

RECEPTOR TARGETED GENE DELIVERY USING FOLATE LIGAND CONJUGATED CATIONIC LIPOSOMES

by

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

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ABSTRACT

Gene therapy has become an important strategy to treat several human diseases, including cancer, viral infections and inherited disorders. In response to this growing trend, a number of gene delivery vectors have been manufactured both to facilitate nucleic acid uptake by target cells and also to promote the transport of genetic materials into the nucleus. The success of gene therapy however depends on the efficient delivery of therapeutic genes into target cells both *in vitro* and *in vivo*. Cationic liposomes represent a class of non-viral vectors that have shown the ability to bind and deliver DNA cargo to defective cells efficiently. This study has focused on the development of a novel folate-targeted cationic liposome-mediated gene delivery system. This receptor is overexpressed on numerous cancer cell types and offers a convenient docking point for subsequent cellular uptake of folate decorated liposome-DNA complexes by receptor mediation.

In this study, a total of six cationic liposome preparations comprising either cationic cholesterol cytofectin *N,N*-dimethylpropylamidodisuccinylcholesterylformylhydrazide (MSO9) or 3β [*N*(*N*₁,*N*₁-dimethylaminopropylsuccinamidoethane)-carbamoyl]-cholesterol (SGO4) were formulated by mixing the fusogenic neutral helper lipid, dioleoylphosphatidylethanolamine (DOPE) as a common constituent. DSPE-PEG₂₀₀₀ was also used in formulations for possible *in vivo* development of PEGylated, targeted liposomes. The targeting ligand folate was appended to the distal end of liposome-anchored DSPE-PEG₂₀₀₀, for prominent display and optimal receptor recognition.

Transmission electron micrographs revealed liposomes to be unilamellar, spherical shaped vesicles with a narrow size range (50 - 80 nm in diameter). Agarose gel retardation studies demonstrated complex formation between cationic liposomes and plasmid DNA, whilst serum nuclease protection assays showed that the liposome formulations were capable of protecting the complexed DNA in lipoplexes against serum nuclease digestion. Ethidium bromide dye displacement studies yielded information on the compaction or condensation efficacy of the liposomes with respect to the cargo plasmid. In addition, particle sizes determined by dynamic light scattering confirmed the suitability of lipoplexes for future *in vivo* applications in which extravasation is essential. Importantly, these liposome:DNA complexes were found to exhibit minimal growth inhibition levels in HEK293, HeLa and KB cells. Further investigations were carried out to determine the optimal transfection activity of

complexes in the folate receptor-positive cell lines (HeLa and KB). The plasmid containing the transgene firefly luciferase (pCMV-luc) was used in transfection studies. Results showed that folate targeted liposomes, irrespective of cytofectins MSO9 or SGO4 achieved highest transfection activities *in vitro*, specifically via receptor mediation. Lower transfection activity was observed for by untargeted PEGylated and unPEGylated liposomes compared to that of the folate targeted liposomes, strongly implicating folate receptor-mediation in the uptake of ligand-displaying lipoplexes. This was further confirmed by flow cytometry analysis. Furthermore, zeta potential values obtained for targeted complexes revealed low negative surface charge, thus minimizing the possibility of electrostatic interaction between lipoplexes and target cells. The cytofectin, MSO9, achieved 10 fold greater transfection activity than the cytofectin SGO4 although they are closely related, differing only in their spacer lengths. Competition assays using free folate (200 μ M) to confirm folate receptor mediated lipoplex uptake in the HeLa, and KB cells revealed a dramatic decline in transfection activity due to the excess free folate binding to and blocking access to the folate receptors on the cell membrane. The two novel PEGylated lipoplexes designed for folate receptor-mediated uptake by transformed mammalian cells display very favourable physicochemical characteristics, low cytotoxicity and promising transfection profiles *in vitro*. Therefore further investigation of the cationic liposome formulations examined in this study *in vivo* is warranted.

PREFACE

The experimental work described in this thesis was carried out in the Discipline of Biochemistry, School of Life Sciences, University of KwaZulu-Natal, Durban, South Africa from August 2011 to November 2014, under the supervision of Dr. Moganavelli Singh and co-supervision of Professor Mario Ariatti.

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

DECLARATION 1 – PLAGIARISM

I, **Sridevi Gorle** declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.
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3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
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DECLARATION 2 – PUBLICATIONS

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

Publications in Peer Reviewed Journals/Conference Proceedings

1. **S. Gorle**, M. Ariatti and M. Singh. “Novel serum-tolerant lipoplexes target the folate receptor efficiently”. *European Journal of Pharmaceutical Sciences*. 59, (2014) 83-93.
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LIST OF ABBREVIATIONS

- AA – Amino acid
- AIDS – Acquired immune deficiency syndrome
- ASGP-R – Asialoglycoprotein receptor
- BCA – Bicinchonimic acid
- ^{13}C NMR – Carbon nuclear magnetic resonance
- CCD - Cationic cholesterol derivative
- CDOPE – *N*-citraconyl-dioleoylphosphatidylethanolamine
- CHCl_3 – Chloroform
- CH_2Cl_2 – Methylene dichloride
- CHEMS – Cholesterol hemisuccinate
- Chol – Cholesterol
- Chol-T – 3-[*N*-(*N'*,*N'*-dimethylaminopropane)-carbamoyl]cholesterol
- CNS – Central nervous system
- Con NH_4OH – Ammonium hydroxide
- Cryo-TEM – Cryo-Transmission electron microscopy
- DAG – Diacylglycerol
- DC – Chol (3-(*N*-(*N'*,*N'*-dimethylaminoethyl)carbamoyl) cholesterol
- DCC – Dicyclohexylcarbodiimide
- DCU – Dicyclohexylurea
- DLS – Dynamic light scattering
- DMAPA – Dimethylaminopropylamine
- DMEM – Dulbecco's minimum essential medium
- DMF – Dimethylformamide
- DMSO – Dimethylsulphoxide
- DNA – Deoxyribonucleic acid
- DOGS – Dioctadecylamidoglycylspermine
- DOPE – Dioleoylphosphatidylethanolamine
- DOSPA – 2,3-Dioleoyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N,N*-dimethyl-1-propaniminium trifluoroacetate.

DOTAP – 1,2-Di-oleyl-3-trimethyl-ammonium-propane
DOTMA – *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride
DSPE-PEG₂₀₀₀ – Distearoylphosphatidylethanolamine polyethylene glycol 2000
DSPE-PEG FOL – Distearoylphosphatidylethanolamine polyethylene-glycol₂₀₀₀folate
DSPE-PEG₂₀₀₀-NH₂ - Distearoylphosphoethanolamine[amino(polyethylene glycol)-2000]
EBV – Epstein barr virus
EDTA – Ethylenediaminetetraacetic acid
EPR – Enhanced permeability retention
EtBr – Ethidium bromide
FBS – Foetal bovine serum
FCS – Foetal calf serum
F-PEG₂₀₀₀-DSPE – Folate-poly(ethylene glycol)-distearoyl-phosphatidylethanolamine
conjugate
FR – Folate receptor
GFP – Green fluorescent protein
GPI – Glycosyl phosphatidylinositol HEPES – 2-[4-(2-hydroxyethyl)-1-piperazinyl]
ethanesulphonic acid
¹H NMR – Proton nuclear magnetic resonance
H_{II} – Formation of membrane destabilizing hexagonal inverted phases
HBS –HEPES buffered saline
HEK293 – Human embryonic carcinoma cell line
HeLa – Human cervical carcinoma cell line
HEPES – 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulphonic acid
HR-MS – High resonance mass spectroscopy
HSV-tk – Herpes simplex virus thymidine kinase
IgE – Immunoglobulin E
KB – Human nasopharyngeal cell line
L α – Lamellar phases
LUV – Large unilamellar vesicles
MEM – Minimal essential medium
MeOH – Methyl alcohol

MLV – Multilamellar vesicles
Mp – Melting point
mRNA – Messenger RNA
MSO8 – Cholesterylformylhydrazidehemisuccinate
MSO9 – *N,N*-dimethylpropylamidodisuccinylcholesterylformylhydrazide
MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N₂H₄ – Hydrazine
NHS – *N*-hydroxysuccinamide
NHSF – NHS ester of folic acid
NHS-FOL – *N*-hydroxysuccinamidefolate
NOD mice – Non-obese diabetic mice
N/P – Nitrogen to phosphate ratio
NP-F – Folate linked nanoparticle
PA – Phosphatidic acid
PAGA – Poly[α -(4-aminobutyl)-L-glycolic acid]
pAMAM – Polyamidoamine dendrimer
PBS – Phosphate buffered saline
PC – Phosphatidylcholine
pCMV-luc – plasmid DNA
PCS – Photon correlation spectroscopy
PDI – Polydispersity index
pDMAEMA – Poly(2-dimethylamino)ethyl methacrylate
PEG – Polyethylene glycol
PEI – Polyethyleneimine
PG – Phosphatidyl glycerol
PL – Phospholipid
PLL – Poly-L-lysine
PS – Phosphatidylserine
RES – Reticuloendothelial system
RFC – Reduced folate carrier

RNA – Ribonucleic acid

RNAi – RNA interference

SCID – Severe combined immuno deficiency

SDS – Sodium dodecyl sulphate

SGO1 – 3β [*N*(2-aminoethyl)-carbamoyl]cholesterol

SGO2 – 3β [*N*(hemisuccinamidoethane)-carbamoyl]cholesterol

SGO4 – 3β [*N*(*N*¹,*N*¹-dimethylaminopropyl-succinamido-ethane)-carbamoyl]
cholesterol

siRNA – Small interfering RNA

SUV – Small unilamellar vesicles

TLC – Thin layer chromatography

T_m – Main transition temperature

Tris – Tris(hydroxymethyl)aminomethane

XRD – X-ray diffraction

THIS THESIS IS DEDICATED TO MY PARENTS
MR. SATYAM GORLE & MRS. KRISHNAVENI GORLE

CHAPTER ONE

INTRODUCTION

1. Gene Therapy - Principle

Gene therapy presents a unique approach to medicine as it can be adapted for the treatment of both genetic and acquired diseases such as cancer, cardiovascular diseases, AIDS, neurodegenerative disorders (Lasic, 1997) and urological diseases (Goins et al., 2009). Gene delivery depends upon the binding or encapsulation of a gene of interest, which is then preferably delivered to target cells. After uptake, the DNA has to be released into the cell so that transcription and translation may take place to afford the protein of interest. To accomplish effective gene delivery, a number of barriers must be overcome at every step of this process in order to enhance gene activity. Although the objectives and principles of gene therapy have been well-defined over the last decades, its application as a versatile, therapeutically efficacious approach has not yet met all expectations. Nowadays, it is clear that gene therapy may not only lead to its key goal of supplanting a deficient gene, but it could also lead to a modulation of the expression of genes acting on the physiology of malicious cells (Swartz et al., 2012). Moreover, by means of gene therapy, new functions might be integrated into cells, hence serving a therapeutic purpose (Hughes, 2005). Thus in a modern concept and broader sense, gene therapy addresses the potential use of nucleic acids, which includes plasmid DNA, antisense oligonucleotides siRNA or miRNA, to modify the expression of genes in cells for therapeutic purposes (Cornford et al., 2009).

The ‘naked’ plasmid DNA is unstable under *in vivo* circumstances due to rapid degradation by serum nucleases. Therefore, improvement of the stability of gene delivery vectors plays an important role in gene therapy. Carriers or ‘vectors’ are essential to provide effective DNA condensation and to protect the DNA or RNA from degradation and finally facilitating their uptake into specific cells (Luo et al., 2000; Lechardeur et al., 2005).

The primary challenge for gene therapy is to develop a method that delivers a therapeutic gene (transgene) safely and efficiently to target cells where gene expression can be achieved.

Gene delivery may be affected by viral and non-viral procedures. An ideal gene delivery vector needs to meet three major criteria: (i) it should protect the transgene against degradation by nucleases in the blood system and intercellular matrices, (ii) it should transport the transgene safely across the plasma membrane and into the nucleus of target cells, and (iii) it should have no detrimental effects to the cells.

1.1. Gene Therapy Approaches

Gene therapy can be accomplished *ex vivo* or *in vivo*. In *ex vivo* gene therapy, cells are removed from the host organism, inoculated with the therapeutic gene and then reintroduced into the organism (Antonio et al.; 2006). With the recent advances in this field, DNA can be easily injected directly into the nucleus while siRNA is transferred to the cytoplasm (Yoichi et al., 2008; Atul et al., 2009). The ability to specifically deliver nucleic acids to the desired cell is clearly gainful. However, this method is not without limitations. Only cells that can be removed and efficiently reintroduced in a functional way can be preserved using *ex vivo* gene therapy. To date, nearly all *ex vivo* gene therapy studies address diseases of the circulatory system (Cavazzana-Calvo et al., 2000; Youngren et al., 2013). Due to the nature of *ex vivo* gene therapy, most diseases cannot be treated in this way.

In contrast, *in vivo* gene therapy involves treatment of cells in their natural environment, in a living organism, and is applicable to nearly all diseases. Nucleic acids (most frequently complexed to a vector) are administered either topically, by direct injection into a tissue, or by systemic intravenous injection (Amiji, 2005). The nucleic acids must then effectively reach the desired cells by crossing the cell membrane, and upon reaching the desired cellular compartment, effectively unpack from the vector before they can exhibit a therapeutic effect. Although this method of gene therapy is broadly applicable and therapeutic nucleic acids have been established and tested, the lack of a safe and efficient delivery vector has limited *in vivo* gene therapy successes.

1.2. Gene Therapy in Cancer

Cancer gene therapy is the best studied application of gene therapy. During the past two decades, more than 600 clinical studies in gene therapy have been assessed, almost 70% of

which were in the area of cancer treatment (Breyer et al., 2001; Yang et al., 2011; Li et al., 2013). The transformation of normal cells into neoplastic ones involves multiple alterations at their genetic level (Bertram, 2000). Due to the complex nature of cancer, cancer gene therapy includes many therapeutic approaches, which fall into two main groups (Figure 1.1).

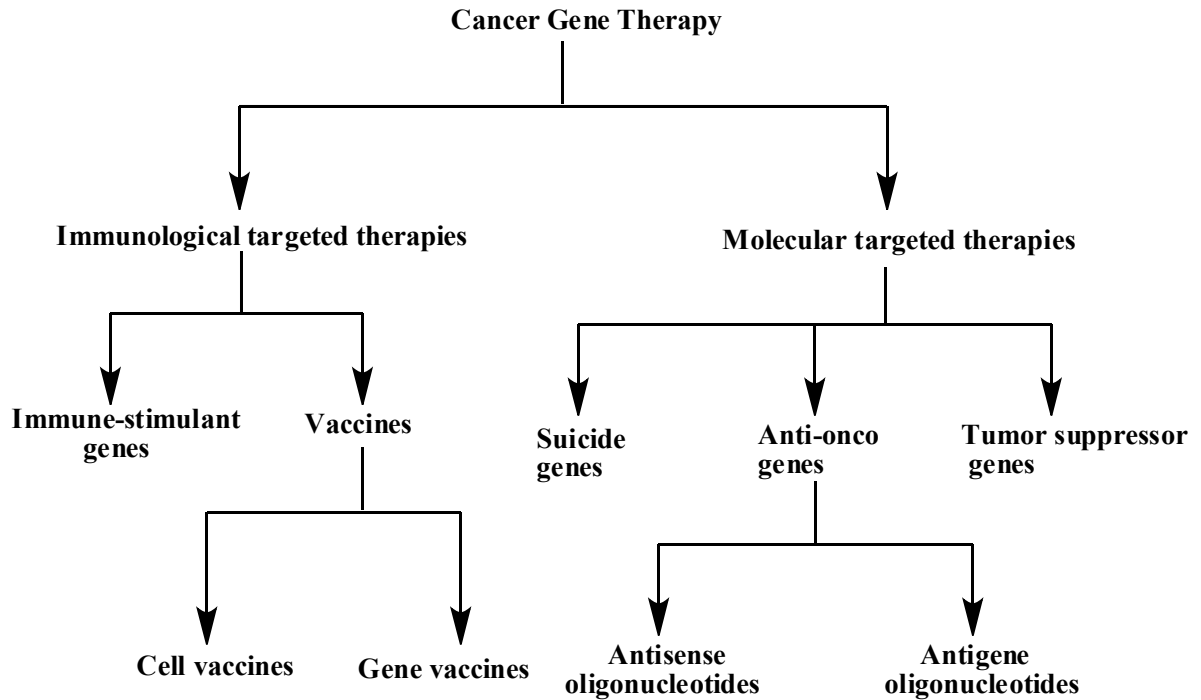


Figure 1.1: Molecular and immunologic therapies.

Up regulation or down regulation of some genes is the basis of tumour initiation and progression. Oncogenes and tumour suppressor genes are two gene groups that counter-balance each other and play a key role in cancer development. While tumour suppressor genes prompt apoptosis (programmed cell death), oncogenes enhance cell proliferation. The biological activity of oncogenes can be modulated and suppressed using anti-oncogenes, such as oligonucleotides, that can bind to a precise sequence of the RNA (antisense oligonucleotides) or the DNA (antigene oligonucleotides). The main representative of the tumour suppressor gene family is the *p53* gene which is responsible for detection of DNA damage followed by repair initiation or apoptosis induction (Wynand and Bernd, 2006). As mutational alterations in the *p53* gene occur in almost 40% of all tumours, successful transfection of *p53* into cancerous cells can result in tumour growth inhibition and regression. Suicide gene strategy which combines chemotherapy

and gene therapy is another molecular approach in cancer gene therapy. This strategy relies on the conversion of a non-toxic prodrug into its active cytotoxic metabolite within cancerous cells.

The conversion is facilitated by non-mammalian enzymes which are overexpressed in neoplastic cells as a result of effective transfection with their genes. Other molecular approaches in cancer gene therapy are, inhibition of angiogenic inducers (vascular endothelial growth factor and angiopoietine), introduction of angiogenic inhibitors (angiostatin, endostatin) or transferring of multiple drug resistance genes and thereby overcoming the dose limiting toxicity of traditional chemotherapy (El-Aneed, 2004).

Cancer cells are immunogenic in nature, with cancer antigens being intracellular molecules. However regular immune response is not enough to destroy tumour cells. The capability of cancer cells to escape the resistant system is associated with the secretion of immunosuppressive factors, down-regulation of antigen expression or major histocompatibility complex molecules and the deficiency of co-stimulation. Genetic immunotherapy can be employed mainly to boost T-cell mediated immune response against cancer. The most frequently used approach to do so, involves the transfer of the genes of immune stimulant molecules such as cytokines and more specifically interleukin-12 (Barajas et al., 2001). The production of interleukin-12 by tumour cells facilitates the immune response by the activation of many components in the immune system, in particular cytotoxic T lymphocytes and natural killer cells. An alternative method to induce the desired immune reaction against cancer cells is direct genetic vaccination by antigen encoding genes. Injected intravenously or intramuscularly, the DNA enters the local cells (fibroblasts, myocytes) which then produce and secrete the antigen. Antigen presenting cells capture the new antigen and migrate to lymphoid organs, initiating the desired immune response. Cell vaccines on the other hand are produced by *in vitro* engineering of antigen presenting cells in a way that enables them to actively present tumour antigens. The success of gene therapy largely depends on the efficiency of delivery of the DNA to its target cell/tissue. The therapeutic gene of interest must form stable complexes with vector of choice (viral or non-viral), be relatively non-toxic to the cells and be efficient in gene transfer and expression in the target cell (El-Aneed, 2004).

1.3. Gene Transfer Vectors – An Overview

Since the sequencing of the human genome use of gene products as a medicine for inherited and acquired diseases has gained importance in biomedical research. The ‘Human genome project’ has provided a vast amount of information about the human genome and at the same time predicted a plethora of genes with therapeutic potential (Taylor et al., 2010). Yet, mere identification and isolation of the therapeutic gene need not necessarily lead to successful gene therapy. Reliable delivery of the genetic material into eukaryotic cells followed by suitable expression of the desired gene is a bottleneck for gene transfer even in *in vitro* systems (van Gaal et al., 2011). Delivery is even more problematic *in vivo* where targeting and safety of the gene vectors, as well as prompt elimination from circulation present additional challenges for successful gene transfer. Accordingly, intensive research efforts have focused on creating safe, efficient, and reliable strategies to convey nucleic acids into eukaryotic cells (Amiji, 2005; Lee et al., 2005).

1.3.1. Bacterial Gene Delivery Systems

The use of bacteria as gene transfer vectors has been exploited in tumour gene therapy. The hypoxic microenvironment in tumours can facilitate colonization by anaerobic bacteria (Theys et al., 2001). Auxotrophic bacteria have also been tested *in vitro*, but require tumour-specific nutrition factors for their replication (Li et al., 2001). To confirm the maximum safety with these therapeutic bacterial vectors the transgene expression should be as specific as possible. One approach to reach this safety level is the use of a radiation induced promoter and thereby exploiting the natural conflict to irradiation. The therapeutic gene that is under control of the radiation-inducible promoter is only expressed in bacteria that colonize irradiated tissues (Nuyts et al., 2001).

1.3.2. Viral Gene Delivery Carriers

Viruses are the most effective gene delivery vectors known today due to their capacity to transfer and protect foreign genes, cross the cellular membrane, escape endosomes, and to achieve efficient gene expression (Walther, 2000). Due to their natural ability to infect cells efficiently in terms of the number of transfected cells, several viruses, such as retroviruses, adenoviruses, adeno-associated viruses and herpes viruses, have been investigated for *in vivo*

viral facilitated gene delivery. For example, retroviral vectors can introduce genes permanently into somatic cells by integration into the cell's chromosomal DNA. Retroviruses only infect replicating cells, though the resultant permanent integration of therapeutic genes minimizes the ability to modify or to terminate therapy in response to any contrary side effects or to cure the disease. In addition, the permanent integration of genes into host chromosomes may result in activation of oncogenes or inactivation of tumour suppressor genes (Arnold and Anna, 2010). In contrast, adenoviruses efficiently infect non-dividing cells and do not integrate genes into the host genome (Forde et al., 2015; Tomlinson, 1996; Young et al., 2006). Different mechanisms exist for the interaction of viruses with cells, depending on the type of virus. The protein capsid of a virus is able to bind to proteins in the cellular membrane, gain entry by internalization, and recycle its membrane proteins. Other viruses have a protein-lipid capsid that can fuse and enable the virus to pass through the cell membrane (Amir et al., 2011). Viruses have inherent mechanisms to avoid lysosomal trafficking, by promoting the fusion of the viral envelope with the endosomal membrane and therefore, causing the release of the virus into the cytoplasm (Sun and Zhang, 2010). These unique abilities of viruses led to the first clinical trial in gene therapy in 1990, where retroviral vectors were used to introduce the adenosine deaminase gene into the white blood cells of patients suffering from severe combined immunodeficiency (SCID) (Tomlinson, 1996; Te-Lang and Dongming, 2011). Viral vectors are able to mediate gene transfer with high efficiency of up to 90% with the possibility of long term as well as stable gene expression, satisfying two out of three criteria described above. However, acute immune response, immunogenicity, and insertional mutagenesis especially after repeated applications in clinical trials have raised serious safety concerns about some commonly used viral vectors.

1.3.3. Non-Viral Gene Delivery

The development of a safe and efficient non-viral gene delivery system remains a hurdle toward the successful application of gene therapy to treat human disease (Rettig and Rice, 2007). Although viral delivery systems are much more efficient in the delivery of genetic material, there are concerns regarding toxicity, high costs of producing therapeutic doses, immunogenicity and possible integration of viral genetic material into the human genome (Mancheno-Corvo, 2006). Chemically defined non-viral vectors can potentially avoid these drawbacks of viral delivery and can be developed and manufactured similarly to traditional pharmaceuticals. The recent

discovery of RNA interference has expanded the scope of gene therapy to include applications for knocking down or otherwise modulating gene expression (Julian et al., 2012). Protein regulation by RNAs and antigen expression by DNA vaccines are two additional applications of gene therapy that hold great promise if the delivery of oligonucleotides to nucleus can be successfully achieved.

Non-viral vector systems, including cationic lipids, polymers, dendrimers, and peptides, all offer potential routes for compacting DNA for systemic delivery. However, unlike viral analogues that have an evolved means to overcome cellular barriers and immune defense mechanisms, non-viral gene carriers consistently exhibit significantly reduced transfection efficiency as they are hindered by numerous extra and intracellular obstacles (Liu et al., 2011). However, biocompatibility and potential for large scale production makes these compounds increasingly attractive for gene therapy (Meredith and Eric, 2009). As a result, a significant amount of research in the past decade has focused on designing cationic compounds that can form complexes with DNA and can avoid both *in vitro* and *in vivo* barriers for gene delivery (Meredith et al., 2009).

1.3.3.1. Non-Viral Physical Gene Delivery Methods

Several physical methods have been developed to enhance DNA delivery efficiency, including electroporation, pressurized intravascular delivery, sonoporation, laser irradiation, and magnetofection (Mehier-Humbert et al., 2005a; Mehier-Humbert and Guy, 2005b). Electroporation increases the permeability of cell membranes to plasmid DNA by exposing the target cells to a series of electrical pulses. Pressurized intravascular delivery has been used to successfully transfect cells in a variety of tissue types including liver and skeletal muscle. Sonoporation, which usually involves the use of a low-dose ultrasound or laser irradiation leads to transient formation of small pores in the cell membrane, enhancing permeability to the nucleic acid. Magnetofection involves the application of a magnetic field to enhance the uptake of plasmid DNA coupled with magnetic nanoparticles. Most of these physical methods enhance the entry of DNA into cells by overcoming barriers posed by the cell membrane, but are often associated with significant cytotoxicity. Furthermore, the challenges associated with intracellular transport to the nucleus remain to be addressed (Guo et al., 2003).

1.3.3.2. Cationic Polymers

Cationic polymers have a common chemical characteristic, a polyamine, which is positively charged at physiological pH due to its high pKa. These positive charges are neutralized upon condensation of DNA into the appropriate form for cellular uptake. The use of cationic polymers for gene transfer was pioneered with the use of poly-L- Lysine (PLL) and it is still widely investigated as conjugates with targeting ligands and other functional peptides (Kerbel and Hawley., 1995; Lakshmi and Cato, 2007; Trinchieri, 2003; Taylor, 1982; Vandercappellen et al., 2008).

However, a number of substantial limitations of PLL based systems are difficult to overcome, which has led to the search of alternative cationic polymers. These include polyethyleneimine (PEI), chitosan, poly (2-dimethylamino) ethyl methacrylate (pDMAEMA), polyamidoamine (pAMAM) dendrimer, poly [α -(4-aminobutyl)-L-glycolic acid] (PAGA). In particular, PEI is the most efficient polymeric gene delivery carrier due to its buffering effect in the endosome compartment, and presents as a possible alternative to cationic liposomes despite its rigid structure and high tendency for aggregation (Defu et al., 2013; Raugi and Lovett, 1987).

1.3.3.2.1. PEI

Polyethylenimine (PEI) is a stable, easy to handle, inexpensive cationic polymer (Lungwitz et al., 2005). It has gained significant attention as a non-viral gene delivery system through condensation of DNA into compact particles, uptake into the cells, release from the endosomal compartment into the cytoplasm, and uptake of the DNA into the nucleus (Kircheis et al., 2001). PEI mediated gene delivery is based on the electrostatic interactions of the polycation with the negatively charged phosphate groups of DNA. The DNA condensation is therefore a function of the cation-to-anion ratio, i.e. the PEI nitrogen-to-DNA phosphate (N/P) ratio. Condensation protects the DNA from degradation by nucleases, and the compact particles can be taken up by cells via natural processes such as adsorptive endocytosis, pinocytosis and phagocytosis (Forrest et al., 2004). The complexation and condensation performance is dependent on several polymer characteristics, such as molecular weight, number and charge density, in addition to the composition of the complexes, e.g. the ratio of polymer to DNA. The physicochemical properties of PEI, different molecular sizes, condensation ability,

hydrodynamic diameter, surface charges and the stability of the PEI: DNA complexes may be important factors to be considered to achieve a higher transfection efficiency of the polycation vectors.

1.3.3.2.2. pAMAM dendrimer

The polyamidoamine (pAMAM) dendrimers with extremely branched spherical structures were tested as gene delivery carriers, but initially low/poor gene transfection activities were achieved with these vectors. pAMAM dendrimers were developed further to improve stability at physiological pH. They have shown good transfection efficiency *in vitro* (Christine et al., 2005; Rak et al., 1996). In recent *in vivo* studies, a pAMAM dendrimer was coupled with the Epstein Barr virus (EBV) based plasmid vector and investigated for its potential to deliver the herpes simplex virus thymidine kinase (HSV-tk) suicide gene into Ewing's sarcoma bearing mice (Yin et al., 2012). The EBV/dendrimer system significantly suppressed tumour growth and prolonged the survival of mice.

1.3.3.2.3. Chitosan

Chitosan is a natural cationic polysaccharide, consisting of D-glucosamine and N-acetyl-D-glycosamine. Chitosan is nontoxic and biodegradable, and is therefore a good candidate for a non-viral gene delivery vector. In particular, chitosan:DNA complexes were reported to be efficient in transfecting intestinal epithelial cells, most likely due to the nucleo adhesive properties of chitosan. In a recent study, oral administration of the chitosanyl dominant peanut allergen gene (pCMV-Arah2) complexes, substantially reduced the peanut antigen induced murine anaphylactic responses, which was associated with reduced levels of plasma histidine, IgE and vascular leakage (Folkman, 2007; Leong, 2004).

1.3.3.2.4. pDMAEMA

Poly (2-dimethylamino)ethyl methacrylate (pDMAEMA) is a water soluble cationic polymer containing tertiary amine groups. Like those of PEI, these tertiary amines act as a proton sponge in the acidic endosome compartment, inducing the osmotic swelling and endosome rupture (Sutapa et al., 2011). This endosomolytic property of pDMAEMA resulted in high transfection efficiency in COS-7 cell lines *in vitro* (Park et al., 2006). In addition,

pDMAEMA/DNA complexes were found to be quite stable in physiological buffer solution over a period of ten months (Car et al., 2014).

1.3.3.2.5. PAGA

Unlike other synthetic cationic polymers, poly[α -(4-aminobutyl)-L-glycolic acid] (PAGA), a biodegradable analogue of PLL, is rapidly degraded in aqueous solution to give L-Oxylysine as a final product (Marie et al., 2008). PAGA is being studied as a cytokine gene delivery carrier for the treatment of diabetes and cancer. When PAGA interleukin-10 gene complexes were systemically administered into NOD mice, the insulinitis was markedly reduced, compared to the use of the naked gene (Gasparini and Harris, 1995). Also PAGA was found to efficiently deliver another cytokine gene encoding interleukin-12 into subcutaneous tumour bearing mice significantly reducing the tumour growth (Ellis and Fidler, 1995; Maheshwari et al., 2002).

1.3.3.3. Cationic Peptides

Cationic peptides employed for gene transfer are amphiphilic peptides which can undergo conformational changes in acidic environments, escaping the endosomal/lysosomal pathways. They contain the positively charged amino acids (histidine, lysine and/or arginine) such that they can effectively condense DNA. The helical KALA peptide (derived from the influenza HA-2 subunit, which enables the virus to infuse into the cell membrane) is one of the early cationic peptides used successfully for gene delivery in cultured cells (Hongtao et al., 2010). In spite of the cationic amino acids of lysine (7 AA) present in the KALA peptide (30 AA), it was corroborated that the arginine residues with 4 cationic amino acids in another α -helical peptide (16 AA residues) were enough to condense DNA and deliver it to the cytoplasm (Bhawna et al., 2005). The efficiency of the peptide vector also depends upon the hydrophobic portion that plays a major role in aggregation and endosomal escape (Haines et al., 2001; Raj et al., 2012). The relationship between peptide aggregation and efficient gene delivery is not well understood. DNA release into the cytoplasm can also be enhanced by the introduction of cysteine moieties into the peptide backbone, resulting in the formation of reducible disulphide bonds within the DNA/peptide complex (McKenzie et al., 2000). The reduction occurs after the internalization of

the delivery complex. As with other vector systems receptor mediated gene transfer can be achieved through ligand attachment (Niidome et al., 2000).

Peptide gene carriers have been mainly explored *in vitro* (Kim et al., 2003; Eric et al., 2008). Their *in vivo* behaviour is still under investigation. Recently successful transfection in the lungs using a peptide vector was obtained after intravenous administration into mice. It was, however, 10–40 folds less efficient than liposomes and PEI vectors (Rittner et al., 2002; Baoum et al., 2012; Letoha et al., 2013).

1.3.3.4. Cationic Lipids

Cationic lipids are especially attractive as they can be easily prepared and extensively characterized. Further, each of their constituent parts can be modified, thereby facilitating the elucidation of structure-activity relationships. A great number and an impressive variety of synthetic vectors have been prepared and their transfection efficiency evaluated not only in experimental studies, but also in clinical trials for treatment of diseases such as cancer (Montier et al., 2008; Roth and Cristiano, 1997) and cystic fibrosis (Martin et al., 2005; Michael et al., 2001). To date although some positive results have been attained, the overall outcome indicates a need for further research (Niidome and Huang, 2002; Parvizi et al., 2013; Tagalakis et al., 2013). There are possible correlations between the length, saturation, type of hydrophobic moiety and transfection efficiency. Intracellular DNA release strategies that can be triggered as a function of the incorporation of cellular environmentally sensitive groups (pH, redox and enzyme sensitive) within the linker moiety are also under investigation.

The length and type of the aliphatic chains incorporated into cationic lipids significantly affect their transfection efficiency. Vectors are often prepared in a series differing in their hydrophobic domain. The hydrophobic domain has also been functionally modified by the inclusion of highly fluorinated alkyl chains which are both hydrophobic and lipophobic, a characteristic which may better protect the lipoplex from unwanted interactions. At first, a series of closely related fluorinated analogues of DOGS (with either both chains fluorinated to varying degrees or just a single chain fluorinated with the other remaining a regular alkyl chain) were

prepared in order to chart the effect of the hydrophobic/lipophobic balance on the transfection efficiency (Vierling et al., 2001).

A cationic lipid is a positively charged amphiphile, which generally contains the following structural domains: i) a hydrophilic head group which is positively charged, usually via the protonation of one (monovalent lipid) or several (multivalent lipid) amino groups; ii) a hydrophobic portion composed of a steroid or of alkyl chains (saturated or unsaturated); iii) a linker (connecting the cationic head group with the hydrophobic anchor) whose nature and length may impact on the stability and the bio-degradability of the vector; and iv) spacer (Figure 1.2). Modifications of the hydrophobic domain have shown that optimal vector structure is often dependent on this moiety, which can fall into various structural classes and variants. Finally, labile linkers have been introduced which are sensitive to various biological stimuli, inducing DNA release at defined time points during the intracellular trafficking of the lipoplex (Hasegawa et al., 2002; Hoekstra et al., 2007).

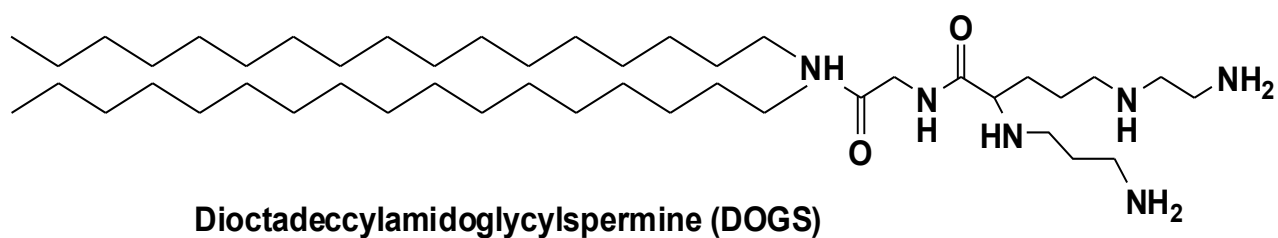
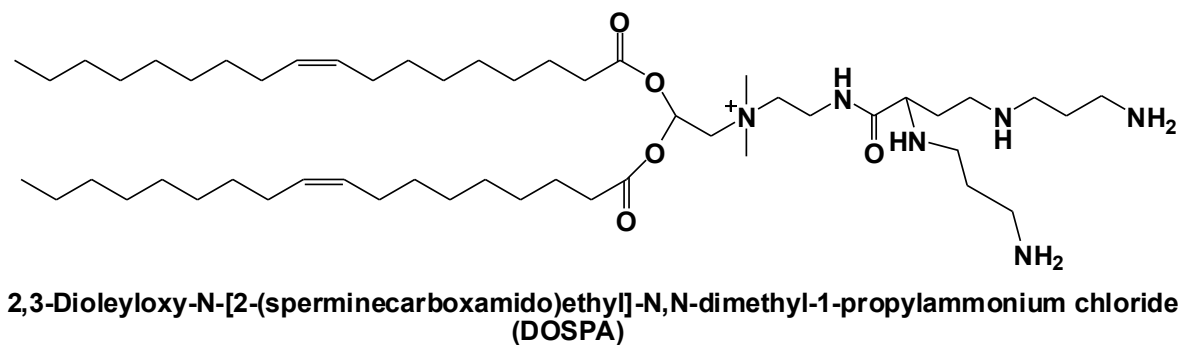
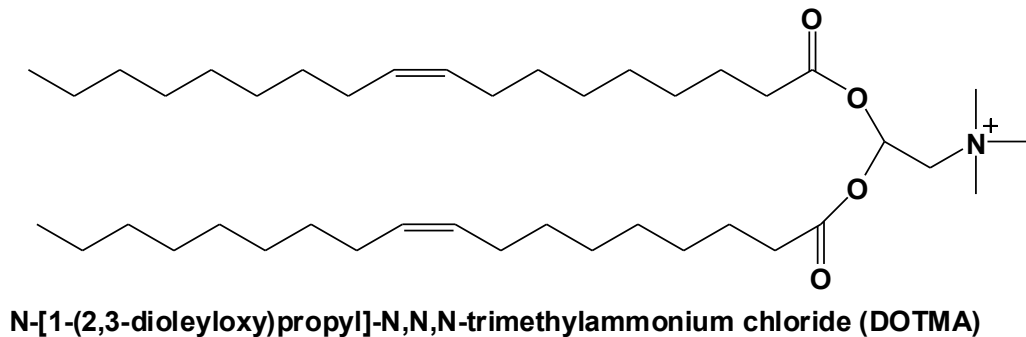
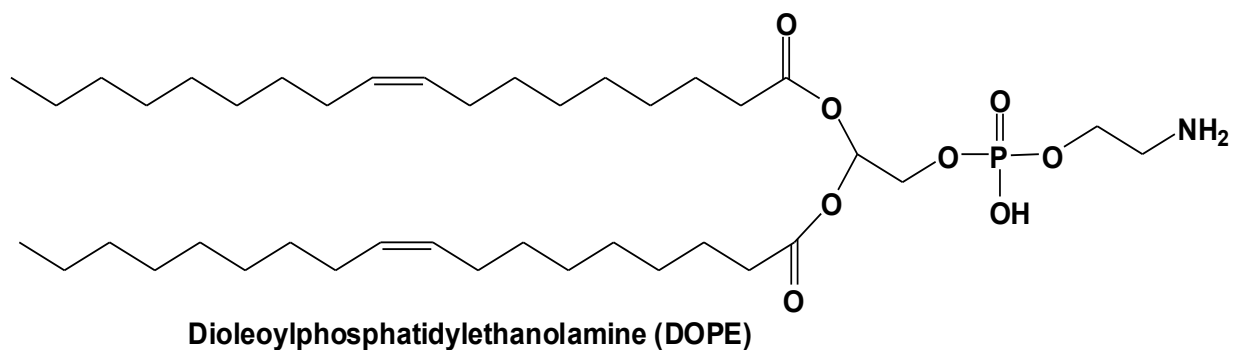
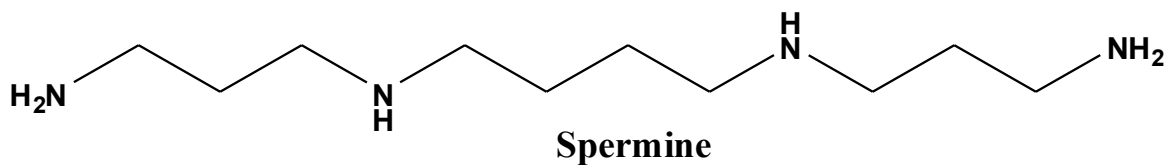


Figure 1.2: Typical cationic lipids used in gene delivery.

