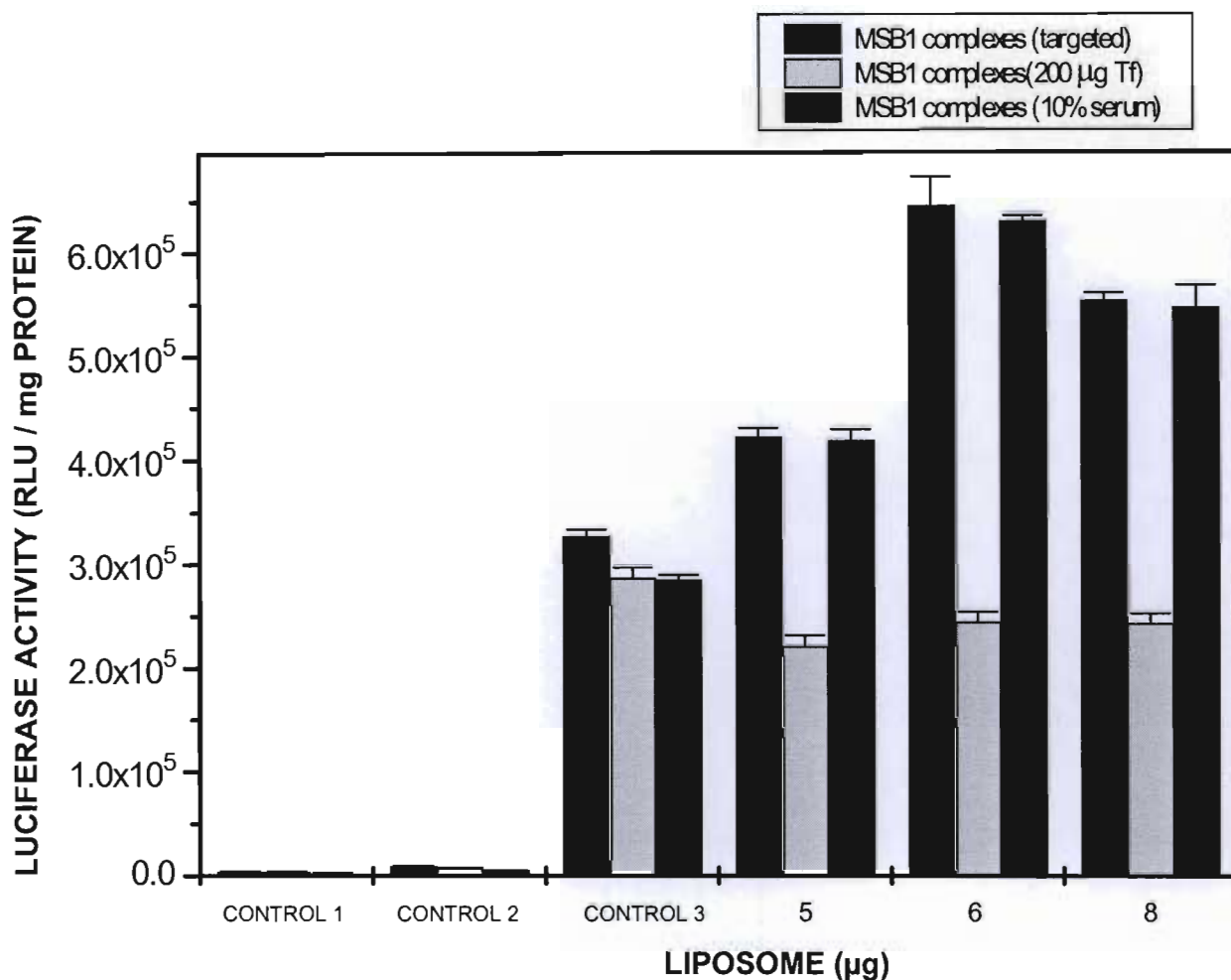
Figure 3.26 : Transfection studies of streptavidin(biotransferrin):liposome MSB1:pGL3 DNA complexes (with and without incubation in 10% serum and in the presence of 200 μg freeTf) in HeLa cells *in vitro*. Liposome MSB1 was varied (0, 5 , 6 , 8 μg) while the DNA (1 μg) and streptavidin (biotransferrin) (10 μg) was kept constant in a total volume of 0.5 ml MEM. Control 1 contains only cells, control 2 contains naked DNA and no liposome, and control 3 contains liposome MSB1:DNA (6:1) at optimal non-targeted ratio (absence of streptavidinbiotransferrin). Data are presented as means ± S.D (n = 4).

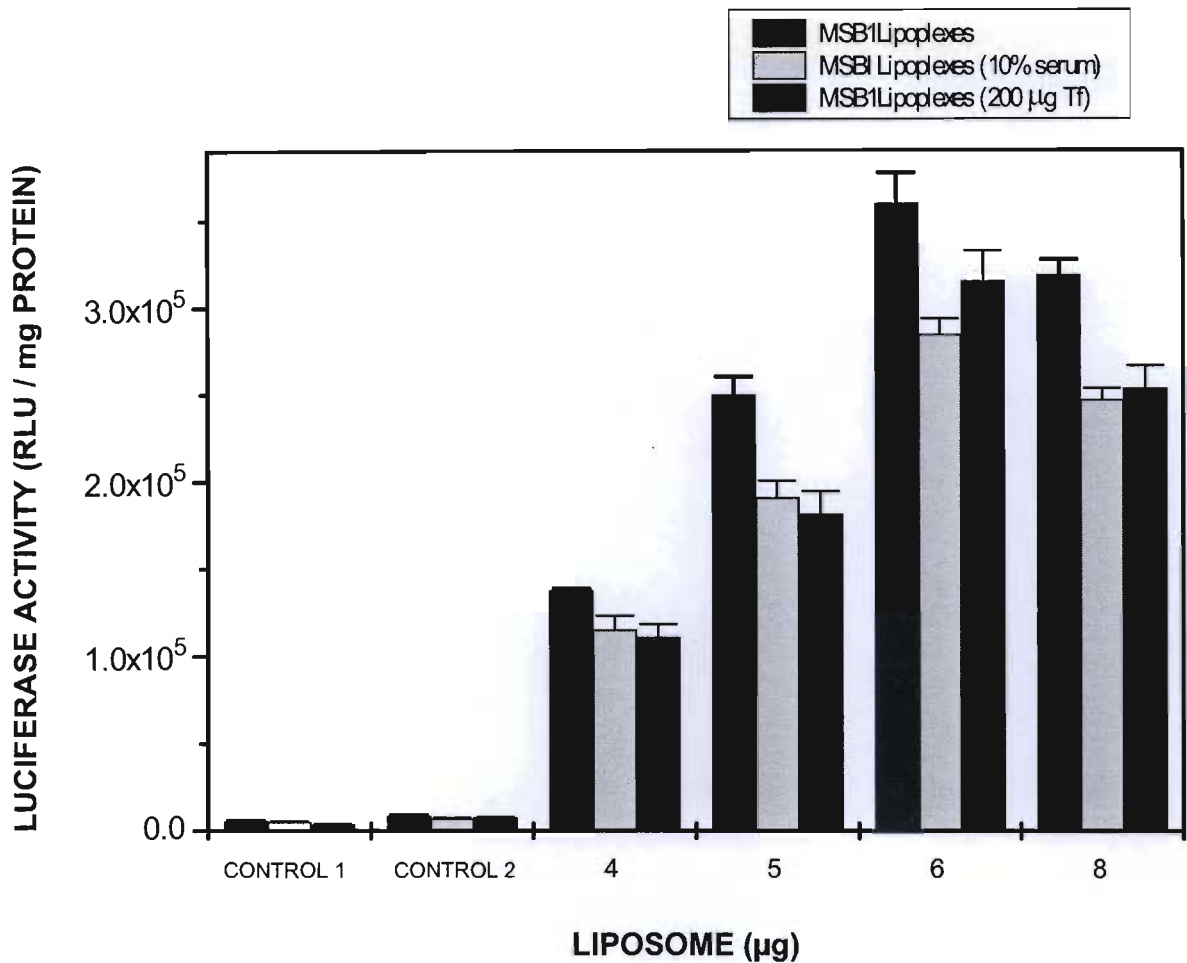**Figure 3.27 :** Transfection studies of streptavidin(biotransferrin):liposome MSB2:pGL3 DNA complexes (with and without incubation in 10% serum and in the presence of 200 µg freeTf) in HeLa cells *in vitro*. Liposome MSB2 was varied (0, 5 , 6 , 8 µg) while the DNA (1 µg) and streptavidin (biotransferrin) (10 µg) was kept constant in a total volume of 0.5 ml MEM. Control 1 contains only cells, control 2 contains naked DNA and no liposome, and control 3 contains liposome MSB2:DNA (6:1) at optimal non-targeted ratio (absence of streptavidinbiotransferrin. Data are presented as means ± S.D (n = 4).
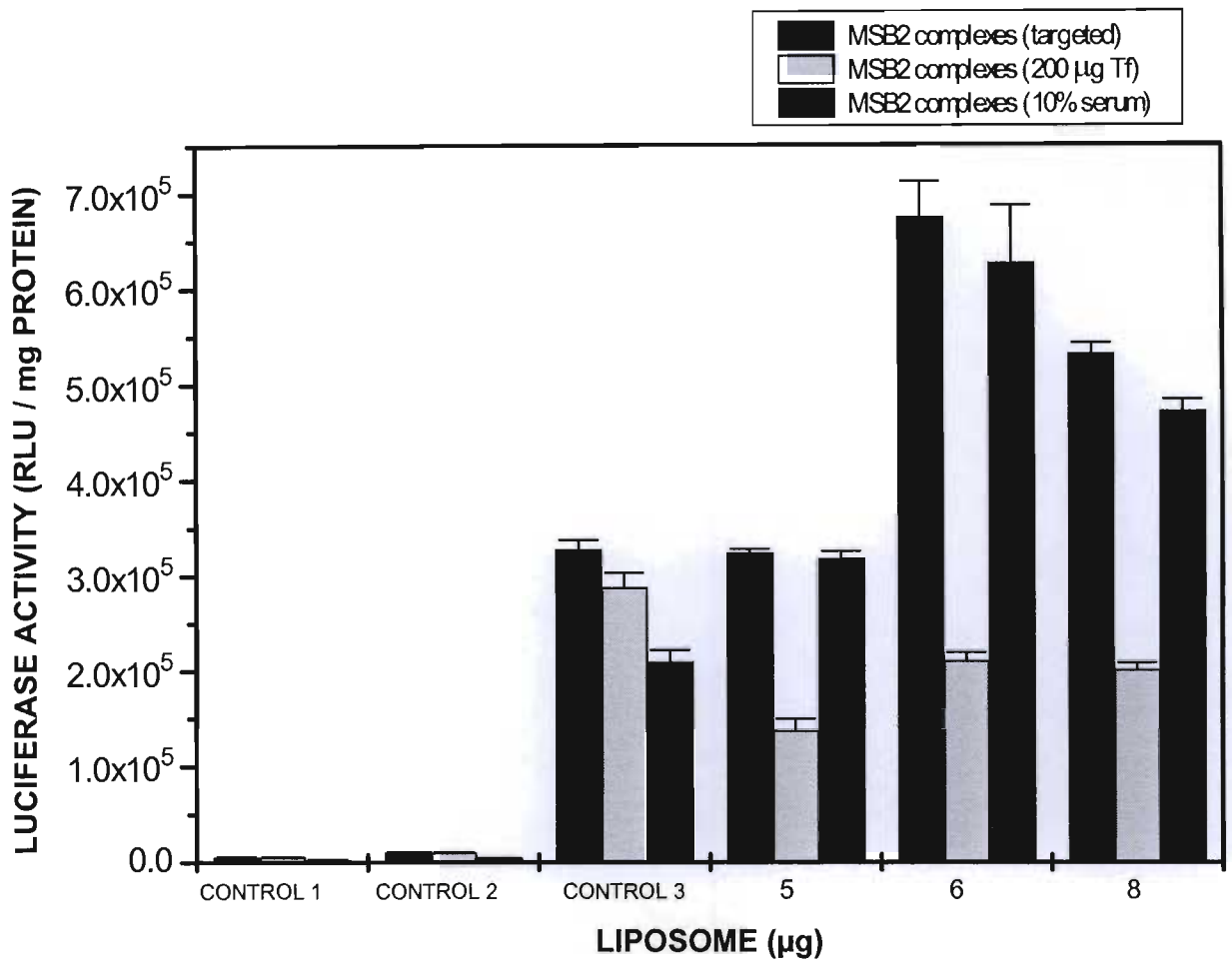
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The untargeted biotinylated liposomes (Figures 3.24-3.25), both showed a 20-25% drop in luciferase activity when transfection was conducted in 10% serum and a drop of about 10% upon addition of 200 µg free iron-Tf added to each well prior to transfection, even at their optimal liposome:DNA (6:1 $^w/_w$) mixing ratios and a (+/-) charge ratio of 0.7. This could be due to the excess Tf that bound to the cell surface transferrin receptors coating the cells, thereby reducing transfection levels to a certain extent. The possibility that excess transferrin present in the medium destabilized the lipoplexes, in part, with some displacement of the plasmid DNA cannot be discounted.

The Tf:liposome:DNA complexes (Figure 3.26-3.27) showed almost a two fold increase in transfection activity compared to the unconjugated liposomes, suggesting that the presence of the Tf component facilitates the uptake of the Tf:liposome:DNA complex into the HeLa cells by receptor mediated endocytosis. The partial uptake of these liposomes by receptor-mediated endocytosis was further confirmed by the transfection of these targeted liposomes in the presence of excess (200 µg) free iron-Tf, where the luciferase activity dropped dramatically. The uptake of transferrin-targeted liposomes can be restricted by competition for the cognate receptor site by free transferrin. The addition of the free transferrin reduced the transfection activity with the Tf coupled liposomes by about 60-70% (+/- charge ratio of 0.7), to levels close to those obtained for the untargeted liposomes. Hence it can be proposed that any increase in transfection was due to the presence of the Tf ligand and the process of receptor-mediated endocytosis.

The transfection levels obtained in this competition assay could be due to normal non-receptor-mediated lipofection processes and not due entirely to receptor mediation. It has been reported that any increase in transfection is eliminated when transfections are conducted in the presence of excess free transferrin (Bellocq et al., 2003). Some investigators have reported using up to 10 mg/ml of free transferrin in their competition assays with about a 72% drop in gene expression (Joo and Kim, 2002), while others have shown almost a total inhibition of luciferase activity (Schoeman et al., 1995).

The results obtained for our ternary complexes in the presence of 10% FBS did not vary significantly from the optimal transfection activity noted for complexes transfected in the absence of serum. Transfection levels dropped from 2-7%. This is a very small reduction in transfection and could be due to other contributing factors such as cell density and destabilization of complexes by serum components. Hence these ternary complexes seem to be quite stable in serum-containing medium. This finding is in contrast with corresponding experiments using untargeted lipoplexes where transfection activities were reduced by 12% at the optimal liposome:DNA ratio of 6:1 (^{W}/$_W$) (Figures 3.24, 3.25, 3.26 and 3.27). It has been suggested that coating of lipoplexes with neutral or negatively charged proteins (eg. transferrin) may constitute a promising strategy to modulate their colloidal stability and transfection efficiency in the presence of serum (Pedroso de Lima *et al.*, 2001). The use of serum-free medium was found to decrease the binding of diferric Tf to the cell surface by 30-60% whereas addition of fetal bovine serum to cells resulted in a rapid (2-3 minutes) concentration dependent increase in binding activity (Ward and Kaplan, 1986).

It is reported that Tf-lipoplexes always mediate higher gene expression compared to plain lipoplexes in the presence or absence of serum. A five fold increase in transfection activity was also observed for complexes in 60% FBS (Tros de Ilarduya and Düzgüneş, 2000). Tf-lipoplexes are said to be superior to plain lipoplexes in transfecting HeLa cells in the presence of high concentrations of serum (Tros de Ilarduya *et al.*, 2002). The inhibitory effect of serum seems to be more evident at lower charge (+/-) ratios of the Tf-complexes, while this inhibitory effect is overcome at higher charge ratios (+/-). Furthermore it was reported that prolonged incubation of Tf-liposome complexes prior to addition to the cells renders them serum resistant and that maturation of the complex is an essential element of serum-resistance. Serum resistance is also dependent on the charge ratio (+/-) and the incubation period (Tros de Ilarduya and Düzgüneş, 2000).

This molecular basis of serum resistance and the enhancement by serum is not completely understood. It is possible that the interaction of the transferrin-lipoplexes with the cell surface receptors facilitates uptake by the host cells, while plain lipoplexes

are coated with serum proteins and hence inhibited from interacting electrostatically with the cell surface. The presence of Tf on the lipoplexes could also hinder the complexation of serum proteins with the cationic lipids (Düzgüneş et al., 2003).

Although liposomes resemble biomembranes in an in vivo system, they still appear as foreign objects for the host cell and they are recognized by the mononuclear phagocyte system (MPS), after interaction with plasma proteins. Hence liposomes are cleared from the bloodstream by the MPS and trapped by the reticuloendothelial system (RES) where macrophage-like cells reside (Oku et al., 2000). The transfection results obtained in serum are important since these ternary complexes exhibited relative stability under these conditions, and could have a potential for in vivo transfer.

To increase the number of transferrin receptors for targeting purposes one could have grown cells in iron deficient medium for a period prior to transfection. This regulation of expression of Tf receptors by iron supply has been seen in many cell types in culture by using iron chelators such as desferrioxamine (Cotton et al., 1990), to reduce the iron supply in the medium (Taylor et al., 1991). Receptor density can also be increased by interfering with heme synthesis using succinyl acetone, or by stimulating the degradation of heme with cobalt chloride treatment (Cotton et al., 1990). It was found that in Hela cells, the intracellular heme content may be an important signal controlling transferrin receptor number (Ward et al., 1984). The rate of endocytosis of Tf was found to be extremely slow in HeLa cells (Girão da Cruz et al., 2001).

It is proposed that transferrin enhances the transfection efficiency of a standard lipofection formulation by preventing DNA condensation, and facilitating endocytosis and nuclear targeting (Joshee et al., 2004). However the efficiency of Tf-lipoplexes in primary cells at high serum concentrations was enhanced using protamine, which condenses the DNA and promotes cellular entry, and increased gene expression by increasing nuclear localization of DNA. This was seen in both cultured cells and in various tissues in mice after intravenous administration (Tros de Ilarduya et al., 2002). The use of pH-sensitive fusogenic peptides together with the cationic liposome and

targeting ligand was found to mediate efficient gene transfer to human macrophage cells *in vitro*, by promoting endosomal destabilization (Simões *et al.*, 1999).

The spacer length of the biotinylated liposomes seems to have little impact on the transfection activity of the two liposomes with MSB2 liposomes being slightly better in transfecting cells by only about 5% at the optimal charge ratio. Transfection also increased with increased liposome: DNA charge ratio until the same optimal transfection ratio was reached for each complex. On further increase of the +/- charge ratio transfection activity declined as was the case with MS09, MS10 and MS11 lipoplexes in Chapter 2.

3.5   CONCLUSION

We have confirmed in these studies that transferrin-mediated endocytosis can be successfully exploited to target genes to the transferrin receptors on HeLa cells *in vitro*, using cationic liposomes as DNA carriers, and shown that the biotin-streptavidin interaction can be used to anchor the targeting ligand to the liposomal membrane bilayer. This could be extended to *in vivo* systems where Tf receptors are overexpressed, especially in some cancers. This targeted delivery system appears to be efficient and shows marked stability in 10% fetal bovine serum. The investigation of this system *in vivo* therefore seems warranted. Although the streptavidin component may exhibit spurious binding to integrins and related cell surface molecules (Das *et al.*, 2002), it is possible that in the assemblies studied here, the streptavidin component may be partially concealed and the implicated Arg-Tyr-Asp (RYD) sequence may be sufficiently adumbrated.

Unfortunately the correlation of *in vitro* and *in vivo* efficacies is a fragile one, although *in vitro* experiments are carried out with strict controls. For clinical application one would have to ensure that the enhanced targeting and transfection efficiency of the liposomes

achieved for *in vitro* experiments be maintained. For example, the tumour suppressor gene p53 has been delivered successfully into tumours *in vivo*, but its expression is low due to a lower transfection efficiency when compared to viral vectors (Qian *et al.*, 2002). However, the influence and the complexities of the intrinsic effects and non-specific interactions would be difficult to predict in any *in vivo* system. Our challenge would be to optimise the various parameters of this current targeted gene delivery system for possible future *in vivo* studies.

CHAPTER FOUR

GLYCOTARGETING TO HepG2 CELLS *IN VITRO*

4.1   INTRODUCTION

Selective delivery of biologically active substances is designed to overcome nonspecific biodistribution of drugs or genes and to increase their local concentration at the target tissue. Certain strategies are based on carrier-mediated delivery to selective tissues and organs through ligands or modified vehicles such as liposomes that are recognized by receptors while others address molecules present on target cells. Gene transfer plays an important role in the development of new therapeutic concepts for liver diseases. Various gene and cell based therapies are emerging at a rapid pace, because they promise to be less invasive, less costly and at least as effective as currently established therapy protocols. Hence gene therapy models are being developed for a wide spectrum of liver diseases, including hereditary liver disorders, malignant liver diseases and viral hepatitis (Ott *et al.*, 2000). The characterization of sugar residues on cancer cells with lectins is used as a prognostic tool for cancer malignancy (Palliard, 1999).

The liver is one of the most important target tissues for gene therapy, given that it has a central role in metabolism (eg. lipoprotein metabolism in various cholesterolemias) and the secretion of circulating proteins (eg. clotting factors in hemophilia). The liver is a unique organ and harbours several other properties including a dual blood supply (80% portal and 20% systemic) for inflow and one outflow system viz. the hepatic vein. It is a regenerative organ and over 70% of the cells are hepatocytes (Galun, 2003). Hepatocytes are parenchymal cells in the liver and can be damaged in various pathological processes. Hence the delivery of therapeutic agents for attenuation of liver injury, inhibition of viral hepatitis replication or modification of hepatocyte-related metabolism should ideally be to the liver, and especially to the hepatocytes (Wu *et al.*, 2002). The endothelium of liver sinusoids (specialized capillaries) serve as an active

barrier between the circulating blood and hepatocytes. Liver sinusoidal epithelial cells lack a basal lamina and comprise unique pores (fenestrae), which act as filters for fluids, solutes, and particles that are exchanged between the sinusoidal lumen and the hepatocytes. These cells are also effective in the uptake of a wide variety of substances from the blood by receptor-mediated endocytosis. Some of these substances can be actively transferred across the endothelial barrier to surrounding tissues by a process called transcytosis (Cormier *et al.*, 2004).

Because its malfunction leads to critical metabolic damage affecting the entire body, the liver is a principal target organ for gene therapy. The efficacy of the particular therapy will depend largely on the ability to target specifically liver cells for corrective gene transfer. Receptor-mediated gene transfer is one of the most promising approaches to the problem, provided the relevant receptors are selectively expressed by hepatocytes (Hisayasu *et al.*, 1999). Targeted delivery of DNA via receptors has been successfully applied using protein ligands to the hepatic asialoglycoprotein receptor (ASGP-R). The ASGP-R is a cell-surface receptor that is highly represented on hepatocytes. Thus, genes targeted to this receptor can be delivered in a highly selective manner to the liver. The asialoglycoprotein receptor on mammalian hepatocytes provides a unique means for the development of liver-specific carriers, such as liposomes, recombinant lipoproteins, and polymers for drug or gene delivery to the liver, especially to the hepatocytes (Wu *et al.*, 2002).

Asialoglycoprotein receptors on the plasma membranes of hepatocytes have been a focus of research interest for receptor-mediated targeting since Wu and Wu (1987) first introduced DNA into hepatocellular carcinoma cells and rat liver (Wu and Wu, 1988), in the form of a complex with asialoglycoprotein and polylysine. Significant expression of the luciferase gene in the pRSVL vector was found after targeting of a cationic lipid (DOGS)-asialoorosomucoid-DNA complex, to the hepatocyte like cell line, HepG2 (Mack *et al.*, 1994). An amphiphilic gelling agent was also added to asialoorosomucoid-polylysine conjugates to enhance receptor-mediated gene delivery to HepG2 cells *in vitro* (Cho *et al.*, 2000). Furthermore, the use of protamine to enhance targeted gene delivery by asialofetuin-lipoplexes to the asialoglycoprotein receptor both in cultured

cells *in vitro* and in the liver of mice *in vivo* was reported (Arangoa *et al.*, 2003). Liposomes have shown substantial potential in the targeting of specific cell types of the liver and may improve the targeting efficacy in the treatment of a variety of liver diseases, including inherited disorders such as hemophilia and low density lipoprotein (LDL) receptor deficiency (Wu and Zern, 1999).

