NOVEL CATIONIC LIPOPLEXES: CHARACTERIZATION IN CELL CULTURE *IN VITRO* AND *IN VIVO*

by

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As the candidates supervisor I have approved this thesis/dissertation for submission.

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ABSTRACT

Amongst the more promising non-viral DNA vehicles are liposomes, with those derived from cationic lipids showing significant potential, despite moderate transfection levels *in vivo*.

This study has investigated the effect of liposome-anchored ionophore crown ethers on lipoplex-mediated gene transfer *in vitro* and *in vivo*. Several liposomes were constructed to include the cytofectin 3β[N(N',N'-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T), the co-lipid dioleoylphosphatidylethanolamine (DOPE), and 5% (mole/mole) of the cholesteryl crown ethers RUI-128 (aza-18-crown-6) or RUI-129 (aza-15-crown-5). Liposome size and lamellarity were established by transmission electron microscopy. All liposome preparations were shown to bind, condense and protect DNA avidly in the respective band shift, ethidium displacement and nuclease protection assays.

Lipoplex targeting to hepatocytes may be achieved via the asialoglycoprotein receptor (ASGP-R), which is abundantly expressed on the human hepatoblastoma cell line HepG2. Therefore six additional liposomes were formulated to include 5% (mole/mole) of the cholesteryl galactosyl RUI-90 (Gal) and cholesteryl glucosyl RUI-92 (Glu) ligands. Their hepatotropic gene delivery was examined in the HepG2 cell line using the pCMV-luc plasmid. Transfection studies in the human embryonic kidney cell line HEK293 (ASGP-R-negative) revealed an increase in transgene activity in lipoplexes displaying the RUI-129 cholesteryl derivative. No ionophore-mediated enhancement of transfection activity was observed in HepG2 cells although Chol-T:DOPE, Chol-T:DOPE:RUI-128 and Chol-T:DOPE:RUI-129 liposomes achieved very high transfection levels, exceeding those of their hepatocyte targeted counterparts. Liposome-anchored crown ethers have been shown to potentiate *in vitro* transfection activity of lipoplexes in the HEK293 cell line. The novel cholesteryl glycosyl derivatives were, however, unable to enhance the targeted entry of lipoplexes into HepG2 cells.

The three most effective preparations from *in vitro* studies were taken forward for *in vivo* assessment in NMRI mice at the University of the Witwatersrand Molecular Medicine and Haematology unit. Three groups of mice were employed for the evaluation of Chol-T:DOPE, Chol-T:DOPE:RUI-129 and Chol-T:DOPE:RUI-129-Gal lipoplexes with the Psi-CHECK plasmid. Mice treated with hydrodynamic injection and untreated animals made up two control groups. Luciferase activity was determined on examination of the harvested liver homogenates. All liposomes showed modest, but significant transfection activity ($p<0.05$) and were well tolerated. The assemblies examined therefore warrant further development.

PREFACE

The experimental work described in this dissertation was carried out in the School of Biochemistry, Microbiology and Genetics, University of KwaZulu-Natal, Durban, from January 2009 to December 2010, under the supervision of Professor M. Ariatti and co-supervision of Dr. M. Singh and Professor P. Arbuthnot.

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

DECLARATION 1 – PLAGIARISM

I Alisha Sewbalas declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
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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

Dr M Singh, Professor M Ariatti, Miss A Sewbalas. (2010). '*Crown Ethers potentiate Transfection activity of Cationic Lipoplexes in Mammalian Cells'*. HUMAN GENE THERAPY. 21:1358 –1396

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LIST OF ABREVIATIONS

Å	Ångström
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
CE	Crown ether
CE 1	RUI-129
CE 2	RUI-128
Chol-T	3 β [N-(N', N'-dimethylaminopropane)-carbamoyl] cholesterol
CL	Cationic liposomes
CL – DNA	Cationic liposome – DNA complex
CMV	Cytomegalovirus
CS	Chondroitin sulphate
DC-Chol	3β[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol
DMRIE	Dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DOGS	Dioctadecylamidoglycyl – spermine
DOPE	Dioleoylphosphatidylethanolamine
DOSPA	2,3 – dioleyoxy-2(6carboxylspermyl)-propylamide
DOTAP	N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulphate
DOTMA	N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride
EDTA	Ethylenediamine tetra-acetic acid
EIV	Ether injection vesicles
FBS	Foetal bovine serum
FPV	French press vesicles
GAGs	Glycosaminoglycans
HA	Hyaluronan
HBS	Hepes buffered saline
HEK 293	Human embryonic kidney cell line

HepG2	Human hepatocellular carcinoma cell line
HS	Heparin sulphate
IFN-γ	Interferon-γ
IL-6	Interleukin-6
IL-12	Interleukin-12
MEM	Minimum essential medium
MoMLV	Moloney murine leukemia virus
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NR	Neutral red assay
NMRI	Naval medical research institute
PBS	Phosphate buffered saline
pIC	Poly inosine-cytosine
REV	Reverse-phase evaporation
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSV	Rous sarcoma virus
RLU	Relative light units
RUI-90	Deacetylated galactosyl ligand $(M + H)^+$ $C_{37}H_{61}N_4O_7$
RUI-92	Deacetylated glucosyl ligand $(M + H)^+$ $C_{37}H_{61}N_4O_7$
RUI-128	Aza-18-crown-6 ether $(M + H)^+$ $C_{40}H_{70}NO_7$
RUI-129	Aza-15-crown-5 ether $(M + H)^+$ $C_{38}H_{66}NO_6$
SDS	Sodium dodecyl sulphate
siRNA	Small interfering RNA
SV40	Simian virus 40
TNF-α	Tumour necrosis factor-α
UV	Ultraviolet

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THIS THESIS IS DEDICATED TO MY LATE GRANDFATHER,
MR. D. SEWBALAS.

CHAPTER ONE

1. INTRODUCTION

1.1. THE NEED FOR GENE THERAPY: THE EARLY YEARS AND BEYOND

Of all non-communicable diseases cancer has been implicated as the foremost cause of mortality, accounting for approximately 7.1 million global deaths annually. Over half of these have been observed in developing countries with cancer also being identified as the primary cause of death in high income or industrialized countries (Morille *et al.*, 2008).

Application of conventional cancer treatments is often insufficient in the face of aggressive, resistant, invasive tumours which have complex mechanisms of propagation. This obstinacy to conventional therapies of chemotherapy, radiotherapy or even hormonal therapy makes cancer an ideal model for therapeutic treatment at the molecular level (Morille *et al.*, 2008). Consequently new approaches are being sought with particular focus given to the investigation of genes involved in cancer development. Our current and extensive knowledge of the genetic basis of many human diseases has provided the impetus for molecular manipulation of disease through the employment of recombinant DNA methodologies. The chief goal for cancer therapy is to bring about treatment through exploitation of therapeutic information encoded in the DNA sequence (Lim, 1999; Rozema, 2008). Besides this, many of the systemic diseases facing us presently are as a consequence of deficiencies in enzymes or factors resulting from defects or the absence of specific genes (Sharma and Sharma, 1997; Rozema, 2008). Treatment at the source of infection has thus made gene therapy an invaluable commodity for tumour eradication (Morille *et al.*, 2008).

Gene therapy that involves the introduction of exogenous DNA into somatic cells needs vectors with specific requirements that allow, reasonably effective transfection, a controllable period of expression as well as cell or spatial specificity in addition to the minimization of safety concerns (Holladay *et al.*, 2009). Morille and collaborators (2008) showed that cancer gene therapy in particular requires specific vector types such as those in Table 1.1, which can meet the four specific criteria listed:

i. Deactivation of oncogene expression,
ii. Enhancement of tumour suppressor expression to promote cancer cell apoptosis,
iii. Inhibition of neo-angiogenesis, and
iv. Immune system stimulation against the neoplasm.

Table 1.1. Gene delivery vector systems for cancer gene therapy (Adapted from Lim, 1999; Mountain, 2000; Zuckerbraun and Tzeng, 2002).

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 Large capacity	Repeat dosing infective owing to strong immune responses Transgene size limit of 7.5 kb Large genome is difficult to manipulate Transient delivery Immunogenicity
Retrovirus	Long term stability for prolonged expression High transfection efficiency *ex vivo* Substantial clinical experience Low immunogenicity Chromosomal integration	Transient delivery and low transfection efficiency *in vivo* Transgene size limit of 8 kb Transfects only dividing cells Random DNA insertion Risk of replication
Adeno-associated virus	Efficiently transfects a wide variety of cells *in vivo* Very prolonged expression *in vivo* Low immunogenicity and non-pathogenic in humans Easily manipulated Stable integration	Transgene limit of 4.5 kb Manufacture in large quantities is very difficult Little clinical experience Safety concern of mutagenesis Repeat dosing affected by neutralizing 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 Relatively unstable
Cationic liposomes	Relatively simple manufacture and storage Efficient transfection *ex vivo* and *in vitro* Low immunogenicity Transfects all cell types	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* Relatively non-pathogenic Retargeted transfection demonstrated	Inefficient transfection *in vivo* Very short duration of expression No clinical experience

Pathologically, cancer has been found capable of averting its own destruction through the variety of inherent mechanisms. Medical intervention in this regard has resulted most often only in prolonging a patient's life from months to possibly years. Presently surgery involving the bulk removal of primary tumours is useful for the treatment of certain types of metastases (Dass and Choong, 2006). Cancer gene therapy can be classified into two completely distinct therapeutic strategies for gene delivery into targeted cells, i.e., corrective and cytoreductive gene therapy. Corrective gene therapy looks at the reversal or inhibition of the pathophysiology of cancer via the introduction of wild-type genes into pre-neoplastic or neoplastic cells. Cytoreductive gene therapy alternatively involves the augmentation of a tumour specific immune response to stimulate malignant cell death or the *in vivo* transfer of genes in the same way as cytotoxic drug introduction (Lim, 1999).

In predominantly healthy tissue a single cancerous cell may develop after a sufficient number of mutation events. Thereafter by drawing on the available nutrient resources the cancerous cell may ensure its rapid growth. Tumour cells require oxygen, glucose and amino acids for their replication, and if presented with sufficient nutrients will metastasize and replace the remaining healthy tissue. The effectiveness of cancer gene therapies is reliant on the ability of the applied treatment to kill neoplastic cells while minimally affecting normal tissue (Brannon-Peppas and Blanchette, 2004). Comprehensive investigations have revealed that the arrested development in cancerous growth-related effects may be achieved through carrier induction of growth inhibitory or pro-apoptotic genes to the targeted tumour (Mäkelä, 2008). This and other findings have further fueled investigations for future cancer therapeutics, however, irrespective of the therapuetic strategy employed, successful delivery systems which are safe and effective are still elusive and remain a primary goal of gene therapy.

Faith in gene therapy as a true therapy of the 21st century stems from its primary role, to purge disease at its cause rather than most available drug systems which only afford symptomatic relief. The ability to correct genetic abnormalities in the relatively simplistic treatment of either heritable or acquired diseases has cemented the indispensability of gene therapy to medicinal applications. Originally gene therapy was applied to the treatment of inherited disorders of adenosine deaminase deficiency, cystic fibrosis, Gaucher's disease, familial hypercholesterolemia, hemophilia and Duchenne muscular dystrophy. More recently though it has also been applied to the treatment of acquired diseases such as the acquired immunodeficiency virus, cancers, heart disease, arthritis and neurodegenerative disorders such as Parkinson's and Alzheimer's upon identification of the genetic basis in each case (Nishikawa and Huang, 2001). As with most potential therapeutics there are disadvantages which need to be overcome to allow development. Some notable disadvantages are linked to patient specificity, cell immunogenicity and cost

considerations due to greater difficulty in cell manipulation, manufacturing and quality control (Mountain, 2000; Chaudhuri, 2002).

Simply put, gene therapy involves the introduction of genetic material into cells for the purpose of altering the course of a medical condition or disease at the molecular level. Some of the nucleic acids investigated for their therapeutic potential as disease modifying agents in cancer gene therapy include, plasmid DNA (pDNA), synthetic nucleic acids or antisense oligonucleotides, aptamers, small interfering RNA (siRNA), or other double stranded RNA's like poly inosine-cytosine (pIC). Plasmid DNA vectors are predominantly employed to produce a 'gain in gene function' via the simple replacement or substitution of specific genetic function in the targeted cell through intra-nuclear delivery and can result in an amended diseased state. For successful application to human diseases gene therapy must tackle a few challenges for instance the relevent therapeutic gene needs to be identified together with the promoter and regulatory sequences that will drive transgene expression as well as efficient target site delivery of therapeutic genes (Russ and Wagner, 2007; Uddin, 2007; Rao, 2010). In addition the characteristics of the chosen vector determine the efficiency and specificity of transfection and transduction, the time taken for expression of the transgene and the immune response of the host to the vector. The treatment and successful execution of present antiviral therapy in many liver diseases is inadequate despite biomedical progress (Uddin, 2007).

As with gene delivery, many difficulties face the dose and target specific distribution of therapeutic drugs in attempts to halt disease progression through alteration of disease pathology. Success has been achieved with many patients using the conventional therapies; however, much research is still required to understand what causes the initiation of the cancer and ultimately its metastasis. Further insight is required to enhance the delivery mechanisms of therapeutics to tumours *in vivo* (Dass and Choong, 2006).

The liver has maintainted its status as an ideal target for gene transfer partly due to its level of accessability and partly because it is the site of a variety of well characterized diseases. Despite efforts employing both viral and non-viral systems for nucleic acid delivery, success in liver gene delivery systems for the treatment of genetic disorders is still hampered by the absence of an 'ideal' vector system. Some of the goals of liver directed gene delivery systems are replacement of absent genes, normal gene overexpression, interference of gene expression or the repair of a genetic defect (Richardson *et al.*, 2002).

The therapeutic prospects of nucleic acid therapy are constantly expanding due to recent discoveries such as that of RNA interference (RNAi), which is thought, at least in principle, to enable selective gene silencing. The number of obstacles facing effective gene therapies have been made more apparent by their inability to bring about specific gene transfer to their targets, such as non-germ cell tissue in sufficient

quantities to result in correction of the disease (Kay *et al.*, 1997). Ever since the initiation of gene therapy in clinical trials it has brought with it a plethora of information on the genetic basis of a multitude of diseases (Naik *et al.*, 2009).

1.2. CARRIER SYSTEMS FOR GENE THERAPY

The basic principles behind gene therapy provide a relatively straightforward manner of evaluating gene and protein function and regulation from the *in vitro* to *in vivo* stage. The drawback to the application of gene therapy is not the administered nucleic acid, but rather the vehicle employed. The vehicles employed for gene therapy are classified as either biological or non-biological. These systems of gene delivery will be examined in further detail.

1.2.1. Viral Systems As Gene Therapy Vectors

The production of a viral vectors involves the separation of coding genes and *cis*-acting sequences into distinct nucleic acid molecules so as to prevent their reconstitution into active viral particles via recombination. The combination of these *cis*-acting sequences with the therapeutic gene can then be introduced into the same cells producing replication-defective particles capable of specific transduction of novel genetic material into targeted cells. For the therapy to be successful an adequate quantity of the therapeutic gene needs to be delivered to the target tissue without substantial toxicity (Kay *et al*., 2001). However, in spite of, numerous manipulations to eradicate toxicity, many limitations including immunogenicity, endogenous viral recombination, oncogenic effects and their restricted capacity to convey transgenic materials still occur. A general advantage associated with viral systems is that the transfection machinery enables the DNA to effectively access the cytosol, while DNA conveyed by non-viral vectors requires an event that will allow escape into the cytoplasm of the cell prior to reaching the lysosome (Wattiaux *et al.*, 2000).

Viral-based vectors (retroviruses, adenoviruses, herpesviruses, lentiviruses, and hybrid/ retroadenoviruses, etc) have been successfully used in gene delivery systems. Most earlier applications of gene therapy have drawn in the use of retroviral vectors due to their capacity for stable gene intergration associated with efficient cellular entry. Of their limitations two concerns in particular have lead to their limited use in clinical trials. The first relates to the potential for the generation of an infectious wild type virus after a recombination event and the second to random target cell integration of the viral sequence

leading to a tumourigenic or cytotoxic event. Some other associated problems limiting viral vector use are the requirement of a helper virus, limited host range, cytotoxicity, immunogenicity and other known safety issues (Thierry *et al.*, 1997). Even if improbable, insertional mutagenesis has the potential to modulate the expression of vital genes including tumour suppressors and cell-cycle mediators and is therefore highly significant in terms of its application (Richardson *et al.*, 2002).

The essential strategy involved with the application of viruses, which are considered natural vehicles for gene delivery, is the removal of a few significant viral genes and their replacement with the therapeutic gene of interest. In doing so a replication-defective particle or viral vector is produced which is advantageous in efforts to overcome the undesired toxic effects of viral-based vectors. The identification of an optimal or ideal viral vector for either transient or permanent gene expression is an essential consideration for therapeutic gene delivery (Eming *et al.*, 2007). Viruses, being structurally and symetrically highly complex due to millions of years of evolution, require their intrinsic properties to be altered so as to regulate their safety, effectiveness and stability as vectors for human therapeutics (Mäkelä, 2008).

Of these vectors the retro- and adeno viruses have been the foremost viral vector systems employed thus far. Retroviruses undergo efficient random intergration into the host genome upon cellular entry thus facilitating stable expression and mainentance of the transgene. Even though these vectors may produce lasting *in vivo* transgene expression, some systems have also been identified to display transient expression of the gene of interest. A major advantage of using these vectors is their capacity for large inserts of foreign DNA effective transient transduction of quiescent as well as proliferating cells. In addition they are capable of producing high titres. Adenoviral vectors pose a lower risk for mutagenesis since there is no host integration of the viral DNA. The recombinant adenoviral vectors have also revealed the capability for the highest levels of gene delivery *in vivo*. Alternatively, lentiviral vectors are reliant on active nuclear transport to enable transduction of non-dividing cells. Some viral systems rely on co-infection with an unrelated helper virus to produce infection. One such vector is the adeno-associated virus which belong to the *Parvoviridae* family of the genus *Dependovirus*. Due to their non-pathogenicity, stability, low immunogenicity, and potential for site specific integration they have become leading vectors in gene therapy. In addition to these, other vectors derived from the herpesviruses, alphaviruses and poxviruses have been and still are utilized in a variety of gene therapeutic applications (Mäkelä, 2008).

Of the viral vectors used for clincal trials the recombinant retroviruses based on the Mouse Moloney Leukemia virus have been utilized most frequently. These vectors contain viral regulating sequences with

all viral genes removed which results in only dividing cells being transduced. Since the cells *in vivo* are predominantly in the quiescent state these vectors are less effective unless the cells are stimulated in the target organ (Kay *et al.*, 1997). Further clinical evaluations have shown non-viral vectors to be the safer alternative for future gene transfer processes, unfortunately no single vector is ideal for all types of gene transfer experiments. Although cationic lipids have been empirically established as producing higher levels of transfection, further research is required for identification of both potential barriers and solutions for the enhancement of transfection (Uddin, 2007).

1.2.2. Non-Viral Sysytems As Gene Therapy Vectors

It is possible to transfer genes directly into mammalian cells without the use of viral systems. In this regard the gene of interest may be incorporated into bacterial plasmid DNA together with a mammalian promoter, enhancer, and other sequences that ensure gene expression. This plasmid DNA may then be incorporated into lipid vesicles such as liposomes, complexed with proteins for tissue specific targeting, or complexed with polymers including poly(L-lysine) and polyethyleneimine. Besides these chemical methods of introducing the DNA into the mammalian cells for expression of the desired gene, many physical techniques have been applied over the years (Rozema, 2008).

Although non-viral systems produce lower transduction efficiency and a reduced duration of transgene expression compared with their viral counterparts, they show the following advantages:

- Safety,
- Simplicity of preparation, and
- High capacity for gene encapsulation.

The immense promise of gene theapy will not achieve its full potential until the development of a safe and efficient gene delivery vector has been resolved (Chen *et al*., 2007). Despite the numerous advantages of non-viral vectors promoting their extensive investigation over the use of the naturally evolved viral vectors, their most significant disadvantage is their diminished capacity for genomic intergration (Richardson *et al.*, 2002). These systems are typically transient, resulting in the relatively short-lived expression of transgenes which is ineffective for the treatment of diseases where prolonged expression is advantageous (Jo and Tabata, 2008).

Over the past 40 years non-viral methodologies have been establised as popular research tools, not only for the treatment of disease, but also for the elucidation of gene structure, regulation and function. Pivotal

to the success of DNA delivery in this regard is their transfection capabilities. Despite efforts to improve transgene expression through the implementation of strong promoters and enhancers, significant enhancement of transfer is difficult to realize due to the limited number of DNA molecules that eventually reach the nucleus (Luo and Saltzman, 2000).

Non-viral or synthetic vector research has increased in response to severe set backs associated with the highly efficient engineered viruses (Ewert, *et al*., 2006; Eming *et al.*, 2007). The techniques used for non-viral vector mediated gene delivery have been classified into two groups: (i.) Physical methods of naked DNA delivery and (ii.) Chemical carrier mediated delivery. Although both these methods enable effective and target specific delivery of genes with little or no toxicity, the chemical techniques are considered the more desirable (Suda *et al.*, 2009).

1.2.3. Naked DNA Delivery and the Physical Methods Employed

To achieve nucleic acid delivery to a specific site, physical targeting involving a series of physical techniques that produce localized action may be employed (Russ and Wagner, 2007). The use of physical stimulus can enhance plasmid DNA delivery into cells by transporting the DNA into close proximity to the cell membrane or through the temporary permeabilization of the membrane (Jo and Tabata, 2008). One of the earliest, although relatively ineffective methods of producing significant levels of transfection is the use of calcium phosphate co-precipitation (Lim H., 1999).

Nucleic acids are in general large molecules displaying a multitude of negative charges resulting in ineffective and non-specific cellular uptake together with meager bioavailabiilty due to their highly hydrophilic nature, size and interactions with serum proteins once in the cytoplasm (Gao *et al*., 2007). These limitations have hindered their use as therapeutic agents. Modifications have been applied to nucleic acids in an effort to overcome these limitations by imparting on the nucleic acid various novel properties including improved nuclease resistance and better penetration through plasma membranes (Yan and Tram, 2007).

The use of naked DNA in the absence of a carrier vector is the simplest and safest means of systemic DNA administration. Plasmids were initially identified for the transfer of antibiotic resistance genes between markers and can be identified as circular DNA molecules. The replacement of these regions of antibiotic resistance with recombinant genes enables their use in the preparation of a number of proteins for use in pharmacological situations (Zuckerbraun and Tzeng, 2002). Some sites for direct plasmid DNA injection have included tumours, skin, skeletal muscle, thyroid, heart muscle, liver and urological organs.

Although a convenient method of administration the area and level of expression post injection are limited due to rapid serum nuclease degradation and clearance via the mononuclear phagocyte system.

This system of direct plasmid injection is one of the most practical for the introduction of DNA into targeted tissues. Furthermore support of this approach has come in the form of a novel and elegant technique known as 'puncture-mediated' or 'micro-seeding' where multiple perforations are produced through the use of oscillating solid micro-needles. This technique was determined to be more efficacious than that using the single dermal injection (Ciernik *et al*., 1996 and Eriksson *et al*., 1998; cited in Eming *et al.*, 2007).

Many of the physical manipulations shown in Figure 1.1 and listed below have been applied to increase the efficacy of gene transfer although these technologies and are known to induce transient injuries or defects on cell membranes to allow diffusion of DNA into the cells (Gao *et al*., 2007).

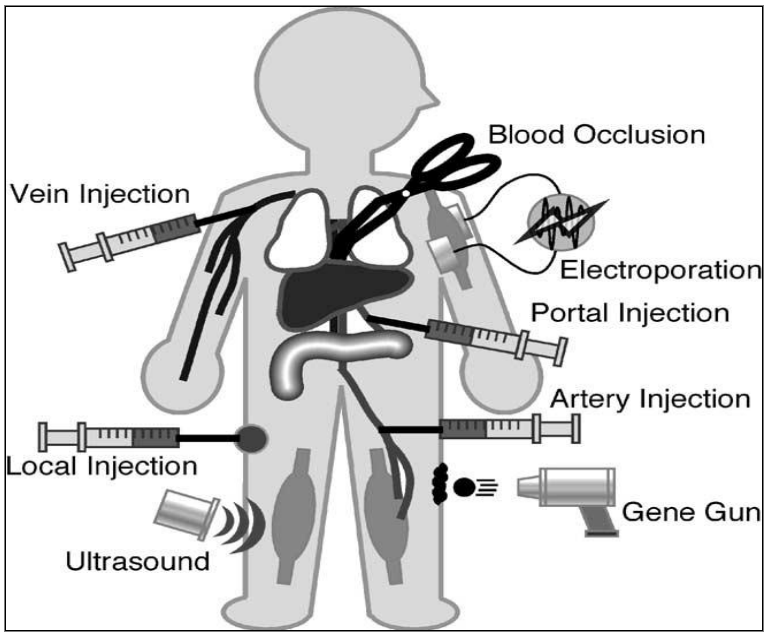


Figure 1.1: An outline of some non-viral gene delivery technologies and the different routes of naked DNA injection (Niidome and Huang, 2002).

1.2.3.1. Electroporation:

Since the first application of electroporative mechanisms in cultured cells in 1982 they have achieved extended application to *in vivo* plasmid DNA delivery. This mechanical technique initiates the reversible permeabilization of cell membranes through the use of a controlled electric field thus allowing DNA to move along a gradient and gain cellular entry. This process has proved advantageous through evasion of

the endocytic pathway. It is also considered a safe, simple and inexpensive method which has been applied efficiently for DNA translocation *in vitro*. Gene expression attained by injection of naked DNA in conjunction with electroporation has shown increases up to 1000 fold compared with the injection alone (Nishikawa and Huang, 2001; Russ and Wagner, 2007). Its use allows for both improved post injection gene uptake as well as enduring expression in numerous tissues with muscle tissue being one of the best candidates for its application.

The use of short intense electric pulses has improved the efficacy of non-permeable drug uptake (Nishikawa and Huang, 2001). Hellar and colleagues (2000) demonstrated the use electroporation in the mediation of DNA delivery to hepatocellular carcinomas which although rare in Western countries are common in Africa and Asia and are often associated with chronic viral hepatitis (Russ and Wagner, 2007).

Several types of electrodes have been developed for *in vivo* administration. Needle electrodes for deep tissues, surface electrodes, and electroporation catheters which are applied for hollow organs such as blood vessels (Kawakami *et al*., 2008). The use of the syringe electrode has recently been reported to produce comparable levels of transfection to those achieved by conventional electrodes while using far lower electric field strengths which therefore minimizes tissue damage (Russ and Wagner, 2007).

Although considered to be the most effective of gene transfer methodologies it is unfortunately constrained by high levels of cellular mortality as a result of the cellular exposure to high voltage as well as difficulties encountered in optimization (Luo and Saltzman, 2000). The amount and distribution of DNA within the treated tissue prior to electroporation has an important impact on *in vivo* transfection efficiency. One of the first drawbacks found with the *in vivo* application of electroporation is the restricted range between electrodes of approximately 1 cm which makes transfection of a large area rather difficult. The fact that the process requires surgical intervention to introduce the electrodes deep into the internal organs is a further problem. Finally irreversible damage to tissue could result from thermal heating brought about by the application of high voltage or the influx of calcium ions in response to cell membrane disruption (Gao *et al*., 2007).

1.2.3.2. Gene gun:

This process is also termed biolistic particle delivery and can achieve the simultaneous introduction of DNA into a large number of cells (Luo and Saltzman, 2000). This method proves highly efficient in its ability to bring about direct penetration of tissues or cells. The process involves gold particles coated with the DNA destined for delivery being shot and consequently directly passing through the cell membrane

entering the cytoplasm or even nucleus. The particles utilized for this biological and ballistic delivery need to be non-toxic, non-reactive and smaller than the diameter of the target cell.

This form of administration like electroporation provides the advantage of evading the endosomal compartment; however, an associated disadvantage is the shallow level of tissue penetration that is achieved (Niidome and Huang, 2002). By altering the pressure of the high-pressure helium propellant, the loaded particles can be directed to the individual epidermal layers. This proves advantageous for achieving optimal gene expression, as it results in an accumulation of the gold particles in the basal epidermal cell layer (Eming *et al.*, 2007).

After surgical exposure, the skin, liver and muscle have been successfully transfected. *In vivo* gene gun application predictably has revealed low levels of expression that persist for only a short period (Nishikawa and Huang, 2001). Nevertheless other reports have identified the liver and brain as target sites for *in vivo* application (Kawakami *et al.*, 2008). Particle bombardment has been employed widely for DNA vaccination in which the local administration of DNA is sufficient to achieve an immune response (Luo and Saltzman, 2000).

1.2.3.3. Ultrasound:

This technique can be used to manipulate the cell membrane, increasing permeability to plasmid DNA and other macromolecules. Ultrasound induced cell permeability as introduced in the mid 1990s is in clinical application for both therapeutic and diagnostic objectives. To date this physical manipulation has been applied to vascular cells, muscle and foetal mice. Irradiation of the injected tissue with ultrasonic waves has been shown to improve observed gene expression. Its flexibility and safety makes this a valuable method of gene delivery for clinical use (Niidome and Huang, 2002). This technique has been successfully applied to both the *in vitro* and *in vivo* situations.

The application of naked DNA in combination with ultrasound systemically has proved inefficient. This could potentially be ascribed to serum nuclease digestion of the DNA as well as low concentrations of the DNA within the environment of sonoporated cells. Furthermore the use of combinations of ultrasound together with transfection agents with the capacity for high levels of expression, have been revealed as a pathway full of potential for the future of ultrasound enhanced liposomal gene targeting (Huang., 2008). Ultrasound has also been used in conjunction with ultrasound contrast agents or microbubbles that lower the cavitation threshold through ultrasound energy and together can produce further increases in the level of gene expression achieved (Niidome and Huang, 2002).

Acoustic cavitations in this process are known to induce non-thermal effects, where cavitation is a consequence of the gaseous inclusion and ultrasonic wave interaction producing cavitation bubbles. This results in cell membrane damage and increased reversible permeability to macromolecules and cell survival. This method of gene delivery is referred to as sonoporation and has so far achieved good expression in the muscle, carotid artery and solid neoplasms (Kawakami *et al.*, 2008).

The efficiency of this technique is determined by the frequency, its output strength, the duration of the treatment as well as the quantity of plasmid DNA to be introduced, and can be improved by enhanced membrane fluidity (Gao *et al*., 2007).

1.2.3.4. Magnetofection:

This process has indicated potential for both *in vitro* and *in vivo* gene targeting. For nucleic acid delivery, this technique involves the reversible attachment of nucleic acids to super-paramagnetic nanoparticles. These particles are then focused via a high-energy magnetic field to the target site. This process is known to encourage swift transfection with high levels of gene expression. The magnetic field in this technique does not affect the uptake of lipoplexes or polyplexes which are physically associated with paramagnetic particles. The magnetic forces are, however, suggested to produce an accelerated accumulation of complexes at the surface of the cell without inducing cell traction. The external application of a strong magnetic field to an experimental animal model in the region of the liver has achieved improved liver targeting in recent studies (Russ and Wagner, 2007).

1.2.3.5. Hydrodynamic injection:

This injection of plasmid DNA in the absence of a carrier system results in the transient overflow of a DNA solution at the inferior vena cava which exceeds the cardiac output. The subsequent flow of solution in retrograde to the liver creates a significant increase in intra-hepatic pressure, expansion of the liver as well as the reversible disruption of the fenestrae. This delivery strategy has broad applicability to any water-soluble compound, small colloidal particles, or viral particles due to its non-specific nature. This method circumvents the need for delivery via an endocytotic pathway; however, a key challenge remains its translation to the human model (Gao *et al*., 2007).

Practically it involves the rapid tail vein injection of naked DNA- saline solution at high volume. Tail vein injection of naked DNA into investigated mouse models has shown little to no gene expression produced in the major organs by virtue of the prompt nuclease digestion and RES (reticuloendothelial system) clearance together with low extravasation *in vivo* (Nishikawa *et al.*, 2001). Due to flexibility and large volume capacity, the liver has been proposed as the primary site for accumulation of injected DNA.

An additional mechanism proposed for efficient liver expression is the rupture of the endothelial barrier due to the force of pressure (Niidome and Huang, 2002).

1.2.3.6. Photochemical internalization:

This technology improves gene delivery to specific light-exposed target sites. Upon endocytosis of gene carrier systems acidic amphiphilic photo-sensitizers position themselves in the endosome forming associations with the gene carrier. At this point they produce illumination leading to chemical damage and tearing of the endosomal membrane allowing the release of the carrier complexes into the cytoplasm, this together with other investigations *in vitro* have revealed the potential of this method for taking gene therapy to the next level (Russ and Wagner, 2007).

1.2.3.7. Occlusion:

Restriction of blood flow at the vena cava, hepatic artery or portal vein can be used to induce high levels of liver gene expression upon receptor or non-receptor mediated internalization of DNA into hepatic sites. Gene transfer may further be affected by the time that the plasmid DNA is retained. Weak binding of DNA at the hepatic cell surface allows blood flow to easily wash away injected DNA. The interruption of blood flow permits the stable binding and cellular internalization of DNA (Niidome and Huang, 2002). Trangene expression has been shown to date to be decisively increased in mouse liver by the transient occlusion of blood flow swiftly after the peripheral intravenous injection of plasmid DNA-saline solution (Nishikawa and Huang, 2001).

1.2.3.8. Hyperthermia:

A controlled elevation of temperature to the target area has been applied to treatment of tumours and is already in clinical use (Russ and Wagner, 2007). The application has been employed to eliminate tumour growth and inhibit the progression of cancer disease. Hyperthermia generally is applied as one of three different strategies: local hyperthermia, regional hyperthermia or whole body hyperthermia. Local hyperthermia limits the application of heat to the tumour site, while regional hyperthermia may be applied to an entire organ or limb and the final strategy involves the application of heat to the entire body. The system makes use of microwaves, high frequency radiowaves, special radiant heat systems and even the application of high energy magnets over the area of tumour. There are unfortunately major risks to the host tissue therefore the restricted application of heat to the volume of the tumour is a potential solution to maximize the toxic effect to the areas of malignancy and not the normal cells (Campbell, 2007). Some associated side effects observed have been direct cytotoxicity, immunomodulatory effects in addition to radiation and chemotherapy sensitizing effects (Russ and Wagner, 2007).

1.2.3.9. Laser irradiation:

This technique involves a laser beam focused onto target cells through the use of a lens. This process results in the formation of relatively large pores in the cell membrane which re-seal and repair themselves fairly swiftly. The formation of these pores allows for the entry of DNA while the cell membranes capability to repair and re-seal allows for its retention. The mechanism employed by this process to improve gene expression *in vivo* is still unclear. This, however, is not often applied due to its expensive nature, the physical size of the laser and difficulties encountered in attempts to control an appropriate environmental temperature (Kawakami *et al.*, 2008).

1.2.4. Gene Delivery Via Chemical Carriers

Chemical derived gene transfer vectors can be manipulated for specific function and may be grouped as follows:

i. DNA complex forming carriers that condense and protect DNA against digestion;
ii. Carriers designed for targeted cell specific DNA delivery;
iii. Cytosol or nucleus delivery enhancing carriers;
iv. Carriers designed for cytosolic dissociation; and
v. Carriers that facilitate continuous and controlled expression in tissues

The earliest method applied for the chemical introduction of DNA involved the use of a high salt concentration with polycationic proteins for improved cellular entry of nucleic acids. From that point many other chemicals have been introduced such as DEAE-dextran (Warden and Thorne, 1968) and calcium phosphate which interact and form complexes with DNA that become deposited on the cell surface for internalization (Luo and Saltzman, 2000). Of the many novel carriers designed lipids and polymers have garnered the most attention in gene delivery.

1.2.4.1. Polymer for gene therapy:

Cationic polymer-based gene delivery systems are effective DNA condensing agents and may thus provide more protection to the DNA cargo when compared to the lipid based systems discussed later (Li and Huang, 2000). There are two essential types of cationic polymers employed in nucleic acid delivery, natural polymers such as chitosan and atelocollagen and synthetic polymers such as poly(L-lysine)

(PLL), poly(ethyleneimine) (PEI) and dendrimers. In general the natural polymers are non-toxic and display adequate mucoadhesive, biocompatibility and biodegradability in contrast to synthetic polymers which provide flexibility and offer the ability for tailoring to fit the size and topology of plasmid DNA (Kawakami *et al.*, 2008). These carriers upon complexation with DNA form a polyplex (Felgner *et al.,* 1997) capable of cell directed gene transfer. Of these the most extensively evaluated are poly(L-lysine) and poly(ethyleneimine) (El-Aneed, 2004).

PEI is highly versatile, being produced as either a branched or linear polymer of varying chain lengths. Its versatility together with its capacity for increased transgene expression in a diverse range of cell lines has placed it above most other non-viral gene delivery systems. Once involved in the endocytic pathway PEI-DNA complexes are presented with a decrease in the pH of their environment from neutral to pH 5.0 whereupon they are destabilized. Polyethylenimine is considered to act as an internal buffer within endosomes due to its 'proton sponge' nature (Chen *et al*., 2007). This buffering capacity at any encountered pH has been attributed to this branched polymer having every third amino nitrogen protonated (El-Aneed, 2004). Despite this PEI polymers exhibit high levels of toxicity due to their polycationic nature and their non-biodegradability *in vivo* (Morille *et al*., 2008).

Poly(L-lysine) (PLL) due to its peptide structure and associated biodegradability has proved advantageous *in vivo*. This cationic polymer was the first employed for gene transfer and until only the past decade was one of the most utilized. The low molecular weight poly-L-lysines, however, possess a reduced ability to form stable complexes resulting in the need for implementation of high molecular PLL for systemic gene delivery. Although these cationic polymers enter the cell via the same mechanism as PEI polyplexes their transfection efficiency is not comaparable (Morille *et al*., 2008). Co-application of lysosomotropic agents such as chloroquine can reduce lysosomal degradation. Due to their apparent low levels of transfection these polymers are rarely applied alone or in the absence of some form of chemical alteration. One commonly employed modification is the introduction of PEG to increase the circulation half-life and consequently the efficacy of transfection (El-Aneed, 2004). For *in vivo* gene delivery polyethylene glycol-poly-L-lysine (PEG-PLL) micelles that form self-assembling particles with DNA have been developed (Niidome and Huang, 2002). DNA delivery using the polycation poly(L-lysine) can be linked to asialoorosomucoid for hepatocyte targeting or transferrin for direction to dividing cells.

Some poly-L-lysine derivatives have been identified in recent reports to be water soluble and biodegradable and capable of releasing condensed DNA on hydrolysis. A few of these reported derivatives have shown higher-level efficiency of gene transfection *in vitro* accompanied by lower cytotoxicity than that found with poly-L-lysine. Temperature fluctuations have been reported to initiate

swelling or de-swelling of hydrated polymers. Consequently thermosensitive polymers have been produced to control the release of entrapped DNA (Niidome and Huang, 2002).

Dendrimers are highly branched, spherical polymers of which the most commonly used are polyamines, polyamides. The most abundant dendimer found is the polyamidoamine (PAMAM) due its capacity for high level transfection. Dendrimers possess primary amines on their surface which are involved in DNA binding and compaction promoting cellular uptake while the tertiary amines present inside act as proton sponges enabling endosomolysis. Their properties of size, modifiable surface functionality, multivalency, water solubility and their internal cavity that is available makes them attractive for drug delivery (Cho *et al.*, 2008).

In an effort to overcome some of the drawbacks faced in applying dendrimers to gene therapy, Holladay and others (2009) proposed a system of partially degraded dendrimers capable of condesing DNA forming complexes which may then be entrapped within a three-dimensional collagen matrix. The supposition put forward was that the collagen matrix can serve as a reservoir for the complexes and thus produce a slower rate of transfection together with prolonged transgene expression. Those cells transfected most significantly were the highly migratory and rapidly dividing cells while those cells that show slower rates of proliferation produced lower levels of transfection activity (Holladay *et al.*, 2009).

1.2.4.2. Peptide for gene therapy:

The amphiphilic peptides employed here are capable of undergoing conformational changes when presented with an acidic environment to thus enable endosomal or lysosomal escape. The presence of positively charged amino acids, such as histidine, lysine, arginine allow for effective condensation of DNA. The incorporation of cysteine residues into the backbone of the peptide can increase the release of DNA into the cytoplasm due to the formation of reducible disulphide bonds within the complex (El-Aneed, 2004). Those carriers containing incorporated redox-sensitive thiols condense DNA in an exceedingly stable complex and are capable of strong *in vitro* transfection activity due to the spontaneous oxidation of the thiol group. Amplification of expression without affecting DNA uptake has been achieved in this system via peptide cross-linking inferring the reduction of the disulphide bond as a key trigger for intracellular release. Stealth activity peptides such as polyethylene glycol and glycopeptides containing sulfhydryl cross-linking for *in vivo* gene targeting have previously been synthesized (Niidome and Huang, 2002).

There are four basic requirements for peptide-based local gene delivery systems namely, DNA binding, condensation, nuclear transfer of DNA and release of active DNA for the nucleus. An appropriate balance

needs to be maintained between the size and stability of peptide DNA complexes as their large size and potent charge can result in cytotoxicity, antigenicity and insolubility (Trentin *et al.*, 2005).

Protamine is an arginine rich peptide that condenses DNA prior to cationic lipid complexation. Liposome-protamine-DNA (LPD) complexes can facilitate endocytosis and enhance the circulating half-life *in vivo* as a result of their small size. Asialofetuin is a liver targeting ligand that used to coat Liposome-protamine-DNA and other cationic DNA complexes for the targeting of liver parenchymal cells (HepG2) allowing increased uptake of entrapped DNA (Uddin, 2007).

1.2.4.3. Lipid for gene therapy:

Since the unveiling of liposomes in 1987 as potential carriers for gene delivery, lipid-mediated gene transfer has maintained its position as the foremost technique for cellular gene delivery. A wealth of cationic lipids including cationic derivatives of cholesterol and diacylglycerol, and quaternary ammonium detergents, additionally polyamine lipid derivatives have been reported (Niidome and Huang, 2002).

A variety of lipofection agents or cytofectins have been developed and evaluated in cell culture, animal models and clinical trials. The term cytofectin is used in reference to positively charged lipid molecules that have the ability to promote the functional entry of macromolecules, polynucleotides as well as small molecules into dividing cells while being able to evade degradation by the lysosome (Felgner *et al.*, 1994). These systems afford the user the advantages of simplicity and efficiency of formulation, commercial availability and the capacity for specific tailoring. The presence of the cationic head group is essential for binding the negatively charged phosphate groups on the DNA backbone (Gao *et al*., 2007). Differentiation of these lipids as outlined below and in Figure 1.2 is based on the number of postive charges in their head group as well as the structure of their hydrophobic moiety:

- Monovalent aliphatic groups that posses a single amine function present in the head group, e.g. DOTMA, DMRIE and DOTAP,
- Multivalent aliphatic lipids with polar headgroups having various amine functions including the spermine group, e.g. DOGS – a class of cationic amphiphile that possesses a cationic spermine group connected to a double chain lipophilic group through an amidoglycyl spacer arm.
- Cationic cholesterol derivatives or cytofectins, e.g. DC-Chol

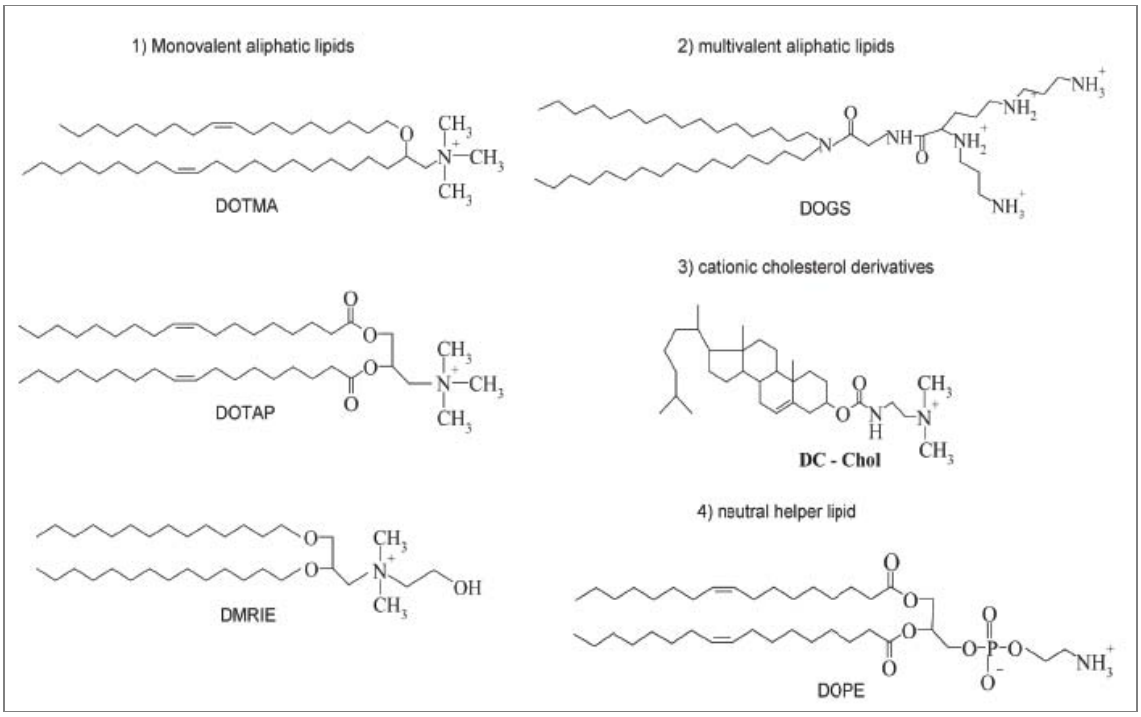


Figure 1.2: Structures of some cationic lipids currently applied in gene therapy and the helper lipid DOPE (Adapted from Morille *et al.*, 2008).

DOTMA and DOGS and their various derivatives have been widely applied in the delivery of DNA to eukaryotic cells. Through their head group containing one to a possible four positive charges these lipids introduce hydrophobic aliphatic groups to the DNA (Cotton and Wagner, 1993). DOPE assumed to posses a weak surface hydration when combined with the lipopolyamine DOGS, may produce a stable bilayer structure due to the inverted cone shape adopted by the molecules (Thierry *et al.*, 1997).

Cationic lipids are chiefly produced from two distinct hydrophobic segments, DOTMA for example, consists of two long hydrocarbon chains that make up its hydrophobic anchor, while DC-Chol and Chol-T employ a cholesterol backbone. Modification at the molecular level is important to the transfection and DNA complexing capability of the cationic lipid. Tang and Hughes (1999) showed in their work that a single tailed cationic lipid could also be used in a carrier system for DNA transfer. An additional structural parameter for consideration is the head group-anchor linker, as it plays a vital part in effecting gene transfer. In this regard the ether linkage as found in DOTMA was shown to be more effective *in vivo* than the ester link used in DOTAP. The ether linkage was also shown to be favourable when linking a cationic ammonium head group with a cholesteryl backbone. The same components appended by either an ester or urethane linker proved to be less effective (Dileep *et al*., 2001; Bajaj *et al.*, 2008). However, the introduction of an ester bond within the alkyl chain has been found to minimize the toxicity associated with cationic lipids.

Helper lipids such as dioleoylphosphatidylethanolamine (DOPE) or cholesterol are known to introduce additional stability and/or fusogenicity to the cationic carrier (Kawakami *et al.*, 2008). Phosphatidylethanolamine has been applied to many cytofectin liposome formulations for optimal activity. The phosphatidylethanolamine is believed to elicit its effect due to an ability to undergo transition from a bilayer or lamellar phase to the inverted hexagonal phase. DOPE in particular has displayed this effect through promotion of membrane fusion and destabilization in cationic lipid-mediated nucleic acid transfer. A number of other lipids such as DOTAP, DOTMA and polymers have also shown the ability to promote DNA complex-cell or endosomal membrane fusion (Resina *et al.*, 2009). Zwitterionic lipid DOPE alone is reported to be ineffective for transfection, possibly due to its difficulty to form a union with the nucleic acid molecule. At neutral pH DOPE cannot produce stable uniform formulations, yet when combined with cytofectins stable liposomes are formed. Vesicles that possess DOPE are known to experience a drop in net positive charge as the pH is increased from 7.0 – 9.0 (Felgner *et al.*, 1994).

Moreover DOPE is known to assist in membrane destabilization of the either cell or endosome. In this regard the amine group of DOPE may prefer the cationic lipid interaction with the negative phosphate groups on the DNA. This association can thus interfere with the existing interactions between the DNA and cationic amphiphile. Therefore formulations consisting of phosphatidylethanolamine are suggested to be more susceptible to cellular factors promoting disassembly (Zuhorn *et al.*, 2002).

Liposomes have been described as vesicles consisting of an entirely enclosed aqueous inner core surrounded by the natural membrane resembling phospholipid bilayer membrane. In general these vesicles have been applied to formulations of poorly soluble therapeutic agents for either parenteral or oral administration (Schnyder and Huwyler, 2005). Being the most studied nanoparticles, liposomes are also able to improve the pharmacokinetics and pharmacodynamics of associated drugs (Wang *et al.*, 2008). The cationic lipids DC-Chol and DOTAP are generally employed in liposome formulations because of their ability to form stable lipoplexes on electrostatic interaction with DNA or oligonucleotide (ODN) molecules (Naik *et al.*, 2009). To become more widely useful as transfection agents the efficiency of the cationic liposome – DNA complexes needs to be improved, which requires increased understanding of the transfection mechanism as well as the chemical and physical parameters that influence it (Ewert *et al*., 2006).

Neutral liposome formulations provide an alternative to the use of cationic liposome – DNA complexes where they are capable of entrapping the DNA within the vesicles. This system may also be coupled to targeting vectors or coated with PEG and can as a result be characterized by high levels of DNA stability

under the physiological conditions in addition to an extended half life (Schnyder and Huwyler, 2005). Liposomal half-life can be improved through coupling with gangliosides or polyethylene glycol (PEG) derivatized lipids. The conventional liposomes coated with PEG, an inert and biocompatible polymer, form sterically stablized liposomes that display a limited affinity for cells *in vitro* and the non-diseased tissues *in vivo* making it a safe alternative for drug delivery systems (Schnyder and Huwyler, 2005).

A further strategy is the utilization of pH-sensitive liposomes which assist in the release of liposomal drugs or nucleic acids within the cytoplasm as these liposomes are associated with the spontaneous discharge of their contents upon encountering a decrease in pH. These liposomes are in general formulated in conjunction with DOPE or ionizable anionic lipids such as CHEMS (cholesterolhemisuccinate) and can be applied to the conventional as well as long-circulating, sterically stabilized liposomes (Schnyder and Huwyler, 2005).

1.2.4.3.1. Additional applications of liposomes

A number of drugs, chemotherapeutic agents in particular, have a small therapeutic window, limited clinical application and are compromised by a dose limiting toxic effect. Consequently the therapeutic effectiveness of these drugs requires their formulation in a beneficial manner. Vesicular systems as pharmaceutical carriers are typically extremely well ordered assemblies consisting of one or more concentric lipid bilayers resembling biological membranes which are ubiquitous structures that encapsulate and organize cells and organelles (Sharma and Sharma, 1997; Biju *et al.*, 2006).

Drugs entrapped in liposomes at either the phospholipid bilayer or in the aqueous internal core, are expected to be transported without undesired degradation or pronounced side effects to the recipient. The positional entrapment of the drug is believed to be dependent on the lipophilicity of the therapeutic drug or agent (Sharma and Sharma, 1997; Goyal *et al.*, 2005).

The successful development liposome-drug delivery systems (Kozubek *et al.*, 2000) for the treatment of a significant number of diseases in both animal and human models underscores the future potential of these systems for clincal and veternary applications. Both unilamellar and multilamellar liposomes ranging in size from 50 – 700 nm have been approved as vehicles for the safe and effective delivery of drugs such as doxorubicin, an anthracycline antibiotic drug used in cancer chemotherapy. Moreover they have been applied as molecular imaging agents for both ultrasound and magnetic resonance imaging (MRI) (Wickline *et al.*, 2006).

Liposomes have been applied to topical ocular drug delivery as well as for the oral administration of insulin (Biju *et al.*, 2006). Besides being applied as drug carriers for topical treatments liposomes have

also been used as active ingredients in skin care products. As such they may either improve penetration of the active component or function through the diffusion of their lipidic ingredients into the skin (Hatziantoniou *et al.*, 2007).

Liposomes have matured into sophisticated models for use as not only cosmeticeuticals, but more importantly pharmaceutical products. The primary aim of liposomal application is to obtain selective localization brought about by either passive or active targeting of the drug molecule at the site of disease be it tumour or inflammation. For the liposomes to be completely effective they must conform to four general requirements:

i. Stable and sufficient drug or agent loading
ii. Prolonged cytoplasmic circulation.
iii. Extravasation at the point of infection or disease.
iv. Target cell translocation of active drugs.

Passive targeting is known to exploit the innate ability of specific cells such as Kupffer cells and macrophages to phagocytose foreign particles such as liposomes while active liposomal drug targeting may be achieved through the attachment of certain antibodies to the vesicles producing immunoliposomes. Active targeting has the ability to enhance target site affinity of the carrier system in contrast to the effect of passive targeting which is known to minimize the non-specific associations with non-targeted sites through the reticuloendothelial system (RES) (Hattori and Maintani, 2005). For the active targeting of a tumour site, ligands corresponding to over-expressed growth factors and surface receptors can be coupled to the nucleic acid delivery systems. The serum glycoprotein transferrin is one ligand applied for targeted delivery to the transferrin receptor. Expression within the tumour is found to occur primarily at sites in close proximity to structures resembling primitive blood vessels (Russ and Wagner, 2007).

Since liposomes that display extravascular leakage will result in poor therapeutic effect at the specific site of disease, the liposomal system must be designed such that stability is retained through storage and *in vivo* plasma circulation, but is in part lost upon interaction with the targeted site of action (Barenholz, 2001). Neoplasms have unique physiological attributes that may be exploited for enhanced nucleic acid or drug delivery. In fact recent works have revealed the propensity of cationic liposomes to accumulate or concentrate within the tumour vessels. Consequently cationic liposome-mediated gene delivery can be considered a valuable pathway for the delivery of antiangiogenic and antivascular drugs to tumour endothelial cells. It has been suggested that cationic liposomes achieve significantly higher levels of localization at tumour sites compared to normal vascular networks. A potential explanation is that the

erratic and lethargic blood flow within the tumour may promote increased association of cationic liposomes with the angiogenic anionic sites (Campbell *et al.*, 2002).

Drug-targeting strategies have been categorized as either 'prodrug' or 'carrier mediated' systems. Prodrugs once at their site of action become converted from being drug-related precursors to their active form. Carrier systems on the other hand entails either covalent or non-covalent association of the drug molecules with the targeting moiety thus enabling cellular targeted drug transfer. With the primary application of drug targeting being for therapeutic and diagnostic uses they aim to induce the distribution of drugs throughout the body (Russ and Wagner, 2007).