1.3.3.4.1. Cationic Head groups in Liposomal Formulations

DNA binding by the vector requires a head group which is capable of sustaining a positive charge at physiological pH. The charge is most often located on amino groups, as was the case for the ‘early’ vectors. The vast majority of cationic lipids for gene delivery rely on the charge accommodated on a nitrogen atom, as there appears to be a relationship between the hydration of such mono-ammonium head groups and the transfection activity. In essence, the greater the imbalance between the cross sectional area of the head group (small end) and the hydrophobic moiety (large end) i.e., the more cone shaped the cationic lipid and the more unstable the resulting lipid assembly. Hence there is a greater likelihood of undergoing fusion with anionic vesicles. Lipoplex instability is presumed to result in improved transfection, as fusion, between the cationic lipoplex and the endosomal membrane leads to DNA release into the cytoplasm (Xu and Szoka, 1996; Joanna et al., 2004; Gao and Huang, 1995).

1.3.3.4.1.1. Multivalent Head Groups

As multivalent cationic lipids may form liposomes with a greater surface charge density than their monovalent counterparts, they are generally expected to be better at DNA binding and delivery. One such approach was the incorporation of natural polyamines e.g. spermidine and spermine that have the ability to interact with the inner groove of B-DNA (Schmid and Behr, 1991). Triaminespermidine in cholesteryl spermidine (Zonghua et al., 2010), and tetraaminespermine in the lipid DOGS (Behr, 1993) are early representative examples. The presence of protonation sites with different pKa values in DOGS may actually result in buffering of the endosomal acidification, thereby protecting the DNA from degradation and facilitating its escape from the endosome. The results suggested that the tetra methylene portion of spermine might be able to bridge between the complementary strands of DNA, whereas a polyamine with a trimethylene central spacer would only interact with adjacent phosphate groups on the same DNA strand. As these branched structures have the advantage of avoiding the folding problems of linear polyamine chains, they can include additional protonation sites without affecting DNA binding. Importantly, with more protonation sites per molecule, the resulting lipoplexes can thus achieve the same charge density with lesser amounts of the cationic lipid in the formulation. This may lessen the drawback of cationic lipid associated cytotoxicity. Indeed, as cationic lipids may exhibit some degree of cytotoxicity, an optimal set of conditions resulting in high transgene

expression and acceptable toxicity need to be found (Darya et al., 2009; Sunil et al., 2004; Loeffler and Behr, 1993).

The hydrophobic and hydrophilic moieties of cationic lipids are commonly linked using carbamate, amide, ester or ether bonds. The linker bond mediates the stability of the cationic amphiphile. Although no particular bond emerges as consistently optimal in structure-activity studies across different vector types, ether linked vectors seem to be more stable (Ghosh et al., 2000). However they are more toxic than ester linked lipids which may also be more easily cleaved within the cell (Bora et al., 2012). Carbamates are thought to achieve a reasonable balance between stability and toxicity and are therefore more frequently used (Gao and Huang, 1991).

1.3.3.4.2. Cationic Lipid Mediated Gene Transfection - Basic Principles

Cationic lipids were first introduced by Felgner et al., (1987), following early attempts to transfer DNA via encapsulation in liposomes (Nicolau and Sene, 1982; Pezzoli and Candiani, 2013). Thus, the first reported lipid was DOTMA, which consists of a quaternary amine connected to two unsaturated aliphatic hydrocarbon chains via ether groups. Synthesis of the multivalent lipopolyamine DOGS was reported soon afterwards (Osama et al., 2006) and DC-Chol (3-(*N*-(*N*, *N*'-dimethylaminoethyl)carbamoyl)cholesterol) with cholesterol as the hydrophobic portion closely following (Gao and Huang, 1991). The transfection activity of cationic lipids (especially those which cannot form bilayers alone) can be increased by their formulation as stable liposomes with the neutral co-lipid DOPE. Inclusion of DOPE is presumed to enhance endosomal escape of the lipoplexes into the cytoplasm as DOPE is thought to have fusogenic properties important for endosomal membrane disruption (Vidal and Hoekstra, 1995). The use of these initial lipids demonstrated the transfection ability of cationic lipids. However, this 'proof of principle' stage has been followed by a highly challenging period. Indeed, progress in improving the level of transfection efficiency up to that required for therapeutic use has been slow. This is possibly linked to an unclear structure-activity relationship in vector design, and to the related incomplete understanding of the highly complex series of steps involved in transfection. Thus, the development of novel lipids is justified, a novel cationic lipid being not just a 'me too' addition, but rather opening new possibilities for differently influencing those

steps. Accordingly, numerous novel vectors were developed, representing a wide variety in structures and thus numerous potential mechanisms by which better transfection levels might be obtained.

1.3.3.4.3. Lipids in Liposome Formulations

1.3.3.4.3.1. Phospholipids (PLs)

Phospholipids (PLs) classically found in high amounts in cell membranes of living matter, are important components in liposome formulations. PLs comprises of two fatty acids connected to a polar head group, with either glycerol (Figure 1.3) or sphingomyelin as the backbone. PLs are amphipathic molecules, and have both hydrophobic and hydrophilic groups. The two hydrocarbon chains constitute the hydrophobic tails, while the phosphate group and its polar attachment constitute the hydrophilic group (Cooper and Hausman, 2009). PLs can consist of different head and tail groups that affect the surface charge and bilayer permeability of liposomes (Perrie and Rades, 2010).

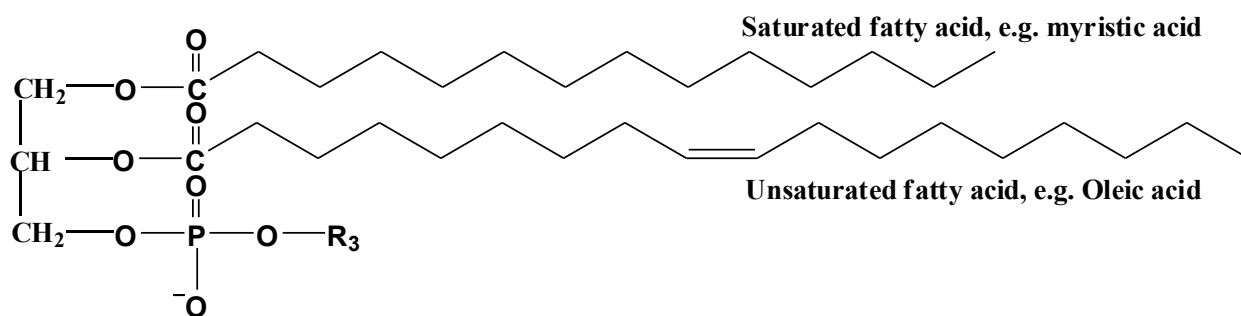


Figure 1.3: Structure of a glycerophospholipid.

Phosphatidylcholine (PC) is a common phospholipid employed in liposomes, and can be obtained from both natural and synthetic sources. PC is zwitterionic and consists of a hydrophilic head group with a quaternary ammonium moiety choline, which is linked to a glycerol via a phosphoric ester (Brandl, 2001).

The rigidity of the liposome membrane depends on the packing of the hydrocarbon chains of the lipid molecules. The hydrocarbon chain length and degree of saturation of the acyl chains influences at which temperature [the main transition temperature (T_m)], the membrane

transforms from a fully extended and closely packed ‘gel phase’ to a liquid crystalline disordered ‘fluid phase’. In general, fluid membranes are more permeable to solutes than rigid bilayers (Brandl, 2001).

The charge of the lipid used in liposome formulation dictates the surface charge of the liposomes. The surface charge of liposomes can be designed by replacing phosphatidylcholine (PC) partly with negatively or positively charged phospholipids, which induces electrostatic repulsion and stabilization against liposome fusion (Ogihara et al., 2010). The surface features of liposomes may also be restructured by modifying lipids with hydrophilic moieties e.g. polyethylene glycol (PEG), to membrane bilayers (Brandl, 2001).

1.3.3.4.3.2. Cholesterol (Chol) and Other Lipids

Cholesterol (Chol) is also commonly used in liposome formulations, and its incorporation into the lipid bilayer has a major influence on the liposome properties. The presence of Chol in the lipid bilayer enhances its stability and leads to the formation of highly ordered rigid membranes with fluid like characteristics (Lee and Lee, 2005).

The molecular structure of cholesterol (Figure 1.4) with the four hydrocarbon rings reveals its strongly hydrophobic character. The presence of the hydroxyl group (OH) attached to position 3 makes that part of the molecule weakly hydrophilic (Cooper and Hausman, 2009). Chol can be incorporated into lipid bilayers at concentrations of up to 50 mole %, without forming a bilayer on its own. Due to its amphipathic properties, Chol inserts itself into the bilayer with its OH-group orientated towards the aqueous environment, and the rigid hydrophobic tail interacts with the other hydrophobic components of the bilayer (Perrie and Rades, 2010).

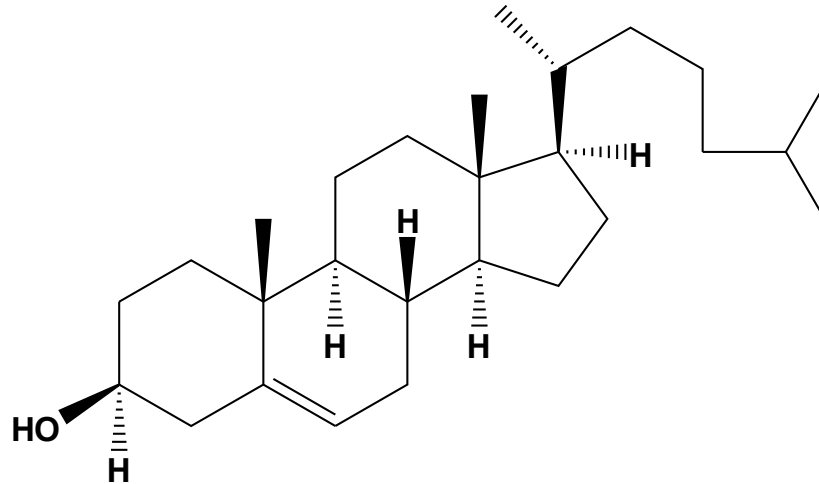


Figure 1.4: General structure of cholesterol (Chol).

1,2-Di-oleyl-3-trimethyl-ammonium-propane (DOTAP) is another example of a lipid used in liposome formation. DOTAP is a cationic lipid with two unsaturated fatty acyl chains. It consists of propane as backbone and a hydrophilic trimethylammonium head group as shown in Figure 1.5.

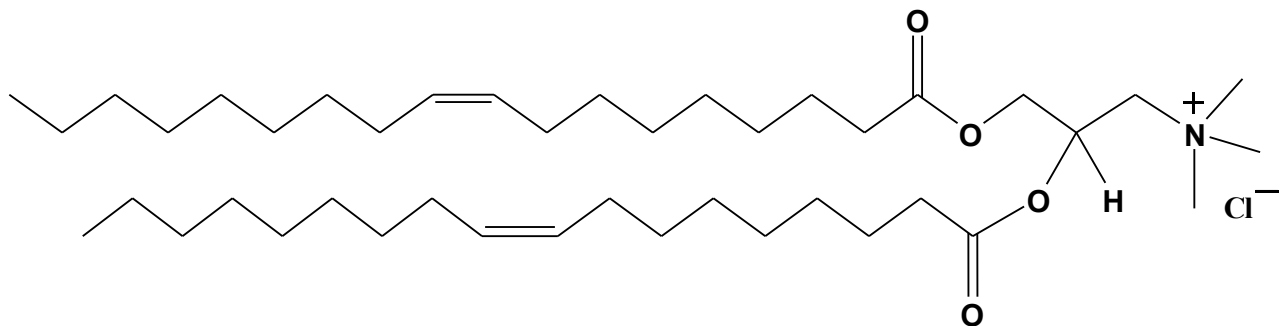


Figure 1.5: General structure of DOTAP.

1.3.3.4.4. Classification of Liposomes

Classifications of liposomes can be based upon their size and lamellarity or charge. Different sizes of liposomes depend on their composition and their method of preparation. Liposomes based on size can be categorized into three main types.

- Small unilamellar vesicles (SUVs) are vesicles consisting of a single bilayer, and can theoretically be as small as about 20 nm. They are more suited to parenteral

administration compared to multilamellar vesicles (MLVs) because of their size homogeneity. Their small size results in lower amount of encapsulation of hydrophilic drugs.

- Large unilamellar vesicles (LUVs) are vesicles with size in the order of 100 nm, consisting of one single lamella. They can entrap a higher amount of hydrophilic drugs due to their larger aqueous core compared to SUVs (Perrie et al., 2010).
- MLVs: These are vesicles having a size range from 100 nm to several micrometers, depending on the method of preparation. They consist of a large number of concentric lamella. Due to their lamellarity they are more suited for the incorporation of lipophilic molecules compared to hydrophilic substances.

Liposomes are generally classified according to their charge;

1.3.3.4.5. Cationic Liposomes

The positive charge on the liposomal surface ensures their binding to the negatively charged cellular membranes. Cationic liposomes react spontaneously with the negatively charged DNA molecules (self-assembling system), forming complexes with almost all DNA molecules participating in the reaction. It has been shown that two processes are involved in the complex formation. A fast exothermic process, attributed to the electrostatic binding of DNA to the liposome surface. A subsequent slower endothermic reaction which is likely to be due to the fusion of the two components and their rearrangement into a new structure (Pector et al., 2000). Incorporation of small amounts of anionic lipid into liposomes leads to DNA association with the inner surface of the liposomal membrane, which protects DNA against enzymatic degradation (Zhdanov et al., 2002).

1.3.3.4.6. Anionic and Neutral Liposomes

Due to the toxicity issues of the early cationic lipids, there has been exploration into the feasibility of anionic or zwitterionic lipids as potentially safe nucleic acid delivery vectors (Mozafari and Omri, 2007). The nucleic acid entrapment and delivery efficiency with these lipids on their own, however, is debatable due to the absence of complexation-enhancing electrostatic interactions between lipids and the nucleic acid. This is because the nucleic acid is

negatively charged and the lipids are either anionic or neutral in nature. Therefore, such delivery systems require a third moiety to achieve intense association to form lipoplexes. Foged et al., (2007) attempted preparing siRNA associated anionic liposomes without utilizing a bridging agent. Consequently, the prepared formulations showed poor encapsulation efficiency (7–9 %) with no activity in HeLa cells. Halder et al., (2006) prepared neutral liposome (DOPC) associated lipoplexes that indicated efficient knockdown of the focal adhesion kinase gene in an ovarian tumour mice model. The tumour growth inhibition was observed for 4 days with overall reduction in tumour weight by 72 %. Although in this case, neutral liposomes were efficient, there may be potential issues with their long-term colloidal stability due to the absence of the repulsive forces between the particles.

1.3.3.4.7. Production of Liposomes

As liposomes are formed by the spontaneous interaction between phospholipids and water (with agitation of some form – Torchilin, 2007), the importance when producing liposomes lies in the ability to form vesicles of the right size and structure with the highest entrapment efficiency (New, 1990). A wide variety of methods have been employed to produce liposomes which include mechanical dispersion techniques, dried-reconstituted vesicles, and solvent dispersion techniques (ethanol/ether injection vesicles and reverse phase evaporation vesicles). Most methods of producing liposomes can be said to consist of three stages, which include the drying down of lipids from organic solvents, dispersion of the lipids in aqueous media and subsequent purification of the resultant liposomes.

1.3.3.4.7.1. Thin Film Hydration

The thin film hydration method involves using a lipid solution in an organic solvent such as chloroform. The lipid solution is then subjected to rotary evaporation to remove the solvent and produce a thin lipid film deposited on the side of the flask. Any residual solvent can then be removed by drying the film under a stream of nitrogen. The film is then rehydrated with an aqueous buffer that is above the T_m of the lipid mixture. The flask is then agitated through hand shaking (sometimes glass beads are used) and/or vortexing, which displaces the thin film from the walls of the flask causing the production of liposomes. The liposomal suspension produced consists of a heterogeneous suspension with liposomes over 1 μm in size. Consequent processing

that includes sonication and extrusion can then be completed to produce a more homogenous formulation with a specific size range (SUVs, LUVs etc) (Sriram and Rhodes, 1995).

1.3.3.4.7.2. Sonicated Vesicles

To produce a homogenous SUV liposomal sample, it is necessary to use a method which imparts energy at a high level on the lipid suspension (New, 1990). Initially, Huang, (1969) produced SUVs with an approximate diameter of 25 nm by using a probe sonication method whilst Schroeder et al., (2009) produced similar SUVs using an ultrasonic bath for a prolonged period of time (1–1.5 h).

Probe sonication can be used when suspensions require high energy in a small volume e.g. high concentration of lipid or the use of a viscous aqueous phase. As a consequence of this process, heat is given off and therefore it is essential that the suspension is maintained at a constant temperature by the use of a cooling bath to prevent any lipid degradation. A further issue that may arise through probe sonication is the possible contamination of the sample through the degradation of the probe, suspending Ti particles within the sample. Subsequent centrifugation or gel permeation chromatography can be used to separate any small MLVs that remain in the SUV population.

The ultrasonic bath method is more suitable to dilute lipid concentrations of a higher volume and as there is a lower energy input, there is subsequently a lower risk of lipid degradation due to heat. The risk of contaminants entering the solution is reduced as the formulation can be maintained in a sealed container throughout the sonication process. The main drawbacks of the bath sonication method include the need for prolonged sonication time and the final liposome size may not be entirely homogenous. Thus there may be a requirement for centrifugation (Samad et al., 2007).

1.3.3.4.7.3. Membrane Extrusion

The use of membrane filters to reduce the size of liposomes has been investigated extensively with two main methods having evolved. The first method uses what is described as a tortuous path membrane which consists of a number of fibers criss-crossed over each other,

which leads to specific channels through which liposomes are forced. The channel or pore size is controlled by the density of the fibers used in the membrane manufacture. The drawback of this method is that larger liposomes can become stuck within the membrane channels and this therefore blocks the filter. The more widely used method is that of the nucleopore membrane which consists of uniform pores through a thin sheet of polymer. A variety of membranes can be obtained, with pore sizes ranging from 50 μm to 10^{-3} μm . Liposomes which exceed the membrane pore size will be broken down into smaller vesicles when extruded through the membrane. The liposomes which are only marginally larger than the pore size may be able to change their conformation and squeeze through the pore. Hence, despite a number of extrusions a small percentage of the liposomes will be larger than the pore size itself (Takeuchi et al., 2001, 2005).

1.3.3.4.8. The Role of Liposome/Lipoplex Size

The rate of the opsonisation and clearance of injected liposomes from the blood circulation by the reticulo endothelial system (RES) is dependent on the composition and size of the complexes (Silva et al., 2014). The RES is part of the immune system and its main function is to eliminate foreign materials from the body (Kevin and Helen, 2007; Perrie and Rades, 2010). The RES is made up of cells such as blood monocytes and Kupffer cells, reticular cells (lymph node, bone marrow, and spleen). Shortly after intravenous injection, liposomes become coated by serum proteins called opsonins. Once they are opsonised, they will rapidly be phagocytosed by the RES cells, but a major component of the injected liposomes will accumulate in the liver and spleen (Maurer et al., 2001).

Generally large liposomes (>200 nm in diameter) are rapidly opsonised and taken up by the RES, and disappear from the blood circulation within a short period, primarily ending up in the spleen. Opsonisation decreases with a decrease in liposome size. Small liposomes have a relatively larger surface area, and will have a lower density of opsonins on the membrane surface which results in lower uptake by macrophages (Younsoo et al., 2005). Liposomes with a size of 70 to 200 nm will have a greater chance to escape from the RES and remain in the circulation longer enabling them to reach their target. Due to extravasations through the fenestrated capillary walls in the liver, the small liposomes (< 70 nm in diameter) show shorter circulation time. The

structure and architecture of the blood capillary walls vary in different organs and tissues. There are structural differences between healthy and tumour capillaries, and blood supply to the organs and tissues is somewhat different (Brandl, 2001).

1.3.3.4.9. The Role of Surface Charge and Membrane Characteristics

The organization of lipids in the liposome membrane plays a major role in the physical membrane properties such as permeability, elasticity, surface charge, binding properties of proteins, and is as important as liposome size in their clearance (Sok et al., 2012)..

Neutrally-charged liposomes with tightly packed membranes tend to remain longer in the circulation and exhibit increased drug retention, compared to charged systems. Protein opsonisation onto the liposome surface is reduced due to the tightly packed and rigid membrane. The presence of Chol liposome formulations may change the packing of the phospholipids to a more stably ordered and rigid membrane which may avoid drug leakage. Moreover, this could also reduce binding of opsonins on the liposomes and improve stability and retention of liposomes *in vivo* (Dan et al., 2007). Certain plasma proteins have an affinity for liposomes, which is enhanced if the liposome is charged. In particular, cationic systems are expected to quickly interact with various components in systemic circulation, resulting in a shorter half-life *in vivo* (Maeda et al., 2009). It is also known that anionic liposomes containing negatively-charged lipids such as phosphatidylserine (PS), phosphatidic acid (PA) and phosphatidylglycerol (PG) are quickly taken up by macrophages and thus disappear from the circulation within a short time (Ichihara et al., 2014; Massing and Fuxius, 2000).

1.3.3.4.10. Long Circulating/Stealth Liposomes

In order to avoid rapid clearance by the RES after intravenous injection and to enable liposomes to remain in circulation for prolonged periods, the attachment of polyethylene glycol (PEG) on the liposome surface has been employed. PEG is a ‘hydrophilic’ polymer varying in molecular weight depending on the number of monomer repeat units. The polymer creates a steric barrier with the flexible chains forming ‘mushroom’ (an array of macromolecular chains attached to a surface or tethered polymers) which extends out from the surface (Figure 1.6), thereby preventing interaction of opsonins and uptake by phagocytic cells. These liposomes are

commonly known as ‘stealth liposomes’, and have good solubility properties in aqueous media (Torchilin, 2007 & 2012). PEG is non-biodegradable; it does not form any metabolites, has a very low toxicity profile and does not accumulate in the RES (Perrie and Rades, 2010).

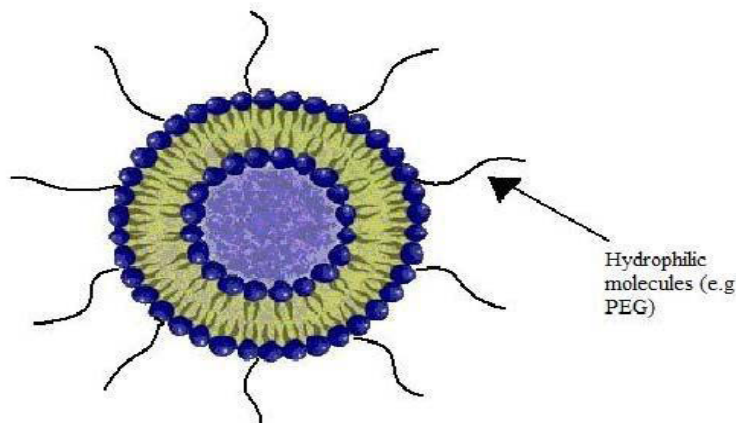


Figure 1.6: Illustration of sterically stabilized liposome surrounded with PEG. Adapted from www.intechopen.com.

1.3.3.4.11. Cationic Liposome:DNA interaction

The first step in the preparation of vector/DNA aggregates suitable for gene transfer is the condensation of the DNA, which is driven by an electrostatic interaction between the cationic liposome and the polyanionic DNA (Banerjee et al., 2004). Spontaneous self-assembly into nanometer scale particles (lipoplexes, nanobioparticles) result, leading to shielding of the DNA from the nucleases of the extracellular medium. Use of an excess of cationic amphiphile (quantified by the lipid/DNA ratio resulting in a mean theoretical charge ratio of the lipoplex (+/-)) gives the lipoplex surface a positive charge, which is presumed to mediate subsequent cellular uptake by interaction with negative cell surface structures such as heparin sulphate (Zhi et al., 2010). As a result of non-specific endocytosis, the lipoplex is encapsulated in intracellular vesicles, although fusion based cellular uptake is not totally excluded. The DNA must then avoid degradation in the late endosome/lysosome compartment by escaping from the (early) endosome into the cytoplasm (Sahay et al., 2010). Trafficking of the DNA through the cytoplasm precedes uptake by the nucleus of the target cell, followed by transgene expression (Figure 1.7). In the nucleus, the DNA appears to be separated from its vector. Microinjection experiments have

suggested that gene expression does not occur if the DNA remains condensed in lipoplexes (Al-Dosari and Gao, 2009). The efficiency of cationic lipids for gene transfection can be evaluated in terms of gene delivery (percentage of transfected cells) or gene expression (amount of transgene protein produced). The efficiency of any transfection reagent also strongly depends on the cell system chosen for its evaluation (transformed cell lines or primary cells *in vitro*, *in vivo* administration) (Kiefer et al., 2004). Efficiency gains *in vitro* do not automatically lead to higher efficiencies *in vivo*. To determine the relative efficiency of compared cationic lipids is crucial, and so quantifying the efficiency of cationic lipid systems needs to take into account their intended use, i.e., in the experimental or clinical setting.

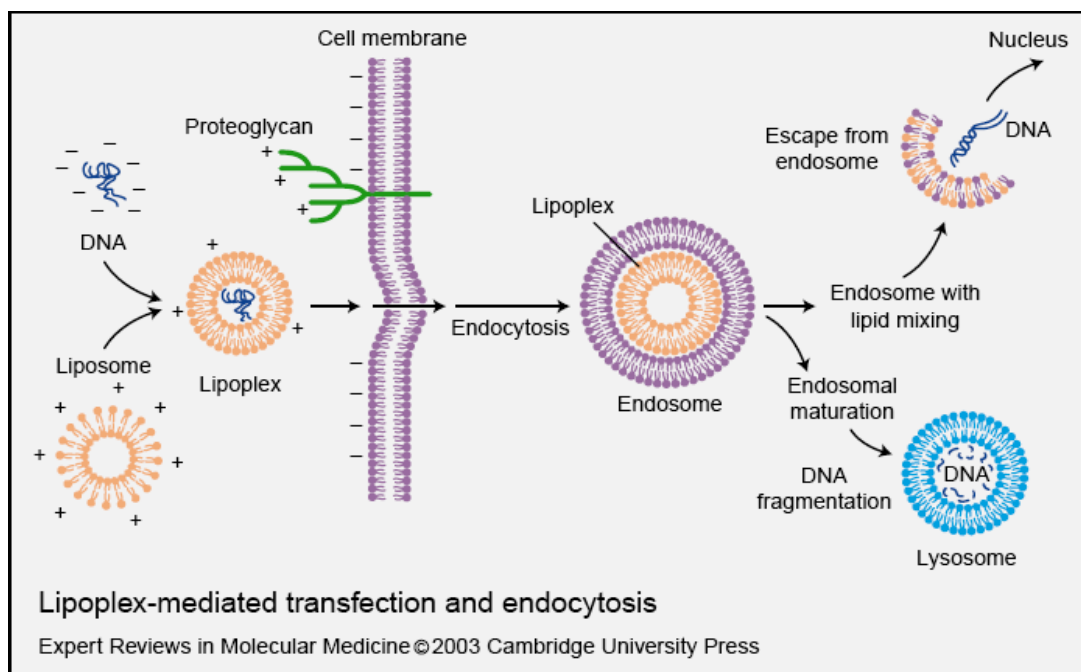


Figure 1.7: Representation of cellular delivery of DNA with surfactant vectors. Cellular internalization is an important step in gene delivery. As shown, internalization is achieved here through endocytosis. Adapted from Parker et al., (2003).

1.4. Targeted Gene Delivery

1.4.1. Active vs Passive Targeting

The term passive targeting is usually defined as a method to deliver drugs based on the ability of the drug carrier to circulate for longer times in the blood stream and accumulate in pathological tissues. ‘Active targeting’ is also called ligand based targeting, which is based on the ligand-receptor recognition enabling the binding of ligand-conjugated carriers to the target tissues. In the case of cancer therapy, the delivery of gene materials with non-targeted agents (passive targeting) is achieved mainly by the enhanced permeability and retention (EPR) effect (Figure 1.8). The endothelial cells of tumour neo-vasculature are poorly organized with large fenestrations, causing macromolecules to leak extensively into the tumour tissue. Additionally, macromolecules are retained easily in the tumours because of the low venous return in the tumour and poor lymphatic clearance (Cabral et al., 2011). This preferential accumulation through the EPR effect is the so-called ‘passive targeting’, which is characteristic of non-targeted agents. On the other hand, active targeting describes the active binding of the drug or gene delivery vectors to the cell surface through receptor-mediated endocytosis, facilitating the retention and cellular uptake (Figure 1.9). The introduction of targeting ligands should enhance the tissue, cell, or sub-cellular specific delivery efficiency through active targeting, when compared to its corresponding non-targeted counterpart agents. To achieve the cell specific active targeting, several ligand based systems have been designed to target specific cancer cells (Shi et al., 2011). This is particularly important for intracellular delivery to facilitate bioactivity.

The successful targeting requires at first the identification of the structures on the cell surface which could provide a selective uptake into the cell. Secondly, for active targeting, gene delivery agents are coupled with a ligand which is expected to interact with a specific target on the cell surface (Merkel et al., 2011). Folate as a ligand has been used as a targeting moiety for lung tissue (Brannon-Peppas and Blanchette, 2012). The pulmonary epithelium is a key point of the administration of bio-macromolecules and could prove to be an attractive approach for local and systemic therapies.

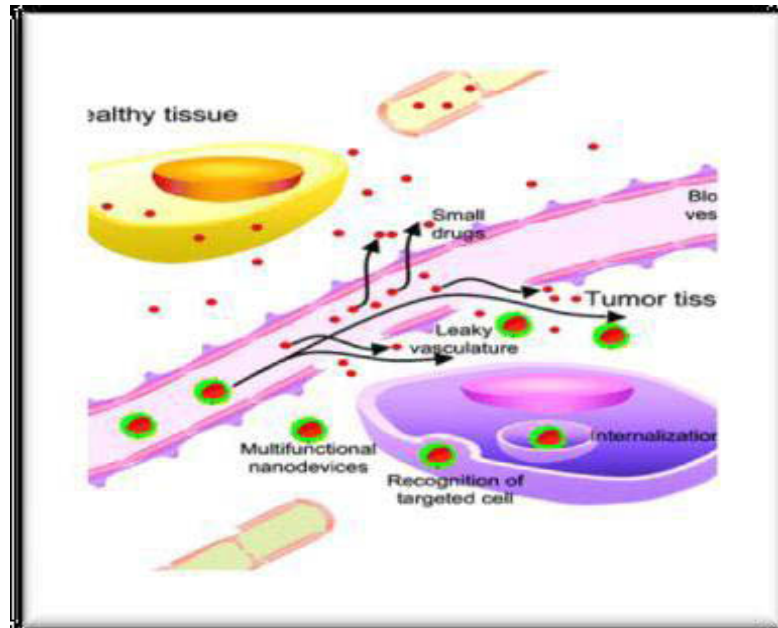


Figure 1.8: Enhanced permeability and retention (EPR). Adapted from Matsumura and Maeda, (1986).

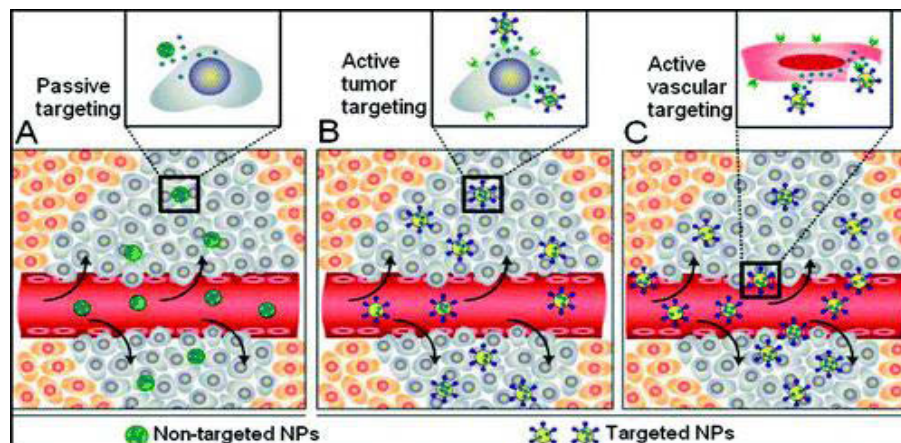


Figure 1.9: Passive vs active targeting. (A) Non-targeted NPs (B) The presence of targeting ligands on the surface of NPs (C) Targeted NPs. Adapted from Farokhzad and Langer, (2009).

1.4.2. Conjugates of Targeting Moieties and Lipids

Targeted gene delivery systems have attracted great attention due to their potential in directing the therapeutic genes to the specific target cells. They may also help minimize adverse effects such as cytotoxicity or immune reactions, as well as maximizing the efficacy of the therapeutic response. The targeted delivery of the lipoplexes may be achieved through the incorporation of targeting moieties (e.g. ligands) into liposomes by direct formulation, with no covalent bond to any lipid (Seol et al., 2000); conjugated to the helper lipid (Dauty et al., 2002), or connected directly to the cationic lipids (Kawakami et al., 2000a; Gaucheron et al., 2001a). For lipoplexes modified with a targeting moiety such as folate (Dauty et al., 2002), galactose (Singh et al., 2007; Kawakami et al., 2000a; Gaucheron et al., 2001a), mannose (Kawakami et al., 2000b), antibodies (Duan et al., 2008) and transferrin (Seol et al., 2000; Sakaguchi et al., 2008; Singh and Ariatti, 2006a; Singh et al., 2006b) the uptake can be receptor mediated and enhanced (Zhang et al., 2010).

1.4.3. Folic acid, as a Ligand for the Selective Targeting into Tumour Cells

Targeting of the folate receptor (FR) has received much attention over the years, since the folate receptor is a tumour marker that is over expressed in many cancer cells, including cancers of the ovary, kidney, uterus, testis, brain and colon. In addition, folic acid is a relatively small molecule (MW ~ 441 Da), and has the advantages of being stable and non-immunogenic compared to monoclonal antibodies (Siti et al., 2010), while retaining a relatively high receptor affinity. Reddy and co-workers (2002) showed that a folate moiety attached to a lipid membrane anchor via a cysteinyl-PEG₃₄₀₀ spacer, greatly increased specific cellular uptake to FR overexpressing cancer cells and transfection efficiency compared to the unmodified cationic liposome. Recently, Yoshizawa et al., (2008) developed a folate-linked nanoparticle (NP-F), composed of cholesteryl-3 β -carboxyamidoethylene-N-hydroxyethylamine, Tween 80 and folate-poly(ethylene glycol)-distearoyl-phosphatidylethanolamine conjugate (F-PEG₂₀₀₀-DSPE), which delivered synthetic siRNA with high transfection efficiency and selectivity into nasopharyngeal tumour (KB) cells.

Folic acid (Figure 1.10) is a vitamin essential for one-carbon transfer reactions in several metabolic pathways. Folic acid is vital for the biosynthesis of nucleotide bases with the vitamin being consumed in higher quantities by dividing cells. Normal cells transport biological folates across the plasma membrane using two membrane associated proteins, the reduced folate carrier (RFC) or the folate receptor (FR). The RFC is found in almost all cells and establishes the primary pathway responsible for uptake of physiological folates. The FR is found predominantly on polarized epithelial cells and activated macrophages (Gordon and Martinez, 2010; Martinez et al., 2009) and preferentially binds and internalizes oxidized folates via receptor-mediated endocytosis (Leamon and Reddy, 2004). Although low concentrations of RFCs are sufficient to source the folate requirements of most normal cells, the FR is frequently overexpressed on cancer cells, facilitating the malignant cell to compete successfully for the vitamin when supplies are limited (Jennifer and Robert, 2000; Leamon and Low, 2001).

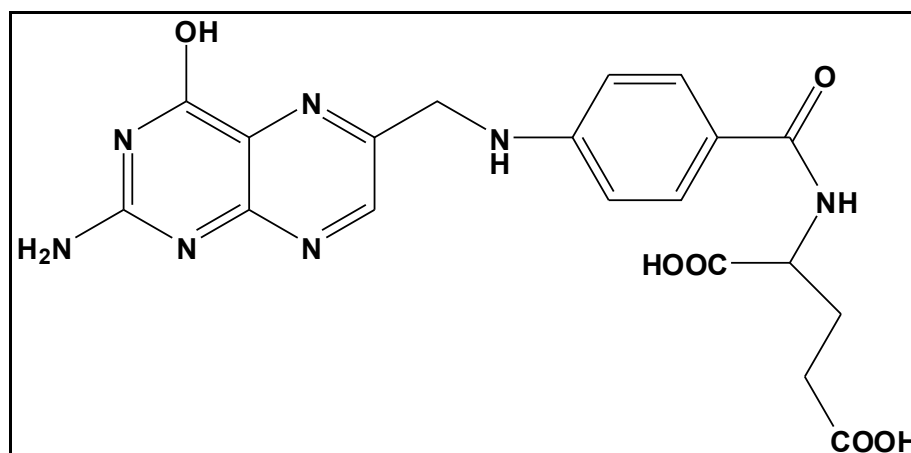


Figure 1.10: Structure of folic acid.

In humans, three genes encoding functional folate receptors termed FR α , FR β , and FR γ (also known as FOLR1, FOLR2, and FOLR3, respectively) were identified (Kelemen 2006; Gabizon et al., 2004; Gabizon et al., 2006; Gabizon et al., 2010; Gabizon et al., 2012). FR α and FR β are anchored at the plasma membrane via GPI anchor, whereas FR γ is secreted due to the lack of a signal sequence for GPI anchor attachment (Lu and Low, 2012; Hala et al., 2009; Salazar and Ratnam, 2007). FR α is generally exposed on the apical surface of polarized epithelial cells, predominantly in the proximal tubule cells of the kidney and the choroid plexus

(Kamen and Smith, 2004). FR β is expressed in normal myelopoiesis, in the placenta, spleen, and thymus (Wibowo et al., 2007). FR γ is secreted from lymphoid cells in the spleen, bone marrow and thymus. FR α is involved in folate transecytosis in the kidney and transfer into the central nervous system (CNS); however, the biological functions of FR β and FR γ remain unclear. FR β , however, has the ability to deliver folate and folate derived molecules into activated macrophages or leukemic cells. Moreover, FR targeted therapies are likely to be effective in the treatment of various types of cancer and inflammatory diseases due to high levels of FR α or FR β in disease affecting cells. In particular, FR α is constantly overexpressed in non-mucinous adenocarcinomas of ovary, breast, kidney, uterus, cervix, colon, and malignant pleural mesothelioma, ependymal brain tumours, testicular choriocarcinoma, and nonfunctioning pituitary adenocarcinoma. FR β expression is increased in certain leukemia, and is most commonly seen in acute myelogenous leukemia and chronic myelogenous leukemia as well as in activated synovial macrophage cells involved in the pathogenesis of rheumatoid arthritis and other inflammatory diseases like Crohn's disease and psoriasis (Felicia et al., 2005).

