Cationic Liposome Mediated Transfection With / Without a Targeting Component

By

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Submitted in partial fulfilment to the requirements for the degree of a Masters in Medical Science in the Pfizer Molecular Biology Research Facility, Doris Duke Medical Research Institute, Faculty of Medicine, Nelson R. Mandela School of Medicine, University of Kwa-Zulu Natal.

Declaration

I declare that this dissertation is my own unaided work. It is being submitted for the degree of a Masters in Medical Science, in the University of Kwa-Zulu Natal – Nelson R. Mandela School of Medicine, Durban. This dissertation has not been submitted for any other degree or examination at any other university.

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Abbreviations

AAV	Adeno-associated virus
AH	Aminohexyl
AOM	Asialoorosomucoid
Arm	Armadillo
ATCC	American Tissue Culture Collection
ATP	Adenosine-tri-phosphate
Bp	Base pair
BDMA	Benzyldimethylamine
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CH_3Cl	Chloroform
Chol-T	3β[N-(N',N'-dimethylaminopropane)carbamoyl]cholesterol
DC-Chol	3β[N',N'-dimethylaminoethane)-carbomoyl]cholesterol
DER 736	Diglycidyl ether of polypropylene glycol
DMAE	Dimethylaminoethanol
DMRIE	(+/-)-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl1-2,3-bis(tetradecyloxy)-1-prpoanaminium bromide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid

DNAses	Deoxyribonucleases
DOPE	Dioleoylphosphatidylethanolamine
DOSPER	1,3 -dioleoyloxy-2 (6-carboxyspermyl) - propylamide
DOTAP	N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulphate
DOTMA	N-[1-(2,3-dioleyloxy) propyl]- N,N,N- trimethylammonium chloride
dpm	Disintegration per minute
Ds	Double stranded
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetra-acetic acid di-sodium salt dihydrate
ERL 4206	Vinylcylophene Dioxide
FBS	Fetal bovine serum
GAP-DLRIE	(+/-)-*N*-(3-aminoprpyl)-*N*,*N*-dimethyl1-2,3-bis(dodecyloxy)-1-prpoanaminium bromide
H	Hydrogen
H_2O	Water
H_2SO_4	Sulphuric acid
HBS	Hepesbuffered saline
HCL	Hydrochloric acid
HeLa	Cervical cell line
HepG2	Hepatocellular carcinoma cell line
HOAc	Glacial acetic acid

HPLC	High performance liquid chromatography
[^{3}H]	Tritium
[^{3}He]	Helium
IBB	Importin β binding
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate
KOAc	Potassium acetate
kbp	Kilo base pair
kDa	Kilo Dalton
LB	Lauria bertani
MEM	Minimal essential medium
MLV	Murine leukemia virus
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaH_2PO_4	Sodium phosphate
NaOAc	Sodium acetate
NaOH	Sodium hydroxide
NH_2	Amino group
NLS	Nuclear localization sequence
NSA	Noneyl succinic anhydride
OD	Optical density
OsO_4	Osmium tetroxide
PBS	Phosphate buffered saline

pRSVL	Polyadenylation site and ampicillin resistance gene
RAAV	Recombinant adeno-associated virus
R_f	Relative to front
RLU	Relative light unit
RNA	Ribonucleic acid
RNAses	Ribooxynucleases
rpm	Rate per minute
RSV-LTR	Rous Sarcoma Virus Long Terminal Repeat
SDS	Sodium-dodecyl sulphate
SDS-PAGE	Sodium-dodecyl sulphate polyacrylamide gel electrophoresis
Ss	Single stranded
TBE	Tris Borate EDTA
TEM	Transmission electron microscopy
TLC	Thin Layer Chromatography
Tris	(2-Amino-2-(hydroxymethyl)-1,3-propanediol)
Tris-EDTA	2-Amino-2-(hydroxymethyl)-1,3-propanediol)ethylenediaminetetra-acetic acid di-sodium salt dihydrate
Tris-HCL	[Tris(Hydroxymethyl) Aminomethane Hydrochloride]
Triton X-100	Iso-octylphenoxypolyethooxyethanol containing approximately 10 mols of ethylene oxide
UV	Ultra-violet
WPE	Epoxy equivalent

X-SCID	X-linked severe combined immunodeficiency

Abstract

The transfer and expression of genes in cells is an important technique for basic research and gene therapy of human disease. A model for gene therapy has been investigated making use of a transfection complex consisting of three components, the DNA i.e. the gene to be transferred and expressed; a gene delivery vehicle *viz.* a cationic liposome and a cell specific targeting ligand, asialoorosomucoid (AOM).

Cationic liposomes are positively charged liposomes that have been prepared from synthetic lipids and have been shown to complex or bind to DNA via electrostatic attraction. They have shown potential as an efficient non-viral gene delivery vehicle in human gene therapy. In this investigation, a novel cationic liposome consisting of 3β [N-(N',N'-dimethylaminopropane)carbamoyl] cholesterol (Chol-T), dioleoylphosphatidylethanolamine (DOPE) and biotinylcholesteryl formylhydrazide was prepared and assessed as a mediator of DNA delivery in a mammalian cell culture system *viz.* the HepG2 cell line. The cationic liposome was synthesised and characterised by electron microscopy.

Foreign DNA may be specifically delivered to target cells by a carrier system which makes use of the recognition of the asialoglycoprotein AOM by cognate receptors on the HepG2 cell plasma membrane. The positively charged AOM was biotinylated and due to this biotinylation, binds streptavidin which contains specific binding sites for biotin. The cationic liposome itself contains biotin residues in its bi-layer which in

turn binds streptavidin resulting in a ternary complex. Further, due to the DNA binding capability of the cationic liposome, a transfection complex is produced consisting of the three components.

The experiments were based on the following concepts :

(i) Hepatocytes possess a unique receptor that binds to and internalises galactose-terminal asialoglycoproteins by receptor mediated endocytosis.

(ii) Due to electrostatic attraction, DNA binds to cationic liposomes forming soluble complexes.

(iii) Through the biotin-streptavidin reaction, the biotinylated AOM is attached to the cationic liposome containing biotin forming complexes enabling targeted delivery of the DNA.

(iv) DNA containing the pGL3 gene for the luciferase enzyme was used and following transfection experiments, the luciferase assay was performed to ensure successful transfection.

The complexes were tested on the hepatocellular carcinoma cell line, HepG2, which possess the asialoglycoprotein receptor. Transfection studies were conducted using a transient expression system, the luciferase assay system. Some degree of success in the transfection of HepG2 cells was observed. Results obtained in this study suggest that transfection using our targeted transfection complex consisting of cationic liposomes and cell specific targeting ligands does in fact transfect cells by receptor mediation.

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INTRODUCTION

Gene therapy is a relatively new application of genetic engineering and molecular therapy. The underlying principle of gene therapy is the introduction of a functional gene into affected tissue, so that the defective, disease-causing gene may be replaced or complemented, in order to correct or overcome the defect. Several non-viral approaches are being investigated and transfection efficiencies remain variable. Cationic liposomes have become increasingly popular as a DNA delivery agent. Recently targeting capabilities have been built into complexes with limited success especially in delivery to liver cells. In this study complexes consisting of DNA, cationised asiaoloorosomucoid and activated cationic liposomes was formulated for the directed delivery of corrective DNA into hepatocytes through a process of receptor mediation. Such research is of importance in the development of gene therapy protocols for diseases and disorders of the liver.

The aim of the study is to develop a safe and efficient mechanism for gene transfer using cationic liposomes as gene transfer vehicles in a cell culture model.

CHAPTER 1

LITERATURE REVIEW

1.1 Gene Therapy

1.1.1 The Importance of Gene Therapy

Gene therapy is defined as the introduction of nucleic acids into cells for the purpose of altering the course of a medical condition or disease (Kay *et al.*, 1997; Bunnell and Morgan, 1998; Kimmelman, 2003; Robson and Hirst, 2003). Human genetic information is encoded in the DNA of the cell. It has been shown that genetic diseases arise as a result of genetic alterations in DNA, usually brought about by genotoxic conditions. Gene therapy utilises molecular processes that correct such alterations. This ability to correct RNA or DNA sequences can be used to repair mutant genetic instructions so that inherited disorders can be permanently and effectively treated. Pathogenic genes associated with cancer (oncogenes) and infectious diseases can also be corrected in this manner (Sullenger, 2003). Gene therapy therefore holds considerable potential for the treatment of both hereditary genetic disorders and infectious diseases.

In recent years, there have been a number of technological breakthroughs allowing for clinical trials in gene therapy to be initiated. Such trials are underway for a variety of conditions such as cystic fibrosis, sickle cell disease, cancer (Sullenger, 2003), muscular

dystrophy (Gruenert *et al.*, 2003), infectious diseases and AIDS (Bunnell and Morgan, 1998; Patil and Rhodes, 2000). Gene therapy now appears to be a realistic goal.

However, as research in gene therapy proceeded, a number of difficulties became evident. Safety considerations pose an obstacle in the path of its progress. Of particular concern is the risk of reactivating potentially oncogenic virus vectors (Weissman, 1992). Insertional mutagenesis in which a hazardous mutation is caused by the introduction of foreign DNA sequences into the genome of the recipient creates added risks to gene therapy by affecting the germ-line of the participant (Kimmelman, 2003). Also, a person's prior exposure to a virus similar to the vector can influence their response to gene transfer. Another weakness is seen when animal models are used for estimating doses that are maximally tolerated in humans. This is because viruses that are pathogenic in humans often have different risk profiles in animals (Kimmelman, 2005). Another obstacle to the advancement of gene therapy is encountered in obtaining high-titre viral vectors as well as finding vectors that infect the appropriate range of human cells. Furthermore, another major limiting factor to the success of gene therapy is the inability to transfer the appropriate gene into a target, non-germ-cell tissue, such that an appropriate amount of gene product (usually a protein) is produced to correct the disease leading to the stable expression of the transferred DNA (Weissman, 1992; Kay *et al.*, 1997).

In order for gene therapy to advance and be successful, these hurdles need to be overcome. These include the ability to deliver the therapeutic gene to the target cell

intact, persistent levels of transgene expression that is sufficient to correct the disease phenotype and the lack of unwanted side-effects that are associated with the exposure to vectors (Jane *et al.*, 1998).

The underlying principle of gene therapy is the introduction of a functional gene into affected tissue in order to complement or replace the defective gene, either physically or functionally (Chaudhuri, 2002). This process relies on efficient gene transfer. Once the desired gene has been identified, it is inserted into the appropriate cell by a process known as transfection or transduction (Singh, 1998). During transduction, a modified virus infects the cell by introducing a viral genome that contains the gene of interest. Transfection is a genetic engineering technique in which DNA is introduced into cultured cells. Transfection uses chemical or physical methods and is thought to be a safer and more efficient technique (Singh, 1998).

1.2 Gene Delivery Systems

There are two major classes of vehicles for gene transfer. These are viral and non-viral vectors (Kay *et al.*, 1997). They all have advantages and limitations. It is believed by some researchers that viruses are most successful because they have evolved for millions of years to become efficient vesicles for transferring genetic material into cells. However, some of the side effects of viruses and possible previous exposures resulting in the host becoming resistant to transduction may hinder the use of viruses in gene therapy (Kay *et*

al., 1997). Furthermore, the production of viral vectors require mass cell cultures making clinical grade production difficult. This is not required of non-viral vectors which in this regard have the obvious advantage (Noguiez-Hellin *et al.*, 1996; Langer, 2000).

Ideally, a vector should efficiently transduce quiescent cells *in vivo*, be capable of accommodating large transgenes and produce prolonged and, in some cases, regulated transgene expression in immunocompetent hosts. Furthermore, vectors should result in minimal or no local tissue inflammation or damage and no long-term neutralising immunity (Leiden, 2000).

The class of vector to be used is based on the above considerations. Once this vector has been designed, one of two general approaches may be used for gene transfer. The *ex vivo* approach is where cells are removed, genetically modified and transplanted back into the same recipient. *In vivo* therapy is accomplished by the transfer of genetic materials directly into the patient. The former method is more complex making it less feasible for wide-scale application. Hence the latter is preferable in most situations (Kay *et al.*, 1997).

1.2.1 Viral Gene Delivery Systems

Viral vectors are infectious, attenuated recombinant viruses. Three different classes of viral vectors have been used. These include retroviruses, adenoviruses and adeno-associated viruses (AAV).

1.2.1.1 Retroviruses

Retroviruses are derived from the murine leukaemia virus (MLV) (Porter *et al.*, 1998) and are enveloped viruses that have surface glycoproteins which interact with specific receptors (Innes *et al.*, 1990). These viruses have a genome consisting of RNA which is converted into DNA upon entry into the cell. This DNA then integrates into the DNA of the host cell. Retroviruses have a very small genome (approximately 10 000 bases) containing only three genes of their own (Figure 1.1). These genes can be readily replaced by the gene of interest, thereby producing a recombinant virus i.e. a virus containing genes from various sources. This recombinant virus retains viral regulatory sequences, and a sequence that allows for the packaging of the nucleic acid genome into a virus particle. The recombinant virus is then introduced into a "packaging cell line" which expresses those virus genes that have been deleted from the recombinant virus, thus allowing it to be packaged into a viral particle. The viral particles produced in this way can efficiently deliver the gene of interest by infecting cells. However, since the recombinant virus cannot produce any of the retrovirus's own proteins, no infectious viral particles will be produced by cells infected in this manner, rendering the virus safe for gene delivery. As retroviruses are easy to manipulate and can deliver a gene safely and efficiently, they have been widely used in gene therapy procedures. Another reason for retroviral-mediated gene transfer being the most widely used method of transfection is due to its high efficiency of gene transfer (Porter *et al.*, 1998).

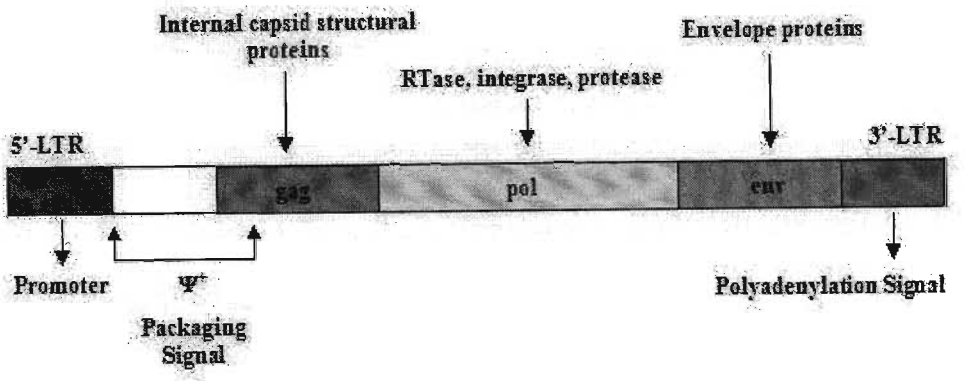


Figure 1.1 : Schematic representation of the retrovirus genome showing the three structural genes

Interestingly, such viruses are particularly applicable to cancer, since these vectors only transduce dividing cells (Kay *et al.*, 1997). As cancer cells are rapidly dividing, this actually makes it possible to target the genes in these cells. This transduction of tumour cells is an important advantage with regard to safety.

These viruses are unsuitable for gene delivery to the non-dividing cells. Although efficient at transduction into cultured cells, most cells *in vivo* however are quiescent at any point in time. This makes the vector less useful for *in vivo* therapies unless the cells in the target organ are stimulated to cycle. After retroviral infection, gene expression depends on viral integration and cell division except in the case of lentiviruses. Thus, as the tumour grows in an organ consisting mostly of quiescent cells, the expression of the transferred gene is limited only by the dividing tumour cells thereby preserving the surrounding normal cells (Noguiez-Hellin *et al.*, 1996).

Another limitation of retroviruses is the relatively low concentration of virus that is produced (Kay *et al.*, 1997). A further disadvantage is that recombination of such retroviral vectors *in situ* may lead to the development of a replication competent virus. In addition these sequences display the tendency to insert next to oncogenes, thereby promoting the possibility of activation (Smith *et al.*, 1993). In 2003, 2 children treated for the rare immune disorder, X-linked severe combined immunodeficiency (X-SCID) by retroviral vectors developed a leukaemia-like disease. Genetic analysis of the malignant cells showed that the retroviral vector used to carry a functional copy of the defective gene into the bone marrow stem cells had inserted into the patients' DNA instead and activated an oncogene which is associated with childhood leukaemia (Relph *et al.*, 2004).

1.2.1.2 Adenoviruses

Human adenoviruses are responsible for mild illnesses such as upper respiratory infections. Adenoviruses are double stranded DNA virus that have a genome of 35 000 bases, and express more than 50 gene products throughout its life-cycle. It is three-and-a-half times larger than the retroviruses and is therefore somewhat more difficult to manipulate. Although adenoviruses have a larger genome than retroviruses, they still cannot accommodate large genes that need to be used in gene therapy, however these vectors allow for efficient transfer both *in vitro* and *in vivo* (Noguiez-Hellin *et al.*, 1996). They can be concentrated to a higher titre and do not integrate into the host's chromosomal DNA (Kay *et al.*, 1997), thus infecting and expressing their genes in both

dividing and non-dividing cells (Relph *et al.*, 2004). There is however the risk of transgene dissemination outside of the tumour (Noguiez-Hellin *et al.*, 1996).

When the E1 region of this vector is eliminated, space is made for placing sequences with therapeutic expression and due to the absence of the transactivating E1a protein (Figure 1.2) the virus cannot replicate. Thus no viral spread will occur after gene transfer (Kay *et al.*, 1997).

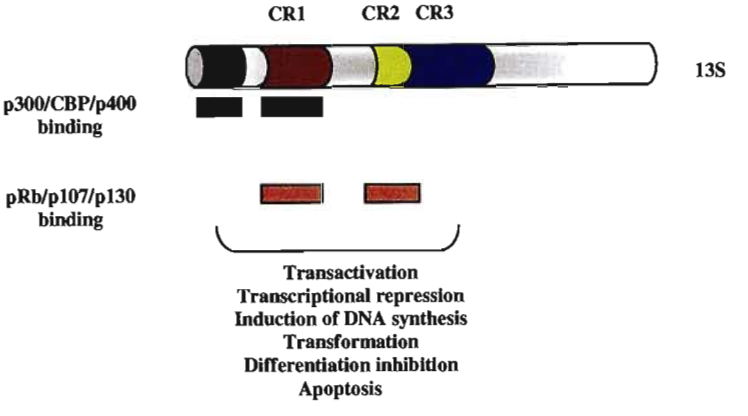


Figure 1.2 : Schematic representation of the adenovirus E1a protein
(adapted from www.mblab.gla.ac.uk/~john/ nltimgs/img017.jpg)

Although a number of genes have been inserted into disabled adenovirus vectors, there have been some safety problems with side effects being observed in clinical trials. Moreover, gene delivery with adenovirus vectors appears to produce intense inflammation and provoke long-term cell- and antibody-mediated immunity which can result in the elimination of the viral vector from the body, thereby leading to loss of the

therapeutic gene (Leiden, 2000). These vectors therefore remain the most immunogenic of all viral vectors (Relph *et al.*, 2004).

1.2.1.3 Adeno-Associated Viruses (AAV)

The adeno-associated virus (AAV) is a non-pathogenic virus of the Parvovirus family that stably integrates as a provirus into a defined region on chromosome 19. *In vitro* manipulation is not required as this stable integration into the host genome does not require cell division (Singh, 1998). These viral vectors were initially thought to be the safest of all viral vectors as the virus does not naturally cause disease in humans and rarely integrates into the genome. However, a study by Kay *et al.* (2003) conducted in mice showed that the vector becomes integrated into regions of DNA containing genes (active regions) more often than into non-coding regions. This could possibly cause similar cellular defects to those that lead to cancer in the SCID patients (Relph *et al.*, 2004).

Recombinant adeno-associated virus (rAAV) vectors contain small single stranded DNA genomes. These vectors have recently been shown to transduce brain, skeletal muscle and liver by injection into quiescent tissue or vasculature. These vectors have been used to achieve therapeutic and in some cases curative concentrations of clotting factor IX in mice without toxicity for at least nine months by *in vivo* delivery (Kay *et al.*, 1997).

Unfortunately, the disadvantage of using these vectors is the limited amount of DNA that can be packaged, preventing larger cDNAs, genes or complex regulatory *cis* elements from being used (Kay *et al.*, 1997). These vectors can only accommodate transgene cassettes of < 4.5 kb. In addition, it is difficult to produce high titre stocks and not all cell types can efficiently be transduced *in vivo*. However, AAVs are much less inflammatory than adenoviruses and appear to be capable of programming long-term expression in immunocompetent animals *in vivo* (Leiden, 2000).

In addition to retroviruses, adenoviruses and adeno-associated viruses there are a number of viral vectors that are currently being derived in the laboratory. These include vectors based on Epstein-Barr virus, herpes, simian virus 40, papilloma, non-human lentiviruses and hepatitis viruses. It is hoped that these laboratory-derived vectors would possess properties that offer advantages to clinical gene therapy that are not yet realised (Kay *et al.*, 1997).

In some countries such as the United Kingdom however, current viral vectors still remain the vehicles of choice for gene transfer (Relph *et al.*, 2004), however drawbacks associated with the use of such vectors as previously outlined have prompted investigators to explore and develop alternative methods of gene delivery (Pedroso de Lima *et al.*, 2003).

1.2.2 Non-Viral Gene Delivery Systems

The non-viral gene delivery system is an alternative method for gene transfer. Non-viral vectors have the advantage over viral ones in that they are less immunogenic and have no potential for viral infection (Carrière *et al.*, 2003; Hirko *et al.*, 2003). Further, they have the potential for transferring and expressing large pieces of DNA into cells (Francescangeli *et al.*, 2003). These non-viral gene systems can either be non-targeted or targeted to a specific cell type (Singh, 1998).

These methods are either physical or chemical by which DNA can be taken up into cells. These methods are detailed as follows.

1.2.2.1 Calcium Phosphate or DEAE-Dextran Mediated Transfection

Calcium phosphate or DEAE-dextran mediated transfection employs a precipitation method to form calcium-phosphate : DNA or dextran : DNA insoluble particles respectively. Some of these insoluble particles become internalised within the host cells by phagocytosis (Singh, 1998). Thereafter some of the DNA escapes digestion by serum nucleases as it is complexed with the calcium-phosphate or dextran and is therefore protected. After the transfected DNA enters the cytoplasm of the cell by endocytosis it is transferred to the nucleus resulting in gene expression.

Transfection is dependant on the cell type and up to 20% of the cell population may be transfected at any one time. Due to its high efficiency, this is the method of choice for experiments that require transient expression of the foreign DNA in a large number of cells. The calcium-phosphate method, especially the use of *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid has proven to be the only efficient method for the cotransfection of multiple plasmids into a wide variety of cell types. Calcium phosphate-mediated transfection may also be used to establish cell lines that carry integrated copies of the foreign DNA arranged in head-to-tail tandem arrays (Sambrook *et al.*, 1989). A previous study (Mack *et al.*, 1998) developed a DEAE-dextran DNA transfection method that mediated reproducible transfection of primary cultured adherent human macrophages which is usually difficult to transfect. They identified three factors that contributed to the success of transfection. These included the DEAE-dextran concentration, the quantity of DNA per transfection and the incubation time of the macrophages with the transfection medium. However useful these methods may be, they have a highly disruptive effect on cellular membranes and are not applicable for *in vivo* manipulation (Smith *et al.*, 1993).

1.2.2.2 Polycations

DNA transfection can be achieved by using polycations and dimethylsulfoxide (DMSO) (Singh, 1998). Polycations enhance the adsorption of retroviruses to cells by interacting with the negative charges on both the virions and cell surfaces forming a bridge between

the two. Thus in the presence of a polycation, DNA is adsorbed onto the cell surface (Singh, 1998).

The polycation, polybrene allows for the efficient and stable introduction of low-molecular weight DNA such as plasmid-DNA into cell lines (e.g. CHO cells), which may be resistant to transfection by other methods (Sambrook *et al.*, 1989). Similarly, the attachment of polycationic adjuncts have been used to chemically modify several types of porous surfaces such as cotton cloth, paper, wood, silk and wool to provide them with antifungal characteristics. This was achieved as a result of disruption to the cell envelope of the bacteria leading to death of the cell (Cohen *et al.*, 2004).

1.2.2.3 Protoplast Fusion

This is a method in which protoplasts, derived from bacteria carrying high numbers of copies of a plasmid of interest, are directly mixed with cultured mammalian cells. Once fusion of the cell membranes has occurred, the bacterial contents enter the cytoplasm of the mammalian cells and the plasmid DNA is transfected into the nucleus. This process yields multiple copies of the plasmid DNA tandemly integrated into the host chromosome (Sambrook *et al.*, 1989). An example of the use of this method was shown previously where membrane-bound alkaline phosphatase was used as a reporter enzyme in a miniprotoplast fusion assay (Caporale *et al.*, 1990). It was demonstrated that large numbers of protoplast fusions could be done simultaneously and successfully to assay for

activity encoded for by an expression vector. This method was also utilised to fuse the suspension-derived protoplasts of a sugar beet breeding line with the mesophyll-derived protoplasts of three breeding lines (Gurel *et al.*, 2002).

1.2.2.4 Electroporation

Electroporation results in the uptake of DNA into cells through nanometer-sized pores in the plasma membrane. These pores are created by applying high-voltage electric pulses to cells. This allows DNA to be transported directly to the cell cytoplasm. Alternatively, the redistribution of membrane components that accompanies the closure of pores may aid in the transportation of the DNA. This method may be used for the transient expression of cloned genes as well as for the establishment of cell lines carrying integrated copies of the gene of interest (Sambrook *et al.*, 1989).

It was suggested that pore formation is driven by local electric field gradients at the water/lipid interface of the phosphlipid bilayers. Water molecules move in these field gradients increasing the probability of water defects penetrating into the bilayer interior. Such water defects cause a further increase in the local electric field thereby accelerating the process of pore formation (Tieleman, 2004).

Electroporation has been used in a variety of fields, including agriculture where naked DNA was introduced into protoplasts resulting in the production of transgenic rice

(Ignacimuthu *et al.*, 2000). In addition, this method proved to be successful in the transfection efficiency of various cell types (Sambrook *et al.*, 1989).

1.2.2.5 Direct Microinjection

Direct microinjection into the nuclei is a method that does not expose the DNA to the cellular compartments such as low-pH endosomes (Sambrook *et al.*, 1989).

An example of the use of this method is seen in the introduction of foreign molecules into the mammalian oocyte and fertilised embryos. This direct transfer of genetic material has provided information as to how the female germ cell functions during development. This method also allowed investigators to study the physiology of the embryos, as well as to alter the genetic capabilities of the transformed animals (Ebert, 1998).

This manual microinjection method yields high transformation efficiencies but the technique employed is tedious and requires considerable skill (Singh, 1998). Furthermore, it cannot be used to introduce DNA on a scale large enough for biochemical analysis. It is therefore used primarily to establish cell lines carrying integrated copies of the DNA of interest (Sambrook *et al.*, 1989).

1.2.2.6 Lipofection

This non-viral method of gene delivery relies on complexes formed from liposomes which are lipid (fat) molecules and DNA. Liposomes are spherical bilayered artificial membrane vesicles into which DNA or RNA is encapsulated. Alternatively the DNA is coated with a synthetic cationic lipid. The complexes termed "lipoplexes" are formed when liposomes containing positively charged cationic lipids are mixed with negatively charged polynucleic acids (Ferrari *et al.*, 2002). These complexes are nonimmunogenic, nonpathogenic, biodegradable and easy to prepare. In addition, they have also been shown to pass through the blood-brain barrier (Ryhanen *et al.*, 2003). The successfulness of these complexes depends on the structure-activity relationship of the cationic liposome/DNA complex (Pedroso de Lima *et al.*, 2003). This method effectively links both chemical and biological principles (Smith *et al.*, 1993).

This gene delivery system proceeds through a series of adverse extracellular and cellular environments in order to successfully deliver genetic material into the nucleus of a eukaryotic cell. Nuclease activities occur in these extracellular environments and cellular compartments and it is therefore essential that the DNA be protected throughout the delivery process. Typically, multivalent cationic molecules are used. These molecules compact the DNA by neutralising the anionic phosphates of the DNA backbone providing protection of the DNA from the nucleases (Murphy *et al.*, 2001).

The liposome then fuses with the cell membrane and enters the cell by a process of endocytosis (Sambrook *et al.*, 1989).

1.3 Receptor-Mediated Endocytosis

Receptor-mediated endocytosis is an effective method for the delivery of genes into target cells. It is a natural process that cells utilise for the uptake of proteins and peptides (Singh, 1998). Receptor-mediated endocytosis represents a major pathway whereby nutrients, hormones, enzymes and viruses enter cells. These are ligands that bind to receptors at the cell surface, undergo internalisation and are thereafter eventually sorted to specific destinations (Murray *et al.*, 2000). Previous studies have used specific ligands such as asialoglycoproteins and transferrin as DNA-protein complexes for DNA delivery *in vitro* and *in vivo* (Christiano *et al.*, 1993a). The targeted delivery of DNA to specific cell types by this process is one of the many delivery systems that are suitable for and are fast becoming popular in gene therapy.

A schematic representation of receptor-mediated endocytosis is detailed in Figure 1.3. The first step in this process involves binding of the ligand to its cognate cell surface receptor found on the plasma membrane of the cell. The receptors then undergo a conformational change and concentrate in depressions called clathrin coated pits in the plasma membrane. These clathrin coated pits undergo movement resulting in the invagination and "pinching off" of the membrane as a coated vesicle. This process

utilises G-proteins and adenosine-tri-phosphate (ATP). The vesicles then fuse with the acidic endosomal compartments. The receptor and ligand dissociate and segregate. Once the ligands are released from the receptors, the receptors may be destroyed in a degradative pathway, where the endosomes fuse with the lysosomes and the ingested material is degraded by lysosomes. The receptors may be recycled to the plasma membrane. The pathway undertaken depends on the type of ligand-receptor pair and low endosomal pH, which may or may not trigger dissociation of the ligand and the receptor (Singh, 1998; Langer, 2000).

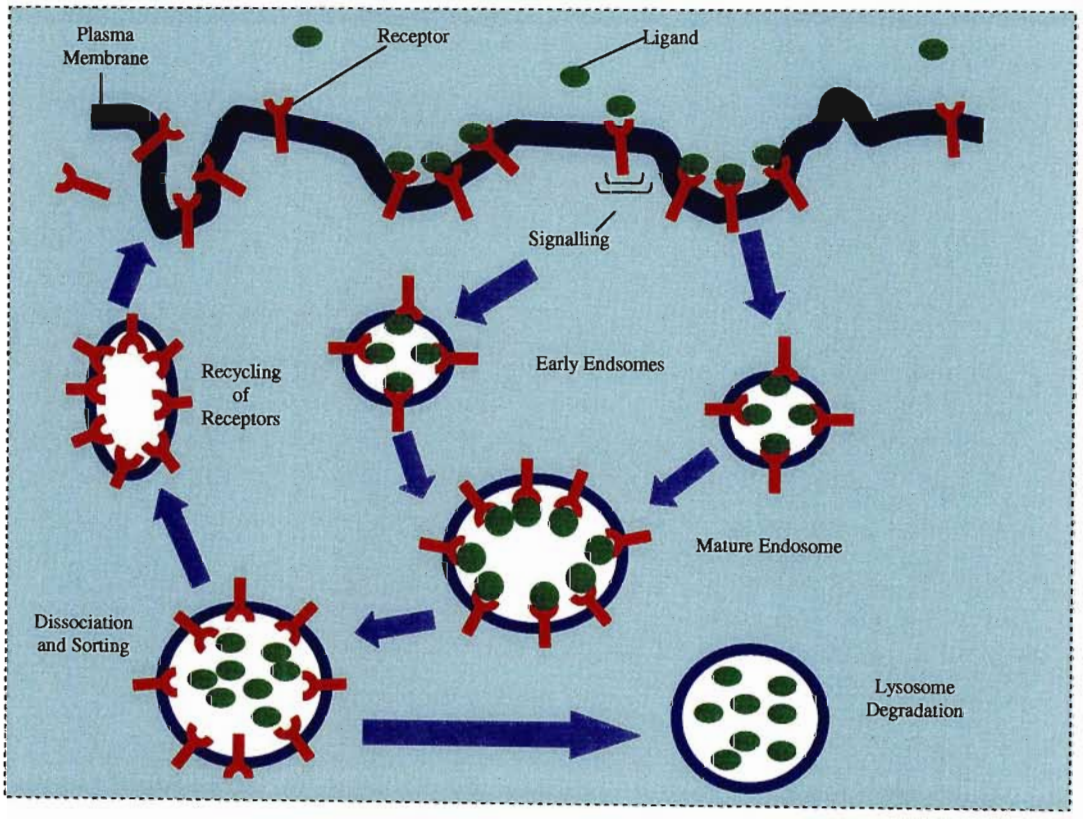


Figure 1.3 : Schematic representation of the process of receptor-mediated endocytosis

DNA is incorporated into the nucleus of the cell during this process. However, the effective delivery of the DNA to the cells via the process of receptor-mediated endocytosis depends on the escape of the DNA from the endosome during transport (Singh, 1998). However, for gene expression to be ensured, the DNA must enter the nucleus. The DNA may be released from the endosome in several ways. For example, the replication-deficient adenovirus binds DNA on the outside of the cell surface, disrupts the endosome and allows the vector into the cytoplasm after it has been internalised by receptor-mediated endocytosis. Receptor-mediated gene transfer has also been enhanced by the use of endosome disruptive peptides as well as by targeted cationic liposomes which bind to cognate receptors on the cell surface thereby achieving long term expression of the DNA that is complexed to them (Singh, 1998).