In their role as carrier vesicles liposomes are able to convey many pharmaceutically active molecules such as chelating agents, anti-neoplastic and antimicrobial drugs, vaccines, steroids and genetic material (Sharma and Sharma, 1997). Many attempts have been undertaken to introduce targetable characteristics to the liposome including their non-covalent association with cell specific antibodies, coating with heat aggregated immunoglobulins, covalent coupling of poly- or monoclonal antibodies, glycoprotein bearing liposomes as well as those containing glycolipids (Goyal *et al.*, 2005). In targeted lung transfection, cationic liposome carriers have primarily been used for the treatment of cystic fibrosis both *in vitro* and *in vivo*. Despite their moderate levels of *in vivo* gene expression off-setting their advantages, the continued production of a wide variety of cytofectins for lipoplex-mediated gene delivery, indicates that the suitability of cytofectins varies with their application (Wheeler *et al.*, 1996; Ferrari *et al.*, 2001).

Since the first application of liposomes as immunological adjuvants to elevate the immune response to the diphtheria toxoid, they have been employed as non-toxic adjuvants with bacterial, viral, protozoan, neoplastic and other antigens which can be introduced into the aqueous volume or bilayer depending on their lipophilicity. In the early days of liposomal therapeutics, the liposome vesicles containing inactivated hepatitis A virions were successfully applied as a vaccine for human use. Their limitations include stability, reproducibility of batch formulations, control of particle size, substantial batch production sizes and stunted circulation half life of vesicles (Sharma and Sharma, 1997).

Besides their potential to be applied as drug, antigen and gene delivery carriers for transfer to specific cells, liposomes have been developed to contain the ultrasound imaging gas perfluoropropane. These liposome-based technologies are referred to as novel liposome bubbles. The use of these bubble liposomes in conjunction with ultrasound could produce minimally invasive and specific tumour targeted gene delivery (Suzuki *et al.*, 2008). Moreover they have also found application capability in antimicrobial therapy where the simple *in vitro* administration of liposomes coupled with a specific antibiotic could be active against the bacterial infection. This application is in response to the need presented by antibiotics

which can only act against intracellular infections if they are able to penetrate phagocytic cells and since liposomes are reknowned for their localization in organs where many microorganisms exist such as the liver and spleen. They are highly effective at directing the antibiotics to these organs. In addition to their imperative role in gene therapy, liposome formulations have achieved success in the potentiation of DNA mediated vaccination (Goyal *et al.*, 2005).

Recently developed liposome-protamine-DNA complexes have shown superiority over conventional lipoplexes providing a possibility for the production of future conjugates of modified cationic lipids capable of DNA compaction and cell penetration (Uddin, 2007).

1.2.4.4. Crown ethers and their cationic amphiphile associations

One of the barriers to cellular transport of drugs, chemicals or nucleic acids is the membrane of the cell. This ubiquitous membrane protects the cell contents from invasion by inappropriate chemicals or foreign molecules and furthermore limits potential leakage of cellular components. Moreover the plasma membrane must also maintain the equilibration of closely related ions and their concentrations. In this regard the sodium cation must be maintained at a concentration of 150 mM outside and 10 mM inside the cell, while the potassium ion must be kept at 5 mM outside and 150 mM inside the cell. In most instances these cations, in addition to some organic species such as sugars are transported via the assistance of complex channel-forming proteins. In contrast to channel-forming compounds that produce pores in the cell membrane through which cations and water align, cation carriers complex with the cation and ferry it across the membrane (Gokel and Mukhopadhyay, 2001).

Since their conception by Charles Pederson in the late 1960's, crown ethers have received recogniton for their role as ionophore antimicrobial agents. This is significant as interruption of the Na^+/K^+ balance is potentially fatal to the cell, furthermore the use of an ionophore to transport ions that are hydrophilic in nature through the hydrophobic membrane can result in bacterial death (Hardinger, 2003). In investigations of crown ether based transport of alkali metal cations across membranes it was revealed that in almost all cases a carrier mechanism was involved (Gokel and Mukhopadhyay, 2001).

Crown ethers have shown a preferance for binding at the basic sites within a peptide. This complexation with peptides occurs at varying amounts resulting in peptides of all sizes and charged states. Thus peptides that were originally similar could be distinguished on the basis of their size, charge and mobility (Hilderbrand *et al.*, 2006).

Several substituted macrocyclic polyethers have been synthesized with varying ring sizes and functional groups producing diverse levels of enhanced cation binding and rates of transport without affecting the

flexibility of the molecule. Of these the triaza crown ethers have displayed impressive affinity for protonated amines in comparison to oxo-, mono- and diaza crown ethers which show greater affinity for potassium (Vogel and Wengel, 2002). Many of these amphiphilic polyaza crown ethers when grafted at opposite ends of an oligonucleotide backbone can produce significant levels of DNA duplex stablization. Much of the research into the introduction of cation-transporting capability into bilayer membranes has involved the large unilamellar vesicles and naturally occuring ionophores. The mechanism of cation transport has been established to be different when measured across a lipid bilayer and when investigated across a model liquid membrane. Due to their low water solubility monoaza-crown ethers with amide linked-lipophilic side arms cannot be introduced into liposomal membranes after the liposomes have been synthesized (Xie *et al.*, 1994). These azacrown ethers are in general prepared with tosyl or toluenesuphonyl groups to protect the nitrogen atoms from further substitution (Gokel *et al*., 2004).

The introduction of an aza-group allows for control of the crown ether function by means of a figurative on-off switch, where the open cavity enables greater ion affinity. By means of this simple action of switching a light source on or off the selective removal of ions from solution can be regulated (Kim *et al*., 2008). Crown ethers as larger fused ring structures are only capable of partial freezing of the ribose pseudo-rotational cycle, however, if this effect is retained, these compounds could potentially impart antimicrobial, antifungal or even antiviral activity. This so-called 'freezing' refers to the restriction of the ribose-like moiety in a receptor- preferred conformation for an improved pharmacological profile (Coppola *et al.*, 2008). The most commonly used substituents in crown ethers are benzene rings which where discovered by Pedersen in his identifiction of dibenzo-18-crown-6 ((Pedersen, 1967; Gokel *et al*., 2004).

Structurally crown ethers consist of an electron rich cavity made up of oxygen lone pairs that enable binding with cations and they have an external hydrophobic 'blanket'. The crown ethers may be named based on size of the ring, shape and the number of oxygens, for example the 15-crown-5 and 18-crown-6 ether compounds. The size of the crown ether cavity is considered to be proportional to the size of the ring. Consequently selectivity of ion complexation may be reliant on the size of the cavity and the ion to be bound such as the sodium ion that has a radius of approximately 1.94 Å and the potassium ion having a radius of 2.66 Å. Conflict exists between earlier reports of 15-crown-5 cavity size and more recent evaluations, which reveal a cavity diameter of 1.7 – 2.2 Å and consequently stongest binding of the sodium ion. The potassium ion on the other hand is firmly bound by the 18-crown-6 with a cavity diameter of 2.6 – 3.2 Å. The crown ether – cation complex can attain stability when the ratio of crown cavity size to cation diameter is at approximately 1 with ratios under 0.7 producing low levels of stability (Darwish and Uchegbu, 1997; Hardinger, 2003). Under suitable conditions the 15-crown-5 and 18-crown-

6 ether compounds are capable of binding Ca^{2+} having an ionic diameter of 2.7 Å. Additionally, an increase of sodium ions can induce an increase in the intracellular levels of the calcium ion released by the sarcoplasmic reticulum. This elevation of calcium has been implicated as part of the ROS system of crown ethers, allowing physiological function (Boojar and Goodarzi, 2006).

The cation selectivity and binding ability of crown ethers is assumed to be dependent on the following parameters:

- Cavity size – cation diameter relationship;
- The number of donor atoms;
- Crown ether conformation, either rigid or flexible; and
- Crown ether lipophilicity.

Some factors that determine the extent of their ability for metal cation binding are the size of the crown ether cavity, the nature of the heteroatoms (oxygen, nitrogen or sulphur), the substituents present in the macrocycle and the solvent employed. One of the most accepted methods of determining the cation complexing ability of crown ethers is by using solvent extraction techniques. Comparative investigations of the relationship between the size of the crown ether cavity and the cation or 'Hole Size relationship', have shown optimal binding if both are of the same size. In this area of research two concepts of molecular switching and sensing have evolved. With molecular switching alterations occur which can either allow or inhibit cation binding in host systems that may or may not have had the ability. Earlier investigations have revealed that the position of crown ether attachment has the ability to influence its preference of complexation (Izumi *et al.*, 1986).

Being capable of complexing alkali metal ions, crown ethers, cyclams and cyclenes have been applied as metal carriers in research in analytical chemistry and radiomedicine. Another potential use of crown ethers that has drawn them further into the spotlight is their antitumour activity (Muzzalupo *et al.*, 2007).

Crown ethers in their simplest form are cyclic polyethers resembling polyethylene glycols composed of alternating ethyleneoxy ($-CH_2CH_2O-$) units and heteroatoms, typically ether oxygens. Two ethyleneoxy units afford dioxane, while six repeating units give 18-crown-6. Macrocycles repeating this unit more than four times are general referred to as crown ethers rather than by their systematic names because of their ability to complex cations (Gokel *et al*., 2004). The grafting of a lipophilic long-chain alkyl group to a crown ether results in the production of a crown ether surfactant known as a bolaform which is capable of forming micelles that are similar to those of traditional nonionic surfactants in aqueous solution (Turro and Kuo, 1987). Bola amphiphiles produced from two aza-15-crown-5 or aza-18-crown-6 headgroups

separated by a 12 carbon spacer were shown by DeWall and co-workers (1997) to generate lipid monolayers of a stable nature. Upon dispersion and sonication in aqueous solution, these molecules self-assemble into unilamellar vesicles that express prolonged stability in the face of various temperatures and changes in ionic strength (Muzzalupo *et al.*, 2007).

In general these macrocycles are toxic and severe irritants to tissues being absorbed through the skin, respiratory and intestinal tracts. These compounds are also able to expand their reactivity range by their characteristic solubilization of inorganic molecules in the presence of organic solvents (Pedersen, 1967). The dibenzo- and dicyclohexyl-crown ethers are the most broadly applied, however, others in this group include the 'azacrowns' and 'thia crowns' where one or more oxygen atoms may be replaced by an NH or S group respectively. Still others that exist and are in use are the cryptands and lariat ethers. Crown ethers are also known to complex protonated amino acids (DeWall *et al*., 1997).

In terms of their toxicity, crown ethers are expected to increase in toxicity with increasing ring size, also the unsubstituted crown ethers show only moderate toxicity. Chelators such as crown ethers are considered polydentate ligands that can fill more than one point in the co-ordination sphere of the ion, in particular the metal ions, primary amines and single protons (Rozema, 2008). Although widely acknowledged as toxic compounds, many studies have shown crown ethers not to interfere with the integrity of the genetic material in mammalian cells. It was demonstrated using biomarkers that exposure of the rat lung to crown ethers could produce oxidative stress in which the local production of oxidative free radicals is significant. This oxidative stress can give rise to high levels of reactive oxygen species (ROS) which attack the cellular membrane lipids or DNA in tissues and can potentially cause oxidative injury leading to a number of disorders. This effect can be enhanced by the movement of crown ethers across the cell membrane producing structural alterations to the membrane (Boojar and Goodarzi, 2006).

The introduction of crown ethers into molecules or vehicles capable of biological activity has potential to afford the novel tools of labelling and structural probing of biomolecules (Ossowski *et al.*, 2005). In this regard their strong association with protonated primary amines and binding of lysine and arginine side arms has been exploited in a group of receptors called 'molecular mouse traps'. It has further been reported that the aza-18-crown-6 molecule displayed greater protein affinity than the 18-crown-6 molecule (Gokel *et al*., 2004).

Amphotropic compounds are capable of producing thermotropic liquid crystals which may coalesce into ordered supramolecular assemblies including micelles, monolayers, liposomes and lyotropic liquid crystalline phases. Crown ethers have received more interest for application in ionoselective membranes due to their capability to form monolayer and bilayer membranes. However, stable and well ordered

membranes produced in this manner have not yet been established (Qian *et al.*, 1996). The cation-crown ether complexation in general occurs in the solvent from which the two-dimensional complex may crystallize. The rate of complexation and release of cations by the crown ethers is notably faster in polar solvents (Gokel *et al*., 2004). In water the aza-crown ether amphiphiles self assemble to produce micelles or niosomes even in the absence of nitrogen donor atoms (Hardinger, 2003). When prepared with cholesterol, aza-15-crown-5 produced a solid state structure upon crystallization. Moreover the presence of a hydrophobic tail and polar head groups allow for the development of stable vesicles on sonication, which, being rigid, suggests a high level of bilayer organization. A wide variety of crown ethers have been shown to produce aggregates commonly or more specifically they form liposomes.

Colloidal drug delivery systems have received a great deal of attention with regard to micellar solutions, vesicle, liquid crystal dispersions and nanoparticles. Alternatives to phospholipid based vesicles are being investigated for potential exploitation due to the large number of non-ionic surfactants currently available and capable of entrapping or complexing both hydrophobic and hydrophilic solutes. The main reason behind this is the associated improved costing, fewer problems with storage and better stability of these systems (Muzzalupo *et al.*, 2007). Furthermore the presence of a hydrophobic exterior and hydrophilic interior results in the crown ether being able to enhance the solubility of large molecules (Bauer *et al.*, 2004).

The *in vivo* use of liposome vector systems requires the application of lipoplexes or liposome-DNA complexes which are large and in general possess an excess of positive charge. These characteristics under the *in vivo* conditions could lead to significant disruption of the cellular ionic equilibrium which has potential to inactivate protein structures and surface lectins inhibiting their optimal functionality. Since there is no way of maintaining control of the amount of metal salts on the surface of complexes consisting of neutral liposomes and DNA, it is possible that the net charge at the surface cannot be controlled. To circumvent this, work has been carried out to introduce crown ethers at the head of the amphipathic lipids so as to exploit their ability to coordinate metal ions. In these investigations the crown ethers were assumed to bring about interactions with the metal cations of suitable strength so as to afford the lipoplex the required stability to reach the target cells, but still lose the cation and retard DNA release upon cytoplasmic entry until it reaches the nucleus (Bruni *et al.*, 2009).

The aza crown ethers have also been applied to polymers as ring opening reagents (Nakaya and Li, 1999). Besides their regular application in chemistry, crown ethers have also been applied to regulate enzymes, interact with and cleave DNA as well as antimicrobial agents in biological systems (Gokel *et al*., 2004).

1.3. CELLULAR ENTRY MECHANISMS, BARRIERS AND SOLUTIONS

The cellular membrane is a relatively lipophilic deliminating structure that restricts the entry of large, hydrophilic or charged molecules such as non-viral gene vectors. Yan and Tram (2007) noted that the delivery methodologies for nucleic acid therapeutics require three concepts to be addressed namely: total uptake, kinetics of uptake and subcellular distribution, since the phospholipid bilayer poses a potent obstacle to the movement of ions. Nucleic acid internalization via this membrane is, in general, inefficient by virtue of the charge repulsion that exists between the negative charges on the nucleic acid and the cell surface. This ineffective and slow rate of their internalization poses a further barrier as their prolonged exposure to physiological conditions creates the potential for enhanced nuclease degradation.

Endocytosis proposed for the entry of most non-viral agents is considered a mechanism for cellular internalization of macromolecules and solutes through the employment of membrane-bound vesicles. There are a number of endocytic pathways of internalization that have been proposed each of which pose their own obstacles to gene therapy (Figure 1.3). In terms of the kinetics of the mechanism three modes have been identified, namely: fluid phase refering to the bulk uptake of solutes in proportion to their extracellular concentration and is a non specific and low efficiency process; adsorptive endocytosis and lastly, receptor mediated endocytosis which involves macromolecules being concentrated and bound to the cell surface prior to their internalization (Yan and Tram, 2007).

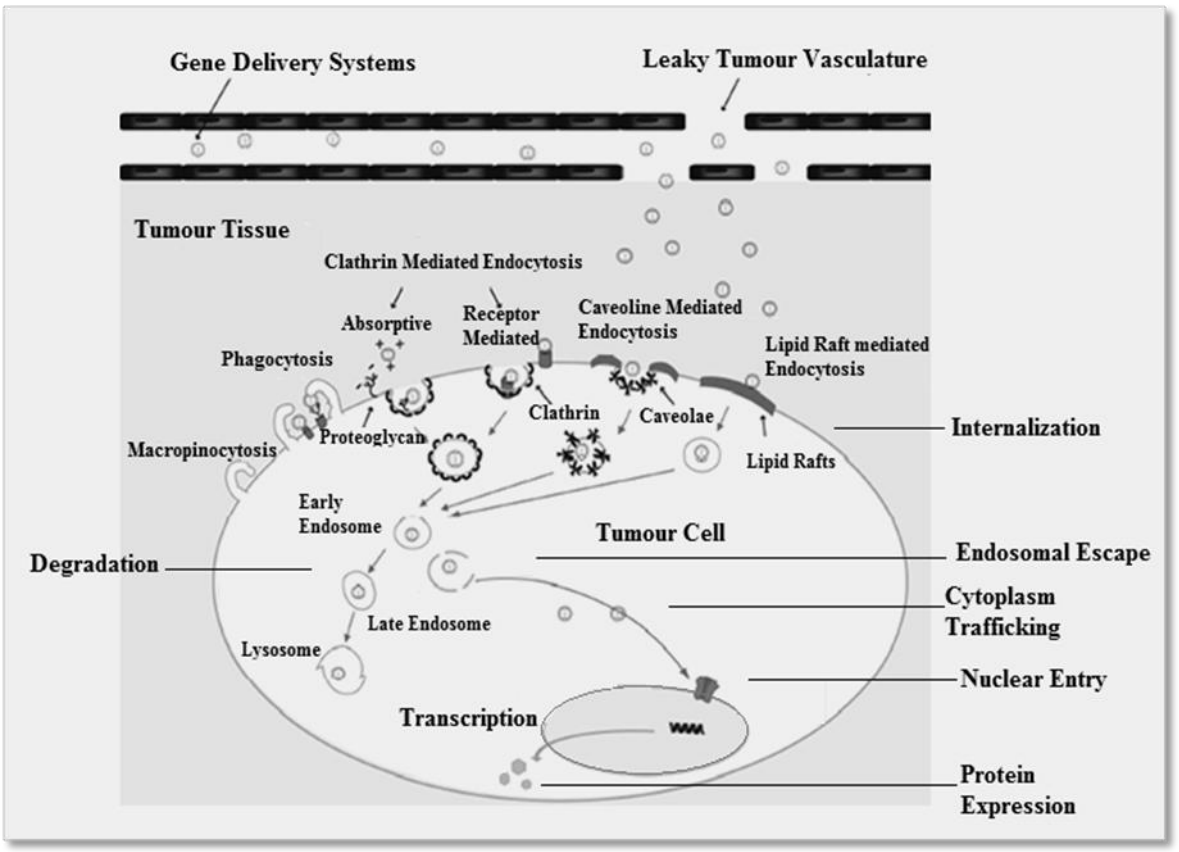


Figure 1.3: Schematic representation of the different hurdles encountered by a gene delivery system through the various internalization pathways into a tumor cell (Morille *et al.*, 2008).

Two broad classes that have been established for endocytosis are phagocytosis and pinocytosis (Khalil *et al.*, 2006). Mechanistically endocytosis encompasses diverse and highly regulated pathways. Where phagocytosis is restricted to specialized mammalian cells and is responsible for the regulation of cellular entry of large particles, pinocytosis entails fluid and solid internalization in all types of cells (Mäkelä, 2008). The latter of the two has been further divided into the four morphologically distinct pathways listed below:

1.3.1. Clathrin Mediated Endocytosis

Clathrin mediated endocytosis (CME) is the major and best characterized of the pathways and is known to take place constitutively in mammalian cells. The process involves the continous uptake of essential nutrients, antigens, growth factors, integral membrane receptors (Mäkelä, 2008) and pathogens (Khalil *et al.*, 2006).

This receptor recognition, binding and internalization forms the first port of call for the clathrin mediated pathway of endocytosis. The first step in this process is the binding of a ligand to a specific cell surface receptors. Two of the most common ligands taken up by this pathway are the cholesterol laden low desity lipoprotein (LDL) and the iron laden transferrin (Tf) which are recognized by their specific receptors. The occurrence of the internalization signal within the cytoplasmic tail of the receptor together with clathrin assembly on the plasma membrane's cytoplasmic face, results in the production of the clathrin coated pits. Thereafter ligand-receptor complexes accumulate in the coated pits which develop into intracellular vesicles upon invagination and pinching off from the plasma membrane. The GTPase dynamin has been implicated as the responsible factor for mediation of invagination and pinching off of the maturing vesicle from the cell membrane (Mäkelä, 2008). These clathrin coated vesicles thus convey the complexes into the cell (Khalil *et al.*, 2006).

In this endocytic mechanism the depolymerization or uncoating of the vesicles' clathrin coat produces an early endosome which goes on to fuse with endosomes already existing thus forming a late endosome which finally fuses with lysosomes. This is a highly regulated and energy dependent process where assembly of the polygonal clathrin lattice having a proposed diameter of 120 nm (Mäkelä, 2008) on the plasma membrane is imperative for intracellular vesicle formation and detachment (Khalil *et al.*, 2006).

Clathrin independent pathways such as caveolae-mediated endocytosis have also been proposed where caveolae are static invaginations of the plasma membrane which delineate cholesterol and sphingolipid rich microdomains. In comparison to CME this is a slow process that also has relatively low capacity (Mäkelä, 2008). The use of several inhibitors has been exploited to establish that lipoplex mediated transfection is achieved through clathrin-mediated endocytosis (Hoekstra *et al.*, 2007).

1.3.2. Caveolae Mediated Endocytosis

The Caveolae mediated endocytotic pathway uses Caveolae which are regarded as small flask, tubular, flat or detached vesicle structures that form hydrophobic membrane microdomains primarily composed of cholesterol and glucosphingolipids. Functionally the caveolae are involved in a number of celluar processes such as cholesterol regulation and transport of glycosphingolipids. They are found therefore in a diverse range of cell types, but are predominantly located in endothelial cells. Cholesterol is considered essential to internalization via caveolae therefore drugs that bind specifically to cholesterol may disrupt this internalization. The exploitation of this pathway may prove to be fruitful as it is not known to involve lysosomes and by extension any potential lysosomal degradation of the molecule being conveyed (Khalil *et al.*, 2006).

1.3.3. Macropinocytosis

The formation of large vesicles of variable morphology produced through an actin derived invagination of the plasma membrane is referred to as macropinocytosis which accompanies cell surface ruffling. Since the endocytic vesicles are distinctly large they can provide an efficient pathway for the non-selective endocytosis of solute macromolecules. Furthermore it shows the advantage of having an inherently leaky constitution allowing easy escape from micropinosomes and the capability of circumventing lysosomal degradation (Khalil *et al.*, 2006).

1.3.4. Phagocytosis

Phagocytosis has been known to be conducted predominantly by specialized cells such as macrophages, monocytes, dendritic cells and neutrophiles in mammalian cells where they are responsible for the imperative removal of relatively large pathogens or debris from sites of tissue damage or inflammation (Mäkelä, 2008). Internalization via this active process involves various sequential and complicated steps. This may be initiated by an interaction between the phagocyte specific receptors and ligands present on the particle to be taken up, which triggers actin assembly and the production of cell surface extensions. These projections then engulf the particle forming phagosomes which become part of an endocytic pathway that result in an ultimate mature phagolysosome where the particles may be degraded (Khalil *et al.*, 2006; Mäkelä, 2008).

1.3.5. Receptor Mediated Endocytosis

Despite receptor internalization taking place primarily via clathrin mediated endocytosis some other pinocytic routes are available for selective receptor-mediated endocytosis. Receptor mediated transfer of genetic material is considered an advantageous and promising mode of disease treatment (Khalil *et al.*, 2006). A wide variety of ligand grafted cationic vectors have consequently been synthesized and investigated for the intracellular presentation of plasmid DNA. However, transgene expression at sites other than sites of disease is unfavourable when the plasmid DNA is constructed using viral promoters such as those from the cytomegalovirus or SV40 which are known to produce inflated levels of gene expression (Ahearn and Malone, 1999). Subsequently specific promoters including that for the α-fetoprotein gene have been applied to cell-specific gene transfer systems (Aramaki *et al.*, 2003).

Lectins are receptor proteins which specifically recognize and bind carbohydrates which may be a sugar or glycan moiety of glycosylated proteins or lipids. These lectins have been revealed to actively direct their specifically bound ligand to either the endosome, lysosome or Golgi apparatus. Two strategies have been put forward for receptor-mediated drug delivery. The first proposes a potential lectin-drug molecule

association that could target cell surface glycoproteins or glycolipids followed by interaction with the glyco-protein or lipid allowing drug internalization. A second possibility invovles the drug molecules being associated with a specific glycan moiety which is then recognized by surface lectins. Endocytosis of the lectins is dependent upon their structure and sugar binding specificity (Yan and Tram, 2007).

Ashwell and Morell (1974, cited in Tozawa *et al*., 2001) were responsible for the discovery of the asialoglycoprotein receptors which are found abundantly expressed on the sinusoidal surface of liver parenchymal cells or hepatocytes. This hepatic receptor was shown to be capable of mediating the swift clearance of glycoproteins displaying desialylated galactose or acetylgalactosamine hexoses as well as lipoproteins and apoptotic cells from circulation (Tozawa *et al*., 2001; Wu *et al.*, 2002).

The human asialoglycoprotein receptors (ASGP-R) are hetero-oligomeric complexes consisting of two homologous subunits called hepatic lectin (HL) 1 and 2 to enable high affinty-binding. The two subunits are both composed of an N-terminal cytoplasmic domain, a transmembrane segment, a stalk domain and finally a C-terminal domain for carbohydrate recognition. The hepatic lectin 1 is the major subunit exclusively containing the signal for endocytosis and being seven times more abundant than the second subunit. The HL2 subunit on the other hand, is a significant element for basolateral (sinusoidal) organization of hetero-oligomeric receptor molecules (Tozawa *et al*., 2001).

A wide array of physiological roles have been proposed for this pathway in the past, one such postulation is the clearance of lipoproteins and chylomicron remnants through the low density lipoprotein (LDL) receptor. In their potential role in host defence, the receptor may also be involved in vertebrate protection against pathogenic organisms that exploit the neuraminidase for host invasion. It is believed that the action of C-type lectin ASGP-R may have originally evolved for protection against viral and bacterial manipulation. Patients with liver cirrhosis notoriously display an accumulation of serum asialoglycoproteins in conjunction with a decrease in receptor expression (Tozawa *et al*., 2001; Pathak *et al.*, 2008).

Of the ligands exploited for cell selective expression of the transgene both *in vitro* and *in vivo* the galactose has been the most investigated (Pathak *et al.*, 2008). The asialoglycoprotein receptor displays a prerequisite for calcium ions (Ca^{2+}) and the presence of three conserved disulphide bonds within the extracellular galactose binding domain. Consequently these hepatic lectins are able to bind non-reducing galactose and N-acetylgalactosamine residues of desialylated tri- or tetra-antennary N-linked glycans. The non-reducing terminal of oligosaccharide moieties present on glycoproteins are usally capped by sialic acid residues with neuraminidases removing the terminally positioned residues. The remaining galactose residues will be exposed and thus recognized by the asialoglycoprotein receptors. The specific hydrogen

bonding of the 3 and 4-hydroxyl groups with the carboxylate and amide side chains of key amino acids allows for receptor specificity to the D-galactose ad D-mannose residues (Wu *et al.*, 2002).

This binding and recognition is dependent on receptor structural organization on the cell membrane as this has not been observed for isolated receptors. This has thus been referred to as the 'cluster effect' by some reseachers. In addition the recognition of the synthetic glycosides is also dependent on the length of the sugar residues' spacer being maintained at a minimum of 15Å. Moreover in addition to asialoglycoprotein receptors, hepatocytes have been identified to express the major facilitative glucose transporter isoform, GLUT2. This glucose transporter affinity has been employed for targeting carriers to the liver (Russ and Wagner, 2007). Asialofetuin and galactosylated albumin have often been employed for the evaluation of hepatocyte-targeting effect through the asialoglycoprotein receptor both *in vitro* and *in vivo*. The presence of asialofetuin or galactosylated albumins has the ability to inhibit the effect of hepatocyte-targeting as well as the capability to decrease the efficiency of transfection (Zhang *et al.*, 2007).

1.3.5.1. The cellular internalization and trafficking barrier

Endocytosis is considered a multistep pathway comprising cellular internalization, endosome formation and fusion with lysosomes and finally lysis. The low pH and presence of enzymes within endosomes and lysosomes will bring about the destruction of the DNA and associated particles. The DNA that survives this must then dissociate from their complexes for nuclear entry. The relatively poor delivery of DNA generally identified for carrier mediated endocytosis is relative to this multistep process where the number of DNA molecules for expression is reduced at each step during its translocation to the nucleus (Luo and Saltzman, 2000; Wattiaux *et al.*, 2000). DNA – carrier dissociation has been evaluated to be a significant factor in the efficacy of nuclear delivery, moreover the precise quantification of delivered DNA and the ultimate efficiency of transfection are essential for a thorough understanding of the hurdles to gene therapy technologies (Hoekstra *et al.*, 2007).

The mechanism involved in cytoplasmic trafficking to the nucleus is relatively unknown, however, a potential prospect for future synthetic systems is the exploitation of those used by viral vectors and bacterial pathogens. These particles are able to traverse the periphery of the cytoplasm to reach the nucleus by means of an intracellular transport system composed of a network of actin or tubulin filaments (Klink *et al.*, 2004). Serum is assumed to exhibit its inhibitory effect either through interfering in the lipoplex-cell membrane interaction or by the presence and association of serum proteins with the complexes thus limiting their ability to effectively deliver the transgene for expression. Of these the lipoproteins have been identified as the predominant component responsible for this effect of serum

(Nchinda *et al.*, 2002). Ineffectual binding and cellular internalization is a major limiting parameter in liposomal mediated gene therapy (Chaudhuri, 2002).

1.3.5.2. The endosomal barrier

The endosome is produced by the pinching off of invaginated plasma membrane consisting of anionic phospholipids. The endosome thus presents an environment that comprises anionic lipids and low pH of approximately 6.2 (Mäkelä, 2008). The positive charge of the cationic amphiphile-DNA carriers is neutralized by mixing with the anionic lipids thus displacing the DNA. Lipid mixing may also prove beneficial, enabling membrane fusion and release of DNA into the cytoplasm for nuclear transport. The lipid phase present in the liposome – DNA complexes has been identified as crucial for escape of DNA from the endosome (Hirashima *et al.*, 2007). It is also believed that the mechanism of escape from the endocytic vesicle will rely on membrane destabilization or endosomolysis (Chaudhuri, 2002).

Endosomes are considered accountable for the separation of internalized cargo to either the recycling pathway where it is sent back to the surface of the plasma membrane or transferred to the late endosome. Following this some pathogens and downregulated receptors are transported to lysosomes where they are degraded by acid hydrolases. Late endosomes are known to have a lower luminal pH of 5.5 – 5.0, discrete protein composition, as well as cisternal and vesicular regions (Mäkelä, 2008).

A few gene carriers like polyethyleneimine have displayed an inherent buffering capacity that produces osmotic swelling in the endosomal cavity. The vector or cationic polymer becomes protonated at low pH triggering an influx of chloride ions together with protons. This leads to an influx of water with ultimately swelling and perturbation of the endosomal membrane together with the concomittant release of the entrapped vectors for nuclear targeting. This phenomenon is referred to as the ‘proton sponge effect’ and was initially proposed by Behr and co-workers (Behr *et al*., 1997; Khalil *et al.*, 2006; Shigeta *et al.*, 2007). Lysosomotropic subtances that can accumulate in the lysosome and bring about destabilization of the membrane may also be utilised. The entry of these agents via the endocytic pathway can bring about an osmotic imbalance and consequent swelling resulting from an influx of water (Wattiaux *et al.*, 2000).

Anionic lipids such as cardiolipin present in mitochondria have also been identified to be located mainly in endosomal membranes and the cytoplasmic-facing monolayer of the plasma membrane. A potential trigger for the initiation of membrane destabilization is the presence of phosphatidylethanolamine in lipid structures having high curvature. Since bilayer destabilization leads to accumulation of anionic lipids at the interaction point, the process of fusion once initiated is deemed autocatalytic (Xu and Szoka, 1996).

Another theory involves the release of compacted DNA within the endosome from the cationic lipid producing osmotic stress which may rupture the endosomal membrane and allow cytosolic release of DNA (Gershon *et al*., 1993). Release of DNA at the cell surface, in comparison may not be able to produce sufficient membrane stress required to induce rupture (Xu and Szoka, 1996).

1.3.5.3. Nuclear localization and entry

DNA translocation to the nucleus is important for expression of the therapeutic protein through the transcription machinery. Two methods that could aid in this situation are nuclear pore trafficking or nuclear breakdown via mitotic activity (Chaudhuri, 2002).

Since 1962 it has been understood that both active and passive transport either into or out of the nucleus occurs via nuclear envelope embedded pores. These embedded nucleopore complexes are made up of three distinct domains, a central domain forming an aqueous channel through the nuclear envelope, a nuclear domain of nuclear filaments forming a basket-like structure joined to a nuclear ring and a cytoplasmic ring comprising an estimated 50 different nucleoporin proteins and attached to long cytoplasmic filaments.

Pouton and colleagues (2007) looked at the two categories of nuclear localizing signals, i.e. classical and non-classical sequences for carrier-cargo complex nuclear direction. Classical nuclear pore sequences exist as short stretches of basic amino acids, the most well examined of these are those resembling the SV40 large tumour antigen. The non-classical variety do not possess these stretches of basic amino acids and the most established example is the primarily hydrophobic 38-residue M9 sequence of the human mRNA-binding protein, hnRNP A1. The two primary physical constraints for the delivery of cargo to the nucleus are passage through the cytoplasm and passage through the nuclear membrame. This is as a result of the molecular sieving effect experienced in both cases restricting the size of the cargo able to gain entry. The larger molecules must consequently form an association with those molecules forming a part of the sieve so as to overcome this physical impediment.

A simpler alternative for the nuclear entry of DNA is via certain cellular mechanisms. During mitosis some cellular mechanisms activate the separation of chromosomal DNA into two nascent daughter nuclei upon nuclear envelope breakdown. This nuclear breakdone thus presents an opportunity for internalization and activation of the desired therapeutic effect (Cotton and Wagner, 1993).

1.3.6. Lipoplex Endocytosis

Of the numerous methods of gene transfection available, lipofection or cationic liposomal gene transfection has been determined to be a promising means of foreign gene delivery to target cells. Thus a variety of cationic liposomes have been developed (Hirashima *et al*., 2007). Despite little knowledge pertaining to their mechanism of action, cationic liposomes have been employed for DNA transfection both in cell culture and animal models (Xu and Szoka, 1996).

Barriers to be encountered in liposome-mediated gene transfection may be classified into two groups of *systemic* and *cellular* barriers. Biological barriers interfere with the specific localization to the affected sites after administration of lipoplexes. Lipoplexes associated with an excess of positive charges are implicated in non-specific interactions promoting promiscuous attachment to both biological surfaces and systemic molecules which *in vitro* may be advantageous for gene expression and internalization. Once applied to *in vivo* conditions, however, these non-specific electrostatic interactions may potentiate reduced stability and targeting ability of lipoplexes (Chaudhuri, 2002).

Cationic lipoplex mediated gene deivery is controlled by factors determined by the cationic liposome formulation, thermodynamic state as well as the cellular properties (Uddin, 2007). To navigate intracellular barriers systemic gene therapy requires that vectors be designed to:

- Safely and efficiently convey extracellular DNA to the cell nucleus,
- Overcome physiological obstacles to internalization,
- Escape the endosomal vesicle,
- Transfer the nucleic acid from the cell cytosol to the nucleus.

Cellular internalization of the lipid-DNA complexes may take place via either of two possible pathways, direct fusion and endocytosis. Firstly unless a specific cell surface receptor targeted compound exists on the lipoplex transfection will involve electrostatic interaction driven lipoplex-cell surface association. Only approximately 2% of the cell population are thought to potentially take part in this internalization via direct fusion (Zhdanov *et al.*, 2002). To preclude any non-specific interactions at the cell surface a diminution of the charge ratio is essential (Wasungu and Hoekstra, 2006).

Inspite of earlier suppositions, Clancy and Sorscher (1999) showed that the second pathway of endocytosis was in fact the required path of internalization for the majority of complexes. This process ends in either destruction of the endosome or the transported complex (Zhdanov *et al.*, 2002). Of the

various endocytic pathways, clathrin-mediated endocytosis has been reported for a few cationic lipids with application to diverse cell types (Wasungu and Hoekstra, 2006).

At the point of DNA complex endosomal entrapment, a fraction of the bound nucleic acid dissociates from the cationic amphiphile and manages to free itself from the early endosome to enter the cytoplasm. Depending on the nucleic acid cargo different functions can be undertaken. For the expression process, transcription, mRNA export, translation and proper protein folding is essential. While nuclear trafficking is required for DNA, mRNA will perform its function directly in the cytoplasm (Murray *et al.*, 2001). Once nuclear entry has been attained, the ultimate event in the transfection process is the expression of the gene (Ahearn and Malone, 1999).

It is in general understood that the transfection of anionic and neutral liposomes is not impressively effective, since they achieve moderate activity *in vitro* and relatively poor expression *in vivo*. The complexes prepared on the basis of neutral phospholipids are able to avoid, in part, a few of the drawbacks associated with the application of cationic liposomes. One of these is their extensive and tight binding of DNA limiting its release and further transport to the nucleus (Zhdanov *et al.*, 2002).

An important aspect in the evaluation of the mechanisms involved in cationic liposome-DNA complexes (lipoplexes) is to determine the proteins and other molecules involved with lipoplex internalization that are present at the cell surface. Proteoglycans present at the cell surface are responsible for an assortment of functions ranging from the production of the extracellular matrix to cell to cell contact and communication. Furthermore they have shown the ability to mediate mechanisms of gene delivery into cultured cells that involve polylysine or cationic liposomes, which implies their potential role in lipoplex uptake *in vivo*. Despite this supposition the use of heparin sulphate *in vitro* has proved capable of releasing DNA from these complexes indicating that the glycosaminoglycan-bearing proteoglycans present in the biological milieu may prevent the uptake of these complexes. Sialic acid is another significant anionic surface molecule that when exhausted produces a similar effect to that produced by the depletion of proteoglycans, which results in the inhibition of *in vivo* lipoplex internalization. However, in contrast to the depletion of proteoglycans *in vitro* which prevents internalization, sialic acid depletion in this environment may enhance the transfection (Mounkes *et al.*, 1998).

1.3.6.1. Liposome-receptor targeting

By definition targeting incorporates any strategy to bring about improved specificity of delivery and expression of the gene at the site of the tumour. Past targeting strategies employed have included targeted delivery of nucleic acids as well as transductional and transcriptional targeting to the intracellular site of

tumour cells. Transductional targeting includes all systems for improving intracellular release and transport of genes to the target cell nucleus. Upon gaining nuclear entry, only the presence of adequate promoter and/or enhancer elements in the expression cassette will allow gene expression (Russ and Wagner, 2007).

Ever since glycotargeting was first demonstrated in the early 1970's as a prospective method for directing therapeutic genes to protein receptors found at the site of localization, the potential of the application of carbohydrate ligand targeted drug delivery systems has been well established. The chief biological mechanism exploited for glycotargeting is receptor-mediated endocytosis (RME). The application of glycoconjugates also termed sugar-macromolecule conjugates may be classified into two types, the first in which the macromolecule is itself the drug or therapeutic agent and the second where the macromolecule has an essential part in assisting the drug or therapeutic agents delivery. Multivalent glycans are generally necessary for efficient functioning of glycotargeting since the mono- and bivalent ligands do not have a high enough binding affinity (Yan and Tram, 2007).

A large amount of high affinity cell surface receptors are exclusivley expressed on liver parenchymal cells for binding and internalization of asialoglycoproteins. Over the years there have been a number of attempts to manipulate liposomes and other carrier systems to display ASGP-R sepcific ligands. Moreover it was revealed that a few synthetic galactose polymer ligands were more effective at ASGP-R complexation than its natural asialofetuin ligand. Additionally specific strategies have been employed in the design and development of these receptor-specific ligand bound vectors, involving the covalent coupling of a neoglycoprotein to the vectors' surface resulting in conjugates with extended sugar epitopes and multisaccharides at their surface (Wu *et al.*, 2002). Collections of triantennary β-galactoside arranged on cholesteryl esters have proved stable and capable of producing high levels of ASGP-R-mediated uptake together with reduced glycolipid exchange with other lipid compartments (Singh and Ariatti, 2003). A critical pitfall in the application of glycotargeting is the potential overloading of the sugar density upon which a higher proportion of cellular uptake will take place non-specifically probably via the Kupffer cell receptors (Davis and Robinson, 2002).

Wu and others in the late 1980's demonstrated successful liver gene transfer in *in vivo* investigations upon administration of poly-L-lysine (PLL)-asialoorosomucoid linked complexes. Galactose presenting complexes may avoid serum protein interaction due to their electric neutrality. The galactosylation of cationic liposomes thus appears to be an effective mechanism to achieve effective hepatocyte targeting (Kawakami *et al.*, 2008). A variety of galactose and lactose terminated compounds including asialoorosomucoid, galactosylated poly-L-glutamic acid, asialofetuin glycopeptide, lactosylceramide and

others which have been applied as ligands for liver targeting liposomes, polymers and nanoparticles (Zhang *et al.*, 2007). While the reductive amination of galactose with lactose results in the production of galactosyl residue, the reductive amination of galactose with galactose results in an acyclic modification which lectins find difficult to recognise (Davis and Robinson, 2002).

Further modifications, this time, in the length of the spacer between the sugar and the backbone of the polymer can increase the uptake of these molecules and their contents (Davis and Robinson, 2002). This improvement in transfection has been observed with an increase in the length of the spacer that links the cholesterol and galactosyl moieties together. Furthermore the affinity of ASGP-R binding appears to show significant dependence on the galactose and N-acetylgalactosamine residues in addition to the space between the respective galactose residues (Singh and Ariatti, 2003). Shigeta and co workers (2007) developed a carrier Gal-C4-Chol that was modified to contain an imino group for electrostatic binding of plasmid DNA as well as a galactose residue for specific recognition by asialoglycoprotein receptors exclusively expressed on mammalian hepatocytes.

Glycosylated liposomes have also been developed and their effect determined for liver targeting whereby the use of cationic lipoplexes composed of glycosylated cholesterol were capable of producing a 10 fold enhancment in liver gene expression. Liver cancer is another significant target for gene therapy with hepatocellular carcinoma being attributed as a leading cause of deaths across the world (El-Aneed, 2003). Other potential ligands for drug delivery that act as enhancers of penetration and drug adsorption are bile salts and glycyrrhizin as a result of their specific affinities for hepatocytes (Kawano *et al.*, 2002).

It is interesting to note that in addition to the effect of the physiochemical properties of the carrier vector, the structure of the ligand grafted to the carrier also plays an influential role in determining the amount delivered through systemic administration to the receptor positive cells. For receptor mediated cellular internalization of carbohydrates both receptor recognition and cell surface accessibility play a major role. Hashida and co-workers (2001) explained that for effective hepatocyte targeting the size of galactosylated-cationic liposome-DNA complexes must be condensed to a diameter of 150 nm. Since charge is also a siginificant determining factor for cationic liposome based gene delivery, the positive charge that is displayed should not be too elevated as this will enhance non-specific interactions. The *in vitro* and *in vivo* transfection efficiencies are also influenced by the lipidic composition of theses complexes. Control of these parameters consequently are essential to the *in vivo* gene transfer mediated by glycosylated liposome carriers. The Gal-C4-Chol cationic lipoplexes have been found to be efficiently recognized and internalized by the HepG2 asialoglycoprotein receptors leading to expression of the transgene (Hashida *et al.*, 2001).

1.4. *IN VIVO* STUDIES AND REQUIREMENTS

The liver makes up approximately one-fiftieth of the total adult body weight and recieves about 25% of cardiac output. The liver system consists of both biliary and blood circulation systems. The biliary system is responsible for connecting the apical surface of all hepatocytes to the duodenum via the canals of Hering. The sinusoid is the terminus for all blood vessels in the liver and is known to possess a discontinous endothelium. Furthermore the absence of a basement membrane permits improved permeability and translocation of molecules less than 100 nm in size to the basolateral surface of the cells. Thus the liver is one of the most amenable organs to gene delivery, as it assumes a pivotal role in a number of acquired and genetic disorders (Hara *et al.*, 1997; Suda *et al.*, 2009).

The liver acts as a protein factory for serum circulating polypeptides and numerous enzymes. It comprises different cell types such as hepatocytes (liver parenchymal cells) which are the predominant cell type, sinusoidal endothelial cells and Kupffer cells resident in the macrophages of the liver. An estimated 80% of the liver mass is composed of hepatocytes which are the main targets for gene transfer in the treatment of several genetic disorders. These cells are round in shape and contain a nucleus and a significant amount of cellular organelles associated with metabolic and secretory functions. To provide support for the metabolic functions there exists an abundance of mitochondria acting as an energy supply. Since the liver is involved in the circulation of blood, this makes the liver parenchymal cells the attractive prospects that they are for the treatment of a multitude of liver associated diseases through the application of gene therapy (Pathak *et al.*, 2008).

Liver transplantation has been established as a successful treatment for hepatic failure caused by acute or chronic liver disease. As a treatment modality, liver transplantation, is instigated by a shortage of donor tissue thus alternative approaches must be developed (Kren *et al.*, 2002). For the successful treatment prolonged expression of the transgene is essential, however, this has been most successfully achieved using retroviral vectors in reimplantation and partial hepatectomy (Hara *et al.*, 1997).

The first liposome systems applied to liver targeted gene delivery were anionic liposomes which only achieved low level transfection and transient gene expression. However, they showed that intravenous administration of liposomes for liver specific gene transfer could be realized (Heller *et al.*, 1996). Cationic liposomes were thus applied for this purpose and have been successful *in vitro* while evaluations for successful *in vivo* application are ongoing. After the success achieved with DC-Chol numerous cationic cholesterol derivatives were synthesized and investigated. From this it became clear that the linker and cationic head group of these amphiphilic molecules are critical to their ability to produce expression of the transgene and toxicity. Due to the need for repetitive dosing as a result of their limited

period of gene expression, complexes containing a carbamate ester linker are generally recommended. Ding and colleagues (2008) provided both *in vitro* and *in vivo* confirmation that lipoplexes displaying a slight excess positive charge were in fact more effective than the negatively charged or almost neutral lipoplexes in producing higher levels of gene expression within tumour tissues.

The use of large-scale screening of available liposome carriers for *in vivo* gene transfer has enabled the elucidation of a vast array of highly active compounds. DMRIE for example has achieved successful transfer of therapeutic and reporter genes to solid tumours in both preclincal and clinical trials while several lipids including DOTMA have been reported to elicit potent reporter gene signals in a variety of tissues. Consequently much screening and empirical testing is needed for the development of cationic liposomes specific for particular *in vivo* applications. These cationic lipids may also be introduced into synthetic artificial viruses to enable non-viral vector targeting (Clancy and Sorscher, 1999).

It has been suggested that the use of highly stabilized lipoplex structures can produce elevated levels of transfection *in vivo*. Furthermore those possessing a spaghetti like structure at optimal transfection concentrations and diameters ressembling that of the nuclear pore may potentially be the ideal cationic lipoplex for active transfection *in vivo*. This potential arises from the similarity of the spaghetti structures to the microvilli where they are highly curved with small radii, thus allowing for effortless adherence and fusion to flat cells. Additionally the excess of positive charge at their surface promotes fusion with both the cell and nuclear membranes thus enabling DNA transfer through the cytosol and to the nucleus (Ma *et al.*, 2007). It has been reported previously that the salt concentration can play a significant role in the stability of galactosylated lipoplexes for enhanced *in vivo* hepatocye specific gene expression, where the presence of an essential quantity of sodium chloride for the prepartion of lipoplexes could stablize their final form in accordance with the surface charge regulation theory (Pathak *et al.*, 2008).

The introduction of genes into experimental animal models and in human trials which produces either general or tissue specific expression may permit the specific manipulation of biological processes *in vivo* with the intention of treating disease and protection against pathogens through induced immune response. The administered lipoplex is thought to also undergo alterations with regard to its physical properties, the extent of which is based on the level of interaction with the biological fluids. Some routes investigated by radiologists for therapeutic gene delivery to the liver are direct percutaneous injection, and transcatheter intra-arterial or intraportal administration (Choi *et al.*, 2002).

Kawakami and colleagues (2008) identified that a lower charge ratio of the lipoplex is favoured for intraportal administration of galactosylated DNA lipoplexes for ASGP-R targeting. This is due to the interaction with the biological mileu which has posed significant issues to both *in vivo* and *in vitro*

conditions. Moreover the association of both polyplexes and lipoplexes with erythrocytes results in an accumulation in the lung for expression (Kawakami *et al.*, 2008). In contrast intratumoural injection involves little interaction with the biological fluids and there are few or no changes to the physical properties of the lipoplex. Intravenous administration comparatively results in altered size, structure and net charge of the lipoplex prior to reaching the target cell (Li and Huang, 2000). Cationic formulations composed of Gal-C4-Chol were shown to produce improved transgene expression in liver parenchymal cells exclusively on portal vein administration (Wu *et al.*, 2002). It has been reported that the tail vein injection of 25 μg of plasmid DNA with the luciferase reporter into rodents could produce nanogram levels of the gene product per milligram of protein in the tissue extract (Kay *et al*., 1997).

Viral or synthetic carrier mediated gene transfer mechanisms can take place either by *ex vivo* or *in vivo* methods of cellular entry. *Ex vivo* refers to the incubation and transfection of the vectors with human cells derived directly form the patient which are then returned to bring about a therapeutic event. The *in vivo* model (Figure 1.4) on the other hand involves systemic delivery either by general administration or local injection directly into the tumour (Safinya *et al.*, 2006). In general both viral and non-viral vectors are not liver specific, although the exceptions of recombinant adenoviruses and some targeted vectors do exist. Consequently these vectors must be injected into the liver peritoneal cavity; infused into the portal or hepatic vein; or instilled into the bile duct, if they are to realize effective delivery. Despite the intravenous administration, liposomes show an innate affinity for the liver, without the presence of a targeting ligand and uptake by the liver is variable (Choi *et al.*, 2002).

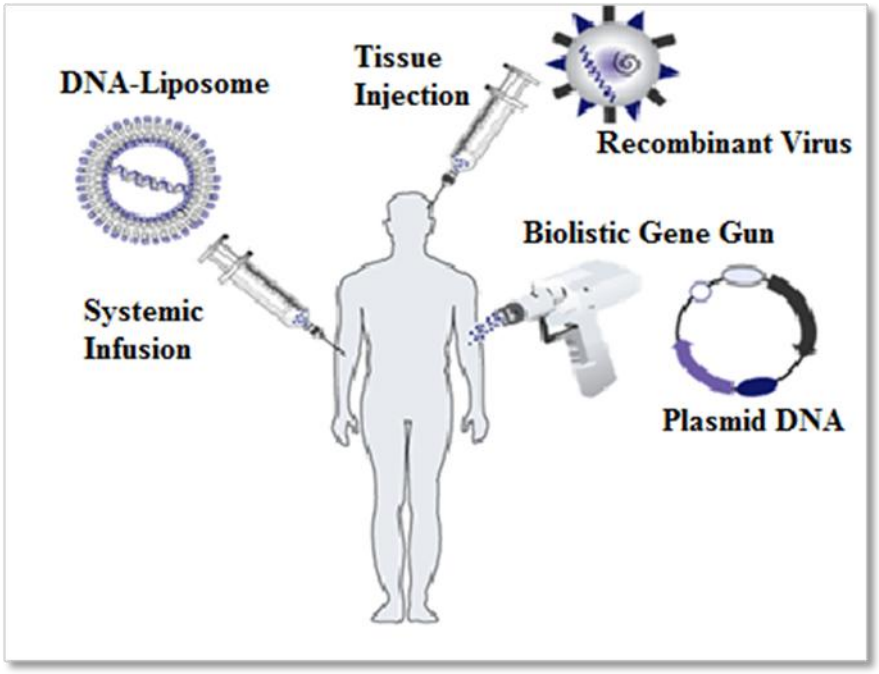


Figure 1.4: In vivo gene therapy performed by targeting the gene delivery system to the desired cell type in the patient using either physical **tissue injection** (brain tumour) or biolistics (dermal DNA vaccination), or potentially using **systemic infusion** of cell-specific receptor-mediated DNA carriers (reconstructed liposomes or viruses) (Miesfeld, 2001).

The *in vivo* requirements for efficient transfection, in the most part, differs from that achieved in cell culture and consequently some of the more effective complexes for *in vivo* studies are lost at the level of *in vitro* cell culture analyses (Nchinda *et al*., 2002). An inherent problem with *in vivo* gene targeting, is the low transfection levels which are difficult to detect and analyze. Hence, there is a need for an accurate and sensitive measurement system for these low level systems (Ino *et al.*, 2005).

Targeting to the hepatocytes is not only attractive, but also a practical consideration as they express an abundance of hepatic lectins on their sinusoidal plasma membrane domain facing the capillaries. They display in particular the asialoglycoprotein receptor presented at an approximate $10^5 – 5x10^5$ binding sites per cell (Pathak *et al.*, 2008). In addition, their physicochemical properties play a determining factor for the amount transferred to the target site upon systemic administration. The establishment of delivery systems with biological and physicochemical properties necessary for site directed delivery of plasmid DNA, is important for *in vivo* expression. Stablility *in vivo* of the DNA-carrier complexes is reliant on the size of both nucleic acids and polycationic carriers. For effective recognition by asialoglycoprotein receptors, the carriers employed should contain a significant amount of galactose residues in their structures. Despite the grafting of the sugar moiety to the carrier vector, Nishikawa and co workers (1998)

showed that successful delivery of the plasmid DNA could not be realized as the cationic complexes may distribute to various cell types upon systemic administration. For administration to mice the complexes were designed to possess a weak negative charge which could emphasize the specific hepatocyte uptake via receptor-mediated endocytosis. During receptor-mediated endocytosis the larger sized particles rarely reach the hepatocytes as the number of fenestrae having a diameter of larger than 200 nm in the liver sinusoid is very few (Nishikawa *et al*., 1998).

Both extracellular and intracellular problems to gene delivery exist *in vivo* (Finsinger *et al.*, 2000). To maintain stability of the liver, it is typically protected against foreign genetic material via the existence of intra- and extracellular barriers. One barrier to both viral and non-viral systems upon administration to the lung is the apical membrane of the intact epithelial airways, as this is the most fortified boundary providing protection from the hostile environment (Klink *et al.*, 2004). The lung poses as the first obstacle facing liver targeted gene therapy, however, the promise of gene therapy may yet be achieved through the use of gene therapy to the lung for the successful treatment of cystic fibrosis nad lung neoplastic disease (Ding *et al.*, 2008).