4.1.1   The HepG2 Cells and Asialoglycoprotein Receptors

The human hepatoma cell line, HepG2, is used as a model *in vitro* gene delivery system for hepatocellular carcinomas and for the study of hepatocyte function. Hence to gain further knowledge on hepatocyte function in health and disease and to develop potential gene transfer therapies for hepatocellular carcinomas, successful transfection of HepG2 cells must be demonstrated.

The HepG2 cells are one of the two human cell lines that were isolated from liver biopsies of hepatoblastoma and hepatocellular carcinoma (Aden *et al.*, 1979). HepG2 cells are known to produce many plasma proteins, viz. α-fetoprotein, albumin, transferrin, haptoglobulin, fibrinogen, α-1-acid glycoprotein, β-lipoprotein, plasminogen, and ceruloplasmin. Albumin and α-fetoprotein are the major secretory products of the HepG2 cell line. This tumour-derived cell line has the same biosynthetic capabilities of normal liver parenchymal cells (Knowles *et al.*, 1980). Of the two human hepatoma derived cell lines, the Hep3B and the HepG2, the latter does not synthesize the hepatitis B virus surface antigen (HBsAg), which is thought to be the probable cause of hepatocellular carcinomas (Szmuness, 1978).

HepG2 cells are known to possess approximately 225 000 asialoglycoprotein receptors per cell (Schwartz et.al., 1981). In growing cells approximately 85% of the functional receptors are on the cell surface and the remaining 15% are internal. The maximal rate of ligand uptake at 37°C is approximately 30 000 molecules per cell per minute. Hence each functional receptor can bind and internalise more than 50 ligand molecules during

a 6 hour period, or 1 ligand every 8 minutes (Schwartz *et al.*, 1982). The asialoglycoprotein receptor recycles via endocytosis and is localized to the sinusoidal face of the hepatocyte plasma membrane (Saxena *et al.*, 2003). Due to its unique location within the microvilli clefts at the sinusoidal hepatocyte surface, abundance and high internalization capacity, the asialoglycoprotein receptor is widely used as a target for specific delivery of genes or therapeutic agents to hepatocytes (Rensen *et al.*, 2001). Some of the physiological and pathophysiological functions of this receptor are the removal of desialylated serum glycoproteins and apoptotic cells, clearance of lipoproteins, and acting as sites of entry for hepatotropic viruses (Tozawa *et al.*, 2001).

The hepatic asialoglyprotein receptor binds glycoproteins that have carbohydrate chains terminating in galactose and N-acetyl galactosamine residues, and endocytose the ligands for intracellular degradation (Wu *et al.*, 2002). The human receptor is a hetero-oligomeric complex with two homologous subunits (H1 and H2), each possessing a galactose binding site. The mature and fully glycosylated forms of the H1 and H2 subunits are approximately 46 and 50 kDaltons, respectively (Saxena *et al.*, 2002). These two receptor polypeptide subunits are similar in their primary structure to those receptors of the rat, that have three subunits, viz. RHL-1, RHL-2 and RHL-3 (Sawyer and Doyle, 1990). Both the surface and internal receptors are hetero-oligomers and retain their structure upon ligand internalization or receptor modulation (Herzig and Weigel, 1990). Each polypeptide subunit of the ASGP-R can bind at least a single galactose or N-acteylgalactosamine residue (Rensen *et al.*, 2001). The specificity and affinity of ligand binding depends on the number and spatial arrangement of the galactose binding sites within the receptor complex. Although both subunits of the receptor are required for high affinity binding, binding can occur in the absence of one of the two units. For example, the asialoorosomucoid (ASOR) and asialofetuin (ASF) are able to bind to COS-7 cells expressing the major subunit H1 in the absence of the second subunit H2 (Bider *et al.*, 1995). The H2 subunit is a minor subunit of the receptor and contains weak signals for endocytosis and recycling of ligands. Hence it cannot facilitate endocytosis in the absence of subunit H1. The H2 subunit is expressed

at a 3 fold higher concentration than the H1 subunit in the human asialoglyprotein receptor (Saxena *et al.*, 2002).

In humans the asialoglyprotein receptor is found mainly on hepatocytes, although they do occur extrahepatically in the thyroid, small and large intestines, the testis and possibly in the mesangial cells in the kidneys (Seow *et al.*, 2002). Hepatocytes were also targeted using liposomes conjugated to the epidermal growth factor (EGF), since many carcinoma cells show a high expression of EGF receptors (Ishii *et al.*, 1989). In hepatocytes the general sugar preference of the asialoglycoprotein receptors is N-acetylgalactosamine (GalNAc) over galactose (Rensen *et al.*, 2001) whereas the macrophage receptor binds both ligands with equal affinity (Iobst and Drickamer, 1996).

The actual receptor-mediated endocytosis of asialoglycoprotein and galactosylated macromolecules is characterized by the vectorial movement of ligand-containing vesicles through the cytoplasm. The ligand-receptor complex is internalized from the cell surface into an acidic late endosomal compartment where the receptor and ligand dissociate and segregate, resulting in movement of ligand-containing vesicles towards lysosomal fusion, while receptor-containing vesicles recycle. It was demonstrated that the endosomal vesicles bind directly to microtubules in a manner that suggests that microtubules play a role in segregation of the ligand and receptor (Goltz *et al.*, 1992). This was supported by evidence that the vesicles that contain the endocytosed ligand, asiaoloorosomucoid, can translocate for distances of up to 20 μm or more and the ligands are released upon addition of ATP. The velocity of this movement is oscillatory (Murray *et al.*, 2000).

4.1.2 Glycotargeting

Lectins are present on the surface of many cells. Many lectins actively recycle from the membrane to the endosomes and efficiently take up glycoconjugates in a sugar-dependent manner. Based on this, glycoconjugates especially those obtained by chemical means, are regarded as being good carriers of drugs, oligonucleotides, or genes (Monsigny et al., 1998). The term "glycotargeting" refers to the targeting by carrier molecules possessing carbohydrates that are recognized and internalized by these cell surface mammalian lectins. Receptor-mediated endocytosis is primarily the mechanism that is targeted. Glycosylation of liposomes can be achieved by coating with glycoprotein or incorporation of synthetic glycolipids (Kawakami et al., 2002). The use of sugar macromolecule conjugates or glycoconjugates may be divided into two types viz. those in which the macromolecule is itself the drug or therapeutic agent, or, those in which the macromolecule plays an important role in the delivery of a particular drug or therapeutic agent (Davis and Robinson, 2002). Many types of glycotargeting vehicles have been designed based on the covalent attachment of saccharides to proteins, polymers and other aglycones (Wadhwa and Rice, 2003). Carbohydrate-conjugated polycations or lipids are considered the most effective transfection system for receptor-mediated transfer (Düzgüneş et al., 2003).

Glycosylated polylysines have been reported to show specificity in the delivery of nucleic acids into cells that express a given surface membrane lectin (Monsigny et al., 1999). Galactose, has also been incorporated into DNA-polylysine complexes to selectively target DNA to hepatocytes that bear galactose receptors (Plank et al., 1992, Perales et al., 1997; Hashida et al., 1998; Hisayasu et al., 1999). Galactose-PEI-DNA complexes were also targeted to HepG2 cells in vitro with limited gene expression (Kunath et al., 2003). Proteins that have been chemically modified to harbor a sugar residue are called 'neoglycoproteins' (Palliard, 1999). Such neoglycoproteins were prepared by the glycosylation of bovine serum albumin (BSA) and targeted to rat hepatocytes (Kawaguchi et al., 1981), to parenchymal cells in mice (Nishikawa et al., 1992) and to solid tumour-bearing mice (Noboru et al., 2001). Quaternary complexes

prepared from galactosylated BSA, poly-L-lysine, DNA and a fusogenic peptide, achieved selective entry into HepG2 cells with high reporter gene expression (Han *et al.*, 1999). Furthermore, cholesterol with an attached triantennary galactose cluster bound via a PEG type spacer was prepared and used to bind to the asialoglycoprotein receptor of the liver parenchymal cells (Biessen *et al.*, 1994). Triantennary galactose clusters were also incorporated into high density lipoproteins for targeting to liver hepatocytes *in vivo* (Van Berkel *et al.*, 1985), and into electroneutral lipospermine/DNA complexes and targeted to HepG2 cells *in vitro* (Schuber *et al.*, 1998).

In previous studies we have shown successful receptor-mediated gene delivery to HepG2 cells by ternary assemblies containing cationic liposomes and cationised asialoorosomucoid (Singh *et al.*, 2001b), and using complexes containing DNA, cationized asialoorosomucoid and activated cationic liposomes (Singh *et al.*, 2003). Galactose modified α-helical peptides were utilized by some investigators to transfer genes into human hepatoma cells (HuH-7), and showed that transfection efficiency increased with increased number of modified galactose residues (Niidome *et al.*, 2000). Low molecular weight chitosan was also galactosylated for hepatocyte targeting in vitro (Gao *et al.*, 2003). Sterylglucoside containing cationic liposomes were utilized for liver-directed gene transfer into the HepG2 cell line and *in vivo* into mice following intravenous injection, with significant gene expression (Hwang *et al.*, 2001; Maitani *et al.*, 2001). In mammals these terminal sugars (galactoses) are recognised by the high affinity hepatic asialoglycoprotein receptors that then removes them from circulation, and internalise them within the hepatocytes and catabolises them in the lysosomes (Ashwell and Morel, 1974).

In addition to the hepatocyte asialoglycoprotein receptor, the glycoprotein receptors present on the Kupffer cells in the liver also have an affinity for monosaccharide ligands such as galactose or N-acetylgalactosamine, and may function in a similar fashion (Fadden *et al.*, 2003).

4.2   OUTLINE OF CHAPTER

An attempt is made here to produce a synthetic targeted cationic liposome-mediated DNA carrier system for an *in vitro* study in a human hepatoma cell line (HepG2). Targeted transfection is expected to be facilitated by the formulation of glucopyranosyl- and galactopyranosylcholesteryl derivatives with the cationic cholesterol derivative MS09 and the helper lipid, DOPE, to produce liposomes with a targeting potential. The sugar moiety present in the liposomal bilayer is intended to bear a specificity towards the membrane lectins in a configuration-dependent manner, while the cationic component is included to bind electrostatically to the negatively charged plasmid DNA. Conventional cationic liposomes have no inherent cell-specific targeting properties, and thus would not be expected to exhibit tissue-specificity *in vivo*. Hence it is hoped that the use of the these novel liposome formulations bearing the ligands D-galactopyranose and D-glucopyranose in an α- or β-glycosidic link with the cholesteryl anchor may help mediate the targeted transfection to the HepG2 cells line that displays specific cell surface (asialoglycoprotein) receptors.

Not only is anomeric preference by the asialoglycoprotein receptor for the D-galactopyranosyl entity being examined, but also the epimeric specificity relating to the C-4' configuration in the pyranosyl ring is being tested by formulating separate liposomes assembled in identical proportions to the galacto-liposomes, but containing glucopyranosyl cholesterol in both an α- and a β-glycosidic link. The pGL3 plasmid DNA is once again used to detect transient expression in these HepG2 cells, since these cells lack luciferase activity.

4.3. MATERIALS AND METHODS

4.3.1 MATERIALS FOR CHEMICAL SYNTHESIS

β-D-glucose pentaacetate was obtained from Sigma-Aldrich (St.Louis, MO, USA). The α,β-galactose pentaacetate was supplied by Pfanstiehl Laboratories, Inc. (Waukegan, IL, USA). Glacial acetic acid, sodium bromide, hexane, cadium carbonate, cholesterol, ethanol, ethylacetate, p-anisaldehyde, sulphuric acid, dichloromethane, sodium bicarbonate, calcium chloride, toluene, silica gel 60 (70-230 mesh) and silica gel $60F_{254}$ chromatography plates were all purchased from Merck (Darmstadt, Germany). All other reagents used were of analytical grade. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Gemini 300 instrument.

Solvent system C = hexane:ethylacetate (8:3 $^v/_v$)

Solvent system D = formic acid:ethylacetate:CHCl$_3$ (1:5:4 v/v/v)

4.3.2 SYNTHESIS OF GLUCOPYRANOSYL AND GALACTOPYRANOSYL CHOLESTEROL DERIVATIVES :

        (a)   Cholesteryl-β-D-galactopyranoside

        (b)   Cholesteryl-α-D-galactopyranoside

        (c)   Cholesteryl-β-D-glucopyranoside

        (d)   Cholesteryl-α-D-glucopyranoside

The cholesteryl glycosides were prepared by a modified Koenigs-Knorr procedure in which the respective C-1 bromo-tetra-O-acteyl monosaccharide in a 2:1 mole ratio with cholesterol was further activated by cadmium carbonate, a halophilic heavy metal salt promoter (Rogers and Thevan, 1986).

4.3.2.1 Bromination of Sugars

To each peracetylated sugar (glucose pentaacetate and galactose pentaacetate, 20 mmoles, 7.8 g), was added glacial acetic acid (100 ml). This was carried out in a glass flask with an inlet and outlet stem, connected to a sintered glass pad to facilitate bromination of the sugars. Samples were chilled to 4°C and bromination was initiated by the addition of H_2SO_4 dropwise into a flask containing sodium bromide, and the generated HBr channeled into the respective samples via the inlet stem. The sugars were each brominated for a period of 15 minutes, with reaction mixtures turning a yellow to orange colour due to the presence of bromine in samples. The reaction mixtures were then maintained at room temperature for 2-3 hours and thereafter placed at 4°C until the bromination reaction had reached completion as determined by TLC using silica gel $60F_{254}$ chromatography plates and solvent system C. The plates were sprayed with a p-anisaldehyde spray (p-anisaldehyde: H_2SO_4:ethanol , 5:5:90 v/v/v).

At the end of bromination (i.e quantitative conversion of the sugar pentaactetates), the coloured, but clear solutions were each added to beakers containing crushed ice (400 ml) respectively. These sugar-ice slurries were stirred and placed in separating funnels. Dichloromethane (130 ml) was added to the slurries to dissolve the sugar components. Extractions of the dichloromethane layers with distilled water (2 x 200 ml) and 1% $NaHCO_3$ (100 ml) were carried out to remove residual acid. Extracted samples were added to flasks containing $CaCl_2$ and dried overnight at 4°C. The $CaCl_2$ was removed by filtration and reaction mixtures evaporated by rotary evaporation. Samples were co-evaporated with toluene and then stored in 25-30 ml toluene. Each product yielded one major green coloured spot (R_f 0.56) on TLC plates developed in solvent system C and sprayed with p-anisaldehyde reagent.

4.3.2.2 Synthesis and isolation of:
    (a)   **Cholesteryl-β-D-tetra-O-acetylgalactopyranoside**
    (b)   **Cholesteryl-α-D-tetra-O-acetylgalactopyranoside**
    (c)   **Cholesteryl-β-D-tetra-O-acetylglucopyranoside**
    (d)   **Cholesteryl-α-D-tetra-O-acetylglucopyranoside**

Cadmium carbonate (4 g, 22.7 mmoles) was added to cholesterol (10 mmoles, 3.86 g) in a round bottomed flask to absorb the liberated HBr. Toluene (75 ml) was added to the mixture and the flask was attached to the 'Dean and Stark' apparatus and immersed in an oil bath. The reagents were heated to boiling with continuous stirring and excess water was removed azeotropically in the 'Dean and Stark' apparatus. The 2,3,4,6-tetra-O-acetyl-α(β)-D-glucopyranosylbromide and 2,3,4,6-tetra-O-acetyl-α(β)-D-galacto-pyranosylbromide (approximately 20 mmoles) in 30 ml toluene was added dropwise into the boiling cholesterol-toluene-CdCO$_3$ mixture. The reactions which yielded more water (removed azeotropically) were monitored against a cholesterol standard by TLC in solvent system C and using the p-anisaldehyde spray. The solutions turned bluish-purple and the Cd(Br)$_2$ precipitated out. Upon completion of the reactions, the mixtures were filtered through celite using a Hartley funnel. The precipitates were washed with toluene. The resultant honey coloured filtrates were kept in a deep freezer (-10°C). Each product yielded one major greenish-blue spot (R$_f$ 0.31), and a minor product (R$_f$ 0.47) when examined by TLC (solvent system C, p-anisaldehyde spray).

The reaction mixtures, cholesteryl-α(β)-D-tetra-O-acetylglucopyranoside (25 ml) and cholesteryl-α(β)-D-tetra-O-acetylgalactopyranoside (20 ml) in toluene were then evaporated in a rotary evaporator and residual toluene removed by co-evaporation with ethanol (2 x 3 ml). The final residue was dissolved in solvent system C (30 ml). A silica gel 60 column (70-230 mesh) with a total volume of 91 ml (2.1 cm x 26.5 cm) was set up. Aliquots of the reaction mixture were applied to the column which was then eluted with solvent system C. Fractions (5.5 ml) were collected after the first 20 ml for tetra-O-acetylglucosylcholesterol and 40 ml for tetra-O-acetylgalactosylcholesterol of the eluate were discarded. The presence of each of the final products was monitored by TLC as described above. The required fractions were pooled and evaporated in a rotary

evaporator. The α-anomers of the sugars eluted earlier than the β-anomers. Products were finally recrystallized from ethanol.

Cholesteryl-β-D-tetra-O-acetylgalactopyranoside (Appendix 1K).

Mp 157-159°C; ^{1}H NMR (300MHz, CDCl$_3$) : δ 0.65 (s,3H,C-CH_3), 0.83 (d, 6H, J=6.6Hz, CH-CH_3), 0.89 (d, 3H, J=6.5, CH-CH_3), 0.96 (s, 3H, C-CH_3), 1.96-2.12 (12H, 4 x CO-CH_3), 3.47 (m, 1H, Chol-H$_{3α}$), 3.86 (t, J=7.0, H-5'), 4.05-4.19 (m, 2H, H-6'a, H-6'b), 4.52 (d, 1H, J=8.0 Hz, H-1'), 4.99 (dd, 1H, J=10.4, 3.4 Hz, H-3'), 5.16 (dd, 1H, J=10.4, 7.9 Hz, H-2'), 5.35 (m, 2H, H-4, Chol-H$_6$).
^{13}C NMR (75 MHz, CDCl$_3$) : sugar region : δ 100.29 (C-1'), 69.07 (C-2'), 70.55 (C-3'), 67.03 (C-4'), 71.02 (C-5'), 61.30 (C-6')ppm.
Cholesteryl moiety : δ 11.86 (C-18), 18.72 (C-21), 19.36 (C-19), 21.05 (C-11), 22.57 (C-26), 22.83 (C-27), 24.29 (C-15), 28.23 (C-16), 29.52 (C-2), 23.82 (C-23), 28.02 (C-25), 31.86 (C-8), 31.94 (C-7), 35.78 (C-20), 36.18 (C-22), 36.71 (C-10), 39.52 (C-24), 39.74 (C-12), 37.19 (C-1), 38.96 (C-4), 42.32 (C-13), 50.14 (C-9), 56.74 (C-14), 56.14 (C-17), 80.35 (C-3), 122.19 (C-6), 140.33 (C-5) ppm.

Cholesteryl-α-D-tetra-O-acetylgalactopyranoside (Appendix 1H).

Mp 194-197°C; ^{1}H NMR (300MHz, CDCl$_3$) : δ 0.65 (s,3H,C-CH_3), 0.84 (d, 6H, J=6.6 Hz, CH-CH_3), 0.89 (d, 3H, J=6.5, CH-CH_3), 0.98 (s, 3H, C-CH_3), 1.97-2.12 (12H, 4 x CO-CH_3), 3.43 (m, 1H, Chol-H$_{3α}$), 4.07 (t, 2H, J=7.0, 5.4, H-6'a, H-6'b), 4.32 (m, 1H, H-5'), 5.43 (m, 1H, H-1') ppm.

Cholesteryl-β-D-tetra-O-acetylglucopyranoside (Appendix 1J).

Mp 160-163°C (Seo *et al.*, 1978 reported 160-164°C); ^{1}H NMR (300MHz, CDCl$_3$) : δ 0.65 (s,3H,C-CH_3), 0.83 (d, 6H, J=6.6 Hz, CH-CH_3), 0.88 (d, 3H, J=6.5, CH-CH_3), 0.96 (s, 3H, C-CH_3), 2.00-2.05 (12H, 4 x CO-CH_3), 3.45 (m, 1H, Chol-H$_{3α}$), 4.08 (m, 2H, H-6'a, H-6'b), 3.65 (dd, 1H, J=7.5,2.3, H-5'), 4.93 (dd ,1H, J=9.5,8.0, H-3'), 4.57 (d, J=7.9 Hz, H-1'), 5.18 (t, 1H, J=9.4, H-2'), 5.33 (d, 1H, J=5.0, Chol-H$_6$), 5.05 (t, 1H, J=9.6, H-4').
^{13}C NMR (75 MHz, CDCl$_3$) : sugar region : δ 99.64 (C-1'), 71.68 (C-2'), 72.90 (C-3'), 68.50 (C-4'), 71.47 (C-5'), 62.09 (C-6')ppm.
Cholesteryl moiety : δ 11.85 (C-18), 18.72 (C-21), 19.36 (C-19), 21.04 (C-11), 22.57 (C-26), 22.83 (C-27), 24.28 (C-15), 28.23 (C-16), 29.44 (C-2), 23.81 (C-23), 28.02 (C-25), 31.85 (C-8), 31.94 (C-7), 35.78 (C-20), 36.18 (C-22), 36.71 (C-10), 39.52 (C-24), 39.74 (C-12), 37.19 (C-1), 38.91 (C-4), 42.32 (C-13), 50.14 (C-9), 56.74 (C-14), 56.14 (C-17), 80.09 (C-3), 122.18 (C-6), 140.34 (C-5) ppm.

Cholesteryl-α-D-tetra-O-acetylglucopyranoside (Appendix 1I)

Mp 201-203°C (Seo *et al.*, 1978 reported 202-204°C) ^{1}H NMR (300MHz, CDCl$_3$) : δ 0.65 (s,3H,C-CH_3), 0.84 (d, 6H, J=6.5 Hz, CH-CH_3), 0.89 (d, 3H, J=6.5, CH-CH_3), 0.96 (s, 3H, C-CH_3), 1.99-2.06 (12H, 4 x CO-CH_3), 3.43 (m, 1H, Chol-H$_{3α}$), 4.06-4.21 (m, 2H, H-6'a, H-6'b), 5.32 (d, 1H, Chol-H$_6$), 5.46 (t, 1H, H-1').
^{13}C NMR (75 MHz, CDCl$_3$) : sugar region : δ 94.23 (C-1'), 71.04 (C-2'), 70.22 (C-3'), 68.74 (C-4'), 67.21 (C-5'), 67.07 (C-6')ppm.
Cholesteryl moiety : δ 11.86 (C-18), 18.72 (C-21), 19.38 (C-19), 21.04 (C-11), 22.57 (C-26), 22.84 (C-27), 23.82(C-23), 24.29 (C-15), 28.02 (C-25), 28.24 (C-16), 31.86 (C-8), 37.20(C-1), 39.52 (C-24), 39.73 (C-12), 42.31 (C-13), 50.09 (C-9), 56.13 (C-17), 56.70 (C-14), 122.21 (C-6), 140.30 (C-5) ppm.

4.3.2.3   Synthesis of :

    (a)   Cholesteryl-α-D-glucopyranoside

    (b)   Cholesteryl-β-D-glucopyranoside

    (c)   Cholesteryl-α-D-galactopyranoside

    (d)   Cholesteryl-β-D-galactopyranoside

The tetra-O-acetyl α- and β-anomers were quantitatively deprotected separately to produce the corresponding gluco- and galacto-cholesterol derivatives. All reaction mixtures were monitored by TLC in solvent system D. Solvent (CHCl$_3$, ethanol) was removed by rotary evaporation at 25°C and the residue was triturated in water at 4°C to remove sodium acetate and excess sodium ethoxide. The powdery white precipitate was suspended in water and stored overnight at 4°C. thereafter water was removed by filtration and products were dried *in vacuo* in a Büchi-TO pistol drier (60°C). All products produced one spot (R$_f$ 0.21).