As the FRs aid as markers for diseased cells in cancers and inflammatory disease, the development of three different types of FR targeted therapeutics based on antibodies, folate-conjugates, and anti-folates are being pursued. Monoclonal antibodies against FR α and FR β can promote the clearance of folate receptor positive cells by the immune system. Folate conjugates used to deliver cytotoxic cargo or imaging agents or DNA therapeutics to FR positive cells, and FR targeted anti-folates would possibly eliminate cytotoxic side effects of current anti-folates transported to normal cells via reduced folate carriers (RFC) (Low and Kularatne, 2009).

While overexpression of FR on many cancer cells recognises the receptor as a potential target for a variety of ligand and antibody directed cancer therapeutics (Peer et al., 2007), the FR is further qualified as a tumour specific target, since it generally becomes accessible to intravenous drugs only after malignant transformation. This is, because the FR is selectively expressed on the apical membrane surface of certain epithelial cells, making it inaccessible to blood borne substances and thus protected from FR directed therapeutics delivered in plasma. Conversely, upon epithelial cell transformation, cell polarity is lost and the FR becomes available to targeted moieties in circulation. It is possible that due to this dual mechanism for

tumour specificity, folic acid has become a popular molecule for targeting therapeutics to cancer cells. The desirability of folate was further enhanced by its high binding affinity ($K_d \sim 10^{-10}$ M), low immunogenicity, ease of modification, small size (MW ~ 441.4), compatibility with a variety of organic and aqueous solvents, low cost, stability during storage, and availability (Park et al., 2005). To date, many chemical and biological therapeutic agents have been successfully conjugated to folic acid, most of which have shown enhanced delivery to FR-positive tumour cells both in *in vitro* and *in vivo* (Kamaly et al., 2009).

1.4.3.1. FR Targeted Liposomal Delivery

Along with efforts to develop folate conjugated anticancer targeted gene therapy agents, progress has been made in the field of folate targeted gene therapy, where both viral (retro and adeno) and non-viral (liposomal or polylysine based) vectors have been examined (Li et al., 2001). As might be expected, when liposomal vectors are used for targeted gene delivery, they encounter the same obstacles as drug encapsulating liposomes, including problems such as serum stability, tumour penetration, vector internalization, and endosomal escape following tumour cell uptake. The solutions to these problems, however, are very different from those for targeted liposome encapsulated anti-cancer agents. Firstly, encapsulation of bulky, negatively charged polynucleotides require a very different set of components and methods than those used with low molecular weight drugs. Secondly, unloading of liposome-entrapped genes following cell surface binding and endocytosis requires formation of pores much larger than those needed for escape of small molecules. And lastly, genes (unlike many drugs) must gain access to the nucleus before their therapeutic activities can be expressed. As a result, folate targeted liposomal vectors must also include features that enable transfer of the genetic material from the cytoplasm into the nucleus. Unlike low molecular weight drugs which can be encapsulated in liposomes of virtually any size, naked DNA is much too bulky to be encapsulated into the small liposomes. This size limitation is crucial, as the well characterized routes for particle endocytosis generally have size limits of 100-200 nm (Liu et al., 2010). As a result, DNA condensation becomes necessary for its delivery into cells through receptor-mediated endocytosis. DNA condensation is generally attained by complexation with high molecular weight polycations (polylysine, polyethylenimine, and polyamidoamine dendrimers), or liposomes in ratios that can allow retention of the electrostatic charge (Pack et al., 2005). For instance, a slight excess of positive charge has been

found useful for encapsulation of DNA:polylysine particles into folate-targeted anionic liposomes (Lu et al., 2012; Medina-Kauwe et al., 2005). The net anionic character of the complex has been shown to reduce non-specific binding to mammalian cell surfaces, thereby allowing transgene expression to be determined primarily by the distribution of the FR.

Endosomal escape mechanisms have also contributed significantly to the efficiency of folate targeted gene therapy. Unlike cationic liposomes and lipoplexes, which can fuse with most plasma membranes and release their contents directly into the cytoplasm, FR-targeted vectors enter endosomal compartments from which they must escape for transfection to occur. For this purpose, mixtures of DOPE and cholesterol hemisuccinate (CHEMS) have proven useful in formulating liposomes that are stable at neutral or basic pH, but fusogenic at acidic or endosomal pH values (Khalil et al., 2006). Folate-targeted liposomal vectors constructed from these fusogenic components transfect cells in orders of magnitude better than non-fusogenic lipids of similar composition. Similarly the use of a “caged” pH-sensitive lipid, *N*-citraconyl-dioleoylphosphatidyl-ethanolamine (CDOPE), that releases its head group at endosomal pH values and thereby becomes a fusogenic DOPE, also augments folate mediated gene expression (Yoo and Park, 2004). Since an improvement in folate-targeted gene therapy is also seen after incorporation of a pH dependent fusogenic peptide into liposomal vectors (Reddy and Low, 2000), it can be concluded that some type of pH triggered endosomal unloading mechanism must be included to enhance the efficiency of folate targeted gene therapy (Xi and Grandis, 2003). Finally, incorporation of a nuclear localization sequence into the encapsulated polynucleotide can also modestly increase the transfection activity of an FR-directed vector, suggesting that facilitated transport of the genetic material from the cytoplasm into the nucleus may also contribute to the efficiency of targeted gene therapy (Reddy et al., 1999).

Although some targeted liposomes do not display greater tumour accumulation than non-targeted liposomes, folate targeted gene therapy vectors have been found to promote much higher levels of tumour specific gene expression than non-targeted vectors. Presumably, as noted above, the folate derivatization enhances vector internalization, without significantly affecting deposition or retention of the large particles in the tumour (Xu et al., 2013). Not only was transgene expression limited to malignant tissues, but most cells in each tumour mass were

observed to express the gene. Furthermore, systemic delivery of a folate targeted p53 cationic gene therapy vector was found to greatly improve the therapeutic efficacy of conventional chemo and radio-therapeutic agents against FR-positive human tumour xenografts, yielding complete cures of subcutaneous cancers of the breast, prostate, and head and neck where the chemo and radio therapeutic agents alone exerted little effect. As expected from studies with other cationic liposomes, the major limitation associated with the PEG coating method was proposed where the pre-condensed DNA-cationic lipid structure could be protected by a layer of PEG, with folic acid at the distal ends of the PEG to facilitate tumour cell targeting (Jennifer and Robert, 2000). Leamon et al., (2003) have also observed significantly improved tumour-specific transgene expression following derivatization of their liposomal vectors with a PEG-tethered folic acid. Not only was tumour expression greatly enhanced, but with one particular vector composition, gene expression in other target tissues was either low or absent. Although many variables were examined in these latter studies, vector size and charge emerged as the most critical parameters to optimize for folate mediated gene expression.

1.5. Receptor Mediated Endocytosis

Targeting is usually achieved by conjugating a high affinity ligand to the carrier that provides preferential accumulation of the latter for instance, in a tumour bearing organ, in the tumour itself, in individual cancer cells or intracellular organelles. The overexpression of receptors or antigens in many human cancers lends itself to efficient drug/gene uptake via receptor mediated endocytosis (Figure 1.11). Folate and transferrin are widely applied ligands for liposome targeting because their cognate receptors are frequently overexpressed in a range of tumour cells (Kakudo et al., 2004; Hilgenbrink and Low, 2005). Liposomes tagged with various monoclonal antibodies have also been delivered to many targets such as the colon, prostate, brain and breast cancer tissues (Park et al., 2001).

The performance of non-viral vectors could be optimized by targeting them to distinct cellular internalization pathways, considering that not every pathway may be equally effective in releasing a therapeutic biomolecule in the cytosol. This step is critical for nucleic acid delivery, to increase the possibility of nuclear transport and the ultimate expression of the delivered genes (Bareford and Swaan, 2007).

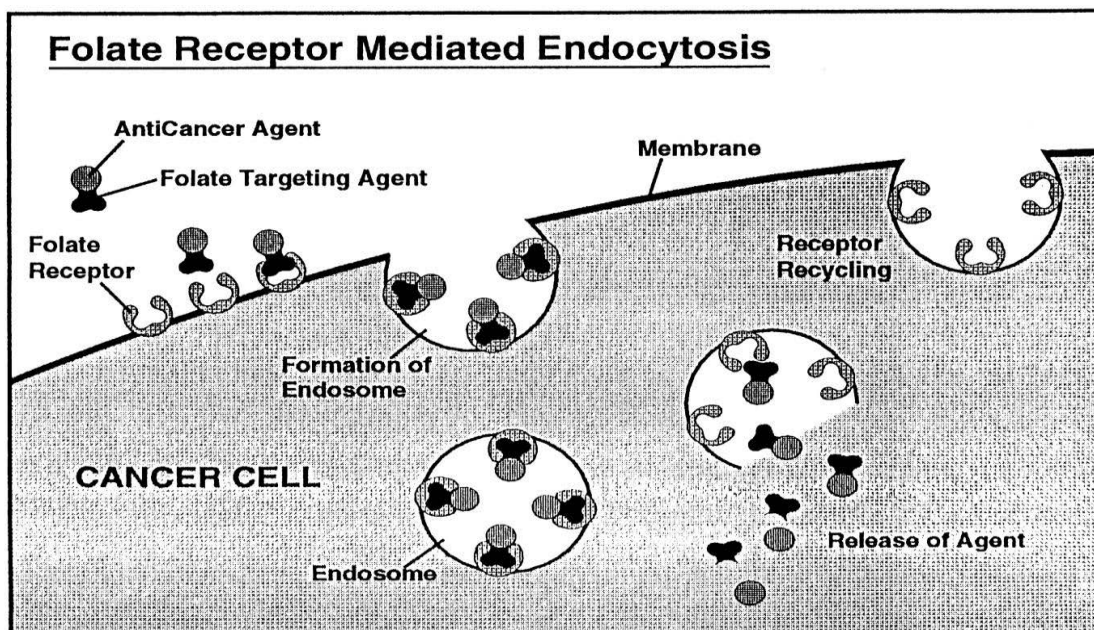


Figure 1.11: Diagrammatic representation of the folate receptor mediated endocytosis pathway. Covalent conjugates of drugs, macromolecules, or imaging agents linked to folic acid via the vitamin's γ -carboxyl group bind to the folate receptor with equal affinity to free folic acid ($K/50$ DpM). Following endocytosis and vesicular trafficking, much of the material is released into the cell cytoplasm. The unbound folate receptor may then recycle to the cell surface. Adapted from Wang and Low, (1998).

1.6. *In vitro* Liposomal Targeting

In vitro liposomal targeting is a commonly used method for targeting specific cells as it offers more assertion and is also more easily regulated than in the *in vivo* applications. It can be challenging as it needs specialization in culture techniques, as well as requiring a mitotic cell population. The *in vitro* targeting provides the advantages of working with cells in a culture environment and liposomes can be effectively delivered to the target cells (Singh, 1998). These *in vitro* methods are very useful for developing and improving techniques *in vivo* (Poste et al., 1984).

1.7. *In vivo* Liposomal Targeting

Successful liposomal targeting *in vivo* is an enviable aim. *In vivo*, gene transfer requires several important conditions to be fulfilled. The gene/drug delivery vector should have a high

specificity to the tumour tissue, and should only enter cells through selective targeting. The therapeutic candidates (drug/DNA) must escape degradation by nucleases and the complex must be internalized into the cell for successful unloading of the therapeutic gene/drug. Furthermore, the vector must not be toxic to the cells and the DNA should be preserved for transcription and eventual expression of the protein (Lesage et al., 2002). However, there are some other obstacles that the vector system must overcome for successful *in vivo* therapeutic transfer into the tumours (Nishikawa and Huang, 2001). In the case of systemic administration, the vector complex must avoid the reticulo endothelial system (RES) and should be able to escape from circulation with minimal interaction with anionic serum proteins. Thereafter, the vector complex should finally bind and enter the target cell and deliver the therapeutic material into the cell nucleus. Lipoplexes that are injected intravenously are mainly internalized by the spleen, liver, and macrophages of the RES (Singh, 1998).

Accessibility of the ligand-carrying lipoplexes is one of the main obstacles of the specific target tissue (Cavallia et al., 2013). Nevertheless, there have been prominent reports on liposome mediated targeting *in vivo*. One of the first *in vivo* studies carried out by Wu and Wu, (1988) showed that the DNA delivery by asialoglycoprotein-poly-L-Lysine conjugate was established in mammalian hepatocytes via the asialoglycoprotein receptor. Loney et al., (2008) also reported the use of cationic liposomes into targeted tissues in animals.

1.8. Lipofection – Barriers and Carriers

In order to be expressed in a eukaryotic cell, foreign DNA has to reach the nucleus where the transcription machinery is located. Successful lipofection depends upon overcoming the barriers posed by the extracellular environment and intracellular structures (Zuhorn et al., 2002; Chou et al., 2011; Atul et al., 2009; Belting et al., 2005). Overcoming these barriers by cunning lipoplex design based on lipoplex structure-function relationships is the cornerstone of research to enhance lipofection efficiency (Zuhorn et al., 2002; Chesnoy and Huang, 2000; Elouahabi and Ruyschaert, 2005). In this section barriers to lipofection as well as structural and functional features of lipoplexes that assist to overcome these barriers will be discussed.

1.8.1. Extracellular Barriers

The extracellular environment is hostile to lipofection both *in vitro* and *in vivo*. When complexed with cationic liposomes DNA is fairly well protected from action of degrading enzymes (Sania et al., 2004; Dash et al., 2011) and nucleases (Bhattacharya and Mandal, 1998) as it is condensed and enveloped by a lipid layer (Daniel et al., 2005; Vladimir and Klemen, 2011; Sternberg et al., 1994). However, cell culture medium contains a number of serum proteins, e.g. albumin, lipoproteins, and macroglobulins, which could interfere with interaction between lipoplexes and cells thus causing reduced transfection (Masottid et al., 2009; Tandia et al., 2003). Interaction with serum components prior to encounter with cells has been shown to influence lipoplex structure diverting its intracellular processing (Zuhorn et al., 2002). Serum effects on lipofection efficiency seem to be dependent on the chemical structure of the cationic lipid, liposome formulation, and cationic lipid/DNA ratio of the lipoplexes (Masottid et al., 2009; Simberg et al., 2003; Tandia et al., 2005). However a detailed understanding of lipoplex serum interactions is yet to be completely elucidated.

The number of *in vivo* extracellular barriers for liposome mediated transgene delivery is multiplied as physiological processes, such as complement activation and the reticulo endothelial system rapidly clear lipoplexes from the circulation (Kaul and Amiji, 2005; Dass, 2004). Targeting lipoplexes into desired tissues instead of nonspecific transfection in liver or lungs (Jafari et al., 2012; Thomas and Klivanov, 2003) presents an additional challenge for *in vivo* lipofection. The most popular strategy to prolong circulation times of lipoplexes and simultaneously incorporate targeting molecules onto the surface of the gene delivery complex, involves the coating of lipoplexes by lipids with a conjugated PEG moiety (Wanga and Thanou, 2010; Tam et al., 2000). A major drawback of these "stabilized plasmid-lipid particles" is their low transfection efficiency *in vitro* due to the stabilizing PEG-coating. However, lipofection efficiency could be at least partly recovered by introducing a positively charged moiety to the distal end of the PEG chain (Chen et al., 2000).

1.8.2. The Plasma Membrane

The plasma membrane is the main barrier of a eukaryotic cell through which the lipoplex has to penetrate to enter the intracellular space. Association of lipoplex onto the cell surface is

driven by a net positive charge of the lipoplex and occurs most likely via electrostatic binding to proteoglycans of the external leaflet of plasma membrane (Mislick and Baldeschwieler, 1996; Eliyahu et al., 2005; Rehman et al., 2013; Mounkes et al., 1998). Essentially, the cytotoxicity of the lipoplex may be related to the charge ratio of the lipoplex and may arise when cationic amphiphiles mix with lipids of the cellular membrane (Zuhorn et al., 2002; Dass, 2004). Initially it was suggested that internalization of lipoplexes proceeds through direct fusion with the plasma membrane (Lechardeur and Lukacs, 2002). Currently, a wealth of data indicates that endocytosis is the most vital internalization route for lipoplexes (Rehman et al., 2012; Jones et al., 2013).

More specifically, it has been suggested that small lipoplexes with diameter < 200 nm enter cells via clathrin-dependent endocytosis while larger complexes prefer the caveolae-mediated route (Hoekstra et al., 2007). It has been further shown that the former pathway is the one that leads to a more efficient lipofection (Zuhorn et al., 2007). However, considering great variability in the chemical structures of cationic lipids and diversity of eukaryotic cells it is likely that details of the endocytotic pathway may vary markedly depending on the cell type.

1.8.3. Cytoplasm and Endosomal Escape

Internalized plasmid DNA has to find its way to the nucleus from the cytoplasm. After endocytosis the cargo entrapped in liposomes trafficks to the endosomes and eventually locates in the lysosomes whose environment is detrimental for the DNA (Xia and Low, 2010; Torchilin, 2006). Hence, escape of the DNA from the endosomal compartment is absolutely required for successful gene delivery. It has been shown that anionic lipids present in the endosomal membranes readily mix with the cationic lipids of lipoplexes resulting in interlipidic ion-pairing and subsequent release of nucleic acid from the complex (Walter and Tejraj, 2010; Ikramy et al., 2006; Zelphati and Szoka, 1996). Moreover, formation of membrane destabilizing hexagonal inverted (H_{II}) instead of lamellar (L_{α}) phases (Figure 1.12) correlates with efficient lipofection (Mok and Cullis, 1997; Munoz-Ubeda et al., 2012). Consistent with the above, most efficient lipoplex formulations contain H_{II} -phase forming lipids, such as dioleoylphosphatidylethanolamine (DOPE) (Zuhorn et al., 2007) or diacylglycerol (DAG) (Balazs and Godbey et al., 2011). Cationic lipids with effective shape favouring the H_{II} -phase are more effective in lipofection than similar molecules preferring lamellar packing (Smisterova et al., 2001).

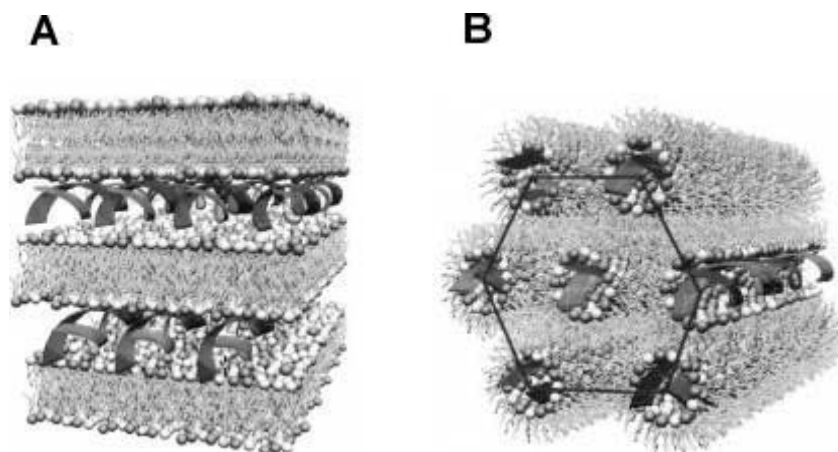


Figure 1.12: Schematic illustration of lamellar L_{α} (Panel A) and inverted hexagonal H_{II} (Panel B) phases of lipoplexes. Ribbons depict DNA complexed with cationic lipid membrane. Adapted from Koltover et al., (1998).

Endosomal escape is often the result of the destabilizing action of lipids of the lipoplex on the endosomal membrane. Interestingly, when mixed in nearly isoelectric stoichiometry, pairing of cationic and anionic lipids result in a lamellar to hexagonal phase transition even though both of the lipids adopt a lamellar phase in isolation (Lewis and McElhaney, 2000). This suggests that mixing of anionic and cationic lipids could induce endosomal membrane destabilization by stimulating the H_{II} phase (Hafez et al., 2001). To enhance endosomal escape lipoplexes have been designed that disrupt the endosomal membrane and release DNA due to the low pH of the endosomes by virtue of pH dependent transitions (Budker et al., 1996; Fielden et al., 2001). However, despite promising preliminary results, pH-sensitive lipoplexes have so far demonstrated only limited success in lipofection efficiency in practice.

1.8.4. The Nuclear Membrane

The nuclear membrane is the last and probably the most challenging barrier for successful gene expression. Data on nuclear entry of the plasmid DNA is somewhat controversial but some basic principles are known. DNA complexed with cationic lipid is not expressed if it is directly injected into nucleus thus suggesting that DNA has to be released from lipoplex somewhere in the cytoplasm, e.g. after fusion with intracellular membrane structures (Mehier-Humbert and Guy, 2005; Zhao and Yung, 2008; Sanna et al., 2014). However, the half-life of free DNA in the cytoplasm is approximately 50-90 minutes (Uttam et al., 2010), setting a rather

narrow time-window for nuclear entry of the plasmid after dissociation from the lipoplex. A close correlation between onset of transgene expression and mitosis in synchronized cell cultures has been established (Mortimer et al., 1999; Merdan et al., 2002) revealing fragmentation of the nuclear membrane during mitosis to facilitate DNA entry into the nucleus. However, non-dividing cells can be transfected by lipoplexes albeit with low efficiency indicating that mitosis is not absolutely required for nuclear entry (Zuhorn et al., 2002). Injection of highly condensed DNA complexed with polyethyleneimine (PEI) into cytoplasm results in efficient transgene expression suggesting that properly packed DNA particles can access the nucleus (Zhao and Yung 2008; Suh et al., 2003). Furthermore, condensation and subsequent conformational changes of DNA induced by cationic liposomes could be significant not only for protection of DNA in the extracellular space but also for nuclear entry and transcriptional activity of the nucleic acid (Vladimir and Klemen, 2011; Braun et al., 2003; Gelbart et al., 2000; Akao, 1996).

1.9. Advantages of Cationic Liposomes

Cationic liposomes which may resemble traditional pharmaceuticals (Hirko et al., 2003; Chen and Haung, 2005) shows low immunogenicity (Goncalves et al., 2004). They also contain a wide diversity of both hydrophobic and hydrophilic diagnostic or therapeutic agents, provide a larger drug payload per particle, protect encapsulated agents from metabolic processes, and allow for a high degree of cooperative binding to target cell antigens. The lipid composition of the bilayer can be further 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). Furthermore, enhanced formulations may prevent them from being cleared by the complement and repeated administrations *in vivo* may be accomplished without adverse consequences (Templeton, 2002).

1.10. Disadvantages of Cationic Liposomes

Disadvantages may include low transfection efficiency and cell toxicity in some cases (Torchilin, 2012; 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 inhibits their interaction with the cell surface and or their

internalisation (Zuhorn and Hoekstra, 2000). This inhibitory consequence of serum on liposome mediated gene delivery (lipofection) can be overcome by using lipoplexes with high lipid:DNA charge ratios. Nanoparticles of DNA condensed 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 various cell lines (Karmali and Chaudhuri, 2007).

1.11. OUTLINE OF THESIS

The aim of the study was to investigate a novel cationic liposome based approach for folate targeted delivery of nucleic acids to human carcinoma cell lines overexpressing the folate receptor.

In this thesis, the *in vitro* delivery of plasmid DNA complexed to novel cationic liposomes prepared with cationic cholesterol cytofectins *N*, *N*-dimethylpropylamidodisuccinylcholesteryl formylhydrazide (MSO9) or 3β [*N*(*N*¹,*N*¹-dimethylaminopropylsuccinamidoethane)-carbamoyl]-cholesterol (SGO4), and with the co-lipid DOPE was investigated. Cationic liposomes were also coated with poly ethylene glycol (PEG) which is said to provide stealth capability and protects liposomes from non-specific opsonization as well as to increase circulation time in the blood system *in vivo*. PEGylated liposomes all contained 2 mole percent PEG. The vitamin, folic acid (also known as vitamin B9) was appended to DSPE-PEG₂₀₀₀ and formulated into FR-targeted liposomes. All liposome preparations including their lipoplexes were characterized using transmission electron microscopy and zeta-sizing. Examination of the ability of cationic liposomes to bind and condense the plasmid DNA was carried out by band shift assays using agarose gel electrophoresis and ethidium bromide intercalation assays respectively. Nuclease protection assays were performed to assess the ability of the cationic liposomes to effectively bind and protect the plasmid DNA from serum nuclease degradation. Cytotoxicity and transfection studies were carried out in the receptor negative HEK293 (human embryonic kidney), and the receptor positive HeLa (human cervical carcinoma) and KB (human nasopharyngeal) cell lines. The protocols for these assays are outlined in chapter two. Cell viability studies were conducted using the MTT assay which is based on the mitochondrial activity of viable cells. Cationic liposome mediated transgene expression was assessed using the

luciferase reporter gene assay. Competition assays using free folate ligand was performed to confirm receptor mediated gene transfection by folate-targeted cationic lipoplexes. Quantitative lipoplex uptake was examined by flow cytometry in FR-positive cell lines.

Briefly, chapter two looks at all the materials and methods utilized in this study. Chapter three details all the results obtained, followed by a detailed discussion of these results. Lastly, chapter four provides a short conclusion for this study.

1.12. Aims and Objectives

Aim

The aim of this study was to synthesize novel cationic liposomes with and without a targeting ligand folic acid, and to evaluate the effects of this targeted cationic liposome vectors *in vitro* culture system.

Objectives

- To synthesize novel cationic cholesterol derivatives for incorporation into cationic liposomes.
- To prepare unPEGylated and PEGylated liposomes with DSPE-PEG₂₀₀₀ (2 mole percentage).
- To formulate folic acid-labelled moiety for incorporating into cationic liposomes to establish targeting aspect.
- Liposome and lipoplex characterization using electron microscopy and zeta measurements.
- To examine the nucleic acid binding, interaction and protection abilities of the liposomes by using band shift assays, ethidium bromide dye displacement assays and nuclease protection assays.
- To examine the effect of cytotoxicity of complexes *in vitro*.
- To study the gene expression phenomenon using luciferase reporter gene assays *in vitro*.
- Quantitative analysis of gene transfection by using flow cytometry approach *in vitro*.

CHAPTER TWO

MATERIALS AND METHODS

2. Materials

Dioleoylphosphatidylethanolamine (DOPE), folic acid and Bicinchoninic acid (BCA) assay reagents were purchased from the Sigma-Aldrich Co., St. Louis, USA. Distearoylphosphatidylethanolamine polyethylene glycol 2000 (DSPE-PEG₂₀₀₀) and DSPE-PEG₂₀₀₀ NH₂ were purchased from Avanti Polar Lipids, Alabaster, USA. Cholesterylchloroformate, 3-dimethylaminopropylamine; 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulphonic acid (HEPES) and ethidium bromide were purchased from Merck, Darmstadt, Germany. Cationic cytofectins MSO9, *N,N*-dimethylpropylamidodistearoyl-cholesteryl-formylhydrazide, SGO4 and 3β[*N*(*N*¹,*N*¹-dimethylaminopropylsuccinamidoethane)-carbonyl]-cholesterol were synthesized according to reported procedures (Singh and Ariatti, 2006a). Ultra-pure DNA grade agarose was obtained from Bio-Rad Laboratories, Richmond, USA. The pCMV-luc DNA and pCMV-luc GFP were obtained from Plasmid Factory, Bielefeld, Germany. HEK293 cells were obtained from the University of the Witwatersrand Medical School, HeLa cells were purchased from Highveld Biological (Pty) Ltd. (Lyndhurst, RSA) and, KB cells were obtained from the Institute of Biological Chemistry, Academia Sinica, Nankang, Taipei, Taiwan. Eagles Minimum Essential Medium (EMEM) containing Earle's salts and L-glutamine, trypsin-versene and penicillin (5000 units/mL)/streptomycin (5000 µg/mL) were purchased from Lonza BioWhittaker, Walkersville, USA. The Luciferase Assay kit was purchased from the Promega Corporation, Madison, USA. All tissue culture plastic consumables were purchased from Corning Incorporated, New York, USA. All other reagents were of analytical grade.

2.1. Methods

2.1.1. Synthesis of Cationic Cholesterol Derivatives

2.1.1.1. Preparation of Cationic Cholesterol Derivative MSO9

MSO9 was synthesized in four steps beginning with the preparation of cholesteryl-formylhydrazide. All four steps described previously by Singh and Ariatti (2006a) are discussed in brief. The reaction scheme for the synthesis of the cytofectin MSO9 is outlined in Figure 2.1.

2.1.1.1.1. Preparation of Cholesterylformylhydrazide MSO4

To a solution of hydrazine (240 mg, 7.5 mmol) in chloroform: methanol (3:0.6 mL) was added a solution of cholesterylchloroformate (1.13 g, 2.5 mmol) in chloroform. This was carried out with stirring at 0 °C. Following 24 h at room temperature, the solution was concentrated *in vacuo*, followed by recrystallization of the resulting crystalline mass from chloroform: methanol (4:1 v/v) to yield the product.

2.1.1.1.2. Preparation of Cholesterylformylhydrazidehemisuccinate (MSO8)

MSO4 (89 mg, 0.2 mmol) and succinic anhydride (20 mg, 0.2 mmol) were dissolved in 2 mL DMF: pyridine (1:1 v/v) and the reaction was continued overnight at room temperature. The solvent was removed by rotary evaporation in a Büchi Rotavapor-R to yield the product of white crystals from absolute ethanol.

2.1.1.1.3. Preparation of *N*-hydroxysuccinimide ester of cholesterylformylhydrazide-hemisuccinate

MSO8 (82 mg, 0.15 mmol), dicyclohexylcarbodiimide (DCC) (62 mg, 0.3 mmol) and *N*-hydroxysuccinimide (NHS) (35 mg, 0.3 mmol) were dissolved in 1 mL of dimethylformamide (DMF). The reaction was monitored by TLC (results not shown). After 48 h, the dicyclohexylurea crystals were removed by filtration. The solvent was then removed by evaporation and the resulting crude product was dissolved in chloroform: water mixture (1:1 v/v). The water layer, containing excess *N*-hydroxysuccinimide, was removed. The chloroform layer was extracted with water (10 mL) and concentrated to dryness *in vacuo*. The residue was extracted with petroleum ether (60-80 °C, 10 mL) to remove excess DCC. The product was obtained as white crystals.

2.1.1.1.4. *N,N*-dimethylpropylamidossuccinylcholesterylformylhydrazide (MSO9)

The *N*-hydroxysuccinimide ester of cholesterylformylhydrazidehemisuccinate (MSO8) (53 mg, 0.083 mmol) and dimethylaminopropylamine (DMAPA) (36 mg, 0.35 mmol) were dissolved in 15 mL water: pyridine: DMF (13: 7: 10 v/v/v) and TLC was used to monitor the reaction (results not shown). Purification of the product was carried out on silica gel 60 F₂₅₄ TLC plates developed in chloroform: methanol (95: 5 v/v) solvent system.

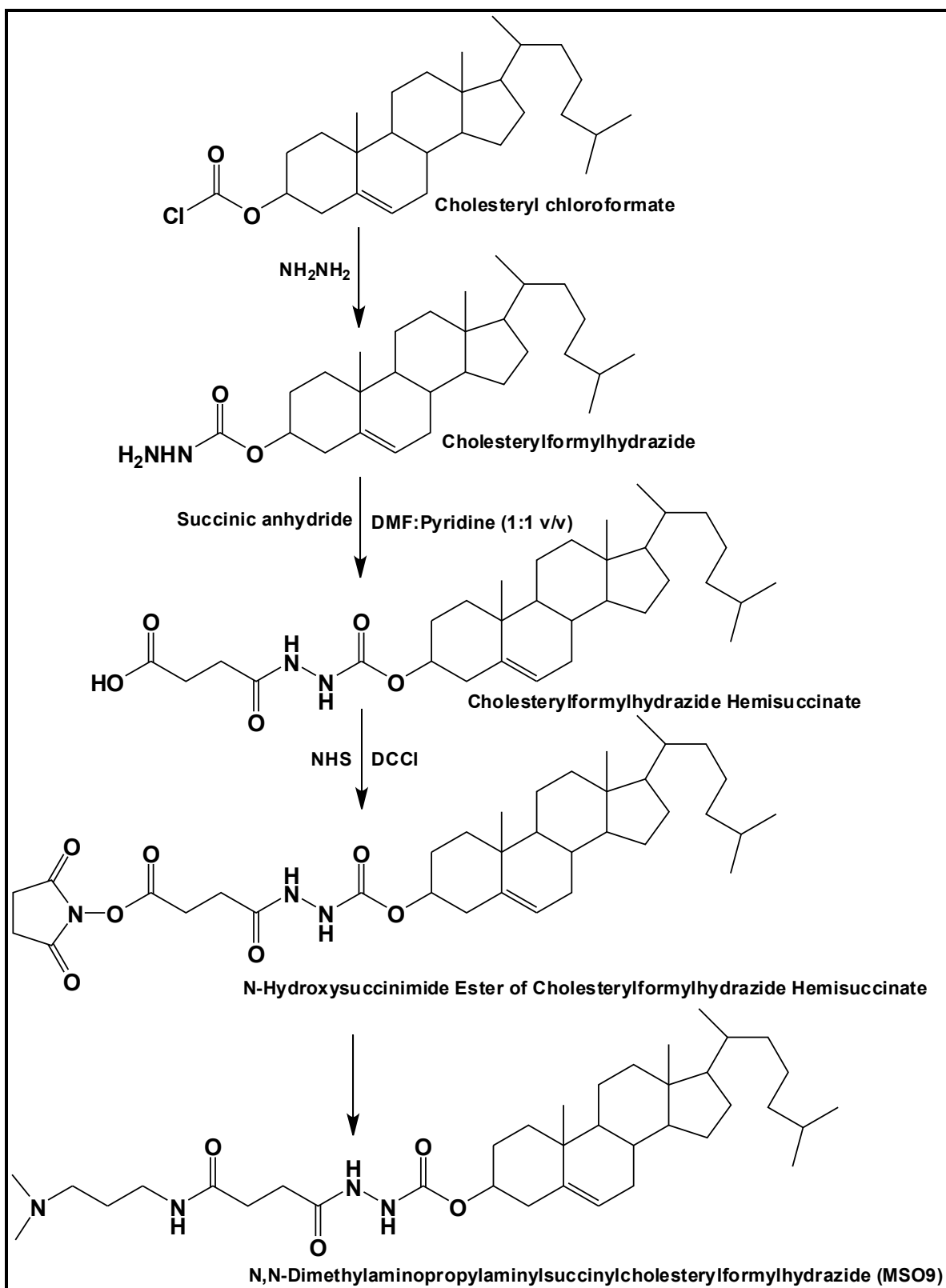


Figure 2.1: Scheme for the synthesis of cationic cholesterol derivative *N,N*-dimethylpropylamino-succinylcholesterylformylhydrazide (MSO9) (Singh and Ariatti, 2006a).

2.1.1.2. Synthesis of Cationic Cholesterol Derivative 3 β [N(N¹,N¹-dimethylaminopropyl--succinamido-ethane)-carbamoyl]cholesterol (SGO4)

SGO4 was synthesized in three steps, as discussed below. The reactions involved in the synthesis of cytofectin SGO4 are outlined in Figure 2.2.

2.1.1.2.1. Preparation of 3 β [N(2-aminoethyl)-carbamoyl]cholesterol (SGO1)

To ethylene diamine (2.25 g, 37.5 mmol) in dry CH₂Cl₂ (40 mL) was added a solution of cholesterylchloroformate (2.0 g, 4.45 mmol) dropwise over 5 minutes. After 48h at room temperature the reaction mixture was extracted with 3 \times 150 mL H₂O. The CH₂Cl₂ layer was dried over anhydrous Na₂SO₄ and evaporated to a white powder. Thereafter, the product was recrystallized from cyclohexane. The product was purified further by column chromatography on a silica gel 60 column (2.2 \times 23 cm). The column was initially eluted with chloroform (50 mL) and then with CHCl₃: MeOH: Con.NH₄OH (95:4:1) (100 mL) followed finally with CHCl₃:MeOH:Conc.NH₄OH (90:10:1). Product fractions were evaporated and the title compound was obtained in a crystalline form from cyclohexane.

Yield: 67% (705 mg); Mp: 168–170 °C; ¹H NMR (400MHz, CDCl₃): δ 0.67 (s, 3H, H-18'), 0.86 (d, 6H, *J* = 7 Hz, overlapping 2 Hz, H-26', H-27'), 0.91 (d, 3H, *J* = 6.5 Hz, H-21'), 1.00 (s, 3H, H-19'), 1.0–2.1 (m, 28H, cholesteryl), 2.35 (m, 2H, H-4'), 2.85 (m, 2H, H₂NCH₂), 3.25 (q, 2H, *J* = 5.7 Hz, H₂NCH₂CH₂), 4.49 (m, 1H, H-3'), 5.37 (d, 1H, *J* = 4.9 Hz, H-6') ppm. ¹³CNMR (100MHz, CDCl₃): 11.9 (C-18'), 18.7 (C-21'), 19.3 (C-19'), 21.0 (C-11'), 22.6 (C-26'), 22.8 (C-27'), 23.9 (C-23'), 24.3 (C-15'), 28.0, 28.2 (C-2', C-16', C-25' overlapping), 31.9 (C-7', C-8' overlapping), 35.8 (C-20'), 36.2 (C-22'), 36.6 (C-1, C-10'), 37.0 (C-1'), 38.6 (C-24'), 39.5, 39.7 (C-4', C-12'), 42.3 (C-13'), 50.0 (C-9'), 56.2 (C-17'), 56.7 (C-14'), 74.4 (C-3'), 122.5 (C-6'), 156.5 (NHCOO), 139.8 (C-5'). HR-MS (ESI-QTOF +ve): *Anal.* Calcd. for C₃₀H₅₃O₂N₂: (M+H) 473.4113, Found 473.4290.

2.1.1.2.2. Preparation of 3 β [N(hemisuccinamidoethane)-carbamoyl]cholesterol (SGO2)

3 β [N(2-aminoethane)-carbamoyl]cholesterol (237 mg, 0.5 mmol) in CH₂Cl₂ (5 mL) was added dropwise to a solution of succinic anhydride (60 mg, 0.6 mmol) in pyridine (1 mL). After 24 h, a gel-like product was formed. A further aliquot of succinic anhydride (60 mg, 0.6 mmol)

and 1 mL of DMF was added to obtain a clear solution. After 24 h a quantitative yield of product was obtained. The product was recrystallized from ethanol.

