THE USE OF CHOLESTEROL-GALACTO COMPOUNDS IN LIVER DIRECTED GENE DELIVERY

By

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ABSTRACT

Gene therapy has to date gained immense interest as a potential method for treating genetic disorders such as Parkinson's and cystic fibrosis. The liver being a central organ of metabolism is susceptible to several metabolic disorders which could be targeted through liver directed gene delivery. The most common diseases being viral hepatitis and hepatocellular carcinoma towards which this study is focused. Non-viral vectors have gained wide interest as the vector of choice for delivering genes to organs such as the liver, because of their large-scale production potential, easy preparation, low cost and are relatively non-immunogenic to target cells / organs.

This study utilized non-viral cationic liposomes targeted to hepatocytes via ligand-receptor recognition. A total of six cationic liposomes (targeted acetylated /non-acetylated, nontargeted, pegylated, non-pegylated) were prepared according to the lipid film hydration method. The liposomes and liposome:DNA complexes were characterized using transmission electron microscope (TEM) and Zeta sizing to determine morphology, lamellarity and size. Results showed spherical, unilamellar cationic liposomes and lipoplexes in the size range of 50-200 nm in diameter. Band shift assays showed that these liposomes have strong DNA binding capabilities which was further confirmed by the ethidium bromide intercalation assays. Nuclease protection assays showed that liposomes were able to protect the integrity of the DNA cargo. From the MTT cell viability assays, low cytotoxicity was observed for all liposomes with cell survival as high as 80 % in most cases. Higher transfection activities were noted in the hepatocellular carcinoma receptor positive cell line (HepG2), for targeted non-acetylated liposomes, compared to the acetylated liposomes. The ligand competition assay and the use of the human embryonic kidney receptor negative cell line (HEK293) confirmed that the complexes entered the cells via receptor mediated endocytosis. Furthermore, it was confirmed that the acetylation of the galactose ligands hindered the process of receptor mediation.

Overall, these liposomal formulations are serum tolerant, have low cytotoxicity and are able to selectively target and transfect hepatocytes *in vitro*. Hence, they have the potential as future non-viral gene delivery vehicles and with further optimisation can be tested *in vivo*.

DECLARATION – PLAGIARISM

I, Nkosiyethu Knowledge Mkhwanazi, declare that:

1. The research reported in this thesis, except where otherwise indicated, and 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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5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and the References sections.

Signed _____

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CHAPTER ONE

1. INTRODUCTION / LITERATURE REVIEW

1.1 GENE THERAPY

Gene therapy entails the delivery of functional genes to target cells in whole organisms, in order to correct genetic mistakes or to provide the cells with a new functional gene (Khalil *et al.,* 2006). There are several methods of achieving corrections in the patient cells. One way is by targeting genes through homologous recombination and the other is by adding a therapeutic gene, a process known as augmentation. Different levels of gene therapy have been reported to date. Germline gene therapy, involves correcting the genetic disorders through direct manipulation of germline cells (Katragadda *et al*., 2010). In this method, the offspring will carry the corrected genotype. Ethical, legal and moral reasons have prevented the application of this technology in human subjects. Transgenic techniques have been used on other species viz. delivery of genes from somatic cells to the nuclei of germ cells, during metaphase stage; e*x vivo* alteration of egg cells following *in vitro* fertilization, and manipulation of mouse embryonic stem cells during *in vitro* culture using different gene delivery systems.

A second level of gene therapy is somatic gene therapy, where genes are inserted into diploid cells, and the genetic material not carried through to it progeny cells (Khalil *et al.*, 2006). This is a safer method since it only affects the target cells. Future offsprings of the targeted individual are not affected in this method. A significant problem associated with this method is that it cannot be used for long term expression because the somatic cells in the tissues die and are ultimately replaced by new cells. Also gene transportation is a limiting factor in this method. However, it is still used to treat various disorders e.g. psoriasis (Zibert *et al*., 2011). Somatic gene therapy can be differentiated into three delivery techniques; *ex vivo, in situ* and *in vitro*. In *ex vivo* delivery, genetic material is first taken from the target tissue or bone marrow, manipulated *in vitro,* and then used to transfect the target cell or tissue. This type of somatic gene therapy is not immunogenic to target tissues (Katragadda *et al.*, 2010).

However, there are limitations associated with this type of delivery; one being the small number of cells taken from the target tissue that remain viable. In *in situ* delivery**,** genetic

material is administered directly into the target tissue. This delivery method has been employed for the delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene using lipid and adenoviral vectors. This gene is delivered directly to a target specific site in the respiratory tract for the treatment of cystic fibrosis. This delivery method however suffers from low transfection efficiency. *In vivo* delivery uses a vector that carries a recombinant gene or DNA to the target site (Katragadda *et al.*, 2010). This type of delivery has gained much support as being a useful method of delivery. A limitation associated with this method could be the inadequate targeting of vectors to the correct tissue sites, although ligands are continually being developed to improve the targeting efficiencies of vectors used in gene/ drug delivery.

For successful gene therapy, therapeutic genes must be safely and successfully delivered to targeted cells or tissues and must be efficiently expressed at therapeutical levels. Over the past 20 years, gene therapy has gained wide interest as a method for treating diseases. It is a potential method for treating monogenic disorders such as Parkinson's disease and cystic fibrosis. It also serves as an alternative technique to traditional chemotherapy methods which are used to treat various types of cancer (Mintzer and Simanek, 2009). Gene therapy is considered as a potential medical revolution, although there are still associated obstacles that need to be overcome. One of the obstacles faced is the complexity of the biological system. Rapid developments in gene therapy are as a result of a growing understanding of the molecular mechanisms that define the development of corrective gene delivery systems. These two areas are important in gene delivery. The systemic gene delivery systems are important for therapeutic use to cells which cannot be accessed using percutaneous injection. It is also important in addressing metastatic manifestations which may be located elsewhere in the body. The therapeutic genes are usually contained in plasmids. When used to replace non-functioning genes, it offers more understanding on the physiological function of genes. Delivery is effected using vectors which protect nucleic acid material to be transferred (Katragadda *et al*., 2010).

1.2 GENE DELIVERY VECTORS

A good plasmid DNA vector should be able to replicate autonomously, be easy to isolate and purify, be easily introduced into the targeted cells, have suitable marker genes present, and have unique target sites for various restriction enzymes so gene inserts can be integrated into

it. For expression of the inserted gene, the plasmid should have the appropriate promoters and operators. Since direct naked DNA delivery is inefficient due to biological barriers, gene carriers which protect the DNA from being degraded by enzymes have been advanced. This has led to development of two diverse methods of delivering genes to cells or tissues, viz. i) the non-viral method and ii) the viral method. Both methods ensure increased transfection efficiency as shown in Figure 1.1(Katragadda *et al.*, 2010).

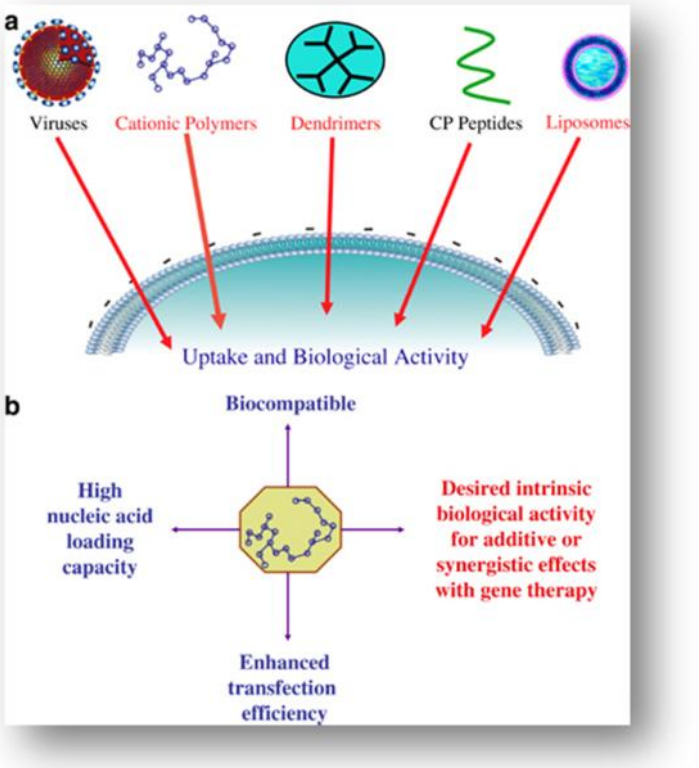

Figure 1.1: Different types of gene delivery vectors: Adapted from http://www.nature.com/gt/journal/v13/n9/figtab/3302692f1.html [accessed 10 October 2013].

1.2.1 VIRAL METHODS

Viral vectors have all the requisites which offer efficient binding to target cells, easy internalisation and uptake into nucleus where the DNA is released. Viral vectors are often associated with toxicity and immunogenicity, and hence may be potentially harmful to targeted cells or tissue. They are also limited with respect to scale-up procedures. Viral vectors include retroviruses, lentiviruses, adenoviruses, herpes viruses and the adeno-associated viruses. These viral vectors have different properties which are essential for the delivery of the genes or

molecules to the target cells or organs. Both the advantages and disadvantages need to be looked at before considering a specific viral vector/s for gene delivery. Table 1.1 summarizes the different viral vectors which are currently studied and employed in gene therapy (Katragadda *et al.*, 2010).

Table 1.1: Comparison of different viral systems.

Viral vector	Size	DNA insert Size	Infection	Expression	Potential Limitations
Retrovirus	7–11 kb (ssRNA)	8 kb	Dividing cells	Stable	Insertional Mutagenesis
Lentivirus	8kb (ssRNA)	9 kb	Dividing and non-dividing cells	Stable	Insertional mutagenesis
Herpes simplex virus	150 kb (dsDNA)	30–40 kb	Dividing and non-dividing cells	Transient	Lack of gene transcription after latent infection
Adenovirus	36 kb (dsDNA)	8 kb	Dividing and non-dividing cells	Transient	Strong antiviral immune response limits repeat administration
Adenoassociated virus (AAV)	8.5 kb (ssDNA)	5 kb	Dividing and non-dividing cells	Stable: integration in one spot of hostgenome	Helper virus required for replication: difficult to produce pure stocks of AAV free of helper virus
Vaccinia virus	190 kb (dsDNA)	25 kb	Dividing cells	Transient	Potential cytopathic effects

1.2.2 NON -VIRAL METHODS

Most non-viral transfer methods allow for targeting strategies and permit targeting of genes to specific cells. Research endeavours to improve this approach are ongoing as many problems have arisen over the years. In some cases, there has been expression of undesired proteins which are harmful to patients, and also down-regulation of gene expression leading to tumours. (Katragadda *et al*., 2010). Generally, non-viral vectors compact DNA for delivery, but

transfection efficiency remains lower than that of viral methods due to the many cellular barriers and immune defences that must be overcome. Non-viral vectors are biocompatible and have a large-scale production potential, enabling them to be widely used in the laboratory. In *in vivo* studies, free DNA is degraded by serum nucleases in the blood when injected intravenously. Due to this, most research has been engaged in designing cationic compounds that will form protective complexes with DNA. These compounds have been designed to be able to avoid gene delivery barriers (Mintzer and Simanek, 2009). Examples include: (i) Cationic lipids, (ii) Cationic polymers, (iii) Gold nanoparticles, (iv) Magnetic nanoparticles, (v) Quantum dots, (vi) Silica nanoparticles, (vii) Fullerenes, (viii) carbon nanotubes (CNTs), and (ix) Supramolecular systems.

(i) Cationic lipids are amphiphilic systems containing a cationic head group attached by a linker to a hydrophobic lipid moiety. The head group is positively charged and binds the nucleic acid phosphate groups. They can also be grouped into various subgroups according to their basic structural characteristics (Liu *et al.*, 2003). There are monovalent aliphatic lipids which have a single amine function in their head group, e.g. N[1-(2,3 dioleyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTMA), 1,2-dioleyl- 3 trimethylammoniumpropane (DOTAP), and N-(2-hydroxyethyl)-N,N-dimethyl 2,3-bis(tetradecycloxy-1-propanaminium bromide (DMRIE). There are also multivalent aliphatic lipids which have polar head groups with several amine functions such as the spermine group, e.g. dioctadecylamido-glycylspermine (DOGS) (Wasungu and Hoekstra, 2006).

Cationic lipids have a polar head group, which compacts DNA, a lipidic chain, for self-association through hydrophilic interaction forming micelles or liposomes; and a linker, that interconnects the two. A lipoplex is a complex formed by interaction of the cationic lipids and DNA, and resembles a multilayered structure with compacted DNA (Xun *et al.*, 2014).

(ii) Cationic polymers can be classified into two main groups. The first group are natural polymers e.g. proteins, peptides and polysaccharides, and the second group are synthetic polymers e.g. polyethyleneimine (PEI), dendrimers, and polyphosphoesters (Mintzer and Simanek, 2009). Cationic polymers interact

with DNA by means of electrostatic interactions, due to the positively charged amine and ammonium functions. The N/P ratio is a ratio of the number of amine groups found in the vector versus the number of phosphates in the DNA. This ratio controls the structure and size of the complex formed between the DNA and the cationic polymers (Mintzer and Simanek, 2009).

Chitosan is a natural polymer which due to its non-toxic nature is favoured for both *in vitro* and *in vivo* studies. However, it has shown low delivery efficiency in some cell lines (Liu *et al*., 2003). It complexes with DNA and has been widely employed in oral and nasal therapy since it is mucoadhesive. Novel chitosan derivatives such as aminoethyl- chitin and thiolated- chitosan have now been employed to improve the transfection efficiency. Intracellular barriers have been recently avoided by conjugating chitosan to folic acid, so that folate receptor mediated endocytosis can occur. PEI is another polycation commonly employed in gene delivery (Liu *et al*., 2003), however it has shown higher cytotoxicity than other polymers *in vitro*.

(iii) Gold nanoparticles: Gold is an inert metal which is not oxidized at lower temperatures, especially temperatures below its melting point. It does not interact with atmospheric oxygen and most other chemicals. Hence, these properties enable gold to be easily manipulated in the presence of atmospheric conditions (Mintzer and Simanek, 2009). Since gold nanoparticles can be synthesized by citrate reduction in an aqueous solution, nanoparticle sizes can be tuned reliably and routinely from 1 nm to 200 nm (Katragadda *et al*., 2010).

(iv) Magnetic Nanoparticles: Recently, much attention has been focussed on developing the nanoparticles. These are metallic clusters which have a good drug delivery and gene therapy potential. They are able to carry large amounts of DNA into targeted cells (Katragadda *et al*., 2010).

(v) Quantum Dots: These are semi-conduction nanomaterials which present bright fluorescence, narrow emission, broad UV excitation, and high photostability.

These offer various advantages compared to normally employed organic dyes and are useful for imaging and sensing (Katragadda *et al*., 2010).

(vi) Silica Nanoparticles: Silica is a major component of most natural materials including glass and sand. It is employed in biomedicine due to its ability to be functionalised. In gene delivery, the surface of the silica nanoparticles is often functionalized with amino-silicanes (Katragadda *et al*., 2010).

(vii) Fullerenes are water-insoluble carbon molecules. Fullerenes have been functionalized by Nakamura *et al* (2000) with two diamine side chains. It has shown transfection efficiency comparable to that of commercially available reagents. Complexes formed between fullerenes and DNA are usually absorbed by cells via phagocytosis.

(viii) Carbon nanotubes (CNTs) are cylindrical fullerenes. CNTs have been functionalized with an amino-terminal oligoethylene glycol chain. These complexes associate with DNA and have high delivery efficiencies and low cytotoxicity.

(ix) Supramolecular systems: It has been shown that highly saccharide-functionalized porphyrin and calix resorcarene derivatives have high specificity toward hepatic cells. These derivatives combine with plasmid DNA to form glycoviruses, which transfect HeLa cells with high efficiency. Series of calix[n]arenes have been functionalized with guanidinium groups 59, 60 and alkyl chains which resulted in condensation of DNA and high transfection (Katragadda *et al*., 2010).

There are other non-viral methods which can be employed to transfect cells / deliver genes during gene therapy. These include the physical methods which allow for direct transfer of naked DNA or nucleic acids into the cytoplasm without using any foreign substance such as lipids. One such example is the method of microinjection. Microinjection is normally used for single cell manipulations. Nucleic acids are injected to cells such as oocytes using a micromanipulator and a microscope. This technique provides various advantages such as high

transfection efficiency but larger number of cells cannot be transfected using this method (Katragadda *et al*., 2010).

Another physical method is the biolistic particle delivery (BPD) where nucleic acids are delivered into cultured cells. This method can be used to transfect cells *in vivo* and is only employed for vaccination and agriculture purposes. BPD is fast, simple and can be used to transfect both dividing and non-dividing cells but it has a high mortality rate and therefore requires a large number of cells. A third physical method is electroporation which is a frequently used technique because it can transfect large DNA fragments with good efficiencies, however this technique leads to high mortality rates in animals.

1.2.2.1 LIPOSOMES

Liposomes, the basic structure of which can be seen in Figure 1.2, are one of the most widely employed non-viral vectors in gene therapy. There are different biological properties (Table 1.2) and applications of liposomes in science (Table 1.3), which have been studied recently and has led to scientists conducting more research on these carrier vectors.

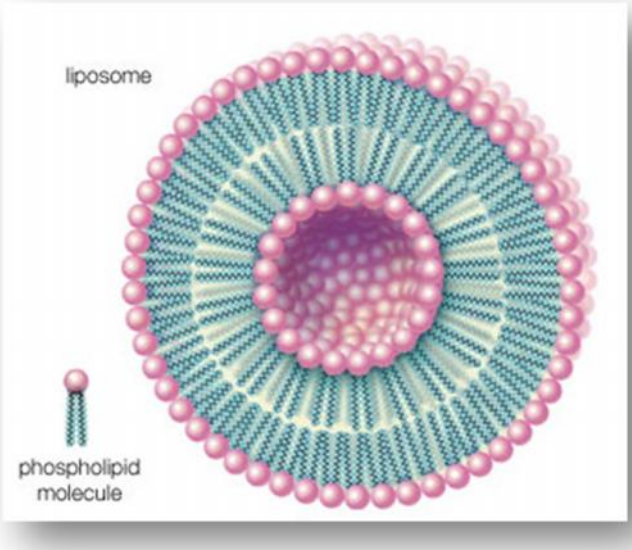

Figure1.2: Liposome structure: Adapted from http://formulation.vinensia.com/2011/06/liposome-definition-structure-and.html [Accessed 10 October 2013].

Table 1.2: Biological properties of liposomes.

PROPERTIES	EXPLANATION
Biocompatibility	Liposomes are biocompatible
Solubility	They have the ability to entrap water-soluble (hydrophilic) and insoluble (hydrophobic) genes into their internal water compartment and the membrane, respectively.
Protection	Those included into genes are protected from the inactivating effect of external conditions.
Delivery	They give a unique opportunity for the delivery of genes into target cells.
Size, charge and surface	These can be easily changed by adding new ingredients to the lipid mixture before the liposome is prepared or through the variation of methods used to prepare the liposome.

Table 1.3: Applications of liposomes in science.

DISCIPLINE	APPLICATION
Biochemistry	Reconstitution of membrane proteins into artificial membranes
Biology	Model biological membranes, cell function, fusion, recognition
Biophysics	Permeability, phase transitions in two-dimensions, photophysics
Chemistry	Photochemistry, artificial photosynthesis, catalysis, microcompartmentalization
Mathematics	Topology of two-dimensional surfaces in three-dimensional space governed only by bilayer elasticity
Medicine	Drug-delivery and medical diagnostics, gene therapy
Pharmaceutics	Studies of drug action
Physical Chemistry	Colloid behaviour in a system of well-defined physical characteristics, inter- and intra-aggregate forces.
Physics	Aggregation behaviour, fractals, soft and high-strength materials

1.2.2.1.1 LIPOSOMES IN DRUG DELIVERY

The use of liposomes in drug delivery (Table 1.4) is a promising technique but it also presents certain problems, such as fast removal from the blood and uptake of liposomal samples by cells of the reticulo-endothelial system, mostly in the liver. Therefore, studies have been undertaken to develop methods to minimise this problem. One of the developments include using liposomes with immunoglobulin ligands attached to their surface, which are referred to as immunoliposomes. The surface ligands are generally able to recognize and transport the cargo to the target cells (Torchilin, 2005). The commonly used ligands to target liposomes are immunoglobulins (Ig) of the IgG class together with their fragments. These do not affect the integrity of the liposome, as it does not bind covalently or via hydrophobic insertion into the liposome. There are various techniques which have been used to increase circulation half-lives. These include coating the liposome with polyethylene glycol (PEG) (Figure 1.3) which protect the liposomes by interfering with their recognition by opsonins *in vivo*. The use of long-circulating liposomes has been studied over many years and have been applied in both *in vitro* and *in vivo* biomedical studies. Protective polymers such as PEG are flexible thereby allowing a small number of surface –grafted polymer molecules to form an impermeable layer over the surface of the liposome. They are dose dependent, have increased bioavailability and are non-saturable (Torchilin, 2005).

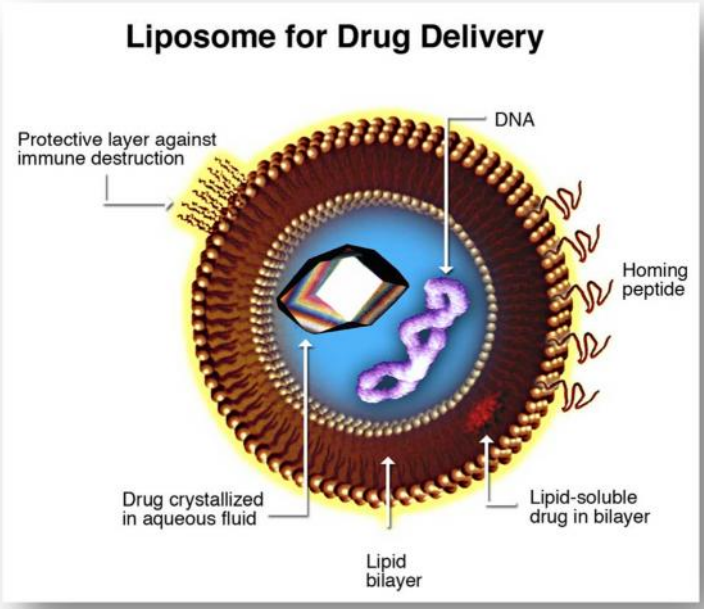

Figure 1.3: Structure of a typical liposome in drug delivery: Adapted from http://en.wikipedia.org/wiki/Liposome#mediaviewer/File:Liposome.jpg [Accessed 10 October 2013].