Receptor-mediated endocytosis is characterised by the movement of ligand-containing vesicles through the cytoplasm (Goltz *et al.*, 1992). The sorting of endocytic material is achieved along a pathway of semi-stable tubulo-vesicular membranous structures that display intracellular localisation and appearance. It has previously been suggested that a relationship exists between these structures and microtubules which plays a critical role in endocytosis and endocytic processing. The transport from early to late endosomes is therefore microtubule dependent and microtubules have been shown to promote fusion of endocytic vesicles (Figure 1.4) (Murray *et al.*, 2000).

Also, the plasma membrane is directly linked and functionally integrated with the underlying actin-based cytoskeleton which forms the cell "cortex". Vesicular trafficking

at the plasma membrane therefore requires the active rearrangement of cortical actin filaments to remove a barrier to vesicular fusion and budding events. Alternatively, these actin filaments and actin-based motor proteins may possibly be required to direct vesicle budding and fusion events through the cell cortex at the plasma membrane (Lamaze *et al.*, 1997).

This endocytic pathway is more or less linear and can be disrupted by drugs such as nocodazole, colchicine and chloroquine that affect microtubule polymerisation (Goltz *et al.*, 1992; Christiano *et al.*, 1993b; Murray *et al.*, 2000). Cytochalasin D is a drug that destabilises actin filaments thereby inhibiting receptor-mediated endocytosis (Lamaze *et al.*, 1997). This evidence suggests that microtubules and actin filaments participate in the sorting and directed movement of endocytic vesicles and is therefore required for receptor-mediated endocytosis. A further requirement for receptor mediation is cellular ATP suggesting that this process is ATP-dependent (Figure 1.4) (Schmid and Carter, 1990; Goltz *et al.*, 1992).

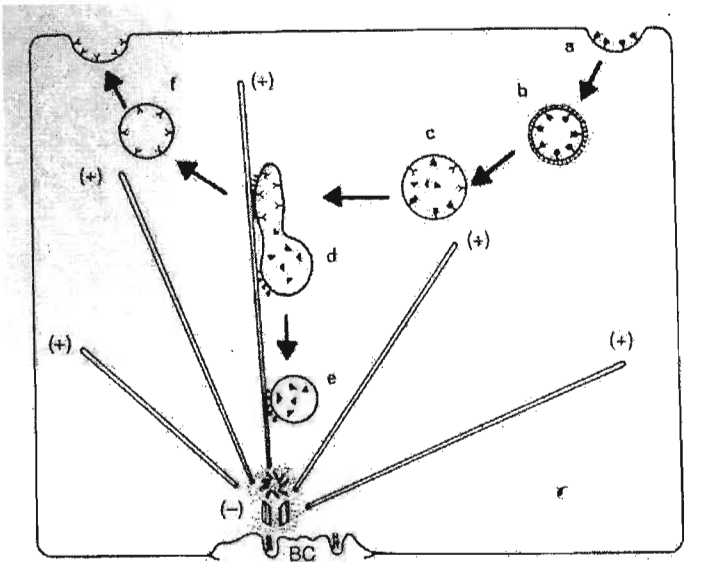


Figure 1.4 : Schematic representation of the hypothetical pathway of vesicle sorting along microtubules during receptor-mediated endocytosis of asialoorosomucoid in hepatocytes (adapted from Goltz *et al.*, 1992)

Asialoorsomucoid binds to its receptor at the basolateral surface of the hepatocyte. When receptor-mediated endocytosis is initiated, receptor-ligand complexes in coated pits (a) are internalised into coated vesicles (b), which subsequently lose their clathrin coats (c). Microtubules in hepatocytes are organised in a polarised manner, possibly as shown. Maturing endocytic vesicles associate tightly with microtubules (d) such that their receptor-containing domains cannot be released by ATP addition; at the same time, although independently, receptor and ligand dissociate. Ligand-containing vesicles are segregated (e) and can be released from microtubules in an ATP-sensitive manner. We propose that while acid-mediated dissociation of ligand and receptor is proceeding, one pole of the vesicle bulges as it is moved along the microtubule by a minus end-directed motor (e.g. cytoplasmic dynein) (d) toward lysosomes, leaving the immobilised receptor domain behind. Dissociated ligand distributes into this vesicular space proportionally to its volume and fission follows. Finally receptor containing vesicles recycle (f). BC, bile canaliculus.

1.4 Liposomes

Liposomal particles were discovered in the early 1960's by Alec Bangham and consequently known as "bangosomes". Liposomes have been used as membrane models and vehicles for the delivery and transfer of various substances (Smith *et al.*, 1993). To

date they have been used to deliver DNA into a variety of cell types in a number of clinical trials. Apart from nucleic acids, liposomes have also been used to encapsulate and deliver a variety of other materials such as drugs and viral particles to cells (Innes *et al.*, 1990; Singh, 1998).

1.4.1 Structure of Liposomes

Liposomes are self-closed spherical structures composed of curved lipid bilayers which entrap part of the solvent in which they freely float, into the interior (Francescangeli *et al.*, 2003). Liposomes vary from tens of nanometers to tens of microns in diameter (Smith *et al.*, 1993). These vesicles consist of one or more concentric lipid bilayers that alternate with aqueous compartments within which is entrapped a variety of lipid-soluble and water-soluble substances respectively (Singh, 1998; Francescangeli *et al.*, 2003). The continuous bilayer of lipid molecules within which the aqueous compartments lie are usually phospholipids. Phospholipids consist of a pair of fatty acids, a hydrophobic tail and a hydrophilic head group and forms the largest structural component of biological membranes. In aqueous solutions the hydrophobic tails self associate to exclude water whilst the hydrophilic heads interact with the liquid. This results in the lipid bilayer forming a continuous membrane enclosing a small volume of water and this is surrounded by a continuous phase. Typically, the furthest distance between two opposing lipids in the bilayer is approximately 6 nm, although this distance is dependant on the ionic activity of the aqueous solvent. A single bilayer of this type is termed unilamellar.

However, multilamellar liposomes also occur. These consist of nested rings of bilayers, somewhat resembling the layers in an onion. These particles can easily be manipulated by replacing the aqueous internal volume or the lipid components thereby altering the construction and subsequently the properties of the liposome. The design of the liposome delivery vehicle can therefore be tailored towards different goals (Smith *et al.*, 1993).

1.4.2 Classes of Liposomes

Generally, there are two classes of liposomal transfection reagents, those that are anionic and those that are cationic.

Transfection using anionic liposomes requires that the DNA be entrapped in the aqueous space of the liposome. This process is time consuming and encapsulation efficiency is variable. Hence this method is not widely used in gene transfer.

Cationic liposomes however do not require the DNA to be entrapped. This is because these lipids are positively charged and are able to interact spontaneously with the negatively charged DNA to form clusters of aggregated vesicles along the nucleic acid. At a critical liposome density, the DNA is condensed and becomes encapsulated within the lipid bilayer. However, there is also some evidence that cationic liposomes do not actually encapsulate the DNA, but instead bind along the surface of the DNA maintaining its original shape and size. In addition, all cationic lipids possess an amine group apart

from the hydrophobic group. It is the amino group that binds the DNA electrostatically while the hydrophobic group facilitates the assembly of the cationic lipids into the bilayer vesicles (Bajoghli, 2004). Ionic interaction of DNA with the cationic liposome results in dehydration and condensation of the DNA, inducing a structural transition from the native B-form to C-form. C-form DNA may further collapse into a tertiary structure known as the chiral Ψ-DNA phase under extreme dehydration and high condensation (Patil and Rhodes, 2000). Since the negative charges of the DNA backbone interact with the positive charges of cationic lipids, this creates a DNA-liposome complex where the DNA is also able to interact stably with the lipid on the outer surface of the liposome (Smith *et al.*, 1993) (Figure 1.5). Such complexes are termed lipoplexes. Lipoplexes i.e. cationic liposomes complexed with DNA have been shown to be promising non-viral delivery systems because of their ability to mimic certain characteristics of natural viruses by acting as efficient chemical carriers of extracellular DNA across outer cell membranes and nuclear membranes (Francescangeli *et al.*, 2003).

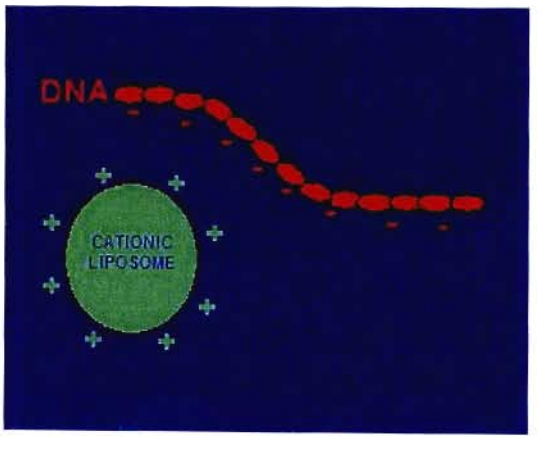


Figure 1.5 : Schematic representation of interactions between cationic liposomes and DNA

1.4.3 Cationic Liposomes

The cationic lipids of the complex can further be classified into two classes. Both these classes have been synthesised and show good transfection efficiency mostly *in vitro* with established cell lines. The first class has two alkyl chains in each cationic lipid molecule, whereas the other type uses cholesterol as its backbone. Both these types contain either mono- or multiple amino groups as the cationic function group to form complexes with DNA via electrostatic interactions i.e. they vary from monocation head groups to polycation head groups and are formulated in an aqueous liposome suspension with a helper/co-lipid (Hofland *et al.*, 1996).

The transfection activity of these cationic lipids such as 1,2 dioleoyl-3-(trimethylammonio)propane or dioctadecylamidoglycylspermine may be improved with the inclusion of a helper lipid/co-lipids such as dioleoylphosphatidylethanolamine (DOPE) or cholesterol in a liposome composition (Kay *et al.*, 1997; Ghosh *et al.*, 2000; Murphy *et al.*, 2001; Braun *et al.*, 2003). This was also suggested in another study (Porter *et al.*, 1998) where the rate of transduction of target cells with retroviral vectors was enhanced by cationic liposomes and the greatest effect was seen with the formulation 3β [N',N'-dimethylaminoethane)-carbomoyl] cholesterol (DC-Chol) / dioleoylphosphatidylethanol- amine (DOPE). Furthermore, optimal cationic liposome/co-lipid combination usually varies with the application. For example, (+/-)-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl1-2,3-bis(tetradecyloxy)-1-propoanaminium bromide (DMRIE):1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) has been shown to be

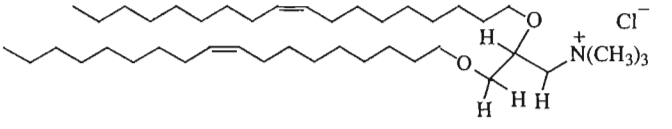
very effective for *in vivo* transfection of a variety of tumour types, while (+/-)-*N*-(3-aminoprpyl)-*N*,*N*-dimethyl1-2,3-bis(dodecyloxy)-1-propoanaminium bromide (GAP-DLRIE):DOPE is better for functional gene delivery to salivary epithelial cells and to the lung following intranasal administration (Ferrari *et al.*, 2002). The success of the use of liposomes seems therefore, to depend on their formulation and method of preparation (Vitas *et al.*, 1996), the application for which it is used (Ferrari *et al.*, 2002) and, a slight excess of cationic lipid in the DNA/lipid complexes i.e. the lipoplexes (Kay *et al.*, 1997). Furthermore, increasing the ratio of N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulphate (DOTAP) or cholesterol to DNA was seen to lead to a dose dependent enhancement of transfection efficiency in the presence of high serum concentrations (Nchinda *et al.*, 2002). Several physical or chemical properties of cationic lipids have been proposed to be responsible for their ability to transfect cells. The number of positive charges of the cationic lipid, the linkage between the hydrophobic and cationic portion of the molecule and the structure of the hydrophobic moieties have been demonstrated to influence transfection efficiencies (Ryhanen *et al.*, 2003).

1.4.3.1 Cationic Liposome-DNA Complexes

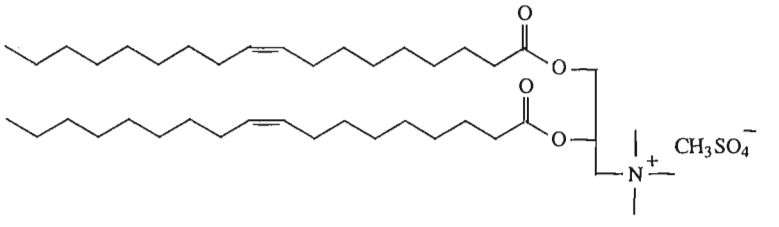
Cationic liposome – DNA complexes are termed lipoplexes. These are membranous structures that are capable of transducing genes into cells, ultimately leading to the expression of the genes (transfection) both *in vitro* and *in vivo* (Zuhorn and Hoekstra, 2002). Lipoplexes range from 50 nm to just over 1 μm in size and it has been suggested

an increase in the size of the lipoplex improves transfection *in vitro* (Bajoghli, 2004). The driving force for lipoplex formation is the removal of the counterions from the lipid surface by the DNA. The inclusion of helper lipids such as dioleoylphosphatidylethanolamine (DOPE) in the liposomal formulation facilitates this removal by weakening the binding of the ions to the cationic surface (Zuhorn and Hoekstra, 2002). These complexes do not face any electrostatic barrier in penetrating the negatively charged biological cell surfaces and are endocytosed by the plasma membrane (Chaudhuri, 2002). A positively charged lipoplex therefore is necessary for binding to the target cells prior to internalisation by endocyosis. Once again the helper lipids are important as they aid endosomal escape for onward transport to the nucleus (Bajoghli, 2004). Cationic liposomes also protect DNA from attack by the en-route deoxyribonucleases (DNAses) and are designed to compact DNA for favourable cellular uptake (Chaudhuri, 2002).

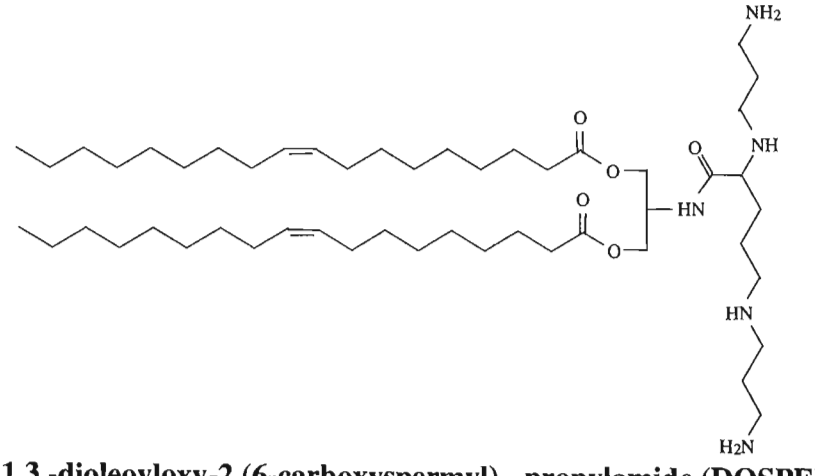
Intense efforts towards developing safe and efficient cationic liposomes as delivery molecules for use in gene therapy are commercially available as cationic lipid-based transfection kits (Chaudhuri, 2002). The molecular structures of some of these commercially available cationic transfection lipids are shown in Figure 1.6.



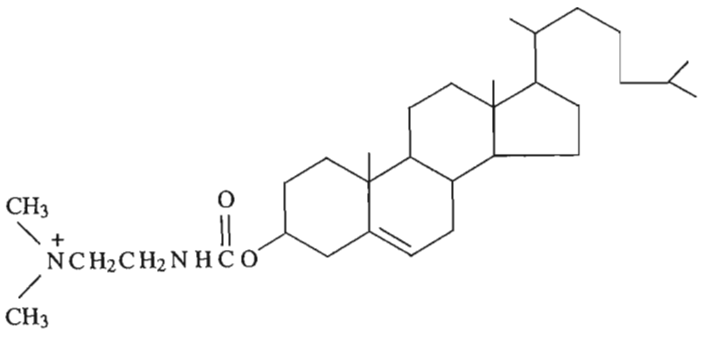
(a) N-[1-(2,3-dioleyloxy) propyl]- N,N,N- trimethylammonium chloride (DOTMA)



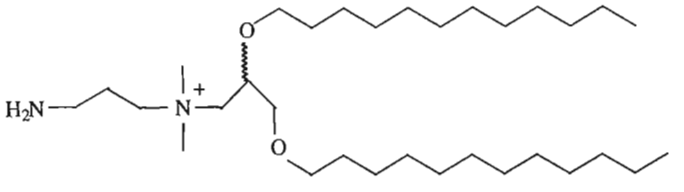
(b) N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulphate (DOTAP)



(c) 1,3 -dioleoyloxy-2 (6-carboxyspermyl) - propylamide (DOSPER)



(d) 3β[N',N'-dimethylaminoethane)-carbomoyl]cholesterol (DC-Chol)



(e) (+/-)-*N*-(3-aminoprpyl)-*N*,*N*-dimethyl1-2,3-bis(dodecyloxy)-1-prpoanaminium bromide (GAP-DLRIE)

Figure 1.6 : Structures of some of the commercially available efficient transfection lipids

The powerful DNA condensing and high transgene expression properties of cationic liposomes, both *in vitro* and *in vivo*, have been reported in numerous investigations. A recent study (Chaudhuri, 2002) showed that lipoplexes injected into the tail vein of mice, successfully transfected lung endothelial cells with reasonably high efficiencies. Direct instillation into the lungs have also demonstrated similar findings in the airway epithelial cells. Gene expression and the appropriate physiological effects have been observed following lipoplex-mediated administration of the cystic fibrosis transmembrane receptor gene into the lungs and nose in patients with cystic fibrosis.

Furthermore, the use of cationic liposomes in gene therapeutic strategies for cancer have recently progressed into phase II clinical studies, and in such studies it was shown that only brief expression of the transgene was required for killing tumour cells (Chaudhuri, 2002). Other studies have demonstrated that transfecting complexes containing cationic liposomes can be targeted to parenchymal cells (Singh and Ariatti, 2003). Further, liposomes may be used for the transport of drugs into phagocytic cells infected with *Brucella abortus* (Vitas *et al.*, 1996). Enzymes can be encapsulated into liposomes by induction of a specific interaction between the enzyme and the lipid surface (Colletier *et al.*, 2002).

1.4.3.2 Advantages and Disadvantages of Cationic Liposomes

Cationic liposomes which may resemble traditional pharmaceuticals (Hirko *et al.*, 2003; Chen *et al.*, 2004) display low immunogenicity (Goncalves *et al.*, 2004) and this contributes to its use as a non-viral vector. They contain a wide variety of both hydrophobic and hydrophilic diagnostic or therapeutic agents, provide a larger drug payload per particle, protect encapsulated agents from metabolic processes, and allow a high degree of cooperative binding to target cell antigens. Also the lipid composition of the bilayer can be modified to obtain other desirable properties, including prolonging circulatory half-life, the ability to complex with nucleic acids to mediate gene delivery or genetic regulation, and the capacity to deliver encapsulated contents to the cytosol through the endosome/lysosome pathway (Spragg *et al.*, 1997). Further, improved

formulations prevent them from being cleared by the complement and repeated administrations *in vivo* may be performed without adverse consequences (Templeton, 2002).

Disadvantages include low transfection efficiency and cell toxicity (Hofland *et al.*, 1996; Hirko *et al.*, 2003). Another limitation is that cationic liposomes form aggregates with serum proteins bearing negative charges (Singh and Ariatti, 2003). Binding of serum proteins to lipoplexes prevents their interaction with the cell surface and / or their internalisation (Zuhorn and Hoekstra, 2002). This inhibitory effect of serum on liposome-mediated gene delivery (lipofection) can be overcome by using lipoplexes with high lipid-to-DNA charge ratios.

Condensing DNA with biocompatible polycations prior to mixing with the cationic liposomes have been shown to render the DNA resistant to DNAse activities and to enhance the transfection efficiencies of cationic liposomes in different cell lines (Chaudhuri, 2002).

Since the pioneering development of the glycerol backbone-based transfection lipid (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), there has been the design and syntheses of numerous cationic transfection lipids exhibiting higher transfection efficiencies (Chaudhuri, 2002). Cationic liposomes have emerged as promising non-viral vectors for the delivery of DNA molecules *in vitro* (Patil and Rhodes, 2000).

1.4.4 Mechanism of Liposomal Action

The mechanism involved in the cellular uptake of lipoplexes is endocytosis, a process occurring after non-specific charge-mediated binding to cellular receptors has taken place. The net positive charge of the lipoplex ensures its efficient binding to the cell surface. However, increasing the positive charge of the lipoplex enhances the cytotoxic effect. This induces cellular damage because the cationic lipids intercalate into the cellular membranes. Cellular damage can be an important event accompanying transfection. Due to the membranous nature of lipoplexes, it has been proposed that their cargo would gain intracellular access via fusion with the plasma membrane. However, lipid mixing assays did not demonstrate a correlation between fusion of lipoplexes with the plasma membrane and their transfection efficiency. Instead, current data support the view that once binding of the complexes to the cell surface has occurred, internalisation is required for productive transfection and nucleic-acid release takes place at the level of endosomal compartments (Zuhorn and Hoekstra, 2002).

Following arrival of the lipoplex in the endosomal compartment, the DNA is dissociated from the complex and released into the cytosol. This release of DNA is a result of the interaction of acidic (phospho-) lipids with the lipoplexes. Interactions between cationic amphiphiles and anionic (phospho-) lipids are driven by ion-pairing and hydrophobic forces. These interactions are stronger than the interactions relying on electrostatic forces involved in cationic lipid-DNA complex formation and stabilisation. It has also been suggested that the release of DNA from lipoplexes coincides with their fusion with the

endosomal membrane and that a tight interaction between the lipoplex surface and the endosomal membrane was essential to ensure that the DNA does not dissociate prematurely from the complex. This may result in its processing into the degradation pathway with the lysosomes as the endpoint (Zuhorn and Hoekstra, 2002).

Once the actual internalisation of the lipoplexes has occurred, an important step in the transfection process is the release of the lipoplex and / or its DNA into the cytoplasm in order to evade lysosomal degradation. The membranous nature of the lipoplex seems to be crucial at this stage in that it allows the exchange of lipids between the endosomal membrane and the lipoplex which results in membrane perturbations which is a prerequisite in the endosomal escape of the DNA. Subsequent to its release into the cytoplasm, the DNA has to be transferred into the nucleus and this nuclear import of DNA is most likely a protein-mediated process. In addition, the uptake of DNA into the nucleus may be facilitated at the time of nuclear envelope disassembly during mitosis. During this process the nuclear envelope is fragmented to facilitate the transfer of DNA into the nucleus (Zuhorn and Hoekstra, 2002). In the case of non-dividing cells, transfection is particularly low due to the diffusion barrier constituted by the nuclear envelope. In such cases, the efficiency of gene transport to the cell nucleus is increased as proteins destined to the nucleus possess at least one nuclear localisation sequence (NLS). This allows them to interact with a nuclear import receptor, namely importin β q. When complexed to importin β q, proteins are delivered to the cell nucleus via nuclear pore complexes. Most proteins interact with importin β q via an adapter imoprtin α. Importin α consists of two functional domains, a short basic amino-terminal domain that is

responsible for importin β q binding (IBB domain) and a central NLS-binding domain that is built of armadillo (arm) repeats. Nuclear localisation sequences have been electrostatically or covalently coupled to plasmid DNA to provide an interaction with importin α and it has been hypothesised that the plasmid DNA / importin α complexes would then interact with importin β q and be transported to the nucleus by a facilitated diffusion pathway via the protein nuclear import system (Carrière *et al.*, 2003). Attaching nuclear localisation sequences to synthetic gene delivery systems therefore enhance the nuclear delivery of DNA in non-dividing cells (Zuhorn and Hoekstra, 2002).

1.4.4.1 Liposomal Targeting

Liposomes do not possess specific receptors for the attachment to and entry of target cells. In order to accomplish this, a system containing the liposome-DNA complex together with a ligand can be used. An efficient DNA targeting vehicle therefore, should consist of three components. These include a tissue specific ligand for target binding, a polycation for complexation of the negatively charged DNA and a lipophylic part that will assist the DNA in entering the cell cytoplasm. As demonstrated in previous studies, a peptide ligand (transferrin) is used to deliver DNA in combination with liposomes to erythroid cells in bone marrow that express the transferrin receptor (Singh, 1998). Alternatively, liposomes can be conjugated to antibodies. For example, a liposome combined with an antibody against a mouse major histocompatibility antigen can more efficiently deliver DNA into target cells than liposome-DNA complexes alone.

Liposomes complexed to asialofetuin, a natural ligand for receptors of liver cells showed greater uptake by the liver in comparison with unmodified liposomes (El-Aneed, 2003). Furthermore, liposomes complexed to targeting ligand glycolipid molecules bearing galactose residues, bound to receptors on liver cells efficiently (Wen *et al.*, 2004). Hence, depending on the ability of the antibody or ligand to facilitate cell specific docking, the antibody or ligand can be targeted to a specific tissue (Singh, 1998).

Another example of a ligand is the asialoglycoprotein asialoorosomucoid (AOM). These are naturally occurring glycoproteins and their major role is to clear glycoproteins and lipoproteins from the circulation (El-Aneed, 2003). Asialoglycoproteins are lectins. Liposomes have been appropriately constituted for galactose-specific recognition which is achieved by the inclusion of galactosyl derivatives of cholesterol or palmitoylated asialofetuin in the membrane bilayer (Singh *et al.*, 2001). This allows them to recognise lectins thereby recognising asialoglycoproteins. This specificity of the receptor for the terminal D-galactose unit is achieved through hydrogen bonding of the sugar 3- and 4-hydroxyl groups with receptor amide and carboxylate side chains (Singh and Ariatti, 2003). Basically, the asialoglycoprotein receptor contains a carbohydrate-recognition domain that binds to galactose derivatives (El-Aneed, 2003). The AOM receptor has been used in targeting DNA to HepG2 cells *in vitro* and liver cells *in vivo* (Christiano *et al.*, 1993a) since it is predominantly expressed on the sinusoidal surface of parenchymal cells in hepatocytes (Singh and Ariatti, 2003) and in the model human hepatocellular carcinoma cell line HepG2 (Singh *et al.*, 2001). A hepatocyte has approximately 500 000 asialoglycoprotein receptors (Christiano *et al.*, 1993a) and the HepG2 cell line displays

approximately 225 000 asialoglycoprotein receptors per cell (Singh and Ariatti, 2003). Such receptors have a high affinity for the galactose terminating triantennary N-linked heteroglycans of asialoglycoproteins (Figure 1.7).

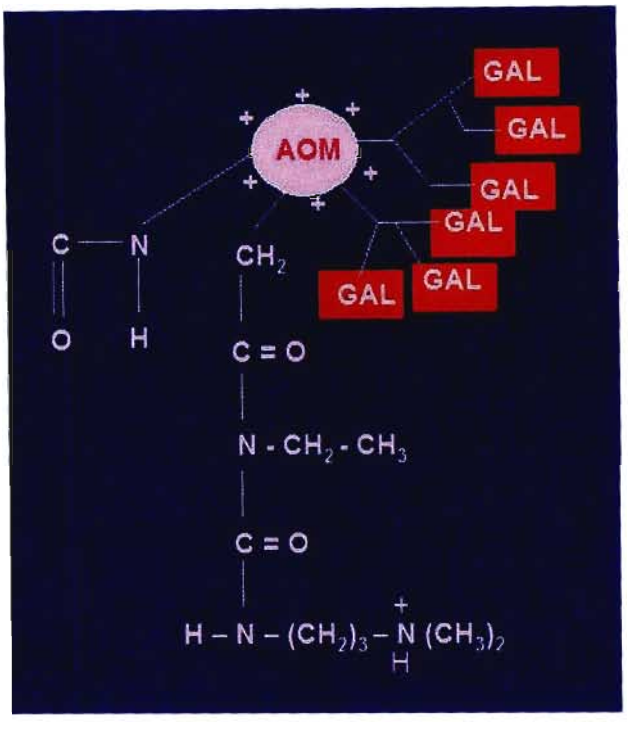


Figure 1.7 : Schematic representation of AOM showing galactose terminating triantennary *N*-linked heteroglycans

As demonstrated by Wu and Wu in 1987 and then again in 1988, polyplexes comprising the ligand AOM cross-linked to the polycation poly-L-lysine and DNA have been used to direct genes to HepG2 cells and to rat liver *in vivo* respectively (Wu and Wu, 1987; Wu and Wu, 1988). The asialoglycoprotein receptor is specific for the terminal D-galactose unit of the polycation. This is achieved as stated earlier through the hydrogen bonding of sugar 3- and 4-hyroxyl groups with receptor amide and carboxylate side chains. The binding of the DNA was achieved by electrostatic attraction. The asialoglycoprotein

receptor is therefore required and responsible for the binding and delivery of DNA (Singh *et al.*, 2001; Singh and Ariatti, 2003). This is brought about by receptor-mediated endocytosis of the asialoglycoproteins in hepatocytes and is characterised by the vectorial movement of ligand-containing vesicles through the cytoplasm. The ligand-receptor complex is internalised from the cell surface into an acidic endosomal compartment where the receptor and ligand dissociate and segregate. This results in the movement of ligand-containing vesicles toward lysosome-fusion while the receptor-containing vesicles recycle (Goltz *et al.*, 1992). Singh *et al.* (2001), have shown that cationised AOM, cationised liposomes and plasmid DNA form complexes that also transfected the same cell line by receptor mediation.

1.5 Outline of Thesis

The development of transfecting systems for *in vivo* application in gene therapy is a rapidly growing field of study. The need to improve on delivery systems has led to the synthesis of new untested vectors. In this project a novel cationic cholesterol derivative *viz.* biotinylcholesteryl formylhydrazide was incorporated in the synthesis of cationic liposomes as DNA carrier systems. A further attempt is made to develop a system for *in vitro* targeting of foreign DNA into a hepatocyte cell line, HepG2 using the formulated cationic liposomes conjugated to DNA and an AOM ligand. The method of uptake of DNA by the HepG2 cells via the asialoglycoprotein receptor was explored. This was based on the fact that these hepatocytes possess the asialoglycoprotein receptor that can

bind and internalise galactosylated glycoproteins such as AOM. The AOM is a specific protein that binds to cognate surface receptors of hepatocytes. This was added in the hope of improving transfection efficiency by specific cell targeting. The ligand was obtained in the form of an orosomucoid (α-1-acid glycoprotein), and was first desialylated to produce the AOM. Desialylation of the protein removed the sialic acid residues, rendering the protein more positively charged and therefore more inclined to bind to the negatively charged DNA. This protein was further biotinlyated. Biotin binds the to streptavidin, forming irreversible bonds (Sato *et al.*, 2000; Lehtolainen *et al.*, 2002). This resulted in a complex which then reacted with the biotinylated cationic liposome, allowing for binding of the liposome to the ligand. The cationic liposome in turn bound to the negatively charged DNA backbone by electrostatic attraction resulting in a ternary vector.