Yield: 84% (165 mg); Mp: 168-170 °C; ¹H NMR (400MHz, C₅D₅N): δ 0.67 (s, 3H, H-18'), 0.90 (d, 6H, *J* = 6.6 Hz, H-26', H-27'), 1.0-2.1 (m, 28H, cholesteryl), 2.56 (m, 2H, H-4'), 2.85 (t, 2H, *J* = 6.9 Hz, H-2), 3.06 (t, 2H, *J* = 6.8Hz, H-1), 3.68 (t, 2H, *J* = 6.5 Hz, H-5), 3.74 (t, 2H, *J* = 5.5 Hz, H-6), 4.82 (m, 1H, H-3'), 5.38 (bs, 1H, H-6'). ¹³C NMR (100MHz, C₅D₅N): 12.0 (C-18'), 19.0 (C-21'), 19.4 (C-19'), 21.3 (C-11'), 22.7 (C-27'), 23.0 (C-26'), 28.2, 28.5, 28.7 (C-2', C-16', C-25'), 31.5 (C-5), 32.1, 32.2 (C-7', C-8'), 36.1 (C-22'), 36.5 (C-10'), 36.8 (C-1'), 39.2 (C-1), 39.7, 39.9 (C-4', C-12'), 42.5 (C-13'), 50.3 (C-9'), 56.4 (C-17'), 56.8 (C-14'), 74.1 (C-3'), 122.6 (C-6), 140.4 (C-5), 157.3 (NHCOO), 172.7 (C-4), 175.5 (C-7). HR-MS (ESI-QTOF +ve): *Anal.* Calcd. for C₃₄H₅₇N₂O₅ (M+H) 573.4273, Found 573.4338.

2.1.1.2.3. Preparation of 3β[*N*(*N*¹,*N*¹-dimethylaminopropylsuccinamidoethane)-carbamoyl]-cholesterol (SGO4)

To a solution of 3β[*N*(hemisuccinamidoethane)-carbamoyl]cholesterol (114 mg, 0.2 mmol) and *N*-hydroxysuccinamide (32 mg, 0.28 mmol) in pyridine (2 mL) was added a solution of dicyclohexylcarbodiimide (55 mg, 0.26 mmol) in pyridine (0.8 mL). A catalytic amount of 4-dimethylaminopyridine (2.4 mg, 0.02 mmol) was also included in the reaction mixture. After 24 h, 3-dimethylaminopropylamine (51 mg, 0.5 mmol) was added to the mixture which was stored in the dark at room temperature for 24 hours. Dicyclohexylurea crystals were removed by filtration. The filtrate was concentrated and the product was purified by silica gel 60 column (2.0×17.0 cm) chromatography. Equilibration and elution were performed using CHCl₃:MeOH:Con. NH₄OH (43:7:1).

Yield: 61% (90 mg); Mp: 216-218 °C; ¹H NMR (400MHz, CDCl₃):δ 0.68 (s, 3H, H-18'), 0.86 (d, 6H, *J* = 6.7 Hz, overlapping 2 Hz, H-26', H-27'), 0.91 (d, 3H, *J* = 6.5 Hz, H-21'), 1.00 (s, 3H, H-19'), 1.00-2.21 (m, 27H, cholesteryl), 2.27 (s, 6H, H-13, H-14), 2.42 (t, 2H, *J* = 6.3 Hz, H-4), 2.50 (s, 4H, H-6, H-7), 3.30-3.36 (m, 6H, H-1, H-2, H-9), 4.48 (m, 1H, H-3'), 5.37 (d, 1H, *J* = 4.9 Hz, H-6') ppm. ¹³C NMR (100MHz, CDCl₃): 11.9 (C-18'), 18.7

(C-21'), 19.3 (C-19'), 21.0 (C-11'), 22.6 (C-26'), 22.8 (C-27'), 23.8 (C-23'), 24.3 (C-15'), 28.0, 28.2 (C-2', C-16', C-25' overlapping), 31.8 (C-10), 31.9 (C-7', C-8' overlapping), 35.8 (C-5'), 36.2 (C-22'), 36.6 (C-1, C-10' overlapping), 37.0 (C-1'), 38.6 (C-24'), 39.5, 39.7 (C-4', C-12'), 42.3 (C-13', C-9 overlapping), 45.2 (C-13, C-14 overlapping), 50.0 (C-9'), 56.1 (C-17'), 56.7 (C-14'), 58.3 (C-11), 74.5 (C-3'), 122.5 (C-6'), 139.8 (C-5'), 156.8 (NH \underline{C} OO), 172.1 (C-7), 173.0 (C-4). HRMS (ESI-QTOF +ve): *Anal.* Calcd. for C₃₉H₆₉N₄O₄ (M+H) 657.5324, Found 657.5516.

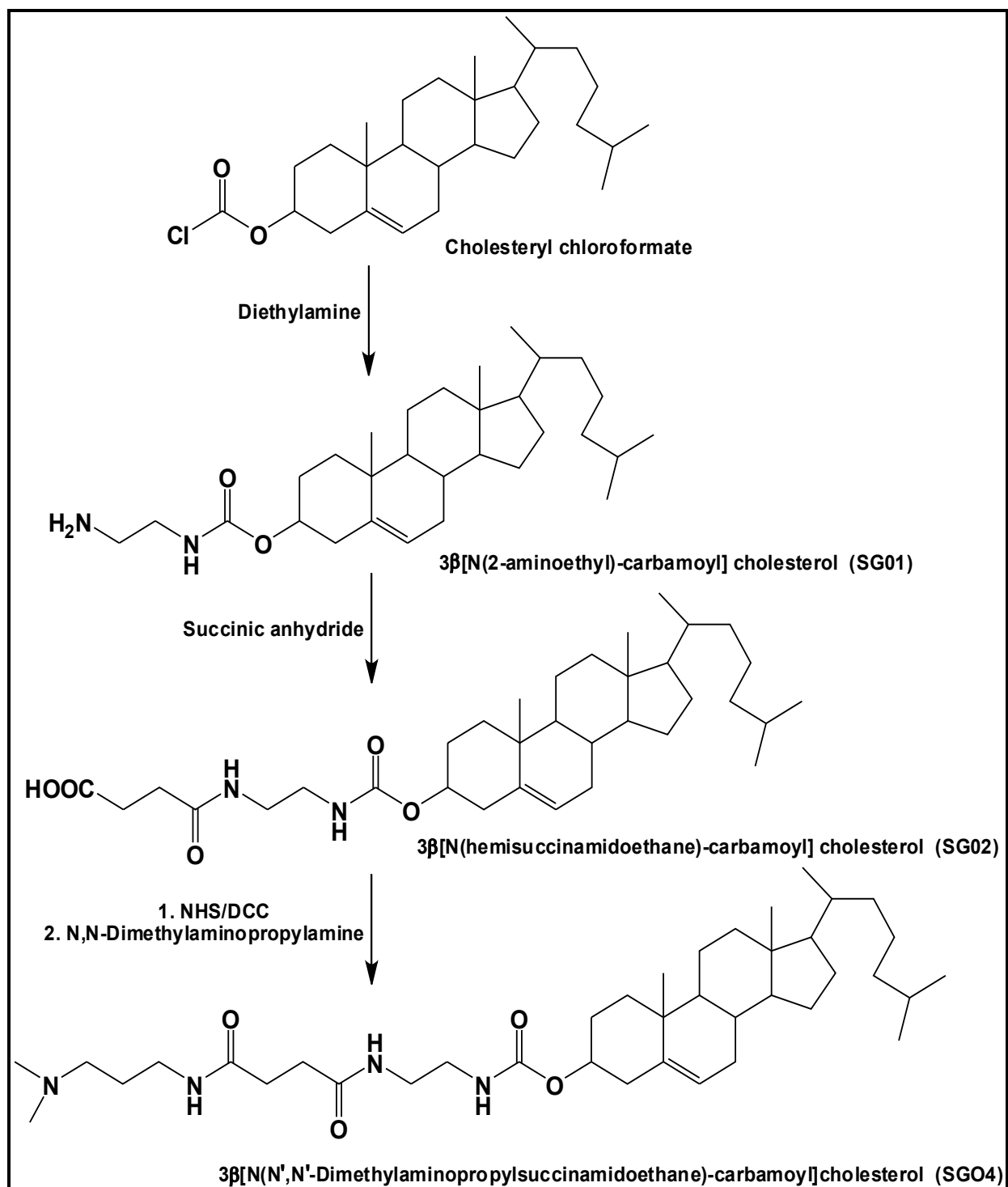


Figure 2.2: Scheme for the synthesis of cationic cholesterol derivative 3β[N(N',N'-dimethylaminopropyl succinamidoethane)-carbamoyl]-cholesterol (SGO4).

2.1.2. Synthesis of DSPE-PEGFOL for Folate Targeted Liposome

DSPE-PEGFOL was synthesized (Figure. 2.3) as an adaptation of previously reported protocols (Yoshida et al., 2006; Liu et al., 2011). To folic acid (100 mg, 0.226 mmol) dissolved in dimethyl sulfoxide (5 mL) were added equimolar amounts of *N*-hydroxysuccinimide (NHS) (28.8 mg, 0.25 mmol), and dicyclohexylcarbodiimide (DCC) (51.5 mg, 0.25 mmol). The reaction mixture was incubated overnight at room temperature. The insoluble dicyclohexylurea (DCU) was filtered off from the reaction mixture. To a solution (44 μ L) of the NHS ester of folic acid (NHSF, 2 μ mol) was added DSPE-PEG₂₀₀₀NH₂ (2.79 mg, 1 μ mol). To obtain a solution, dimethylsulfoxide (DMSO) (40 μ L) and pyridine (40 μ L) were added. The solution was left overnight and pyridine was removed by rotary evaporation. Water (500 μ L) was added to the reaction mixture and the solution was centrifuged at 12000 rpm (SANYO MSE) for 5 min at room temperature, to remove the trace insolubles. The supernatant was dialyzed (MW cutoff of 2000 Da) against water (three x 500 mL). The dialyzed product was analyzed by UV-Spectroscopy. The dialyzed product (DSPE-PEG₂₀₀₀FOL) was stored at -20 °C before use.

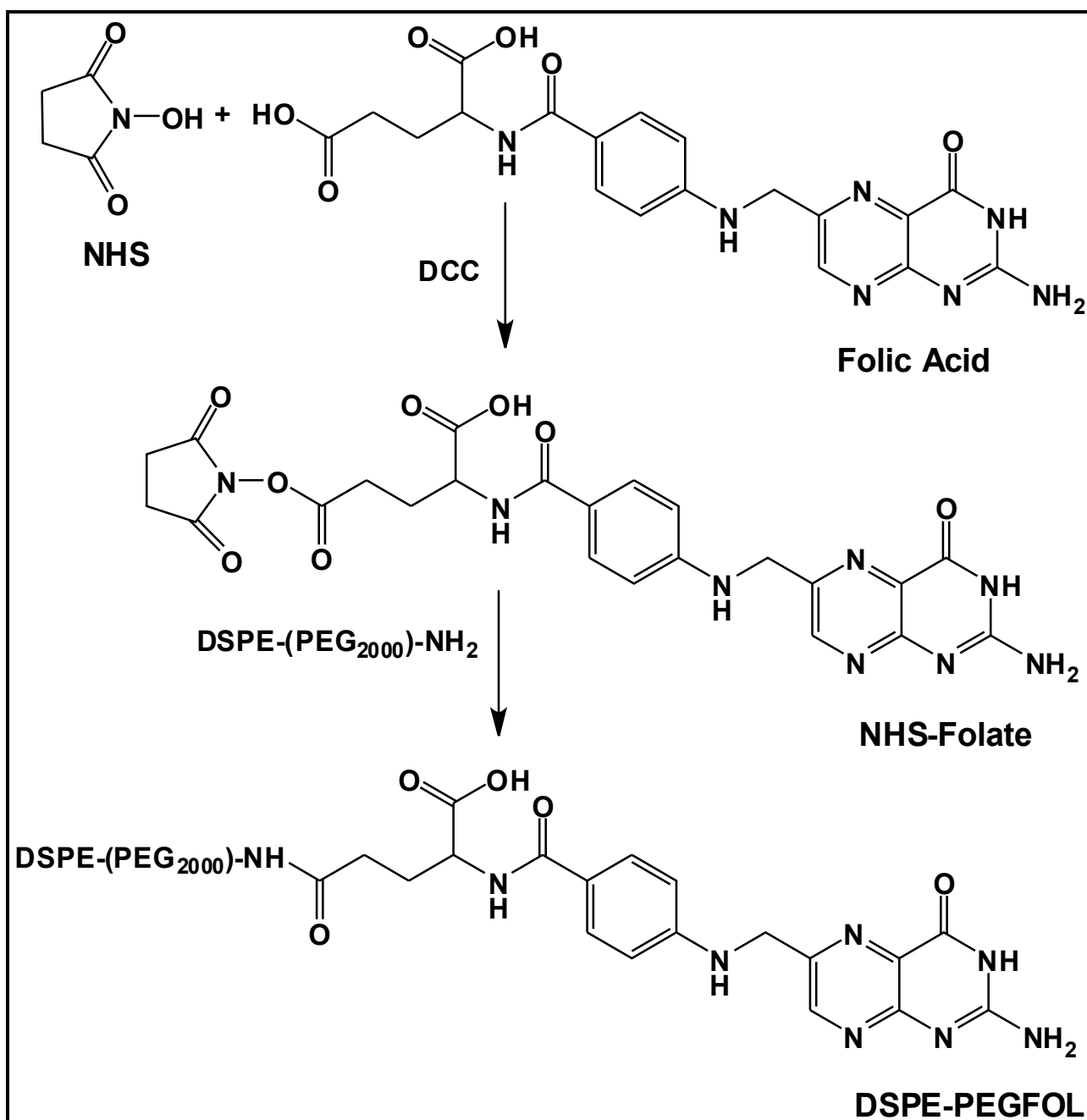


Figure 2.3: Scheme for the synthesis of DSPE-PEGFOL.

2.1.3. Preparation of Cationic Liposomes

Cationic liposomes with or without folate were synthesized by the thin film rehydration method adapted from Gao et al., (1991). Six cationic liposomes were prepared with the cytofectins MSO9 or SGO4 with the quantities of each lipid component shown in Table 2.1. The liposome preparations were made up to a total of 2 μmol of lipid in 0.5 mL of HEPES buffer (150 mM NaCl, 20 mM HEPES, pH 7.5) (HBS). The targeting ligand folic acid appended to PEG₂₀₀₀ was used in the targeted cationic liposomes. Additionally, distearoylphosphatidylethanolamine polyethylene-glycol₂₀₀₀ (DSPE-PEG₂₀₀₀) was formulated into liposomes to obtain both a folate targeting and stealth capability in liposome formulations. Each lipid was first dissolved in chloroform (1 mL) and rotary-evaporated (Rotavapor-R at 25 °C) to afford a thin film deposit on the inner wall of a test tube. The sample was dried further under high vacuum in a drying pistol for 20-30 min. Thereafter, the resultant thin lipid film was rehydrated in 0.5 mL of sterile HBS and finally the mixture was briefly vortexed and sonicated for 5 minutes using a Transonic bath type sonicator at 20 °C. Liposome suspensions were routinely stored at 4 °C.

Table 2.1: Components of cationic liposomes.

Cationic Liposome Formulation	Mass (mg)					Molar Ratio (μmole)				
	MSO9	SGO4	DOPE	DSPE-PEG ₂₀₀₀	DSPE-PEGFOL	MSO9	SGO4	DOPE	DSPE-PEG ₂₀₀₀	DSPE-PEGFOL
MSO9:DOPE	0.63	---	0.74	---	---	1	---	1	---	---
MSO9:DOPE: 2%PEG ₂₀₀₀	0.63	---	0.71	0.11	---	1	---	0.96	0.04	---
MSO9:DOPE: 2%PEG ₂₀₀₀ : DSPE- PEGFOL	0.63	---	0.71	0.11	0.03	1	---	0.96	0.04	0.01
SGO4:DOPE	---	0.65	0.74	---	---	---	1	1	---	---
SGO4:DOPE: 2%PEG ₂₀₀₀	---	0.65	0.71	0.11	---	---	1	0.96	0.04	---
SGO4:DOPE: 2%PEG ₂₀₀₀ : DSPE- PEGFOL	---	0.65	0.71	0.11	0.03	---	1	0.96	0.04	0.01

2.1.4. Calculation of Nitrogen to Phosphate (N:P/+/-) Ratio

The required amounts of cationic liposome and plasmid (pCMV-luc) DNA were calculated according to the desired N/P ratio based on previous reports by Zanta et al., (1997). The N/P ratio is the ionic balance of the liposome:pDNA in lipoplexes. The positive charge of the liposome arises from the liposome amine nitrogen (N). The negative charges in the plasmid DNA backbone arises from the phosphate groups of the deoxyribonucleotides. The average molecular weight of a nucleotide was assumed to be 350 g/mol. The lipoplexes of plasmid DNA and liposome were prepared by mixing of both components leading to complex formation due to electrostatic interactions. The lipid:DNA charge ratio was calculated as the molar ratio of lipid cytofectin molecule with one positive charge per molecule, to the phosphate of the nucleotide of DNA.

2.1.5 Preparation of Lipoplex

Lipoplexes were prepared by simply mixing the desired liposome suspension with the plasmid DNA gently in a final volume of 10 μ L of HBS to obtain the desired (+/-) charge ratios from 1:1 to 7:1. Thereafter, the lipoplexes were incubated at room temperature for 30 minutes to mature. The preparation of complexes was carried out in a class-II bio-hazard hood under sterile solutions.

2.1.6 Characterization of Liposomes and Lipoplexes

The chemical and physical characteristics of liposomes determine their *in vivo* and *in vitro* behaviour. The quality control of liposomal dispersions is essential since they are used extensively as vehicles for gene/ drug delivery and their properties will determine their fate *in vivo*. Two of the most important parameters are particle size and zeta potential. Liposome size is important, as it is one factor that determines the uptake of the vector into cells. Also for a possible *in vivo* use, small size has to be ensured to avoid complications such as micro embolism in blood vessels. Surface charge of the particles in dispersion is an important parameter as the potential of liposomes plays a role, for example in stabilizing liposomes against aggregation or fusion and in the interaction between liposomes and charged drugs. It also has an impact on the behaviour of liposomes *in vivo* (Cevc, 1993). Any subsequent modification of the liposome surface in terms of charge can also be monitored by measurement of the zeta potential. The

techniques used for detecting lipoplex formation, and protection of plasmid DNA by cationic liposomes are described below.

2.1.6.1. Agarose Gel Electrophoresis

Cationic liposome:pDNA complexes were formed using pCMV-luc (6.2 kbp) at a final DNA concentration of 0.5 μg in 10 μL HBS and at liposome:DNA (+/-) charge ratios from 1:1 to 7:1 as shown in Table 2.2. The complexes were incubated for 30 min at room temperature for lipoplex maturation. Thereafter, 3 μL of gel loading buffer (50% glycerol, 0.5% bromophenol blue, 0.5% xylene cyanol in 2X gel buffer) was added to the incubation mixtures which were then loaded onto 1% agarose gels containing ethidium bromide (0.5 $\mu\text{g}/\text{mL}$). The gel running buffer was composed of 36 mM Tris-HCl, 30 mM sodium phosphate and 10 mM EDTA (pH 7.5). Electrophoresis was performed at 50V for 90 min and images were captured using the VacutecSyngene G: Box gel documentation system (Syngene, Cambridge, UK) at an 800 milli second exposure time.

Table 2.2: Liposome:DNA complexes set up for gel retardation studies.

Lipoplex Formulation (+/-)	Well Number							
	1	2	3	4	5	6	7	8
MSO9:DOPE	0	1:1	2:1	3:1	4:1	5:1	6:1	7:1
MSO9:DOPE:2%PEG₂₀₀₀	0	1:1	2:1	3:1	4:1	5:1	6:1	7:1
MSO9:DOPE:2%PEG₂₀₀₀:DSPEPEG₂₀₀₀FOL	0	1:1	2:1	3:1	4:1	5:1	6:1	7:1
SGO4:DOPE	0	1:1	2:1	3:1	4:1	5:1	6:1	7:1
SGO4:DOPE:2%PEG₂₀₀₀	0	1:1	2:1	3:1	4:1	5:1	6:1	7:1
SGO4:DOPE:2%PEG₂₀₀₀:DSPEPEG₂₀₀₀FOL	0	1:1	2:1	3:1	4:1	5:1	6:1	7:1
pCMV-luc (μg)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

2.1.6.2. Serum Nuclease Protection Assay

Lipoplexes were analysed to determine the protection offered to the DNA against nuclease attack by the different liposome preparations. Varying amounts of cationic liposomes (Table 2.3) were added to a constant amount of pCMV-luc DNA (1 µg). This was made up to a final volume of 10 µL with HBS. The samples were allowed to incubate for 30 minutes at room temperature. Thereafter, 10% (v/v) foetal bovine serum (FBS) was added to the lipoplexes. Two controls were employed, a negative control containing only pCMV-luc DNA and a positive control containing pCMV-luc DNA and 10% FBS. The samples were then incubated for 4 h at 37 °C. After the incubation period, the chelator EDTA was added to the samples to a final concentration of 10 mM to stop the nuclease reaction. Sodium dodecyl sulphate (SDS) was then added to a final concentration of 0.5% (w/v), to liberate DNA from the complexes for migration into the gel. The samples were then incubated for a further 20 minutes at 55°C. Thereafter, the samples were subjected to electrophoresis on a 1% agarose gel for 90 min at 50 volts and images captured using the VacutecSyngene G: Box gel documentation system as described in 2.1.6.1.

Table 2.3: Nuclease protection assessment by cationic liposomes.

Cationic Liposome Formulation	Liposome:DNA Ratio (+/-)			pCMV-luc (µg)
MSO9:DOPE	2:1	3:1	4:1	1
MSO9:DOPE:2%PEG₂₀₀₀	1:1	2:1	3:1	1
MSO9:DOPE:2%PEG₂₀₀₀:DSPEPEG₂₀₀₀FOL	2:1	3:1	1:1	1
SGO4:DOPE	2:1	3:1	4:1	1
SGO4:DOPE:2%PEG₂₀₀₀	2:1	3:1	4:1	1
SGO4:DOPE:2%PEG₂₀₀₀:DSPE-PEG₂₀₀₀FOL	2:1	3:1	4:1	1

2.1.6.3. Ethidium Bromide Intercalation Assay

The binding of DNA with the cationic liposomes was further studied using the EtBr intercalation assay. EtBr is an intercalating agent, and acts as the fluorescent probe providing reproducible and efficient evaluation of lipoplex formation. The displacement of EtBr, upon lipid interaction with the DNA, is reflected as a drop in the fluorescence signal, since unbound EtBr does not fluoresce as strongly (Gopal et al., 2011). The level of condensation is essential,

particularly at the point of endosomal escape for delivery of DNA therapeutic agents (Lasic, 1997). All fluorescence measurements were carried out using a Glomax multi⁺ detector system (Promega, Biosystems, Sunnyvale, USA). The excitation wavelength was 525 nm and the emission wavelength was kept at 580 nm. Initially, 2 μL of ethidium bromide (0.2 μg), from a 100 $\mu\text{g}/\text{mL}$ diluted stock solution, was added to 100 μL of HBS buffer (pH 7.4) in black 96-well plates and the baseline fluorescence was determined as 0%. The 100% relative fluorescence was achieved by adding 2.4 μL (1.2 μg) of pCMV-luc plasmid DNA to the HBS-ethidium bromide mix. Thereafter, liposome (1 μL) preparations were added stepwise to the mixture and readings were taken after each addition until a plateau was reached. Contents in the wells were mixed thoroughly for even distribution and also to attain the complete DNA compaction or condensation. The fluorescence intensity obtained upon each addition of liposome was normalized relative to the fluorescence signal of the DNA–EtBr complex, which was taken as 100%.

2.1.6.4. Cryo-Transmission Electron Microscopy (Cryo-TEM)

Morphology and size of liposomes as well as lipoplexes were determined by cryo-TEM. The cationic liposome suspensions were diluted to a 1:20 ratio, and the lipoplexes were diluted 1:100 with sterile Hepes buffered saline (HBS). A 1 μL droplet of the diluted cationic liposome or lipoplex suspension was deposited onto a copper grid. To this, was added 1 μL of 1% (^{w/v}) uranyl acetate and the grid was allowed to dry for 2 minutes. After removing the excess suspension with a filter paper, the grid was placed in liquid nitrogen and then transferred into a GATAN cryo-holder maintained at -170 °C. This was then introduced into the TEM for observation at -150 °C. Images were obtained under cryogenic conditions and investigated at 100 kV in a JEOL JEM1010 electron microscope (Tokyo, Japan). The micrographs were captured on a MegaViewIII camera, and SIS i-TEM software facilitated measurements of liposomes on calibrated images.

2.1.6.5. Photon Correlation Spectroscopy

Particle size, size distribution as well as the particles surface charge of dispersions can be estimated using photon correlation spectroscopy (PCS), also known as dynamic light scattering (DLS) technique. It is the analysis of the time dependence of intensity fluctuations in scattered

laser light (helium, neon or argon) due to the Brownian motion of particles in solution/suspension (Ostrowsky, 1993). Intensity of the stray light fluctuates as the particles in dispersion show Brownian motion. Small particles diffuse more rapidly than large particles, and the rate of fluctuation of scattered light intensity varies accordingly. Therefore, the hydrodynamic diameter of the particles may be deduced. Results of this calculation include two parameters: the Z-average as mean calculated hydrodynamic diameter describing the size of particles and the polydispersity index (PDI) describing the particle size distribution (Hope et al., 1986).

The sizes of the liposomes and lipoplexes, size distribution and zeta potential were measured in a Malvern Zetasizer Nanoseries (Malvern Inst, Worcestershire, UK) at 25 °C. The cell types chosen for Z-average measurement were a DTS0012 – polystyrene disposable sizing cuvette and DTS-1061 for zeta potential measurements. These types of cells allows for determination of an appropriate amount of sample in the range of 0.5 to 1 mL. For liposomes, the samples were prepared in a 1:20 dilution (50 µL sample + 950 µL) in sterile HBS, and for lipoplexes, the samples were prepared in a 1:100 dilution (10 µL of preformed lipoplex sample + 990µL) in sterile HBS. Three measurements were taken at setting position 4.65 and using run time and attenuator (intensity adjustment) around 6-8 as recommended by the analysis software.

2.1.7. Growth and Maintenance of Cells

2.1.7.1. Propagation of Cells

HEK293, HeLa and KB cells were grown in EMEM (Lonza) containing 10% FBS and penicillin (5000 units/mL)/streptomycin (5000 µg/mL). Cells were monitored on a regular basis and medium changed when necessary. Cells were trypsinised and split into desired ratios once they had reached confluence. For the trypsinisation procedure, the medium from the cells was decanted, and the cells washed with 5 mL phosphate buffered saline (PBS) (150 mM sodium chloride, 27 mM potassium chloride, 1 mM potassium dihydrogen phosphate, 6 mM di-sodium hydrogen phosphate, pH 7.5). Thereafter, 1 mL trypsin-EDTA mixture (0.25% ^{w/v} trypsin, 0.1% ^{w/v} EDTA) was added and trypsinisation was observed under a Nikon TMS inverted microscope (Nikon, Tokyo, Japan) (approximately 1-2 minutes). Once cells had rounded off, approximately 1-2 mL of medium (EMEM + 10% FBS + antibiotics) was added to the cells, and the flask was

gently tapped against the palm of the hand in order to dislodge the cells. The cells were split as desired into 25 cm³ flasks, containing 4 mL medium. The cells were incubated at 37 °C and medium changed at the required intervals. Once cells had reached confluency they were once again trypsinised and split as required or cryopreserved and stored in liquid nitrogen or a -81 °C Biofreezer (Nuair) for future use.

2.1.7.2. Cryopreservation and Reconstitution of Cells

Confluent cells were washed with PBS and trypsinised as in 2.2.7.1. The cells were then pelleted by centrifugation at 1000 rpm for 3 minutes in a Eppendorf centrifuge. The cells were then resuspended in 0.9 mL medium and 0.1 mL dimethylsulfoxide (DMSO). The cell suspension was dispensed into a cryogenic ampoule which was placed in a NalgeneMR Frosty cryogenic container and in a -80 °C biofreezer for slow freezing, at a rate of 1 °C/minute. The ampoules of frozen cells were then transferred into liquid nitrogen for long term storage or to cryoboxes for short term storage in the -81 °C biofreezer.

The cells were reconstituted when required in the following manner. The ampoule containing the cells was removed from liquid nitrogen/biofreezer, and immediately placed in a 37 °C water bath. Directly after thawing, the ampoule was wiped with ethanol, and the cells were pelleted by centrifugation (1000 rpm for 3 minutes). The supernatant was discarded into a waste bottle, and the cells were resuspended in 1 mL fresh medium (EMEM + 10% FBS + antibiotics). The medium was changed after 24 hours, and the cells were monitored with frequent medium changes until they reached confluency.

2.1.8. Plasmid DNA Amplification

The pCMV-luc DNA (Plasmid factory) (Figure. 2.4) was successfully amplified in the Department of Biochemistry, University of KwaZulu-Natal according to a standard protocol. The DNA purity and concentration were quantified using a Thermo Electron Corporation Biomate 3 spectrophotometer at 260 and 280 nm and adjusted to 0.5 µg/µL. The isolated DNA was analyzed on a 1% agarose gel against a control DNA sample to confirm purity and integrity. Moreover, the quality of DNA in terms of three forms of bands, namely, supercoil, circular and linear was also verified on 1% agarose gel.

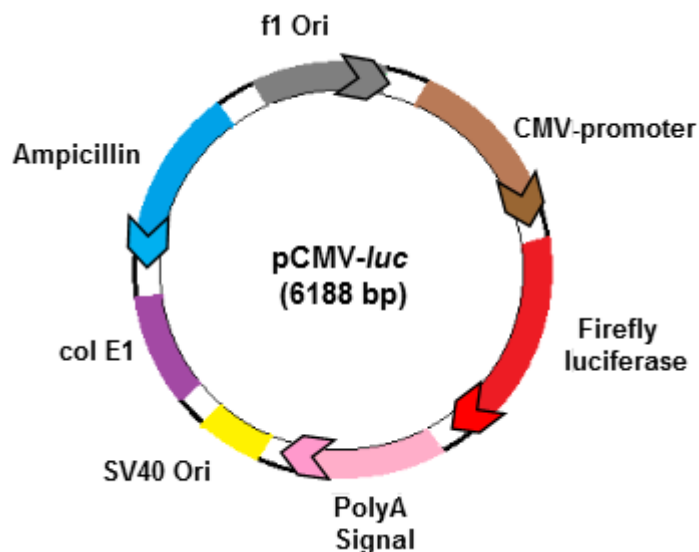


Figure 2.4: Map of pCMV-luc control vector showing the SV40 promoter and firefly luciferase (luc) gene (Plasmid Factory, Bielefeld, Germany).

2.1.9. Cell Viability Assay

The viability of the cells in the presence of varying amounts of cationic liposome was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay described by Mosmann, (1983). Cells were seeded at a density of 2.5×10^4 cells/well in 48-well plates, and incubated at 37 °C in a humidified atmosphere containing 5% CO₂ for 24 h before treatment. The liposome:pCMV-luc complexes were prepared as defined in Table 2.4 and incubated at room temperature for 20-30 minutes. Thereafter, they were added to each well containing 0.3 mL of EMEM medium (containing 10% FBS, streptomycin 100 µg/mL), and penicillin (100 U/mL) and incubated for further 48 h at 37 °C. After incubation, the medium was discarded and 0.2 mL of fresh complete medium and 0.2 mL of 5 mg/mL MTT solution in phosphate buffered saline (PBS) was added to the sample wells and incubated for an additional 4 h at 37 °C. Thereafter, the MTT containing medium was aspirated and 200 µL of DMSO was added to dissolve the yellow formazan crystals formed by viable cells. The absorbance was measured at 570 nm to determine cell viability as percentage of untreated control cells. The data are presented as means ± standard deviation.

Table 2.4: Lipoplex ratios used in cell viability and transfection studies.

Cationic Liposome Formulation	Liposome:DNA Ratio (+/-)		
MSO9:DOPE	2:1	3:1	4:1
MSO9:DOPE:2%PEG₂₀₀₀	1:1	2:1	3:1
MSO9:DOPE:2%PEG₂₀₀₀:DSPEPEG₂₀₀₀FOL	2:1	3:1	1:1
SGO4:DOPE	2:1	3:1	4:1
SGO4:DOPE:2%PEG₂₀₀₀	2:1	3:1	4:1
SGO4:DOPE:2%PEG₂₀₀₀:DSPEPEG₂₀₀₀FOL	2:1	3:1	4:1

2.1.10. Transfection Assay

Cells were trypsinised and 2.5×10^4 cells/well were seeded into a 48-well plate, keeping the volumes constant to 0.3 mL complete medium (EMEM + 10% FBS + antibiotics) per well and incubated at 37 °C, 5% CO₂ for 24 h. Prior to transfection, the medium was removed and replenished with fresh complete medium. Transfection mixtures were prepared as indicated in Table 2.4 and added to the cells. Two controls were set up and included untreated cells, and cells exposed to naked plasmid DNA only. Cells were incubated for a further 48 h at 37 °C. Each transfection experiment was conducted in triplicate. The resulting luciferase expression was quantified using the Promega luciferase assay system.

2.1.10.1. Luciferase Assay

The luciferase assay was carried out using the Promega luciferase assay kit. The luciferase assay reagent (20 mM Tricine, 1.1 mM Magnesium carbonate hydroxide pentahydrate, 2.7 mM Magnesium sulphate, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 μM coenzyme A, 470 μM luciferin, 530 μM ATP), was prepared by adding 10 mL of the luciferase assay buffer to one vial of lyophilised luciferase assay substrate. The cell culture lysis reagent (5X) (25 mM trisphosphate, pH 7.8; 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid, 10% (v/v) Glycerol, 1% (v/v) Triton X-100), was diluted with distilled water to obtain a 1x stock.

Initially, cells were prepared by first removing the growth medium and carefully washing twice with cold PBS (0.3 mL). Thereafter, 80 μ L of 1X cell lysis reagent was added to the wells to cover the cells and the multi-well plate was then placed on a Scientific STR 6 platform shaker for 15 minutes at 30 rev/min. Thereafter, the cell debris was dislodged from the wells and the resultant suspension was centrifuged in Eppendorf microcentrifuge at 12000 x g for 5 seconds to pellet the cellular debris. The cell free extracts were collected to measure luciferase activity. This was done by adding 50 μ L of luciferase assay reagent to 20 μ L of cell free extract at room temperature, mixing immediately and placing the reaction mixture in a GloMax Multi⁺ detection system (Promega). To produce the most uniform and reproducible data the reaction mixtures were completely mixed prior to measurement. The light produced was measured and normalized against the protein content in the cell lysates that was determined using the bicinchoninic acid (BCA) assay (Sigma).

2.1.11. FA-Competition Studies

To elucidate and confirm the cellular mechanisms of gene delivery by folic acid conjugated lipoplexes, the following experiment was designed. Folate receptor positive KB and HeLa cells were cultured in the same conditions as that for the transfection tests. Cells were seeded into 48-well plates at 2.5×10^4 cell density per well and incubated for 24 h. Thereafter, the medium was changed and excess/free folic acid (200 μ M) was added to cells in each well and cells incubated for 20 min at 37 °C to allow the free folic acid to bind to the receptor on the cell surfaces. Following this incubation the FA-targeted lipoplexes prepared as in Table 2.5 with predetermined ratios were added to the cells containing excess/free folic acid, and incubated at 37 °C for 48 h in the presence of 5% CO₂. Thereafter, the medium was aspirated and the cells harvested for luciferase activity measurements.

Table 2.5: Competition assays of lipoplexes.

Formulation	Liposome/DNA Ratio (+/-)		
MSO9:DOPE:2%PEG ₂₀₀₀ :DSPEPEG ₂₀₀₀ FOL	2:1	3:1	4:1
SGO4:DOPE:2%PEG ₂₀₀₀ :DSPEPEG ₂₀₀₀ FOL	2:1	3:1	4:1

2.1.12. Flow Cytometry

The quantitative cell uptake of lipoplexes was analyzed further by flow cytometry. Cells which are FR-positive (HeLa, KB) were seeded in 12-well plates at a seeding density of 2.5×10^4 cells/well and incubated for 24 h under the same culture conditions described in above sections (37 °C, 5% CO₂). Thereafter, complexes at high transfection N/P ratios as shown in Table 2.6 were prepared, added to the cells and were incubated for additional 48 h. The pCMV-luc GFP DNA (1 µg) was used in this study for complex formation with liposomes. At the end of incubation, medium from the wells was discarded and cells were gently washed two times with PBS buffer (0.3 mL) to eliminate non-specific binding. Thereafter, cells were treated with Trypsin-EDTA (0.1 mL) for 2-5 minutes until every cell had detached from the well, and subsequently centrifuged at 1000 rpm for 5 minutes to give cell pellets. The pellets were then dispersed in PBS (1 mL) then analyzed using Accuri C6 flow cytometer (BD Biosciences). Gating was performed on forward scatter-side scatter plots (FSC-SSC) to eliminate the dead cells and cell debris. Fluorescence parameters from at least 10,000 cells were recorded. Data were analyzed using FlowJo software program (Tree Star, Inc, Ashland, OR). Fluorescence displayed quantitatively as mean fluorescence intensity of the gated cell population in comparison to cells not expressing GFP.

Table 2.6: Flow cytometry assays of lipoplexes.

Formulation	HeLa	KB
	Liposome:DNA Ratio (+/-)	Liposome:DNA Ratio (+/-)
MSO9:DOPE	4:1	3:1
MSO9:DOPE:2%PEG	3:1	3:1
MSO9:DOPE:2%PEG:DSPEPEG ₂₀₀₀ FOL	4:1	3:1
SGO4:DOPE	4:1	2:1
SGO4:DOPE:2%PEG	2:1	3:1
SGO4:DOPE:2%PEG:DSPEPEG ₂₀₀₀ FOL	3:1	3:1

2.1.13. Statistical Analysis

Statistical significance of differences between data was evaluated by one-way ANOVA and Tukey's multiple comparison tests. Differences were considered statistically significant at $p < 0.05$.

CHAPTER THREE

RESULTS AND DISCUSSION

3. Synthesis of Cationic Cholesterol Derivatives

Two cationic cholesterol derivatives (CCDs), namely MSO9 and SGO4 were used in this study. These two CCDs show similar chemical structural properties which include a cholesterol anchor, a carbamoyl linker bond and a dimethylamino head group. The structural difference between the two cationic cholesterol derivatives lies in the spacer arm where CCD MSO9 has an 11 atom spacer arm and SGO4 has a 13 atom spacer length as illustrated in Figures 3.2.1 and 3.2.2 respectively.

3.1. Synthesis of *N, N*-dimethylpropylamidosuccinylcholesterylformylhydrazide (MSO9)

The synthesis of cationic cholesterol derivative MSO9 was achieved in four steps (Figure 2.1). In the first step, cholesterylformylhydrazide (MSO4) was afforded by the reaction of cholesteryl chloroformate with hydrazine (N_2H_4) (electron donor). This may be described as a dehydrohalogenation reaction.