Table 1.4 Liposomes as drug carriers in the pharmaceutical industry: Adapted from http://www.remedicajournals.com/liposomes/drug delivery [Accessed 09 November 2013]

Liposome utility	Current applications	Disease states treated
Accumulation	Prostaglandins	Cardiovascular diseases
Drug Depot	Lungs, sub-cutaneous, intra-muscular, ocular	Wide therapeutic applicability
Drug protection	Cytosine arabinoside, interleukins	Cancer
Enhanced Penetration	Topical vehicles	Dermatology
Extravasation	Leaky vasculature of tumours, inflammations, infections	Cancer, bacterial infections
Site-Avoidance	Amphotericin B – reduced nephrotoxicity, doxorubicin – decreased cardiotoxicity	Fungal infections, cancer
Solubilization	Amphotericin B, minoxidil	Fungal infections
Specific Targeting	Cells bearing specific antigens	Wide therapeutic applicabilit
Sustained-Release	Systemic antineoplastic drugs, hormones, corticosteroids, drug depot in the lungs	Cancer, biotherapeutics
RES Targeting	Immunomodulators, vaccines, antimalarials, macophage-located diseases	Cancer, MAI, tropical parasites

Recent studies have focused on the attachment of PEG in a detachable fashion so that the liposome can be selectively taken up by target cells (Torchilin, 2005). When these PEGylated liposomes accumulate at the site of interest, PEG is removed under conditions of decreased pH in tumours. Detachment of PEG is through mild thiolysis of the dithiobenzylurethane linkage found between PEG and the amino-containing substrate. There have also been studies which have focused on other polymers that could be used to prepare long circulating liposomes, e.g. poly[N-(2-hydroxypropyl) methacrylamide)], poly-N-vinylpyrrolidones, L-amino-acid-based biodegradable polymer–lipid conjugates and polyvinyl alcohol (Torchilin, 2005).

1.2.2.1.2 LIPOSOMES IN GENE DELIVERY

Liposomes are one of most common non-viral systems for delivering genes into target cells or organs. Preparing cationic lipid based liposomes is easy, cost effective and they are relatively non-immunogenic to cells. Liposomes are however still not thoroughly understood and there are several studies underway which are focused on their function and structure in gene delivery and the level of transfection efficiency they promote. DNA can be encapsulated in anionic or neutral liposomes or electrostatically bound to cationic liposomes bringing about condensation of DNA. Recent studies reported the use of polycationic liposomes for gene delivery. Polycationic liposomes are liposomes with cetylated polyethelene imine groups (Torchilin, 2005). The polycation anchor into the membrane using the cetyl residues and thereby bind to DNA using their positive charges. These liposomes have a high transfection efficiency. Reports on delivery of small interfering RNA using cationic liposomes have also been recently published (Hattori *et al.*, 2013).

1.2.2.1.3 ADVANTAGES AND DISADVANTAGES OF LIPOSOMES

1.2.2.1.3.1 ADVANTAGES

Liposomes have the ability to increase the therapeutic index of delivered drugs such as actinomycin-D thereby increasing the efficiency of the drugs. Since anionic /neutral liposomes encapsulate genes or drugs to be delivered, they tend to increase the stability of the genes (Dua *et al.*, 2012). Liposomes are biocompatible and relatively non-immunogenic to the target organ. The non-toxicity and the ability of them to be completely biodegradable is an advantage of using them for both *in vitro* and *in vivo* purposes. Reports show that using liposomes reduces the toxicity of the drug or gene which is encapsulated within it. Liposomes are also known to play a role in the reduction of exposure of sensitive tissues to the toxic agent. These liposomes have the flexibility to bind to site-specific ligands (Akbarzadeh *et al.*, 2013).

1.2.2.1.3.2 DISADVANTAGES

Generally, unmodified liposomes have a short half-life. It was also reported that using liposomes can be a disadvantage because the phospholipids can sometimes undergo hydrolysis and oxidation-like reactions (Dua *et al.*, 2012).

1.2.2.2 DELIVERY PATHWAYS AND CELLULAR BARRIERS

When designing a non-viral system for gene delivery, it is necessary to identify all the cellular barriers which has to be overcome by the vector during the gene delivery process. Firstly, the DNA must be able to interact with this vector since the half-life of naked DNA is increased by the vector which protects DNA from degradation by proteases (Pathak *et al.,* 2009). DNA condensation can be achieved by using cationic compounds that form electrostatic interactions with the DNA phosphate group, and can interact with the lipids using hydrophobic interactions. DNA collapses when neutral lipids such as PEG are highly concentrated, resulting in the DNA molecules forming compacted particles via non-specific interactions with an exclusion of solvent volume. When complexes are formed, they then have to cross the plasma membrane of the target cells (Katragadda *et al*., 2010). Therefore, a ligand-containing vector should be able to undergo internalisation by processes such as receptor-mediated endocytosis. If there is no ligand then the complex can be degraded by serum nucleases, or as in many cases the complexes are taken up by an adsorption process. There are different ligands which maybe be employed e.g. folic acid, transferrin and epidermal growth factor (EGF), for cancer cells. After binding and entry into the target cell /tissue, the complex should be able to escape from the endosome into the cytosol. PEI, a common polymer in gene delivery, can be used to achieve this, as it destabilizes the endosomal membrane and allows the complex to escape through a process known as the proton sponge effect (Katragadda *et al*., 2010).

1.3 RECEPTOR MEDIATED ENDOCYTOSIS

Receptor mediated endocytosis involves the internalization of molecules including viruses. This process is highly dependent on the molecules interaction with a specific binding protein (receptor) on the cell membrane (Gaidarov *et al*., 1999). There are different major types of events which occur during the internalization of molecules. The ways in which cells internalize molecules can be seen in Figure 1.4.

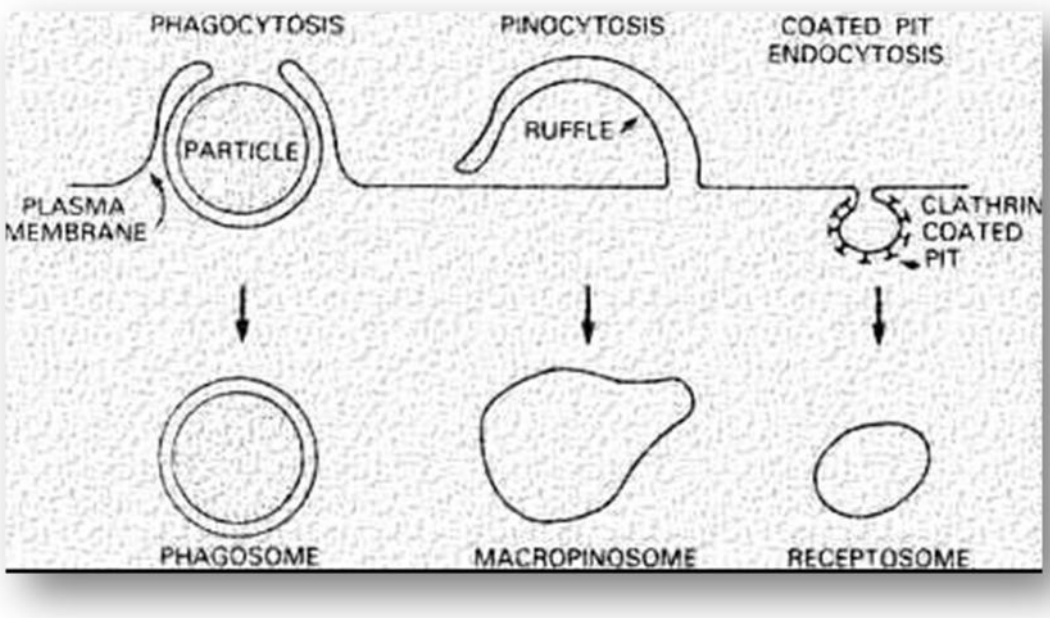

Figure 1.4: Events which occur during internalization of molecules in cells: Adapted from http://www.cytochemistry.net/cell-biology/recend.htm#internalization [Accessed 14 July2014].

During phagocytosis, bacteria or other cells are internalised without the need of a receptor. In the case of pinocytosis, soluble material is generally internalised by the cells, also without the need for receptors. In these processes, the complexes or molecules are captured into the vacuole (phagosomes or macropinosomes). However, during receptor-mediated endocytosis, endosomes are formed into which the complexes or molecules are enclosed (Gaidarov *et al*., 1999). Specific binding to the receptor is important for this receptor-mediated endocytosis to occur. This process is shown as the coated pit endocytosis in Figure 1.4. The coated pit is a specific region found on the cell membrane and is coated with clathrin which ensures stability and assists during transportation. During endocytosis, this coated pit region forms a vesicle which loses its clathrin coat and joins with other coated pits to form a receptosome (Figure 1.4) (Gaidarov *et al*., 1999).

1.3.1 PATCHING AND CAPPING

During receptor-mediated endocytosis, the ligand binds to its specific receptor on the surface of the cells. The complex formed between receptor and ligand (ligand-receptor complex) is stored in the clathrin coated pits. Different things occur in different cells, however, in most cells the coated pits with complexes concentrate in a certain cell area and these appear as

patches of label on the surface of the cell, thus the process is termed patching (Gaidarov *et al*., 1999). These then merge and form a cap at one pole inside the cell, the process is called capping. This process of pre-concentrating enables the cell to control the amount of fluid that is taken up by the vesicle.

1.3.2 FORMATION AND FUNCTION OF CLATHRIN-COATED PITS

During receptor-mediated endocytosis, the clathrin coats mount the pit and form clathrin coated pits (Figure 1.5). Formation of these molecules causes the pit to invaginate, and help stabilise the vesicles that bud from the cell membrane. The clathrin-coated pits are known to move in the membrane plane.

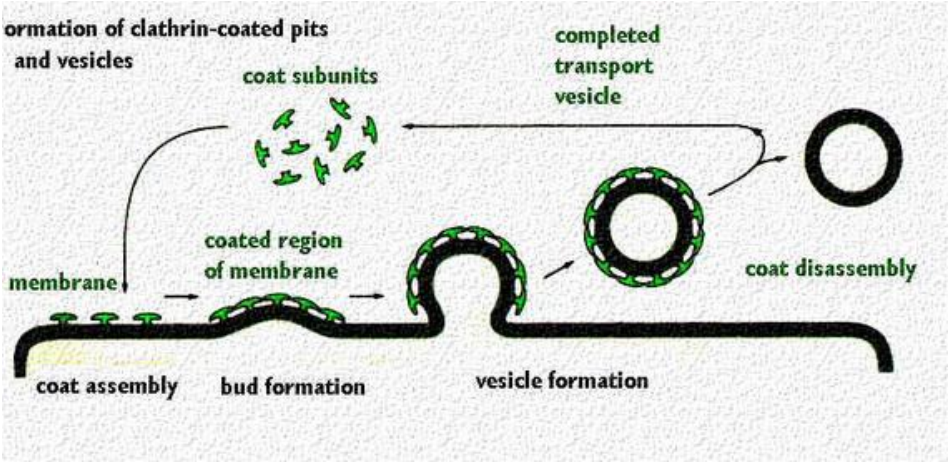

Figure 1.5: Formation of clathrin-coated pits during receptor- mediated endocytosis: Adapted from Adapted from http://www.cytochemistry.net/cell-biology/recend.htm#internalization [Accessed 14 July2014].

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Studies have shown that there is an organised movement of the clathrin coated pits. One of the studies involved transfecting cells with a green fluorescent protein carrying plasmid DNA which was attached to the clathrin light chain (Gaidarov *et al*., 1999). This study showed the disappearance and appearance of clathrin coated pits at different intervals. The clathrin coat is lost from the pits which then form vesicles in an energy dependent process resulting in the formation of the endosomes or receptosomes.

In the cytoplasm, the vesicles lose their coat and fuses with other similar vesicles in a process known as the hymotypic fusion. During early endosome formation, most of the incoming components from the membrane are recycled back from the early endosome to the membrane of the cell while a large fraction of the molecules are taken to the late endosome (Figure 1.6). The late endosome is formed by the continual drop in pH to about 5-6. The late endosomes are important because they contains digestive enzymes which degrade most lipids and proteins and also recycle some receptors. Finally, the endosome fuses with the lysosomes which are the highly acidic vacuoles containing hydrolases, forming endolysosomes where molecules are digested (Gaidarov *et al*., 1999).

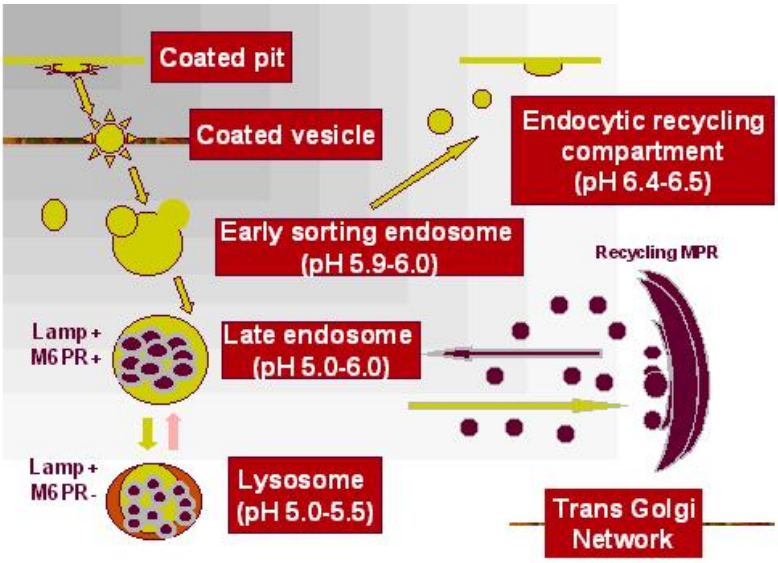


Figure 1.6: Formation of early, late endosome and lysosome during receptor mediated endocytosis: Adapted from http://www.cytochemistry.net/cell-biology/recend2.htm [Accessed 14 July 2014].

One example where receptor mediated endocytosis is used by the cells to take up molecules involves the uptake of the low density lipoprotein (LDL) which was first published in 1994 (Alberts *et al.*, 1994). This model shows the uptake of cholesterol bound to LDL by the cell. In this study the receptors were recycled and the LDL-cholesterol ligand was taken up with cholesterol being released into the cell where it was metabolised. One of the most commonly studied receptors in receptor mediated endocytosis is the asialoglycoprotein receptor which is found almost exclusively on the surface the liver cells (Gaidarov *et al*., 1999).

1.4 ASIALOGLYCOPROTEIN RECEPTOR (ASGP-R)

The asialoglycoprotein receptor (also known as the hepatic lectin) is a C-type, calcium ion-dependent lectin which is expressed only on the surface of the parenchymal hepatocytes (Rensen *et al*., 2001). These ASGP-R receptors are distributed randomly over the sinusoidal plasma membrane which faces the capillaries. ASGP-R was discovered by Ashwell and Morell in 1974. The fact that the ASGP-R is highly expressed in the hepatocytes and is an efficient ligand for endocytosis makes it an important target for liver gene delivery (Wu *et al.,* 2002). The ASGP-R specifically binds to glycoproteins which have terminal galactose or the N-acetyl-galactosamine residues, and to desialylated tri or tetra-antennary N-linked glycans (Wu *et al.*, 2002).

1.4.1 ASGP-R STRUCTURE

Hepatocytes contain approximately 100 000 – 500 000 ASGP-R binding sites per cell. The ASGP-R found in humans has two homologous subunits, which are H1 and H2 (46 kDa and 50 kDa, respectively) (Wu *et al*., 2002). These subunits are type II membrane proteins, comprising of a cytoplasmic N terminus made up of 40 amino acid residues, an exoplasmic C terminus made up of approximately 230 amino acid residues and a transmembrane domain. Found in these domains is a stalk segment made up of about 80 amino acid residues and also a carbohydrate recognition domain. It is due to the multiple interactions of this recognition domain that the receptor has the high ligand binding specificity and affinity (Meier *et al.*, 2000). These subunits form a non-covalent hetero-oligomeric complex by the alpha-helical coiled coil domains with an estimated ratio of 2.5:1. The subunits are single spanning membrane proteins with a calcium-dependent D-galactose/ D-N-acetylgalactosamine recognition domain (Rensen *et al.*, 2001). The recognition of the carbohydrate moiety is mediated through the H1- subunit, while the H2- subunit accounts for the functional configuration of the native receptor.

1.4.2 STRUCTURE OF THE CARBOHYDRATE RECOGNITION DOMAIN (CRD) OF H1 SUBUNITS

Beta-strands of the CRD form a protein core as they are arranged in a bent plane form with the alpha-helices located on either side of the plane. There are three sheets which extend in

different directions from the protein core (Meier *et al.*, 2000). The protein core is formed by the beta 3 strand which is at the middle of the glycine residue 212 and is bent at an angle of about 90 degrees. The glycine loop protrudes from the surface of the protein, and thus forms the sugar-binding site. The CRD has an N-terminus which has a disulphide bridge connection. Also present on this structure are three calcium ion binding sites. This CRD region is responsible for the high binding affinity and specificity for carbohydrates as is shown in Figure 1.7. Here the blue strips represent the beta-strands while the green balls represent the calcium ions and the yellow lines showing the disulphide bridges (Meier *et al.*, 2000).

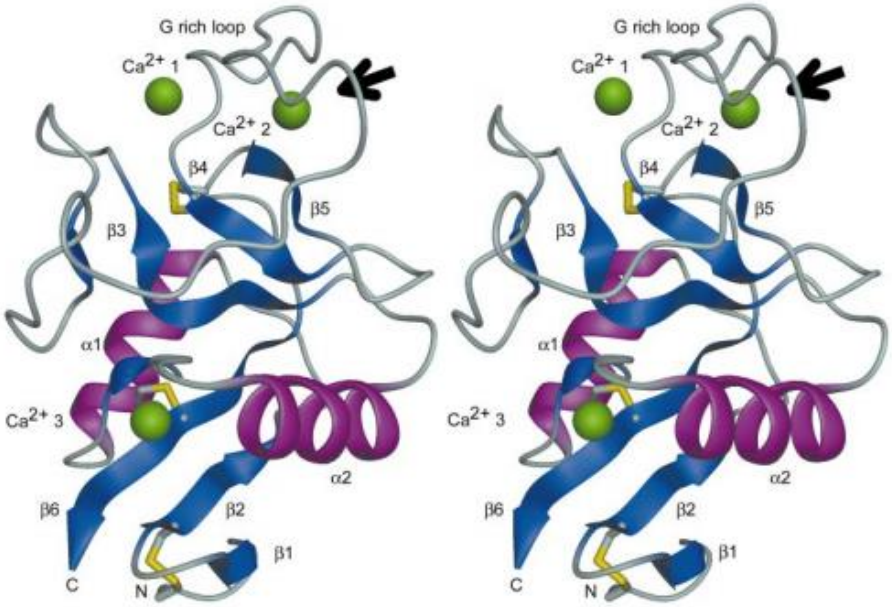


Figure 1.7: Stereoscopic ribbon diagram of the H1-CRD of ASGP-R: Adapted from Meier *et al.*, (2000).

1.4.3 THE GALACTOSE-BINDING SITE

This is the region that defines the sugar-binding site and has a polpeptide chain which stretches continuously from Arg236 to Cys268. There is also a glycine-rich loop and has a beta-turn between Thr258 and Arg262. This region therefore has three segments which are in contact with the calcium ions. In this site, there are two metal ions separated by 8.5 Å. The three segments are located within metals and the glycine-rich loop extends and attaches to the Asp215-Trp220 loop. Two of the three segments have a beta-strand conformation. These characteristics of the galactose-binding site of the asialoglycoprotein receptor are shown clearly in Figure 1.8 (Meier *et al.*, 2000).

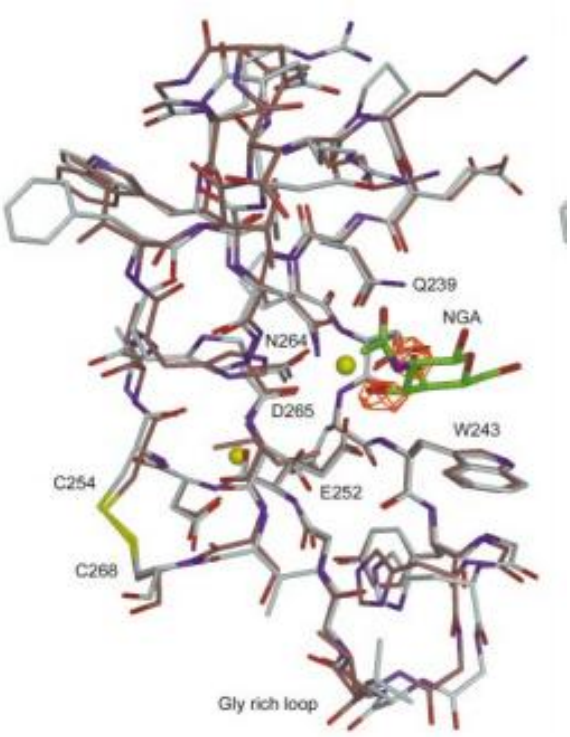


Figure 1.8: Overlay of the sugar-binding site of the H1-CRD of the ASGPR (grey) containing an N-acetyl galactosamine molecule as ligand (green): Adapted from Meier *et al.*, (2000).

1.4.4 PHYSIOLOGICAL ROLE OF ASGP-R

The function of the ASGP-R is to maintain serum glycoprotein homeostasis by recognition, binding, and the entry of asialoglycoproteins by endocytosis. Studies have revealed that patients with liver cancer have high levels of asialoglycoproteins, arising from impaired function of ASGP-R (Geffen and Spiess, 1992). Furthermore, the ASGP-R is responsible for the uptake of glycoproteins which is essential for the liver. An example of such a glycoprotein is immunoglobulin A (IgA) that contains terminal Gal and Gal*N*Ac residues on its oligosaccharide chains (Brown and Koppel, 1986). During endocytosis the receptor clears the ligand proteins from the circulation, and these ligand proteins are eventually degraded, and the receptor is recycled back to the cell surface. In this case, the ASGP-R provides a degradative pathway for serum glycoproteins. According to a report by Treichel *et al*. (1997), certain viruses such as the hepatitis B virus and the Marburg virus may cause infection using these receptors. However, these receptors play a major role in the delivery of genes and drugs.

