CATIONIC LIPOSOME MEDIATED TARGETED GENE DELIVERY WITH AND WITHOUT PEGYLATED ACCESSORIES

by

NICOLISHA NARAINPERSAD

Submitted in fulfilment of the academic requirements for the degree of
Master of Science in the School of Biochemistry, Genetics and Microbiology,
University of KwaZulu-Natal
Durban

December 2009

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

Supervisor: Dr M. Singh Signed_____ Date:_____

Co-Supervisor: Professor M. Ariatti

ABSTRACT

As a consequence of safety issues encountered by the use of viral vectors in gene therapy, there has been a steady increase in the development and application of non-viral vectors, especially liposomes. Cationic liposome mediated delivery is one of the most promising non-viral delivery methods. These liposomes are prepared from synthetic lipids, are positively charged and interact favourably with DNA through electrostatic interactions. Cationic liposomes have also shown immense potential in the targeting of specific cell types such as HepG2 (hepatocellular carcinoma) cells, a model *in vitro* gene delivery system for the study of hepatocyte function. However, these liposomes also have a number of limitations *in vivo*. In an attempt to overcome these restrictions, a hydrophilic polymer, polyethylene glycol (PEG) is incorporated into the cationic liposome. This covalent attachment of (PEG) to the liposomal surface is thought to sterically stabilise liposomes, promote biological stability, inhibit aggregation, decrease toxicity and immunogenicity, prevent interaction with serum proteins and complement and thus prolonging the circulation time of liposomes *in vivo*. The versatility and simplicity of cationic liposomes have made them vitally significant non-viral gene delivery vehicles for human gene therapy.

In this investigation novel untargeted and targeted glycosylated liposomes with and without PEG were synthesised to evaluate their gene transfer activities *in vitro* to potentially develop a suitable gene delivery system for future *in vivo* applications. A constant molar quantity of the cationic cholesterol derivative, 3 β [N-(N', N'-dimethylaminopropane)-carbamoyl] cholesterol (CHOL-T) was mixed with dioleoylphosphatidylethanolamine (DOPE) and a galactose/glucose derivative to produce targeted cationic liposomes. PEG liposomes were prepared in the same way with the addition of distearoylphosphoethanolamine polyethylene glycol 2000 (DPSE-PEG_{2000}), 2% on a molar basis.

Supported by transmission electron microscopy characterisation, we present evidence that the pegylation of liposomes affects the DNA binding capability and transfection efficiencies of the cationic liposomes in addition to protecting the plasmid DNA in lipoplexes from serum nuclease degradation. Optimal DNA : liposome binding ratios were obtained from gel retardation studies and confirmed by ethidium bromide intercalation assays. These complexes were then tested on the human hepatoma cell line, HepG2, to determine toxicity and assess

transfection efficiencies. From results obtained in this study, it appears that both cationic and pegylated cationic liposomes are well tolerated by cells *in vitro.* The results further suggest that targeting by use of glycolipids incorporated into the structure of the liposome increases transfection, while pegylation of cationic liposomes marginally decreases the transfection efficiency of the lipoplexes to HepG2 cells *in vitro*.

PREFACE

The experimental work described in this dissertation was carried out in the School of Biochemistry, Genetics and Microbiology, University of KwaZulu-Natal, Durban from May 2007 to December 2009, under the supervision of Dr. Moganavelli Singh and co-supervision of Professor M. Ariatti.

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

FACULTY OF SCIENCE AND AGRICULTURE

DECLARATION 1 – PLAGIARISM

I, Nicolisha Narainpersad, declare that

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

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

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

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sourced have been quoted, then:
    a. Their words have been re-written but the general information attributed to them has been referenced
    b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and the References sections.

Signed _____

FACULTY OF SCIENCE AND AGRICULTURE

DECLARATION 2 – PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis *(include publications in preparation, submitted, in press and published and give details of the contributions of each author to the experimental work and writing of each publication.)*

Publication 1: Peer Reviewed Published Abstract – Appendix B

Narainpersad N., Singh M. and Ariatti M. (2009). *'Pegylated cationic liposome mediated targeted gene delivery'*. Human Gene Therapy. **20**: 1542.

Experimental work: N. Narainpersad
Writing of publication: M. Singh and M. Ariatti

Signed_____

ACKNOWLEDGEMENTS

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

- Dr. M. Singh, for her invaluable supervision and guidance during the course of this study.

- Professor M. Ariatti, for his invaluable guidance, supervision and vast array of scientific knowledge.

- Dr. J. Wesley-Smith, Department of Electron Microscopy, University of KwaZulu-Natal, for the use of the TEM unit, Formvar coated grids and his immeasurable assistance with electron microscopy.

- Dr. S. Singh, Department of Chemistry, University of KwaZulu-Natal, for the generation of the infrared data for the chemical compounds.

- Mr A. Rajh, Science Photographer, University of KwaZulu-Natal, for assistance with poster presentations.

- The National Research Foundation (NRF), for the scholarship that enabled me to complete my studies.

- The University of KwaZulu-Natal, for financial assistance.

- Miss S. Dorasamy, for her support, invaluable assistance with this project and for always being a good friend.

- Miss A. Sewbalass and Miss J. Pillay, for their support, kind words and moments of light relief.

- My family and friends, for their constant support, encouragement, assistance and kindness during the course of this study.

- Mr N. Naidoo, for his unwavering encouragement, support, understanding and patience during the last year and a half.

- All the staff of the Department of Biochemistry, University of KwaZulu-Natal.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ATP	Adenosine-5’-Triphosphate
BCA	Bicinchoninic acid
CAP-β-Gal	Cholesteryl 3β-N-(4-aminophenyl-β-D-galactopyranosyl) carbamate
CAP-β-Glu	Cholesteryl 3β-N-(4-aminophenyl-β-D-glucopyranosyl) carbamate
CCF	cholesteryl chloroformate
CHOL-T	3 β [N-(N’, N’-dimethylaminopropane)-carbamoyl] cholesterol
DC-CHOL	3β[N,N’,N’-dimethylaminoethane)-carbamoyl] cholesterol
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DOGS	Dioctadecylamidoglycyl-spermine
DOPE	Dioleoylphosphatidylethanolamine
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
DSPE-PEG	Distearoylphosphatidylethanolamine polyethylene glycol
EDTA	Ethylenediaminetetraacetic acid
FBS	Foetal bovine serum
HEPES	2-[4-(2-hydoxyethyl)-1–piperazinyl] ethanesulphonic acid
HBS	HEPES buffered saline
LUV	Large unilamellar vesicle

MEM	Minimum Essential Medium
MPS	Mononuclear phagocyte system
PBS	Phosphate buffered saline
pDNA	Plamid DNA
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
PEI	Polyethylenimine
PLL	Poly-L-lysine
RES	Reticuloendoethelial system
Rpm	Revolutions per minute
SUV	Small unilamellar vesicle
TLC	Thin layer chromatography
Tris	Tris(hydroxymethyl)aminomethane

CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

1.1 GENE THERAPY: AN OVERVIEW

Gene therapy, the use of nucleic acids as therapeutically useful molecules, offers a fundamental approach to the treatment of a variety of diseases of acquired or genetic origin (Smith, 1999; Huang *et al*., 1999). Gene therapy provides a new paradigm for the treatment of human disease and is considered by many as a potential revolution in medicine. This is because the ultimate goal of gene therapy is to eliminate the causes of diseases by adding, correcting or replacing genes whereas most current treatment of disease is by only treating symptoms not causes (Mountain, 2000; Huang *et al.,* 1999). The basis of this therapy requires the introduction of a functional gene or other nucleic acid molecule with an information sequence, into the cell to achieve the desired therapeutic effect and the gene therefore serves as the drug (Lasic, 1997).

The basic ideas for gene therapy were first expressed in the 1960's when Amos (1961) found that the uptake of nucleic acid molecules into cultured cells was enhanced when RNA was complexed with protamine (Huang *et al.,* 1999). Following that initial discovery, several other articles were published which clearly established that complexation of DNA or RNA with agents such as gelatin, methylated protein, polylysine and polyarginine increased transfection and/or infectivity (Huang *et al.*, 1999). In 1972 a discussion of gene therapy was offered in which it was proposed that a set of ethicoscientific criteria be formulated to guide the clinical application and development of gene therapy techniques (Friedmann and Roblin, 1972). Since then a number of advances and discoveries, such as the development of retroviral vectors with almost 100% transfection efficiency in 1982, have been made leading to the first therapeutic study involving gene therapy in 1990. This study was conducted in patients with adenosine deaminase deficiency. Mullen and co-workers used a retrovirus to insert the gene that codes for adenosine deaminase into lymphocytes *ex vivo*. A summary of some of the key events in the development of gene therapy is seen in Table 1.1.

TABLE 1.1: Summary of key events in the development of gene therapy (Adapted from Huang *et al.,* 1999).

YEAR	EVENT
1956	Viral genomes can be permanently incorporated in cell genomes
1961-2	Foreign DNA can integrate stably into mammalian cellular genomes
1972	A discussion of gene therapy was offered
1981-2	Retroviral vectors were developed to transfer foreign DNA to essentially 100% of exposed mammalian cells
1987	Synthesis of cationic lipid, DOTMA
1990	Adenosine deaminase gene therapy trial was initiated
1992	First gene therapy trial using DC-CHOL/DOPE cationic liposomes
1990s	Other gene delivery systems were developed
2000s	Other clinical trials were initiated using a variety of gene delivery systems

In the last two decades a number of gene therapy trials, involving genetic diseases such as cystic fibrosis, acquired diseases such as cancer and infectious diseases such as AIDS, have been initiated (Nishikawa and Huang, 2001). Some examples of diseases targeted by gene therapy are given below (Table 1.2).

TABLE 1.2: Examples of some disease applications of gene therapy (Lasic, 1997).

DISEASE
Cystic fibrosis
Sickle cell anemia
Gaucher's disease
Duchenne muscular dystrophy
Cancer
AIDS
Parkinson's disease
Alzheimer's disease
Arthritis
Atherosclerosis

Up to March 2009, there are 1405 approved, ongoing or pending clinical trials worldwide (www.wiley.com.uk/genetherapy/clinical) (Figure1.1). Almost two-thirds of these trials are directed at cancer with most of the remainder targeted to monogenetic disorders, cardiovascular and infectious diseases.

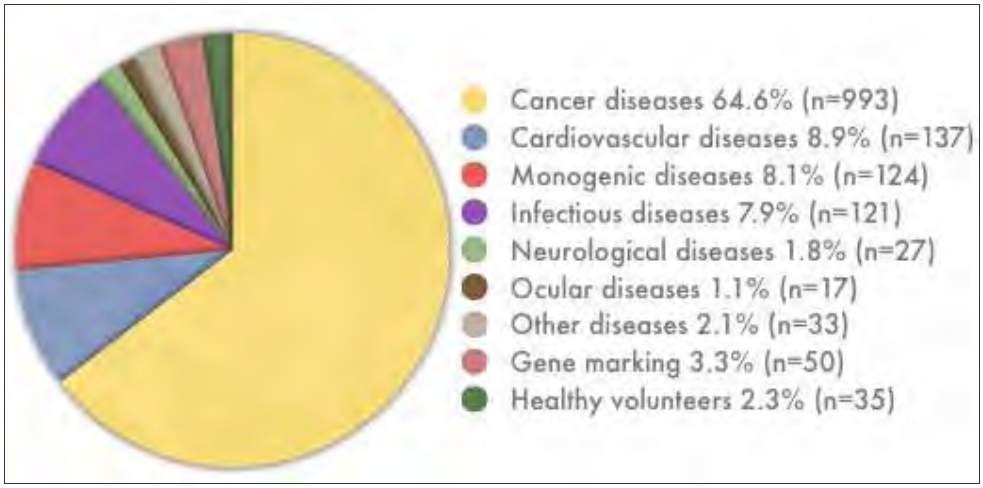


Figure 1.1: Indications of diseases/disorders addressed by worldwide gene therapy clinical trials (www.wiley.com.uk/genetherapy/clinical).

Almost 50 years after its 'discovery', gene therapy has had modest success. In 2007 University College London's Institute of Opthalmology reported a clinical trial for inherited retinal disease using a recombinant adeno-associated virus. The subject showed a moderate increase in vision and no apparent side effects (Bainbridge *et al.,* 2008). However there are still major obstacles facing effective gene therapy. Some of these include identification of diseases and access to target tissue, gene defect identification, stable and prolonged expression of newly introduced genes, immune response of the body, long and short term toxicity and probably the most limiting of all, the development of a vector that can selectively and efficiently deliver a gene to target cells with minimal toxicity (Smith, 1999; Li and Ma, 2001).

The goal in recent years has been to overcome these hurdles, however the one major limitation that still has yet to be resolved is the lack of a safe and efficient vector. This has led to a great deal of interest in research into finding the ideal gene delivery system.

1.2. GENE DELIVERY VECTORS

In order for genes to be expressed they must be in the nucleus of the cell however a gene is incapable of entering a cell by itself for two reasons: it is too large and secondly it is surrounded by anionic charges (Ropert, 1999). Therefore a vector is needed to 'carry' the DNA. An ideal vector should be safe, stable, easy to produce and proficient at achieving efficient, extended and cell or tissue specific gene expression (Li and Ma, 2001). Gene delivery systems generally fall into two categories: viral and non-viral. Viral vectors include all viruses used in the delivery of genes while non-viral vectors encompass all other methods of gene delivery. The advantages and disadvantages of these vectors are detailed in Table 1.3.

TABLE 1.3: Summary of advantages and disadvantages of gene delivery systems (Adapted from Mountain, 2000).

Vector	Advantages	Disadvantages
Adenovirus	Very high transfection efficiency *ex vivo* and *in vivo* Transfects both dividing and non-dividing cells Substantial clinical experience Efficient retargeted transfection demonstrated	Repeat dosing infective owing to strong immune responses Transgene size limit of 7.5 kb Manufacture and storage are moderately difficult Short duration of expression
Retrovirus	Fairly prolonged expression High transfection efficiency *ex vivo* Substantial clinical experience Low immunogenicity	Low transfection efficiency *in vivo* Transgene size limit of 8 kb Transfects only dividing cells Safety concerns of mutagenesis
Adeno-associated virus	Efficiently transfects a wide variety of cells *in vivo* Very prolonged expression *in vivo* Low immunogenicity	Transgene limit of 4.5 kb Manufacture is very difficult Little clinical experience Safety concern of mutagenesis Repeat dosing affected by neutralising antibodies
Naked DNA	Manufacture, storage are simple and safe Very low immunogenicity Very good safety profile Clinical efficacy demonstrated in critical limb ischemia	Very short duration of expression in most tissues Very inefficient transfection *in vivo* Retargeting transfection very difficult
Cationic liposomes	Relatively simple manufacture and storage Efficient transfection *ex vivo* and *in vitro* Low immunogenicity Good safety profile	Inefficient transfection *in vivo* Short duration of expression Little clinical experience Retargeting transfection difficult
Cationic polymers	Relatively simple manufacture and storage Efficient transfection *ex vivo* Low immunogenicity Good safety profile Retargeted transfection demonstrated	Inefficient transfection *in vivo* Very short duration of expression No clinical experience

1.2.1 Viral Vectors

Viral vectors are replication-deficient viruses with part of their viral sequence replaced by therapeutic genes (Li and Ma, 2001). Generally most viral vectors are highly efficient gene transfer vehicles as they contain all of the necessary characteristics for successful gene transfer such as cell adhesion, membrane translocation, efficient transcription and translation (Cristiano, 1998). In addition, their ability to stably integrate exogenous DNA into host chromosomes and their high specificity are among the major reasons why viral vectors were employed in more than 70% of clinical gene therapy trials (Singh *et al*., 2006a; Walther, 2000). However several limitations are inherent in their use. Depending on the type of viral vector, these could include toxicity, low viral titres, and provocation of mutagenesis and carcinogenesis in hosts (Huang *et al.,* 1999; Liu and Huang, 2002).

Retroviruses are the most extensively used vectors. They are single-stranded RNA viruses which are constructed into viral vectors by replacing the genes required for replication with therapeutic genes. This type of viral vector randomly incorporates the gene directly into the host chromosome leading to safety concerns such as mutagenesis and carcinogenesis (Lasic, 1997; Smith, 1999). Adenoviruses are less hazardous as they do not integrate into the host chromosome so gene expression is short-lived (Huang *et al.,* 1999). This vector produces high viral titres but suffers in that it induces an inflammatory response from the host making repeat doses impossible (Huang *et al.,* 1999). These limitations can be overcome by the use of 'gutless' or 'helper dependent' adenoviruses, which lack all viral coding sequences (Jǿzkowicz and Dulak, 2005). Adeno-associated viruses are small non-pathogenic DNA viruses that require a helper virus to replicate. They infect both dividing and non-dividing cells and like retroviruses, they can integrate into the host chromosome. The major drawbacks of adeno-associated viruses are that they have a small transgene capacity of less than 5 kb and produce low titres (Smith, 1999; Huang *et al.,* 1999). Other viral vectors include Herpes simplex virus (HSV), the main disadvantage of which is its cytotoxicity; lentiviruses which suffers from low infectivity and pox viruses which, although they have a large transgene capacity, are immunogenic and provide only transient expression (Huang *et al.,* 1999).

As mentioned above, the safety concerns related to the use of these viruses in humans far outweigh their advantages making non-viral delivery systems an attractive alternative.

1.2.2 Non-viral Vectors

Advantages of non-viral vectors include their simplicity of use, lack of specific immune response, low acute toxicity and ease of large scale production (Li and Huang, 2000; Huang *et al.,* 1999). This type of gene delivery vehicle has the potential to provide nucleic acid-based therapeutics that strongly resembles traditional pharmaceuticals in that the products should be capable of repeat dosage with minimal toxicity, production of large quantities with acceptable cost, high reproducibility and stable to storage (Davis, 2002). Non-viral gene delivery vectors can be non-targeted or targeted to a specific cell or tissue type (Singh, 1998). Several non-viral delivery methods exist and they can be broadly divided into two categories, naked DNA delivery by a physical method or DNA delivery by complexation with a cationic carrier (Nishikawa and Huang, 2001). These methods will be discussed in brief.

1.2.2.1 Naked DNA Injection

The direct transfer of DNA into the nucleus of cells by microinjection is the simplest system for DNA delivery (Nishikawa and Huang, 2001). The DNA used can be produced on a large scale by cultivation of plasmid harbouring *Escherichia coli.* Direct DNA transfer by this method is not feasible as it cannot be done on a large scale, gene expression levels are low and it is fairly limited to only a few tissues i.e. the skeletal muscle, the heart muscle, liver and solid tumours (Singh, 1998; Huang *et al.,* 1999). However it has an application as 'DNA vaccines' (Li and Ma, 2001).

1.2.2.2. Gene Gun Method

This method is also referred to as the ballistic method, the particle acceleration method or the micro-projectile method (Lasic, 1997). This physical method involves shooting gold particles coated with pDNA into target cells or tissues with a gene gun (Nishikawa and Huang, 2001). The gene gun method allows for DNA to penetrate directly into the cell thereby bypassing enzymatic degradation by the endosomal pathway (Li and Huang, 2000). A major drawback of this type of gene delivery is that the target tissues have to be surgically exposed and there is only low level of gene product (Huang *et al.,* 1999; Nishikawa and Huang, 2001). A possible application for the gene gun method is genetic or DNA vaccinations.

1.2.2.3 Electroporation and Nucleofection

Electroporation was first described in the 1965 by Coster. This method involves the application of short, intense bursts of controlled electrical pulses to induce transient membrane breakdown of the target cells, thereby allowing the DNA to enter the cytoplasm. After initial permeabilisation, the pores on the membrane close, trapping the DNA inside the cell (Nishikawa and Huang, 2001). To date, this technique has been applied to the skin, liver and muscles but the parameters associated with optimal gene expression differ from tissue to tissue making implementation of this method on a large scale difficult (Nishikawa and Huang, 2001).

Nucleofection is a further development of electroporation. It follows the same principle as electroporation however this cell-type specific technique depends on less harmful electrical pulses and specialised solutions optimised for specific cell types (Gresch *et al.,* 2004).

1.2.2.4 Cationic Polymers

High molecular weight polymers bearing cationic groups have been used to condense DNA via electrostatic interaction and thus facilitate gene transfer (Huang *et al.,* 1999; Mountain, 2000). The complexes formed between cationic polymers and DNA are referred to as 'polyplexes' (Felgner *et al.,* 1997). The complexation between the polymer and DNA protects the nucleic acid molecule from degradation. These polymers can enhance the uptake of plasmid DNA by cells by non-specific adsorptive endocytosis (Nishikawa and Huang, 2001). Commonly used polymers include polyethylenimines (PEI), poly-L-lysine (PLL), poly-L-ornithine and chitosan (Nishikawa and Huang, 2001; Oku *et al.,* 2001). These polymers can be readily chemically synthesised, making them amenable to scalable synthesis.

1.2.2.5 Liposomes

Liposomes have been described as vesicular colloidal particles that are composed of self-assembled amphiphilic molecules (Lasic, 1997). These 'liposomal particles' were first developed by Bangham in 1965 and due to their resemblance to cell membranes they have been extensively used as model membrane systems (Smith *et al.,* 1993).

Liposomes are vesicles consisting of one or more concentric bilayers alternating with aqueous compartments, within which a variety of lipid soluble or water soluble substances can be enclosed (Bangham *et al.,* 1972). They are usually composed of biodegradable, reusable phospholipids, however their design and structure is dependent on their intended function (Singh, 1998). Due to their relatively simple design and ease of formulation techniques, these vectors are easily synthesised on a large scale. Until their fairly recent application to gene therapy, liposomes were widely used as carriers for a variety of drugs (Wang *et al.,* 2006). Other applications of liposomes include a role in cosmetics and the possible use of liposomes in genetic vaccinations (Lasic, 1997; Gregoriadis *et al.,* 2002). As vehicles for the delivery of nucleic acid molecules, liposomes offer a protective biocompatible and biodegradable delivery system that can enhance their cellular uptake. Liposome mediated gene delivery is known to exhibit a number of desirable advantages over viral vectors such as lack of mutagenesis, reproducibility and ease of use, significant transgene expression and decreased immunogenicity and toxicity, thus safety in their use (Koumbi *et al.,* 2006; Percot *et al.,* 2004). Liposomes can be divided into four different classes as defined with respect to their functionality (Figure 1.2).

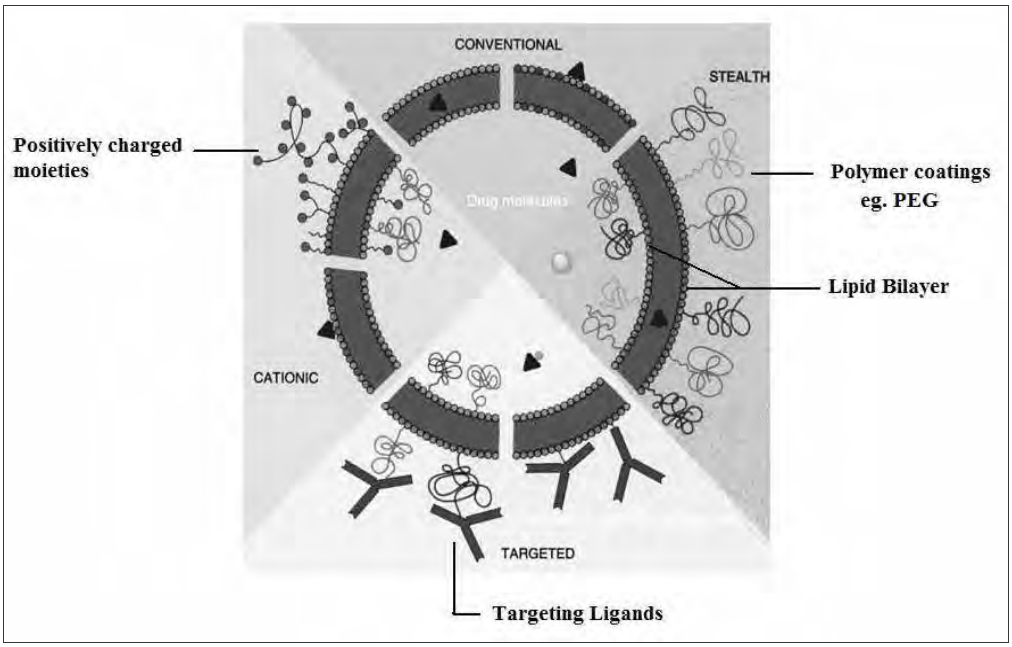


Figure 1.2: Four classes of liposomes as defined by their functionality (Adapted from Lasic, 1997).

Conventional or anionic liposomes are characterised by their non specific interactions with the environment (Lasic, 1997). They are composed of neutral or anionic phospholipids. This

type of liposome encapsulates macromolecules such as plasmid DNA within their aqueous environment. As a rule, they are not considered very efficient gene delivery vectors as they have relatively small packing capacities that limit the size of plasmid DNA that can be entrapped (Zhdanov *et al.,* 2002).

Targeted liposomes embody a relatively new concept. This type of liposome has ligands or targeting moieties such as monoclonal antibodies, peptides, lectins, growth factors, glycoproteins, receptor ligands or simple molecules such as carbohydrates attached to the liposome allowing for targeting to a specific cell type or tissue (Lasic, 1997; Immordino *et al.,* 2006). The other two liposome classes, stealth and cationic liposomes will be discussed below.

Liposomes interact with cells through four different mechanisms (Figure 1.3). These can be distinguished as (1) Adsorption, with extracellular release of the liposomal contents; (2) Adsorption with lipid exchange; (3) Endocytosis and (4) Fusion of the vesicle with the cell membrane (Torchilin, 2003). The two types of adsorption lead to the contents of the liposomes entering the cell without the uptake of the intact liposome. In the first type, the water soluble contents are released into the extracellular environment with the subsequent passive or active transport of the molecules into the cell. The second type involves the selective transfer of lipophilic material from the liposomal membrane to the cell membrane.

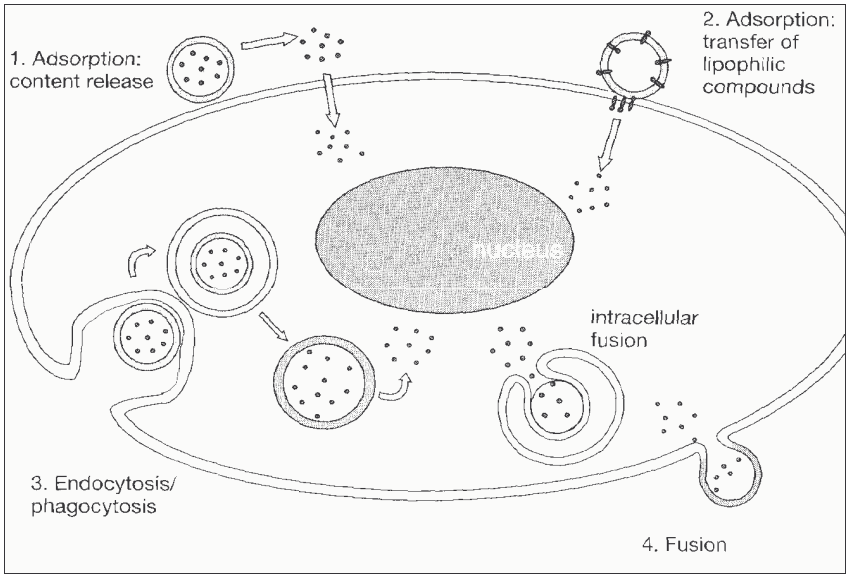


Figure 1.3: Four mechanisms of liposome-cell interactions by which liposomes can deliver their contents (Torchilin, 2003).

Endocytosis is generally receptor mediated and is discussed in detail in 1.3 below but briefly, upon binding the liposome undergoes endocytotic internalisation followed by intracellular degradation via the endolysosomal pathway and subsequent intracellular release of the liposomal content. The process of fusion involves the complete mixing of the liposomal membrane with the cell membrane and, thereby releasing the contents of the liposome into the cytoplasm of the cell (Torchilin, 2003). The occurrence of any of these interactions depends largely on the characteristics of the liposome, such as size, composition, charge, the presence of targeting ligands, and the type of cell (Torchilin, 2003).

1.2.2.5.1 Cationic Liposomes

Cationic liposomes were not extensively studied in the first 20 years of liposome research due to their apparent high toxicity (Lasic, 1997). However since 1987 when Felgner and colleagues reported the first successful *in vitro* transfection with their synthesised cationic lipid, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) (Figure 1.4 (a)), a number of other cationic lipids have been produced and have proven to be significant tools in gene delivery (Li and Ma, 2001; Singh, 1998). These include, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulphate (DOTAP) (Figure 1.4 (b)); 3β[N,N',N'-dimethylaminoethane)-carbamoyl] cholesterol (DC-CHOL) and dioctadecylamidoglycyl-spermine (DOGS) to name a few. All cationic lipids have the same basic structure, a polar head group, a hydrophobic tail and a linker region connecting the two (Lonez *et al.,* 2008).

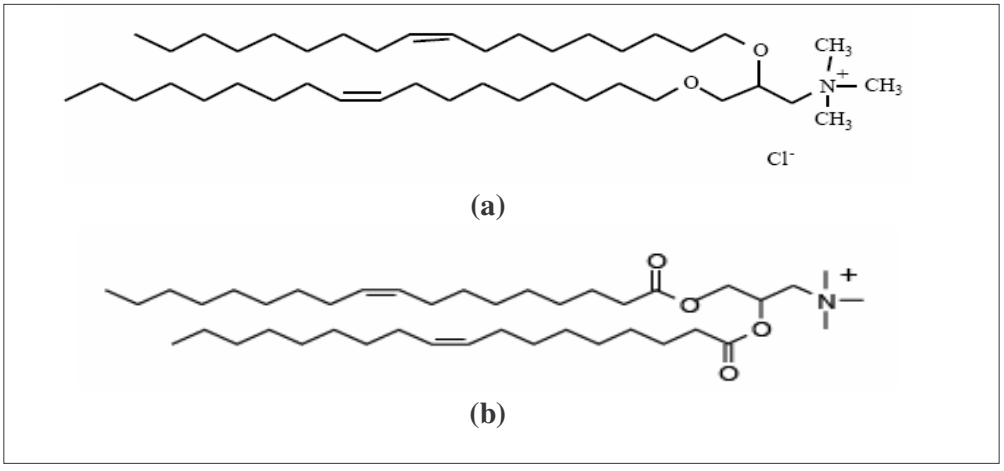


Figure 1.4: Structure of cationic lipids, **(a)** DOTMA and **(b)** DOTAP (Martin *et al.,* 2005; Immordino *et al.,* 2006).

A cationic gene delivery system comprises three components: the cationic lipid, a neutral helper lipid such as dioleoylphosphatidylethanolamine (DOPE) and the plasmid DNA (Huang *et al.,* 1999). The cationic lipid forms the cationic liposome in conjunction with a neutral co-lipid. These positively charged liposomes can interact with negatively charged DNA through electrostatic forces to form a cationic liposome-DNA complex, known as a 'lipoplex' (Felgner *et al.,* 1997). Several studies have suggested that successful gene transfer involves: 1) packaging of DNA, 2) adhesion of complex to the cell surface, 3) internalisation of DNA, 4) DNA escape from endosomes and 5) DNA expression in cell nuclei (Hui *et al*., 1996). Therefore it seems that cationic liposomes are fairly successful gene delivery vehicles as since their first deployment in this role in 1987, numerous cationic liposomes have been synthesised and used for the delivery of nucleic acid molecules into cultured cells, in animals and even in patients undergoing phase I and II clinical trials (Lonez *et al.,* 2008).

Cationic liposomes are the most extensively employed non-viral gene transfer agents (Singh *et al.,* 2006a) as they offer distinct advantages over other non-viral methods. There is virtually no size limitation on the DNA to be transferred and owing to their positive charge, they interact favourably with the negatively charged cell membrane (Li and Ma, 2001). There are, however, several drawbacks related to the use of cationic liposomes *in vivo*. These include their undesired interaction with negatively charged serum proteins and components leading to opsonisation, complement activation and their rapid clearance from circulation and accumulation in cells of the mononuclear phagocyte system (MPS) (Rejman *et al.,* 2004; Zalipsky *et al.,* 1996).

1.2.2.5.2 Stealth Liposomes

As mentioned above, once liposomes have been administered *in vivo*, their biological fate is governed by their interaction with several components within the blood system that can cause liposomal 'destabilisation' or binding to specific proteins that can lead to removal by the macrophages of the MPS. The introduction of 'stealth' liposomes has attempted to address this undesirable biological fate of liposomes.

The use of stealth or 'sterically stabilised' liposomes is a fairly recent development in gene therapy. These sterically stabilised liposomes are so named as their surface is coated with a hydrophilic polymer such as polyethylene glycol (PEG) (Lasic, 1997). The 'first generation'

of long-circulating liposomes contained the monosialoganglioside, GM_1 as a surface component as it was believed that it was responsible for the liposomal survival in circulation (Allen, 1994). In fact liposomes with GM_1 incorporated onto their surface displayed blood circulation times of several hours (Immordino *et al.,* 2006). However, problems associated with the clinical availability of GM_1 led to the search for a viable substitute and the development of 'second generation' formulations which contained surface coating with polyethylene glycol (PEG) (Allen, 1994).

PEG is a neutral crystalline, hydrophilic, thermoplastic polymer with a high solubility in both water and organic solvents, a lack of toxicity and immunogenicity, nonbiodegradability and ease of excretion from living organisms (Ishida *et al*., 2008; Zalipsky, 1995). PEG can be conjugated to various lipids such as cholesterol and phosphatidylethanolamine (PE). PEG-cholesterol was not found to be very effective in prolonging liposome circulation *in vivo* (Allen *et al.,* 1991). PE is the lipid more commonly used. It can be conjugated to PEG via a succinate, carbamate, amide or a direct linkage, the carbamate linkage being regarded as superior (Parr *et al.,* 1994). The incorporation of the lipid derivative of PEG, distearoylphosphatidylethanolamine polyethylene glycol (DSPE-PEG) (Figure 1.5), into the bilayer of liposomes improves their stability, inhibits protein adsorption to the surface of the liposome and opsonisation *in vivo* thereby preventing liposomal recognition by the reticuloendothelial system (RES) consequently leading to an increase in the liposome circulation time (Gabizon, 2001; Garinot, *et al.,* 2007). The pegylation of novel hepatocyte-targeted and non targeted pegylated cationic liposomes is described in Chapter two. The 'stealth effect' is related to the ability of PEG polymer chains grafted onto the liposome to prevent the close approach of the abovementioned molecules to the liposomal surface (Needham and Kim, 2000). It has been hypothesised that PEG brings about these benefits by attracting a water shell to the liposome surface thus providing a steric barrier against opsonins and the macrophages of RES (Ishida *et al.,* 2008; Managit *et al.,* 2003). Other theories to explain the mechanism of pegylated liposomes include the role of surface charge and hydrophilicity of the polymer and the presence of a 'conformational cloud' of liposome-grafted polymer chains on the liposome surface (Torchilin, 2003). To date, the clinical applications of stealth liposomes have been fairly limited. Pegylated liposomal doxorubicin (PLD) (DOXIL/Caelyx) is the only stealth liposome formulation to be approved in the USA and Europe for the treatment of Kaposi's sarcoma and recurrent ovarian cancer. However

there are three more formulations that are currently undergoing trials for the treatment of several types of cancer (Immordino *et al.,* 2006).

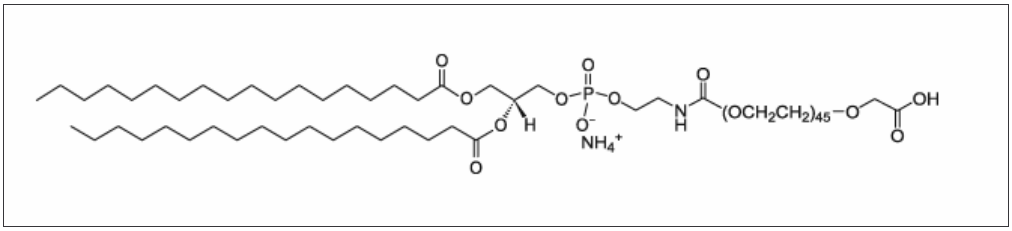


Figure 1.5: Structure of DSPE-PEG (www.avantipolarlipids.com).

1.2.3 The Biophysical Aspects of Pegylated Liposomes

There are two principal approaches to the preparation of pegylated liposomes. The first, which affords 'pre-pegylated' liposomes, involves the incorporation of a lipid derivative of the PEG polymer into the structure of the liposome. In the second, 'post pegylated' liposomes result from the incubation of pre-formed non-pegylated liposomes with the PEG-lipids (Peeters *et al.,* 2007; Shi *et al.,* 2002). The lipid derivatives used for these two types of pegylated liposomes are generally different. For pre-pegylated liposomes, the lipid utilised is commonly a phosphatidylethanolamine while for 'post pegylated' vesicles, ceramides are used (Shi *et al*., 2002; Peeters *et al*., 2007). This lipid selection is based on transfection efficiencies of various preparations reported by Shi *et al.* in 2002.

Regardless of 'post or pre-pegylation', the behaviour of pegylated liposomes depends on the characteristics and properties of the specific PEG molecule linked to the liposomal surface (Immordino *et al.,* 2006). Allen (1994) reported that targeted liposomes with PEG components of higher molecular mass such as 5000 Da sterically hinder the ability of antibodies or ligands to bind to their target cognate receptor while those of lower molecular mass, e.g. 750 Da, had decreased circulation time *in vivo*. It was therefore suggested that PEG components of approximately 2000 Da were optimal for receptor recognition as well as the provision of an effective steric barrier to promote long half-lives in circulation (Allen, 1994; Song *et al.,* 2002). PEG 2000 with various lipid derivatives have been utilised by

several authors (Song *et al.*, 2002; Rejman *et al.,* 2004; Remaut *et al.,* 2007; Peeters *et al*., 2007; Shi *et al*., 2002).