A further concern with systemic gene therapy is direct tumour targeted delivery (Wolschek *et al.*, 2002). In addition to its regular degradative effect, serum may penetrate into the lipoplex structure interfering with the packaging of the DNA. This results in nucleic acid unfolding with subsequent protrusion at the lipoplex surface thus causing interference with further cell surface interactions down the line (Wasungu and Hoekstra, 2006). Lipoplex association with negatively charged blood components causes the production of large aggregates which have the potential to be adsorbed onto the surface of circulating blood cells, trapped in a thick mucous layer or embolized in microvasculature thus inhibiting their localization at their intended target (Gao *et al*., 2007).

Non-viral vectors that produce effective gene transfer *in vivo* have yet to be realized. To produce systems for broad range applicability transgene expression must be extended. The generally transient expression produced by non-viral vectors may be attributed to their inability for either intergration or extrachromosomal replication and maintenance (Mountain, 2000).

1.5. THESIS OUTLINE

Amongst its many potential applications, gene therapy is also being investigated for the treatment of both benign and malignant tumours. Thus the development of novel vectors that are safe and effective for both the *in vitro* and *in vivo* application is receiving much attention.The requisite high titre administration of some viral vectors is known to produce severe hepatic inflammation while others pose challenges of random chromosomal DNA intergration. Non-viral transfer of nucleic acids has proven safe and nontoxic in the treatment of human melanoma. Of the different vector systems, cationic liposomes have been revealed to be effective therapeutically in benign and malignant diseases in tested animal models (Hui *et al*., 1997 cited in Mohr *et al*., 2001).

In this thesis the delivery of plasmid DNA using various cationic liposome formulations both *in vitro* and *in vivo* has been evaluated. Cationic liposomes were produced with a cationic cholesterol derivative Chol-T in association with the neutral lipid DOPE. Furthermore macrocyclic crown ethers were incorporated with the cytofectin and DOPE to produce liposomes. A distinct obstacle encountered by approaches to gene therapy is the ability to achieve effective delivery of functional therapeutic genes into the target cells (Hwang *et al.*, 2001). In order to assist in this regard both targeted and untargeted liposomes were synthesized by incorporation of unprotected glucosyl and galactosyl moeities. These were prepared with the primary aim of specifically targeting the asialoglycoprotein receptors present on the liver hepatocytes.

The first step in the investigation is the formulation of the above mentioned liposomes and the characterization of their structure, size and distribution assessed by transmission electron microscopy using a negative staining – vitrification protocol. The liposome – DNA complexes were analyzed in much the same manner, however, the interaction between the liposome and DNA was also assessed by agarose gel electrophoretic retardation and ethidium bromide displacement assays. Thereafter the *in vitro* trasfection efficiency of plasmid DNA complexes with cationic cytofectin and crown ether containing liposomes at different mixing ratios were empirically examined in the human hepatocellular carcinoma cell line HepG2 (ASGP-R positive) and the human embryonic kidney cell line HEK293 (ASGP-R negative) in terms of their physiochemical properties. The transfection activity and growth inhibition of these cationic liposomes were determined using the luciferase reporter gene assay with the pCMV-Luc plasmid and crystal violet growth inhibition assay respectively. This was also assessed *in vivo* in NMRI (Naval Medical Research Institute) mice over a three day period.

The primary goal of this investigation was to produce cationic liposomes capable of high transfection activity *in vitro* that could be successfully applied to the *in vivo* situation, in the hope of overcoming the descrepancies often found to exist between the two models. Improvement in liver targeting through the application of receptor specific ligands as well as a better understanding of the effects of the crown ether compounds to potentiate transfection of the cationic liposome-DNA complexes were additional goals in this study.

CHAPTER TWO

2. LIPOSOMES SYNTHESIS AND STRUCTURAL CHARACTERIZATION

2.1. INTRODUCTION

The principal mechanism of intracellular liposomal drug delivery has shown a strong dependence on liposomal composition. This and other factors provide a means for liposomal classification into five catergories listed below and of which four are represented in Figure 2.1:

i. Conventional liposomes;
ii. pH sensitive liposomes;
iii. Cationic liposomes;
iv. Immunoliposomes; and
v. Long-circulating or stealth liposomes.

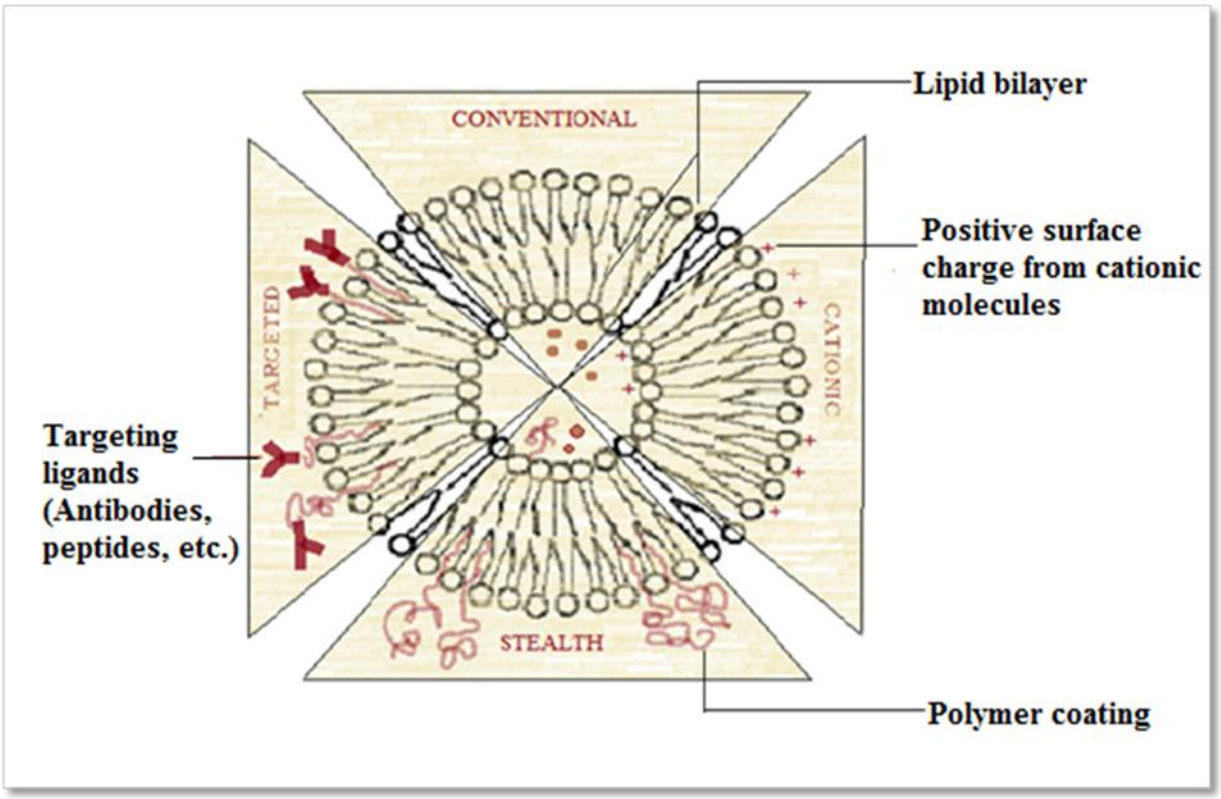


Figure 2.1: Liposome classes as distinguished by their function and functional groups (Adapted from Lasic, 1997).

During their formative years liposomes were prepared from a variety of lipids which were analogous to those in biological membranes (Kozubek *et al.*, 2000). This innate correlation with natural cell membranes has made liposome vectors the favoured biocompatible drug delivery system (Figure 2.2). Liposomes are established as microscopic vesicles composed of single or multiple bilayers encasing a watery or aqueous interior (Sharma and Sharma, 1997; Chaudhuri, 2002). Liposomes that comprise lipid layers with an internal water phase have the capacity to encapsulate both water soluble and lipophilic drugs; and their behavior *in vivo* may be controlled through surface modification (Maitani *et al*., 2001).

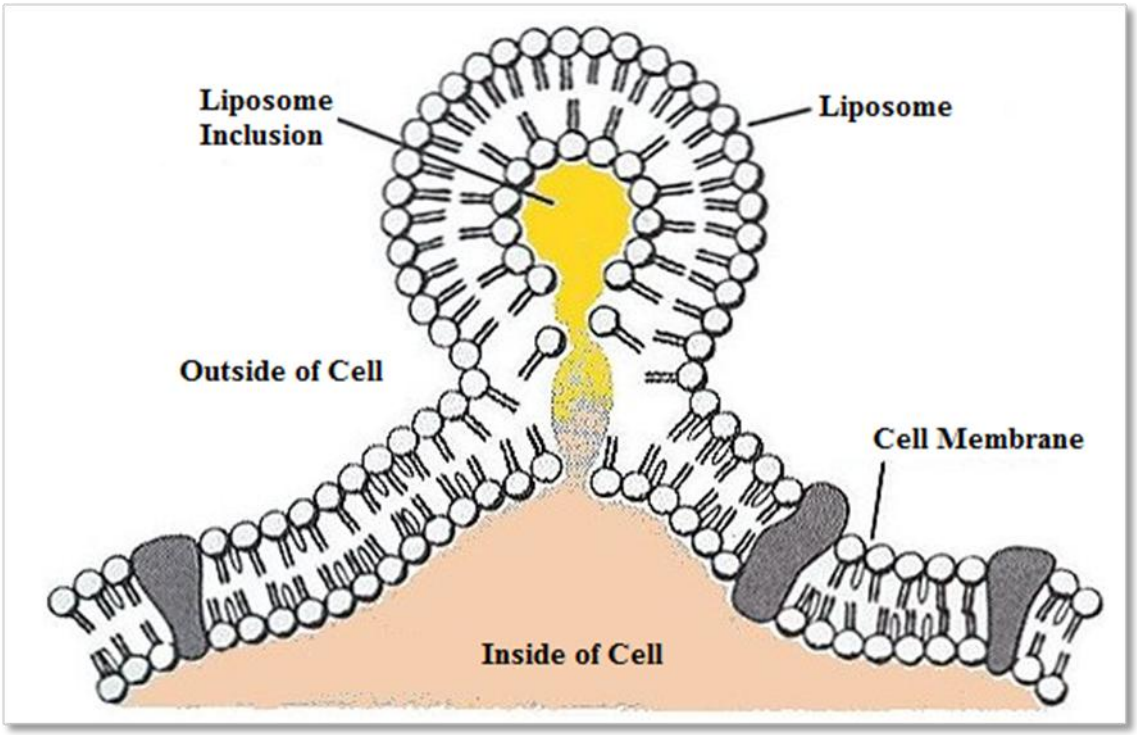


Figure 2.2: Showing acceptance of the liposome into the cell and the membrane similarity between both the liposome and cell (Adair, 2005)**.**

Liposomes have been placed into three categories of classification, viz:

i. Multilamellar vesicles (MLV) (0.1 – 0.5 μm);
ii. Large unilamellar vesicles (LUV) (> 0.06 μm); and
iii. Small unilamellar vesicles (SUV) (0.02 - 0.05 μm).

Description of liposome preparations traditionally involve the parameters of size and lamellarity rather than method of preparation whether they be described as reverse-phase evaporation (REV), French press vesicles (FPV) or ether injection vesicles (EIV) (Vemuri and Rhodes, 1995). The production of these various liposomes as classified above is illustrated in Figure 2.3.

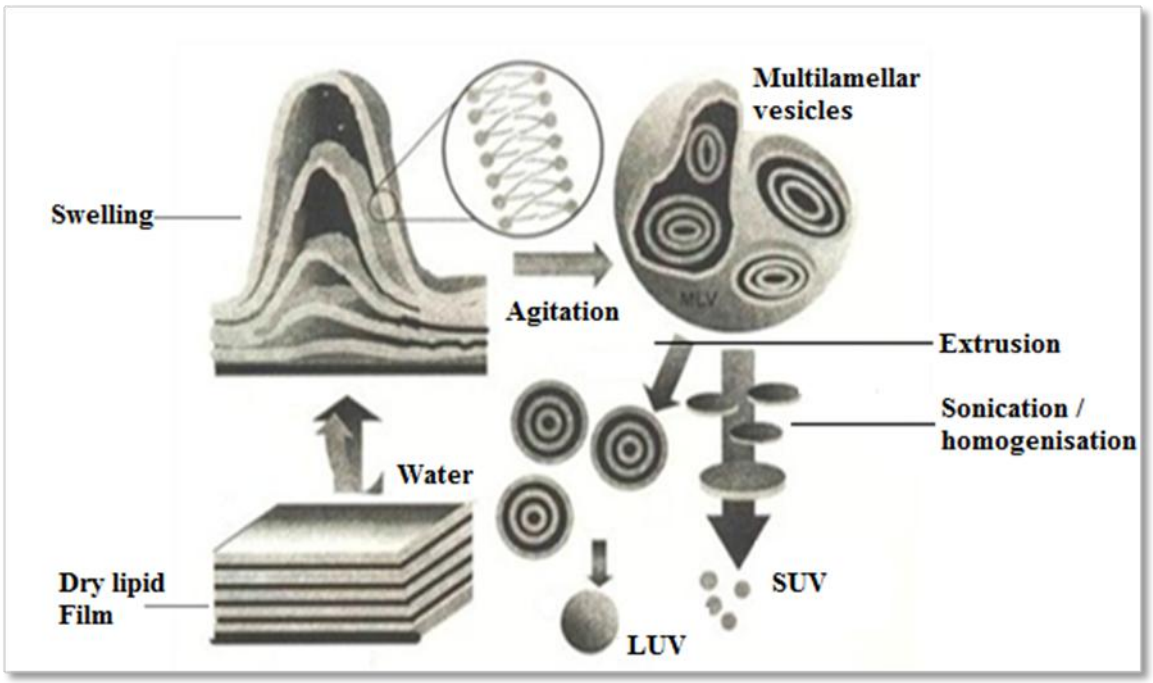


Figure 2.3: Stepwise mechanism and processing for the generation of various types of vesicles (Patel, 2006).

Thin film rehydration is one of the most commonly applied methods of preparation involving the hydration of a thin film of lipids with an aqueous buffer above the lipid transition temperature. This procedure results in heterogeneous populations having diameters between 1 – 5 μm. Although widely used and simple to apply, an associated disadvantage is the relatively poor encapsulation of hydrophilic drugs. Liposomes can be prepared to display high levels of entrapment through the freeze-drying of pre-formed SUV and dispersion in an aqueous solution containing the drug under investigation. Furthermore by performing liposome hydration using a buffer in the presence of an organic solvent, the efficiency of encapsulation achieved by these liposomes can be improved (Sharma and Sharma, 1997).

A variety of techniques are available for the production of these large vesicles such as solvent injection using ether or ethanol, detergent dialysis, calcium induced fusion as well reverse phase evaporation (Batzri and Korn, 1975; Skoza and Papahadjopoulos, 1978; Sharma and Sharma, 1997). In comparison to multilamellar vesicles unilamellar liposomes have the capacity to carry large volumes of solution in their cavity. Additional advantages of unilamellar liposomes include a reduction in the amount of lipid employed resulting from the larger capacity for drug encapsulation as well as reproducible rates of drug discharge (Vemuri and Rhodes, 1995).

In general large unilamellar liposomes may experience reduced physical stability in the presence of a deficiency of surface charge leading to self aggregation. Neutral liposomes due to the anionic nature of

the cellular surface cannot associate successfully necessitating cellular drug entry through extracellular release from the liposome. The size of liposomes is an important factor in determining the proportion that is cleared by RES where small liposomes are opsonized to a lesser degree than the larger liposomes. Accumulation of liposomes in the tumour tissues has been attributed to a reduction in liposomal size and partly to the enhanced half life of circulating small liposomes. Many techniques have been employed to bring about size reduction of MLV or LUVs including sonication (bath or probe type), extrusion or micro-fluidization (Sharma and Sharma, 1997). The bath sonicator has shown numerous advantages of use including temperature control and product processing in an aseptic environment. Previous attempts at sonication of MLVs to produce the small unilamellar vesicles were performed under the effects of nitrogen or argon, alternatively injection of ether or ethanol can be used in their preparation (Vemuri and Rhodes, 1995).

Cationic liposomes have, since their introduction into the world of gene therapy established themselves as the foremost synthetic vectors available (Lin *et al*., 2003). The unilamellar liposomal vesicles are produced through a variety of different methods including; sonication, detergent removal or through membrane extrusion to provide uniform liposomes (Dass and Choong, 2006; Uddin, 2007). The head group of cationic amphiphiles, typically consist of primary, secondary, tertiary amines or quaternary ammonium salts. However, some studies have also explored the potential of incorporating imidazole or guanidino groups (Lv *et al*., 2006; Kim *et al.*, 2009). The presence of the cationic charge on the amphiphilic lipid distinguishes it from its natural phospholipid structural analogue which may be negative charged or neutral depending on the pH of the environment (Rao, 2010).

Cholesterol and its derivatives have also often been introduced into liposome formulations to improve bilayer stability. Their inclusion has improved membrane fluidity and stability in the presence of blood, plasma or other biological fluids, and minimized permeability to water soluble molecules (Vemuri and Rhodes, 1995). Owing to its stable association with the liposomal membrane, cholesterol is often chosen as the hydrophobic anchor in liposome formulations (Hashida *et al.*, 2001).

Despite the wide array of techniques available for liposome formulation only a handful may afford a large enough capacity for water-soluble drugs (Vemuri and Rhodes, 1995). The preparations of liposomes involves the dissolution of the respective components in an organic solvent followed by subsequent drying to produce a thin film. Thereafter a liposome suspension can be obtained through the dispersion of the film in an aqueous solution at a critical hydrating temperature which should be above the phase transition temperature of the employed phospholipid (Biju *et al.*, 2006).

In the case of lipid mixtures prepared in the presence of organic solvents, both the lipid mixture and contaminants present in the solvent are concentrated on solvent evaporation. Liposomal stability is of primary importance at all points of their preparation, from initial processing, storage and administration. In this regard the fusion and breakage during storage results in the undesired vesicular drug leakage. The pH, buffer used, solvent system and ionic strength play a siginificant part in influencing the stability of liposome formulations. and liposome shelf-life. Some organic solvents employed for the facilitation of uniform lipidic dispersion are freon, methylene chloride, methanol, ethanol and chloroform and their combinations. When determining the solvent for use, factors such as safety, solubility and purity need to be considered. Chloroform although efficient for lipid dispersion, contains the carcinogen, carbon tetrachloride. Therefore when intended for human applications it is of the utmost importance to ensure complete removal of the solvent (Vemuri and Rhodes, 1995).

Liposomal formulations may have short shelf lives, due to the level of chemical and physical stability which is dependent on the liposomal composition. This can be explained by the chemical oxidation of fatty acids in phospholipids which result in reaction products that may alter bilayer permeability. The storage stability of liposomes may be increased through preservation by lyophilization of the liposomes. To ensure suitable quality control liposomes need to undergo characterization promptly upon preparation as well as during storage. The size and size distribution of liposomes have been previously successfully examined using freeze-fracture electron microscopy and dynamic light scattering to monitor vesicle stability (Sharma and Sharma, 1997).

This chapter involves the preparation of nine novel cationic liposomes and crown ether (CE) cationic liposomes with and without targeting moieties. All contain the cationic cholesterol derivative, 3 β [N-(N', N'-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) and the neutral lipid dioleoylphosphatidylethanolamine (DOPE). Characterization of all liposome preparations was performed by transmission electron microscopy.

2.2. MATERIALS AND METHODS

2.2.1. Materials

Dioleoylphosphatidylethanolamine (DOPE) was purchased from the Sigma Chemical Company, St Louis, USA. Chol-T cationic lipid 3β[N-(N',N'-dimethylaminopropane)-carbamoyl] cholesterol was synthesized at the University of KwaZulu-Natal Department of Biochemistry, Westville, South Africa. The aza-15-crown-5, aza-18-crown-6 ether preparations crown ethers (CE) 1 and 2 respectively as well as the RUI-90 and RUI-92 unprotected galactosyl and glucosyl targeting preparations were synthesized at the University of Witwatersrand, Johannesburg, Department of Chemistry. The 2-[4-(2-hydoxyethyl)-1–piperazinyl] ethanesulphonic acid (HEPES) was purchased from Merck, Darmstadt, Germany. The Uranyl acetate (ACS) of molecular weight 425.15 Daltons, depleted of radioactivity was obtained from Ted Pella Inc. All other chemicals were of analytical grade.

2.2.2. Methods

2.2.2.1. Preparation of cationic liposomes

The cationic liposomes were prepared in accordance with an adaptation of the protocol employed by Goa and Huang (1991). This was due to the cationic liposomes investigated displaying only a slight variation in chemical structure compared to that originally synthesized by Goa and Huang. The components used are presented in Table 2.1. The liposome preparations were made up to contain a total of 4 μmoles of lipid in 1 ml of HEPES buffered saline (HBS). All liposome preparations contained the cytofectin 3 β [N-(N', N'- dimethylaminopropane)-carbamoyl] (Chol-T) that was originally synthesized in our laboratory from cholesteryl chloroformate according to the scheme presented in Figure 2.4. The structures for the crown ethers RUI-128 and RUI-129 as well as the targeting moieties RUI-90 and RUI-92 are shown in Figure 2.5. The general method for the synthesis of CE 1(RUI-129) and CE 2 (RUI-128) are shown in Figure 2.6. The 1,3-dipolar cyclo-addition reaction or Click reaction were performed by using $Cu(II)SO_4$ in presence of sodium-ascorbate using dichloromethane:water (3:1) to afford the novel triazole linked cholesterol derivatives. These were then deacetylated using K_2CO_3 in methanol (10 ml) to produce the unprotected targeted sugar moieties. This scheme is presented in Figure 2.7.

The lipid mixtures were prepared in the presence of the chloroform. The liposome lipidic components were dissolved in chloroform, mixed and deposited as thin films in test tubes by rotary evaporation of solvent *in vacuo* (Büchii Rotavapor-R). Samples were dried under high vacuum in a drying pistol for

several hours. The thin film of the samples was then rehydrated in a total volume of 1 ml containing sterile HBS (20 mM HEPES and 150 mM NaCl, pH 7.5). All preparations were vortexed and hydrated over night. Following this, preparations were sonicated for 5minutes in a bath sonicator (Labotech Transsonic 460/H, 220 – 240 V/AC) at a frequency of 35 kHz to produce unilamellar liposomes.

Table 2.1: The total lipid composition of the prepared cationic liposomes

CATIONIC LIPOSOME	Chol-T		DOPE		RUI-128		RUI-129		RUI-90		RUI-92	
	MOLAR RATIO (µmoles)	MASS (mg)	MOLAR RATIO (µmoles)	MASS (mg)	MOLAR RATIO (µmoles)	MASS (mg)	MOLAR RATIO (µmoles)	MASS (mg)	MOLAR RATIO (µmoles)	MASS (mg)	MOLAR RATIO (µmoles)	MASS (mg)
Chol-T	2	1.03	2	1.49	-	-	-	-	-	-	-	-
Chol-T-Gal	1.8	0.926	2	1.49	-	-	-	-	0.2	0.135	-	-
Chol-T-Glu	1.8	0.926	2	1.49	-	-	-	-	-	-	0.2	0.135
Chol-T/RUI-128	2	1.03	1.8	1.34	0.2	0.135	-	-	-	-	-	-
Chol-T-RUI-128-Gal	1.8	0.926	1.8	1.34	0.2	0.135	-	-	0.2	0.135	-	-
Chol-T-RUI-128-Glu	1.8	0.926	1.8	1.34	0.2	0.135	-	-	-	-	0.2	0.135
Chol-T-RUI-129	2	1.03	1.8	1.34	-	-	0.2	0.126	-	-	-	-
Chol-T-RUI-129-Gal	1.8	0.926	1.8	1.34	-	-	0.2	0.126	0.2	0.135	-	-
Chol-T-RUI-129-Glu	1.8	0.926	1.8	1.34	-	-	0.2	0.126	-	-	0.2	0.135

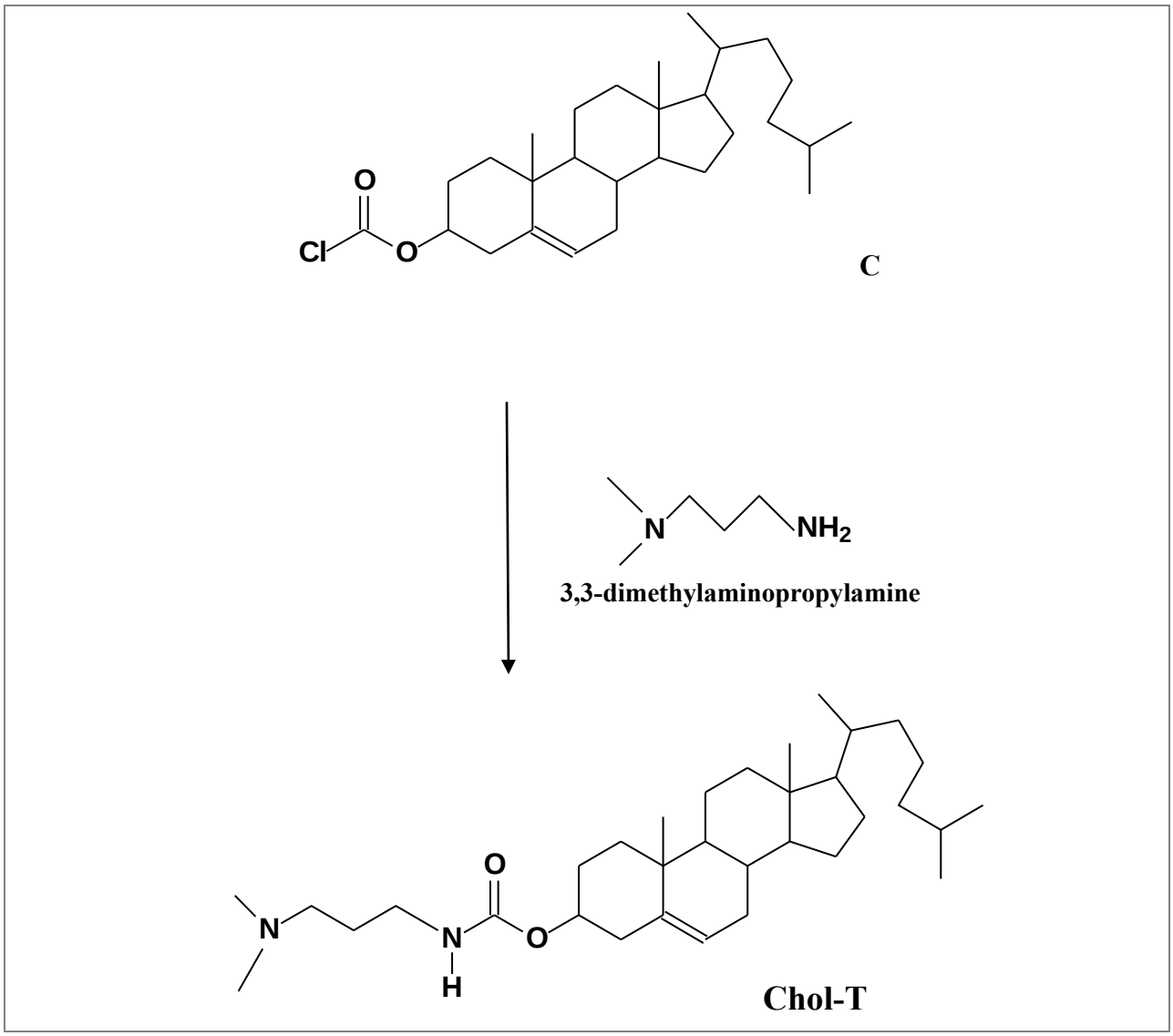


Figure 2.4: Reaction scheme showing the preparation of the cationic cholesterol derivative Chol-T from cholesteryl chloroformate (**C**) and 3-dimethylaminopropylamine.

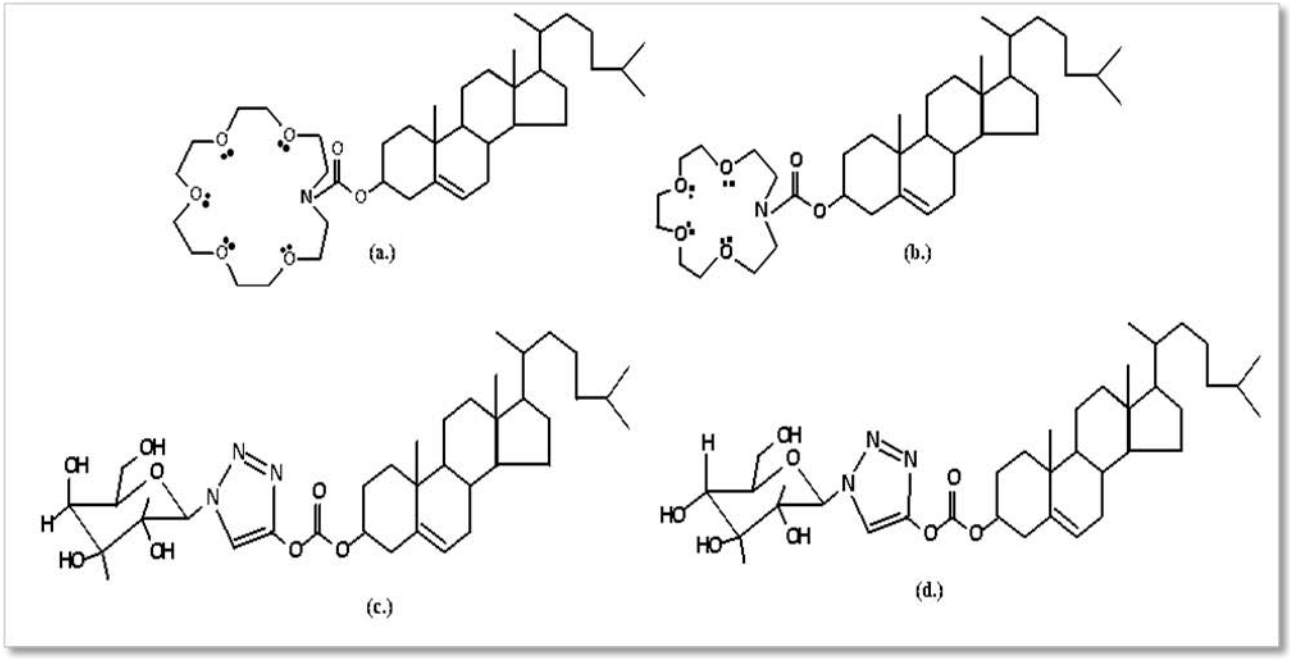


Figure 2.5: Structures of crown ethers and targeting components used for the various liposome preparations: **(a)** Aza-18-crown-6 (RUI-128), **(b)** Aza-15-crown-5 (RUI-129), **(c)** cholesteryl galactosyl (RUI-90) and **(d)** cholesteryl glucosyl (RUI-92). (Compounds synthesized and provided by Islam, R.U., Professor C.B. de Koning, Professor W. Van Otterlo and Professor P. Arbuthnot).

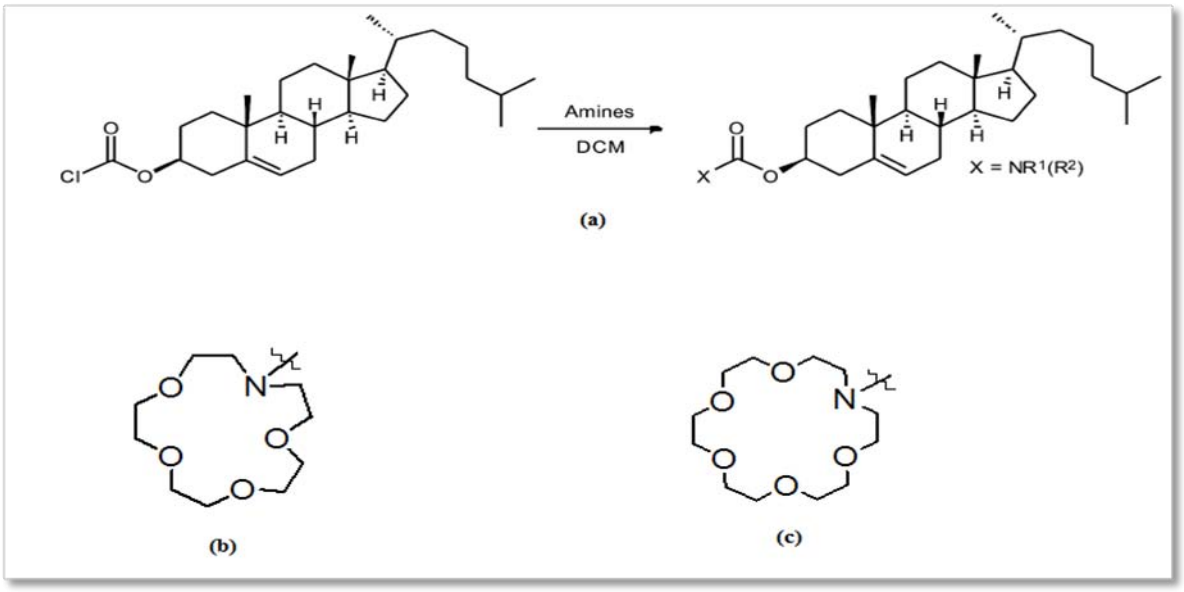


Figure 2.6: **(a)** Chemical synthesis scheme of the structural moieties **(b)** and **(c)** attached to **(x)** cholesterol derivatives. **(b)** = CE 1 (RUI-129) and **(c)** = CE 2 (RUI-128) (Adapted from Islam *et al*., 2009).

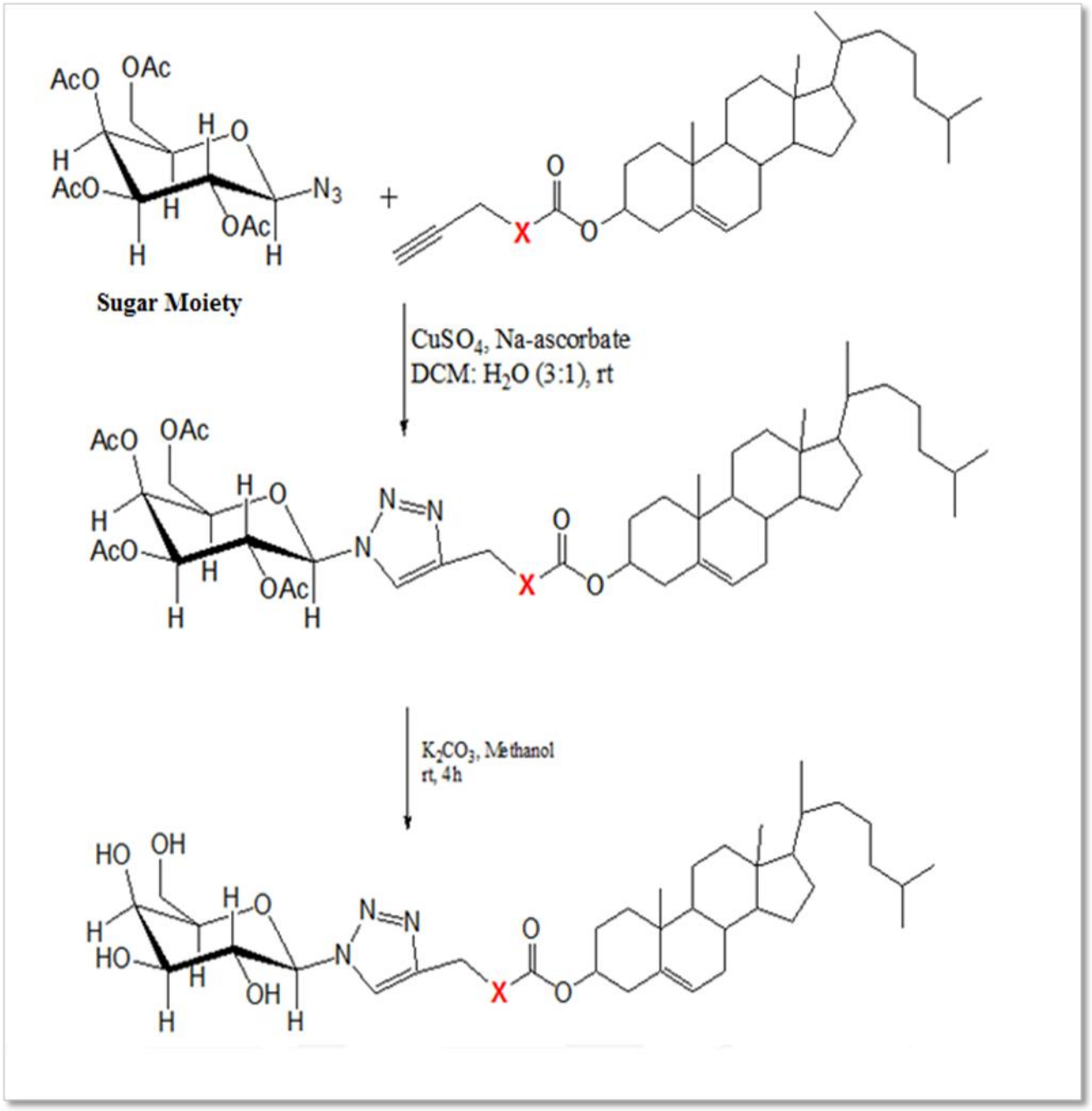


Figure 2.7: Schematic showing the click reaction for the production of the deacetylated sugar moieties conjugated to cholesterol derivatives. Showing the introduction of $Cu(II)SO_4$ (0.1mmol) and sodium-ascorbate (0.1mmol) to an alkyne (1mmol) and azide (1.2 mmol) solution in DCM:water (3:1). Upon drying with $MgSO_4$ the crude solid was purified by column chromatography using 50% ethyl acetate: hexane. K_2CO_3 in methanol was used for deacetylation producing unprotected sugar moieties conjugated to cholesterol by a triazole linker. **X** = NH, Sugar moiety= Galactose and glucose (synthesis provided by Islam, R.U. and co workers at the University of Witwatersrand – unpublished).

2.2.2.4. Characterization of liposomes by transmission electron microscopy (TEM)

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 with a 1 µl aliquot of a saturated solution of the uranyl acetate negative stain. The coated grids were then allowed to stand for 3 – 5 minutes after which the excess liquid was removed with filter paper. For the crown ether liposome formulations the grids were coated with carbon prior to use. The samples were immediately vitrified by plunging into liquid nitrogen cooled propane gas, using a spring-loaded Leica CPC system. Grids were then transferred to a Gatan cryotransfer system and viewed using a JEOL 1010 TEM without warming above -150ºC. The electron micrographs, obtained from this negative staining – vitrification methodology, were subjected to statistical analysis to assess the size and dimensions of the liposome formulations.

2.2.2.5. Statistical analysis

Particle sizes of the different liposome preparations were analyzed using a matched student t-test. P-values of less than 0.05 were considered to be of statistical significance.

2.3. RESULTS AND DISCUSSION

2.3.1. Preparation of Cationic Liposomes

Cationic liposomes were prepared successfully using the method described in section 2.2.2.1. The two major ingredients in all liposome preparations were the cytofectin Chol-T and the neutral zwitterionic phospholipid dioleoylphosphatidylethanolamine (DOPE) which is employed as a helper or co-lipid. The Chol-T was synthesized according to a procedure reported by Singh and co-workers (2001). Being described as cationic amphiphiles this implies the presence of both a hydrophobic and hydrophilic region within the lipid. The cationic amphiphile examined here has a cholesterol ring anchor, a carbamoyl linker bond and a monovalent dimethylamino head group (Wasungu and Hoekstra, 2006). The incorporation of cationic lipids into liposomal membranes is known to adjust the vascular structure. Cholesterol derivatives such as this are in general unable to produce stable bilayers in liposome formulations unless they include neutral lipids such as DOPE or cholesterol in their preparation (Hattori and Maintani, 2005). Cholesterol, for example is responsible for controlling membrane fluidity and permeability (Obata *et al.*,

2009). The use of DOPE or cholesterol for application of cationic lipids in transfection has been reported to induce the conversion of the lamellar lipoplex phase into the hexagonal conformation which enhances the effectiveness of transgene expression. The ratio of helper lipid to cationic lipid determines toxicity and transfection efficiency (Morille *et al*., 2008).

Pedersen (1967) showed that of the solvents used for the solublization of crown ethers, formic acid, chloroform, ethylene dichloride and pyridine were the best, while ethers, methanol, water and other alcohols were moderate to very poor solvents for these compounds. In our preparation of cationic liposomes with crown ether (CE) functionalities chloroform was used as the organic solvent resulting in complete dissolution of the crown ethers. Our preliminary investigations showed that the CE's although known to form micellar structures, were unable to form liposomes in the absence of cationic lipids (results not shown).

For the targeted liposome formulations RUI-90 and RUI-92 targeting moieties were employed in the liposomes. The ligands were designed to specifically target the asialoglycoprotein receptors (ASGP-R) of liver hepatocytes. In their synthesis the targeting moieties were prepared using click chemistry to bridge the sugar moiety to the cholesteryl anchors. In this study, the novel unprotected galactosyl and glucosyl cholesterol derivatives were formulated in cationic liposomes to specifically target the ASGP-R on the hepatocyte derived cell line HepG2 in culture.

2.3.2. Characterization of Liposomes by Transmission Electron Microscopy

Transmission electron microscopy using the negative staining-vitrification protocol (outlined in section 2.2.2.4.) revealed the sizes and unilamellar structure of the different cationic liposome preparations (Figures 2.8 to 2.10). Cationic liposomes showed a broad size range of 30 nm to 650 nm in diameter. The average diameters of the different liposome preparations are presented in Table 2.2.

Table 2.2: Mean diameters of the various liposome formulations determined by transmission electron microscopy.

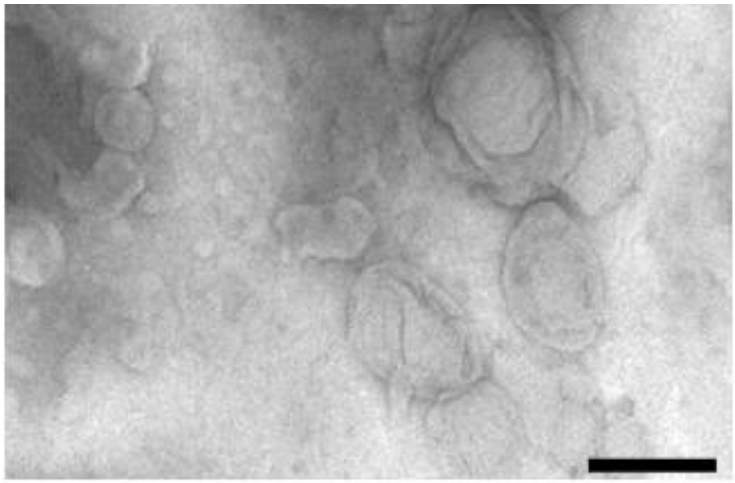
LIPOSOME	MEAN DIAMETER (nm)	STANDARD DEVIATION (nm)
Chol-T	68.40	39.31
Chol-T-Gal	37.24	21.05
Chol-T-Glu	64.75	30.62
RUI-128	528.52	89.73
RUI-128-Gal	96.20	19.8
RUI-128-Glu	37.36	5.74
RUI-129	124.28	23.98
RUI-129-Gal	54.06	12.04
RUI-129-Glu	80.71	3.80

Note: Data presented as mean ± standard deviation (n = 5).

From the results it is clear that those liposome preparations incorporating the galactosyl and glucosyl targeting ligands display a reduced diameter size. It can also be noted that the CE 2 formulation consisting of the aza-18-crown-6 ether moiety produced distinctly large liposomes. This may be attributed to the large crown ether (CE) cavity that it possesses, having an average cavity size of 2.6 – 3.2 Å. The Chol-T and CE 1 liposomes displayed liposome diameters that fall within the range of 30 – 530 nm. The relatively low standard deviations indicate only a slight divergence in particle size, while those displaying higher standard deviations show greater size variation within a liposome population. Preparations were not extruded through defined pore size membranes.

A result may be considered statistically significant if the p-value is below the preset threshold of 0.05. In this regard both targeted preparations of Chol-T showed no significant size variation compared with the untargeted Chol-T liposome ($p > 0.05$). On the other hand all crown ether (CE) liposomes showed statistically significant differences in size between the targeted and untargeted preparations ($p < 0.05$). Moreover a comparison of the Chol-T liposome and both CE liposomes revealed statistically significant size variations between it and each of the crown ether liposomes. This provides us with comparative information of the different liposomes in terms of their size distribution.

All liposome preparations showed a generally spherical morphology, with some showing a deformed nature which could be attributed to manner in which the samples were frozen, rendering the liposomes immobilized and in some instances deflated. Artifacts seen in some of the liposome images are attributed to the cryo-TEM process. Moreover any stippling in the micrographs can be attributed to the propane employed in the above mentioned freezing protocol or the carbon coating used on Formvar grids employed for viewing the crown ether preparations. The carbon coating was essential for the examination of crown ether preparations as the ether present in the formulation was observed to corrode the delicate Formvar grids, making it impossible to view the micrographs. Although TEM provides a more than adequate preliminary examination of the size and relative distribution of liposomes in a given sample there are many techniques available that can provide more significant information on the distribution of the liposomal population. Two such techniques are dynamic light scattering and zeta-sizing, which were unavailable for this study.



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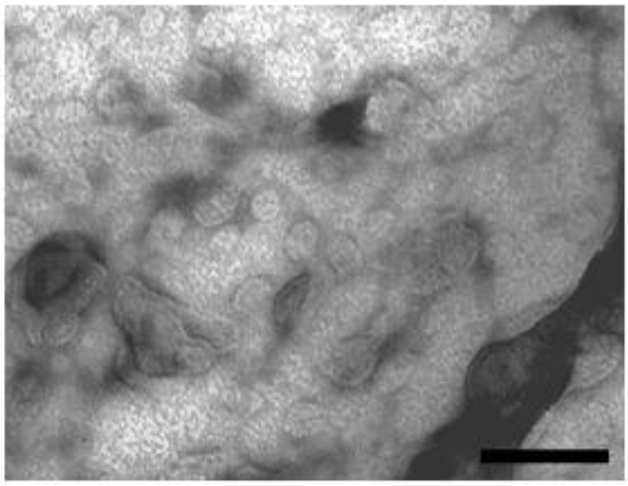

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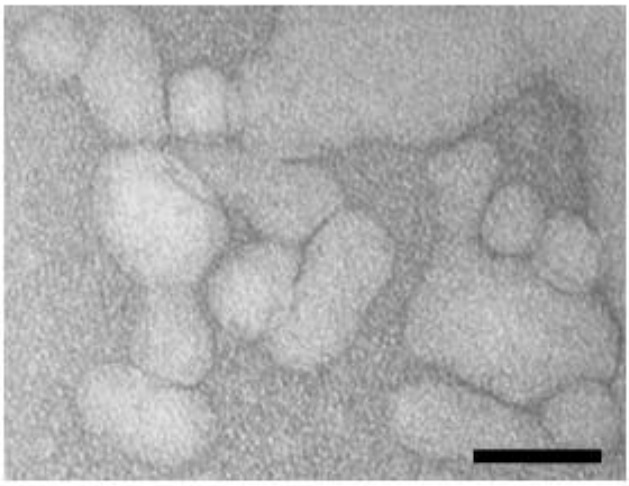
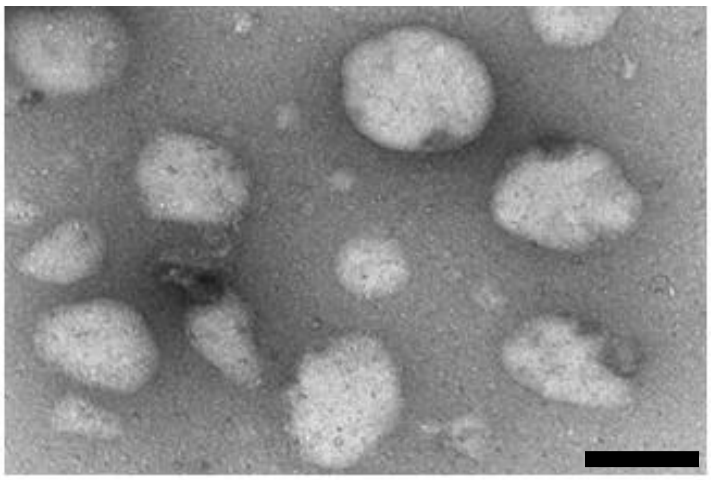


(c.)

Figure 2.8: Transmission electron micrographs of cationic liposomes. **(a)** Chol-T, **(b)** Chol-T -Gal**, (c)** Chol-T –Glu. Bar = 100 nm.



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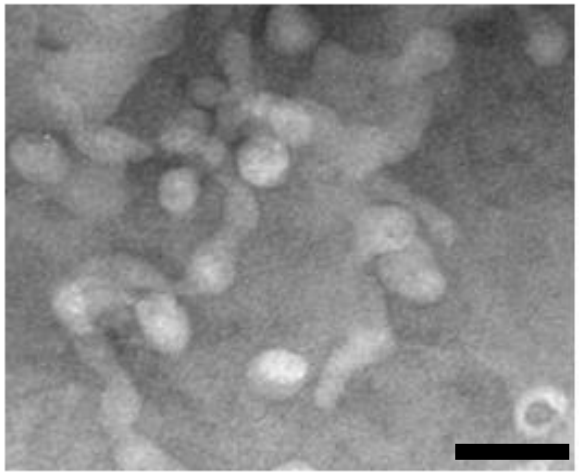

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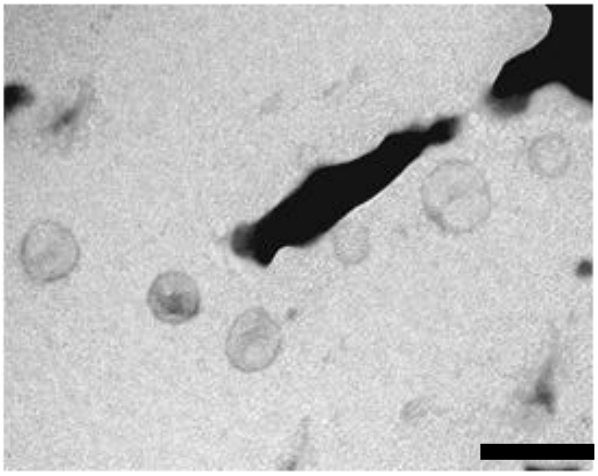
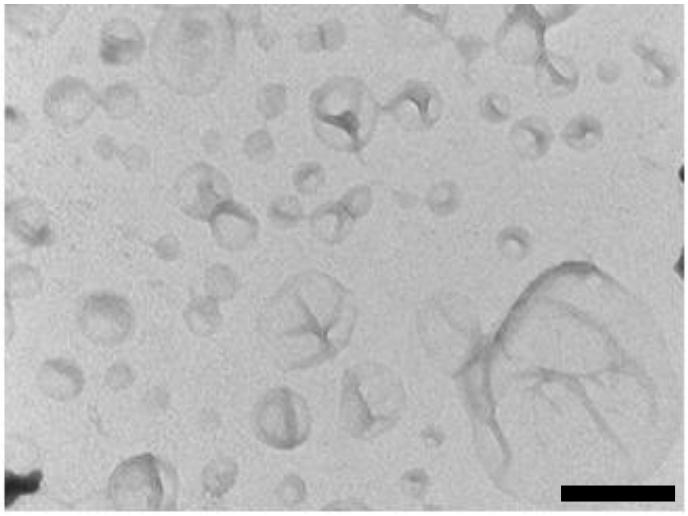


(c.)

Figure 2.9: Transmission electron micrographs of cationic liposomes. **(a)** RUI-128, **(b)** RUI-128-Glu**, (c)** RUI-128-Gal. Bar (a.) = 500 nm, (b.) = 100 nm and (c.) = 200 nm.



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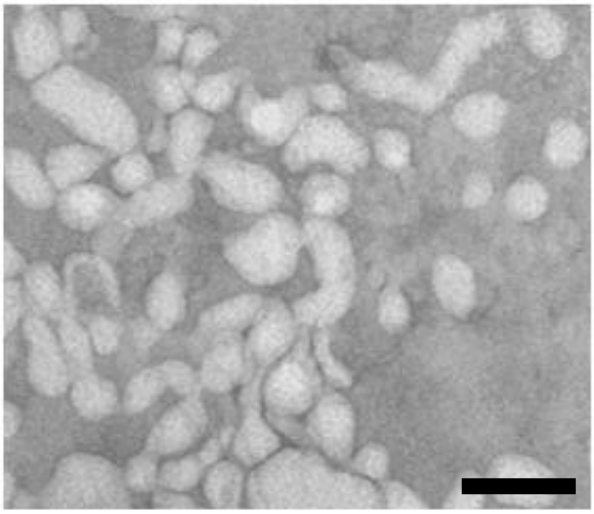

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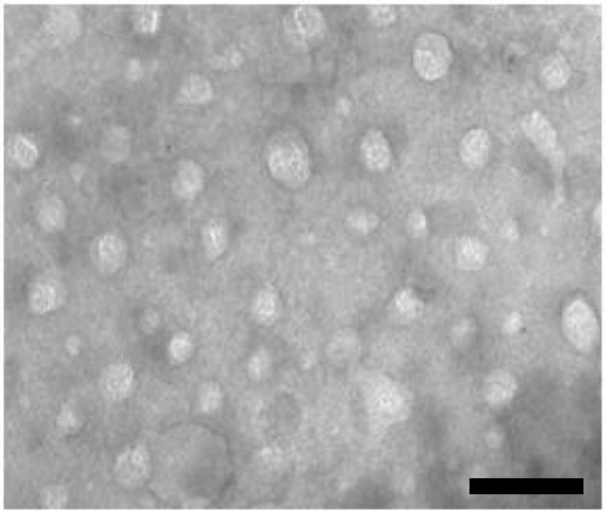


(c.)

Figure 2.10: Transmission electron micrographs of cationic liposomes. **(a)** RUI-129, **(b)** RUI-129-Glu**, (c)** RUI-129-Gal. Bar (a,c) = 200 nm and (b.) = 100 nm.

CHAPTER THREE

3. PREPARATION AND CHARACTERISATION OF LIPOSOME – DNA COMPLEXES OR LIPOPLEXES

3.1. INTRODUCTION

Plasmids are exciting polynucleotides that have been the primary focus of research and application in gene therapy due to the flexibility of their production which offers numerous advantageous prospects. Their capacity to be engineered to incorporate large DNA segments enables transfection of a large open reading frame or combinations of genes (Ahearn and Malone, 1999).

The spontaneous association of cationic liposome head groups with plasmid DNA is the first step in the transfection process mediated by cationic lipid molecules and results in the formation of a heterogeneous lipoplex (Figure 3.1) (Reimer *et al*., 1997; Ferrari *et al.*, 2001). Monocationic lipids notwithstanding their need for a relatively high minimum positive charge, can achieve effective DNA binding through self assembly resulting from their hydrophobic tail alignment. These vectors can be regarded as multipurpose constructs as they provide a multivalent surface to which the plasmid DNA can bind electrostatically, thus offering protection and charge neutralization. This neutralization consequently leads to the collapse of the plasmid DNA structure producing small distinct particles that are compliant with the requirements of the endocytotic pathway (Reimer *et al*., 1997; Uduehi *et al.*, 2001).