(a)   Cholesteryl-α-D-glucopyranoside (MSαGLU)

Chloroform (0.5 ml) and sodium ethoxide (0.15 ml, 0.33 mmoles) was added to the tetra-O-acetylglucocholesterol (19.5 mg, 0.03 mmoles). The resultant mixture turned cloudy and was kept at room temperature overnight in a cupboard before proceeding as described above,

^{13}C NMR (75 MHz, C_5D_5N) : sugar region : δ 98.81 (C-1'), 73.18 (C-5'), 72.50 (C-3'), 71.39 (C-4'), 70.62 (C-2'), 62.21 (C-6') ppm.
Cholesteryl moiety : δ 11.76 (C-18), 18.72 (C-21), 19.24 (C-19), 21.04 (C-11), 22.47 (C-26), 22.72 (C-27), 23.87 (C-23), 24.27 (C-15), 27.98 (C-25), 28.28 (C-16), 31.84 (C-8), 37.14 (C-1), 39.47 (C-24), 39.73 (C-12), 42.24 (C-13), 50.08 (C-9), 56.10 (C-17), 56.60 (C-14), 121.61 (C-6), 140.98 (C-5) ppm.

(b)   Cholesteryl-β-D-glucopyranoside (MSβGLU)

Chloroform (2 ml) and sodium ethoxide (0.5 ml, 1.1 mmoles) was added to the tetra-O-acetylglucocholesterol (100 mg, 0.14 mmoles). The resultant mixture turned cloudy and was kept at room temperature overnight in a cupboard. A white amorphous product was obtained by the procedure outlined above.

Mp 256-260°C (Seo *et al.*, 1978 reported 257-260°C). ^{1}H NMR (300 MHz, C_5D_5N) : δ 0.54 (s,3H,C-CH_3), 0.77 (d, 6H, J=6.6 Hz, CH-CH_3), 0.85 (d, 3H, J=6.5, CH-CH_3), 4.85 (d, 1H, J=7.6, H-1').
^{13}C NMR (75 MHz, C_5D_5N) : sugar region : δ 102.84 (C-1'), 76.51 (C-5'), 75.03 (C-3'), 72.32 (C-2'), 69.86 (C-4'), 62.03 (C-6') ppm.
Cholesteryl moiety : δ 11.76 (C-18), 18.72 (C-21), 19.20 (C-19), 21.05 (C-11), 22.47 (C-26), 22.72 (C-27), 23.87 (C-23), 24.26 (C-15), 27.98 (C-25), 28.29 (C-16), 31.82 (C-8), 37.26 (C-1), 39.48 (C-24), 39.73 (C-12), 42.24 (C-13), 50.12 (C-9), 56.10 (C-17), 56.60 (C-14), 121.66 (C-6), 140.77 (C-5) ppm.

(c)   Cholesteryl-α-D-galactopyranoside (MSαGAL)

Chloroform (0.5 ml) and sodium ethoxide (0.15 ml, 1.1 mmoles) was added to the tetra-O-acetylgalactosylcholesterol (14.8 mg, 0.02 mmoles). The resultant mixture turned cloudy and was kept at room temperature overnight in a cupboard. A non-crystalline white powder was obtained by work-up as described.

^{1}H NMR (300 MHz, CDCl$_3$, C$_5$D$_5$N) : δ 0.58 (s,3H,C-CH_3), 0.81(d, 6H, J=5.6, CH-CH_3), 0.85 (d, 3H, J=7.0, CH-CH_3), 0.89 (s, 3H, C-CH_3), 5.29 (d, 1H, J=3.8, H-1').

^{13}C NMR (75 MHz, CDCl$_3$, C$_5$D$_5$N) : sugar region : δ 98.29 (C-1'), 76.51 (C-5'), 75.03 (C-3'), 72.32 (C-2'), 69.86 (C-4'), 62.03 (C-6') ppm.

Cholesteryl moiety : δ 11.87 (C-18), 18.81 (C-21), 19.31 (C-19), 21.11 (C-11), 22.60 (C-26), 22.85 (C-27), 23.96 (C-23), 24.35 (C-15), 28.07 (C-25), 28.35 (C-16), 31.91 (C-8), 37.16 (C-1), 39.57 (C-24), 39.81 (C-12), 42.33 (C-13), 50.13 (C-9), 56.18 (C-17), 56.70 (C-14), 121.63 (C-6), 140.89 (C-5) ppm.

(d)   Cholesteryl-β-D-galactopyranoside (MSβGAL)

Chloroform (1.5 ml) and sodium ethoxide (0.35 ml, 0.76 mmoles) was added to the tetra-O-acetylgalactosylcholesterol (69 mg, 0.10 mmoles). The resultant mixture turned cloudy immediately and was kept at room temperature overnight in a cupboard. Work-up was as described.

Mp 270-273°C. ^{1}H NMR (300 MHz, CDCl$_3$, C$_5$D$_5$N) : δ 0.58 (s,3H,C-CH_3), 0.84 (d, 6H, J=4.0 Hz, CH-CH_3), 0.88 (d, 3H, J=6.5, CH-CH_3), 4.35 (d, 1H, H-1').

^{13}C NMR (75 MHz, CDCl$_3$, C$_5$D$_5$N) : sugar region : δ 102.26 (C-1'), 74.84 (C-2'), 78.63 (C-3'), 71.43 (C-4'), 78.09 (C-5'), 62.61 (C-6') ppm.

Cholesteryl moiety : δ 11.88 (C-18), 18.80 (C-21), 19.31 (C-19), 21.11 (C-11), 22.60 (C-26), 22.86 (C-27), 23.95 (C-23), 24.35 (C-15), 28.08 (C-25), 28.35 (C-16), 31.89 (C-8), 37.30 (C-1), 39.57 (C-24), 39.81 (C-12), 42.33 (C-13), 50.18 (C-9), 56.18 (C-17), 56.69(C-14), 121.75 (C-6), 140.65 (C-5) ppm.
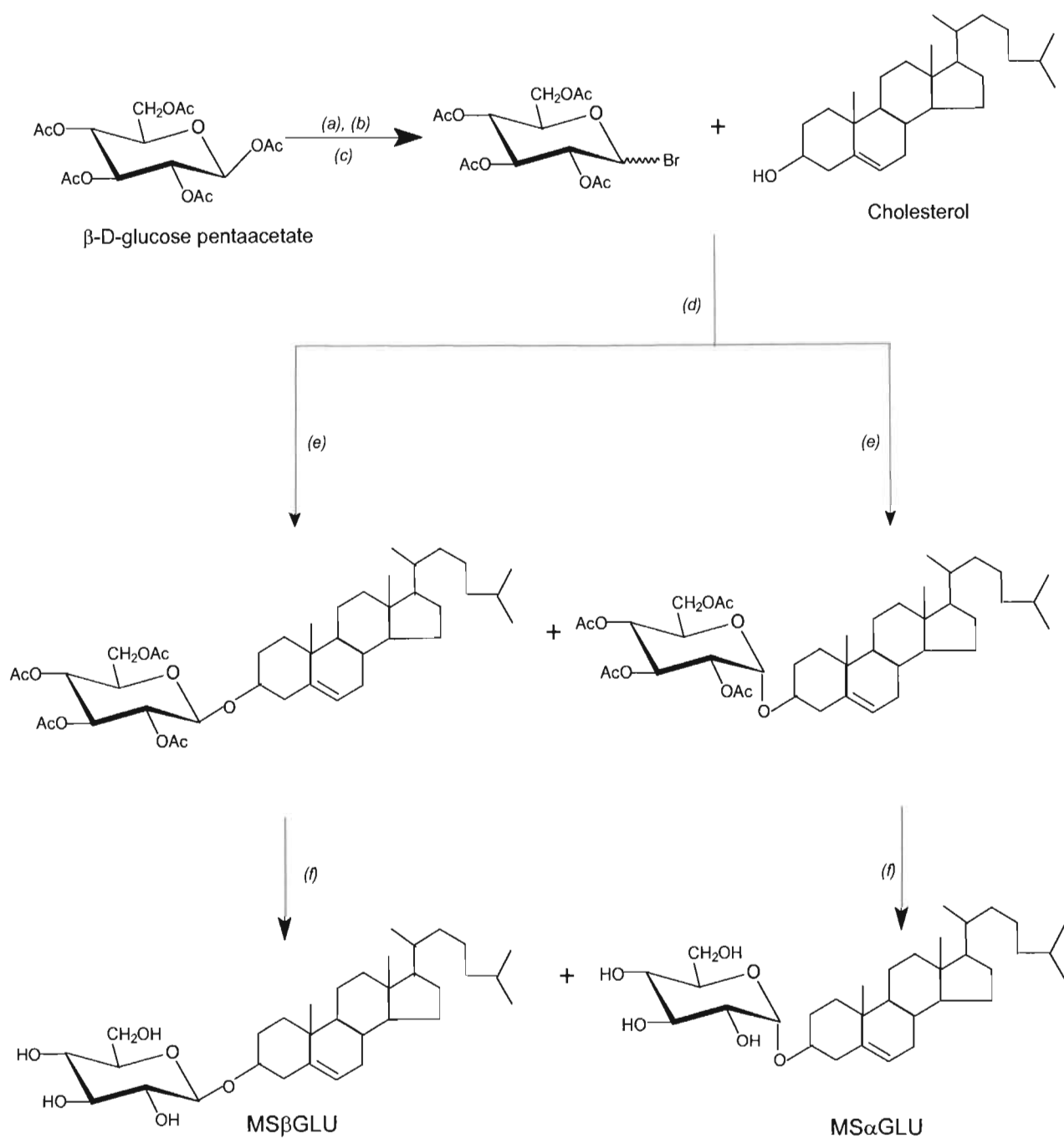
Figure 4.1 : Scheme for the synthesis of cholesteryl-α-D-glucopyranoside and cholesteryl-β-D-glucopyranoside.

a = glacial acetic acid/H_2SO_4 /NaBr, 15 minutes.

b = dichloromethane/$CHCl_3$; c = dH_2O / 1% $NaHCO_3$ / $CaCl_2$, 4°C.

d = $CdCO_3$ / toluene / cholesterol ; e = column chromatography.
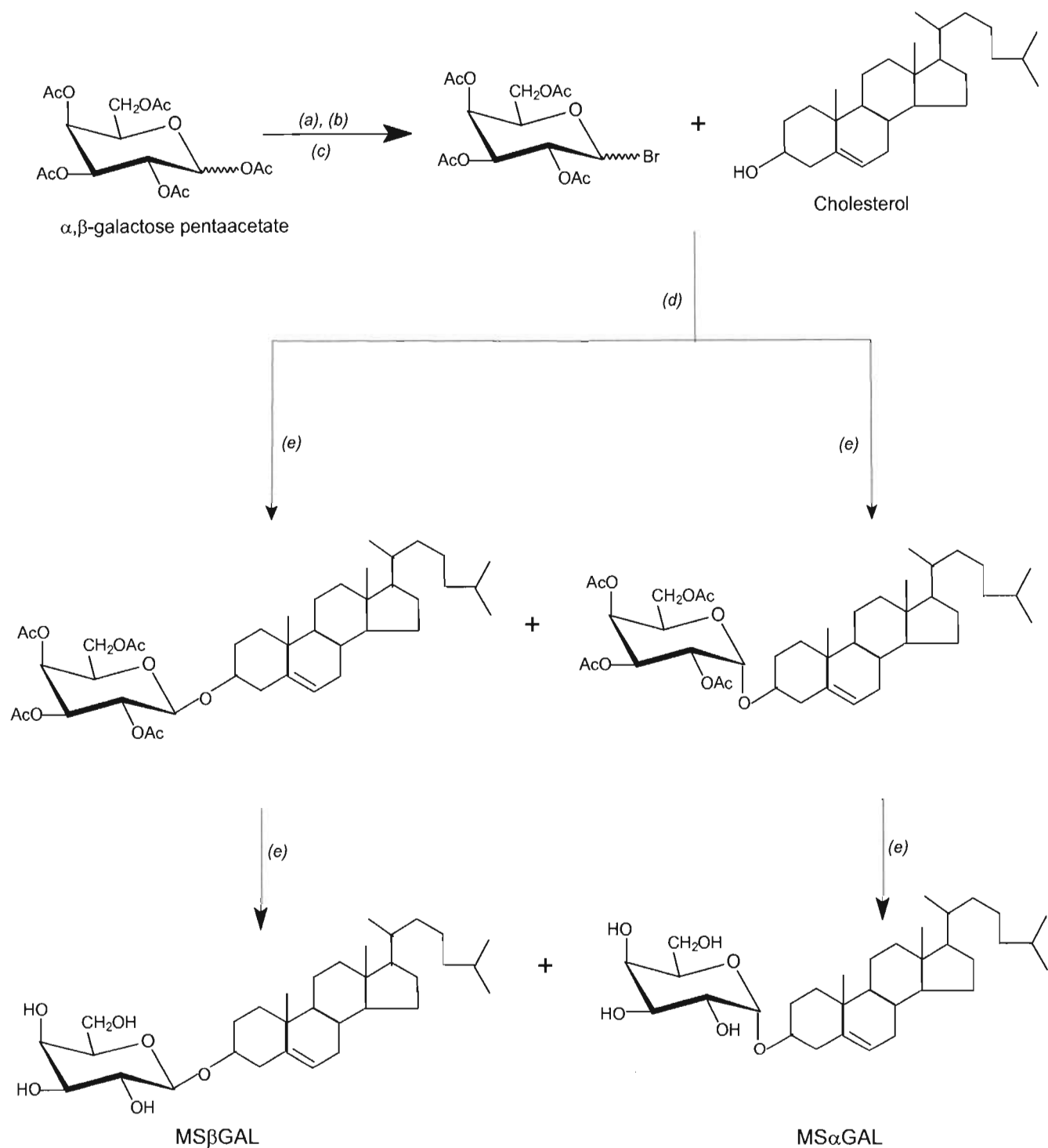
f = $CHCl_3$ / sodium ethoxide.

Figure 4.2 : Scheme for the synthesis of cholesteryl-α-D-galactopyranoside and cholesteryl-β-D-galactopyranoside
a = glacial acetic acid/H_2SO_4 /NaBr, 15 minutes.
b = dichloromethane/$CHCl_3$; c = dH_2O / 1% $NaHCO_3$ / $CaCl_2$, 4°C.
d = $CdCO_3$ / toluene / cholesterol ; e = column chromatography.
f = $CHCl_3$ / sodium ethoxide.

4.3.3   **PREPARATION AND CHARACTERISATION OF CATIONIC CHOLESTERYLGLUCO- AND GALACTOPYRANOSYL LIPOSOMES**

4.3.3.1   Materials

DOPE was purchased from Sigma-Aldrich (St.Louis, MO, USA). 2-[4-(2-hydroxyethyl)-piperazunyl]-ethanesulfonic acid (HEPES), sodium chloride and chloroform were obtained from Merck (Darmstadt, Germany). Formvar coated copper grids were prepared by the Electron Microscope Unit, University of KwaZulu Natal (Pietermaritzburg Campus). Cationic cholesterol derivative (MS09), was prepared as described in Chapter 2. All other reagents were of analytical grade.

4.3.3.2   Formulation of Gluco- and Galactopyranosylcholesterol-Containing Liposomes

Liposome components for each liposome MSαGLU, MSβGLU, MSαGAL and MSβGAL were formulated in a 4:4:1 molar ratio of MS09:DOPE: MSαGLU/MSβGLU/MSαGAL/MSβGAL with DOPE kept at 2 μmol (Table 4.1).

Table4.1 : Liposomal components of Biotinylated liposomes MSαGLU/MSβGLU and MSαGAL/MSβGAL

LIPOSOME FORMULATION	MOLAR RATIOS FOR RESPECTIVE LIPIDS (μmoles)			
	DOPE	MS09	MSαGLU/MSβGLU	MSαGAL/MSβGAL
Liposome MSαGLU/MSβGLU	2	2	0.5	-
Liposome MSαGAL/MSβGAL	2	2	-	0.5

The gluco- and galactopyranosylcholesterol derivatives were first dissolved in pyridine (200 μl) and then added to the other liposomal components. Thereafter $CHCl_3$ (1 ml) was added to dissolve all components and liposomes prepared as described in chapter two, section 2.2.5.2.

4.3.3.3 Characterisation of Gluco and Galacto- Containing Liposomes using Transmission Electron Microscopy:

To one drop of the respective liposome suspension on parafilm was added 0.5% uranyl acetate. This was mixed and allowed to stand for three minutes. The matt surface of formvar coated grids was brought into contact with the liposome - uranyl acetate mixture for 3 minutes. Thereafter discs were air dried overnight and viewed in a Jeol 1010 transmission electron microscope at 60 kV.

4.3.4 LIPOPLEX FORMATION

4.3.4.1 Materials

pGL3 control vector was purchased from Promega Corporation ,Madison,USA. and amplified previously according to manufacturers protocol. pBR322 plasmid DNA was supplied by Roche Diagnostics, Germany. Agarose was obtained from Bio-rad Laboratories, Richmond, CA, USA. Tris-HCl, NaH_2PO_4, HEPES, NaCl, EDTA, SDS, glycerol, bromophenol blue, xylene cyanol, , uranyl acetate and ethidium bromide were purchased from Merck, Darmstadt, Germany. Fetal calf serum was obtained from Delta Bioproducts, Johannesburg, South Africa. All other reagent were of analytical grade. Formvar coated grids were prepared by the Electron Microscope Unit, University of KwaZulu Natal (Pietermaritzburg Campus).

4.3.4.2   Gel Retardation Assays

Varying ratios of the four different liposome:DNA complexes were set up as shown in Table 4.2.   All liposome:DNA complexes were incubated for 30 minutes at room temperature for lipoplex formation. At the end of the incubation, the procedure outlined in section 2.2.6.2 was followed closely.

Table 4.2 :   MSαGLU/MSβGLU and MSαGAL/MSβGAL liposome:DNA complexes. Incubation mixtures (10 μl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained liposome (0 - 5 μg) and pGL3 DNA (0.5 μg), for each of the four liposome formulations respectively.

LIPOPLEX COMPONENTS	1	2	3	4	5	6	7	8
pGL3 DNA (μg)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Liposome (μg)	-	1	1.5	2	2.5	3	3.5	4

4.3.4.3   Dye Displacement Assays

This assay was conducted as outlined in section 2.2.6.3, for all four liposome formulations. Having calibrated the spectrofluorimeter for 0% and 100% fluorescence as described (2.2.6.3), aliquots of each liposome (3 μg), were added, stepwise to the respective solutions until 45 μg of each liposome had been added. Each solution was mixed thoroughly after each addition and fluorescence was measured in a Shimadzu RF-551 spectrofluorometric detector, at an excitation wavelength of 520 nm and an emission wavelength of 600 nm , and at high sensitivity. Results were plotted relative to 100% fluorescence.

4.3.4.4   Transmission Electron Microscopy of lipoplexes

The procedure carried out was the same as described in chapter two, section 2.2.6.5.

4.3.4.5   Nuclease Digestion Assays

Liposome:DNA complexes were set up as in Table 4.3 for all four liposome formulations.

Table 4.3 :   MSαGLU/MSβGLU and MSαGAL/MSβGAL liposome:DNA complexes. Incubation mixtures (10 μl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained cationic liposome (0 - 14 μg) and pGL3 DNA (1 μg).

LIPOPLEX COMPONENTS	1	2	3	4	5	6	7	8
pGL3 DNA (μg)	1	1	1	1	1	1	1	1
Liposome (μg)	-	-	1	2	4	5	6	7

Samples were each made up to a total volume of 10 μl with 20 mM HEPES, 150 mM NaCl, pH 7.5.   Digestion, electrophoresis of samples and gel photography were carried out as described in section 2.2.6.6.

4.3.5 CELL CULTURE AND TRANSFECTION STUDIES

4.3.5.1 Materials

Asialofetuin was obtained from Sigma-Aldrich, USA. All other materials used in the culture of HepG2 and HeLa cells and their transfection are those listed in section 2.2.7.1.

4.3.5.2 Growth and Maintenance of Cell Lines

The HepG2 and HeLa cell lines were propagated in MEM with 10% FCS and antibiotics, and maintained as described in chapter two, section 2.2.7.2.

4.3.5.3 Growth Inhibition Assays

HepG2 and HeLa cells were trypsinised and seeded into a 24 well plate at varying seeding densities. The seeding densities were 2.2×10^4 cells/well for HeLa cells and 1.8×10^4 cells/well for HepG2 cells, for liposomes MSαGLU and MSβGLU, and 2×10^4 cells/well for HeLa cells and 1.9×10^4 cells/well for HepG2 cells, for liposomes MSαGAL and MSβGAL. Wells in row 1 received no cells and were used to obtain a blank (0) reading. Cells were incubated for 24 to 36 hours and allowed to attach to the wells and grow to semi-confluency. The liposome:DNA [pGL3 DNA (1 μg)] complexes for all four of the liposome formulations were set up as outlined in Tables 4.4. The reaction mixtures were each made up to a constant volume with 20 mM HEPES, 150 mM sodium chloride, pH 7.5, and incubated at room temperature for 30 minutes.

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Table 4.4 : MSαGLU/MSβGLU and MSαGAL/MSβGAL liposome:DNA complexes. Incubation mixtures (15 μl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained each liposome (0–7 μg) and pGL3 DNA (1 μg).

LIPOPLEX COMPONENTS	1	2	3	4	5	6
pGL3 DNA (μg)	-	0	1	1	1	1
Liposome (μg)	-	0	4	5	6	7

The cells were prepared as described previously by first removing the growth medium and replacing it with 0.5 ml of serum free medium. The reaction complexes were then added to the wells containing the cells. The assays were carried out in quadruplicate. After a 4 hour incubation at 37°C, the medium was replaced with complete medium (MEM + 10% FCS + antibiotics). The cells were incubated again at 37°C for a further 48 hours and quantified as explained in chapter two, section 2.2.7.3.

4.3.5.4   Transfection Studies
        (a)   Liposome MSαGAL : pGL3 DNA complexes
        (b)   Liposome MSβGAL : pGL3 DNA complexes
        (c)   Liposome MSαGLU : pGL3 DNA complexes
        (d)   Liposome MSβGLU : pGL3 DNA complexes

Transfection of all four lipoplexes were conducted using two cell lines viz. the HepG2 cell line that contains the asialoorosomucoid receptor, and the HeLa cell line which does not, and may be considered an appropriate control cell line. All cells were trypsinised and seeded at varying densities in 24 well plates. The cells were allowed time to attach to the wells and to grow to semi-confluency. The transfection complexes (a – d) were then set up in quadruplicate for both cell lines as shown in Tables 4.5. The transfection efficiencies of all four liposome:DNA complexes (a – d) in the HepG2 cell line was evaluated in the absence of serum, the presence of 10% serum and in the presence of an excess (300 μg) asialofetuin. Furthermore transfection was monitored in the HeLa cell line as a control.

Table 4.5 : Transfection complexes as added to 24-well plates. Control 1 contained only cells, control 2 contained cells and pGL3 DNA (1 µg) only. Test samples 1 to 4 contained varying amounts of each liposome (4,5,6 and 7 µg respectively). pGL3 DNA was kept constant at 1 µg. All complexes were made up in a final volume of 15 µl with 20 mM HEPES, 150 mM sodium chloride (pH 7.5).

Reaction Components	Control 1	Control 2	Test 1	Test 2	Test 3	Test 4
HeLa / HepG2 Cells	+	+	+	+	+	+
Liposome (µg)	-	-	4	5	6	7
pGL3 DNA (µg)	-	1	1	1	1	1

(i)   Liposome MSαGAL/ MSβGAL : pGL3 DNA complexes

Transfection complexes were added to 24-well plates containing cells at a seeding density of 1.9×10^4 cells/well for HeLa cells, and 1.8×10^4 cells/well for HepG2 cells. For transfection in the presence of 10% FCS and competing (300 µg) asialofetuin, the seeding densities for the HepG2 cells were 2×10^4 cells/well and 1.9×10^4 cells/well respectively.

(ii)   Liposome MSαGLU/ MSβGLU : pGL3 DNA complexes

Transfection complexes were added to 24-well plates containing cells at a seeding density of 2×10^4 cells/well for HeLa cells, and 1.9×10^4 cells/well for HepG2 cells. For transfection in the presence of 10% FCS and competing (300 µg) asialofetuin, the seeding densities for the HepG2 cells were 2×10^4 cells/well and 1.8×10^4 cells/well respectively.

4.4 RESULTS AND DISCUSSION

4.4.1 PREPARATION OF CHOLESTERYL GLYCOSIDES

Four cholesteryl glycosides (MSαGLU, MSβGLU, MSαGAL and MSβGAL) were synthesized for the study of their asialoorosomucoid receptor-targeting potential when incorporated into the bilayer of cationic liposomes. The hydrophobic perhydrocyclopentanophenanthrene fused ring system of cholesterol interacts with the other hydrophobic membrane components in the liposome bilayer while the 3β-OH group, which would ordinarily project into the hydrophilic environment, has been used to forge a glycosidic link with the α and β anomers of D-glucose and D-galactose. The syntheses were effected by a Koenigs-Knorr procedure from the appropriately protected bromo-sugar and cholesterol under anhydrous conditions (Rogers and Thevan, 1986). During the course of this project, Wimmer and co-workers reported a similar procedure for the preparation of glucoside and galactoside derivatives of cyclic (secondary) alcohols in good yield using cadmium carbonate as promoter under anhydrous conditions (Wimmer et al., 2004).