1.5 THE LIVER AS A TARGET FOR GENE DELIVERY

The liver (Figure 1.9) is an essential target organ for gene delivery as it plays a central role in metabolism and is also important in serum protein production. Many debilitating metabolic diseases associated with deficiency of hepatocyte derived gene products have been reported. These disease can be targeted through delivery of therapeutic genes to the liver (Hara *et al*., 1997). Furthermore, diseases such as hepatoma and viral hepatitis can be targeted using hepatic gene therapy. There are important factors which contribute to the improvement of hepatic gene therapy, one of them being the degree of development of an ideal delivery system which can efficiently introduce the therapeutic genes into the hepatocytes. Various methods have been used to deliver genes into the liver, albeit with some disadvantages.

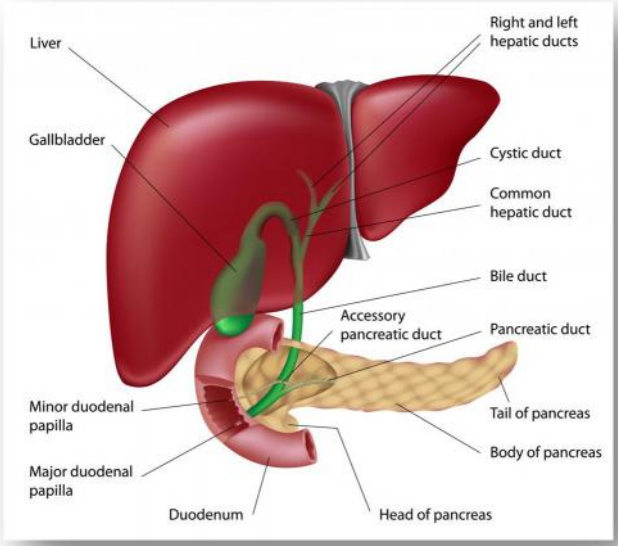


Figure 1.9: Image of the liver: Adapted from http://lookfordiagnosis.com/mesh_info.php?term=liver+diseases&lang=1 [Accessed 10 October 2013].

Viral vectors such as the retroviral vectors have been used to introduce genetic material into the liver. Retroviral vectors however do not infect quiescent cells, and for this reason they have been used mostly for ex vivo delivery of genes, e.g. they have been used in transduction of hepatocytes which have been cultured and subsequently re-implanted into the host liver (Hara *et al*., 1997).

On the other hand, direct introduction of genetic material into the liver requires improved propagation activity of the hepatocytes. Propagation can be achieved through partial hepatectomy. In some cases, adenoviral vectors have been used to deliver genes into the liver. The introduction of these vectors by injection into the portal vein leads to increased levels of foreign gene expression. However, transient expression is usually observed and repetitive injection is not conducive because of the high immunogenicity of the adenoviral vector. The non-viral gene transfer methods which have been described earlier (section 1.2.2) include injection of plasmid DNA alone, but in most cases plasmid DNA is complexed with polymers such as polylysine or with liposomes (Hara *et al*., 1997). These are still being studied, although they have the disadvantage of producing low levels of gene expression which is insufficient for therapeutic use. In the systemic circulation, there are lipoproteins which carry cholesterol and other lipids.

Studies have recently shown that reconstituted chylomicron remnants which have been produced from commercially-available lipids are taken up by hepatocytes following intravenous injection. These remnants are produced during the circulation of blood where the triglycerides-rich lipoproteins are remodelled to chylomicron remnants through the hydrolysis of core triglycerides by lipoprotein lipases and by binding apolipoproteins (Hara *et al*., 1997). The liver parenchymal cells then take up the remnants through the apolipoprotein-specific receptors. Non-viral particles resulting from inclusion of beneficial DNA into reconstituted chylomicron remnants could be a competent vector for gene delivery to the liver (Hara *et al*., 1997). There have been studies conducted on the inclusion of DNA into reconstituted remnants through hydrophobic DNA complex formation with quaternary ammonium derivatives of cholesterol. Studies showed that when these were introduced into the liver, there was high expression of foreign genes. The therapeutic potential of the novel vector has then been shown in humans, with the expression of the 1-antitrypsin (hAAT) gene in the liver (Hara *et al*., 1997).

1.6 CHOLESTEROL

Cholesterol is a naturally occurring lipid which is metabolized in the body. The development of novel cholesterol based cationic lipids gained momentum after the gene delivery using DC-Chol lipid was successful demonstrated in 1991 by Gao and Huang. The T-shaped headgroups of cationic lipids are more effective in delivering genes compared to the linear type. Reports

have shown that the T-shape spermine and spermidine cholesteryl carbamate have a 50-100 fold higher efficiency both *in vitro* and *in vivo* when compared to the level of gene expression achieved by DC-Chol (Wang *et al.*, 2002). The advantages of lipids and polycations were combined by synthesizing lipopolylysine. Poly (L-lysine) with molecular weight 3300 Da was mixed with the N-hydroxysuccinimide ester of dipalmitoylsuccinylglycerol in dimethyl sulfoxide. These were synthesized using the solid phase synthesis method. Reports have also shown that preparing liposomal formulations with cationic amphiphiles and using DOPE enhances gene delivery. Cetyl groups were also used as hydrophobic lipid anchors onto PEI and later used to prepare polycation liposomes for gene delivery (Mahato, 2005).

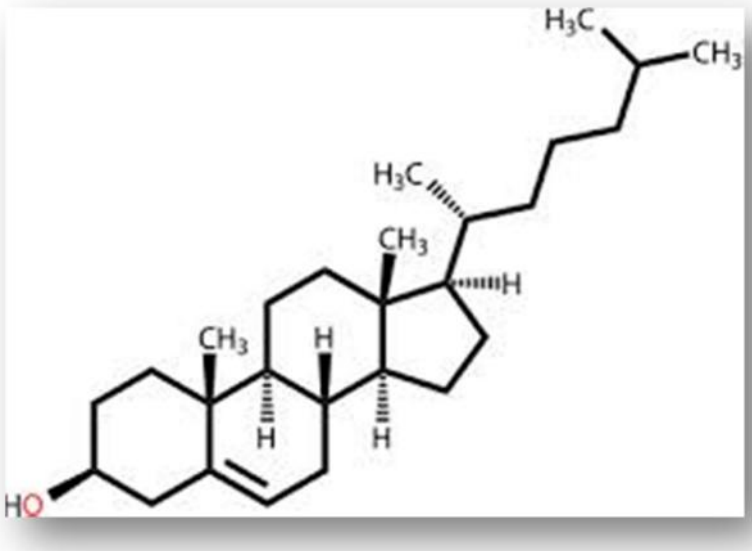


Figure 1.10: Structure of cholesterol: Adapted from http://www.diagnosisdiet.com/food/cholesterol/ [Accessed 10 October 2013].

1.7 OUTLINE OF THESIS

1.7.1 AIMS AND OBJECTIVES:

The aim of this project was to formulate and utilize non-viral hepatocyte targeted cationic liposomes. The galactose ligand incorporated into the liposomal formulation was used for the ligand-receptor recognition. The human hepatocellular carcinoma cells (HepG2) which has the asialoglycoprotein receptor which recognises and binds to the galactose moiety was used as the target cell line while a ASGP-R negative (HEK293) cell line was used as a control. The objectives were:

- to prepare cationic liposomes (PEGylated/targeted/acetylated) using novel cholesterol derivatives according to the lipid film hydration method.
- to characterize the liposomes and liposome-DNA complexes (lipoplexes) using transmission electron microscope (TEM) and Zeta sizing.
- to perform DNA binding studies using gel retardation, nuclease digestion and ethidium bromide intercalation assays.
- to assess cytotoxicity to cell lines *in vitro* using the MTT assay.
- to evaluate luciferase activity using the luciferase reporter gene assay.
- to perform competition assays to confirm lipoplex entry is by receptor mediated endocytosis.

Chapter one includes the introduction and literature survey on gene therapy concentrating on non-viral gene delivery vehicles viz. liposomes. Chapter two provides a brief introduction and detailed account of the synthesis and preparation of the different cationic liposome formulations and their characterisation using electron microscopy and zeta sizing. Chapter three includes a short introduction, description and discussion of the different assays performed to determine liposome: DNA binding viz. gel retardation, nuclease protection, and ethidium bromide intercalation studies. Chapter four outlines the cell culture assays undertaken *in vitro*. This includes an introduction to cell culture, including results and discussions of the MTT cell viability assays and the luciferase transgene activity. Lastly a brief conclusion is provided taking into account the results obtained and future work that needs to be done.

CHAPTER TWO

2. LIPOSOME PREPARATION AND CHARACTERISATION

2.1 INTRODUCTION

Liposomes can be defined as hollow spheres of lipids or vesicles which have a similar lipidic composition to the cell membrane. The term liposome is taken from the Greek words, 'lipos', which means fat and 'soma', which means body. Dr Alec D Bangham was the first scientist to describe liposomes in 1961, and published this data in 1964 (Bangham and Horne, 1964). This discovery of the liposome was done at the Babraham Institute (Cambridge), where Dr Bangham and R.W. Horne were simply testing the new electron microscope at the institution. These two scientists added negative stain to the dry phospholipid and hence discovered liposomes. The liposome resembled the plasmalemma, and pictures taken using that microscope were the first real images of a liposome.

The structure of the membrane involves a lipid bilayer which encloses an aqueous compartment. The bilayer is made up of phospholipids with the hydrophobic tails on the inside and the hydrophilic head groups on the outside (Figure 2.1). The hydrophilic head groups, are attracted to water and hydrophobic tails commonly made up of long hydrocarbon chains are repelled by water. Generally, the two head groups in the bilayer face opposite directions while the tails face each other. This structural form makes it a bilayer while the formation of a monolayer structure is termed a micelle. Phospholipids found in the plasma membrane are usually phosphatidylethanolamine and phosphatidylcholine. However, liposomes can have naturally- derived phospholipids which have mixed lipid chains i.e. phosphatidylethanolamine or they can be made of pure surfactant components such as DOPE (dioleoylphosphatidylethanolamine).

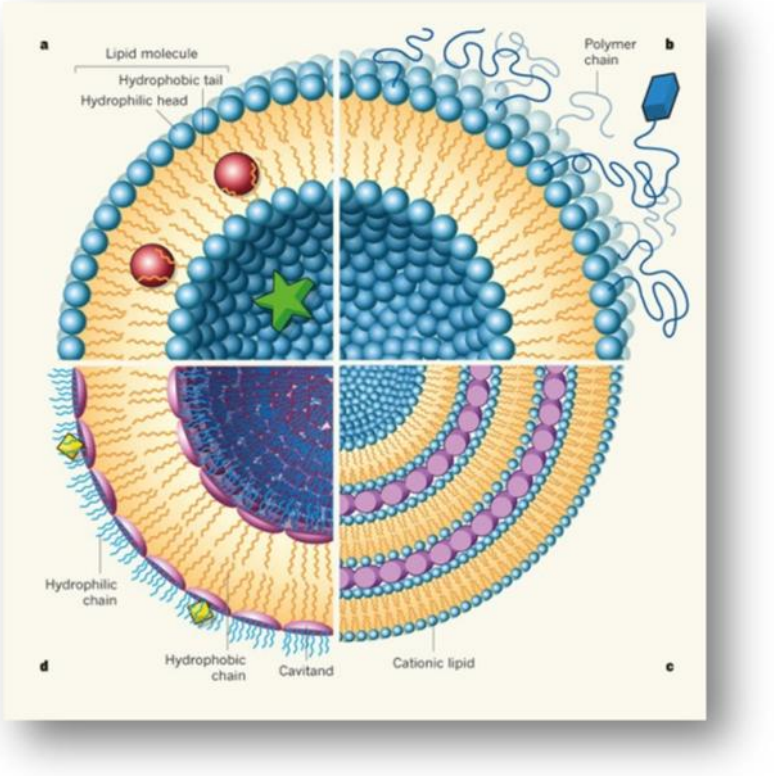


Figure 2.1: Diagram showing the different modifications of a liposome: Adapted from http://www.nature.com/nature/journal/v489/n7416/fig_tab/489372b_F1.html [Accessed 12 October 2013].

There are different types of liposomes which can be classified according to their structural parameters, preparation method and their composition and applications as seen in Table 2.1 and Figure 2.2 respectively.

Table 2.1 Classification of liposomes based on their structural parameters.

Vesicle Types	Diameter Size	Number of lipid bilayers
Small unilamellar vesicles (SUV)	20-100nm.	One lipid bilayer
Large unilamellar vesicles (LUV)	>100nm.	One lipid bilayer
Multilamellar vesicles (MLV)	>0.5μm.	Five to twenty lipid bilayers
Oligolamellar vesicles (OLV)	0.1-1μm.	Approximately five lipid bilayers
Multivesicular vesicles (MMV)	>1μm.	Multi-compartmental structure

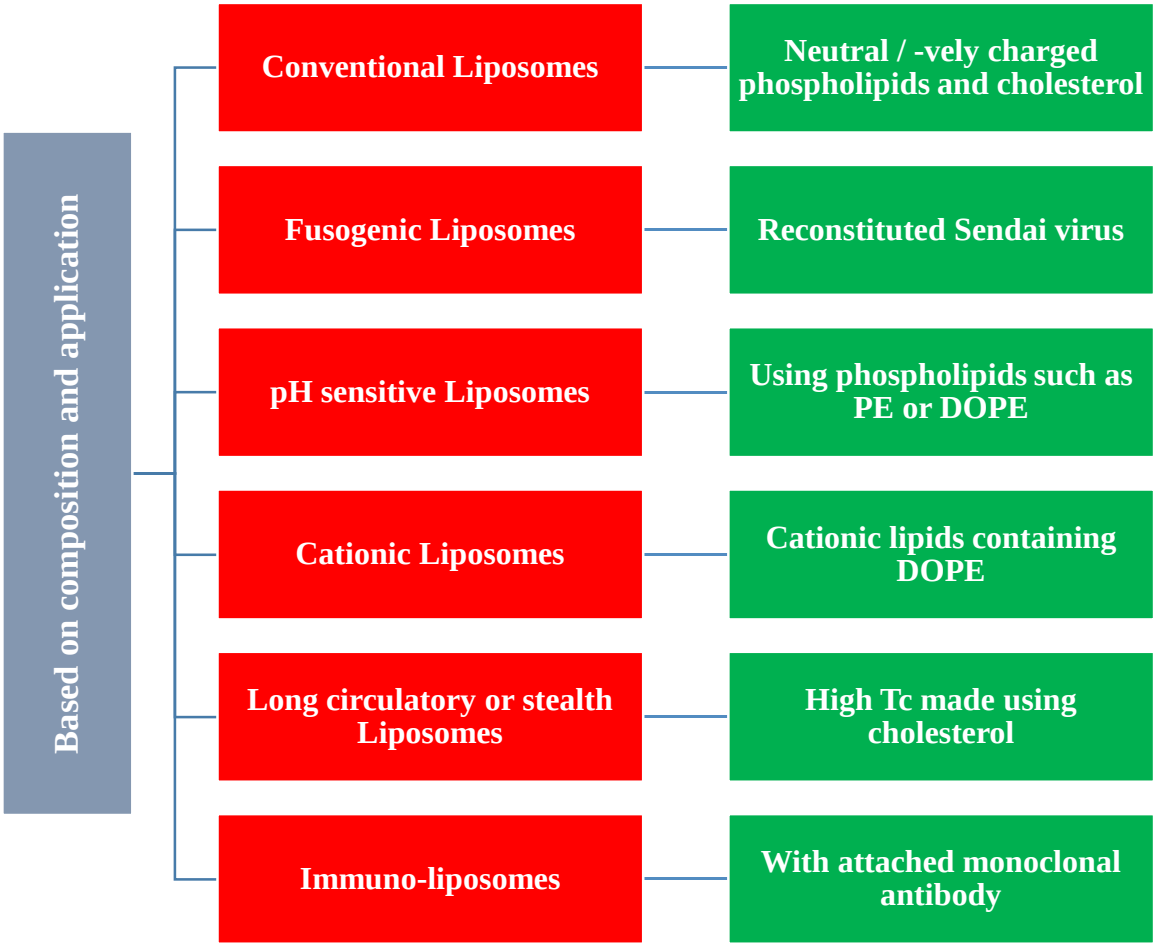


Figure 2.2: Classification of liposomes based on composition and application: Adapted from Dua *et al*., (2012).

2.1.1 HANDLING OF LIPOSOMES

There are a few important things to take note of when handling or using liposomes in the laboratory. Since liposomes are prepared using lipids, this makes them susceptible to oxidation because the lipids which are used during preparation are unsaturated. Also chloroform which is commonly used when preparing liposomes, is a volatile solvent which tends to evaporate when exposed to the atmosphere. Hence, it is important to store liposome preparations in an inert nitrogen atmosphere and it should be in a closed container away from light.

2.1.2 LIPOSOMES AND CELLULAR ENTRY

There are four ways with which liposomes interact with cells in order to gain entry.

- ✓ Endocytosis: this is a process where liposomes enter cells through phagocytosis and occurs commonly with cells of the reticulo endothelial system e.g. neutrophils and macrophages
- ✓ Adsorption: liposomes are taken up by the cell through electrostatic or nonspecific weak hydrophobic forces or through specific interactions with the cell-surface components.
- ✓ Cell fusion: liposome fuses with the plasma membrane of the cell by inserting its lipid bilayer into the plasma membrane, and the liposome contents is released into the cytoplasm at the same time.
- ✓ Lipid transfer into cell membranes: lipids are transferred into the cellular or subcellular membranes and vice versa (Gaurav and Tejal, 2011)

2.1.3 METHODS OF PREPARATION OF LIPOSOMES

There are different methods which can be used to prepare liposomes. These includes lipid hydration or thin-film hydration method, solvent spherule method, sanitation method (sonication), French pressure cell method, solvent injection methods, and detergent removal methods amongst others (Gaurav and Tejal, 2011)**.** Thin-film hydration provides a quick and easy protocol to prepare concentrated and homogeneous suspensions of small unilamellar liposomes. The liposomes prepared by thin-film hydration are suitable for laboratory-scale animal experiments. It involves the formation of a thin film of dried lipids on the walls of a round bottom glass flask. This is followed by the hydration of these lipids in aqueous solutions such as phosphate buffered saline (PBS) to form a suspension of lipids that could be either unilamellar or multilamellar. In many cases, this is followed by the passage of the lipid suspension through polycarbonate filters of specific pore sizes. The shear force due to the lipid passage through the membrane produces unilamellar liposomes. Liposome sizes are based on the pore size of the filters through which the lipids are filtered. If one needs to prepare liposomes for large scale purposes, microfluidization should be employed (Sorgi and Huang, 1996).

This chapter reports on the preparation of six novel cationic liposomes. All these liposomes contain the cholesterol derivative, 3 β [N-(N', N'-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) and the neutral lipid dioleoylphosphatidylethanolamine (DOPE). Three of which are PEGylated and acetylated and the other three are non-acetylated and not PEGylated. Characterization of all liposome preparations was performed by transmission electron microscopy.

2.2. MATERIALS AND METHODS

2.2.1. MATERIALS

Chol-T cationic lipid 3β[N-(N',N'-dimethylaminopropane)-carbamoyl] cholesterol was synthesized following a previously published procedure (Singh *et al*., 2001). Dioleoylphosphatidylethanolamine (DOPE) was purchased from the Sigma Chemical Company, St Louis, USA. The acetylated and non-acetylated galacto compounds were synthesized in the Department of Chemistry, University of the Witwatersrand, South Africa. HEPES buffered saline (HBS) was purchased from Merck, Darmstadt, Germany. 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene-glycol)-2000] ammonium salt (PEG2000) was purchased from Avanti Polar Lipids, Alabaster, USA.

2.2.2 METHODS

2.2.2.1 LIPOSOME PREPARATION

The six cationic liposomes were prepared using the thin film hydration method. The components and quantities used are presented in Table 2.2. The liposome preparations were made up to contain a total of 4 μmoles of lipid in 1 ml of HBS. All six liposomes contained DOPE and the cytofectin 3 β [N-(N', N'- dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) (Figure 2.3 A). The structures of the acetylated and deacetylated compounds used are shown in Figure 2.3.B Chloroform was employed during the preparation of liposomes to solubilise the liposome lipidic components. This was then mixed and deposited as thin films on the walls of the quick fit tubes by rotary evaporation of the suspension in *vacuo* (Büchi Rotavapor-R). Samples were dried under high vacuum in a drying pistol for 24 hours. The thin film 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 overnight. Following this, preparations were sonicated for 5 minutes in a sonicator (Labotech Transsonic 460/H, 220 – 240 V/AC) at a frequency of 35 kHz to produce unilamellar liposomes.