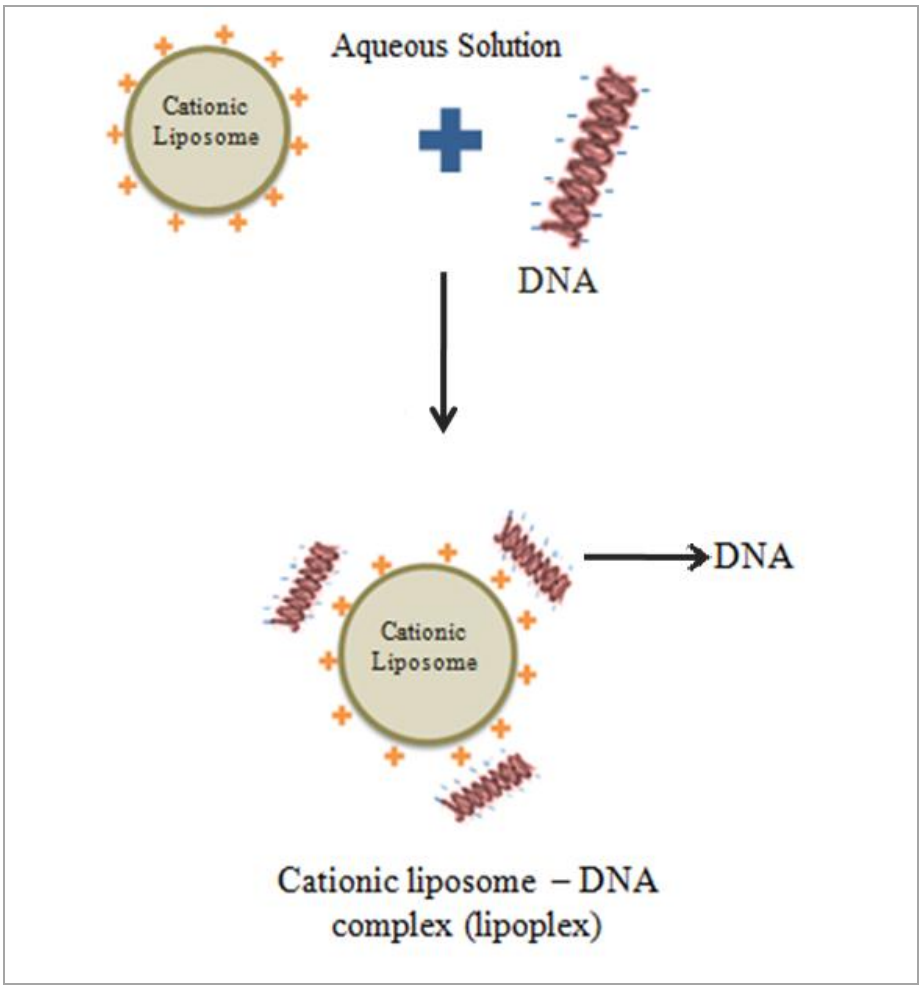


Figure 3.1: Illustration of the formation of cationic liposome-DNA complex showing electrostatic binding of positively charged liposome to negatively charged DNA. (Diagram not drawn to scale).

Typically lipoplexes are prepared by combining DNA and liposome solutions at room temperature and have been drawing attention ever since Felgner and co workers (1997) described their potential use in gene therapy. Lipoplexes may be applied to *in vitro* and *in vivo* systems although endogenous DNAse digestion has been observed for DNA in lipoplexes tested in clinical trials (Obata *et al*., 2009). The specificity and structural requirements for effective transfection are determined by variation associated with liposomal arrangement and are dependent upon both the size and charge ratio of the lipoplex (Russ and Wagner, 2007). The final size of the complex produced is proportional to the mass of the nuleic acid compacted by the vector (Uddin, 2007). Although size is a critical factor to be considered particularly for *in vivo* studies a perfect complex size has not been agreed upon with earlier reports indicating sizes larger than 200 nm were effective and more recent efforts showing the opposite to be true (Rao, 2010).

A positive ratio of liposome to DNA results in positively charged complexes that will repel each other remaining as distinct entities. The DNA association may, however, be limited by the presence of half the positive charges on the interior of the bilayer, resulting in a higher liposome to DNA ratio (Singh and Ariatti, 2003). However, when the complexes are close to the isoelectric point or at lipid concentrations above 0.1 mM large aggregates will form on interaction due to van der Waals forces of attraction overcoming the weak electrostatic forces of repulsion (Lasic *et al*., 1997; Colosimo *et al.*, 1999). Continuous aggregation of lipoplexes results in poor stability over time and necessitates their administration promptly on preparation. The stability of these complexes is influenced by the physical factors such as particle size, zeta potential, the DNA/liposome ratio and the ionic strength of the medium. These factors are assumed to have marked effects on complex formation and ultimately the efficiency of transfection (Wasungu and Hoekstra, 2006; Uddin, 2007).

In order to develop and optimize cationic lipid formulations capable of effective transfer of therapeutic nucleic acids, many studies have concentrated on the structure-function relationship of cationic lipids and DNA (Zuhorn *et al.*, 2002). DNA is known to carry 20 phosphate groups per helical pitch of 34.1 Å and on complete condensation will have its counter-ions released and replaced by the cationic lipid as illustrated in Figure 3.2 (Rädler *et al*., 1997; Wagner *et al*., 2000). This results in an increase in entropy that drives the reaction and compensates for changes in positive enthalpy that is known to occur upon interaction of pure cationic lipids with DNA (Barenholz, 2001; Pozharski and MacDonald, 2002; Goncalves *et al*., 2004). The formation of the liposome-DNA complex is a highly dynamic process involving two steps, the first of which is a fast exothermic process ascribed to the electrostatic binding of the DNA to the surface of the liposome. The second process involves a slow endothermic reaction which may potentially be attributed to the subsequent fusion of the two entities and the associated structural rearrangements (Zhdanov *et al.*, 2002).

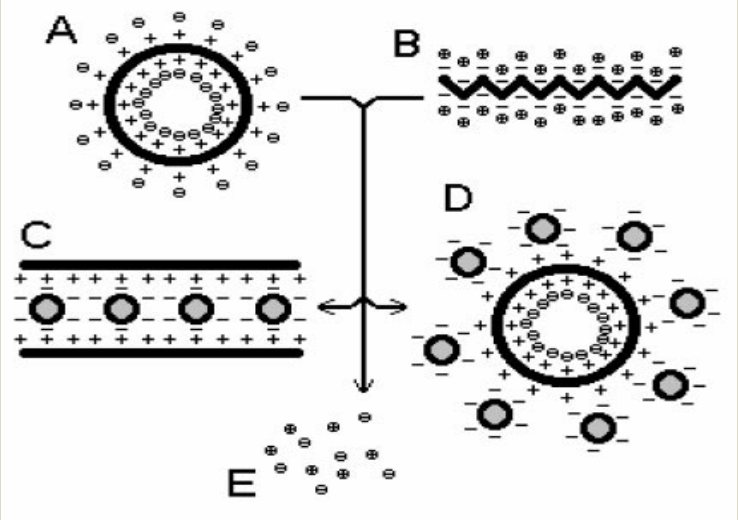


Figure 3.2: Diagram of cationic lipid-DNA complex formation, illustrating the presence of loosely bound counter-ions in the vicinity of both cationic liposomes and DNA. (A) Unilamellar cationic lipid vesicle. (B) Naked DNA. (C) Multilamellar complex. (D) DNA coated cationic lipid vesicle. (E) Released counter-ions resulting in a gain in entropy (Pozharski and MacDonald, 2002).

During lipoplex formation it is understood that the DNA molecule undergoes several changes on condensation and charge neutralization. In addition there is liposome restructuring and fusion producing large aggregates and involving release of its aqueous contents (Khalil *et al.*, 2006; Hoekstra *et al.*,, 2007). Liposomal restructuring was confirmed by electron microscopy presenting elongated rod-like structures together with globular particle aggregates (Gershon *et al*., 1993). After investigation of these aggregates using cryoelectron microscopy and small angle X-ray scattering studies it was found that an internal multilamellar structure existed, within which the hydrated DNA and lipid bilayers alternate (Lasic *et al*., 1997; Radler *et al*., 1997; Goncalves *et al*., 2004).

Despite being widely used for gene transfer, the physical and chemical characteristics of cationic liposome – DNA (CL – DNA) complexes are not well distinguished. A plethora of morphological structures have, however, been revealed using freeze fracture microscopy including beads on a string (Gershon *et al*., 1993) (Figure 3.3), spaghetti and meatball, multilamellar (L_{α}^{C}) and the inverted hexagonal (H_{II}^{C}), in addition to the recently identified map-pin and sliding columnar phase structures (Ma *et al.*, 2007). Questions pertaining to the relationship between the efficiency of delivery as well as the micro- and macroscopic structure of the complex together with the physical mechanisms of complex formation are still rife despite the great strides made in both their biomedical and biophysical studies (Pozharski and MacDonald, 2002).

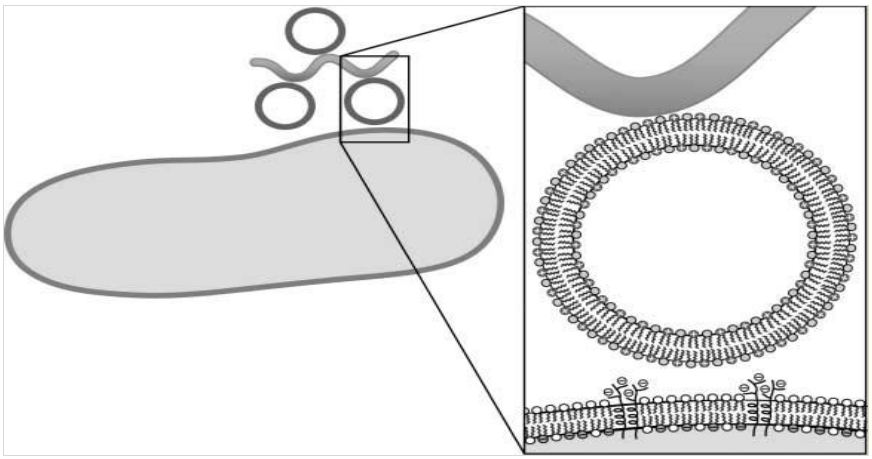


Figure 3.3: Cartoon of a „beads-on-a-string' (i.e. cationic liposomes attached to a string of DNA) complex electrostatically bound to the anionic surface of an animal cell. The enlargement shows a Cationic liposome bound to a section of negatively charged DNA on one side and the plasma membrane with cell surface proteoglycans and negatively charged sulphated groups on the other side (Safinya, 2006).

The "beads on a string" structure involving a strand of DNA distinctly decorated with attached liposomes was one of the first models described and was confirmed by electron microscopy (Gershon *et al.*, 1993; Rädler *et al*., 1997). Thereafter „spaghetti and meatball' structures were proposed that are believed to comprise semi-fused liposomes connecting DNA and tube-like structures that may be representative of lipid coated DNA. DNA stability in addition to the mechanism of nuclear delivery and expression may be affected by the strength of the ionic/hydrophobic interactions of the lipid with DNA. It may be necessary to develop lipid based carriers that yield complexes which are stable against circulating enzymes yet permit the dissociation of lipid from the DNA after entry into the cell through the maintenance of strong ionic/hydrophobic interactions (Reimer *et al*., 1997; Xu *et al*., 1999).

The inverted hexagonal (H_{II}^{C}) and the more abundant lamellar (L_{α}^{C}) liquid crystal phases (Figure 3.4) are equilibrium structures for the CLs – DNA complexes and although capable of comparative transfection, their mechanisms vary greatly as a consequence of their structures. Over and above the rigidity and membrane composition, the structure of the DNA complexes is determined by the molecular shape and preferred lipid curvature. Evidence for this can be seen in the tendency of DOPE to promote the H_{II}^{C} phase, due to its cone-shaped molecular structure (Ewert *et al*., 2006). Whether or not the cationic liposome-DNA complex will take on its preferred morphology is known to be dependent on the chemical nature of the zwitterionic or helper lipid (Khalid *et al.*, 2008). The DNA in this regard is identified in a transmembrane orientation to avoid exposure to the lipid tails, while interaction with the lipid headgroup results in the deformation of the bilayer in the vicinty to DNA. The presence of the neutral helper lipid

DOPE can cause looser binding of the cationic lipid with DNA as can be identified on ethidium bromide intercalation analyses (Wasungu and Hoekstra, 2006). The lamellar form of lipoplexes results in a condensed globular structure containing DNA monolayers sandwiched between cationic bilayers and is characterized by uniform inter-helical spacing. Alternatively the inverted hexagonal phase comprises cationic lipid coated DNA monolayers on a two dimensional hexagonal lattice (Morille *et al*., 2008).

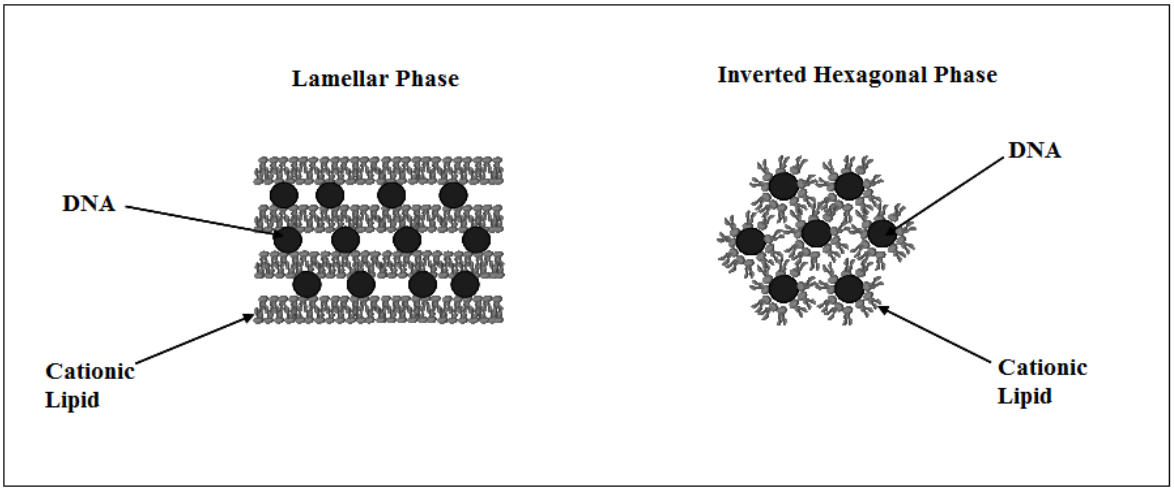


Figure 3.4: Schematic representation of lamellar or inverted hexagonal phase structure in the formation of lipid-DNA complexes (lipoplexes) (Morille *et al.*, 2008).

Since zwitterionic and negatively charged liposomes are unable to effectively encapsulate DNA, the DNA must either be made more lipophilic or carried by cationic liposomes. The liposome –DNA association is believed to occur in one of two ways. The first involves the DNA being electrostatically adsorbed onto the cationic liposome surface and is referred to as the „external model', while the second termed the „internal model' suggests that the DNA is encased in a lipid envelope (Ma *et al.*, 2007). It is thus generally accepted that cationic lipids will offer their DNA cargo more protection than the non-cationic lipids as enclosure within a liposome depends on a good entrapment efficiency to provide optimal protection (Legido and Abell, 1998).

For our own investigations synthesized cationic liposomes were used mixed with plasmid DNA. The resulting lipoplexes were further characterized in respect to their size, shape, degree of condensation, binding and protection of DNA against serum nuclease digestion. Agarose gel electrophoresis was employed to examine the electrostatic interactions between the positive charge of cationic liposomes and the negatively charged anionic DNA molecules which lead to, binding and compaction of the nucleic acid into the lipoplex (Hasegawa *et al*., 2002). The level of DNA condensation within the lipoplexes was

estimated using the ethidium dye displacement protocol. Serum is considered to dramatically affect the biophysical and transfection properties of lipoplexes. It is assumed to lead to significant aggregation causing further associations and disintergration with the subsequent release and destruction of DNA (Li and Huang, 2000; Wattiaux *et al.*, 2000). Cationic liposomes such as those investigated are known to afford DNA in lipoplexes protection against DNAse I digestion; however, upon complex dissociation this protection is lost (Xu and Szoka, 1996). Moreover, the presence and morphology of the lipoplexes was verified using transmission electron microscopy (TEM).

3.2. MATERIALS AND METHODS

3.2.1. Materials

Ultra pure DNA grade agarose was acquired from Bio-Rad Laboratories, Richmond, USA. pBR322 DNA was purchased from Roche Diagnostics, Mannheim, Germany. Ethidium bromide was obtained from Merck, Darmstadt, Germany. All other chemicals were of analytical grade.

3.2.2. Methods

3.2.2.1. Gel retardation assays

In essence the agarose gel mobility shift assay is used to analyze this ability of the positive charges present on liposome molecules to neutralize the negative charges of the DNA phosphate backbone producing electroneutral complexes that cannot migrate during electrophoresis (Huang *et al*., 1998). The charge ratio as can be determined through analysis of mobility shift assays as well as zeta potential are significant in understanding the interaction between the DNA and the cationic lipid within the complex as well as with the cell as a whole. When the charge ratio approaches 1 there is aggregation of the complex together with a reduction in zeta potential (Zhdanov *et al.*, 2002).

3.2.2.1.1. Gel retardation of:

a) Chol-T : DNA,

b) Chol-T-Gal: DNA and,

c) Chol-T-Glu: DNA

A 1% agarose gel was prepared by dissolving 0.2 g of agarose in 18 ml of 18 Mohm water. To this ethidium bromide was introduced at 1µg/ml. This was then left to set for a minimum of 45- 60 minutes.

A fixed amount of pBR322 DNA (0.5 µg) was added to increasing amounts of Chol-T cationic liposome (0, 1, 1.5, 2, 2.5, 3, 3.5, 4 µg), respectively. This was made up to a final volume of 7 µl with HBS. Similar incubation mixtures were set up with the cationic RUI-90 and RUI-92 targeted liposomes at increasing amounts of (0, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 µg). 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 thereafter viewed under UV transillumination and images captured using the Vacutec Syngene G: Box gel documentation system.

3.2.2.1.2. Gel retardation of:

a) RUI-128 : DNA,

b) RUI-128-Gal: DNA and,

c) RUI-128-Glu: DNA

The gel retardation of these liposomes was carried out using the procedure as outlined above in section 3.2.2.1.1. A constant amount of pBR322 DNA was introduced to each sample tube containing increasing amounts (0, 1, 1.5, 2, 2.5, 3, 3.5, 4 µg) of the crown ether cationic liposome RUI-128. The same principle was applied to the two RUI-90 (Gal) and RUI-92 (Glu) targeted liposomes at the liposome amounts of (0, 2, 2.5, 3, 3.5, 4, 4.5, 5 µg). These samples were then incubated at room temperature for 30 minutes. Thereafter samples were incubated and subjected to electrophoresis as described in 3.2.2.1.1.

3.2.2.1.3. Gel retardation of:

a) RUI-129 : DNA,

b) RUI-129-Gal: DNA and,

c) RUI-129-Glu: DNA

The reaction mixtures were prepared according to section 3.2.2.1.1 where varying amounts of RUI-129 : DNA (0, 2, 2.5, 3, 3.5, 4, 4.5, 5 µg), RUI-129-Gal (0, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 µg) and RUI-129- Glu (0, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 µg) were incubated with 0.5 µg of pBR322 plasmid DNA and incubated at room temperature for approximately 30 minutes. The samples were subjected to electrophoresis as in 3.2.2.1.1.

3.2.2.2. Nuclease protection assay

Lipoplexes were preformed and analyzed to determine the protection offered by the different liposome preparations to the DNA against nuclease attack. Varying amounts of cationic liposomes (Table 3.1) were added to a constant amount of pBR322 DNA (1 µg). This was made up to a final 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%, based on published methods obtained from literature (Huang et al., 1998). Two controls were employed, a negative control containing only pBR322 DNA and a positive control containing only pBR322 DNA and FBS. The samples were then incubated for 4 hours at 37°C. After the incubation period, the chelator ethylenediaminetetraacetic acid (EDTA) was added to the samples to a final concentration of 10 mM to stop the nuclease reaction and sodium dodecyl sulphate (SDS) to a final concentration of 0.5% ($^{w}/_{v}$), to liberate DNA for migration. The samples were thereafter incubated for a further 20 minutes at 55°C. 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: Nuclease protection assays. Component ratios of liposomes as indicated were incubated with pBR322 DNA (1 µg) and FBS (10%, v/v).

LIPOSOME PREPARATION	LIPOSOME AMOUNT (µg)			DNA (µg)
Chol-T	3	4	5	1
Chol-T: Gal	5	6	7	1
Chol-T: Glu	5	6	7	1
RUI-128	6	7	8	1
RUI-128: Gal	7	8	9	1
RUI-128: Glu	9	10	11	1
RUI-129	5	6	7	1
RUI-129:Gal	3	4	5	1
RUI-129: Glu	3	4	5	1

3.2.2.3. Ethidium bromide intercalation assay

In order to ascertain the ability of the liposomes to compact DNA an ethidium displacement or ethidium bromide intercalation assay was carried out using a Shimadzu RF – 551 Spectrofluorometric Detector which was set at an excitation wavelength of 520 nm and an emission wavelength of 600 nm. . Ethidium bromide, a widely used label for fluorescent detection of DNA, intercalates between base pairs of the DNA double helix producing an intense fluorescence (Xu and Szoka, 1996). The level of condensation is important particularly at the point of endosomal escape for delivery of DNA therapeutic drugs (Lasic *et al*., 1997).

Initially 10 µl (1 µg) of an ethidium bromide stock solution (100 µg/ml) was added to 500 µl of HBS in a quartz microcuvette and measured to obtain a baseline relative fluorescence reading of zero. The 100% relative fluorescence was set by introducing 24 µl (6 µg) of pBR322 DNA to the HBS-ethidium bromide mixture. Thereafter 1 µl aliquots (approximately 2.5 µg) of liposome preparation were systematically

added to the mixture and readings taken until approximately 12 - 25 μg of each liposome preparation had been added or until a plateau in readings had been reached. In order to ensure an accurate reading the solution was mixed to promote full dispersion of the liposome in the solution thereby encouraging complete compaction of the DNA. The results obtained were plotted relative to 100% fluorescence. This procedure was employed for all liposome preparations discussed.

3.2.2.4. Transmission electron microscopy

Characterization of cationic liposome – DNA complexes was carried out as outlined in chapter 2 section 2.2.2.3.

3.2.2.5. Statistical analysis

A comparison of the particle sizes of the different lipoplexes was analyzed using a matched student t-test. P-values of less than 0.05 were considered to be of statistical significance.

3.3. RESULTS AND DISCUSSION

3.3.1. Gel retardation assays

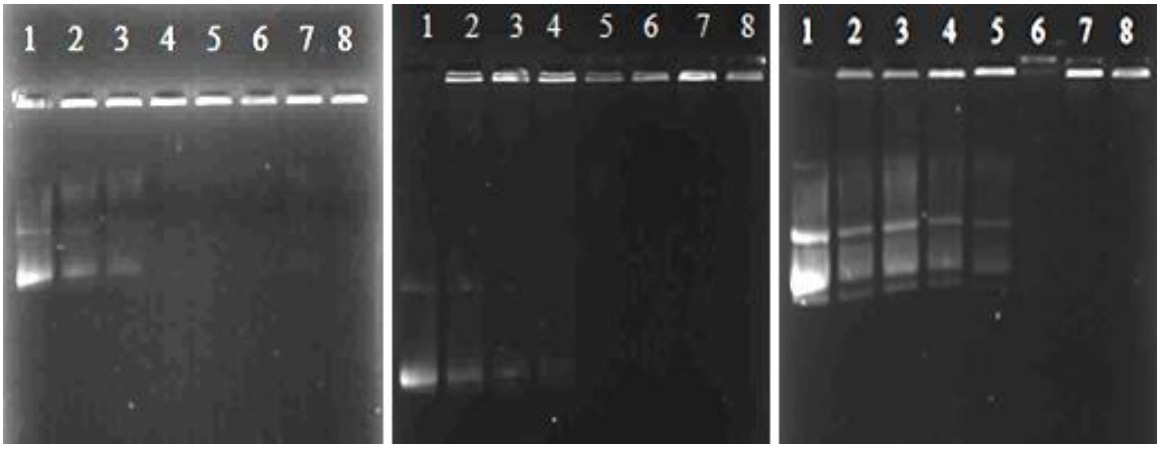
3.3.1.1. Gel retardation of:

(a) Chol-T : DNA,

(b) Chol-T: Gal: DNA and,

(c) Chol-T: Glu: DNA

The results for the agarose gel electrophoresis of these three liposome formulations can be seen in Figure 3.5. The optimal ratio of plasmid DNA binding achieved by each liposome formulation is given in Table 3.2.



(a) **(b)** **(c)**

Figure 3.5: Agarose Gels showing the liposome – DNA binding by the different cationic liposome preparations. Reaction mixtures of samples in lanes 1 – 8 consisted of varying amounts of liposome in 20 mM HEPES, 150 mM NaCl (pH 7.5), shown below. pBR322 DNA was maintained at a constant 0.5 µg.

(a.) Chol-T, (0, 1, 1.5, 2, 2.5, 3, 3.5, 4 µg)

(b.) Chol-T-Gal, (0, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 µg) and

(c.) Chol-T-Glu (0, 1, 1.5, 2, 2.5, 3, 3.5, 4 µg)

Table 3.2: DNA – Liposome ratios of complete and optimal binding

Liposome Preparation	**Optimal Retardation conditions**		
	Liposome (µg)	**DNA: Liposome Ratio (w/w)**	**DNA: Liposome Charge Ratio (-ve/+ve)**
Chol-T	2	1 : 4	1 : 1.1
Chol-T-Gal	3	1 : 6	1 : 1.6
Chol-T-Glu	3	1 : 6	1 : 1.6

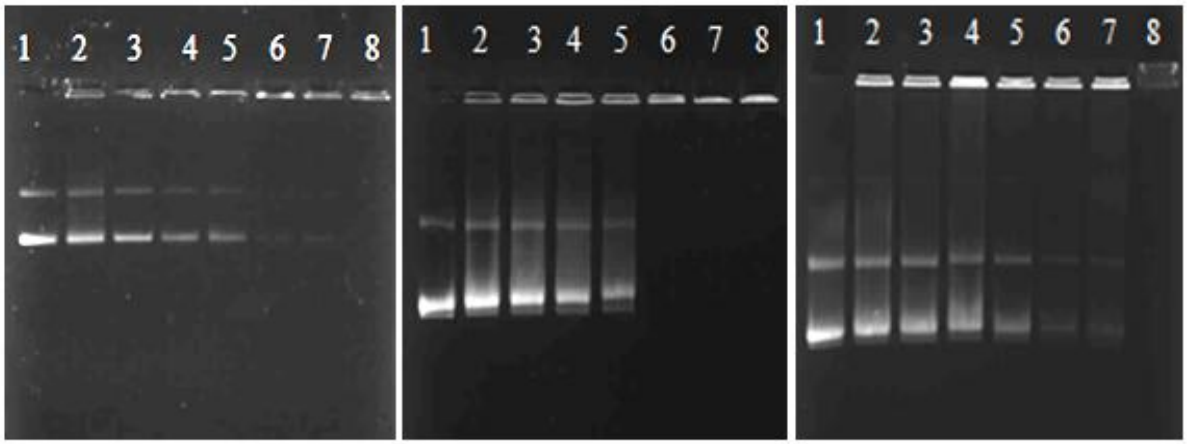
3.3.1.2. Gel retardation of:

(a) RUI-128 : DNA,

(b) RUI-128: Gal: DNA and,

(c) RUI-128: Glu: DNA

Figure 3.6 below shows the results of the agarose gel retardation studies of these three liposome formulations. The retardation information gathered has been compiled in Table 3.3.



(a) **(b)** **(c)**

Figure 3.6: Agarose Gels showing liposome – DNA binding by the different cationic liposome preparations (a – c). Reaction mixtures of samples in lanes 1 – 8 consisted of varying amounts of liposome in 20 mM HEPES, 150 mM NaCl (pH 7.5), shown below. pBR322 DNA was maintained at a constant 0.5 µg.

(a) RUI-128, (0, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 µg)

(b) RUI-128-Gal, (0, 2, 2.5, 3, 3.5, 4, 4.5, 5 µg) and

(c) RUI-128-Glu (0, 2, 2.5, 3, 3.5, 4, 4.5, 5 µg)

Table 3.3: DNA – Liposome ratios of complete and optimal binding

Liposome Preparation	**Optimal Retardation conditions**		
	Liposome (µg)	**DNA: Liposome Ratio (w/w)**	**DNA: Liposome Charge Ratio (-ve/+ve)**
RUI-128	3.5	1 : 7	1 : 1.9
RUI-128-Gal	4	1 : 8	1 : 1.8
RUI-128-Glu	5	1: 10	1 : 2.3

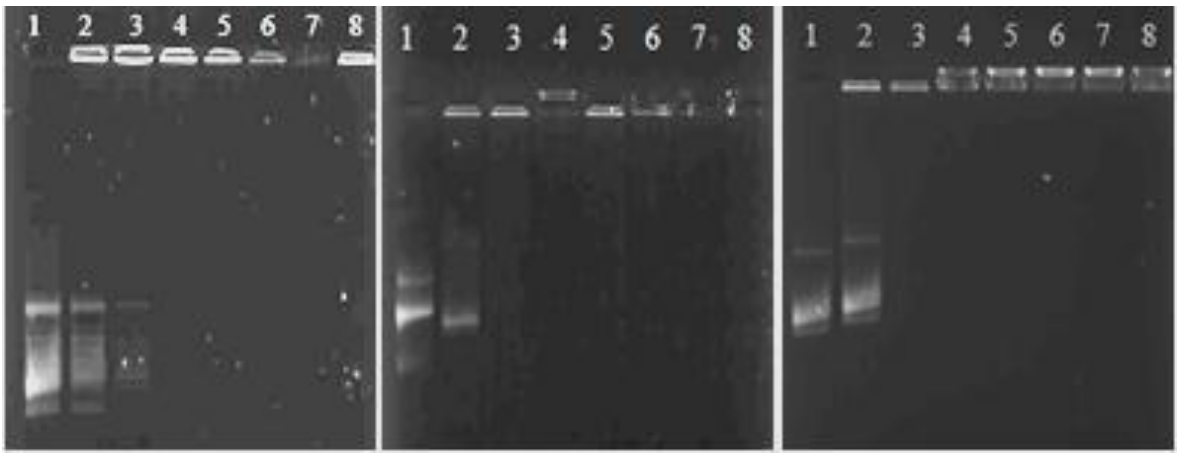
3.3.1.3. Gel retardation of:

(a) RUI-129 : DNA,

(b) RUI-129: Gal: DNA and,

(c) RUI-129: Glu: DNA

Figure 3.7 shows the results of the agarose gel electrophoresis of the above samples. The charge and DNA binding ratios are outlined in Table 3.4.



(a) (b) (c)

Figure 3.7: Agarose Gels showing liposome – DNA binding by the different cationic liposome preparations. Reaction mixtures of samples in lanes 1 – 8 consisted of varying amounts of liposome in 20 mM HEPES, 150 mM NaCl (pH 7.5), shown below. pBR322 DNA was maintained at a constant 0.5 µg.

(a) RUI-129, (0, 2, 2.5, 3, 3.5, 4, 4.5, 5 µg)

(b) RUI-129-Gal, (0, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 µg) and

(c) RUI-129-Glu (0, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 µg)

Table 3.4: DNA – Liposome ratios of complete and optimal binding

Liposome Preparation	**Optimal Retardation conditions**		
	Liposome (µg)	**DNA: Liposome Ratio (w/w)**	**DNA: Liposome Ratio (-ve/+ve)**
RUI-129	3	1 : 6	1 : 1.97
RUI-129-Gal	2	1 : 4	1 : 0.93
RUI-129-Glu	2	1 : 4	1 : 0.93

From the results it is evident that all liposome formulations are capable of binding DNA successfully. For all gel retardation studies (Figure 3.5 – 3.7) the first lane of all gels contained the control pBR322 DNA alone, displaying the three conformations of undigested DNA migrating through the gel. These bands moving slowest to fastest were the nicked, linearised and supercoiled DNA. This plasmid DNA migrates freely toward the cathode of the electrophoresis apparatus. Upon introduction of liposome at varying amounts, less DNA is able to move through the agarose gel matrix. This inhibition of DNA migration will proceed to a point at which the DNA is completely bound or retarded by the liposome preparation. This is known as the point of electroneutrality, where as mentioned earlier all negative charges are completely titrated by the cationic liposome in what is termed an electroneutral complex. This is evidenced by the DNA retained in the wells of the agarose gel. Beyond this point the complexes possess an excess of positive charge and are drawn toward the anode. Aggregated complexes may also float out of wells.

The targeted formulations of both Chol-T and RUI-128 were required at higher quantities for DNA binding as shown in Tables 3.2 and 3.3. This was in contrast to the targeted RUI-129 liposomes which were able to completely bind DNA at lower concentrations than the untargeted variety (Table 3.4).

The Chol-T preparation (Figure 3.5.a.) showed the ability to form electroneutral complexes at a DNA ratio of 1:4 using 2 μg of liposome to completely neutralize the negative charges of 0.5 μg of plasmid pBR322 DNA. This is in contrast to earlier studies (Singh *et al*., 2001) that have reported slightly higher ratios of DNA retardation with the Chol-T liposomes. This interaction between the cationic liposome and the plasmid DNA backbone is known to affect the organization of the solvent present around the lipoplex ultimately resulting in the restriction of the number of sites available for plasmid DNA binding (Ferrari *et al.*, 2001). This could account for some preparations resulting in more or less restriction of these binding sites depending on their composition and interaction with the surroundings. The resulting potential shielding effect could be responsible for the higher amount of liposome required for optimal binding of the DNA by the targeted preparations. The targeting ligands in this regard may potentially adumbrate some of the positive charge centers. Moreover evaluation of the RUI-128 crown ether (CE) liposomes showed a larger concentration of the targeted preparations being employed for optimal DNA retardation. However, on closer evaluation of their respective charge ratios, almost all formulations whether targeted or not required close to 2 positive charges to neutralize 1 negative. The large cavity of the crown ether head may play a role in this potential masking of the positive charges. The aza-18-crown-6 employed is known for its ability to bind large cations, potassium in particular. Its bulky nature may play an, as yet, ill defined role in DNA binding.

In contrast the RUI-129 liposomes with their smaller crowns provide less inhibitory shielding potential. Remarkably the targeted formulations here were able to achieve effective DNA retardation at a ratio of 1:4, lower than that achieved by the untargeted liposome. It may be possible that rearrangements on formulation has resulted in more of the cationic Chol-T cytofectin being present at the liposome surface leading to these preparations being able to bind DNA at lower concentrations.

In all liposome preparations there exists an excess of the neutral lipid DOPE in contrast to the other components. It is thus possible that this excess could cause a greater internalization of the Chol-T during formulation. If this were to be the case, then this effect would lead to different consequences with respect to the different formulations. The targeted Chol-T-Gal and Chol-T-Glu having lower amounts of Chol-T in their formulation than the untargeted Chol-T liposome require more liposome to completely retard the pBR322 plasmid DNA. Once again the same could be said for the targeted and untargeted RUI-128 liposomes, which showed a higher amount of positive charges required for optimal DNA binding. Finally on application of these suppositions to the RUI-129 CE preparations we find that despite this possible effect of DOPE in the formulations, the targeted liposomes were able to produce optimal DNA retardation using very low amounts of liposome. Therefore with respect to these particular preparations, the strong binding of the CE with small cations (Na^+) could assist significantly in improving the interaction of the liposomes with the DNA through the formation of an additional cationic head. The same may occur for the RUI-128 liposomes; however, their crown could prove more inhibitory than advantageous as a result of the potential steric hindrance associated with the much larger cavity size.

The information extracted from these agarose gel electrophoresis studies will be used to produce transfection complexes for transfection experiments in the HEK293 and HepG2 cell lines (Chapter 4). The resulting lipoplexes show sensitivity to the DNA-liposome charge ratio where low charge ratios are associated with poor transfection due to possible nucleic acid exposure. Although cationic liposomes are capable of negative charge neutralization the quantity of DNA required and the optimal ratio of DNA phosphate to cationic lipid for efficient transfection vary for the different cell types (Reimer *et al*., 1997). As such the reaction complexes were formulated at the optimal DNA: liposome ratio as well as at a ratio before (slightly negative) and one after (slightly positive).

3.3.2. Nuclease Protection Assay

The results from the serum nuclease protection studies as carried out by agarose gel electrophoresis can be seen in Figures 3.8 – 3.10.

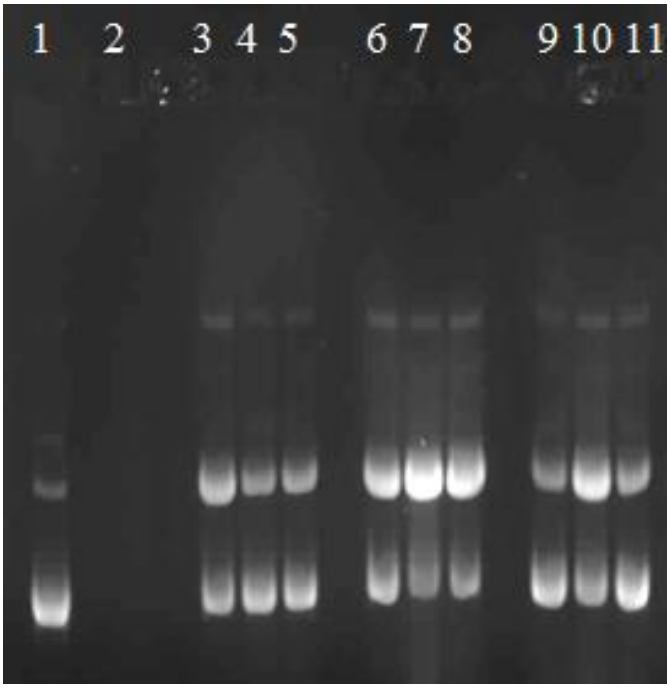


Figure 3.8: Serum nuclease protection assay of the DNA-cationic liposome complexes containing Chol-T, Chol-T-Gal and Chol-T-Glu, respectively.

Lane 1: pBR322 DNA (1 μg).

Lane 2: Naked pBR322 DNA (1 μg) in the presence of 10% (v/v) serum for 4 hours.

Lanes 3-5: DNA-cationic liposome complexes with Chol-T (3, 4 and 5 μg).

Lanes 6-8: DNA-cationic liposome complexes with Chol-T/ Gal (5, 6 and 7 μg).

Lanes 9-11: DNA-cationic liposome complexes with Chol-T/ Glu (5, 6 and 7 μg).

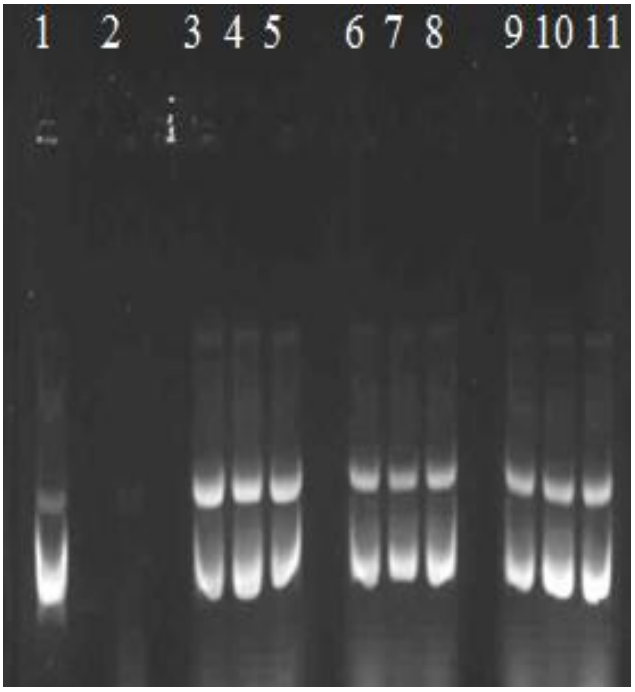


Figure 3.9: Serum nuclease protection assay of the DNA-cationic liposome complexes containing RUI-128, RUI-128-Gal and RUI-128-Glu, respectively.

Lane 1: pBR322 DNA (1 μg).

Lane 2: Naked pBR322 DNA (1 μg) in the presence of 10% (v/v) serum for 4 hours.

Lanes 3-5: DNA-cationic liposome complexes with RUI-128 (6, 7 and 8 μg) with serum 10%.

Lanes 6-8: DNA-cationic liposome complexes with RUI-128-Gal (7, 8 and 9 μg) with 10% foetal bovine serum.

Lanes 9-11: DNA-cationic liposome complexes with RUI-128-Glu (9, 10 and 11 μg) with 10% foetal bovine serum

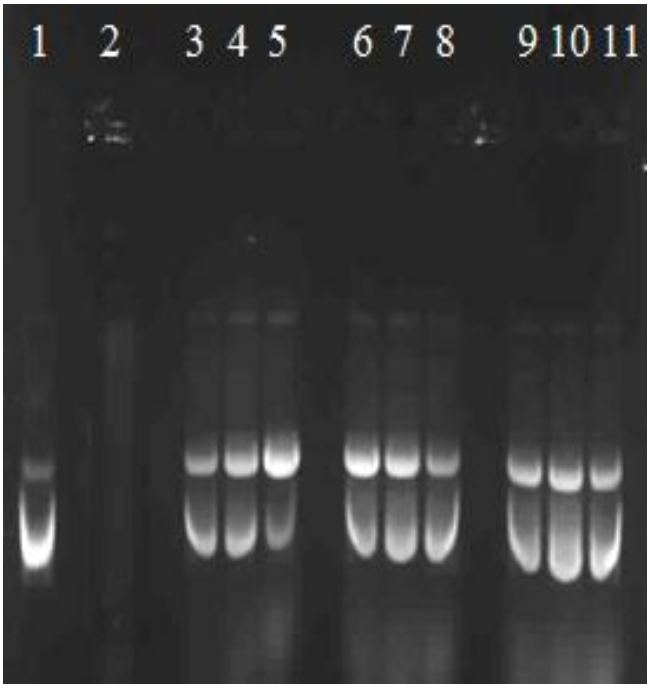


Figure 3.10: Serum nuclease protection assay of the DNA-cationic liposome complexes containing RUI-129, RUI-129-Gal and RUI-129-Glu, respectively.

Lane 1: pBR322 DNA (1 μg).

Lane 2: Naked pBR322 DNA (1 μg) in the presence of 10% (v/v) serum for 4 hours.

Lanes 3-5: DNA-cationic liposome complexes with RUI-129 (5, 6 and 7 μg).

Lanes 6-8: DNA-cationic liposome complexes with RUI-129-Gal (3, 4 and 5 μg).

Lanes 9-11: DNA-cationic liposome complexes with RUI-129-Glu (3, 4 and 5 μg).

In this analysis the different liposomes were tested for their ability to protect the integrity of the DNA to which they are complexed. The ratios for this were determined by the gel retardation or mobility shift studies in section 3.3.1. This assay provides information as to the ability of the complexes to successfully navigate the conditions of the *in vivo* system where they will be faced with different cellular barriers, particularly serum nucleases.

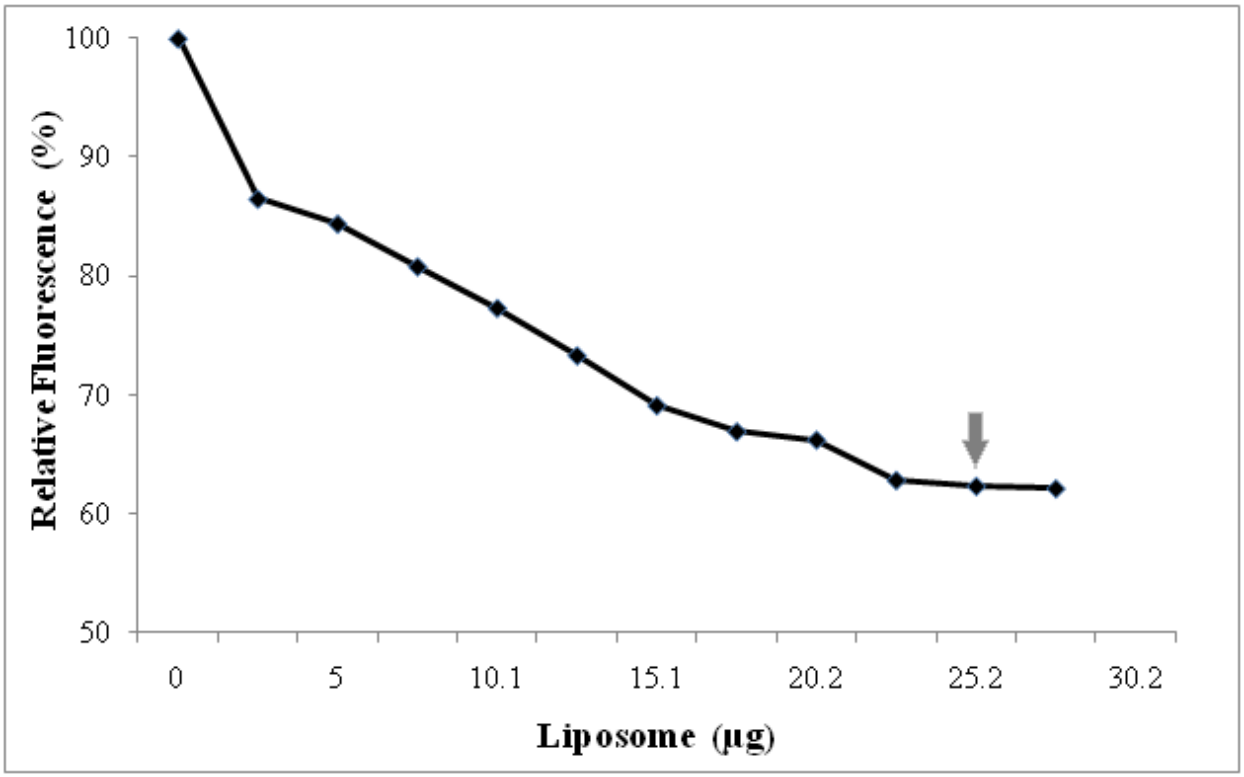
Two controls were employed for all three agarose gels shown above, viz. pBR322 plasmid DNA alone, and pBR322 plasmid DNA in the presence of 10% foetal bovine serum. The reasoning behind these controls is to identify the extent of the serum nuclease degradation on naked DNA compared to that of undigested DNA. The DNA is complexed with varying amounts of liposome and incubated in 10% serum (Huang *et al*., 1998). Once liberated the DNA migrates through the agarose gel so that its integrity or extent of degradation may be observed.

From this study it is evident that the introduction of liposomes to the DNA provides complete protection against the effect of nucleases present in the serum (Figure 3.8 – 3.10). All liposomes afforded the DNA protection at all three ratios analyzed, i.e., at, before and after electroneutrality or the optimum DNA: liposome ratio. Lane 2 in all gels gives a clear indication of the effect of serum on DNA, where all DNA has been degraded. In contrast lane 1, with no serum shows intact plasmid DNA. Since the liposomes were able to release the DNA in its original form we can confirm their ability to protect DNA hence underscoring their value as suitable transfection agents. The banding pattern observed for DNA released from all investigated lipoplexes shows a clearly defined third band, which may be identified as the nicked or open circular conformation of the DNA. High charge ratios of liposome : DNA correlate with a high capacity to circumvent the serum effected impediment on lipofection (Chaudhuri, 2002). A possible explanation for this protection could be the degree of binding and compaction involved in the formation of the highly organized supramolecular structure of the lipoplex and the electrostatic forces responsible for it.

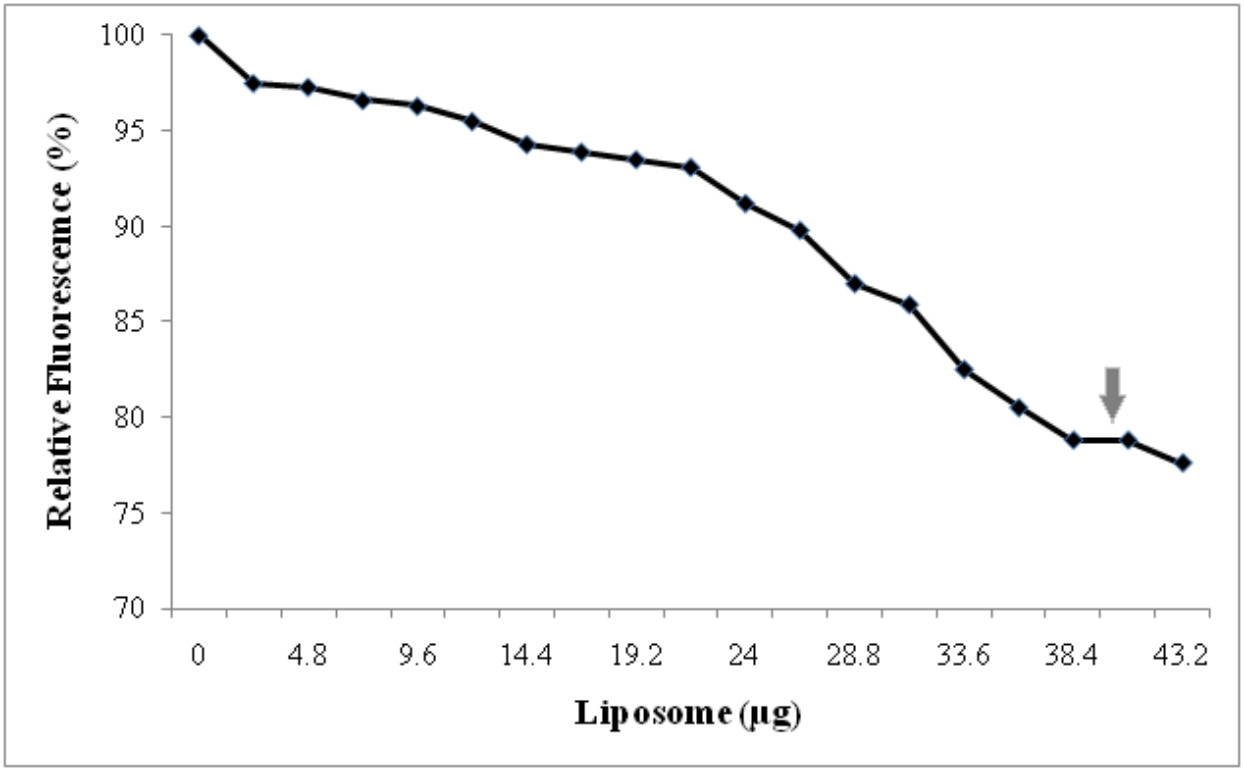
Nuclease digestions were conducted at 37 ^{o}C for 4 hours, the same conditions under which cells are exposed to lipoplexes in transfection studies *in vitro*. Hence it can be taken from this study that all the liposomes formulated show a good degree of serum stability and protection of DNA under these conditions and in the presence of 10% foetal bovine serum.

3.3.3. Ethidium Bromide Intercalation Assay

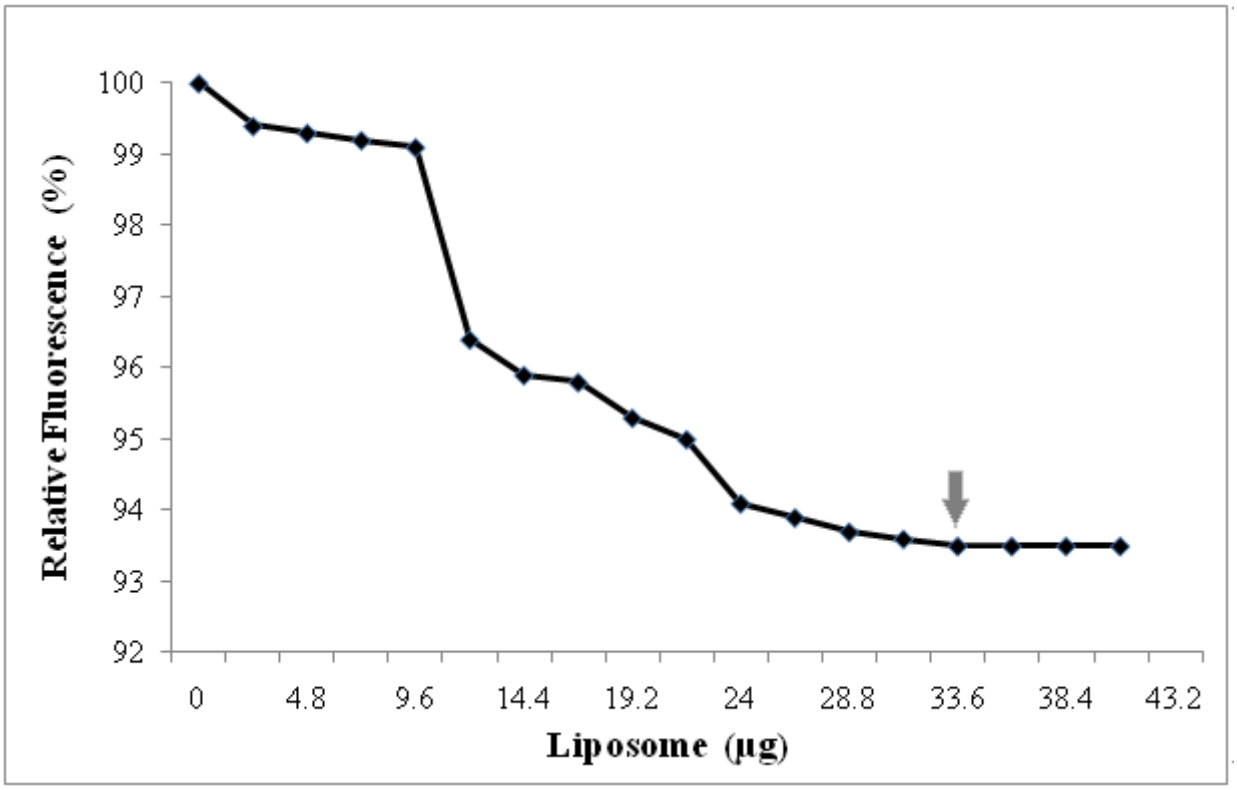
DNA is known to be condensed by liposomes in lipoplexes, a property that was confirmed with the ethidium bromide dye displacement protocol as outlined in section 3.2.2.3. The results from this study on introduction of liposomes to a solution of DNA and ethidium bromide can be seen in Figures 3.11 – 3.13.