The liposome DNA interactions were assayed using gel retardation, ethidium bromide displacement, and nuclease digestion assays. Transfection was conducted on HepG2 cells with and without an excess (100 fold) of the ligand asialofetuin and using a commercially available transfection, reagent FuGene 6.

The efficiency of the cationic liposome mediated transfections were assessed using the luciferase reporter gene assay to achieve transient expression. This type of tissue-specific targeting interaction may have broad applications with a variety of gene delivery systems.

CHAPTER 2

MATERIALS AND METHODS

2.1 Ethical Approval

This study has been approved by the Faculty of Medicine Ethics Committee.

2.2 Preparation of DNA

The luciferase expression vector i.e. the pGL3 luciferase control vector (Promega Corporation, Madison, USA) is a 5256 kbp expression vector that contains a Rous Sarcoma Virus Long Terminal Repeat (RSV-LTR) promoter-driven luciferase reporter gene from the firefly *Photinus pyralis*, SV40 small T antigen and polyadenylation site and ampicillin resistance gene (pRSVL) (Figure 2.1). This luciferase reporter gene is a modified coding region that has been optimised for monitoring transcriptional activity in transfected eukaryotic cells using the luciferase assay.

This vector was amplified in the host cell *Escherichia coli* JM 109 and then isolated.

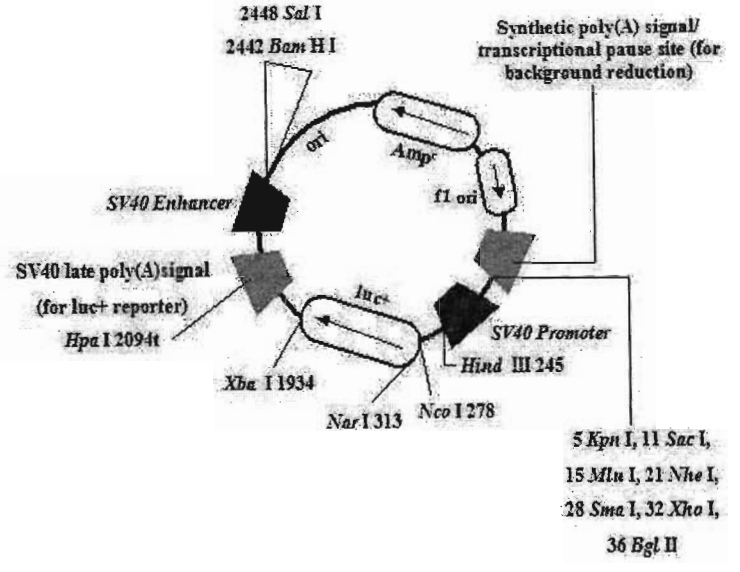


Figure 2.1 : Diagrammatic representation of pGL3 control vector circle map

Additional description: *luc+*, cDNA encoding the modified firefly luciferase; Amp^r, gene conferring ampicillin resistance in *E.coli*; f1 ori, origin of replication derived from filamentous phage; ori, origin of replication in *E.coli*. Arrows within *luc+* and the Amp^r gene indicate the direction of transcription, the arrow in f1 ori indicates the direction of ssDNA strand synthesis (adapted from Promega Technical Manual No. 033).

2.2.1 Preparation of Lauria Bertani (LB) Agar Plates Containing Ampicillin

Lauria Bertani (LB) medium (25 ml) containing agar (pH 7.0) and ampicillin (Appendix A) were carefully poured into 90 mm petri dishes under aseptic conditions. The plates were left to stand until their contents hardened. The lids were placed in position and the plates sealed with parafilm. The plates were then stored in an inverted position in autoclave bags at 4°C.

2.2.2 Transformation of *E.coli* with Plasmid DNA

2.2.2.1 Preparation of Competent Cells

One loop of JM 109 cells was aseptically inoculated into LB medium (3 ml) (Appendix A). This was incubated at 37°C in a shaking incubator (Innova 4000) for 16 hours. This overnight *E.coli* culture was then diluted to 100 ml with LB medium and again incubated with shaking at 37°C until the density was equivalent to an optical density (OD) of 0.375 at 590 nm (Ultrospec 1100 Pro). The culture was immediately placed on ice for 10 minutes to stop any further reaction. Thereafter the cells were centrifuged at 6000 rpm for 30 minutes at 4°C (Sigma 3K-2). The supernatant was discarded and the resultant pellet was gently resuspended in 50 mM cold calcium chloride ($CaCl_2$) and 10 mM Tris-HCl ([Tris(hydroxymethyl) aminomethane hydrochloride]) (pH 8.0) (10 ml) (Appendix B). Centrifugation then followed at 6000 rpm for 30 minutes at 4°C and the pellet was resuspended gently in cold 50 mM $CaCl_2$ and 10 mM Tris-HCl containing 15% glycerol (pH 8.0) (2.5 ml) (Appendix B). The tubes were then stored at 4°C for 24 hours to increase competency. The pellet was thoroughly dissolved by vortexing (Vortex-2 genie). The resultant competent cells (50 µl) were then aliquotted into eppendorf tubes and stored at -70°C.

2.2.2.2 Heat Shock Transformation

Frozen competent cells (50 µl) were allowed to thaw slowly on ice. The pGL3 control vector (2 µl) was added to the cells and gently mixed. This was incubated on ice for 10 minutes. The tube was then placed into a 42°C water bath for 2 minutes and then placed in ice for 15 minutes. LB medium (900 µl) was then added to the tube and incubated for 1 hour at 37°C in a shaking incubator. The resulting DNA-competent cell mixture (100 µl) was spread onto LB agar plates containing the antibiotic ampicillin (Appendix A). The plates were incubated overnight at 37°C (Memmert). The resultant colonies were then picked up from the agar plates using a pipette tip and placed into tubes containing LB medium and ampicillin (4 ml). This was incubated overnight in a shaking incubator at 37°C.

2.2.3 Isolation of Plasmid DNA : Quick DNA Plasmid Mini Prep

Approximately 1.5 ml of the overnight culture was aliquotted into an eppendorf tube. This was then centrifuged at 12 000 rpm for 30 seconds (Sorvall MC 12V). The supernatant was discarded and the pellet resuspended in sucrose/tris (25 g sucrose, 5 ml 1M Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol), (pH 8.0) (15 µl) (Appendix B) and mixed gently using a pipette. Tris lysing mix (5 ml Triton X-100 (iso-octylphenoxypolyethoxyethanol containing approximately 10 moles of ethylene oxide), 5 g sucrose, 5 ml 1 M Tris (pH 7.0); 10 ml 0.5 M EDTA (ethylenediaminetetra-acetic acid di-sodium salt dihydrate) (100 µl) (Appendix B) was added and the tube gently inverted.

Thereafter lysozyme mixture (10 mg/ml in sucrose/tris) (10 µl) (Appendix B) was added and the tube was once again inverted at room temperature for 5 minutes. The tube was then placed into a boiling water bath for 4 minutes and centrifuged at 12 000 rpm for 15 minutes at 4°C. The resultant pellet was then removed with a toothpick. Approximately 12 µl of 2.5 M potassium acetate (KOAc) (pH 4.8) (Appendix B) together with isopropanol (100 µl) was added to the supernatant. This was incubated at -80°C for 10 minutes and thereafter centrifuged at 12 000 rpm for 10 minutes. The supernatant was then discarded and the pellet washed with 80% ethanol (80 µl). The pellet was allowed to dry and was then resuspended in Tris-EDTA (2-amino-2-(hydroxymethyl)-1,3-propanediol) ethylenediaminetetra-acetic acid di-sodium salt dihydrate) buffer (10 mM Tris, 1 mM EDTA, pH 7.2) (30 µl) (Appendix B). This resultant isolated plasmid DNA was then stored at -70°C.

2.2.4 Confirmation of Plasmid DNA

The purity and concentration of the pGL3 DNA was determined using two methods *viz.* spectrophotometry and agarose gel electrophoresis.

Spectrophotometric (Gene Quant II) readings were obtained at 260 nm indicating the concentration of the DNA, the absorbance value, and the ratio.

The pGL3 control vector, obtained from Promega was used as a control in agarose gel electrophoresis (Appendix C) against the isolated DNA samples. Agarose gel electrophoresis was then performed on a 2% gel containing ethidium bromide (Appendix C) at 100 V for approximately 1 hour. Gels were then viewed (Hoefer) and photographed

(ChemiDoc XRS, Biorad). The results obtained from this procedure enabled comparisons to be made between the isolated DNA samples and the control pGL3 control plasmid vector DNA.

2.3 Liposome Preparation

The cationic liposome prepared and utilised in this project consisted of three components. These were biotinylcholesteryl formylhydrazide (Sigma Chemical Company, USA), 3β[N-(N',N'-dimethylaminopropane)carbamoyl]cholesterol (Chol-T) (Sigma Chemical Company, USA) and dioleoyl-L-α-phosphatidylethanolamine (DOPE) (Sigma Chemical Company, USA).

2.3.1 Synthesis of 3β[N-(N',N'-dimethylaminopropane)carbamoyl]cholesterol (Chol-T)

To a solution of N,N-dimethylaminopropylamine (0.379 ml, 3 mmol) in chloroform (CH_3Cl) (1 ml) at 0^o was added cholesteryl chloroformate (449 mg, 1 mmol). This constituted a reaction mixture, a sample of which was retained for Thin Layer Chromatography (TLC) (Appendix D). After 1 hour, the solvent was removed by rotary evaporation (Büchii Rotavapor-R). The residue was re-dissolved in absolute ethanol and then placed in the freezer resulting in the formation of crystals (Singh *et al.*, 2001). TLC (Appendix D) was then performed to confirm the synthesis of Chol-T. Three samples

were spotted : a standard Chol-T sample, the reaction mixture of Chol-T taken prior to crystallisation and Chol-T crystals obtained after crystallisation.

2.3.2 Synthesis of Cationic Liposomes

Biotinylcholesteryl formylhydrazide (0.08 μmol), Chol-T (1.92 μmol) and DOPE (2 μmol) were dissolved in CH_3Cl (1 ml) constituting a total reaction mixture containing 4 μmoles of lipid. The solutes were then deposited as a thin film on the inner wall of a test tube by evaporating the solvent in vacuo at 20°C using a Büchii Rotavapor-R. This film was rehydrated overnight in a sterile HEPES (20 mM, pH 7.5, 1 ml) containing sodium chloride (NaCl) (150 mM) (Appendix B). Thereafter the suspension was briefly vortexed and then sonicated in a bath sonicator (Branson 1210) for 5 minutes at 20°C. This produced a liposome suspension which was then stored in a parafilm-sealed tube at 4°C overnight. The sample was then sonicated for 5 minutes to produce unilamellar liposomes (Singh and Ariatti, 2003).

2.3.3 Characterisation of Liposomes by Transmission Electron Microscopy (TEM)

Samples for transmission electron microscopy were prepared by mixing the liposome suspension (50 μl) with 5% ($^{w}/_{v}$) bovine serum albumin (BSA) (100 μl) (Boehringer

Mannheim, Germany). This was then diluted with 0.1 M Tris-HCl buffer (pH 7.2) (100 µl) followed by the addition of 25% ($^{v}/_{v}$) glutaraldehyde (50 µl) (Merck, Darmstadt, Germany). This was incubated for 20 minutes resulting in the formation of a lemon coloured gel. Thereafter the gel was diced using a scalpel and transferred into a small vial containing osmium tetroxide (OsO_4) (Merck, Darmstadt, Germany). The vial was capped and the sample was stored in the dark for 24 hours. The OsO_4 was removed and the sample was dehydrated for 15 minutes each in increasing concentrations of ethanol (70%, 90%, 100%). Once the alcohol was removed, propylene oxide (Merck, Darmstadt, Germany) was added to the gel and this was left to incubate for 20 minutes. Upon removal of the propylene oxide, propylene oxide : Spurr's resin (1:1, $^{v}/_{v}$) was aliquotted into the vial containing the sample and was incubated for 20 minutes. This was then removed and Spurr's resin (TAAB Laboratories, United Kingdom) (Appendix E) was added ensuring that the sample was completely immersed. The sample was then incubated in an oven (60°C) for 45 minutes. The resin was thereafter removed. As this was not done in vacuo the sample was once again incubated in Spurr's resin at 60°C for 45 minutes. Thereafter the sample was removed from the vial using an orange stick and placed into a polythene capsule (size 00, BEEM). Within this capsule, the sample was embedded in Spurr's resin and incubated in an oven (60°C) for 48 hours allowing the resin to harden (Singh and Ariatti, 2003). The resultant blocks were sectioned with glass knives using a Reichert Ultracut ultramicrotome. Ultrathin sections (50-60 nm) were collected onto uncoated copper 200 mesh grids. Sections were double stained with uranyl acetate (Merck, Darmstadt, Germany) and lead citrate (Merck, Darmstadt, Germany) for 2-3 minutes respectively.

The stained sections were viewed on the Joel 1010 Transmission Electron Microscope and images were photographed using Kodak fine grain electron sensitive plate film or digitised.

2.4 Preparation of Asialoorosomucoid (AOM)

The AOM was prepared by desialylation of the orosomucoid.

2.4.1 Desialylation of the Orosomucoid

The orosomucoid (Sigma-Aldrich Chemical Company, St. Louis, MO, USA) was desialylated using the insoluble neuraminidase enzyme (from *Clostridium perfringens)* (Sigma-Aldrich Chemical Company, St. Louis, MO, USA). This enzyme is attached to beaded agarose, type IVA, 0.625 units/ml. The method used was conducted under aseptic conditions and was adapted from that of Kawasaki and Ashwell (1977) (Singh, 1998). Approximately 3 units of enzyme was washed three times with sterile distilled water (5 ml) and twice with sterile 0.1 M sodium acetate (NaOAc) (pH 4.5) (5 ml) (Appendix B). For each washing step, the sample was centrifuged in a sterile 12 ml centrifuge tube (Sterilin) for 10 minutes at 3000 rpm. Finally, the insoluble enzyme pellet was resuspended in 0.1 M NaOAc (pH 4.5) (Appendix B) (7.5 ml). The orosomucoid (25 mg, 0.625 μmoles) was dissolved in 0.1 M NaOAc (pH 4.5) (Appendix B) (17.5 ml) and

filter sterilised using a Millex GV-low binding 0.22 μm filter (Millipore Corporation, USA), into a McCartney bottle. The insoluble enzyme preparation (7.5 ml) was then added to the orosomucoid solution (17.5 ml). This digestion mixture was then placed on a Wheaton roller culture apparatus and gently rotated over a period of 24 hours at 37°C.

Samples (75 μl) of this digestion mixture were removed hourly for 8 hours. These samples were diluted to 150 μl using 0.1 M NaOAc (pH 3.6) (Appendix B). The samples were then passed through a Millex GV4-0.22 μm-low binding filter and were retained for High performance liquid chromatography (HPLC) analysis (2.4.2). After 24 hours, the reaction mixture was removed from the Wheaton roller culture apparatus and another 75 μl sample was removed and treated in the same way as the other samples for HPLC analysis. A further 75 μl sample was retained to be assayed for sialic acids using the Thiobarbituric assay (2.4.3). The remainder of the reaction was centrifuged at 3000 rpm for 10 minutes. The supernatant (protein) was retained and the pellet (insoluble enzyme) was washed three times with sterile distilled water (4 ml). The supernatant was then dialysed for 4 days against distilled water with frequent water changes. The dialysates were then cooled in a Cryocool Immersion Cooler CC-80 and then lyophilised over 24 hours using a Xerotec vacuum freeze dryer to a fluffy white precipitate.

The desialylation of the orosomucoid was confirmed by agarose gel electrophoresis. Proteins are usually run on sodium-dodecyl sulphate polyacrylamide gels by sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). However, the use of a 1% agarose gel was sufficient.

Orosomucoid (5.5 mg) was dissolved in 0.1 M NaOAc (pH 4.5) (Appendix B) (3.85 ml). Varying concentrations of this were run against the same concentrations of the dialysates and comparisons were made. Agarose gel electrophoresis (Appendix C) was performed on a 1% gel containing no ethidium bromide (Appendix C) using Laemmli 2X concentrate (Merck, Darmstadt, Germany) as the gel loading buffer at 70 V for approximately 1 hour. Gels were stained with Coomassie Brilliant Blue (Merck, Darmstadt, Germany) and then destained with methanol/acetic acid solution (Merck, Darmstadt, Germany) (Appendix F). Gels were then viewed and photographed using white light (ChemiDoc XRS, Biorad).

The results obtained from this procedure enabled a distinct banding pattern to be established between the orosomucoid and the dialysates.

2.4.2 High Performance Liquid Chromatography (HPLC) Analysis of the Digestion Samples

The samples were analysed using a TSK-GEL-DEAE-5PW anion exchange column (Tosohaas), and a Bischoff isocratic HPLC pump (Appendix G). The UV absorbance was measured at 254 nm using a Dupont 860 Absorbance Detector. The column was washed and equilibrated with 0.1 M NaOAc buffer (pH 3.6) (Appendix B). Samples (50 µl) were injected into the sample loop and passed through the column which was eluted with the equilibration buffer at a flow rate of 1 ml/minute. Sensitivity was set at 3 and chart speed

at 1 cm/minute. Fractions (0.5 ml) were collected at 0.5 minute intervals using an Isco fraction collector at a constant flow rate of 1 ml/minute.

2.4.3 The Thiobarbituric Assay for Sialic Acids

The method used to detect and measure the amount of sialic acid released was adapted from Warren (1959). This procedure detects the released sialic acids only and not the bound sialic acid. To a sample containing up to 0.05 µmoles of N-acetylneuraminic acid (sialic acid) in a volume of 0.2 ml, periodate solution (0.2 M sodium periodate in 9 M phosphoric acid) (0.1 ml) (Appendix B) was added. The tubes were shaken and kept for 20 minutes at room temperature. Arsenite solution (10% $^{w}/_{v}$ sodium arsenite in 0.5 M sodium sulphate-0.1 N sulphuric acid solution) (1 ml) (Appendix B) was added. The tubes were shaken until the resulting yellow-brown colour disappeared. Thiobarbituric acid solution (0.6% $^{w}/_{v}$ 2-thiobarbituric acid in 0.5 M sodium sulphate) (3 ml) (Appendix B) (Merck, Darmstadt, Germany) was added. The tubes were shaken and heated for 15 minutes in a boiling water bath. The samples were then cooled in cold water for 5 minutes. The entire 4.3 ml of aqueous solution was extracted with an equal volume of cyclohexanone (Merck, Darmstadt, Germany). The tubes were then shaken and centrifuged for 3 minutes at 3000 rpm. The upper red organic phase was removed and the OD determined at 549 nm. A blank tube (0.2 ml distilled water) and three standard samples (0.01, 0.03 and 0.05 µmoles sialic acid) were prepared following the same

procedure as for the sample. These standard samples were used to produce a standard curve for determination of the released sialic acid residues from the digestion mixture.

2.4.4 [^{3}H] Aminohexanoyl Biotinylated Asialoorosomucoid

This method was used to determine the binding ratio of aminohexanoyl biotin to the AOM molecule and was adapted from Schoeman *et al.* (1995). To a solution of 0.48 ml of AOM (2.8 mg, 0.07 μmoles) in 0.15 M NaCl, 0.5 M sodium bicarbonate ($NaHCO_3$) (0.1 ml) was added. [^{3}H] aminohexanoyl-biotin-N-hydroxysuccinimide (0.11 mg, 0.25 μmoles) (11 μl) (Merck, Darmstadt, Germany) in dimethylformamide (Merck, Darmstadt, Germany) was added. The reaction mixture was kept at 20°C in the dark for 2 hours and thereafter dialysed at 5°C against 0.15 M NaCl with three changes of saline. The [^{3}H] biotinylated AOM was stored at -10°C. The radioactivity was determined using a Packard 1900 TR scintillation spectrophotometer / counter (Appendix H). The ratio of biotin to AOM was then calculated.

2.4.5 Aminohexanoyl Biotinylated Asialoorosomucoid

Once the ratio of biotin to AOM was established the above coupling reaction was performed again using non-radioactive aminohexanoyl-biotin-N-hydroxysuccinimide.

To a solution of AOM (0.5 ml, 2.44 mg, 0.061 μmoles) in 0.15 M NaCl was added 0.5 M $NaHCO_3$ (0.1 ml) and aminohexanoyl-biotin-N-hydroxysuccinimide (0.083 mg, 0.183 μmoles) (9 μl) in dimethylformamide. The reaction mixture was kept at 20°C in the dark for 2 hours and thereafter dialysed at 5°C against 0.15 M NaCl with three changes of saline. The biotinylated AOM was stored at -10°C.

2.4.6 Protein Determination of Aminohexanoyl Biotinylated Asialoorosomucoid and [^{3}H] Aminohexanoyl Biotinylated Asialoorosomucoid

The protein concentration of aminohexanoyl biotinylated AOM and [^{3}H] aminohexanoyl biotinylated AOM was determined using the Bicinchoninic Acid (BCA) Protein assay kit (Sigma Chemical Company, USA) (Appendix I).

2.4.7 Streptavidin-Biotinylated Asialoorosomucoid Interactions

2.4.7.1 Preparation of Streptavidin Aminohexanoyl Biotinylated Asialoorosomucoid

Streptavidin (1 mg, 0.016 μmol) (Sigma Chemical Company, USA) was dissolved in 817 μl 0.2 M NaCl, 0.005 M Tris-HCl, pH 7.6 (Appendix B). An equimolar amount of

aminohexanoyl biotinylated AOM (0.64 mg, 0.061 μmol, 183 μl) was then added to this, i.e. a molar ratio of 1:1. This constituted a concentration of 1.64 mg/ml i.e. 1.64 μg/μl. Approximately 500 μl was removed and equally aliquotted into 3 smaller tubes of 167 μl each and stored at -20°C. To the remaining 500 μl, sterile glycerol (500 μl) was added constituting a concentration of 0.82 μg/μl. This was then further equally aliquotted (333 μl) into 3 tubes and stored at -20°C.

2.4.7.2 AH (Aminohexyl) Sepharose 4B – Biotinylation

To 50 mg AH Sepharose powder (Sigma Chemical Company, USA), 0.1 M $NaHCO_3$ (1.5 ml) was added and allowed to swell. This was washed by pulse centrifugation and the supernatant discarded to remove dextran and lactose contained in the powder. This washing step was done three times. Thereafter, 0.1 M $NaHCO_3$ containing 0.1 M NaCl (1.2 ml) was added. Aminohexanoyl-biotin-N-hydroxysuccinimide (1.4 mg, 0.00308 μmoles) (50 μl) in dimethylformamide was added. This was rocked for 2-4 hours and thereafter stored overnight at 4°C. Washing then followed under a vacuum using 0.1 M $NaHCO_3$ containing 0.1 M NaCl. The biotinylated AH Sepharose 4B was then stored in 0.1 M $NaHCO_3$ containing 0.1 M NaCl (1.5 ml) with 1 mM EDTA to prevent growth of micro-organisms.

2.4.7.3 AH (Aminohexyl) Sepharose Assay for [^{3}H] Streptavidin Aminohexanoyl Biotinylated Asialoorosomucoid

In this experiment, streptavidin is combined with [^{3}H] aminohexanoyl biotinylated AOM and binding is observed radioactively. To a solution of streptavidin (180 μg; 0.003 μmoles) in 0.15 M NaCl (50 μl) was added [^{3}H] aminohexanoyl biotinylated AOM (0.003 μmoles; 3086 dpm) in saline (30 μl). After 45 minutes incubation at room temperature, the mixture was added to the biotinylated AH sepharose (50 μl gel; 0.1 μmoles with respect to biotin). After a further incubation at 30 minutes, the gel was sedimented at 11 000 rpm in a microcentrifuge. The supernatant (80 μl) was mearsured out and counted for radioactivity by liquid scintillation in a Packard 1900 TR scintillation spectrophotometer / counter (Appendix H).

2.5 Gel Retardation Assays

2.5.1 DNA-Liposome Interactions

Reaction mixtures with a total volume of 20 μl were prepared using 1 μg pGL3 plasmid DNA, increasing amounts of the liposome preparation (1-7 μg) and HBS (20 mM HEPES; 150 mM NaCl, pH 7.5) (Merck, Darmstadt, Germany) (Appendix B). The mixtures were incubated for 25-30 minutes at 20°C. Samples were then subjected to agarose gel electrophoresis on 1% agarose gel containing ethidium bromide (Appendix

C) at 70 V for approximately 1 hour. Gels were then viewed (Hoefer) and photographed (ChemiDoc XRS, Biorad).

2.5.2 DNA-Liposome-Asialoorosomucoid Interactions

Reaction mixtures with a total volume of 25 µl were prepared using 1 µg pGL3 plasmid DNA, liposome preparation (3 and 4 µg), streptavidin aminohexanoyl biotinylated AOM preparation (9-11 µg) and HBS (20 mM HEPES, 150 mM NaCl, pH 7.5) (Appendix B). The mixtures were incubated for 25-30 minutes at 20°C. Samples were then subjected to agarose gel electrophoresis on 1% agarose gel containing ethidium bromide (Appendix C) at 70 V for approximately 1 hour. Gels were then viewed (Hoefer) and photographed (ChemiDoc XRS, Biorad).

2.6 Ethidium Bromide Intercalation Assay

This assay is also referred to as the ethidium bromide displacement assay and was carried out to clarify intercalative activity of compounds.

2.6.1 DNA-Liposome Interactions

To a cuvette was added 500 µl HBS (20 mM HEPES, 150 mM NaCl, pH 7.5) (Appendix B) and 10 µl 100 mg/ml ethidium bromide (Merck, Darmstadt, Germany). This was used to set the baseline value for spectrofluorometric readings. The spectrofluorometric detector (SHIMADZU RF – 551 Spectrofluorometric detector) was set to an excitation wavelength of 520 nm and an emission wavelength of 600 nm. Approximately 6 µg of pBR322 DNA was added and a reading was obtained. Aliquots (1 µl) of liposome was added to the solution until a total volume of 30 µg was added. Solutions were mixed thoroughly after each addition. Results were plotted relative to 100% fluorescence.

2.6.2 DNA-Liposome-Asialoorosomucoid Interactions

To a cuvette was added 500 µl HBS (20 mM HEPES, 150 mM NaCl, pH 7.5) (Appendix B) and 10 µl 100 mg/ml ethidium bromide. This was used to set the baseline value for spectrofluorometric readings. The spectrofluorometric detector was set to an excitation wavelength of 520 nm and an emission wavelength of 600 nm. Approximately 6 µg of pBR322 DNA complexed to 24 µg liposome suspension was added and a reading was obtained. Aliquots (4 µl) of streptavidin aminohexanoyl bioyinylated AOM was added to the solution until a total volume of 65.6 µg was added. Solutions were mixed thoroughly after each addition. Results were plotted relative to 100% fluorescence.

2.7 Characterisation of Interactions of the Components of the Transfection Complex

2.7.1 Characterisation of DNA-Lipoplex Complex (Lipoplex) by Transmission Electron Microscopy (TEM)

Once the DNA-liposome binding ratio was obtained lipoplexes at this binding ratio as well as ratios lower and higher were prepared and incubated for 25-30 minutes at 20°. To one drop of the respective lipoplex suspension on parafilm was added 0.5% uranyl acetate. This was mixed and allowed to stand for three minutes. The matt surface of formvar-coated grids were then brought into contact with the lipoplex - uranyl acetate mixture for 3 minutes. Thereafter discs / grids were air dried overnight, viewed in a Jeol 1010 transmission electron microscope at 60 kV and photographed using Kodak fine grain electron sensitive plate film or digitised.

2.7.2 Characterisation of DNA-Liposome-Asialoorosomucoid Complex by Transmission Electron Microscopy (TEM)

Once the DNA-liposome-AOM binding ratio was obtained complexes at this binding ratio as well as the ratios higher and lower were prepared and incubated for 25-30 minutes at 20°C. Samples were prepared as in 2.7.1.

2.8 Nuclease Digestion Assay

Reaction mixtures with a total volume of 25 µl were prepared using 1 µg plasmid DNA, liposome preparation (1-6 µg), streptavidin aminohexanoyl biotinylated AOM preparation (9 µg) and HBS (20 mM HEPES, 150 mM NaCl, pH 7.5) (Appendix B). The mixtures were incubated for 30 minutes at 20°C. Fetal Bovine Serum (FBS) (Gibco-BRL, Life Technologies Ltd., Inchinnan, Scotland) (10%) was then added to all tubes except the control containing only DNA. These tubes were incubated at 37°C for 4 hours. EDTA was added to a final concentration of 10 mM. Thereafter sodium-dodecyl sulphate (SDS) (Sigma Chemical Company, USA) was added to a final concentration of 0.5% ($^{v}/_{v}$). Samples were then incubated for 20 minutes at 55°C and thereafter subjected to agarose gel electrophoresis on 1% agarose gel containing ethidium bromide (Appendix C) at 70 V for approximately 1 hour. Gels were then viewed (Hoefer) and photographed (ChemiDoc XRS, Biorad).

2.9 Tissue Culture Studies

2.9.1 Maintenance and Growth of HepG2 cells

Cells were obtained from the ATCC (American Tissue Culture Collection) and purchased from Highveld Biologicals (Pty) Ltd., Johannesburg, South Africa. On receipt of the cells

(2 x 25 cm^2 flasks), the flasks were incubated overnight at 37° C (LTE A Searle Company Qualitemp 80 TC). The original medium was thereafter removed and fresh medium (Minimal Essential Medium (MEM) (Highveld Biologicals (Pty) Ltd., Johannesburg, South Africa) + 10% Fetal Bovine Serum (FBS))(Appendix J) was added. Alternatively, cells can be trypsinised and split into desired / recommended ratios. Cells were observed daily and the medium changed when necessary.

2.9.1.1 Trypsinisation Procedure

Cells are normally trypsinised when they become confluent or almost confluent. Trypsinisation allows for the breaking down of intracellular protein bonds. 1-2 hours prior to trypsinisation, the following were removed from the refrigerator and warmed to 37°C : Medium (MEM, Phosphate Buffered Saline (PBS), Trypsin/Versene (0.5% trypsin) (Whittaker M.A. Bioproducts, Maryland, USA)(Appendix J). Bottles were wiped down with 70% ethanol and placed in the laminar flow hood. The Bunsen burner was ignited and trypsinisation was carried out as follows:

The medium from the culture vessel / flask was decanted into a sterile waste bottle. The neck of the flask was flamed. The cells were then rinsed with 5 ml PBS (Appendix J). The rinse medium i.e. PBS was removed and approximately 1 ml trypsin/versene was added. Trypsinisation was then allowed to proceed at room temperature or 37°C for 1-5 minutes. Rounding off of the cells was then observed using an inverted microscope

(Nikon TMS 0.2 A Japan). Thereafter the flask was tapped firmly to dislodged cells. Cells were then reconstituted with 2 ml medium (MEM) containing serum (Appendix J) in order to inhibit the enzyme. Cells were then split according to a predetermined ratio (1:3, 1:2, 1:5) into separate 25 cm^2 flasks each containing 5 ml medium with serum (MEM +10% FBS) (Appendix J). This was done by using a 1 ml pipette and drawing up the cell suspension and expelling it repeatedly to evenly distribute the cells prior to dividing them into flasks. The neck of the flasks was flamed before screwing on the lids tightly and placing them in the incubator at 37°C. The cells were re-trypsinised after 3-5 days once cells reached confluency.

2.9.1.2 Cryopreservation Procedure

Flasks containing cells at or near confluency were selected. Cells were dislodged by trypsinisation and pelleted by centrifugation at 1000 rpm for 5 minutes. Cells were then resuspended in 0.9 ml MEM (with serum) (Appendix J) and 0.1 ml of cryoprotective medium, dimethylsulfoxide (DMSO) (Merck, Darmstadt, Germany). This was mixed well and aliquotted into suitable cryovials for freezing in liquid nitrogen or a -80°C biofreezer. All vials were sealed with plastic prior to freezing. The freezing process was started within one hour of adding the cryoprotective medium. The cells were frozen at a rate of 0.5-2 degrees per minute to a temperature of about -70°C using a cold probe. Cells were stored long term in liquid nitrogen and short term at -80°C in a biofreezer.