The most commonly used percentages of PEG-lipids employed by authors to form pegylated liposomes are in the 1.9 to 10 mol % range (Ross *et al.,* 1999; Lee *et al.,* 2005; Peeters *et al.,* 2007; Meyer *et al.,* 1998). It was suggested that pegylated liposomes with PEG-lipids within this range exhibit the best biological activity (Peeters *et al.,* 2007). At percentages lower than approximately 2 mol %, PEG-lipids no longer provide liposomes with an effective steric barrier and at percentages of more than 15 mol %, the PEG may cause the disruption of the bilayer and the formation of micelles (Lee *et al.,* 2005; Ross *et al.,* 1997; Kenworthy *et al.,* 1995a). For PEG 2000, this threshold percentage or saturation limit is believed to be around 8 mol % (Tirosh *et al*., 1998). Therefore the optimum range for PEG content seems to be 2 – 10 mol % *in vivo*.

Dependent on both the molecular weight of the PEG polymers attached to the liposomal surface via their lipid anchors and the graft density of these polymers, two distinguishable regimes for their configuration on the liposome surface have been proposed: mushroom regime and brush regime (de Gennes, 1980; Needham *et al.,* 1997). These two regimes have different physical characteristics. The mushroom regime forms at low grafting densities where contiguous polymer chains do not interact laterally while the brush regime arises when adjacent chains overlap laterally at high grafting densities (Needham *et al.,* 1997; Ishida *et al.,* 2008). The degree of surface coverage of the vesicle is determined by the molecular weight of the PEG as well the graft density (Ishida *et al.,* 2008). The 'mushrooms' project out of the bilayer at about 3 – 5 nm while the 'brushes' are longer at 5 – 10 nm (Barenholz, 2001) (Figure 1.6). Kenworthy *et al.* (1995b) further proposed that the de Gennes low grafting density regime be further subdivided into a 'mushroom regime' where the polymer chains from opposing bilayers interact at the midpoint between the opposing bilayers and an 'interdigitated regime' where the chains from opposing bilayers interdigitate. It has already been suggested by Moghimi in 2006 that liposomes with PEG polymer chains in the brush regime are highly resistant to clearance by macrophages but those in the non-overlapped mushroom conformation are still susceptible to attack by phagocytic cells.

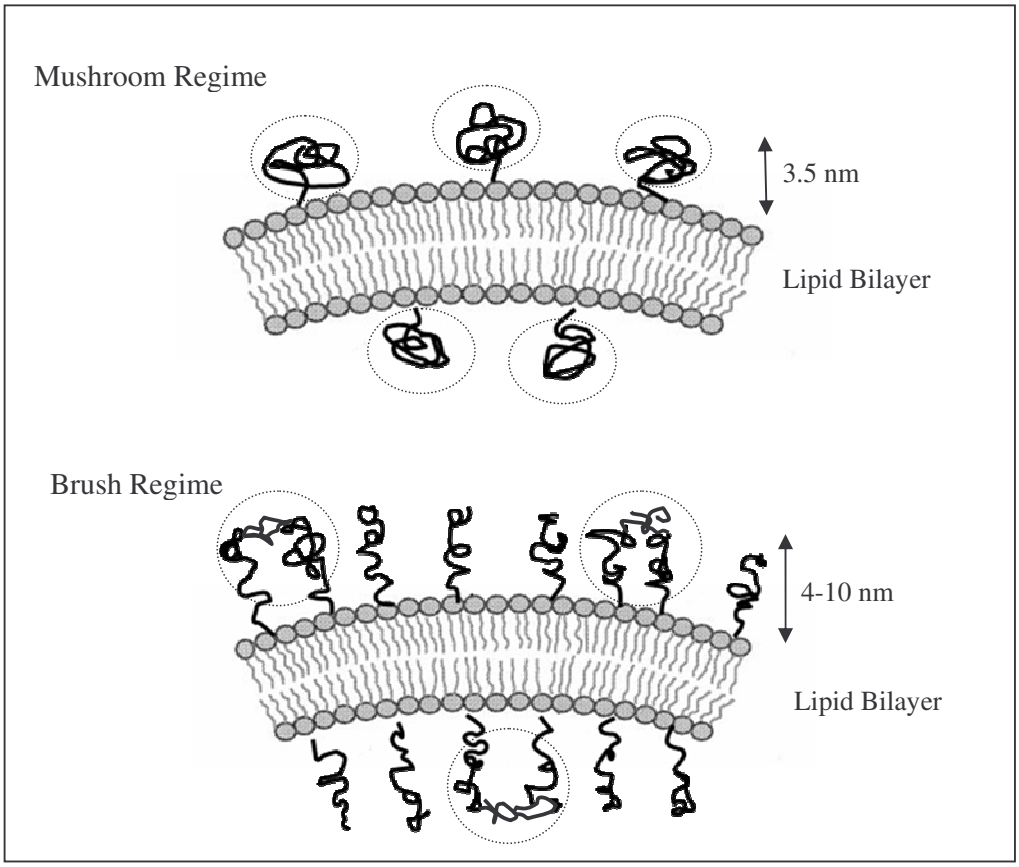


Figure 1.6: Schematic diagram of a PEG-grafted bilayer at concentrations of PEG-lipid up to 7 mol % forming the non-overlapped mushroom regime and at concentrations of PEG-lipid over 10 mol % forming the overlapped brush regime (Adapted from Barenholz, 2001; Tirosh *et al.,* 1998).

As mentioned in 1.2.2.5.2 above, the PEG polymer chains provide steric stabilisation to liposomes by attracting a water shell around them. Using differential scanning calorimetry it was discovered that this water shell contained 134 ± 4 molecules of water bound to one molecule of free PEG 2000. For PEG 2000 attached to a lipid molecule organised in micelles or bilayers, the amount of water bound increases to 210 ± 6 molecules for each PEG polymer (Tirosh *et al.,* 1998). This accumulation of water molecules around the polymer chain results in the release of water from the lipid headgroup region of the PEG-lipid. The grafted PEG chain induces the elimination of this water which in turn diminishes the effective size of the polar headgroup subsequently reducing bilayer defects and enhancing the lateral packing of the acyl chains (Tirosh *et al.,* 1998; Barenholz, 2001). Tirosh *et al.* (1998) assumed that this dehydration of the lipid headgroup region in combination with the increase of the hydration of

the outer layer by PEG polymer chains grafted in the brush regime is responsible for the increasing thermodynamic stability of the liposomes at 5 – 7 mol %. PEG, chemically attached to the headgroup of the PEG-lipid, undergoes steric exclusion from the liposomal surface. This configuration of the polymer chains is related to the mechanism by which PEG induces dehydration of the headgroup region (Ishida *et al.,* 2008; Barenholz, 2001). This PEG-induced drying and its subsequent effect on the packing of hydrophobic acyl chains are factors that contribute to steric stabilisation of pegylated liposomes (Barenholz, 2001).

1.3 TARGETED DELIVERY BY RECEPTOR MEDIATED ENDOCYTOSIS

Receptor mediated endocytosis is a multistep natural process that cells use to take in a variety of substances such as growth factors, peptide hormones, blood serum proteins, antibodies, glycoproteins, vitamins and viruses (Wolfe, 1995). Receptor mediated endocytosis is an attractive method for gene delivery, by non-viral vectors, to specific target cells. Non-viral vectors like cationic liposomes exploit this pathway to deliver DNA to a particular cell type by incorporating into their structure a targeting ligand specific to a receptor expressed by the target cell.

The first step in receptor mediated endocytosis is the binding of the ligand to its cognate cell surface receptor (Figure 1.7). This leads to a conformational change in the receptor and the resultant clustering of the receptor-ligand complex in coated pits with the subsequent invagination and pinching of these pits to produce a coated endocytic vesicle (Wolfe, 1995; Singh, 1998). As the vesicle forms, it loses its surface coat to form endosomes. In the endosome, the receptor-ligand undergoes sorting and uncoupling. The receptors and ligands have different fates. The receptors are either destroyed in a degradative pathway or recycled back to the surface of the cell membrane. The late endosome becomes acidic and fuses with lysosomes leading to the ingested material being degraded (Wolfe, 1995; Singh, 1998).

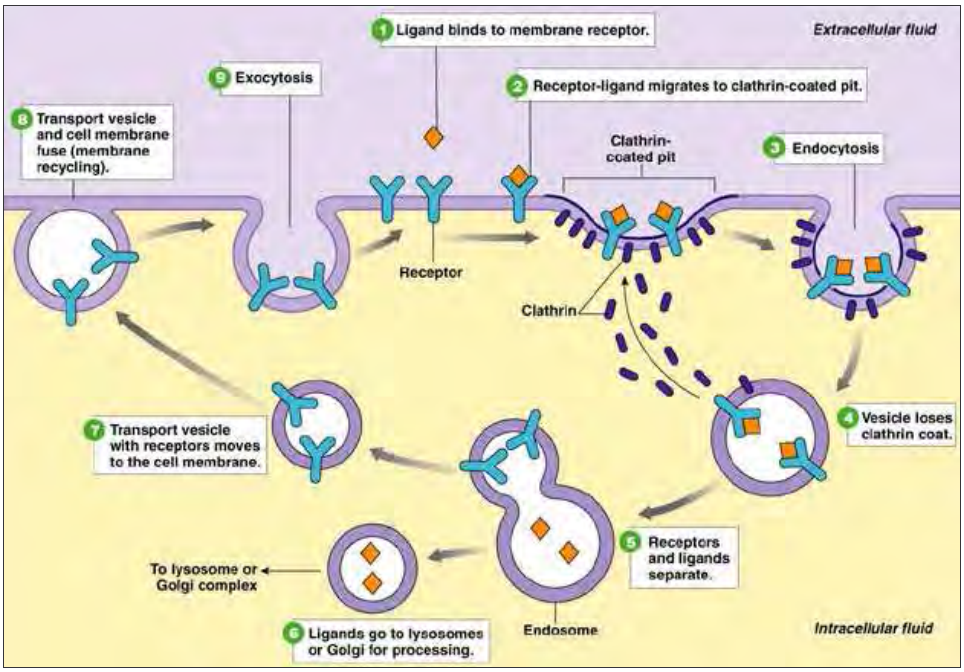


Figure 1.7: Illustration of the major steps of receptor mediated endocytosis (www.colorado.edu/intphys/class).

The pathway that targeted liposomes follow is essentially the same as that described above with a few variations. In order to achieve gene transfer, the DNA has to escape the endosome to avoid lysosomal degradation (Hoekstra e*t al.,* 2007). This is facilitated by the destabilisation of the endosomal membrane by a number of methods but mostly by the incorporation of DOPE in the structure of the liposome (Lui and Huang, 2002). Once in the cytosol, the DNA has to enter the nucleus for transcription (Figure 1.8). The mechanism of this step is not well understood but it has been suggested that the DNA enters the nucleus through nuclear pores in the nuclear envelope (Li and Ma, 2001).

For untargeted cationic liposomes, it was proposed by Felgner *et al.* in 1987 that entry into cells was facilitated by fusion of the liposome with the cell membrane (Huang *et al.,* 1999). However evidence by Friend *et al*. (1996) suggested that endocytosis is the main intracellular pathway by which cationic lipoplexes enter cells. This process is the same for targeted liposomes with the main exception being the initial step in the transfection process, where the cationic lipoplex – cell surface interaction is driven by electrostatic interactions instead of binding between the a receptor and a ligand (Wasungu and Hoekstra, 2006) (Figure 1.8).

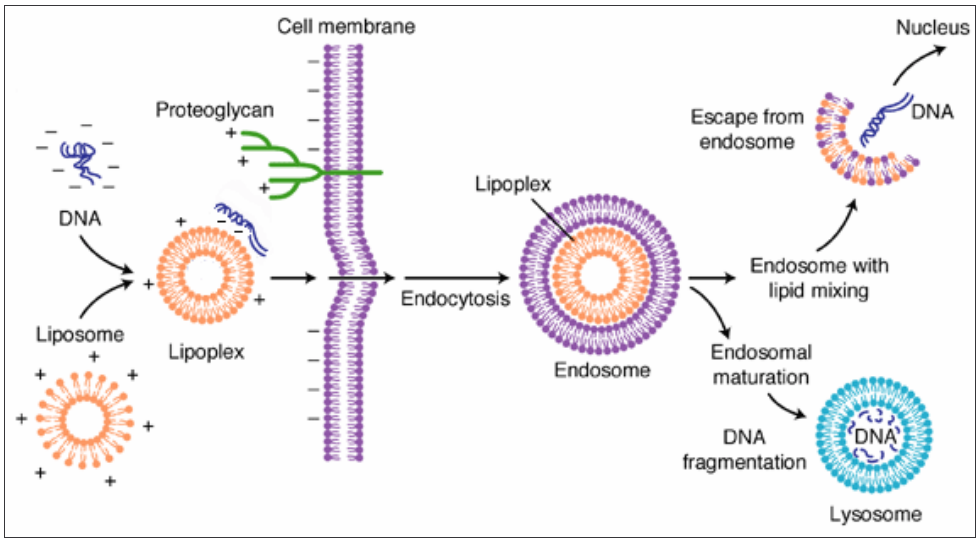


Figure 1.8: Illustration of lipoplex mediated endocytosis and transfection (Parker *et al.,* 2003).

1.3.1 Liposomal Targeting by Receptor Mediated Endocytosis

Liposome gene delivery has been considered non-specific as liposomes generally do not possess specific moieties for attachment to and subsequent entry into target cells. This lack of tissue or cell specificity has severe limitations for liposome applications as administration of these vectors result in their elimination or extensive accumulation in the lungs and liver (Hashida *et al.,* 2001). This limitation can be circumvented by incorporating into the liposomal structure, a targeting ligand or antibody conjugate. Hence the ligand or antibody can be targeted to a specific cell type or tissue.

The use of cationic lipids improves the efficiency of cell-specific transfection by receptor mediated endocytosis as the size and type of nucleic acid is not restricted and they interact favourably with the negatively charged cell membrane (Singh, 1998). There are a number of cells that exclusively express certain receptors providing attractive opportunities for cell-specific gene delivery with liposomes. These include receptors for carbohydrates such as the asialoglycoprotein receptor on hepatocytes, the mannose receptors on liver endothelial cells and macrophages, transferrin receptors on proliferating malignant cells and epithelial growth factor receptors on tumour cells (Hashida *et al.,* 2001; Tros de Ilarduya *et al.,* 2002; Lasic, 1997).

Selective localisation can be achieved using either 'passive' or 'active' targeting. 'Passive' targeting is a process that refers to the natural localisation patterns of liposomes when administered into the body and is dependent on the physical properties of the liposome and the route of administration. 'Active' targeting, on the other hand attempts to direct the liposome to a specific cell type by the addition of a 'homing device' such as an antibody or receptor ligand, into the liposomal structure, as described earlier (Singh, 1998, Barenholz, 2001). Liposomal targeting has been attempted *in vitro* as well as *in vivo.*

1.3.1.1 Liposomal Targeting *in vivo*

Successful liposomal targeting is a desirable goal. For gene transfer *in vivo*, several important conditions need to be fulfilled. The vector must have a high specificity and only selectively enter target cells, the therapeutic DNA must be protected from degradation, the lipoplex must be internalised into the cell and the functionality of the DNA preserved for expression of the gene and finally the vector complex must not be cytotoxic (Lesage, *et al.,* 2002).

Depending on the route of administration, there are a number of barriers that the gene delivery system has to overcome for successful gene transfer *in vivo* (Nishikawa and Huang, 2000). If the complex is administered systemically, the vector has to evade the RES, escape from circulation with minimal interaction with blood proteins, bind to and enter its target cell and finally deliver the DNA to the nucleus of the cell for expression. Liposomes injected intravenously are mostly taken up by the liver, spleen and macrophages of the RES (Singh, 1998).

The main concern with these ligand-bearing liposomes is the accessibility of the intended target tissue (Lasic, 1997). Yet despite this, there have been reports of successful *in vivo* targeting by liposomes. An asialoglycoprotein-poly-L-lysine conjugate for DNA delivery was introduced into mammalian hepatocytes via the asialogylcoprotein receptor *in vivo* (Wu and Wu, 1988) and Lonez *et al.* (2008) reported the use of targeted cationic liposomes in animals and in patients enrolled in phase I and II clinical trials.

1.3.1.2 Liposomal Targeting *in vitro*

This is a commonly used technique for targeting specific cells as it provides more assurances and is more easily regulated than the *in vivo* application. It can be problematic as it requires specialisation in tissue culture techniques, and it requires a mitotic cell population. *In vitro* targeting offers the advantages of working with well characterised cells in culture and liposomes can be added directly to the target cells (Singh, 1998). These *in vitro* methods can be used in developing and optimising techniques to be utilised *in vivo* (Poste *et al.,* 1984).

Thus significant expression of the luciferase gene in the pGL3 vector was found after targeting of lipoplexes with biotinylated transferrin accessories to transferrin-receptor positive HeLa cell (Singh *et al.,* 2006b). Singh *et al.* in 2007 reported an increase in transfection when galactosylated liposomes were delivered to asialoglycoprotein receptor-positive HepG2 cells.

1.4. OUTLINE OF THESIS

Gene transfer to hepatocytes is of great therapeutic potential as hepatocytes are one of the most physiologically active cells in the body being responsible for the synthesis of a wide variety of proteins, which play important roles in the functioning of the body (Kawakami *et al.,* 1998). In this thesis we have investigated the *in vitro* delivery of pegylated and non-pegylated hepatocyte-targeted cationic liposomes in the hepatocyte-derived human cell line, HepG2. Untargeted liposomes were also prepared for comparison. This targeting of foreign DNA was achieved by incorporating into the structure of cationic liposomes, targeting ligands, cholesteryl 3β-N-(4-aminophenyl-β-D-galactopyranosyl) carbamate (CAP-β-Gal) and cholesteryl 3β-N-(4-aminophenyl-β-D-glucopyranosyl) carbamate (CAP-β-Glu). We explored the method of uptake of DNA by HepG2 cells via the asialoglycoprotein receptor based on the fact that hepatocytes exclusively express large numbers of this cell surface receptor that binds and subsequently internalises asialoglycoproteins expressing galactose and N-acetylgalactosamine at the non-reducing termini of their heteroglycans.

Cationic liposomes as a gene delivery tool *in vivo* have a number of limitations including a short lifespan of lipoplexes, their inactivation by serum proteins and rapid clearance by the RES. To circumvent these limitations, the polymer polyethylene glycol is incorporated into

liposomes to provide a steric barrier that inhibits opsonisation and extends the circulation time of liposomes *in vivo* (Song *et al.,* 2002).

In chapter two the preparation of two cholesteryl glycolipids intended to provide the targeting elements of liposomes is described. In addition, the preparation of all liposomes used in this study and their characterisation by transmission electron microscopy is presented. In chapter three, the interaction of all liposomes with plasmid DNA is explored by agarose gel electrophoretic retardation and ethidium bromide displacement assays. Moreover the stability of lipoplex DNA in the presence of 10% (v/v) foetal bovine serum is explored. The evaluation of lipoplex cytotoxicities and their targeted and untargeted pegylated and unpegylated lipoplex mediated transfection of HepG2 cells using the pGL3 plasmid and the luciferase assay are the focus of chapter four.

The main aim of this study was to elucidate the effect of pegylation on targeted cationic liposomal DNA delivery to HepG2 cells in culture and to determine which targeting ligand and at what percentage the best transfection activity was obtained *in vitro.* The use of pegylation in this study is envisaged to prolong circulation time and prevent opsonisation of liposomes in the blood system, hence a more efficient delivery of a gene of interest. This type of tissue-specific targeting may have broad applications *in vivo.*

CHAPTER TWO

PREPARATION AND CHARACTERISATION OF LIPOSOMES

2.1 INTRODUCTION

This chapter focuses on the preparation of ten novel cationic liposomes and pegylated cationic liposomes with and without targeting elements, all containing the cationic cholesterol derivative, 3 β [N-(N', N'-dimethylaminopropane)-carbamoyl] cholesterol (CHOL-T). In addition, pegylated cationic liposomes contained distearoylphosphatidylethanolamine polyethylene glycol 2000 (DSPE-PEG_{2000}), a phospholipid derivative of the hydrophilic polymer polyethelene glycol (Mr = 2000). All liposomes were characterised by transmission electron microscopy.

Liposomes are lipid-based vesicular artificial macromolecular complexes usually composed of biodegradable, reusable amphiphilic molecules (Zhdanov *et al.,* 2001). These molecules have a hydrophilic group, the polar head, and a hydrophobic portion, which is the non polar tail. Therefore these amphiphilic molecules self-assemble and form ordered structures, such as lipid bilayers, in aqueous solutions (Lasic, 1997). These lipid bilayers exist such that the polar surfaces shield the non-polar interior. However, as it is energetically unfavourable to have hydrophobic edges adjacent to water, the bilayer sheets self close to form liposomes with the water both inside and outside the bilayer (Singh, 1998; Lasic, 1997) (Figure 2.1). These liposomes were once referred to as smectic mesophiles (Bangham, 1992).

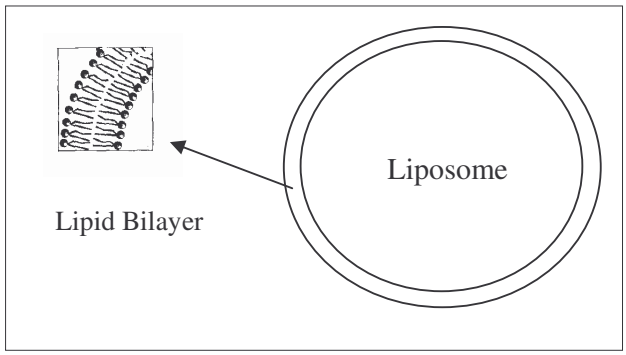


Figure 2.1: Illustration of a typical liposome showing the lipid bilayer.

Liposomes can be used for a variety of medical and non-medical purposes including the eventual delivery of RNA, DNA, drugs and other macromolecules to eukaryotic cells and may be prepared in a number of ways. The various preparations differ in the method by which 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. Sonication to prepare small unilamellar vesicles (SUVs) (Johnson *et al.,* 1971)

iii. Extrusion by filters to form large unilamellar vesicles (LUVs) or SUVs (Lasic, 1997)

iv. Homogenisation for mass production of liposomes (Lasic, 1997)

v. Ethanol injection technique (Batzri and Korn, 1973; Campbell, 1995)

vi. Ether injection technique (Deamer and Bangham, 1976)

vii. Detergent depletion (Torchilin, 2003)

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

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

The latter method was adapted and employed for the preparation of cationic liposomes utilised in this study.

Cationic lipids can be incorporated into liposome structures to form cationic liposomes. These cationic lipids are positively charged amphiphilic molecules that contain a cationic polar head group (usually an amine), attached via a linker (ester, ether, amide etc.) and spacer region to a usually double hydrocarbon chain or a cholesterol derivative (Lonez *et al.,* 2008; Wasungu and Hoekstra, 2006). Examples of commonly used cationic lipids can be seen in Figure 2.2. Cationic liposomes generally contain the phospholipid, dioleoylphosphatidylethanolamine (DOPE) as a neutral helper lipid (Figure 2.3). It is the most common helper lipid used in liposome preparations (Ramezani *et al.,* 2009). It has been suggested that DOPE has a dual role. Firstly, it helps in liposome formation and secondly, once the lipoplex is internalised in cellular endosomes, it assists in endosomal escape of DNA by destabilising the membrane (Lonez *et al.,* 2008; Percot *et al*., 2004).

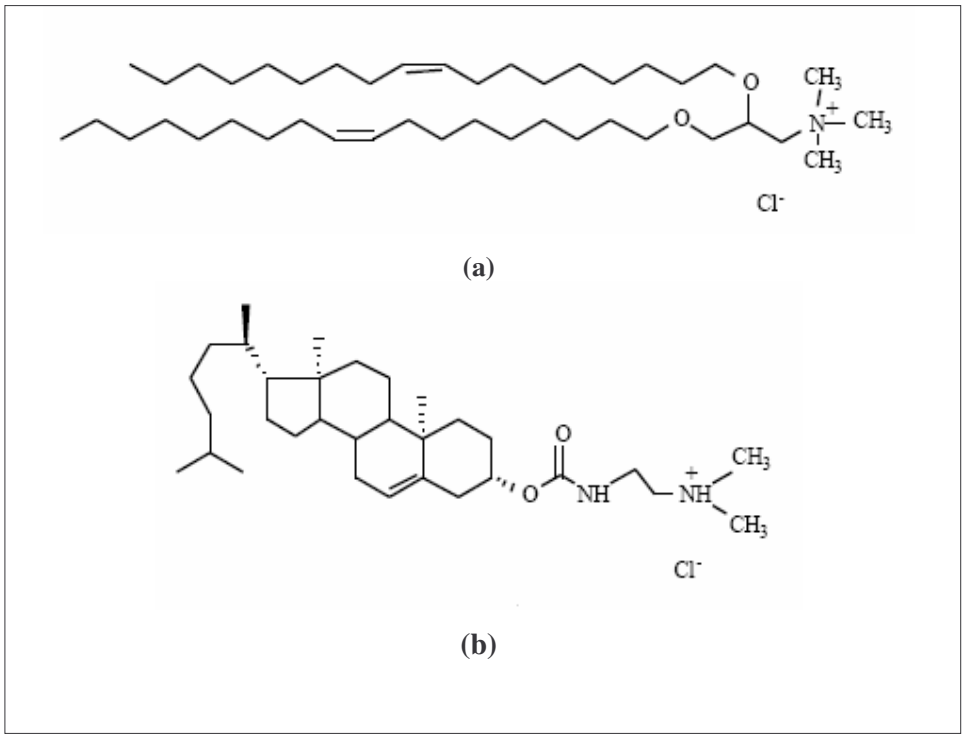


Figure 2.2: Examples of commonly used cationic lipids, **(a)** DOTMA and **(b)** DC-CHOL (Martin *et al.,* 2005).

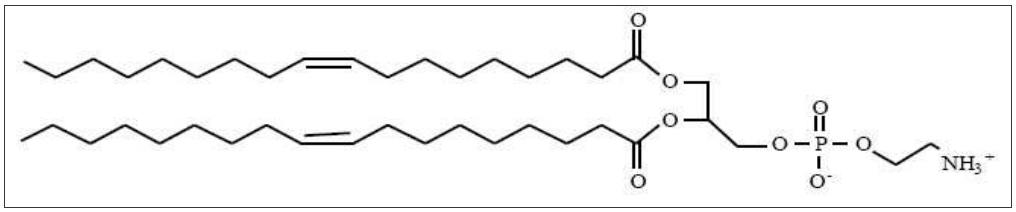


Figure 2.3: Structure of the neutral co-lipid, DOPE (Martin *et al.,* 2005).

Due to the versatility and ease of cationic liposome design, functional groups such as polyethylene glycol (PEG) can be incorporated into liposomes thus conveying stealth properties to the cationic vector, which allows these 'sterically stabilised' liposomes to avoid detection and elimination from the blood by the reticuloendothelial system (Wasungu and Hoekstra, 2006; Allen *et al.,* 1994). The cationic liposomes can be further modified by attachment of targeting moieties thereby providing targeting properties to particular cellular receptors (Wasungu and Hoekstra, 2006). An illustration of such a modified liposome is shown in Figure 2.4.

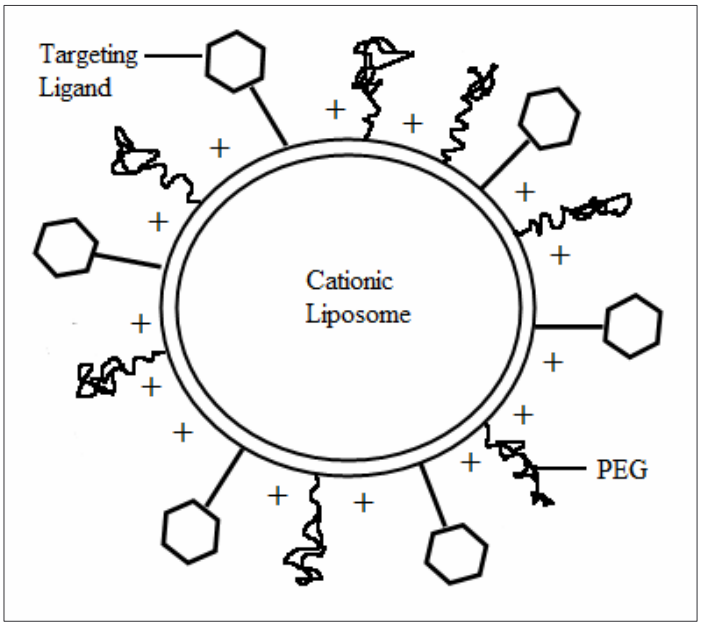


Figure 2.4: Illustration of a cationic liposome showing targeting ligands and PEG coating.

The size and structure of liposomal vesicles obtained is dependent on the method of preparation. Liposomes range from multilamellar to small and large unilamellar vesicles. Shapes of liposomes show great variety from the usual sphere to oval, dumbbell, pear shaped and even tubules (Lasic, 1997). Multilamellar vesicles (MUVs) are considered to be the simplest liposomes to prepare and they fall in the nanometer to micrometer range in diameter. Multilamellar vesicles are composed of multiple concentric lamellae separated by aqueous layers such that the ratio of entrapped volume to lipid is low and consequently macromolecules are not efficiently entrapped (Singh, 1998). Sonication of MUVs leads to the formation of small unilamellar vesicles (SUVs). These vesicles range in size from 20 nm to 200 nm in diameter (Lasic, 1997) and are also considered unsuitable for nucleic acid encapsulation because of the small aqueous volume. Large unilamellar vesicles (LUVs) are used extensively for encapsulation of macromolecules and range in size from 500 nm to several microns (Chapman, 1984).

In the investigation that follows, cholesterol derived ligands and a cationic cholesterol derivative were prepared and utilised to formulate novel cationic and pegylated cationic liposomes. These were subsequently characterised by electron microscopy to determine lamellarity and size distribution. The interaction of these liposomes with DNA was investigated in Chapter three using the gel retardation and dye displacement assays.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Dioleoylphosphatidylethanolamine (DOPE) was purchased from the Sigma Chemical Company, St Louis, USA. Distearoylphosphatidylethanolamine polyethylene glycol 2000 (DSPE-PEG_{2000}) was purchased from Avanti Polar Lipids, Alabaster, USA. Cholesteryl chloroformate, 3-dimethylaminopropylamine; p-dimethylaminocinnamaldehyde, 2-[4-(2-hydoxyethyl)-1–piperazinyl] ethanesulphonic acid (HEPES), 4-aminophenyl-β-D-galactopyranoside, 4-aminophenyl-β-D-glucopyranoside and silica gel 60 F_{254} chromatography plates were purchased from Merck, Damstadt, Germany. All other chemicals were of analytical grade.

2.2.2 Methods

2.2.2.1 Preparation of Cholesterol Derived Ligands

A solution containing cholesteryl chloroformate (33 mg, 74 µmoles); 2 ml dimethylformamide (DMF) and either 4-aminophenyl-β-D-galactopyranoside (p-NH_2-Ø-Gal) or 4-aminophenyl-β-D-glucopyranoside (p-NH_2-Ø-Glu) (20 mg, 74 µmoles) was made. To this solution was added 33 µl triethylamine. This reaction mixture was allowed to dissolve and left overnight at room temperature. After the incubation period, the sample was tested by thin layer chromatography (TLC) against the starting glycoside, either p-NH_2-Ø-Gal or p-NH_2-Ø-Glu on silica gel 60 F_{254} in a chloroform:methanol (4:1 $^{v}/_{v}$) solvent system. The plate was sprayed with 33% sulphuric acid and gently heated on a hot plate until coloured spots appeared. This served to confirm the presence of the cholesterol derived galactose derivative, cholesteryl 3β-N-(4-aminophenyl-β-D-galactopyranosyl) carbamate (CAP-β-Gal) and glucose derivative, cholesteryl 3β-N-(4-aminophenyl-β-D-glucopyranosyl) carbamate (CAP-β-Glu), in the sample mixture (Figure 2.5).

The solvent (DMF) was evaporated by rotary evaporation using a Büchii Rotavapor-R. The resulting residue was washed with water and allowed to stand at 4ºC for 2 hours, after which the mixture was filtered. The product was dried further by rotary evaporation. Ether was then added to the product, which was left overnight at 4ºC. This mixture was centrifuged at 3000 rpm in a MSE bench top centrifuge for 5 min. The resultant pellet was dried by rotary evaporation to yield a whitish coloured powdery product. The products were analysed by TLC as described above in a chloroform:methanol (4:1 $^{v}/_{v}$) solvent system (Figures 2.7 and 2.8); and by infrared spectrometry.

CAP-β-Gal:

IR: 3333 (m, OH, CONH); 2933, 2900, 2867 (m, CH, CH_2, CH_3); 1698 (m, urethane); 1603 (m, benzene ring); 1510 (s, benzene ring); 1414 (m, O–H); 1381 (w, $C(CH_3)_2$); 1214 (s, C–O); 1050 (s, C–OH); 833 (m, aromatic C–H) cm^{-1}

CAP-β-Glu:

IR: 3371 (m, O–H); 2927, 2868, 2850 (m, CH, CH_2, CH_3); 1693 (urethane); 1605 (m, benzene ring); 1511 (m, benzene ring); 1413 (m, O–H); 1379, 1366 (w, $C(CH_3)_2$); 1229 (s, C–O); 1042 (s, C–OH); 829 (s, aromatic C–H) cm^{-1}

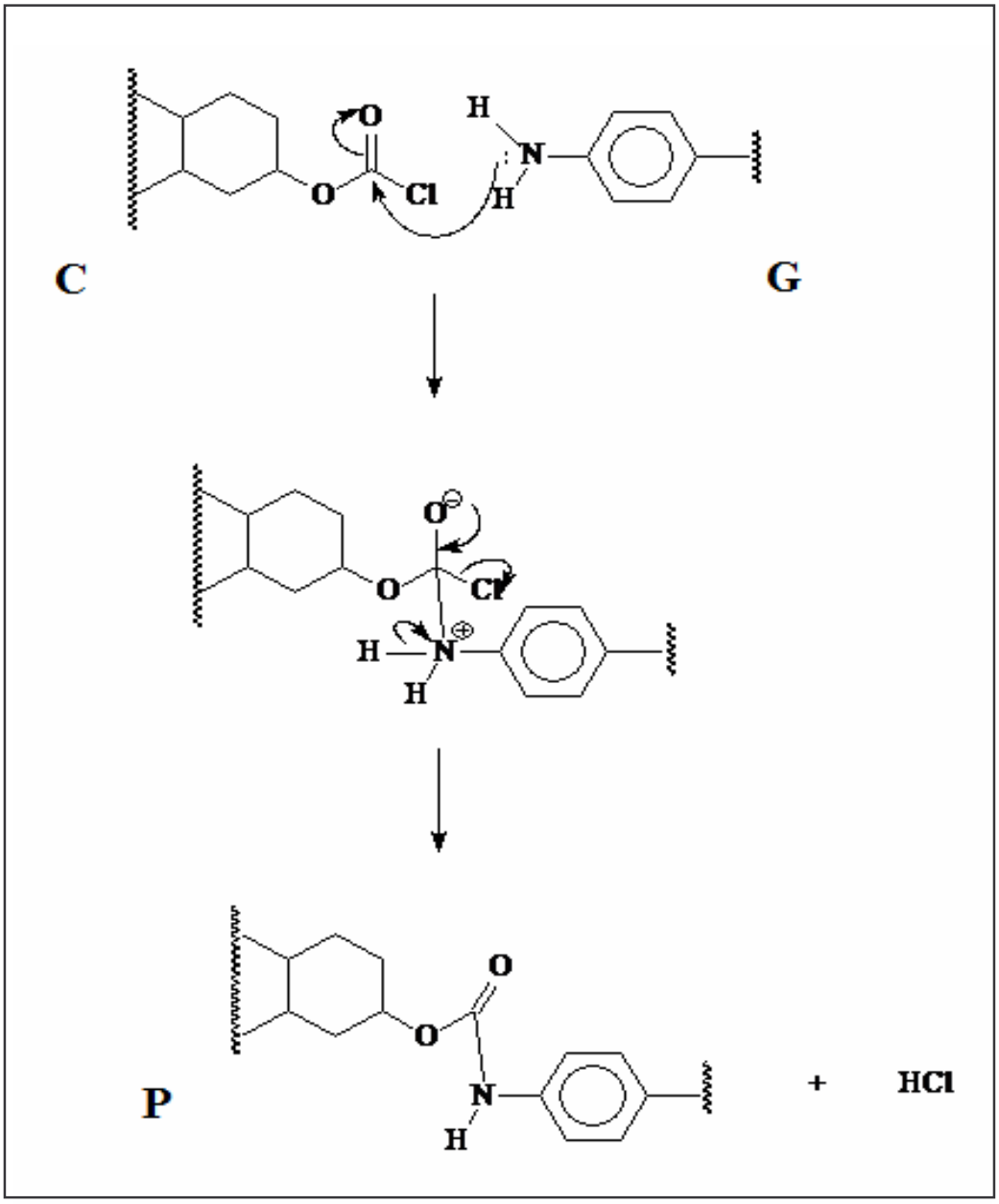


Figure 2.5: Synthesis reaction scheme of cholesterol derived ligands, cholesteryl 3β-N-(4-aminophenyl-β-D-galactopyranosyl) carbamate (CAP-β-Gal) or cholesteryl 3β-N-(4-aminophenyl-β-D-glucopyranosyl) carbamate (CAP-β-Glu) (**P**) from starting products cholesteryl chloroformate (**C**) and 4-aminophenylglycosides (**G**).

2.2.2.2 Preparation of Cationic Cholesterol Derivative 3 β [N-(N', N'-dimethylaminopropane)-carbamoyl] cholesterol (CHOL-T)

The CHOL-T used in this study was previously synthesised in our laboratory as follows:

To a solution of cholesteryl chloroformate (90 mg, 0.2 µmoles) in 1 ml dichloromethane was added 3-dimethylaminopropylamine (62.8 µl, 0.11 µmoles). This synthesis reaction was allowed to proceed for 1 hour at room temperature (Figure 2.6) and monitored by TLC (results not shown). The solvent (dichloromethane and excess 3-dimethylaminopropylamine) was subsequently removed by rotary evaporation in a Büchii Rotavapor-R. The resultant residue was dissolved in absolute ethanol and allowed to crystallise overnight at -4ºC. The product was then recrystallised, filtered under a stream of dry nitrogen gas and further dried by rotary evaporation to yield whitish coloured crystals.