In the second step, the cholesterylformylhydrazide (MSO4) amino group was treated with succinic anhydride ($C_4H_4O_3$) in the presence of a dimethylformamide:pyridine solvent system (1/1 v/v) to yield the intermediate compound cholesterylformylhydrazide hemisuccinate (MSO8). This step of MSO8 synthesis involves the expansion of the spacer segment by succinylation. MSO8 is more polar due to the presence of peptide and acidic carboxyl functionalities.

In step three, the *N*-hydroxysuccinimide ester of cholesterylformylhydrazide hemisuccinate (NHS ester of MSO8) was prepared by reacting MSO8 with equimolar ratios of DCC:NHS (1:1), in preparation for the addition of a cationic head group. The MSO8 carboxyl group was first activated by DCC, followed by reacting with *N*-hydroxysuccinimide (NHS) to generate the reactive ester of MSO8. In step four, the pairing of amine group dimethylaminopropylamine (DMAPA) to the carboxyl group on the NHS ester of MSO8 by the

amido link was executed to afford the final compound, MS09, with loss of *N*-hydroxysuccinimide.

3.2. Synthesis of 3 β [*N*(*N*¹,*N*¹-dimethylaminopropylsuccinamidoethane)-carbamoyl]-cholesterol (SGO4)

Cationic cholesterol derivative SGO4 was obtained in good yield in a convenient three step process (Figure 2.2). In the first step, cholesteryl chloroformate was reacted with the aliphatic ethylene diamine to afford the compound 3 β [*N*(2-aminoethyl)-carbamoyl]cholesterol (SGO1), formed as a result of nucleophilic attack on to the carboxyl group of cholesteryl chloroformate. This constituted the first step in the construction of a spacer segment.

The second step was a succinylation reaction. Succinylation aids in lengthening the spacer fraction by adding a succinyl group (CO-H₂C-H₂C-CO) to SGO1. Thereafter SGO1 was treated with succinic anhydride (C₄H₄O₃) in the presence of pyridine:DMF (1:1 v/v) to obtain 3 β [*N*(hemisuccinamidoethane)-carbamoyl]cholesterol (SGO2). SGO2 is more polar than SGO1 due to the presence of an aliphatic peptide bond, a carbamoyl link and a carboxylic group.

The final step in the reaction entailed the establishment of a cationic head group on to the SGO2 intermediate for binding to anionic DNA. The final product, SGO4 was obtained by two sub-steps. The reaction between SGO2 and NHS/DCC-DMAPA in a pyridine solvent system gave a product SGO4 (61% reaction yield) with a carbamate bond. Every step was monitored by TLC, and the final product gave a single spot at R_f=0.07 (CHCl₃:MeOH:Conc.NH₄OH) (43:7:1 (v/v/v)) (Figure 3.1) and HRMS 657.55 (M+H) peak. The infrared spectrum was also consistent with the structure assignment (results shown in Appendix-A).

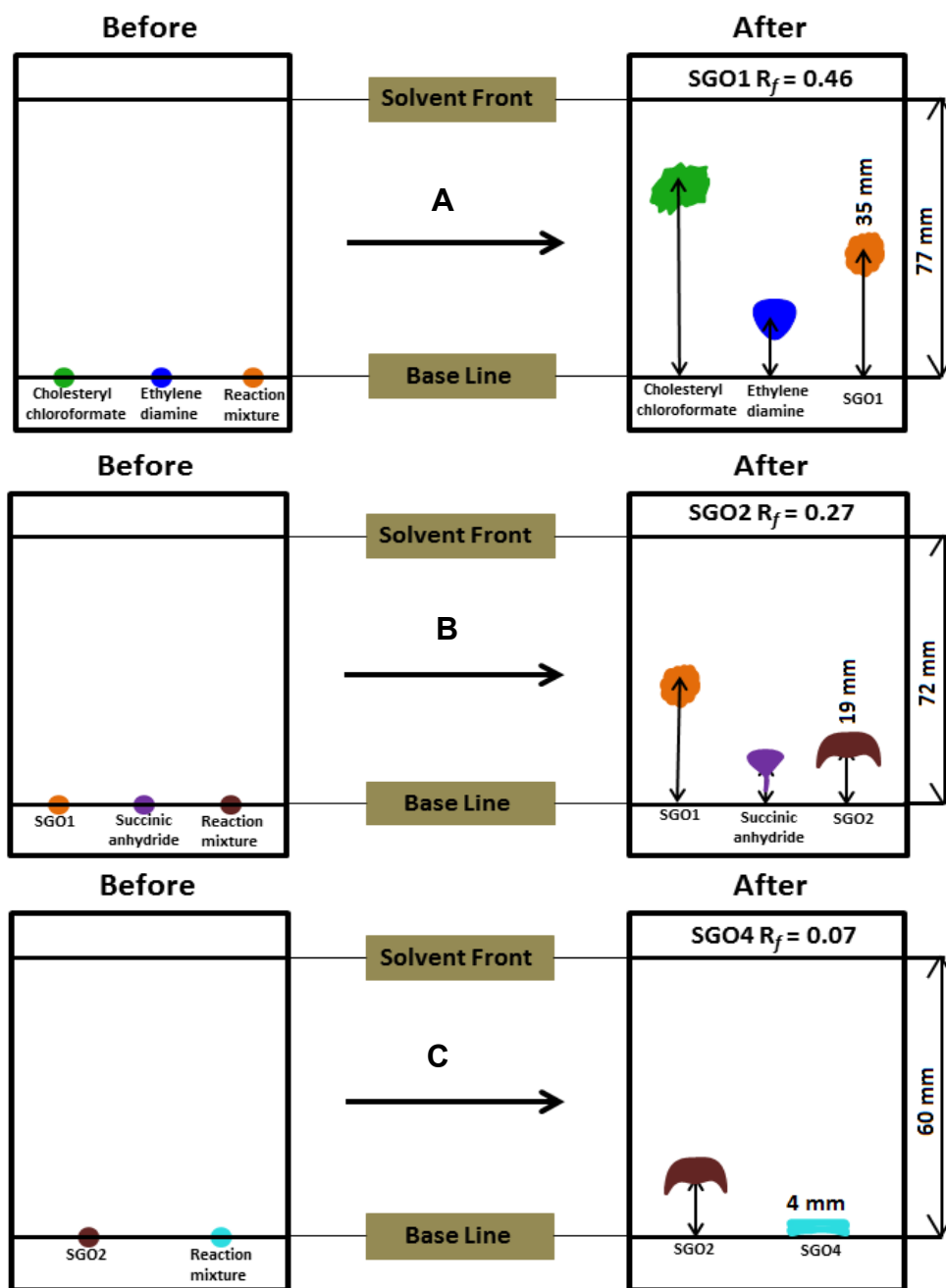


Figure 3.1: Representative TLC plates showing reaction progress of synthesis of SGO4 cationic cholesterol derivative. A. SGO1 B. SGO2 C. SGO4. Solvent system used was: $\text{CHCl}_3:\text{MeOH}:\text{Conc. NH}_4\text{OH}$ (43:7:1 (v/v/v)).

3.3. Synthesis of DSPE-PEG₂₀₀₀FOL for Targeted Liposome Formulation

Figure 2.3 outlines the synthesis of the targeting moiety wherein, folate was conjugated to DSPE-PEG₂₀₀₀, a PEG derivative of phosphatidylethanolamine. The specific ligand-receptor interactions are utilized in this study to deliver nucleic acids in a receptor specific manner using folate conjugated liposomes. Folate was chosen since the folic acid receptor constitutes a useful target for tumour specific delivery, especially in malignancies of the ovary, but also because this small size molecule is poorly immunogenic and easily available (Lu et al., 2006; Bharali et al., 2005). DSPE can be regarded as a buoy intended to be non-covalently entrapped into the nano-carrier wall. It is often utilized to insert PEG into nanoparticles or liposomes (Wang et al., 2012; Liu et al., 2014; Fondell et al., 2011; Xu et al., 2009).

The DSPE-PEGFOL polymer was successfully synthesized in two steps. In the first step a reactive intermediate, *N*-hydroxysuccinamidefolate (NHS-FOL), an active ester was prepared by a dehydration reaction. This was achieved by activating the carboxyl group on folic acid with *N*-hydroxysuccinimide (NHS) in the presence of DCC (coupling agent). NHS is water soluble and DCC is soluble in petroleum ether, resulting in simplified purification processes for the intermediate and final products.

The second step was to attain the intended compound DSPE-PEGFOL by the covalent attachment of DSPE-PEG₂₀₀₀ with a distal amine group to the succinate ester NHS-FOL. Covalent attachment of folate to PEG was deemed to be advantageous as the ligand would not only be prominently displayed, but the spacer, PEG₂₀₀₀ enjoys low cytotoxicity (Gorle et al., 2014), water solubility (Vinayak et al., 2005) and low immunogenicity (Mohs et al., 2005). Moreover, the presence of PEG to form a shell at the outside of the carrier provides functional end groups for the attachment of the targeting ligand through a flexible tether (Otsuka et al., 2012). NHS ester of folate (NHS-FOL) was coupled with amino DSPE-PEG₂₀₀₀ in DMSO:pyridine solvent system (1:1 v/v) to yield the final product DSPE-PEGFOL. The successful formation of DSPE-PEG-FOL was confirmed using U.V spectrometry (results shown in Appendix- A).

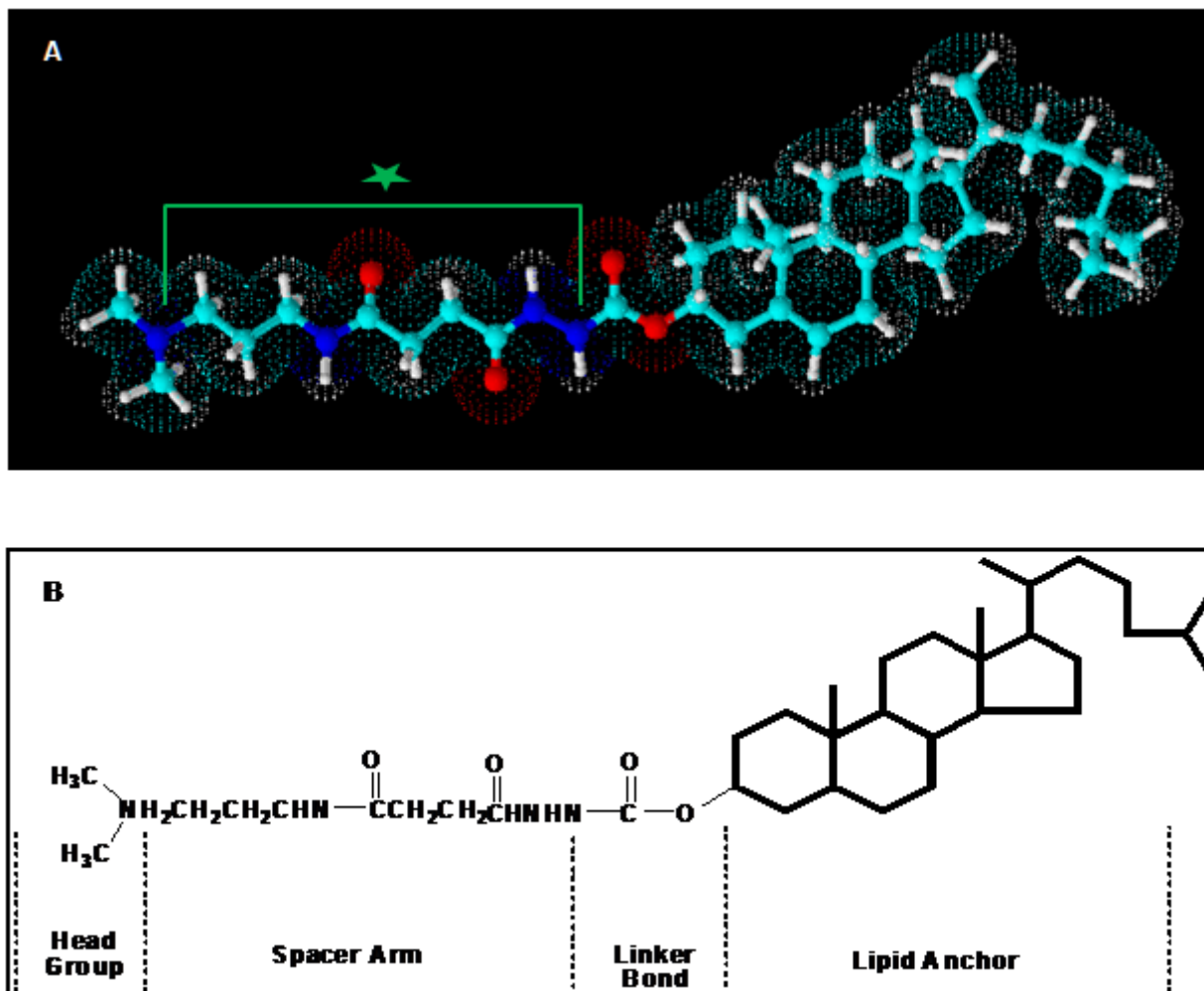


Figure 3.2.1: Structure of cationic cholesterol derivative MSO9. **A.** 3D model **B.** MSO9 displaying the four key structural parts. ★ → Spacer length = 12.45 Å

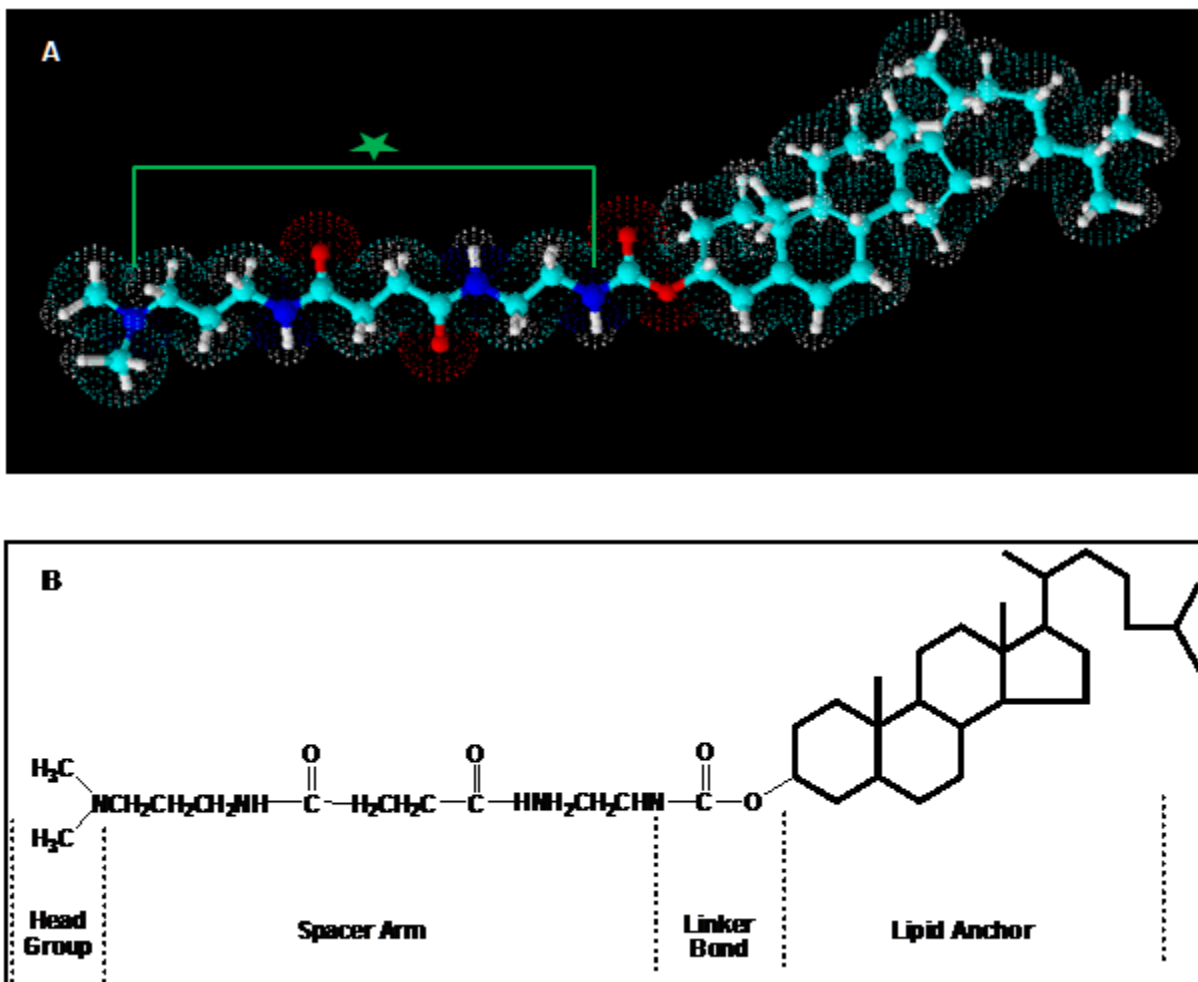


Figure 3.2.2: Structure of cationic cholesterol derivative SGO4. **A.** 3D model **B.** SGO4 displaying the four key structural parts. ★ → Spacer length = 14.95 Å

3.4. Preparation of Cationic Liposomes

3.4.1. Conventional/Untargeted Liposome Preparation

Liposomes are microscopic sphere-shaped particles (Figure 3.4) in which membranes, consisting of one or more lipid bilayers, encapsulate a fraction of the solvent in which they are suspended into their interior (Jimenez-Rojo et al., 2014). The cationic liposome preparation was achieved by utilizing the method described in section 2.1.3. Liposomes in this study were prepared by using two cationic cholesterol cytofectins MSO9 and SGO4. The synthesis of the former cytofectin was previously reported by Singh and Ariatti, (2006a) and the latter was newly synthesized successfully by adapting the method by Singh and Ariatti, (2006a) with a simple modification, using active methylene group containing amine (ethylene diamine) instead of hydrazine. The common structural features of these two cationic lipids include a positively charged tertiary amino head group, a spacer arm, linker bond and a hydrophobic lipid domain (Figure 3.2.1 and Figure 3.2.2). The head group usually contains simple or multiple amine groups and is responsible for the interactions between liposome and DNA, and the lipoplex and the cell membrane (Lavigne et al., 2004).

Cholesterol ($C_{27}H_{45}OH$), which is a cell membrane component, can adapt membrane fluidity, elasticity and permeability by closing the gaps created by imperfect packing of other lipid species when proteins are embedded in the membrane (Bermudez et al., 2002). Cholesterol-based cationic lipids have been used as the main lipid component of liposomes for the transport of genes (Tagami et al., 2007) and chemical drugs (Al-Jamal and Kostarelos, 2007) since they are generally less toxic than other lipids (Tomasinsig et al., 2009). Furthermore, cholesterol enables the formation of vesicles with reduced aggregation and greater stability (Liang et al., 2008). The linker bond is responsible for the chemical stability and biodegradability of a cationic lipid. The backbone in the cationic lipid acts as a connector between the hydrophobic domain and the cationic head group. The chemistry of the actual connector (linker) has most often been of the carbamate or amide variety, both of which are chemically stable and biodegradable. The connecting moieties can be designed to be ‘tunable’; i.e. they can be stable during formulation, storage, administration and initial circulation, but can be degraded rapidly at the desired site, e.g. in an endosome (Singh and Ariatti, 2006a & 2006b; Tros de Ilarduya et al., 2010). Obata and co-workers suggested that the gene expression level is influenced by the length and type of spacer

unit (Obata et al., 2009). The length of the spacer arm may not be very critical, however, the cytotoxicity and transfection potential of the cationic liposome may be influenced by the nature and length of the spacer arm (Oh and Park, 2009; Palermo et al., 2012). The fusogenic potential between liposomes and biomembranes depends on the nature of the spacer arm (i.e. length and hydrophobicity), which plays an important role in intracellular DNA delivery (Obata et al., 2009). A longer spacer segment in the cationic cytofectin will decrease the steric hindrance between hydrophilic head group and the lipophilic cholesterol anchor system which will then result in effective interaction of the cationic lipid with the nucleic acid (Singh, 1998). MSO9 and SGO4 are monovalent cationic lipids and are able to compact nucleic acids less strongly when compared to multivalent cationic lipids. However, it has been said that a high number of positive charges in the head group results in an attraction with the nucleic acid that is so strong that the release of the nucleic acid is hindered resulting in low transfection efficiency (Miller, 1998; Yang et al., 2013; Safari and Hosseinkhani, 2013).

All six cationic liposomes in this study (both PEGylated and unPEGylated) were prepared with the neutral co-lipid DOPE as a common component. Cationic lipids are often mixed with so-called helper lipids, such as dioleoylphosphatidylethanolamine (DOPE) or cholesterol; both lipids potentially promote the conversion of the lamellar lipoplex phase into a non-lamellar structure, which presumably rationalizes their ability to improve cationic lipid mediated transfection efficiency (Wasungu and Hoekstra, 2006). DOPE is believed to help in membrane fusion as well as deterioration of the cellular and endosomal membrane (Resina et al., 2009). Additionally, DOPE profoundly affects the polymorphic features of lipoplexes in that it promotes the transition from a lamellar to a hexagonal phase, and its presence causes neutralization of cationic charges by the negative charges on phosphodiester backbone of DNA (Vijayanathan et al., 2002; Srinivasan and Burgess, 2009). The presence of DOPE in lipoplexes helps in DNA release into the cytoplasm by disrupting the lipid membrane of the endosomes. This molecular parameter will thus be important for DNA dissociation and hence eventual transfection efficiency (Xu et al., 2013; Gao et al., 2011). Liposomes prepared in the absence of DOPE have been shown to have poorer rates of cell internalization and transfection (Xu et al., 1996). In addition, the incorporation of DOPE is assumed to decrease the toxicity effect of the cationic liposomes.

3.4.2. Preparation of PEGylated and Targeted Cationic Liposomes

The pre-PEGylation of the cationic liposome was achieved using a 2 mole percentage of DSPE-PEG₂₀₀₀ and reducing the percentage of DOPE in the relevant liposome preparation to maintain a total of 4 mM total lipid. Polyethylene glycol (PEG) is typically conjugated to distearoylphosphatidylethanolamine (DSPE) via a carbamate linkage that results in a net negative charge on the phosphate moiety (Mora-Huertas et al., 2010). PEG is an inert, biocompatible polymer which forms a protective layer over the liposome surface and slows down liposome recognition by opsonins and therefore subsequent clearance of liposomes (Torchilin, 2005). The repulsive characteristic feature of PEG prevents the liposomes from being aggregated and therefore favours the formation of small liposomes (Lin and Thomas, 2003).

Novel targeted liposome formulations were prepared by incorporating the targeting moiety, folic acid (0.01 μ M). This ligand was intended to specifically target the folate receptor (FR) bearing tumour cells (Figure 3.3). To attain better selectivity of PEG shielded liposomes, it is advantageous to attach the targeting ligand via a PEG spacer, so that the ligand is appended outside of the polymer, which will decrease steric hindrance when binding to the target tissues (Torchilin, 2005). The PEG spacer between the lipid and the targeting ligand may position the targeting moiety suitably and provide stealth property to the liposome (Hossen et al., 2010). The folate receptor is a 38-KDa glycosyl phosphatidylinositol GPI-anchored glycoprotein with highly restricted normal tissue distribution and amplified expression in a wide variety of human tumours, including over 95% of non-mucinous ovarian carcinomas (Tomassetti et al., 1999). Liposomes incorporating a lipophilic folate derivative, such as folate-PEG-DSPE or folate-PEG-Chol, have been shown to efficiently deliver antitumour agents into FR-bearing tumor cells via receptor-mediated endocytosis (Chiu et al., 2006; Wu et al., 2006). Targeting by folic acid has been studied by several researchers, showing the use of the ligand as a good targeting molecule for gene delivery *in vitro* and *in vivo*. Specifically, folate-targeted cationic liposomes have proven to be efficient vectors in a variety of cancer cells (Urbiola et al., 2013).

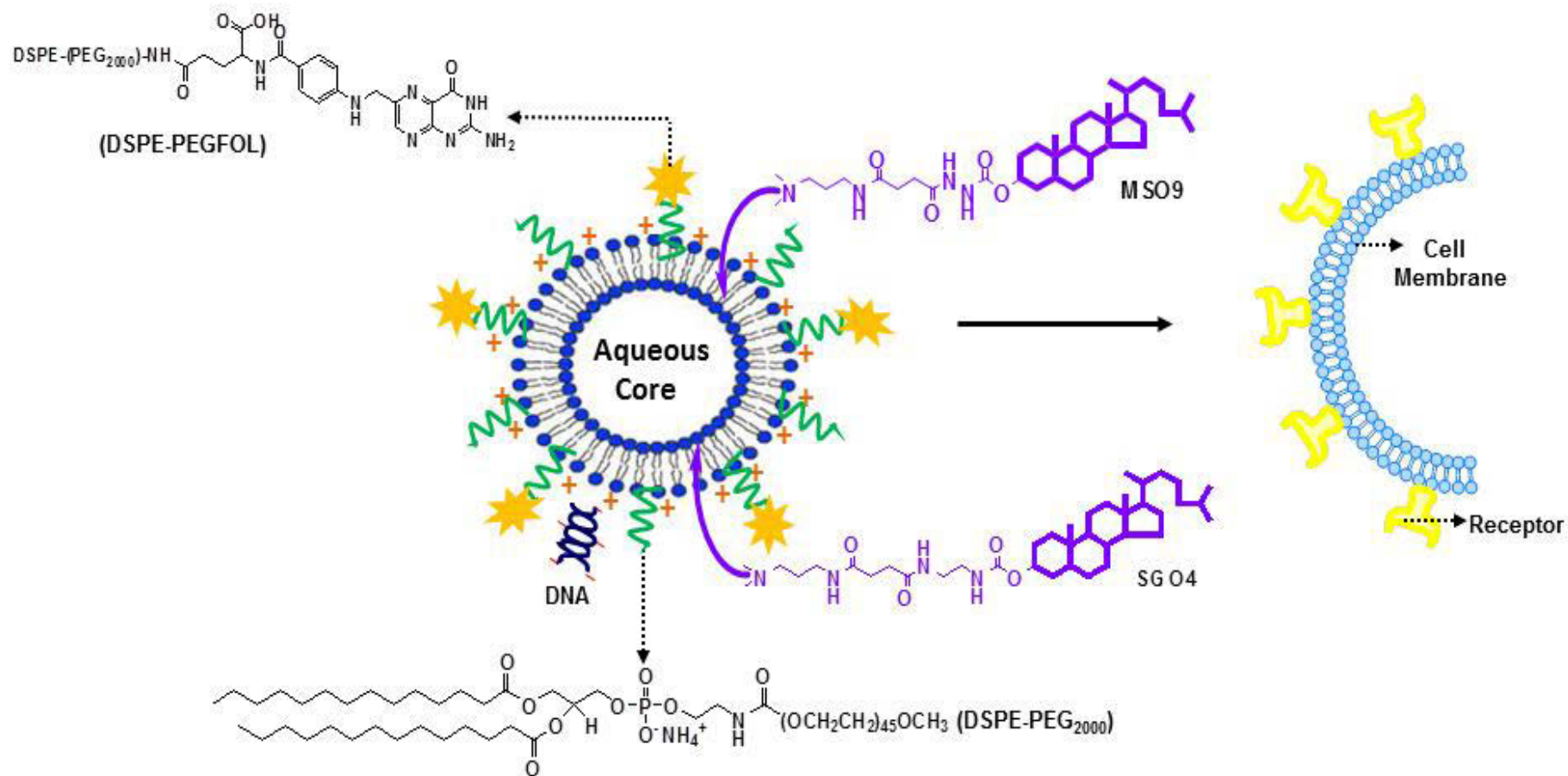


Figure 3.3: Graphic representation of the cationic liposome mediated receptor targeted gene delivery used in this study. Cationic liposome interacts with DNA to form the lipoplex via electrostatic interaction. PEGylation was established by the addition of DSPE-PEG₂₀₀₀ in the liposome formulation, targeting aspect by the conjugation of folic acid to DSPE-PEG. The gene carrying liposome enters the cell by binding to the receptors on the cell membrane. (Diagram not drawn to scale)

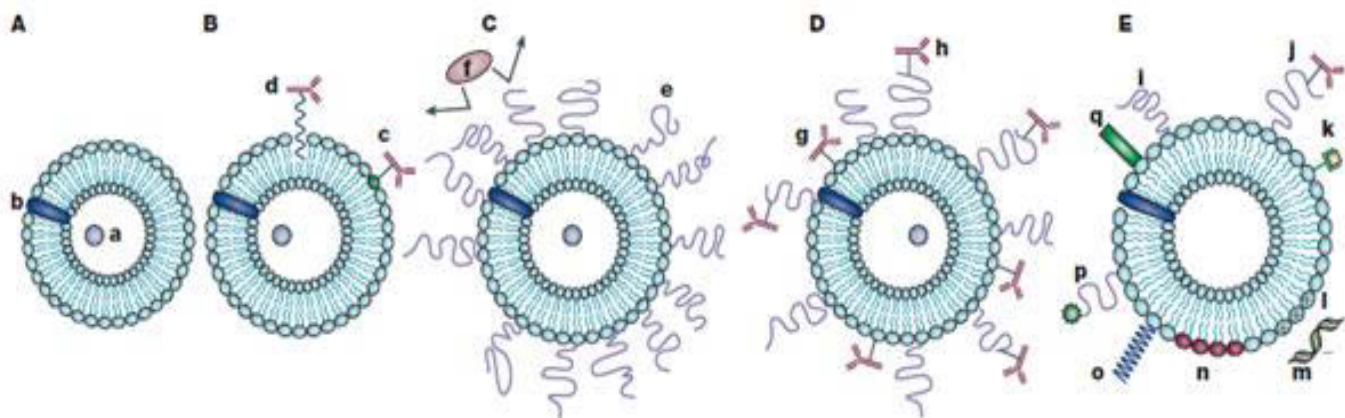


Figure 3.4: Evolution of liposomes. **A.** Early traditional phospholipids ‘plain’ liposomes with water soluble drug (a) entrapped into the aqueous liposome interior and water-insoluble drug (b) incorporated into the liposomal membrane. **B.** Antibody targeted immunoliposome with antibody covalently coupled (c) to the reactive phospholipids in the membrane, or hydrophobically anchored (d) into the liposomal membrane after preliminary modification with a hydrophobic moiety. **C.** Long circulating liposome grafted with a protective polymer (e) such as PEG, which shields the liposome surface from the interaction with opsonizing proteins (f). **D.** Long circulating immunoliposome simultaneously bearing both protective polymer and antibody, which can be attached to the liposome surface (g) or, preferably, to the distal end of the grafted polymer chain (h). **E.** New-generation liposome, the surface of which can be modified by different ways. Among these modifications are: the attachment of protective polymer (i) or protective polymer and targeting ligand, such as antibody (j); the attachment/incorporation of the diagnostic label (k); the incorporation of positively charged lipids (l) allowing for the complexation with DNA (m); the incorporation of stimuli-sensitive lipids (n); the attachment of stimuli-sensitive polymer (o); the attachment of cell-penetrating peptide (p); the incorporation of viral components (q). Adapted from Torchilin, (2005).

3.5. Formation of Liposome:DNA Complex (Lipoplex)

A lipoplex is a typical complex produced upon the electrostatic interaction of negatively charged DNA with the positively charged liposome (Figure 3.5). Lipoplexes were prepared by using varying amounts of MS09 and SGO4 cationic liposomes complexed with plasmid DNA expressing the luciferase reporter gene (pCMV-luc, 6.2 kb) to obtain a range of N/P (+/-) ratios as shown in section 2.1.6.1.

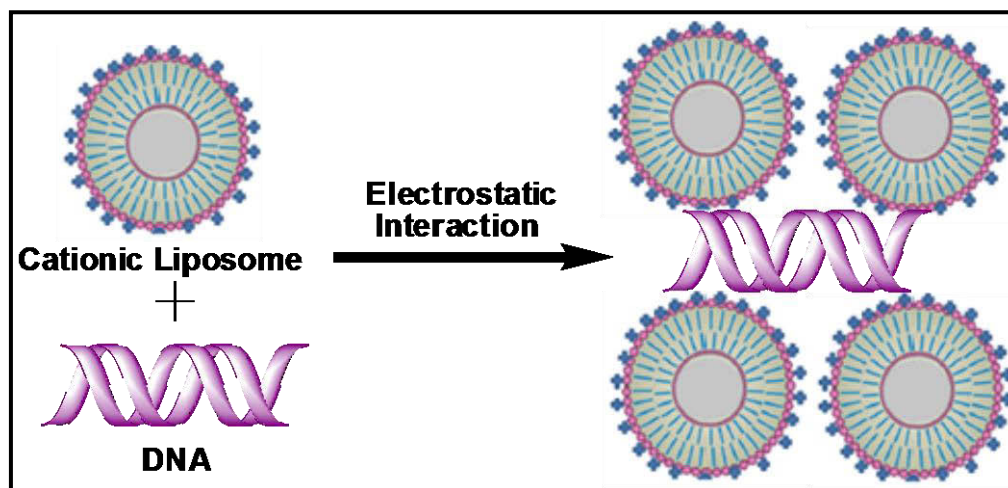


Figure 3.5: Schematic representation of liposome, DNA complex (lipoplex) formation. DNA is sandwiched between liposomes forming the liposome:DNA complex (lipoplex) via electrostatic interactions.

The phosphate group on the deoxyribose rings of DNA confers a net negative charge to the molecule, limiting the potential for electrostatic interaction with the anionic lipids in the cell membrane. Accordingly, efficient protection and transportation of DNA into cells is required as the DNA is vulnerable to enzyme degradation in serum and would not reach the cell nucleus on its own (Sakolvaree et al., 2007). Cationic lipids are highly soluble in aqueous solution, forming positively charged micellar structures termed liposomes. Intriguingly, cationic carrier molecules can complex with DNA thus neutralizing its electrostatic charge, thereby promoting cell-membrane-DNA interaction (Parker et al., 2003). It is postulated that two processes are involved in the complex formation. A fast exothermic process attributed to the electrostatic binding of DNA to the liposome surface, and a subsequent slower endothermic reaction which is likely to be caused by the fusion of the two components and their rearrangement into a new structure. During this complex forming process, the homogenous and physically stable lipoplex suspensions are formed (Zhdanov et al., 2002).

Immediately after complexation or at low DNA concentrations, multiple liposomes appear to abut with DNA sandwiched between. Different complexes emerged later, which might vary depending on charge ratio, lipid formulation and mode of preparation. Condensed lipoplexes are seen with diameters of 100 – 200 nm, and also elongated, ‘spaghetti’ shaped, lipoplexes, which are thought to represent DNA surrounded by a lipid uni and/or bilayer. Large aggregates or ‘spaghetti’ lipoplexes are also observed, and thought to comprise numerous lipid and DNA molecules (Tros de Ilarduya et al., 2010). X-ray diffraction (XRD) studies have revealed that different lipoplex structures exist. Two types of structures were observed in plain lipoplexes (Tros de Ilarduya et al., 2010; Nishiyama and Kataok, 2006; Lasic et al., 1998) viz a multilamellar structure (Figure 1.12A), with DNA monolayer sandwiched between the cationic membranes, and an inverted hexagonal structure (Nishiyama and Kataok, 2006) (Figure 1.12B), occasionally called the inverted ‘honeycomb’ phase, with DNA encapsulated within the cationic lipid monolayer tubes (Tros de ilarduya et al., 2010). The lipoplex is perceptive to the cationic to anionic (+/-) charge ratios of the complex and the sizes of the liposome and DNA (de Lima et al., 2003).

3.6. Characterization of Liposomes

3.6.1. Transmission Electron Microscopy

Transmission electron microscopy with negative staining was used to (outlined in section 2.1.6.4) investigate the morphology of cationic liposomes produced by the thin film method. The liposomes appeared to vary in shapes (Figures 3.6.1 and 3.6.2). The images showed that all liposomes (unPEGylated and PEGylated) were generally discrete and round structures (Figures 3.6.1 and 3.6.2), ranging in size from 50 to 100 nm in diameter (Table. 3.1). For convenient reading purposes, MSO9:DOPE and SGO4:DOPE liposomes will be referred as untargeted liposomes, MSO9:2%PEG and SGO4:2%PEG are as PEGylated, MSO9:2%PEG:DSPE-PEGFOL and SGO4:2%PEG:DSPE-PEGFOL will be described as targeted liposomes throughout the discussion.

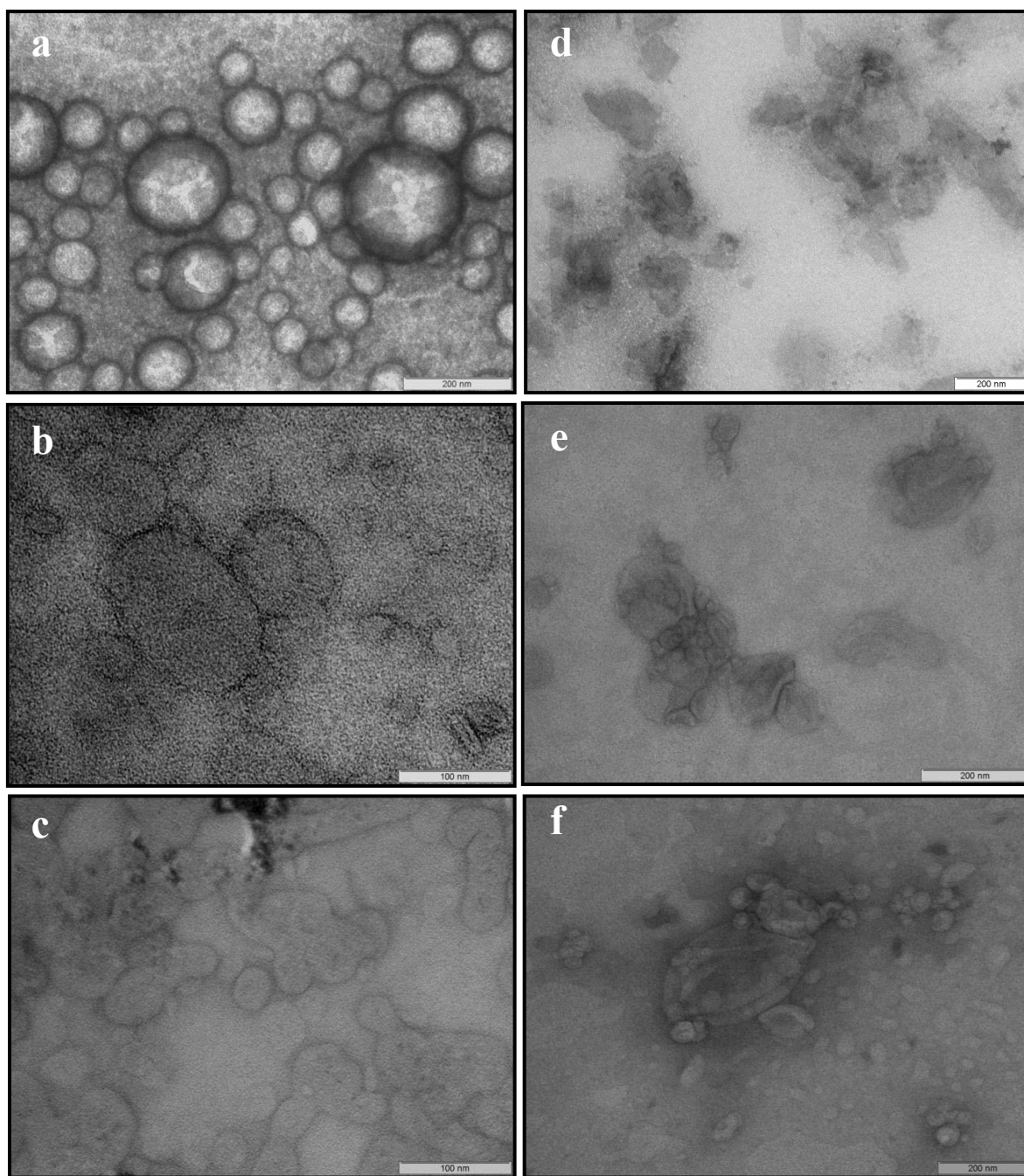


Figure 3.6.1: Transmission electron micrographs of MSO9 cationic liposome. (a,b,c) unPEGylated, PEGylated and targeted MSO9 liposomes respectively. (d,e,f) unPEGylated, PEGylated and targeted MSO9 lipoplexes respectively. Lipoplexes at their optimal gel retardation charge ratios were employed for TEM investigation. Scale bar represents 200 nm.