2.9.1.3 Reconstitution Procedure

The vials containing the cells were removed from liquid nitrogen / the biofreezer wearing protective gloves. Vials were then placed into a 37°C water bath and thereafter disinfected with 70% ethanol. Vials were opened aseptically in a laminar flow hood and poured into tubes. Cells were centrifuged at 1000 rpm for 5 minutes. This resulted in the cells forming a pellet at the base of the tube. Old medium from cells was decanted and 1 ml fresh medium containing serum was added. This was then transferred to a new flask containing 5 ml medium and serum. Flasks were then placed into the incubator at 37°C. The cells were allowed to attach to the flask overnight before replacing the medium. This was done so that any remaining cryoprotective medium which is harmful to the cells and any dead cells that may be present was removed.

2.10 Transfection Studies in Tissue Culture : Gene Transfer Experiments

2.10.1 Growth Inhibition Assay

HepG2 cells were trypsinised and seeded into a 24-well plate at a seeding density of 2 x 10^4 cells/well. Cells were incubated for 24 hours and allowed to attach to the wells and grow to semi-confluency. Reaction mixtures with a total volume of 25 µl were prepared using 1 µg plasmid DNA, liposome preparation (2-5 µg), streptavidin aminohexanoyl biotinylated AOM preparation (9 µg) and HBS (20 mM HEPES, 150 mM NaCl, pH 7.5)

(Appendix B). This was done in quadruplicate. These complexes were then incubated for 30 minutes at 20°C. The 24-well plate was removed from the incubator (37°C) and the cells were prepared by first removing the growth medium (MEM) containing FBS and replacing it with serum free medium (MEM) (0.5 ml). The reaction complexes were then added into the corresponding wells containing the HepG2 cells within the 24-well plate and this was incubated for 4 hours at 37°C. The serum free medium was then removed and replaced with complete medium containing FBS and antibiotics (0.5 ml). This was then incubated at 37°C for a further 48 hours. Thereafter, the cells were washed twice with PBS (Appendix J) and stained with 200 µl crystal violet solution (0.5% $^{w}/_{v}$ crystal violet, 0.8% $^{w}/_{v}$ NaCl, 5% $^{v}/_{v}$ formaldehyde, 50% $^{v}/_{v}$ ethanol) for 20 minutes. The stain was then removed and the cells were washed extensively with water. The 24-well plate was then dried for 24 hours and the stain was extracted with 2-methoxyethanol (0.5 ml) over a 36 -hour period with gentle rocking (10 rev/minute) on a platform shaker (Stuart Scientific STR 6). Absorbance values for the samples were then read at a wavelength of 550 nm (Philip PU 8700 series UV / visible spectrometer).

2.10.2 Evaluation of Gene Expression

2.10.2.1 Transfection of HepG2 Cells with Transfection Complexes

HepG2 cells were trypsinised and seeded into a 24-well plate at a seeding density of 2 x 10^4 cells/well. Cells were incubated for 24 hours and allowed to attach to the wells and

grow to semi-confluency. Reaction mixtures with a total volume of 25 µl were prepared using 1 µg plasmid DNA, liposome preparation (2-5 µg), streptavidin aminohexanoyl biotinylated AOM preparation (9 µg) and HBS (20 mM HEPES, 150 mM NaCl, pH 7.5) (Appendix B). This was done in quadruplicate. These complexes were then incubated for 30 minutes at 20°C. Cells were prepared by first removing the growth medium containing FBS, washing them with PBS (Appendix J), and replacing it with serum free medium (0.5 ml). The reaction complexes were then added into the corresponding wells containing the HepG2 cells within the 24-well plate and this was incubated for 4 hours at 37°C. The serum free medium was then removed and replaced with complete medium with 10% FBS and antibiotics (0.5 ml). This was then incubated at 37°C for 48 hours. The luciferase assay was then carried out as in 2.10.2.4.

2.10.2.2 Competition Assay

HepG2 cells were trypsinised and seeded into a 24-well plate at a seeding density of 2.2 x 10^4 cells/well. Cells were incubated for 24 hours and allowed to attach to the wells and grow to semi-confluency. Reaction mixtures were set up in quadruplicate as in 2.10.2.1. These complexes were then incubated for 30 minutes at 20°C. Cells were prepared by first removing the growth medium containing FBS, washing them with PBS (0.5 ml) (Appendix J), and replacing it with serum free medium (0.5 ml). The reaction complexes were then added into the corresponding wells containing the HepG2 cells within the 24-well plate and this was incubated for 4 hours at 37°C in the incubator. The serum free

medium was then removed and replaced with complete medium containing 10% FBS and antibiotics (0.5 ml). This was then incubated at 37°C for 48 hours. The luciferase assay was then carried out as in 2.10.2.4.

2.10.2.3 Transfection using FuGene 6

FuGene 6 was obtained from Roche Diagnostics, Mannheim, Germany. HepG2 cells were trypsinised and seeded into a 24-well plate at a seeding density of 2 x 10^4 cells/well. Cells were incubated for 24 hours and allowed to attach to the wells and grow to semi-confluency. The FuGene / DNA complexes were set up in quadruplicate using a 3:1, 4:1, 5:1 and 6:1 FuGene : DNA ratio. Cells were prepared by first removing the growth medium containing FBS, washing them with PBS (0.5 ml) (Appendix J), and replacing it with serum free medium. The complexes were then incubated for 15 minutes at 20°C in the serum free medium to a total volume of 100 µl. These reaction complexes were then added into the corresponding wells containing the HepG2 cells within the 24-well plate and this was incubated for 4 hours at 37°C. The serum free medium was then removed and replaced with complete medium. This was then incubated at 37°C for a further 48 hours. The luciferase assay was then carried out as in 2.10.2.4.

2.10.2.4 Luciferase Assay

The luciferase assay was carried out using the Promega Luciferase Assay System (Promega Corporation, Madison, USA). The luciferase assay reagent (20 mM tricine, 1.1 mM magnesium carbonate hydroxide pentahydrate, 2.7 mM magnesium sulphate, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 µM coenzyme A, 470 µM luciferin, 530 µM adenosine-tri-phosphate (ATP)), was prepared by adding 10 ml of the given luciferase assay buffer to one vial of lyophilised luciferase assay substrate. The cell culture lysis reagent (5X) (25 mM tris-phosphate, pH 7.8; 2 mM dithiothreitol, 2 mM 1.2-diaminocyclohexane-N,N,N',N'-tetra-acetic acid, 10% $^{v}/_{v}$ glycerol, 1% $^{v}/_{v}$ triton X-100), was diluted with distilled water to produce a 1X stock. Both reagents were equilibrated to room temperature.

The cells were prepared by removing the growth medium. The cells were then washed twice with PBS (Appendix J) (0.5 ml) being careful not to dislodge any of the attached cells. A minimal amount of 1X cell culture lysis reagent (60 µl) was added to cover the cells in each well. The plate was then rocked gently (10 rev/minute) on a platform shaker for 15 minutes. The attached cells were then dislodged from the wells and the cell solution was transferred to a microcentrifuge tube. The cells were spun briefly for approximately 30 seconds in an eppendorf microcentrifuge at 13 000 rpm to pellet the large debris. The cell free extracts (supernatants) were retained to be assayed for luciferase activity. To 20 µl of cell free extract at room temperature was added 100 µl of luciferase assay reagent. The reaction mixture was immediately mixed and placed in a

luminometer (Lumac Biocounter 1500). The light produced was measured for a period of 10 seconds. The protein determination of the cell free extracts was determined using the Bicinchoninic Acid (BCA) Protein assay kit (Sigma Chemical Company, USA) (Appendix I).

CHAPTER 3

RESULTS

3.1 Preparation of DNA

3.1.1 Preparation of Competent Cells

Spectrophotometric readings at 590 nm were recorded at half hour intervals and were as follows (Table 3.1):

Table 3.1 : Spectrophotometer readings (590 nm)

	Optical Density ($OD_{590\ nm}$)
1	0.082
2	0.137
3	0.315
4	0.425

An $OD_{590\ nm}$ reading of 0.415 exceeds the optimum $OD_{590\ nm}$ reading (0.375). However, *E.coli* have a tolerance level of 0.60. It is therefore acceptable to proceed with an $OD_{590\ nm}$ reading of 0.415.

3.1.2 Heat Shock Transformation

Plating resulted in the formation of colonies on the LB agar plates containing ampicillin. The presence of colonies on the plate (Figure 3.1) indicated the presence of the Amp^r gene concluding that the *E.coli* cells were in fact transformed with the plasmid.

Figure 3.1 : Photograph of LB agar ampicillin plates with colonies

Five plates were used and the colony counts in each of these plates were as follows: 372; 436; 452; 764 and 841. An average colony count of 573 was obtained.

3.2 Confirmation of Plasmid DNA

Following isolation of plasmid DNA as per protocol, spectrophotometer readings and agarose gel electrophoresis were carried out to confirm that the pGL3 control plasmid vector DNA was indeed isolated.

Spectrophotometric readings at 260 nm produced the following: an absorbance of 34.5 for sample in lane 2 and 23.7 for sample in lane 3 (Figure 3.2). The concentrations obtained of the samples prior to dilution were 1.725 µg/µl for sample in lane 2 and 1.185 µg/µl for sample in lane 3 (Figure 3.2). The ratios (260 nm: 280 nm) obtained were 1.367 and 1.172 respectively.

The pGL3 control vector obtained from Promega was used as a control and agarose gel electrophoresis (Appendix C) was carried out using the isolated DNA as test samples. Interpretation of the ethidium bromide gel (Figure 3.2) is as follows.

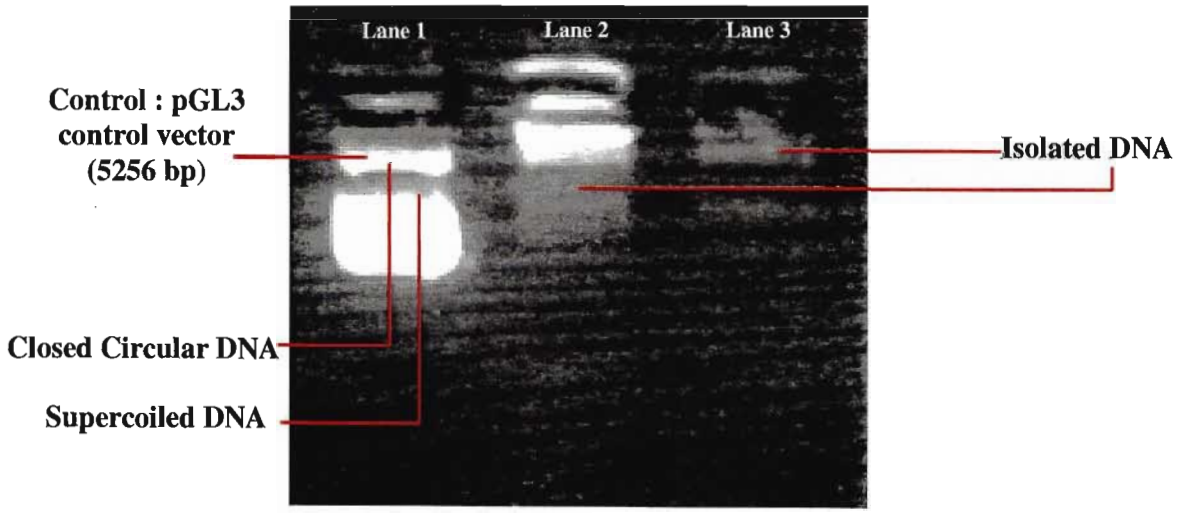


Figure 3.2: Ethidium bromide gel of pGL3 control vector vs. isolated DNA

Lane 1 contains the control and lanes 2 and 3 contain diluted samples of decreasing concentrations respectively. The presence and positions of bands in lanes 2 and 3

indicate the presence of the plasmid DNA as they correspond to the control concluding that isolation was successful.

3.3 Synthesis of Liposomes

3.3.1 Confirmation of Synthesis of 3β[N-(N',N'-dimethylaminopropane) carbamoyl] cholesterol (Chol-T) by Thin Layer Chromatography (TLC)

TLC enabled us to confirm the synthesis of the desired product Chol-T by comparison of a standard Chol-T sample, the reaction mixture of Chol-T taken prior to crystallisation and Chol-T crystals obtained after crystallisation i.e. the end product. The result can be seen in Figure 3.3.

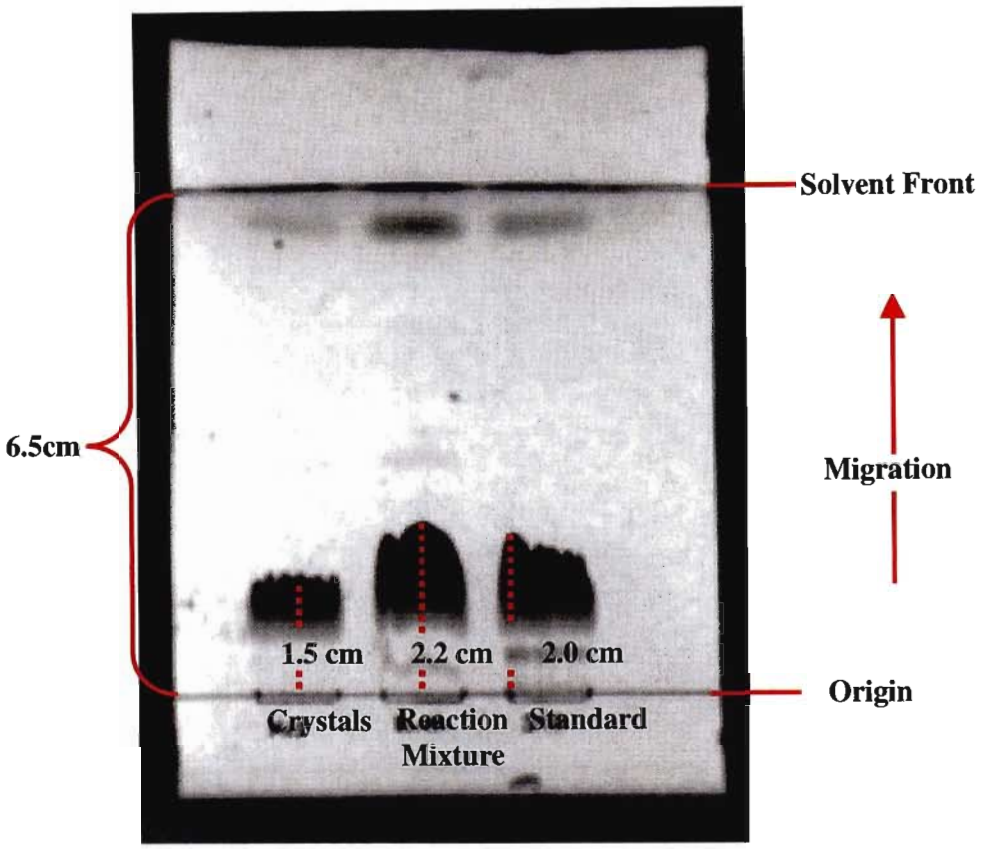


Figure 3.3 : TLC plate showing samples of Chol-T

The R_f values calculated were 0.307 for the standard Chol-T sample, 0.338 for the reaction mixture of the Chol-T sample prior to crystallisation and 0.230 for the Chol-T crystals after crystallisation.

The R_f values are very similar for all three samples. In addition, the migration patterns also show similarity. These indicate successful synthesis of Chol-T.

3.3.2 Characterisation of Liposomes by Transmission Electron Microscopy (TEM)

Transmission electron microscopy confirmed the presence of liposomes dispersed within a proteinaceous debris (Figures 3.4 and 3.5). Liposomes were observed occurring either singly (Figure 3.4) or in clusters (Figure 3.5). Ultrastructurally they appeared as spherical (Figure 3.4) to irregularly shaped moieties (Figure 3.6). The internal cavities showed electron-lucent homogenous areas which are typical of lipid droplets (Figure 3.5). The liposomes were encapsulated by bi-layered membranes (Figure 3.7) and were in the size range of 100 – 300 nm.

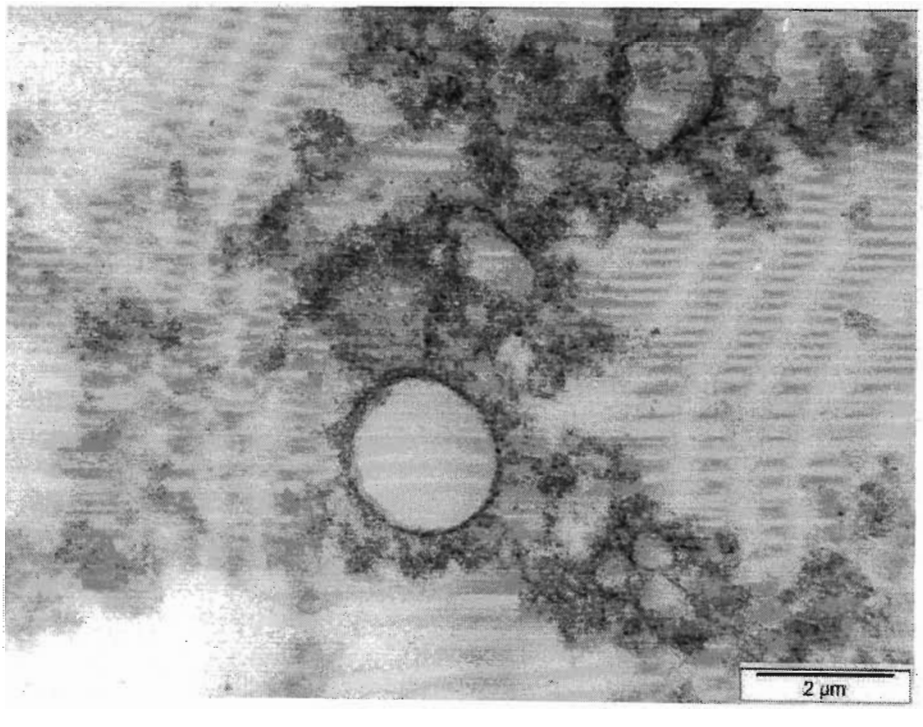


Figure 3.4 : TEM of liposome occurring singly (x 20 k)

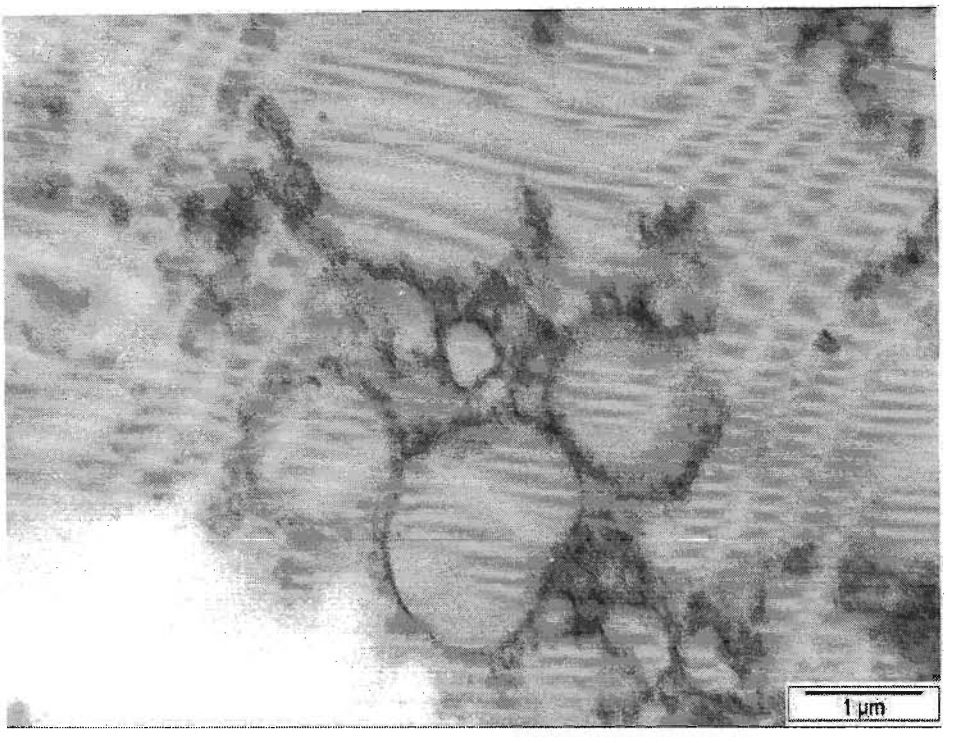


Figure 3.5 : TEM of lipsomes occurring as clusters (x 40 k)

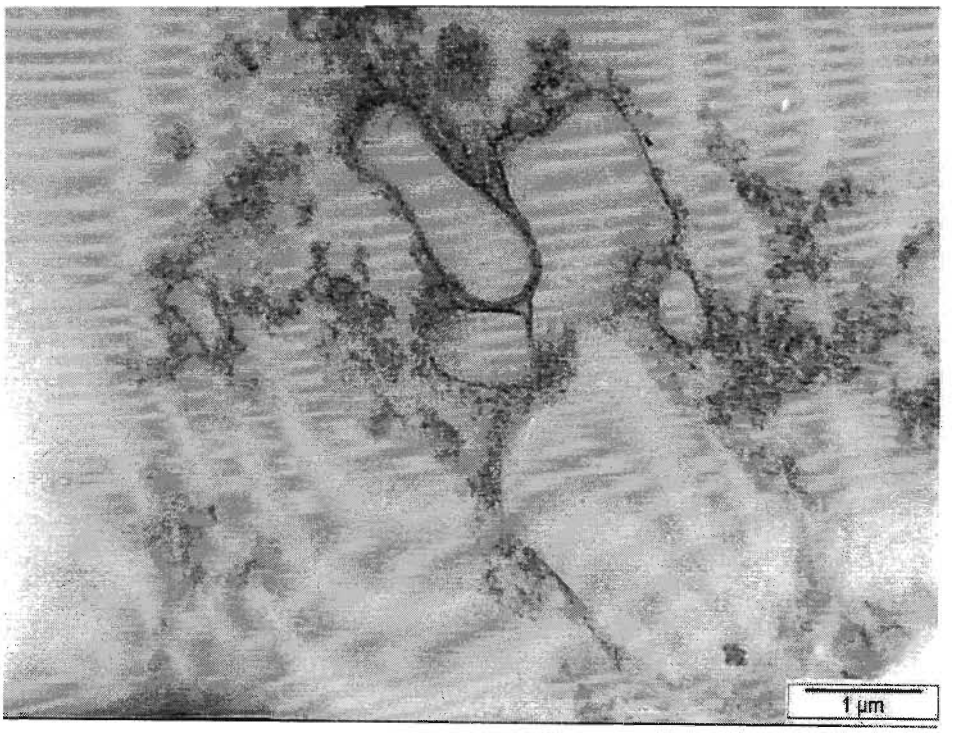


Figure 3.6 : TEM of irregularly shaped moieties of liposomes (x 30 k)

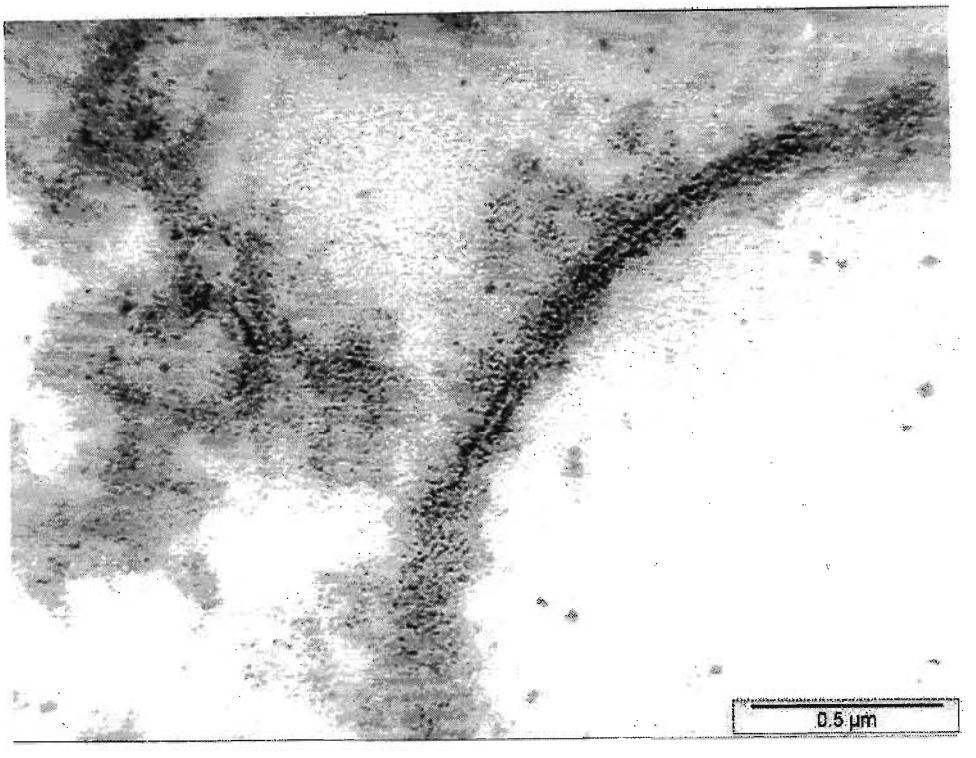


Figure 3.7 : TEM showing bilayered membrane of liposome (x 120 k)

3.4 Preparation of Asialoorosomucoid (AOM)

3.4.1 Desialylation of the Orosomucoid

Confirmation of desialylation of the orosomucoid was obtained by agarose gel electrophoresis, HPLC analysis and the thiobarbituric acid assay for sialic acids. For agarose gel electrophoresis, varying concentrations of the orosomucoid were run against the same concentrations of the dialysates and comparisons were made (Figure 3.8).

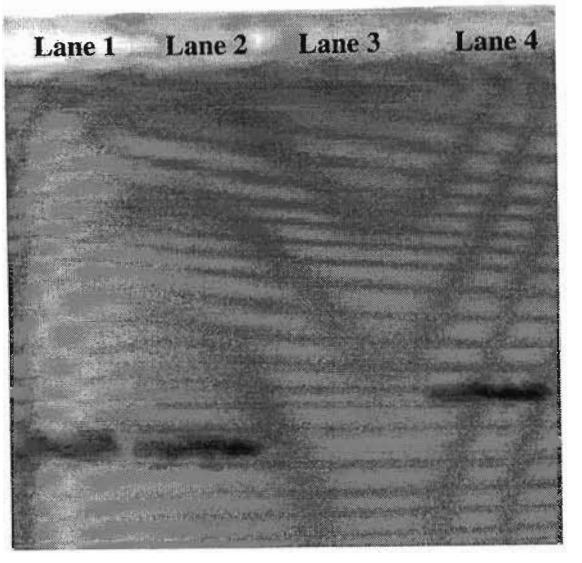


Figure 3.8 : Distinct banding pattern confirming desialylation of the orosomucoid
Lanes 1 and 2 contain orosomucoid, 20 µl and 10 µl respectively. Lane 3 contains 10 µl of the dialysate. Lane 4 contains 20 µl of the dialysate.

The negatively charged orosomucoid, like DNA migrated forward towards the positive electrode. The dialysate i.e. AOM carries a less positive charge due to the removal of sialic acid residues, and therefore migrated a shorter distance as compared to the orosomucoid which migrated a further distance away from the well as seen in Figure 3.8. Both protein bands can be seen within the gel matrix indicating that the protein is negatively charged and has migrated in the electric field due to this charge (lanes 1 and 2). No bands are seen in lane 3 probably because the concentration was not high enough to be detected. A band is clearly visible in lane 4 indicating that protein is indeed present. However, this band has migrated a shorter distance as compared to those of lanes 1 and 2 indicating that the protein in this lane carries a less negative charge. A difference in the banding pattern is thus established confirming that desialylation has been successful.

3.4.2 High Performance Liquid Chromatography (HPLC) Analysis of the Digestion Samples

HPLC analysis revealed that the orosomucoid was successfully desialylated. The chromatograms obtained show differences in the retention time of the orosomucoid and the AOM. The retention times of the AOM was less than that of the standard orosomucoid. The chromatogram showed that the orosomucoid peaked at a retention time of 3 minutes and 23 seconds (Figure 3.9) and AOM i.e. the desialylate peaked earlier at 1 minute and 86 seconds (Figure 3.10). Furthermore, a mixture of AOM together with orosomucoid produced two successive peaks confirming this theory. Figure 3.11 shows two peaks, the first peak represents the AOM and the second peak represents the orosomucoid.

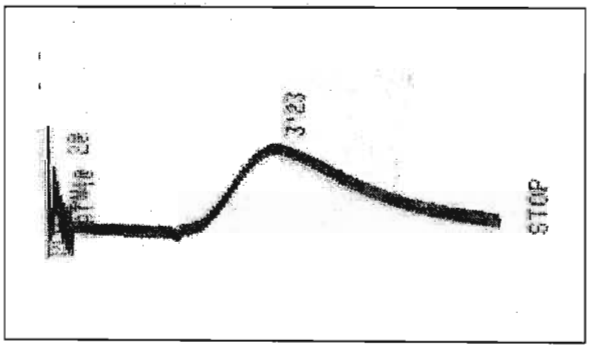


Figure 3.9 : Chromatogram showing HPLC analysis of orosomucoid

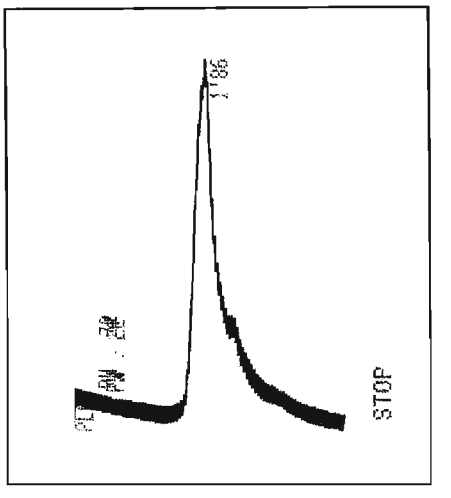


Figure 3.10 : Chromatogram showing HPLC analysis of AOM

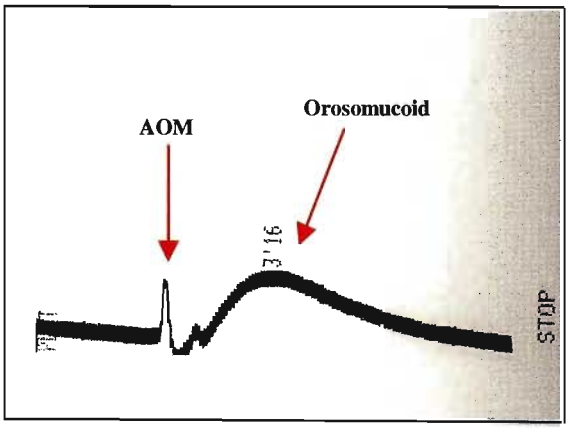


Figure 3.11 : Chromatogram showing HPLC analysis of mixture of AOM and orosomucoid

3.4.3 The Thiobarbituric Assay for Sialic Acids

This assay further confirmed the desialylation of the orosomucoid. The sialic acid released was detected and measured using the thiobarbituric acid assay. Spectrophotometer readings were taken to detect the OD of the sample. Three standard samples (0.01, 0.03 and 0.05 μmoles) were used and the readings (Table 3.2) taken as comparison for that of the sample.

Table 3.2 : Spectrophotometer readings of standards(549 nm)

Standard (μmoles)	Optical Density ($OD_{549\ nm}$)
0.01	0.16
0.03	0.2
0.05	0.7

The optical density obtained for the sample was 0.119. A standard curve for sialic acids (Figure 3.12) was constructed using the standard readings (Table 3.2). The OD of the sample (0.119) corresponded to approximately 0.0089 μmoles of *N*-acetylneuraminic acid on Figure 3.12.