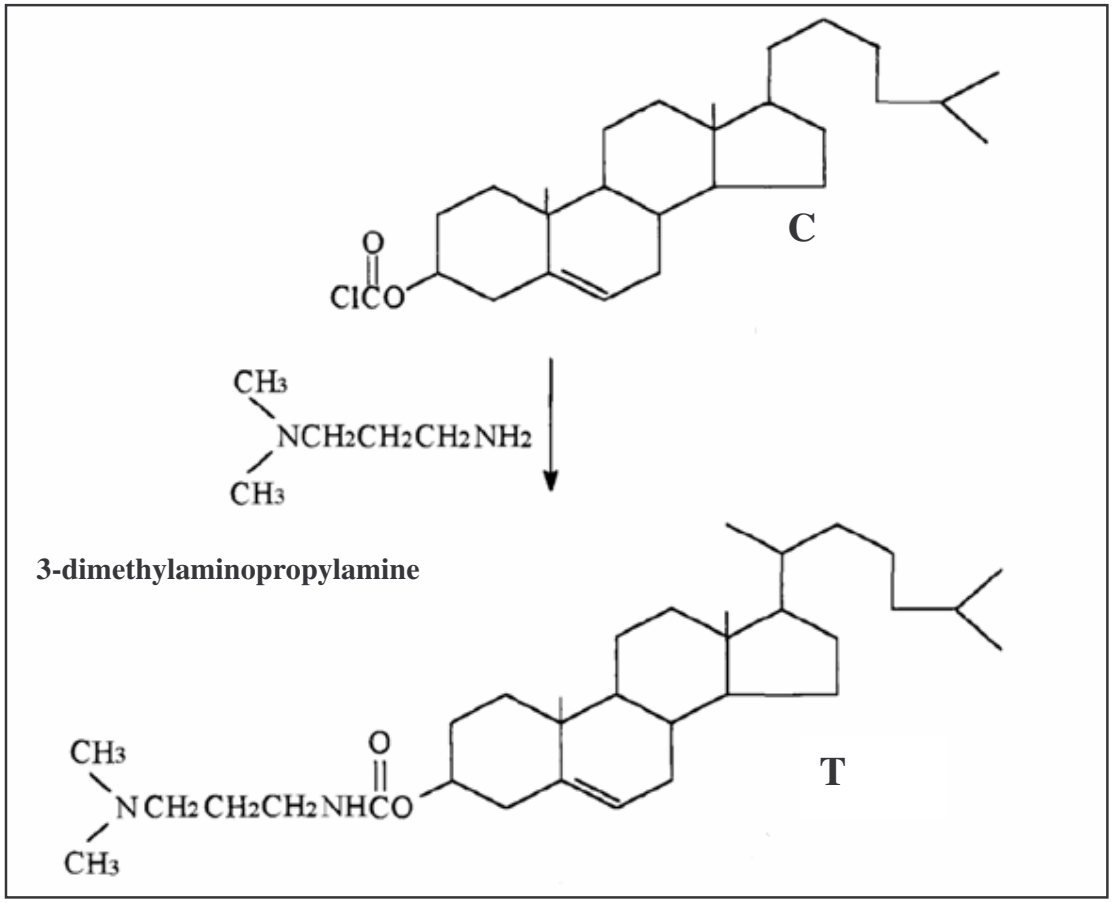


Figure 2.6: Synthesis reaction scheme of cationic cholesterol derivative CHOL-T (**T**) from starting material cholesteryl chloroformate (**C**) with the addition of 3-dimethylaminopropylamine.

2.2.2.3 Synthesis of Cationic and Pegylated Cationic Liposomes

Both the cationic liposomes and the pegylated cationic liposomes were synthesised by a method adapted from the one employed by Gao and Huang (1991). The relative quantities of the components of both the cationic and pegylated cationic liposomes were as set out in Table 2.1 and 2.2 respectively.

TABLE 2.1: Lipid composition of cationic liposomes.

CATIONIC LIPOSOME PREPARATIONS	**MOLAR RATIOS (µmoles)**			**MASS (mg)**		
	CHOL-T	DOPE	CAP-β-Gal / CAP-β-Glu	CHOL-T	DOPE	CAP-β-Gal / CAP-β-Glu
NN	2	2	-	1.03	1.49	-
NN – 5% CAP-β-Gal	2	1.8	0.2	1.03	1.34	0.14
NN – 10% CAP-β-Gal	2	1.6	0.4	1.03	1.19	0.27
NN – 5% CAP-β-Glu	2	1.8	0.2	1.03	1.34	0.14
NN – 10% CAP-β-Glu	2	1.6	0.4	1.03	1.19	0.27

TABLE 2.2: Lipid composition of pegylated cationic liposomes.

PEGYLATED CATIONIC LIPOSOME PREPARATIONS	**MOLAR RATIOS (µmoles)**				**MASS (mg)**			
	CHOL-T	DOPE	DSPE-PEG_{2000}	CAP-β-Gal / CAP-β-Glu	CHOL-T	DOPE	DSPE-PEG_{2000}	CAP-β-Gal / CAP-β-Glu
PEG-NN	2	2	0.08	-	1.03	1.43	0.22	-
PEG-NN – 5% CAP-β-Gal	2	1.8	0.08	0.2	1.03	1.28	0.22	0.14
PEG-NN – 10% CAP-β-Gal	2	1.6	0.08	0.4	1.03	1.13	0.22	0.27
PEG-NN – 5% CAP-β-Glu	2	1.8	0.08	0.2	1.03	1.28	0.22	0.14
PEG-NN – 10% CAP-β-Glu	2	1.6	0.08	0.4	1.03	1.13	0.22	0.27

The total reaction mixture contained 4 µmoles of lipid. The cationic liposomes (NN, NN – 5% CAP-β-Gal, NN – 10% CAP-β-Gal, NN – 5% CAP-β-Glu and NN – 10% CAP-β-Glu) were prepared using a constant molar quantity (2 µmoles) of the cationic lipid preparation, CHOL-T. The remaining 2 µmoles of each preparation comprised dioleoylphosphatidylethanolamine (DOPE) and either 5% or 10% of CAP-β-Gal or CAP-β-Glu.

The pegylated cationic liposomes (PEG-NN, PEG-NN – 5% CAP-β-Gal, PEG-NN – 10% CAP-β-Gal, PEG-NN – 5% CAP-β-Glu and PEG-NN – 10% CAP-β-Glu) were prepared as described in 2.2.2.3 with the addition of a constant molar quantity of distearoylphosphatidylethanolamine polyethylene glycol 2000 (DSPE-PEG_{2000}) (2% on a molar basis).

Each cationic liposome and pegylated cationic liposome lipid solution in $CHCl_3$ was evaporated using a Büchii Rotavapor-R to produce a thin film deposit on the inside of a test tube. The samples were further dried in a drying pistol for 1,5 hours. Thereafter the thin film layers were rehydrated in 1 ml of sterile HEPES buffered saline (HBS) (20 mM HEPES and 150 mM NaCl, pH 7.5). The mixtures were then vortexed, sonicated for 5 minutes and left overnight at 4ºC. Thereafter the samples were sonicated prior to use.

2.2.2.4 Characterisation of Liposomes by Transmission Electron Microscopy (TEM)

Cationic and pegylated cationic liposome preparations were diluted 1 : 5 with HBS to promote fluidity of the samples. Aliquots of 1 µl of each diluted sample were placed on Formvar coated grids and the excess blotted off with filter paper. The samples were immediately vitrified by plunging into liquid ethane cooled by liquid nitrogen, using a spring-loaded Leica CPC system. Grids were then transferred to a Gatan cryotransfer system and viewed using a JOEL 1010 TEM without warming above -150ºC.

2.3 RESULTS AND DISCUSSION

2.3.1 Preparation of Cholesterol Derived Ligands

The thin layer chromatograms confirmed the presence of the desired products, cholesteryl 3β-N-(4-aminophenyl-β-D-galactopyranosyl) carbamate (CAP-β-Gal) and cholesteryl 3β-N-(4-aminophenyl-β-D-glucopyranosyl) carbamate (CAP-β-Glu) in the reaction mixture (Figures 2.7 to 2.9).

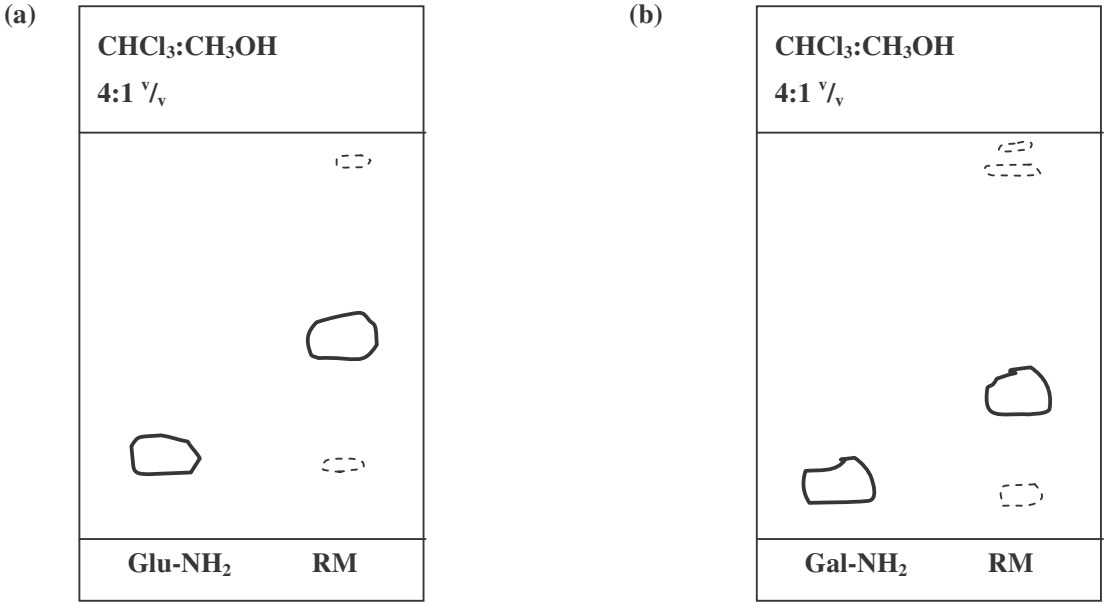


Figure 2.7: Thin layer chromatography of **(a)** standard 4-aminophenyl-β-D-glucopyranoside (Glu-NH_2) and reaction mixture (RM) in the synthesis of CAP-β-Glu and **(b)** standard 4-aminophenyl-β-D-galactopyranoside (Gal-NH_2) and the reaction mixture (RM) in the synthesis of CAP-β-Gal in a chloroform:methanol (4:1 $^{v}/_{v}$) solvent system. (silica gel 60 F_{254}).

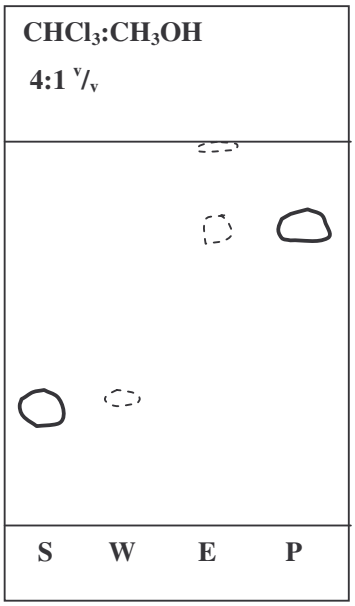


Figure 2.8: Thin layer chromatography of the prepared sample of CAP-β-Glu in a chloroform:methanol (4:1 $^{v}/_{v}$) solvent system where

S = Standard p-NH_2-Ø-Glu
W = Supernatant of the water wash
E = Ether extract in ethanol
P = Product in DMF

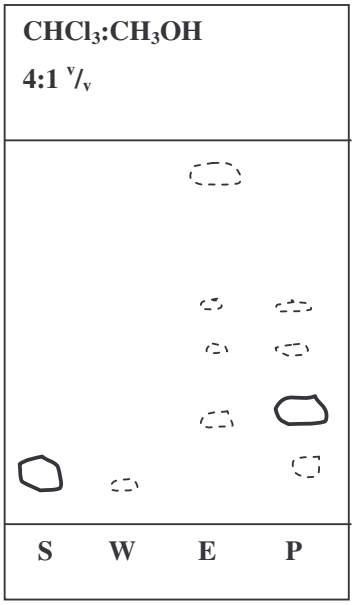


Figure 2.9: Thin layer chromatography of the prepared sample of the CAP-β-Gal in a chloroform:methanol (4:1 $^{v}/_{v}$) solvent system where

S = Standard p-NH_2-Ø-Gal
W = Supernatant of the water wash
E = Ether extract in ethanol
P = Product in DMF

Concentrated sulphuric acid was sprayed onto the thin layer chromatograms to aid in colour development and visualisation. Dehydration of sterols, such as free cholesterol and cholesteryl chloroformate by the acid results in a purple/pink colour while dehydration of free sugars yields a black/brownish colour. Figures 2.8 and 2.9 illustrate the cleaning up process of the desired products. The water wash step was done to dissolve out any excess unreacted sugar which was seen as a brownish black spot indicating the sugar (W in Figures 2.8 and 2.9). The ether was used to dissolve out the unreacted cholesteryl chloroformate (CCF), which appeared as a purple spot near the solvent front. The ether extract also contained some residual product. The product lanes contained the desired crystalline products as well as negligible amounts of unreacted sugar and CCF which suggested that the purification process was successful.

The cholesteryl glycosides CAP-β-Gal and CAP-β-Glu were prepared from cholesteryl chloroformate and the p-aminophenyl-β-D-glycopyranosides. Thus the carbonyl carbon of the chloroformate undergoes a nucleophilic attack by the aryl amino group of the glycoside to afford a urethane link, which was confirmed by the strong bands at 1698 and 1693 cm^{-1} in the infrared spectra of the galacto and gluco products respectively. The reactions, which were quantitative, were facilitated by the relatively high basicity of the p-amino group on the phenolic ring ($pK_b = 8.50$) (Sykes, 1970).

2.3.2 Synthesis of Cholesterol Derivative (CHOL-T)

3 β [N-(N', N'- dimethylaminopropane) - carbamoyl] cholesterol (CHOL-T) was previously successfully synthesised (Singh, 2001). This cationic lipid has the general structure of most cationic lipids used today, i.e. it has four basic components: a hydrophobic lipid anchor, spacer arm, linker bond and a positively charged head group (Huang *et al.*, 1999)
(Figure 2.10).

The cationic lipid in this study has a cholesterol ring anchor, a carbamoyl linker bond and a monovalent dimethylamino head group. The cholesterol anchor brings rigidity to the liposome structure but it also seems to have an influence on membrane fluidity and the consequent effects on lipid mixing within the bilayers as well as determining other physical properties of the lipid bilayer (Huang *et al*., 1999; Lesage *et al.,* 2002).

The linker bond of the cholesterol derivative is a carbamoyl bond and influences the chemical stability and biodegradability of the cationic lipid (Huang *et al*., 1999). The spacer arm length may play a role in promoting DNA interaction with the charged head group i.e. a longer spacer arm would result in the decrease of steric hindrance between the polar head group and the hydrophobic cholesterol ring system (Singh, 1998).

The type of head group plays an important role in determining the transfection efficiency and cytotoxicity of the formulated liposomes. Monovalent cationic lipids, like CHOL-T, condense DNA less strongly than multivalent cationic lipids *in vitro*, however the presence of too many positive charges on the head group may result in an extremely secure interaction with the DNA and the subsequent decrease in transfection activity as this impedes the disassociation of the pDNA at the molecular level (Huang *et al.,* 1999). Cholesterol derivatives with a tertiary amino group, such a CHOL-T, generally have stronger transfection activities than quaternary derivatives (Farhood *et al.,* 1992).

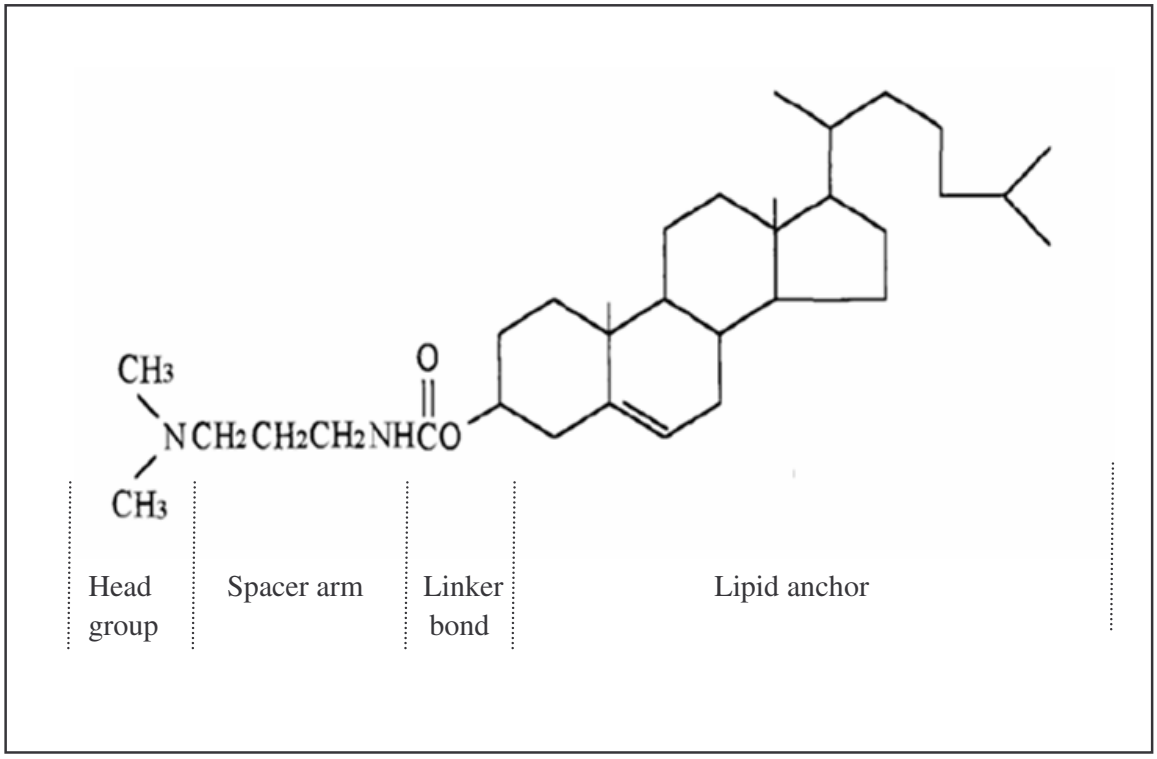


Figure 2.10: Structure of cationic lipid CHOL-T showing the four structural components.

2.3.3 Synthesis of Cationic Liposomes

Cationic and pegylated cationic liposomes were successfully synthesised using the method described. Both the cationic and pegylated cationic liposomes were prepared using a 50% mole composition of the cationic lipid preparation, CHOL-T.

The other major ingredient in all liposome preparations is the cephalin dioleoylphosphatidylethanolamine (DOPE). DOPE is a neutral zwitterionic phospholipid and is often used in liposome preparations as a helper or co-lipid. It tends to form a heterodimer with the cationic lipid through the interaction between the negatively charged phosphate on DOPE and the tertiary ammonium group on the cationic lipid (Felgner *et al.,* 1994). It is thought that this lipid improves cationic lipid mediated transfection efficiency by potentially promoting the conversion of the lamellar lipoplex phase into a non-lamellar or hexagonal phase (Wasungu and Hoekstra, 2006; Liu *et al.,* 2002). DOPE has a small head group and two bulky fatty acyl chains that gives it an inverted cone shape (Figure 2.3) that contributes to the fusogenic capacity of DOPE that allows this neutral lipid to assist in endosomal membrane destabilisation and the subsequent release of DNA from the endosome (Li and Ma, 2001; Percot *et al.,* 2004).

The targeting moieties, CAP-β-Gal and CAP-β-Glu, form part of both the cationic and the pegylated cationic liposomes. These are incorporated into both types of liposome preparations at a concentration of 5% and 10% (Table 2.1 and 2.2). The ligands were designed to specifically target the asialoglycoprotein receptors of liver hepatocytes. Hepatocytes exclusively express large numbers of these receptors thus substances can be targeted directly to them using asialoglycoproteins as ligands (Grove *et al.,* 1998). In this study, novel cholesterol derived glycolipids, CAP-β-Gal and CAP-β-Glu were synthesised and incorporated into liposomes to target the asialoglycoprotein receptors on the hepatocyte derived cell line HepG2 in culture.

The final constituent of the pegylated liposomes is the distearoylphosphatidylethanolamine polyethylene glycol 2000. This lipid was incorporated into all of the pegylated cationic liposomes (PEG-NN, PEG-NN – 5% CAP-β-Gal, PEG-NN – 10% CAP-β-Gal, PEG-NN – 5% CAP-β-Glu and PEG-NN – 10% CAP-β-Glu) at a concentration of 2 mol %. DSPE-PEG_{2000} has a distearoylphosphatidylethanolamine lipid anchor covalently attached via a

carbamate linkage to polyethylene glycol of molecular weight 2000 (Rejman *et al.,* 2004). DSPE was used as a bilayer anchor as it was cited as the most effective anchor for PEG in terms of stability of the linkage and minimisation of lipid exchange (Bradley *et al.,* 1998). PEG is a hydrophillic polymer that when incorporated into a liposomal bilayer by its lipid anchor, extends 3 – 10 nm from the liposomal surface thus providing a steric barrier that stabilises liposomes, reduces their interaction with biological macromolecules and inhibits opsonisation by proteins allowing for longer circulation times in blood (Song *et al.,* 2002; Hong *et al.,* 1997). Explanations of the phenomenon of 'sterically stabilised' liposomes relate to the role of the hydrophilicity of pegylated liposomes and the 'cloud' of polymer chains that surround the surface of pegylated liposomes (Torchilin *et al.,* 2003).

2.3.4 Characterisation of Liposomes by Transmission Electron Microscopy

Transmission electron microscopy revealed the unilamellar nature and sizes of the different cationic and pegylated cationic liposome preparations (Figures 2.11 and 2.12). Cationic liposomes range in size from 100 nm to 500 nm while the pegylated cationic liposomes ranged in size from 33 nm to 250 nm in diameter. The morphologies observed for all liposome preparations were generally spherical however some revealed a deformable nature. No significant difference in size was noted between the targeted and untargeted liposomes ($p < 0.05$). Artefacts seen in some of the liposome images are attributed to the cryoTEM process. Pegylated cationic liposomes seem to be much smaller in size as compared to their non-pegylated counterparts. This observation is consistent with a study conducted by Lee *et al.* (2005). It was proposed that the PEG molecules on the surface of the liposome have a repulsive characteristic that leads to inhibition of aggregation between liposomes during preparation thus forming smaller sized vesicles (Lee *et al.,* 2005). It has been suggested that liposome size or their hydrodynamic radii play an important part in the liposomal clearance rates in blood (Campbell *et al.,* 2001). The size of liposomes is also related to their removal by the RES. Smaller liposomes with a less fluid membrane are not easily opsonised by complement proteins in circulation when compared to larger liposomes (Oku *et al.,* 2000). Lee *et al*. (2005) also suggested that liposome size is one of the major factors in determining how liposomes accumulate in the body. It should be noted that while the TEM used in this study provides a preliminary evaluation of liposomal size, more information on the actual distribution of the liposomal population in a sample can be obtained by using techniques such as dynamic light scattering and zeta-sizing.

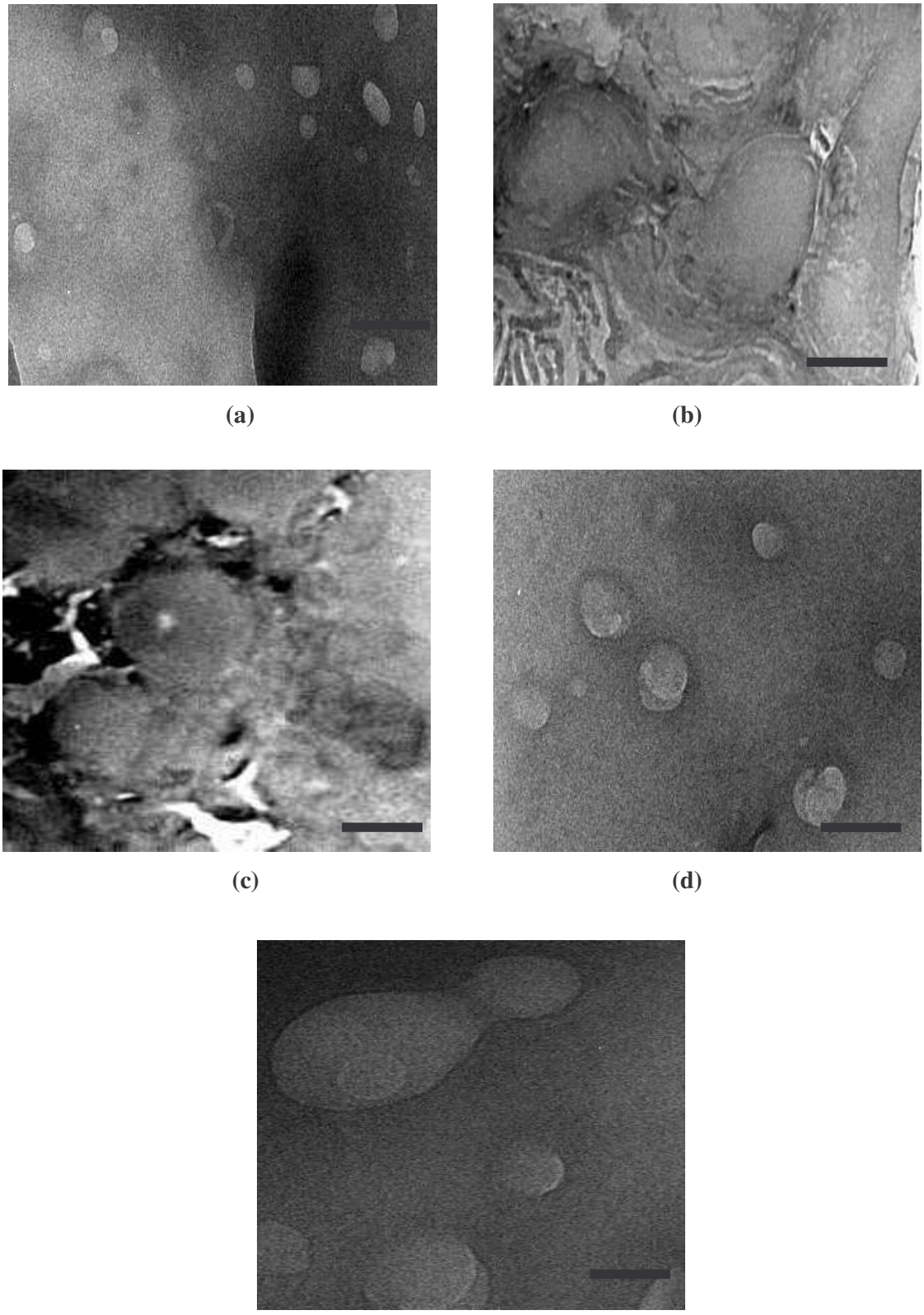
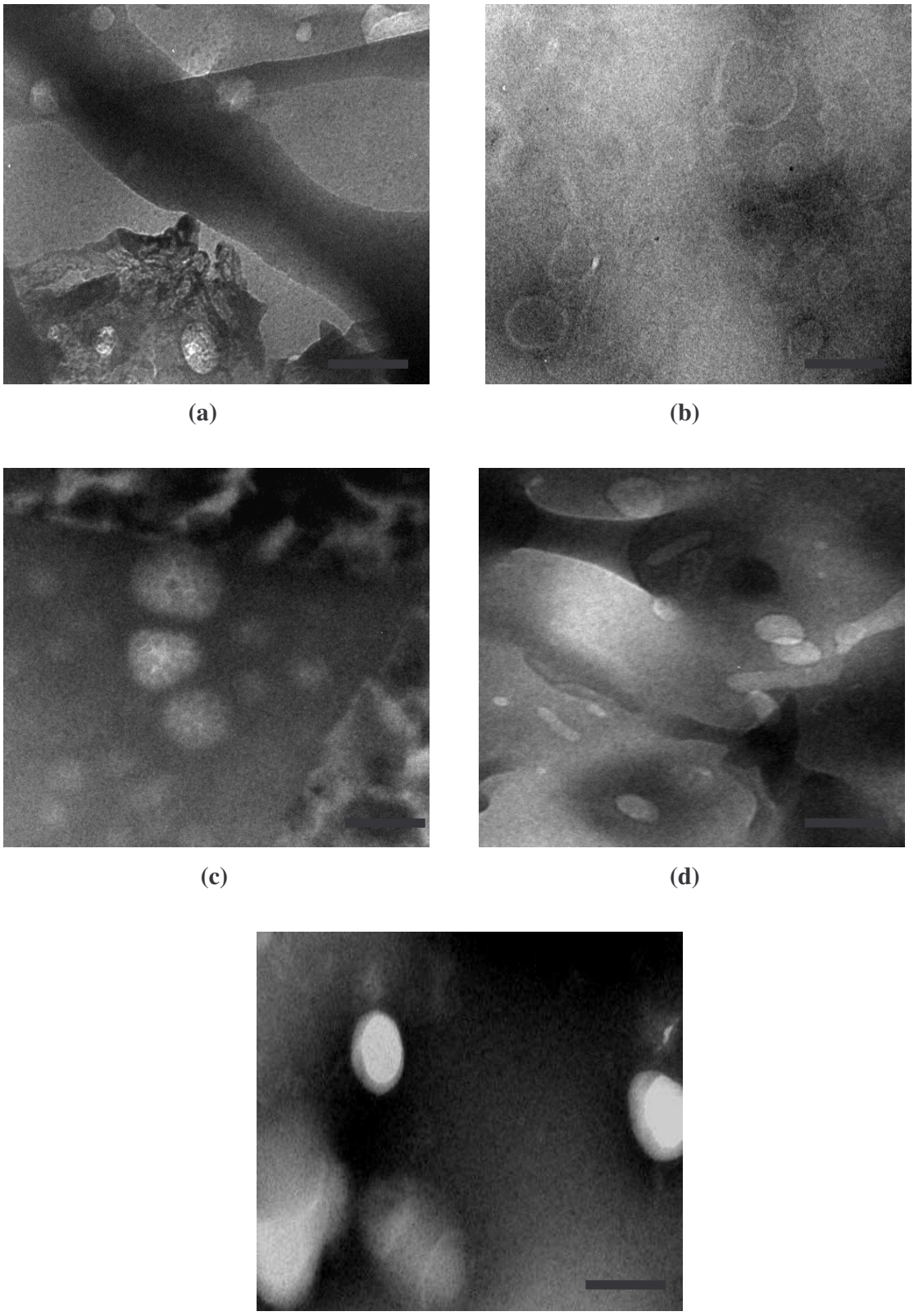


Figure 2.11: Transmission electron micrographs of cationic liposomes.
Bar = 200 nm expect (a,c) (500 nm). **(a)** NN**; (b)** NN – 5% CAP-β-Gal**;** **(c)** NN – 10% CAP-β-Gal; **(d)** NN – 5% CAP-β-Glu and **(e)** NN – 10% CAP-β-Glu



(a) **(b)**

(c) **(d)**

(e)

Figure 2.12: Transmission electron micrographs of pegylated cationic liposomes. Bar = 200 nm expect (b) (500 nm). **(a)** PEG-NN; **(b)** PEG-NN – 5% CAP-β-Gal; **(c)** PEG-NN – 10% CAP-β-Gal; **(d)** PEG-NN – 5% CAP-β-Glu and **(e)** PEG-NN – 10% CAP-β-Glu

CHAPTER THREE

PREPARATION AND CHARACTERISATION OF LIPOSOME-DNA TRANSFECTION COMPLEXES

3.1 INTRODUCTION

The term 'lipoplexes' was first used by Felgner *et al*. (1997) to describe the complex formed by the interaction of plasmid DNA (pDNA) with cationic liposomes or lipids. These so-called 'lipoplexes' have proven to be a viable alternative in the search for new DNA delivery agents. In this chapter, the interaction of pegylated and non-pegylated cationic liposomes with plasmid DNA is described and discussed.

The exact mechanism involved in the formation of cationic liposome-DNA constructs and structure of the lipoplexes is still inadequately understood. What is known and holds true today is that complexation is achieved by simply mixing and incubating the cationic liposome preparation and the pDNA (Felgner *et al*., 1987). This process is dependent on a number of factors including charge ratio, liposome concentration and nature of preparation, plasmid size and temperature to name a few (Wasungu and Hoekstra, 2006; Barenholz, 2001). It is generally accepted that the cationic liposomes interact with pDNA through charge attraction (Li and Ma, 2001). The first step involves an electrostatic reaction between the positively charged amine headgroup of the cationic lipid and the negatively charged phosphate backbone of the pDNA resulting in the formation of a liposome-DNA complex (Ropert, 1997) (Figure 3.1). The second step includes the rearrangement of the liposome and pDNA such that the pDNA is compacted or condensed in a way that it is effectively shielded by the lipids of the liposome (Zhdanov *et al*., 2002; Wasungu and Hoekstra, 2006).

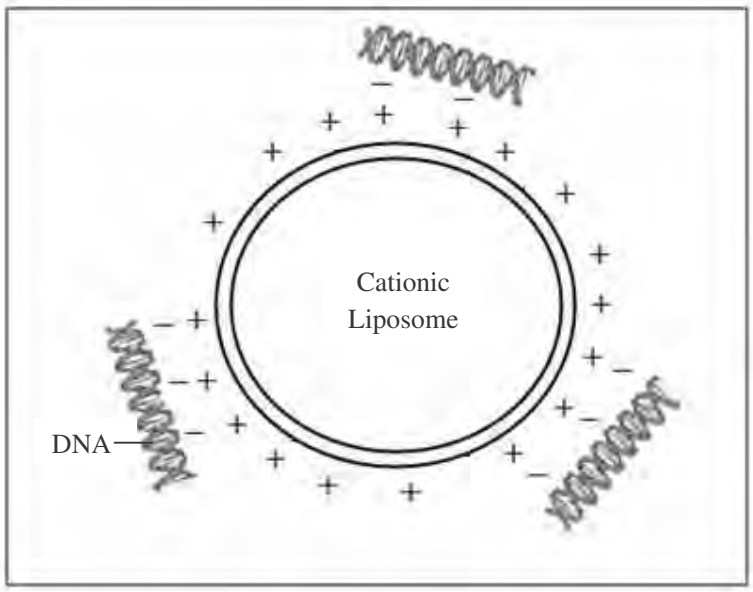


Figure 3.1: Illustration of cationic liposome-DNA complex showing electrostatic binding of positively charged liposome to negatively charged DNA.

There are essentially two hypothetical models of liposome-DNA interactions that have been elucidated by the use of fluorescence, atomic force and electron microscopy techniques. These two models are the electrostatic model and the coated electrostatic model (Singh, 1998; Smith *et al*., 1993). The original electrostatic model was proposed by Felgner and Ringold in 1989 and is based on the probability that electrostatic forces underlie the successful interactions between DNA and cationic lipids. This model proposed that four liposomes were bridged by one plasmid DNA molecule (Figure 3.2). Although four liposomes may interact with one plasmid, the small size of the plasmid would bring the four liposomes in such close proximity that they would probably repel each other and may disassociate from the DNA. This model was never confirmed experimentally (Huang *et al*., 1999).

The coated electrostatic model suggests that the DNA is coated by lipid bilayers due to the DNA entrapped between the bilayers during liposome-DNA interactions. Gershon *et al*. in 1993 used electron microscopy to reveal that DNA thickens and shortens, hence condensing, when interacting with cationic liposomes thus resembling beads on the string (Lasic, 1997) (Figure 3.3). At a critical cationic lipid to DNA ratio all the DNA is coated by lipid. At this point two processes occur; viz. DNA induced membrane fusion and the cooperative collapse of DNA known as condensation or compaction which seems to protect the DNA (Smith *et al*., 1993; Huang *et al*., 1999).

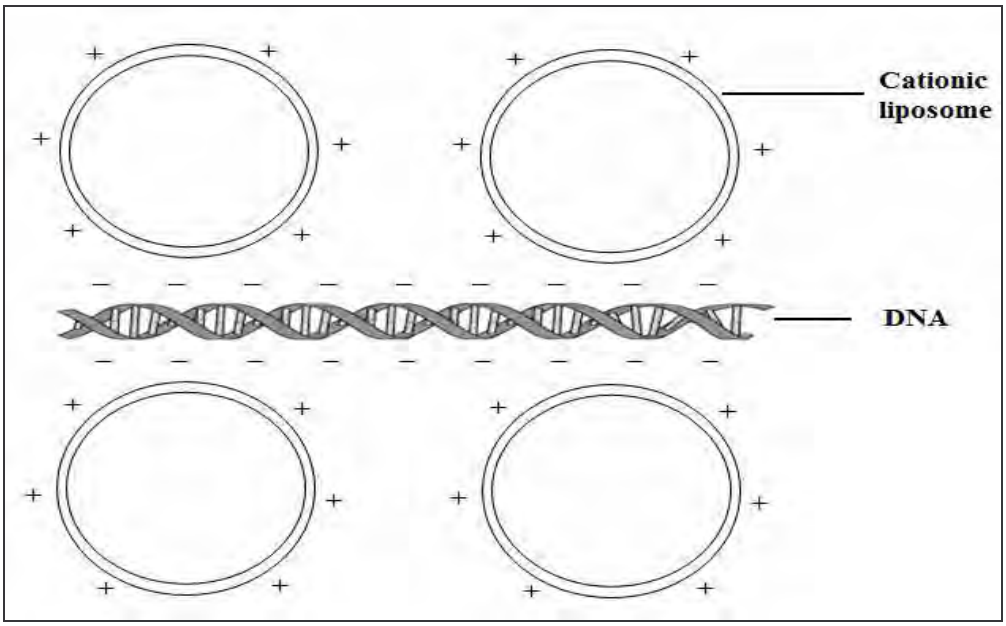


Figure 3.2: The original electrostatic model of liposome-DNA complexes proposed by Felgner and Ringold, (1989).

Observations by freeze-fracture electron microscopy revealed partially fused spherical aggregates with a halo of fibres. This gave rise to the so-called 'spaghetti and meatballs' model where the 'spaghetti' structures are the DNA covered by a lipid bilayer and the 'meatballs' are liposome-DNA aggregates (Lasic, 1997).