Structures were confirmed by reference to ^{1}H and ^{13}C NMR spectra recorded in CDCl$_3$ for the intermediate cholesteryl-tetra-O-acetylglycosylpyranosides and in C$_5$D$_5$N (deuteropyridine) for the deprotected final products. ^{13}C signal assignments for the cholesteryl moieties were very similar to one another in the eight cholesteryl glycosides generated and closely resembled assignments reported previously in a study of glycosidation shifts in ^{13}C NMR spectra of related sterylglycosides (Seo et al., 1978). ^{1}H NMR spectra of the carbohydrate components were very similar to those reported by Agrawal (1992), while the ^{13}C NMR spectra of the tetra-O-acetylglucoside and galactoside components were very similar to those reported by Seo et al., 1978. ^{1}H peak assignments for the tetra-O-acetyl-β-D-galactoside entity were virtually identical to those reported by Ren et al., 2001, in a study of galactosylated dendrimers containing multiple primary amino groups.

4.4.2 PREPARATION AND CHARACTERISATION OF GLUCO- AND GALACTOPYRANOSYLCHOLESTEROL CONTAINING LIPOSOMES

Each of the four liposomes contained the cationic cholesterol derivative MS09 (chapter two), the neutral lipid DOPE and one of the four prepared glyco-containing cholesterol derivatives viz. cholesteryl-α-D-glucopyranoside, cholesteryl-β-D-glucopyranoside, cholesteryl-α-D-galactopyranoside, and, cholesteryl-β-D-galactopyranoside.

Liposomes prepared here are constituted from MS09, DOPE and cholesterylglycosides. The glycoside component has been limited to 9% on a $^w/_w$ basis, a level at which it is estimated that sufficient targeting character will be expressed. Other ratios were not explored. It is apparent, however, that the sugar density in a liposome/glycolipid is critical. It was observed that at a loading ratio of 5% ($^w/_w$) the liposome/glycolipid particles are efficiently processed by the asialoglycoprotein receptor, whereas at a loading ratio of 50% ($^w/_w$) uptake was not blocked by excess asialofetuin, suggesting a less specific uptake of the liposome/glycolipid particles (Davis and Robinson, 2002). A large number of galactose or glucose residues could interfere with the electrostatic interaction of the liposome with DNA. Investigations by Nishikawa *et al.*, 1998, using poly-L-lysine showed that the optimal modification of the poly-L-lysine with galactose was between 14%-16%.

These liposomes are bifunctional with each liposome possessing a cationic component (MS09) for binding to the DNA molecule and a terminal galactose or glucose component for targeting to the galactose or asialoglycoprotein receptor on the HepG2 cells (Figure 4.3). All liposome formulations were stable at 4°C for several weeks and showed no aggregation or precipitation of components.
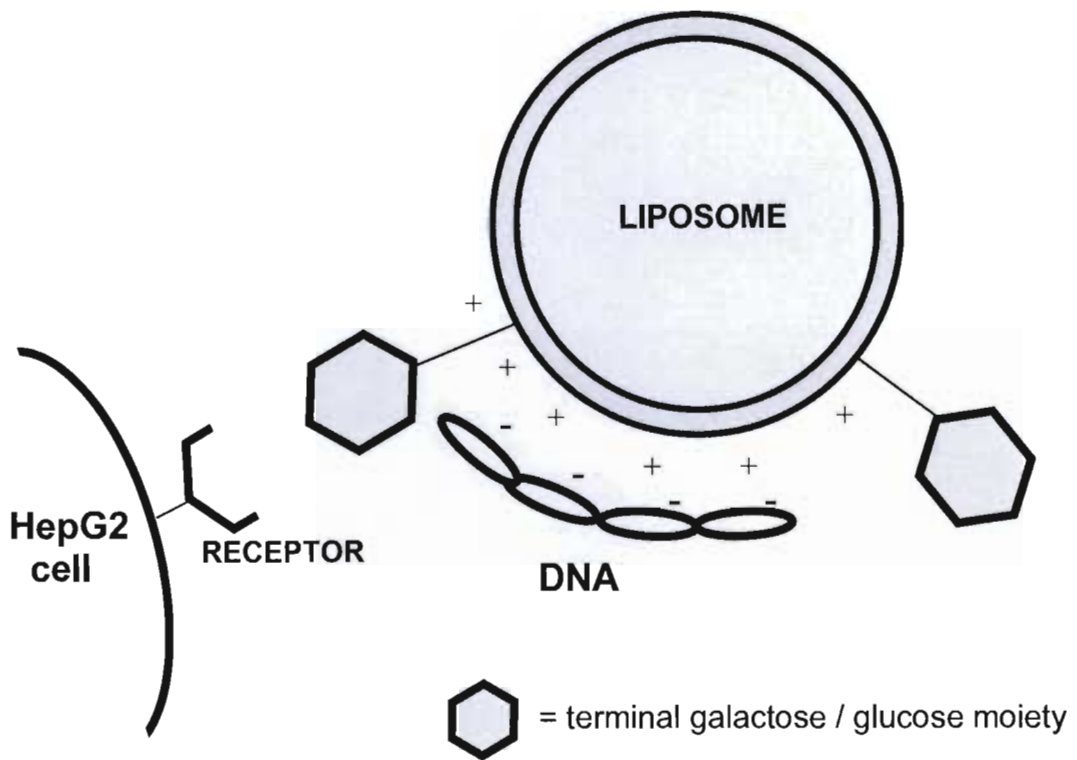
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Figure 4.3 : Schematic representation of the cationic gluco / galacto containing liposomes and their interaction with the negatively charged DNA molecule and the cell surface receptor on the HepG2 cells. Diagram not drawn to scale.

4.4.2.1 Characterisation of Gluco and Galacto-Containing Liposomes using Transmission Electron Microscopy

Transmission electron microscopy revealed the formation of predominantly spherical or oval vesicles and confirmed the unilamellar nature of all liposome preparations. Liposome MSαGAL sizes varied from 100 nm to 200 nm, liposome MSβGAL from 50 nm to 150 nm, liposome MSαGLU from 50 nm to 200 nm, and MSβGLU from 100 to 200 nm (Figures 4.4 to 4.7).
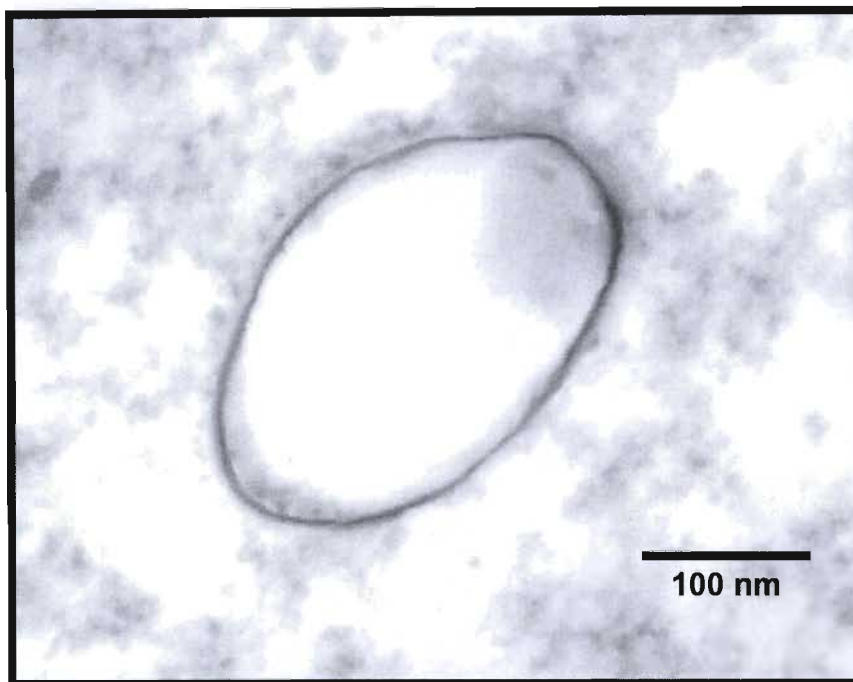
Figure 4.4 : TEM of liposome MSαGAL. Liposome size varies from 100 nm to 200 nm.
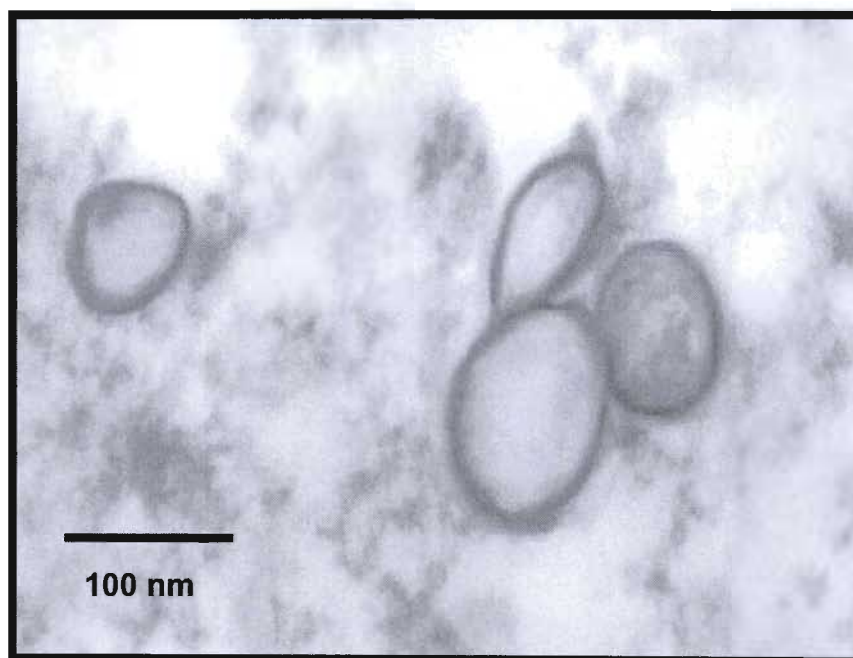
Figure 4.5 : TEM of liposome MSβGAL. Liposome size varies from 50 nm to 150 nm.
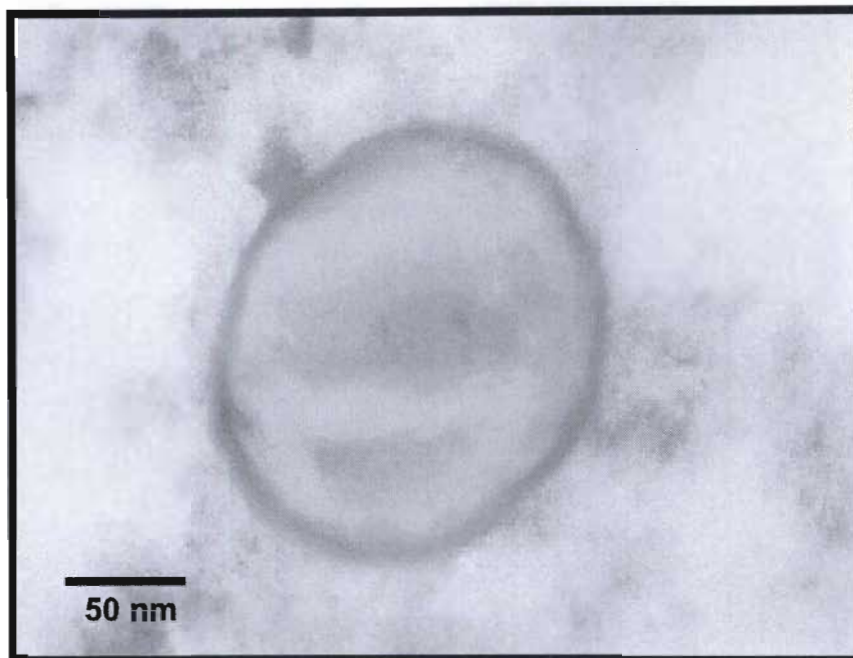
Figure 4.6 : TEM of liposome MSαGLU. Liposome size varies from 50 nm to 200 nm.
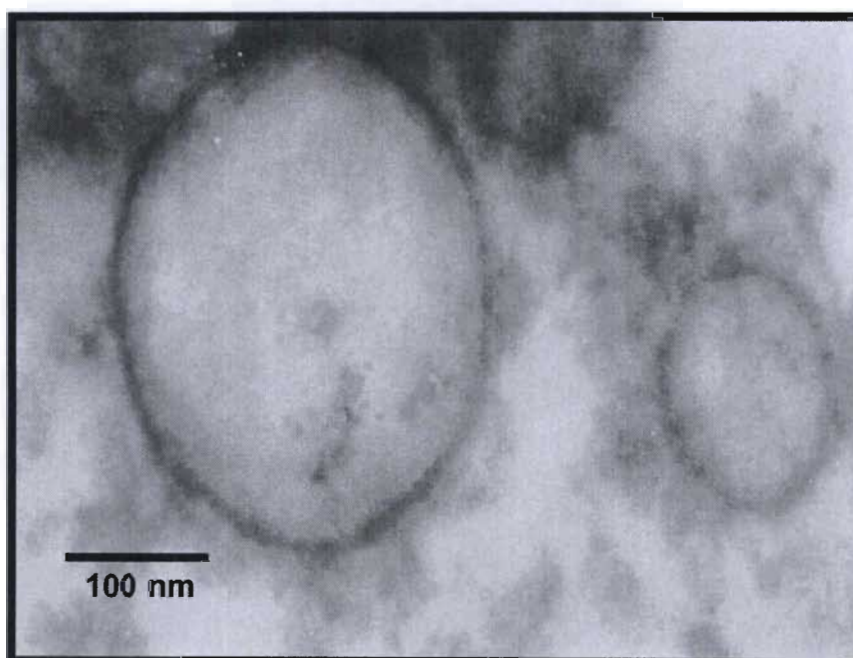

Figure 4.7 : TEM of liposome MSβGLU. Liposome size varies from 100 nm to 220 nm.

4.4.3 LIPOPLEX FORMATION OF LIPOSOMES

4.4.3.1 Gel Retardation Assays

In order to determine the optimal ratio for binding between the liposome and the DNA, gel retardation assays are commonly performed. These can be seen in Figures 4.8-4.9.
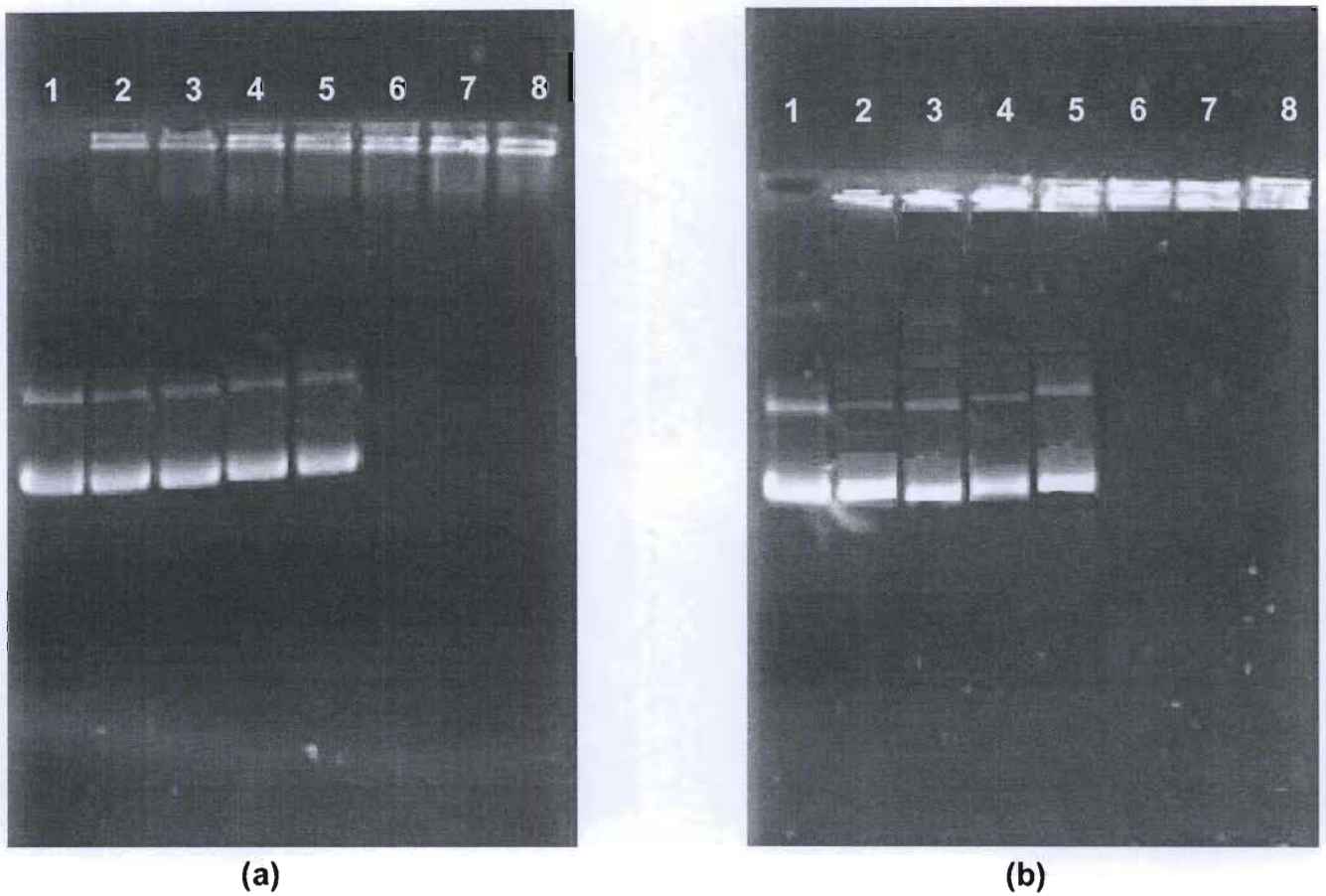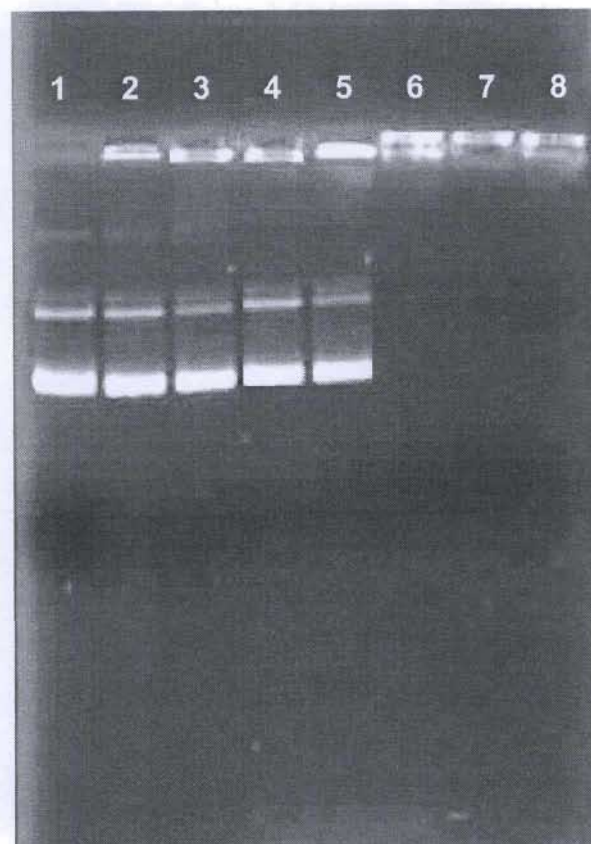

(a)   (b)

Figure 4.8 : Gel retardation study of (a) MSαGAL liposome : DNA complexes and (b) MSβGAL liposome : DNA complexes.
Incubation mixtures (10 μl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained varying amounts of liposome in lanes 1-8 (0,1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 μg), while the pGL3 DNA was kept constant at 0.5 μg.

Figure 4.9 : Gel retardation study of (a) MSαGLU liposome : DNA complexes and (b) MSβGLU liposome : DNA complexes.
Incubation mixtures (10 µl) in 20 mM HEPES, 150 mM NaCl (pH 7.5) contained varying amounts of cationic liposome in lanes 1-8 (0,1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 µg), while the pGL3 DNA was kept constant at 0.5 µg.

No migration of the plasmid DNA was seen at and above the liposome:DNA ratio of 6:1 ($^W/_w$) equivalent to a charge ratio (+/-) of 0.7. This lack of migration is an indication that the plasmid DNA is completely bound by the cationic component of the liposome, resulting in large complexes that do not migrate into the gel.

4.4.3.2   Dye Displacement Assays

These results strongly support the notion that all four lipoplexes which have been formulated to contain the synthesized glycolipids readily bring about displacement of the intercalated ethidium bromide upon stepwise introduction of liposomes to the plasmid DNA. This process is largely driven by the association of DNA phosphodiester negative charges forming ion pairs with the cationic headgroups of MS09 (Figures 4.10 and 4.11)

The fluorescence decreased steadily until the point was attained which seemed to correlate with total retardation seen for lipoplexes in gel retardation assays. The maximum displacement of ethidium bromide as measured by fluorescence was approximately 45% for liposome MSαGAL, 36% for MSβGAL2, 47% for MSαGLU and 38% for MSβGLU) at the total retardation ratio of liposome:DNA of 6:1 ($^W/_w$) and a corresponding charge ratio (+/-) of 0.7. As can be seen, MSβGAL and MSβGLU liposomes both displayed higher displacement of the dye than MSαGAL and MSαGLU. This could be due to the fact that they are both β-anomers and somehow allow greater accessibility to the DNA than their corresponding α-anomers.