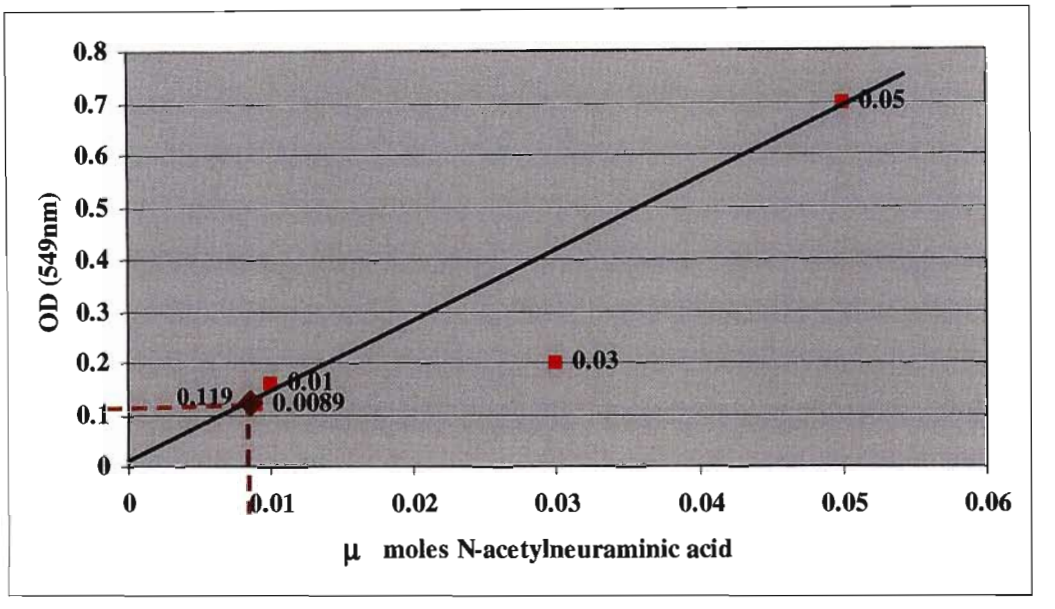


Figure 3.12 : Standard curve – for *N*-acetylneuraminic acid

The number of moles of sialic acid released per 75 μl sample was determined from the standard curve to be approximately 0.0089 μmoles.

In addition, the amount of *N*-acetylneuraminic acid present in a given sample can be calculated theoretically from the Equation :

$$\mu\text{moles } N\text{-acetylneuraminic acid} = \frac{V \times OD_{549\ nm}}{57}$$

where, V is the final volume of the test solution and 57.00 is the molecular extinction coefficient (Warren, 1959).

Hence, from the equation, the amount of *N*-acetylneuraminic acid present in the sample was determined to be 0.0089 μmoles using a volume of 4.3ml and a $OD_{549\ nm}$ reading of 0.119.

The result obtained from the standard curve is the same as that obtained theoretically thus confirming the amount of sialic acid released.

3.4.4 [^{3}H] Aminohexanoyl Biotinylated Asialoorosomucoid

This procedure allowed for determination of the binding ratio of aminohexanoyl biotin to the AOM molecule.

A 13.5 µl sample of [^{3}H] aminohexanoyl-biotin-N-hydroxysuccinimide contained 149 µg. The number of moles can be calculated using the Equation :

$$n = \frac{m}{MM}$$

where n is the number of moles, m is the mass and MM is the molar mass.

Biotin has a molar mass of 454. Therefore n = 0.328 µmoles. After counting, an activity of 166579 dpm was obtained for [^{3}H] aminohexanoyl-biotin-N-hydroxysuccinimide, therefore the specific activity of [^{3}H] aminohexanoyl-biotin-N-hydroxysuccinimide is 166579 dpm/0.328 µmoles. This is equivalent to 507863 dpm/µmole.

A 150 µl sample of AOM treated with [^{3}H] aminohexanoyl-biotin-N-hydroxysuccinimide contained 600 µg. The number of moles was calculated to be 0.015 µmoles. An activity of 15430.3 dpm was obtained for AOM treated with [^{3}H] aminohexanoyl-biotin-N-hydroxysuccinimide. Using this value and the specific activity of 507863 dpm/µmole obtained for [^{3}H] aminohexanoyl-biotin-N-hydroxysuccinimide the number of moles of [^{3}H] aminohexanoyl-biotin-N-hydroxysuccinimide in the sample containing AOM treated with [^{3}H] aminohexanoyl-biotin-N-hydroxysuccinimide was calculated to be 0.0303 µmoles [^{3}H] aminohexanoyl-biotin-N-hydroxysuccinimide. If 0.015 µmoles AOM treated with [^{3}H] aminohexanoyl-biotin-N-hydroxysuccinimide corresponds to 0.0303 µmoles [^{3}H] aminohexanoyl-biotin-N-hydroxysuccinimide, 1 µmole of AOM is equivalent to 2.02 µmole of [^{3}H] aminohexanoyl-biotin-N-hydroxysuccinimide.

It can therefore be deduced and concluded that to 1 molecule of AOM, 2 aminohexanoyl biotin molecules have bound.

3.4.5 Protein Determination of Aminohexanoyl Biotinylated Asialoorosomucoid and [^{3}H] Aminohexanoyl Biotinylated Asialoorosomucoid

The protein concentration of aminohexanoyl biotinylated AOM and [^{3}H] aminohexanoyl biotinylated AOM was determined using the Bicinchoninic Acid (BCA) Protein assay kit (Sigma) (Appendix I).

A standard curve (Figure 3.13) was constructed using BSA standards and the protein concentration in the unknown/test samples i.e. aminohexanoyl biotinylated AOM (a) and [^{3}H] aminohexanoyl biotinylated AOM (b) was determined by plotting the absorbance values obtained i.e. 0.309 and 0183 respectively, versus the BSA protein standard concentrations.

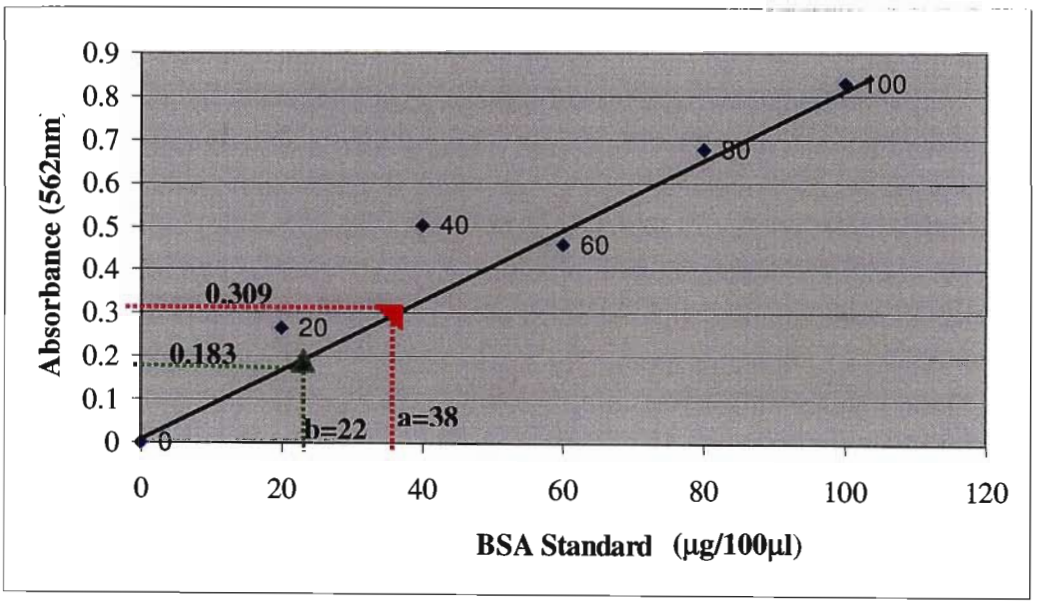


Figure 3.13 : Standard Curve produced from BCA protein assay data

The protein concentration of aminohexanoyl biotinylated AOM (a) and [^{3}H] aminohexanoyl biotinylated AOM (b) was determined from the standard curve to be approximately 38 and 22 µg/100µl of protein respectively.

The standard curve (Figure 3.13) therefore indicates the unknown/test sample aminohexanoyl biotinylated AOM ($abs_{562\ nm}$ = 0.309) contains 38 μg/100μl of protein and the unknown/test sample [^{3}H] aminohexanoyl biotinylated AOM ($abs_{562\ nm}$ = 0.183) contains 22 μg/100μl of protein.

The actual concentration of protein present in the unknown/test sample was calculated using the values obtained from the standard curve as the concentration in 20 μl and a total volume of 700 μl as the volume obtained after dialysis in the actual sample.

The protein concentration of aminohexanoyl biotinylated AOM was calculated to be 1330 μg and that of [^{3}H] aminohexanoyl biotinylated AOM was calculated to be 770 μg.

3.4.6 AH (Aminohexyl) Sepharose Assay for [^{3}H] Streptavidin Aminohexanoyl Biotinylated Asialoorosomucoid

The experiment was designed to provide a "proof of concept" for the approach adopted in this study to anchor AOM to cationic liposomes. Thus AOM was biotinylated and pre-incubated with streptavidin before introduction to biotinylated AH sepharose. Results show that 60 % of the [^{3}H] aminohexanoyl biotinylated AOM was gel bound (1850 dpm). Hence streptavidin appears to have formed an effective bridge between tritiated aminohexanoyl biotinylated AOM and the biotinylated affinity gel.

3.5 Gel Retardation Assays

3.5.1 DNA-Liposome Interactions

The formation of complexes between plasmid DNA and cationic liposomes was demonstrated in a gel retardation assay. To confirm that the DNA bound to the cationic liposome, a gel retardation assay was performed. During this procedure, the plasmid DNA and the cationic liposome were allowed to interact and agarose gel electrophoresis was thereafter carried out. A control constituting only pGL3 plasmid DNA was run against varying amounts of cationic liposomes while the amount of DNA was kept constant. Binding of DNA to liposome resulted in the retardation of migration of plasmid DNA through the gel matrix as a consequence of the formation of large complexes that remained within the well.

The results obtained are seen in Figure 3.14.

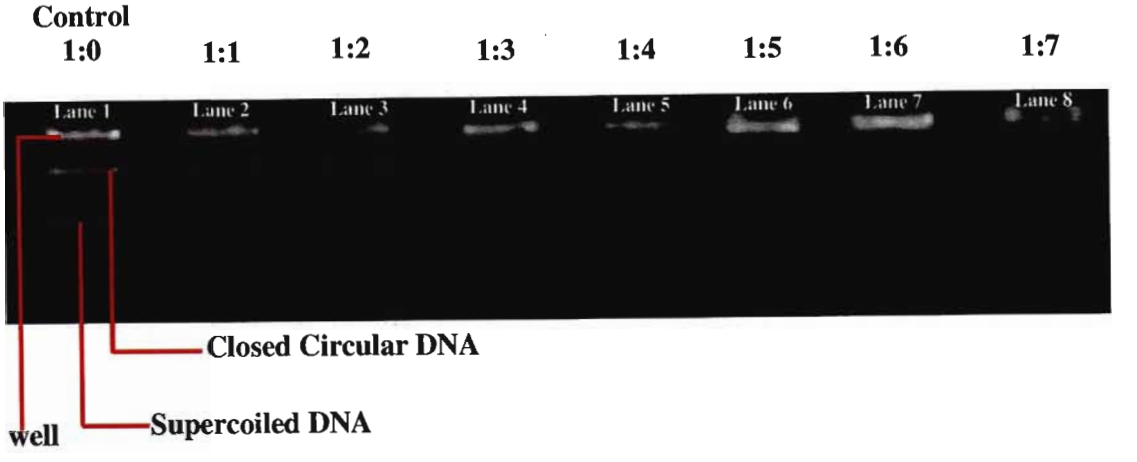


Figure 3.14 : Ethidium bromide gel depicting results of DNA-liposome interactions
(Ratios expressed as pGL3 plasmid DNA : Cationic liposome)

Lane 1 contained the control i.e. only pGL3 plasmid DNA. As no liposome was present no binding occurred resulting in migration of the DNA through the gel matrix. Lanes 2 and 3 show DNA bands within the gel matrix indicating that the concentration of liposome in these lanes was not high enough to bind the DNA completely. Lanes 4 - 8 show that the DNA has remained within the wells and has not migrated through the gel matrix indicating that binding of the DNA to the liposome has occurred. This resulted in the formation of complexes that were too large to migrate out of the wells through the gel i.e. retardation of migration. Thus, liposome-associated DNA migration in an electric field is retarded and very large, electroneutral complexes fail to enter the gel matrix. It is clear from Figure 3.14 that all the DNA is liposome-associated at a plasmid/liposome ratio of 1:3. Thus a binding ratio of plasmid DNA:cationic liposome of 1:3 was obtained.

3.5.2 DNA-Liposome-Asialoorosomucoid Interactions

The formation of complexes between pGL3 plasmid DNA, cationic liposomes and the streptavidin aminohexanoyl biotinylated AOM was demonstrated in a gel retardation assay. During this procedure the plasmid DNA, the cationic liposome and the streptavidin aminohexanoyl biotinylated AOM were allowed to interact and agarose gel electrophoresis was thereafter carried out. A control constituting only pGL3 plasmid DNA was run against varying amounts of cationic liposomes (3 and 4 μg) and streptavidin aminohexanoyl biotinylated AOM (9-11 μg) while the amount of DNA was kept constant. Binding of all three components i.e. the DNA, the liposome and the streptavidin aminohexanoyl biotinylated AOM resulted in the retardation of migration of plasmid DNA through the gel matrix as a consequence of the formation of large complexes that remained within the well.

The results obtained are seen in Figure 3.15.

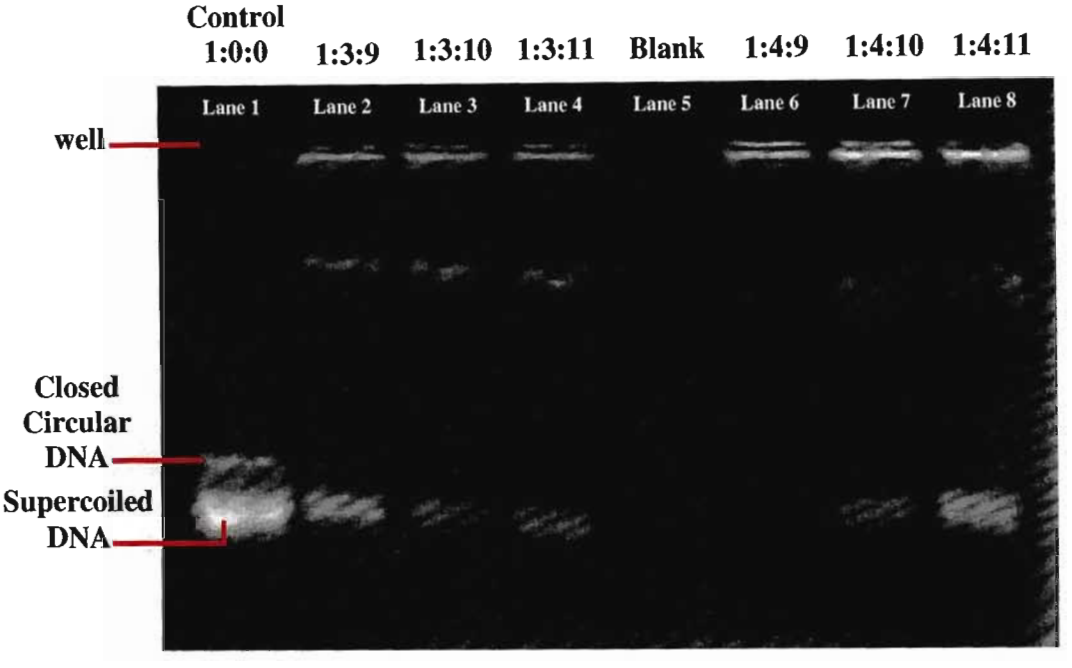


Figure 3.15 : Ethidium bromide gel depicting results of DNA-liposome-AOM interactions

(Ratios expressed as pGL3 plasmid DNA : Liposome : AOM)

Lane 1 contained the control i.e. only pGL3 plasmid DNA. As no liposome or streptavidin aminohexanoyl biotinylated AOM were present no binding occurred resulting in migration of the DNA through the gel matrix. Lanes 2, 3 and 4 contained DNA, liposome and streptavidin aminohexanoyl biotinylated AOM in the ratios of 1:3:9; 10 and 11 respectively. These lanes show DNA bands within the gel matrix indicating that migration of the DNA occurred and complexes did not form. The DNA was free to migrate through the gel matrix. Lane 5 was a blank, therefore nothing is visible in this lane. Lane 6 contained DNA, liposome and streptavidin aminohexanoyl biotinylated AOM in a 1:4:9 ratio. Lanes 7 and 8 contained DNA, liposome and streptavidin aminohexanoyl biotinylated AOM in the ratios 1:4:10 and 11 respectively. Migration of

the DNA can be seen in these lanes, however, lane 6 shows a noticeable difference where only one band can be seen in the gel matrix as compared to lanes 7 and 8 which show 2 bands. In addition, the presence of DNA bands are also visible within the wells. This DNA that has remained within the wells has not migrated through the gel matrix indicating that binding of the DNA to the liposome and the streptavidin aminohexanoyl biotinylated AOM has occurred. This resulted in the formation of large complexes that did not migrate out of the wells through the gel i.e. retardation of migration. Hence, retardation occurred in lane 6 indicating that a complex was formed. Thus a binding ratio for the transfection complex of 1:4:9 was obtained.

3.6. Ethidium Bromide Intercalation Assay

3.6.1 DNA-Liposome Interactions

The percentage fluorescence was calculated from the absorbance values obtained using the absorbance value obtained for the sample containing HBS, ethidium bromide, DNA (6 μg) and liposome (2.5 μg) as being 100 percent.

The percentage fluorescence was then plotted against the liposome quantity (μg) (Figure 3.16). As shown in the graph, fluorescence intensity i.e. percentage fluorescence diminished with increasing concentration of liposome indicating binding to DNA displacing the ethidium bromide.

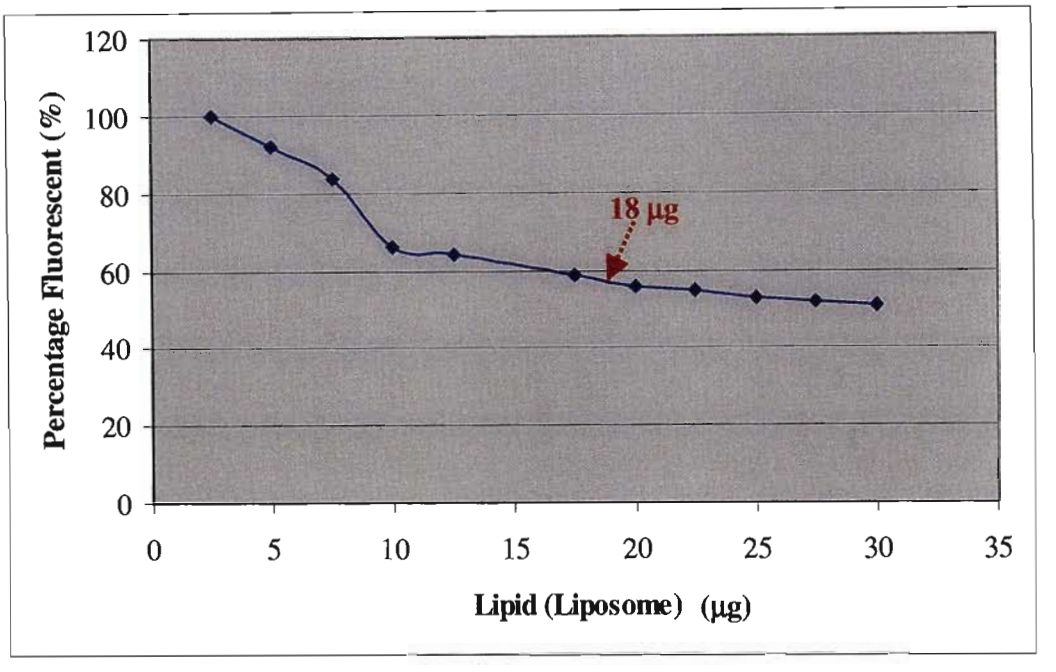


Figure 3.16 : Ethidium bromide intercalation curve of DNA-liposome interactions
The liposome concentration at which the DNA was liposome bound was determined to be 18µg.

Ideally, to confirm the results obtained from the gel retardation assay i.e. a DNA : liposome binding ratio of 1:3, a ratio of 6:18 should show binding since 6 µg DNA was used. This ratio is equivalent to the ratio 1:3. The DNA : liposome binding ratio of 1:3 was confirmed from this graph as the percentage fluorescence showed a decrease and stabilised after 18 µg liposome was added. This confirmed the theory that the ethidium bromide was diplaced by the lipid indicating that binding at a DNA : liposome ratio of 1:3 was obtained.

3.6.2 DNA-Liposome-Asialoorosomucoid Interactions

The percentage fluorescence was calculated from the absorbance values obtained using the absorbance value obtained for the sample containing HBS, ethidium bromide, DNA (6 μg), liposome (24 μg) and AOM (6.56 μg) as being 100 percent.

The percentage fluorescence was then plotted against the AOM quantity (μg) (Figure 3.17). As shown in the graph, fluorescence intensity i.e. percentage fluorescence diminished with increasing concentration of AOM indicating binding to the liposome displacing the DNA.

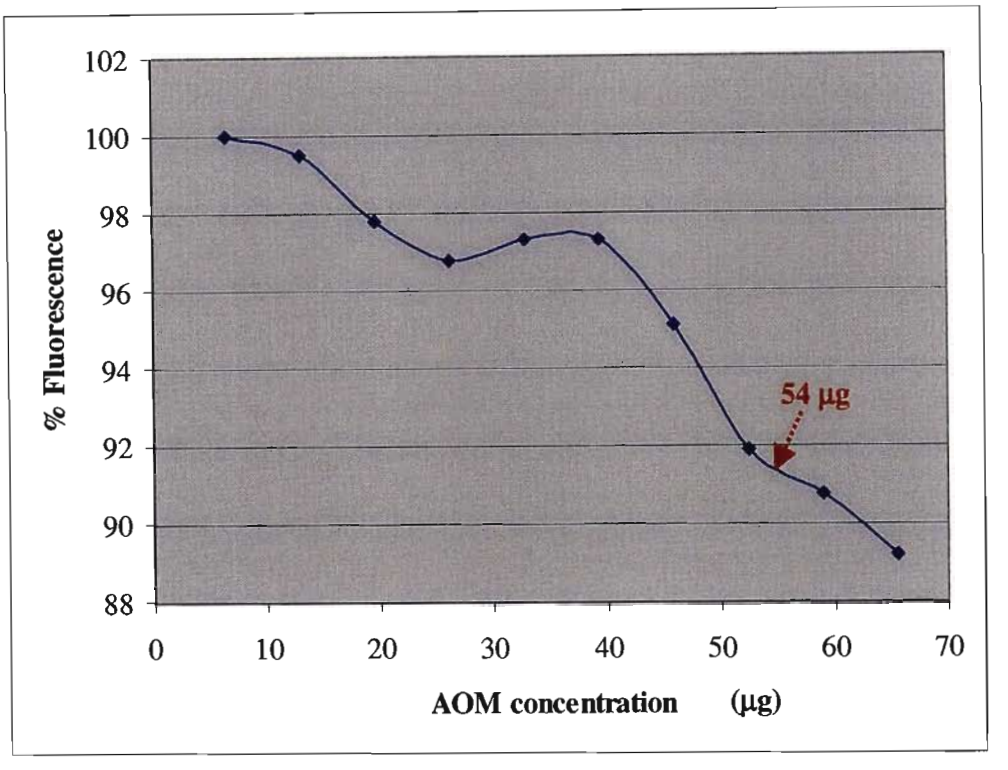


Figure 3.17 : Ethidium bromide intercalation curve of DNA-liposome-AOM interactions

The AOM concentration at which the AOM was liposome bound was determined to be 54µg.

The DNA : liposome : AOM binding ratio of 1:4:9 was confirmed from this graph as the percentage fluorescence showed a decrease although the readings did not vary extensively as the AOM concentrations increased. This confirmed the theory that the protein i.e. the AOM bound to the liposome and as its concentration was increased it displaced some of the DNA. This binding ratio obtained is equivalent to the binding ratio of 1:4:9 that was obtained from the gel retardation assay.

3.7 Characterisation of Interactions of the Components of the Transfection Complex

3.7.1 Characterisation of DNA-Liposome Complex (Lipoplex) by Transmission Electron Microscopy (TEM)

Ultrastructurally, the lipoplexes appeared as clusters of closely packed spherical vesicles and resembled a cluster of blackberries (Figure 3.18). The lipoplexes showed a dependence on the DNA : liposome ratio. Previous experiments showed that the DNA is liposome associated at a DNA : liposome binding ratio of 1:3. In this experiment, three DNA : liposome ratios were observed i.e. 1:2 (Figure 3.19); 1:3 (Figure 3.20) and 1:4 (Figure 3.21). All three ratios exhibited binding of the DNA to the liposome but significant aggregation was observed closer to electroneutrality i.e. a ratio of 1:3 leading to larger structures in which once again spherical vesicles were a dominant feature. This is seen at high power magnifications where the lipid components of the complex is clearly identified as characterised by the lipid nature of the complex (Figures 3.22 and 3.23) The results suggest that the development of larger complexes is seen when the liposome concentration is increased. Lipoplex sizes varied and ranged from 100 – 400 nm. However, these complexes aggregated to one another forming larger structures as can be seen in the Figures.

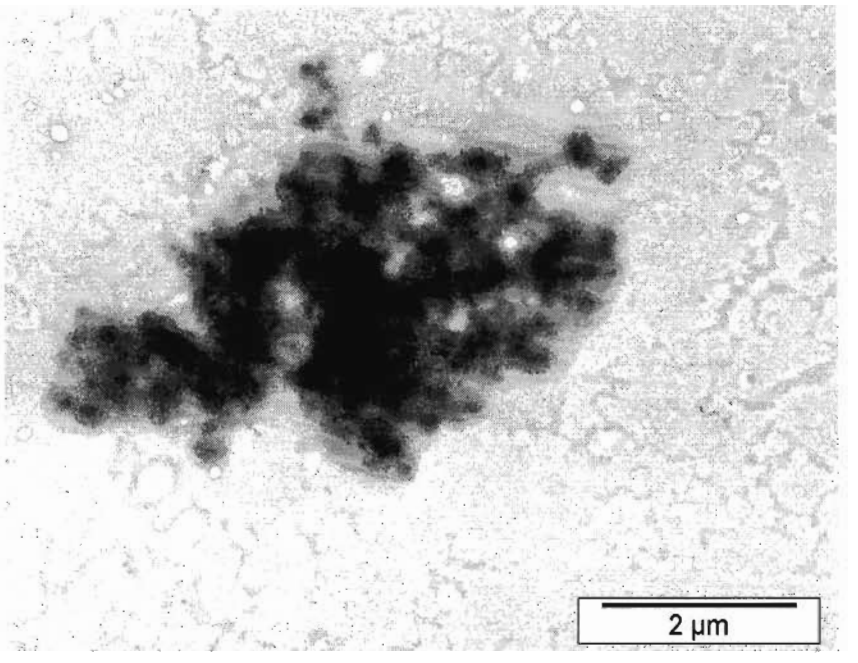


Figure 3.18 : TEM showing lipoplex resembling cluster of blackberries (x 15 k)

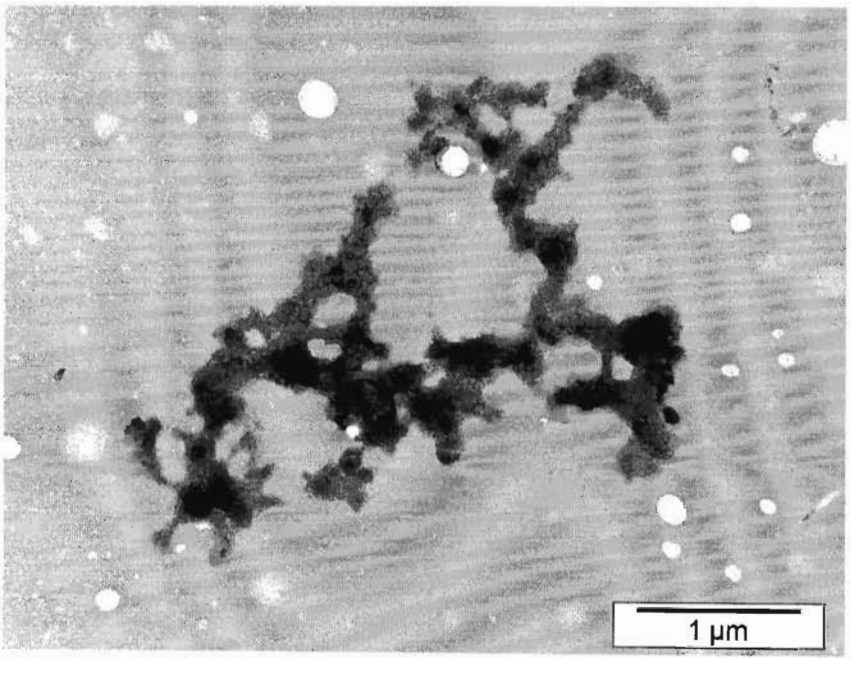


Figure 3.19 : TEM showing lipoplex; DNA:liposome ratio 1:2 (x 25 k)

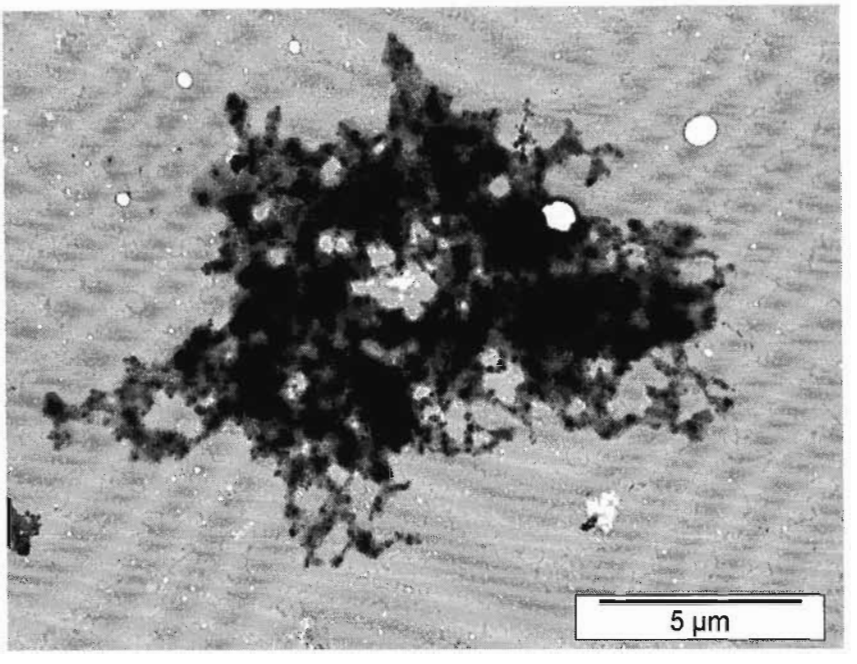


Figure 3.20 : TEM showing lipoplex; DNA:liposome ratio 1:3 (x 6 k)

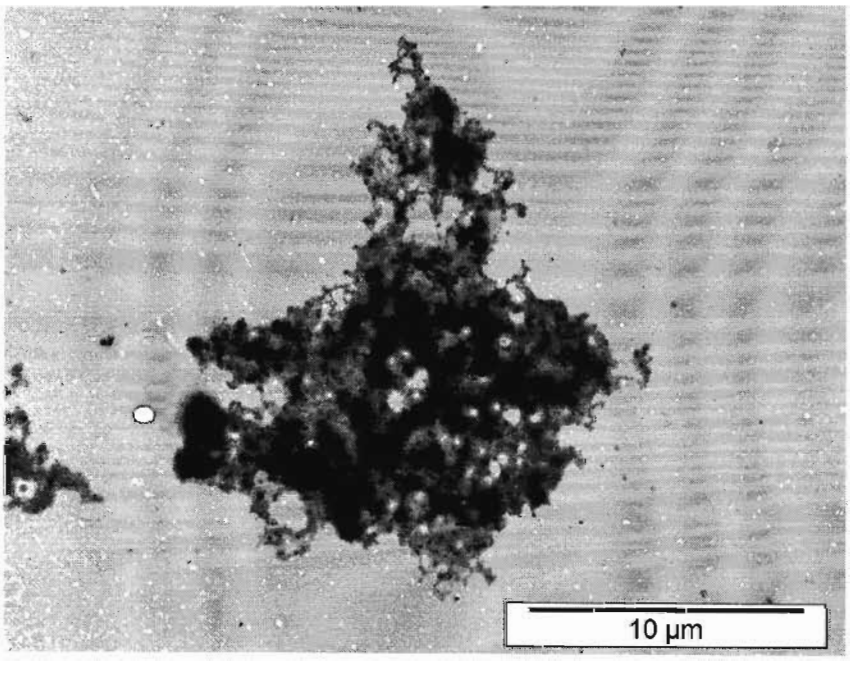


Figure 3.21 : TEM showing lipoplex; DNA:liposome ratio 1:4 (x 4 k)