It is possible that none of the above models are correct (Lasic, 1997). However two fairly recent studies by Radler *et al*., 1997 and Lasic, 1997 on lipid-DNA interactions using small-angle X-ray scattering lend support to the coated electrostatic model (Li and Ma, 2001).

In this study liposome-DNA complex formation was monitored by gel retardation assays. The gel retardation or electrophoretic mobility shift assay was originally devised by Fried and Crothers for studying protein-DNA interactions (Scott *et al*., 1994). It was adapted for cationic liposome studies and the underlying principal of this assay is that the formation of complexes between plasmid DNA and cationic liposomes results in DNA electrophoretic migration retardation. The amount of liposome-associated DNA that is retained in the wells increases with increasing liposome concentration until complete DNA complexation is achieved and the charge ratio corresponding to this complexation can be thus estimated (Percot *et al*., 2004). This liposome-DNA association was also explored in an ethidium bromide dye displacement assay. This method was adapted from that described by

Tros de Ilarduya *et al*. (2002). When ethidium bromide, a monovalent DNA-intercalating cationic fluorophore binds to naked DNA, a marked enhancement of fluorescence is noted (Tros de Ilarduya *et al*., 2002; Singh *et al*., 2006a). This fluorescence is quenched when DNA-associated ethidium bromide is displaced by higher affinity compounds such as cationic liposomes or by condensation of DNA structure (Xu *et al*., 1996; Tros de Ilarduya *et al*., 2002). DNA degradation is known to be a major limiting factor in the application of gene transfer and any synthetic vector used has to be able to effectively protect the DNA (Obata *et al*., 2009). The role of lipoplexes in protecting the DNA from attack by serum nucleases was assessed by *in vitro* nuclease protection assays using agarose gel electrophoresis.

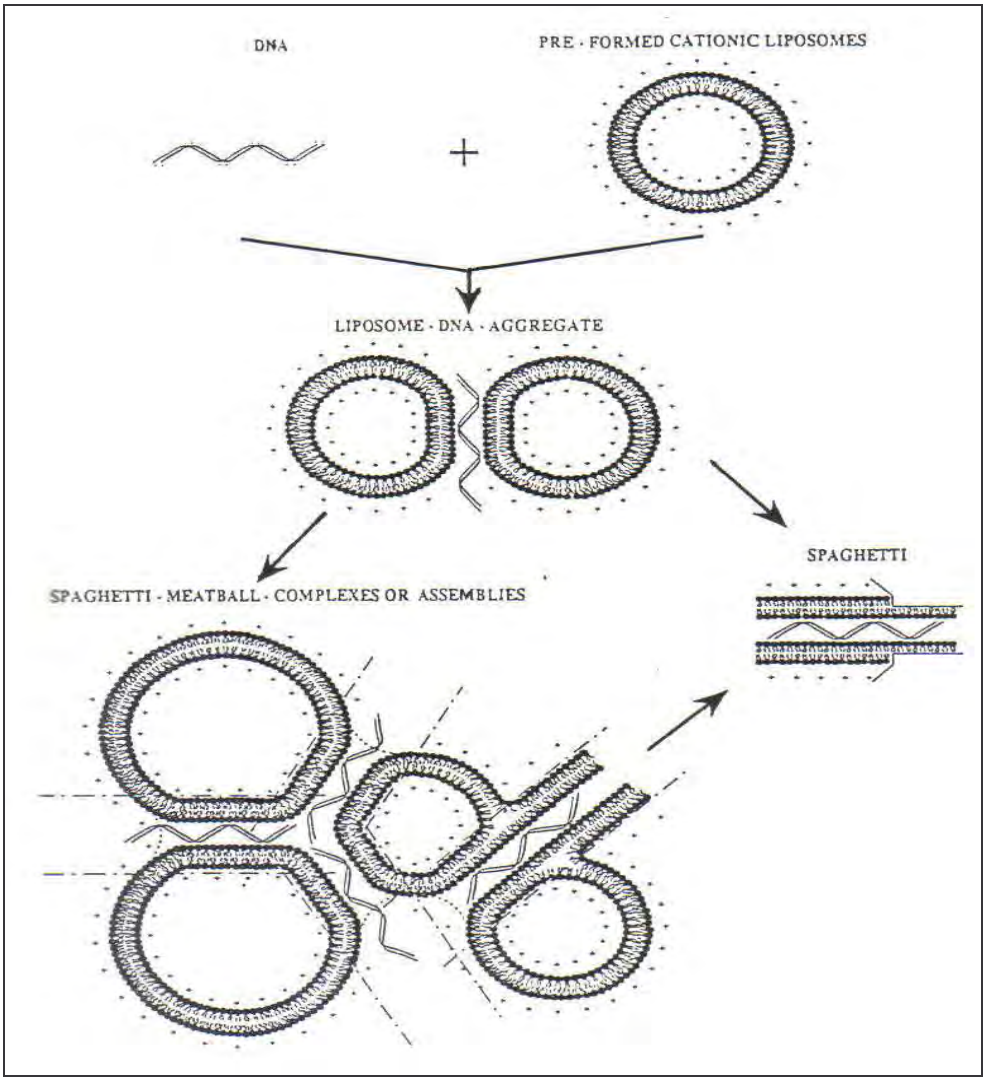


Figure 3.3: Scheme based on freeze fracture electron micrographs, depicting the interaction of negatively charged DNA and cationic liposomes forming liposome-DNA aggregates resembling 'spaghetti and meatball-like' complexes (Sternberg *et al*., 1994).

3.2 MATERIALS AND METHODS

3.2.1 Materials

Molecular biology grade agarose was acquired from Bio-Rad Laboratories, California, USA. pBR322 DNA was purchased from Roche Diagnostics, Mannheim, Germany. Ethidium bromide was obtained from Merck, Damstadt, Germany. All other chemicals were of analytical grade.

3.2.2 Methods

3.2.2.1 Gel Retardation Assay

A fixed amount of pBR322 DNA (0.5 µg) was added to increasing amounts of cationic liposome (0, 4, 5, 6, 7, 8, 9, 9.5 µg), or pegylated cationic liposomes (0, 1, 2, 3, 4, 5, 6, 6.5 µg) respectively. This was made up to a final volume of 6 µl with HBS. Complexes were allowed to incubate for 30 minutes at room temperature. Thereafter 2 µl of gel loading buffer (50% glycerol, 0.05% bromophenol blue, 0.05% xylene cyanol) was added to all samples. The samples were subjected to electrophoresis on 1% agarose gels in a Bio-Rad electrophoresis tank containing electrophoresis buffer (36 mM Tris-HCl, 30 mM sodium phosphate, 10 mM EDTA pH 7.5), for 90 minutes at 50 volts. The gels were stained thereafter with ethidium bromide (1 µg/ml) for 20 minutes and viewed under UV transillumination and images captured using the Vacutec Syngene G:Box gel documentation system.

3.2.2.2 Nuclease Protection Assay

Varying amounts of cationic liposome and pegylated cationic liposome (Table 3.1), as determined from retardation studies, were added to a constant amount of pBR322 DNA (1 µg). This was made up to a volume of 10 µl with HBS. The samples were allowed

to incubate for 30 minutes at room temperature. Foetal bovine serum (FBS) was thereafter added to the complexes to a final concentration of 10%. A negative control was set up containing only pBR322 DNA with no liposome or FBS. A positive control contained only pBR322 DNA and FBS. The reaction mixtures were then incubated for 4 hours at 37ºC. After the incubation period, ethylenediaminetetraacetic acid (EDTA) was added to the samples to a final concentration of 10 mM and sodium dodecyl sulphate (SDS) to a final concentration of 0.5% ($^{w}/_{v}$). The samples were incubated for a further 20 minutes at 55ºC. Thereafter the samples were subjected to electrophoresis on a 1% agarose gel (as per 3.2.2.1) for 120 minutes at 50 volts. Following electrophoresis, the gel was stained with ethidium bromide (1 µg/ml) for 20 minutes, and images captured using the Vacutec Syngene G:Box gel documentation system.

TABLE 3.1: Varying amounts of liposome used from each preparation for the nuclease protection assay. DNA was constant at 1 µg.

LIPOSOME PREPARATION	**LIPOSOME AMOUNT (µg)**			
NN	8	9	10	-
NN – 5% CAP-β-Gal	14	15	16	-
NN – 10% CAP-β-Gal	16	17	18	-
NN – 5% CAP-β-Glu	12	13	14	-
NN – 10% CAP-β-Glu	14	15	16	17
PEG-NN	6	7	8	9
PEG-NN – 5% CAP-β-Gal	8	9	10	11
PEG-NN – 10% CAP-β-Gal	6	7	8	9
PEG-NN – 5% CAP-β-Glu	8	9	10	11
PEG-NN – 10% CAP-β-Glu	6	7	8	9

3.2.2.3 Ethidium Bromide Intercalation Assay

This assay was conducted on a Shimadzu RF – 551 Spectrofluorometric Detector set at an excitation wavelength of 520 nm and an emission wavelength of 600 nm. Initially 10 µl (1 µg) of stock ethidium bromide solution (100 µg/ml) was added to 500 µl of HBS in a quartz microcuvette and measured to obtain a baseline relative fluorescence of 0. Subsequently 24 µl (6 µg) of pBR322 DNA was added to the mixture and the reading taken was assumed to represent 100% relative fluorescence. Thereafter 2 µl aliquots, approximately 5 – 5,5 µg, of liposome preparation were systematically added to the mixture until approximately 70 µg of liposome preparation was added and/or a plateau in readings was reached. The solution was thoroughly mixed after each addition so as to promote dispersion before each fluorescence reading. The results were plotted relative to the 100% fluorescence value. This procedure was conducted for all liposome preparations.

3.3 RESULTS AND DISCUSSION

3.3.1 Gel Retardation Assay

The results of the agarose gel electrophoresis using varying amounts of both cationic and pegylated cationic liposome preparations, respectively, together with a constant amount of DNA (0.5 µg) can be seen in Figures 3.4 and 3.5.

The results show binding between the negatively charged DNA and the cationic or pegylated cationic liposome preparations tested. The plasmid DNA, pBR322 (Lane 1), produced the expected two bands, the supercoiled form (bottom band) and the closed circular form (top band) of DNA. As the liposome concentration in each preparation increased, more DNA was bound by the liposomes and hence less DNA entered the gel. The DNA that did not enter the gel can be seen intensely stained by ethidium bromide in the wells. Complete retardation of the DNA occurs at various DNA : liposome ratios for the different cationic and pegylated cationic liposome preparations as set out in Table 3.2.

TABLE 3.2: DNA : Liposome ratios at which all plasmid DNA is lipoplex – associated.

LIPOSOME PREPARATION	RETARDATION	
	Liposome Amount (µg)	DNA:Liposome Ratio (w/w)
NN	5	1:10
NN – 5% CAP-β-Gal	8	1:16
NN – 10% CAP-β-Gal	9	1:18
NN – 5% CAP-β-Glu	7	1:14
NN – 10% CAP-β-Glu	8	1:16
PEG-NN	4	1:8
PEG-NN – 5% CAP-β-Gal	5	1:10
PEG-NN – 10% CAP-β-Gal	4	1:8
PEG-NN – 5% CAP-β-Glu	5	1:10
PEG-NN – 10% CAP-β-Glu	4	1:8

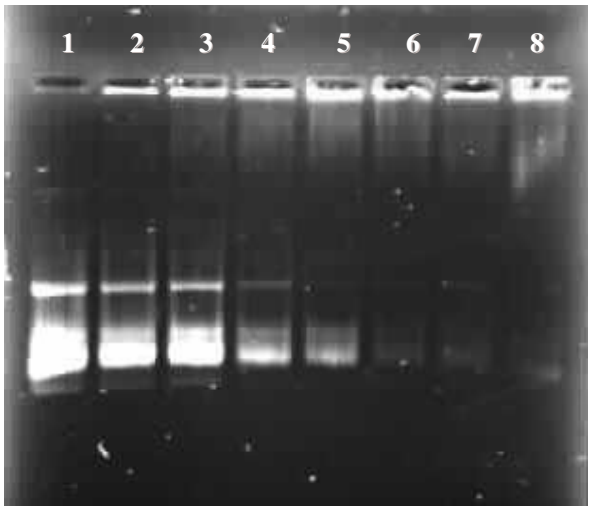


Fig 3.4 (a)

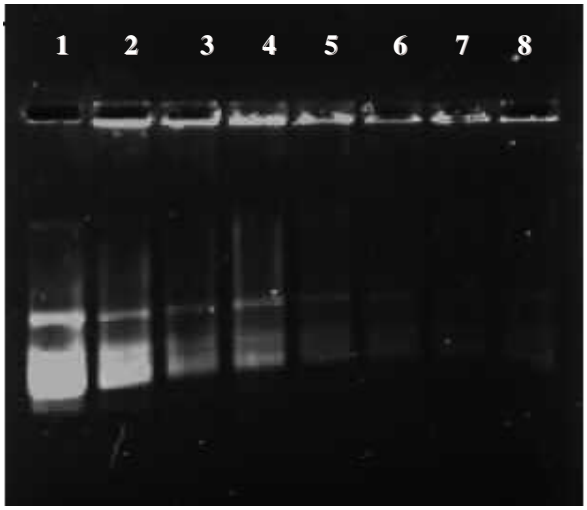


Fig 3.4 (b)

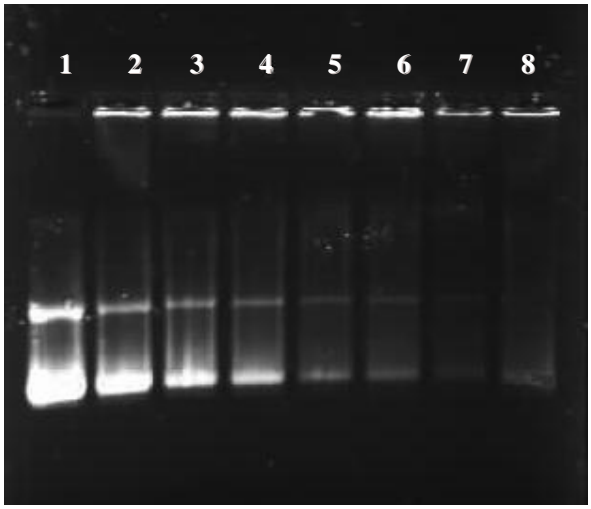


Fig 3.4 (c)

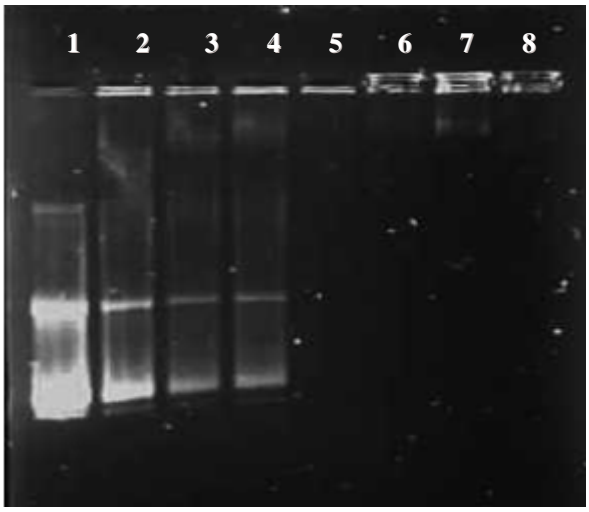


Fig 3.4 (d)

Fig 3.4 (e)

Figure 3.4: Gel retardation analysis of the binding interaction of varying amounts of cationic liposome preparations containing 0 – 10% CAP-β-Gal or CAP-β-Glu in a total of 6 µl reaction mixture.

(a) varying amounts of NN in lanes 1-8 (0, 1, 2, 3, 4, 5, 6, 6.5 µg);

(b) varying amounts of NN – 5% CAP-β-Gal in lanes 1-8 (0, 4, 5, 6, , 8, 9, 9.5 µg);

(c) varying amounts of NN – 10% CAP-β-Gal in lanes 1-8 (0, 4, 5, 6, 7, 8, 9, 9.5 µg);

(d) varying amounts of NN – 5% CAP-β-Glu in lanes 1-8 (0, 4, 5, 6, 7, 8, 9, 9.5 µg) and

(e) varying amounts of NN – 10% CAP-β-Glu in lanes 1-8 (0, 4, 5, 6, 7, 8, 9, 9.5 µg),

while the pBR322 DNA was kept constant at 0.5 µg per well.

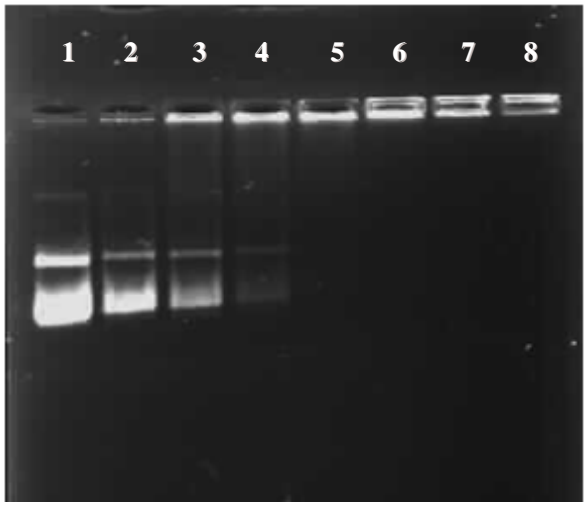


Fig 3.5 (a)

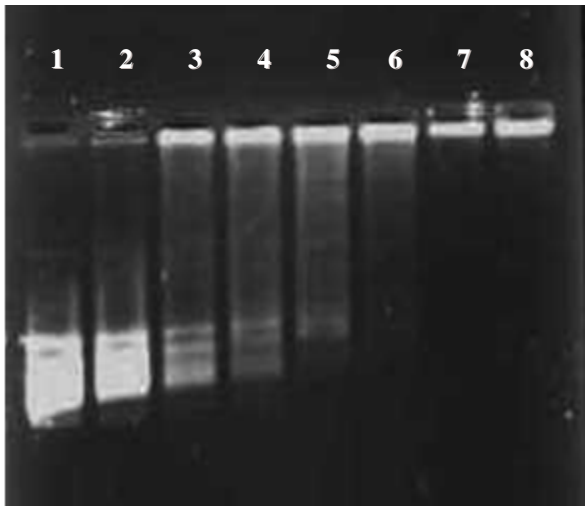


Fig 3.5 (b)

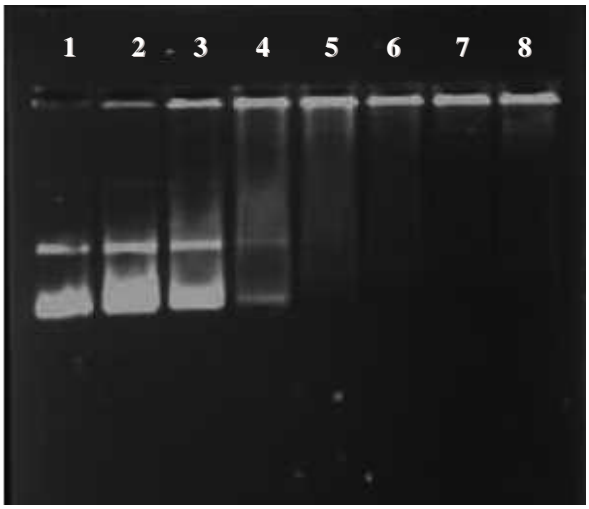


Fig 3.5 (c)

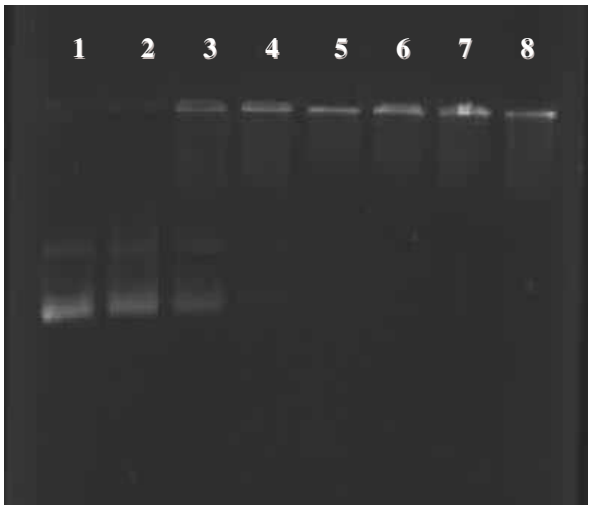


Fig 3.5 (d)

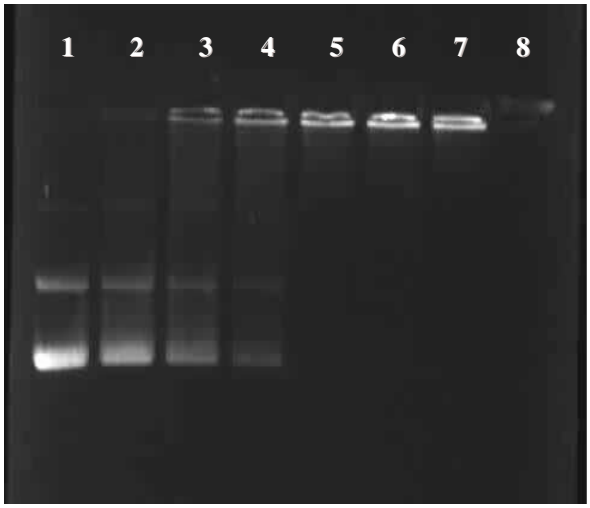


Fig 3.5 (e)

Figure 3.5: Gel retardation analysis of the binding interaction of varying amounts of pegylated cationic liposome preparations containing 0 – 10% CAP-β-Gal or CAP-β-Glu in a total of 6 µl reaction mixture.

(a) varying amounts of PEG-NN in lanes 1-8 (0, 1, 2, 3, 4, 5, 6, 6.5 µg);

(b) varying amounts of PEG-NN – 5% CAP-β-Gal in lanes 1-8 (0, 1, 2, 3, 4, 5, 6, 6.5 µg);

(c) varying amounts of PEG-NN – 10% CAP-β-Gal in lanes1-8 (0, 1, 2, 3, 4, 5, 6, 6.5 µg);

(d) varying amounts of PEG-NN – 5% CAP-β-Glu in lanes 1-8 (0, 1, 2, 3, 4, 5, 6, 6.5 µg) and

(e) varying amounts of PEG-NN – 10% CAP-β-Glu in lanes 1-8 (0, 1, 2, 3, 4, 5, 6, 6.5µg)

while the pBR322 DNA was kept constant at 0.5 µg per well.

Complete retardation suggests that the negative charges of the DNA are completely titrated by the positive charges of the cationic and pegylated cationic liposomes. This yields electroneutral complexes that may not migrate through the agarose gel matrix and can be seen stained by ethidium bromide in the wells. At higher DNA : liposome ratios precipitates may form and float out of the sample wells thus evading detection even after staining (Singh, 1998). Agarose gel electrophoresis is used to demonstrate the binding efficiency or complex formation between the DNA and cationic liposomes. Naked DNA, in the absence of liposome, would migrate in the gel matrix, however in the presence of increasing concentrations of either cationic and pegylated cationic liposome, the DNA is retarded and finally retained in the wells. As can be seen from the results (Figures 3.4 and 3.5) all cationic and pegylated cationic liposomes successfully bound the DNA, at various ratios.

From the results obtained it seems that, for the non-pegylated cationic liposomes, an increase in the amount of targeting ligand, CAP-β-Gal and CAP-β-Glu, caused a corresponding increase in the amount of liposome needed to bind 0.5 µg pBR322 DNA. Liposome preparations, NN required 5 µg, NN – 5% CAP-β-Gal required 8 µg while NN – 10% CAP-β-Gal required 9 µg. Preparations NN – 5% CAP-β-Glu and NN – 10% CAP-β-Glu required 7 µg and 8 µg respectively, to bind the same amount of DNA (0.5 µg). It can also be seen that liposome preparations with the CAP-β-Gal ligand, NN – 5% CAP-β-Gal and NN – 10% CAP-β-Gal had marginally higher DNA : liposome ratios than those containing the corresponding percentage of the CAP-β-Glu ligand, NN – 5% CAP-β-Glu and NN – 10% CAP-β-Glu, i.e. 1 : 16 (w/w) and 1 : 18 for NN – 5% CAP-β-Gal and NN – 10% CAP-β-Gal compared to 1 : 14 and 1 : 16 for NN – 5% CAP-β-Glu and NN – 10% CAP-β-Glu respectively. From the above, it may be postulated that cationic liposomes containing no or a lower percentage of targeting derivative have an apparently greater external positive charge as lower amounts of liposome are required to retard a given amount of DNA (Table 3.2). Furthermore, cationic liposomes carrying the CAP-β-Glu ligand seem to have more positive charges available than cationic liposomes with the CAP-β-Gal ligand.

Cationic liposomes with the higher percentage of targeting ligand (NN – 10% CAP-β-Gal and NN – 10% CAP-β-Glu) appear to be less positively charged, as estimated by retardation studies, than the preparations with the lower percentage of targeting ligand (Table 3.3). A possible reason for the trend observed could be due to the shielding effect of these targeting ligands. These ligands have lipid anchors that form part of the lipid bilayer of the liposome

and targeting head groups which structurally protrude out of the bilayer of the liposomes and may possibly be blocking or shielding the positive charges of the liposomes thereby decreasing the 'available' overall positive charge of the liposome and thus interfering with the binding of the cationic liposomes to the negatively charged DNA. A similar shielding effect was observed in a study by Meyer *et al.* (1998) in which the polymer blocked negative charges of liposome-bound oligodeoxyribonucleotides. As the concentration of both targeting ligands, CAP-β-Gal and CAP-β-Glu, increased from five to ten mole percent, more ligands were incorporated into the structure of the liposome, more positive charges were blocked and the binding interaction was consequently reduced and therefore more cationic liposome was required to bind the same amount of DNA.

The conclusion that the cationic liposomes with the CAP-β-Glu ligand have a greater number of positive charges than liposomes with the CAP-β-Gal ligand is probably erroneous. A more plausible explanation for the observations could be that more CAP-β-Gal ligand is externalised during the cationic liposome formation resulting in more CAP-β-Gal ligands on the surface of the liposome and therefore more of the shielding effect observed for those preparations thus less 'available' positive charge for binding DNA. Conversely, more CAP-β-Glu ligands could be internalised resulting in less ligands on the surface of the liposomes and consequently less of a shielding effect and a greater DNA binding efficiency. Differences between the two groups of targeted liposomes are nevertheless small and may not be significant.

Gel retardation results for the pegylated cationic liposome preparations suggest a different trend however. As can be seen in Table 3.2, and in contrast with the non-pegylated cationic liposome preparations, an increase in the amount of targeting ligand caused a small decrease in the amount of liposome needed to bind 0.5 µg of DNA. Liposome preparations, PEG-NN required 4 µg, PEG-NN – 5% CAP-β-Gal required 5 µg while PEG-NN – 10% CAP-β-Gal required 4 µg and preparations PEG-NN – 5% CAP-β-Glu and PEG-NN – 10% CAP-β-Glu required 5 µg and 4 µg respectively, to bind the same amount of DNA (0.5 µg). From these results it can be suggested that pegylated liposome preparations with no or a higher percentage of targeting derivative should theoretically have a greater amount of positive charge as they require less liposome to bind the same amount of DNA (0.5 µg). Moreover, no difference is observed between the pegylated cationic liposomes containing CAP-β-Gal and

those that have CAP-β-Glu in their structure. A similar observation was noted by Singh *et al.* (2007).

TABLE 3.3: The charge ratios of DNA to liposome for each of the cationic and pegylated cationic liposome preparations.

LIPOSOME PREPARATION	CHARGE RATIO (DNA : LIPOSOME) (-ve : +ve)
NN	1 : 2.5
NN – 5% CAP-β-Gal	1 : 4.2
NN – 10% CAP-β-Gal	1 : 4.7
NN – 5% CAP-β-Glu	1 : 3.6
NN – 10% CAP-β-Glu	1 : 4.2
PEG-NN	1 : 2.0
PEG-NN – 5% CAP-β-Gal	1 : 2.4
PEG-NN – 10% CAP-β-Gal	1 : 2.0
PEG-NN – 5% CAP-β-Glu	1 : 2.4
PEG-NN – 10% CAP-β-Glu	1 : 2.0

Pegylated cationic liposomes with a lower percentage of targeting derivative i.e. PEG-NN – 5% CAP-β-Gal and PEG-NN – 5% CAP-β-Glu appear to have a slightly greater number of external positive charges, as estimated by retardation studies (Table 3.3), than liposomes with a higher percentage of targeting derivative (PEG-NN – 10% CAP-β-Gal and PEG-NN – 10% CAP-β-Glu). A possible reason for this observation could be that pegylated cationic liposomes with lower percentage of targeting ligand have a greater amount of DOPE (5 mole %) in their structure than their higher percentage counterparts. Consequently, this excess of DOPE could have resulted in more of the cationic lipid, CHOL-T being internalised during liposome preparation thereby reducing the number of 'available' positive charges and thus interfering with the binding of negatively charged DNA to the pegylated cationic liposome.

In summary, it is noted that more cationic liposomes than pegylated cationic liposomes are required to fully retard a given mass of pDNA. A plausible explanation for this observation

could be that the presence of PEG in the structure of the pegylated cationic liposomes could have caused a disproportionate amount of the targeting derivatives to become situated on the inner leaflet of the bilayer during liposome formation thus negating the shielding effect of the targeting moieties and increasing the availability of positive charges. The other possible reason could be that PEG caused more of the cationic lipid, CHOL-T, to be externalised during liposome preparation thus affording a greater amount of positive charge to the pegylated cationic liposome.

The data obtained has been used to facilitate the design of lipoplexes intended for the transfection of Hep G2 cells described in chapter four. Hence DNA : liposome ratios covering a range from slightly below to slightly above the ratio corresponding to electroneutrality have been examined.

3.3.2 Ethidium Bromide Intercalation Assay

Cationic liposome preparations are known to displace DNA- associated ethidium bromide (Xu *et al.*, 1999). In accordance with this all liposome preparations were able to successfully displace intercalated ethidium bromide from the pBR322 DNA. This was demonstrated by the continual decrease in fluorescence upon the stepwise addition of both cationic and pegylated cationic liposomes to the reaction mixture as illustrated in Figures 3.6 and 3.7 respectively. Varying amounts of both cationic and pegylated cationic liposomes were added to the reaction mixture, forming a liposome-DNA complex, until the fluorescence values reached a plateau, referred to as the point of inflection. This point represents the DNA : liposome ratio at which the liposome maximally displaces the ethidium bromide and completely binds or condenses the DNA. For cationic liposome preparation NN, 60 µg of liposome was added before this point was reached which gives a DNA : liposome ratio of 1 : 10 (w/w) that corresponds to the ratio obtained in the gel retardation study (Table 3.2). The remaining cationic liposome preparations, NN – 5% CAP-β-Gal; NN – 10% CAP-β-Gal; NN – 5% CAP-β-Glu and NN – 10% CAP-β-Glu revealed decreases in ethidium bromide fluorescence until points of inflection were obtained at DNA : liposome ratios of 1 : 10.8; 1 : 10.8; 1 : 10.8 and 1 : 10 respectively. These ratios differed slightly from those obtained through the gel retardation studies and this could be attributed to the extreme sensitivity of this assay resulting in complete condensation of the plasmid DNA at a lower ratio than expected.

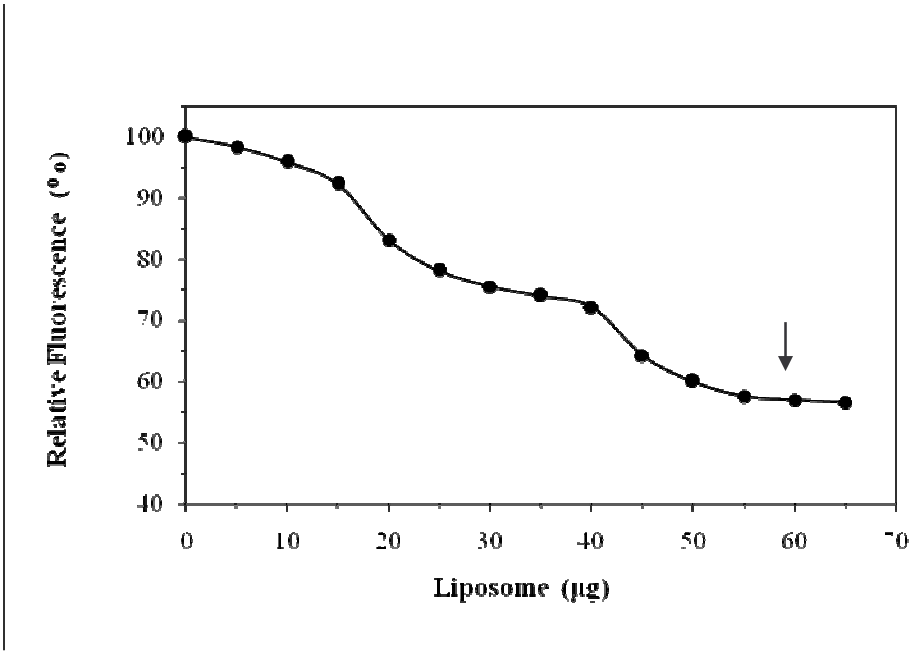


Fig 3.6 (a)

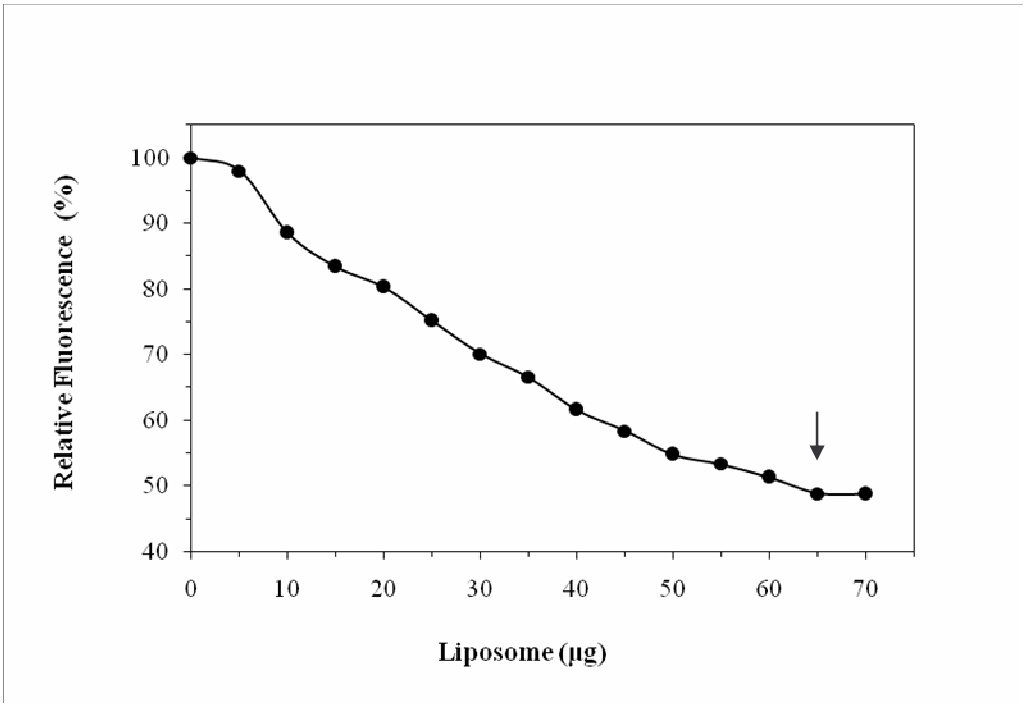


Fig 3.6 (b)

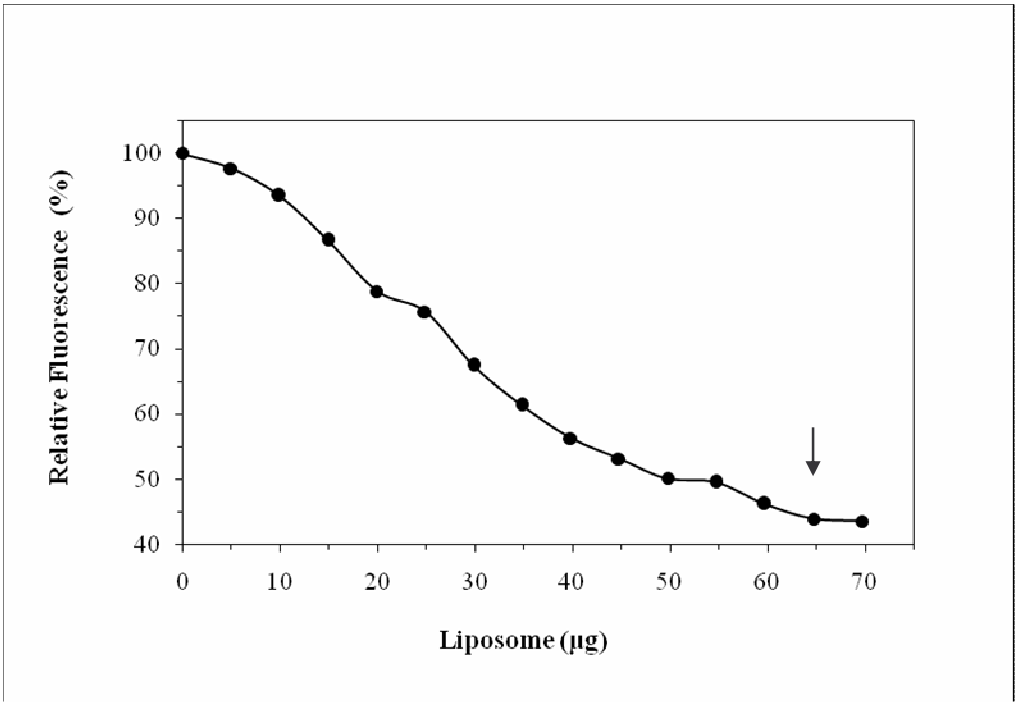


Fig 3.6 (c)