Relative Fluorescence (%)
100
90
80
70
60
50
0
5
10.1
15.1
20.2
25.2
30.2
Liposome (µg)

(a)

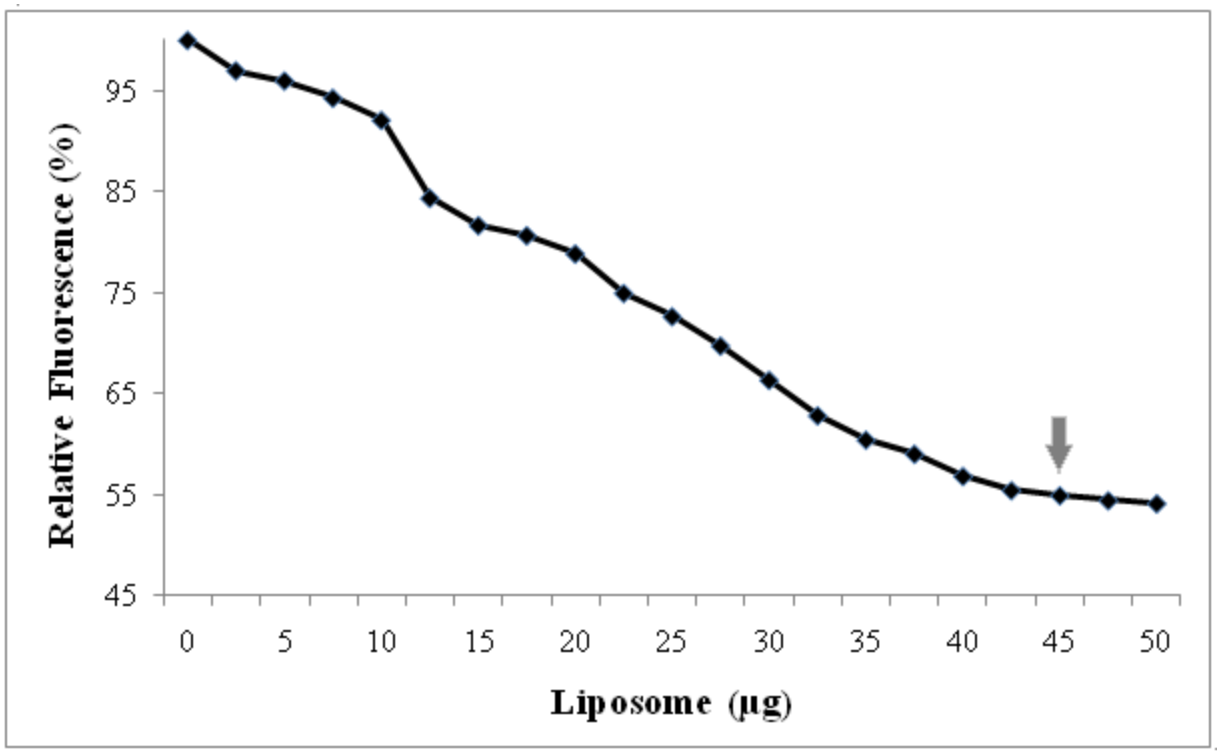

Relative Fluorescence (%)
100
95
90
85
80
75
70
0
4.8
9.6
14.4
19.2
24
28.8
33.6
38.4
43.2
Liposome (µg)

(b)

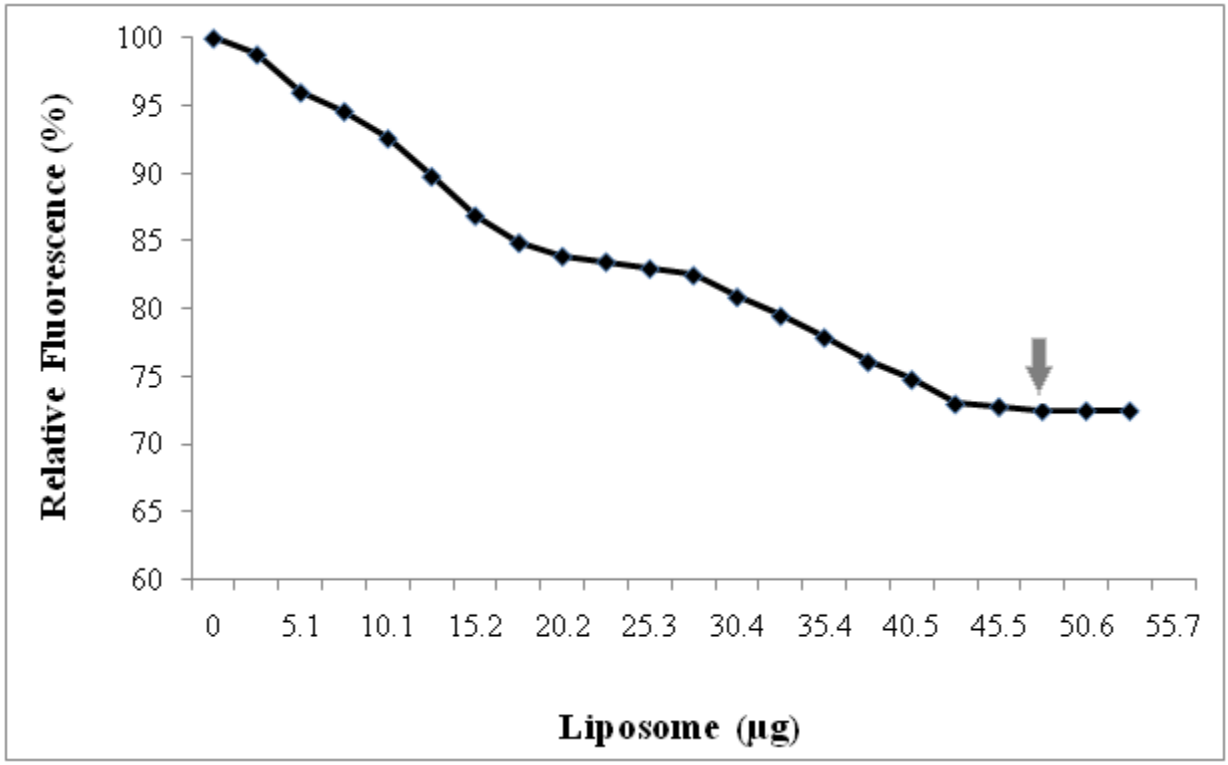


(c)

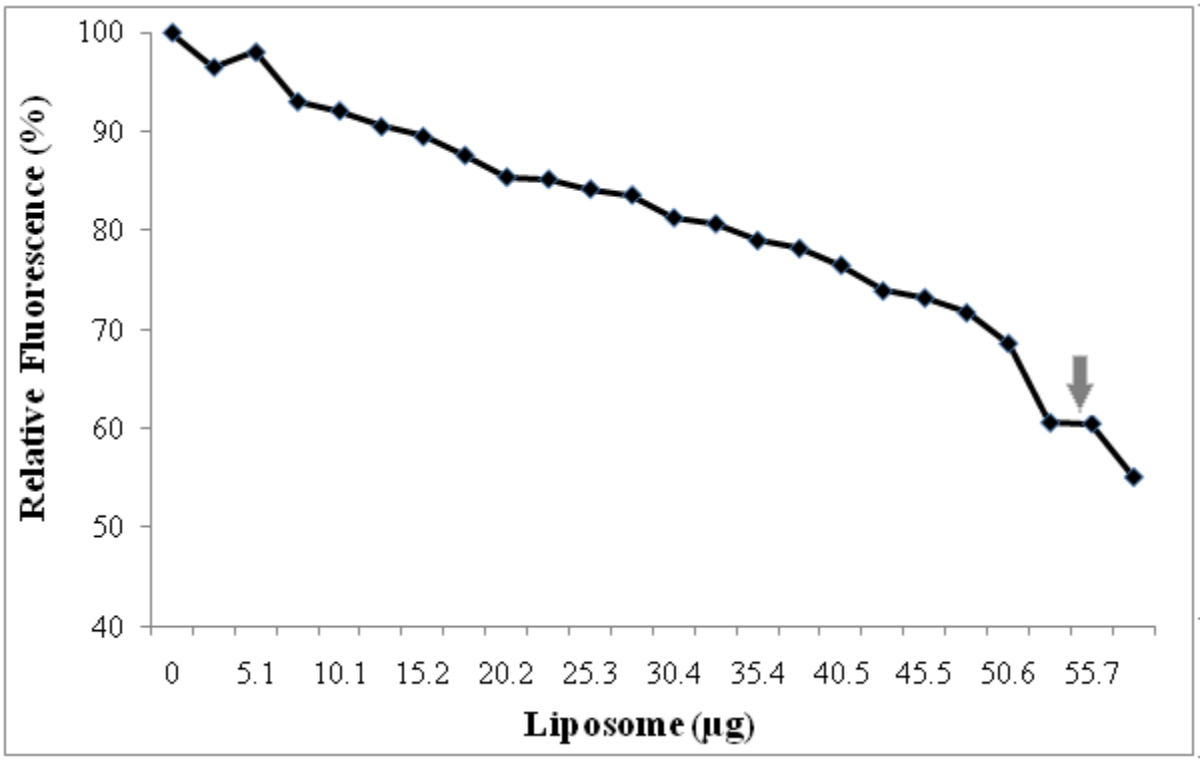
Figure 3.11: Ethidium bromide intercalation assay for cationic liposomes. The volume of incubation mixtures was 500 µl and contained 6 µg pBR322 and increasing amounts of liposome. **(a)** Chol-T **(b)** Chol-T-Gal and **(c)** Chol-T-Glu.


Relative Fluorescence (%)
95
85
75
65
55
45
0
5
10
15
20
25
30
35
40
45
50
Liposome (µg)

(a)

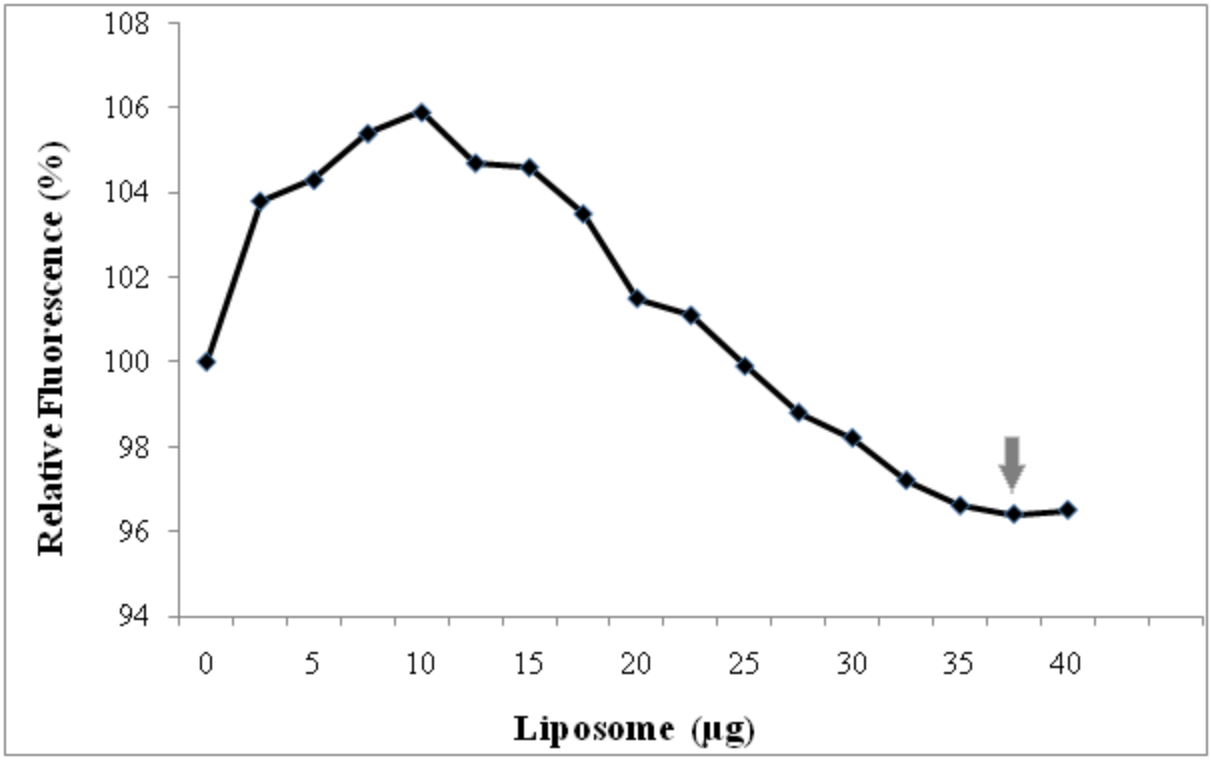

Relative Fluorescence (%)
100
95
90
85
80
75
70
65
60
0
5.1
10.1
15.2
20.2
25.3
30.4
35.4
40.5
45.5
50.6
55.7
Liposome (µg)

(b)

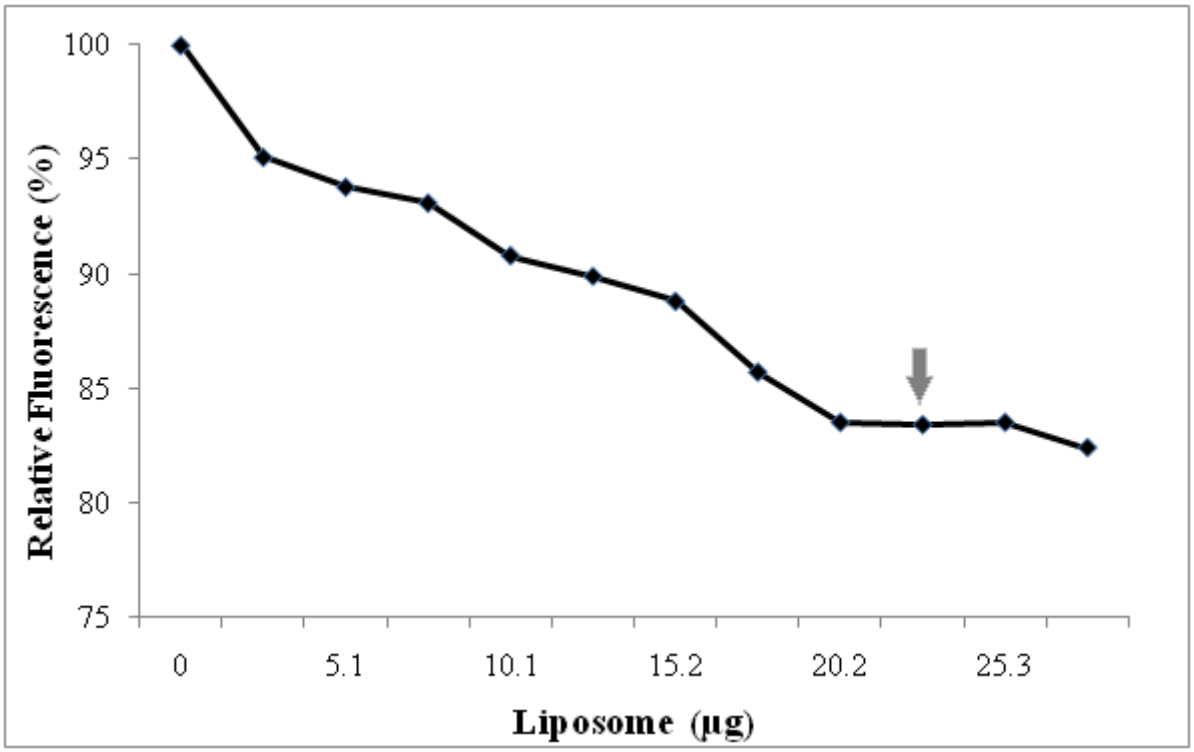


(c)

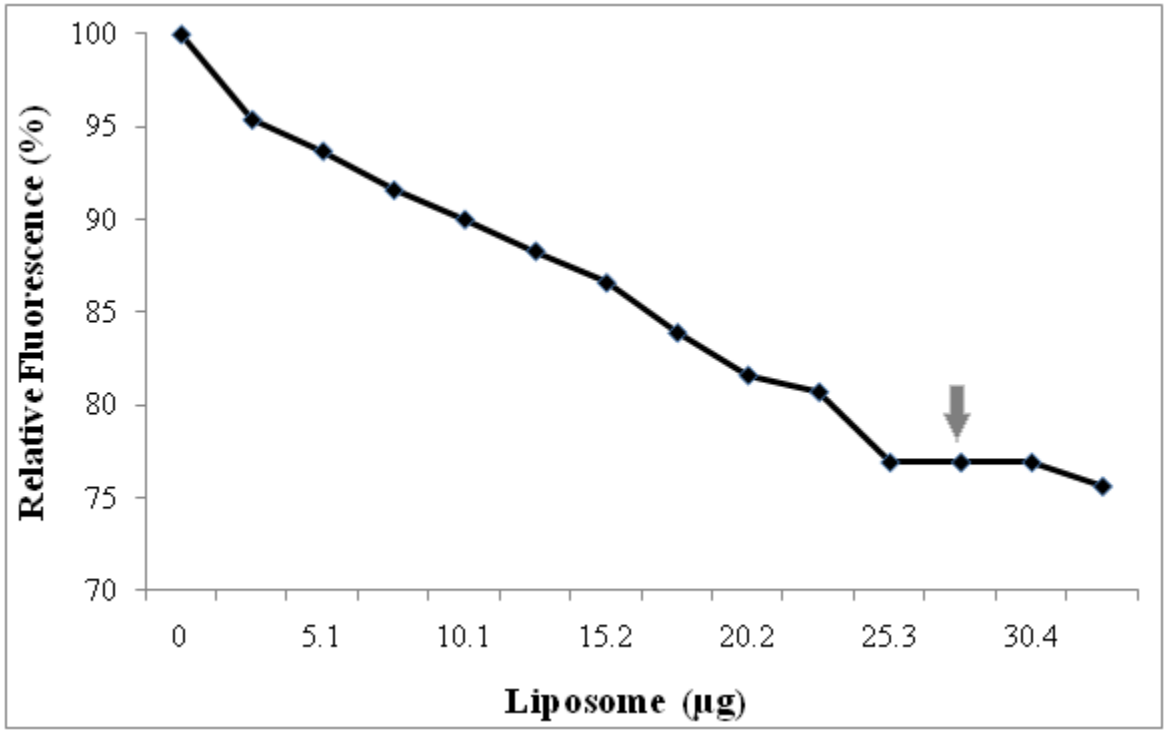
Figure 3.12: Ethidium bromide intercalation assay for cationic liposomes. The volume of incubation mixtures was 500 µl and contained 6 µg pBR322 and increasing amounts of liposome. **(a)** RUI-128 **(b)** RUI-128-Gal and **(c)** RUI-128-Glu.


108
106
104
102
100
98
96
94
Relative Fluorescence (%)
0
5
10
15
20
25
30
35
40
Liposome (µg)

(a)


100
95
90
85
80
75
Relative Fluorescence (%)
0
5.1
10.1
15.2
20.2
25.3
Liposome (µg)

(b)



(c)

Figure 3.13: Ethidium bromide intercalation assay for cationic liposomes. The volume of incubation mixtures was 500 µl and contained 6 µg pBR322 and increasing amounts of liposome. **(a)** RUI-129 **(b)** RUI-129-Gal and **(c)** RUI-129-Glu.

As mentioned earlier, this assay can be used to analyze the interaction of liposomes and DNA within lipoplexes. In this assay the ethidium ions intercalate between the base pairs of the DNA double helix and emit an intense fluorescence at 600 nm when excited at 500 nm. Consequently the liposome-DNA association can be estimated by the relative decrease in fluorescence due to the liposome interacting with the DNA and displacing the ethidium dye (Hasegawa *et al*., 2002; Ueno *et al.*, 2007). The decrease in the initial levels of fluorescence is brought about by the tight liposome/DNA interaction leading to DNA encapsulation restricting intercalation of the ethidium bromide stain (Hirashima *et al*., 2007).

In our studies all liposome preparations were able to successfully displace the ethidium dye and bind the plasmid DNA. To the initial solution of plasmid DNA-ethidium bromide solution increasing amounts of the different liposomes were introduced which formed complexes with the DNA. This was continued until fluorescence decay reached a plateau or point of inflection. This point of leveling off represents the DNA: liposome ratio at which there is maximal DNA condensation and dye displacement.

All cationic liposomes prepared produced a decline in the fluorescence emitted to this point of inflection with the DNA: liposome ratios identified here showing relatively good correlation with those achieved in the gel retardation studies section 3.3.1., Tables 3.2 – 3.4. The Chol-T, Chol-T-Gal and Chol-T-Glu liposomes were found to maximally condense DNA at DNA: liposome ratios of 1: 4.62, 1: 1.2 and 1: 1. The RUI-128, RUI-128-Gal and RUI-Glu preparations produced a reduction in fluorescence emitted down to a plateau corresponding to the DNA: liposome ratios of 1: 8.3, 1: 6.64 and 1: 6.68. Finally the RUI-129, RUI-129-Gal and RUI-129-Glu liposomes showed the ability to completely complex DNA at ratios of 1: 6.6, 1: 4.2 and 1: 5.06. The slight differences observed between the two assays could be attributed to the highly sensitive nature of this assay. Alternatively it could be put down to the differences that exist between this assay and the agarose gel retardation assay. Although in general this may be considered as a corroborative study for the gel mobility shift analyses, some discrepancies do exist. In this regard the retardation studies are based on charge neutralization involving the complete association of the cationic liposome positive charges with the negative charges of the DNA backbone. In contrast this assay has its primary focus based on the condensation of DNA.

In this assay the percentage of ethidium bromide displaced, as identified in Figures 3.8 – 3.10, is a reflection of the degree of DNA compaction. It is important to note also that the degree of compaction should not be too high as this is indicative of the DNA being bound too tightly and as such may limit the later steps of the transfection process when complex dissociation is essential. On the other hand it should also not be too low as this may induce early dissociation and subsequent DNA degradation. The Chol-T, Chol-T-Gal and Chol-T-Glu formulations showed a 40%, 25% and less than 10% decrease in fluorescence respectively at their points of inflection. The RUI-128, RUI-128-Gal and RUI-Glu preparations demonstrated an approximate 40%, 30% and 40% decrease in fluorescence respectively. The RUI-129, RUI-129-Gal and RUI-129-Glu CE liposomes produced a decrease in fluorescence of approximately 10%, 20% and 30% respectively. The RUI-129 liposome also showed a slight increase in fluorescence prior to displacing the ethidium bromide fluorophore.

This increase could be due to the stain continuing to intercalate between the DNA even on addition of the first few micrograms of liposome. The relatively low percentage of fluorescence reduction is indicative of a low degree of DNA compaction. Despite the low concentration of liposome required for DNA complexation, the actual condensation of the DNA is not effective and may result in the early disengagement of the DNA from the liposome carrier, which may or may not be advantageous depending on the conditions presented.

3.3.4. Transmission Electron Microscopy

Transmission electron microscopy using the negative staining-vitrification protocol (outlined in Chapter 2 section 2.2.2.4 and section 3.2.2.4) revealed cationic liposome-DNA complexes at varying sizes and distribution (Figures 3.14 – 3.16). Cationic lipoplexes display a wide range in size from 50 nm to 300 nm in diameter. The mean diameter sizes of the different CL-DNA complexes can be seen in Table 3.5 below.

Table 3.5: The mean diameters of the various lipoplex suspensions examined.

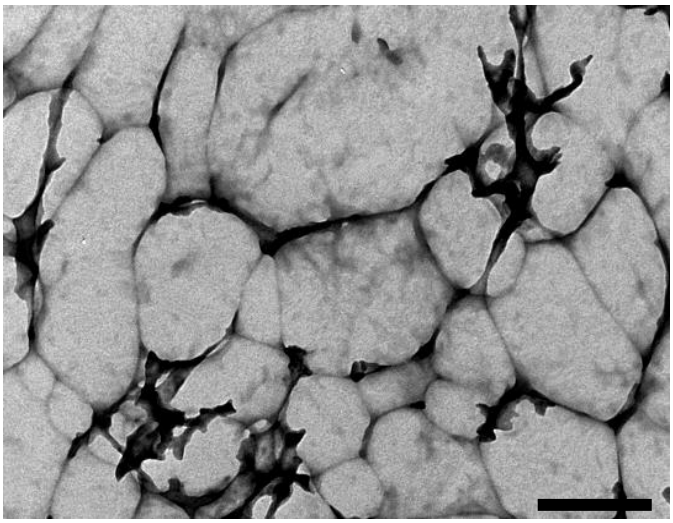
LIPOSOME	MEAN DIAMETER (nm)	STANDARD DEVIATION (nm)
Chol-T	185.70	53.01
Chol-T-Gal	169.30	42.23
Chol-T-Glu	50.48	12.47
RUI-128	101.03	25.06
RUI-128-Gal	130.55	102.6
RUI-128-Glu	171.22	27.82
RUI-129	228.84	34.82
RUI-129-Gal	183.65	28.11
RUI-129-Glu	131.61	57.04

Note: The data are presented as a means, ±S.D. (n = 5).

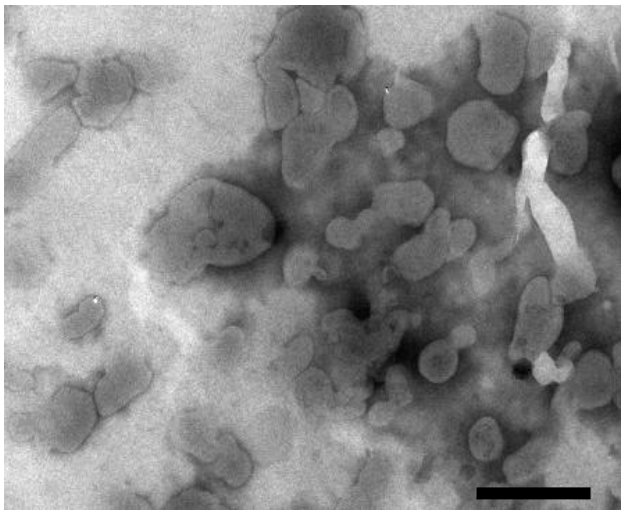
From the results tabulated above it is seen that all cationic liposome-DNA complexes were in the size range acceptable for application *in vivo*, but with the DNA being only moderately condensed by the different liposome preparations (Figures 3.11 – 3.13). The large size of the RUI-129 lipoplex, in particular, may be problematic for *in vivo* transfection experiments. The generally low standard deviations observed indicate a satisfactory size distribution of most of the lipoplex suspensions. Those lipoplexes displaying higher standard deviations show greater size variation within a liposome population, of these the RUI-128-Gal complex showed greatest size variation.

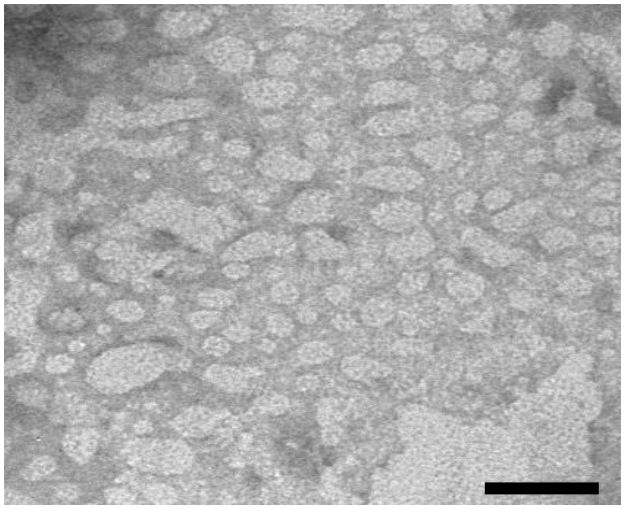
No significant divergence in size was observed between the Chol-T lipoplex preparation and the Chol-Gal targeted complexes; however, statistically significant variation did exist on comparison with complexes that were Chol-T-Glu targeted ($p < 0.05$). The same was observed for the RUI-128 lipoplexes, where statistically significant size differences were evident against the RUI-128-Glu complexes but not the RUI-128-Gal. Furthermore both targeted RUI-129 formulations on statistical analysis showed p-values of less than 0.05 when compared to the targeted preparation. This is indicative of significant variations in the sizes of the lipoplexes produced by the different formulations. Lastly a statistical comparative of the Chol-T lipoplex and both CE liposome – DNA complexes demonstrated that the differences in lipoplex size was of statistical significance ($p < 0.05$).

All preparations displayed what could be termed a spherical morphology, however, a large number of complexes were observed to have a deformed nature similar to some liposomes, however, more distinct. The deformity and any artifacts can be accredited to the cryo-TEM freezing protocol, rendering the liposomes immobilized, deflated or constricted. The carbon coating used for the evaluation of crown ether preparations could also account for the mottled effect that can be seen in some of the preparations. Although this technique provides a valuable understanding of the size and distribution of the DNA-liposome complexes, the measurement of particle zeta potential would be useful for further investigations.



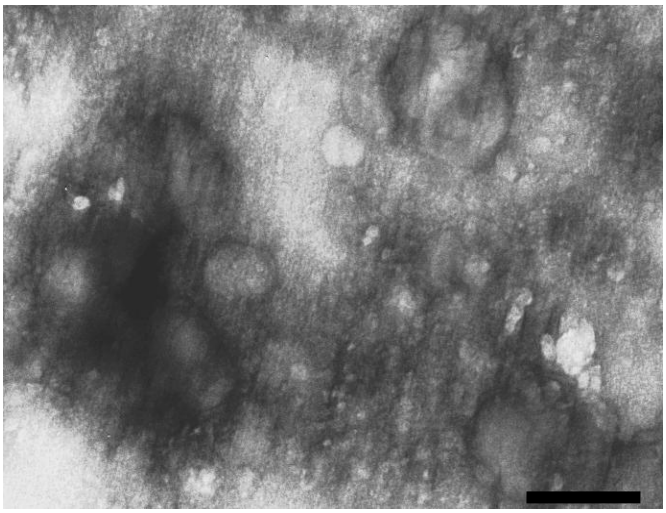
(a)



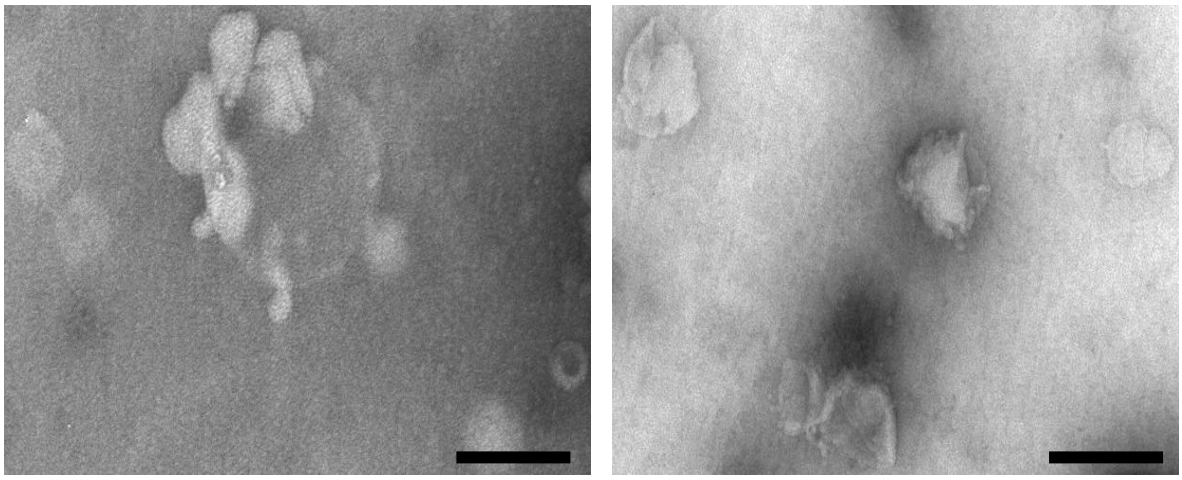


(b) (c)

Figure 3.14: Transmission electron micrographs of cationic liposome – DNA complexes, (a) Chol-T – DNA; (b) Chol-T-Gal – DNA and (c) Chol-T-Glu – DNA. Bar (a, c) = 200 nm (b) = 500 nm.

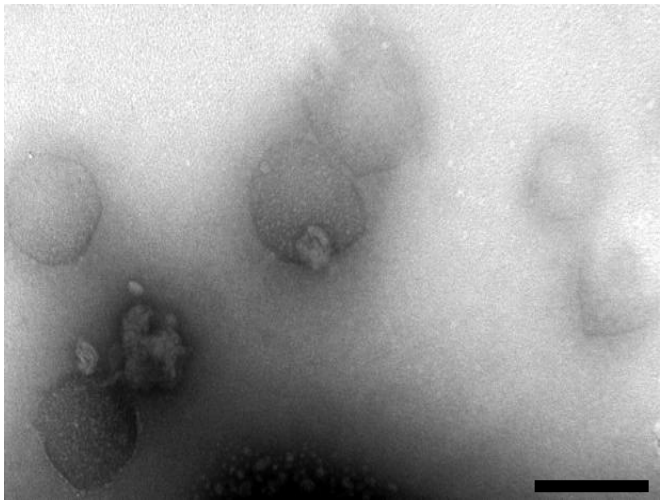


(a)

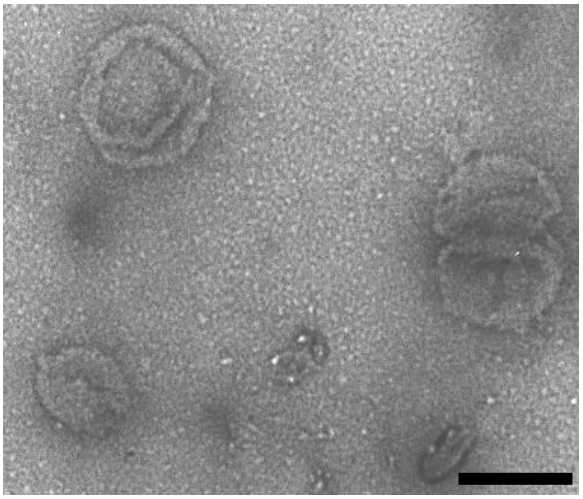


(b) (c)

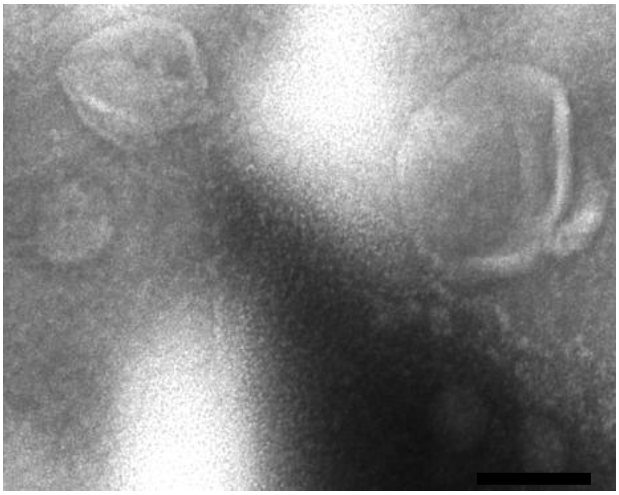
Figure 3.13: Transmission electron micrographs of cationic liposome – DNA complexes, (a) RUI-128 – DNA; (b) RUI-128-Gal – DNA and (c) RUI128-Glu – DNA. Bar = 200 nm.



(a)



(b)



(c)

Figure 3.16: Transmission electron micrographs of cationic liposome – DNA complexes, (a) RUI-129 – DNA; (b) RUI-129-Gal – DNA and (c) RUI129-Glu – DNA. Bar = 200 nm.

CHAPTER FOUR

4. TRANSFECTION AND GROWTH INHIBITION STUDIES IN CELL CULTURE

4.1. INTRODUCTION

The *in vivo* applicability of liposome-based formulations for drug and nucleic acid targeting necessitates the meticulous *in vitro* analysis for evaluation of loading capacity, entrapment stability under physiological conditions as well as the efficiency of loading (Schnyder and Huwyler, 2005). The analysis of transfection efficacy is an essential tool to the determination of the effectiveness of a particular gene therapy protocol. This is because it examines the quantity of exogenous DNA that is transported into cells and its subsequent expression of the gene (Safinya *et al.*, 2006). 'Transfection', the introduction and consequent expression of genes within cells has allowed for developments in therapeutic and cellular biology through the exploitation of this technique for the treatment of disease (Hoekstra *et al.*, 2007). 'Lipofection' more particularly looks at the cationic liposome carrier mediated introduction of functional nucleic acids into cells in culture (Gao, 2006)

In an attempt to rationally design and improve lipid based delivery systems an understanding of the interaction between the cationic liposome – DNA complex and the surface of the mammalian cell is essential (Ahmed *et al.,* 2005). Complexes consisting of a mixture of oppositely charged DNA and cationic liposomes mimic natural viruses in their ability to act as synthetic carriers of extracellular DNA across outer cell and nuclear membranes for gene delivery (Reimer *et al*., 1997). The use of cationic liposomes may enhance transfection by providing an overall positive charge to the cationic liposome – DNA complex enabling attachment to the anionic cell surface (Radler *et al*., 1997). Besides the application of cationic liposomes, many condensing agents have been employed for the transport of relatively large DNA molecules such as polyvalent cations, polycations, and cationic polyelectrolytes. The use of these mediators of transfection allows for improved penetration by DNA due to compaction, modification of its superficial structure and hydrophobic properties as well as restricted particle size (Zhdanov *et al.*, 2002). An evaluation of the DNA transport properties of different liposome and lipid formulations allows for better understanding of the cationic lipids and liposomes. Moreover the DNA internalization and expression of the transgene are influenced by the intrinsic physiochemical properties of lipoplexes and the cell-type specific factors (Colosimo *et al.*, 1999).

In the preparation of our synthetic gene transfection systems, cationic liposomes prepared with and without galactosyl and glucosyl ligands for cell specific targeting were mixed with plasmid DNA. Gene expression plasmid systems are known to possess either the full gene or minigene cDNA coding sequence

as well as numerous other genetic elements. A minimal requirement for the transcription unit of the protein encoding therapeutic gene is the presence of a 5'enhancer or promoter and a downstream polyadenylation signal. Many promoters that have origins in eukaryotic viruses have achieved broad scale application and include the cytomegalovirus (CMV), simian virus 40 (SV40), Moloney murine leukemia virus (MoMLV), and the Rous sarcoma virus (RSV) (Hattoria and Maitani, 2005). The pCMV-Luc plasmid DNA was used in lipoplexes with several different ratios of cationic liposome to DNA prior to use in culture. This reporter plasmid is employed to allow for the detection of transient expression within these cell lines which normally do not exhibit luciferase activity. Consequently the presence of this activity post gene delivery is indicative of successful transgene expression. The pCMV-Luc plasmid encodes the luciferase gene under the control of the cytomegalovirus promoter-enhancer (Figure 4.1) (Elouahabi *et al*., 2003).

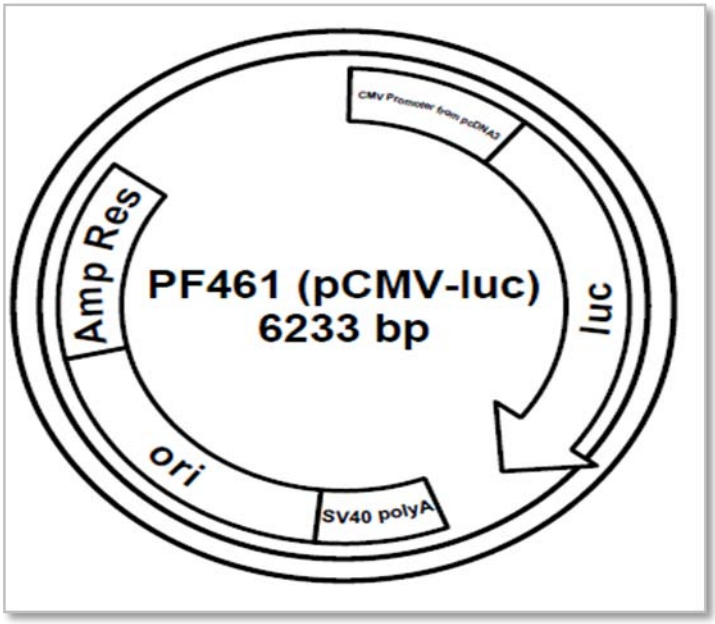


Figure 4.1: Plasmid map of pCMV-Luc control vector with a CMV promoter from pcDNA3 (Schmeer, 2008).

The luciferase gene was isolated from the North American firefly *Photinus pyralis.* It is an ATP-dependent luciferase that has been applied over a broad scale as a reporter enzyme for cell directed gene expression assays. This luciferase offers high sensitivity and large dynamic range of bioluminescence. However, as a catalytic enzyme of the bimolecular reaction between D-luciferin (Figure 4.2) and ATP which are small substrate molecules, inhibition by a variety of low-molecular-weight heterocyclic compounds is not unexpected. This enzyme as a reporter is expressed intracellularly and in contrast to other available reporters has a short protein half-life.

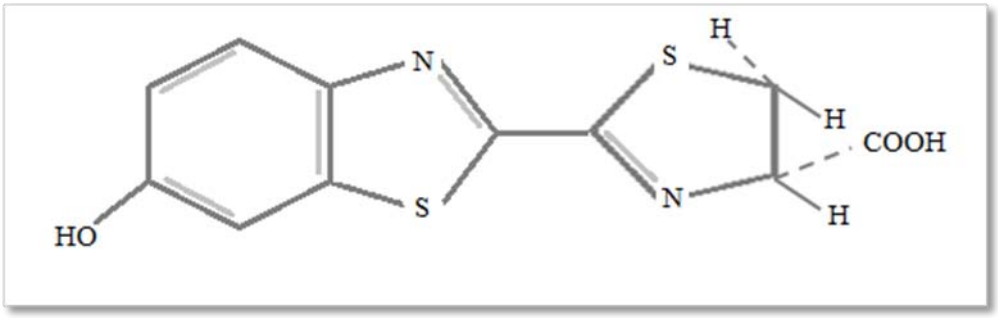


Figure 4.2: Structure of firefly D-(-)-Luciferin.

Two of the most important physiochemical factors implicated in cationic lipofection are size and lipidic composition. The effect of these factors and others were evaluated in both the human embryonic kidney (HEK293) and human hepatoblastoma (HepG2) cell lines through transfection of lipoplexes having different liposome/DNA molar ratios. Cationic liposome–mediated transfection may also be influenced by the presence of particular cell surface molecules such as lectins which provide points of targeting for the vector system. The importance of achieving successful gene transfection for therapeutic purposes cannot be over emphasized and due to its need for improved DNA uptake and expression, specific ligands as well as antibodies have been introduced for cell specific targeting.

It has often been overlooked in the past that the coupling ligand may not necessarily be a polypeptide, but also a sugar residue as found on glycoproteins and lipids. These sugar residues such as galactose are recognized by lectins present such as the asialoglycoprotein receptors present on the surface of hepatocytes (Nishikawa and Huang, 2001). This lectin was used in the first successful attempt at gene glycotargeting (Paillard, 1999). Moreover this receptor mediated hepatocyte derived cellular targeted delivery of therapeutic DNA is imperative for the development of gene therapy protocols for human liver diseases and disorders (Singh and Ariatti, 2003). Among the receptors investigated for ligand targeting, the transferrin receptor has been one of the most frequently used, owing to its overexpression in a variety of tumour cells. The asialoglycoprotein receptor (ASGP-R), however, has shown the greatest gene targeting potential. Besides being abundantly expressed on the HepG2 cell line, it is also renowned for both a high ligand affinity and rapid rate of internalization. The ASGP-R and other carbohydrate receptors such as the mannose receptor found on macrophage and liver endothelial cells thus allow for the requisite cell specific delivery in liposome carrier systems (Qi *et al*., 2005). Furthermore in addition to galactose other low molecular weight ligands, monoclonal antibodies, or macromolecules can be used to enhance target cell specific uptake of DNA (Figure 4.3) (Nishikawa and Huang, 2001).

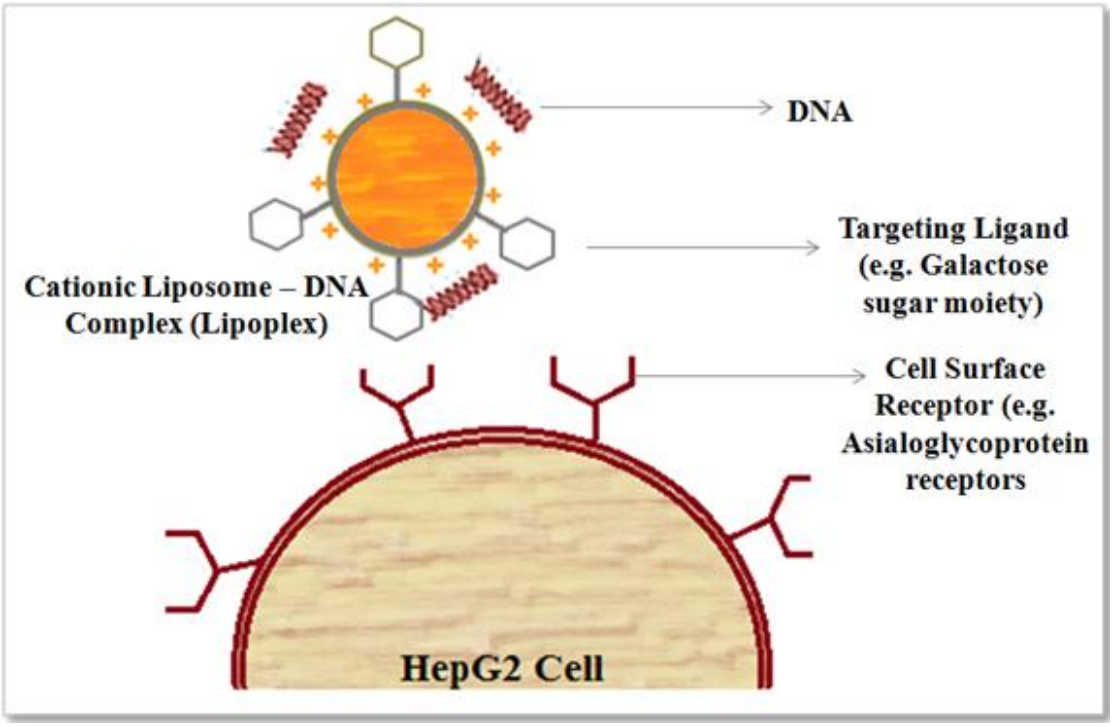


Figure 4.3 : Illustration portraying the interaction between a targeted cationic lipoplex and a HepG2 cell. (Diagram not drawn to scale).

Other cell surface molecules that may also play an important role are the polyanionic glycosaminoglycans (GAGs) which interact with lipoplexes thus inhibiting transgene expression. Negatively charged sulphated proteoglycans, also present on the cell surface, may be responsible for mediating the electrostatic attraction between positively charged lipoplexes and mammalian cells (Bloomfield, 1991). In addition to the quantity of cell surface heparin sulphate (HS), hyaluronan (HA) and chondroitin sulphate (CS), the type of cells and carrier employed also play a role in the inconsistencies of cellular uptake of the lipoplexes. Significant levels of variance in tansfection have been reported for the same lipoplex formulation within different cell lines (Bailey and Cullis, 1997; Uddin, 2007).

Most analyses of liver directed gene regulation in human cells has been in either primary cultures of normal hepatocytes or a restricted class of human hepatoma cell lines. Primary cells are thought to more closely represent the human liver metabolism compared to the HepG2 cells in that they are more sensitive toward promutagens and express the phase I oxidative, reductive and hydrolytic enzymes at higher levels. Morphologically the two cell types differ with the primary hepatocytes displaying a cubic shape with often two nuclei, while the HepG2 cells display a more epithelial-like morphology containing one nucleus and a chromosome number ranging from 48 – 54 chromosomes per cell (Wilkening *et al.*, 2003). Thus human hepatocytes may be considered an ideal system for *in vitro* analysis of toxicological and

pharmaceutical studies of the human liver. These hepatocytes are long-lived cells that undergo low rates of proliferation in culture and supply a stable environment for prolonged gene expression (Suda *et al.*, 2009).

The HepG2 cell line which is a well differentiated human hepatoma derived cell line was originally isolated from liver biopsies of hepatoblastoma and hepatocellular carcinomas (Aden *et al*., 1979; Morris *et al.*, 1982). Hepatoma cell lines such as HepG2 and Hep3B retain a differentiation status that is characteristic of the adult liver. The HepG2 cells used are typified for their expression of liver specific genes encoding plasma protein and detoxification enzymes. These cells have also aided in our understanding of complement biosynthesis at either the hepatic or extrahepatic levels. Many of these liver specific genes are regulated by nuclear proteins which are strongly localized in the liver as well as liver-derived cell lines (Le Jossic *et al.*, 1996; Morris *et al.*, 1982). This cell line has also been recognized for its retention of a great number of liver-specific phenotypes and possesses neither the hepatitis B surface antigen or intergrated viral DNA (Lee *et al.*, 1992).

Due to the low to moderate transfection levels obtained *in vivo* using low doses of cationic liposomes the application of higher doses is most often required, often resulting in increased levels of toxicity. This fine balance between efficacy and toxicity has brought into focus the question of vehicle safety (Dass and Choong, 2006). The trasfection efficiency and toxicity that is produced by the gene delivery vectors employed, has been reported by Huang and co-workers (1998) to show dependence on the cell lines used for the investigation undertaken. The development of *in vitro* assays of cytoxicity has been propelled by the need to be able to evaluate the potential detrimental effect of a large number of compounds as well as to limit the experimental investigation in the animal model. There are three essential principles on which cytotoxicity investigations *in vitro* are based:

i. The first looks at the measurement of metabolic activity where a reduction here could provide an early indication of cellular damage.
ii. Membrane integrity is a parameter for the determination of cytotoxicity involving measurement of the lactate dehydrogenase in the extracellular medium. When cells are damaged they become leaky and as a result the extracellular presence of lactate dehydrogenase is indicative of cellular damage.
iii. The final type of assay involves the detection of cell viability or cell number as the dead cells in adherent cell lines are known to detach and as such are removed from the system in the culture medium.

It has been reported that evaluation of metabolic activity provides a significantly early warning of cellular injury while investigations on membrane integrity are often too late, identifying a far more serious injury and potentially cell death (Noabbiodiscoveries, www.noabbiodiscoveries.com).

The cytoxicity induced by chemicals can be evaluated through various assays of cultured cells. One such method is the quantitative colorimetric neutral red assay (NR) which measures the uptake of the neutral red dye that is known to build up within the lysosomes of unaffected cells. A far more popular technique employed widely is the MTT assay which is based on the uptake and reduction of the yellow soluble MTT tetrazolium by mitochondrial succinic dehydrogenase to form an insoluble blue MTT formazan product. Possibly the simplest and yet highly reproducible analyses of cytotoxicity is the crystal violet staining assay which employs a colorimetric determination of stained cells to measure the reduction in cellular growth rate. Despite being convenient means of evaluating the cellular toxicity of chemicals they each have their own drawbacks to their application. Use of the neutral red assay can result in underestimation of the cytotoxic effect as a result of lysosomal swelling, while MTT formazan product formation may also be compromised by some reducing agents and respiratory chain inhibitors of mitochondrial MTT reduction. Results obtained using the crystal violet assay may vary if the culture is continued past confluence. Furthermore due to the non-specificity of the crystal violet staining assay, all adherent cells will be considered viable. These disadvantages make the need for test methods that employ a variety of different cytotoxicity markers evident (Chiba *et al.*, 1998).

Using the results obtained from *in* vitro cytotoxicity and transfection studies in the ASGP-R positive cell line HepG2 and the ASGP-R negative cell line HEK293, three liposome formulations were chosen for *in vivo* evaluation. It is important to note, however, that although *in vivo* experimentaion is the next step following *in vitro* cell culture analysis, a correlation in transfection activity does not always exist between the two systems (Uddin, 2007).

4.2 MATERIALS AND METHODS

4.2.1. Materials

HEK293 cells were obtained from Prof. P. Arbuthnot at the University of Witwatersrand. HepG2 cells together with 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, trypsin-versene and Penicillin (5000 units/ml) /Streptomycin (5000 μg/ml) were purchased from Lonza BioWhittaker, Walkersville, USA. The pCMV-Luc control vector was obtained from the Plasmid Factory, Bielefeld, Germany. The Luciferase Assay kit was purchased 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. Preparation of culture medium

MEM was prepared in 1 litre batches using 18 Mohm water and was supplemented with $NaHCO_3$ (10 mM), HEPES (20 mM), penicillin (5000 units/ml) and streptomycin (5000 μg/ml). The pH was adjusted to 7.3 – 7.4 before the final volume adjustment. The medium was filtered through a Millipore 0.22 μm bell filter unit into autoclaved 250 ml wide-neck blue cap bottles. This was carried out in a laminar flow hood using a Cole-Palmer Masterflex (model 7017-12) peristaltic pump to drive the medium through the filter. The medium was stored for 24 hours at room temperature in a laminar flow hood after filtration. The medium was then stored long term at 4°C. Sterile foetal bovine serum was introduced into the medium to a final concentration of 10% (v/v) prior to use.

4.2.2.2. Maintenance and cell culture

All media preparation and other cell culture work were performed in a class 1 laminar flow hood. All cells were visually inspected frequently. Frequent medium changing is important for maintaining the pH balance and for eliminating waste products. Cells were subcultured when they were in a semi-confluent / confluent state. In general, mammalian cells should be handled gently and not vortexed, vigorously pipetted or centrifuged at greater than 1500 x g (Wolf, 2006).

4.2.2.3. Reconstitution, subculture and cryopreservation of cells

An ampoule of cells was removed from the biofreezer and placed in a 37°C water bath to thaw. Following disinfection of the cryogenic vial with 70% ethanol, it was opened aseptically in the laminar flow hood. To pellet cells the cell suspensions were centrifuged at 1000 rpm (800 x g) for 5 minutes. The medium was decanted and the pellet resuspended in 1 ml of fresh medium (MEM +10% FCS). Cells were transferred to a new flask containing 5 ml of medium containing serum. Flasks were incubated at 37°C.

Cells were allowed to attach to the flask before the medium was replaced every 2-3 days. A colour change in the medium from pink to orange was used as an immediate visual indicator of medium usage and the need for replacement.

4.2.2.3. (a) Trypsinization and subculturing

MEM and trypsin-versene were removed from the refrigerator and warmed to 37°C prior to use. After the cells had reached semi- or complete confluency, spent culture medium was decanted. Cells were rinsed with 5 ml of PBS (phosphate buffered saline). PBS was decanted and 1 ml of trypsin- versene was added to just cover the cells in the flask. Trypsinization was allowed to proceed at 37°C for 1 – 5 minutes, until rounding off of the cells was observed with a Nikon TMS inverted light microscope (100 x magnification). The activity of trypsin-versene was inhibited by the addition of approximately 2 – 3 ml of complete medium (MEM + 10% FBS). Culture flasks were tapped against the palm to dislodge cells. Cells were then split according to a predetermined desired ratio into separate flasks / multiwell plates or alternatively cryopreserved.

4.2.2.3. (b) Cryopreservation of cells

Following dislodging by trypsinization as described in 4.2.2.3. (a.), cells were pelleted by centrifugation at 1000 rpm for 5 minutes. Pelleted cells were resuspended in 0.9 ml complete medium and 0.1 ml of a cryoprotective medium, typically dimethylsulphoxide (DMSO). Cells were then aliquoted into suitable cryogenic vials which were vacuum sealed prior to freezing. Cells were frozen at a rate of -1^{o} C / minute to a temperature of -50°C using a cold probe. Vials were then stored at -80°C in a NUAIRE biofreezer.