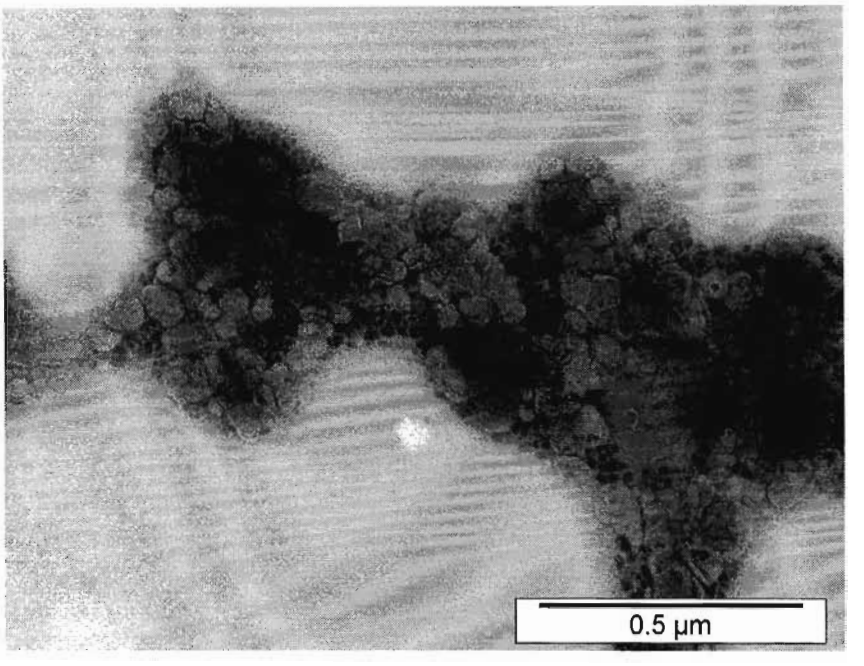


Figure 3.22 : TEM showing lipid nature of lipoplex (x 80 k)

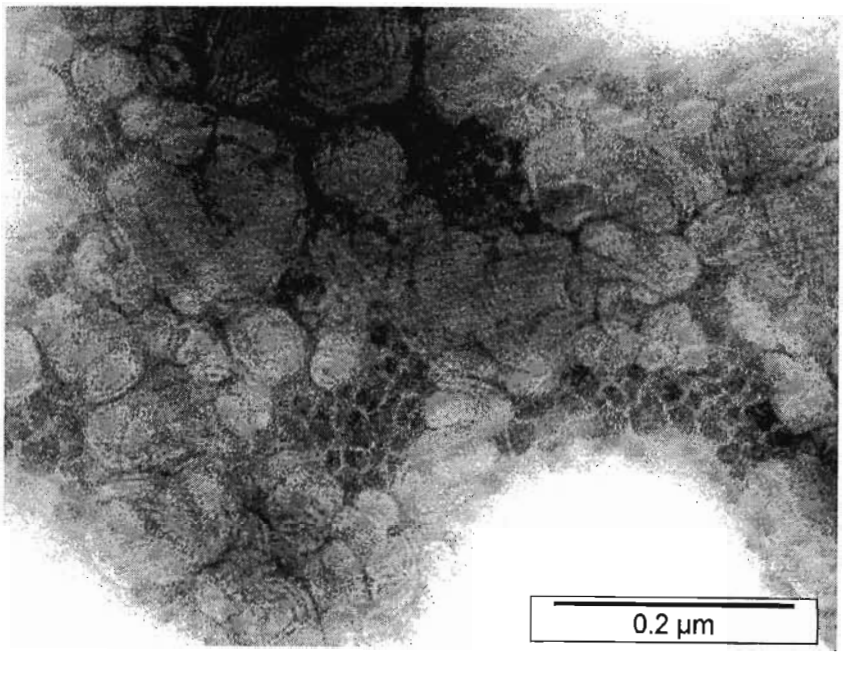


Figure 3.23 : TEM showing lipid nature of lipoplex (x 200 k)

3.7.2 Characterisation of DNA-Liposome-Asialoorosomucoid Complex by Transmission Electron Microscopy (TEM)

Ultrastructurally, the complexes appeared similar to lipoplexes as clusters of closely packed spherical vesicles and resembled a cluster of blackberries (Figure 3.24). However, there is a noticeable difference in that the proteinaceous component of the complex is clearly visible as compared to its lipid counterpart. This is seen at high magnification where the proteinaceous nature of the complex is clearly distinguished (Figure 3.25). Like the lipoplexes, the transfection complexes showed a dependence on the DNA : liposome: AOM ratio. Previous experiments showed that the lipoplexes are protein associated at a DNA : liposome : AOM binding ratio of 1:4:9. In this experiment, two DNA : liposome : AOM ratios were observed i.e. 1:3:9 (Figure 3.24) and 1:4:9 (Figure 3.26). Both ratios exhibited binding of the lipoplexes to the protein but significant aggregation was observed at the higher liposome concentration i.e. a ratio of 1:4:9 leading to larger structures. The results suggest that the development of larger complexes is seen once again when the liposome concentration is increased. Complex sizes varied and ranged from 200 – 500 nm, but due to the aggregation of these complexes, much larger structures formed as seen in the Figures.

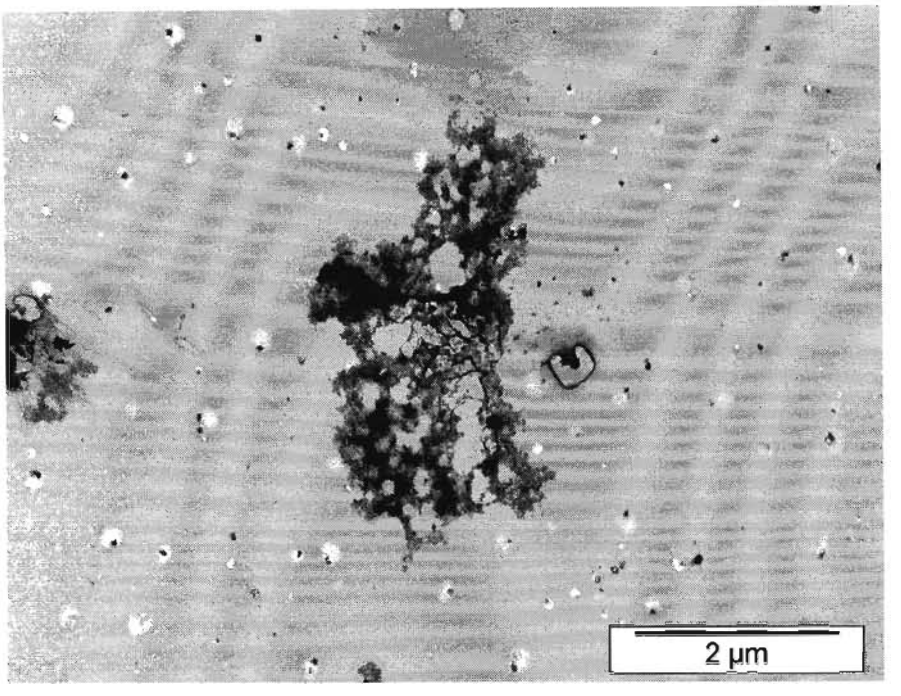


Figure 3.24 : TEM showing transfection complex resembling cluster of blackberries; DNA:liposome:AOM ratio 1:3:9 (x 15 k)

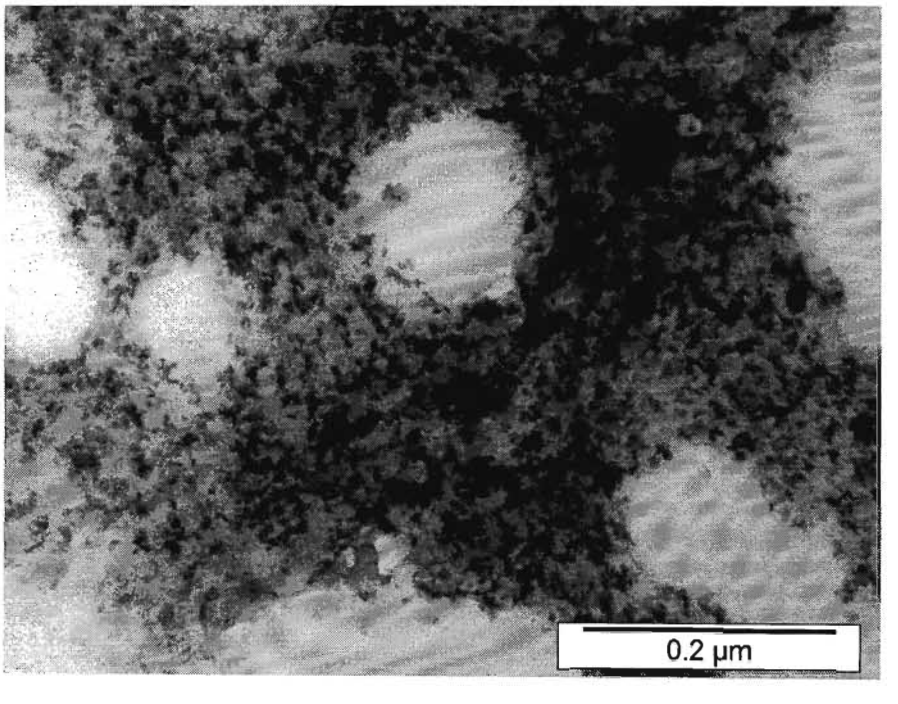


Figure 3.25 : TEM showing proteinaceous nature of transfection complex (x 200 k)

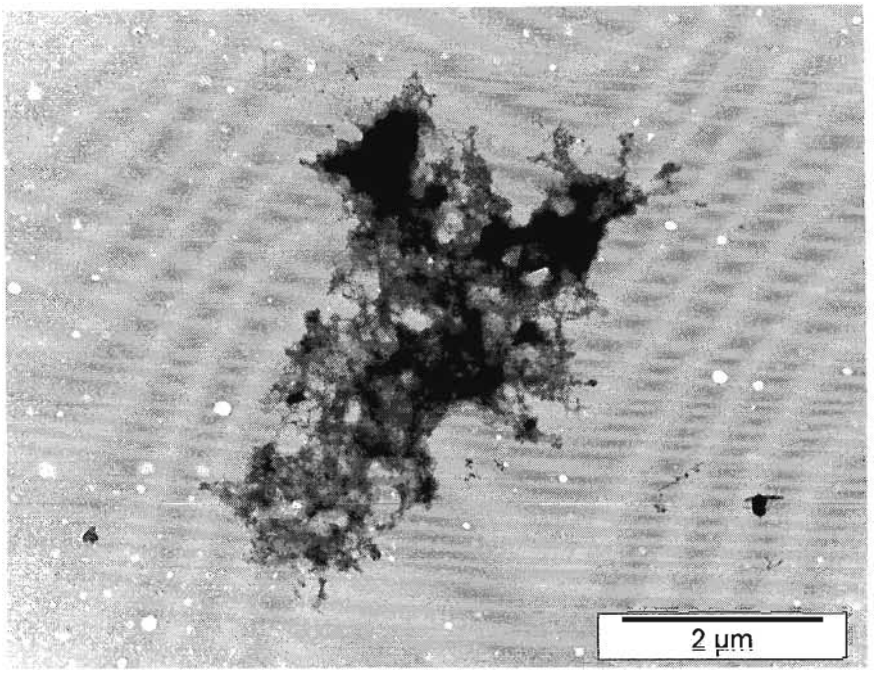


Figure 3.26 : TEM showing transfection complex; DNA:liposome:AOM ratio 1:4:9 (x 15 k)

3.8 Nuclease Digestion Assay

The results obtained are seen in Figure 3.27.

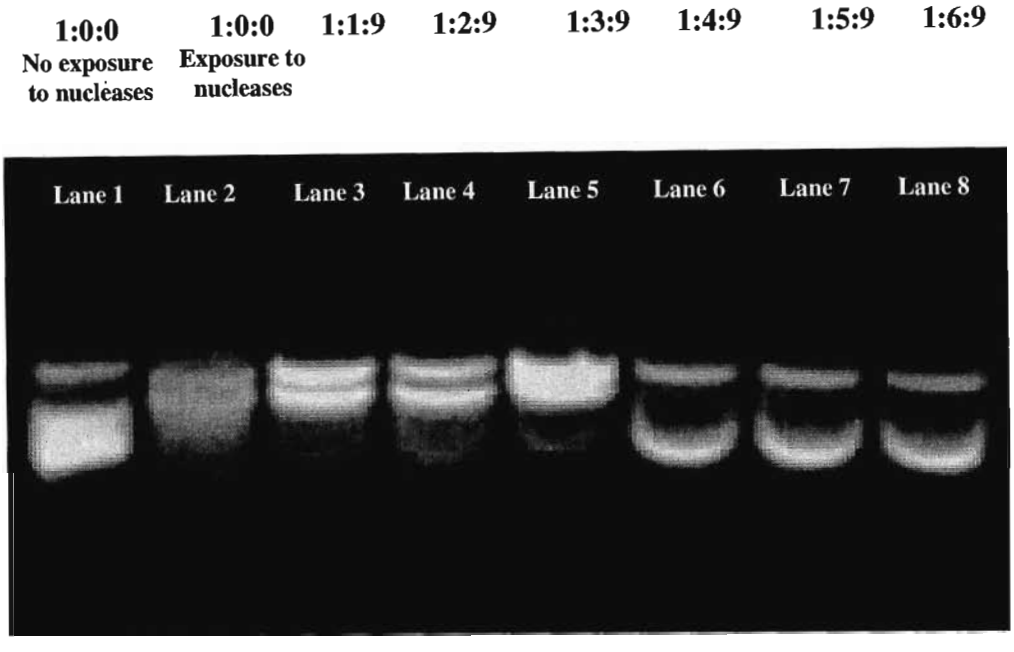


Figure 3.27 : Ethidium bromide gel depicting results of nuclease digestion
(Ratios expressed as pGL3 plasmid DNA : Liposome : AOM)

Lane 1 contained DNA only and this DNA was not exposed to / treated with nucleases. Therefore it is expected that this DNA would not be digested. This expectation is confirmed by the presence of highly concentrated DNA bands.

Lane 2 contained DNA only that was exposed to / treated with nucleases. No liposome or streptavidin aminohexanoyl biotinylated AOM were present. Therefore no complexes would have formed leaving free DNA that was not protected. Exposure to nucleases resulted in the degradation of the DNA and this is seen in Figure 3.27,where the DNA in

lane 2 appears degraded and are not clearly visible. Ideally, however no bands should have been present.

Lanes 3, 4 and 5 contained DNA, liposome and streptavidin aminohexanoyl biotinylated AOM in the following ratios respectively : 1:1:9; 1:2:9 and 1:3:9. DNA bands are seen in these lanes indicating that the DNA was not completely degraded but protected within complexes formed by the liposomes and the streptavidin aminohexanoyl biotinylated AOM.

Lanes 6, 7 and 8 contained DNA, liposome and streptavidin aminohexanoyl biotinylated AOM in the ratios 1:4:9; 1:5:9 and 1:6:9 respectively. The DNA is therefore protected and this is evident in Figure 3.27 as lane 6 (ratio 1:4:9) shows DNA bands that have not been digested by the action of the nucleases. This is also evident in lanes 7 and 8 (ratios 1:5:9 and 1:6:9 respectively) indicating that the DNA is protected and has not been digested.

From the results obtained, the ratio of 1:4:9 of DNA, liposome and streptavidin aminohexanoyl biotinylated AOM proves to be an optimum ratio where the DNA is protected by the action of the nucleases.

3.9 Tissue Culture Studies

3.9.1 Maintenance and Growth of HepG2 cells

The HepG2 cells were successfully propagated in growth medium (MEM), 10% fetal bovine serum and antibiotics (Figure 3.28). Cell growth however, at times was relatively slow with cells reaching confluency after 4 to 6 days. HepG2 cells were subdivided in 3:1 or 2:1 splits after trypsinisation.

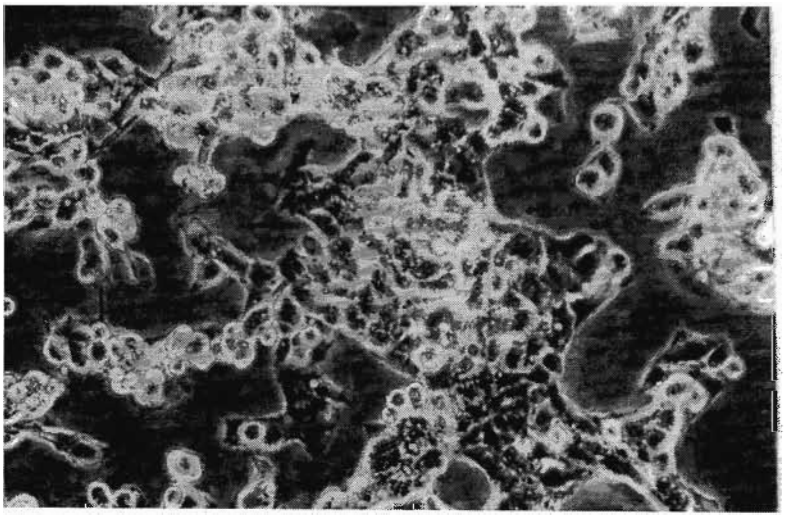


Figure 3.28 : Monolayer of HepG2 cells at semi-confluency

3.10 Transfection Studies in Tissue Culture : Gene Transfer Experiments

3.10.1 Growth Inhibition Assay

The growth of HepG2 cells were observed after being exposed with the transfection complexes with varying liposome concentrations. The liposome showed little toxicity to the HepG2 cells in culture. Maximum cell death recorded was about 41% (Figure 3.29).

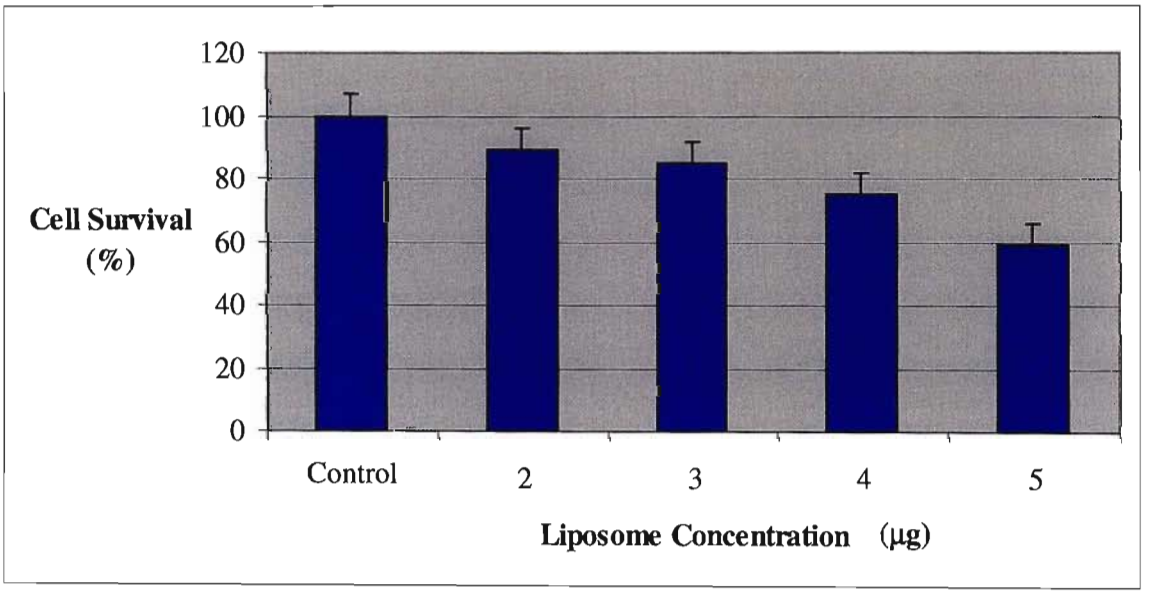


Figure 3.29 : Toxicity of liposome:pGL3 DNA:AOM complexes to HepG2 cells in culture

Liposome concentration was varied (2-5 µg / 25 µl), while the DNA 1 µg / 25 µl) and AOM 9 µg / 25 µl) were kept constant. A control sample (no liposome), contained only HepG2 cells was assumed to have 100% survival. Data presented as means ± S.D (n = 4).

It can be noted from Figure 3.29 that as the concentration of the liposome is increased, the cell survival is decreased exhibiting an inversely proportional relationship. Hence the toxicity of the respective liposome complexes to the HepG2 cells is also increased.

It is concluded therefore, that complexes in which the DNA and AOM ratio was constant at 1 μg : 9 μg and in which the liposome content varied between 2 and 5 μg were shown to be relatively non toxic to HepG2 cells under transfection conditions. In particular, complexes and compositions chosen for transfection studies inhibited cell growth as liposome concentration was increased.

3.10.2 Evaluation of Gene Expression

3.10.2.1 Transfection of HepG2 Cells

Gene transfer experiments were conducted with assemblies containing 1 μg pGL3 DNA, 2-5 μg liposomes and 9 μg AOM. The complexes showed some degree of luciferase activity (Figure 3.30) indicating that the complexes successfully transfected HepG2 cells in culture. Results presented in Figure 3.30 show an increase in transfection activity measured in relative light units (RLU) per mg soluble cell protein as the liposome content of the complexes increases from 2 – 5 μg. The competition assay involved the inclusion of excess asialoglycoprotein. The HepG2 cells containing the asialoglycoprotein receptors were treated with asialofetuin prior to incubation with the transfection complexes.

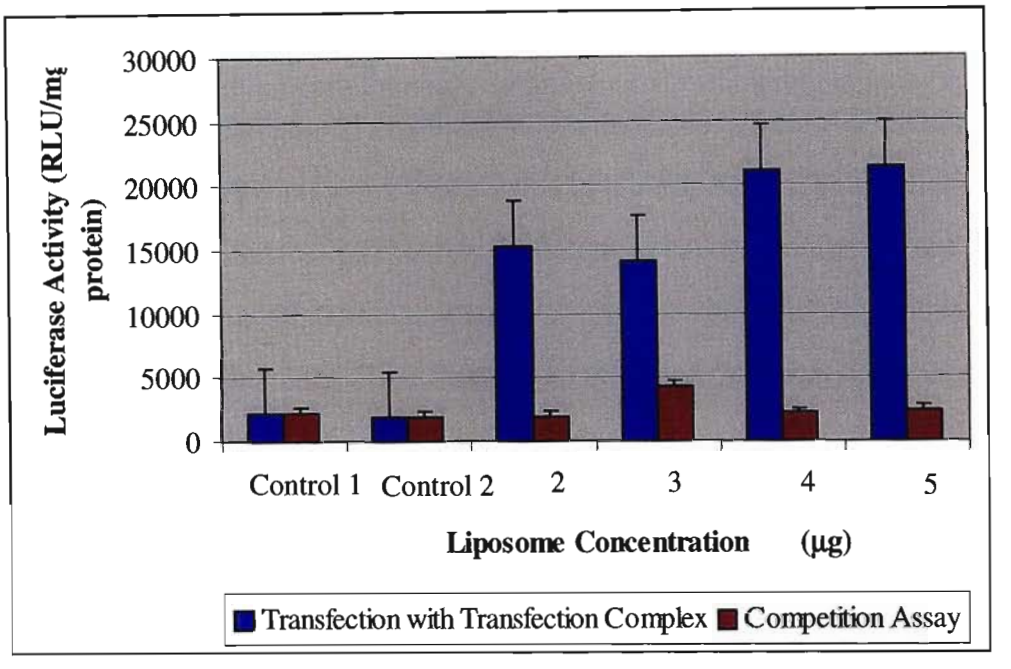


Figure 3.30 : Transfection of HepG2 cells

Cells were incubated and transfected with transfection complexes containing pGL3 plasmid DNA (1 µg / 25 µl), AOM (9 µg / 25 µl) and increasing amounts of cationic liposomes (2-5 µg / 25 µl). Competition assays were conducted in the presence of excess asialofetuin. Transfection was monitored by the Luciferase Assay. Data presented as means ± S.D (n = 4).

Control 1 represents a sample containing only HepG2 cells. Control 2 contained HepG2 cells and DNA only. Results show that the luciferase activity increased with increasing liposome concentration indicating that transfection was successful. In addition, in the presence of excess asialoglycoprotein, the transfection activities showed a marked reduction.

3.10.2.2 Transfection using FuGene 6

Results show that the luciferase activity ranged from 17357 to 27614. These results were comparatively high (Figure 3.31) and a slight degree higher than those obtained from transfection using the transfection complex which ranged from 14162 to 21453 RLU/mg protein.

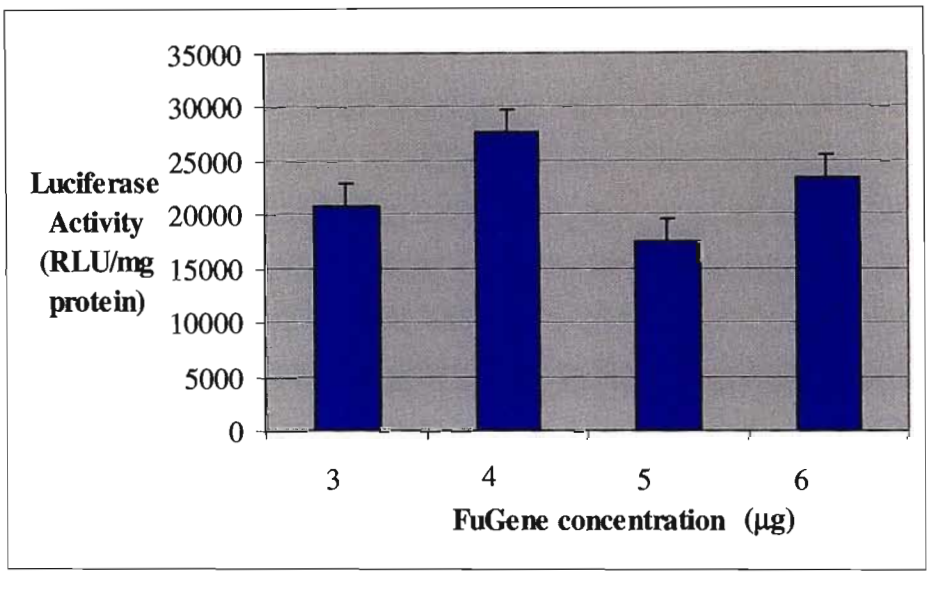


Figure 3.31 : Transfection of HepG2 cells by FuGene:pGL3 DNA complexes
FuGene concentration was varied (3-6 µg), while the DNA (1 µg / 25 µl) was kept constant. Transfection was monitored by the Luciferase Assay. Data presented as means ± S.D (n = 4).

CHAPTER 4

DISCUSSION

4.1 Preparation of DNA

4.1.1 Transformation of *E.coli* with Plasmid DNA

Transformation is the uptake of naked DNA by a cell and the incorporation of this DNA into the recipient chromosome in a heritable form. The supplied JM 109 cells did not contain the vector and were not competent cells. A competent cell is a cell that has undergone physical or chemical treatment in order to be transformed. Transformation occurs when the DNA molecule (in this case the pGL3 plasmid vector) is introduced into a compatible host cell. Therefore in order for the *E. coli* cells to be transformed with the plasmid DNA they were made competent.

4.1.1.1 Preparation of Competent Cells

The *E. coli* cells were made competent by treatment with CaCl. Competency is a complex phenomenon and is dependent on several conditions, for example, bacteria need to be at a certain phase of growth. The OD at which these bacterial cells display optimum competency is 0.375 at a wavelength of 590 nm. This is because it is at this OD reading

that the population of cells is in the log / exponential phase of growth and it is at this phase where the cell walls are permeable to allow penetration of the plasmid (Figure 4.1).

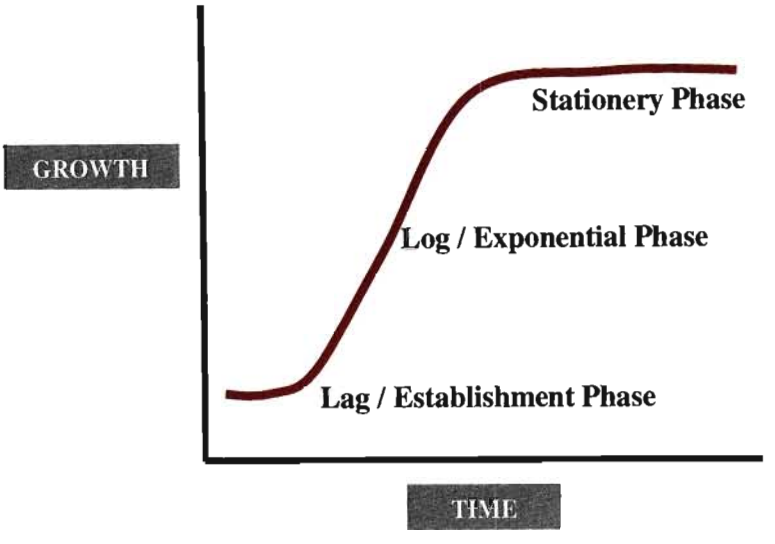


Figure 4.1 : Growth curve of *E.coli*

4.1.1.2 Heat Shock Transformation and Plasmid Isolation

Once the *E.coli* cells were made competent, they were transformed with the pGL3 control plasmid vector. The vector was then allowed to amplify within the host *E.coli* cells. The presence of colonies on the plate (Figure 3.1) indicated the presence of the Amp^r gene concluding that the *E.coli* cells were in fact transformed with the plasmid.

Plasmids are almost always purified from cultures that have been inoculated with a single bacterial colony that has been picked up from an agar plate. Because plasmid vectors can replicate to such a high copy number, they can be purified in large yield from small culture volumes.

Following isolation of plasmid DNA as per protocol, spectrophotometer readings and agarose gel electrophoresis were carried out to confirm that the pGL3 control plasmid vector DNA was indeed isolated.

From Figure 3.2, it can be seen that the sample with the higher concentration value i.e. the sample in lane 2 produced brighter bands within the gel as compared to the sample with the lower concentration value (lane 3). Ratios of 1.367 and 1.172 were obtained for the samples in the respective lanes i.e. lanes 2 and 3. The ratio provides an estimate of the purity of the nucleic acid. Pure preparations of DNA have a ratio of 1.8 indicating pure DNA without protein or RNA contamination (Sambrook *et al.*, 1989). Hence the sample in lane 2 was of a purer grade than that of lane 3 as the ratio obtained for the former was closer to 1.8 than that obtained for the latter.

The presence and positions of bands in the lanes containing the test samples i.e. lanes 2 and 3 (Figure 3.2) indicate the presence of the pGL3 plasmid DNA as they correspond to the control (lane 1) (Figure 3.2). As explained above, due to the difference in the concentrations, the brightness of the bands in each lane varied as these were diluted samples of decreasing concentrations. Two distinct bands can be seen in the lanes

containing the test samples. However, separation of plasmid DNA usually produces 4 bands within the gel matrix. This includes a linear fragment closest to the well followed by open circular DNA, closed circular DNA and then the supercoiled molecule. The two bands seen in the gel matrix are the supercoiled DNA molecule and the closed circular DNA. Restriction digests were not performed on the plasmid DNA therefore linear and open circular DNA bands are not seen. Most plasmids exist as supercoiled molecules. This occurs because the double helix of plasmid DNA is partially unwound during plasmid replication by enzymes called topoisomerases. This enzyme alters the conformation of covalently closed circular DNA e.g. plasmid DNA molecules by adding or removing supercoils. The supercoiled conformation can be maintained only if both polynucleotide strands remain intact. Hence the more technical name of covalent closed circular DNA. If one of the polynucleotide strands is broken, the double helix reverts to the normal relaxed state and the plasmid then takes on the alternative conformation called open circular DNA (Sambrook *et al.*, 1989).

4.2 Synthesis of Liposomes

Liposomes are widely used in gene therapy as DNA delivery vehicles. Cationic liposomes in particular are positively charged molecules that are able to interact spontaneously with the negatively charged DNA forming stable complexes that can be delivered to target cells (Singh and Ariatti, 2003).