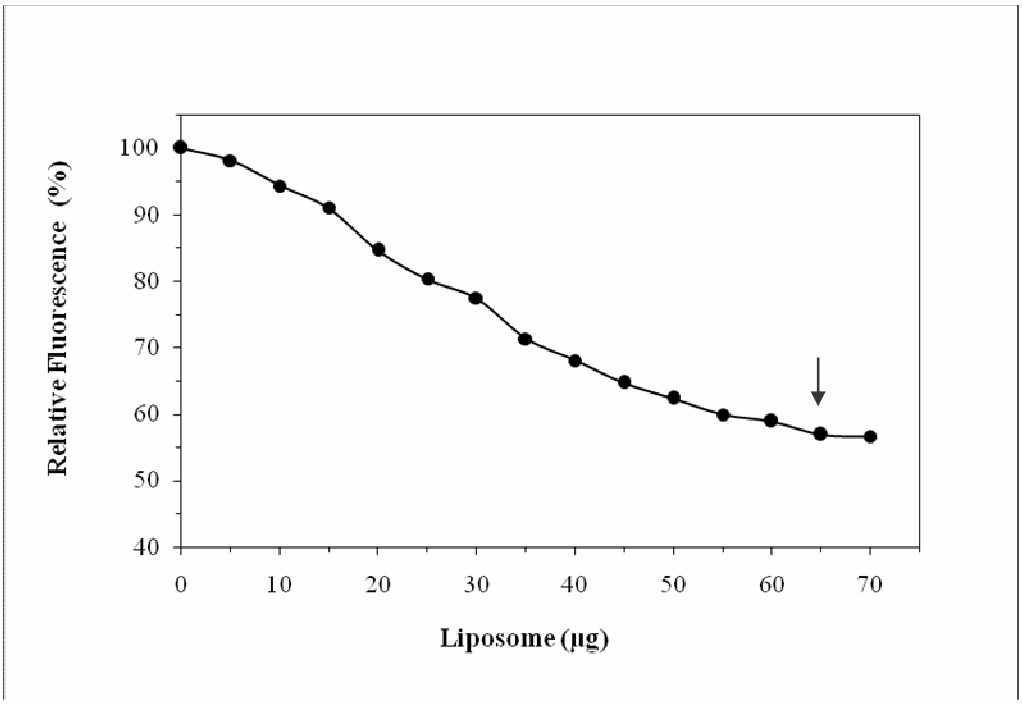


Fig 3.6 (d)

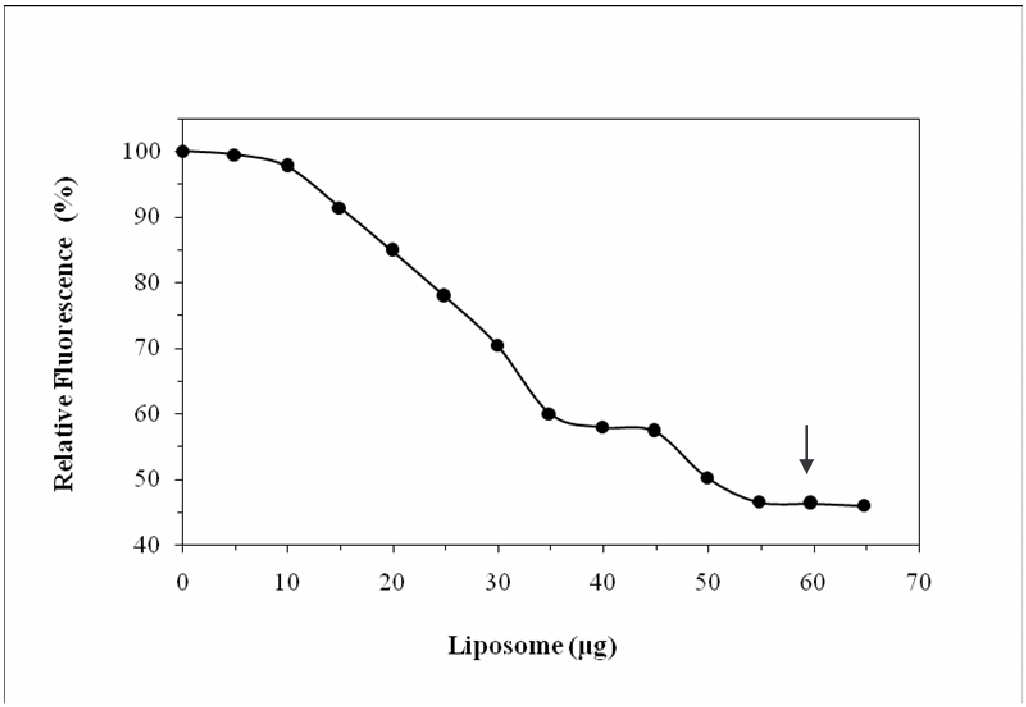


Fig 3.6 (e)

Figure 3.6: Ethidium bromide intercalation assay for cationic liposomes in a total of 500 µl incubation mixtures containing 6 µg pBR322 and increasing amounts of liposome in 2 µl (±5 µg) aliquots. **(a)** NN**; (b)** NN – 5% CAP-β-Gal**; (c)** NN – 10% CAP-β-Gal; **(d)** NN – 5% CAP-β-Glu and **(e)** NN – 10% CAP-β-Glu

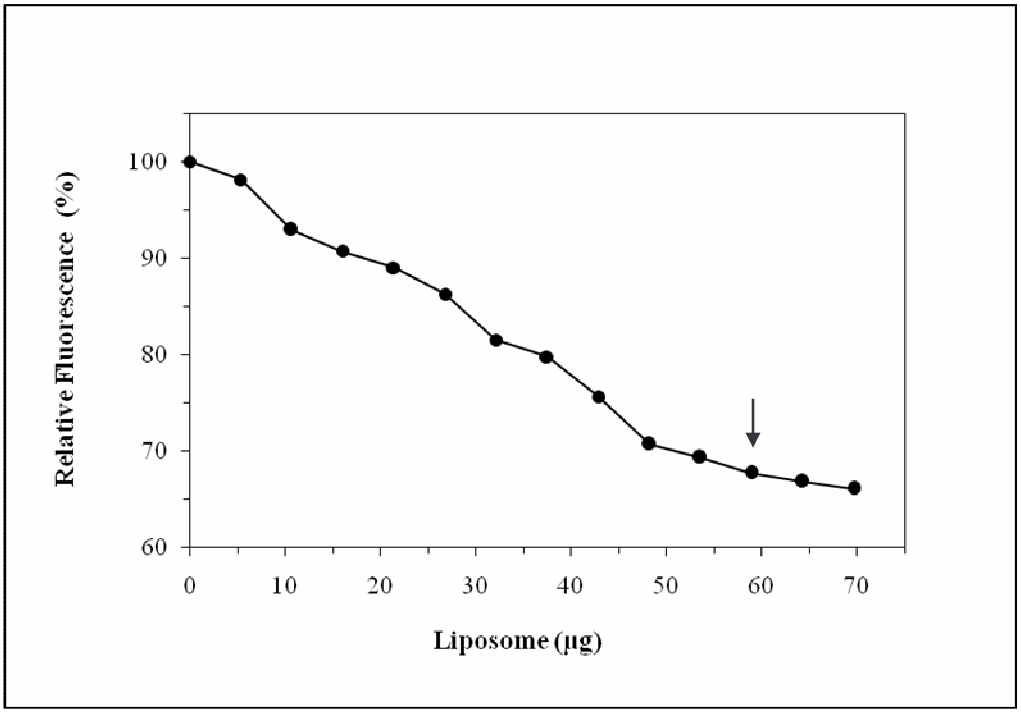


Fig 3.7 (a)

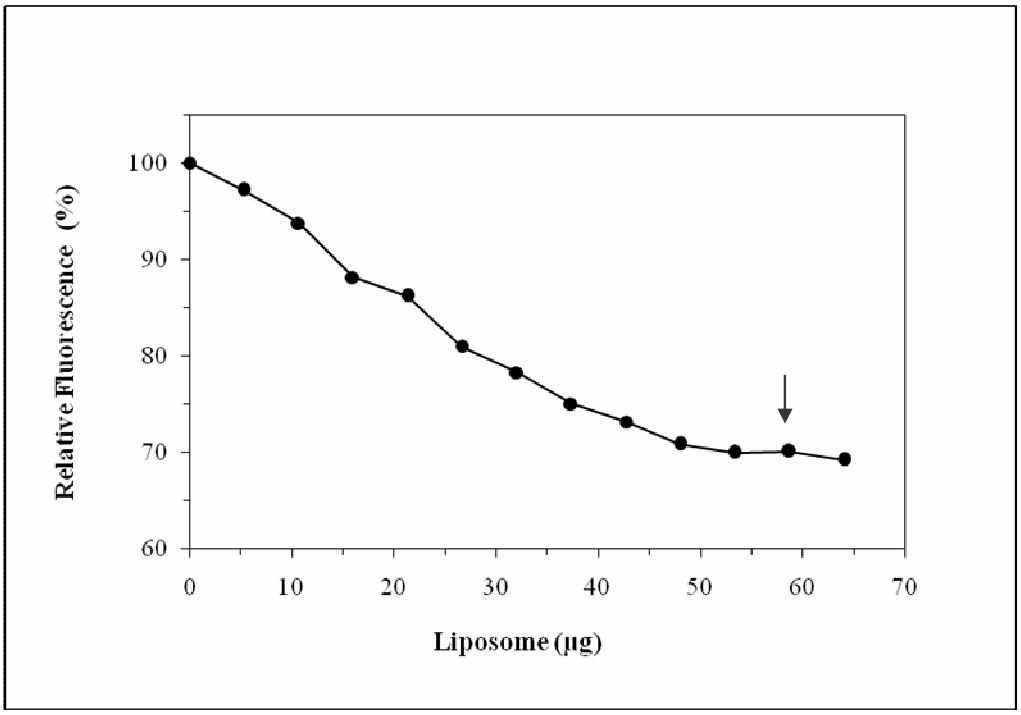


Fig 3.7 (b)

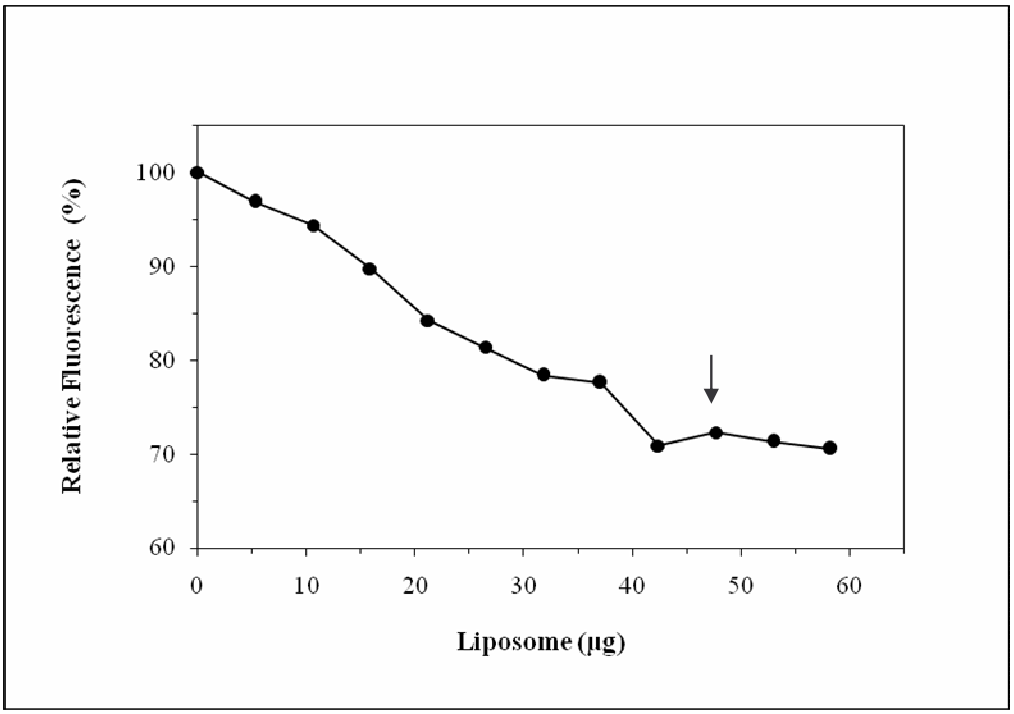


Fig 3.7 (c)

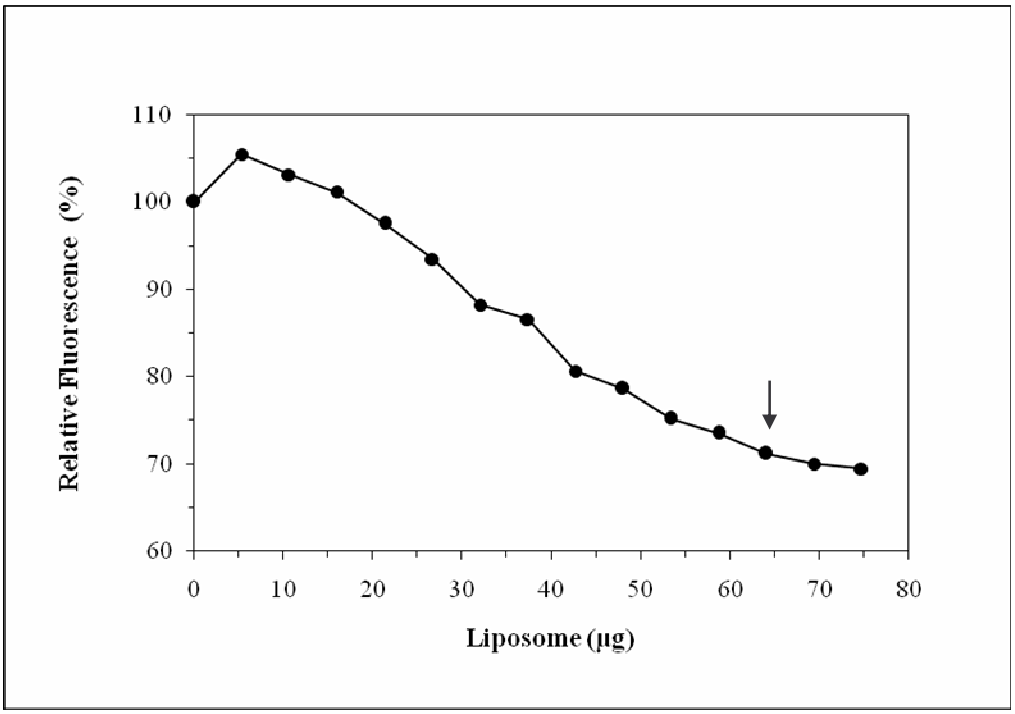


Fig 3.7 (d)

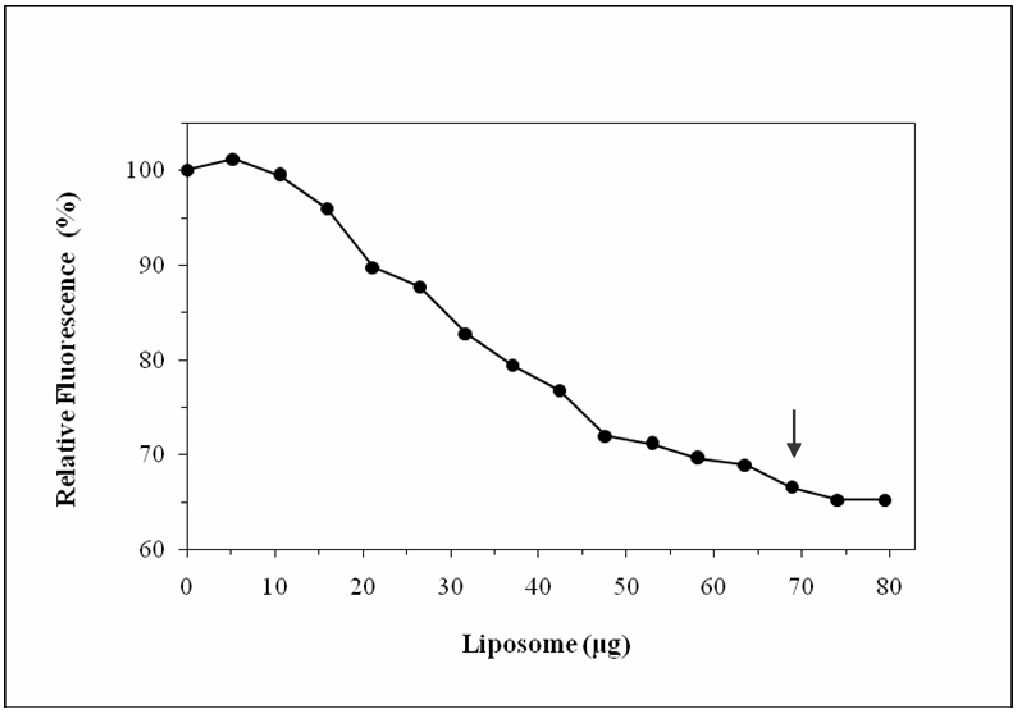


Fig 3.7 (e)

Figure 3.7: Ethidium bromide intercalation assay for pegylated cationic liposomes in a total of 500 µl incubation mixtures containing 6 µg pBR322 and increasing amounts of liposome in 2 µl (±5.3 µg) aliquots. **(a)** PEG-NN; **(b)** PEG-NN – 5% CAP-β-Gal; **(c)** PEG-NN – 10% CAP-β-Gal; **(d)** PEG-NN – 5% CAP-β-Glu and **(e)** PEG-NN – 10% CAP-β-Glu

Another reason for this discrepancy is that retardation studies are based on charge neutralisation i.e. the point at which the negative charges of the DNA are completely titrated by the positive charges of the cationic liposome where this assay is based on DNA condensation by the cationic liposome. From the results, it was observed that cationic liposome preparation NN displaced approximately 40% of the intercalated ethidium bromide while preparations NN – 5% CAP-β-Gal; NN – 10% CAP-β-Gal; NN – 5% CAP-β-Glu and NN – 10% CAP-β-Glu approximated 50%, 55%, 40% and 55% of ethidium bromide displacement. This reflects a generally higher degree of DNA compaction in targeted lipoplexes.

For all the pegylated liposome preparations, the DNA : liposome ratio obtained at the point of inflection roughly corresponded to the ratios obtained from the retardation studies (Table 3.2)

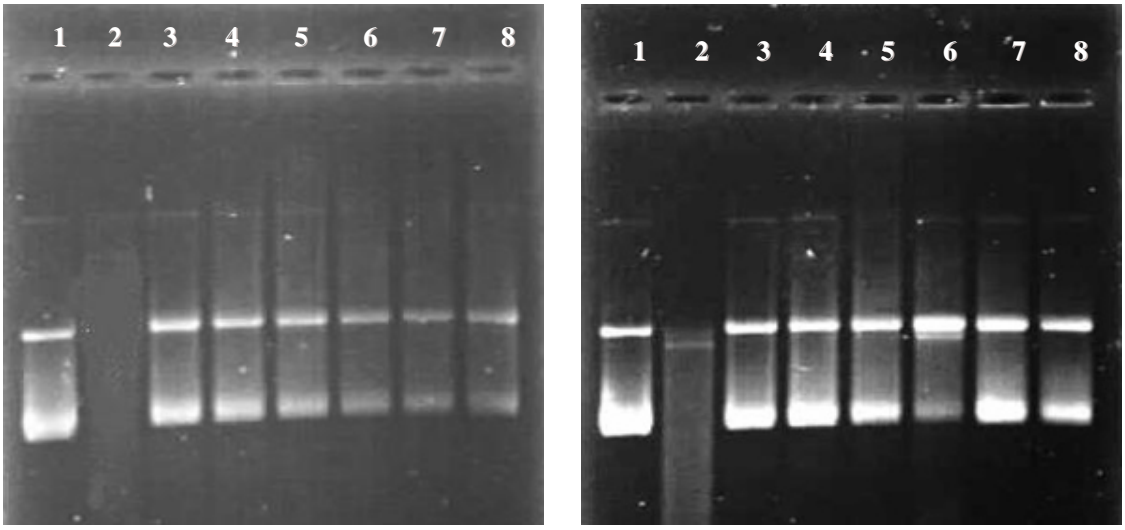
For pegylated cationic liposome preparation, PEG-NN this ratio was 1 : 9.8 while pegylated liposome preparations PEG-NN – 5% CAP-β-Gal, PEG-NN – 10% CAP-β-Gal, PEG-NN – 5% CAP-β-Glu and PEG-NN – 10% CAP-β-Glu had ratios of 1 : 9.8; 1 : 7.9; 1 : 10.6 and 1 : 11.4 respectively. All the pegylated liposome preparations displaced approximately 30 – 35% of intercalated ethidium bromide. For pegylated cationic liposomes containing the CAP-β-Glu targeting derivative, an initial increase in fluorescence was observed upon addition of the first few micrograms of liposome preparation. This could be due to these particular preparations causing the ethidium bromide to bind more strongly to the pDNA until more liposome preparation was added which displaced the ethidium bromide as expected.

Overall, it was noted that cationic liposome preparations displaced ethidium bromide in the range of 40 – 55% while pegylated cationic liposomes did the same in a range of 30 – 35%. It can therefore be suggested that pegylated cationic liposomes displace this intercalating dye to a lesser degree reflecting a lower degree of compaction. Another observation noted was that for all liposome preparations assayed, a further increase in the amount of liposome added after the point of inflection did not result in a corresponding decrease in fluorescence suggesting the plasmid DNA did not undergo additional condensation beyond this point. However at this point and beyond a slight turbidity of the solution was noted. Previously Gershon and colleagues in 1993 showed this phenomenon with a DOTMA/DOPE liposome composition.

3.3.3 Nuclease Protection Assay

The results of the agarose gel electrophoresis assay using varying amounts (Table 3.1) of both cationic and pegylated cationic liposome preparations, respectively, together with a constant amount of DNA (1 µg) can be seen in Figures 3.8 and 3.9.

The integrity of DNA in serum containing media is vitally important in gene delivery systems. Nucleic acid degradation by serum nucleases such as DNase 1 is of particular concern as inefficient protection of DNA by a gene delivery vehicle is an undesirable trait in vector systems (Obata *et al.,* 2009). The results show that all liposome preparations, both cationic and pegylated cationic protect pBR322 DNA from possible degradation by serum nucleases in medium containing 10% FBS.



(a) **(b)**

Figure 3.8: Nuclease protection assay of cationic liposome-DNA complexes of **(a)** NN and NN – 5% CAP-β-Gal and **(b)** NN – 10% CAP-β-Gal and NN – 5% CAP-β-Glu in 10 µl reaction mixture with pBR322 DNA constant at 1 µg.
Lane 1: Untreated marker pBR322 DNA (1µg)
Lane 2: Unprotected pDNA in the presence of 10% FBS

(a) Lane 3-5: Varying amounts of NN (8, 9, 10 µg) with pDNA in 10% FBS
Lane 6-8: Varying amounts of NN – 5% CAP-β-Gal (14, 15, 16 µg) with pDNA in 10% FBS
(b) Lane 3-5: Varying amounts of NN – 10% CAP-β-Gal (16, 17, 18 µg) with pDNA in 10% FBS
Lane 6-8: Varying amounts of NN – 5% CAP-β-Glu (12, 13, 14 µg) with pDNA in 10% FBS

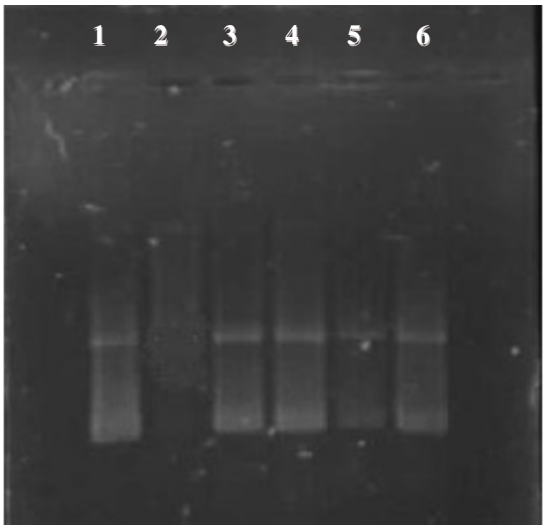


Figure 3.8(c): Nuclease protection assay of cationic liposome-DNA complexes of NN – 10% CAP-β-Glu in 10 µl reaction mixture with pBR322 DNA at 1 µg.
Lane 1: Untreated marker pBR322 DNA (1µg)
Lane 2: Unprotected pDNA in the presence of 10% FBS
Lane 3-6: Varying amounts of NN – 10% CAP-β-Glu (14, 15, 16 and 17 µg) with pDNA in 10% FBS

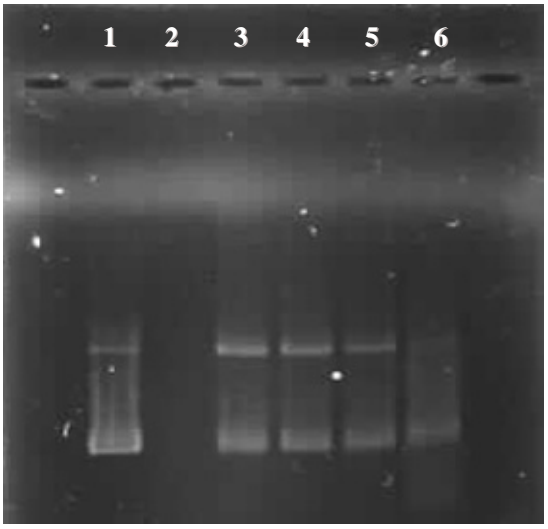


Fig. 3.9 (a)

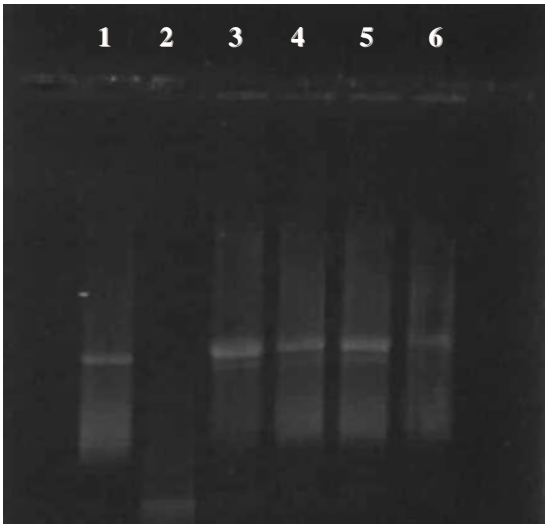


Fig 3.9 (b)

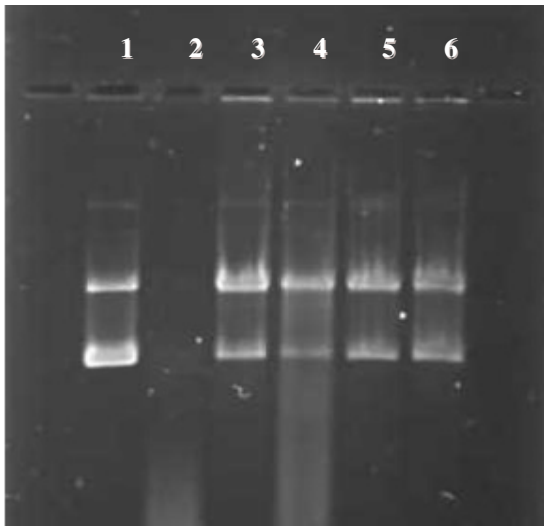


Fig 3.9 (c)

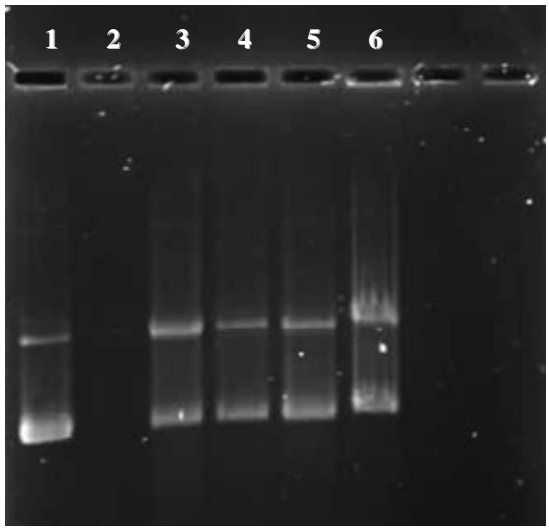


Fig 3.9 (d)

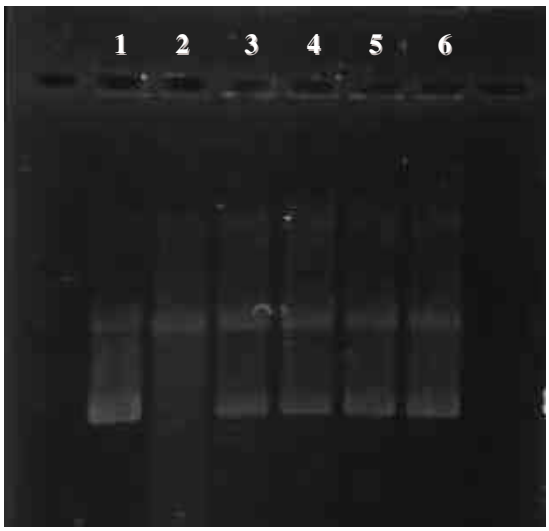


Fig 3.9 (e)

Figure 3.9: Nuclease protection assay of varying amounts of pegylated cationic liposome-DNA complexes containing 0 – 10% CAP-β-Gal or CAP-β-Glu in 10 µl reaction mixture with pBR322 DNA at 1 µg.
Lane 1: Untreated marker pBR322 DNA (1µg)
Lane 2: Unprotected pDNA in the presence of 10% FBS

(a) Lane 3-6: Varying amounts of PEG-NN (6, 7, 8 and 9 µg) with pDNA in 10% FBS

(b) Lane 3-6: Varying amounts of PEG-NN – 5% CAP-β-Gal (8, 9, 10 and 11 µg) with pDNA in 10% FBS

(c) Lane 3-6: Varying amounts of PEG-NN – 10% CAP-β-Gal (6, 7, 8 and 9 µg) with pDNA in 10% FBS

(d) Lane 3-6: Varying amounts of PEG-NN – 5% CAP-β-Glu (8, 9, 10 and 11 µg) with pDNA in 10% FBS

(e) Lane 3-6: Varying amounts of PEG-NN – 10% CAP-β-Glu (6, 7, 8 and 9 µg) with pDNA in 10% FBS

Ideal DNA : liposome ratios, as determined by retardation studies, were utilised for this study. As seen in Figures 3.8 and 3.9 plasmid DNA was protected over a range of ratios and not just at the optimum DNA : liposome ratio (electroneutral) obtained from the retardation studies and shown in Table 3.2 for all cationic and pegylated liposome preparations. The protection of plasmid DNA by the complexes could be due to the stability of the complexes or to the electrostatic forces between the positively charged liposomes and the negatively charged DNA that leads to the formation of highly organised supramolecular structures where DNA is condensed or compacted and thus protected against nuclease degradation (Singh *et al.,* 2006a). Hence, in lane two of Figures 3.8 and 3.9, the naked pDNA is completely degraded by nucleases whereas the DNA associated with cationic and pegylated cationic liposomes is protected and undegraded. This is consistent with a study by Remaut *et al*. (2005) in which it was suggested, by Fluorescence Correlation Spectroscopy, that both non-pegylated and pegylated cationic liposomes possessed the ability to protect DNA. It is interesting to note that at pegylated cationic liposome : DNA ratios higher than the optimum ratio (Figure 3.9 a-e, lane 6) the DNA in some cases appear to be slightly degraded. This could indicate that pDNA is not effectively protected at ratios higher than the lowest ratio at which DNA is completely bound to the pegylated cationic liposome.

From the results obtained, it can be deduced that all cationic and pegylated cationic liposomes form lipoplexes with plasmid DNA and afford the nucleic acid protection against degradation by serum nucleases over a period of four hours at 37ºC in the presence of 10% FBS.

CHAPTER FOUR

CELL CULTURE AND TRANSFECTION STUDIES

4.1 INTRODUCTION

The ability to introduce and express genes in cells, or 'transfection' is a major technique in cell biology research. The key to the success for any gene transfer strategy is the development of a safe and effective vector that can deliver and efficiently express the gene in a specific cell population (Nishikawa and Huang, 2001). Gene transfer can be conducted both *in vitro* and *in vivo*. A number of different vectors and targeting methods can be used to target a specific cell receptor in cultured cell lines during *in vitro* transfection. Targeted gene delivery *in vitro* has enjoyed much success using a variety of delivery vehicles including cationic liposomes. However *in vivo* transfection using cationic liposomes has met with limited success due to the problems encountered, namely rapid elimination due to interactions with proteins and the reticuloendoethelial system and DNA degradation (Thompson *et al.,* 2005; Obata *et al.,* 2009).

The aim of this study was to develop a synthetic gene transfer system based on cationic liposome mediated transfection that could be selectively targeted to a specific cell type. An attempt is made to produce a targeted cationic and pegylated cationic liposome vector system for an *in vitro* study in a human hepatoma cell line (HepG2). Targeting was facilitated by the incorporation of galactose and glucose glycolipids, respectively, into the structure of the cationic liposomes. Pegylation was achieved by the addition of DSPE-PEG_{2000} into the bilayer of liposomes. This targeted gene delivery system was prepared by complex formation between the plasmid pGL3 and cationic liposome preparations, NN, NN – 5% CAP-β-Gal, NN – 10% CAP-β-Gal, NN – 5% CAP-β-Glu, NN – 10% CAP-β-Glu, and pegylated cationic liposomes, PEG-NN, PEG-NN – 5% CAP-β-Gal, PEG-NN – 10% CAP-β-Gal, PEG-NN – 5% CAP-β-Glu, and PEG-NN – 10% CAP-β-Glu respectively. The targeting and pegylation properties of these liposomes would be desirable for application into *in vivo* gene therapy thus it is hoped that these properties help mediate transfection to the asialoglycoprotein receptors expressed on HepG2 cells. The pGL3 plasmid DNA was used to detect transient expression in the cell line

as these cells lack the luciferase activity which, if measurable after gene delivery, would indicate successful gene transfer.

The HepG2 cell line used in this study is one of several cell lines isolated from the liver biopsies of hepatoblastoma and hepatocellular carcinomas (Aden *et al.,* 1979). They are known to exhibit the same biosynthetic capabilities of normal liver parenchymal cells (Knowles *et al.,* 1980) and thus produce a large number of serum proteins namely, albumin, transferrin, α-fetoprotein, fibrinogen, hapatoglobulin, α-1-acid glycoprotein, β-lipoprotein, plasminogen and ceruloplasmin (Knowles *et al.,* 1980).

Hepatocytes, including the hepatocyte-derived cell line HepG2, exclusively express high affinity asialoglycoprotein receptors that bind circulating glycoproteins that have heteroglycan chains that end in galactose and N-actylgalactosamine (asialoglycoproteins) and subsequently internalise them (Kawakami *et al.,* 1998). This receptor, predominately expressed on the sinusoidal surface of hepatocytes, is responsible for the clearance of glycoproteins with terminal desialylated galactose or acetylgalactosamine residues via receptor mediated endocytosis (Wu *et al.,* 2002). In human hepatocytes, the receptor is a transmembrane glycoprotein hetero-oligomer composed of two structurally different subunits, H1 and H2 with a molecular mass of 41 kD (Wu *et al.,* 2002). Each subunit possesses a galactose binding site however the complex must be assembled to ensure the high affinity uptake of asialoglycoproteins (Sawyer and Doyle, 1990).

The determination of transfection efficiency of the gene delivery system is of great importance. For liposome based gene transfer systems, plasmids containing 'reporter' genes are used. The gene expression in transfected cells is studied by linking a promoter sequence to the 'reporter' gene within the plasmid (Torchilin, 2003). Most plasmid based vectors also contain a eukaryotic replication origin. Some commonly used reporter genes include firefly luciferase, green fluorescent protein, β-galactosidase and alkaline phosphatase (Torchilin, 2003). The pGL3 control vector incorporating the firefly luciferase gene (*luc*+), was used in this study. This plasmid contains both SV40 promoter and enhancer sequences (Promega Technical Manual) (Figure 4.1). Firefly luciferase, a monomeric 61 kD protein was cloned from the North American firefly (*Photinus pyralis*). The advantage of luciferase is that is has a very high specific activity but it requires costly luciferin, ATP, oxygen and Mg^{2+} as substrates (Torchilin, 2003). The structure of firefly luciferin is seen in Figure 4.2.

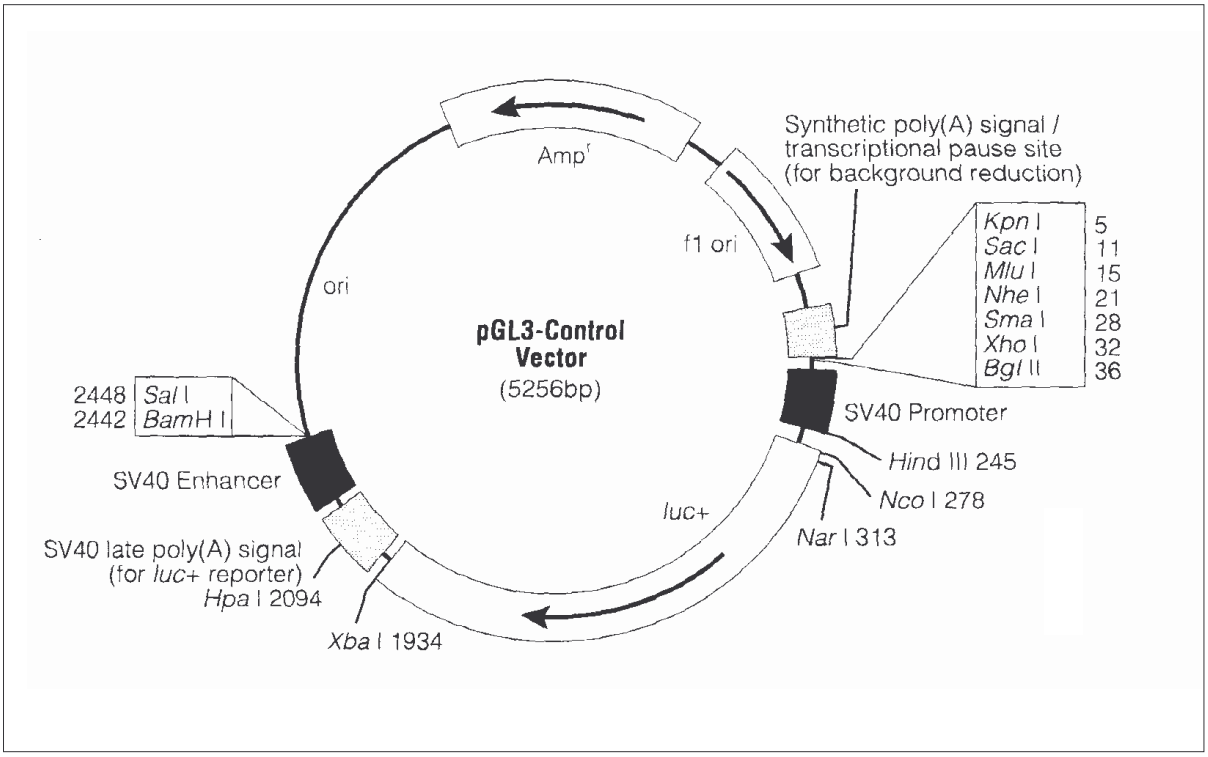


Figure 4.1: Construct of the pGL3 control vector showing the luciferase (*luc*) gene and the SV40 promoter and enhancer. (Promega Technical Manual – pGL3 luciferase reporter vectors).