4.2.2.4. Amplification of pCMV control vector

The pCMV-Luc control vector was amplified in the Department of Biochemistry, University of KwaZulu-Natal according to a standard protocol. The DNA purity and concentration was determined

spectroscopically using a Thermo Electron Corporation Biomate 3 spectrophotometer. The isolated DNA was analyzed on a 1% agarose gel against a control DNA sample to confirm purity.

4.2.2.5. Cell viability studies

Table 4.1: DNA : cationic liposome ratios used for reaction complexes investigated in growth inhibition and transfection studies.

LIPOSOME PREPARATION	DNA : LIPOSOME RATIO (w/w)		
Chol-T	1 : 3	1 : 4	1 : 5
Chol-T-Gal	1 : 5	1 : 6	1 : 7
Chol-T-Glu	1 : 5	1 : 6	1 : 7
RUI-128	1 : 6	1 : 7	1 : 8
RUI-128-Gal	1 : 7	1 : 8	1 : 9
RUI-128-Glu	1:8	1:9	1:10
RUI-129	1 : 5	1 : 6	1 : 7
RUI-129-Gal	1 : 3	1 : 4	1 : 5
RUI-129-Glu	1 : 3	1 : 4	1 : 5

4.2.2.5.1. Growth inhibition studies in the HEK293 and HepG2 cell lines:

HEK293 and HepG2 cells were trypsinized and seeded into two 48 well plates at seeding densities of 1.32×10^4 cells/well and 2.52×10^4 cells/well respectively. These plates were incubated at 37°C for 24 hours. The cells were prepared by removing the growth medium and replacing it with serum-free medium. All liposome solutions were sonicated for 30 seconds prior to formation of reaction complexes with the pCMV-Luc DNA. Varying concentrations of these complexes were thereafter introduced to the prepared wells of the cell culture plates. The assays were carried out in triplicate. The cells were incubated at 37°C for 4 hours. Following the incubation period, the serum-free medium was removed and replaced with complete (serum-containing) medium. Cells were incubated for a further 48 hours at 37°C. Thereafter the

medium was removed from the wells and the cells were washed twice with PBS. Cells were stained upon addition of 200 µl of crystal violet solution (0.5% w/v crystal violet, 0.8% w/v sodium chloride, 5% v/v formaldehyde, 50% v/v ethanol) for 20 minutes. Stain was then removed and the cells washed extensively with water. The multiwell plates were dried for 24 hours and the stain extracted from the cells using 2-methoxyethanol over a period of 36 hours, with gentle rocking (10 rev/min) on a Stuart Scientific Stiz 6 platform shaker. Absorbance values for the samples were then read on a UV/visible spectrometer at 550 nm (Biomate 3).

4.2.2.6. Transfection studies

4.2.2.6.1. Transfection of the HEK293 and HepG2 cell lines

HEK293 and HepG2 cells were trypsinized and evenly seeded into two 48-well plates at densities of 2.1 x 10^5 cells/well and 2.88 x 10^4 cells/well respectively. The cells were allowed to attach to the wells and grow to semi-confluence. The transfection complexes were prepared as in 4.2.2.5.1 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.

4.2.2.6.2. Luciferase assay

The luciferase assay was carried out using the Promega Luciferase Assay kit. In principle this assay involves the expression of luciferase in the cytoplasm resulting in the formation and decarboxylation of the luciferase.luciferyl-AMP intermediate to essentially produce oxyluciferin and light, shown in Figure 4.17. The use of Coenzyme A, as in the Promega luciferase assay protocol allows for intense light emission that remains constant for several minutes. 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 lyophilized luciferase assay substrate. The cell culture lysis

reagent (5x) (25 mM tris-phosphate, pH 7.8; 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane – N, N, N'N'- tetra-acetic acid, 10% ($^{v}/_{v}$) glycerol, 1% ($^{v}/_{v}$) triton X-100), 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. Approximately 80 µl of 1x cell lysis reagent (Promega) was added to each well to cover cells; the plate was then placed on a Scientific STR 6 platform shaker for 15 minutes at 30 rev /min. Attached cells once dislodged from the wells were 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 (Promega) 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 emission 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 HEK293 and HepG2 Cell Lines

Cells were successfully propagated and maintained in complete MEM for the entire duration of study. The sucessful propagation of both cell types can be seen in the representaive cell lines shown in Figure 4.4. The initial rate of cellular growth was slow, requiring 5 – 6 days to reach confluency. Thereafter a vast improvement in cell growth was evident upon further propagation. This increase in growth is believed to be as a result of the growth factors secreted by the cells growing in the medium. Although both cell lines showed an early lag in cell growth the effect was more obvious in the HepG2 cell line which is known to display this particular characteristic. Moreover this initial retarded growth could also be attributed to the cells having been exposed to extended periods of cryopreservation prior to reconstitution. It could also potentially be due to prolonged exposure to trypsin-versene during the cryopreservation protocol or extensive tapping of the cell culture flask resulting in cellular shearing and death. Once growing at an appreciable rate the cells were trypsinized and subdivided in 1 : 2 or 1 : 3 splits depending on the extent of growth in each flask.

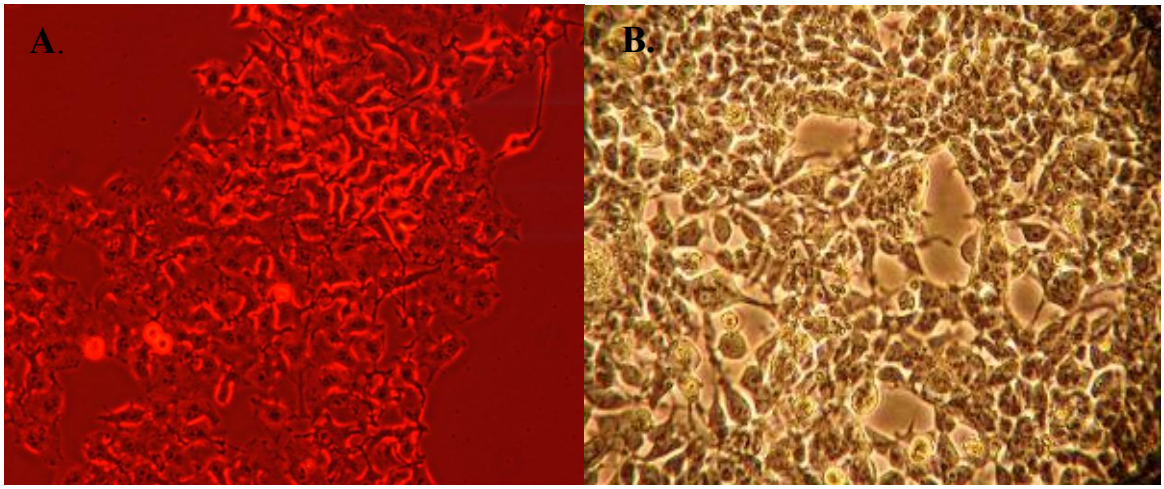


Figure 4.4: Monolayer of cells at semi-confluence viewed under an Olympus fluorescence microscope (100 x) A. HepG2 cells and B. HEK293 cells

4.3.2. Amplification of pCMV-Luc Control Vector

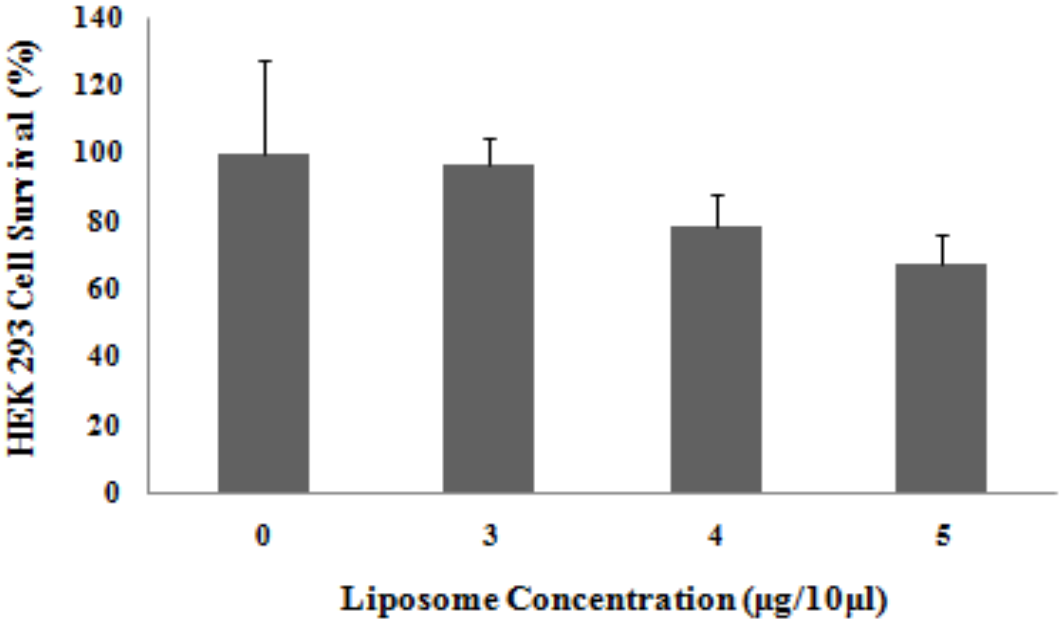
The pCMV-Luc control vector was successfully amplified. The concentration of the plasmid DNA was measured by UV absorption at 260 nm and adjusted to 0.25 µg/µl. The plasmid DNA showed relatively high purity with the 260 nm/ 280 nm absorption ratio between 1.8 and 2.

4.3.3. Cell Viability Studies

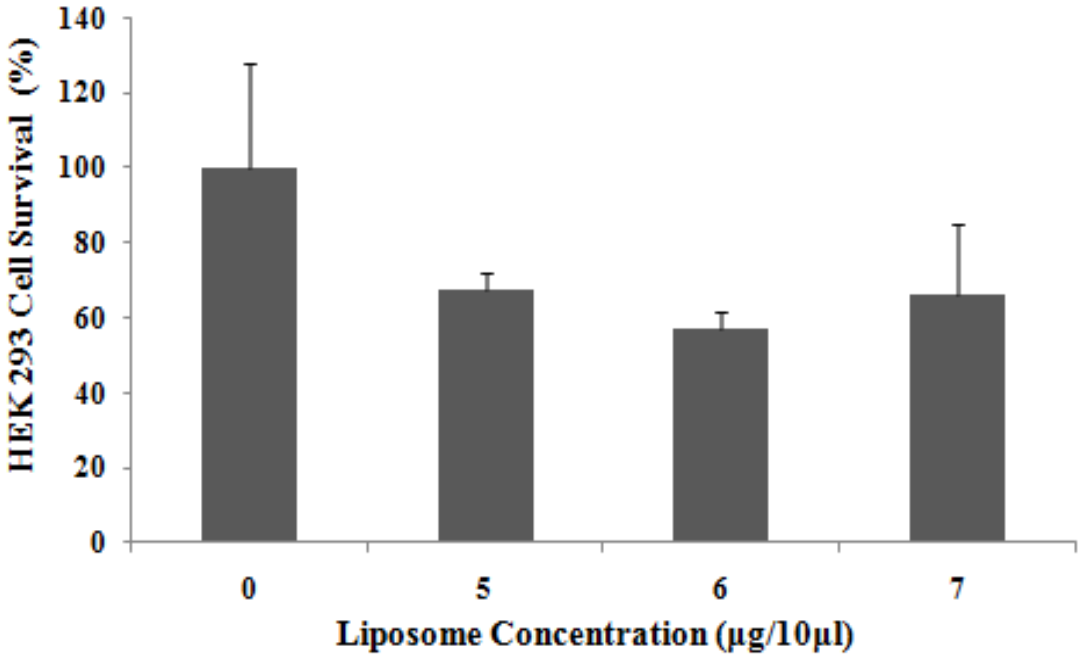
In order to determine the potential risk of the therapeutic compounds or drugs in humans a number of toxicity studies have been carried out using animal models. These may not always accurately reflect toxicities in humans and consequently human cell culture models have been established and employed for toxicity assays (Wilkening *et al.*, 2003).

The effets on cell survival caused by the lipoplexes was determined by the crystal violet assay in both the human embryonic kidney (HEK293) and HepG2 cell lines. The results of these cytoxicity evaluations can be seen in Figures 4.5 – 4.7 and Figures 4.8 – 4.10 respectively.

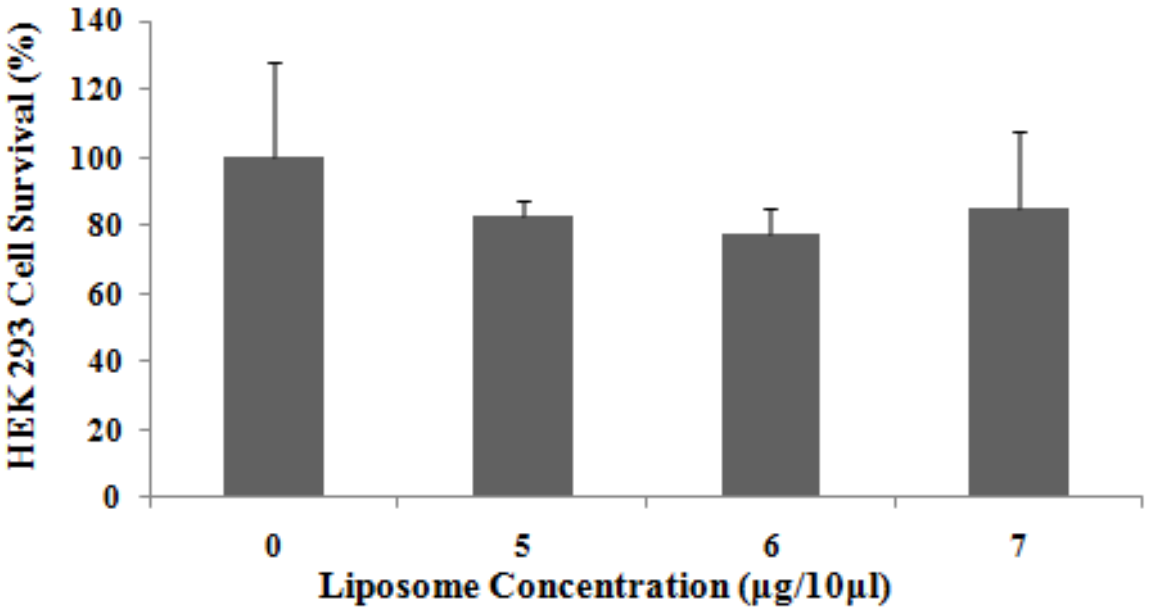
4.3.3.(a) Growth inhibition assay for the HEK293 cell line:



(a)



(b)



(c)

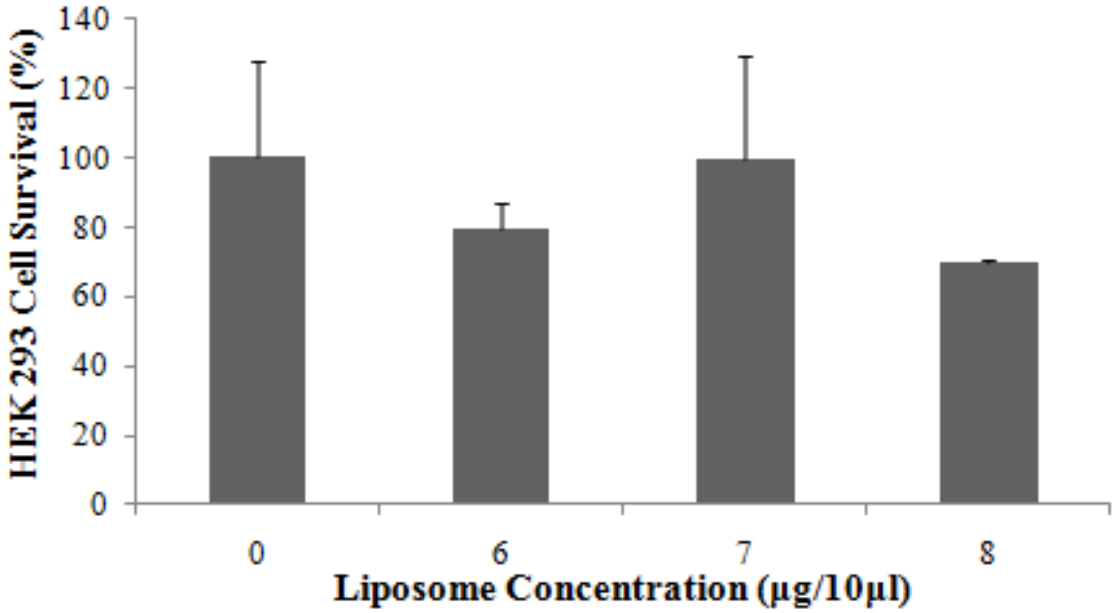
Figure 4.5: Growth inhibition studies of cationic liposome: pCMV-Luc DNA complexes to HEK293 cells *in vitro.* DNA was kept constant at 1 µg while varying amounts of liposome used are listed below.

(a) Chol-T (0, 3, 4 and 5 µg/10 µl)

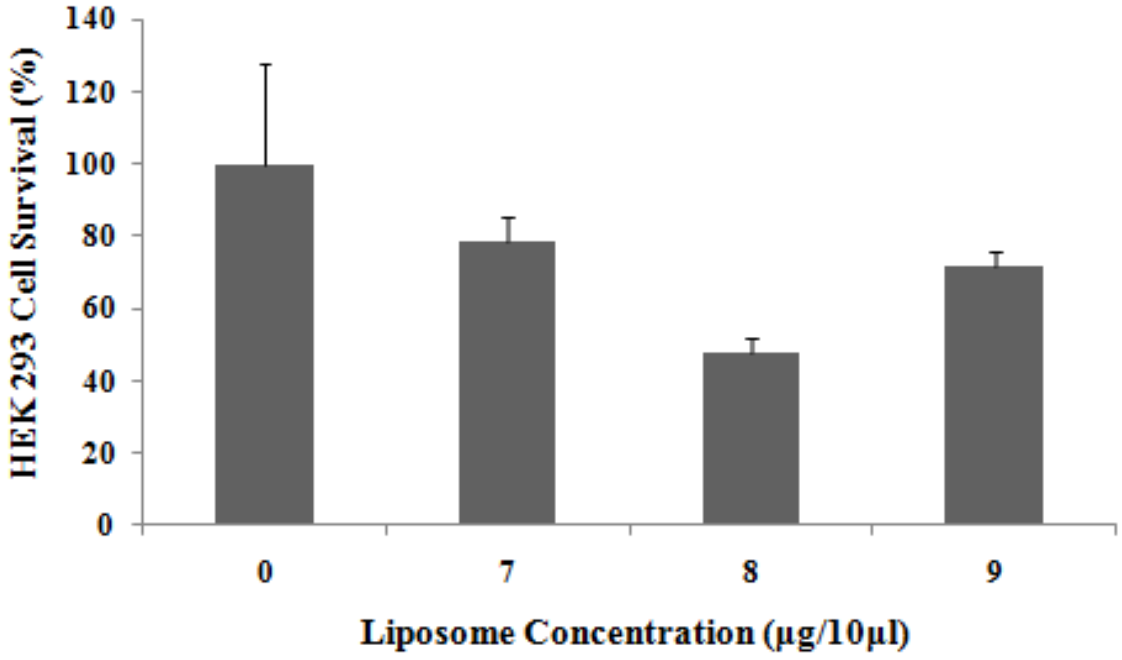
(b) Chol-T-Gal (0, 5, 6 and 7 µg/10 µl)

(c) Chol-T-Glu (0, 5, 6 and 7 µg/10 µl)

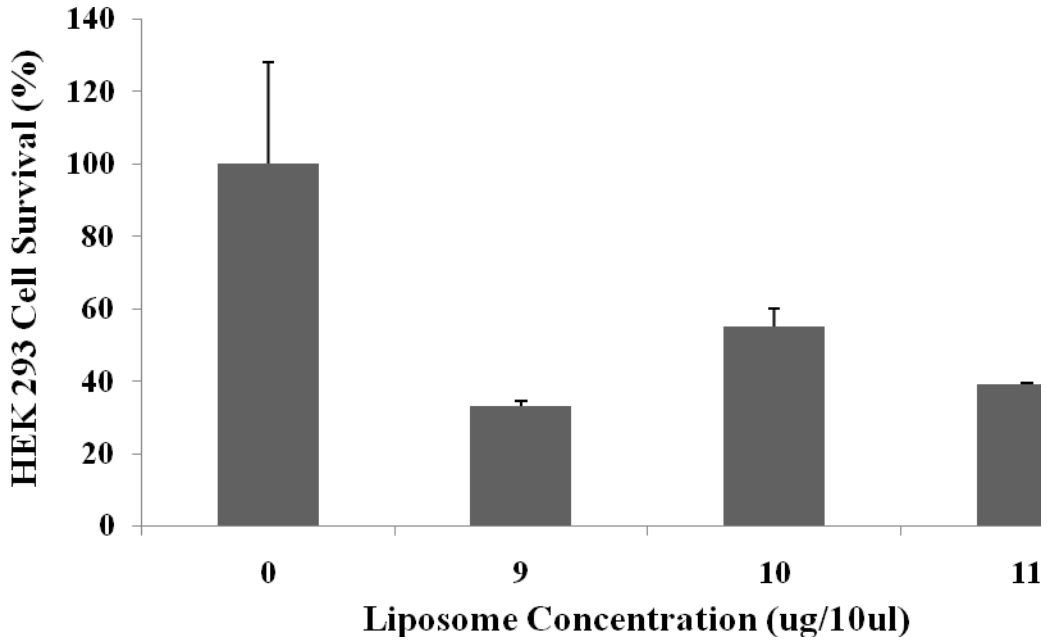
Data are presented as a percentage of the control sample (no liposomes) and are represented as means ±S.D. (n = 3).


HEK 293 Cell Survival (%)
140
120
100
80
60
40
20
0
0
6
7
8
Liposome Concentration (µg/10µl)

(a)


HEK 293 Cell Survival (%)
140
120
100
80
60
40
20
0
0
7
8
9
Liposome Concentration (µg/10µl)

(b)



(c)

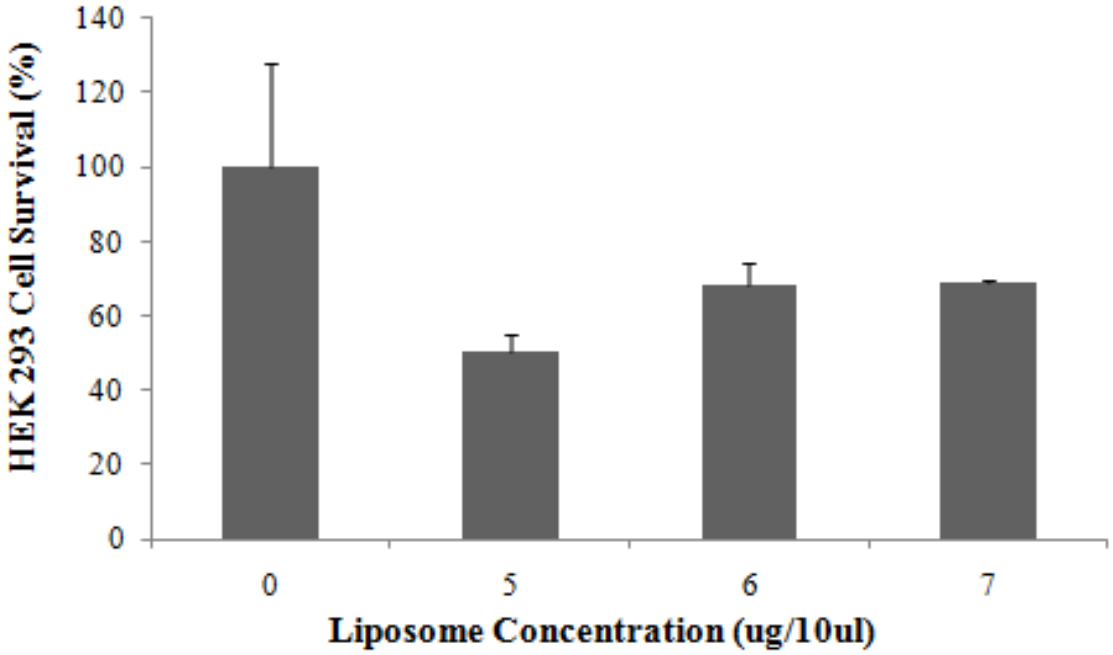
Figure 4.6: Growth inhibition studies of cationic liposome: pCMV-Luc DNA complexes to HEK293 cells *in vitro.* DNA was kept constant at 1 µg. Liposomes were introduced at varying amounts.

(a) RUI-128 (0, 6, 7 and 8 µg/10 µl)

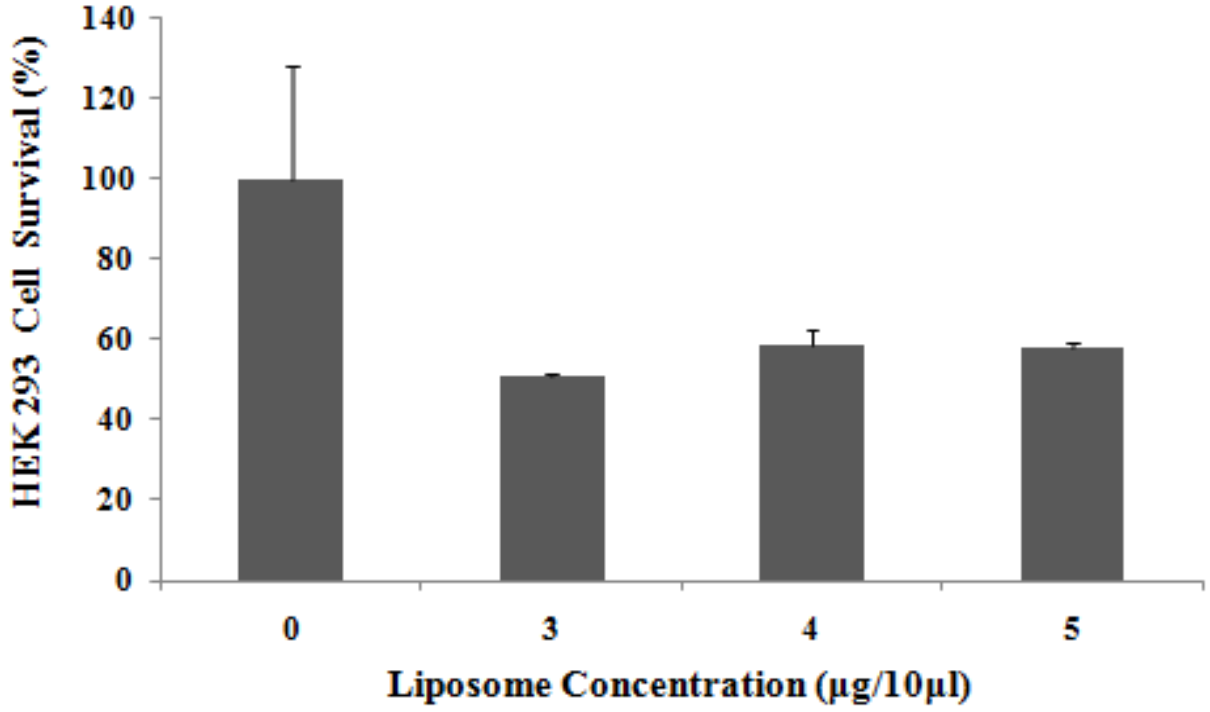
(b) RUI-128-Gal (0, 7, 8and 9 µg/10 µl)

(c) RUI-128-Glu (0, 9, 10 and 11 µg/10 µl)

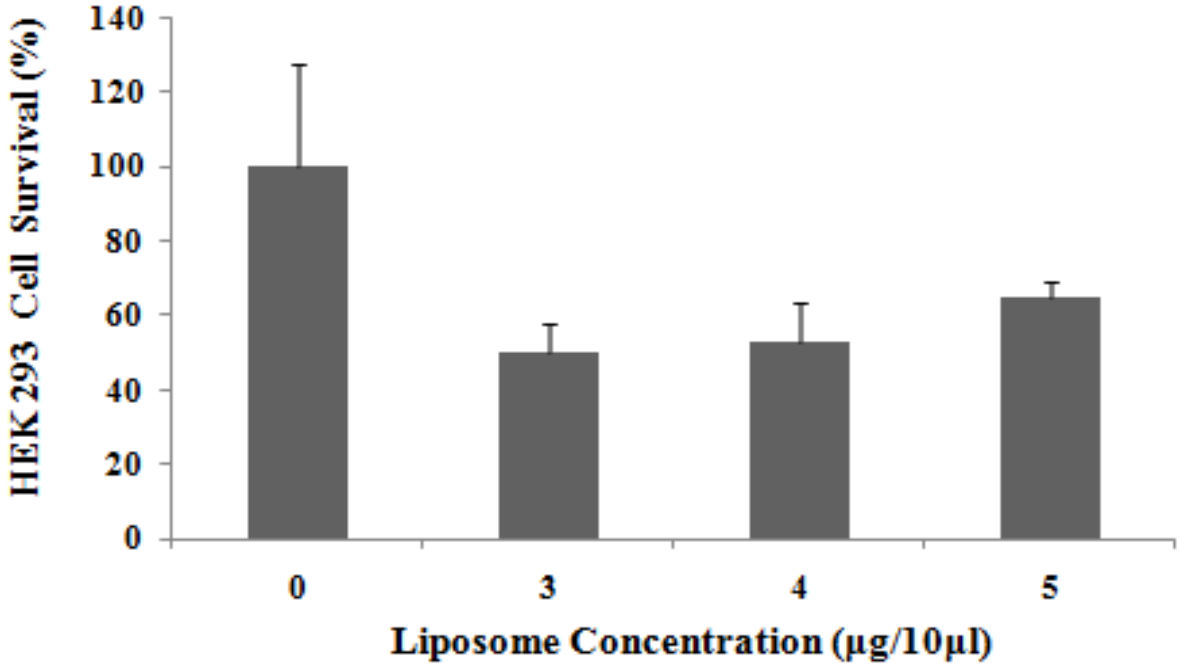
Data are presented as a percentage of the control sample (no liposomes) and are represented as means ±S.D. (n = 3).


HEK 293 Cell Survival (%)
140
120
100
80
60
40
20
0
0
5
6
7
Liposome Concentration (ug/10ul)

(a)


HEK 293 Cell Survival (%)
140
120
100
80
60
40
20
0
0
3
4
5
Liposome Concentration (µg/10µl)

(b)



(c)

Figure 4.7: Growth inhibition studies of cationic liposome: pCMV-Luc DNA complexes to HEK293 cells *in vitro.* DNA was kept constant at 1 µg while liposomes were introduced at varying amounts.

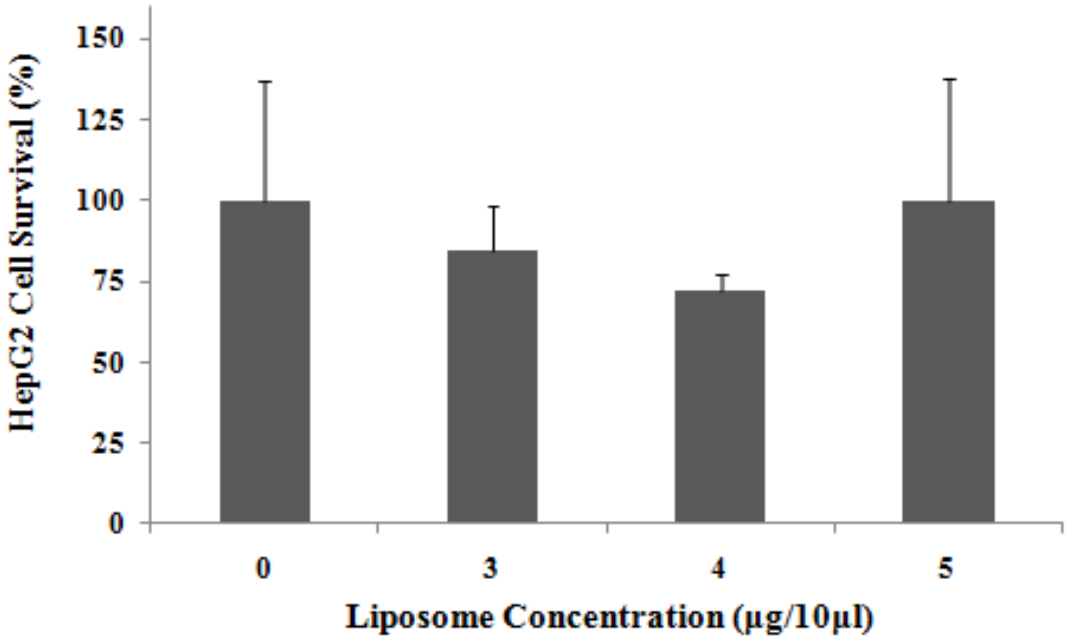
(a) RUI-129 (0, 5, 6 and 7 µg/10 µl)

(b) RUI-129-Gal (0, 3, 4and 5 µg/10 µl)

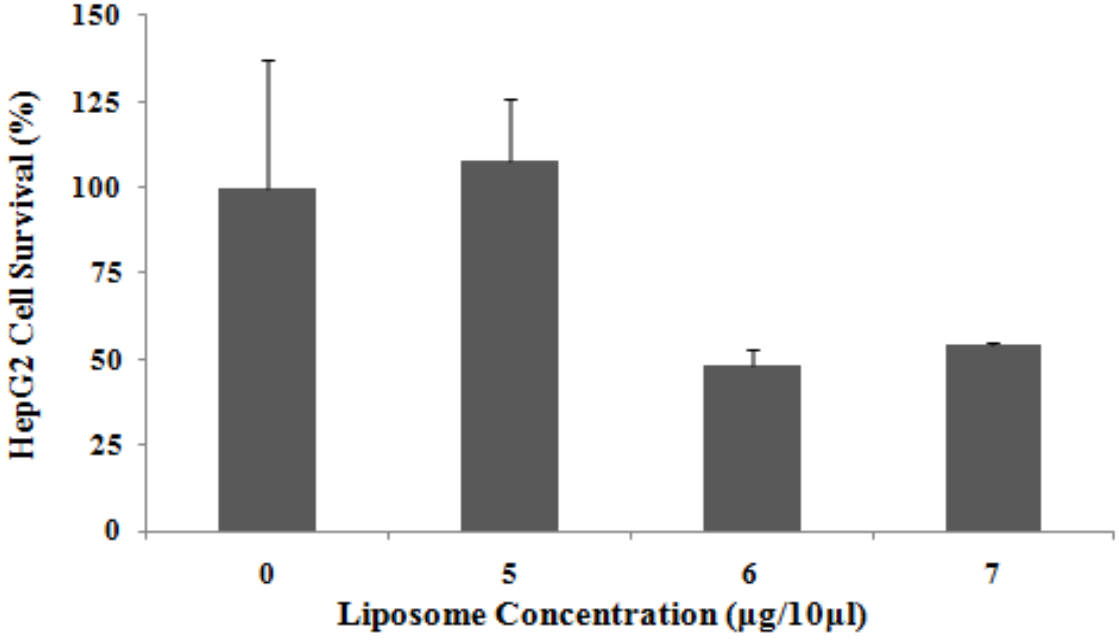
(c) RUI-129-Glu (0, 3, 4and 5 µg/10 µl)

Data are presented as a percentage of the control sample (no liposomes) and are represented as means ±S.D. (n = 3).

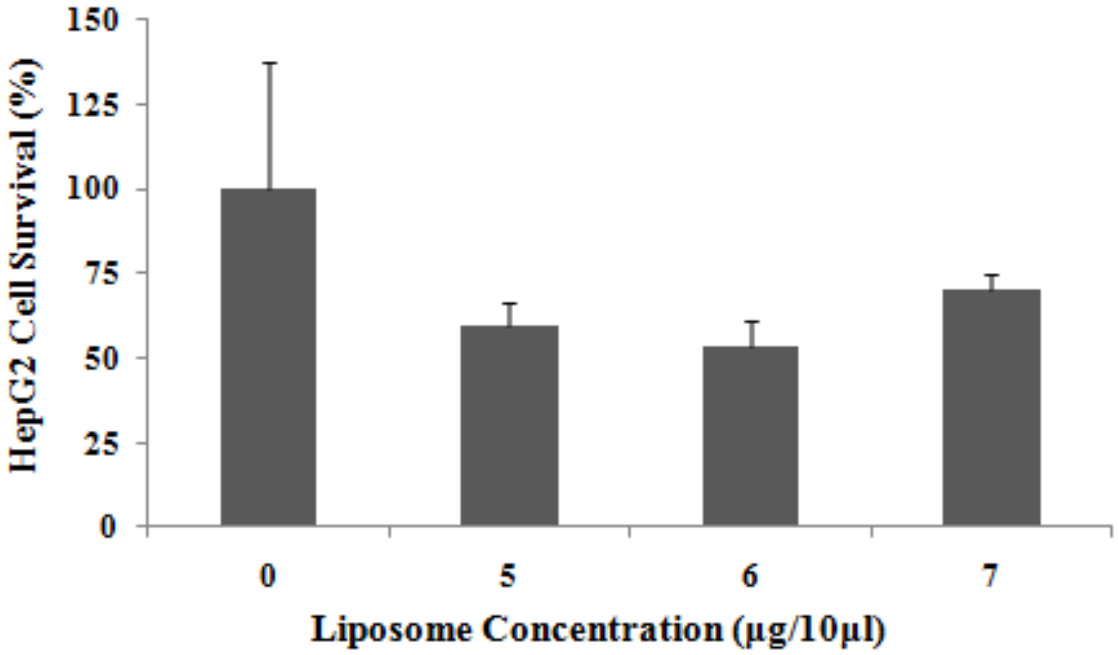
4.3.3.(b) Growth inhibition assay for the HepG2 cell line:



(a)



(b)



(c)

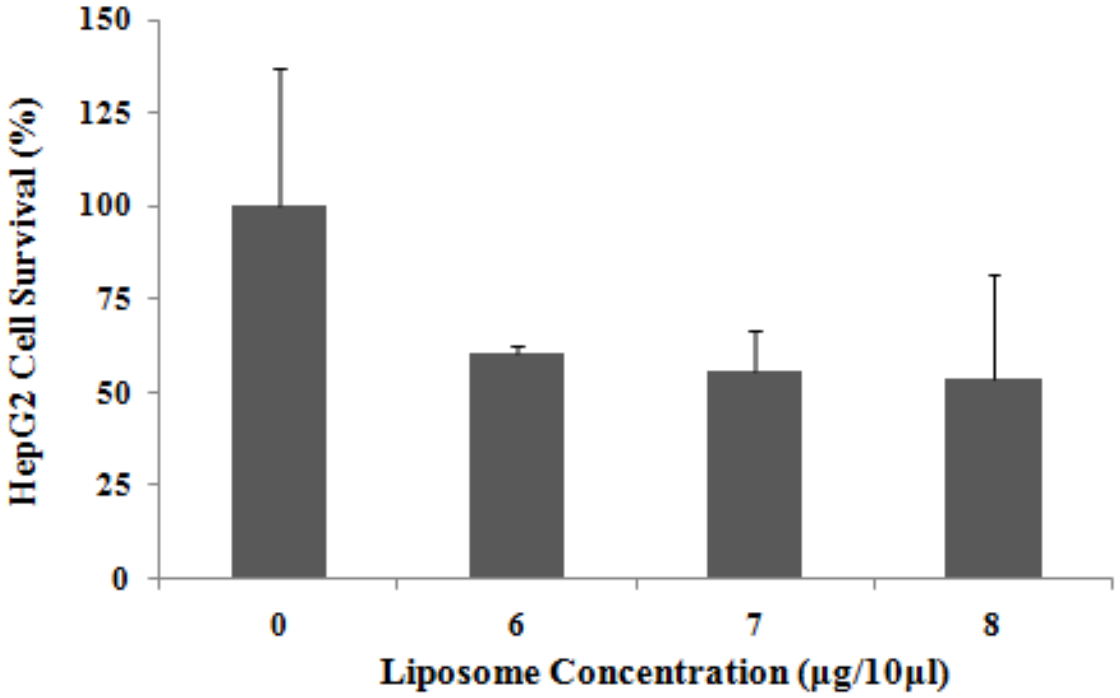
Figure 4.8: Growth inhibition studies of cationic liposome: pCMV-Luc DNA complexes to HepG2 cells *in vitro.* DNA was kept constant at 1 µg. Liposomes were used at increasing amounts.

(a) Chol-T (0, 3, 4 and 5 µg/10 µl)

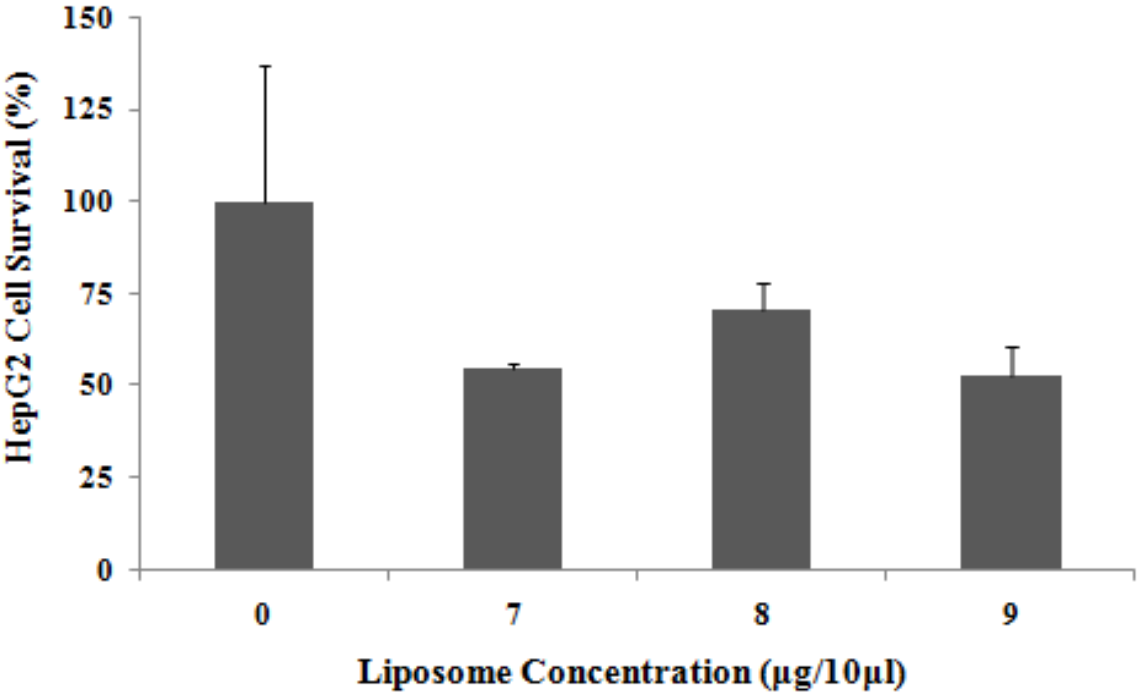
(b) Chol-T-Gal 0, (5, 6 and 7 µg/10 µl)

(c) Chol-T-Glu (0, 5, 6 and 7 µg/10 µl)

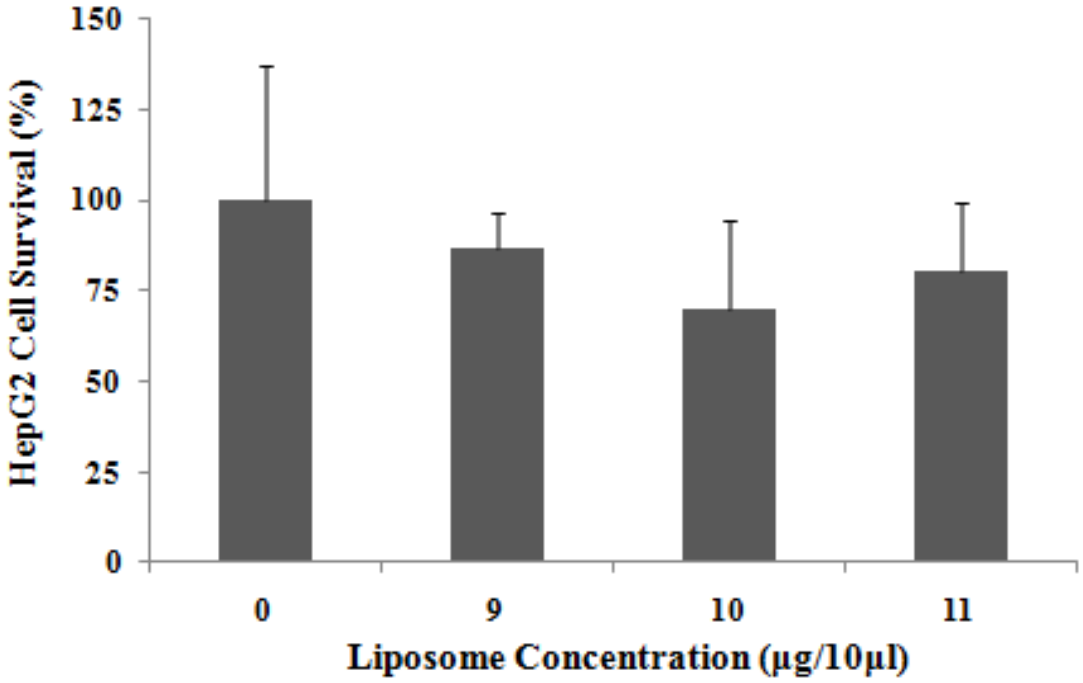
Data are presented as a percentage of the control sample and are represented as means ±S.D. (n = 3).


150
125
100
75
50
25
0
HepG2 Cell Survival (%)
0
6
7
8
Liposome Concentration (µg/10µl)

(a)


150
125
100
75
50
25
0
HepG2 Cell Survival (%)
0
7
8
9
Liposome Concentration (µg/10µl)

(b)



(c)

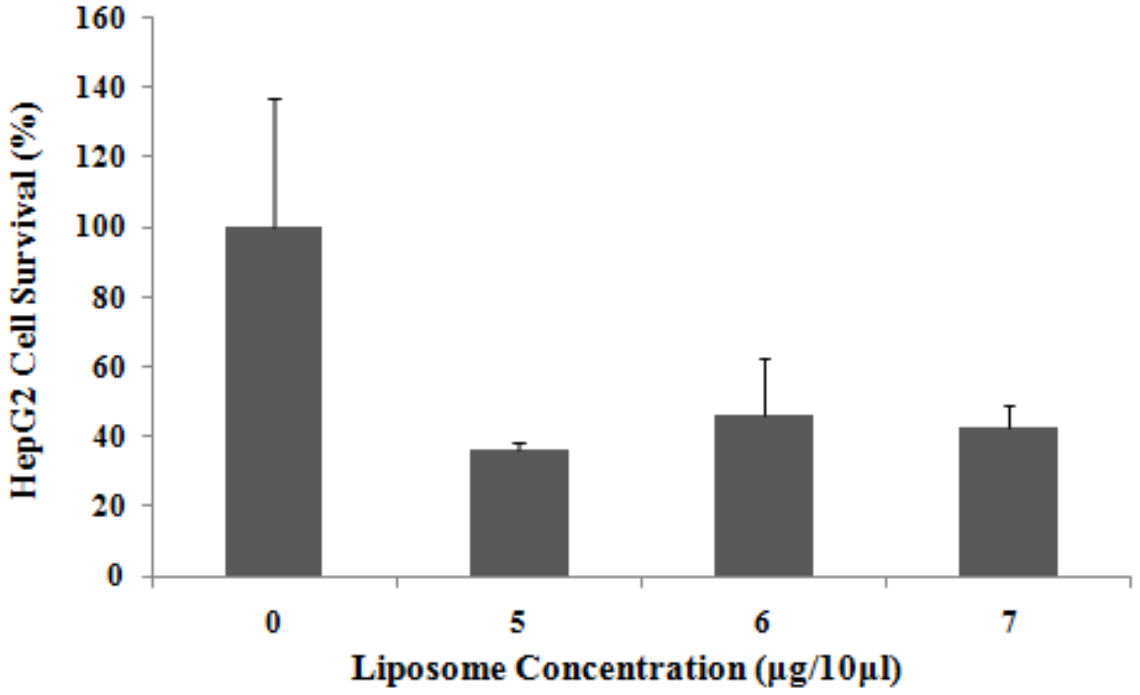
Figure 4.9: Growth inhibition studies of cationic liposome: pCMV-Luc DNA complexes to HepG2 cells *in vitro.* DNA was kept constant at 1 µg. Liposomes amounts were varied.

(a) RUI-128 (0, 6, 7 and 8 µg/10 µl)

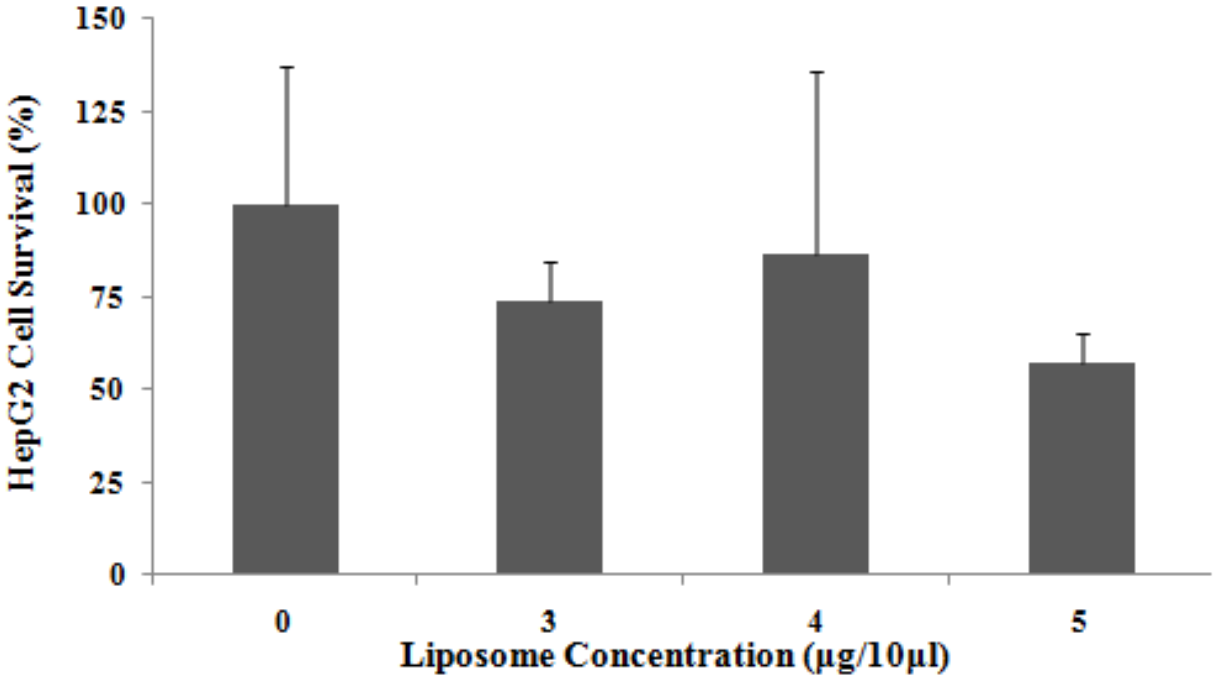
(b) RUI-128-Gal (0, 7, 8and 9 µg/10 µl)

(c) RUI-128-Glu (0, 9, 10 and 11 µg/10 µl)

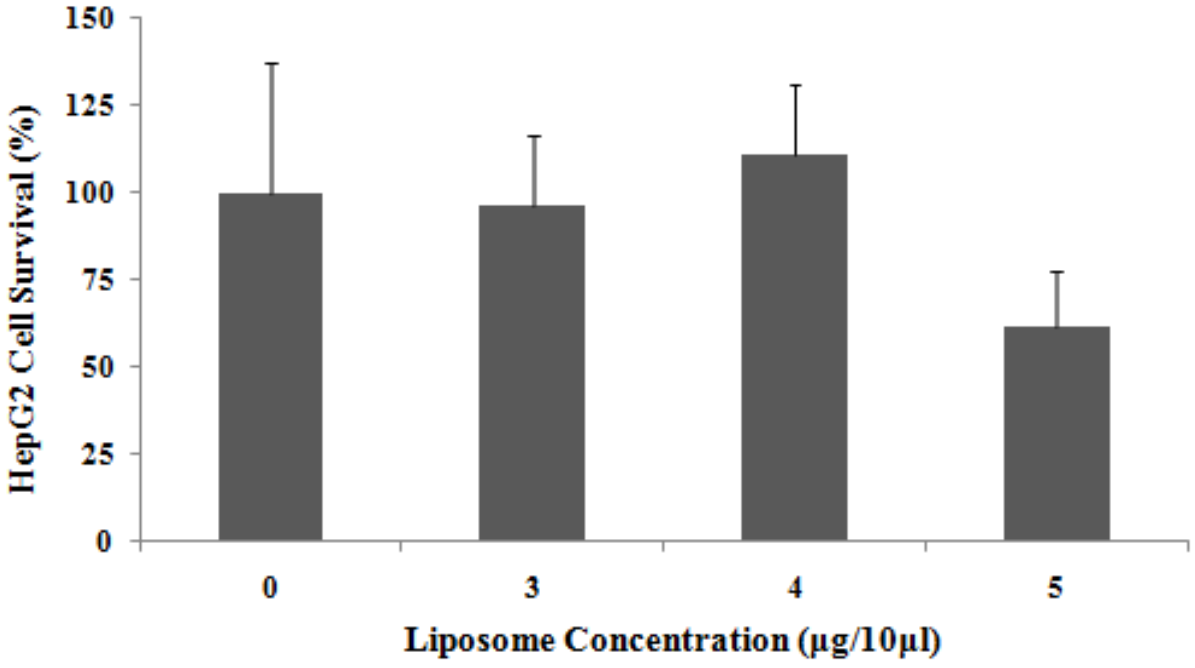
Data are presented as a percentage of the control sample and are represented as means ±S.D. (n = 3).


160
140
120
100
80
60
40
20
0
HepG2 Cell Survival (%)
0
5
6
7
Liposome Concentration (µg/10µl)

(a)


150
125
100
75
50
25
0
HepG2 Cell Survival (%)
0
3
4
5
Liposome Concentration (µg/10µl)

(b)



(c)

Figure 4.10: Growth inhibition studies of cationic liposome: pCMV-Luc DNA complexes to HepG2 cells *in vitro.* DNA was kept constant at 1 µg with liposomes used at varying amounts.

(a) RUI-129 (0, 5, 6 and 7 µg/10 µl)

(b) RUI-129-Gal (0, 3, 4and 5 µg/10 µl)

(c) RUI-129-Glu (0, 3, 4and 5 µg/10 µl)

Data are presented as a percentage of the control sample and are represented as means ±S.D. (n = 3).