Direct gene transfer for the treatment of human diseases requires a vector that can be administered safely, efficiently and repeatedly. Cationic liposomes represent one of the few examples that meet these requirements. Currently, more than a dozen cationic liposome formulations have been reported. These liposomes bind and condense DNA spontaneously to form complexes with high affinity to cell membranes. Endocytosis of the complexes followed by the disruption of the endosomal membrane appears to be the major mechanism of gene delivery. The effectiveness and safety of this DNA delivery method has been established in many studies. The simplicity, efficiency and safety features have rendered the cationic liposome an attractive vehicle for human gene therapy (Singh, 1998; Hirko *et al.*, 2003; Singh and Ariatti, 2003; Goncalves *et al.*, 2004).

4.2.1.Confirmation of Synthesis of 3β[N-(N',N'-dimethylaminopropane) carbamoyl] cholesterol (Chol-T) by Thin Layer Chromatography (TLC)

TLC is a simple, quick and inexpensive procedure that gives a quick answer as to how many components are in a mixture. TLC is also used to support the identity of a compound in a mixture when the R_f of a compound is compared with the R_f of a known compound. TLC thus enabled us to confirm the synthesis of Chol-T by comparison of a standard Chol-T sample, the reaction mixture of Chol-T taken prior to crystallisation and Chol-T crystals obtained after crystallisation i.e. the end product.

The R_f values calculated were 0.307 for the standard Chol-T sample, 0.338 for the reaction mixture of the Chol-T sample prior to crystallisation and 0.230 for the Chol-T crystals after crystallisation. These R_f values are very similar indicating that all three samples have a similar retention factor. In addition, the migration patterns also show similarity. These indicate that all three samples are very similar to one another and are the same compound. Hence, the successful synthesis of Chol-T was achieved.

However similar the three compounds may be, it can be seen that the reaction mixture of the Chol-T sample exhibits most impurity as slight migration can be seen close to the solvent front (Figure 3.3). The standard Chol-T sample shows some degree of impurity as well as indicated by migration close to the solvent front but not as much as that of the reaction mixture. The Chol-T crystals show only slight migration near the solvent front, much less that the other two samples indicating a much purer, cleaner sample.

4.2.2 Characterisation of Liposomes by Transmission Electron Microscopy (TEM)

The results obtained from the micrographs were in keeping with results previously published. Liposome preparations from Chol-T, DOPE and the *N*-hydroxysuccinimide ester of cholesteryl hemisuccinate (4:5:1, molar ratio) was shown to have similar characteristics but these liposomes however were found to be unilamellar in the size range 200 - 500 nm (Singh and Ariatti, 2003) as opposed to the observations in this study

where the liposomes ranged from 100 – 300 nm in size and encapsulated by bi-layered membranes.

4.3 Preparation of Asialoorosomucoid (AOM)

The ultimate aim of gene therapy is the introduction and incorporation of therapeutic DNA into a cell so that it can be expressed. Liposomes are DNA delivery vehicles and their duty is to transport the DNA to the target cell but they do not possess specific receptors for the attachment to and entry of these cells. In order to accomplish this, a system containing the liposome-DNA complex together with a ligand can be used. The asialoglycoprotein AOM is one such ligand.

4.3.1 Desialylation of the Orosomucoid

Orosomucoid is a serum glycoprotein with a molecular weight of approximately 40 000 daltons. It contains a number of sialic acid residues which were removed by desialylation producing AOM which is essential for the protein to be efficiently endocytosed via the asialoglycoprotein receptor of the HepG2 cells (Singh, 1998). The sialic acid residues on the orosomucoid molecule are negatively charged. In order for this molecule to bind DNA which also carries a negative charge, these sialic acid residues need to be removed producing a stable molecule AOM which is positively charged and electrostatically binds

DNA. This procedure is called desialylation and its purpose therefore, is to remove sialic acid residues from the orosomucoid by the action of the neuraminidase enzyme which removes the terminal *N*-acetyl neuraminic acid residues from oligosaccharides and glycoproteins thus producing AOM which is the targeting ligand that will be used in the transfection complex.

Desialylation was first confirmed by agarose gel electrophoresis comparing varying concentrations of the orosomucoid against the same concentrations of the dialysates.

In the electric field, the negatively charged orosomucoid, like DNA migrated forward towards the positive electrode. The dialysate i.e. the positively charged AOM migrated a shorter distance as compared to the orosomucoid which migrated a further distance away from the well as seen in Figure 3.8. For the orosomucoid samples of differing concentrations, both protein bands can be seen within the gel matrix indicating that the protein is negatively charged and has migrated in the electric field due to this charge (lanes 1 and 2) (Figure 3.8). The sample containing 10 µl of the dialysate (lane 3) (Figure 3.8) showed no visible bands probably because the concentration was not high enough to be detected. However, the sample containing 20 µl of the dialysate (lane 4) (Figure 3.8) produced a band that was clearly visible indicating that protein was indeed present. This band has migrated a shorter distance as compared to those of lanes 1 and 2 indicating that the protein in this sample carries a less negative charge. However, since the distance away from the wells for both the orosomucoid and the dialysate is not so substantial comparatively, it is presumed that complete desialylation did not occur. A difference in

the banding pattern is however seen confirming that some degree of desialylation did occur.

4.3.2 High Performance Liquid Chromatography (HPLC) Analysis of the Digestion Samples

HPLC analysis is a separation technique resulting in a chromatogram showing the separation of samples which are expressed as peaks that are drawn automatically by a chart recorder as the different substances pass the detector. A chromatogram is a graph that monitors the signal in the detector over time. As the sample is detected by the instrument, the signal increases, and the chromatogram displays a "peak." Each peak in the chromatogram indicates the presence of the substance in the sample. Each peak is labelled with retention time. Retention time indicates how long it takes for a compound to come out of the HPLC column (Ebbing, 1996).

Here, HPLC analysis was used to monitor and confirm the desialylation of the orosomucoid. This was evident by the time in which the samples peaked i.e. the retention time, the time necessary for maximum elution of the solute. This is explained as follows : the steel column through which the sample passes is positively charged. Orosomucoid is negatively charged. Orosomucoid remains in the column due to this negative charge. When a salt buffer passes through the column, the orosomucoid is eluted. Desialylation of the orosomucoid produced AOM by removal of the negatively charged sialic acid

residues thereby giving it a less negative charge. Thus, AOM would leave the column earlier than the orosomucoid as the buffer passes through, i.e. it would be eluted earlier than the orosomucoid producing a peak occurring earlier than that produced by the orosomucoid. Considering this, the retention time of the AOM should be less than that obtained from the orosomucoid.

The above theory was confirmed with orosomucoid peaking at a retention time of 3 minutes and 23 seconds (Figure 3.9) and AOM i.e. the desialylate peaking earlier at 1 minute and 86 seconds (Figure 3.10). Furthermore, a mixture of AOM together with orosomucoid produced two successive peaks confirming this theory where the first peak represents the AOM and the second peak represents the orosomucoid (Figure 3.11). All chromatograms however, show the orosomucoid as a much thicker peak as compared to that of the AOM. A probable reason for this is that the orosomucoid was a heterogeneous mixture with molecules possessing various numbers of sialic acid residues. The AOM showed a much thinner, neater peak probably because the mixture was homogenous.

4.3.3 The Thiobarbituric Assay for Sialic Acids

This procedure was used to monitor successful desialylation. The sialic acid released was detected and measured using the thiobarbituric acid assay. This assay detects only the released and not the bound sialic acids. This is a colorimetric assay which has been developed for sialic acids in which sialic acids are oxidised with sodium periodate in

concentrated phosphoric acid. The periodate oxidation product i.e. β-formylpyruvic acid is then coupled with thiobarbituric acid and the resulting chromophore is extracted into cyclohexanone as it is soluble in cyclohexanone. A chromophore is a chemical group capable of selective light absorption resulting in the colouration of certain organic compounds. This assay is reproducible, sensitive (extinction co-efficient = 57.000 for *N*-acetylneuraminic acid), and considerably more specific than other methods (Warren, 1959).

From the standard curve constructed (Figure 3.12), using the $OD_{549\ nm}$ reading i.e. the absorbance value obtained at 549 nm which was 0.119, 0.0089 μmoles of *N*-acetylneuraminic acid was obtained. This was the amount of sialic acid that was detected and released. Further, this result was confirmed using the equation where the amount of *N*-acetylneuraminic acid present in the sample was determined to be 0.0089 μmoles. The result obtained from the graph (Figure 3.12) is the same as that obtained theoretically from the equation, thus confirming the amount of sialic acid released. The initial sample however, contained approximately 0.05 μmoles of *N*-acetylneuraminic acid (sialic acid). The amount of sialic acid released was substantially low as compared to this value indicating that not all of the sialic acid was released and a large amount remained bound to the orosomucoid. Thus the orosomucoid was not completely desialylated although a desialylation did occur as seen by agarose gel electrophoresis and by HPLC analysis.

If the amount of thiobarbituric acid reagent used in the assay was decreased to 2.5 ml, a slightly higher optical density would have been obtained resulting in a greater amount of

sialic acid being released. This is because 2.5 ml is the minimum amount which will give a molecular extinction coefficient of 57.000 (Warren, 1959).

4.3.4 [^{3}H] Aminohexanoyl Biotinylated Asialoorosomucoid

This procedure allowed for the determination of the binding ratio of aminohexanoyl biotin to the AOM molecule.

One AOM molecule has approximately 11 lysine amino acid residues. The amino acid lysine has one free ε amino (NH_2) group attached to it. Therefore the AOM molecule has 11 NH_2 groups attached to it. When reacted with aminohexanoyl biotin, one of the hydrogen (H) molecules from one of the NH_2 groups reacts with the active ester i.e. the *N*-hydroxysuccinimide ester of aminohexanoyl biotin forming a water-soluble compound which is removed by dialysis. The remaining H molecule then binds the aminohexanoyl biotin molecule by an amide bond forming aminohexanoyl biotinylated AOM. This explanation is seen in Figure 4.2.

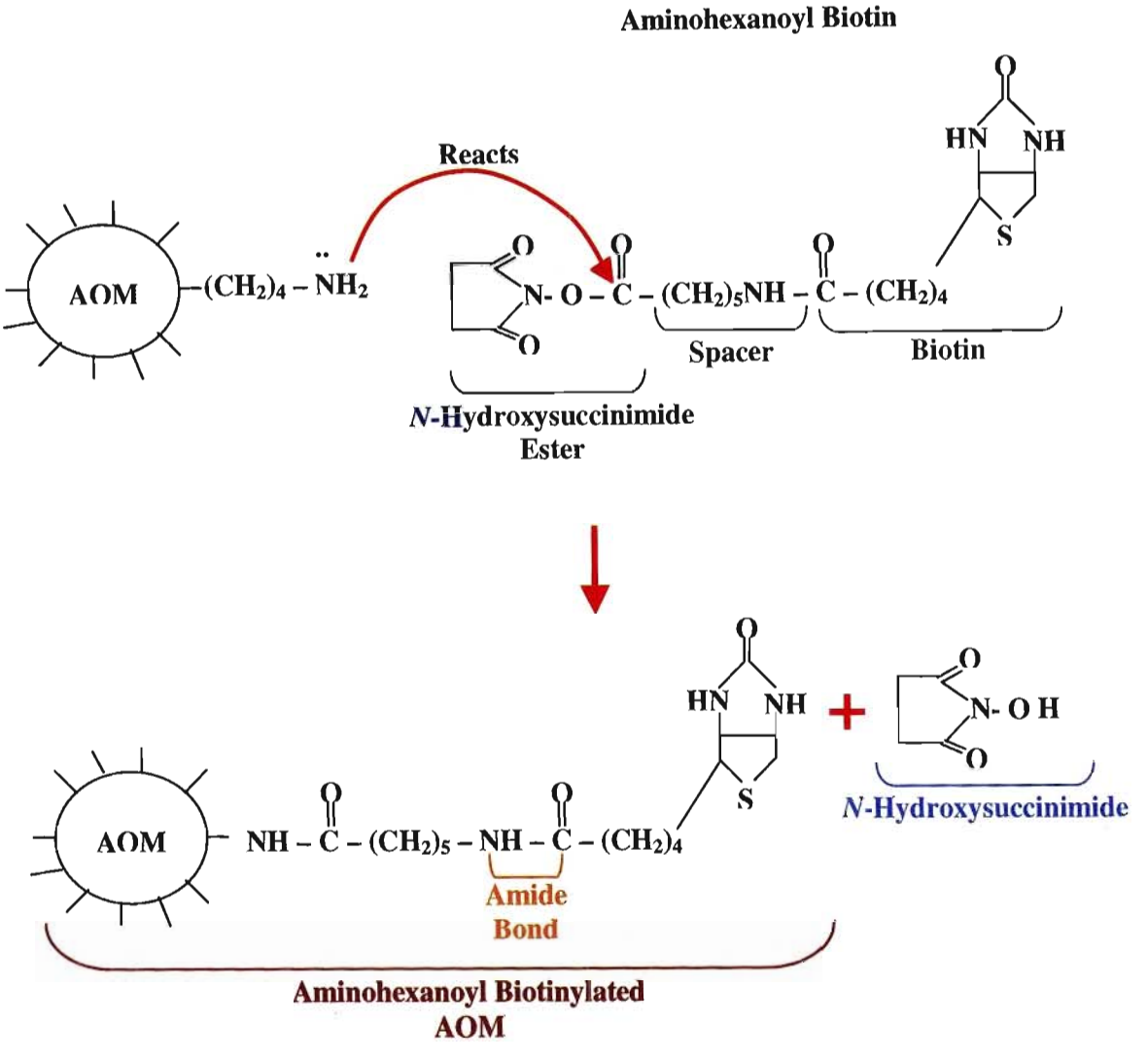


Figure 4.2 : Chemical reaction of AOM and Aminohexanoyl Biotin

In this experiment however, the aminohexanoyl biotin molecule was labelled with tritium (^3H) as this is an unstable isotope of hydrogen and is therefore radioactive. Tritium decays to emit β (Beta) particles and helium (^3He). β particles are weak energy sources that travel approximately 30 cm in air (β-radiation) and are detected indirectly using a

scintillation counter. For every β particle that is emitted, 1 photon is produced. This photon is measured in a scintillation counter which enables it to be determined as the number of disintegrations per minute (dpm). Using these values obtained and the equation, the specific activities could be calculated ultimately resulting in the deduction and conclusion that 1 molecule of AOM bound 2 aminohexanoyl biotin molecules as shown in the results. In theory, due to the presence of 11 NH_2 groups, one would expect 11 aminohexanoyl biotin molecules to bind and thus if maximum binding had occurred, 1 molecule of AOM would have bound 11 aminohexanoyl biotin molecules. It is unclear why this was not the case. The reason for keeping the biotin content low in the aminohexanoyl biotinylated AOM ensured that little structural alterations of the protein occurred and it prevented cross-linking of streptavidin-bound biotinylated AOM. This reasoning was adopted previously where streptavidin was combined with transferrin containing 1-2 biotin moieties in a 1:1 mole ratio (Schoeman *et al.*, 1995).

4.3.5 Protein Determination of Aminohexanoyl Biotinylated Asialoorosomucoid and [^{3}H] Aminohexanoyl Biotinylated Asialoorosomucoid

The protein concentration of aminohexanoyl biotinylated AOM and [^{3}H] aminohexanoyl biotinylated AOM was determined to be 1330 μg and 770 μg of protein respectively. It can be seen that for some reason the radioactive sample had a much lower protein concentration. It is unclear as to why there is such a vast difference in the concentrations.

In addition we assume that these values do not account for pure protein content as AOM is a glycoprotein and is not constituted exclusively of protein.

4.3.6 Streptavidin-Biotinylated Asialoorosomucoid Interactions

The cationic liposomes used as the delivery agent in this project contains biotinylchoesteryl formylhydrazide. The use of this particular cholesterol derivative is advantageous because biotin interacts and binds to protein avidin and streptavidin. Avidin is a 68 kDa tetrameric glycoprotein composed of four identical subunits. Each subunit is glycosylated, and contains one binding site for biotin. Streptavidin is a neutral bacterial analog of avidin which occurs in a non-glycosylated form. Due to its glycosylation state, non-specific binding of biotin by avidin has been observed. Streptavidin and avidin both possess great affinity for biotin, with which they form irreversible bonds (Sato *et al.*, 2000; Lehtolainen *et al.*, 2002). In addition, the targeting component AOM was also biotinylated and the streptavidin-biotin interaction was utilised thereby forming a bridge allowing the cationic liposomes and AOM to attach to one another resulting in part of the transfection complex.

4.3.6.1 AH (Aminohexyl) Sepharose Assay for [^{3}H] Streptavidin Aminohexanoyl Biotinylated Asialoorosomucoid

The AH-sepharose gel beads were biotinylated in a previous experiment (2.4.7.2). AH Sepharose 4B contains a 6-carbon "spacer" group with four amino groups available for coupling. These amino groups bind biotin in a similar way as does the amino groups of AOM (Figure 4.3).

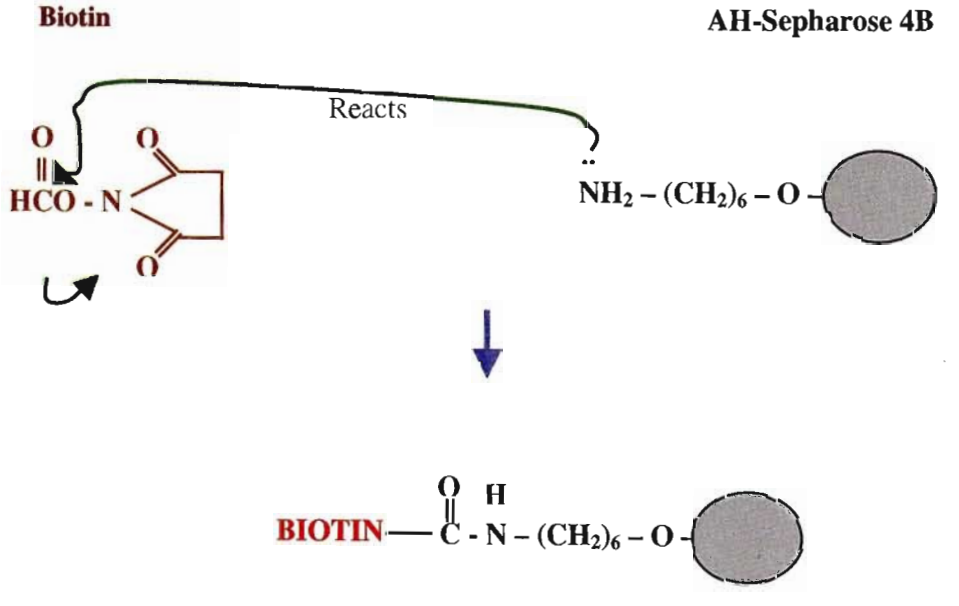


Figure 4.3 : Chemical reaction showing biotinylation of AH-sepharose 4B

As mentioned, streptavidin has four binding sites for biotin. When streptavidin and the radioactive ([^{3}H]) aminohexanoyl biotinylated AOM are allowed to interact, the biotin of the radioactive aminohexanoyl biotinylated AOM binds to streptavidin forming a large

complex since both these components are large glycoproteins. The biotinylated AH-sepharose gel beads, when brought into contact with the complex binds to the complex in a similar biotin-streptavidin chemical reaction (Figure 4.4).

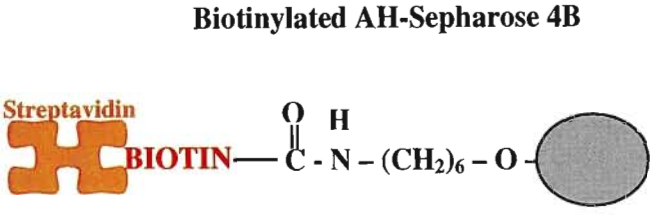


Figure 4.4 : Diagrammatic representation (not to scale) of biotinylated AH-sepharose 4B

Upon centrifugation, most of the complex now bound to the AH-sepharose gel beads resulted in a pellet leaving the supernatant fairly complex free. Due to the low radioactivity (1850 dpm) of the supernatant, streptavidin appears to have formed an effective bridge between the tritiated aminohexanoyl biotinylated AOM and the biotinylated affinity gel (Figure 4.5) indicating and reinforcing the bond created between biotin and streptavidin. In theory therefore, due to the presence of the complex within the pellet, higher radioactivity, should be detected here.

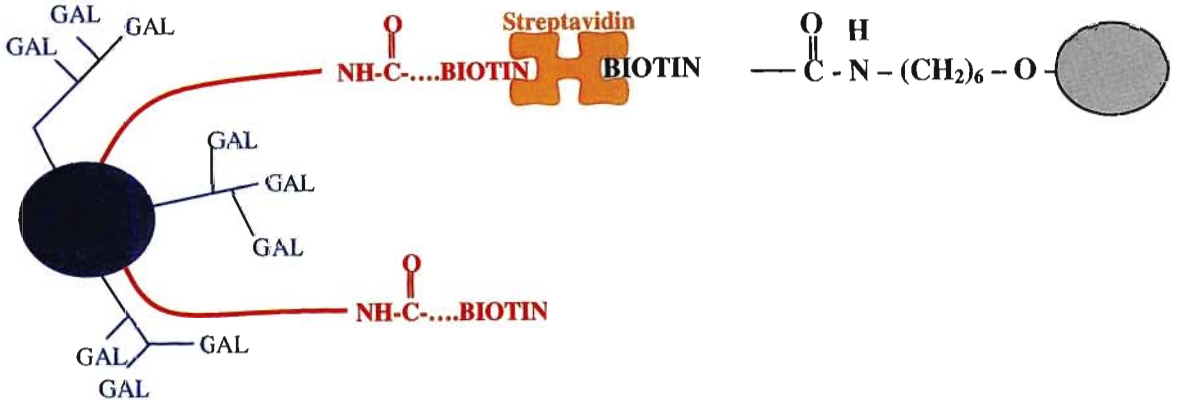


Figure 4.5 : Diagrammatic representation (not to scale) of complex formed between biotinylated AH-sepharose 4B, streptavidin and aminohexanoyl biotinylated AOM

4.4 Gel Retardation Assays

Gel retardation assays are binding assays that are used to assess binding ratios of the different components of the transfecting complex. Various interactions were investigated i.e. the plasmid DNA with the cationic liposome and the plasmid DNA, cationic liposome together with the AOM. Binding of components resulted in the retardation of migration of plasmid DNA through the gel matrix as a consequence of the formation of large complexes that remained within the well.

4.4.1 DNA-Liposome Interactions

From the results obtained, the DNA is liposome-associated at a plasmid : liposome ratio of 1:3. Thus a binding ratio of plasmid DNA : cationic liposome of 1:3 was obtained. This ratio is similar to that obtained in previous studies. For example, Singh *et al.* in 2003 have shown a plasmid : liposome binding ratio of 0.5:5. Although this would suggest that when DNA is fully associated with cationic liposomes there is an excess of positive charges due to the liposome over the negative DNA charges, it is suggested that this finding may be attributed to the bulky nature of the cationic liposomes and superhelical DNA which may prevent close contact of the two species. Further, approximately half of the positive charges which are on the interior surface of the 4 nm thick bilayer will have limited interaction with the DNA. Similar observations were made where it was stated that interactions between cationic liposomes and oligonucleotides involve the binding of positive charges on the exterior surface of liposomes with the negative charges of the oligonucleotides (Cao *et al.*, 2000; Singh and Ariatti, 2003).

4.4.2 DNA-Liposome-Asialoorosomucoid Interactions

From the results obtained, a binding ratio of plasmid DNA : cationic liposome : streptavidin aminohexanoyl biotinylated AOM of 1:4:9 was obtained. Generally, one would expect that as the components of the complex were increased the complex would increase in size and retardation of migration would occur. However, from the results

obtained, an increase in the concentration of streptavidin aminohexanoyl biotinylated AOM as seen in lanes 7 and 8 of Figure 3.15, showed that migration of the DNA did in fact occur. An explanation for this migration is as follows. The complexes consist of three components *viz.* the DNA, the liposomes and the streptavidin aminohexanoyl biotinylated AOM (Figure 4.6). The liposome contains biotin and the AOM has been biotinylated. Streptavidin binds biotin in the liposome and the biotinylated AOM is already complexed to streptavidin.

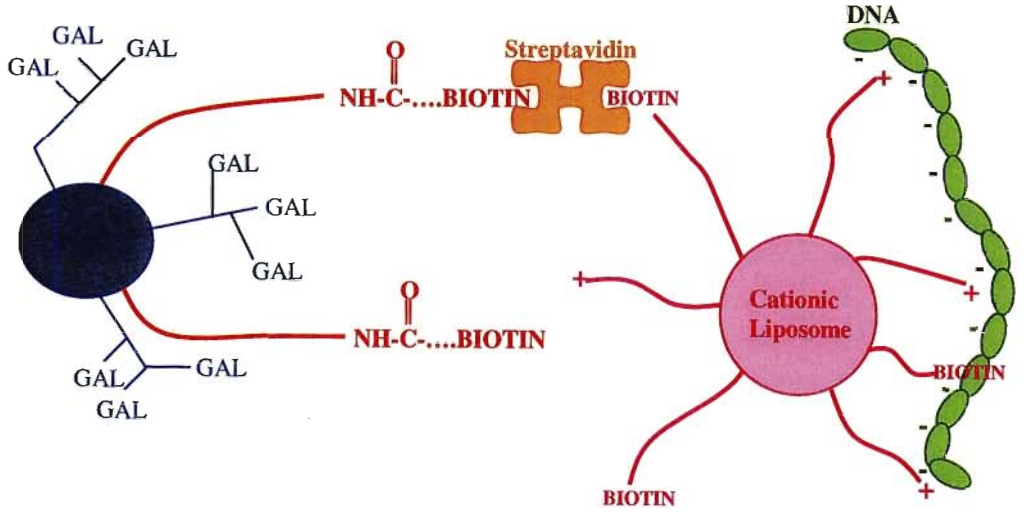


Figure 4.6 : Diagrammatic representation of transfection complex

In this experiment, when the positively charged liposome is reacted with the negatively charged DNA, a complex between these two components is formed. It was previously shown that DNA-cationic liposome complexes form at a DNA:cationic liposome binding ratio of 1:3. This liposome containing biotin in turn reacts with and binds to the

streptavidin of the streptavidin aminohexanoyl biotinylated AOM at a DNA : cationic liposome : streptavidin aminohexanoyl biotinylated AOM binding ratio of 1:4:9. When the concentration of streptavidin aminohexanoyl biotinylated AOM is increased above 9 μg, the protein component of the entire complex i.e. the streptavidin aminohexanoyl biotinylated AOM is very high due to the high molecular weights of AOM and streptavidin which are 40 000 and 60 000 repectively. This high protein content results in the displacement of the DNA causing a decrease in the amount of DNA that is bound and hence migration of the DNA occurs. In addition, lanes 6, 7 and 8 of Figure 3.15 show that the DNA is retarded as indicated by the presence of bands within the wells, however, due to the displacement of the DNA by excess proteins especially in lanes 7 and 8 DNA bands are visible within the gel matrix but of considerably lower concentrations as compared to those in the wells as indicated by the clarity of the bands i.e. those within the wells appear more highly concentrated.

4.5 Ethidium Bromide Intercalation Assay

4.5.1 DNA-Liposome Interactions

This assay was performed to confirm the DNA : liposome binding ratio obtained from the gel retardation assay. The ethidium bromide binds to / intercalates between the bases of double stranded DNA. When the liposome concentration is increased the ethidium bromide is displaced, and, when the adequate DNA : liposome binding ratio is reached,

the DNA is now completely bound to the liposome. This can be seen by diminished fluorescence activity i.e. a decrease in fluorescence and stabilisation of the absorbance readings.

From the results obtained from the graph (Figure 3.16), fluorescence intensity i.e. percentage fluorescence diminished with increasing concentration of liposomes. This was expected as this result indicated that the ethidium bromide was in fact displaced by the cationic liposomes. Ethidium bromide is a dye that exhibits fluorescence. The percentage fluorescence decreased and then stabilised as the ethidium bromide was being displaced with the liposomes. In addition, the binding ratio of the DNA to the cationic liposome was confirmed to be 1:3.

4.5.2 DNA-Liposome-Asialoorosomucoid Interactions

This assay was performed to confirm the DNA : liposome : streptavidin aminohexanoyl biotinylated AOM binding ratio obtained from the gel retardation assay. The ethidium bromide binds to / intercalates between the bases of double stranded DNA. When the streptavidin aminohexanoyl biotinylated AOM concentration is increased the ethidium bromide is displaced and when the adequate DNA : liposome : streptavidin aminohexanoyl biotinylated AOM binding ratio is reached, the DNA is now completely bound to the liposome and streptavidin aminohexanoyl biotinylated AOM. This can be

seen by diminished fluorescence activity i.e. a decrease in fluorescence and stabilisation of the absorbance readings.

From the results obtained from the graph (Figure 3.17), fluorescence intensity i.e. percentage fluorescence diminished with increasing concentration of AOM. This was expected as this result indicated that the DNA was displaced as the protein bound to the cationic liposomes. Ethidium bromide is a dye that binds to DNA and exhibits fluorescence. The percentage fluorescence decreased as the DNA was being displaced with the protein. In addition, the binding ratio of the DNA : cationic liposome : AOM was confirmed to be 1:4:9.

4.6 Characterisation of Interactions of the Components of the Transfection Complex

The introduction of DNA into cells may be achieved by the non-viral approach of liposome-mediated gene transfer. Cationic liposomes composed of a cationic lipid and a neutral co-lipid form lipoplexes (liposome-DNA complexes) mainly through electrostatic attraction between positively charged head groups on the membrane bi-layer with the negatively charged phosphodiester backbone of the nucleic acids. It is thought that lipoplexes exhibit some fusogenic behavior, which causes destabilisation of the cell membrane allowing their entry into the cell followed by release from endosomes. A targeting capability may be built into the DNA-liposome complex by the inclusion of cell-specific ligands resulting in ternary complexes (Moodley *et al.*, 2002). An example

of a ligand, and that which was investigated in this study, is the asialoglycoprotein AOM which displays cell specificity to hepatocytes. Once lipoplexes are assembled, they carry a net positive charge (Goncalves *et al.*, 2004). For this reason, since the targeting component AOM is also positively charged, and will therefore not bind to the complex by electrostatic attraction as was the reaction complexing the lipoplexes, the linking reaction of biotin-streptavidin was crucial to this system.

The use of such non-viral systems is advantageous because they are able to mimic certain characteristics of natural viruses in their ability to act as efficient transporters of extracellular DNA across outer cell membranes and nuclear membranes (Francescangeli *et al.*, 2003). In addition, the absence of risk for infection, low immunogenicity as well as the ease of manufacturing such complexes adds to the advantages (Goncalves *et al.*, 2004).

4.6.1 Characterisation of DNA-Liposome Complex (Lipoplex) by Transmission Electron Microscopy (TEM)

In this study, the lipoplexes were characterised microscopically exhibiting their binding capability. The results obtained are in keeping with various previous studies where similar structures were obtained (Cao *et al.*, 2000; Moodley *et al.*, 2002). In addition the behaviour of the various ratios also produced similarity to that observed in this study though the liposome composition differed slightly. The lipoplexes varied in size range

(100 – 400 nm) but complexes aggregated forming larger structures. This is similar to that in previous studies where the sizes ranged from 200 – 500 nm (Singh and Ariatti, 2003).

A number of cationic lipids are currently being used for liposome-mediated delivery of DNA into mammalian cells and for each application there are optimal cationic lipids. The relationship between the lipoplex ultrastructure as well as the transfection efficiency also varies between various systems (Moodley *et al.*, 2002).