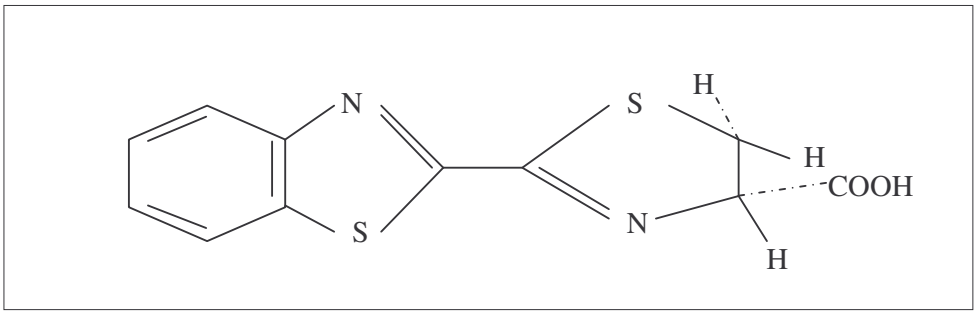


Figure 4.2: Structure of firefly D-(-)-Luciferin.

4.2 MATERIAL S AND METHODS

4.2.1 Materials

HepG2 cells and irradiated foetal bovine serum were obtained from Highveld Biological (PTY) LTD., Lyndhurst, South Africa. Minimum Essential Medium (MEM) containing Earle's salts and L-glutamine together with the Trypsin-Versene and Penicillin/Streptomycin mixtures was purchased from Lonza BioWhittaker, Walkersville, USA. The pGL3 Control Vector and Luciferase Assay kit was obtained from the Promega Corporation, Madison, USA. The Bicinchoninic acid (BCA) assay reagents were purchased from the Sigma-Aldrich Co., St. Louis, USA. All tissue culture plastic consumables were purchased from Corning Incorporated, New York, USA. All other reagents were of analytical grade.

4.2.2 Methods

4.2.2.1 Maintenance of HepG2 Cells

4.2.2.1 (a) Preparation of Culture Medium

MEM powdered medium intended for manufacture of 1 litre of medium was dissolved in 900 ml of 18 Mohm water. To this solution was added 10 mM $NaHCO_3$, 20 mM HEPES and 10 ml antibiotic (5000 units penicillin and 5000 µg streptomycin / ml). The pH of the solution was adjusted to 7.3 – 7.4. The medium was then made up to 1 litre with 18 Mohm water. The growth medium was subsequently filter sterilised using a Cole-Palmer Masterflex (model 7017-12) peristaltic pump through a Millipore 0.22 µm bell filter unit, into autoclaved 250 ml Schott bottles. Before the culture medium could be added to the HepG2 cells for growth, 10% foetal bovine serum was added to produce complete medium.

4.2.2.1 (b) Reconstitution of HepG2 Cells

An ampoule of cryopreserved HepG2 cells was removed from the biofreezer (-80ºC) and placed in a 37ºC water bath to thaw. Once thawed, the vial was wiped with ethanol, aseptically opened and the suspension subjected to centrifugation (1000 rpm for 3 minutes) to pellet cells. The resultant supernatant was discarded and the pellet resuspended in 1 ml of fresh complete medium (MEM + 10% foetal bovine serum + antibiotics). This 1 ml suspension was transferred to a cell culture flask containing 5 ml complete medium (MEM + 10% FBS + antibiotics). The flask was placed in a 37ºC incubator. The medium was changed every two to three days and the cells monitored until they reached confluence

4.2.2.1 (c) Propagation of HepG2 Cells

Once the cells were at or near confluence, they were trypsinised. Briefly, spent medium from the culture flask was discarded into a sterile waste bottle and the cells washed with sterile 5 ml phosphate buffered saline (PBS) (150 mM NaCl, 2.7 mM KCl, 1 mM KH_2PO_4, 6 mM Na_2HPO_4; pH 7.5). Thereafter 1 ml of trypsin-versene was added to the cells for trypsinisation. This process was allowed to proceed for approximately 5 minutes at room temperature and observed under a Nikon TMS inverted light microscope. Subsequently, 2 ml of complete medium (MEM + 10% foetal bovine serum + antibiotics) was added to the flask before the trypsinised cells were dislodged by firm tapping against the palm. The resultant cell suspension was split at a desired ratio into culture flasks, each containing 5 ml of complete medium (MEM + 10% foetal bovine serum + antibiotics). These flasks were incubated at 37ºC and medium changed when necessary. Once cells had reached confluence, they were again trypsinised and split as desired or cryopreserved for future use.

4.2.2.1 (d) Cryopreservation of HepG2 Cells

HepG2 cells at or near confluence were trypsinised as in 4.2.2.1 (c). The cell suspension was centrifuged at 1000 rpm for 3 minutes. The pelleted cells were resuspended in 0.9 ml complete medium (MEM + 10% foetal bovine serum + antibiotics) and 0.1 ml dimethylsulfoxide (DMSO). This suspension was then aliquoted into cryogenic vials, which

were vacuum sealed and frozen at a rate in temperature drop of 1ºC / minute to -50ºC using a cold probe. The cryogenic ampoules of frozen cells were thereafter stored in a -80ºC NUAIRE biofreezer.

4.2.2.2 Growth Inhibition Assay

Confluent HepG2 cells were trypsinised and seeded into a 48 well plate at a seeding density of 3 x 10^4 cells / well. The cells were incubated at 37ºC for 24 hours and allowed to adhere to the wells and grow to semi-confluence. The liposome-DNA complexes were set up as in Table 4.1. A constant amount of pGL3 DNA (1 µg) was added to the complexes and made up to a final volume of 10 µl with HBS. Complexes were allowed to incubate for 30 minutes at room temperature.

Cells were prepared by firstly removing the medium and replacing it with 0.3 ml serum free medium (MEM + antibiotics). Next, the reaction complexes were added to the wells containing cells. The assays were carried out in triplicate. The cells were then incubated at 37ºC for 4 hours. After 4 hours, the serum free medium was replaced with 0.3 ml complete medium (MEM + 10% foetal bovine serum + antibiotics). The cells were incubated again for a further 48 hours. After the incubation period, the cells were washed twice with PBS and stained with 200 µl crystal violet solution (0.5% ($^w/_v$) crystal violet, 0.8% ($^w/_v$) NaCl, 5% ($^v/_v$) formaldehyde, 50% ($^v/_v$) ethanol) for 20 minutes. Thereafter the stain was removed and the cells extensively washed with water. The multi-well plate was subsequently dried for 24 hours and the stain extracted with 0.3 ml 2-methoxyethanol over 36 hours with gentle rocking (10 rev/min) on a Stuart Scientific STR 6 platform shaker. Absorbance values for the samples were then read on a Thermo Electron Corporation Biomate 3 UV / visible spectrophotometer at a wavelength of 550 nm.

4.2.2.3 Amplification of pGL3 Control Vector

The pGL3 control vector was amplified in the Department of Biochemistry, University of KwaZulu-Natal according to the Promega protocol. The DNA purity and concentration was determined spectroscopically using a Thermo Electron Corporation Biomate 3

spectrophotometer. The isolated DNA was run on a 1% agarose gel against a Control pGL3 DNA sample to confirm purity and identify the different forms of DNA.

TABLE 4.1: The DNA : cationic liposome ratios used for the growth inhibition and transfection studies.

LIPOSOME PREPARATION	DNA : LIPOSOME RATIO (w/w)		
NN	1:8	1:9	1:10
NN – 5% CAP-β-Gal	1:14	1:15	1:16
NN – 10% CAP-β-Gal	1:16	1:17	1:18
NN – 5% CAP-β-Glu	1:12	1:13	1:14
NN – 10% CAP-β-Glu	1:14	1:15	1:16
PEG-NN	1:6	1:7	1:8
PEG-NN – 5% CAP-β-Gal	1:8	1:9	1:10
PEG-NN – 10% CAP-β-Gal	1:6	1:7	1:8
PEG-NN – 5% CAP-β-Glu	1:8	1:9	1:10
PEG-NN – 10% CAP-β-Glu	1:6	1:7	1:8

4.2.2.4 Transfection of HepG2 Cells

4.2.2.4 (a) Transfection

HepG2 cells were trypsinised and evenly seeded into a 24-well plate at a density of 5 x 10^4 cells per well. The cells were allowed to attach to the wells and grow to semi-confluence. The transfection complexes were prepared as in 4.2.2.2 in triplicate. The cells were prepared by discarding the medium and replacing it with 0.5 ml serum free medium (MEM + antibiotics). The transfection complexes were then added to the wells containing cells. Two controls were set up, one with wells containing HepG2 cells only and the other having

received only naked DNA (1 µg). The multi-well plates were then incubated at 37ºC for 4 hours. Thereafter the medium was replaced with 0.5 ml complete medium (MEM + 10% foetal bovine serum + antibiotics) and the cells incubated for a further 48 hours at 37ºC. Following the incubation period, the cells were assayed for luciferase activity.

4.2.2.4 (b) Luciferase Assay

The luciferase assay was carried out using the Promega Luciferase Assay kit. 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 luciferase assay buffer to one vial of lyophilised luciferase assay substrate. The cell culture lysis reagent (5x) (25 mM tris-phosphate, 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 1x stock. Both reagents were allowed to equilibrate at room temperature.

The cells were prepared by first removing the growth medium and carefully washing twice with PBS. 80 µl of 1x cell lysis reagent was added to the wells to cover them and the multi-well plate then placed on a Scientific STR 6 platform shaker for 15 minutes at 30 rev /min. Thereafter the attached cells were dislodged from the wells, the resultant cell solution was briefly centrifuged (5 seconds) in an Eppendorf microcentrifuge at 12 000 x g to pellet the debris. The cell free extracts (supernatant) were retained to be assayed for luciferase activity. This was achieved by adding 100 µl of luciferase assay reagent to 20 µl of cell free extract at room temperature, mixing immediately and placing the reaction mixture in a Lumac Biocounter 1500 luminometer. The light produced was measured for a period of 10 seconds. Protein determination was performed on the cell free extracts using the bicinchoninic acid (BCA) assay.

4.3 RESULTS AND DISCUSSION

4.3.1 Maintenance of HepG2 Cells

The HepG2 cells (Figure 4.3), were successfully cultured in MEM + 10% foetal bovine serum + antibiotics) over the period of study. Initial cell growth was slow with cells only reaching confluence after 4 to 5 days, however this is characteristic of the HepG2 cell line. This initial slow growth could also be attributed to the prolonged cryopreservation prior to reconstitution of the cells. As time passed, cell numbers increased probably due to the increased levels of growth factors secreted by the growing cells. The HepG2 cells were trypsinised and subdivided 1 : 3 or 1 : 2 splits every 3 to 4 days.

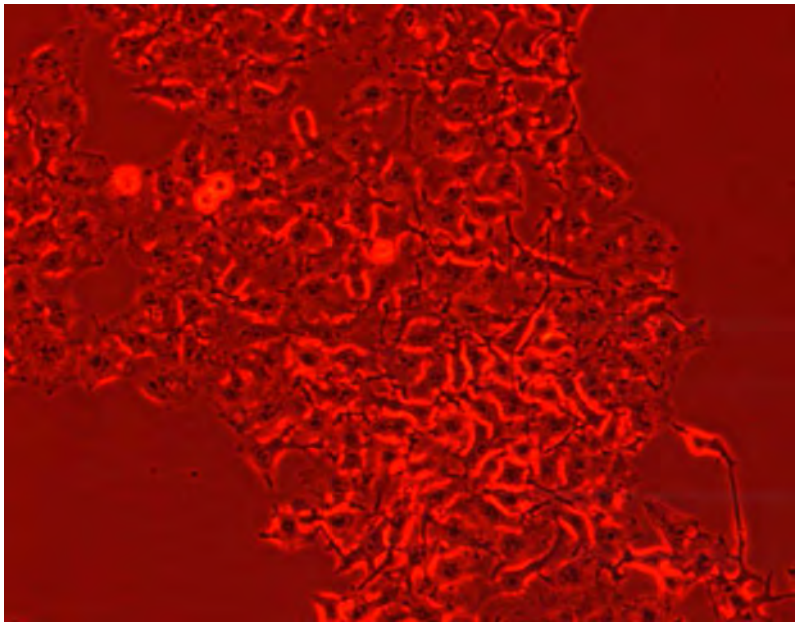


Figure 4.3: Monolayer of HepG2 cells at semi-confluence viewed under an Olympus fluorescence microscope (100x).

4.3.2 Amplification of pGL3 Control Vector

The pGL3 control vector was successfully amplified. The pDNA concentration was measured by UV absorption at 260 nm and adjusted to 0.5 mg/ml. The pDNA showed a high purity as the 260 nm/ 280 nm absorption ratio was between 1.8 and 2.

4.3.3 Growth Inhibition Assay

Cytotoxicity is an important parameter in non-viral gene therapy that is often overlooked. Cationic lipids in high doses have been shown to be cytotoxic (Zhdanov *et al.,* 2001). As many biological systems are negatively charged, there are two possible toxicities that can originate from cationic liposomes and lipids. These toxicities are related to (1) the germicidal action of positively charged surfactants against bacteria, viruses, fungi and invertebrates as well as (2) the interaction of cationic particles with biological molecules that may induce aggregation flocculation, thrombosis and platelet aggregation on a colloidal level (Lasic, 1997). On a molecular level, toxicity of cationic lipids is related to increasing membrane permeability and creation of transmembrane pores (Singh *et al.,* 2006). Toxicity studies are an important feature of non-viral vector based transfection as they determine the safety of these gene delivery vehicles.

Both cationic and pegylated cationic lipoplexes were well tolerated over the entire lipid concentration range tested i.e. from 8 – 18 µg/10µl for cationic liposomes and 6 – 10 µg/10µl for pegylated cationic liposomes (Figures 4.4 and 4.5). This is in agreement with the studies by Kawakami *et al.* (1998) and Percot *et al.* (2004) who observed that DOPE-containing liposomes with galactosylated cholesterol derivatives exhibited low toxicity to HepG2 cells. Maximum growth inhibition for cationic lipoplexes and pegylated cationic lipoplexes was 31% and 34% respectively. Overall, the pegylated cationic liposome preparations showed slightly more cytotoxicity than their non-pegylated counterparts, with the exception of PEG-NN – 10% CAP-β-Gal where the inverse was true. Even at optimum binding ratios, as elucidated by gel retardation studies (Table 3.2), cationic liposomes showed less cytotoxicity than pegylated cationic liposomes. A similar observation was made by Dadashzadeh *et al.* (2008) who noted that pegylated liposomal formulations with the drug, topotecan showed marginally higher toxicity than non-pegylated liposomes. In a study on nanoparticles, it was noted by He *et al.* (2009) that an increase in the molecular weight of PEG corresponded to an increase in cytotoxicity on Chang cell lines. Thus the somewhat higher cytotoxicity observed for pegylated liposomes could be attributed to the presence of the PEG component.

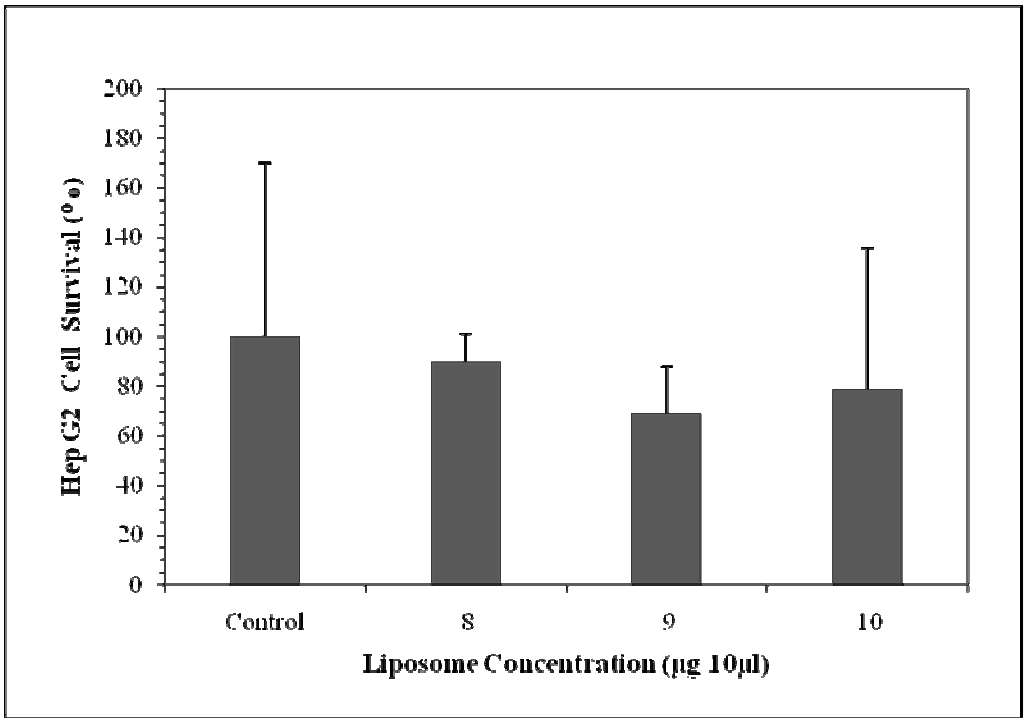


Fig 4.4 (a)

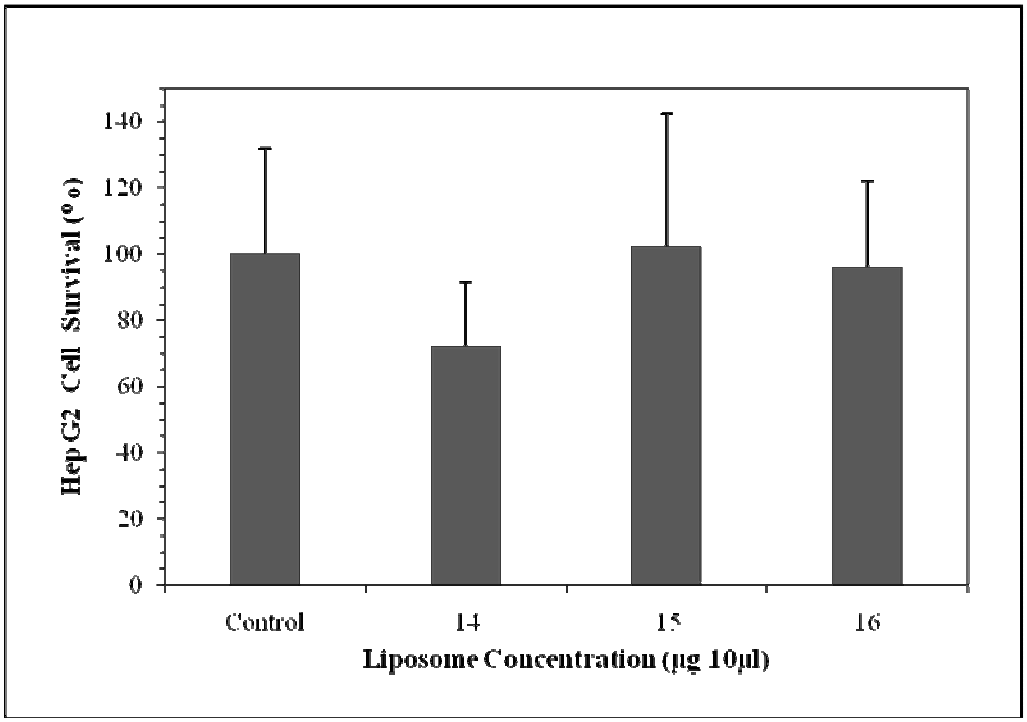


Fig 4.4 (b)

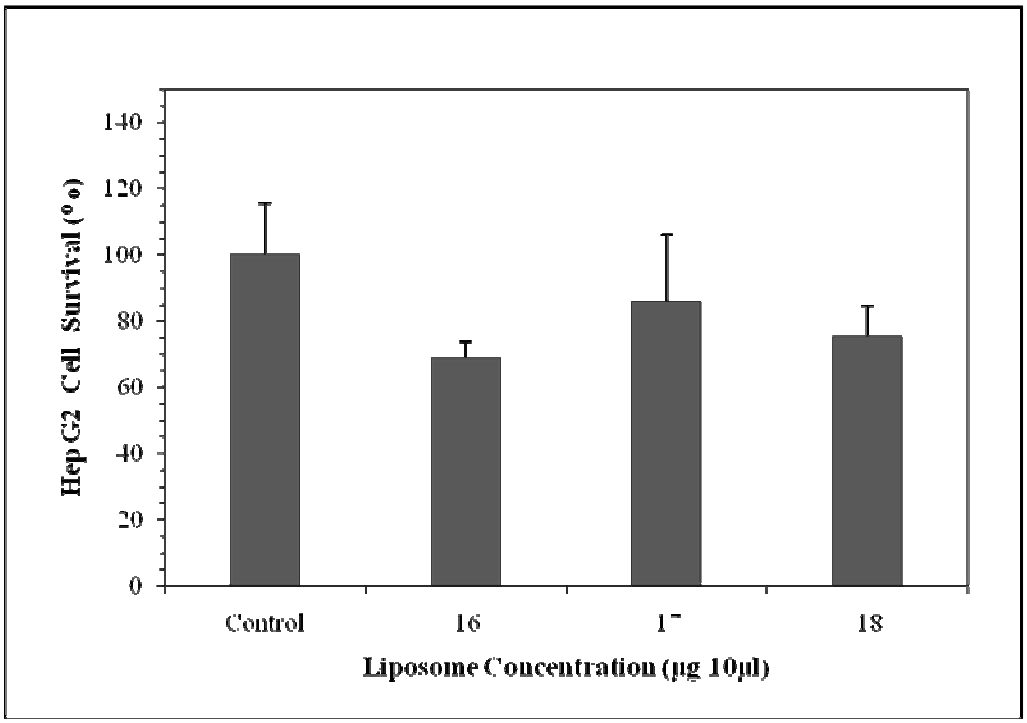


Fig 4.4 (c)

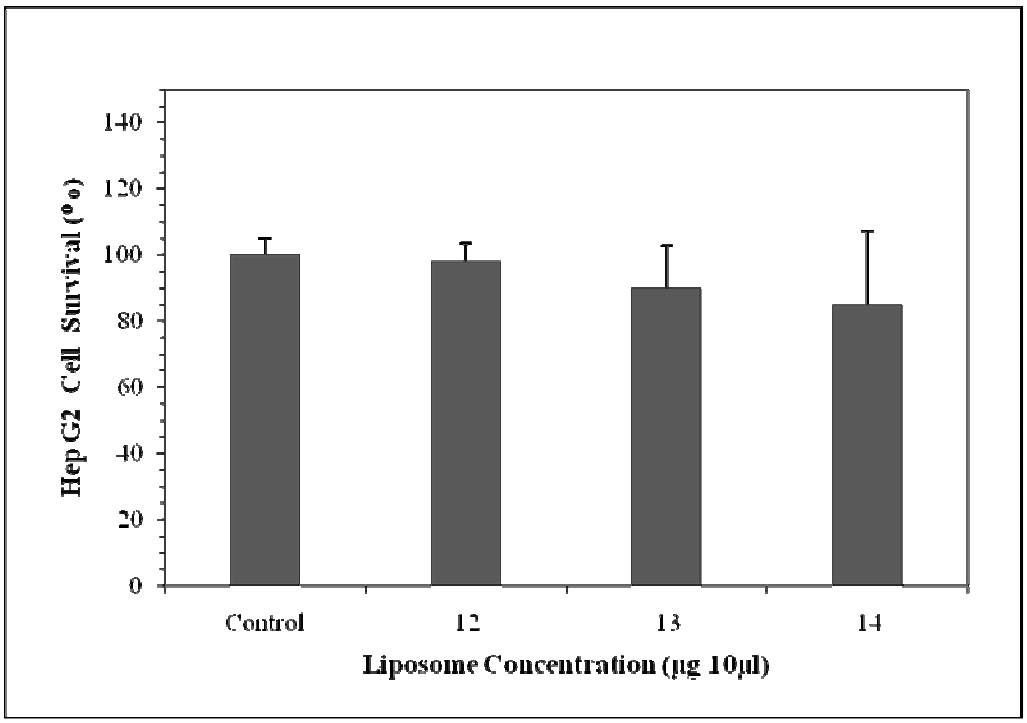


Fig 4.4 (d)

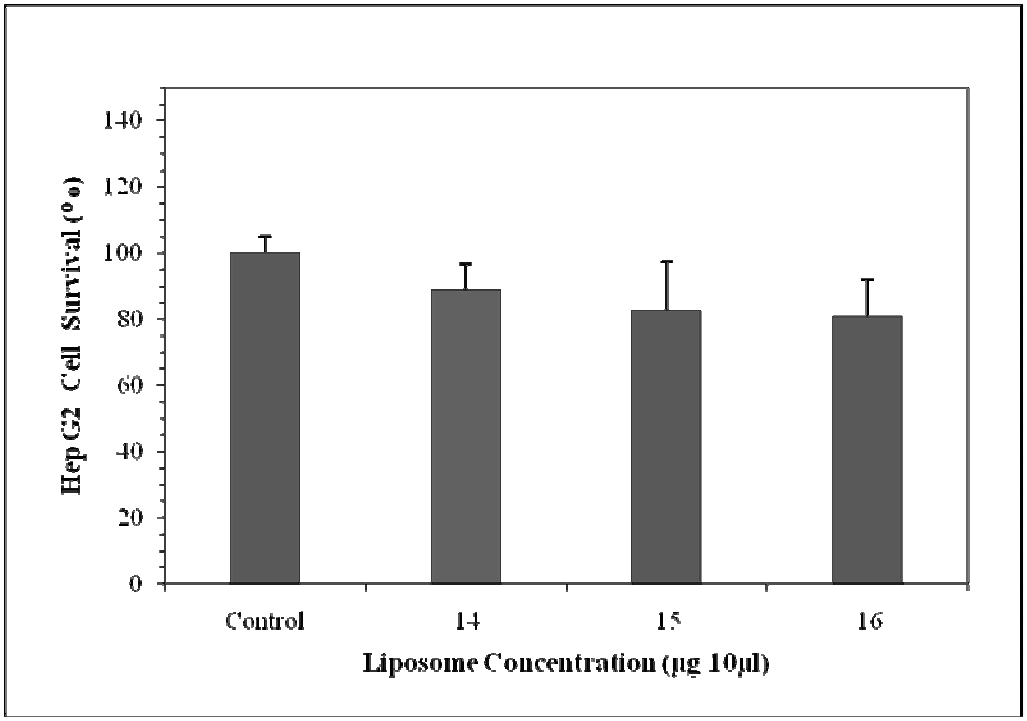


Fig 4.4 (e)

Figure 4.4: Growth inhibition studies of cationic liposome : pGL3 DNA complexes to HepG2 cells *in vitro.*

(a) varying amounts of NN (8, 9 and10 µg/10 µl)

(b) varying amounts of NN – 5% CAP-β-Gal (14, 15 and 16 µg/10 µl)

(c) varying amounts of NN – 10% CAP-β-Gal (16, 17 and 18 µg/10 µl)

(d) varying amounts of NN – 5% CAP-β-Glu (12, 13 and 14 µg/10 µl)

(e) varying amounts of NN – 10% CAP-β-Glu (14, 15 and 16 µg/10 µl)

while the DNA was kept constant at 1 µg in a total volume of 0.5ml of medium (MEM). Data are presented as a percentage of the control sample (no liposome) and are represented as means ±S.D (n=3).

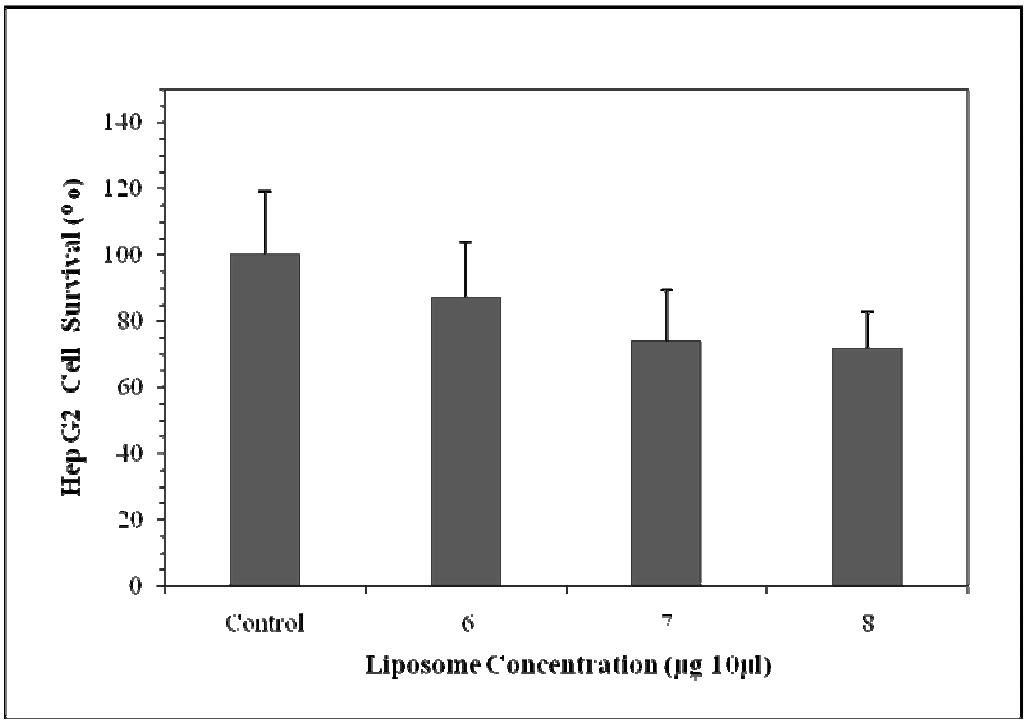


Fig 4.5 (a)

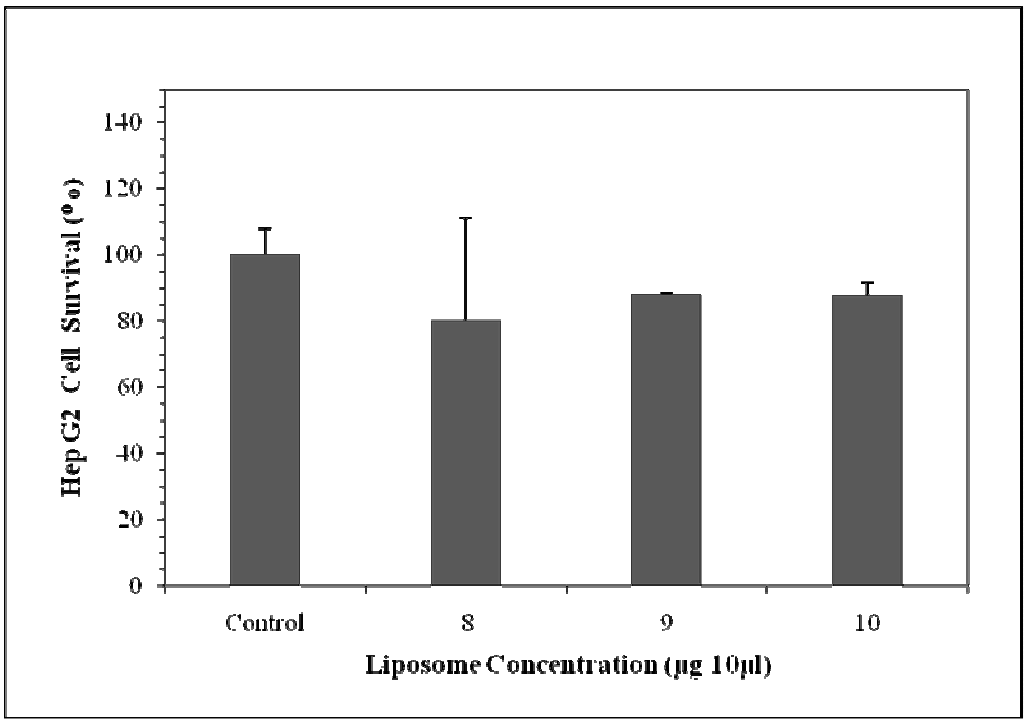


Fig 4.5 (b)

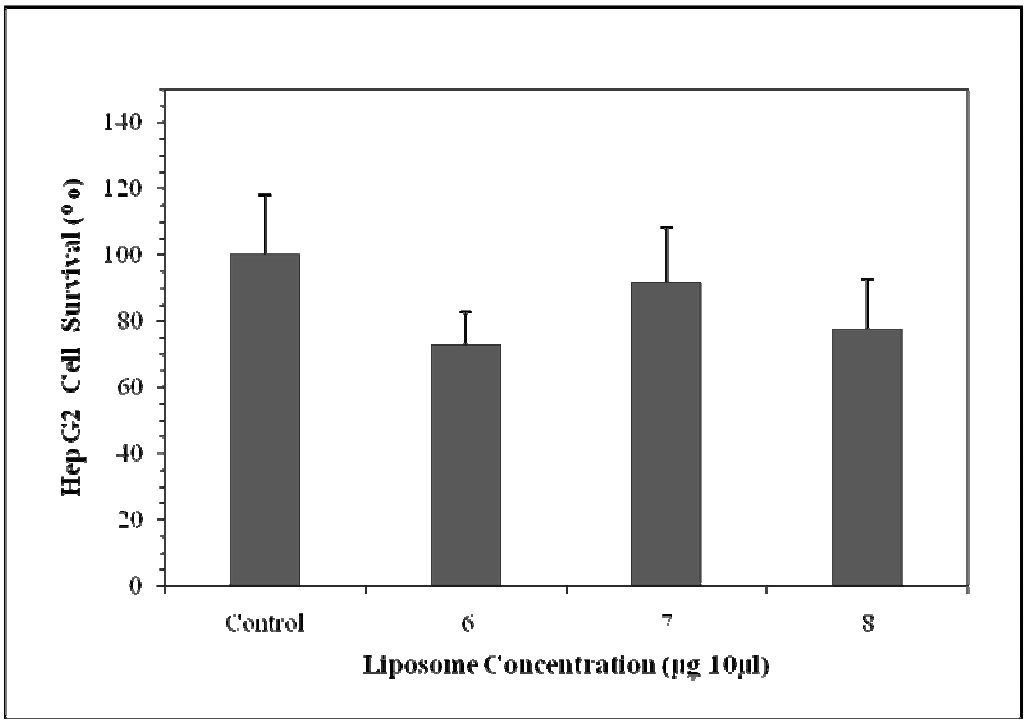


Fig 4.5 (c)

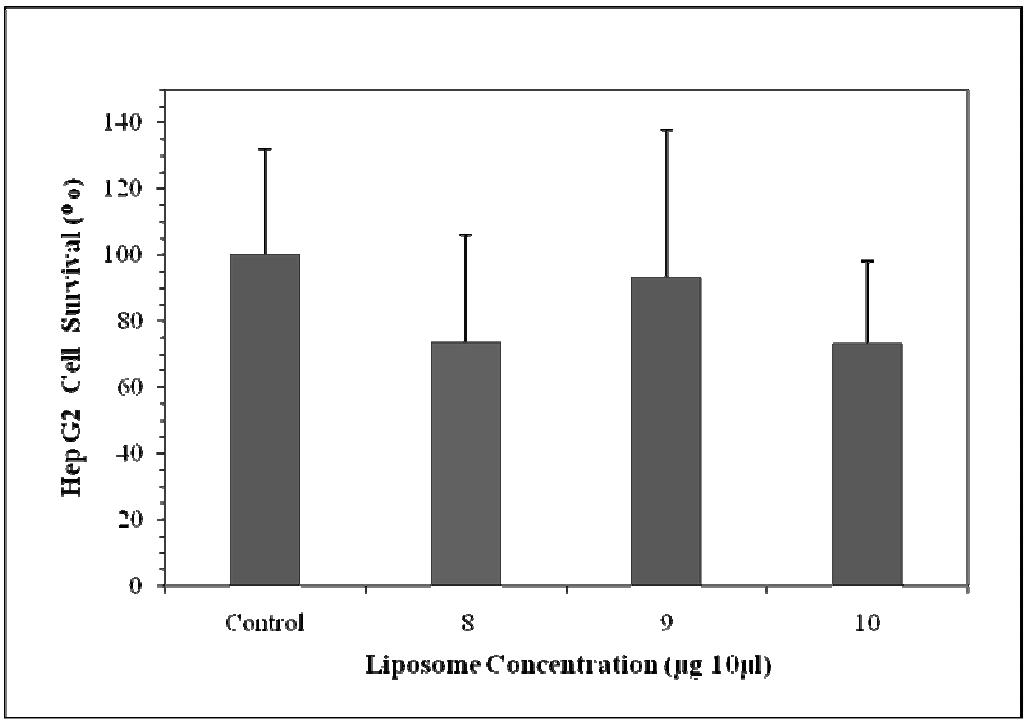


Fig 4.5 (d)

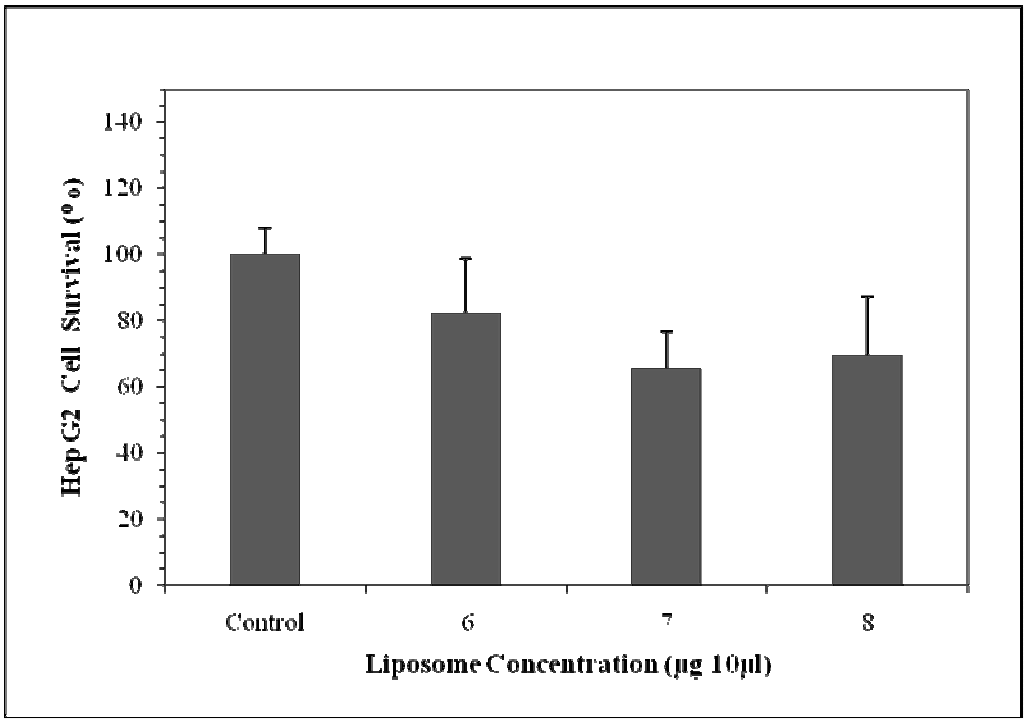


Fig 4.5 (e)

Figure 4.5: Growth inhibition studies of pegylated cationic liposome : pGL3 DNA complexes to HepG2 cells *in vitro.*

(a) varying amounts of PEG-NN (6, 7 and 8 µg/10 µl)

(b) varying amounts of PEG-NN – 5% CAP-β-Gal (8, 9 and 10 µg/10 µl)

(c) varying amounts of PEG-NN – 10% CAP-β-Gal (6, 7 and 8 µg/10 µl)

(d) varying amounts of PEG-NN – 5% CAP-β-Glu (8, 9 and 10 µg/10 µl)

(e) varying amounts of PEG-NN – 10% CAP-β-Glu (6, 7 and 8 µg/10 µl)

while the DNA was kept constant at 1 µg in a total volume of 0.5ml of medium (MEM). Data are presented as a percentage of the control sample (no liposome) and are represented as means ±S.D (n=3).