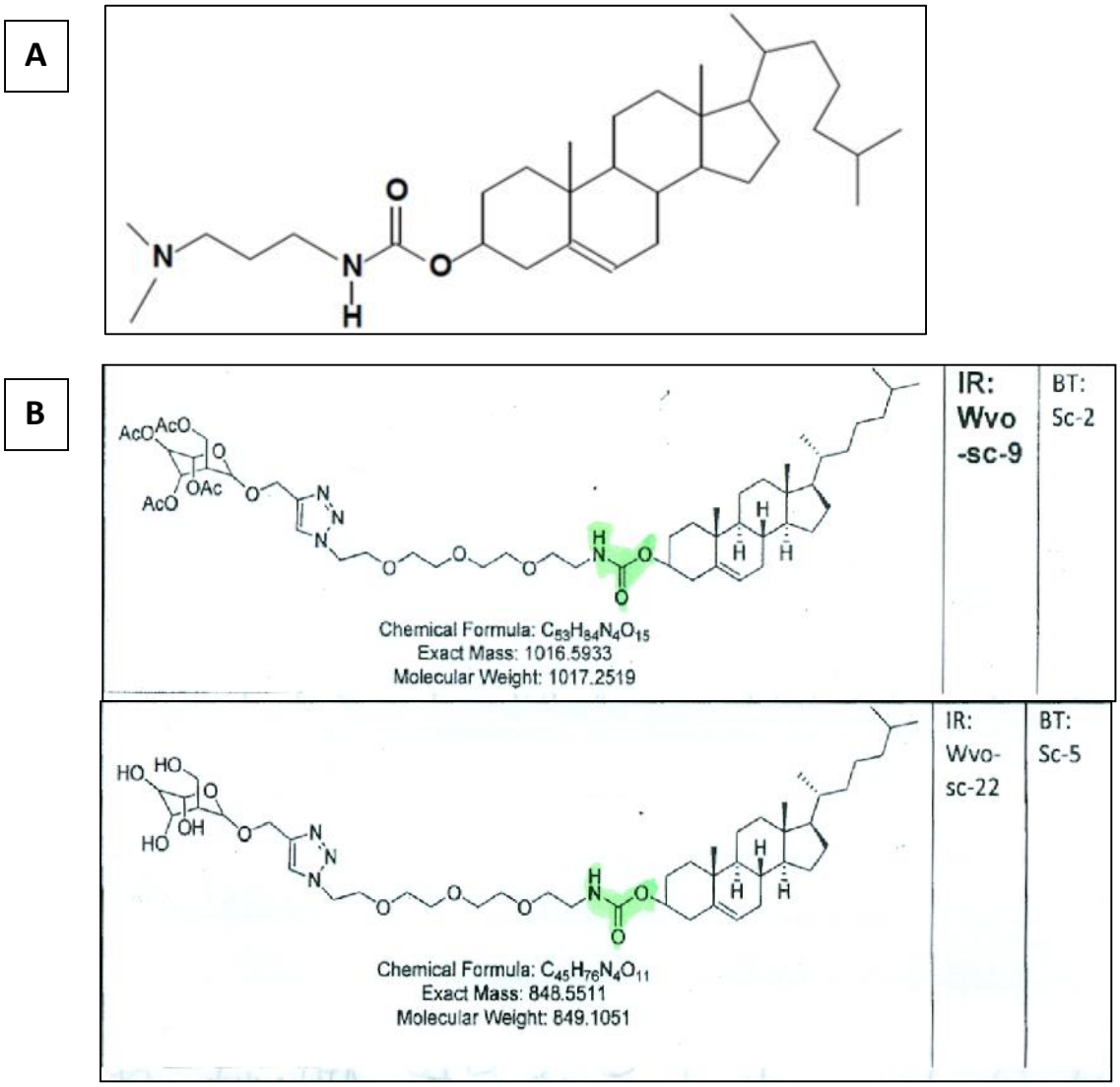


Figure 2.3: A: Structure of cationic cytofectin Chol-T. **B:** The structures of the acetylated Sc-2 (Cholest-5-en-3-yl 2-[2-(2-{2-[4-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-1-oxymethyl)-1*H*-1,2,3-triazol-1-yl]ethoxy}ethoxy)- ethoxy]ethylcarbamate) and deacetylated Sc-5 (Cholest-5-en-3-yl 2-[2-(2-{2-[4-(β-D-galactopyranosyl-1-oxymethyl)-1*H*-1,2,3-triazol-1-yl]ethoxy}ethoxy)ethoxy]ethylcarbamate) compounds.

Table 2.2: Lipid content in the various liposome formulations

Cationic Liposome	DOPE		CHOL-T		ACETYLATED GAL		GAL		DSPE-PEG2000	
	µmoles	MASS (mg)	µmoles	MASS (mg)	µmoles	MASS (mg)	µmoles	MASS (mg)	µmoles	MASS (mg)
1	2	1.49	2	1.03	-	-	-	-	-	-
2	1.8	1.34	2	1.03	-	-	-	-	0.2	0.56
3	1.6	1.19	2	1.03	-	-	0.4	0.34	-	-
4	1.4	1.04	2	1.03	-	-	0.4	0.34	0.2	0.56
5	1.6	1.19	2	1.03	0.4	0.41	-	-	-	-
6	1.4	1.04	2	1.03	0.4	0.41	-	-	0.2	0.56

2.2.3 LIPOSOME CHARACTERIZATION BY TRANSMISSION ELECTRON MICROSCOPY (TEM).

Cationic liposomes in HBS were diluted 1:5 to promote fluidity of the samples. Aliquots (1 µl) of each diluted sample were placed on a Formvar coated grid. Uranyl acetate (0.5%) negative stain was mixed into the sample. Grids were allowed to stand for 3 – 5 minutes. A spring-loaded Leica CPC system was used to plunge samples into liquid nitrogen cooled propane gas. Grids were then viewed under the JEOL 1010 TEM without warming above -150ºC. The electron micrographs were examined to observe the size and dimensions of the liposome formulations.

2.2.4 *ZETA* SIZING

A Malvern Zetasizer Nanoseries (Malvern Instruments, Worcestershire, UK) was used to measure the hydrodynamic diameters of all six cationic liposomes. All cationic liposomes were diluted 1:20 with HBS before measurements were taken. These diluted samples were then

vortexed to completely suspend and ensure even distribution of the liposomes in the HBS. The sizes of the cationic liposomes were measured using the zetasizer at a refractive index of 1.450.

2.3 RESULTS AND DISCUSSION

2.3.1 CATIONIC LIPOSOMES PREPARATION

Cationic liposomes were prepared using the thin film rehydration method as described in section 2.2.2.1. A total of six cationic liposomes were prepared, each containing the cytofectin Chol-T and the neutral co-lipid dioleoylphosphatidylethanolamine (DOPE). DOPE functions as the helper lipids in these cationic liposome preparations to enhance transfection efficiency. Chol-T has a dimethylamino head group which is protonated at physiological pH (pH 6-8). Chol-T therefore shows a positive charge and tends to form stable liposomes when mixed with equal number of moles of DOPE (Singh *et al.,* 2011). DOPE exhibits transitions in structure with changes in pH. Above pH 9, DOPE forms a spherical micelle-like structure, which then transforms to an inverted hexagonal form at an acidic pH. This structural change plays an important role in the fusion and the destabilization of the endosomal membrane. It has been reported that DOPE destabilizes the binding that occurs between the cationic lipids and DNA, thus allowing the DNA to be released from lipoplexes (Mochizuki *et al*., 2012). There have been several reports that investigate the responsiveness of micelles made from DOPE in order to understand the molecular origin of pH dependence of DOPE (Mochizuki *et al.*, 2012).

The structure of the cationic cytofectins consist of a cholesterol ring anchor, a carbamoyl linker bond and a monovalent dimethylamino head group. Some of these liposomes have hydrophilic polymers such as poly(ethylene glycol (PEG) and targeting ligands such as galactose moiety which have an affinity for the asialoglycoprotein receptor (ASGP-R), predominantly found in hepatocytes and hepatocyte -derived cells. The hydrophilic nature of PEG helps inhibit plasma proteins from adsorbing onto the carrier's surface, resulting in an increase in the carrier's systemic circulation time and enhancing the possibility of transfection. In the case of the acetylated liposomes, the effect of *O*-acetylation on the hydroxyl groups of the liposome-bound galactose has yet to be fully explored. The incorporation of cholesterol is important as it plays a vital role in controlling fluidity and permeability of the membrane and hence ensuring stability of the formulation (Obata *et al.*, 2009).

2.3.2 TRANSMISSION ELECTRON MICROSCOPY (TEM) OF LIPOSOMES

TEM uses a high energy beam of electrons to provide specific images of the sample. These electrons interact with the atoms and one can observe features such as the structure, shape and size of the samples. The six cationic liposome formulations used in this study displayed unilamellar, spherical-like morphologies but varied in their sizes from 50 to 200 nm in diameter (Figure 2.4). Studies have revealed that cationic liposomes can be classified according to their structure and size. According to Immordino *et al*., (2006), unilamellar liposomes have a large aqueous core and such liposomes are preferred when encapsulating water-soluble drugs. The differences in sizes of these six cationic liposomes could be due to their varied lipid, polymer and carbohydrate compositions.

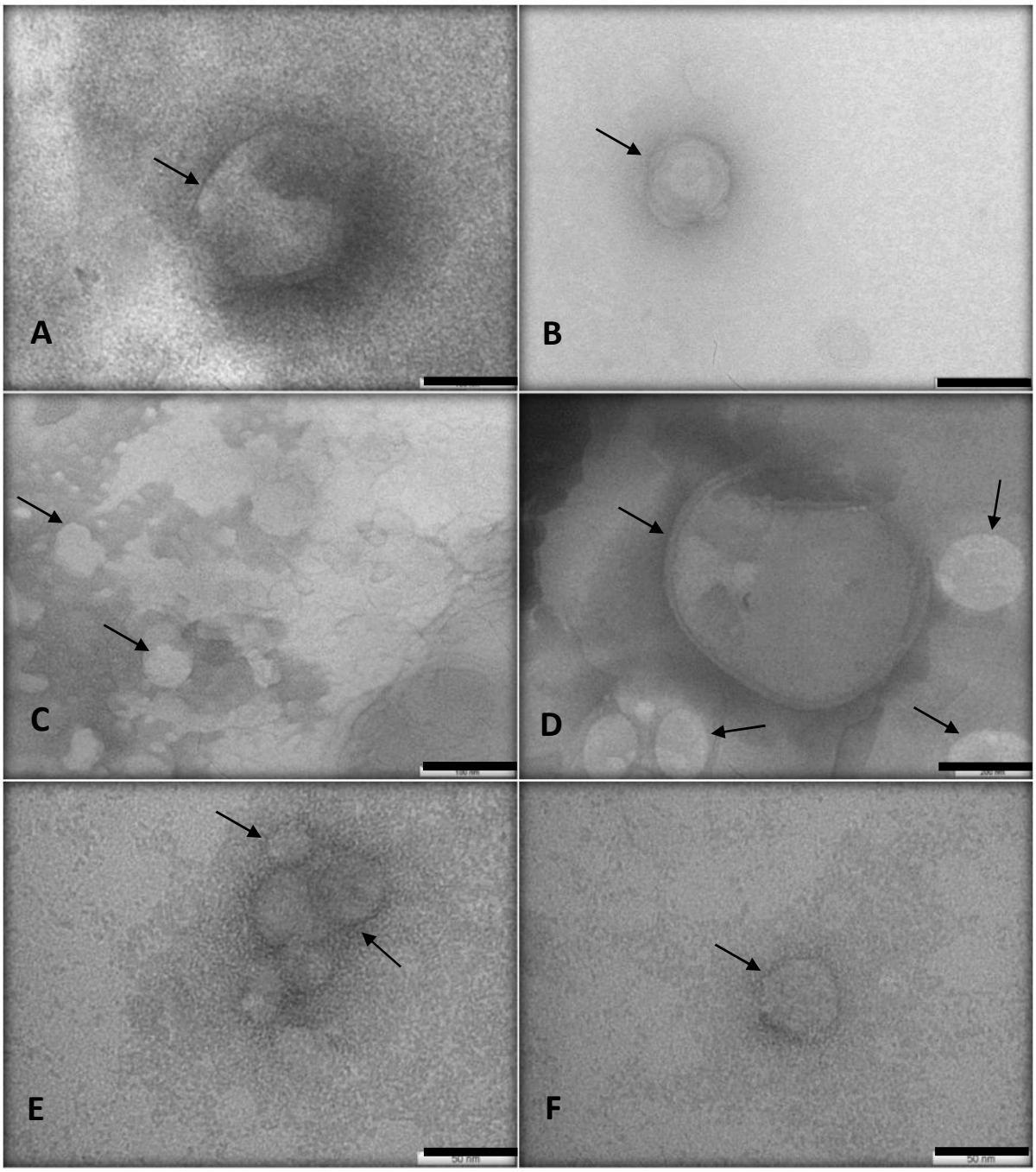


Figure 2.4: Transmission electron micrographs of the six cationic liposomes. The average diameter of each of the liposomes were: **A**: DOPE + Chol-T (Bar = 100 nm); **B:** DOPE + Chol-T + PEG (Bar = 100 nm); **C:** DOPE + Chol-t + Sc-5 (Bar = 100 nm); D: DOPE + Chol-T + Sc-5 + PEG (Bar = 200 nm); E: DOPE + Chol-T + Sc-2 (Bar = 50 nm); F: DOPE + Chol-T + Sc2 + PEG (Bar = 50 nm).

Overall from the TEM images, it was observed that the PEGylated liposomes in all cases appeared smaller in size (70 – 105 nm) than that of the non-PEGlated liposomes (150- 200 nm). This could be due to the PEG chains on the liposome surface which are thought to prevent vesicle aggregation (Immordino *et al*., 2006).

2.3.3 ZETA-SIZING

Table 2.3 shows the hydrodynamic diameters of the cationic liposomes as determined from zeta-sizing. From these results, it was noted that the sizes of the liposomes were varied which is similar to what was seen under TEM. The size of the liposomes overall ranged from 79.99 nm for the targeted PEGylated liposome to 198.9 nm for the targeted acetylated liposome. Here again the results show that the PEGylated liposomes are much smaller than their non-PEGylated counterparts. The polydispersity index (PDI) for all liposomes were around 0.2. A PDI around 0.1 generally signifies a monodisperse system (Cabral *et al*., 2004). The PDI obtained for these liposome are still low, indicating that much of the liposome populations may be monodisperse and only slightly heterogeneous.

Table 2.3: Sizes and Polydispersity of the different cationic liposomes.

LIPOSOME	Z-AVERAGE (d.nm)	PDI
DOPE:Chol-T	157.4	0.224
DOPE:Chol-T:PEG	108.9	0.217
DOPE:Chol-T:Sc-5	144.4	0.216
DOPE:Chol-T:Sc-5:PEG	79.99	0.205
DOPE:Chol-T:Sc-2	198.9	0.450
DOPE:Chol-T:Sc-2:PEG.	105.1	0.227

CHAPTER THREE

3. PREPARATION AND CHARACTERISATION OF LIPOPLEXES

3.1 INTRODUCTION

Cationic liposomes have been widely used *in vitro* to condense DNA, thus forming lipoplexes (Figure 3.1). Lipoplexes may be defined as self-assembling nanosystems. There have been two different models which have been proposed to understand the structures of lipoplexes formed between plasmid DNA and cationic lipids. The first model is known as the external model, where the plasmid DNA is adsorbed onto the cationic liposome surface. The second model; is known as the internal model, where the plasmid DNA is coated by the lipid envelope (Ma *et al.*, 2007).

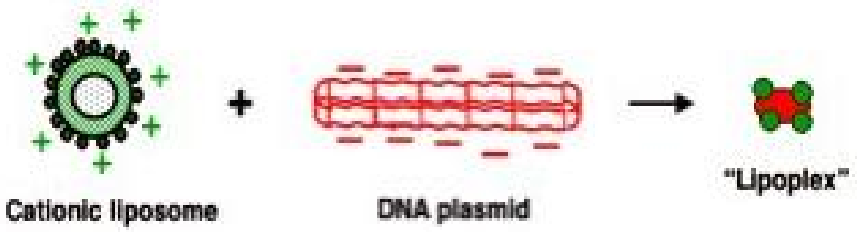


Figure 3.1: Formation of the lipoplex by binding of cationic liposome and plasmid DNA: Adapted from http://www.uweb.engr.washington.edu/research/tutorials/drugdelivery.html [Accessed 09 September 2014].

The second model however involves various steps during the mechanism of the lipoplex formation. These steps have been studied using lipid monolayers, cryo-electron microscopy and atomic force microscopy, and has shown that the first step in the formation of lipoplexes occurs within a millisecond, where the DNA interacts with the amine head groups (positively charged) of the cationic lipids through electrostatic interactions. This interaction on one side or surface of the DNA and the liposome causes packing constraints within the bilayer, resulting in widespread interactions between adjacent bilayers as well. This is followed by the release of the contents of the vesicle which shows that the membrane structure has been broken, causing the hydrophobic edges to be exposed. These edges may serve as the nucleation sites where adjacent structures interact with each other, thus causing more merging of membranes and mixing of lipids. As time goes by, the cationic liposome becomes completely bound around the plasmid DNA. The complex formed has a smooth surface which shows that the DNA has

been properly packaged within the cationic liposome. At this point, lipid mixing stops because sites where there can be potential interaction are blocked. It is important for the cationic lipid to compact the DNA effectively, and to maintain its elasticity. Easily hydrated lipids form fluid aggregates which then undergo a transition to form an inverted hexagonal phase when plasmid DNA is introduced at physiological ionic strength. These lipids often produce high transfection efficiencies. Neutral lipids that are usually incorporated in the cationic liposome, play vital roles in the formation and functioning of the lipoplex.

In this study, the neutral lipid DOPE was used when preparing the cationic liposomes. DOPE is known to have an effect on the polymorphic features of the lipoplex because it can induce the lamellar to hexagonal phase transition. Also, the presence of DOPE results in cationic charges being neutralised by the phosphodiester bond which is negatively charged (Wasungu and Hoekstra, 2006). The lamellar structure is the most common phase that lipoplexes assume with plasmid DNA in between the cationic bilayers. Several factors do however affect the behaviour of the lamellar and hexagonal phase. Figure 3.2 shows different structures which can be assumed by lipoplexes formed by cationic liposomes and plasmid DNA. For a lamellar morphology (Figure 3.2 a), the rods of DNA are located between the lipid bilayers of the cationic liposome. In the hexagonal lattice (Figure 3.2 b), the rods of DNA are coated with the cationic liposome monolayer and lastly, as seen in Figure 3.2 c, the rods of DNA are covered by three honeycombs of cationic lipid micelles and these micelles are also arranged as a hexagonal lattice.

Studies have revealed that the structure of the lipoplex and it stability is dependent upon the molecular characteristics of cationic and helper lipids as well as on the cationic liposome to plasmid DNA ratio. Ionic strength and temperature also have an effect on the behaviour or functioning of the lipoplexes. The lamellar phase lipoplexes are known to be stable over a wide range of temperature and ionic strengths (Ma *et al.*, 2007).

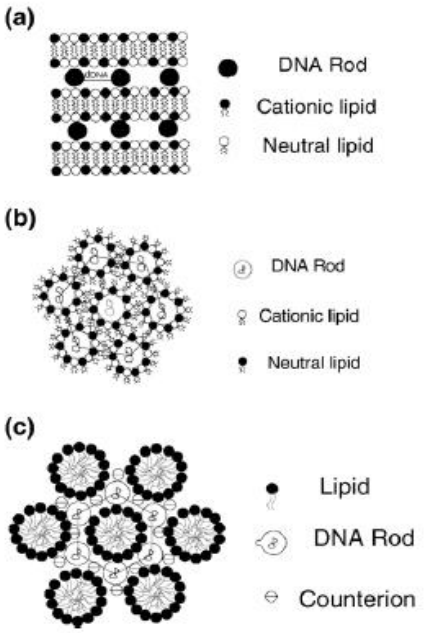


Figure 3.2: Different phases of lipoplexes with (a) a lamellar structure formed from the mixture of cationic lipid and plasmid DNA, (b) an inverted hexagonal structure, and (c) an intercalated hexagonal structure. Adapted from Ma *et al.,* (2007).

Efficient transfection or gene expression is dependent on different factors such as the ability of DNA to be transported successfully to the target site without leakage or being degraded by cytoplasmic enzymes or serum nucleases. Other factors may include easy escape of the DNA from the endosomal compartment. A number of assays have been developed to characterize the liposome-DNA association and the results obtained have been correlated with transfection efficiency. These include gel retardation, nuclease protection and ethidium bromide intercalation assays. These three assays determine whether the cationic liposomes can efficiently bind the plasmid DNA and protect it from the action of nucleases, and indicate the degree of cargo DNA condensation respectively.

3.2 MATERIALS AND METHODS

3.2.1 MATERIALS

Ethidium bromide was acquired from Merck, Damstadt, Germany. Plasmid DNA (pCMV-luc) was obtained from Plasmid Factory, Bielefeld, Germany. Agarose was purchased from Bio-Rad Laboratories, California, USA. All other reagents were of analytical grade.

3.2.2. METHODS

3.2.2.1 GEL RETARDATION ASSAY

Agarose gel electrophoresis was performed using a 1% agarose gel prepared by dissolving 0.2 g of agarose powder in 18 ml of distilled water. The agarose mixture was heated to boiling point in order to dissolve the entire agarose powder. This was followed by cooling to approximately 75 ^{o}C, followed by the addition of 2 ml of 10x electrophoresis buffer (0.36 M Tris HCl, 0.3 M NaH_2PO_4, 0.1 M EDTA, pH 7.5), and 1.5 µl of ethidium bromide solution (1 µg/ml). The agarose solution was allowed to cool further to a temperature of about 60-62 ^{o}C and then poured into a sealed casting tray containing an 8 well comb. Various ratios of cationic liposome-DNA complexes were prepared as follows: plasmid DNA (0.5 µg) was added to increasing volumes of cationic liposome dispersions in HBS (Table 3.1). The plasmid DNA–cationic liposome complexes were subjected to brief vortexing, centrifugation and then incubated at room temperature for 30 minutes. Thereafter, 3 µl of gel loading buffer (40 % sucrose, 0.5 % w/v bromophenol blue) was added to all complexes and samples loaded into the wells of the agarose gel in a Bio-Rad mini-sub electrophoresis apparatus containing 1x electrophoresis buffer (36 mM Tris HCl, 30 mM NaH_2PO_4, 10 mM EDTA pH 7.5). Gels were run at 50 volts for 90 minutes and then viewed under UV transillumination at 300 nm and images were captured using a Vacutec Syngene G: Box gel documentation system (Syngene, Cambridge,UK).

Table 3.1: Various ratios of DNA -cationic liposome complexes preparation.