4.6.2 Characterisation of DNA-Liposome-Asialoorosomucoid Complex by Transmission Electron Microscopy (TEM)

In this study, the transfection complexes were characterised microscopically exhibiting their binding capability. Observations show that binding occurred at both the ratios (1:3:9 and 1:4:9, DNA : liposome : AOM) conducted. On comparing the electron micrographs obtained from these transfection complexes and the lipoplexes, one can see a significant difference in the composition of the complexes. The lipoplexes are more lipid in nature as opposed to the transfection complexes where the protein component is clearly visible. This is due to the addition of the asialoglycoprotein complexed to the glycoprotein streptavidin. In addition, comparing the two ratios, complexes composed of the 1:3:9 ratio are assumed to exhibit a more lipid nature than those of the 1:4:9 ratio. One would argue that this should not be the case as it is the liposome concentration that is varied and

the 1:4:9 ratio should show a higher lipid content. However, it is assumed that because binding of the protein to the lipoplex was optimal at the 1:4:9 ratio, more protein bound and is therefore more clearly visible. This can be seen comparing Figures 3.24 and 3.26 where the ratios are 1:3:9 and 1:4:9 respectively. The results obtained reinforces the importance of the biotin-streptavidin reaction and suggest that the ratio in which the complexes are composed are crucial for the production of complexes for optimal and efficient transfection.

As with the case of lipoplexes, the relationship between the complex ultrastructure as well as the transfection efficiency varies depending on a number of factors.

4.7 Nuclease Digestion Assay

This assay was performed to confirm the optimum ratio in which the DNA is protected within the DNA-liposome-streptavidin aminohexanoyl biotinylated AOM complex. The optimum concentration of streptavidin aminohexanoyl biotinylated AOM was observed to be 9 μg. In this assay therefore, the concentration of streptavidin aminohexanoyl biotinylated AOM was kept constant at 9 μg and the amount of liposome was varied (1-6 μg) whilst the DNA was kept constant at 1 μg. During this procedure the pGL3 plasmid DNA, the cationic liposome and the streptavidin aminohexanoyl biotinylated AOM were allowed to interact and thereafter treated with nucleases contained within the FBS and agarose gel electrophoresis was then carried out. Plasmid DNA with no exposure to

nucleases was run against plasmid DNA exposed to nucleases as well as varying amounts of cationic liposomes (1-6 μg), streptavidin aminohexanoyl biotinylated AOM (9 μg) while the amount of DNA was kept constant.

Nucleases are enzymes that hydrolyse nucleic acids and are often referred to as RNAses and DNAses. These enzymes are secreted by the pancreas and breakdown nucleic acids into individual nucleotides or into chains of three or four nucleotides (Wallace *et al.*, 1996).

This experiment was performed to ensure that the DNA is not digested / degraded by the action of the nucleases and that the DNA is in fact protected within the transfection complex. Free DNA on the other hand, is expected to be degraded when exposed to nucleases contained within serum as it is not protected. This experiment was therefore carried out to ensure that the DNA is indeed protected should this transfection complex be exposed to serum containing nucleases.

From the results obtained, the optimum ratio in which the DNA is protected within the DNA-liposome-streptavidin aminohexanoyl biotinylated AOM complex is 1:4:9. This is also the optimum binding ratio obtained in the DNA-liposome-asialoorosomcoid interactions. Prior to this ratio, ratios of 1:1:9; 1:2:9 and 1:3:9 were observed. The DNA at these ratios was protected by the complex to an extent but not completely as suggested by the absence of the third band of DNA in all these lanes (3-5) (Figure 3.27) as compared to that of lane 1 where three bands are visible. The remaining lanes i.e. lanes 6-8 of Figure 3.27 (ratios 1:4:9; 1:5:9 and 1:6:9 respectively) show significantly more DNA

as compared to that seen in lanes 3-5 indicating that at these ratios the DNA was completely protected. The optimum binding ratio for the transfection complex was 1:4:9. It is at this ratio that all the DNA is bound within the complex and is the optimum ratio at which the DNA is protected from digestion by nucleases.

4.8 Tissue Culture Studies

4.8.1 Maintenance and Growth of HepG2 cells

The liver is an excellent target organ for gene therapy and hence the HepG2 (hepatocellular carcinoma) cell line was utilised in this study. Hepatocytes account for 50% of the cells in the liver and are responsible for the synthesis of most plasma proteins (Singh and Ariatti, 2003). To develop therapies for disorders of the liver, and to test the ability of DNA protein complexes to deliver genes to primary hepatocytes, these HepG2 cells displaying asialoglycoprotein receptors were targeted using complexes containing DNA, a ligand AOM specific for the asialoglycoprotein receptors and cationic liposomes.

The maintenance and growth of the HepG2 cells require tissue culture work and it is imperative that cautionary procedures (Appendix J) be maintained at all times to reduce the risk of contamination.

Sometimes cells at or near confluency may be preserved. Freezing cultures have the following advantages; it requires less work saving time and money, it serves as a backup supply for emergencies and it provides a homogenous population by minimizing culture aging and evolution.

However, freezing damage must be prevented in the following ways; slow freezing must be used to remove all intracellular water, cryoprotective agents must be used to minimise dehydration effects and it must be stored below -130°C to completely stop biological time. When these cells are required they are then resuscitated and allowed to grow in culture flasks.

4.9 Transfection Studies in Tissue Culture : Gene Transfer Experiments

4.9.1 Growth Inhibition Assay

Complexes in which the DNA and AOM ratio was constant at 1 μg : 9 μg and in which the liposome content varied between 2 and 5 μg were shown to be relatively non toxic to HepG2 cells under transfection conditions. Cell growth was inhibited in the range 1.3 - 16.3%. Previous findings showed complexes in which the DNA and AOM ratio was constant at 0.5 μg : 2 μg and in which the liposome content varied between 1 and 6 μg. This study however showed that complexes and compositions chosen for transfection studies inhibited cell growth in the range 17 - 33% (Singh and Ariatti, 2003). Another

study by Singh *et al.* (2001) which also used complexes in which the DNA and AOM ratio was constant at 0.5 μg : 2 μg but the liposome content varied between 1 and 3 μg showed that complexes and compositions chosen for transfection studies inhibited cell growth in the range 7 - 25%. The difference could be attributed to the chemical composition of the liposomes used. Our study made use of liposomes constituted of Chol-T, DOPE and biotinylchoesteryl formylhydrazide, whereas Singh *et al.* (2001) used Chol-T, DOPE and a higher homologue of 3β[N',N"-dimethylaminoethane)-carbomoyl] cholesterol (DC-Chol) and Singh and Ariatti (2003) used Chol-T, DOPE and the N-hydroxysuccinimide ester of cholesterol hemisuccinate.

4.9.2 Evaluation of Gene Expression

Gene expression and other cellular events coupled to gene expression may be studied by genetic reporters. One such reporter is the firefly luciferase. Fireflies contain oxygen and a substance called luciferin. The chemical reaction between the two produces light. The luciferase enzyme helps to speed up the process and this intensifies light emission (Promega Technical Manual Luciferase Assay System). The luciferase assay was performed to ensure that transfection of the HepG2 cells was successful by the emission of light produced by the chemical reactions of the pGL3 reporter gene.

4.9.2.1 Transfection of HepG2 Cells

The HepG2 cells transfected with the transfection complexes showed some degree of luciferase activity.

As a general view, an increase in transfection activity was seen as the liposome concentration was increased. As the liposome concentration was increased, the luciferase activity also increased indicating that there is linear relationship between the two with the highest transfection efficiency seen with 5 μg liposome. Likewise, it was previously shown that the transfection efficiency increased as the liposome concentration was increased from 2-4 μg (Singh and Ariatti, 2003).

Furthermore it was observed that those complexes containing no liposomes exhibited luciferase activity approximately 3 orders of magnitude lower that that obtained with ternary complexes (Singh and Ariatti, 2003) and a similar finding was observed in the present study. This is because even though the pGL3 DNA containing the luciferase gene was present, the HepG2 cells were not transfected as the DNA delivery agent i.e. the liposomes and the cell specific targeting ligand i.e. the AOM were absent.

The observations obtained suggest that as the liposome concentration was increased to optimum concentrations, relatively more protein bound. Hence, the transfection efficiency was greater. Successful transfection occurred where the complexes bound to and entered the HepG2 cells transferring the DNA into the cell. This once again reinforces the importance of the asialoglycoprotein in such a complex as it supports the

theory that transfecting complexes containing the cell specific targeting ligand AOM gain entry into the HepG2 cells by asialoglycoprotein receptor mediation (Figure 4.7).

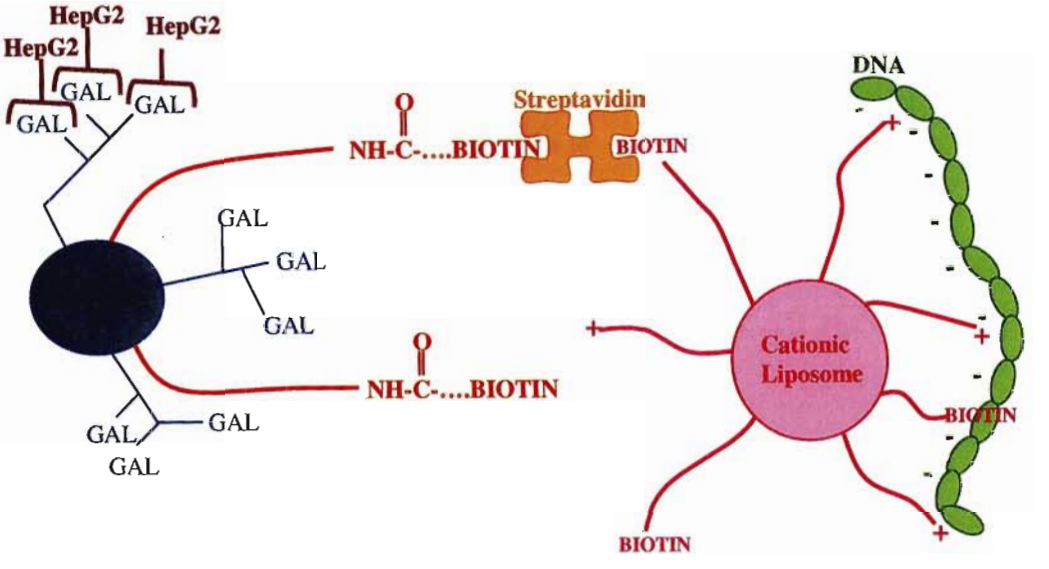


Figure 4.7 : Diagrammatic representation (not to scale) of transfection complex indicating binding of the galactose receptors of AOM to HepG2 cells

Further, it can be seen that the DNA delivery agent (liposome) and the cell specific targeting ligand (AOM) which are specific for receptors on HepG2 are essential for transfection to occur and it is concluded that the presence of the liposome and the AOM is imperative for transfection. In addition, the ratios in which these components co-exist together with the DNA is also of importance.

The above finding was reiterated when excess asialoglycoprotein asialofetuin was added to the cells prior to incubation with the complexes. This assay was performed to observe

if binding of the complexes to the asialoglycoprotein receptor on the HepG2 cells occurred in the presence of another asialoglycoprotein. The use of excess free asialofetuin over the complexed asialoglycoprotein AOM resulted in a marked decrease in transfection. In fact, at a DNA : liposome : AOM ratio of 1:5:9 which afforded the highest activity (21435 RLU/mg protein), the inclusion of competing asialofetuin reduced activity by approximately 85%. The luciferase activity of these were low as compared to transfection in the absence of asialofetuin indicating that the asialofetuin bound to the cell receptors allowing very little AOM of the transfection complex to bind, suggesting that transfection was not as successful. These luciferase activities were much lower than those obtained with the transfection complexes in the absence of asialofetuin, suggesting that much less protein entered the cell by receptor mediation. Hence, the transfection efficiency was sub-optimal. Such an observation reiterates that transfecting complexes gain entry into cells by asialoglycoprotein receptor mediation, stressing the importance of the mechanism of receptor mediation for transfection of the cells.

In addition, the commercial reagent FuGene 6 was used as a comparison to assess transfection efficiency observed using our transfection complex. Results show that the luciferase activities were comparatively high and the readings do not differ extensively from those obtained from transfection using our transfection complex.

However, in comparison with previous studies, the transfection efficiency was considerably lower as indicated by the luciferase activities. The size ranges of the transfection complexes in this study is similar to that obtained previously (Singh and

Ariatti, 2003), but it was noticed that the complexes aggregated to form larger entities and this could account for the lower transfection rate. The mechanism of gene transfer investigated in this study however, proved to be successful as evident by successful transfection.

CHAPTER 5

CONCLUSION

Targeted delivery of DNA to specific cell types by receptor mediated endocytosis has been shown to be a viable mechanism for gene delivery. Ligands such as AOM have been used to target specific cell types followed by entry of the cell and incorporation into the cells DNA via receptor mediation (Wu and Wu, 1987; Wu and Wu, 1988; Christiano *et al.*, 1993b; Singh *et al.*, 2001; Singh and Ariatti, 2003).

Targeting of DNA to a specific cell however, is only a part of the entire transfection process. In order for the corrective DNA to gain entry into the target cell, it needs to be delivered to the cell by a DNA delivery agent. One such agent is the cationic liposome which has been used in a vast array of studies. The DNA and cationic liposome form a complex, the lipoplex via electrostatic attraction. In this study, a novel cationic cholesterol derivative, biotinylcholesteryl formylhydrazide was used as a component in the synthesis of the cationic liposome. The AOM was also biotinylated. Through a series of chemical reactions, the biotin molecules of the two components were complexed to a glycoprotein specific for biotin binding, streptavidin, resulting in a bridge connecting these components.

We have focussed on the use of cationic liposomes, targeted to cell surface receptors as a means of enhancement of the receptor mediated gene delivery. The targeted liposome mediated DNA transfer was effective in transfection of the human hepatocellular

carcinoma (HepG2) cell line as the targeting ligand used is specific for such cells. From the results obtained it can be concluded that the novel cationic liposome prepared, and the mechanism investigated is a potentially valuable tool for gene delivery. Further, future experimentation could be extended to *in vivo* methods and this may be applied to the treatment of genetic diseases and disorders.

These cationic liposomes are quickly and simply prepared and from toxicity studies conducted, are well tolerated by cells *in vitro.* The incorporation of such cationic liposomes in the system investigated therefore, shows considerable promise for gene delivery. Another advantage contributing to the fact that the complexes are not damaging or toxic to the cells would be the use of the asialoglycoprotein which is an endogenous serum glycoprotein.

In this study, DNA containing the pGL3 luciferase gene was targeted to the HepG2 cells that possess the asialoglycoprotein receptor on their surface by forming a complex with cationic liposomes and asialoorosomucoid in a 1:4:9 ratio. The luciferase assay was used to confirm that transfection occurred and the competition assay was conducted to show that the complexes gain entry into the cells by asialoglycoprotein receptor mediation. Another method that could have been performed to confirm further the receptor binding specificity of the complexes would have been to employ the use of another cell line for example the cervical cell line (HeLa) containing no asialoglycoprotein receptors as a control cell line in this project.

The overall objective of this project was to develop a safe and efficient mechanism for gene transfer using cationic liposomes as gene transfer vehicles in a cell culture model. This objective has thus far proven successful and has the advantage in that it is a non-viral mechanism that is relatively non toxic to cells *in vitro*, and unlike viral vectors should theoretically not elicit an immune response *in vivo*. However, future studies will need to be conducted in order to determine this and despite the progress in this field over the years, there are a number of obstacles that need to be overcome before this mechanism will be ready to be used in clinical studies.

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APPENDIX A

Preparation of Media

Lauria Bertani (LB) Medium

For 250 ml :

Tryptone – 2.5 g

Yeast extract – 1.25 g

Sodium Chloride (NaCl) – 2.5 g

Add 235 ml distilled water. pH to 7.0 with sodium hydroxide (NaOH). Adjust volume with distilled water to 250 ml. Autoclave in autoclave bag (121°C, 15 minutes). Cool. Store at 4°C. 125 µl ampicillin (100 000 µg/ml stock) may be added if required.

Ampicillin Stock

To 500 mg add 5 ml distilled water. This affords a concentration of 100 000 µg/ml.

APPENDIX B

Preparation of Solutions and Buffers

10 X TBE (Tris-Borate-EDTA) Buffer

For 1 L

Tris – 108 g

Boric Acid – 55 g

EDTA – 9.3 g

Measure out the above reagents into a conical flask. Add approximately 700 ml distilled water. Dissolve on stirrer at low heat. Adjust volume to 1000 ml with distilled water. Filter and store at room temperature in schott bottle.

50 mM $CaCl_2$, 10 mM Tris-HCl (pH 8.0)

For 100 ml

$CaCl_2$ – 0.735 g

Tris-HCl – 0.15764 g

Measure out the above reagents. Add approximately 60 ml distilled water. pH to 8.0. Adjust volume to 100 ml with distilled water. Autoclave (115°C, 10 minutes). Cool. Store at 4°C.

15% Glycerol in 50 mM $CaCl_2$, 10 mM Tris-HCl (pH 8.0)

For 100 ml

$CaCl_2$ – 0.735 g

Tris-HCl – 0.15764 g

Glycerol- 15 ml

Measure out the above reagents. Add approximately 60 ml distilled water. pH to 8.0. Adjust volume to 100 ml with distilled water. Autoclave (115°C, 10 minutes). Cool. Store at 4°C.

1 M Tris (pH 8.0 / pH 7.5)

For 100 ml

Tris – 12.11 g

Measure out the above reagent. Add approximately 60 ml distilled water. pH to 8.0 / 7.5. Adjust volume to 100 ml with distilled water. Autoclave (115°C, 10 minutes). Cool. Store at 4°C.

Sucrose/Tris

For 100 ml

Sucrose – 25 g

1 M Tris (pH 8.0) – 5 ml

Measure out the above reagents. Adjust volume to 100 ml with distilled water. Autoclave (115°C, 10 minutes). Cool. Store at 4°C.

Triton Lysing Mix

For 100 ml

Triton X-100 – 5 ml

Sucrose – 5 g

1 M Tris (pH 7.5) – 5 ml

0.5 M EDTA – 10 ml

Measure out the above reagents. Adjust volume to100 ml with distilled water. Autoclave (115°C, 10 minutes). Store at room temperature.

2.5 M KoAc (pH 4.8)

For 100 ml

KoAc – 24.5 g

Measure out the above reagent. Add approximately 40 ml distilled water. pH to 4.8 with HOAc. Adjust volume to 100 ml with distilled water. Autoclave (115°C, 10 minutes). Store at room temperature.

Lysozyme Mixture (10 mg/ml in Sucrose/Tris)

Add 10 mg lysozyme to 1 ml Sucrose/Tris. (Only Stable for 1hour).

Tris-EDTA Buffer (pH 7.2)

(10 mM Tris-HCl, 1 mM EDTA)

For 100 ml

Tris-HCl – 0.15764 g

EDTA – 0.037224 g

Measure out the above reagents. Add 60 ml distilled water. pH 7.2. Adjust volume to 100 ml with distilled water. Autoclave (115°C, 10 minutes). Store at room temperature.

HBS (Hepesbuffered saline) (pH 7.5)

20 mM HEPES

150 mM NaCl

pH to 7.5

0.1 M Sodium Acetate (pH 3.6 / pH 4.5)

For 100 ml

Sodium Acetate – 0.8203 g

Sodium Chloride – 0.2922 g

Acetic Acid – 660.55 ml

Measure out the above reagents. Add approximately 60 ml distilled water. pH to 3.6 / 4.5. Adjust volume to 100 ml with distilled water. Autoclave (115°C, 10 minutes). Cool. Store at room temperature.

Periodate Solution

(0.2 M sodium periodate in 9 M phosphoric acid)

For 100 ml

Sodium Periodate – 4.2778 g

Phosphoric Acid – 74.97 ml

Measure out the above reagents. Add approximately 100 ml distilled water. Stir to dissolve. Autoclave (115°C, 10 minutes). Cool. Store at room temperature.

Arsenite Solution

(10% $^{w}/_{v}$ sodium arsenite in 0.5 M sodium sulphate-0.1 N sulphuric acid solution)

For 100 ml

Sodium Arsenite – 10 g

Sodium Sulphate – 16.1105 g

0.1 N Sulphuric Acid – 100 ml

Measure out the above reagents. Stir to dissolve. Autoclave (115°C, 10 minutes). Cool. Store at room temperature.

Thiobarbituric Acid Solution

(0.6% $^{w}/_{v}$ 2-thiobarbituric acid in 0.5 M sodium sulphate)

For 100 ml

2- Thiobarbituric Acid – 0.6 g

0.5M Sodium Sulphate – 100 ml

Measure out the above reagents. Stir to dissolve. Autoclave (115°C, 10 minutes). Cool. Store at room temperature.

0.2 M Sodium Chloride, 0.005 M Tris-HCl (pH 7.6)

For 100 ml

Sodium Chloride – 1.1688 g

Tris-HCl – 0.07882 g

Measure out the above reagents. Add approximately 60 ml distilled water. pH to 7.6. Adjust volume to 100 ml with distilled water. Autoclave (115°C, 10 minutes). Cool. Store at 4°C.

NOTE : All chemicals were of analytical grade unless otherwise stated.

APPENDIX C

Agarose Gel Electrophoresis

Principle

In electrophoresis, the DNA is loaded into a gel and subjected to an electric current. Since the speed of migration of the DNA toward the positive pole is inversely related to its size, the separation of the DNA fragments by size is achieved. Ethidium bromide is a chemical that intercalates with the DNA and fluoresces under ultra-violet light. It is therefore included in the gel mixture and allows the visualisation of the DNA fragments as bands after electrophoresis.

Procedure to make Agarose Gel

For 2% gel

Agarose – 1.4 g

1 X electrophoresis buffer – 70 ml

For 1% gel

Agarose – 0.70 g

1 X electrophoresis buffer – 70 ml

Measure out the above reagents. Add to a conical flask. Plug the top end of the flask using a paper towel. Swirl flask and place into the microwave for approximately 1 minute

until agarose has dissolved. Cool to 50-60°C and add 3.5 µl ethidium bromide. (Ethidium bromide is a powerful mutagen, gloves must be worn at all times).

Set gel mould and insert combs as required. Pour gel to set and remove air bubbles by using a pipette tip and moving the bubbles to the side. Leave gel at room temperature until set (approximately 30 minutes).

Procedure to run gel

- Add 1 X electrophoresis buffer into the gel tank – enough to cover electrodes.
- Remove combs and the sides of the gel mould.
- Place gel with the gel tray into the gel tank. Ensure that the buffer covers the gel.
- Into the wells of a microtitre plate add 2-3 µl gel loading buffer. Bromophenol blue is used for larger DNA products and Orange G is used for smaller DNA products. Also add appropriate amount of sample. Mix and add the total mixture gently into the well of the gel.

  Note: Hold the tip of the pipette in the well and gently press the release button of the pipette.
- Place the lid of the apparatus into position and on making sure that the electrodes are connected appropriately, switch on the power supply.
- Leave gel to run at 100 V for approximately 45 minutes to an hour.
- Turn off the power supply, remove lid and carefully lift out the gel. Blot excess TBE on paper towel and place gel on the illuminator. Remove tray if it does not allow penetration of U-V light. (Gloves must be worn when handling gels).
- View gel and photograph.

APPENDIX D

Thin Layer Chromatography (TLC)

Principle

TLC is a simple, quick and inexpensive procedure that gives a quick answer as to how many components are in a mixture. TLC is also used to support the identity of a compound in a mixture when the R_f of a compound is compared with the R_f of a known compound.

Procedure

- A solvent system is prepared using chloroform and methanol in a 9:1 ratio.
- The TLC is a sheet of glass, metal or plastic containing a thin layer of solid adsorbent such as silica. The origin is marked on the plate and the plate is labeled.
- Samples are spotted at the origin (at the corresponding labels) using a spotting tube.
- The plate is then placed into a beaker containing the solvent ensuring that the origin is at the base of the beaker and that the solvent does not exceed the line of the beaker.
- The beaker is then covered to prevent the chloroform from evaporating.
- The plate is left in the beaker for approximately 15 minutes.
- The plate is then removed and the solvent front marked.
- The plate is then sprayed with sulphuric acid (H_2SO_4).
- The plate is then place on a hot plate.
- Spots are visible as reddish / purple in colour.

APPENDIX E

Preparation of Spurr's Resin

SPI-CHEM™ SPURR LOW VISCOSITY EMBEDDING KIT

Introduction

The Spurr low viscosity system of embedding offers superior penetration of sample material. Among its other convenience features are:

- Less viscous than "Epons" and "Araldites"
- Ease of Mixing
- Long pot life
- Short cure time
- Controllable hardness
- Ease of sectioning (glass or diamond knives)

Ultrathin sections are stable in the electron beam and usually can be mounted on 200 mesh grids without a supporting film. Sections from biological specimens stain readily with uranyl acetate and lead citrate solutions. Good results have also been obtained with phosphotungstic acid stains.

The SPURR SYSTEM is based on four components:

1. Vinylcylophene Dioxide (ERL 4206), a cycloaliphatic diepoxide with a molecular weight of 1402 and an epoxy equivalent (WPE) of 74-78.
2. Diglycidyl Ether of Polypropylene Glycol (DER 736), the flexibiliser, has a

WPE of 175-205. The hardness of the cured resin is controlled by varying the amount of DER 736.

3. Noneyl Succinic Anhydride (NSA), the hardner, has a molecular weight of 224.

   Exposure to atmospheric moisture must be kept to absolute minimum.

4. Dimethylaminoethanol (DMAE), the recommended cure accelerator, gives a longer pot life to the resin mixture. Curing time can be altered by varying the amount of DMAE used. For faster curing, use Benzyldimethylamine (BDMA) or DMP-30.

Formulations

Standard and modified embedding formulations are shown in the following table:

COMPONENT	STANDARD FORMULA FIRM	HARD	SOFT
VCD	10.0	10.0	10.0
DER 736	6.0	4.0	7.0
NSA	26.0	26.0	26.0
DMAE	0.4	0.4	0.4

Mixing of Resin Components

Procedure to prepare the Standard Formula Firm

The preparation and mixing of the resin components must be thorough and always performed in a fume hood.

- Weigh a tared disposable plastic beaker and add 42.4 g to the beam (total weight of components to be used).
- Slowly pour 26 g of NSA into the beaker, adding the last few drops with a spatula or stirring rod.
- Add 10 g ERL 4206 into the beaker from a disposable syringe.
- Add 6 g of DER 736 to the beaker from a disposable syringe.
- Add 4 g of DMAE to the beaker, remove from balance and immediately begin to stir for approximately 3 minutes.

APPENDIX F

Staining gels with Coomassie Brilliant Blue

Principle

Proteins separated on gels can be simultaneously fixed with methanol:glacial acetic acid and stained with Coomassie Brilliant Blue R250, a triphenylmethane textile dye also known as Acid Blue 83. The gel is immersed in a concentrated methanol/acetic acid solution of the dye for several hours and excess dye is then allowed to diffuse from the gel during a prolonged period of destaining.

Procedure

- Dissolve 0.25 mg Coomassie Brilliant Blue R250 in 90 ml methanol:H_2O ($1:1^{v}/_{v}$) and 10 ml glacial acetic acid. Filter the solution through a Whatman No. 1 filter to remove any particulate matter.
- Immerse the gel completely in staining solution and place on a slowly rotating platform at room temperature for a minimum of 4 hours.
- Remove the stain. This stain may be saved for future use. Destain the gel by soaking it in methanol/acetic acid solution (step 1) without the dye on a slowly rotating platform for 4-8 hours, changing the destaining solution three to four times.
- The more thoroughly the gel is destained, the smaller the amount of protein that can be detected by staining with Coomassie Brilliant Blue i.e. prolonged

destaining results in loss of intensity of staining of protein bands (Sambrook *et al.*, 1989).

APPENDIX G

TSK-GEL-DEAE-5PW anion exchange column (Tosohaas), and a Bischoff isocratic HPLC pump

Calibration of HPLC

Equipment warm-up : All electronic equipment is allowed to warm up. During this period, 0.1 M sodium acetate (NaOAc), buffer is passed through the column (approximately 20 minutes at 1.5 ml/min) and continued until the baseline is level at the UV detector's greatest sensitivity. This is to ensure all the methanol is eluted. Methanol is passed through to prevent corrosion of the plumbing when the instrument is not in use.

Initial calibration : Injections of each calibration standard over the concentration range of interest are made into the HPLC. Peak heights or peak areas are obtained for each analyte.

Procedure

- A reservoir solvent of 0.1 M sodium acetate (NaOAc) is used.
- 50 µl of orosomucoid dissolved in 0.1 M sodium acetate (NaOAc), pH 4.5 is injected into the loop and used as a control.
- 50 µl of sample is then injected into the loop.
- A chromatogram is then printed.
- The buffer is then flushed out with water and then methanol.

APPENDIX H

Packard 1900 TR Scintillation Spectrophotometer / Counter

Principle

This instrument is a device that detects nuclear radiation from flashes of light generated in a material by the radiation.

Preparation of samples

- Into a vial 10 ml scintillation fluid (Ready Solv HP Liquid Scintillation Cocktail) was added. This served as a blank.
- Into a second vial containing 150 µl asialoorosomucoid treated with [^{3}H] aminohexanoyl-biotin-N-hydroxysuccinimide, 10 ml scintillation fluid (Ready Solv HP Liquid Scintillation Cocktail) was added.
- Into a third vial containing 13.5µl [^{3}H] aminohexanoyl-biotin-N-hydroxysuccinimide, 10ml scintillation fluid (Ready Solv HP Liquid Scintillation Cocktail) was added.

All fingerprints must be wiped off the vials and excessive aluminum foil is removed as this would cause reflection of light within the instrument affecting the results.

Procedure

- Switch the instrument on and insert vials into rack and close lid.
- Select protocol on system : Tritium [^{3}H]; 1 min counts.
- Allow instrument to count each sample.

APPENDIX I

Bicinchoninic Acid (BCA) Protein Assay Kit (Sigma)

Principle

The Bicinchoninic Acid (BCA) Protein Assay relies on the formation of a Cu^{2+} -protein complex under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{1+}. The amount of reduction is proportional to the protein present. It has been shown that cystine, cysteine, tryptophan, tyrosine, and the peptide bond are able to reduce Cu^{2+} to Cu^{1+}. In alkaline environments BCA forms a purple blue complex with Cu^{1+} providing a basis to monitor the reduction of alkaline Cu^{2+} by proteins (Bicinchoninic Acid (BCA) Protein Assay Kit Manual, Sigma Product Information).

Procedure

- The BCA Working Reagent is prepared by mixing 50 parts Reagent A with 1 part Reagent B. Mix this BCA Working Reagent until it is light green in colour.
- Prepare standards of different concentrations. These bovine serum albumin (BSA) protein standards can range from 200-1000 µg/ml (20-100 µg of total protein). This is accomplished by making serial dilutions starting from the 1mg/ml standard, and then using 0.1 ml of each diluted standard in the assay.
- Prepare the unknown/test samples taking note of the dilution factor.
- Add 2 ml BCA Working Reagent to 0.1 ml of each blank, BSA protein standard and unknown/test sample. The total volume in the test tube is 2.1 ml.
- Vortex gently to ensure the reaction mixtures are thoroughly mixed.

- Incubate at 37°C for 30 minutes.
- If required, allow the tubes to cool to room temperature.
- Transfer the reaction mixtures to cuvettes.
- Measure the absorbance of each of the reaction mixtures at 562 nm (Novaspec® II spectrophotometer).
- Create a standard curve based on either the BSA protein standard concentration or on the amount of protein present in the BSA protein standard.
- Determine protein concentration by comparison of the absorbance of the unknown/test samples to the standard curve prepared using the BSA protein standards.

APPENDIX J

Tissue Culture

Media and Buffers

Preparation of Medium : Minimal Essential Media (MEM)

- One vial/packet MEM (with Earle's salts) is emptied into a 2 L beaker.
- 900 ml autoclaved water is added and thoroughly mixed.
- 10 mM $NaHCO_3$ is added.
- 20 mM HEPES is added.
- 20 ml antibiotic (penicillin/streptomycin mixture) is added.
- Make up to 1 L with autoclaved water and check pH (between 7.3-7.4).
- Filter the medium through a 0.22 µm filter unit with a filling bell into 5 equal 200 ml aliquots in sterile 250 ml wide-neck bottles. This must be carried out in a laminar flow hood using a peristaltic pump to drive the medium through the filter. Store at 4°C.

Preparation of Phosphate Buffered Saline (PBS) (pH 7.3-7.4)

NaCl – 8 g

KCl – 0.2 g

KH_2PO_4 – 0.12 g (anhydrous)

Na_2PO_4 – 0.91 g (anhydrous) or Na_2PO_4. 12 H_2O – 2.29 g

Make up to 1 L with autoclaved water. Adjust pH. Autoclave (115°C, 10 minutes). Cool. Store at 4°C.