4.3.4 Transfection of HepG2 Cells

All cationic liposomes (NN, NN – 5% CAP-β-Gal, NN – 10% CAP-β-Gal, NN – 5% CAP-β-Glu and NN – 10% CAP-β-Glu) and pegylated cationic liposome preparations (PEG-NN, PEG-NN – 5% CAP-β-Gal, PEG-NN – 10% CAP-β-Gal, PEG-NN – 5% CAP-β-Glu and PEG-NN – 10% CAP-β-Glu) showed varying degrees of transfection activity as monitored by luciferase activity (Figures 4.6 and 4.7).

As mentioned in 4.2.2.4 (a), two controls were utilised, HepG2 cells alone and HepG2 cells incubated with naked DNA. These two controls showed low luminescence levels. Transfection using the two untargeted liposomes, NN and PEG-NN demonstrated the lowest luciferase activity of all the liposome preparations (Figures 4.6 (a) and 4.7 (a)). Hence transfection of HepG2 cells was more successful using targeted complexes which suggests asialoglycoprotein receptor recognition of the CAP-β-Gal and CAP-β-Glu targeting moieties.

Eight of the ten liposome preparations performed optimally at DNA : liposome ratios slightly below the ratio at which complete DNA retention by liposomes was seen on gel retardation assays (Table 3.2). This could possibly be due to size differences of lipoplexes at different DNA : liposome ratios (Higuchi *et al.,* 2006). Two liposome preparations, NN – 5% CAP-β-Gal and PEG-NN – 5% CAP-β-Gal did not follow this trend and showed optimal luciferase enzyme activity at the ratio at which all plasmid DNA was lipoplex associated, 1 : 16 (w/w) and 1 : 10 respectively. Of the cationic targeted complexes, NN – 5% CAP-β-Gal had the highest transfection efficiency, followed by NN – 5% CAP-β-Glu, NN – 10% CAP-β-Glu and NN – 10% CAP-β-Gal respectively. These results suggest that galactose density on the liposomal surface affects the transfection efficiency of these cationic liposomes and also supports the notion that liposomes with the lower concentration of targeting ligand incorporated into their structure are more efficient at transfecting HepG2 cells *in vitro*. It can be further observed that cationic liposomes with 5% CAP-β-Gal displayed slightly higher luciferase activity than its 5% CAP-β-Glu counterpart in keeping with reports by Singh *et al.* (2007) which suggested that galactosylated cationic liposomes showed greater transgene activity than glucosylated cationic liposomes when directed to HepG2 cells. This observation was not noted for the cationic liposome preparations containing 10% CAP-β-Gal and 10% CAP-β-Glu. This may be possibly ascribed to the difference in physiochemical properties of these liposome preparations as size and charge of the lipoplexes have been reported to be very

important parameters in gene targeting to hepatocytes *in vitro* and *in vivo* (Shigeta *et al.,* 2007).

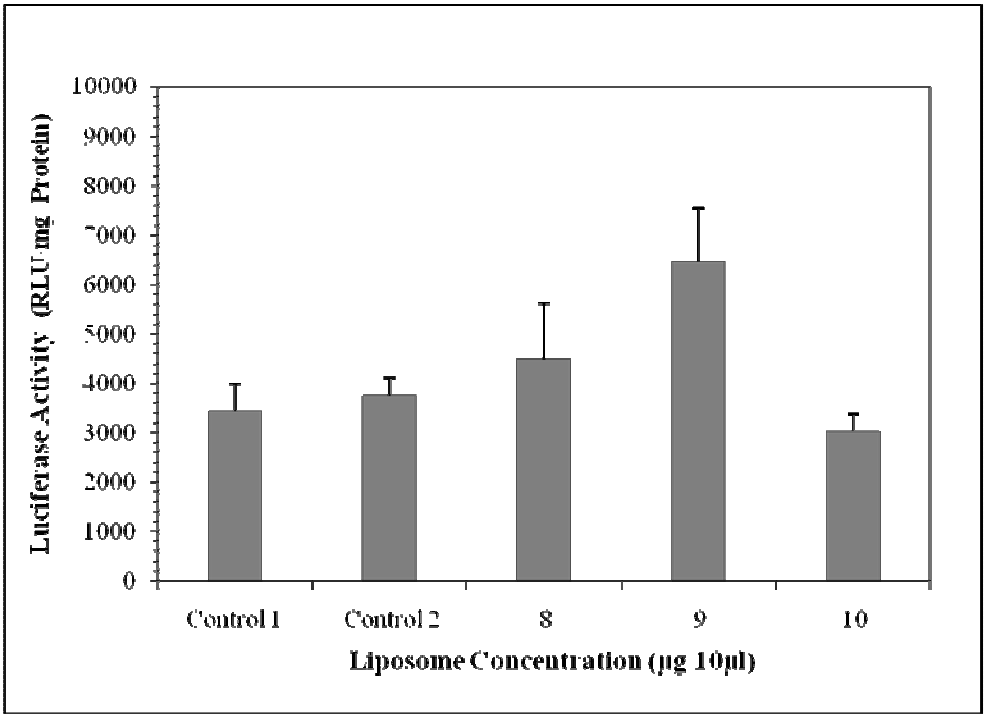


Fig 4.6 (a)

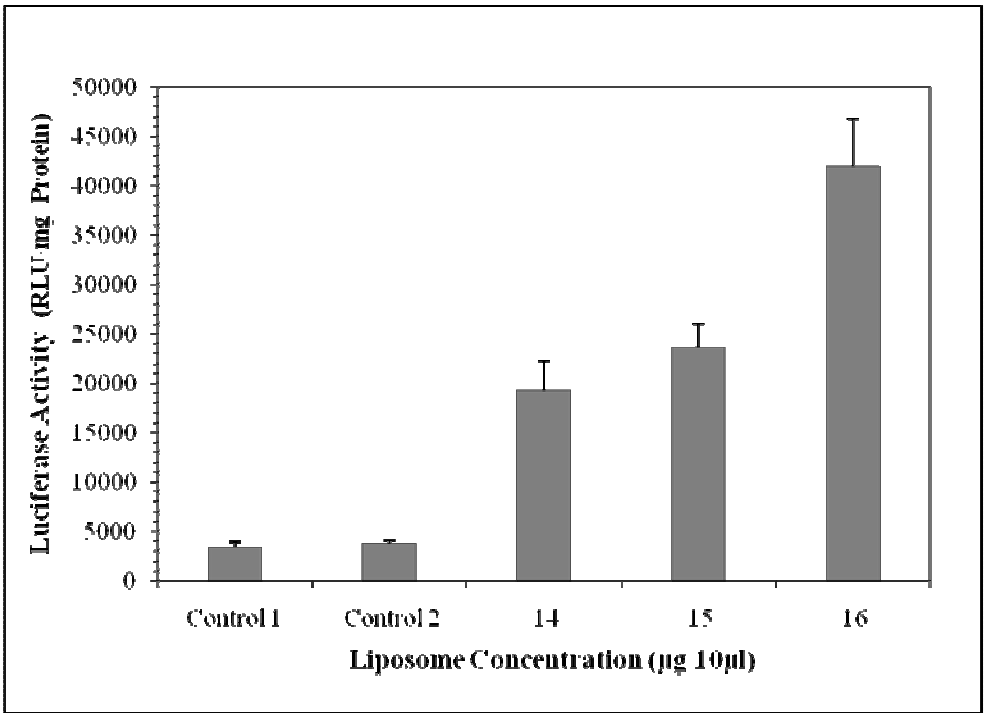


Fig 4.6 (b)

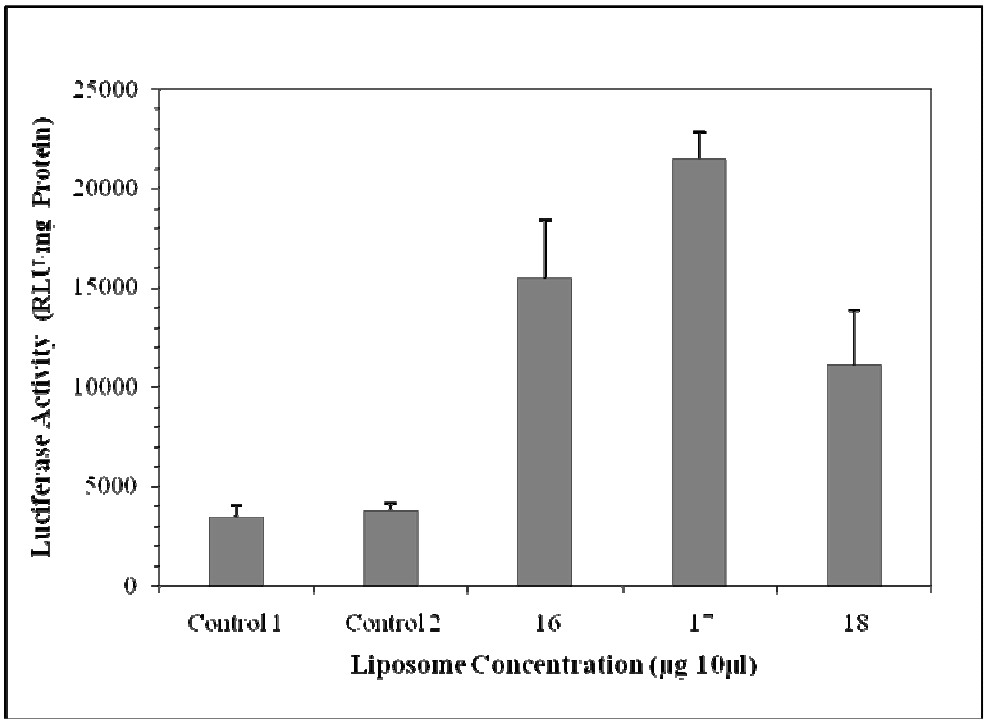


Fig 4.6 (c)

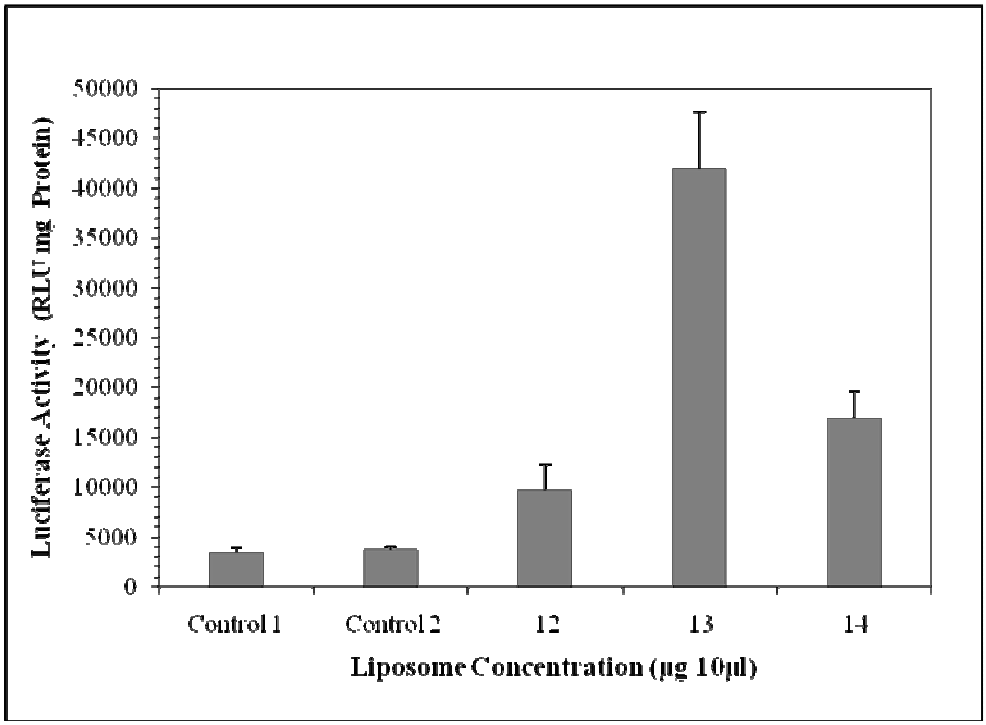


Fig 4.6 (d)

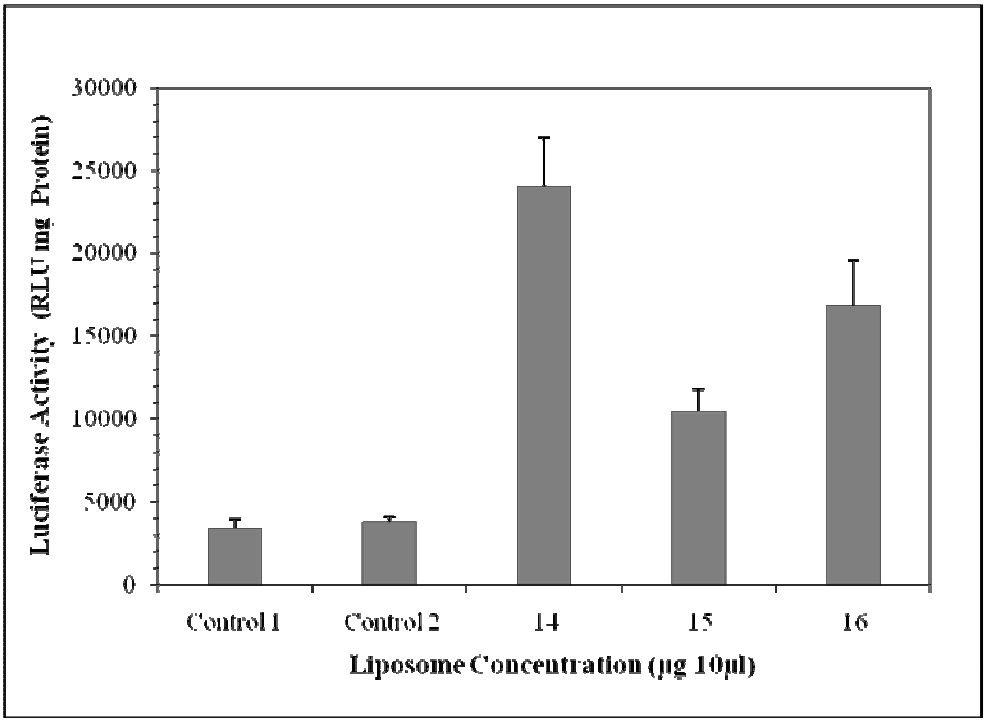


Fig 4.6 (e)

Figure 4.6: Transfection studies of cationic liposome : pGL3 DNA complexes to HepG2 cells *in vitro.*

(a) varying amounts of NN (8, 9 and10 µg/10 µl)

(b) varying amounts of NN – 5% CAP-β-Gal (14, 15 and 16 µg/10 µl)

(c) varying amounts of NN – 10% CAP-β-Gal (16, 17 and 18 µg/10 µl)

(d) varying amounts of NN – 5% CAP-β-Glu (12, 13 and 14 µg/10 µl)

(e) varying amounts of NN – 10% CAP-β-Glu (14, 15 and 16 µg/10 µl)

while the DNA was kept constant at 1 µg in a total volume of 0.5ml of medium (MEM). Control 1 contained only HepG2 cells while Control 2 contained HepG2 cells with naked pGL3 DNA. Data are represented as means ±S.D (n=3).

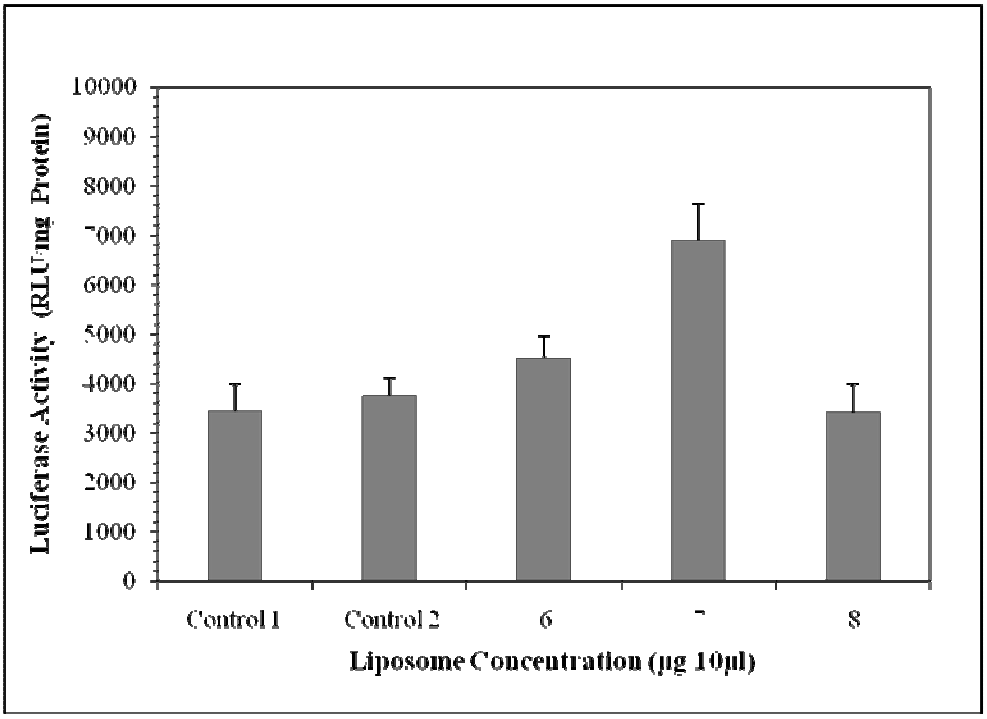


Fig 4.7 (a)

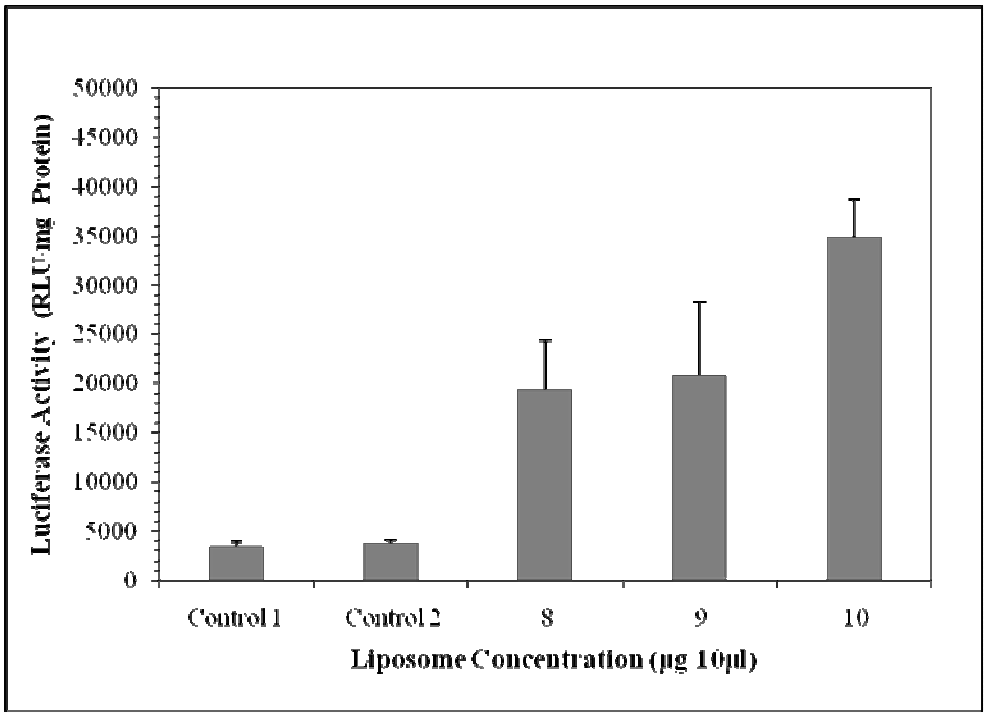


Fig 4.7 (b)

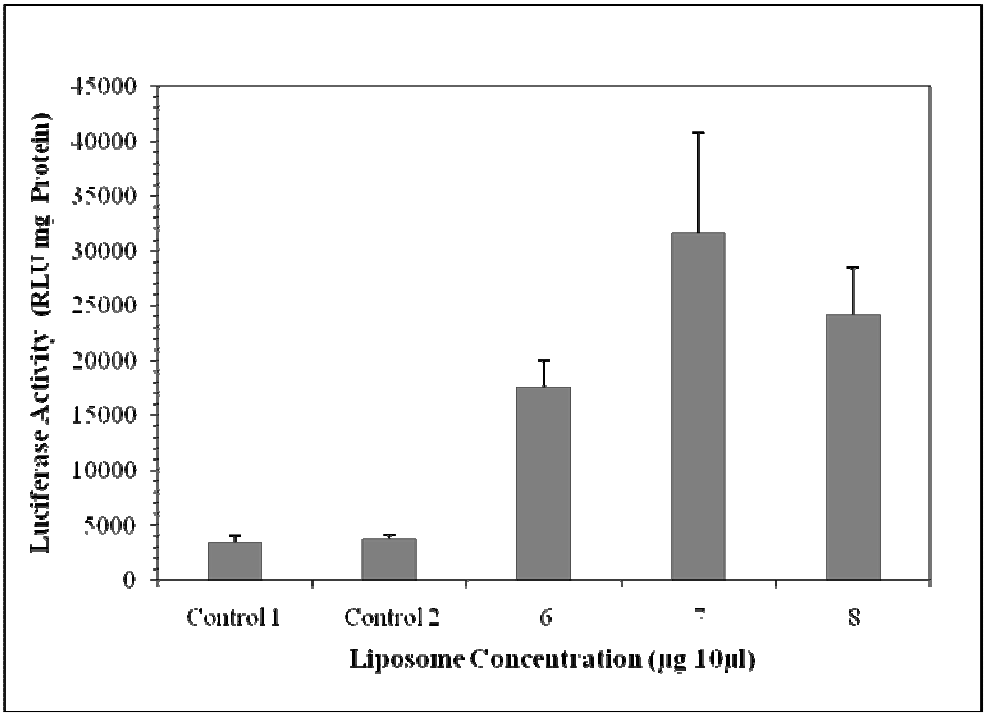


Fig 4.7 (c)

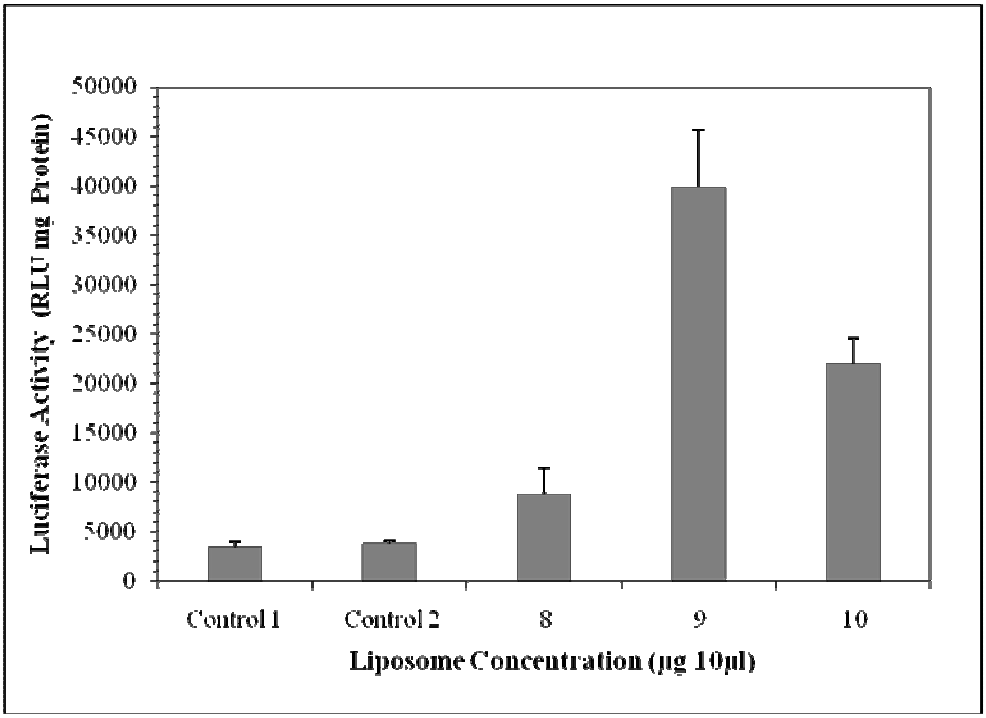


Fig 4.7 (d)

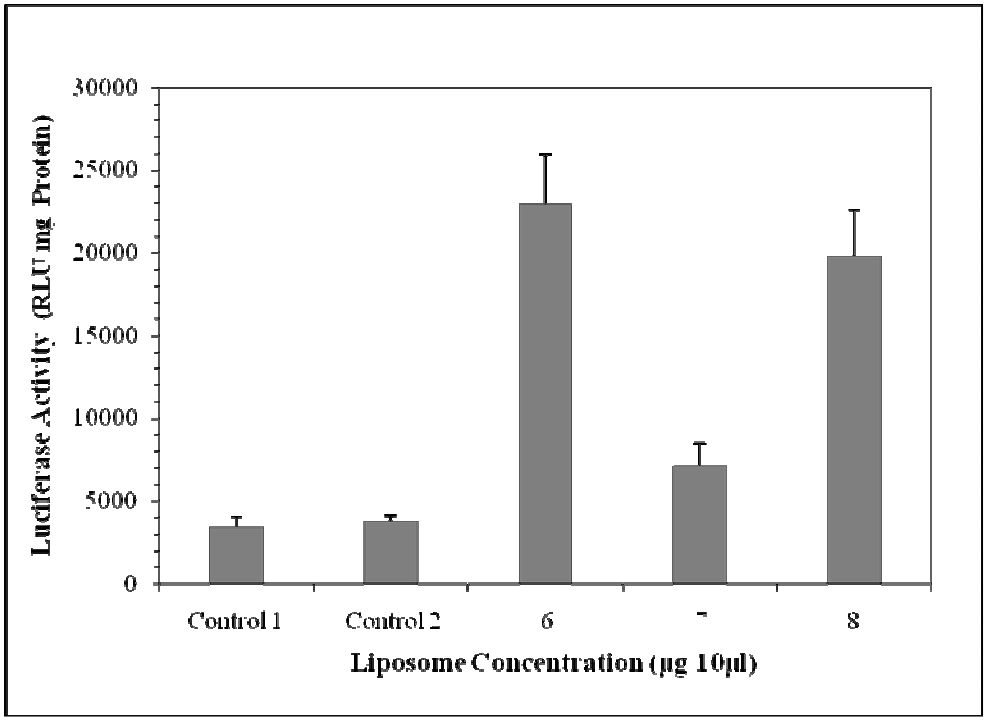


Fig 4.7 (e)

Figure 4.7: Transfection studies of pegylated cationic liposome : pGL3 DNA complexes to HepG2 cells *in vitro.*

(a) varying amounts of PEG-NN (6, 7 and 8 µg/10 µl)

(b) varying amounts of PEG-NN – 5% CAP-β-Gal (8, 9 and 10 µg/10 µl)

(c) varying amounts of PEG-NN – 10% CAP-β-Gal (6, 7 and 8 µg/10 µl)

(d) varying amounts of PEG-NN – 5% CAP-β-Glu (8, 9 and 10 µg/10 µl)

(e) varying amounts of PEG-NN – 10% CAP-β-Glu (6, 7 and 8 µg/10 µl)

while the DNA was kept constant at 1 µg in a total volume of 0.5ml of medium (MEM). Control 1 contained only HepG2 cells while Control 2 contained HepG2 cells with naked pGL3 DNA. Data are represented as means ±S.D (n=3).

In this study, the PEG head group was kept constant at a molecular weight of 2000, a size known to act as an effective steric barrier (Song *et al.,* 2002). As mentioned previously, the attachment of PEG to a gene delivery system such as cationic liposomes has a number of distinct advantages including steric stability, inhibiting excessive aggregation and fusion during the self assembly phase when cationic liposomes interact with anionic pDNA to form lipoplexes and a desired property of gene delivery vehicles, which is increased circulation time of vectors (Song *et al.,* 2002; Ishida *et al.,* 2008). In fact, pegylated liposomes have shown extended circulation times in all mammalian species including mice, dogs, rats and humans (Ishida *et al.,* 2008). It has been suggested by Kenworthy *et al.* (1995b) that PEG 2000 at 2 mol % PEG-lipid would appear to be in the mushroom to interdigitated mushroom conformation.

For targeted pegylated cationic liposomes, the trend observed was almost the same as for targeted cationic liposomes i.e. the liposomes with the lower concentration of CAP-β-Gal or CAP-β-Glu displayed higher transfection efficiency than their counterparts with a higher concentration of targeting moieties. As this trend is observed for both cationic and pegylated cationic liposomes it can be assumed that 5% of the targeting moiety is more effective than 10% of the targeting moiety. In contrast to that observed for cationic liposomes, PEG-NN – 5% CAP-β-Glu showed the highest transfection followed by PEG-NN – 5% CAP-β-Gal, PEG-NN – 10% CAP-β-Gal and PEG-NN – 10% CAP-β-Glu respectively. It has already been stated that it is vitally important to have a high density of targeting residues on the liposomal surface for effective receptor mediated gene transfer (Kawakami *et al.,* 1998). A possible reason for the discrepancy noted could be that the presence of PEG on the PEG-NN – 5% CAP-β-Gal liposome caused more of CAP-β-Gal moiety to be internalised during liposome formation thus decreasing the density of CAP-β-Gal on the liposomal surface and consequently deceasing transfection.

As seen by the results, pegylated cationic liposomes have demonstrated a small but measurable decrease in luciferase activity when compared to their non-pegylated cationic equivalents for the majority of the liposome preparations. According to literature, there are two theories regarding the inhibitory effect of PEG-lipids on DNA transfer. The first possible theory could be that cellular interaction and uptake of the lipoplexes is diminished probably due to steric hindrance imparted by the polymer chains during binding of the complex to the cell surface thus inhibiting endocytosis (Deshpande *et al.,* 2004). However Song *et al.*

(2002) found that intracellular uptake of pegylated lipoplexes was unaffected and suggested that the inhibitory effect of PEG is to be found at the step of endosomal escape of DNA. According to this theory the presence of PEG chains, dependent on the nature of the lipid anchor and the molecular weight of the PEG moiety, prevent close contact between the lipids of the pegylated complex and the endosomal membrane thus DOPE is unable to destabilise the endosomal membrane, by promotion of the inverted hexagonal phase, and pDNA is not released into the cytosol of the cells, prohibiting its delivery to the nucleus (Song *et al.,* 2002; Remaut *et al.,* 2007; Rejman *et al.,* 2004). Instead pDNA remains entrapped in the endosome while it fuses with the lysosome and is subsequently degraded (Remaut *et al*., 2007).

It is uncertain which of these two theories account for the slight decrease in transfection efficiency observed for the pegylated cationic liposomes in this study. However results obtained by Shi *et al.* (2002); Rejman *et al.* (2004), Remaut *et al.* (2007) and Peeters *et al.* (2007) all support the theory proposed by Song *et al.* (2002). Another possible explanation for the results obtained here could involve the use of DSPE as an anchor for PEG. Shi *et al*. (2002) observed that PEG-lipid analogues, such as DSPE-PEG, stabilise the lamellar phase of the bilayer preventing its conversion to the hexagonal phase. This is further supplemented by Rejman *et al.* (2004) who reported that DSPE-PEG provides a firm anchor in the bilayer of the liposomes thereby inhibiting the destabilisation and lipid mixing of the endosomal membrane and the liposome complex thus preventing the escape of DNA and resulting in decreased luciferase activity.

Pegylated cationic liposome preparation, PEG-NN – 10% CAP-β-Gal showed a marginal increase in luciferase activity as compared to cationic liposome, NN – 10% CAP-β-Gal. A potential explanation for this observation could be that there was a greater density of CAP-β-Gal on the surface of the pegylated liposome which would have facilitated greater interaction with the cell surface receptors which could result in increased transfection. This difference is nonetheless small and may not be significant.

The hepatocyte-derived human hepatocellular carcinoma cell line, HepG2 binds the ligand-associated liposomes and internalises them via receptor mediated endocytosis. This process is highly efficient as each cell contains approximately 225 000 asialoglycoprotein receptors, of which 85% are on the cell surface (Schwartz *et al*., 1982). These receptors are highly specific for D-galactose and therefore D-glucose, an epimer of D-galactose, would be expected to be

more poorly recognised by the asialoglycoprotein receptor as demonstrated by cationic liposomes in this study (Singh *et al.,* 2007). Figure 4.8 shows the possible mode of interaction between a targeted pegylated cationic liposome and its target cell, HepG2.

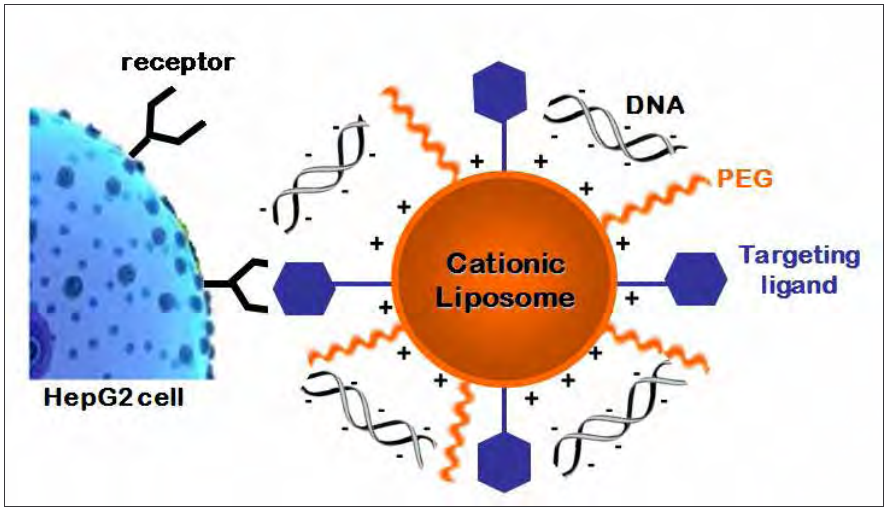


Figure 4.8: Illustration depicting the interaction between a targeted pegylated cationic lipoplex and a HepG2 cell. (Diagram not drawn to scale).

The luciferase assay system was used for the detection of transient gene expression in the HepG2 cells. The chemiluminescent assay for firefly luciferase activity is an easy-to-handle, rapid, extremely sensitive and non-isotopic alternative to other reporter gene assays. Firefly luciferase has a detectable linear range of approximately 10 fg/ml to 1 µg/ml (Torchilin, 2003). This enzyme does not occur in mammalian cells thus background luminescence levels are low hence it serves as an exceptional reporter for transfection of mammalian cells (Torchilin, 2003). Luciferase can function as a genetic reporter immediately upon translation as it does not require post-translational processing for enzymatic activity (de Wet *et al.,* 1987).

The firefly luciferase produces light from luciferin in the presence of ATP and Mg^{2+} :

$$\textbf{Beetle Luciferin + ATP + } O_2 \xrightarrow[Mg^{2+}]{\text{Firefly Luciferase}} \textbf{Oxyluciferin + AMP + Ppi + Light}$$

(Adapted from Torchilin (2003) and Promega Luciferase Assay System Manual)

This overall ATP dependent reaction catalysed by firefly luciferase is an oxidative decarboxylation of beetle luciferin producing light emission at a wavelength of 562 nm. The Promega Luciferase Assay kit used in this study incorporates coenzyme A (CoA) for improved kinetics. The light intensity which is a measure of the rate of catalysis by the luciferase enzyme is constant for several minutes and dependent on temperature, which for luciferase activity is optimum at 20 – 25ºC.