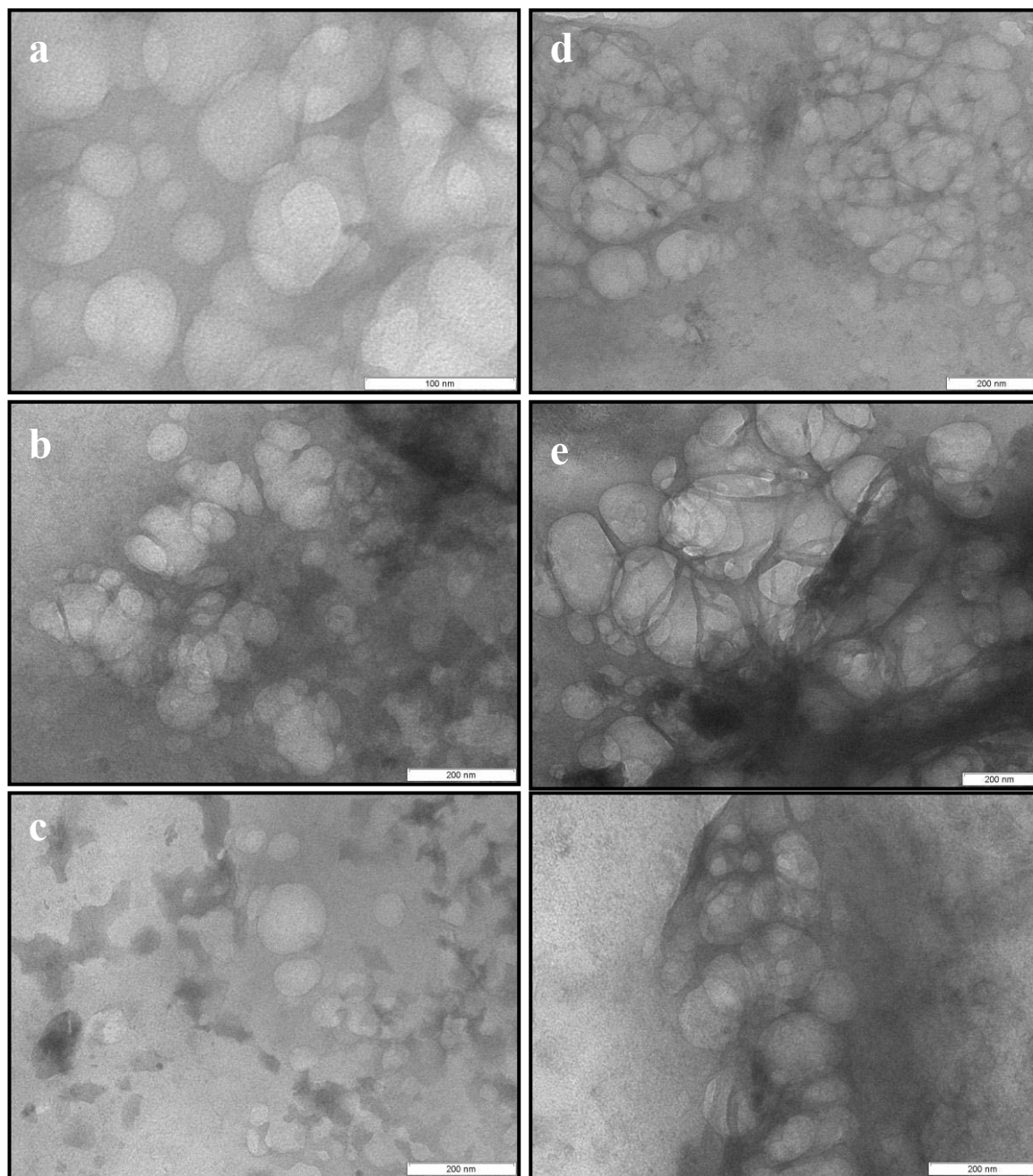


Figure 3.6.2: Transmission electron micrographs of SGO4 cationic liposome. (a,b,c) unPEGylated, PEGylated and targeted SGO4 liposomes respectively. (d,e,f) unPEGylated, PEGylated and targeted SGO4 lipoplexes respectively. Lipoplexes at their optimal gel retardation charge ratios were employed for TEM investigation. Scale bar represents 100 or 200 nm.

The average size measurements obtained (Table. 3.1) from transmission electron microscopy for MSO9 untargeted liposomes was 77.41 nm, MSO9 PEGylated was 73.83 nm and MSO9 targeted was 53.49 nm. Sizes obtained for SGO4 unPEGylated, PEGylated and targeted liposomes were 73.50 nm, 70.85 nm and 70.70 nm, respectively. From the results, it is evident that incorporation of PEG into the liposomes influenced the size of liposomes. The presence of PEG molecules at the surface of liposomes has been reported to cause disaggregation of liposome assemblies resulting in a gradual reduction of liposome size (Kaur et al., 2012). Polyethylene glycol components that are present on the liposome outer surface have a tendency to repel each other, which prevents liposomal aggregation during the preparation process resulting in the construction of smaller sized vesicles (Vermette and Meagher, 2003). Results show that at optimal lipid:DNA charge ratios (+/-), the liposomes in liposome:DNA complexes seemed more compressed than those liposomes not complexed to the pCMV-luc plasmid DNA (Figures 3.6.1-3.6.2). It is thought that the cationic lipoplexes fuse with the negatively charged plasma membrane (Kamiya et al., 2002; Duan et al., 2009) subsequently releasing the DNA in to the cytoplasm (Kamiya et al., 2002).

Table 3.1: Sizes obtained for liposomes prepared with cytofectins MSO9 and SGO4 by TEM.

Liposome Formulation	Average Size (nm) ± SD
MSO9:DOPE	77.41 ± 9.29
MSO9:DOPE:2%PEG	73.83 ± 8.12
MSO9:DOPE:2%PEG:DSPE-PEGFOL	53.49 ± 15.55
SGO4:DOPE	73.50 ± 11.24
SGO4:DOPE:2%PEG	70.85 ± 11.79
SGO4:DOPE:2%PEG:DSPE-PEGFOL	70.70 ± 13.43

All liposome vesicles used in this study showed spherical, unimodal, relatively narrow size distributions in the range of 50 –100 nm. However, no significant differences in sizes between untargeted and targeted liposomes were perceived in the transmission electron micrographs. Morphology and size distribution of all liposome and lipoplex particles were described using TEM. Additionally, size distribution, polydispersity and charge of the particles were also confirmed by zeta sizing and zeta potential parameters using the dynamic light scattering technique (DLS) and will be discussed in subsequent sections.

3.6.2. Band Shift Assay/ Gel Retardation Assay

Band shift or gel retardation assay results of both cationic liposomes and PEGylated cationic liposome preparations are shown in Figures 3.7.1 - 3.7.2. In all cases, increasing weight ratios of cationic liposomes calculated as per the nitrogen to phosphate proportions (N/P or +/-), together with a constant amount of plasmid DNA (pCMV-luc) (0.5 μ g) were used for all the gel retardation assays.

The purpose of this assay was to investigate the efficiency and effect of binding and interaction between the cationic liposomes and the DNA. The gel retardation assay is a commonly used method to evaluate the complex formation of DNA to a non-viral vector. DNA that is completely bound to the liposome would not travel into the agarose gel matrix, since the negative charges on the DNA are completely neutralized by the positive charges on liposomes. On the other hand, free DNA and DNA which is not fully complexed with cationic liposomes would easily travel in the gel towards the anode (positive charge).

The results presented in Figures 3.7.1 - 3.7.2 show the binding of DNA (-ve charge) and the cationic or PEGylated cationic liposomes (+ve charge) as a result of charge neutralization. The naked DNA in lane 1, in the absence of liposome, appears as two bands. The bottom band is the supercoiled form of the DNA while the top band is the relaxed closed circular form. As can be seen in Figures 3.7.1-3.7.2, with the increased charge ratios (N/P), the migration of free DNA into the gel was reduced or retarded. The plasmid DNA which was completely bound to the cationic liposome via electrostatic interactions did not migrate from the wells and remained as deep intensely stained bands. At complete retardation it is said that the cationic liposome and DNA form complexes which do not migrate through the agarose gel matrix when subjected to electrophoresis, hence remain in the wells (Zuber et al., 2003). These complexes (lipoplexes) are visualized in the wells due to staining the gel with ethidium bromide. Sometimes, the lipoplexes may precipitate in the wells causing them to float out of the wells thus avoiding the detection of DNA (Singh, 1998). The optimal retardation ratios of all six liposome formulations are indicated by white arrows on the gels (Figures 3.7.1 and 3.7.2).

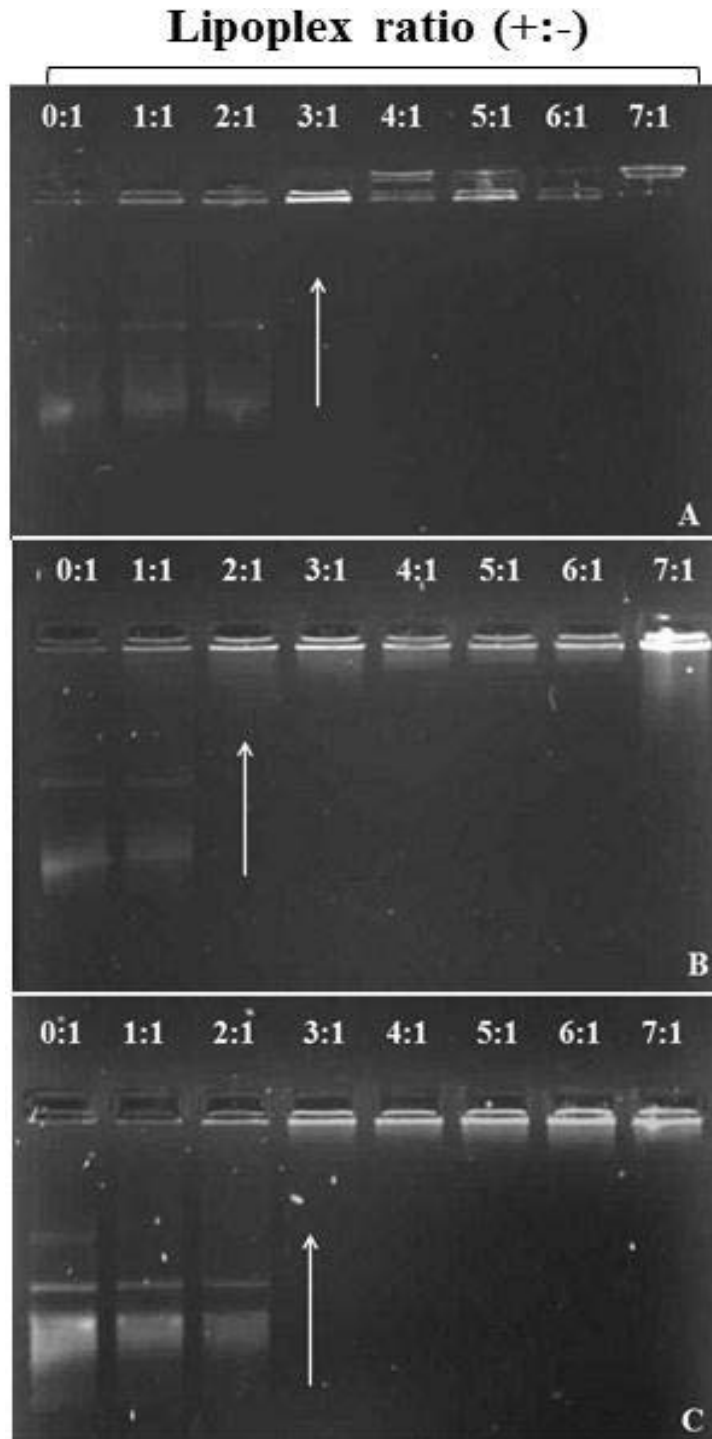


Figure 3.7.1: Gel retardation assays of MSO9. Untargeted **A.** PEGylated **B.** and targeted **C.** liposomes were complexed with pCMV-luc at various charge ratios, and then run through a 1% agarose gel. The mobility of pDNA was visualized by ethidium bromide staining. The charge ratio of liposome:DNA was 1:1, 2:1, 3:1, 4:1, 5:1, 6:1 and 7:1 (lanes 2, 3, 4, 5, 6 and 7 respectively). Lane 1, 0.5 μ g plasmid DNA. The white arrows indicate the optimal endpoint ratios in the gels.

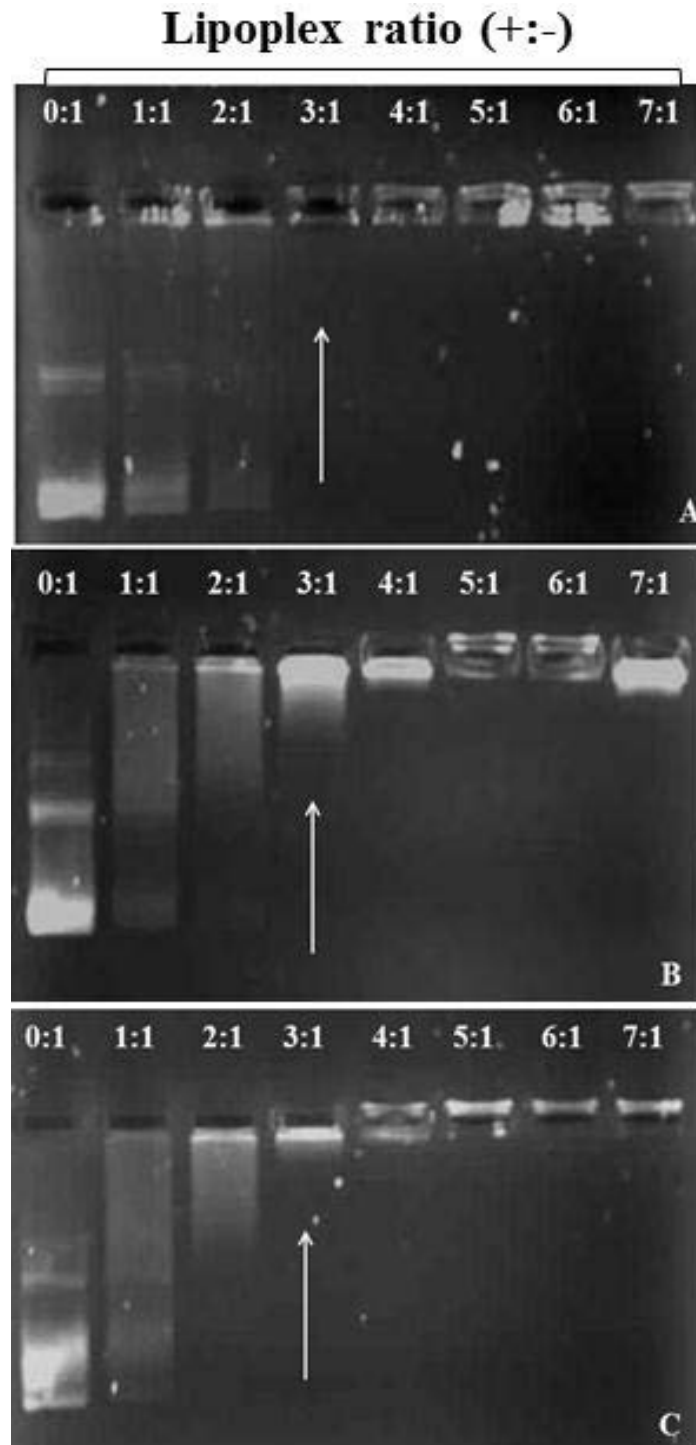


Figure 3.7.2: Gel retardation assays of SGO4. Untargeted **A**, PEGylated **B**, and targeted **C**, liposomes were complexed with pCMV-luc at various charge ratios, and then run through a 1% agarose gel. The mobility of pDNA was visualized by ethidium bromide staining. The charge ratio of liposome:DNA was 1:1, 2:1, 3:1, 4:1, 5:1, 6:1 and 7:1 (lanes 2, 3, 4, 5, 6 and 7 respectively). Lane 1, contained 0.5 μg plasmid DNA. The white arrows indicate the optimal endpoint ratios in the gels.

Agarose gel retardation results showed that all the liposomes were able to successfully bind the DNA and form lipoplexes. As can be seen from the results (Figure 3.7.1), a lower cationic liposome:DNA binding ratio was obtained with the PEGylated MSO9 liposome compared to the MSO9 untargeted and folate targeted liposomes. Complete retardations obtained for untargeted MSO9, PEGylated MSO9, folate targeted MSO9 cationic liposomes were at 6 μg , 4 μg , and 6 μg respectively with the pCMV-luc DNA kept constant (0.5 μg) to achieve these end point weight ratios. Charge ratios calculated (N/P) for these liposome formulations were 3:1, 2:1, and 3:1 respectively. From the results (Figure 3.7.1) it can be noticed that, with the increasing charge ratios the ability of the DNA to bind to the cationic liposome increased, thus providing the tight binding of the DNA in the complex. Cationic liposomes spontaneously interacted with the negatively charged DNA molecules by interaction between the cationic and anionic centers (on phosphate groups of the DNA) forming complexes in a self-assembling manner. When all the DNA negative charges are neutralized by the positive charges of the cationic liposome surface, the DNA is no longer able to migrate into the gel (Lungwitz et al., 2005; Mintzer and Simanek, 2009). As expected, a smaller charge ratio was obtained for the PEGylated MSO9 liposome due to the presence of the PEG molecule. The PEG coating can cause some adumbration of charge centres, as a consequence, the number of positive charges accessible for interaction with the DNA negative charges is reduced.

A similar trend was observed with the SGO4 cationic liposome. However, there was no difference in charge ratios was noticed between the untargeted, PEGylated and folate targeted cationic liposomes. Interestingly, the amount of liposome (6 μg) to fully bind to the 0.5 μg plasmid DNA was the same for all liposome formulations (Figure 3.7.2). The charge ratio for all three SGO4 liposomes obtained was 3:1 (N/P) and this charge ratio was considered as the optimal end point ratio. Interestingly, the PEG component in the SGO4 liposome formulation did not influence the charge ratio.

Agarose gel retardation results were taken into account to develop the complexes for cell culture studies, such as cytotoxicity and transfection in the different cell lines. Therefore, along with the optimal end point charge ratio the infra, supra and optimal liposome:DNA ratios were considered in further studies.

3.6.3. Serum Nuclease Protection Assay

DNA degradation by serum nucleases is one of the most important barriers faced by gene delivery vectors (Hashida et al., 1996). A desirable feature of any delivery vector is its ability to bind and protect the DNA from degradation by nucleases. The ability of the six liposomes prepared with cytofectins MSO9 and SGO4 to protect the DNA from enzymatic degradation was studied using the nuclease protection assay. The results obtained are presented in Figures 3.8.1-3.8.2. EDTA was added to the complexes to stop the action of the enzyme and sodium dodecyl sulphate (SDS) was used to release the bound DNA from the liposome:DNA complex. These complexes were then subjected to agarose gel electrophoresis where the unbound, negatively charged DNA will migrate into the gel during electrophoresis. Protection of DNA from nuclease digestion by cationic liposomes was confirmed by the appearance of undigested intense fluorescent bands seen in the agarose gel when compared to the naked plasmid DNA that was totally digested with the enzyme where no band was visible.

Figure 3.8.1A shows the serum nuclease protection study on MSO9 untargeted (lanes 3, 4, 5) and targeted (6, 7, 8) lipoplexes on agarose gels. Interaction of plasmid DNA with MSO9 liposome was analyzed by examining the fragmentation of uncomplexed or unprotected DNA as a result of the endonuclease activity of serum nucleases. As is evident from the results, uncomplexed or intact DNA was completely degraded by the nucleases (lane 2). In contrast this confirmed the stability of the lipoplex bound plasmid DNA in the presence of serum nucleases (lanes 3-8). The detection of intense fluorescence appeared in lanes 3-8 indicating the tight association between MSO9 untargeted and targeted liposomes and DNA. However, diffused fluorescence detected in the lanes 3-8 is attributed to the leaching of small amounts of DNA from the liposome particles or the partial DNA degradation by serum digestive enzymes. In contrast, the MSO9 liposome:DNA complexes were found to be able to partly protect the complexed DNA against serum nucleases as seen in (Figure 3.8.1A and B) where the supercoiled form of the plasmid DNA in lipoplexes had virtually disappeared. The predominant bands of MSO9 untargeted and targeted lipoplexes, in lanes 3 to 8 (Figure 3.8.1A), could be due to nicking of the supercoiled DNA to the relaxed closed circular forms. From the results it seems that the PEGylated MSO9 liposomes provided greater plasmid protection than the untargeted and targeted liposomes (Figure 3.8.1B). Supercoiled and closed circular forms of the plasmid DNA are visible from the lanes 3 to 6 which suggest that the DNA was protected by the PEGylated MSO9 liposomes.

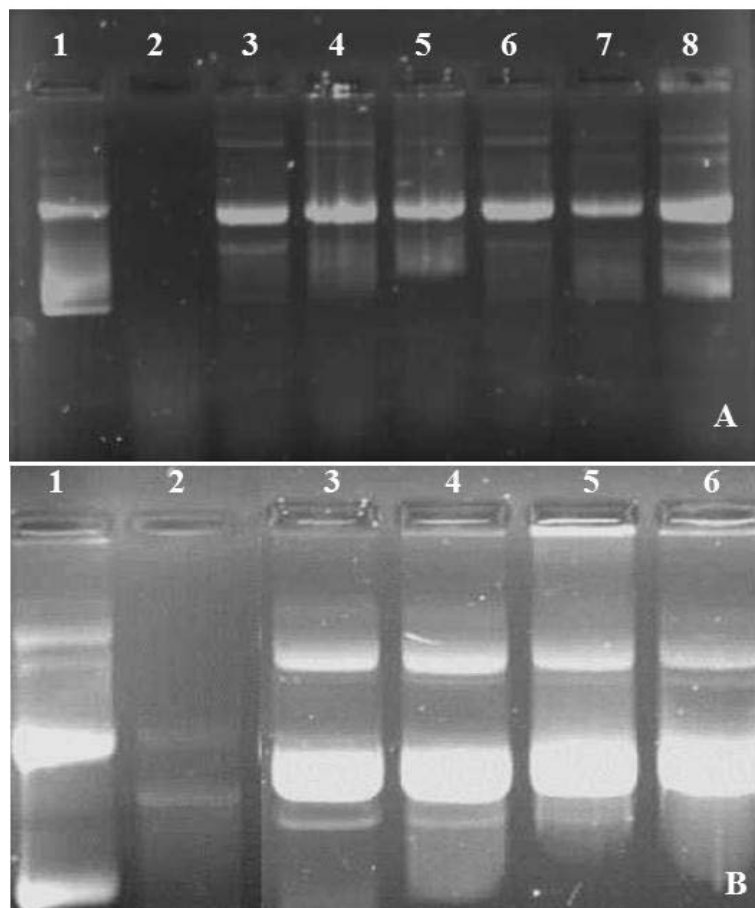


Figure 3.8.1: Serum nuclease protection assay of MSO9 liposomes. Lipoplex stability at infra to supra optimal N/P ratios were studied. **A.** MSO9 untargeted and targeted lipoplexes at 2:1, 3:1, 4:1 ratios (lanes 3, 4, 5 and lanes 6, 7, 8 respectively) **B.** MSO9 PEGylated lipoplexes at 1:1, 2:1, 3:1, 4:1 ratios (lanes 3, 4, 5, 6). In both gels Lane 1 contained free DNA (1 μ g), and Lane 2 shows digested pCMV-luc in the absence of liposome. pCMV-luc was kept at 1 μ g.

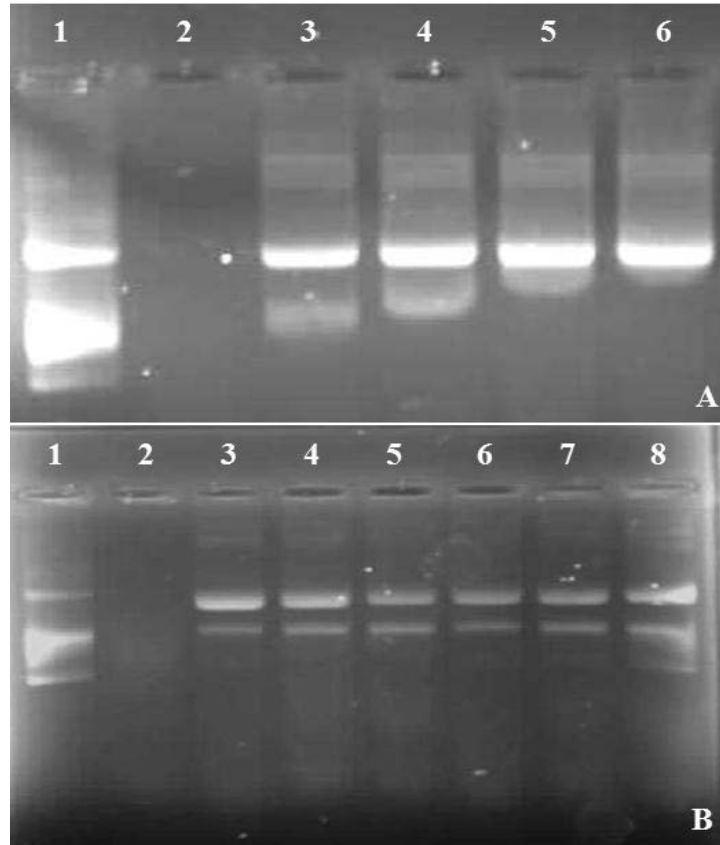


Figure 3.8.2: Serum nuclease protection assay of SGO4 liposomes. Lipoplex stability at infra to supra optimal N/P ratios were studied. **A.** SGO4 untargeted lipoplexes at 2:1, 3:1, 4:1, 5:1 ratios (lanes 3, 4, 5 and 6) **B.** SGO4 PEGylated and targeted lipoplexes at 2:1, 3:1, 4:1 ratios (lanes 3, 4, 5 and lanes 6, 7, 8 respectively). In both gels Lane 1 contains free DNA (1 μ g), and Lane 2 digested pCMV-luc in the absence of liposome. pCMV-luc was kept constant at 1 μ g.

Results presented in Figure 3.8.2 demonstrate that plasmid DNA in different SGO4 lipoplexes tested at various ratios was protected partially from nuclease degradation. Figure 3.8.2 shows the image of free plasmid DNA in the gel after the uncomplexation from SGO4 liposomes in the presence of 10% serum. The high mobility band in lane 1 (Figure 3.8.2A) was attributed to the most compact (supercoiled) form, whereas the other bands were considered to contain the non-supercoiled content in the plasmid i.e. circular, linear. The loss of the supercoiled form due to nicking was detected for the PEGylated and targeted SGO4 lipoplexes (Figure 3.8.2B, lanes 3 to 8) respectively. A significant amount of linear DNA is noticeable in the gel (Figure 3.8.2B) suggesting that the impact of nuclease attack to further degrade the plasmid in lipoplexes from relaxed closed circular to linear form. However, the supercoiled form is visible in the case of SGO4 untargeted lipoplexes (Figure 3.8.2A, lanes 3 to 6). Hence, the results suggested that plasmid DNA complexed with SGO4 liposomes was more stable than the free or intact plasmid DNA in lane 2, which was completely degraded by nucleases present in the serum. The difference in the protective effects between the cationic liposomes studied could be due to the stability, and surface modification, such as the polymer coating of the liposomal membranes.

The DNA protection efficacy was achieved by the introduction of liposome carriers and subsequent liposome:DNA complex formation. Although some plasmid DNA degradation in complexes occurred, it was nevertheless protected partly from nuclease attack through the complexation with the cationic MSO9 and SGO4 liposomes.

3.6.4. Ethidium Bromide Intercalation Assay

Ethidium bromide is an intercalating dye, which is used to study the level of DNA condensation. The higher the level of DNA condensation, the lower is the ethidium bromide uptake and therefore lower fluorescence intensity is detected. Electrostatic interaction between cationic liposomes and anionic DNA causes binding and compaction of liposome:DNA complexes. When ethidium bromide is added to a solution of DNA, it intercalates between the base pairs of the DNA double helix, emitting an intense fluorescence. This fluorescence quenched upon the formation of liposome:DNA complexes. In general, as the ratio of cationic lipid to DNA is increased, ethidium bromide fluorescence decreased, indicating that less DNA is accessible for ethidium bromide intercalation (Fedoreyeva et al., 2011; Kashanian et al., 2008). However, these decreases in fluorescence

intensity with increasing lipid:DNA ratio were found to be markedly dependent on the concentration of ethidium bromide relative to DNA (Hongtao et al., 2006).

Therefore, to assess the interaction of cationic liposomes with DNA, the fluorescence intensity of ethidium bromide for MSO9 and SGO4 lipoplexes as a function of the (cationic lipid:DNA) +/- charge ratio was investigated by the addition of increasing amounts of liposome to a fixed amount of DNA and ethidium bromide in HBS. In this study, results revealed that all liposome formulations successfully displaced the ethidium bromide in the HBS-EtBr-DNA suspension and were capable of condensing the DNA. The liposome was added in 1 μ L aliquots and the point at which the DNA is fully compacted to the liposome was established as the inflection point or plateau. Beyond this point, any further addition of the liposome suspension did not result in further DNA condensation or reduced fluorescence.

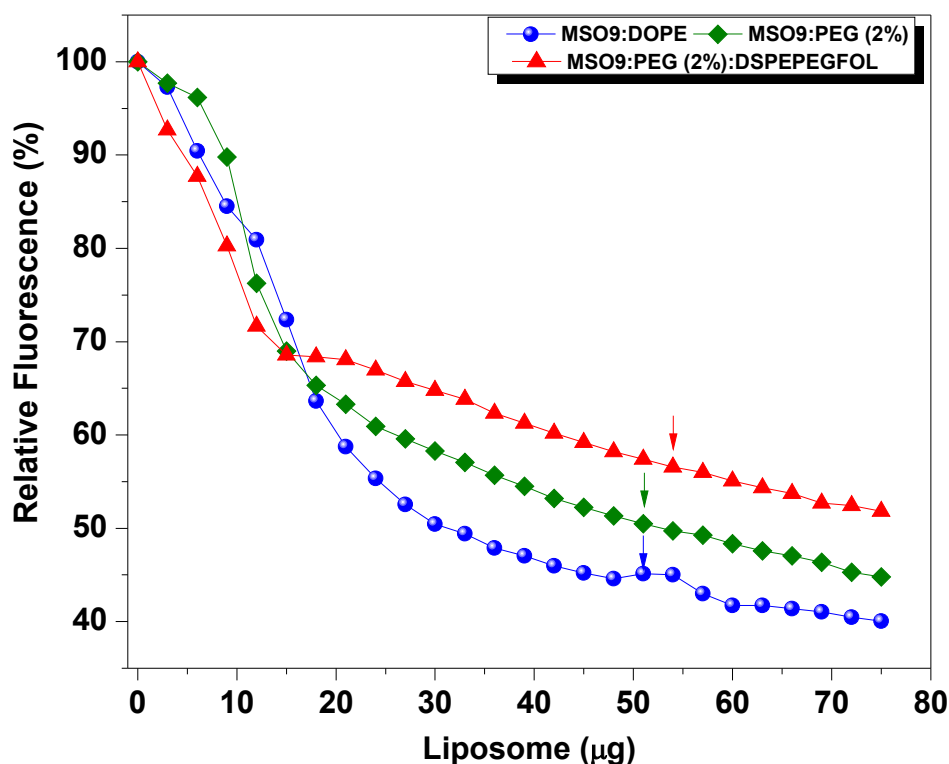


Figure 3.9.1: Ethidium bromide intercalation assay of MSO9 cationic, PEGylated cationic liposomes. The incubation mixtures contained 100 μ L (HBS), 1.2 μ g pCMV-luc DNA. Increasing amount of liposome in 1 μ L aliquots (2.8 μ g total lipid) was added to the wells containing HBS-EtBr mixtures.

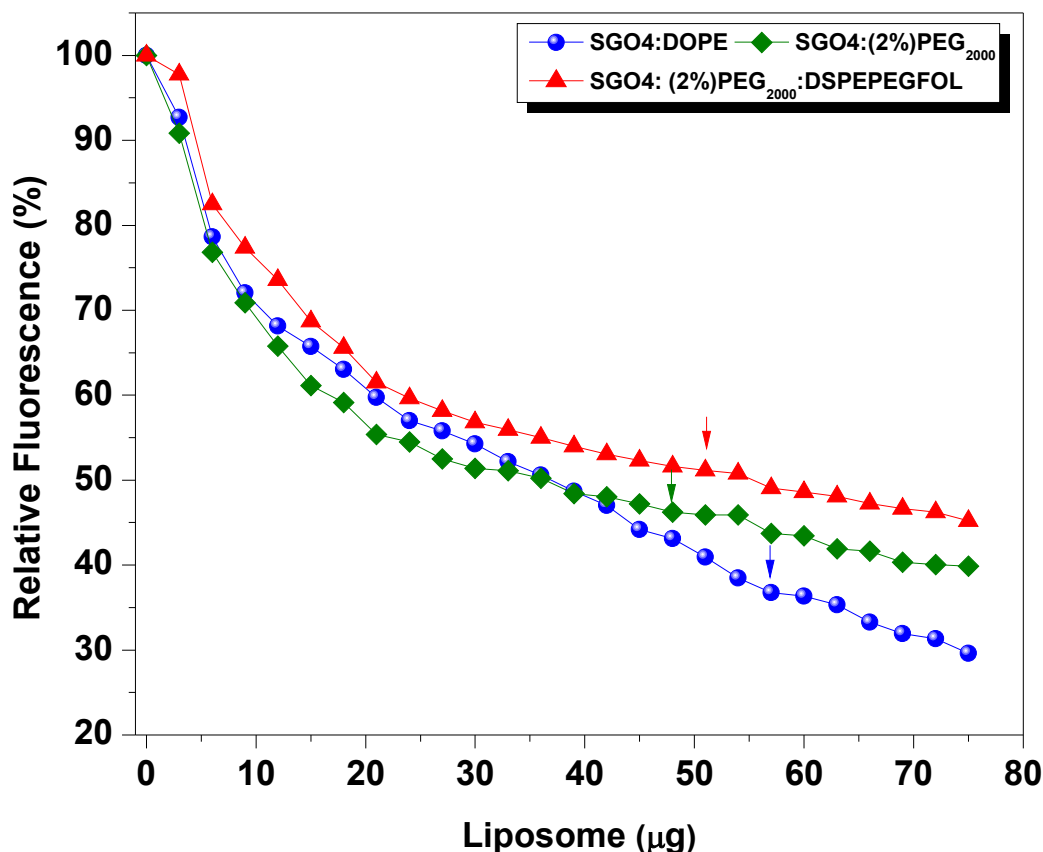


Figure 3.9.2: Ethidium bromide intercalation assay of SGO4 cationic, PEGylated cationic liposomes. The incubation mixtures contained 100 μL (HBS), 1.2 μg pCMV-luc DNA. Increasing amount of liposome in 1 μL aliquots (2.9 μg total lipid) was added to the wells containing HBS-EtBr mixtures.

The results represented in the Figure 3.9.1 showed, the gradual decrease in fluorescence with the increase in liposome concentration. UnPEGylated MSO9 liposome showed an overall fluorescence reduction or displacement of approximately 60%. PEGylated and folate targeted liposomes showed 55% and 45% of fluorescence reduction respectively. From the results (Figure 3.9.1), it can be noted that the relative fluorescence for the unPEGylated MSO9 liposome appeared slightly higher than that for the PEGylated MSO9 liposome. However, folate targeted MSO9 (also PEGylated) liposomes showed a significant decrease of percentage fluorescence when compared to both the MSO9 unPEGylated and PEGylated cationic liposomes. The charge ratios (+/-) obtained from this study for MSO9 unPEGylated, PEGylated and targeted liposomes were 2:1, 2.4:1 and 2.6:1 respectively. The quenching of the ethidium bromide fluorescence is due to plasmid DNA condensation by the liposomes causing displacement of the ethidium bromide.

The fluorescence pattern obtained for SGO4 liposomes seemed to be similar to that of the MSO9 liposomes. Ethidium displacement or fluorescence reduction percentage for untargeted SGO4 liposome was 70%. Approximately 55% fluorescence reduction was observed for PEGylated SGO4, and 50% for the folate targeted SGO4 liposome. From the results (Figure 3.9.2) it is clear that ethidium bromide dye displacement by the PEGylated liposome was reduced slightly compared to the unPEGylated SGO4 cationic liposome. This indicates a lower degree of DNA compaction or condensation and a more tenuous binding of DNA. In the case of folate targeted SGO4 liposome, it was found that a lower ethidium bromide access to DNA than the unPEGylated, PEGylated SGO4 liposome. It should be noted that the degree of compaction has a significant role in the overall transfection process. In order to be able to attain the best transfection results condensation of DNA in the liposome:DNA complex should not be too tight, as the eventual dissociation of the DNA from the liposome within the cell is a crucial aspect for successful transfection. However, on the other hand, a lower degree of DNA compaction may stimulate premature dissociation in the endosome followed by DNA degradation (Grigsby and Leong, 2010; Lechardeur et al., 2005). Liposome(+):DNA(-) charge ratios obtained at endpoints in this study for SGO4 untargeted, PEGylated and targeted liposome were 2.7:1, 2.3:1 and 2.4:1 respectively.

Ethidium bromide dye displacement results obtained are consistent with the agarose gel retardation assay results discussed in section 3.5.2. For both MSO9 and SGO4 the degree of condensation of DNA by the liposomes was highest in the untargeted lipoplexes, followed by the PEGylated and lastly the targeted liposomes. The PEGylated liposomes do show higher EtBr accessibility to pDNA, which could be ascribed to a reduction in pDNA condensation encouraged by PEG in the lipoplexes. Moreover, Zhang and co-workers (2010) suggested that PEGylation reduces the surface charge density of cationic liposomes and show a negative effect on nucleic acid binding affinity which may result in weaker pDNA binding compared to unPEGylated liposomes.