DNA:Liposome Charge ratios (+/-)	1:0	1:1	1:2	1:3	1:4	1:5	1:6	1:7
DNA (0.5 µg/ µl) (µl)	1	1	1	1	1	1	1	1
Diluted liposome (µl)	-	3.75	-	-	-	-	-	-
Undiluted liposome (µl)	-	-	1.5	2.25	3	3.5	4.5	5.25
HBS (µl)	9	5.25	7.5	6.75	6	5.8	4.5	3.75
Total volume (µl)	10	10	10	10	10	10	10	10

3.2.2.2 NUCLEASE PROTECTION ASSAY

Complexes were prepared as described in section 3.2.2.1 but using the sub-optimal, optimal and supra-optimal ratios for each complex. The samples were incubated at room temperature for 30 minutes, followed by the addition of foetal bovine serum (FBS) to the complexes to a final concentration of 10 % (v/v). Two controls were also prepared, one containing plasmid DNA only and a second containing plasmid DNA and FBS. The samples containing FBS were further incubated at 37°C for 4 hours. Following the incubation period, ethylenediaminetetraacetic acid (EDTA) was added to all samples to a final concentration of 10 mM. EDTA acts as the chelating agent for divalent cations thus stopping the nuclease reaction. Sodium dodecyl sulphate (SDS) was then added to a final concentration of 0.5% (w/v) to break the interactive forces between the liposomes and the DNA and to allow the DNA to be released from the lipoplexes. Thereafter, the samples were incubated at 55°C for 20 minutes. Samples were then subjected to agarose gel electrophoresis at 50 volts for 120 minutes. Gels were then viewed and images captured as in section 3.2.2.1.

Table 3.2: Ratios of liposome: DNA used for lipoplex formation.

LIPOSOME PREPARATION	**LIPOSOME AMOUNT (µg)**			**DNA (µg)**
DOPE:Chol-T	7.11µg	10.665	14.22	1
DOPE:Chol-T:PEG	8.79µg	13.185	17.58	1
DOPE:ChoL-T:Sc-5	11.52µg	15.36	17.92	1
DOPE:Chol-T:Sc-5:PEG	8.91µg	13.365	17.82	1
DOPE:Chol-T:Sc-2	11.835µg	15.78	18.41	1
DOPE:Chol-T:Sc-2:PEG.	9.12µg	13.68	18.24	1

3.2.2.3 ETHIDIUM BROMIDE INTERCALATION ASSAY

This assay was conducted at an excitation wavelength of 520 nm and emission was monitored at 600 nm using a GLOMAX multi+ detection system (Promega Biosystems, Sunnyvale, USA). Briefly, 2 µl of stock ethidium bromide solution (1 mg/ml) was added to 100 µl of HBS in 2 wells of a black 96 well plate and measured to obtain a baseline relative fluorescence of 0. Thereafter, 4.8 µl (1.2 µg) of pCMV-luc DNA was added to the mixture and the reading taken was assumed to represent 100% relative fluorescence. Following this, 1 µl (2.37 µg) aliquots of the liposome preparation was added stepwise to the above mixture and readings were taken until a plateau had been reached. Accurate readings were assured by thoroughly mixing samples to promote full dispersion of the liposome in the solution, thus encouraging complete association with the DNA. Results obtained were plotted as relative fluorescence percentages against mass of cationic liposome.

3.2.2.4 TRANSMISSION ELECTRON MICROSCOPY

Cationic liposome – DNA complexes were characterised using transmission electron microscope. Cationic liposomes were diluted to a ratio of 1:5 with sterile HBS. Briefly, 2 µl of the diluted liposome suspension was placed onto a Formvar coated copper grids. To this was added 2 µl of 0.5% uranyl acetate and the excess liquid was blotted using filter paper. The grids were allowed to dry for 2 minutes and immediately vitrified or plunged into liquid propane at –183 °C. The samples were then viewed in a high resolution transmission electron microscope (JEOL1010). The electron micrographs were digitally captured using an Olympus MegaView III camera and SIS iTEM software (Tokyo, Japan).

3.2.2.5 *ZETA* SIZING

Zeta sizing was carried out using the Malvern Zetasizer Nanoseries (Malvern Instruments, Worcestershire, UK). All lipoplexes were diluted 1: 20 in HBS. Readings were measured in the zetasizer at 25 °C and data recorded using the Zetasizer software, version 6.30.

3.2.2.6 STATISTICAL ANALYSIS

The particle sizes of lipoplexes were evaluated using the matched student t-test and the P-values of < 0.05 were considered to be significant.

3.3 RESULTS AND DISCUSSION

3.3.1 GEL RETARDATION ASSAY

Gel retardation assay can also be termed as the electrophoretic mobility shift assay or the band shift assay. This assay has been widely used to detect DNA binding proteins found in crude oil extracts and also used for studying DNA- binding protein activity. This assay was originally devised by Fried and Crothers (1981) when they were studying protein – DNA interaction kinetics. In this assay the liposome-DNA complexes are expected to remain intact and migrate slowly compared to free DNA which will freely enter the gel during agarose gel electrophoresis.

When all DNA is liposome bound, creating an electroneutral complex, the DNA will not be able to enter the gel and the complex will be viewed as an intense fluouresence band in the well. Sometimes complexes float out of the well during electrophoresis and reduces the fluorescence observed (Singh, 2005).

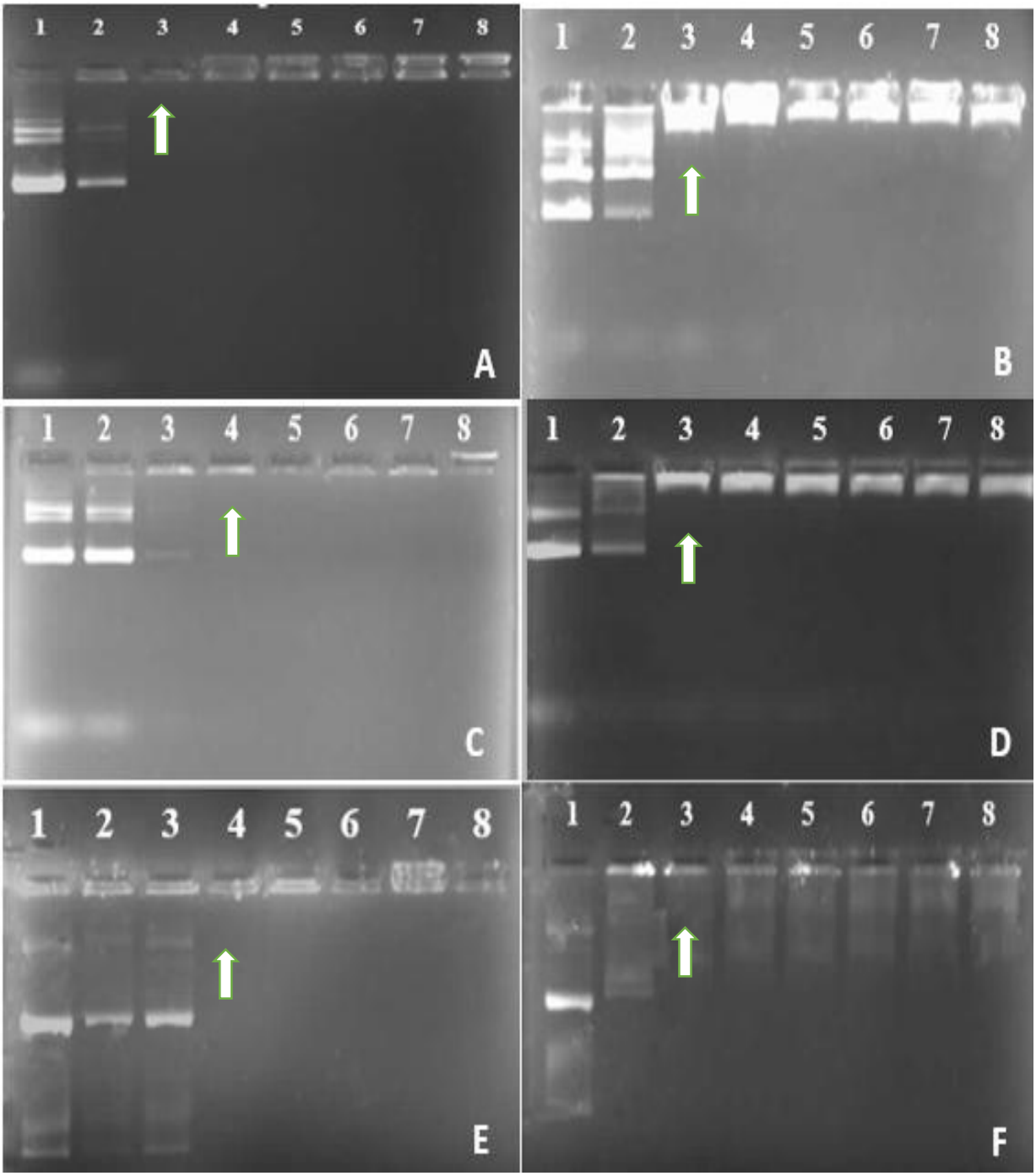


Figure 3.3: Gel retardation assay of lipoplexes. Reaction mixtures (11 µl) comprised of a constant amount of pCMV-luc DNA (0.5 µg) and varying amounts of cationic liposomes in HBS. Lane 1: naked DNA which serves as a control. Cationic liposome: DNA ratios (w/w) from lane 2-8 (1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1) were used for all six lipoplexes, viz. A: DOPE + Chol-T; B: DOPE + Chol-T + PEG; C: DOPE + Chol-T + Sc-5; D: DOPE + Chol-T + Sc-5 + PEG; E: DOPE + Chol-T + Sc-2; F: DOPE + Chol-T + Sc2 + PEG.

Figure 3.3 (A-F) shows the binding abilities of the cationic liposomes to DNA. During agarose gel electrophoresis the control naked DNA (lane 1), which is negatively charged, migrates freely from the cathode (negative) to the anode (positive). Lane two to eight contained a constant amount of plasmid DNA (0.5 μg) mixed with varying amounts of liposomes. From the results, the control displayed all three DNA conformations after electrophoresis. The band closest to the well was the nicked DNA, followed by the linearised DNA and the supercoiled DNA which moved furthest away from the well towards the anode. In Figure 3.3 (A, B, D and F), complete retardation of DNA was observed at DNA: cationic liposome charge ratios of 1:2, whereas in Figures 3.3 (C and E) complete retardation of DNA was observed at DNA: cationic liposome charge ratios of 1:3. Table 3.2 shows the end point ratios of the six liposome formulations.

Table 3.3: Cationic liposome to plasmid DNA charge and mass ratios at complete retardation (end point ratios).

LIPOSOME	CHARGE RATIO (+/-)	MASS RATIO ($^{w}/_{w}$)
DOPE:Chol-T	2:1	7.11: 1
DOPE:Chol-T:PEG	2:1	8.79 : 1
DOPE:Chol-T:Sc-5	3:1	11.52: 1
DOPE:Chol-T:Sc-5:PEG	2:1	8.91 : 1
DOPE:Chol-T:Sc-2	3:1	11.835 : 1
DOPE:Chol-T:Sc-2:PEG.	2:1	9.12 : 1

Overall, good nucleic acid condensation abilities is a prerequisite for all gene delivery vectors in gene therapy. The results above show that the cationic liposomes were capable of binding and condensing DNA successfully.

3.3.2 NUCLEASE PROTECTION ASSAY

One of the problems that have been encounted in gene therapy studies, which has led to the use of the vectors is the possibility of degradation of naked DNA by serum nucleases upon entry into the cytoplasm of the target cells or organs. The nuclease protection assay was carried out to determine the ability of the cationic liposomes to protect the integrity of the nucleic acid

cargo. In this study, serum nucleases were used to perform the protection assays. Nucleases are enzymes that have the ability to cleave the DNA phosphodiester bonds leading to their degradation. Figure 3.4 A-C shows the nuclease protection ability of the six liposome formulations.

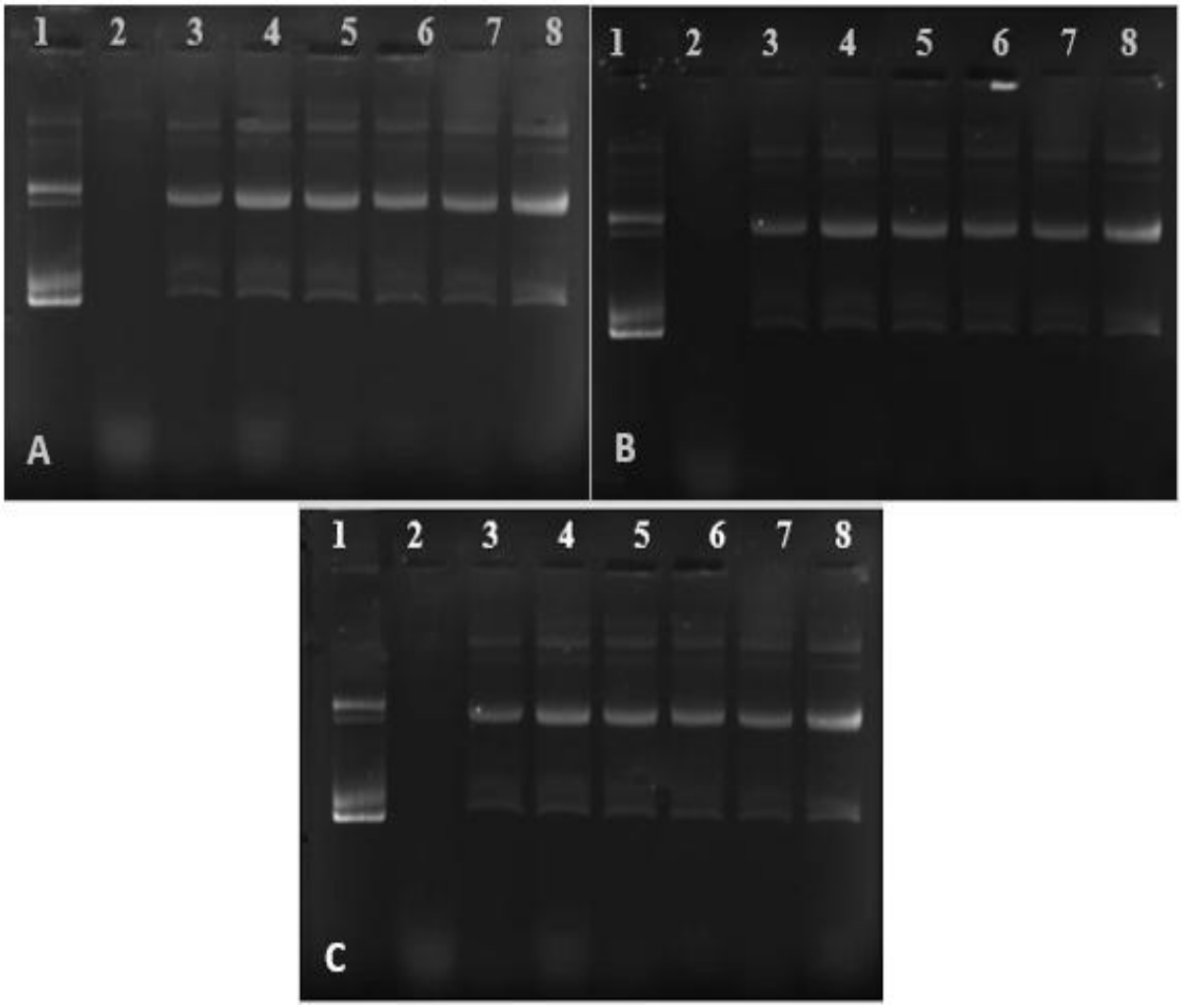


Figure 3.4: Nuclease protection assay of cationic liposome/DNA complexes. Complexes were incubated in the presence of 10 % serum. In all gels, lane 1 contained undigested plasmid DNA, lane 2 contained plasmid DNA treated with serum and Lane 3-8 contained the different lipoplexes incubated with serum.

A: lane 3-5 DOPE + Chol-T, lane 6-8 DOPE + Chol-T + PEG
B: lane 3-5 DOPE + Chol-T + Sc-5, lane 6-8 DOPE + Chol-T + Sc-5 + PEG
C: lane 3-5 DOPE + Chol-T + Sc-2, lane 6-8 DOPE + Chol-T + Sc2 + PEG

In this assay, lipoplexes were prepared as described in section 3.2.2.2.The lipoplexes may play a role in the reduction of the electrostatic repulsion forces between the cell surface and the DNA while also protecting the DNA from being degraded by enzymes such as nucleases within

cytoplasm or serum. From the results above one can observe that the control DNA (lane 1) showed all three forms of DNA which are nicked, linear and supercoiled. However, when the same DNA was treated with serum (lane 2) no DNA bands were observed after gel electrophoresis. A smear in lane 2 was seen in all three figures which is indicative of the degradation of DNA by the enzyme. In the case of the lipoplexes that were treated with serum (lanes 3-8), all three forms of the DNA were observed, although the fluorescence of supercoiled DNA band in Figures 3.4 B and C is very light. This indicates that some of the supercoiled forms may have been nicked by the nucleases to produce the closed circular forms. The use of SDS assisted in the release of the DNA from the respective lipoplexes and hence we were able to visualise the DNA band on the gel. Overall, all liposomes have shown that they are able to bind and protect the integrity of the DNA cargo.

3.3.3 ETHIDIUM BROMIDE INTERCALATION ASSAY

Ethidium bromide is known as the dye that strongly interacts with the double helix of DNA by intercalation. The ethidium bromide intercalation assay is widely employed to assess the strength of the interaction between the plasmid DNA and the cationic liposome. The intercalation of ethidium bromide between the helical strands of DNA can be observed through fluorescence emission. In this study, plasmid DNA was treated with ethidium bromide followed by the addition of the respective cationic liposomes. Figure 3.5 A-C show relative fluorescence as percentages against various amounts of liposome.

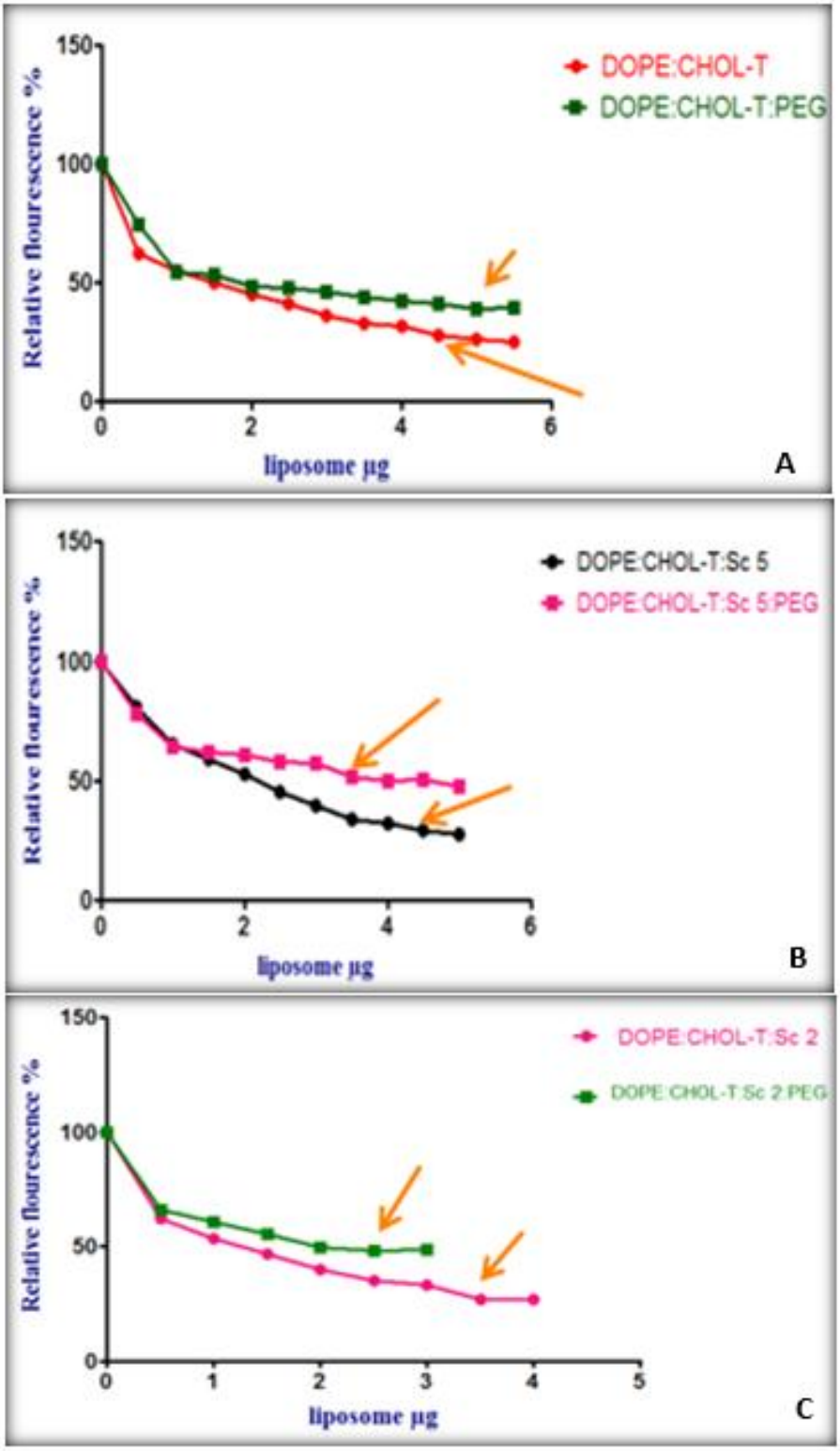


Figure 3.5 Ethidium bromide intercalation assay. Cationic liposomes were added to plasmid pCMV-luc DNA (0.5 µg) pre-treated with ethidium bromide dye. Arrows indicate the point of inflection.

A: DOPE + CHOL-T, and DOPE + CHOL-T + PEG

B: DOPE + CHOL-T + Sc-5, and DOPE + CHOL-T + Sc-5 + PEG

C: DOPE + CHOL-T + Sc-2, and DOPE + CHOL-T + Sc2 + PEG

The ethidium bromide fluorescence is known to be greatly increased or enhanced through its intercalation into the double stranded DNA. However, ethidium bromide fluorescence could be reduced or quenched by the addition of the cationic liposomes which should electrostically bind to the DNA. From the results one can observe (Figure 3.5) that upon sequential addition of each cationic liposome to the plasmid DNA/ethidium bromide/HBS complex, there was a steady decrease in the fluorescence intensity. This confirms that as the cationic liposomes were added, they gradually electrostatically interacted with and bound to the plasmid DNA thereby displacing the intercalated ethidium bromide. There was a slight difference between the non-PEGylated cationic liposomes and those that were PEGylated in that the relative fluorescence decreased about 30-40 % for all the non PEGylated cationic liposomes, compared to about 50% (point of inflection) for the PEGylated cationic liposomes. Also, the PEGylated cationic liposomes reached the plateau faster or sooner than the non-PEGylated liposomes. This difference in final relative fluorescences suggest that the non-PEGylated liposomes compact DNA much better than do the PEGylated liposomes.