CONCLUSION

Receptor mediated targeting is a promising approach to selective gene delivery. The possibility of targeting genes or drugs to specific cells or tissues as well as facilitating their uptake and delivery has made liposomes a versatile carrier system with several potential applications in and out of the medical field. One particular method of receptor mediated targeting exploits the mechanism of sugar recognition that specific cells or tissue types possess (Hashida *et al.,* 1995). One of those cell types, hepatocytes, exclusively express high affinity cell surface receptors that can be specifically targeted by the chemical modification of liposomes with asialoglycoproteins or low molecular weight glycolipids. Managit *et al.* (2003) used a galactosylated cholesterol derivative to modify liposomes with galactose moieties for hepatocyte targeting. The glycolipids, CAP-β-Gal and CAP-β-Glu used in this study serve a similar function as those galactose moieties. Though cationic liposome-DNA complexes are efficient gene delivery vehicles *in vitro*, their use *in vivo* has a number of limitations. In an attempt to potentially circumvent those limitations, the synthetic polymer PEG was employed.

Novel hepatotropic pegylated and non-pegylated liposomes have been prepared from the cytofectin CHOL-T, DOPE, DSPE-PEG_{2000} and the glycolipids CAP-β-Gal and CAP-β-Glu by a thin film hydration – sonication technique. Preparations were characterised by cryoTEM which revealed the presence of deformable vesicular material. All liposome preparation suspensions formed lipoplexes with plasmid DNA as demonstrated in gel retardation and ethidium bromide displacement studies. Moreover liposomes were shown to offer protection from serum nuclease digestion to plasmid DNA in lipoplexes. These complexes exhibited low to moderate inhibition to HepG2 cell proliferation in the concentration range selected for transfection studies using the pGL3 plasmid and the luciferase assay. The latter studies clearly revealed that glycosylated cationic and pegylated cationic liposomes promoted luciferase expression at levels up to one order of magnitude greater than non-glycosylated controls. Highest activity was achieved by the non-pegylated NN – 5% CAP-β-Gal complex although its pegylated counterpart displayed only marginally lower activity. This was a trend observed for most the non-pegylated and pegylated liposome preparations. Further investigation is required to elucidate the exact mechanism responsible for this slight decrease in luciferase activity observed. However, regardless of the mechanism, future strategies that could be employed for the design of a more efficient pegylated gene carrier include, firstly,

the concept of 'post pegylation' and secondly, the utilisation of removable PEG polymer chains. These removable chains can be made to disassociate from the liposome either on contact with the target cell membrane by the use of exchangeable PEG-lipids or in the endosome by employing PEG-lipids that have a pH sensitive link to the lipid derivative (Remaut *et al.,* 2007).

The findings would suggest that the last mentioned liposome preparations i.e. NN – 5% CAP-β-Gal and PEG-NN – 5% CAP-β-Gal, should be evaluated further *in vivo* to establish their potential for therapeutic application. This would be undertaken in further studies. Although gene therapy is a potentially powerful clinical tool, is still lacks unequivocal clinical success. However, some time in the near future, the development of an 'ideal' vector that is a safe, stable, effective and targetable gene delivery system will make the vague notion of gene therapy into a commonplace reality. A better understanding of the cellular and *in vivo* barriers in gene transfer will lead to further improvement of the delivery system and a step closer to gene therapy finally realising its promise.

REFERENCES

Aden D.P., Fogel A., Damjanov S., Plotkin B. and Knowles B.B. (1979). '*Controlled synthesis of HBsAg in a differential human liver carcinoma-derived cell line'*. Nature. **282**: 615-616.

Allen T.M. (1994). *'Long-circulating (sterically stabilised) liposomes for targeted drug delivery'*. TiPS. **15**: 215-220.

Allen T.M., Hansen C., Martin F., Redemann C. and Yau-Young A. (1991). '*Liposomes containing synthetic lipid derivatives of polyethylene glycol show prolonged circulation half-lives in vivo'*. BBA. **1066 (1)**: 29-36.

Amos H. (1961). '*Protamine enhancement of RNA uptake by cultured chick cells'*. Biophysical Research Communications. **5**: 1-4.

Bainbridge J.W.B., Smith A.J., Barker S.S., Robbie S., Henderson R., Balaggan K., Viswanathan A., Holder G.E., Stockman A., Tyler N., Petersen-Jones S., Bhattacharya S.S., Thrasher A.J., Fitzke F.W., Carter B.J., Rubin S.B., Moore A.T., and Ali R.R. (2008). *'Effect of Gene Therapy on Visual Function in Leber's Congenital Amaurosis'*. The New England Journal of Medicine. **358**: 2231-2239

Bangham A.D. (1972). '*Lipid bilayers and Biomembranes'*. Annual Review of Biochemistry. **41**: 753-776.

Bangham A.D. (1992). '*Liposomes: realising their promise'*. Hospital Practice. **27**: 51-62.

Bangham A.D., Standish M.M. and Watkins J.C. (1965). *'Diffusion of univalent ions across the lamellae of swollen phospholipids'*. Journal of Molecular Biology. **13**: 238-252.

Barenholz Y. (2001). *'Liposome application: problems and prospects'*. Current Opinion in Colloid & Interface Science. **6**: 66-77.

Batzri S. and Korn E.D. (1973). *'Single bilayer liposomes prepared without sonication'*. Biochimica et Biophysica Acta. **298**: 1015-1019.

Bradley A.J., Devine D.V., Ansell S.M., Janzen J. and Brooks D.E. (1998). '*Inhibition of liposome-induced complement activation by incorporated poly(ethylene glycol)-lipids'*. Archives of Biochemistry and Biophysics. **357**: 185-194.

Campbell M.J. (1995). *'Lipofection reagents prepared by a simple ethanol injection technique'*. Biotechniques. **18 (6)**: 1027-1032.

Campbell R.B., Balasubramanian S.V. and Straubinger R.M. (2001). *'Phospholipid-cationic lipid interactions: influence on membrane and vesicle properties'*. Biochimica et Biophysica Acta, Biomembranes. **1512 (1)**: 27-39.

Chapman D. (1984). *'Physiochemical properties of phospholipids and lipid-H_2O systems'*. Liposome Technology. **1**: 1-18.

Christiano R.J., Smith L.C., Kay M.A., Brinkly B.R. and Woo, S.L.C. (1993). '*Hepatic gene therapy: efficient gene delivery and expression in primary hepatocytes utilizing a conjugated adenovirus-DNA complex'*. Proceedings of the National Academy of Sciences, USA. **90**: 11548-11552.

Coster H.G. (1965). *'A quantitative analysis of the voltage-current relationships of fixed charge membrane and the associated property of the "punch through"'*. Biophysical Journal. **5**: 669-686.

Dadashzadeh S., Vali A.M. and Rezaie M. (2008). *'The effect of PEG coating on invitro cytotoxicity and in vivo disposition of topotecan loaded liposomes in rats'*. International Journal of Pharmaceutics**. 353**: 251-259.

Davis M.E. (2002). *'Non–viral gene delivery systems'*. Current Opinion in Biotechnology. **13**: 128-131.

de Gennes P.G. (1980). '*Conformations of polymers attached to an interface'*. Macromolecules **13**: 1069-1075.

de Wet J.R., Wood K.V., DeLuca M., Helsinki D.R. and Subramani S. (1987). '*Firefly luciferase gene: structure and expression in mammalian cells'*. Molecular and Cellular Biology. **7 (2)**: 725-737.

Deamer D. And Bangham A.D. (1976). *'Large volume liposomes by ether vaporisation method'*. Biochimica et Biophysica Acta. **443**: 629-624.

Deshpande M.C., Davies M.C., Garnett M.C.,Williams P.M., Armitage D., Bailey L., Vamvakaki M., Armes S.P. and Stolnik S. (2004). *'The effect of poly(ethylene glycol) molecular architecture on cellular interaction and uptake of DNA complexes'*. Journal of Controlled Release **97**: 143-156.

Farhood H., Bottega R., Epand R.M. and Huang L. (1992). *'Effect of cationic cholesterol derivatives on gene transfer and protein kinase C activity'*. Biochimica et Biophysica Acta. **1235**: 289-295.

Felgner J.H., Kumar R., Sridhar C.N., Wheeler C.J., Tsai Y.J., Border,R., Ramsey P., Martin M. and Felgner P.L. (1994). '*Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations'*. Journal of Biological Chemistry. **269 (4)**: 2550-2561.

Felgner P.L. and Ringold G.M. (1989). '*Cationic liposome mediated transfection'*. Nature. **337**: 387-388.

Felgner P.L., Barenholz Y., Behr J.P., Cheng S.H., Cullis P., Huang L., Jesse J.A., Seymour L., Szoka Jr F.C., Thierry A.R., Wagner E. and Wu G. (1997). '*Nomenclature for synthetic gene delivery systems'*. Human Gene Therapy. **8**: 511-512.

Felgner P.L., Gadek T.R., Holm M., Roman R., Chan H.W., Wenz M., Northrop J.P., Ringold J.M. and Danielsen M. (1987). '*Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure'*. Proceedings of the National Academy of Sciences, USA. **88**: 7413-7417.

Filion M.C. and Phillips N.C. (1998). *'Major limitations in the use of cationic liposomes for DNA delivery'*. International Journal of Pharmaceutics. **162**: 159-170.

Fried M.G. and Crothers D.M. (1984). '*Equilibrium studies of the cyclic AMP receptor protein-DNA interaction'*. Journal of Molecular Biology. **172 (3)**: 241-262.

Friedmann T. and Roblin R. (1972). *'Gene therapy for human genetic disease'*. Science. **175 (4025)**: 949-955.

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

Gabizon A.A. (2001). '*Stealth liposomes and tumor targeting: one step further in the quest for the magic bullet'*. Clinical Cancer Research. **7**: 223-225.

Gao X. and Huang L. (1991). *'A novel cationic liposome reagent for efficient transfection of mammalian cells'*. Biochemical Biophysical Research Communications. **179**: 280-285.

Garinot M., Mignet N, Largeau C, Seguin J., Scherman D. and Bessodes M. (2007). '*Amphiphilic polyether branched molecules to increase the circulation time of cationic particles'*. Bioorganic and Medicinal Chemistry. **15**: 3176-3186.

Gershon H., Ghirlando R., Guttman S.B. and Minsky A. (1993). *'Mode of formation and structural features of DNA-cationic liposome complexes used for transfection'*. Biochemistry. **32**: 7143-7151.

Gregoriadis G., Bacon A., Caparros-Wanderley W. and McCormack B. (2002). *'A role for liposomes in genetic vaccination'*. Vaccine **20**: B1-B9.

Gresch O., Engel F.B., Nesic D., Tran T.T., England H.M., Hickman E.S., Körner I., Gan L., Chen S., Castro-Obregon S., Hammermann R., Wolf J., Müller-Hartmann H., Nix M., Siebenkotten G., Kraus G. and Luna K. (2004). *'New non-viral method for gene transfer into primary cells'*. Methods. **33**: 151-163.

Grove R.I. and Wu G.Y. (1998). '*Pre-clinical trials using hepatic gene delivery'*. Advanced Drug Delivery Reviews. **30**: 199-204.

Hashida M., Nishikawa M., Yamashita F. and Takakura Y. (2001). *'Cell-specific delivery of genes with glycosylated carriers'*. Advanced Drug Delivery Reviews. **52**: 187-196.

Hashida M., Nishikawa M. and Takakura Y. (1995). *'Hepatic targeting of drugs and proteins by chemical modifications'*. Journal of Controlled Release. **36**: 99-107.

He L., Yang L., Duan Y., Deng L., Sun X., Gu X. and Zhi-Rong Z. (2009). '*Cytotoxicity and hemocompatibility of a family of novel MeO-PEG-Poly (D,L-Lactic-co-glycolic acid)-PEG-OMe triblock copolymer nanoparticles'*. Journal of Applied Polymer Science. **113 (5)**: 2933-2944.

Higuchi Y., Kawakami S., Fumoto S., Yamashita F. and Hashida M. (2006). '*Effect of the particle size of galactosylated lipoplex on hepatocyte-selective gene transfection after intraportal administration'*. Biological and Pharmaceutical Bulletin. **29**: 1521-1523.

Hoekstra D., Rejman L., Wasungu L., Shi F. and Zuhorn I. (2007). *'Gene delivery by cationic lipids: in and out of an endosome'*. Biochemical Society Transactions. **35 (1)**: 68-71.

Hong K., Zheng W., Baker A. and Papahadjopoulos D. (1997). '*Stabilisation of cationic liposome-plasmid DNA complexes by polyamines and poly(ethylene glycol)-phospholipid conjugates for efficient in vivo gene delivery'*. FEBS Letters. **400**: 233-237.

Huang L., Hung M. and Wagner E. (1999). Nonviral Vectors for Gene Therapy. Academic Press, San Diego, California.

Hui S., Langer M., Zhao Y., Ross P., Hurley E. and Chan K. (1996). *'The role of helper lipids in cationic liposome-mediated gene transfer'*. Biophysical Journal. **71**: 590-599.

Immordino M.R., Dosio F., Cattel L. (2006). '*Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential*'. International Journal of Nanomedicine **1(3)**: 297-315.

Ishida T. and Kiwada H. (2008). *'Accelerated blood clearance (ABC) phenomenon upon repeated injection of PEGylated liposomes'*. International Journal of Pharmaceutics. **354**: 56-62.

Johnson S. M., Bangham A.D., Hill H.W. and Korn E.D. (1971). '*Single layer liposomes'*. Biochimica et Biophysica Acta. **233**: 820-826.

Józkowicz A. and Dulak J. (2005). *'Helper-dependent adenoviral vectors in experimental gene therapy'*. Acta Biochimica Polonica. **52 (3)**: 589-599.

Kawakami S., Yamashita F., Nishikawa M., Takakura Y., Hashida M. (1998). *'Asialoglycoprotein receptor-mediated gene transfer using novel galactosylated cationic liposomes'*. Biochemical Biophysical Research Communications. **252**: 78-83.

Kenworthy A.K., Hristova K., Needham D. and McIntosh T.J. (1995b). '*Range and magnitude of the steric pressure between bilayers containing phospholipids with covalently attached poly(ethylene glycol)'*. Biophysical Journal. **68**: 1921-1936.

Kenworthy A.K., Simon S.A. and McIntosh T.J. (1995a). '*Structure and phase behavior of lipid suspensions containing phospholipids with covalently attached poly(ethylene glycol)'*. Biophysical Journal. **68**: 1903-1920.

Knowles B.B., Howe C.C. and Aden D.P. (1980). '*Human hepatocelluar carcinoma cell lines secrete major plasma proteins and Hepatitis B surface antigens'*. Science. **209**: 497-499.

Koumbi D., Clement J., Sideratou Z., Yaouanc J., Loukopoulos D. and Kollia P. (2006). *'Factors mediating lipofection potency of a series of cationic phosphonolipids in human cell lines'* Biochimica et Biophysica Acta. **1760**: 1151-1159.

Lasic D.D. (1997). Liposomes in Gene Delivery. CRC Press LLC, Boca Raton, Florida.

Lee C., Choi Y., Huh E.J., Lee K.Y., Song H., Sun M.J., Jeong H., Cho C. and Bom H. (2005). *'Polyethylene glycol (PEG) modified ^{99m}Tc-HMPAO-liposome for improving blood circulation and biodistribution: the effect of the extent of pegylation'*. Cancer Biotherapy and Radiopharmaceuticals. **20**: 620-628.

Lesage D., Cao A., Briane D., Lievre N., Coudert R., Raphael M., Salzmann J.L. and Taillandier E. (2002). *'Evaluation and optimisation of DNA delivery into gliosarcoma 9L cells by a cholesterol-based cationic liposome'*. Biochimica et Biophysica Acta. **154**: 393-402.

Li S. and Huang L. (2000). *'Nonviral gene therapy: promises and challenges'*. Gene Therapy. **7**: 31-34.

Li S. and Ma Z. (2001). *'Nonviral gene therapy'*. Current Gene Therapy **1 (2)**:1-35.

Liu L. and Huang L. (2002). *'Development of non-viral vectors for systemic gene delivery'*. Journal of Controlled Release. **78**: 259-266.

Lonez C., Vandenbranden M. and Ruysschaert J. (2008). *'Cationic liposomal lipids: from gene carriers to cell signalling'*. Progress in Lipid Research. **47**: 340-347.

Managit C., Kawakami S., Nishikawa M., Yamashita F. and Hashida M. (2003). *'Targeted and sustained drug delivery using PEGylated galactosylated liposomes'*. International Journal of Pharmaceutics. **266**: 77-84.

Martin B., Sainlos M., Aissaoui A., Oudrhiri N., Hauchecorne M., Vigneron J.P., Lehn J.M. and Lehn P. (2005). *'The design of cationic lipids for gene delivery'*. Current Pharmaceutical Design. **11**: 375-394.

Meyer O., Kirpotin D., Hong K., Sternberg B., Park J.W., Woodle M. C., and Papahadjopoulos D. (1998). *'Cationic liposomes coated with polyethylene glycol as carriers for oligonucleotides'*. The Journal of Biological Chemistry. **273 (25)**: 15621-15627.

Moghimi S.M. (2006). *'The effect of methoxy-PEG chain length and molecular architecture on lymph node targeting of immuno-PEG liposomes'*. Biomaterials**. 27**: 136-144.

Mountain A. (2000). *'Gene therapy: the first decade'*. TIBTECH. **18**: 119-128.

Mullen C.A., Snitzer K., Culver K.W., Morgan R.A., Anderson W.F. and Blaese R.M. (1996). *'Molecular analysis of T lymphocyte-directed gene therapy for adenosine deaminase deficiency: long term expression in vivo of genes introduced with a retroviral vector'*. Human Gene Therapy. **49**: 807-837.

Needham D. and Kim D.H. (2000). *'PEG-covered lipid surfaces: bilayers and monolayers'*. Colloids and Surfaces B: Biointerfaces. **18**:183-195.

Needham D., Stoicheva N., and Zhelev D.V. (1997). *'Exchange of monooleoylphosphatidylcholine as monomer and micelle with membranes containing poly(ethylene glycol)-lipid'*. Biophysical Journal **73**: 2615-2629.

Nishikawa M. and Huang L. (2001). '*Nonviral vectors in the new millennium: delivery barriers in gene transfer'*. Human Gene Therapy. **12**: 861-870.

Obata Y., Saito S., Takeda N. and Takeoka S. (2009). '*Plasmid DNA-encapsulating liposomes: effect of spacer between the cationic head group and hydrophobic moieties of the lipids on gene expression efficiency'*. Biochimica et Biophysica Acta. **1788**: 1148-1158.

Oku N., Tokudome Y., Asai T. and Tsukada H. (2000). *'Evaluation of drug targeting strategies and liposomal trafficking'*. Current Pharmaceutical Design. **6**: 1669-1691.

Oku N., Yamazaki Y., Matsuura M., Sugiyama M., Hasegawa M. and Nango M. (2001). *'A novel non-viral gene transfer system, polycation liposomes'*. Advanced Drug Delivery Reviews. **52**: 209-218.

Parker A.L., Newman C., Briggs S., Seymour L. and Sheridan P.L. (2003). '*Lipoplex-mediated transfection and endocytosis'*. Expert Reviews in Molecular Medicine. **5**: 1.

Parr M.J., Ansell S.M., Choi S. and Cullis P.R. (1994). '*Factors influencing the retention and chemical stability of polyethylene-glycol lipid conjugates incorporated into large unilamellar vesicles'*. BBA. **1195 (1)**: 21-30.

Peeters L., Sanders N.N., Jones A., Demeester J., and De Smedt S.C. (2007). *'Post-pegylated lipoplexes are promising vehicles for gene delivery in RPE cells'*. Journal of Controlled Release. **12**: 208-217.

Percot A., Briane D., Coudert R., Reynier P., Bouchemal N., Lievre N., Hantz E., Salzmann J.L. and Cao A. (2004). *'A hydroxyethylated cholesterol-based cationic lipid for DNA delivery: effect of conditioning'*. International Journal of Pharmaceutics. **278**: 143-163.

Poste G., Kirsh R. and Koestler T. (1984). *'The challenge of liposome targeting in vitro'*. Liposome Technology. **III**: 1-28. Editor: Gregoriadis G. CRC Press Inc., Florida.

Promega Technical Manual – pGL3 luciferase reporter vectors. Promega Corporation.

Promega Luciferase Assay System Manual. Promega Corporation.

Radler J.O., Koltover I., Salditt T. and Safinya C.R. (1997). '*Structure of DNA-cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes*'. Sciences. **275**: 810-814.

Ramazani M., Khoshhamdam M., Dehshahri and Malaekeh-Nikouei B. (2009). *'The influence of size, lipid composition and bilayer fluidity of cationic liposomes on the transfection efficiency of nanolipoplexes'*. Colloids and Surfaces B: Biointerfaces. **72**: 1-5.

Rejmana J., Wagenaar A., Engbertsb J.F.B.N. and Hoekstra D. (2004). *'Characterization and transfection properties of lipoplexes stabilized with novel exchangeable polyethylene glycol–lipid conjugates'*. Biochimica et Biophysica Acta. **1660**: 41-52.

Remaut K., Lucas B., Braeckmans K., Sanders N.N., Demeester J. and De Smedt S.C. (2005). *'Protection of oligonucleotides against nucleases by pegylated and non-pegylated liposomes as studied by fluorescence correlation spectroscopy'*. Journal of Controlled Release. **110**: 212-226.

Remaut K., Lucas B., Braeckmans K., Demeester J. and De Smedt S.C. (2007). '*Pegylation of liposomes favours the endosomal degradation of the delivered phosphodiester oligonucleotides*'. Journal of Controlled Release. **117**: 256-266.

Roper C. (1999). '*Liposomes as a gene delivery system'*. Brazilian Journal of Medical and Biological Research. **32**: 163-169.

Ross P.C. and Hui S.W. (1999*). 'Polyethylene glycol enhances lipoplex-cell association and lipofection'*. Biochimica et Biophysica Acta. **1421**: 273-283.

Sawyer J.T. and Doyle D. (1990). *'Assembly of heterooligometric asialoglycoprotein receptor complex during cell-free translation'*. Proceedings of the National Academy of Sciences, USA. **87**: 4854-4858.

Schwartz A.L., Geuze H.J. and Lodish H.F. (1982). *'Recycling of the asialoglycoprotein receptor: biochemical and immunological evidence'*. Philosophical Transactions of the Royal Society of London Biological Sciences. **300**: 229-235.

Scott V., Clark A.R. and Docherty K. (1994). *'Gel Retardation Assay'*. Methods in Molecular Biology. **31**: 339. Editor: Harwood A.S. Humana Press., New York.

Shi F., Wasungu L., Nomden A., Stuart M.A.C., Polushkin E., Engberts J.B.F.N. and Hoekstra D. (2002). '*Interference of poly(ethylene glycol)–lipid analogues with cationic-lipid-mediated delivery of oligonucleotides; role of lipid exchangeability and non-lamellar transitions'*. Biochemical Journal. **366**: 333-341.

Shigeta K., Kawakami S., Higuchi Y., Okuda T., Yagi H., Yamashita F. and Hashida M. (2007). *'Novel histidine-conjugated galactosylated cationic liposomes for efficient hepatocyte-selective gene transfer in human hepatoma HepG2 cells'*. Journal of Controlled Release. **118**: 262-270.

Singh M. (1998). *'Liposome-asialoorosomucoid complexes and their delivery of an expression vector to HepG2 cells in culture'*. MSc Thesis, University of Durban-Westville.

Singh M. and Ariatti M. (2006a). '*A cationic cytofectin with long spacer meadiates favourable transfection in transformed human epithelial cells'*. International Journal of Pharmaceutics. **309**: 189-198.

Singh M., Hawtrey A. and Ariatti M. (2006b). *'Lipoplexes with biotinylated transferrin accessories: novel, targeted, serum-tolerant gene carriers'*. International Journal of Pharmaceutics. **321**: 124–137.

Singh M., Kisoon N. and Ariatti M. (2001). *'Receptor-mediated gene delivery to HepG2 cells by ternary assemblies containing cationic liposomes and cationised asialoorosamucoid'*. Drug Delivery. **8**: 29-34.

Singh M., Rogers C.B. and Ariatti M. (2007). *'Targeting of glycosylated lipoplexes in HepG2 cells: Anomeric and C-4 epimeric preference of the asialoglycoprotein receptor'*. South African Journal of Science. **103**: 204-210.

Smith J.G., Walzem R.L. and German B. (1993). *'Liposomes as agents of DNA transfer'*. Biochimica et Biophysica Acta. **1154**: 327-340.

Smith A.E. (1999). *'Gene therapy – where are we?*'. Lancet. **354 (Suppl 1)**: 1-4.

Song L.Y., Ahkong Q.F., Rong Q., Wang Z., Ansell S., Hope M.J. and Mui B. (2002). *'Characterization of the inhibitory effect of PEG-lipid conjugates on the intracellular delivery of plasmid and antisense DNA mediated by cationic lipid liposomes'*. Biochimica et Biophysica Acta. **1558**: 1-13.

Sternberg B., Sorgi F.L. and Huang L. (1994). *'New structures in complex formation between DNA and cationic liposomes visualised by freeze-fracture electron microscopy'*. FEBS Letters. **356 (23)**: 361-366.

Sykes P. (1970). A guidebook to mechanism in organic chemistry. **3rd Edition**. Longmans, London.

Szoka Jr F.C. and Papahadjopoulos D. (1978). '*Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation'*. Proceedings of the National Academy of Sciences, USA. **75 (9)**: 4194-4198.

Thompson B., Mignet N., Hofland H., Lamons D., Seguin J., Nicolazzi C., de la Figuera N., Kuen R.L., Meng X.Y., Scherman D. and Bessodes M. (2005). '*Neutral postgrafted colloid particles for gene delivery'* Bioconjugate Chemistry. **16**: 608-614.

Tirosh O., Barenholz Y., Katzhendler J. and Priev A. (1998). *'Hydration of polyethylene glycol-grafted liposomes'*. Biophysical Journal **74**: 1371-1379.

Torchilin V. (2003). 'Liposomes: a practical approach'. **2nd Edition**. Oxford University Press, New York.

Tros de Ilarduya C., Arangoa M.A., Moreno-Aliaga M.J. and Düzgünes N. (2002). *'Enhanced gene delivery in vitro and in vivo by improved transferrin-lipoplexes'*. Biochimica et Biophysica Acta. **1561**: 209-221.

Walther W. and Stein U. (2000). '*Viral vectors for gene transfer: A review of their use in the treatment of human diseases'*. Drugs. **60 (2)**: 249-271.

Wang S., Cheng L., Yu F., Pan W. and Zhang J. (2006). '*Delivery of different length poly(L-lysine)-conjugated ODN to Hepg2 cells using N-stearyllactobionamide-modified liposomes and their enhanced cellular biological effects'*. International Journal of Pharmaceutics**. 311 (1-2)**: 82-88.

Wasungu L. and Hoekstra D. (2006*). 'Cationic lipids, lipoplexes and intracellular delivery of genes'*. Journal of Controlled Release. **116**: 255-264.

Wolfe S.L. (1995). Introduction to cell and molecular biology. Wadsworth Publishing Company, California.

Wu G.Y. and Wu C.H. (1988). *'Receptor-mediated gene delivery and expression in vivo'*. Journal of Biological Chemistry. **263**: 14621-14624.

Wu J., Nantz M.H. and Zern M.A. (2002). '*Targeting hepatocytes for drug and gene delivery: emerging novel approaches and applications'*. Frontiers in Bioscience. **7**: 717-725.

Xu Y. and Szoka Jr F.C. (1996). *'Mechanism of DNA release from cationic liposome:DNA complexes used in cell transfection'*. Biochemistry. **35**: 5616-5623.

Xu Y., Hui S.-W., Frederik P. and Szoka Jr F.C. (1999). '*Physicochemical characterization and purification of cationic liposomes'*. Biophysical Journal*.* **77**: 341-353.

Zalipsky S. (1995). '*Functionalized poly(ethy1ene glycol) for preparation of biologically relevant conjugates'*. Bioconjugate Chemistry. **6**: 150-165.

Zalipsky S., Hansen C.B., Lopes de Menezes D.E.and Allen T.M. (1996). *'Long-circulating, polyethylene glycol-grafted immunoliposomes'*. Journal of Controlled Release. **39**: 153-161.

Zhdanov R.I., Podobed O.V. and Vlassov V.V. (2002). *'Cationic lipid-DNA complexes – lipoplexes – for gene transfer and therapy'*. Bioelectrochemistry. **58**: 53-64.

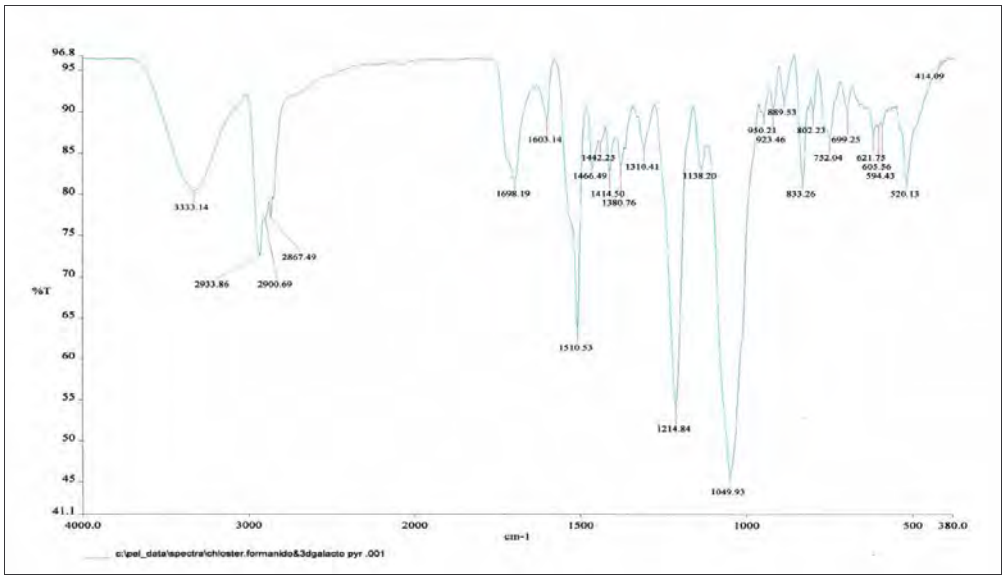
www.colorado.edu/intphys/class

www.avantipolarlipids.com

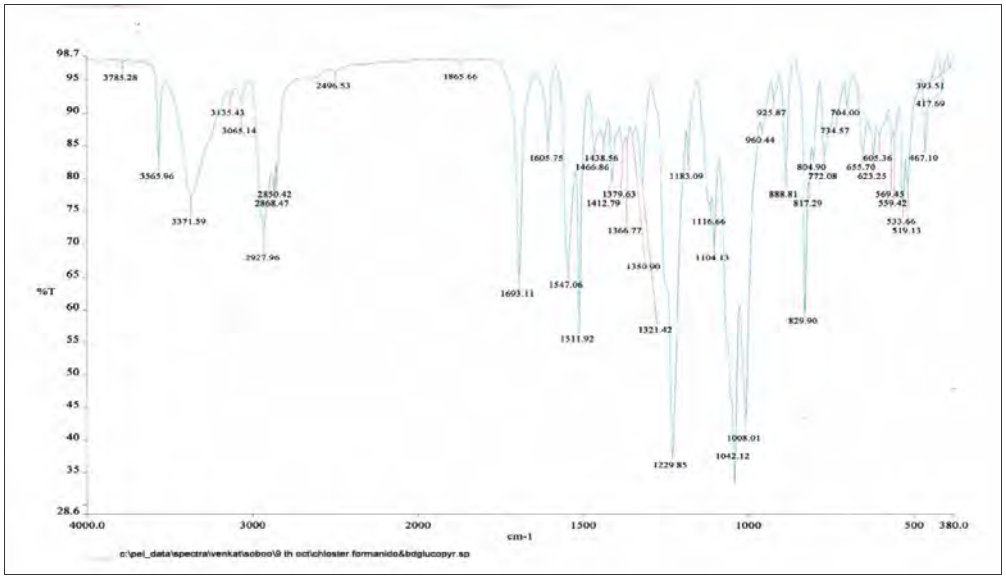
www.wiley.com.uk/genetherapy/clinical

APPENDIX A

INFRARED SPECTRA



CAP-β-Gal: Cholesteryl 3β-N-(4-aminophenyl-β-D-galactopyranosyl) carbamate



CAP-β-Glu: Cholesteryl 3β-N-(4-aminophenyl-β-D-glucopyranosyl) carbamate

APPENDIX B

PUBLICATIONS

Desirable feature of synthetic DNA carriers is their ability to provide cell and tissue DNA uptake specificity by means of receptor-mediated endocytosis. CXCR4 is a receptor of chemokine SDF-1 and is expressed on some types of stem and cancer cells. CXCR4 also interacts with vMIP-II (viral macrophage inflammatory protein II). The aim of this project is to design and to characterize the group of chemokine derived peptides (CDPs) for receptor-mediated gene delivery. Our present study focused on three peptides, two of them is derived from N-terminal sequences of the chemokine SDF-1 and one - from vMIP-II. All peptides are modified with DNA-binding sequence (K8). Control peptide consists of only K8 sequence. Gene delivery by CDP/DNA complexes is glycerol-dependent and the level of luciferase expression with CDPs was comparable with the efficacy of PEI in CXCR4 expressing cell lines (A172, HeLa) and was 10–50-fold higher compared to unmodified peptide. On the contrary CDP transfection efficacy on CXCR4-negative cells (CHO) was much lower than in PEI. Intracellular uptake analysis of biotin-labeled peptides indicated that CDPs entered cells more efficiently than oligolysine. Addition of free CDP to cells during the uptake analysis blocked peptides transport significantly. In summary, the results presented herein show that oligolysine polyplexes can be specifically targeted to cells expressing CXCR4 receptor, by using peptides derived from chemokine SDF-1 and viral protein vMIP-II.

E-mail: ankiselev@yahoo.co.uk

P 345

Muscle-targeted gene transfer enables continuous secretion of GLP-1 and exendin-4 in mice

Aiman Mahmoud*, Stijn Niessen, Ali aldibbiat, Hussain Al-Turaifi, Chris Huggins, JAM Shaw

Institute of Cellular Medicine, Newcastle University, UnitedKingdom

Session: Vector targeting

Glucagon-like peptide-1 (GLP-1) is a potent insulinotropic hormone with an important role in maintaining normal blood glucose but the short half-life of the active peptide, necessitates continuous infusion or regular self-injection limiting clinical use. Exendin-4 is a GLP-1 homologue resistant to DPP IV mediated degradation, increasing its half-life and making it an attractive alternative for Type 2 diabetes therapy. However, two injections are still required every day. The aims of the study were to generate plasmid constructs enabling constitutive secretion of GLP-1 / Exendin-4 and evaluate expression, biosynthesis and secretion in murine muscle in vitro and in vivo. GLP1(7–36); Exendin-4; and reporter eGFP cDNAs preceded by signal sequence were sub-cloned into pVR1012 plasmid. In vitro transfections were undertaken in the C2C12 muscle cell line. In vivo gene transfer was performed in male CD1 mice by direct plasmid injection into hind limb muscles. GLP-1 and Exendin-4 expression/biosynthesis in vitro was confirmed by RT-PCR/cytoplasmic immunofluorescence staining, and constitutive secretion of bioactive GLP1 (260 ± 20 pmol/l) and Exendin-4 (2433 ± 585 pmol/l) demonstrated by specific immunoassay. Biosynthesis following plasmid-mediated gene transfer in mice was confirmed by immufluorescence staining; with glucagon-like peptide secretion into systemic circulation at 7 days (GLP1: 56 ± 28 pmol/L vs eGFP: $13 \pm$ pmol/L; $p = 0.01$), (Exendin-4: 847 ± 295 pmol/L vs eGFP: 6.6 ± 1pmol/; $p = 0.0002$). Glucose levels were unaffected but weight lower with Exendin-4 (40 ± 1 g vs eGFP: 44 ± 1 g; $p = 0.001$). Constitutive secretion of GLP-1 and Exendin-4 has been attained following plasmid-mediated gene transfer to muscle with bioactivity confirmed in vivo.

E-mail: aiman.mahmoud@ncl.ac.uk

P 346

Pegylated cationic liposome mediated targeted gene delivery

Nicolisha Narainpersad, Moganavelli Singh*, Mario Ariatti

Biochemistry, University of KwaZulu-Natal, South Africa

Session: Vector targeting

Background: Cationic liposomes have shown potential in the targeting of specific cells such as HepG2 (hepatocellular carcinoma) cells, a model in vitro gene delivery system to study hepatocyte function. The covalent attachment of the hydrophilic polymer, polyethylene glycol (PEG) to the liposomal surface is thought to sterically stabilise liposomes, promote biological stability, decrease toxicity and immunogenicity and prolong the liposome circulation time in vivo.

Method: Targeted glycosylated lipoplexes with and without PEG were synthesised. An equimolar amount of a cationic cholesterol derivative was mixed with dioleoylphosphatidylethanolamine (DOPE) and a galactose/glucose derivative to produce targeted cationic liposomes. PEG liposomes were prepared similarly with the addition of DPSE-PEG2000. Liposome interaction with the luciferase expression plasmid pGL3 afforded lipoplexes which were characterized by band shift, serum nuclease digestion and ethidium displacement assays. Growth inhibition and transient transfection activities were determined in vitro in the HepG2 cell line.

Results: Cationic liposome pegylation reduces liposome DNA binding capability and transfection activity in vitro. Liposomes showed low cytotoxicities and were able to protect the DNA from nuclease degradation.

Conclusion: Although cationic liposome pegylation decreases transfection activity in vitro, their low toxicity and transfection ability make them promising candidates for in vivo studies.

E-mail: singhm1@ukzn.ac.za

P 347

Targeted cancer gene therapy by clostridium perfringens enterotoxin (CPE) gene transfer