No further addition of liposome was carried out after 45 µg due to the onset of turbidity which would have rendered the readings invalid. From the liposome:DNA ratio of 6:1 ($^W/_w$) the graph seems to reach a plateau as liposome:DNA binding had apparently reached its optimum, resulting in no further compacting of the double stranded nucleic acid taking place.
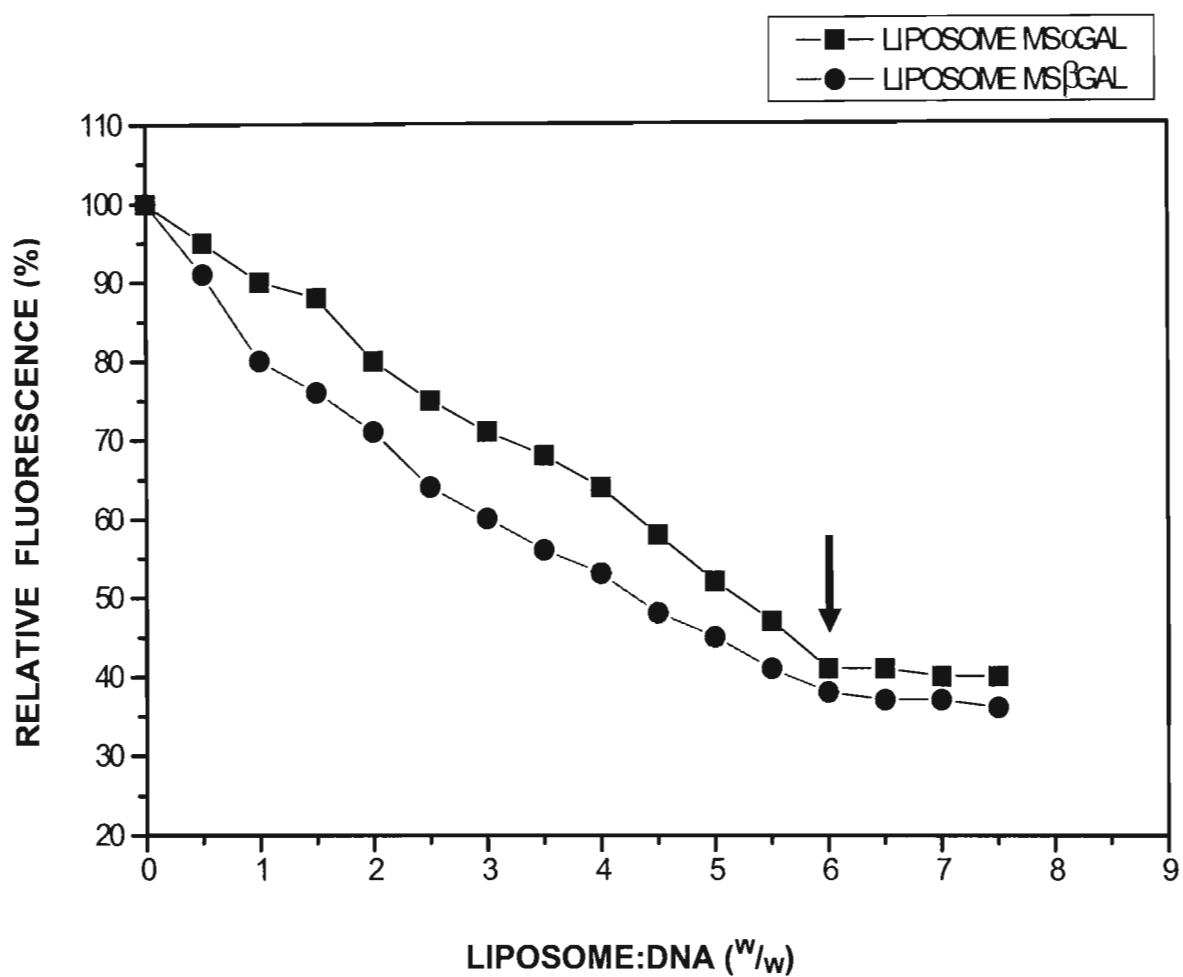
Figure 4.10 : Ethidium bromide intercalation assay for galactosylated liposomes
MSαGAL and MSβGAL at varying liposome:DNA ($^{w}/_{w}$) binding ratios.
Cuvettes contained 6 µg of pBR322 DNA and increasing quantities of
liposomes at pH 7.5 (section 4.2.4.3)

Figure 4.11 : Ethidium bromide intercalation assay for glucosylated liposomes
MSαGLU1 and MSβGLU2 at varying liposome:DNA ($^w/_w$) binding ratios.
Cuvettes contained 6 μg of pBR322 DNA and increasing quantities of
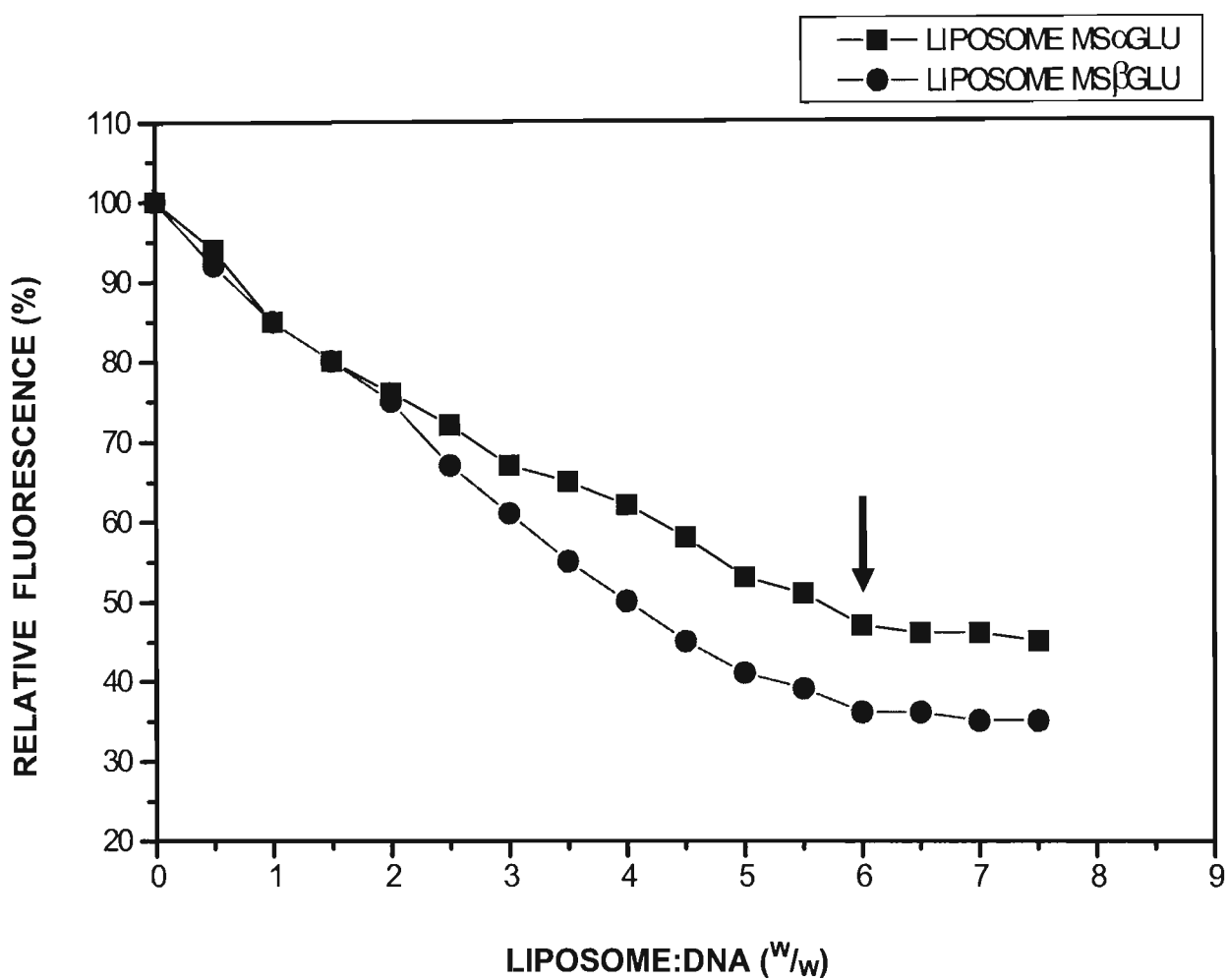liposomes at pH 7.5 (section 4.2.4.3)

4.4.3.3   Transmission Electron Microscopy of lipoplexes

The biodistribution of current carrier systems is generally influenced to a large extent by their charge and importantly by their size. The sizes obtained for the lipoplexes are of great importance especially if these liposomes are to be used for *in vivo* studies. For most gene therapy applications, the target cells will form part of the parenchyma or interstitium of an organ, with access to these cells restricted for complexes after vascular administration. Most complexes will only enter certain sites, e.g. the liver or spleen where suitable gaps or fenestrae are present to allow specific sized complexes to pass through (Schätzlein, 2003). In the case of liver cells, complex sizes are restricted due to the size of these fenestrae. The pore or fenestrae sizes of liver sinusoidal microvessels are between 200 nm and 300 nm in diameter and larger lipoplexes or liposomes of about 300 nm in diameter or greater are accumulated in a time-dependent manner in the liver due to trapping of the liposomes in the reticuloendothelial system (RES) (Oku *et al.*, 2000). Thus the size of lipoplexes is an important property to consider for gene delivery *in vivo*, since it can limit access to target organs and distribution to specific tissues or cells.

It is assumed that the degree of substitution by sugar residues in a liposome could affect the complex / lipoplex size. A high degree of substitution will generally increase the size of the complex and thus limit transfection efficiency (Palliard, 1999). In our liposomes the galactose and glucose components were kept to a minimum. This could account for the smaller sizes of lipoplexes seen under EM. In our study the lipoplex sizes for all four complexes ranged from 50 nm to an upper limit of 200 nm at higer charge ratios (Figures 4.12 to 4.15). At a liposome:DNA ratio of 5:1 ($^{w}/_{w}$), +/- charge ratio of 0.6, the diameter of all four lipoplexes ranged from 50 nm to 100 nm, whereas at a a higher liposome:DNA ratio of 6:1 ($^{w}/_{w}$),+/- charge ratio of 0.7, lipoplex diameters ranged from 80 to 200 nm. Lipoplex morphology resembled aggregates or clusters similar to those of the cationic lipoplexes in section 2.3.3.4.
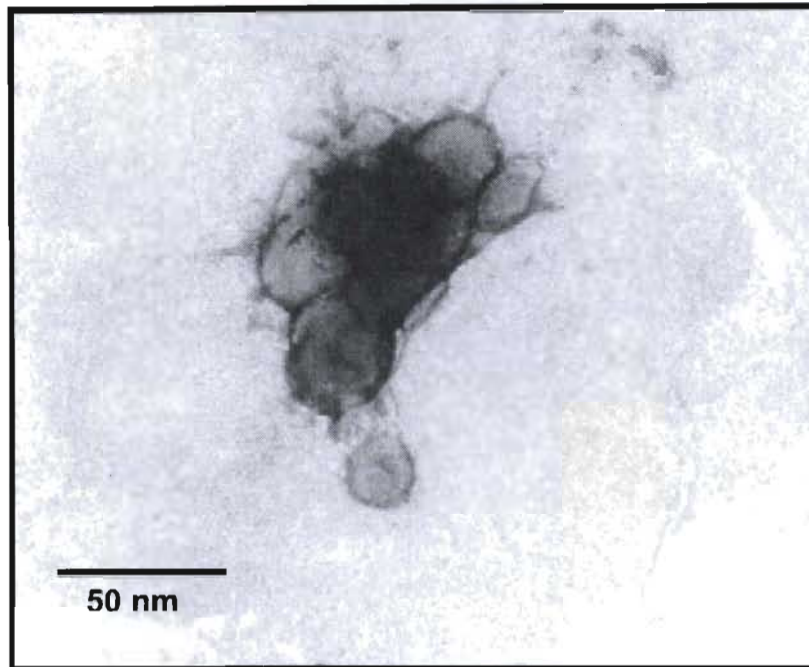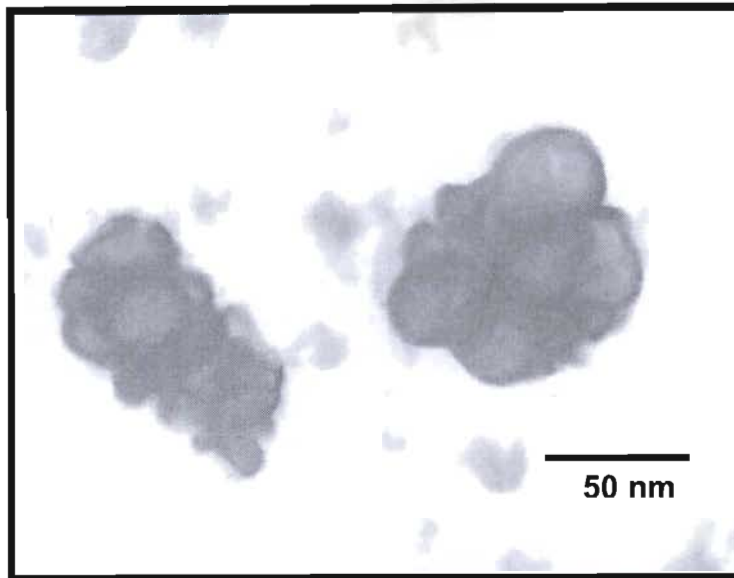
(a)
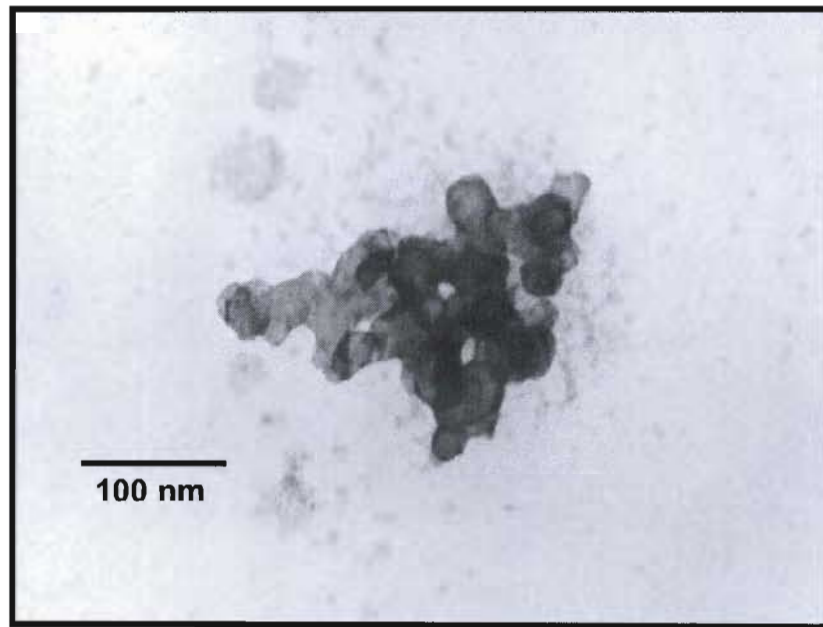
(b)

Figure 4.12 : TEM of complex between liposome MSαGAL and pGL3 DNA at
(a) liposome:DNA ratio of 5:1 ($^w/_w$), and,
(b) liposome:DNA ratio of 6:1($^w/_w$).

(a)

(b)

Figure 4.13 : TEM of complex between liposome MSβGAL and pGL3 DNA at
(a) liposome:DNA ratio of 5:1 ($^{w}/_{w}$), and,
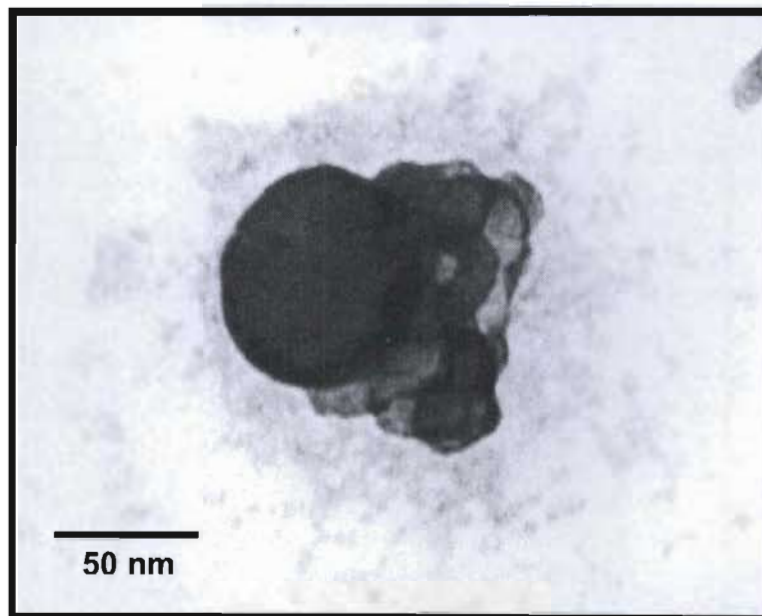(b) liposome:DNA ratio of 6:1($^{w}/_{w}$).

(a)

(b)

Figure 4.14 : TEM of complex between liposome MSαGLU and pGL3 DNA at
(a) liposome:DNA ratio of 5:1 ($^w/_w$), and,
(b) liposome:DNA ratio of 6:1($^w/_w$).

(a)

(b)

Figure 4.15 : TEM of complex between liposome MSβGLU and pGL3 DNA at
(a) liposome:DNA ratio of 5:1 ($^w/_w$), and,
(b) liposome:DNA ratio of 6:1($^w/_w$).
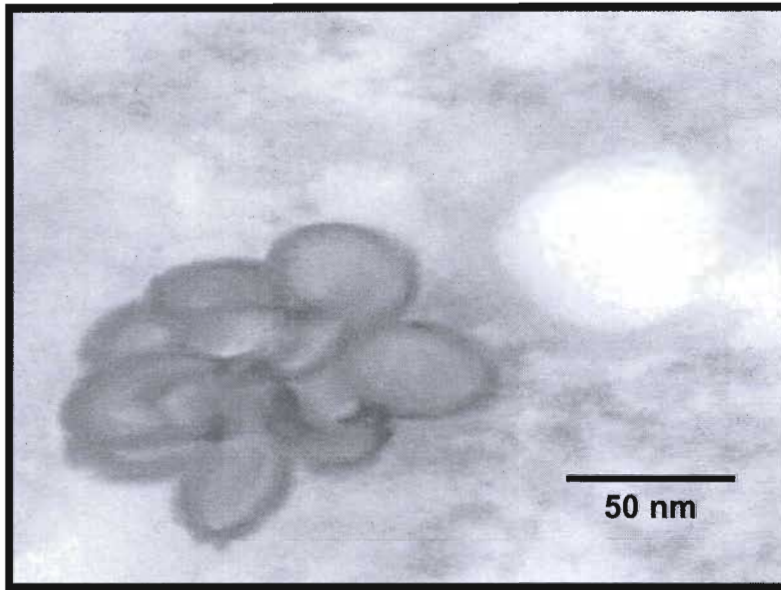
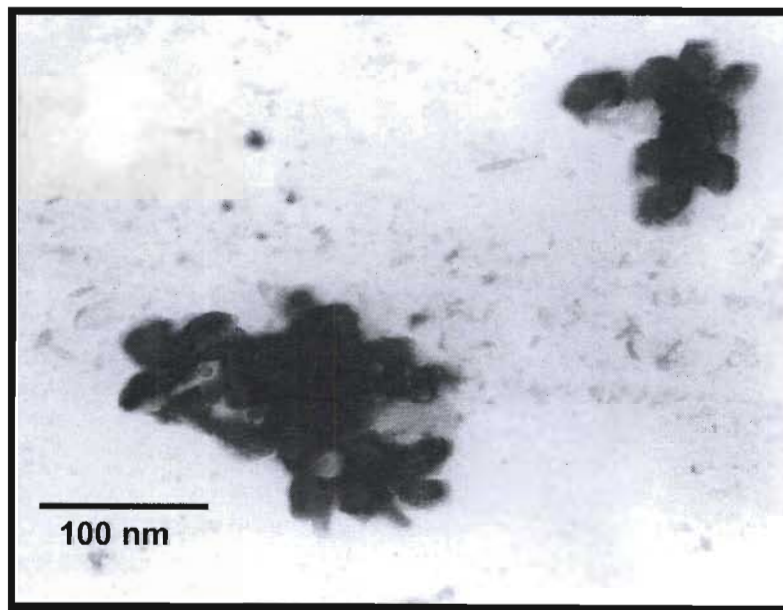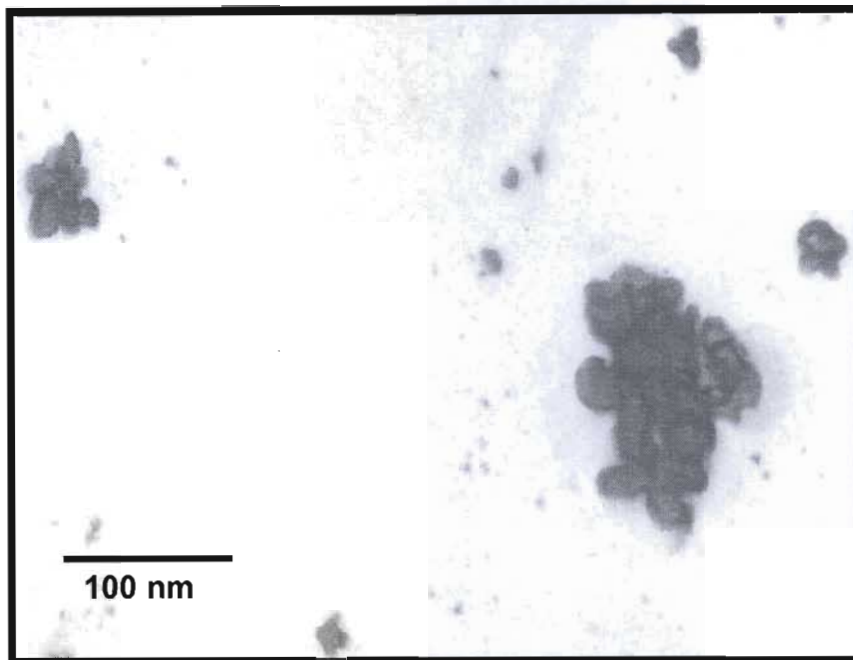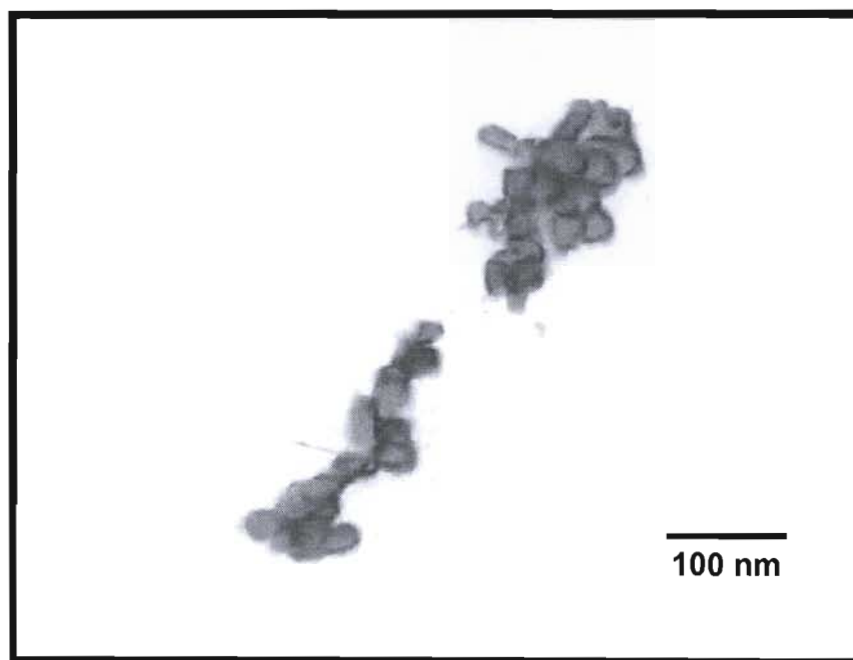Reports on the complex size that can be accommodated by the asialoglycoprotein receptor have been varied. Some studies have suggested that the hepatocyte asialoglycoprotein receptor has an upper size limitation for the uptake of a specific ligand of about 10 nm (Schelepper-Schäfer *et al.*, 1986) or 21-23 nm (Bijsterbosch and van Berkel, 1992) in diameter, depending on the type of artificial ligand used. Another proposal is that glycoparticles with diameters greater than 70 nm are not easily recognized by the asialoglycoprotein receptor (Rensen *et al.*, 2001). Kawakami *et al.*, 2002, suggested that a glycoplex size must be 150 nm or less for hepatocyte targeting. Our lipoplexes at the optimal transfection ratio (see 4.4.4.3) of liposome:DNA (5:1 $^w/_w$) were no larger than 100 nm in diameter and this size seemed to be taken up quite readily by the receptors on the HepG2 cells as determined by the significant gene expression observed at this ratio during transfection studies. Even larger lipoplexes (>100 nm) seen at a liposome:DNA ratio of 6:1 ($^w/_w$), afforded good receptor-mediated gene expression (4.4.4.3). Hence it may be concluded that the size limit of a lipoplex that can be accommodated by the asialoglycoprotein receptor remains an unsettled issue.

For *in vivo* systems, this size preference is assumed to be related to the existence of another receptor for galactosylated proteins in the Kupffer cells of the liver. The Kupffer cell receptor is very efficient in taking up and degrading galactosylated molecules of larger size *in vivo* and competes for the uptake of the galactosylated DNA complex with the asialoglycoprotein receptor on the surface of hepatocytes. Thus, the diameter of the ligand-DNA complex must be relatively small to be effective in transferring genes specifically to hepatocytes by receptor-mediated endocytosis via the asialoglycoprotein receptor (Perales *et al.*, 1997).

The endocytotic vesicles with a clathrin coating are reported to have an internal diameter of about 100 nm in hepatocytes (Molas *et al.*, 2003). Larger complexes could be endocytosed, but a correlation could exist between the structure of the DNA complex and the efficiency and specificity for receptor-mediated gene transfer by the asialoglycoprotein receptor. Furthermore it is suggested that for *in vivo* systems uptake

of particles of about 100 nm is dependent on the blood pressure within the liver (Pouton and Seymour, 2001). It is thought that the belief that 100 nm or smaller vehicles are most effective for systemic delivery is only true for inflexible delivery vehicles. Blood cells of several microns (up to 7000 nm) in size, have no difficulty in circulating in the blood and even in the smallest capillaries, emphasizing the point that flexibility of the vehicle is of great importance. Furthermore increased lipoplex size tends to extend their circulation time *in vivo* (Templeton, 2003).

Since the size and structure of the lipoplex is critical for receptor-mediated gene transfer into target tissues in animals, it is important that the condensation process does not result in the formation of large aggregated, multimolecular DNA complexes but rather into unimolecular complexes of a minimum size. It was observed under EM that when the charge on the DNA is totally neutralized (charge ratio of 1:1) a turbid solution consisting of a fine precipitate of DNA complexes is produced that are large aggregates of smaller units that are hypothesized to be highly condensed, unimolecular DNA complexes (Perales *et al.*, 1997)..

It was concluded that despite the disparity in the sizes reported in the different studies due to utilization of different ligands, assays and assay conditions, the size or volume of the gene or drug to be enveloped by the receptor will be important for the process to be successful and specific (Molas *et al.*, 2003).