3.3.4 TRANSMISSION ELECTRON MICROSCOPY AND ZETA-SIZING

Figure 3.6 A-F show the TEM images for the lipoplexes at the optimum binding ratios.

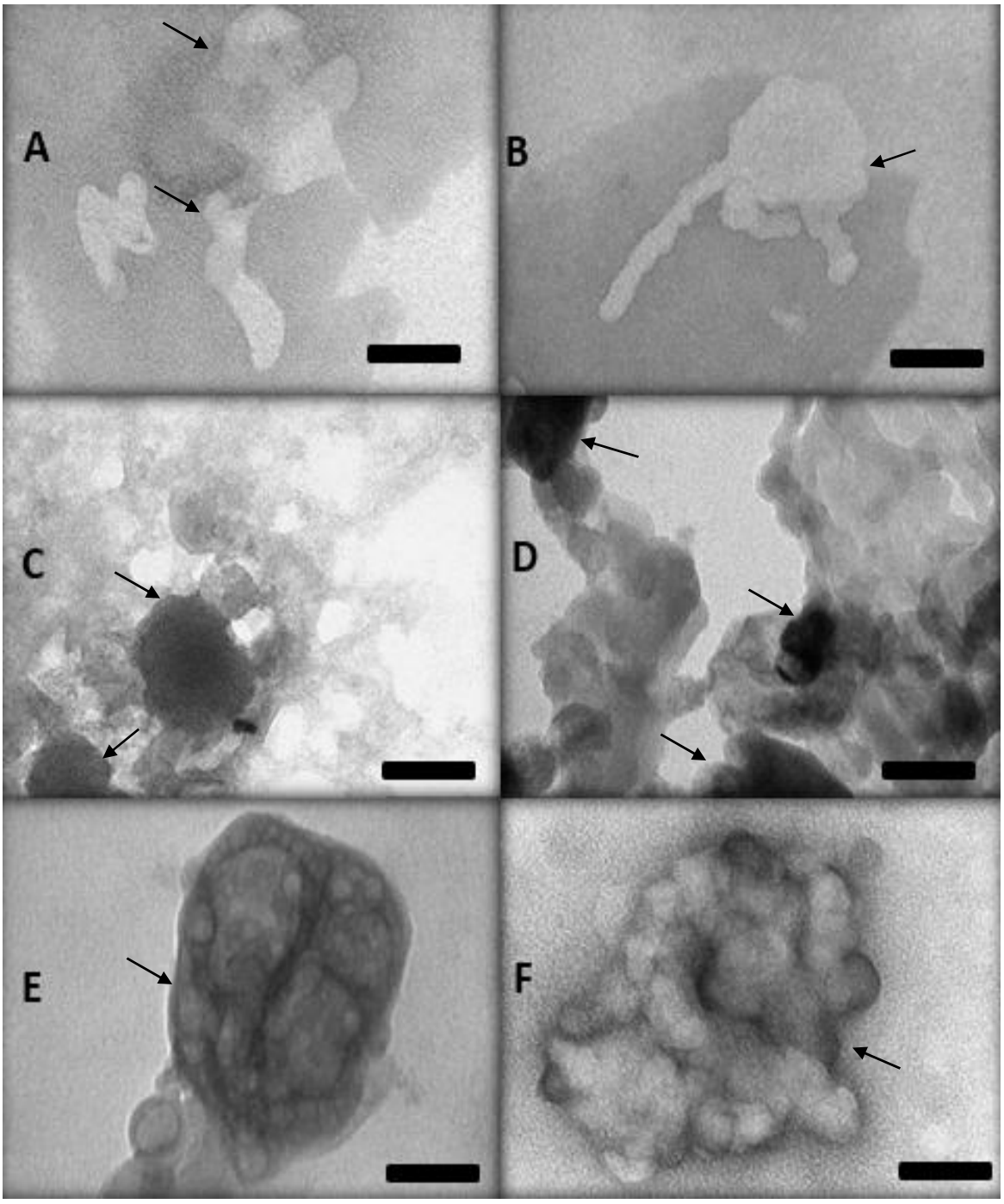


Figure 3.6 Transmission electron micrographs of lipoplexes formed between cationic liposomes and plasmid DNA. A: DOPE + CHOL-T; B: DOPE + CHOL-T + PEG; C: DOPE + CHOL-T + Sc-5; D: DOPE + CHOL-T + Sc-5 + PEG; E: DOPE + CHOL-T + Sc-2; F: DOPE + CHOL-T + Sc2 + PEG. Bar = 100nm

Cryo-transmission electron microscopy was used to characterise the lipoplexes formed between the DNA and the cationic liposomes. The images (Figure 3.6 A-F) obtained revealed different morphological characteristics of these lipoplexes at optimum binding ratios, such as average diameters and sizes. As seen in Table 3.4, the hydrodynamic diameter of the lipoplexes as determined from zeta-sizing, ranged from 96.7 nm for the un-PEGylated, targeted lipoplex to 193.5 nm for the unPEGylated and untargeted cationic liposome.

Table 3.4: *Zeta* sizes of different cationic liposomes-plasmid DNA lipoplexes. Data are presented as means (n = 3).

Liposome	Z-Average diameter (nm)	Polydispersity index (PDI)
DOPE:Chol-T	193,5	0,360
DOPE:Chol-T:PEG	111,7	0.193
DOPE:Chol-T:Sc-5	96,7	0.203
DOPE:Chol-T:Sc-5:PEG	88,8	0.708
DOPE:Chol-T:Sc-2	119,5	0.239
DOPE:Chol-T:Sc-2:PEG.	110,7	0.222

The PDI values ranged from 0.193 to 0.708 suggesting that most of the lipoplexes were polydispersed. However, PDI values closer to 0.1 would indicate a more monodisperse population of lipoplexes. From the images it can be noted that the lipoplexes formed had roughly spherical shapes but differed from one to the other, especially the lipoplexes that were not PEGylated, produced vastly different images and shapes of ipoplexes compared to those that contained PEG. Lipoplexes seemed to appear in the form of clusters of small vesicles. This is particularly true for the PEGylated lipoplexes. Lipoplex sizes are extremely important as those with smaller average sizes may accumulate more easily in the vesicles during uptake compared to larger lipoplexes. Furthermore, it will influence their ability to enter specific cells or tissues with smaller pore sizes eg. in the liver. The diameter of these vesicles is approximately in the 100-200 nm range. Hence, it will be expected that the smaller sized pores in liver tissue will favour the uptake of these smaller lipoplexes than larger lipoplexes (Saffarian *et al*., 2009).

CHAPTER FOUR

4. *IN VITRO* CYTOTOXICITY AND TRANSFECTION STUDIES

4.1 INTRODUCTION

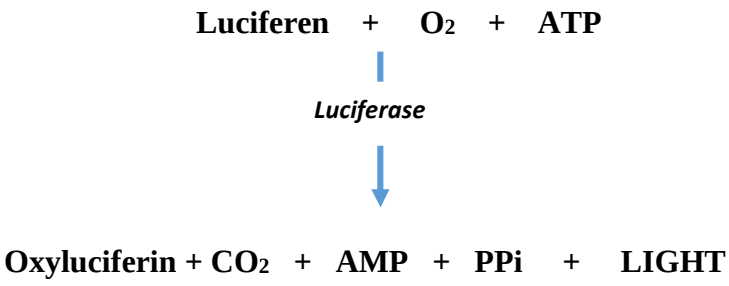
Cytotoxicity is a measure of the degree or level of toxicity of a specific compound to cells. Cells may respond in a variety of ways after being exposed to certain compounds or assemblies such as liposomes. There are different outcomes that may be seen after exposure, such as necrosis, where cells lose the integrity of the cell membrane and die due to cell lysis, or alternatively the cells may stop growing or dividing due to activation of programmed cell death, called apoptosis. Necrosis can be generally seen as swelling, loss of membrane integrity and non-functioning of the cells. Apoptosis on the other hand is due to well-defined cytological and molecular events, which can also be linked to cytoplasmic shrinkage and DNA cleavage. There are several assays available commercially which can be used to determine the degree of toxicity of a specific compound to cells. These assays enable scientists to predict the compounds and the dosage of the compound which will be safe for use in humans. Some researchers employ these assays in order to understand normal and abnormal processes which control cellular metabolism. Two of the most commonly used assays include the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) or the similar MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), cell viability assays (Berridge *et al.*, 2005).

Transfection is a technique used to study the functions of genes and gene products by inhibiting or enhancing specific gene expression within the cell. Transfection methods can be classified into three different groups viz. biological, chemical and physical. (Kim and Berwine, 2010). Transfection methods enable scientists to deliver genes and other nucleic acids to a specific target organ, tissue or cell. A new way to study genes and their products combining mRNA and point directed transfection has also been proposed. Transfection alters or modifies the overall functions of a cell. The exogenous genetic materials which has been introduced into the cell can exist in one of the two different states, depending on it nature, leading to either stable or transient transfection. In order to obtain stable transfection, one needs to introduce genes / genetic material which have markers necessary for selection (transgenes) which are integrated into the genome of the host cell, thereby sustaining gene expression even after completion of host cells replication. However, transient transfected genetic material can be

expressed for a certain period of time and here the exogenous DNA is not integrated into the host genome (Kim and Berwine, 2010). One of the disadvantages associated with transient transfected genes is that they may be lost during cellular division.

Reporter gene assays have been commonly employed to determine transfection activity. A reporter gene produces a specific phenotype during transfection, which enables researchers to differentiate between transformed cells (cells containing gene of interest) from those that are not transformed. This selection of transformed cells from many of those that are not transformed would otherwise be difficult if the reporter gene was not employed. Reporter genes are known to express specific protein products which can be detected and quantified easily in the laboratory. Reporter genes afford measurable activity at the transcription level on elements which are contained within a promoter region of a gene. These are DNA sequences that are associated with the genes coding region which regulate gene transcription and are often termed enhancers. To alter expression of the gene, transcription factors activate or suppress the promoters. During the reporter gene assays, promoters are engineered into a plasmid thereby enabling one to assess the activity at the transcription level through the expression of the reporter gene. The ideal reporter gene must have the following features; it must not be toxic to target cells, it must be easily quantifiable, it should have high ratios of signal-to-noise or low endogenous background, gene product must be resistant to chemicals which are used for processing of the product, and finally, the assay itself must be reliable and sensitive.

One of the most commonly used reporter genes is the firefly luciferase gene. This gene was isolated from the North American firefly *Photinus pyralis*. This organism expresses the luciferase enzyme which permits it to glow in the dark. The enzyme luciferase catalyzes the following reaction (Ignowski and Schaffer, 2004).



4.2 MATERIALS AND METHODS

4.2.1 MATERIALS

The HEK293 cell line was obtained from the Anti-Viral Gene Therapy Unit, Medical School, University of the Witwatersrand, and the HepG2 cell line and dimethylsulfoxide (DMSO) were purchased from Highveld Biological (PTY) LTD, Lyndhurst, South Africa. Luciferase Assay Kit (Luciferase Assay reagent and Cell Culture Lysis reagent) was purchased from Promega Corporation (Madison, USA). Bicinchoninic acid (BCA) assay reagent and asialofetuin were purchased from Sigma-Aldrich Co (St. Louis, USA). Eagles Minimal Essential Medium (EMEM) and Foetal Bovine Serum (FBS) were purchased from Lonza Bio-Whittaker, Walkersville, USA. Phosphate buffered saline (PBS) tablets and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) salt were purchased from Merck (Darmstadt, Germany). Tissue culture plastic wares were all obtained from Corning Incorporated (New York, USA). All other reagents were of analytical grade.

4.2.2 METHODS

4.2.2.1. GROWTH AND MAINTENANCE OF CELLS

Maintenance of cells and cell culture assays were carried out in a Class II Biosafety cabinet which ensured sterility and prevention of contamination. Cells were frequently observed for growth using a Nikon TMS inverted light microscope (100 x magnification) (Nikon Corporation, Tokyo, Japan). Complete medium (EMEM + 10% FBS + 100 µg / ml penicillin, 100 µg/ ml streptomycin) was routinely changed to ensure cell growth. Sub-culturing and cryopreservation of cells was carried out when cells were in a semi-confluent / confluent state.

4.2.2.2 RECONSTITUTION OF CELLS

Previously cryopreserved cells were removed from the biofreezer and immediately placed in a 37 °C waterbath to thaw. Thereafter, the vial was wiped with ethanol and under sterile conditions, the cell suspension was transferred to a sterile microcentrifuge tube and centrifuged at 3000 rpm for 1 min. The supernatant containing the DMSO was then discarded and the pellet were reconstituted in 1 ml of fresh complete medium. This was then transferred to a tissue culture flask containing 4 ml of complete medium. Flasks were incubated in a Steri-cult CO_2 incubator

HEPA Class 100 (Thermo-Electron Corporation, Waltham, Massachusetts, USA) at a temperature of 37 °C. Medium was routinely changed every 2-3 days using standard aseptic techniques and cells were monitored regularly until they reached confluency.

4.2.2.3 TRYPSINIZATION

When the cells had reached confluency, the old medium was removed from the flask, and cells were washed with 5 ml of sterile Phosphate Buffered Saline (PBS) (150 mM NaCl, 2.7 mM KCl, 1 mM KH_2PO_4, 6 mM Na_2HPO_4; pH 7.5). Thereafter, 1 ml of a trypsin/versene mixture was introduced to the cells for the trypsinization process. Cells were observed for rounding off using the Nikon TMS inverted microscope. The process of trypsinization generally took about 3-5 minutes at 37 °C. Thereafter, 2 ml of complete medium was added to the flask containing cells and the flask was tapped gently against the palm of the hand in order to dislodge cells. Cells were then split as desired into 25 cm^2 flasks or plated for assay purposes into a 48-well plate. The cells were then incubated at 37 ° C and medium was changed every 2-3 days using aseptic techniques. Once cells had reached confluency, they were either trypsinized again or plated for assay puposes or cryopreserved either in a -80 biofreezer (Nuaire, Lasec Laboratory and Scientific Equipment) for short term or in liquid nitrogen for long term storage.

4.2.2.4 CRYOPRESERVATION OF CELLS

Cells at or close to confluency were trypsinized as in 4.2.2.3. Cells were then pelleted by centrifugation at 1000 rpm for 3 minutes and the pellet re-suspended in 0.9 ml of complete medium and 0.1 ml of dimethylsulfoxide (DMSO). The cell suspension was briefly vortexed and 1 ml aliquots of the cell suspension were transferred into 2 ml cryovials, which were then placed into a Nalgene Mr. Frosty 1°C freezing container containing isopropanol and stored in the -80 °C biofreezer overnight. Thereafter, the vials were transferred to cryo-boxes and kept in the biofreezer or placed in liquid nitrogen.

4.2.2.5 CYTOTOXICITY STUDIES

Cytotoxicity of liposomes was measured using the MTT cell viability assay. HepG2 and HEK293 cells were trypsinized and seeded into 48 well plates at a seeding density of 1.5 x 10^5 cells per well. Cells were then incubated at 37 °C overnight (24 hours). Lipoplexes were prepared as described in Chapter 3 section 3.2.2.2. Then lipoplexes were incubated for 30 minutes at room temperature. Spent medium was removed from cells and replaced with 0.3 ml of complete medium (EMEM + 10% FBS + 100 µg / ml penicillin, 100 µg/ ml streptomycin). This was followed by addition of the lipoplexes to the cells and incubation at 37 °C for 48 hours. Positive controls were set up using only cells with no lipoplexes added. The assay was carried out in triplicate. After incubation, the medium was removed and cells were washed with 300 µl PBS. Thereafter, 300 µl of MTT solution (5 mg/ml in PBS) and 0.3 ml medium were added to each well containing cells and this was incubated for a further 4 hours at 37 °C. After the incubation period, the MTT solution and medium were removed and replaced with 300 µl of dimethylsulfoxide (DMSO). Absorbance of the solubilized DMSO formazan extracts were measured using a Mindray-MR-96A microplate reader (Vacutec, Hamburg, Germany) at a wavelength of 570 nm.

4.2.2.6. TRANSFECTION STUDIES

HepG2 and HEK293 cells were trypsinized and seeded in 48 well plates at a seeding density of 1.5 x 10^5 cells per well. Cells were then incubated in a 37 °C incubator overnight Lipoplexes were prepared as in Chapter 3 section 3.2.2.2. and at the same ratios added for cytotoxicity (4.2.2.5). Then lipoplexes were incubated for 30 minutes at room temperature. Spent medium was removed from cells and replaced with 0.3 ml of complete medium. This was followed by addition of the lipoplexes to the cells, and incubation at 37 °C for 48 hours. Two sets of controls were set up viz; one containing only cells and a second containing cells with naked plasmid pCMV-luc DNA (0.5 µg). The assay was carried out in triplicate. After incubation period, cells were assayed for luciferase activity (section 4.2.2.6.2).

4.2.2.6.1 COMPETITION ASSAY

The competition assay was carried out only on the targeted HepG2 cell line at the same cell density and following the same protocol as in section 4.2.2.6. for the transfection. The only difference was that cells were first pre-incubated with asialofetuin (200 µg/well) for 30 minutes prior to the addition of the lipoplexes. After the 48 hour incubation, the cells were assayed for luciferase activity (section 4.2.2.6.2).

4.2.2.6.2 LUCIFERASE ASSAY

The Promega Luciferase Assay kit was used to measure the luciferase activity of the cells. A 1x cell culture lysis reagent was prepared from a 5x lysis reagent stock (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) using 18 Mohm water. Cells were prepared by removing the old medium, and washing the cells with PBS (2 x 100 µl). Thereafter, approximately 80 µl of 1x cell lysis reagent was added to the cells. The multiwell plate was then placed on a STR 6 (Stuart Scientific, Staffordshire, UK) platform rocker for 15 minutes at 30 rev/min. The cells were then scraped from the wells and the cell suspension transferred into clean microcentrifuge tubes and microfuged at 12 000 x g for 5 seconds. The supernatants or cell free extracts were then used for the determination of luciferase activity. Briefly, luciferase assay reagent (100 µl) was added to 20 µl of supernatant at room temperature, followed by mixing and reading of luminescence (relative light units) in a GloMax®-Multi Detection System (Promega BioSystems, Sunnyvale, USA) operated by Instinct software. Protein concentration of the cell free extracts were assessed using the bicinchoninic acid colorimetric (BCA) assay determined colorimetrically at 562 nm, as per manufacturer's protocol. The luminescence readings in relative light units (RLU) were normalised against the the protein concentrations and was expressed as RLU/mg protein.

4.2.2.7 STATISTICAL ANALYSIS

Graph pad prism version 5.04 (GraphPad Software Inc, USA) was used to perform the statistical analysis of the results using the student t-test. $P < 0.05$ was considered to be significant.

4.3 RESULTS AND DISCUSSION

4.3.1 GROWTH AND MAINTENANCE OF CELLS

Both cell lines were successfully propagated in complete medium (EMEM + 10% FBS + 100 µg / ml penicillin, 100 µg/ ml streptomycin) in the laboratory. The complete medium used is one of the many examples of growth medium which have been formulated to support the growth of cells in cell culture laboratories. The EMEM contains essential amino acids, salts, glucose and vitamins. Amino acids are compulsory requirements for all cell culture media because they are the building blocks of proteins and are needed for cell proliferation. Since cells cannot synthesize all the amino acids, the essential ones are incorporated into the media. Inorganic salts help in retention of the osmotic balance in the medium and also assists in regulating the integrity of the cell membranes. Glucose provides cells with energy which is necessary for growth. Vitamins are also important for the cell proliferation and are commonly incorporated into the medium since cells cannot sufficiently synthesise their own (Arora, 2013).

Serum provides the necessary growth factors for the survival and growth of cells in the medium. Serum has advantages and disadvantages when added to the medium. The advantages of serum include providing hormones which stimulates cell function and growth, playing a role in cell attachment and spreading, helping to maintain the pH of the medium and to assist in minimising the damage to cells due to viscosity. However, serum may lack uniformity, may contain factors which inhibit growth and could increase the risk of the medium contamination which may lead to cell death.

The addition of antibiotics to the complete medium is important in controlling bacterial and fungal growth. Antibiotics prevent contamination of medium which could result in death of cells (Arora, 2013). Complete medium provided cells with all these essential nutrients discussed above, thus enabling the cells to grow and proliferate. From our observation the HEK293 cells grew and reached confluency quicker than the HepG2 cells. Once confluency was reached, cells were trypsinized using trypsin-versene solution, which contains the enzyme trypsin for detaching the cells and versene or ethylenediamine tetracetic acid (EDTA) for the chelation of the divalent cations such as calcium and magnesium and to increase the activity of the enzyme allowing it to hydrolyse the necessary peptide bonds.

4.3.2 CYTOTOXICITY STUDIES

Some compounds or delivery agents may be toxic to the target organ, cell or tissue and hence not suitable for gene or drug delivery. Hence, it is essential that the compound or vector be assessed for its cytotoxity levels *in vitro*. One method which is commonly used to determine the toxicity of compounds is the MTT cell viability assay. This assay was first described by Mosmann (1983) and is based on mitochondrial activity and integrity which will only be present in viable cells (van Meerloo *et al*., 2011). The MTT-[3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] dye is membrane permeable, and during this assay the dye interacts with the cells and the mitochondrial succinate dehydrogenases metabolizes the MTT to produce dark-purple/blue crystals of formazan as shown in Figure 4.1. These crystals generally accumulate in cells which are not damaged and are proliferating. Hence the quantity of formazan produced is directly proportional to the number of viable cells. These formazan crystals are then dissolved in an appropriate solvent (e.g. DMSO) and the optical density at 570 nm is determined colorimetrically and correlated with cell viability assuming 100% viability of untreated cells.