3.6.5. Size Distribution of the Cationic Liposomes

The ability of liposome:DNA complexes to enter into the cell via clathrin and caveolin-mediated endocytic pathways, which involve the development of coated vesicles and flask-shaped invaginations have been investigated and found to depend largely on particle size (Khalil et al., 2006). The sizes of liposomes and lipoplexes were measured by dynamic light scattering (DLS) at 25 °C (Figure 3.10) using a Malvern Zetasizer Nanoseries

(Malvern Instrument). Lipoplexes at their optimal binding charge ratios (+/-) were considered for the sizing analysis. It can be seen from Table. 3.2 that the profile of the size variation appears markedly different for the various complexes studied.

Table 3.2: Particle sizes of MSO9, SGO4 liposome and lipoplex assemblies investigated using zeta-sizer (Malvern zetasizer nanoseries). Lipoplex size measurements were done at the optimal charge ratios established from the gel retardation assays.

Formulation	Liposome		Lipoplex		
	Mean diameter (nm) ± SD	Poly Dispersity Index	Charge Ratio (+/-)	Mean diameter (nm) ± SD	Poly Dispersity Index
MSO9:DOPE	196 ± 2.17	0.22	3:1	695 ± 112.56	0.57
MSO9:DOPE:2%PEG	121 ± 1.62	0.23	2:1	106 ± 0.46	0.21
MSO9:DOPE:2%PEG:DSPEPEG ₂₀₀₀ FOL	168 ± 2.91	0.33	3:1	191 ± 11.63	0.47
SGO4:DOPE	89 ± 0.84	0.19	3:1	1055 ± 166.2	0.64
SGO4:DOPE:2%PEG	77 ± 0.58	0.17	3:1	195 ± 3.0	0.33
SGO4:DOPE:2%PEG:DSPEPEG ₂₀₀₀ FOL	78 ± 0.83	0.21	3:1	147 ± 0.6	0.15

Sizes obtained for untargeted, PEGylated, and folate targeted MSO9 liposomes were 196 nm, 121 nm, and 168 nm respectively. A clear reduction in size was detected for the PEGylated liposomes. It was noted that the PEGylated liposomes appeared much smaller than untargeted liposomes (Table. 3.2). The incorporation of DSPE-PEG₂₀₀₀ seemed to reduce the particle size of cationic liposomes when compared to the unPEGylated liposomes. Yet again; a significant increase is observed for the sizes of PEGylated cationic liposomes upon the addition of the ligand, folic acid in the folate targeted liposome preparation (Table. 3.2). The MSO9 untargeted liposome:DNA complex prepared at a 3:1 charge ratio had the largest size of (695) nm, compared to the MSO9 PEGylated, targeted lipoplexes which had sizes of 106 nm and, 191 nm respectively. This result suggests that large lipoplexes might form when the charge of lipoplex is neutralized, and the size of lipoplex could decrease when the charge of lipoplex increases from its neutralization point (Zhang et al., 2010). The larger liposomal particles tend to be rapidly taken up by the reticulo-endothelial system (RES) resulting in rapid clearance and a shorter half-life of the lipoplex. PEG is the structural component of lipid vesicles, which is responsible for steric stability. The size reduction observed by PEG attachment can be attributed to reduced electrostatic repulsion, thereby

inhibiting fusion of the liposomes (Kim et al., 2010). The phenomenon of instability in the liposome preparation results in an increase in particle size due to the aggregation of unstable liposomes during the preparation and/or upon storage. It is critical that liposomes have small and uniform sizes in order for them to be effective vectors (Yang et al., 2007).

The mean diameters obtained for SGO4 untargeted, PEGylated and folate targeted liposomes were 89 nm, 77 nm and 78 nm, respectively. The hydrodynamic diameter of SGO4 lipoplexes prepared in the absence or presence of PEG was 1055 nm, 195 nm and 147 nm respectively. A similar trend involving the decrease in size of liposomes and lipoplexes upon PEGylation was afforded. Lipoplex size is considered essential for influencing the entry pathway into the cell, although this may not have a direct correlation on the transfection efficiency (Gopal et al., 2011; Arangoa et al., 2003; Kim et al., 2010). Though the correlation of size with high transfection efficiency is widely debated (Dai et al., 2011), it is known that the size and heterogeneity of the lipoplex increases with increasing lipid to DNA charge ratio and depends on the condensing ability of monovalent lipids.

For both MSO9 and SGO4 untargeted lipoplexes an abrupt increase in diameter was detected (695 nm, and 1055 nm respectively) at a predetermined charge ratio, however, the sizes of the other PEGylated lipoplexes were of the size that is generally found to be suitable for cellular uptake (100-200 nm) especially *in vivo*. Moreover, the poly dispersity index is an important parameter when assessing the homogeneity of colloidal dispersions such as liposomes, particularly for ensuring predictable therapeutic release prompted by a uniform surface area available for diffusion (Pereira- Lachataigneris et al., 2006; Wang et al., 2011). A polydispersity index (PDI) of 0.1 is indicative of monodispersity. A PDI >0.1 indicates that the liposomal population was polydisperse and heterogenous. In this study, PDI was between 0.17 and 0.33, indicating that samples were polydisperse. SGO4 liposomes however were slightly less heterogenous as their PDI values were close to and less than 0.2.

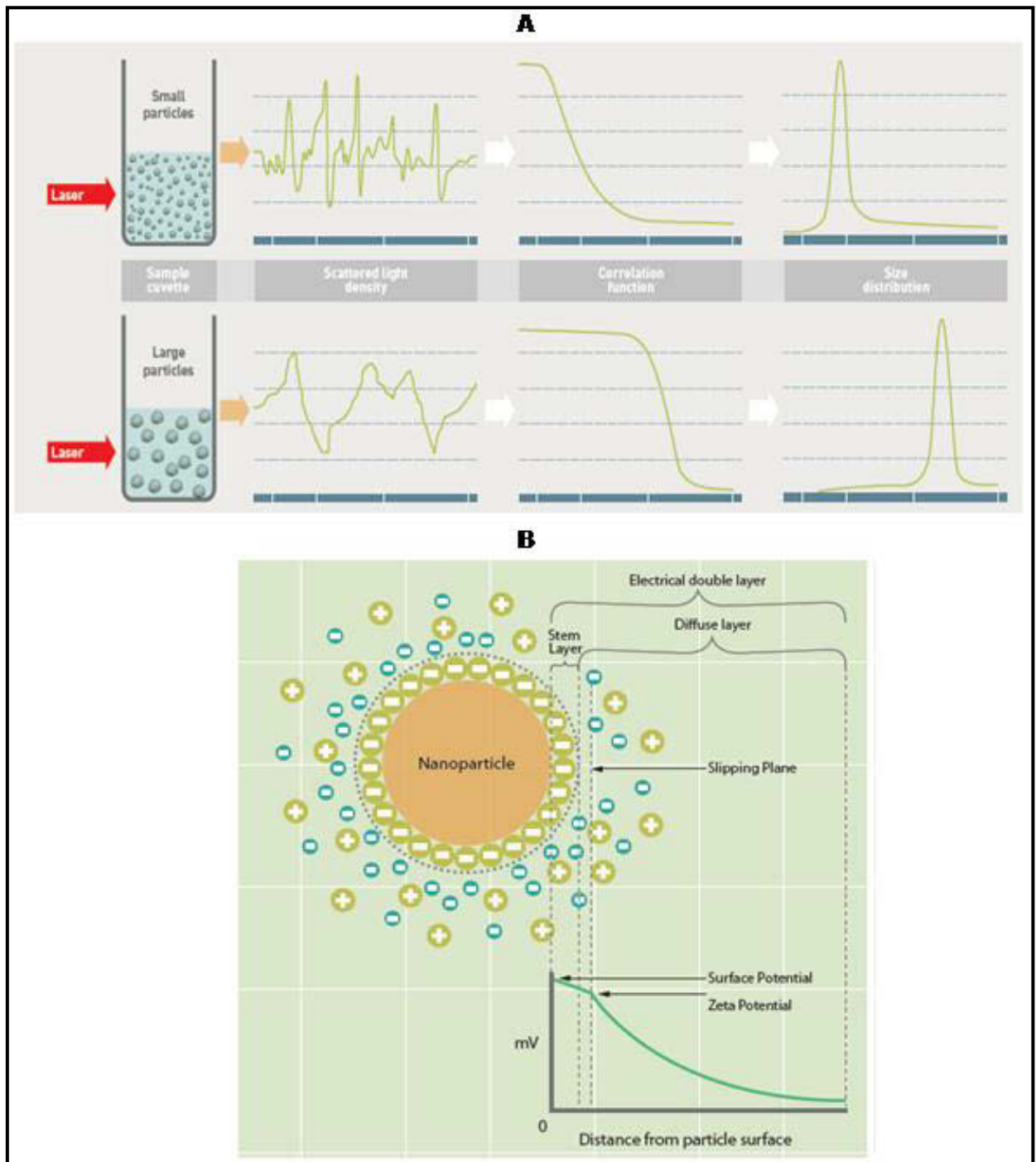


Figure 3.10: Particle sizing and zeta potential analysis using Dynamic Light Scattering technique (DLS). **A.** Particle size and distribution is measured by passing a laser beam through the sample. **B.** Surface charge (zeta potential) of particles in the sample is measured by the moment and velocity of the particles. Adapted from Malvern Material Relationships MRK1839-01.

3.6.6. Surface Charge of Cationic Liposomes using Zeta Potential Measurements

The complexation induced by mixing the negatively charged polyelectrolyte DNA and the positively charged liposome was studied by means of zeta potential measurements to detect the electro neutral points of the liposome:DNA complexes. The presence of a positive charge is essential for pDNA:cationic liposome condensation. The charge neutralization point of the anionic DNA:cationic lipid complex is considered as one of the most fundamental parameters of a lipoplex, being defined as the cationic lipid:DNA ratio at which the charge ratio of the lipoplex equals 1. In these conditions, positive charges of liposomes neutralize the negative charges of the DNA stoichiometrically. Theoretically, this charge neutralization point should be equal to 1, when the number of negative moles from DNA is equivalent to number of moles on the cationic liposome (Cuomo et al., 2012). Though, the glycocalyx in the outer cell membrane has a negative charge which is effective in the lipoplex uptake, it appears that there is no need for lipoplexes to carry positive charges to transport DNA into the cell cytoplasm (Cevher et al., 2012). In fact, the charge ratio value that corresponds to the electrostatic neutrality of the surface charge of the particle depends on various factors such as the hydrophobic forces on the nanoparticle surface which could play an important role and contribute to the charge of the ratio value at neutralization (Vijayanathan et al., 2002).

As seen in Table. 3.3, the zeta potential achieved for the untargeted MSO9 cationic liposome was 24.07 mV while PEGylated and targeted liposomes gave values of 22.23 mV and 19.33 mV respectively. Here it appears that the zeta potential value for the liposomes was reduced when the polymer PEG was introduced in the formulation. This observation may be attributed to the PEG steric shield over the surface of the cationic liposomes which could partly shield the positive charge centres. Zeta potential established for their lipoplexes were 8.07 mV, -3.37 mV and -14.31 mV respectively. A drastic decrease of surface charge in untargeted lipoplexes was noticeable (Table. 3.3). The comprehensive interaction of DNA molecules with the cationic charges present on the liposomal surface led to the complete charge neutralization of the DNA anionic forces by the liposome cationic forces. PEGylated MSO9 and targeted lipoplexes had negative zeta potential values at optimal charge ratios for DNA binding.

Table 3.3: Zeta potential (surface charge) of MSO9, SGO4 liposome and lipoplex assemblies investigated using zeta-sizer. Lipoplex size measurements were done at the optimal charge ratios established from the gel retardation assays.

Formulation	Liposome Zeta Potential (mV)	Lipoplex Charge Ratio (+/-)	Lipoplex Zeta Potential (mV)
MSO9:DOPE	24.07	3:1	8.07
MSO9:DOPE:2%PEG	22.23	2:1	-3.37
MSO9:DOPE:2%PEG:DSPEPEG₂₀₀₀FOL	19.33	3:1	-14.31
SGO4:DOPE	36.07	3:1	1.91
SGO4:DOPE:2%PEG	-1.03	3:1	-1.79
SGO4:DOPE:2%PEG:DSPEPEG₂₀₀₀FOL	-0.38	3:1	-2.09

A positive zeta potential (surface charge) value of 36.07 mV was obtained for the untargeted SGO4 liposome. In contrast, it was seen that the zeta potential for PEGylated SGO4 sharply decreased approaching the surface charge neutralization point and thereafter reversed from positive to a negative value to -1.03 mV. The surface charge of the SGO4 targeted liposome was also found to be negative with a value of -0.38 mV. It is important to note that the negative value attained for targeted SGO4 liposome was close to the electro neutral point and lower than that for the PEGylated SGO4 liposome, suggesting that incorporation of the targeting ligand did not seriously interfere with the charge, resulting in a slightly more negative zeta potential value. From the results obtained it can be said that the negative zeta potential values correspond to a population of cationic molecules, having the surface completely wrapped by the polyethylene glycol. Surface charge values attained for untargeted, PEGylated and targeted SGO4 lipoplexes were 1.91 mV, -1.79 mV, and -2.09 mV respectively. As was seen with liposomes, a trend in decreased in zeta potential value from positive to negative was noted for SGO4 lipoplexes. A comparison between the zeta potential of untargeted SGO4 liposome and the lipoplexes suggests that the decreased surface charge of the lipoplex was due to the shielding effect of the cationic charges of the liposome with anionic charges of DNA as a consequence of electrostatic interaction. It is important to note that, in the case of the targeted SGO4 lipoplexes, a combination of factors; such as the negatively charged folic acid, anionic DNA and the presence of polyethylene glycol offered

them a slight increase in the zeta potential value when compared to their liposome counterparts. These results are consistent with previous reports (Ghonaim et al., 2008).

The general trend observed for both the MSO9 and SGO4 liposomes is a decrease in zeta potential value upon polyethylene glycol grafting onto the cationic surface of liposome. The zeta potential of all cationic liposomes was reduced by the addition of DSPE-PEG.

3.7. Maintenance of Cell Lines

HEK293, HeLa and KB cells were successfully maintained in complete culture medium (EMEM + foetal bovine serum (10%) + antibiotics) throughout the study. Figure 3.11 shows the images upon successful cell propagation of cell lines utilized in this study. Cell growth initially was slow probably due to reconstitution after a long period of cryopreservation, but growth increased gradually over time. This increase in proliferation is most likely due to the increased levels of growth factor secretions by the dividing/growing cells. Once the cells reached the confluent stage, they were trypsinised and subcultured in 1:2 or 1:3 splits every 3 to 4 days, depending on the cell growth in the culture flask.

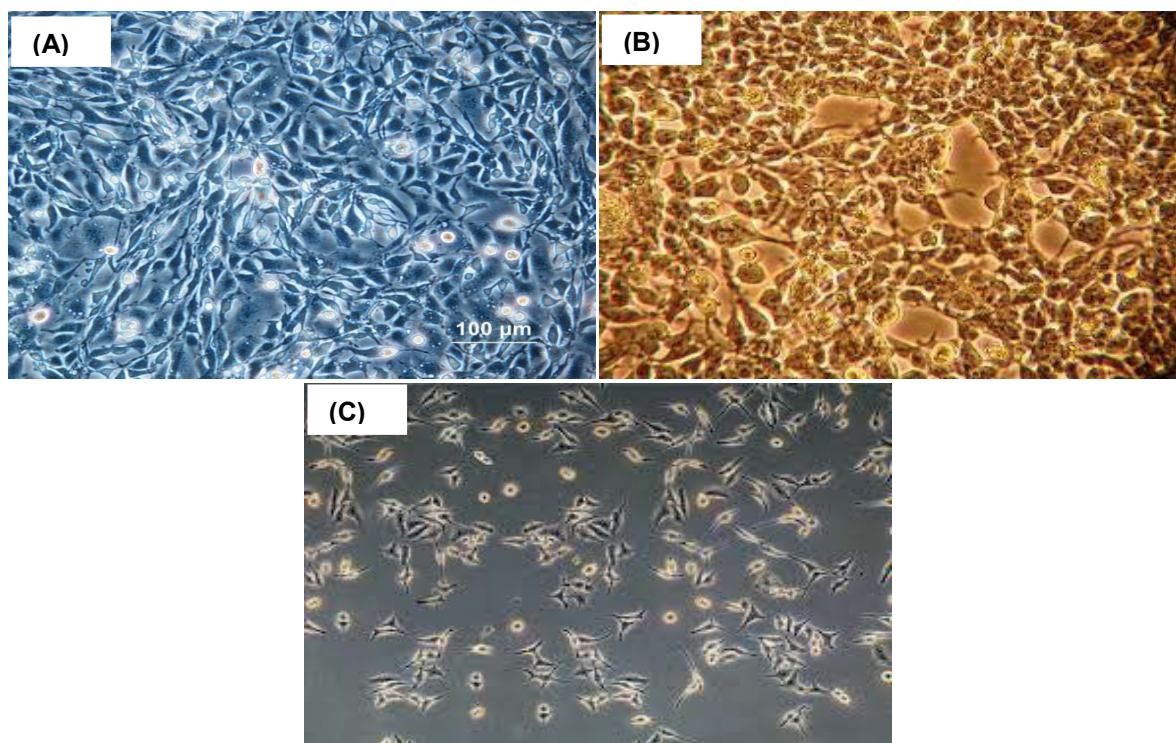


Figure 3.11: Cells viewed under inverted microscope (100 X) at semi-confluent state. **A.** KB Cells (human nasopharyngeal carcinoma), **B.** HEK293 (human embryonic kidney) cells and **C.** HeLa (human cervical carcinoma) cells.

3.8. Cell Viability Assay

The cytotoxicity of the cationic liposomes was evaluated using the MTT assay at charge ratios that encompass their infra and supra optimal retardation end points. The results were expressed as the percentage of cell viability with respect to a control corresponding to untreated cells. All the cationic formulations showed significantly lower cytotoxicity in the presence of 10% FBS. The cytotoxicity of the cationic assemblies was investigated using the HEK293, HeLa and KB cell lines. No appreciable cytotoxicity was observed; whilst over 70% of cells were found to be viable in the presence of each cationic liposome at the charge ratios considered for the study. Cells not treated with liposome:DNA complexes were considered as controls, with a cell viability of 100%.

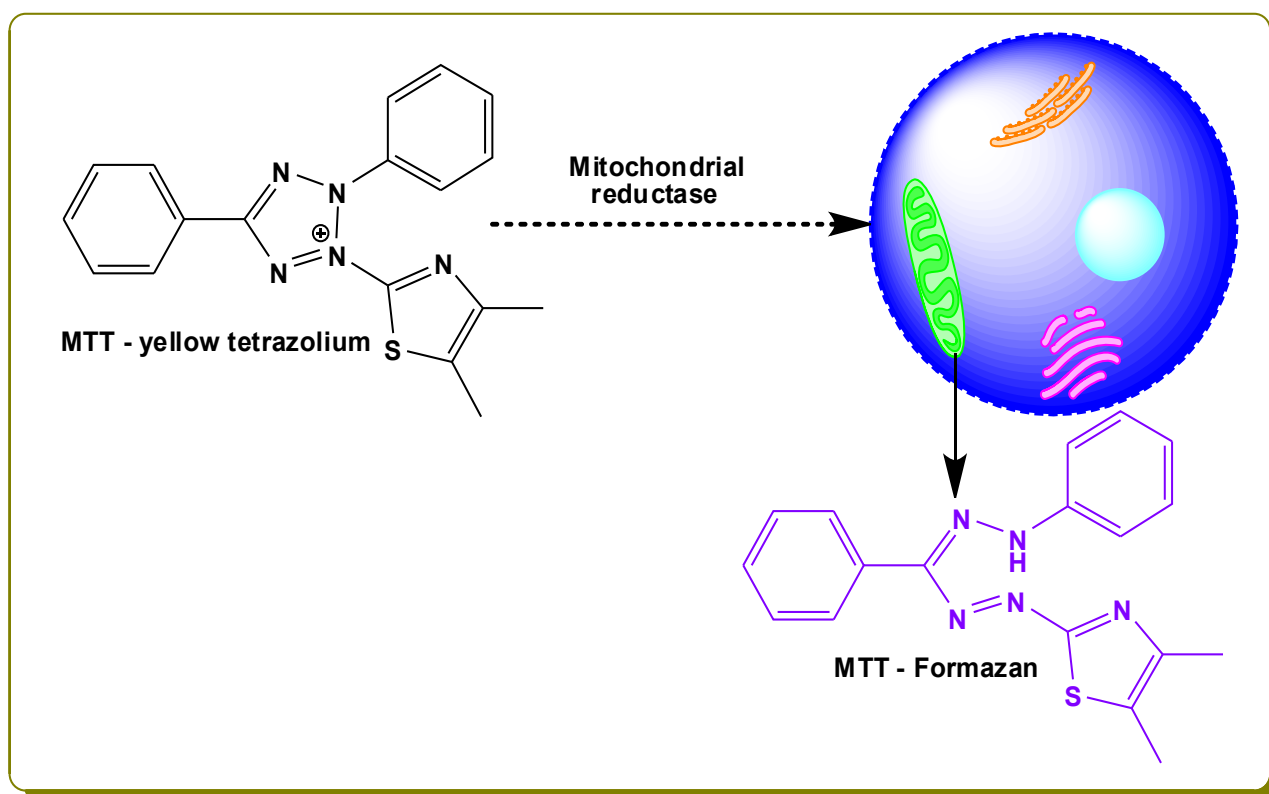


Figure 3.12: Diagrammatic representation (not drawn to scale) of MTT dye activity in mitochondria by metabolically active cells. Mitochondrial reductase converts the MTT (yellow) tetrazolium dye into MTT formazan (purple). The absorbance of this purple formazan can be measured spectrophotometrically at 570 nm.

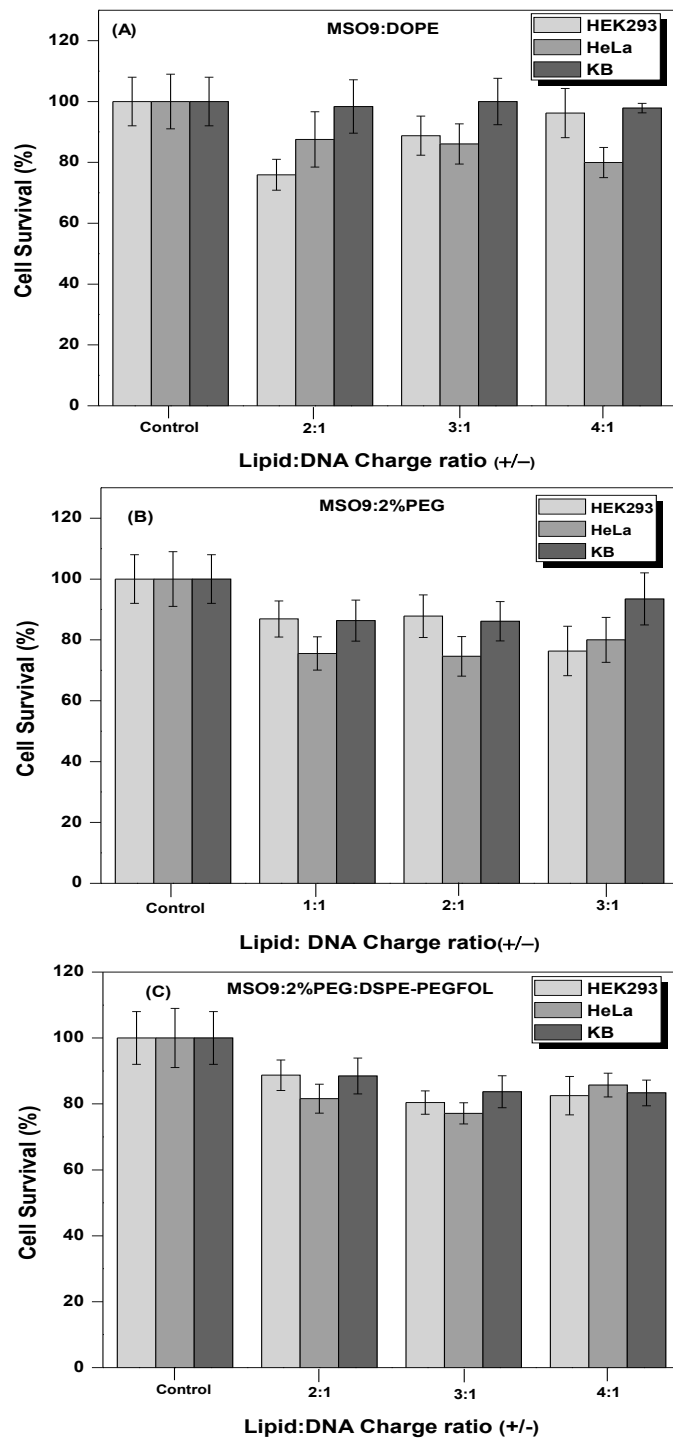


Figure 3.13.1: *In vitro* growth inhibition assay of HEK293, HeLa and KB cells with plasmid DNA complexed to MSO9 cationic liposomes. N/P ratios: **A.** MSO9 unPEGylated lipoplexes from 2:1, 3:1, 4:1 (4, 6, 8 μ g) **B.** MSO9 PEGylated lipoplexes from 1:1, 2:1, 3:1 (2, 4, 6 μ g), **C.** MSO9 targeted lipoplexes from 2:1, 3:1, 4:1 (4, 6, 8 μ g). Control: cells without liposome or DNA treatment. Cell viability was measured as a percentage relative to untreated cells. The data represent the means \pm SD (n=3).

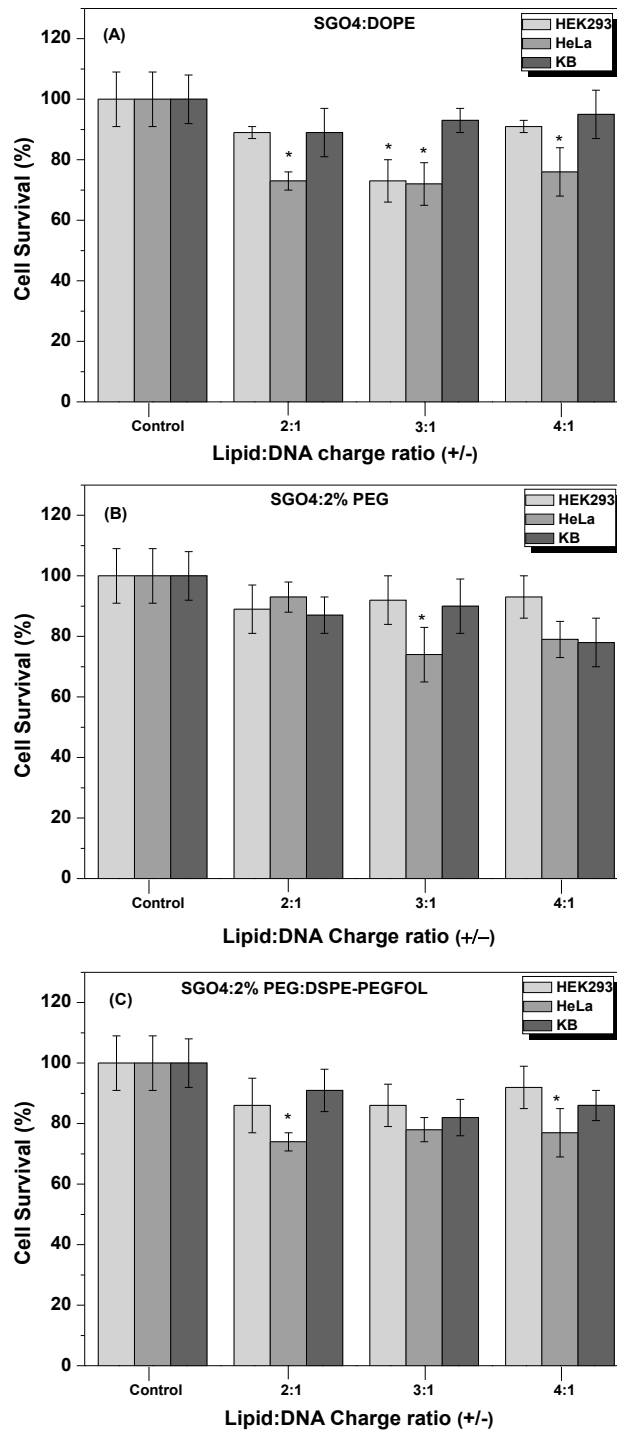


Figure 3.13.2: *In vitro* growth inhibition assay of HEK293, HeLa and KB cells with plasmid DNA complexed with SGO4 cationic liposomes. N/P ratios: **A.** SGO4 unPEGylated lipoplexes from 2:1, 3:1, 4:1 (4, 6, 8 μ g) **B.** SGO4 PEGylated lipoplexes from 2:1, 3:1, 4:1 (4, 6, 8 μ g), **C.** SGO4 targeted lipoplexes from 2:1, 3:1, 4:1 (4, 6, 8 μ g). Control: cells without liposome or DNA treatment were considered as the control. Cell viability was measured as a percentage relative to untreated cells. The data represent the means \pm SD (n=3). *p < 0.05 was considered statistically significant.

The results from this assay clearly show that the MSO9 untargeted liposomes at the three charge ratios 2:1, 3:1 and 4:1 showed minimal toxicity with 75% to 95% cell viability in all three cell lines tested (Figure 3.13.1A). MSO9 PEGylated liposomes had a 75 to 90% cell viability rate at charge ratios of 1:1, 2:1 and 3:1 (Figure 3.13.1B). In the case of the targeted MSO9 liposomes 80% to 85% of the cells were viable (Figure 3.13.1C). It can be noted that HEK293, HeLa, KB cells treated with MSO9 untargeted and PEGylated liposomes were more than 75% viable, an indication that these cationic liposomes were relatively well tolerated by the cells. A reduction in cell viability was observed with MSO9 PEGylated liposomes. It can be seen that lower levels of cytotoxicity was established for the liposomes with shorter spacer units. Generally, the hydrophobicity of the lipids, on the liposomal surface, would be expected to affect the degree of cytotoxicity since the hydrophobic material can facilitate adherence to the cellular membranes by hydrophobic interaction (Obata et al., 2009).

Figure 3.13.2A-C shows the effects of SGO4 liposomes on cell viability in HEK293, HeLa and KB cells. The results show at least 70% to 90% average cell viability (Figure 3.13.2A) for liposome:DNA complexes formulated with unPEGylated untargeted SGO4 liposomes at charge ratios of 2:1 to 4:1. For PEGylated SGO4 lipoplexes, cell viability increased slightly at charge ratios 2:1, 3:1 for all cell lines, but dropped at charge ratio 4:1 (Figure 3.13.2B). Cell viability remained high at 70 to 90% for the targeted SGO4 lipoplexes (Figure 3.13.2C) at charge ratios 2:1 to 4:1. Cationic lipids themselves have been shown to exhibit some cytotoxicity. It is, therefore, possible that the cytotoxicity exhibited by cationic liposome:DNA complexes was partly attributed to the effect of a combination of cationic lipids and the nucleic acid (Moghimi et al., 2005; Wu et al., 2007; Medvedeva et al., 2009). By contrast, no drastic reduction in cell viability was observed for the different liposome formulations. The toxicity of liposomes to cells may be related to the presence of different cationic compounds such as DOGS or DOSPA. After releasing plasmid DNA, the cationic compounds form aggregates with the cellular organelles which lead to cell death (Sarker et al., 2012; Obata et al., 2008).

3.9. Transfection

Liposome:DNA complexes are generally taken up by the process of endocytosis and transported to the lysosomes, where the complexes are degraded. The DNA must be released from the endosome before degradation and enter the nucleus where transcription takes place (Ferrari et al., 2002; Hasegawa et al., 2002). Liposomal uptake is considered to be a critical rate-limiting step and has a strong influence on the transfection efficiency. The transfection efficiency of the different cationic lipoplexes (MSO9 and SGO4) was evaluated on three transformed cell lines; HEK293 (human embryonic kidney cell line), HeLa (human cervical carcinoma), and KB (human nasopharyngeal carcinoma) cell lines at different charge ratios (+/-) as mentioned in section 2.1.9. The gene expression efficiency was quantified by determining the luciferase activity after an incubation period of 48 h using a luciferase assay system as seen in Fig. 3.14.

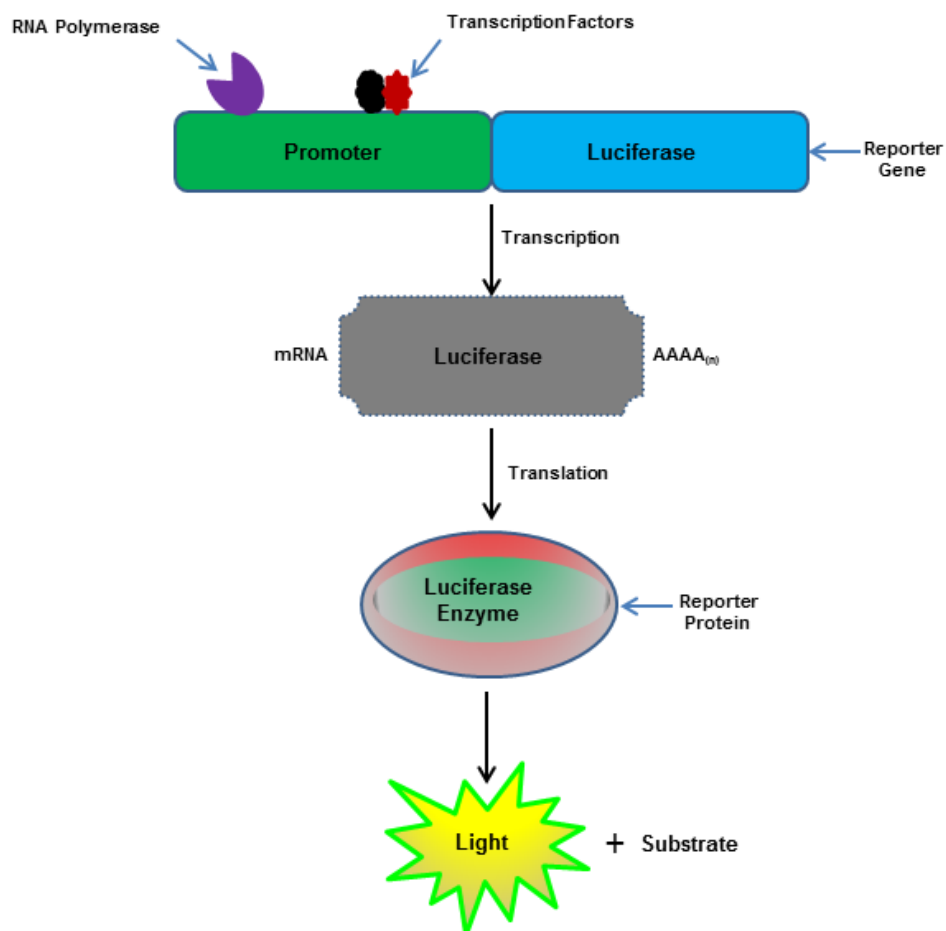


Figure 3.14: Schematic illustration of luciferase assay system used to measure the transgene expression.

Lipoplexes prepared with MSO9 untargeted liposomes at a 4:1 charge ratio exhibited elevated transfection levels whilst lipoplexes at a 2:1 ratio exhibited weaker transfection in HeLa cells. For KB cells, untargeted MSO9 lipoplexes prepared and employed at the optimal ratio (3:1) had a peak gene expression activity at charge ratios of 4:1 and 2:1 respectively (Figure 3.15.1A). On the other hand, PEGylated MSO9 lipoplexes at a 2:1 charge ratio revealed an increase in transfection activity over those with the 1:1 and 3:1 charge ratios in HeLa cells. This finding suggests that favorable lipoplex size and zeta potential values offered the complexes better transfection conditions such as suitability for cellular entry with the size <150 nm in diameter (106 nm, Table. 3.2); and low negative zeta value -3.37 mV (Table. 3.3) which is important for fusion with cellular membrane. In the case of the KB cells, PEGylated lipoplexes with 4:1 and 3:1 ratios achieved almost the same transfection levels, while low transfection was measured with the 2:1 charge ratio (Figure 3.15.1B). This suggests that although DNA association in the lipoplexes is favourable at these charge ratios, any further increase in +/- charge ratio may not afford better transfection activities since the plasmid DNA binding in the lipoplex may be too tight. This tight packing may also result in poor DNA release in cell cytoplasm from the endosome.

Figure 3.15.1C shows the transfection results obtained with MSO9 targeted lipoplexes. The transfection level in HeLa cells at a 2:1 charge ratio was greater than the transfection obtained at 3:1 and 4:1 charge ratios. The highest activity measured at a 2:1 ratio is in agreement with the EtBr displacement result, which also showed the complete plasmid DNA condensation by the liposome at this ratio. Transfection in KB cells by the FR-targeted liposomes continued to be dominant with the 3:1 optimal charge ratio similar to the untargeted assemblies. Luciferase gene expression activity attained by MSO9 targeted lipoplexes was 100 fold higher than the MSO9 untargeted lipoplexes and 10 fold higher when compared to the MSO9 untargeted PEGylated lipoplexes (Figure 3.15.1A-1C). These results suggest that the reason for the higher transfection efficiency of the MSO9 FR-targeted liposome when compared to their untargeted counterparts, is its binding to the folate receptor on the membrane via the attachment of folate ligand, and subsequent internalization of liposome:DNA complexes in the endosomes, resulting in higher efficiency of translocation of the DNA into the nucleus. These results are consistent with the EtBr intercalation assay results. Transfection levels decreased markedly for HEK293 cells for all lipoplexes.

As can be seen in Figure 3.15.2A, gene expression activity of SGO4 unPEGylated liposomes in the HeLa cell line with a charge ratio of 4:1 was significantly higher than those at charge ratios of 3:1 and 2:1 ($p < 0.05$) respectively. Gel retardation results showed that the complete binding of DNA to SGO4 untargeted liposome occurred at the charge ratio of 3:1 (+/-). Therefore, it can be demonstrated that effective DNA binding to the liposome could effectively offer improved gene expression activity. However, there seems to be no direct correlation with the lipoplex size and transfection efficiency, since it was seen that the highest transfection was achieved for the larger of SGO4 untargeted lipoplexes (1055 nm). A large lipoplex may form when the charge of lipoplex is neutralized. The non-viral vectors producing larger particle sizes with nucleic acids were reportedly effective to protect the DNA from nuclease attack, thereby facilitating improved gene transfection (Almofti et al., 2003). Mok and Cullis, (1997) reported that pDNA encapsulated in the stabilized lipid particles inhibited plasmid DNA degradation during intracellular delivery. High gene expression efficiency achieved for the same liposome composition in KB cells was at the lipid-to-DNA charge ratio of 2:1. Indeed, no significant reduction in gene expression levels was obtained with the other charge ratios of 3:1 and 4:1. Transfection activity in HEK293 cells was found to be low compared to that of the HeLa and KB cell lines. Cationic lipids form a stable complex with the fusogenic co-lipid DOPE, which has been reported to have a high tendency to form an inverted hexagonal phase (H_{II}) at acidic pH (Hafez et al., 2001). Formation of the H_{II} phase by DOPE destabilizes the endosomes, resulting in efficient escape from lysosomal degradation and cytoplasmic release of DNA (Chun-Jung et al., 1990; Caracciolo et al., 2009).