Cationic amphiphiles are regarded as toxic both *in vitro* and *in vivo* as they induce several cellular changes including cellular shrinking, a reduction in the number of mitoses and vacuolization of the cytoplasm. Structurally both the hydrophobic and hydrophilic regions of the cationic lipid play a significant role in both transfection and the level of toxicity produced (Mohr *et al*., 2001).

The untargeted Chol-T formulation showed little toxicity with maximal cell death recorded at 33% and 25% in the HEK293 and HepG2 cell lines respectively. The targeted Chol-T-Gal and Chol-T-Glu produced increases in percentage cell death. Chol-T-Gal produced results of 22 and 44 % cell death respectively in each cell line while Chol-T-Glu showed an approximate 40 – 50% cell death in each. The RUI-128 CE preparation upon examination displayed the greatest levels of cell mortality at 30.4% and 43.8% in the HEK293 and HepG2 cell lines respectively. Maximal cell death produced by RUI-128-Gal was at 52.2% and 44.4% in the respective cell lines while the RUI-128-Glu liposome showed cell death of approximately 67% and 26.1% respectively. The RUI-128 (aza-18-crown-6) formulations showed the highest levels of cell mortality from all liposome preparations examined. This could be attibuted to the large cavity size of the crown ether, where increases in the cavity size have been reported to correlate to increases in toxicity. Finally the RUI-129 CE formulation recorded relatively high levels of toxicity with maximum cell death observed at 49.5% and 62.1% respectively. In most cases it was shown that the targeted liposome formulations produced increased levels of cytotoxicity. The galactosyl and glucosyl targeted RUI-129 preparations in contrast to the other examined targeted formulations, showed approximately 50% cell death in the HEK293 cell line and 35% cell death in the HepG2 cell line.

It should also be noted from the results presented in Figures 4.8.c, 4.9.c, 4.10.c that all the glucosyl targeted preparations at the lower DNA: liposome ratios showed cell survival that was close to if not greater than that obtained with the untreated control of HepG2 cells with DNA. In general the increase in cell mortality can be attributed to the increase in liposome concentration. A plausible explanation for this could lie in a potential synergism between the cationic lipid and the plasmid DNA. Their combined effect to produce significant levels of toxicity was evident on comparison to the effect of either the cationic lipid or DNA molecule alone (Dass and Choong, 2006; Ma *et al.*, 2007). The augmentation of this effect by increased concentrations of the cationic lipid in the lipoplex was observed and reported by Felgner *et al.*, (1994). Moreover the steroid backbone of Chol-T is considered more inhibitory to the protein kinase C than the straight chain analogues and consequently is believed more cytotoxic (Mohr *et al*., 2001). Despite this, tertiary amphiphiles such as Chol-T are known to be less toxic in general than the quaternary amines when used in liposome formulations, while cationic lipids with a single hydrophobic tail are regarded as more cytotoxic than their double tailed counterparts (Tang and Hughes, 1999; Lv *et al*., 2006). A possible explaination for this cytotoxicity, almost always associated with cationic liposomes in

lipoplexes, is the induction of pro-inflammatory cytokines (TNF-α, IL-6, IL-12 and IFN-γ) in response to immune cell stimulation by the unmethylated CpG motifs. These CpG sequences are identified to be located in plasmid DNA and in the pCMV-Luc vector employed, these sequences specifically induce production of the tumour necrosis factor-α (TNF-α) (Niidome and Huang, 2002; Elouahabi *et al*., 2003; Kako *et al.*, 2008).

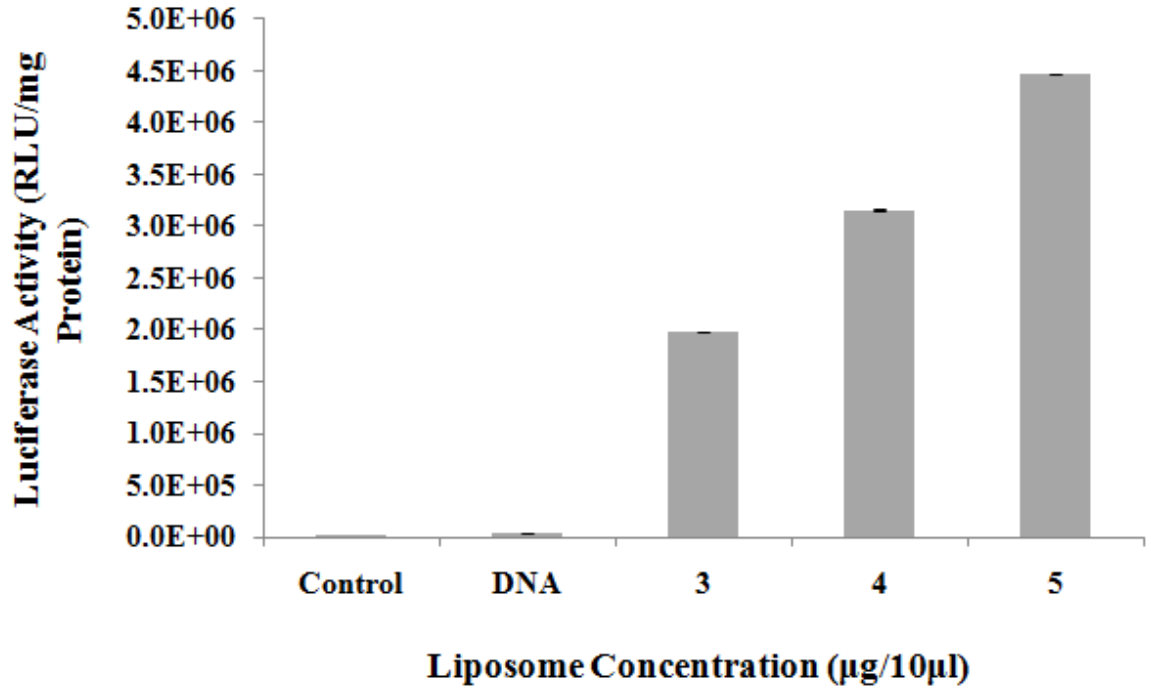
Finally in addition to the cationic lipid altering the membrane surface charge, activity of the ion channels, receptors and enzymes as well as producing membrane perturbations the organic solvent used in liposome formulation is also a concern (Felgner *et al.*, 1994; Tang and Hughes, 1999). Any residual solvent present in the liposome would contribute to the toxicity and stability of the lipid vesicle. These solvents exert their cytotoxic effect either at the molecular level involving enzyme inhibition, protein denaturation and membrane modification, or at the phase level involving nutrient extraction, cell wall disruption, formation of emulsions and cell coating (Mortazavi *et al.*, 2007).

4.3.4. Transfection Studies

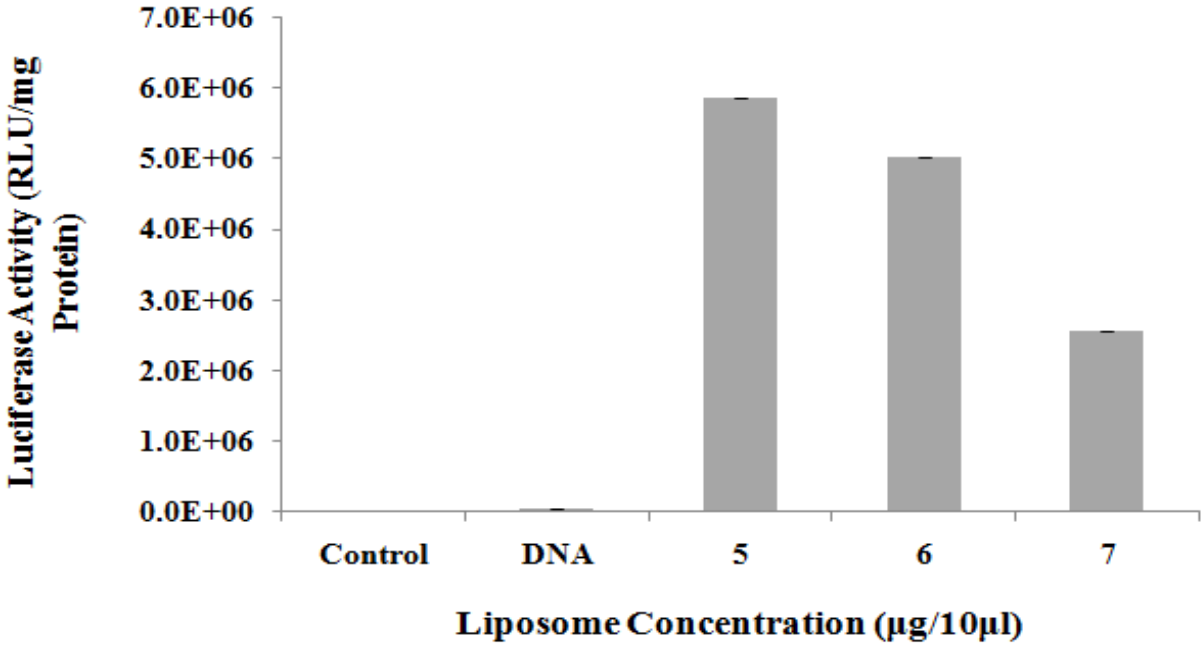
Lipofection mediated by cationic liposomes has been established as a valuable method for gene transfer both *in vitro* and *in vivo*. This process involves the mixing of plasmid DNA in tandem with prepared cationic liposome vesicles for the production of lipoplexes which go on to interact with and be endocytosed by the targeted cells. In general the ratio of positive charge present on the cationic liposomes to the negative charge on the plasmid DNA is a critical determinant involved in producing optimal conditions for transfection (Sakurai *et al*., 2000).

The results of cationic liposome mediated transfection studies in the HEK293 cell line can be seen in Figures 4.11 – 4.13, while transfection into the HepG2 cell line can be viewed in Figures 4.14 – 4.16.

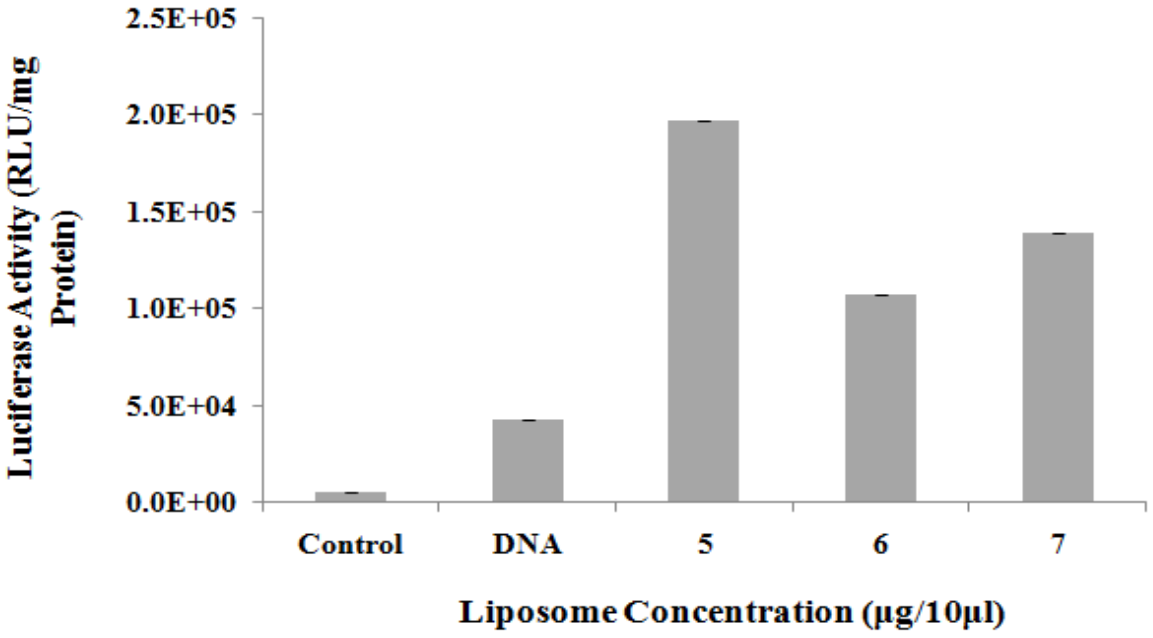
4.3.4. (a) Transfection of the HEK293 cell line



(a)



(b)



(c)

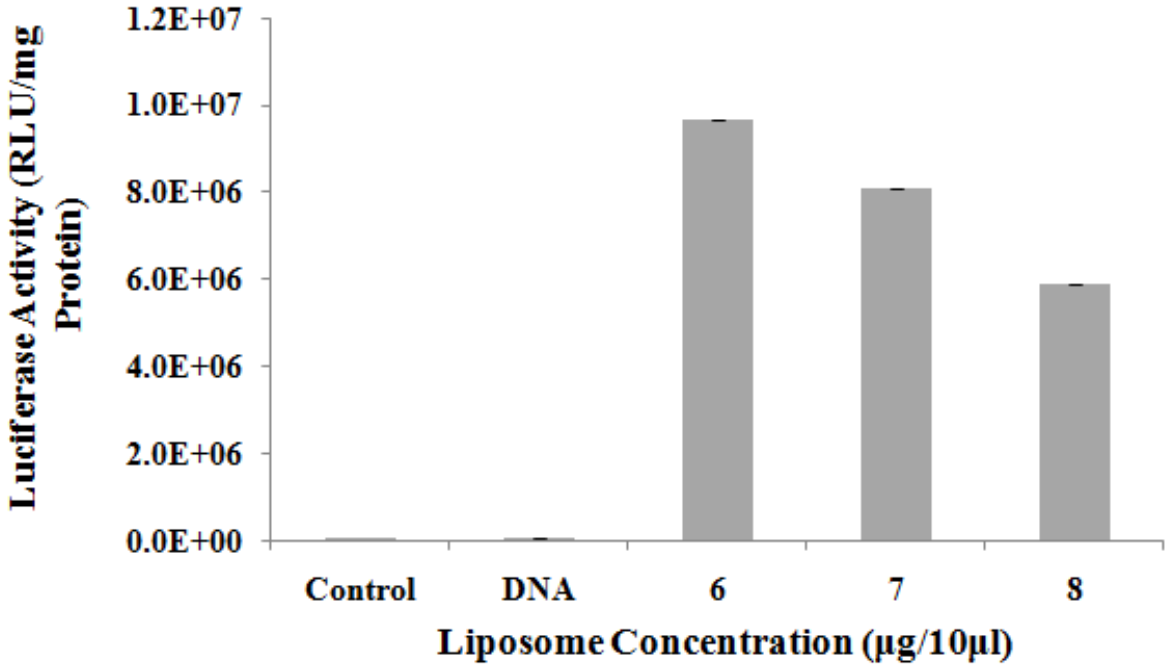
Figure 4.11: Transfection studies of cationic liposome: pCMV-Luc DNA complexes to HEK293 cells *in vitro.* DNA was kept constant at 1 µg. Liposomes were introduced at varying amounts.

(a) Chol-T (0, 3, 4 and 5 µg/10 µl)

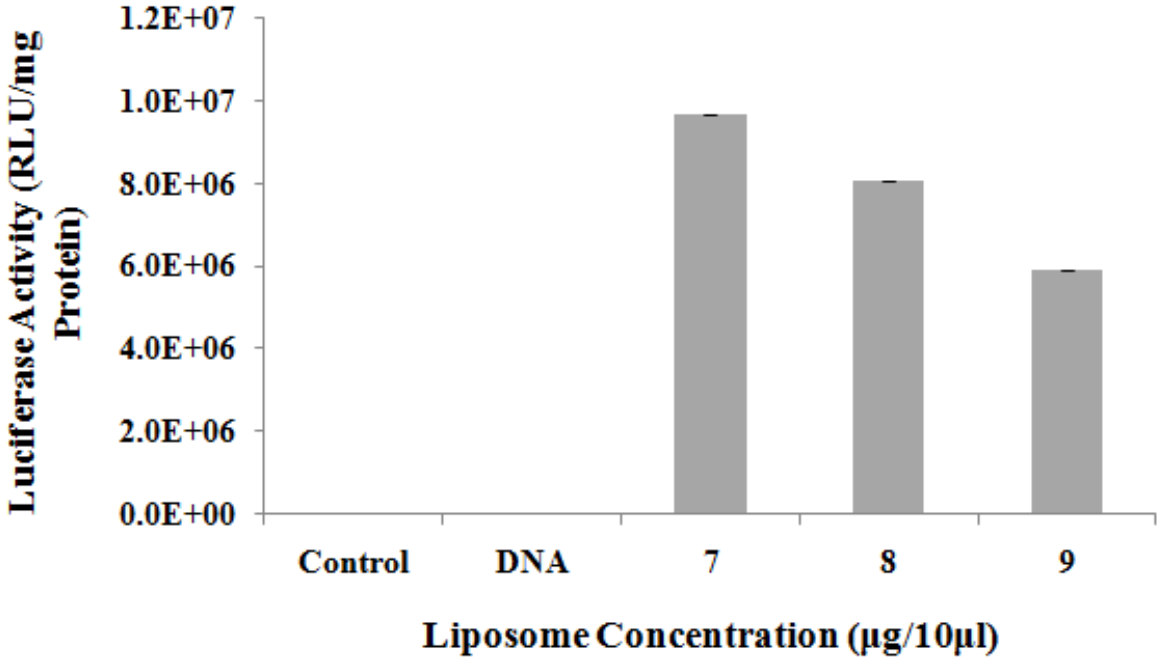
(b) Chol-T-Gal (0, 5, 6 and 7 µg/10 µl)

(c) Chol-T-Glu (0, 5, 6 and 7 µg/10 µl)

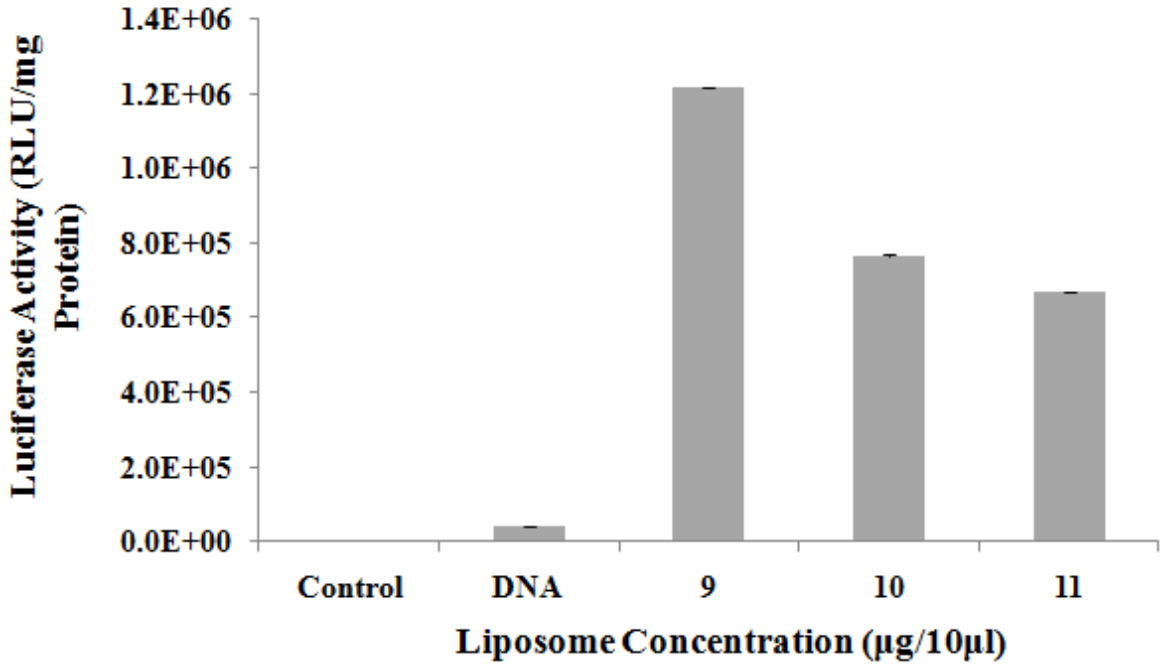
Data are presented as means ±S.D. (n = 3).


Luciferase Activity (RLU/mg Protein)
1.2E+07
1.0E+07
8.0E+06
6.0E+06
4.0E+06
2.0E+06
0.0E+00
Control
DNA
6
7
8
Liposome Concentration (µg/10µl)

(a)


Luciferase Activity (RLU/mg Protein)
1.2E+07
1.0E+07
8.0E+06
6.0E+06
4.0E+06
2.0E+06
0.0E+00
Control
DNA
7
8
9
Liposome Concentration (µg/10µl)

(b)



(c)

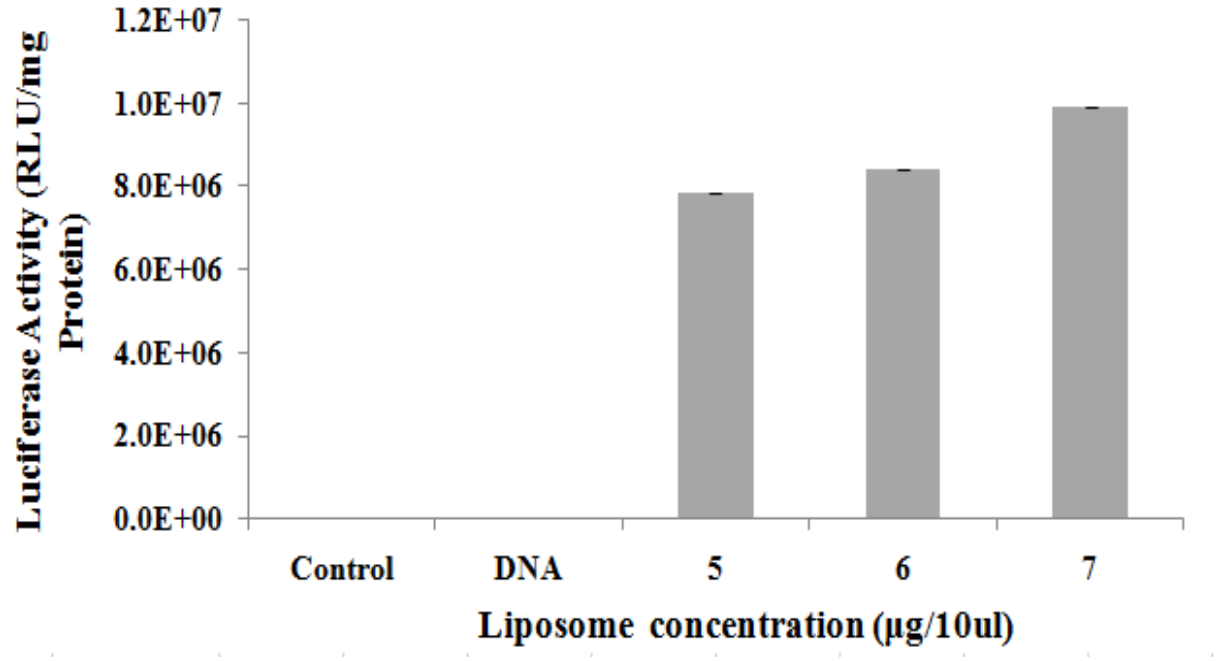
Figure 4.12: Transfection studies of cationic liposome: pCMV-Luc DNA complexes to HEK293 cells *in vitro.* DNA was kept constant at 1 µg. Liposomes were varied.

(a) RUI-128 (0, 6, 7 and 8 µg/10 µl)

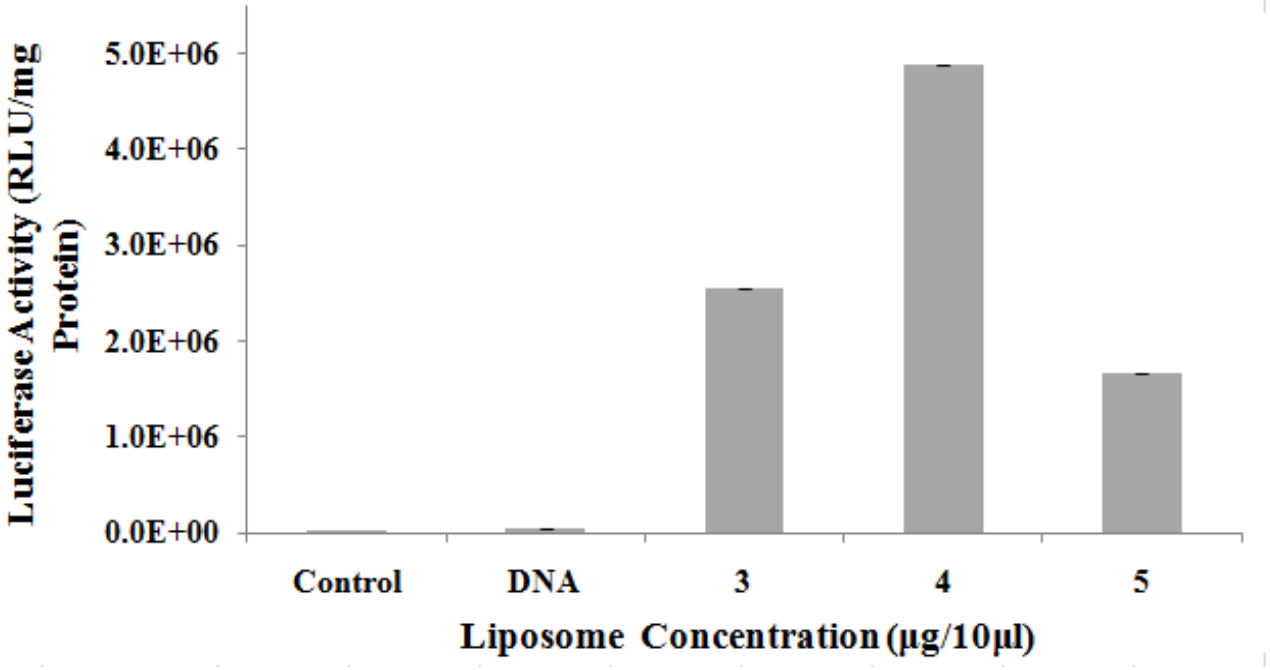
(b) RUI-128-Gal (0, 7, 8 and 9 µg/10 µl)

(c) RUI-128-Glu (0, 9, 10 and 11 µg/10 µl)

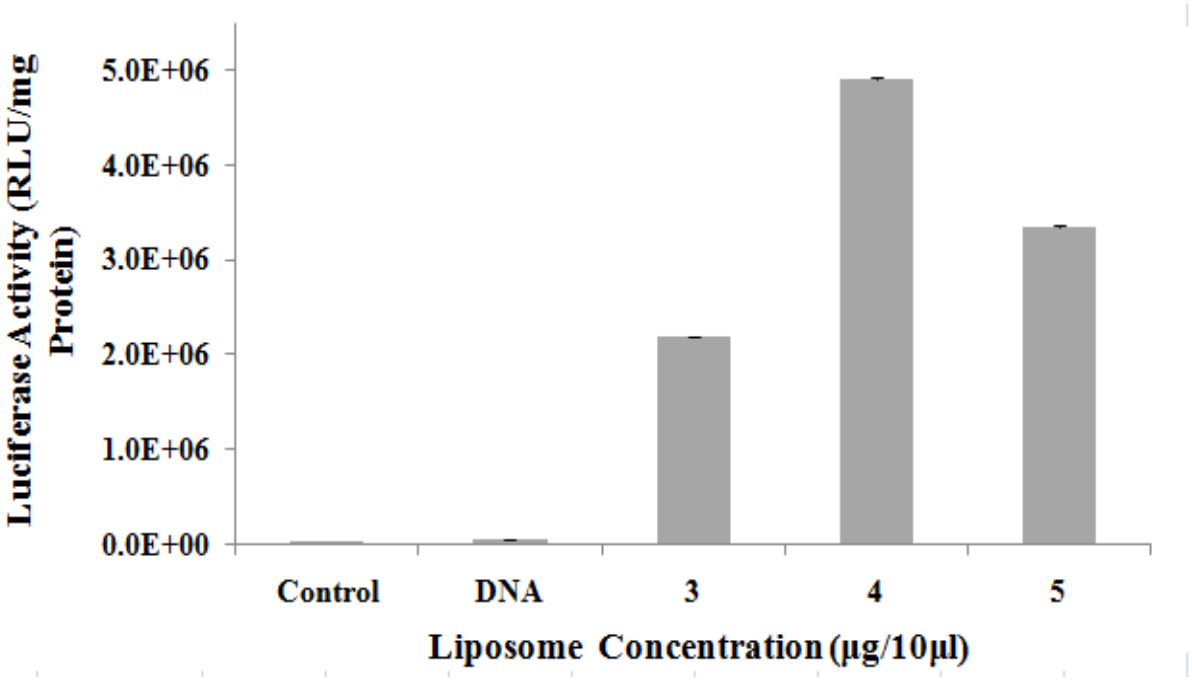
Data are presented as means ±S.D. (n = 3).


Luciferase Activity (RLU/mg Protein)
1.2E+07
1.0E+07
8.0E+06
6.0E+06
4.0E+06
2.0E+06
0.0E+00
Control
DNA
5
6
7
Liposome concentration (µg/10ul)

(a)


Luciferase Activity (RLU/mg Protein)
5.0E+06
4.0E+06
3.0E+06
2.0E+06
1.0E+06
0.0E+00
Control
DNA
3
4
5
Liposome Concentration (µg/10µl)

(b)



(c)

Figure 4.13: Transfection studies of cationic liposome: pCMV-Luc DNA complexes to HEK293 cells *in vitro.* DNA was kept constant at 1 µg. Liposomes were used at varying concentrations.

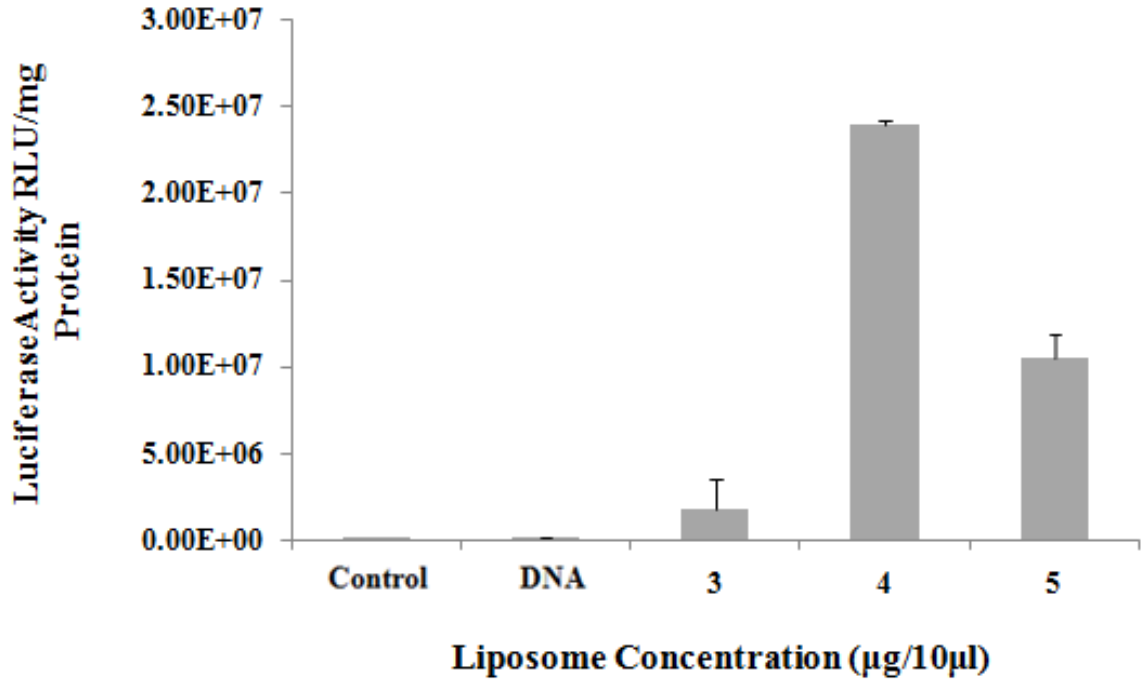
(a) RUI-129 (5, 6 and 7µg/10 µl)

(b) RUI-129-Gal (3, 4 and 5µg/10 µl)

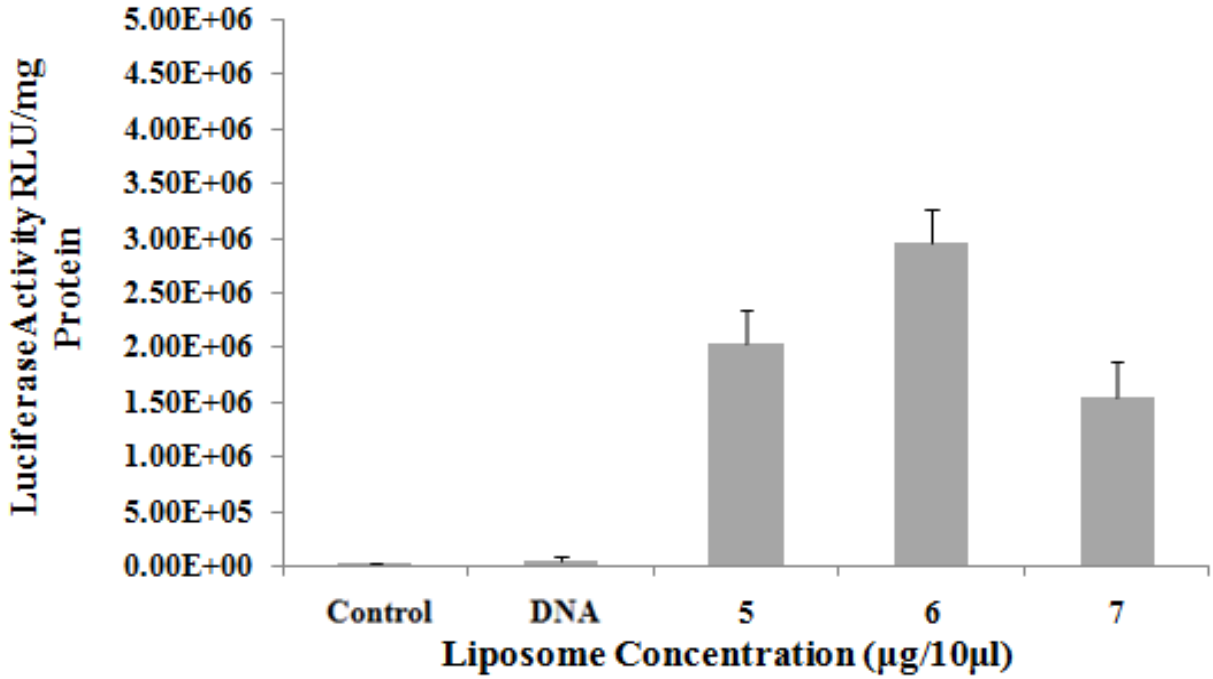
(c) RUI-129-Glu (3, 4 and 5µg/10 µl)

Data are presented as means ±S.D. (n = 3).

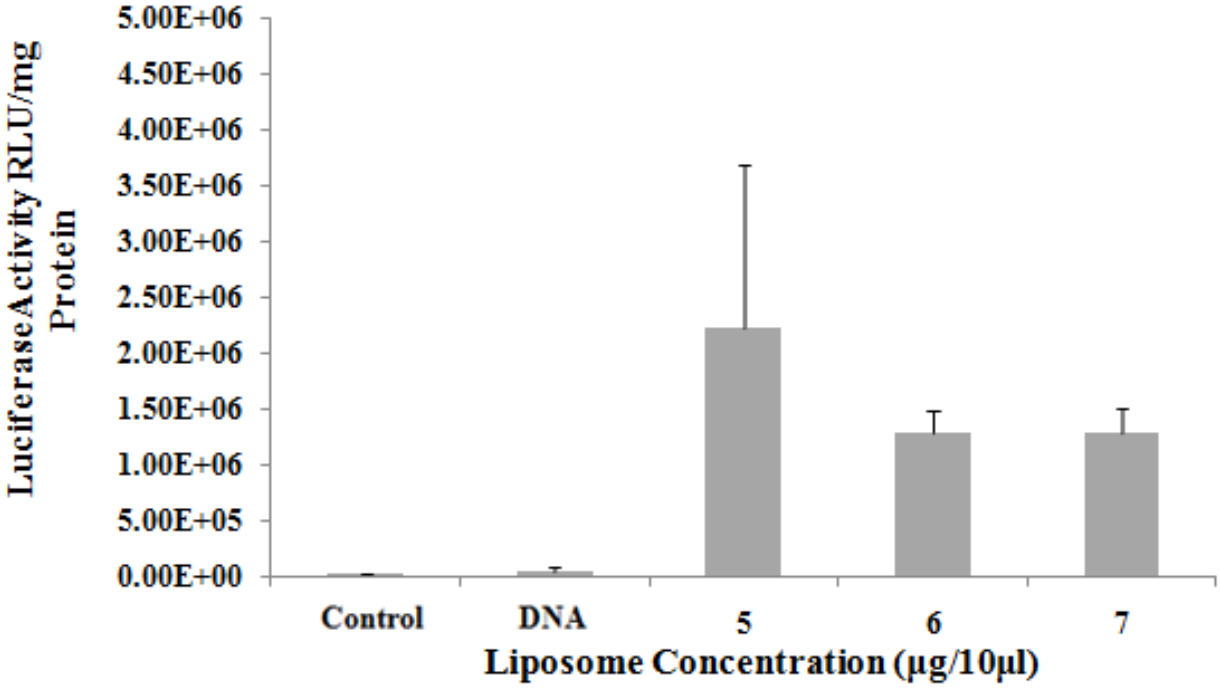
4.3.4. (b) Transfection of the HepG2 cell line



(a)



(b)



(c)

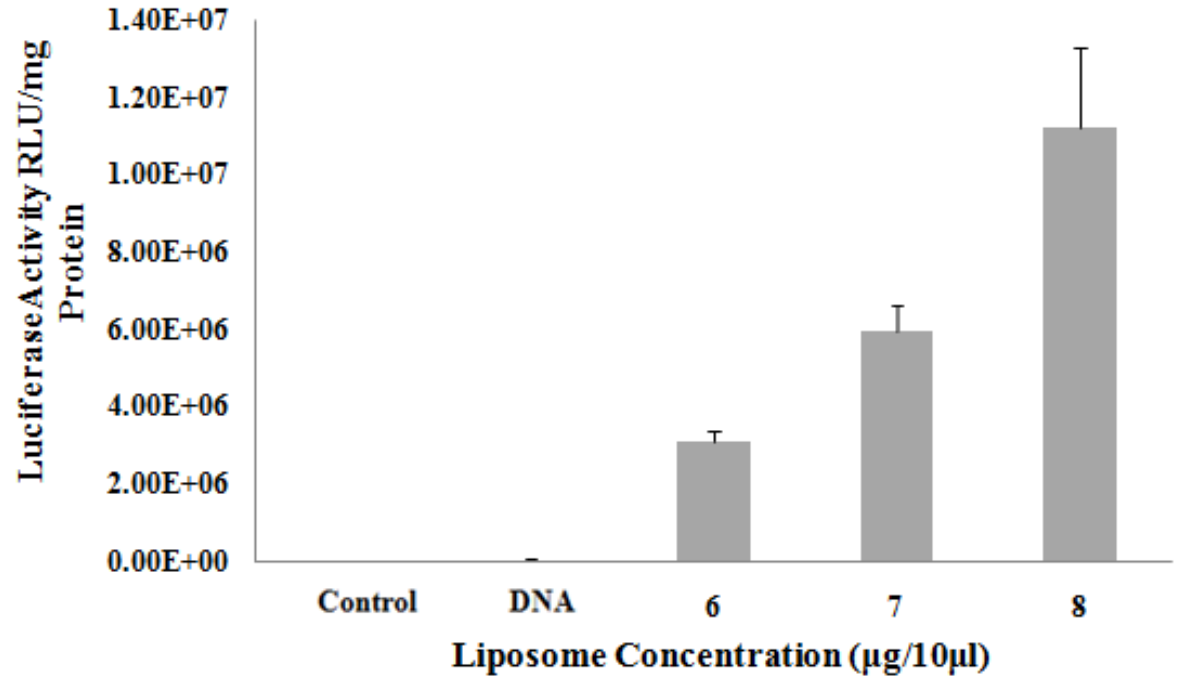
Figure 4.14: Transfection studies of cationic liposome: pCMV-Luc DNA complexes to HepG2 cells *in vitro.* DNA was kept constant at 1 µg with liposomes introduced at varying quantities.

(a) Chol-T (0, 3, 4 and 5 µg/10 µl)

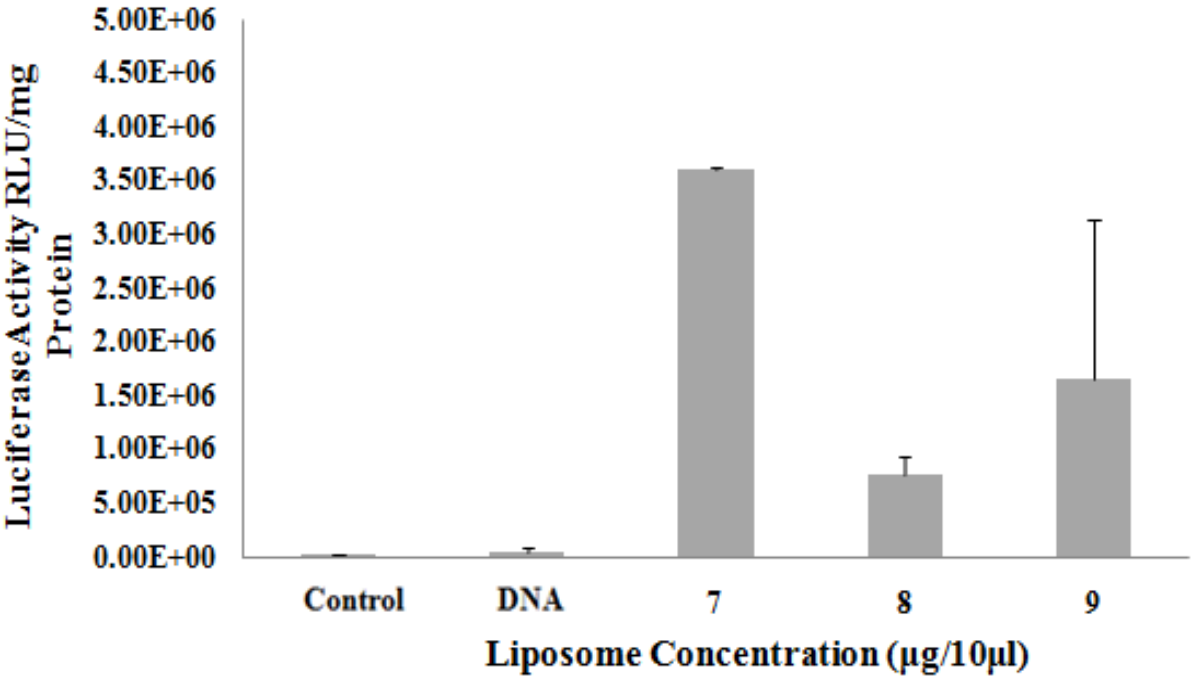
(b) Chol-T-Gal (0, 5, 6 and 7 µg/10 µl)

(c) Chol-T-Glu (0, 5, 6 and 7 µg/10 µl)

Data are presented as means ±S.D. (n = 3).


1.40E+07
1.20E+07
1.00E+07
8.00E+06
6.00E+06
4.00E+06
2.00E+06
0.00E+00
Luciferase Activity RLU/mg Protein
Control
DNA
6
7
8
Liposome Concentration (µg/10µl)

(a)


5.00E+06
4.50E+06
4.00E+06
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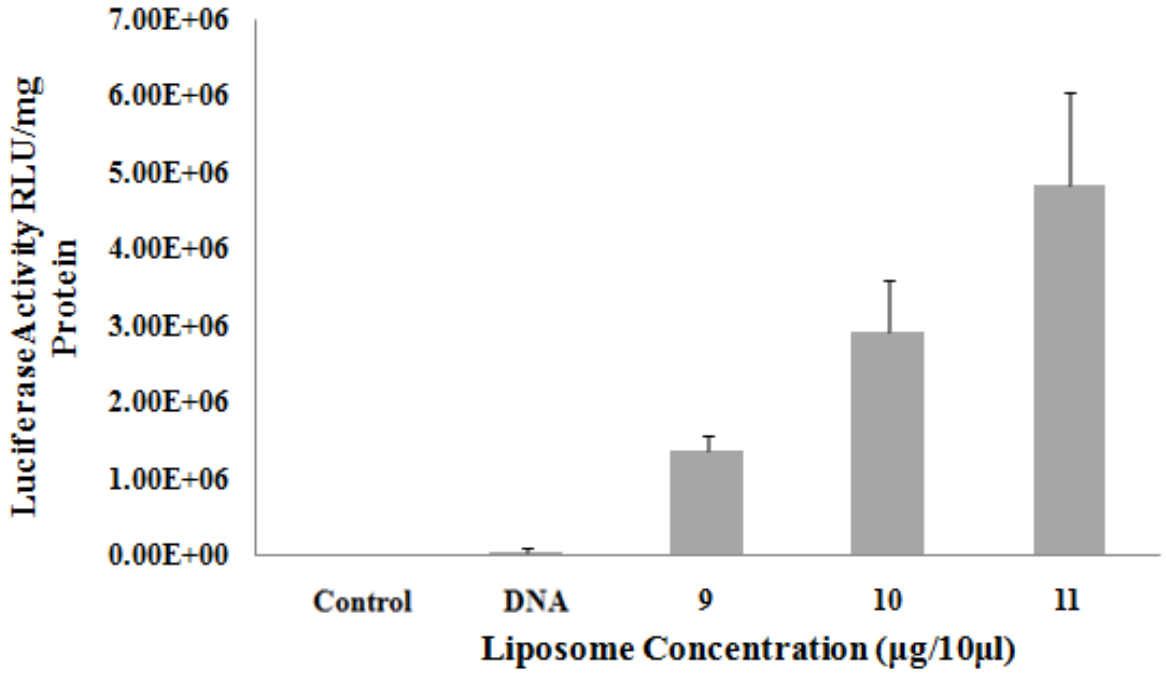
3.00E+06
2.50E+06
2.00E+06
1.50E+06
1.00E+06
5.00E+05
0.00E+00
Luciferase Activity RLU/mg Protein
Control
DNA
7
8
9
Liposome Concentration (µg/10µl)

(b)



(c)

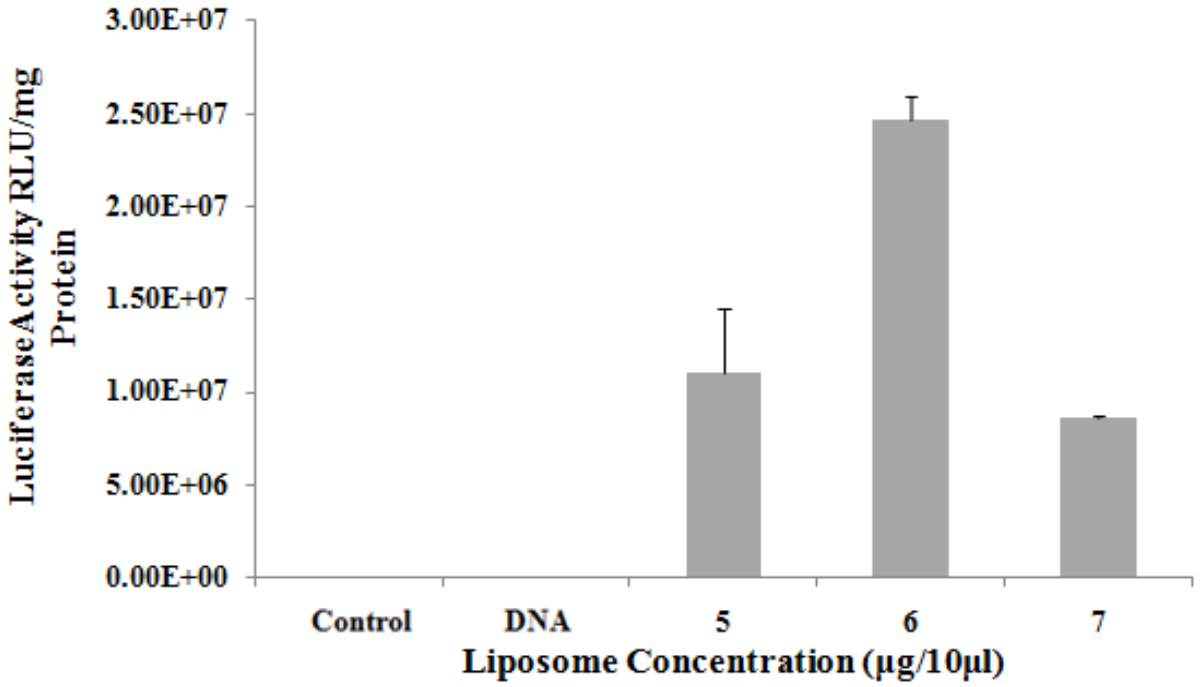
Figure 4.15: Transfection studies of cationic liposome: pCMV-Luc DNA complexes to HepG2 cells *in vitro.* DNA was kept constant at 1 µg while liposomes were introduced at varying amounts.

(a) RUI-128 (0, 6, 7 and 8 µg/10 µl)

(b) RUI-128-Gal (0, 7, 8and 9 µg/10 µl)

(c) RUI-128-Glu (0, 9, 10 and 11 µg/10 µl)

Data are presented as means ±S.D. (n = 3).


3.00E+07
2.50E+07
2.00E+07
1.50E+07
1.00E+07
5.00E+06
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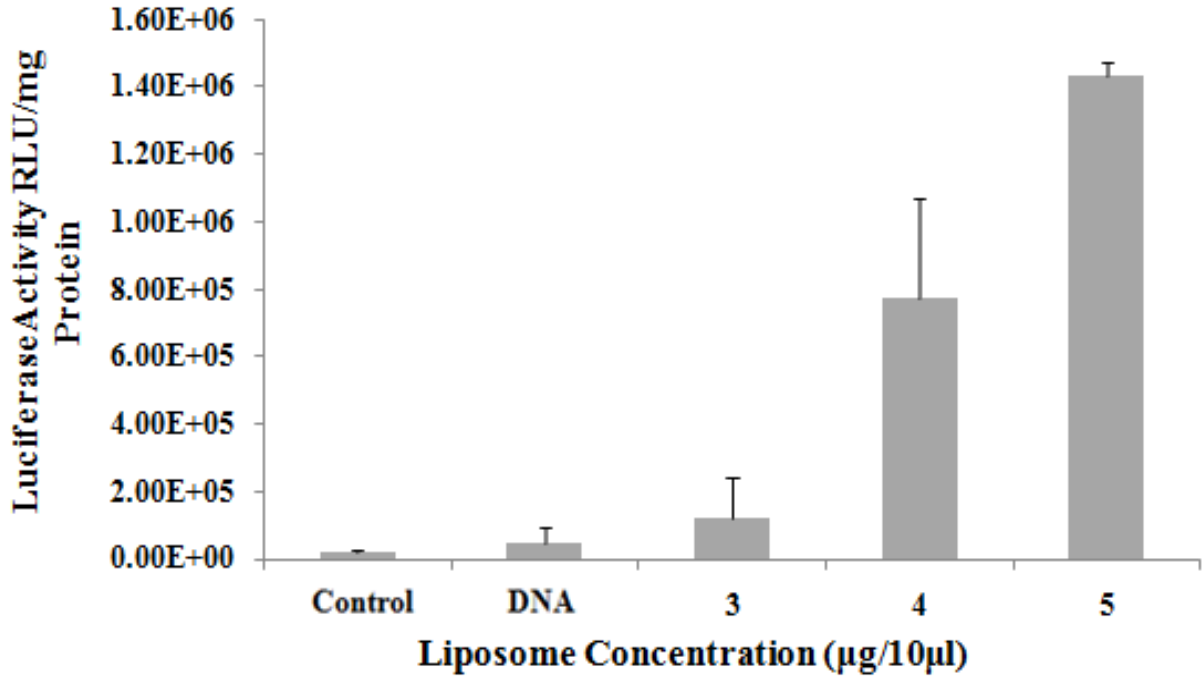
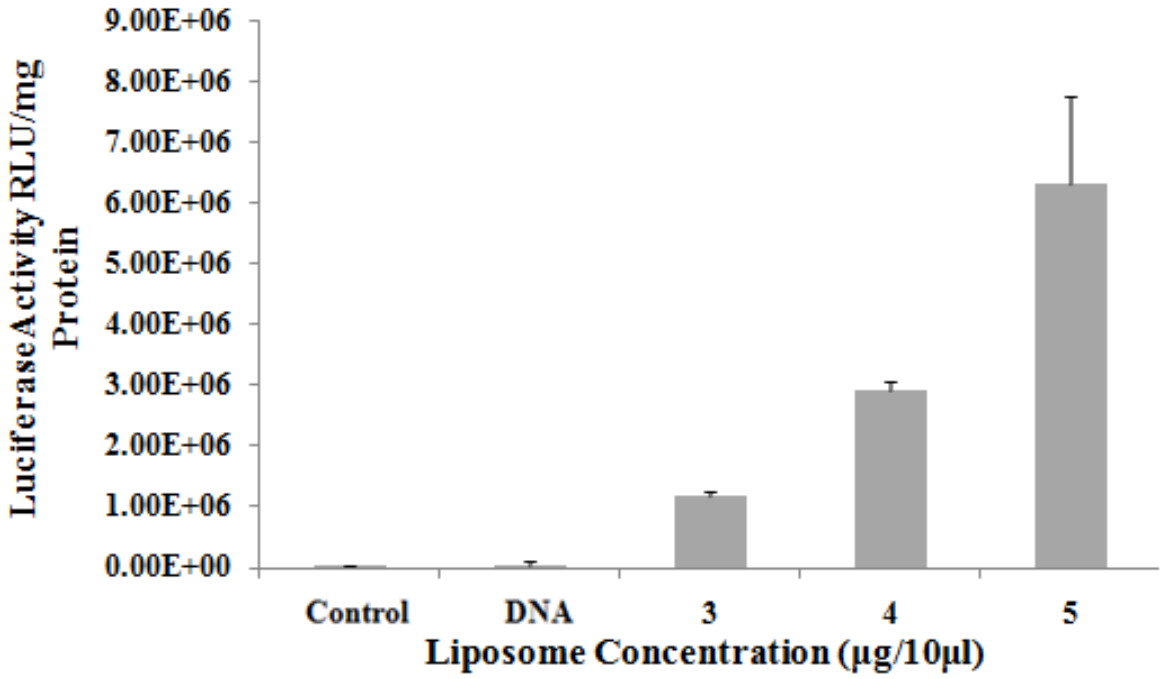
Luciferase Activity RLU/mg Protein
Control
DNA
5
6
7
Liposome Concentration (µg/10µl)

(a)


1.60E+06
1.40E+06
1.20E+06
1.00E+06
8.00E+05
6.00E+05
4.00E+05
2.00E+05
0.00E+00
Luciferase Activity RLU/mg Protein
Control
DNA
3
4
5
Liposome Concentration (µg/10µl)

(b)



(c)

Figure 4.16: Transfection studies of cationic liposome: pCMV-Luc DNA complexes to HepG2 cells *in vitro.* DNA was kept constant at 1 µg. Liposomes were used at varying amounts.