4.4.3.4 Nuclease Digestion Assays

These studies enabled us to further characterise the strong binding interaction between the liposomes and the DNA and to observe what protection is afforded by the cationic liposomes to the bound DNA. The effect of the serum nucleases on each of the four lipoplexes can be seen in Figures 4.16 to 4.18 respectively.

Figure 4.16 : Nuclease protection assay of liposome MSαGAL and pBR322 DNA.
Lane 1 : undigested plasmid pBR322 DNA (1 μg)
Lane 3: unprotected plasmid DNA (1 μg) digested by serum nucleases
Lanes 2-8 : Varying amounts of cationic liposomes (1, 0, 2, 4, 5, 6, 7 μg)
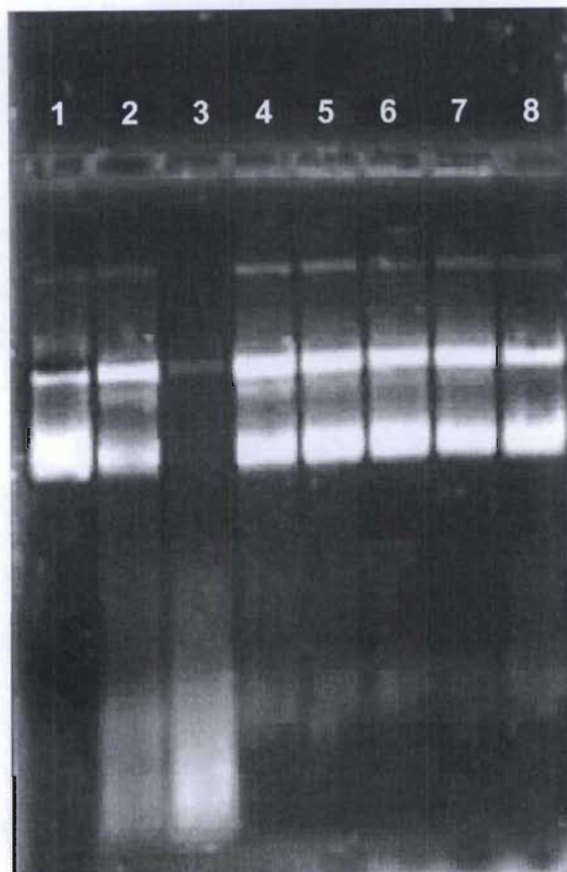with pBR322 DNA (1 μg) and serum (10%).

Figure 4.17 : Nuclease protection assay of liposome MSβGAL and pBR322 DNA.
Lane 1 : undigested plasmid pBR322 DNA (1 µg)
Lane 2 : unprotected plasmid DNA (1 µg) digested by serum nucleases
Lanes 2-8 : Varying amounts of cationic liposomes (0, 1, 2, 4, 5, 6, 7 µg)
with pBR322 DNA (1 µg) and serum (10%).
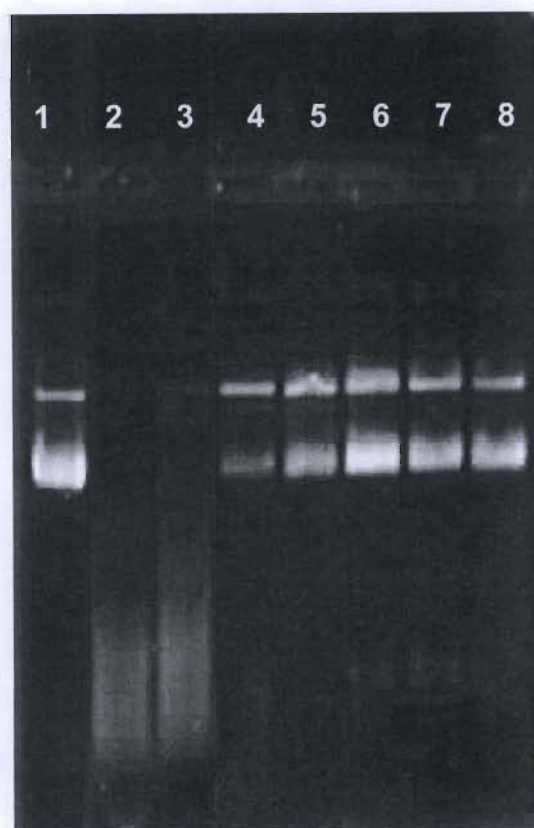
Figure 4.18 :   Nuclease protection assay of (a) liposome MSαGLU and pBR322 DNA,
and (b) liposome MSβGLU and pBR322 DNA.
Lane 1 : undigested plasmid pBR322 DNA (1 μg)
Lane 2 : unprotected plasmid DNA (1 μg) digested by serum nucleases
Lanes 2-8 : Varying amounts of cationic liposomes (0, 1, 2, 4, 5, 6, 7 μg)
with pBR322 DNA (1 μg) and serum (10%).

Generally good protection was afforded by the liposomes to the DNA at most liposome:DNA ratios. However, the greatest degradation of the DNA was noted for lipoplexes with a liposome:DNA ratio of 1:1 ($^w/_w$), especially for The MSαGLU and MSβGLU liposomes. This may be attributed to the low liposome:DNA (+/-) charge ratio of 0.1 at which stage there may be free unbound DNA molecules present. Indeed at such a low ratio it is anticipated that liposome-bound DNA may also be susceptible to serum nuclease digestion as condensation may be incomplete. At higher charge ratios it can be seen that the DNA was well protected within the lipoplexes, although a small but discernable amount of degradation can be seen on the gels. It is noteworthy that this degradation occurs to a lesser degree with liposomes containing the β-glycosides suggesting that the DNA in these lipoplexes may benefit from greater protection. This observation tallies with the finding that the same β-glycoside-containing liposomes displace more ethidium bromide from DNA than their corresponding α-glycosides suggesting, perhaps, greater condensation of the DNA in lipoplexes containing the β-glycosides.

The adsorption of serum proteins can induce a number of effects such as complex destabilization, aggregation or retargeting. The stability of lipoplexes or polyplexes depends on the strength of the electrostatic interaction, the total charge and the charge density of the carrier molecule (Schätzlein, 2003).

In liver tissue a large proportion of acid DNAses are located in the sinusoidal cells (Wattiaux *et al.*, 2000). This will be an important consideration in future *in vivo* studies since these liposomes seem to afford reasonable protection of the DNA to nucleases.

4.4.4 CELL CULTURE AND TRANSFECTION STUDIES

4.4.4.1 Growth and Maintenance of Cell Lines

The HepG2 and HeLa cells (Figure 2.25 and 2.23), were successfully propagated in MEM as described in chapter two. The cells reached confluency after 2-3 days. Both cell lines were routinely subdivided into 3:1 or 2:1 splits after trypsinisation for experimental purposes.

4.4.4.2 Growth Inhibition Assays

High toxicity of liposomes or lipoplexes will limit their application *in vivo*. Toxicity *in vivo* can arise, in part, due to the opsonisation with serum components and their interaction with cells such as macrophages (Monteir *et al.*, 2004). The ultimate goal of the development of any novel gene delivery vector will be to ascertain its efficiency in *in vivo* systems. Hence results obtained in these growth inhibition studies are of extreme importance.

In vivo lipoplex-mediated toxicity is closely associated with charge ratio between the cationic lipid in the liposome and the DNA, as well as the dose of lipoplex administered. Higher charge ratios are toxic to a variety of cell types including cancer cell lines (Dass, 2002). In our *in vitro* studies all four liposome preparations showed extremely low toxicity to both the HepG2 and HeLa cell lines *in vitro* (Figures 4.19 and 4.20).

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Figure 4.19 : Growth inhibition studies of liposomes MSαGAl, MSβGAL, MSαGLU and MSβGLU:DNA complexes to HepG2 cells *in vitro* . All liposomes were varied (0, 4, 5 ,6 ,7 µg) while the DNA (1 µg) was kept constant in a total volume of 0.5 ml MEM. A control sample (no liposome), containing only cells was assumed to have 100% survival. Data are presented as means ± S.D (n = 4).

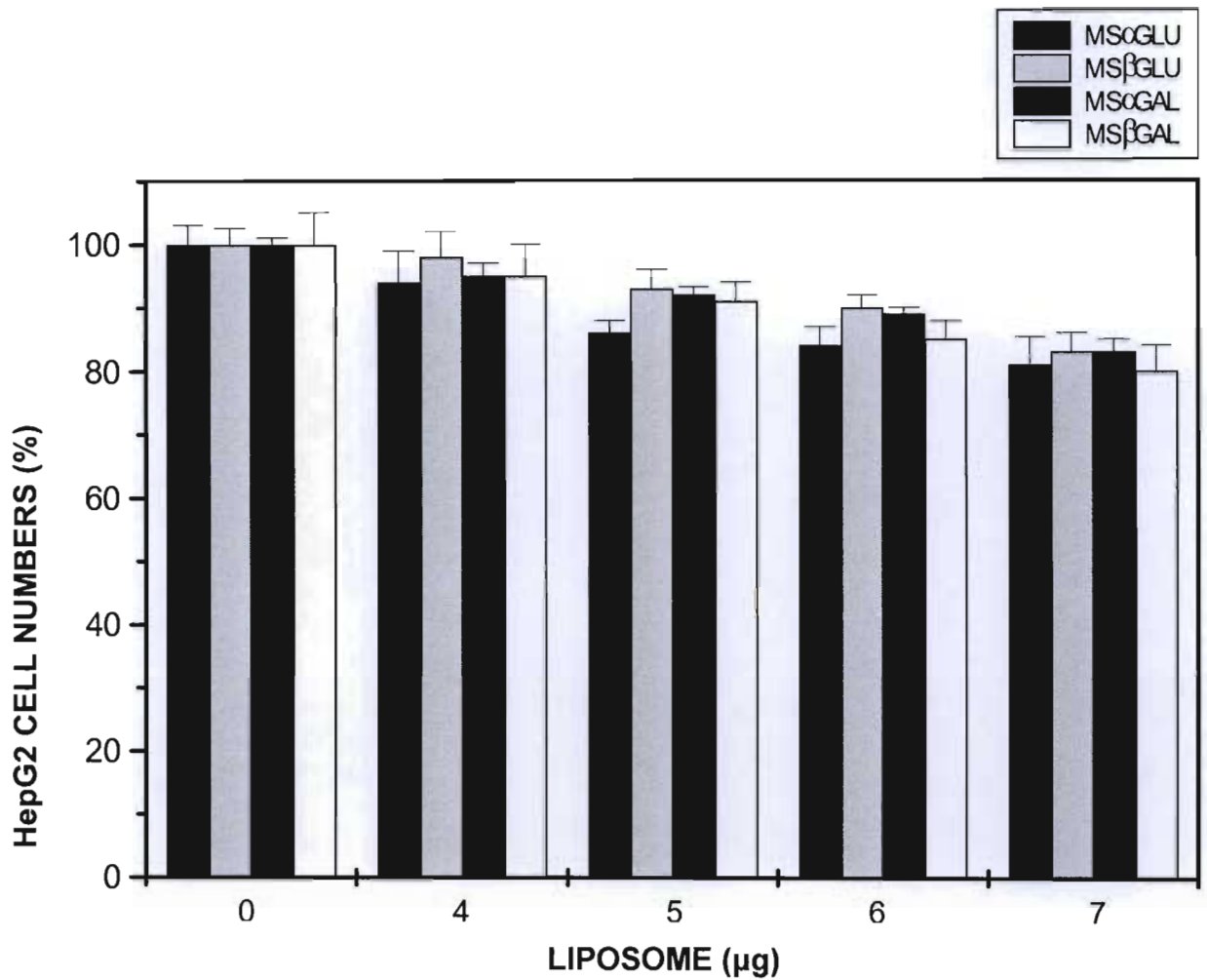**Figure 4.20 :** Growth inhibition studies of liposomes MSαGAl, MSβGAL, MSβGLU and MSβGLU:DNA complexes to HeLa cells *in vitro* . All liposomes were varied (0, 4, 5 ,6 ,7 µg) while the DNA (1 µg) was kept constant in a total volume of 0.5 ml MEM. A control sample (no liposome), containing only cells was assumed to have 100% survival. Data are presented as means ± S.D (n = 4).
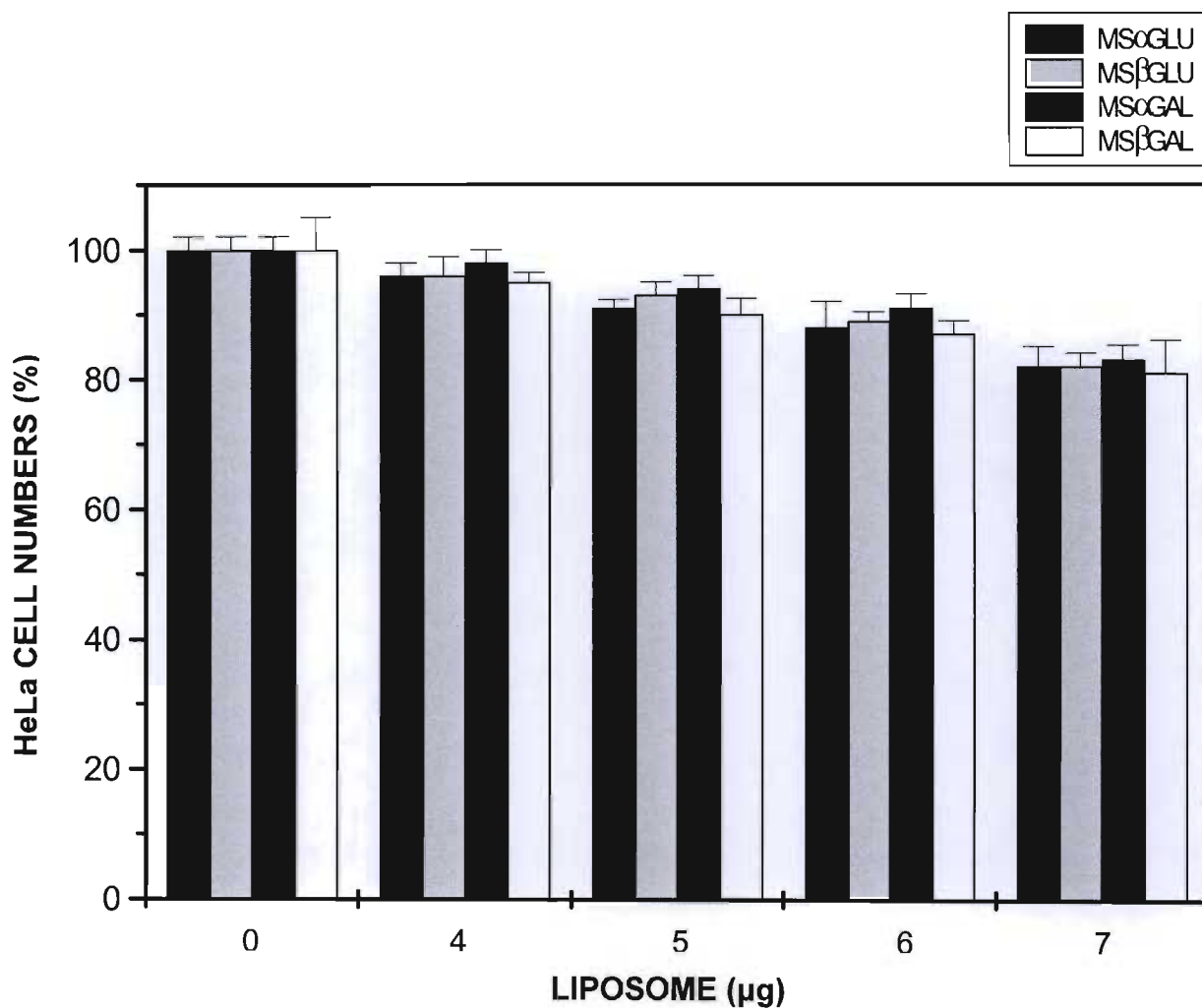
Cells were exposed to the same lipoplex concentrations utilized in transfection studies. Maximum cell death recorded for liposome MSαGAL was 16% for HepG2 cells and 17% for HeLa cells, while for liposome MSβGAL the maximum cell death recorded was 17% for both HepG2 and HeLa cells. Liposome MSαGLU showed a maximum cell death of 18% for HepG2 cells and 17% for HeLa cells and liposome MSβGLU recorded a maximum cell death 16% for HepG2 cells, 17% for HeLa cells. These toxicity levels were observed at the highest liposome:DNA ratio of 7:1 ($^w/_w$). At optimal transfection ratios (5:1) the inhibition level was 8% and 9% for liposomes MSαGAL and MSβGAL respectively, and 14% and 7% for liposome MSαGLU and MSβGLU respectively.

Hence it can be stated that the cytotoxicity levels did not vary much with the different liposome formulations and also with the two different cell lines being utilised. As the liposome concentrations increased so did the inhibition of cell growth by the lipoplexes.

4.4.4.3   Transfection Studies

When galactose is attached to the surface of a transfecting complex, the complex may be internalized by hepatocytes via the asialoglycoprotein receptor at a much faster rate than that of the untargeted vector (Nishida *et al.*, 1992). This could be an advantage in DNA and drug delivery. The duration of gene expression in transiently transfected cells is generally dependent on the proliferation rate of the cells. The results obtained for the transient gene expression of our four glycosylated liposome formulations can be seen in Figures 4.21 to 4.24.