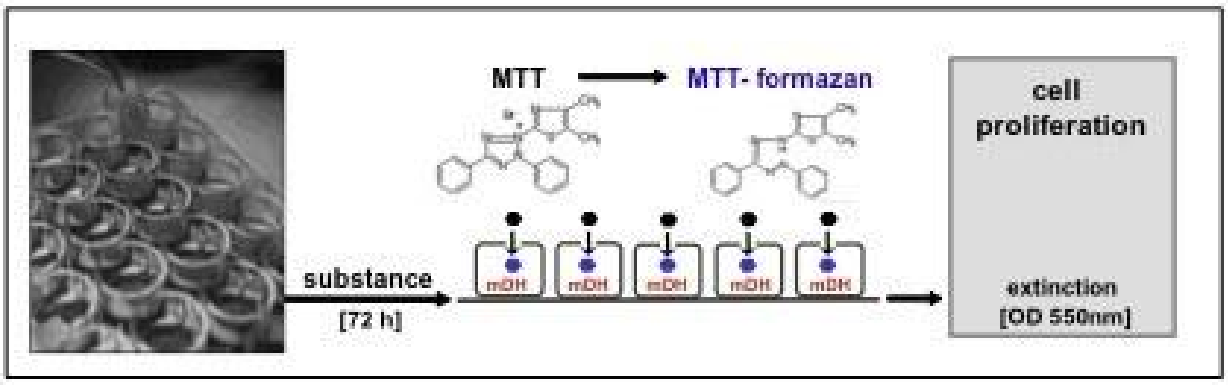


Figure 4.1: Metabolism of MTT-dye (yellow) to MTT-formazan crystals (blue) by mitochondrial dehydrogenases (mDH) of living cells: Adapted from http://www.uni-giessen.de/cms/fbz/fsp/meu/methods/analysis2/cell-proliferation [Accessed 03 July 2014].

The cytotoxicity assay was done in order to assess the toxicity of the cationic liposomes to both HepG2 and HEK293 cell lines. Figures 4.2, 4.3 and 4.4 depicts the levels of cytotoxicity achieved for the six liposomal formulations in the two cell lines. From the graphs it can be seen that these lipoplexes were reasonably well tolerated by the cells, with the HEK293 cells showing greater overall cell survival than the HepG2 cells. Overall, in the HEK293 cells, cell

survival ranged from around 60% for the Chol-T + Sc-5 + PEG liposomes at liposome:DNA charge ratio of 4:1 (Figure 4.3B) to well over 90% for the untargeted Chol-T, Chol-T+ PEG and the Chol-T +Sc2 + PEG liposomes (Figures 4.2A,B,and 4.4B) at a liposome:DNA charge ratio of 2:1. However in the HepG2 cells, cell survival ranged from just over 50% for the Chol-T+Sc2+PEG liposome (3:1) (Figure 4.4A) to over 80% for the Chol-T (charge ratio 2:1), Chol-T+PEG (charge ratio 4:1), Chol-T +Sc5 (charge ratio 4:1), Chol-T +Sc5 + PEG (charge ratio 3:1), and Chol-T +Sc2 + PEG (charge ratio 2:1) (Figures 4.2 A-B, 4.3A-B and 4.4B).

Generally, it could be seen that the control untargeted liposomes (Chol-T and Chol-T+PEG) (Figure 4.2A-B) were least toxic to both cell lines when compared to the liposome formulations that contain the targeting ligands Sc2 and Sc5 (Figures 4.3A-B and 4.4A-B). The highest cell death recorded for the control liposomes was 30% for Chol-T+PEG (charge ratio 2:1) in the HepG2 cells. Furthermore, the HEK293 cells all showed cell survival over 80% for these liposomes.

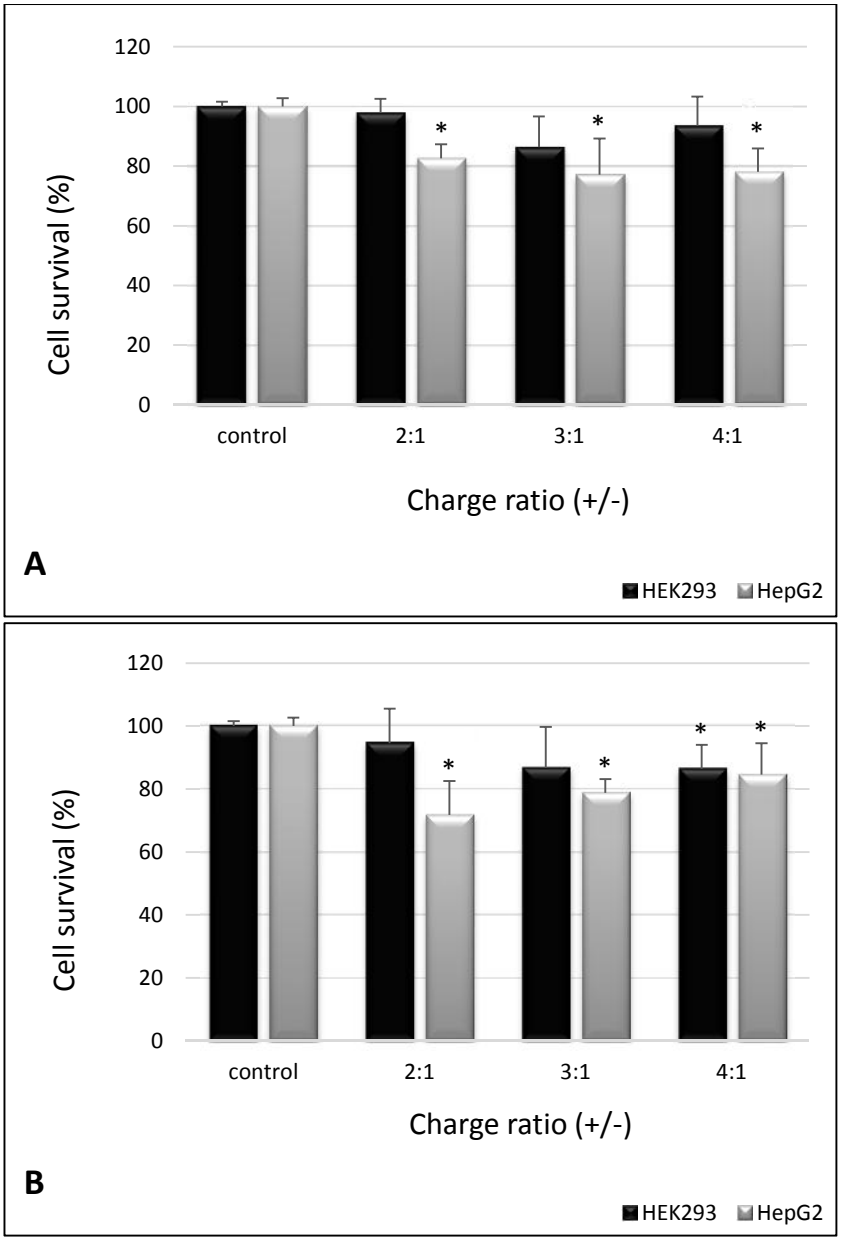


Figure 4.2: Cytotoxicity studies of lipoplexes in HEK293 and HepG2 cell lines *in vitro.* Varying amounts of cationic liposomes were added to a constant amount of DNA (Table 3.2). Data are presented as means ±S.D. (n = 3). There were significant differences between test and control results. * P<0.05, ***P< 0.001.

(A) DOPE + Chol-T): pCMV-Luc DNA

(B) DOPE + Chol-T + PEG: pCMV-Luc DNA

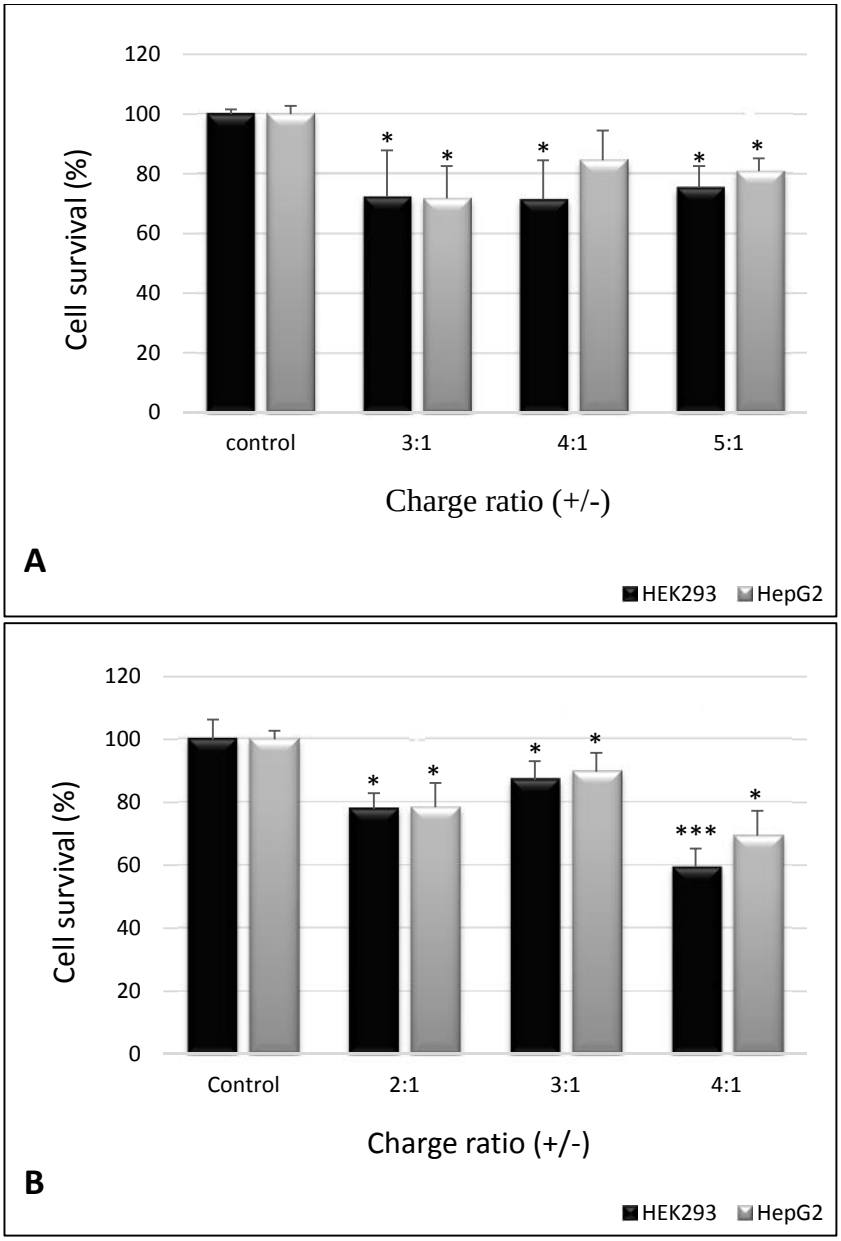


Figure 4.3: Cytotoxicity studies of lipoplexes in HEK293 and HepG2 cell lines *in vitro.* Varying amounts of cationic liposomes were added to a constant amount of DNA (Table 3.2). Data are presented as means ±S.D. (n = 3). There were significant differences between test and control results. * P<0.05, ***P< 0.001.

(A) DOPE + Chol-T + Sc-5): pCMV-Luc DNA

(B) DOPE + Chol-T + Sc-5 + PEG): pCMV-Luc DNA

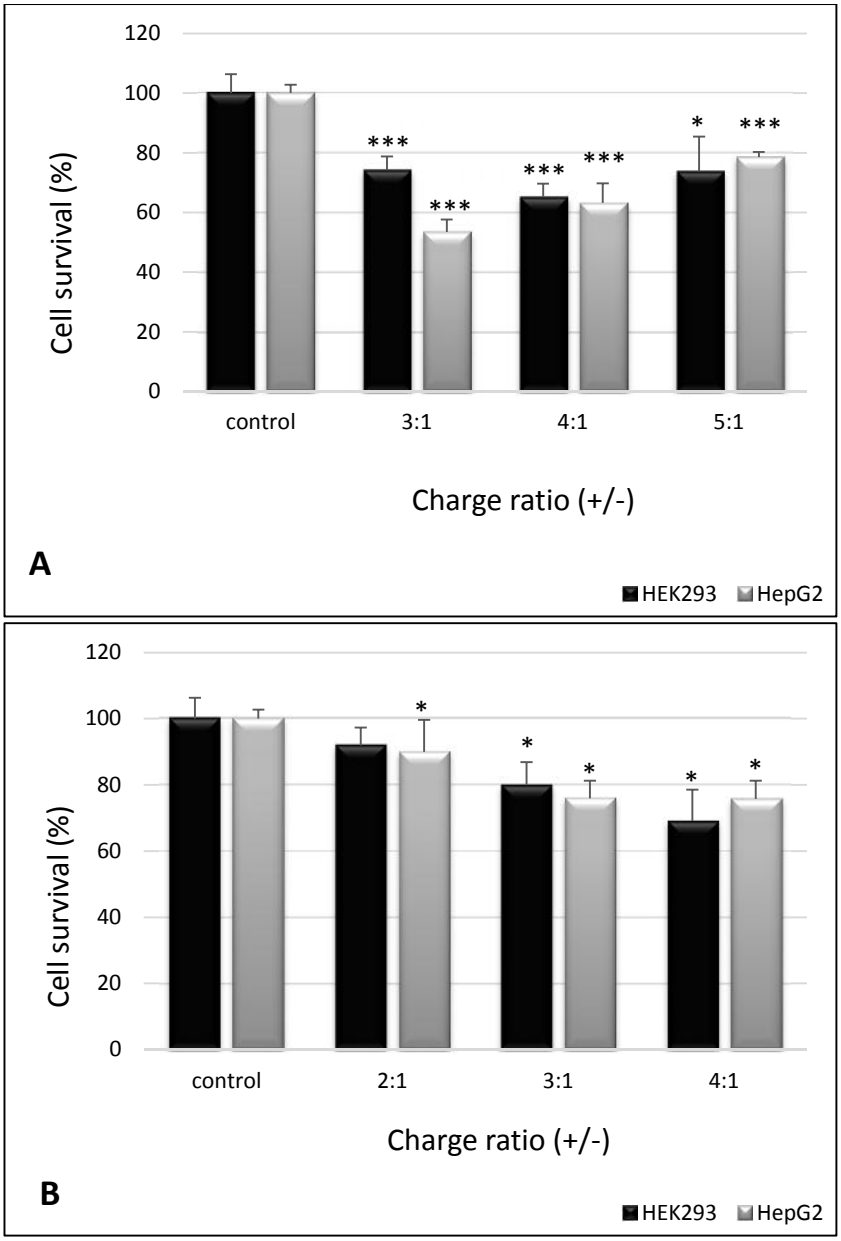


Figure 4.4: Cytotoxicity studies of lipoplexes in HEK293 and HepG2 cell lines *in vitro.* Varying amounts of cationic liposomes were added to a constant amount of DNA (Table 3.2). Data are presented as means ±S.D. (n = 3). There were significant differences between test and control results. * P<0.05, ***P< 0.001.

(A) DOPE + Chol-T + Sc-2): pCMV-Luc DNA

(B) DOPE + Chol-T + Sc2 + PEG): pCMV-Luc DNA

In the case of the ligand containing formulations, there was no significant differences in toxicity levels for the acetylated and non-acetylated formulations. Also, significant to note was that the targeted PEGylated liposomes (Figures 4.3B and 4.4B) seemed to be better tolerated by the cells than their non-PEGylated counterparts, with the exception of Chol-T+Sc5 +PEG at charge ration of 4:1 (Figure 4.3B) where the cell survival is much lower. The results reveal that the toxicity is liposome-dependent and with an increase in the amount of liposome or N/P charge ratios (+/-), the more toxic the cationic liposomes are. The findings of this study are in agreement with the study performed by Romoren *et al* (2005), where incorporation of DOPE increased the liposomes toxicity with the toxicity dependent on the N/P charge ratios (+/-). According to Romoren *et al.* (2004), the pH-sensitive DOPE which is incorporated in the cationic liposomes contribute to the toxicity of liposomes. Lysosomes tend to degrade most complexes during transfection, however since cationic liposomes form the hexagonal phase at acidic pH (pH associated with lysosomes), DOPE may enhance the cationic liposomes toxicity by destabilizing the membrane of a liposome (Romoren *et al*., 2004). Results show that non-acetylated Sc-5 cationic liposomes are less toxic to cells than the acetylated counterpart; these findings are in agreement with previously published articles on mammalian cells. The PEGylated Chol-T and DOPE liposome shows more toxicity to cells compared to the non-PEGylated DOPE and Chol-T liposome, which corroborate findings by Romoren *et al*., 2004. However, in the presence of a target ligand such as Sc-2, PEGyaltion is seen to be less toxic to cells compared to its non-PEGylated counterpart. The results show that even though the cationic liposomes were toxic to cell, the cell viability was above 60% at most charge ratios, which indicate that the cationic liposomes were reasonably well tolerated by both cell lines.

4.3.3 TRANSFECTION STUDIES

Transfection is the method of introducing genetic material such as nucleic acids into the cells in order to alter their genetic function and trait. Prepared cationic liposomes were used to deliver the plasmid pCMV-luc DNA into the receptor negative HEK293 and receptor positive HepG2 cell lines *in vitro*. Cationic liposomes contain phospholipids, which are building blocks of cellular membranes. The structure of a cationic cytofectin is known to possess a positively charged or cationic head group and hydrocarbon or sterol chains. Charged head groups on the cationic liposomes facilitate the interaction of the lipid with the negatively charged phosphate

backbone of the nucleic acids. The cationic lipid, Chol-T (Figure 2.3A) used in this study to deliver plasmid DNA had a positive surface charge which facilitated the electrostatic interaction of the liposome membrane and the plasmid DNA. The complexes thus formed are then taken up by cells through the process of endocytosis which could be receptor mediated in the presence of an appropriate ligand.

Transfection activity was determined using the luciferase reporter gene assay. This assay is commonly used in laboratories to study the expression of genes at the level of transcription and is convenient to perform. The luciferase enzyme that is expressed allows the organism to emit light, a process termed as bioluminescence. The reporter gene that was employed during transfection was the firefly luciferase gene. The study is based on the fact that the transfected cells will emit light or bioluminescence which is measured in a luminometer and expressed as relative light units of luciferase activity as shown in Figures 4.5, 4.6 and 4.7.

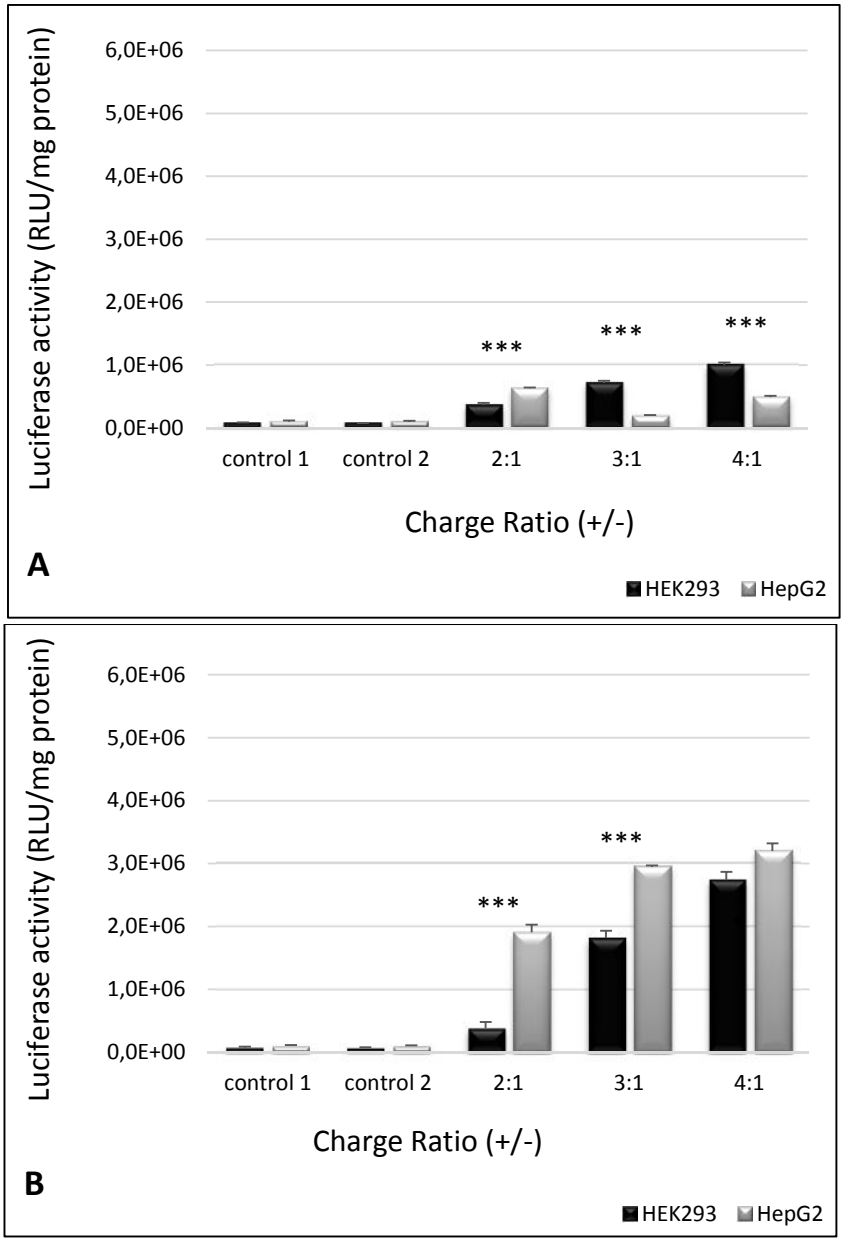


Figure 4.5: Transfection studies of lipoplexes in HEK293 and HepG2 cell lines. Varying amounts of cationic liposomes were added to pCMV-luc plasmid DNA (0.5 µg) in HBS (final volume 10 µl) to achieve +/- charge ratios as indicated A: DOPE + Chol-T and B: DOPE + Chol-T + PEG. Data are presented as means ±S.D. (n = 3). There were significant differences between results in cell lines at individual +/- charge ratios. * P<0.05, ***P< 0.001.