(a) RUI-129 (0, 5, 6 and 7 µg/10 µl)

(b) RUI-129-Gal (0, 3, 4and 5 µg/10 µl)

(c) RUI-129-Glu (0, 3, 4and 5 µg/10 µl)

Data are presented as means ±S.D. (n = 3).

The HepG2 cell line was employed as the targeted test cell line as it expresses an abundance of asialoglycoprotein receptors on the extracellular surface of its plasma membrane. In theory the liposome formulations presenting either galactosyl or glucosyl moieties should be directed to and internalized by these receptors. The HEK293 cell line which is ASGP-R negative was used as a control cell population, against which the activity of the liposome formulations would be tested. Transient gene expression in both these cell lines was detected using the luciferase assay system. This is a chemiluminescent assay for firefly luciferase activity. In comparison with other reporter assays used, the luciferase assay has shown higher levels of sensitivity, speed of reading results without sacrificing the accuracy of results. The overall ATP dependent reaction catalyzed by the firefly luciferase is illustrated in Figure 4.17.

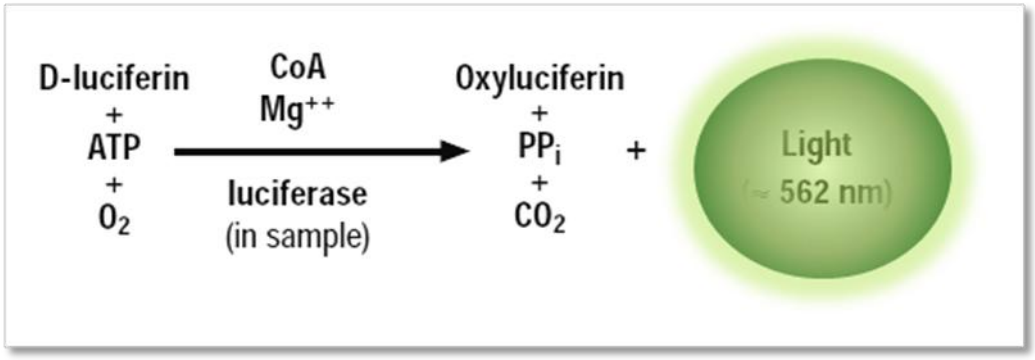


Figure 4.17: Luciferase enzyme reaction (Adapted from Roche Molecular Biochemicals, 2003).

The following trends were observed in the HEK293 cell line using this luciferase assay system. Of the targeted and untargeted Chol-T liposomes, the untargeted Chol-T and targeted Chol-T-Gal preparations showed comparable transfection levels producing higher luciferase enzyme activity than the Chol-T-Glu liposome. The Chol-T, Chol-T-Gal and Chol-T-Glu liposomes showed optimum activity at the DNA: liposome ratio of 1: 5 (w/w) each. A comparative examination of Figure 4.12 (a – c) revealed the RUI-128, RUI-128-Gal and RUI-128-Glu preparations achieved optimal transgene expression at 1: 6, 1: 7 and 1:9 respectively. The untargeted crown ether (CE) formulation showed 3 – 8 fold greater transgene activity than that produced by either targeted liposome. Lastly the RUI-129 untargeted and targeted RUI-129-Gal and RUI-129-Glu CE preparations showed maximal luciferase activity at ratios 1: 6, 1: 4 and 1: 4 (w/w) respectively. Here again the untargeted crown ether formulation promoted the highest activity from all three liposomes.

Overall it can be noted that both untargeted CE formulations achieved the best transfection with the highest levels of luciferase enzyme activity. Moreover it is also clear that for all liposomes investigated the galactosylated cationic liposomes showed greater activity than the glucosylated liposomes in the HEK293 cell line.

On transfection of the HepG2 cell line it was observed that the Chol-T and Chol-T-Glu liposomes showed greatest luciferase activity recorded at their optimal DNA: liposome ratios of 1:4 and 1: 6 (w/w) respectively, as determined by gel retardation studies. The Chol-T-Gal preparation, however, produced this maximal activity at a higher liposome : DNA ratio of 1: 5 (w/w). Maximal transgene expression based on determinations of luciferase enzyme activity was observed for the RUI-128 liposome formulations. Both the RUI-128 and RUI-128-Glu preparations produced optimal activity at the DNA: liposome ratios of 1: 8 and 1: 11 respectively. In comparison the RUI-128-Gal formulation showed greatest effectiveness at a ratio of 1: 7 (w/w). Moreover the RUI-129 and RUI-129-Gal liposomes revealed their maximal luciferase enzyme activity at their optimal DNA: liposome ratios, while the RUI-129-Glu preparation showed its best activity at a ratio of 1: 3 (w/w).

An overview of these results revealed that of the untargeted liposome formulations the RUI-129 CE preparation performed the best followed closely by the Chol-T cationic liposome. The RUI-128 liposome also produced reasonable activity, although not to the extent of the afore mentioned preparations. It was also evident from the results that all the untargeted preparations were more effective at producing high levels of transfection than any of the galactosylated or glucosylated formulations. This was an unexpected result in this targeted cell line.

With no recorded evidence for support the following can only be postulated based on the given working knowledge of the targeting compounds synthesized through click chemistry. The ineffective targeting achieved by these ligand targeted liposomes to the asialoglycoprotein receptors could be affected by the length of the spacer in either the RUI-90 or RUI-92 ligands (glucosyl and galactosyl respectively). Kawakami and colleagues (2001) reported that the success of liposome targeting systems is dependent on the biochemical and physiochemical properties of the glycosylating agents. In this regard it is possible that although linked to a cholesterol anchor, the sugar moiety may not be able to protrude sufficiently out of the liposome membrane to allow effective binding. A significant impediment to transfection by cationic liposomes and other non-viral vectors is the ineffective liberation of DNA into the cytoplasm as well as generally poor nuclear penetration (Niidome and Huang, 2002; Singh *et al*., 2004; Chen *et al*., 2007).

Although it was proposed by De Smedt and colleagues (2005) that complexes with low charge ratios and relatively large diameters produce low levels of transgene expression, in our studies of the different liposome formulations, all having low charge ratios, were capable of successful transfection. The increases in transfection observed with CE preparations could potentially be attributed to cation binding capabilities resulting in an additional positive head which could thus aid in the binding of the lipoplexes to the cell membrane and internalization. Ewert and others (2002) reported improved levels of transfection achieved with a higher head group charge on the multivalent lipids. Moreover the relatively large lipoplex size may be a further contributing factor in this elevated production of transgene expression identified by the levels of luciferase enzyme activity. In this regard the assistance or contribution comes from the larger size of the lipoplexes resulting in their rapid settling onto the surface of the cells in culture (Khalil *et al*., 2006).

Since our experimental evaluations of transfection were carried out in the absence of serum, other factors such as the release of DNA from complexes as well as complex escape from the endosome prior to degradation could play a role in limiting the levels of transfection activity achieved. Furthermore, besides testing for protection against nuclease digestion, the effect of the complexes in the presence of serum is relatively unknown. Further testing under *in vivo* conditions was thus necessary to evaluate the full potential of these targeted and untargeted cytofectin and cytofectin-crown ether systems.

CHAPTER FIVE

5. TRANSFECTION AND TOXICITY ANALYSES *IN VIVO*

5.1. INTRODUCTION

Over the past three decades research relating to the development of *in vivo* nucleic acid delivery vectors has been at its height. Amongst the first non-viral vectors used in this regard were cationic lipids followed closely by cationic polymers. More recent developments have involved the synthesis and implementation of nanoparticles (Rao 2010). One of the major obstacles facing the successful delivery of therapeutic plasmids is the safe and efficient transport to required cells in the *in vivo* situation in spite of advantages such as ease of large scale production (Lasic *et al*., 1997).

Since their introduction cationic liposomes have been synthesized to introduce plasmid DNA to cells in culture, animals and clinical trial patients (Figure 5.1). The inherent flexibility in the application of these systems has allowed for the improvement of novel approaches for the treatment of human diseases (Clancy and Sorscher, 1999). Clinical trials involving earlier generation cationic lipids including DMRIE and DC-Chol revealed that the minimal beneficial effects of transgene expression observed were overshadowed by their toxicity. It is important to note that the observations made in cell culture studies should be treated with great caution as extrapolation of this may not lead to corroborative or similar results with the nucleic acid carrier *in vivo*. Factors important in the *in vivo* situation are the route of administration and the particle diameter (Li and Huang, 2000; Chapel *et al.*, 2004; Dass and Choong, 2006). Furthermore in contrast to the *in vitro* systems which make use of complexes displaying an excess positive charge, the *in vivo* applications have employed both this as well as lipoplexes that exhibit an excess of negative charge (Xu *et al.*, 1999).

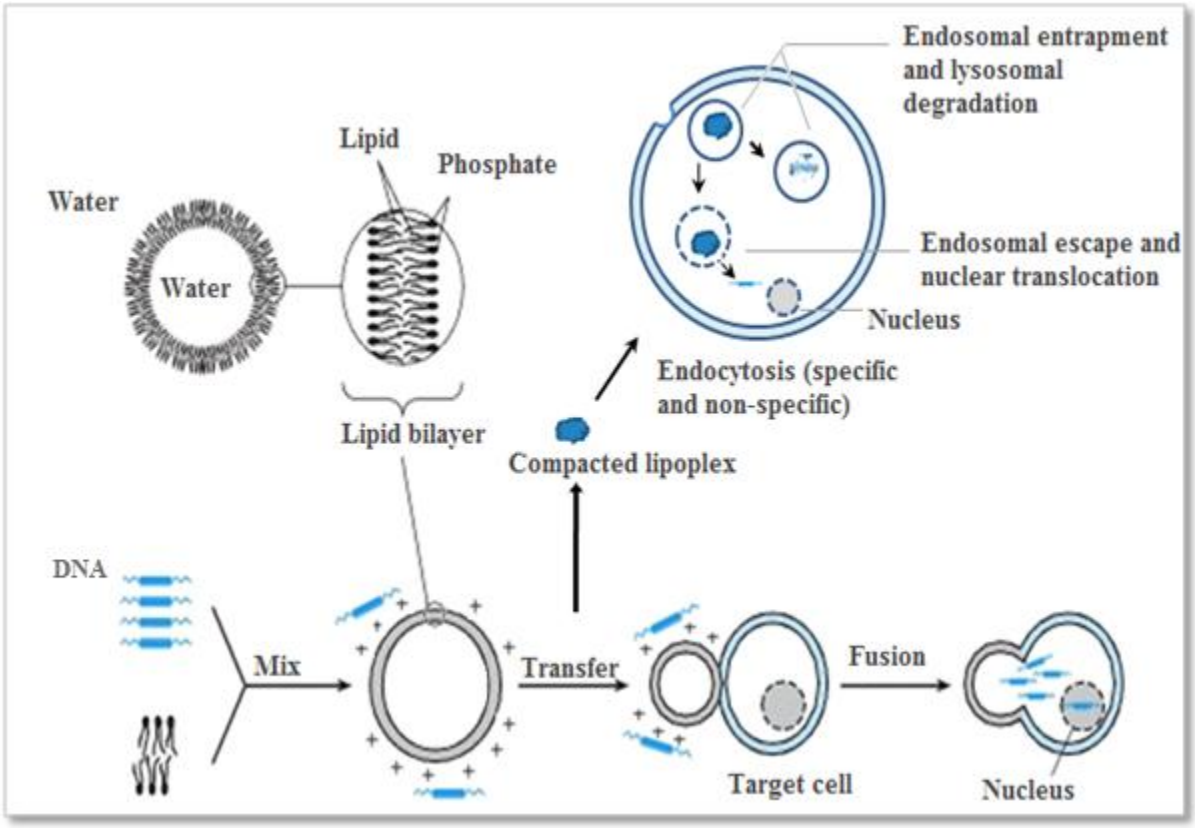


Figure 5.1: Illustration of liposome structure and potential strategies of *in vivo* transfection (Adapted from Bios Scientific Publishers, www.azonano.com. 1999).

The intraportal and intravenous administration of simple cationic liposomes is associated with the difficulty of effective hepatocyte transfection. This is due to the liposomes' natural preference for lung and reticular endothelial system (RES) targeting. In the case of intravenous plasmid DNA injection, the first trap met by the cationic liposome carrier is the capillaries of the lung. Consequently the lung may be considered the point of highest gene expression. Therapeutic undertakings involving specific liver hepatocyte targeting by liposome systems obviates the challenge of enhancing the hepatocyte uptake of liposome carriers while reducing the Kupffer cell uptake (Qi *et al.*, 2005). Systemic DNA injection has proved more advantageous than the largely popular method of local injection as it allows for a far more even distribution of administered DNA in the liver (Niidome and Huang, 2002). Moreover intranasal administration has been identified as a more capable pathway than the intratracheal treatment in producing enhanced expression (Wheeler *et al.*, 1996).

In attempts to overcome this so called 'lung trap' of hepatocyte cell targeting, the size of the carrier molecules can be altered and the surface charge of the complex with DNA reduced, ultimately allowing

improved accumulation of therapeutic material in the liver rather than the lung for effective transfection. Kupffer cells of the liver, mentioned earlier, are part of the RES which is known to commonly clear out liposomes when administered intravenously. The primary site of liposome accumulation has in general been found within the non–parenchymal cells, despite their desired incorporation into the liver parenchymal cells as required for liposomal gene therapy (Maitani *et al*., 2001). The liver was chosen in this study for liposomal targeted for gene delivery *in vivo*.

Cationic liposome-DNA complex mediated transgene expression may most certainly produce antitumour effects (Thurston *et al.*, 1998). The plasmid DNA employed in this study was the Psi-CHECK plasmid encoding the Renilla Luciferase and Firefly Luciferase reporter genes. Succcess of this system for *in vivo* gene expression is dependent on various factors including cholesterol based liposomes, high cationic charge, high DNA dose and the absence of the non-methylated CpGs which are known to induce inflammatory cytokine expression in the immune system (Morille *et al*., 2008). The requirement for transplantation in *in vivo* gene therapy has made clear the need for direct transfer of genes into the target tissues and cell cultures. Consequently novel glycosylated derivatives have been synthesized over the years for use as liver targeting ligands, however, the low antitumoural efficacy of these systems for *in vivo* conditions necessitates the investigation and development of new and effective vectors (Eming *et al.*, 2007; Díez *et al.*, 2009).

The systemic method of administration offers the opportunity for non-invasive interaction with numerous target cells which are otherwise inaccessible for direct administration. The inability for effective cell transfection upon intravenous injection to systemic systems has primarily been ascribed to the interaction between lipoplexes and serum proteins, a barrier that may be surmounted through an increase in lipoplex charge ratio (Mohr *et al*., 2001). Many of the vectors employed today can afford protection to the carried nucleic acid and assist in internalization, however, these carrier systems are limited by their inability to overcome the endothelial barriers as well as to efficiently exit the general circulation (Hattori and Maitani, 2005). The premier obstacle that faces systemically administered lipoplexes is the glycocalyx shield consisting of glycoproteins, glycolipids, glycosaminoglycans, phospholipids, and proteoglycans which position themselves on the luminal side of the vesicular wall (Rao, 2010).

An unfortunate disadvantage in the application of cationic lipid – DNA complexes is their associated toxicity primarily attributed to the systemic induction of inflammatory response to the CpG motifs of the compacted plasmid DNA (Elouahabi *et al*., 2003). Evaluation of potential therapeutic compound toxicity requires a blood sample from the experimetal animal model. For this to be successfully obtained blood must be drawn from the retro-orbital sinus of mice or the retro-orbital plexus of rats (Figure 5.2). When

performed correctly this method can provide moderate to large amounts of blood for analysis, however, incorrect handling or implementation of protocol can result in serious injury to the animal. Rats in comparison to mice have a plexus and thus the potential for tissue damage may be more significant than with mice. Consequently other available alternatives to retro-orbital bleeding should be kept in mind (Figure 5.3) (Guanzini and Barriere, 2009).

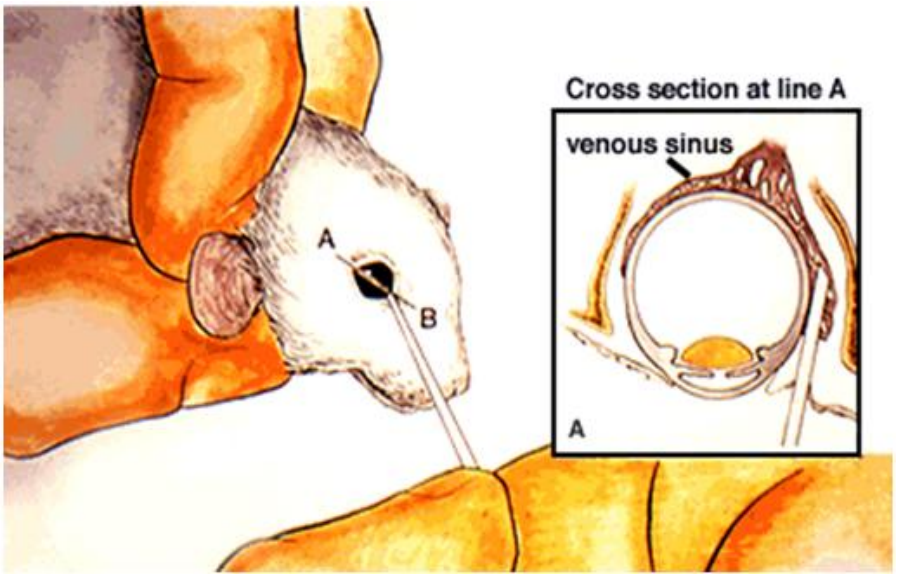


Figure 5.2: Demonstrative illustration of Retro-orbital bleeding of mice (www.spcollege.edu/LabAnimals).

Table 5.1: A summary of different blood sampling techniques (Brown, 2005)

Route	General Anesthesia Required	Speed and efficiency		Sample Quality		Repeated Sampling	Relative Volumes Obtainable	Potential for Complication	Species	Comments
		Mouse	Rat	Mouse	Rat					
Tail Vein or Artery	No	++Vein +++Artery	+++Vein +++Artery	± to +	++ to +++	Yes	Small - medium (Vein) Medium - large (Artery)	Low	Rat, Mouse	Repeatable, Simple, Variable sample quality
Tail Clip	No	+++	+++	+/-	+/-	Yes	1 – 2 drops	Low	Rat, Mouse	Repeatable if gently pull scab
Retro-orbital	Mouse – No Rat – Yes	+++	++	+++	++	Should alternate	Medium to large	Moderate to high	Rat, Mouse	Rapid potential for complication
Saphenous	No	++	++	++	++	Yes	Small to medium	Low	Rat, Mouse	Not as rapid as other techniques, low potential for tissue damage
Jugular	Recommend ed	N/A	+/++	N/A	+++	Difficult	Large	Low	Rat	Limited application, poor for repeated sampling
Mandibular	No	+++	N/A	+++	N/A	Yes	Medium to large	Low	Mouse	Rapid, easy and repeated samples possible

Efforts towards the successful development of lipid-based carriers for systemic transfection is highly significant and consequently unrelenting (Liu and Song, 1998).

5.2. MATERIALS AND METHODS

5.2.1. Materials

All Chemicals utilized were of analytical grade. The NMRI mice are so called as they were transferred to the Naval Medical Research Institute after being inbred for 51 generations. The mice were thereafter in 1979 introduced to the Charles River Laboratories. They were kept at a room temperature of

approximately 21^{o}C on autoclaved sawdust bedding (Volstroff, 2007). The Psi-CHECK plasmid DNA was amplified in transformed *Escherichia coli* cells and purified using the Qiagen Endotoxin Free Kit. The purified plasmid DNA was analyzed on a Nano-drop spectrophotometer.

5.2.2. Methods

5.2.2.1. Lipoplex preparation

The liposomes were prepared at their optimum DNA binding ratios (determined by agarose mobility shift analysis) with the amplified Psi-CHECK plasmid vector. The lipoplexes were made up in four replicates under aseptic conditions as shown in Table 5.1 below. The positive control was employed at 2.5ml to cause an alteration in the hydrostatic pressure of the mouse system, allowing delivery of the DNA. It further provides a good indication that the DNA being used is effective. A negative control of DNA – Sabax sodium chloride solution was not used in this study, however, un-injected mice were examined, representing normal conditions. All liposome preparations were made up in Sabax sodium chloride solution from Adcock Ingram, Johannesburg, South Africa.

Table 5.2: Component ratios of liposome and DNA used for transfection and cytotoxicity assays and the quantities introduced for luciferase analysis by tail vein injection.

Preparation	Amount of Liposome (µg)	Psi-CHECK Plasmid DNA (µg)	Quantity Injected (µl)
Negative Control (Un-injected NMRI Mice)	-	5	-
Positive Control (Hydrodynamic Injection)	-	5	2500
Chol-T Liposome	20	5	200
RUI-129 Liposome	30	5	200
RUI-129 – Gal Liposome	20	5	200

5.2.2.2. Tail vein injection

The NMRI mice were number tagged from 139 - 158 and divided into five groups A - E. The mice are characterized as inbred albino mice and were approximately 25 – 30 g in weight. For tail vein injection,

heat control bags were used to enhance the appearance of the vein so as to ensure accurate injection. Small plastic mice restraints were used to control mice movement.

Lipoplexes of Chol-T, RUI-129 and RUI-129-Gal were injected into groups A, B and C respectively. Group D was subjected to the positive control of hydrodynamic injection, while group E, the negative control was left un-injected.

5.2.2.3. Toxicology

Three days after injection the mice were bled using the retro-orbital bleeding method. The blood samples were left to coagulate, i.e. clot by incubation in refrigerator for approximately 4hrs. These samples were then spun down by quick centrifugation and the supernatant collected and sent for analysis at the HIV testing unit at the University of Witwatersrand School of Medicine, Department of Molecular medicine and Hematology. The AST (Aspartate Aminotransferase) and ALT (Alanine Aminotransferase) levels were examined as a basis for hepatic and heart cytotoxicity analysis.

5.2.2.4. Luciferase reporter gene transfection

The mice were thereafter sacrificed and the livers harvested. To 1 g of the harvested livers an equal volume of Sabax saline solution was introduced and the livers homogenized using the CATx120 mechanical homogenizer having variable speed control. Thereafter 20 µl of the diluted homogenate was introduced into a 96 well plate together with 100 µl of luciferase reagents obtained from Promega. A 2x dilution was used with 2 reagents employed for analysis of the Psi-CHECK plasmid, i.e. both the luciferase and stop glow reagent for the Renilla luciferase encoded in the plasmid. The plate was thereafter analyzed in the Veritas Microplate Luminometer from Turner Biosystems. Protein determination was performed on the cell free extracts using the bicinchoninic acid (BCA) assay using the Pierce BCA kit.

5.2.2.5. Statistical analysis

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

5.3. RESULTS AND DISCUSSION

5.3.1. Lipoplex Preparation

The Psi-CHECK plasmid used in this preparation was successfully amplified. The concentration of the DNA was measured and used as a stock of 1.1293 µg/µl. The plasmid DNA showed relatively high purity with the 260 nm/ 280 nm absorption ratio of 1.91. Lipoplexes were successfully and aseptically prepared using the sodium chloride solution. The DNA-saline solution was prepared at a volume of 2.5 ml as shown in Table 5.1. Despite encoding both the Renilla and luciferase reporters, for consistency in our studies, only the luciferase reporter gene activity was examined as described in section 5.3.3.

5.3.2. Toxicology

The potential for the administration of therapeutic agents or chemicals to produce a toxic or detrimental effect to the cell is often considered relative to the dose injected. The toxicity may be local, i.e., a local effect, where the harmful event takes place at the site of administration, or systemic involving the distribution of the detrimental factors throughout the cirulatory system resulting in the damage of other organs. In addition the toxic effect can be termed either acute or delayed, in relation to the time taken for exposure (Lv *et al*., 2006).

Results from cytotoxicity analysis based on the levels of ALT and AST present in the blood samples obtained through retro-orbital bleeding can be seen in Figure 5.3.

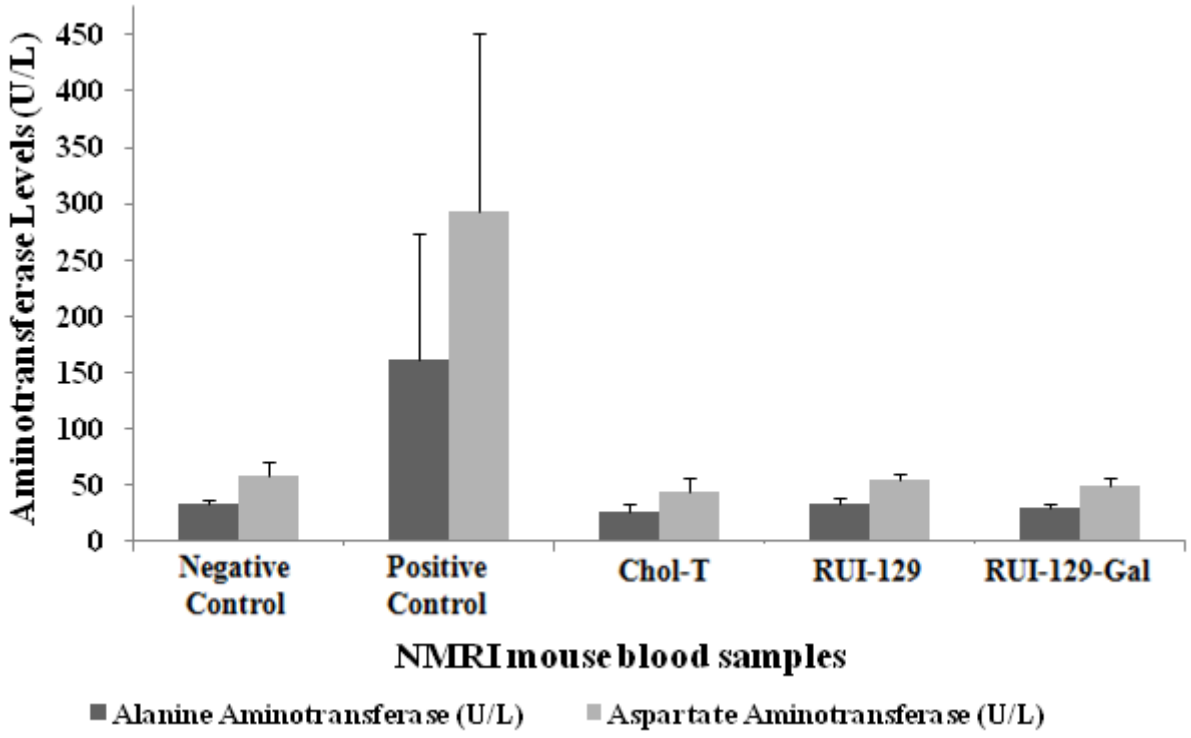


Figure 5.3: ALT and AST levels of different blood samples inoculated with the different liposome preparations. Data presented as a mean ±S.D. (n = 4).

Analysis of the ALT and AST levels conducted in this investigation allows for the determination of the extent of liver damage or toxicity induced by the presence of the liposome – DNA complex. In principle the damage of a specific selection of cells results in the release or leakage of certain enzymes into the blood. These enzymes are thus utilized as indicators of cellular damage. The ALT levels show a marked increase in hepatitis and other acute liver damage. AST, although similar in this regard is not as liver specific as it is predominntly found in other tissues such as the heart and muscle. These enzyme are known as part of the transaminase family and are sometimes referred to as glutamate pyruvate transaminase (GPT) and glutamate oxaloacetate transaminase (GOT). As transaminases these enzymes are also known to facilitate certain intracellular reactions and may also be termed aminotransferases. Fundamentally, injury or inflammation of the liver can be identified by elevated ALT blood levels. Damage to the heart or liver will release additional AST into the blood stream. The levels of AST detected is thus believed to directly related to the degree of tissue damage accrued. In general the normal range of AST and ALT values should be below 40 – 50 (U/L) (Cholestech Corporation, 2007).

From the results shown in Figure 5.3. all the liposomes investigated produced minimal cytotoxic effect in the liver tissue. This was extrapolated from a comparision between the aminotransferase levels produced

in response to the liposomes preparations and those of the uninfected mice in the negative control. The comparable levels of AST and ALT observed between the negative control and the test lipoplexes is indicative of minimal damage being effected by the presence of the different liposomes. The positive control, involving the hydrodynamic injection of the Psi-CHECK plasmid produced high level toxicity. This tissue toxicity was not unexpected due to the drastic changes induced by hydrodynamic injection of a large volume of DNA-saline solution into the bloodstream. Moreover the presence of CpG motifs in the DNA may once again prove their ability to potentiate cytotoxicity.

An overview of these results further indicates higher quantities of AST rather than ALT in the blood samples of the control and test animals examined. The liver in general shows the ability to tolerate most potential toxic effects that may take place due to its regenerative capabilities (Suda *et al.*, 2009).

5.3.3. Luciferase Reporter Gene Transfection

Results from the luciferase reporter gene assay in the NMRI mice can be viewed in Figure 5.4.

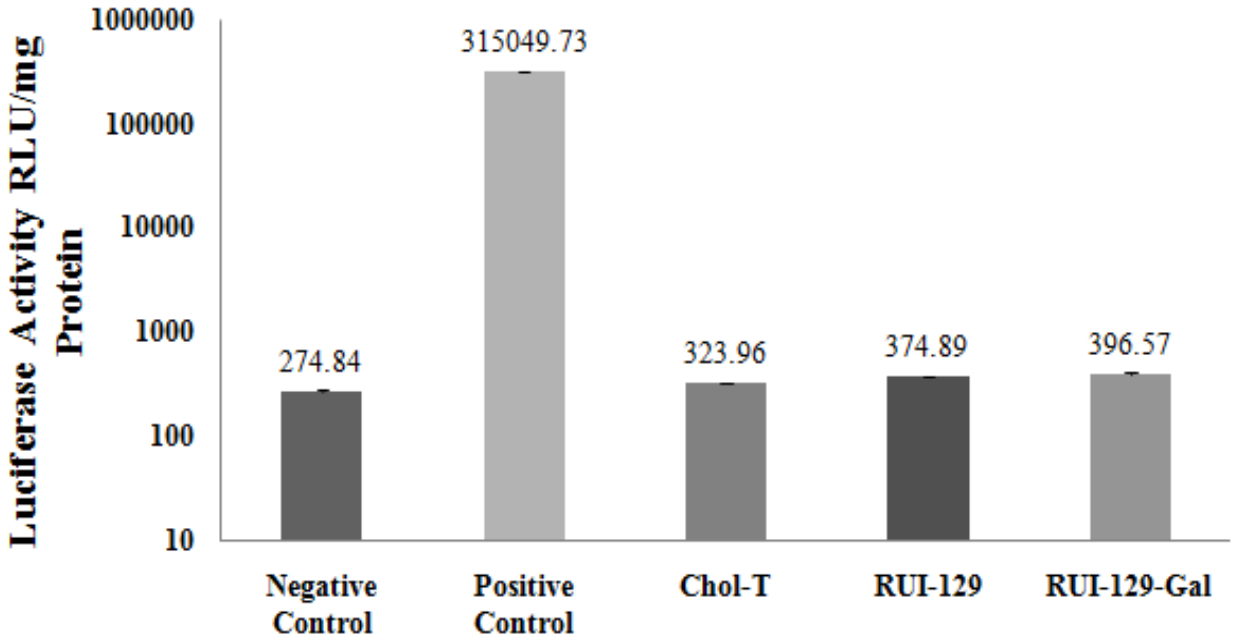


Figure 5.4: Luciferase activity recorded in the different groups of NMRI mice on incubation with the different liposome preparations. DNA was kept standard at 5 µg. The positive control involved injection of DNA in the absence of liposome, while the negative control used was of untreated mice. Data presented as a mean ±S.D. (n = 4).

For an analysis of the luciferase enzyme activity effected by the different liposome formulations, two control systems were employed. The first control used was a group of NMRI mice that were not treated so as to display the normal condition. The second control employed was the hydrodynamic injection of 2.5 ml of a DNA-saline solution into the tail vein of the mice. This control was termed the positive control as it is renowned for the successful production of extremely high levels of transgene expression in the mouse liver. In addition to this elevated *in vivo* gene expression Niidome and Huang (2002) have reported the prolonged maintenance of this therapeutic level for at least 10 months. This postive control produced high levels of transgene expression that were shown to be statistically significant on comparison to the different liposomes examined ($p < 0.05$). Had all preparations been based on the hydrodynamic method of injection, it is possible that an alternative outcome would have resulted from the lipoplexes investigated in this mouse model.

From an examination of the results it should be noted that all three liposome formulations were shown to be relatively poor at directing the plasmid DNA successfully to the liver for transgene expression. Despite this, the low luciferase enzyme activity found with liposome – DNA complexes compared to the background fluorescence in untreated, negative control liver extracts, were shown to be of statistical significance ($p < 0.05$).

There are a great number of barriers presented to the *in vivo* transfection of liposome formulations that limit their effectiveness. The inconsistencies observed here and in earlier reports between the carrier activity *in vitro* and *in vivo* may potentially be attributed to poor condensation of the DNA resulting in the untimely liberation of the plasmid DNA into *in vivo* circulation. Xu and Szoka (1996) proposed that this premature DNA discharge could also be due to the endogenous glycosaminoglycans where the potent release agent heparin can interfere with the *in vivo* transfection. A possible way to comfirm this postulation, could have been through a real time examination of the mice using florescence imaging, so as to identify the location of the florescent DNA within the blood system. Moreover the intravenous introduction of the cationic liposome –DNA complexes in general ends in their rapid uptake by the first capillary system encountered (McCuskey, 1994 cited in Mohr *et al.,* 2001; McCuskey, 2008). This capillary snare could result in poor *in vivo* tissue distribution and consequently poor luciferase transfection activity. Additionally despite the influence of mitotic activity on cationic liposome-mediated delivery *in vitro*, *in vivo* the low number of hepatocytes that undergo mitosis may, to an extent, be held accountable for the reduced frequency of transfected hepatocytes present in non-regenerating liver (Mohr *et al*., 2001).

The relatively large lipoplexes used in this study, having diameters of between 50 – 230 nm achieved successful transfection *in vitro* in the absence of serum. This may be attributed to their swift descent to the cell surface in the culture plate (Khalil *et al*., 2006). Lipoplex size is an important factor in the promotion of gene expression in the liver since the liver fenestrae have an average size of approximately 150 nm and display negative charges (McCuskey, 2008). These larger size cationic lipoplexes may thus not be able to easily negotiate these fenestrae and reach the hepatocytes. Fenestrae avidity that is generally observed for injected liposomes can possibly be ascribed to their expression of positive charge at the lipoplex surface. This liposome–fenestrae association is known to occur via non-specific charge interactions (Thurston *et al.,* 1998).

Smaller particles of 100 nm or less are preferred for *in vivo* systems as they experience a lower rate of circulation clearance. They additionally offer greater potential to reach target cells by virtue of their relative ease in narrow capillary navigation. To further minimize macrophage clearance and achieve improved site targeting, the presence of a hydrophilic surface on the lipoplex is advantageous (Colosimo *et al*., 1999; Brannon-Peppas and Blanchette, 2004; Gao *et al*., 2007).

Although the introduction of cell surface targeting ligands is thought to enhance hepatocyte specific delivery, our findings showed the RUI-129-galactosyl targeted and untargeted liposome formulations capable of comparable levels of transgene expression. A proposed explanation could be related to the Kupffer cells present in the liver expressing galactose specific receptors different to those present of the surface of the liver parenchymal cells (Kawakami *et al*., 2001). Kupffer cells are essentially macrophages anchored to the sinusoidal endothelium at the luminal surface and are thus exposed to the blood stream where they are believed to clear out any foreign particles from circulation. The liposomal carriers could thus potentially interact with the surface of these cells and its receptors resulting in removal from the blood stream before being allowed to deliver the therapeutic gene or drug to the hepatocytes (parenchymal cells) (McCuskey, 2008).

The presence of the helper lipid DOPE in all liposome formulations may further contribute to the differences between the *in vitro* and *in vivo* transfections achieved. The lack of translation between the two systems on this basis may be as a result of the DOPE undergoing aggregation in the blood in response to hydrophobic effects (Lin *et al*., 2003). This poor correlation may also be attributable, in part, to the fact that *in vitro* transfection studies were conducted in the absence of serum (Rao, 2010). Of all sera available, that obtained from the mouse is believed to most closely resemble the human condition. Earlier reports have suggested that in the presence of mouse serum the cationic liposome-DNA complex will

become slightly anionic, experience an increase in size and eventually disintegrate. The rate of this disintegration is dependent on the nature of the co-lipid used in liposome synthesis. DOPE for example assists in rapid disintegration when incorporated in the liposome formulation (Nishikawa and Huang, 2001).

Finally Hartikka and co workers (2000) revealed that the level of DNA degradation that takes place *in vivo* can be reduced through a simple change in the injection medium from the traditional saline or phosphate buffered saline to a phosphate buffer. Additionally the introduction of molecules that provide added stability in the presence of serum and prolonged circulation times may also be useful for further studies.

CONCLUSION

A compound proficient in lipid mediated transfection in one application may not necessarily be suitable for another and consequently a great deal of experimental research is needed so as to ascertain the most favourable conditions for both *in vitro* and *in vivo* lipofection. It is precisely the ability of cationic liposomes to selectively deliver their material to target tissues of tumour endothelium, lungs and liver that make them highly striking commodities for cancer therapy (Dass and Choong, 2006). Delivery vehicles for gene therapy need to be chosen in accordance with their particular therapeutic aim; however, the absence of an ideal vector suitable to all safety and therapeutic needs still eludes us. An adept formulation for the application of therapeutic genes for cancer treatment must be capable of complete protection of encapsulated nucleic acids, endosomal escape and specific site targeting. For this purpose numerous systems have been developed and investigated for efficient therapeutic gene transfer (El-Aneed, 2004).

Our investigations have focussed on the development of novel hepatotropic cationic liposomes prepared from the Chol-T cytofectin, DOPE, the crown ethers (CE's) RUI-128 and RUI-129, and the glycotargeting ligands RUI-90 (galactosyl) and RUI-92 (glucosyl). Formulations of cationic liposomes used for the translocation of plasmid DNA have the zwitterionic molecule DOPE incorporated as it acts as a fusogenic lipid component and can promote an increase in the efficiency of transfection under *in vivo* conditions, although it is more effective *in vitro*. The presence of this neutral lipid in the cationic liposomes may also assist in enhancing the biodistribution at the target site in addition to potentially contributing to the intracellular sorting in the target cells *in vivo* (Han *et al.*, 2008). These liposomes formed by thin film hydration and sonication were distinguished as small unilamellar vesicles with broad size range and distribution by cryo-TEM. The liposome preparations were shown via gel retardation and ethidium bromide displacement studies to form lipoplexes with plasmid DNA, which on nuclease digestion analysis was proven to offer effective serum protection.

Differences found in the structure of cationic cholesterol derivatives directly affects their DNA interaction and is consequently reflected in variations in transfection activity. In our evaluations of lipoplexes in the HepG2 and control HEK293 cell lines, low cell growth inhibition was observed for the Chol-T liposomes, however, the addition of the glycotargeting ligands and crown ether compounds led to distinct increases in the levels of cell cytotoxicity. Luciferase analysis of transfection of the pCMV-Luc control vector revealed the untargeted Chol-T, RUI-128 and RUI-129 liposomes directed to the HepG2 cell line produced approximtely 10, 3 and 20 times greater levels of transgene activity than their glycosylated counterparts. Of all the liposomes the RUI-129 cationic liposome preparation was able to achieve maximal transgene expression in both cell lines, showing that the introduction of the aza-15-crown-5

crown ether could enhance cationic liposome driven transfection. Although the RUI- 128 liposome was capable of cellular transfection its effect was not of the same magnitude as the afore-mentioned preparation, showing that the CE cavity size may play a significant role in determining transfection activity. The Chol-T preparation also showed high transfection levels based on luciferase enzyme activity. From these studies it is evident that the indroduction of the crown ether functionalities was most beneficial to the effectiveness of the cationic liposomes investigated in this study.

Three of the most promising formulations that produced high level transfection results *in vitro* were selected for further evaluation *in vivo*. Chol-T, RUI-129 and RUI-129-Gal liposomes were thus assessed in the NMRI mouse model where they showed low, but mearsurable transfection activity in the liver.

All cationic liposomes tested in the NMRI mouse model showed low levels of therapeutic nucleic acid delivery *in vivo.* However, a significant increase in luciferase reporter gene expression was observable on comparison with control untreated mice. From these studies it was also evident that the introduction of plasmid DNA to the liver was most effectively achieved through hydrodynamic injection. This method, however, was also responsible for far higher levels of tissue toxicity than that obsevered with the cationic liposomes. To address this difficulty, much research has focused on improving the cell specificity of cationic liposome –DNA complexes through engineered surface proteins or targeting ligands such as the asialoglycoproteins, transferrin, the epidermal growth factor, lactose and antibodies (Zheng *et al.*, 2009). The ligands, RUI-90 and RUI-92 intended for hepatotargeting, however, proved to be ineffective in the present study *in vitro*.

In vivo transfection activity of cationic liposomes may be improved through the introduction of certain molecular modifications. The introduction of polyethylene glycol to the liposomal formulation is one such modification that can improve the *in vivo* activity of cationic liposomes by extending the half life of liposomes in circulation. Ligand attachment for receptor targeted delivery may also be achieved through either the direct coupling of the ligand to the phospholipid or by means of fusion with the distal end of the PEG-lipid which proves advantageous as the ligand is more accessible for receptor interaction (Rao, 2010). Reports by Xu and co-workers (1999) that the transfection activities of distinct lipoplexes are more resistant to serum effects than those complexes which are aggregated is a promising observation for *in vivo* gene therapies and provides an alternate approach to transfection using cationic lipoplexes which are subsequently more stable in the presence of serum.

Nucleic acids differ from smaller drugs due to their polycationic nature which renders them more dependent on the nature of their carrier systems if their application in gene therapy is to be successful. The use of RNAi and RNA decoys for regulatory protein coupling and modulation of gene expression, in

addition to work carried out with DNA provides an exciting potential for the future. Non-viral gene delivery systems remain at an early stage of their development, with improvement in these systems showing continuous dependence on an improved knowledge of the cellular and *in vivo* barriers to gene transfer. In spite of the multitude of advantages and applications of non-viral vector systems in transfection, many of them are unable to completely replace viral vectors for high expression in gene therapy protocols. Cationic liposomes have, however, even in the face of ongoing criticism, persisted and have yet to be overtaken by other gene delivery methods or other liposome types. Adaptation and enhancement is required, but we now know that the task is no longer an insurmountable one, when the quantity and quality of research being undertaken is considered.

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APPENDIX A

Chemical and spectral data of the crown ether and deacetylated sugar moieties employed, obtained from Islam, R.U. (unpublished research).

Compound RUI-128:

Molecular Formula: $(M + H)^+$ $C_{40}H_{70}NO_7$

Infrared spectra: 2934, 2866, 1738, 1697, 1466 cm^{-1}

Melting Point: 58-60^{0}C

^{1}H NMR (300 MHz, $CDCl_3$): 0.67(s, 3H, -CH_3), 0.85-0.87(d, 6H, *J* = 6 Hz, -CH_3), 0.90-0.92 (d, 3H, *J* = 6 Hz, -CH_3), 1.00 (s, 3H, -CH_3), 1.04-1.60(m, 21H, Cholesteryl H), 1.69-1.95(m, 5H), 2.17-2.33 (m, 2H), 3.44-3.61(m, 4H), 3.80-3.83(m, 20H), 4.38-4.48(m, 1H), 5.30(s, 1H)

^{13}C (75 MHz, $CDCl_3$): 11.8, 18.7, 19.3, 21.0, 22.5, 22.8, 23.8, 24.2, 27.9, 28.2, 28.2, 31.8, 35.7, 36.1, 36.5, 37.0, 38.6, 39.4, 39.5, 39.7, 42.3, 50.0, 56.1, 56.6, 70.0, 70.4, 70.6, 70.6, 70.8, 74.7, 76.6, 122.4, 139.8, 155.8

Mass: 698 (100%), 654 (20)

Molecular Weight: 676.51500

Compound RUI-129:

Molecular Formula: $(M + H)^+$ $C_{38}H_{66}NO_6$

Infrared Spectra: 2939, 2866, 1738, 1698, 1462 cm^{-1}

Melting Point: 68-70^{0}C

^{1}H NMR (300 MHz, $CDCl_3$): 0.67(s, 3H, -CH_3), 0.85-0.87(d, 6H, *J* = 6 Hz, -CH_3), 0.90-0.92 (d, 3H, *J* = 6 Hz, -CH_3), 1.01 (s, 3H, -CH_3), 1.05-1.63(m, 21H, Cholesteryl H), 1.63-1.98(m, 5H), 2.24-2.38 (m, 2H), 3.37-3.50(m, 4H), 3.63-3.72(m, 16H), 4.45-4.55(m, 1H), 5.35-5.37(d, 1H, J = 4.8 Hz)

^{13}C (75 MHz, $CDCl_3$): 11.8, 18.7, 19.3, 21.0, 22.5, 22.8, 23.8, 24.2, 27.9, 28.2, 28.2, 31.8, 35.7, 36.1, 36.5, 37.0, 38.6, 39.5, 39.7, 42.3, 49.3, 50.0, 56.1, 56.6, 69.7, 70.2, 70.2, 71.0, 71.2, 74.7, 76.6, 122.3, 139.9, 155.8

Mass: 1286 (15%), 632 (100)

Molecular Weight: 632.48810

Compound RUI-90:

Molecular Formula: $(M + H)^+ C_{37}H_{61}N_4O_7$

Infrared Spectra: 3381, 3130, 2941, 2358, 1740, 1682, 1460 cm^{-1}

Melting Point: 188-190^{0}C

^{1}H NMR (300 MHz, $CDCl_3$): 0.64(s, 3H, -CH_3), 0.83-0.85(d, 6H, *J* = 6 Hz, -CH_3), 0.90-0.92 (d, 3H, *J* = 6 Hz, -CH_3), 0.98 (s, 3H, -CH_3), 1.03-1.52(m, 23H), 1.79-1.97(m, 5H), 2.20-2.30(m, 2H), 2.49-2.50(t, 2H, J = 4.2 Hz), 3.45-3.54(m, 3H), 3.67-3.70(t, 1H, J = 4.2 Hz), 3.75(d, 1H, J = 1.8 Hz ….), 3.97-4.01(t, 1H, J = 6.9 Hz), 4.22(s, 2H), 4.30-4.38(m, 1H), 4.63-4.69(m, 1H), 5.33-5.43(m, 1H), 5.43-5.45(d, 1H, J = 6.9 Hz), 7.99(s, 1H)

^{13}C (75 MHz, $CDCl_3$): 11.4, 18.3, 18.8, 20.3, 22.1, 22.4, 23.0, 23.6, 27.2, 27.5, 27.6, 31.1, 31.2, 35.0, 35.4, 35.8, 36.4, 38.1, 38.7, 49.3, 55.4, 55.9, 60.1, 68.1, 69.1, 73.0, 73.4, 78.7, 78.9, 79.0, 87.8, 121.1, 121.6, 139.5, 145.2, 155.5

Mass: EI = 673 (100%)

Molecular Weight: 673.45312

Compound RUI-92:

Infrared Spectra: 3379, 2939, 2359, 1737, 1680, 1457 cm^{-1}

Melting Point: 145-147^{0}C

^{1}H NMR (300 MHz, $CDCl_3$): 0.64(s, 3H, -CH_3), 0.83-0.85(d, 6H, *J* = 6 Hz, -CH_3), 0.90-0.92 (d, 3H, *J* = 6 Hz, -CH_3), 1.04 (s, 3H, -CH_3), 1.08-1.51(m, 22H), 1.79-1.97(m, 5H), 2.20-2.30(m, 2H), 2.49-2.50(s, 2H), 3.67-3.76(m, 2H), 4.21-4.32(m, 3H), 4.30-4.38(m, 1H), 4.34(s, 1H), 5.24(s, 3H), 5.32-5.34(d, 1H, J = 4.8 Hz), 5.47-5.50(d, 1H, J = 9Hz), 8.02(s, 1H)

^{13}C (75 MHz, $CDCl_3$): 11.5, 18.4, 18.8, 20.4, 22.2, 22.5, 23.1, 23.7, 27.2, 27.6, 27.7, 31.2, 31.2, 35.0, 35.5, 35.6, 35.9, 36.4, 38.1, 39.9, 41.7, 49.3, 55.4, 56.0, 60.5, 69.4, 71.8, 73.0, 76.8, 79.7, 87.2, 121.6, 139.5, 145.1, 155.6

Mass: ESI(+) = 673.41(M+), 674.45(M+1)

Molecular Weight: 673.45312

APPENDIX B

Publications:

such as the TNF-Related Apoptosis Inducing Ligand (TRAIL) [1]. TRAIL's role in T1D is not yet clear and further studies are required [2]. Thus, we analyzed the expression profiles of TRAIL ligand and its receptors in CY-accelerated T1D in NOD mice.

Materials and Methods: All mice received a single dose of 200 mg/kg CY. Pancreatic tissues were collected from pre-diabetic and diabetic NOD mice in addition to Non-Obese Diabetes Resistant (NOR) mice prior to immunohistochemical analysis. TUNEL assay was used for the detection of the related apoptotic profiles.

Results: TRAIL expression was significantly decreased in CY-accelerated T1D in NOD mice while no change was observed in the age-matched CY-applied NOR mice. The apoptotic cell count was increased along with diabetes development.

Conclusion: Different effects exerted on the immune system by various diabetes-inducing agents should be taken into consideration in diabetes investigations.

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P 160

Crown Ethers potentiate Transfection activity of Cationic Lipoplexes in Mammalian Cells

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Background: Non-viral gene delivery modalities compatible with application in man are receiving growing attention in light of safety issues surrounding viral vector systems. Amongst the more promising DNA vehicles are liposomes. Those derived from cationic lipids show much potential although transfection levels achieved in vivo are moderate. In this investigation two cholesteryl derived ionophores have been formulated into cationic liposomes and have been examined for their transfection enhancing potential

Methods: Liposome formulations included the cytofectin 3β[N-(N',N'-dimethylaminopropane) carbamoyl] cholesterol (Chol-T), the co-lipid dioleoylphosphatidylethanolamine and 5% (mole/mole) of the cholesteryl crown ethers RUI-128 (aza-18-crown-6) and RUI-129 (aza-15-crown-5). The remaining preparations were developed for liver targeting by including 5% (mole/mole) cholesteryl-β-D-galactopyranoside/glucopyranoside. Liposome interaction with plasmid DNA was characterized by band shift, ethidium displacement and serum nuclease digestion analyses. Both growth inhibition and transfection activities were determined in the human kidney HEK293 and human hepatoma HepG2 cell lines.

Results: All liposomes showed effective DNA binding and protection against serum nuclease degradation. Liposomes containing the crown ether preparations exhibited slightly higher cytotoxicity but enhanced transfection levels in both cell lines. The preparations containing the cholesteryl-β-D-glycopyranosides however showed poor targeting in the HepG2 cell line.

Conclusion: Liposome preparations showed generally low cytotoxicity levels in both cell lines. Crown ether liposomes may have potentiated transfection through endosome destabilization however the absence of targeting may be ascribed to molecular shielding.

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Gene therapy for β-thalassemia using lentiviral vectors encoding either γ-globin or BCL11A/shRNA

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β-thalassemia results from severely reduced or absent expression of the β-chain of adult hemoglobin ($\alpha_2\beta_2$;HbA) causing precipitation of excess α-chains and apoptosis of erythroid precursors. However, increased levels of fetal hemoglobin ($\alpha_2\gamma_2$;HbF), such as in hereditary persistence of HbF (HPFH), ameliorate the severity of β-thalassemia. Furthermore, recent data revealed BCL11A as a major repressor of γ-globin expression in adult erythroid cells (Sankaran et al, Science 322:1839, 2008). In the present study, we compared two, self-inactivating, γ-globin lentiviral vectors termed V5m3-400 (Pestina et al, Mol Ther 17:245, 2009) and GGHI (Papanikolaou et al, Mol Ther 16[Suppl.1]:S278, 2008) with a lentiviral vector comprising a U6-regulated shRNA for BCL11A knockdown (BCL11A/shRNA), using steady-state bone marrow $CD34^+$ cells from adults with β-thalassemia major. An *in vitro* model of human erythropoiesis was used to assay for enhanced production of HbF (Wilber et al, Blood 115:3033, 2010). Gene transfer for all three vectors was _>80% as determined by PCR analysis of CFUe. The mean levels of HbF produced from each vector, measured by HPLC, were 56.4% (V5m3), 63.4% (GGHI) and 49.4% (BCL11A/shRNA). Cells transduced with V5m3-400 demonstrated an average of 45% reduction in apoptotic cells ($Tunnel^+$ assay), while the respective values using Annexin V assay were 20.2% for GGHI and 14.5% for BCL11A/shRNA, suggesting a significant rescue via correction of the globin chain imbalance. These novel data document that both approaches, i.e. gene addition or genetic reactivation, have the potential to provide

APPENDIX C

Animal experimentation was carried out using protocols approved by the University of Witwatersrand Animal Ethics Screening Committee.

AESC 3

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2008/24/03

APPLICANT: Prof P Arbuthnot

SCHOOL: Molecular Medicine & Haematolgy
DEPARTMENT:
LOCATION:

PROJECT TITLE: **Testing anti hepatitis B virus (HBV) efficacy of RNA interference activators in a transgenic mouse model of viral replication**

Number and Species

834 6-weeks Mice
Approval was given for the use of animals for the project described above at an AESC meeting held on 20080527. This approval remains valid until 20100527

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

Inject lamivudine IP, provide dosage amounts and route of delivery in bed letters, the CAS will test the procedure using the saphenous vein, and if successful, the researcher will be guided on how to perform the technique for the study

Signed: _____ Date: 09/06/2008
(Chairperson, AESC)

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ Date: 09/06/2008
(Registered Veterinarian)

cc: Supervisor:
Director: CAS

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AESC 2007

Please note that only typewritten applications will be accepted. Should additional space be required for section "I" and/or "j", please use the back of this form.

ANIMAL ETHICS SCREENING COMMITTEE

MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Patrick Arbuthnot

b. Department: Antiviral Gene Therapy Research Unit, Department of Molecular Medicine & Haematology

c. Experiment to be modified / extended **AESC NO:**

Original AESC number	2008/24/03		
Other M&E's :			

d. Project Title: Testing anti hepatitis B virus efficacy of RNA interference activators in a transgenic mouse model of viral replication.

e. Number and species of animals **originally approved**:		834
f. Number of additional animals **previously allocated on M&Es**:		0
g. Total number of animals **allocated** to the experiment to date:		314
h. Number of animals **used** to date:		314

i. Specific modification / extension requested:
Extension for use of allocated animals approved under original application

j. Motivation for extension:

Experiments originally described in the approved submission to the AESC are ongoing and have been delayed because of technical difficulties encountered in the development of the antiviral sequences. Our intention to carry out the investigations remain a priority and this request is to allow use of the remaining 520 mice that have been allocated to the study during the coming 2 years.

Date: 03 March 2010 Signature: Patrick Arbuthnot

RECOMMENDATIONS:

.....Approved.....

Date: 03/03/2010 Signature: .....
Chairman, AESC