Figure 4.21 : Transfection studies of liposome MSαGAL:DNA complexes with HepG2 cells *in vitro*. Competition experiments with HepG2 cells included 300 μg asialofetuin per well. Liposome MSαGAL was varied (0, 4, 5 , 6 , 7 μg) while the DNA (1 μg) was kept constant in a total volume of 0.5 ml MEM. Control 1 contains only cells and control 2 contains naked DNA and no liposome. Data are presented as means ± S.D (n = 4).
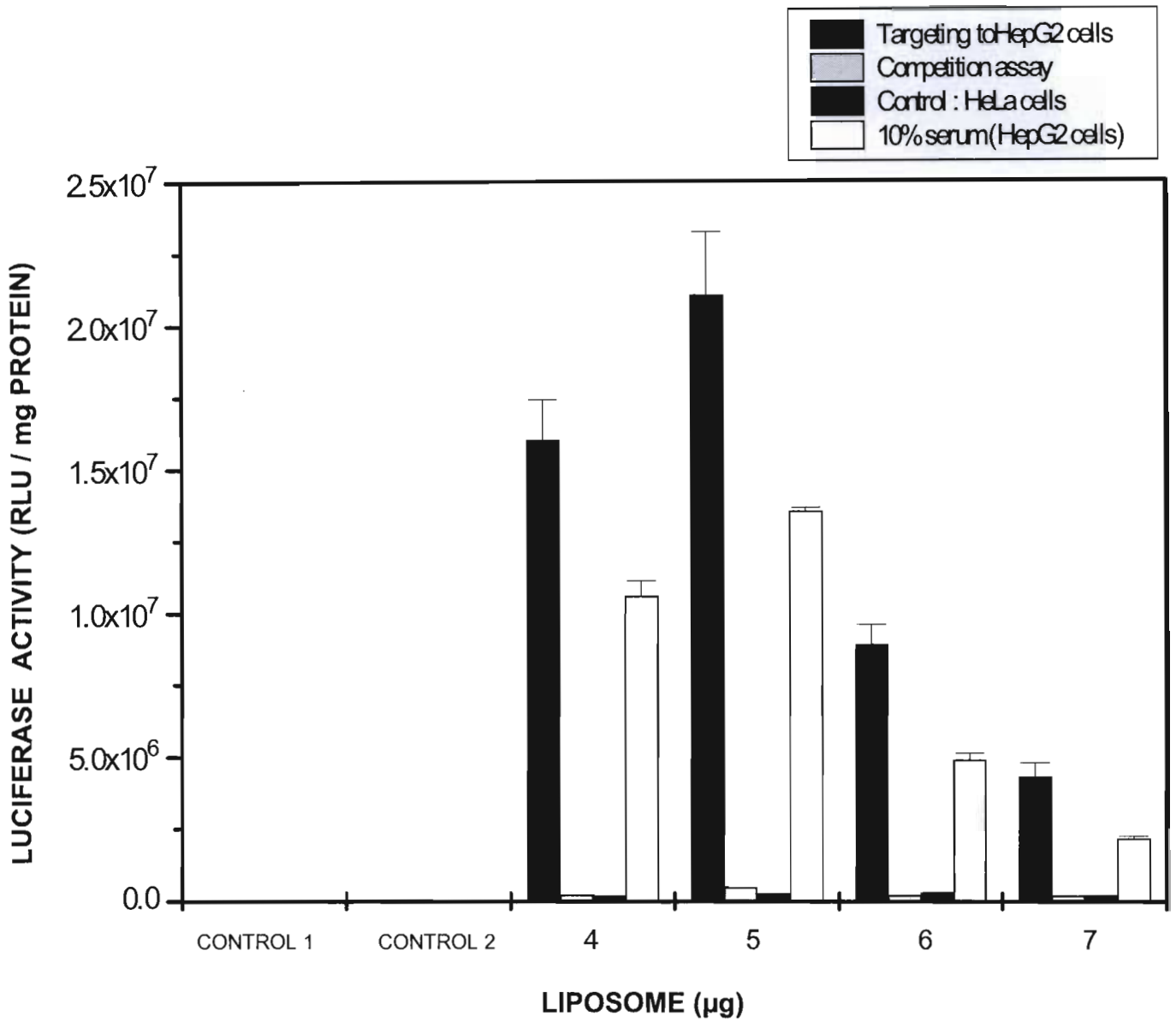
Figure 4.22 : Transfection studies of liposome MSβGAL:DNA complexes to HepG2 cells *in vitro*. Competition experiments with HepG2 cells included 300 μg asialofetuin per well. Liposome MSβGAL was varied (0, 4, 5, 6, 7 μg) while the DNA (1 μg) was kept constant in a total volume of 0.5 ml MEM. Control 1 contains only cells and control 2 contains naked DNA and no liposome. Data are presented as means ± S.D (n = 4).
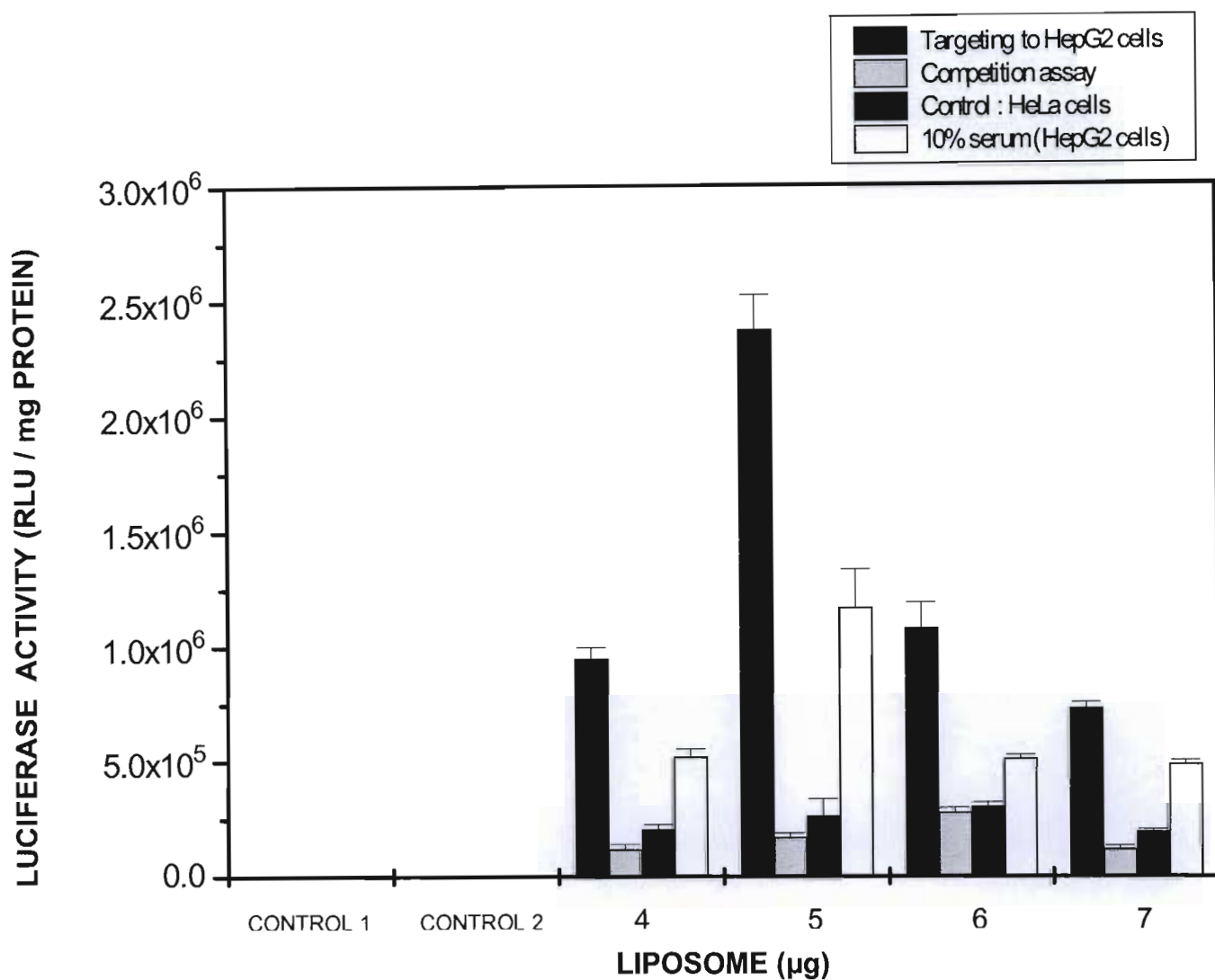
Figure 4.23 : Transfection studies of liposome MSαGLU:DNA complexes to HepG2 cells *in vitro*. Competition experiments with HepG2 cells included 300 µg asialofetuin per well. Liposome MSαGLU was varied (0, 4, 5 , 6 , 7 µg) while the DNA (1 µg) was kept constant in a total volume of 0.5 ml MEM. Control 1 contains only cells and control 2 contains naked DNA and no liposome. Data are presented as means ± S.D (n = 4).
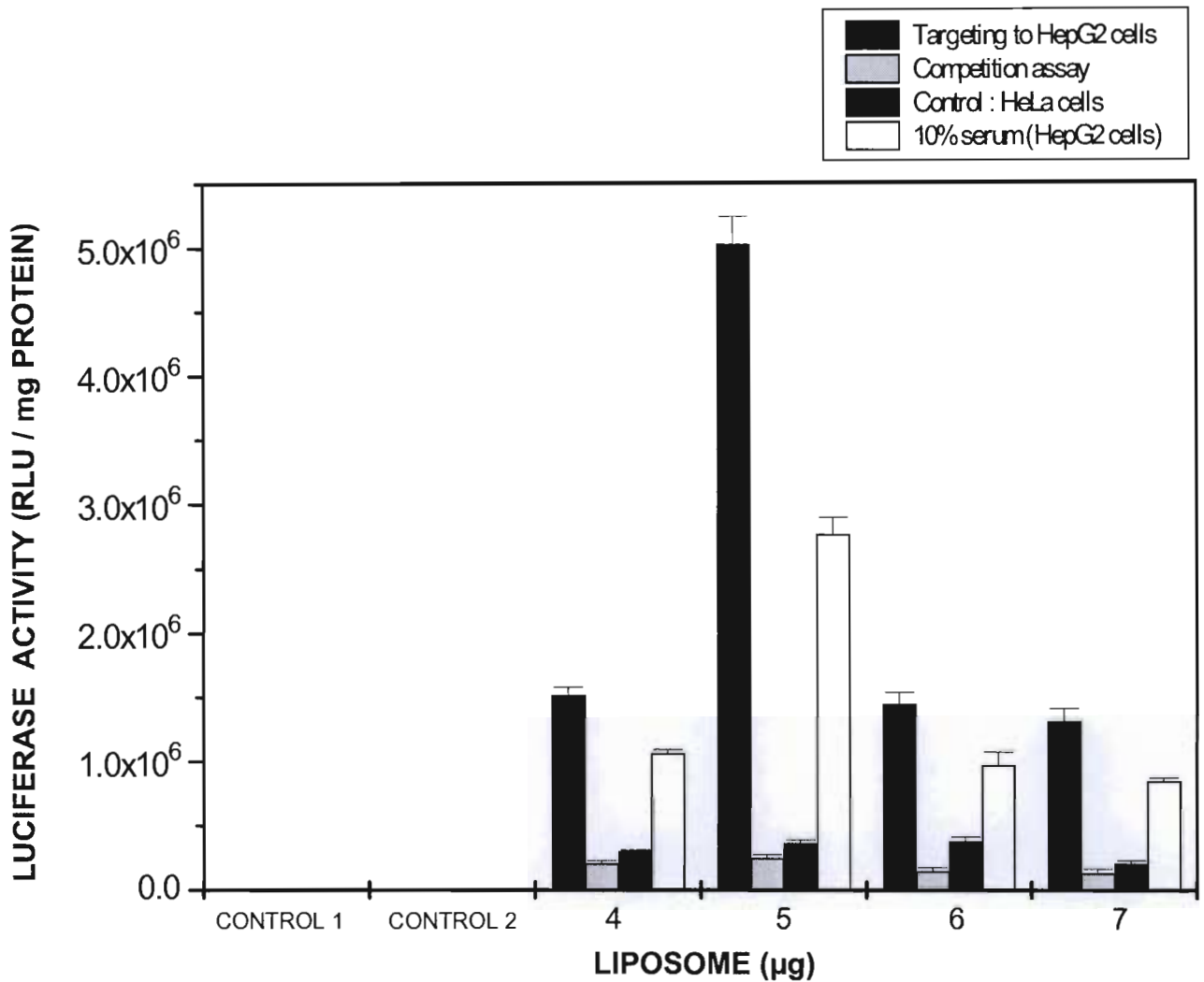
Figure 4.24 : Transfection studies of liposome MSβGLU:DNA complexes to HepG2 cells *in vitro*. Competition experiments with HepG2 cells included 300 μg asialofetuin per well. Liposome MSβGLU was varied (0, 4, 5 , 6 , 7 μg) while the DNA (1 μg) was kept constant in a total volume of 0.5 ml MEM. Control 1 contains only cells and control 2 contains naked DNA and no liposome. Data are presented as means ± S.D (n = 4).
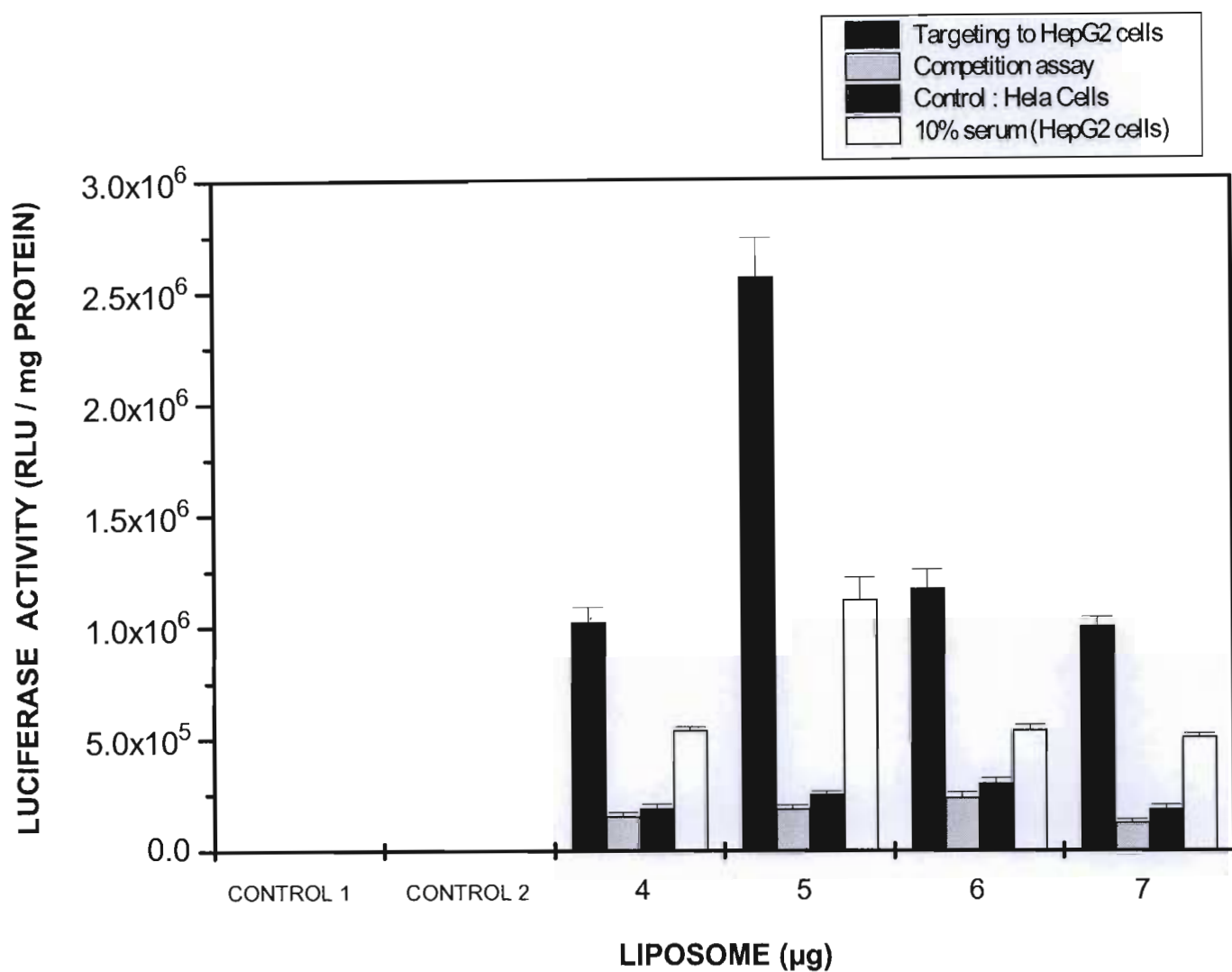
It was reported that the correct structural orientation of the terminal galactose residue in a ligand is important for proper binding to the asialoglycoprotein receptor (Hawtery and Ariatti, 1999). This was clearly evident in the results obtained with the β-galacto-derivative showing the best transfection efficiency when compared to its α-anomer. There was a 4-fold increase in the targeted transfection efficiency of the liposome MSβGAL (21 047 500 RLU/ mg protein) over its α-anomer MSαGAL (5 022 000 RLU/ mg protein). The optimal transfection efficiency of liposome MSαGLU (2 371 000 RLU/ mg protein) and MSβGLU (2 562 632 RLU/ mg protein) were not significantly different.

Furthermore it was observed that there is a preference for liposomes bearing the galactose moieties rather than those bearing the glucose moieties. This is borne out in the large difference in their respective luciferase activities. There was more than an eight fold increase in the activity of liposome MSβGAL over both liposomes MSαGLU and MSβGLU. Liposome MSαGAL showed a 2-fold increase over liposomes MSαGLU and MSβGLU at the optimal transfection ratio of liposome:DNA of 5:1 ($^w/_w$), and +/- charge ratio of 0.6. It can be seen that optimal transfection was not obtained at the liposome:DNA ratio of 6:1($^w/_w$) and (+/-) charge ratio of 0.7, the ratio at which complete retardation of the DNA on agarose gel retardation assays was obtained (see 4.4.3.1) but at a lower ratio of liposome:DNA of 5:1 ($^w/_w$), (+/-) charge ratio of 0.6. This could be due to the fact that the lipoplex size range (50-100 nm in diameter) at the liposome:DNA ratio 5:1($^w/_w$) was somewhat smaller than that (80-200 nm) at the higher range. Hence it would appear that lipoplex size does play an important role in transfection efficiency and in receptor-mediated gene delivery in the system chosen for this study.

From the transfection results obtained here, it can also be concluded that the asialoglycoprotein receptor has the greatest affinity for the MSβGAL (β-anomer), followed by MSαGAL (α-anomer), MSβGLU (β-anomer) and finally MSαGLU (α-anomer). It is generally assumed that the ASGP-R has the following binding affinity : tetraantennary > triantennary > biantennary > monoantennary galactosides (Rensen et al., 2001). Our liposome formulations possessed single galactose units extending outward from the lipid bilayer of the liposome and still showed extremely high

transfection activity when targeted to the HepG2 cells *in vitro*. However one may consider that the monoantennary derivatives synthesized for the present study are in fact affixed to a membrane bilayer, and depending on distances between neighbouring cholesterylglycosides in the outer leaf of the bilayer, they may mimic a multiantennary system resulting in a potentiated interaction between the receptor and the glycosylated lipoplex due to favourable spatial conditions.

It was observed that optimal receptor recognition of synthetic cluster glycosides is also determined by appropriate spacing (of at least 15 Å) between sugar residues (Rensen *et al.*, 2001). However it has been suggested that the transfection activity with glycoplexes is not determined only by the specificity of the lectin expressed at the cell surface membrane but also by intracellular trafficking of the glycoplexes, which could be mediated by lectins present inside the cells (Fajac *et al.*, 1999). It is thought that the main limitation to sugar-lectin interactions is the low specificity due to the limitation of the diversity of the simple sugars, and the large binding site of the lectin that can accommodate several simple sugars and therefore is liable to recognize several glycoconjugates and show little cell specificity (Palliard, 1999). Our results have clearly demonstrated the distinct differences in the transfection activities of the four types of glyco-liposomes. It was reported that galactosylated particles can produce a ten fold increase in transfection activity in hepatoma cells over glucosylated particles, but show no transfection preference in cell lines that lack the asialoglycoprotein receptor (Pun and Davis, 2002).

To test for specific receptor-mediated endocytosis of DNA complexes which include a specific ligand, a competition assay is routinely used that employs an excess of ligand. The use of excess asialofetuin (300 µg) in the competition assay was extremely effective. There was a dramatic decrease in the luciferase activity observed, indicating that the principal means of delivery of the plasmid DNA was by internalization by receptor-mediated endocytosis via the asialoglycoprotein receptor. In all four liposomal formulations the decrease in transfection activity at the optimal transfection ratio was greater than 90% [over 95% (20-40 fold decrease) for liposomes MSαGAL (250 476 RLU/mg protein) and MSβGAL (477 143 RLU/mg protein), and over 92% (14 fold

decrease) for liposomes MSGαLU (170 000 RLU/mg protein) and MSβGLU (186 000 RLU/mg protein)]. In hepatocytes, endocytosis may occur via clathrin coated pits or via fluid-phase endocytosis (Synnes et al., 1999). The luciferase activity observed in the competition assay with excess asialofetuin could be due to this fluid phase endocytosis or 'pinocytosis' process. Excess free asialofetuin up to 4 mg in a 24 well plate, was utililised by some researchers in studies using a DNA/galactosylated polylysine complex, but produced only about a 10% decrease in transfection efficiency. This drop was attributed to the inhibition of specific recognition of the DNA/galactosylated polylysine complex (Han and Yeom, 2000).

Others have reported the use of 1 mg of free asialofetuin (1000 fold excess) in a 48 well plate, with significant levels of inhibition ranging from a 3 to 9-fold decrease in gene expression (Arangoa et al., 2003). Asialofetuin-bound lipoplexes were found to be efficient in targeted delivery to the asialoglycoprotein receptor in cultured liver cells. Asialofetuin, a natural ligand, is a glycoprotein containing three N-linked glycans with tri-antennary sugar chains possessing the Galβ1→3GalNAc sequence. Hence, it can be internalized into hepatoma cells via the asialoglycoprotein receptor (Niidome et al., 2000). Besides galactose and N-acteylgalactosamine present as terminal monosaccharides, mannose units also occur (Hynes et al., 2003). Asialofetuin modified with polyethylene glycol has been utilized as a model system for binding to the galactose receptor of hepatocytes in vitro and in vivo (Roseng et al., 1992), and asialofetuin labeled cationic liposomes were used in receptor-mediated transfer to HepG2 cells (Hara et al., 1995). Furthermore asialofetuin lipoplexes and protamine sulphate-enhanced asialofetuin lipoplexes showed significant levels of gene expression in the liver of mice and in cultured HepG2 cells (Arangoa et al., 2003).

To demonstrate the inhibition of receptor mediated transfection, free galactose up to 20 mM was also utilized by some investigators with significant inhibition (Wen et al., 2004; Hashida et al., 2001; Kawakami et al., 1998), while others utilized up to 100 mM N-acetylgalactosamine with marked inhibition of receptor mediation (Rensen et al., 2001). Previous studies conducted utilizing a 100-fold excess of free asialoorosomucoid

over the bound glycoprotein in ternary complexes in competition experiments was also shown to be successful in indicating cell-specific targeting and receptor-mediated endocytosis in HepG2 cells *in vitro* (Singh *et al.*, 2001b; Singh *et al.*, 2003). Receptors for free galactose are known to be present in liver parenchymal cells, where glucoside or even mannoside residues present on the surface of liposomes, can bring about selective uptake of these liposomes (Behari and Nihal, 2000).

The gene expression levels in HepG2 cells obtained with our four glycosylated liposomes were much higher than those obtained for the untargeted cationic liposome (MS09) in chapter two, despite the slightly lower percentage of MS09 in the formulation of the glycosylated liposomes (44%, $^M/_M$), when compared with the unglycosylated counterpart (50%, $^M/_M$). The β-galactosylated liposome (MSβGAL) produced the greatest difference with a 17 fold increase, followed by the α-galactosylated liposome (MSαGAL) with a 4 fold increase while both the glucosylated liposomes (MSαGLU and MSβGLU) showed a 2 fold increase over the untargeted MS09 liposome at their respective optimal gene expression.

The lipoplexes were also tested for gene transfer efficiency in the HeLa cell line, which was used as a control cell line. The HeLa cells, which do not express the galactose-specific membrane lectin (Bettinger *et al.*, 1999), produced a far lower level of gene expression which was comparable to that achieved in the competition assay. The highest transfection efficiency, however, was not achieved at the ratio for liposome:DNA of 5:1 ($^w/_w$) and (+/-) charge ratio of 0.6 as was required for the HepG2 cell targeting, but was attained at a higher liposome:DNA ratio of 6:1 ($^w/_w$) and a (+/-) charge ratio of 0.7. This could be due to the fact that the lipoplex size at the liposome:DNA ratio 5:1($^w/_w$) of 50 nm to 100 nm in diameter was smaller than those at the higher ratio (80 nm to 200 nm). The differences, however, are marginal and it would not appear that binding to the negatively charged cell surface can explain this difference, since at the highest ratio of liposome:DNA of 7:1 ($^w/_w$) the transfection levels are lower. However the transfection levels achieved in the HeLa cells were reduced by more than 90% when compared to that achieved for the targeted glycosylated

liposomes to the HepG2 cells, at their respective optimal transfection ratios. The luciferase activity observed for the HeLa cells could be due to the normal lipofection process of the liposomes. The high transfection achieved at a higher charge ratio correlated with the complete retardation ratios observed in the gel retardation assays (Figures 4.8 to 4.9) and in the maximum displacement of ethidium bromide achieved in the dye displacement assays (Figures 4.10 and 4.11).

To determine if these liposomes have the capability and the stability to be utilized in future *in vivo* studies, transfection of the HepG2 cell line was also conducted in the presence of 10% serum. There was a 36% drop in transfection for the MSβGAL liposomes, a 45% drop in transfection for the MSαGAL liposomes, a 50% drop for the MSαGLU liposomes and a 56% drop in transfection efficiency for the MSβGLU liposomes. It can be seen that the galactose containing liposomes seem to be more stable in the presence of serum than the glucose containing liposomes. The important conclusion to emerge is that the MSβGAL liposomes that produced the greatest transfection activity showed the least reduction in transfection in the presence of serum. Hence it appears to have the potential to be utilized in further studies directed towards the development of an *in vivo* gene transfer system. It has been emphasized that stability in serum and thus the proper bioavailability of a vector to its specific receptor may be the single greatest limiting factor on the overall gene transfer efficiency *in vivo* (Molas *et al.*, 2003). Albumin, one the serum proteins, is generally the most abundant protein that is found to be associated with both neutral and charged liposomes after recovery from circulation *in vivo*. It has been implicated in destabilization of liposomes (Semple *et al.*, 1998).

Liposomes are generally cleared *in vivo* by the mononuclear phagocyte system (MPS) which comprises the liver, spleen and bone marrow. Most of the liposomes administered intravenously could be located in the liver (Oku *et al.*, 2000). Hence if these liposomes possess galacto-targeting moieties for targeting to liver cell (hepatocyte) receptors, such as the liposomes used in this study, the accumulation and hence binding to the liver cells *in vivo* could increase. Furthermore, the use of a target

site *in vivo* that is part of the RES will be of an advantage for the targeted delivery of genes, drugs or other macromolecules. This was demonstrated by *in vivo* studies in mice using glycosylated liposomes, where after intravenous injection the glycosylated liposomes were rapidly eliminated from the circulating blood and preferentially recovered in the liver (Kawakami *et al.*, 2000).

However in contrast liposomes without glycosylation were retained for a longer time in the circulating blood. Mannosylated , fucosylated and galactosylated liposomes were all taken up by the liver via the asialoglycoprotein receptor in the paranchymal cells, and the mannose and fucose receptors in the non-parenchymal cells respectively. Galactosylated liposomes, if administered at high doses, can also be taken up by the fucose receptors in the non-parenchymal cells, that act as galactose particle receptors (Kawakami *et al.*, 2000). If a target site outside the RES is to be targeted then the liposomal formulations would have to be modified so as to avoid being trapped by the RES. Fenestrations in the liver play an important role for *in vivo* delivery, especially in allowing the genes access to the parenchyma. Increased hydrostatic pressure has been used to widen these pores and to improve delivery (Liu *et al.*, 1999).

It has been suggested that even a high level of expression in a small percentage of cells could reverse a pathological phenotype, as in the case of hemophilia where an expressed protein needs to be secreted (Galun, 2003). Hence, we infer that the HepG2 cells studied, which express the galactose-specific membrane lectin, were efficiently and selectively transfected with the MSαGAL/MSβGAL/MSαGLU/MSβGLU:DNA complexes in a monosaccharide configuration-dependent manner.

4.5 CONCLUSION

We have confirmed in these studies that the galactosyl and glucosyl liposomes are both effective in transfection via the receptor-mediated endocytotic pathway. They were successfully targeted to the asialoglycoprotein receptors on the HepG2 cell surface *in vitro*. Hence it can be seen that the use of targeting liposomes can significantly improve the transfection activity. It is said that although cell culture results obtained in *in vitro* systems, cannot be directly translated to *in vivo* delivery, they can at least indicate which lipoplexes or liposome formulations have the potential to be useful for applications in animal models or humans (Düzgüneş *et al.*, 2001). The galactopyranosylcholesterol, especially the β anomer-containing liposomes, could have the potential to become effective drug or gene carriers. Here, we have also determined the ligand recognition characteristic of the asialoglycoprotein receptor in that it has an affinity or preference in the order : β-galactose > α-galactose> β-glucose > α-glucose, although the difference between the α- and β-glucose anomers was not as great as that seen between the α- and β-galactose anomers.

Results presented in this chapter suggest that the asialoglycoprotein receptor does indeed display an anomeric preference for the galactose moieties present on the liposomal surface in a β-glycosidic link to the cholesterol component. Moreover there appears to be a clear epimeric preference with respect to the C-4 configuration, the position at which D-glucose and D-galactose differ. It is also gratifying to note that a simple glycosidic link between the monosaccharide moiety and the cholesterol anchor without a longer spacer is sufficient to elicit the required specificity and recognition.

Hepatocellular carcinoma represents more than 5% of all cancers in the world with estimated deaths exceeding 500 000 per year (Llovet, 2003). Furthermore, the incidence of hepatocellular carcinoma is on the rise in a number of countries, partly because of an increase in the rate of hepatitis infection. It is expected that as the population ages, the proportion of elderly hepatocellular carcinoma patients is likely to grow (Dohmen *et al.*, 2004). With recent progress in understanding the molecular

changes that underlie cancer development, the prospect of specifically targeting malfunctioning molecules and pathways to achieve more effective and rational cancer therapy is becoming possible (Sawyers, 2004). Furthermore gene delivery via the asialoglycoprotein receptor could be an effective delivery system into the liver *in vivo*. Hence the results obtained in this study bode well for possible future *in vivo* applications.

Glycoconjugates have found major applications in antiviral therapy, immunoactivation, enzyme replacement therapy and gene therapy. It may be concluded that the discovery of new mammalian lectins which endocytose their ligands will lead to the rapid development of new glycotargeting agents founded on the principles of carbohydrate-protein interactions (Wadhwa and Rice, 2003). The preparation of DNA:ligand complexes remains a significant technical obstacle, and improved methods will likely be required to obtain complexes suitable for receptor-mediated gene transfer *in vivo*. It is reported that activated macrophages express the macrophage asialoglycoprotein binding protein (M-ASGP-BP) that recognizes terminal galactose or N-acetylgalactosamine units, and may participate in the interaction between tumoricidal macrophages and tumour cells (Matsumoto *et al.*, 1999). Macrophages also infiltrate solid malignancies to form the tumour solid mass, dominating in areas of hypoxia and necrosis (Greco *et al.*, 2002). Hence, it is hoped that our findings will have further implications in future research on liposome-mediated asialoglycoprotein receptor-directed gene or drug delivery to hepatocytes or to other cells that possess galactose receptors.