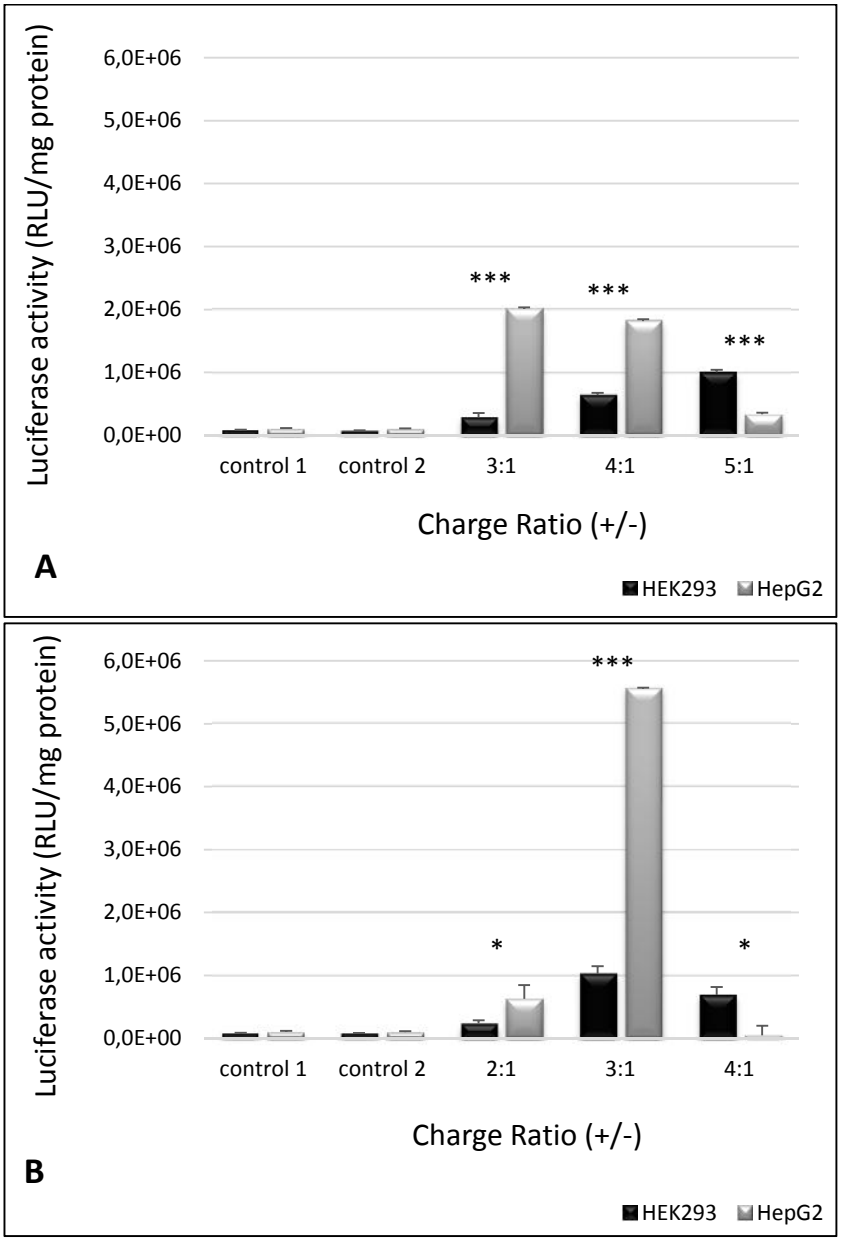


Figure 4.6: Transfection studies of targeted lipoplexes in HEK293 and HepG2 cell lines. Varying amounts of cationic liposomes were added to pCMV-luc plasmid DNA (0.5 µg) in HBS (final volume 10 µl) to achieve +/- charge ratios as indicated A: DOPE + Chol-T + Sc-5 and B: DOPE + Chol-T + Sc-5 + PEG. Data are presented as means ±S.D. (n = 3). There were significant differences between results in cell lines at individual +/- charge ratios. * P<0.05, ***P< 0.001.

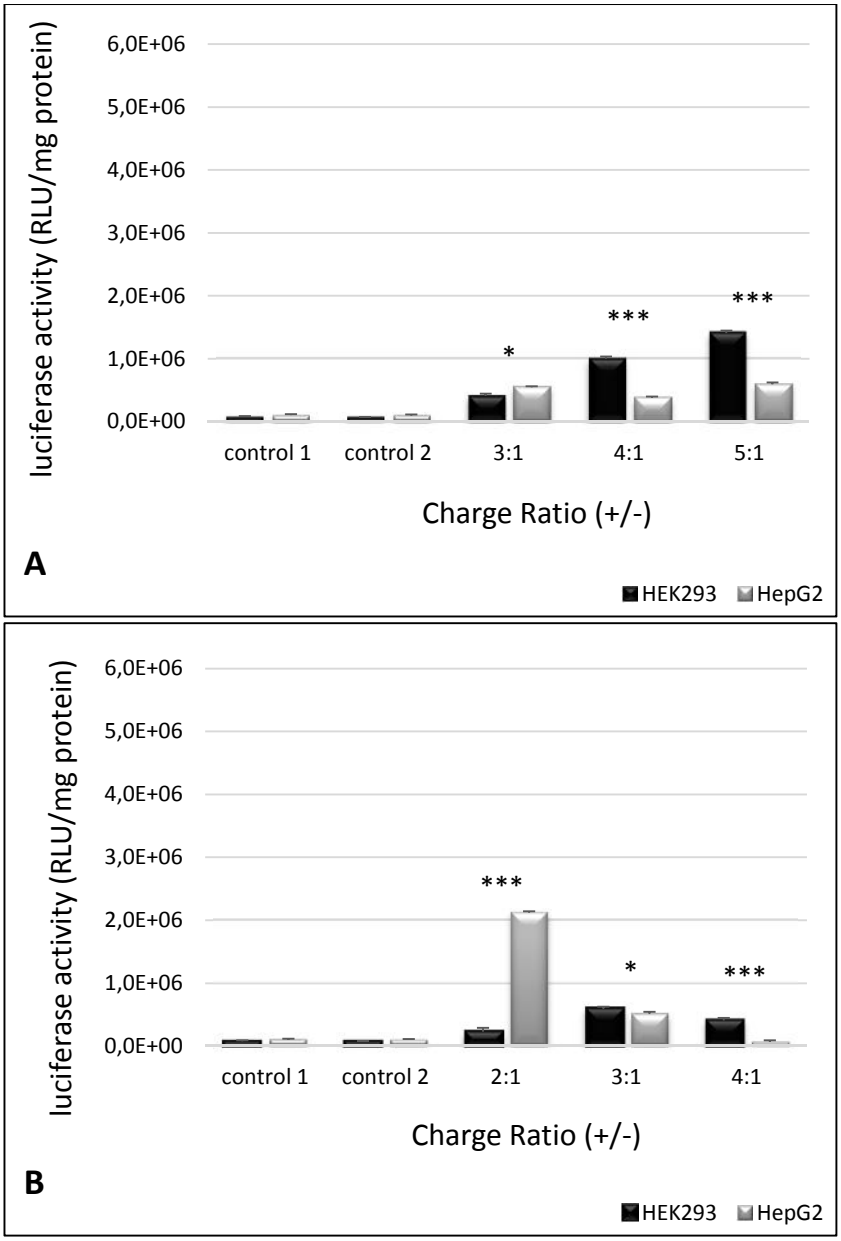


Figure 4.7: Transfection studies of targeted lipoplexes in HEK293 and HepG2 cell lines. Varying amounts of cationic liposomes were added to pCMV-luc plasmid DNA (0.5 µg) in HBS (final volume 10 µl) to achieve +/- charge ratios as indicated A: DOPE + Chol-T + Sc-2 and B: DOPE + Chol-T + Sc2 + PEG. Data are presented as means ±S.D. (n = 3). There were significant differences between results in cell lines at individual +/- charge ratios. * P<0.05, ***P< 0.001.

The luciferase reporter gene was used to quantitatively assess the *in vitro* transfection efficiencies of the lipoplexes in HEK293 cells which lack the asialoglycoprotein receptor (ASGP-R) and HepG2 cells which are ASGP-R-positive. Furthermore, this study also served to determine whether the acetylation on the hydroxyl groups of the galacto-compounds had an effect on the ability of the targeted lipoplexes, in which they are incorporated, to bind the ASGP-R and enter the cell through receptor-mediated endocytosis, thereby enhancing transfection. The effect of PEGylation of the liposomes on the overall transfection activity was also examined.

Figures (4.5, 4.6 and 4.7) shows transfection activity of the six lipoplex formulations at the various charge ratios. Luciferase activity is reflected as relative light unit (RLU) per mg soluble protein. All liposomes showed successful transfection in both cell lines. The degree of luciferase activity is compared to that of the two controls which show almost no luminescence. From the two control untargeted liposomes the PEGylated lipoplex (Figure 4.5 B) produced greater luciferase activity of around 3.1 x 10^6 RLU/mg protein for the HepG2 cells and over 2.5 x 10 RLU/mg protein for the HEK293 cells at the charge ratio of 4:1. Results also show that the HepG2 cells produced higher luciferase activity at all charge ratios compared to the HEK293 cells, although HEK293 cells are generally regarded as easy to grow and transfect readily compared to the HepG2 cells.

For targeted lipoplexes (Sc5 with and without PEG) (Figure 4.6 A-B) the transfection activity was lower that the untargeted control lipoplexes (Figure 4.5 A-B) in both cell lines. Here again the HEK293 cells showed lower transgene activity with maximum activity at a charge ratio of 5:1 (1x10^6 RLU/mg) and lowest luciferase activity at a charge ratio of 3:1 (5 x10^5 RLU/mg) in the non-PEGylated lipoplex. In contrast the HepG2 cells produced higher transfection activity at a charge ratio of 3:1 (2x10^6 RLU/mg) (Figure 4.6 A). However, the targeted PEGylated complexes produced increased transfection activity especially in HepG2 cells, notably at charge ratio of 3:1, where luciferase activity was above 5x10^6 RLU/mg protein (Figure 4.6 B) compared to the low levels seen again for the HEK293 cells.

For the acetylated targeted lipoplexes (Figure 4.7 A-B) the HEK293 cells displayed better transfection activity than the HepG2 cells with the highest transfection level recorded at a charge ratio of 5:1 (1x10^6 RLU/mg) in the non-PEGylated lipoplex(Figure 4.7 A). Results

show that the PEGylated lipoplexes (Figure 4.7 B) produced lower transfection activity in both cell lines with the exception of lipoplexs at a charge ratio of 2:1 which produced luciferase activity above $2x10^6$ RLU/mg protein in the HepG2 cells only.

From the results presented in (Figures 4.5, 4.6 and 4.7), it may be concluded that acetylation and PEGylation affect the ability of the the lipoplexes to transfect cells. De-acetylated cationic liposomes (Sc5 – Figure 4.6) showed higher luciferase activities compared to the acetylated cationic liposomes (Sc2- Figure 4.7). PEGylation was shown to enhance the transfection activities in HepG2 cells but not in the HEK293 cells. The enhanced transfection efficiencies which are observed when PEG is incorporated in cationic liposomes could be due to the fact that the sizes of lipoplexes which were formed between PEGylated cationic liposomes and plasmid DNA were smaller. It is also important to note that the clathrin coated pits, which are the portals for cell entry by receptor mediated endocytosis, are <200 nm in diameter (Saffarian *et al.,* 2009), therefore the smaller targeted PEGylated lipoplexes would gain entry into cells more easily. The acetyl groups on the 2', 3', and 4'positions may cause steric problems making it difficult for the ASGP-R binding site to accommodate the acetylated galactose moiety. According to Lee (1982), for successful binding of the galactose compound to the ASGP-R, the 2-OH (equatorial) and the 3-OH (equatorial) are both needed for binding, but the 4-OH must be in the axial position and the –CH2OCH at C-5 can be modified. Binding is also dependent on pH and Ca^{2+}. Therefore modifying the galacto moiety by introducing the acetyl group on the hydroxyl group (3-OH and 2-OH) may lead to less recognition by the receptor since these –OH groups are required for binding. Hence, this could result in reduced transfection activity compared to that of the de-acetylated cationic liposomes that were used. This was clearly seen for the Sc2 (acetyleated) and Sc5 (de-acetylated) lipoplexes (Figures 4.6 and 4.7).

4.3.4 COMPETITION ASSAY

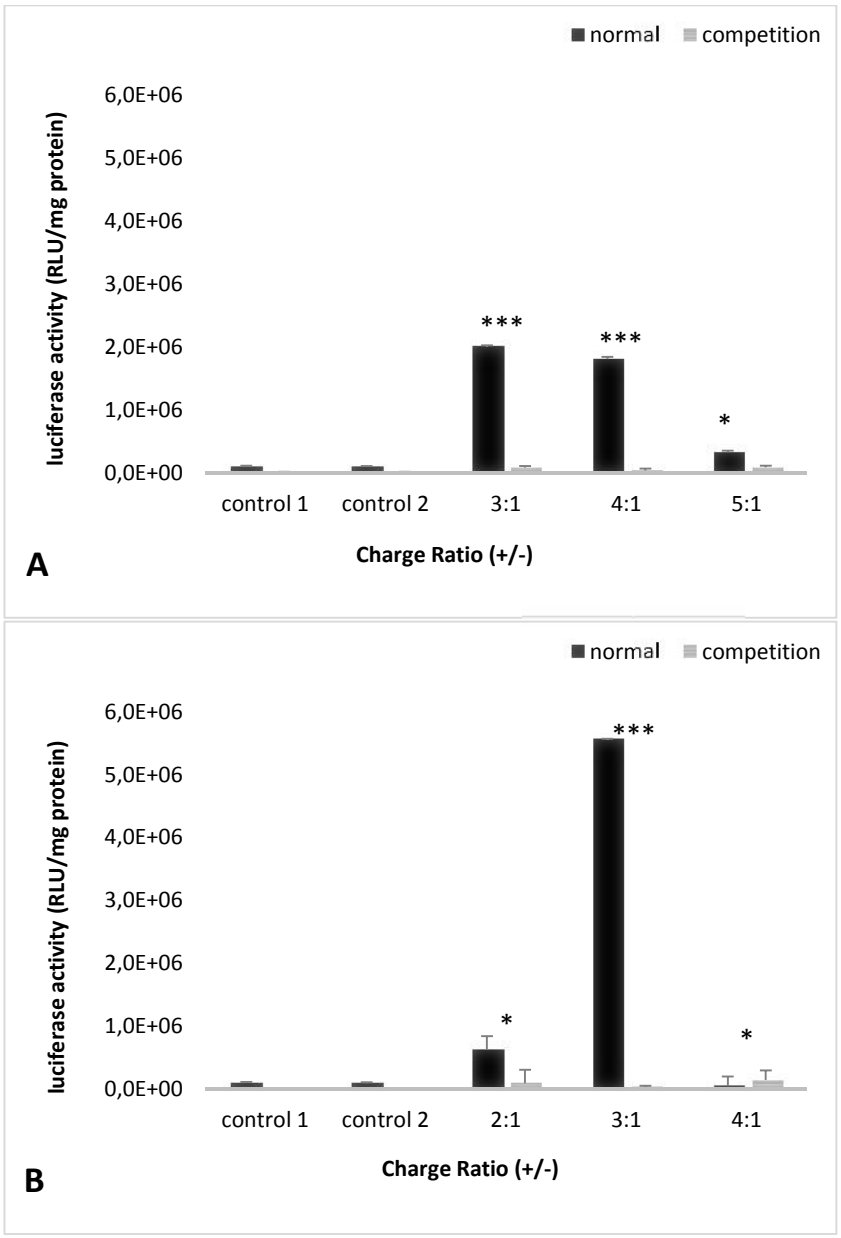


Figure 4.8 Competition transfection assays in HepG2 cells. Cells were pre-incubated with asialofectuin (200 µg/well) for 30 minutes before addition of lipoplexes mixtures (Table 3.2). A: DOPE + Chol-T + Sc-5 and B: DOPE + Chol-T + Sc-5 + PEG. Data are presented as means ±S.D. (n = 3). There were significant differences between results in cell lines at individual +/- charge ratios.* P<0.05, ***P< 0.001.

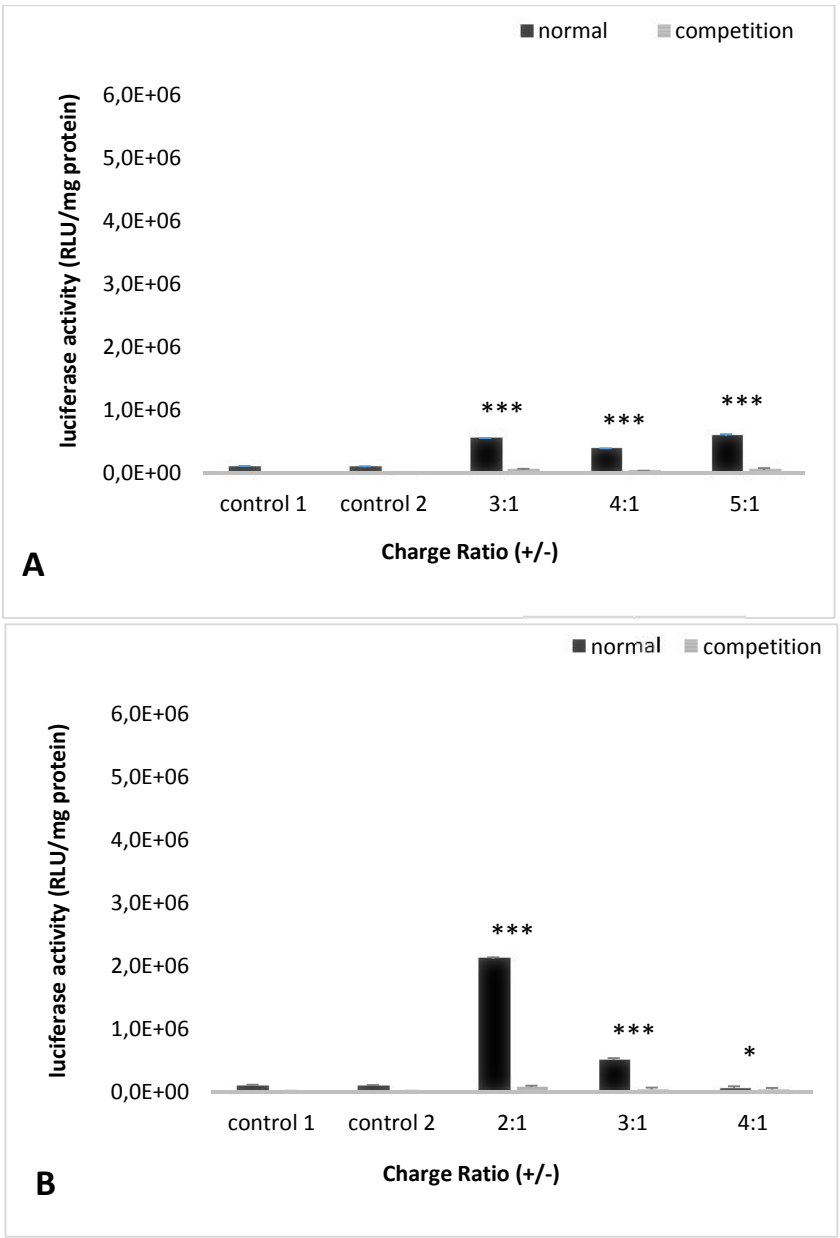


Figure 4.9 Competition transfection assays in HepG2 cells. Cells were pre-incubated with asialofectuin (200 µg/well) for 30 minutes before addition of lipoplexes mixtures (Table 3.2). A: DOPE + Chol-T + Sc-2 and B: DOPE + Chol-T + Sc2 + PEG. Data are presented as means ±S.D. (n = 3). There were significant differences between results in cell lines at individual +/- charge ratios.* P<0.05, ***P< 0.001.

Competition assays were performed to verify that targeted lipoplexes were indeed taken up by the cells ASGP-R-mediated endocytosis process. Results presented in Figure 4.8 (A) showed that at the optimal transfection ratio 3:1 the presence of an excess of the ASGP-R ligand asialofetin effectively reduced the transfection activity by DOPE, Chol-T, Sc-5 lipoplexes. This was also the case with PEGylated lipoplexes formulated with Sc-5 (Figure 4.8 B). Competition assays conducted with Sc-2-containing lipoplexes (Figure 4.9 A-B) also produced a sharp decrease (more than 80%) in transfection activity in the presence of excess asialofectuin. Thses results are similar to those obtained by Singh and coworkers using the galactose ligand and excess asialofetuin in the competition assay. A massive decrease of 90% in luciferase activity was observed (Singh et al., 2007). Although results presented here show that the galacto-containing lipoplexes achieved higher transfection activities than the lipoplexes containing tetra acetylated galactose units, it appears that the latter lipoplexes also gain entry into the HepG2 cells via ASGP-R-mediation.

CONCLUSION

This study was done in order to assess the properties of the cationic liposomes which have been prepared using the thin film hydration method. Stable liposome formulations were achieved with the liposomal components and mole ratios selected for this study. Gel retardation showed that both acetylated and non-acetylated liposomes formed lipoplexes with plasmid pCMV-luc DNA. The plasmid DNA was relatively well protected from serum nuclease degradation as shown by the nuclease protection assay. PEGylation appeared to generate smaller lipoplexes, and the PEGylated lipoplexes carried DNA in a less condensed state as evidenced by ethidium bromide displacement assays. The lipoplexes were relatively non-toxic under the transfection conditions employed in both cell lines. The lipoplexes displaying the ASGP-R ligand β-D-galactopyranose achieved transfection levels up to 250 percent greater than those achieved by lipoplexes decorated with tetra-O-acetylgalactopyranose. PEGylation was accompanied by an increase in transfection activity in HepG2 cells but not in HEK293 cells. This would support the notion that smaller PEGylated lipoplexes are more readily accommodated in the restricted space within coated pits and that cellular uptake in HEK293 cells is perhaps coated pit-independent. The competition assays confirmed ASGP-R mediated uptake of Sc-2 and Sc-5 lipoplexes although there is a clear preference for the non-acetylated galactopyranosyl moiety. These liposomal formulations have the potential as future non-viral gene delivery vehicles and with further optimisation can be tested *in vivo*. Future studies may be carried out to understand more on the behaviour and importance of the acetyl groups on the galacto compounds.

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