5. SUMMARY AND CONCLUDING REMARKS

In order to develop optimum corrective gene transfer to any specific tissue requires the tailoring of the transgene expression system into a suitable delivery vehicle. The actual selection of the various delivery components depend upon the target tissue and the type of disease. Although the efficiency and specificity of non-viral delivery systems have previously not been very high, problems relating to transfection methods are being gradually resolved. Various transfection mediators for effective and directed gene delivery into a variety of cells have been developed. Although liposomes targeted via antibodies or ligands are still being developed for testing in clinical trials, liposomes passively targeted to tumour tissues have been approved for treatment of Kaposi's sarcoma and for clinical trials for other forms of cancer (Düzgüneş and Nir, 1999). Liposome-mediated DNA vaccination is a viable prospect. This system can produce prolonged gene expression which will eliminate the need for booster injections, and the risks associated with live and attenuated vaccines (Chisti, 1999).

Gene therapy has hence provided a novel approach to the treatment of various disorders. As we begin to understand more of the molecular mechanisms involved in these diseases we will be able to utilize this knowledge to target specific cells, tissues and organs. Our detailed knowledge of the human genome sequence will provide promising tools for defining disease-specific targets or genes and their expression patterns. The further development of proteomics and achievement of high-throughput screenings offer a great potential for the discovery of drugs including DNA drugs for gene therapy (Luo, 2004). Hence, with the advent of gene discovery, gene delivery will be a major focus, and non-viral delivery methods such as liposomes will prove increasingly more effective.

Infectious diseases, genetic defects and even traumatic injury may someday be treated with gene therapy and gene therapy strategies. As more effort is directed towards developing vectors and increasing the potential of this technology, the success or failure of these systems will depend on the ability to manipulate and control the genetic

transfer into specific target cells. Progress towards identifying barriers to gene transfer will hopefully lead to more efficient vectors being designed with increased safety and lower immunogenicity, ultimately providing gene transfer techniques that could revolutionise gene therapy for specific diseases (Pilewski, 2002).

Advances in molecular and cellular biology have led to the development of a new class of biotherapeutic macromolecules such as proteins, (poly)peptides and nucleic acids that can be designed to modulate functions of target cells. In order for these biotherapeutics to successfully access their targets within the cytoplasm and nucleus, efficient gene or drug carriers are required for safe and efficient delivery across cellular membrane barriers and into the cytosol of target cells. Hence gene delivery using liposomes is undergoing a transition from simple electrostatic systems to more complicated engineered designs.

The investigations which form the basis for this thesis show that the cationic liposomes synthesized are a viable and interesting alternative to other non-viral gene delivery systems. Results with the cationic cholesterol derivative MS09, in particular, which embodies a basic dimethylamino headgroup at the end of a rather long and relatively polar spacer separating it from the cholesteryl anchor (Chapter 2), have also revealed that the efficiency of these cationic liposomes may be improved by incorporating into the liposome the appropriate ligands directed at cognate receptors expressed by model cell lines. More specifically it has been demonstrated that the biotin-avidin reaction may be applied with success in the construction of a cationic liposome-based DNA delivery system containing MS09 that apparently recognizes the transferrin receptor on HeLa cells (Chapter 3), although it is clear that not all the assemblies enter the cells by this route. In addition, a study of MS09-containing glycosylated liposomes designed to target the asialoglycoprotein receptor, which is located on the extracellular face of the plasma membrane on the human hepatocyte-derived cell line, HepG2, revealed a strong anomeric preference by the receptor for the β-D-galactopyranosyl moiety while it is clear, from the much reduced transfection levels with the glucopyranosyl-containing

liposomes, that the configuration at the C-4 position in the monosaccharide is critical for asialoglycoprotein receptor recognition (Chapter 4).

The next step would be to apply these cationic liposomes to *in vivo* models. Most of the lipoplexes producing optimum transfection efficiency were relatively small in size, ranging from 100-200 nm which is suitable for systemic administration. The transfection in mammalian cells has provided us with an indication of the likelihood of success when applied to *in vivo* studies. The stability of the complexes studied in the presence of serum suggest that they could be promising candidate vehicles for *in vivo* gene delivery. Herein lies our greatest challenge in the optimisation of the various liposomal gene delivery systems to suit the needs for clinical gene delivery.

One of the broad long term objectives of this type of research is to develop a fundamental science base that will lead to the design and synthesis of optimal non-viral DNA carriers for gene therapy and disease control. It is further required to improve the efficiency of delivering large pieces of DNA containing important human genes and their regulatory sequences (> 100 kbp), which at present can only be achieved with synthetic vectors (Safinya, 2001). Various cationic lipid / liposome formulations are likely to continue to attract scientific and commercial interest for the foreseeable future. It is hoped that with further structural and functional studies, a "magic" cationic lipid will emerge that will be equally efficient *in vitro* as it would be *in vivo*.

Liposomes have the capacity to tolerate lyophilisation and later rehydration, which could extend the medicinal usage of liposomal formulations. Hence, liposomes need to be manufactured in a way that permits retention of structure on rehydration after the freeze–drying conditions to which they are subjected (Chisti, 1999). From a technological point of view, vesicles are increasingly used in cosmetic industries as controlled release agents, e.g. in formulations of lotions, gels, creams and ointments, and continue to be explored for their utilization in the food and agricultural industries, apart from impacting on the medical field as drug and gene carriers (Chiruvolu *et al.*, 1994).

If gene therapy is to succeed, the corrective plasmid DNA or constructs must be delivered to cell targets in a form that will preserve function, penetrate the numerous barriers to cell invasion and promote the expression of the therapeutic protein (Birchall *et al.*, 1999). Gene transfer approaches will become increasingly more important for the treatment of malignant diseases and will hopefully result in effective, specific and safe therapies. Gene therapy can be seen as the "light at the end of the tunnel" for many affected by a genetic disorder.

Finally it has been proposed that the promise of better non-viral vectors designed for specific gene therapy applications will only be fulfilled with a greater level of interdisciplinary integration among different research areas, such as polymer chemistry, biophysics, cell biology, molecular biology and medicine amongst others (Molas *et al.*, 2003). This could provide important information for the creation of targeted, effective, and safe DNA therapeutics in the future.

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SELECTED NMR SPECTRA
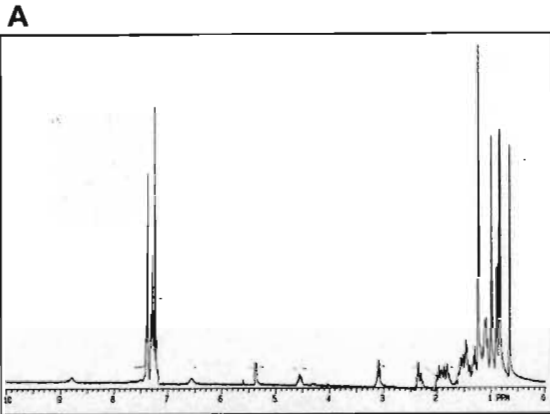
MS01 : N-tritylglycylcholesterylformylhydrazide
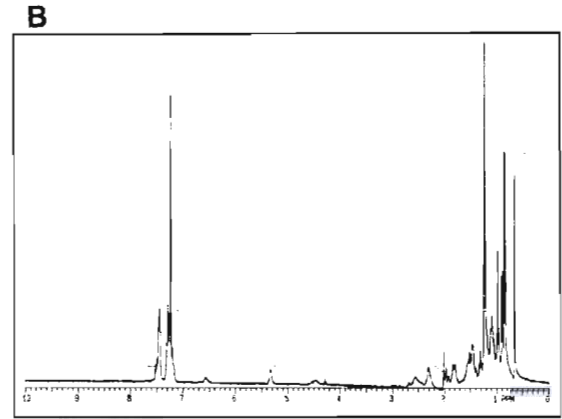
MS02 : N-trityl-β-alanylcholesterylformylhydrazide
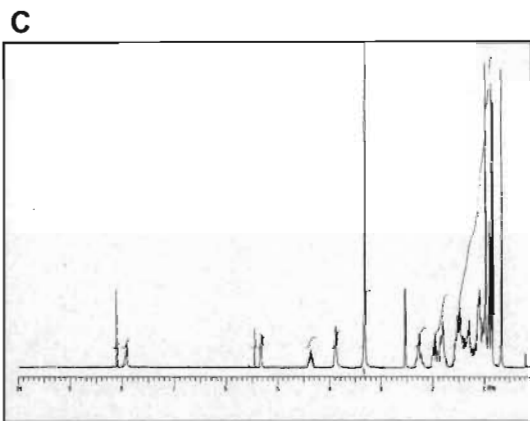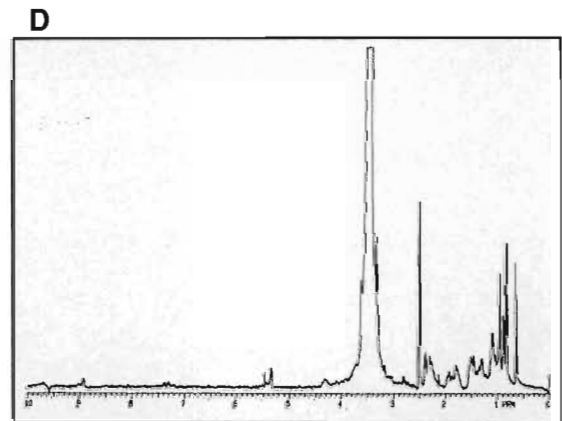
MS04 : Cholesterylformylhydrazide

MS08 : Cholesterylformylhydrazide hemisuccinate
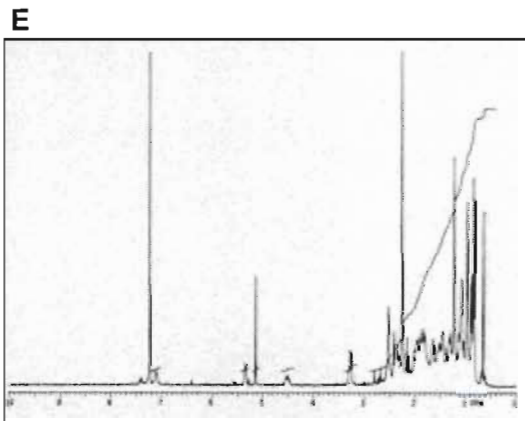
MS09 : N,N-dimethylaminopropylaminylsuccinyl-cholesterylformylhydrazide
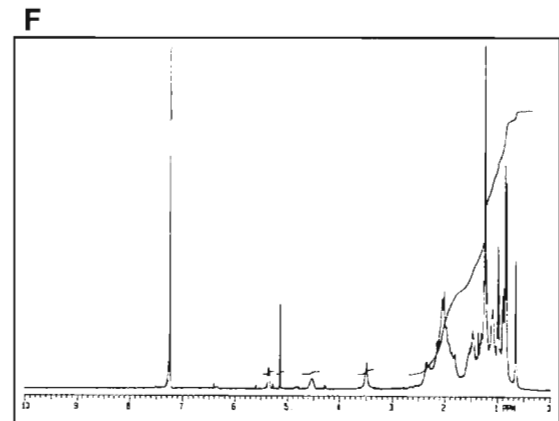
MS10 : Glycylcholesterylformylhydrazide

G
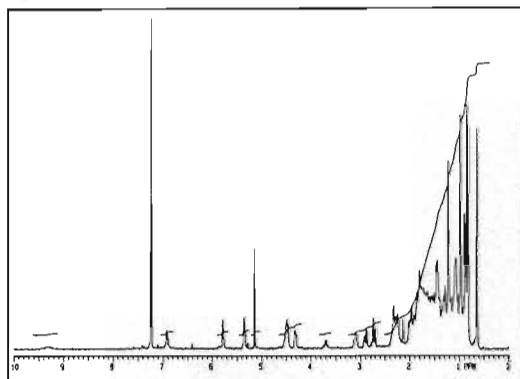
MSB1 : Biotinylcholesterylformylhydrazide

H
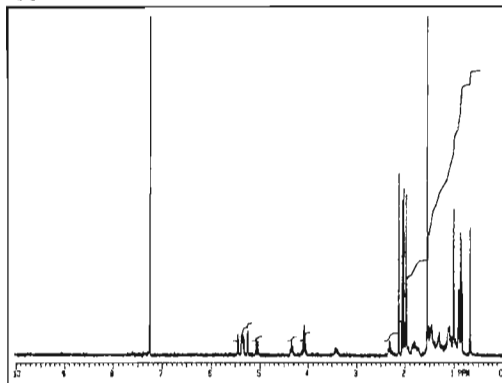
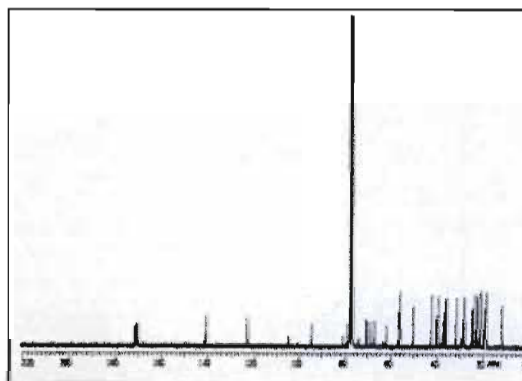Cholesteryl-α-D-tetra-O-acetylgalactopyranoside
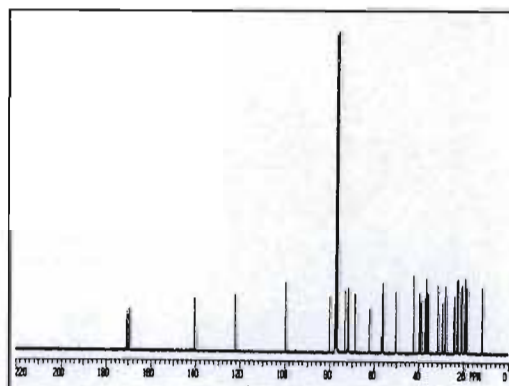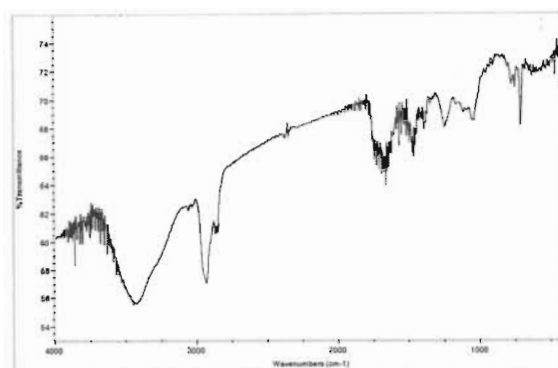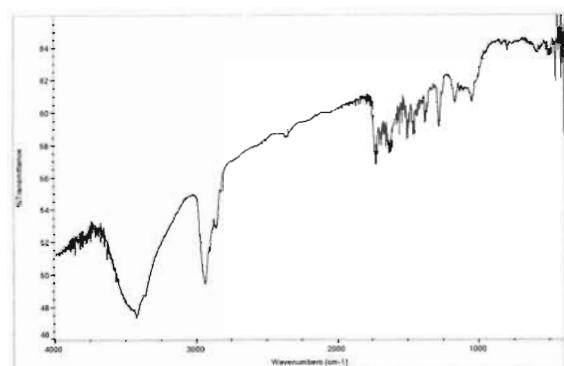
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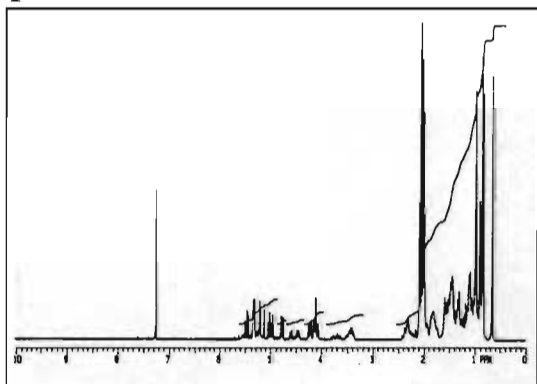
^{1}H NMR   Cholesteryl-α-D-tetra-O-acetylglucopyranoside ^{13}C NMR

J

^{1}H NMR   Cholesteryl-β-D-tetra-O-acetylglucopyranoside ^{13}C NMR

K
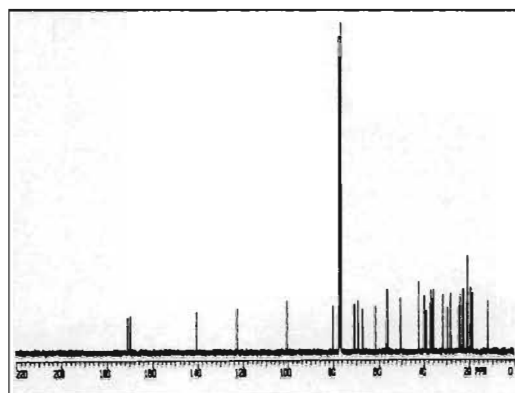
^{1}H NMR   Cholesteryl-β-D-tetra-O-acetylgalactopyranoside ^{13}C NMR

APPENDIX 2

SELECTED INFRA-RED SPECTRA

A
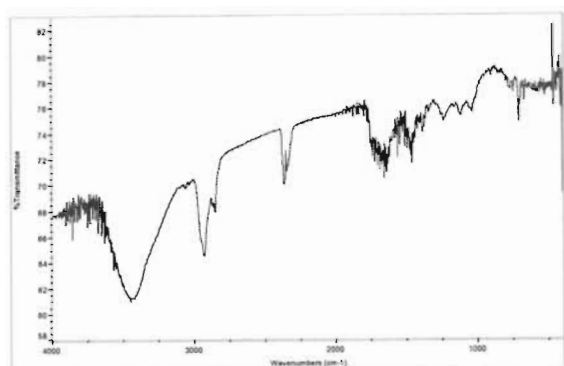
MS01 : N-tritylglycylcholesterylformylhydrazide

B

MS02 : N-trityl-β-alanylcholesterylformylhydrazide

C

MS04 : Cholesterylformylhydrazide

D
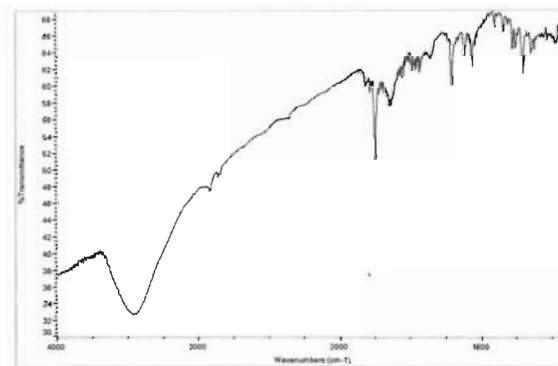
MS05 : N-trityl-β-alanine NHS ester

E
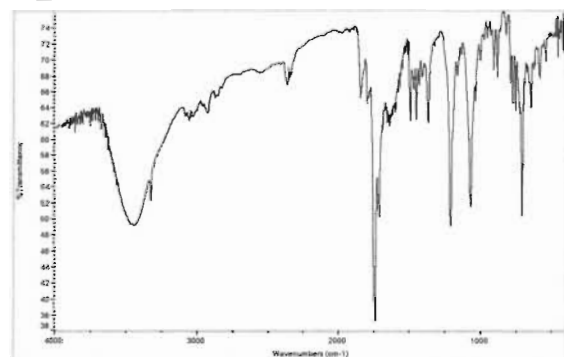
MS06 :   N-trityl-β-alanine

F
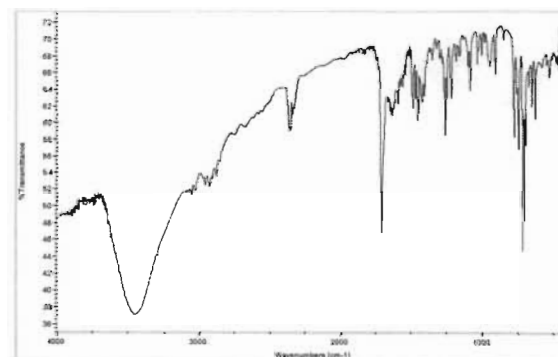
MS07 : N-tritylglycine NHS ester

G
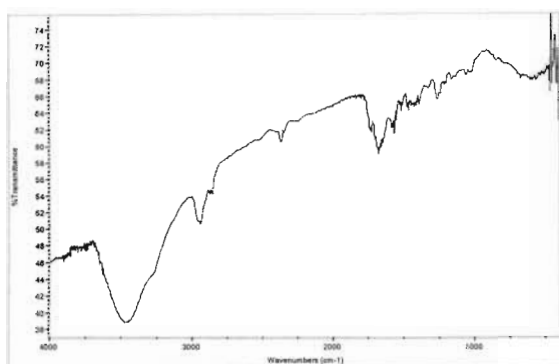
MS08 : Cholesterylformylhydrazide hemisuccinate

H
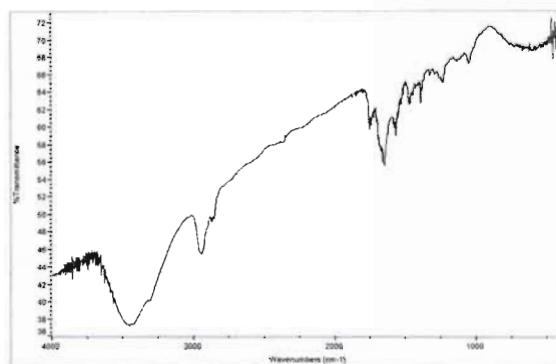
MS09 : N,N-dimethylaminopropylaminylsuccinyl-
cholesterylformylhydrazide
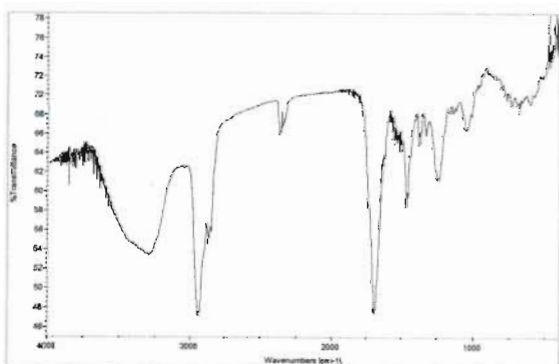
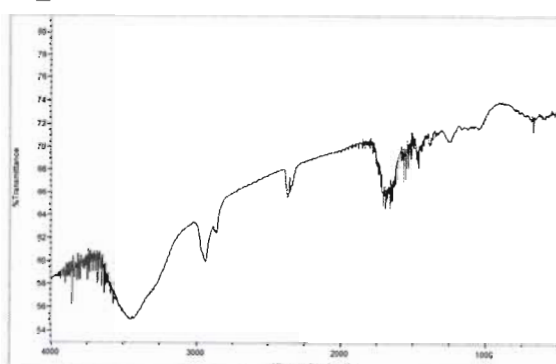**I**
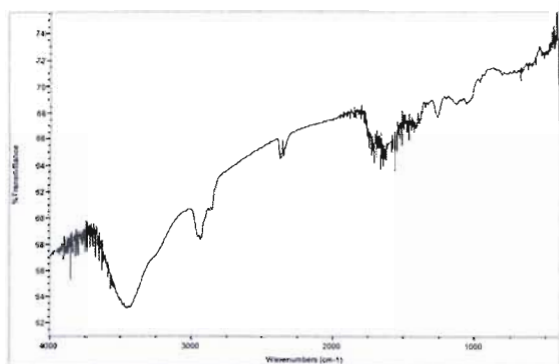
MS10 : Glycylcholesterylformylhydrazide

J
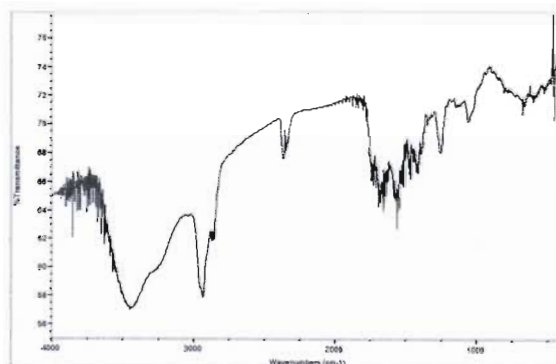
MS11 : β-alanylcholesterylformylhydrazide

K

MSB1 : Biotinylcholesterylformylhydrazide

L

MSB2 : Aminohexanoylbiotinyl chloesterylformylhydrazide