In the case of PEGylated SGO4 liposomes (Figure 3.15.2B), maximum gene transfection activity in the HeLa cells were observed at a 2:1 ($p < 0.05$) liposome:DNA charge ratio, thereafter a slight decrease was perceived for charge ratios 3:1 and 4:1 respectively. In the KB cell line, the highest transfection activity was obtained at a 3:1 charge ratio, whilst the lowest was achieved at a 2:1 charge ratio. The transfection efficiency of SGO4 PEGylated liposomes in both HeLa and KB cell lines was 10 fold greater than that of the untargeted SGO4 liposomal formulation which also displayed low activity at all charge ratios (Figure 3.15.2A). PEG derived liposomes are widely used as long-circulating liposomes (van Vlerken et al., 2007) mainly for systemic delivery of nucleic acids. PEGylation enhances the stability and half-life of nano-carriers in blood circulation by avoiding recognition and clearance by

phagocytic cells of the reticulo endothelial system (RES), therefore contributing to the high gene expression activity (Chaudhari et al., 2012; Torchilin, 2012).

In contrast, PEGylated liposomes which are slightly negatively charged (Table. 3.3), have reduced clearance rate from the circulation and are able to localize in tumours via the enhanced permeability and retention (EPR) effect. Compared to electrostatic interactions, the EPR effect depends on extravasations, which is a relatively slow process. Kim et al., (2007) reported that PEGylated lipoplexes seemed to increase transfection efficiencies even in the presence of serum when compared to liposome mediated transfection methods that lack such surface attachments. Additionally, it is thought that PEG forms a steric barrier around the lipoplexes, which stifles clearance due to reduced macrophage uptake, which may allow the liposome to overcome aggregation problems through mutually repulsive interactions between the PEG molecules. These characteristics increase bioavailability, facilitating higher transfection efficiencies due to improved tissue distribution and larger available concentrations (Barry et al., 1999).

Targeted gene delivery mediated by folate receptors can be evaluated and confirmed by comparing the transfection activities of FR-positive cell lines (HeLa, KB) with FR-negative cell lines (HEK293) (Zhou et al., 2012; Lee et al., 2006). As shown in Figure 3.15.2C, transfection levels were significantly enhanced for the SGO4 targeted-PEGylated lipoplexes ($p < 0.001$) at all charge ratios and in all cell lines. It is interesting to note that the transfection pattern of targeted SGO4 lipoplexes is similar as that of the PEGylated-untargeted SGO4 lipoplexes, nonetheless transfection activity in targeted lipoplexes was increased by 15 %. A charge ratio of 2:1 displayed the highest transfection activity in HeLa, followed by 3:1 and 4:1 respectively. High transfection levels seen in the KB cell line were obtained at charge ratios 3:1, 4:1 and 2:1 respectively. Gratton et al., (2008) indicated that particles with sizes around 150 nm can achieve high internalization levels in HeLa cells. In this study, enhanced gene expression levels were achieved in HeLa cells, with lipoplexes of approximately 150 nm in size. Transfection mediated by the SGO4 folate targeting lipoplex exhibited a 10 fold higher reporter gene activity in both HeLa and KB cell lines when compared to the SGO4 un-targeted liposomes (either unPEGylated untargeted or PEGylated SGO4 lipoplexes) (Figure 3.15.2A-C). Gene expression levels with the SGO4 targeted lipoplexes were generally high at the charge ratios tested (Figure 3.15.2C). The maximum-

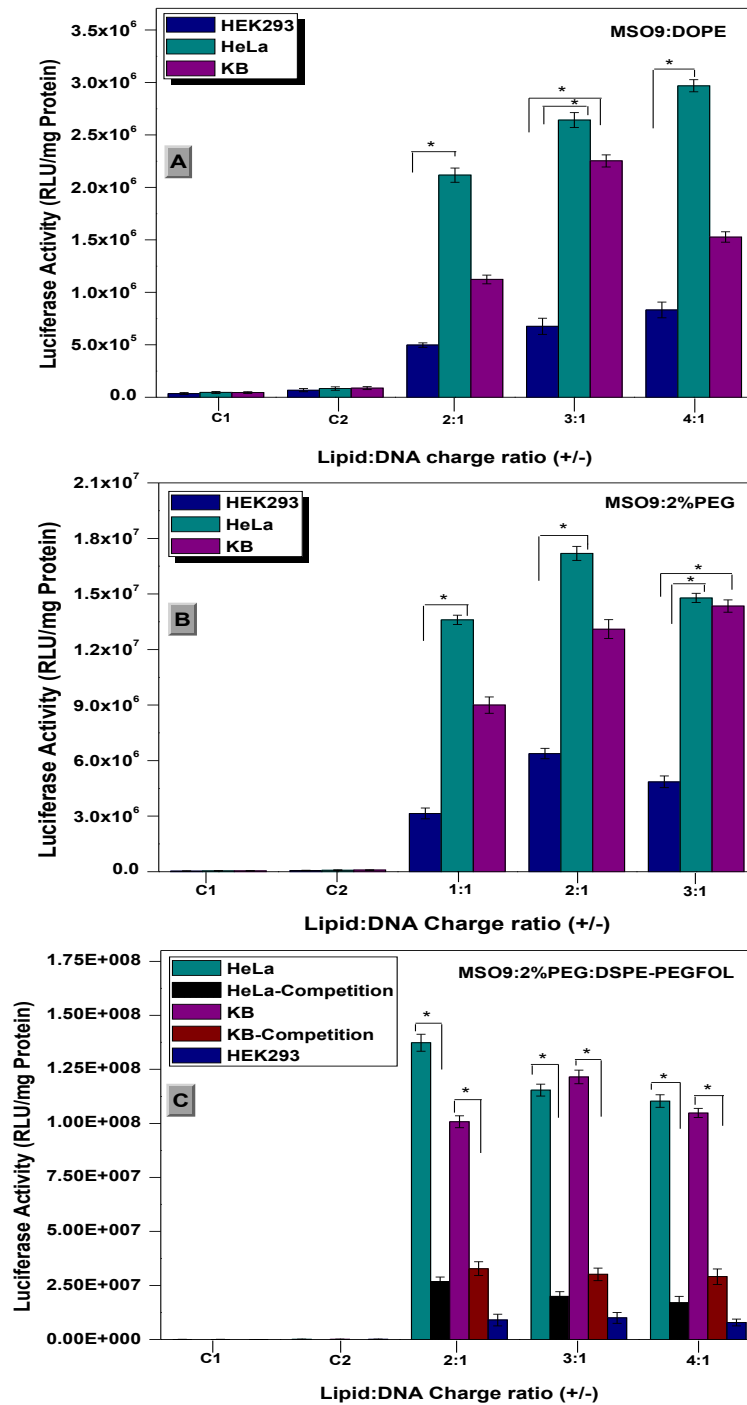


Figure 3.15.1: *In vitro* transfection activity in HEK293, HeLa and KB cells with plasmid DNA complexed with MSO9 cationic liposomes. **A.** MSO9 unPEGylated lipoplexes from 2:1, 3:1, 4:1 (4, 6, 8 μ g) **B.** MSO9 PEGylated lipoplexes from 1:1, 2:1, 3:1 (2, 4, 6 μ g), **C.** MSO9 targeted lipoplexes from 2:1, 3:1, 4:1 N/P ratios (4, 6, 8 μ g), 200 μ M excess folate was used for competition study of folate targeted liposomes. C1: cells without liposome or DNA treatment were considered as the control 1. C2: cells treated in the absence of liposome and the presence of plasmid DNA was considered as control 2. The data represent the means \pm SD (n=3). Gene expression activity was measured in RLU/mg protein. $p < 0.05$ was considered statistically significant.

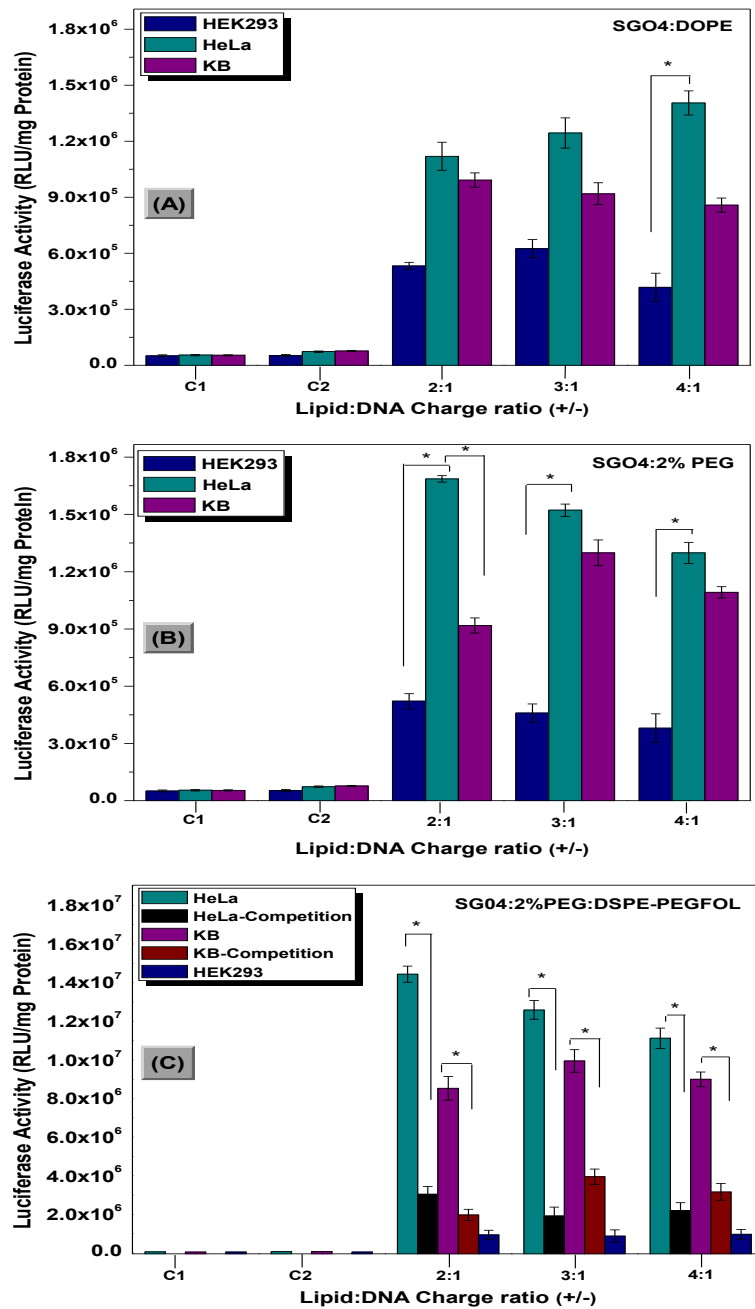


Figure 3.15.2: *In vitro* transfection activity in HEK293, HeLa and KB cells with plasmid DNA complexed with SGO4 cationic liposomes. **A.** SGO4 unPEGylated lipoplexes from 2:1, 3:1, 4:1 N/P ratios (4, 6, 8 μ g) **B.** SGO4 PEGylated lipoplexes from 2:1, 3:1, 4:1 N/P ratios (4, 6, 8 μ g), **C.** SGO4 targeted lipoplexes from 2:1, 3:1, 4:1 N/P ratios (4, 6, 8 μ g), 200 μ M excess folate was used for competition study of folate targeted liposomes. C1: cells without liposome or DNA treatment were considered as the control 1. C2: cells treated in the absence of liposome and the presence of plasmid DNA was considered as control 2. The data represent the means \pm SD (n=3). Gene expression activity was measured in RLU/mg protein. *p < 0.05 was considered statistically significant.

-gene expression activity of SGO4 FR-targeted lipoplexes compared to that mediated by SGO4 untargeted or PEGylated lipoplexes can be explained by the differences in their binding and uptake in the cells. Indeed, the surface charge of the lipoplexes and the existence of folate can influence the interaction of the lipoplexes with the cell membrane. A net positive surface charge on the SGO4 untargeted lipoplexes (Table. 3.3) permits them to engage in electrostatic interactions with the negatively-charged cell membrane, thus promoting their internalization by the cells. Conversely, FR-targeted SGO4 lipoplexes prepared at the charge ratio of 3:1 (+/-) had a negative surface charge (Table. 3.3), indicating that the enhanced effect on binding and internalization of complexes in the cells is presumably attributed to the presence of the folic acid ligand, and not on the establishment of electrostatic interactions with the anionic cell membrane. As a result of these factors (binding and uptake), the surface charge of the lipoplexes and the existence or lack of the folate moiety, the SGO4 targeted lipoplexes showed higher transfection activity than that of the untargeted SGO4, PEGylated lipoplexes (Figure 3.15.2). The transfection enhancement by FA-targeted lipoplexes is not only attributed to the receptor mediated binding and uptake of the lipoplex but also depends on other factors such as, release of DNA from endosome and its translocation into the nucleus. Overall, in all cases (Figure 3.15.2A-C) SGO4 cationic liposomes showed the highest transfection activity in HeLa cells followed by KB cells and HEK293 cells. Decreased transfection activity was noted with targeted lipoplexes for HEK293, as was expected for this folate receptor negative cell line.

Many strategies including receptor mediated endocytosis (Karmali and Chaudhuri, 2007) and ligands for extra cellular matrices (Remy et al., 1995) have been designed to increase the endosomal uptake of the lipoplexes, thus achieving higher transfection levels. Success of such therapies based on nucleic acids as drugs critically depend on their delivery efficiency. Approaches to enhance delivery of lipoplexes to specific cell types commonly employ cell specific ligands as targeting molecules. A number of small molecules such as folic acid (Müller and Schibli, 2011), transferrin (Cardoso et al., 2009, Singh et al., 2006b), haloperidol (Mukherjee et al., 2005), hyaluronic acid (Park et al., 2010) and short peptides (Mäe et al., 2009) have been successfully utilized for targeted transfection.

The results of this study indicate that both (MSO9 and SGO4) folate targeted liposomes had enhanced transfection activity compared to the non-targeted liposomes. The cytofectins MSO9 and SGO4 as mentioned earlier (section 3.1); differ in the length of their

spacer arms (11, 13 spacer arms respectively). This simple structural variation had a minor impact on the overall transfection efficiency with a 10 fold greater transfection activity noted for liposomes prepared with MSO9 than those prepared with SGO4. Moreover, TEM and zeta sizing investigations (Table. 3.1 and 3.2 respectively) revealed that the relative size measurements for the liposomes and lipoplexes containing the cytofectins MSO9 and SGO4 were not significantly affected as a result of the length of the spacer segment. Furthermore, a slight variation in charge ratios was observed for MSO9 and SGO4 lipoplexes for the gel retardation studies suggesting that these cytofectins show rather similar functionalities despite their structural difference. Therefore, it can be hypothesized from these findings that, the cationic liposome formulations (MSO9 and SGO4) grafted with a targeting ligand folate, mediated the efficient uptake of lipoplexes into the cells by means of the folate receptors. On the other hand, commercially available liposomes e.g. Lipofectamine 2000/3000, DOTAP, Fugene were not implemented for the purpose of comparison of transfection results obtained with the liposomes used in this study. This is because they are non-targeting, Turbofect™ (Naicker, 2014), FuGene 6 (Singh, 2006) and Lipofectamine™ (Singh, 2005) were tested previously in our laboratory and showed very comparable results.

3.10. Competition Assay

In order to establish that the folate is indeed mediating the binding of the ligand functionalized stealth liposomes to FR-positive cells, cells were incubated with an excess of free folic acid (200 μ M) for 20 min prior to incubating them with the liposome:DNA complexes as in section 2.2.11 for 48 h at 37 °C. The FR-positive HeLa and KB cells were used to confirm and evaluate that the targeted lipoplexes entered the cell via folate receptor-mediated endocytosis. Cell-specific targeted liposomes may achieve better treatment results than non-targeted liposomes owing to the tumour cell specific endocytosis and the rate of therapeutic (DNA/drug) escape from the endosomes (Xiong et al., 2011). As reported, the folate receptor is over expressed in several types of human cancer, such as brain, kidney, lung, and breast. The FR ligand, folate (or folic acid), is a vitamin required for nucleotide biosynthesis and elsewhere in one carbon metabolism and is utilized in elevated levels to meet the needs of proliferating tumour cells (Wang et al., 2010).

For MSO9 folate targeted lipoplexes, a decline in the transfection level was obtained for receptor positive HeLa and KB cells at all three charge ratios tested (2:1, 3:1, and 4:1) due to the fact that the receptors were blocked as a result of the initial addition of excess folic acid

(Figure 3.15.1C). The decline in transfection activity was highest in the HeLa cells (24%) compared to that of the KB cells (28%) in the targeted MSO9 liposomes. Similar results have been reported by Xiang et al., (2013) when excess folate molecules were used to compete for receptor binding on the cell surfaces. These results support the hypothesis that the targeted MSO9 lipoplexes were proficiently recognized by the folate receptors on the cell surface, which consecutively facilitated the receptor mediated endocytosis mechanism for the folate targeted components. Wang et al., (2010) also showed folate-conjugated nanoparticles were taken up selectively by mammalian cells via receptor mediated endocytosis pathway.

As can be seen in Figure 3.15.2C, the luciferase gene expression was significantly higher ($p < 0.05$) in HeLa and KB cells when transfected with targeted SGO4 lipoplexes than when the complexes were transfected in the presence of excess/free folic acid. In Figure 3.15.2C, it appeared that addition of the excess free folate inhibited the stealth liposomes from being bound to the folate receptor positive HeLa and KB cells. The transfection efficiency of targeted SGO4 lipoplexes in both HeLa and KB cells was extremely low in the presence of excess folate. The results obtained in this study suggest that free/excess folate can prevent cellular uptake of the lipoplexes by competitively binding to the folate receptors on the cell membrane. Therefore, it can be said that folate is mediating the binding of functionalized folate targeted lipoplexes to the receptor positive tumour cells.

3.11. Flow Cytometry

Intracellular uptake of optimized lipoplexes was quantitatively studied using flow cytometry. Figures 3.16.1-3.16.2 are the representative GFP fluorescence intensity histograms of FR-positive HeLa and KB cells transfected with MSO9 or SGO4 untargeted, PEGylated and targeted lipoplexes along with that of the untreated cells as control. Results revealed that all lipoplexes produced fluorescence signals. Therefore, the mean fluorescence intensities of different lipoplexes were compared in order to establish the lipoplexes efficiency in terms of transfection. The results suggested that in general, the fluorescence efficiency was higher in the folate targeted lipoplexes than those of the both types of untargeted lipoplexes (PEGylated or unPEGylated) in all cell lines which is in agreement with the transfection result obtained from the luciferase reporter gene assay.

The GFP fluorescence intensities of MSO9 lipoplexes were significant in both HeLa and KB cells compared to the controls. The order of mean fluorescence intensities observed

in HeLa cells transfected with various MSO9 lipoplexes was as follows: PEGylated-targeted > PEGylated-untargeted > untargeted-unPEGylated lipoplexes with the mean fluorescence intensity of 40354, 33172 and 32369 respectively. It is apparent from Figure. 3.16.1 that the cell uptake of targeted lipoplexes was higher compared to the untargeted lipoplexes. This enforces the idea of the targeted lipoplex being receptor dependent, which is not the case for untargeted lipoplexes as they lack the receptor specificity. In KB cells, the MSO9 lipoplexes showed moderate to stronger fluorescence signals depending on the type of formulation. GFP mean fluorescence intensities noted for the MSO9 untargeted, PEGylated and targeted lipoplexes were 29258, 31056, 32071 respectively. This result of increased fluorescence intensity of the targeted lipoplexes clearly points to the specificity and FR-mediated uptake. Glycosaminoglycans (GAGs) or mucopolysaccharides serve as the primary receptors of many viral vectors, polyplexes and lipoplexes serve as non-viral vectors (Payne et al., 2007). Generally, these early carrier-cell surface GAGs interactions were rather dependent on common surface physico-chemical properties of the complexes, markedly, for instance their surface charges. These early interactions promote the initial binding of the complexes and further cellular internalizations. Subsequent cell uptake (whether caveolin clathrin dependent or other pathways) can be improved in target cells by using specific ligands (for instance folate) through receptor mediated endocytosis (Russell-Jones et al., 2004; Darvishi et al., 2013). Tang and co-workers (2014) noted the increased cell uptake efficiencies at increased FA densities in HeLa cells, and postulated that it may due to the FR-mediated internalization process (Tang et al., 2014). Thus, the results of this study demonstrate the main internalization mechanism of the MSO9 targeted lipoplex is reliable to a FR-facilitated endocytosis pathway, specific to FR-expressing cells such as HeLa and KB cells.

Figure. 3.16.2 shows the mean fluorescence intensities of SGO4 lipoplexes in HeLa (upper panel) and KB cells (lower panel). As illustrated in the figures, fluorescence intensities were significant for the SGO4 targeted lipoplexes in the FR-overexpressed HeLa and KB cells in comparison to SGO4 untargeted lipoplexes, irrespective whether they were PEGylated or unPEGylated. In the HeLa cells, the mean GFP fluorescence intensities of SGO4 lipoplexes were 35284, 37584, and 40678 respectively for untargeted-unPEGylated, untargeted-PEGylated and targeted lipoplexes respectively. In KB cells, uptake of lipoplexes by means of fluorescence intensity was respectively of 22959, 21407 and 24207 for the untargeted, PEGylated and targeted lipoplexes. In both cell lines, the targeted lipoplexes

produced greater fluorescence intensity within the gated cell population area which remained constant for all liposome formulations. This is also supported by the luciferase assay result, and it can be reasonably concluded that the efficient gene delivery mediated by the FA-targeted lipoplexes was a result from the synergistic effect of PEG improved transfection and the FR-targeted delivery. Literature has suggested that folate functionalized liposomal uptake will proceed by receptor-mediated endocytosis in FR-bearing tumour cells; more so, folate targeted liposomes have faster clearance compared to non-targeted liposomes probably due to direct liposome uptake via the liver FR (Gabizon et al., 2004). The fluorescence observed in control untreated cells was credited to the cells auto fluorescence.

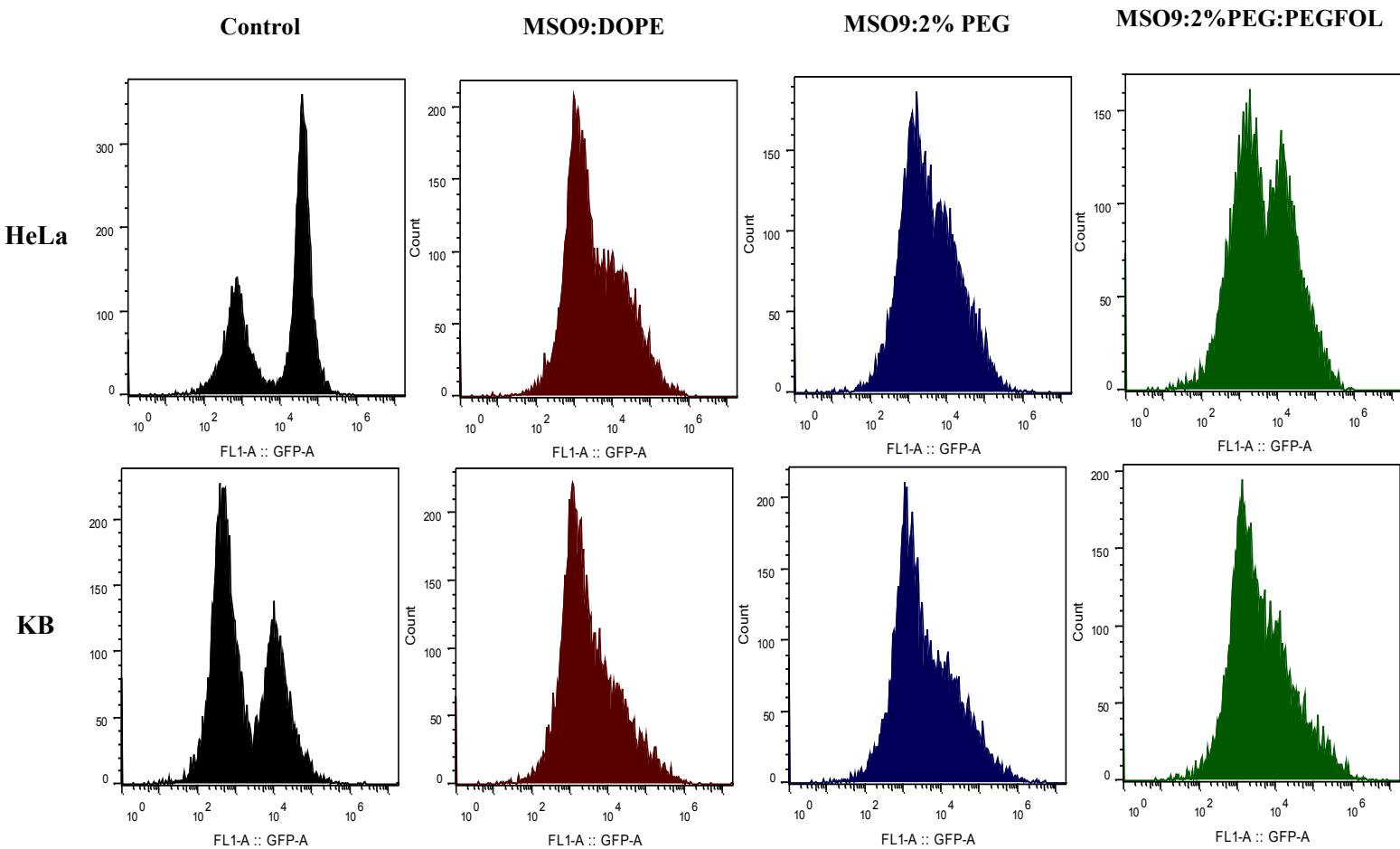


Figure 3.16.1: Quantitative cell uptake analysis of MSO9 complexes by flow cytometry. Fluorescence intensities of untreated cells, untargeted, PEGylated and targeted MSO9 lipoplexes respectively in HeLa (upper row images) and KB (lower row images) cells. Results of the flow cytometry are shown in histograms with the X-axis indicating the mean fluorescence intensity and Y-axis indicating the cell count.

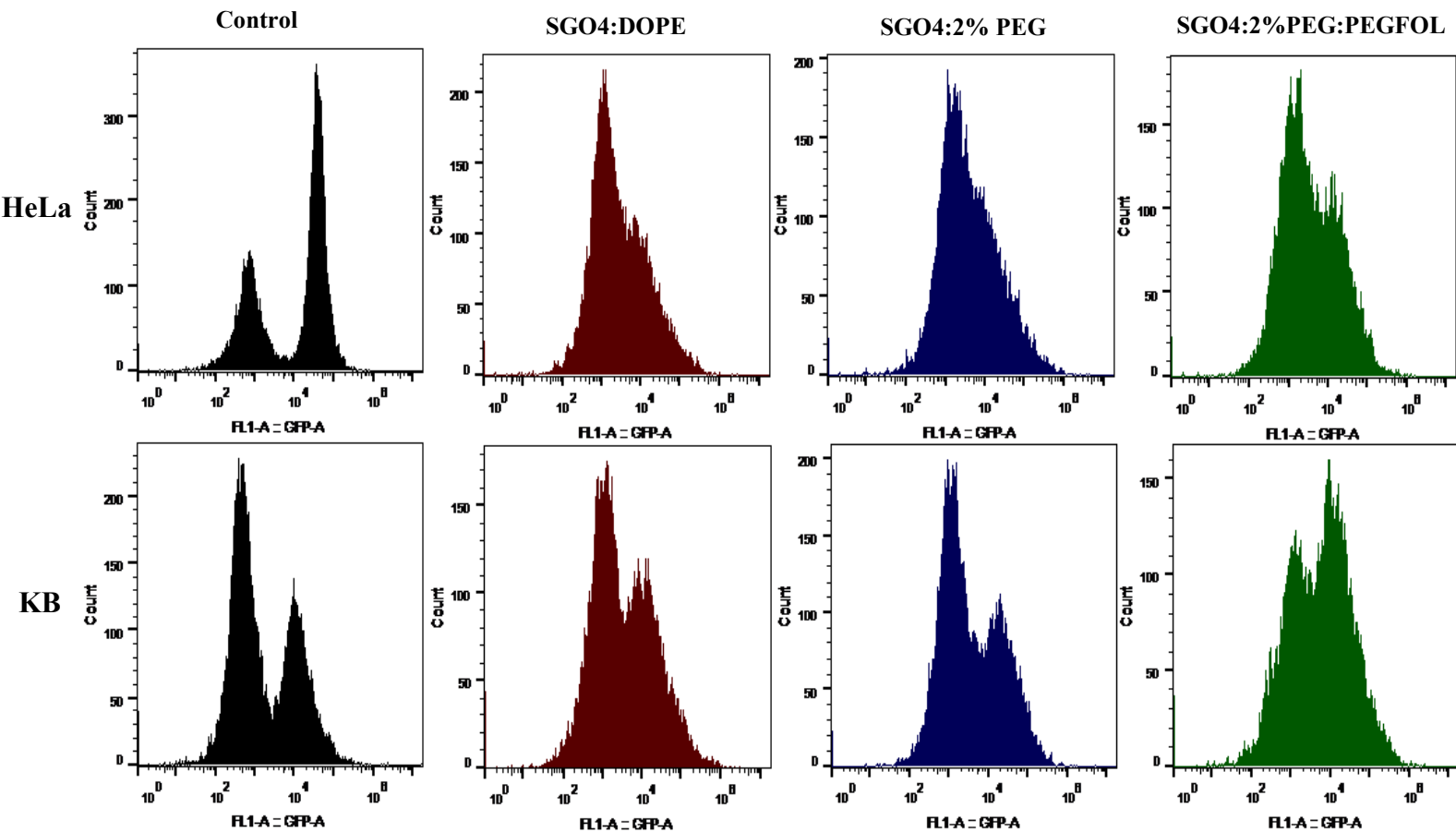


Figure 3.16.2: Quantitative cell uptake analysis of SGO4 complexes by flow cytometry. Fluorescence intensities of untreated cells, untargeted, PEGylated and targeted SGO4 lipoplexes respectively in HeLa (upper row images) and KB (lower row images) cells. Results of the flow cytometry are shown in histograms with the X-axis indicating the mean fluorescence intensity and Y-axis indicating the cell count.

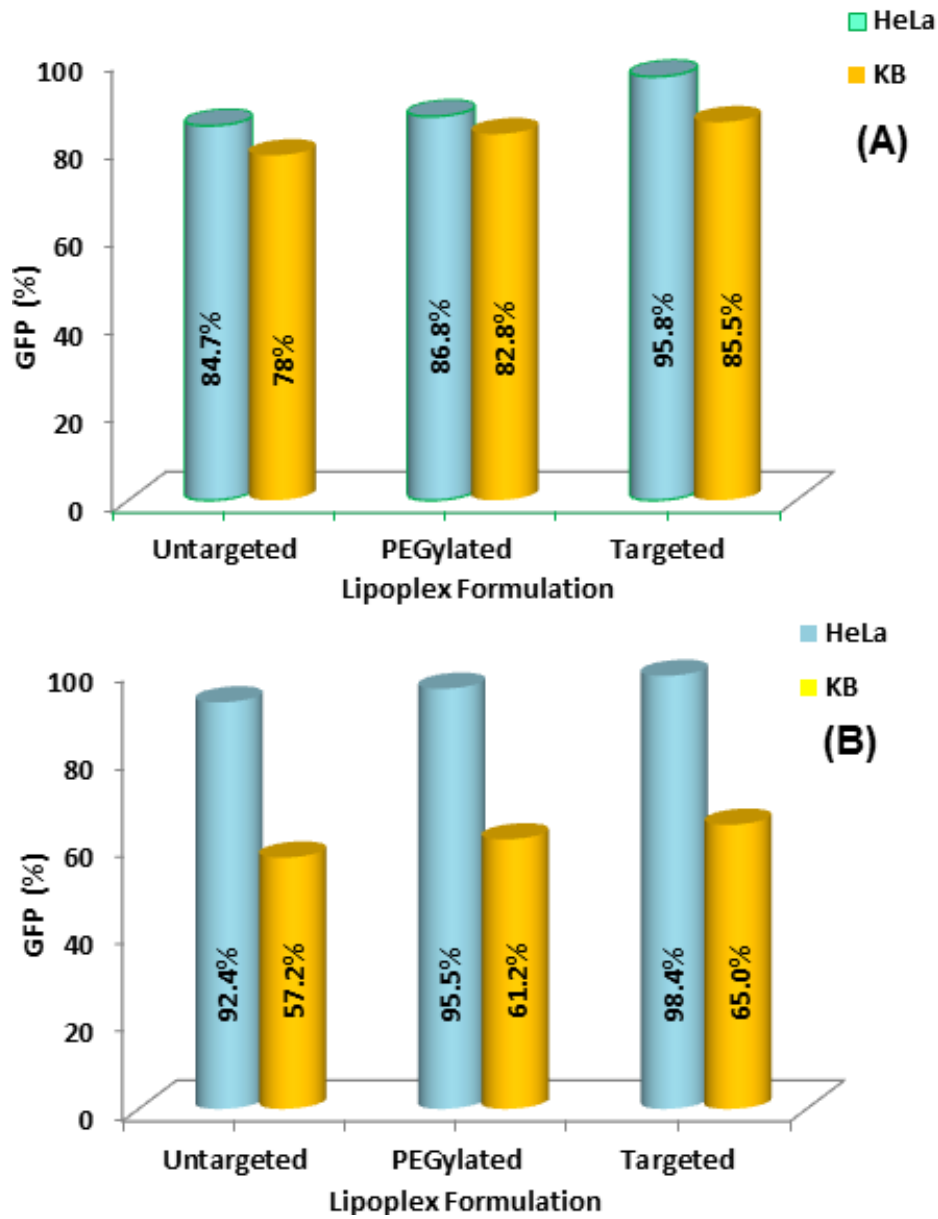


Figure 3.16.3: GFP fluorescence intensity of lipoplexes in HeLa and KB cells. Transfection of (A) MSO9 untargeted, PEGylated and targeted lipoplexes, and SGO4 (B) untargeted, PEGylated and targeted lipoplexes respectively at ratios at optimum transfection activity seen in the luciferase assay.

Figure 3.16.3 demonstrates the transfection efficiency of MSO9 and SGO4 lipoplexes in terms of percentage range. It can be seen clearly from the results that either MSO9 or SGO4 targeted lipoplexes indicated enhanced cell uptake when compared to the untargeted, unPEGylated lipoplexes which is in agreement with the flow cytometry histograms where the targeted lipoplexes exhibited broader fluorescence distribution.

At the optimal transfection ratios of the different MSO9 or SGO4 lipoplexes in each of two cell lines utilized for study, FA-functionalized lipoplexes displayed higher fluorescence intensity than the non-functionalized lipoplexes, corroborating the idea that the FA-lipoplexes are targeted to HeLa and KB cells via FR-mediated endocytosis. These results indicate that FA-targeted MSO9 or SGO4 liposomes can be used to target FR-positive tumour cells to deliver therapeutics of interest. The general transfection efficiency and FR-specificity of these liposome formulations justifies their potential for *in vivo* use, where active targeting together with passive targeting would play a role in tissue accumulation, though the latter will be the main mechanism for non-targeted liposomes.

CHAPTER FOUR

CONCLUSION

Gene therapy exemplifies a pioneering approach for the treatment of certain human diseases, and is anticipated to have a great impact in the field of medicine in the future. Hence, for this purpose safe and effective gene delivery vectors need to be developed. Due to several drawbacks associated with viral vectors with regards to safety, the non-viral vectors are emerging as alternatives for therapeutic applications. However, some non-viral vectors undesirably lack the functional complexity to overcome several barriers that impede successful gene transfer. Efforts to design the essential functionalities into non-viral vectors resulted in modest triumphs, revealing major limitations in our current understanding of non-viral vectors (Zhang et al., 2012). Cationic vectors (polymers, liposomes etc) have been the subject of intensive investigations for researchers in recent years to understand the parameters governing the efficiency of transfection (Jean-Michel et al., 2014). The understanding of mechanisms, like formation of complexes to the intracellular delivery, has led to the design of a plethora of novel non-viral vector systems for gene therapy applications.

In the current study, cationic liposomes, targeted and untargeted (PEGylated and unPEGylated) were successfully formulated with mono-cationic head group containing cytofectins MSO9 and SGO4 and the fusogenic co-lipid DOPE through the thin film hydration-sonication method. The PEGylated and targeted liposomal formulations encompassed a constant amount of two mole percent amino-DSPE-PEG₂₀₀₀ to sustain the expedient properties of PEG. Morphological and physicochemical characterization of all the liposome preparations by cryo-TEM, and dynamic light scattering techniques revealed unilamellar, nano-scaled particles with liposomes appearing as sphere-shaped vesicles and lipoplexes as spherical knots. Size distribution as determined by cryo-TEM and dynamic light scattering were in unison. Successful lipoplex formation with pCMV-luc DNA was confirmed by agarose gel retardation studies, and ethidium bromide dye displacement assay which further confirmed the liposomal ability to condense the plasmid DNA. Furthermore, serum nuclease protection assays showed that the cationic liposomes partially protected the plasmid DNA within the lipoplexes from nuclease degradation. Zeta potential measurements served to further confirm charge neutralization in lipoplex formulations.

Complexes prepared with the MSO9 and SGO4 liposome formulations and pCMV-luc DNA were shown to exhibit moderate cytotoxicity levels in HEK293, HeLa and KB cell lines at selected concentrations chosen for gene transfection studies. This luciferase reporter gene assay showed that transgene activity achieved with PEGylated cationic liposomes was tenfold higher than that obtained with the unPEGylated control liposomes for both MSO9 and SGO4 preparations in the HeLa, and KB cells. Further investigation is required to validate this increased gene expression efficiency by PEGylated liposomes and to establish optimum PEG concentrations to achieve even better transfection efficiencies.

The targeting aspect by the biological ligand, folic acid was successfully established to promote liposome-mediated DNA delivery into target tumour cells. Cationic liposomes formulated with the folate targeting ligand in this study were shown to specifically bind and interact with the folate receptor bearing tumour cells (HeLa and KB) and successfully increase the transfection efficiency by 100 fold compared to untargeted formulations. Furthermore, folate-tagged PEGylated liposomes in the presence of either MSO9 or SGO4 cytofectins also showed higher transfection levels compared to their untargeted counterparts. Receptor-mediated transfection by targeted liposomes was confirmed using competition experiments with free folic acid, where the transfection activity decreased significantly, while flow cytometry revealed effective internalization of folate targeted lipoplexes. All these findings confirm cell-specific uptake of these targeted cationic liposomes via the receptor-mediated endocytosis mechanism.

From the results obtained in this study we can propose that these novel liposomal suspensions have the potential to be promising clinical gene delivery vectors. Further optimization and investigation of the system *in vivo* is warranted, given the observed lipoplex stability, integrity of the cargo DNA, high folate receptor specification and favorable particle size for extravasation into tumour masses. Nonetheless, further derivatisation of the formulations may be needed to reduce non-specific uptake and increase gene expression efficiency. In summary, these novel targeted cationic liposome formulations were able to successfully bind and deliver plasmid DNA to tumour cells *in vitro* primarily via the process of folate receptor mediation. This type of tissue-specific targeting interaction may have broad implications for the development of efficient targeted gene delivery systems. Further studies involve optimization of these delivery vehicles for *in vivo* studies.

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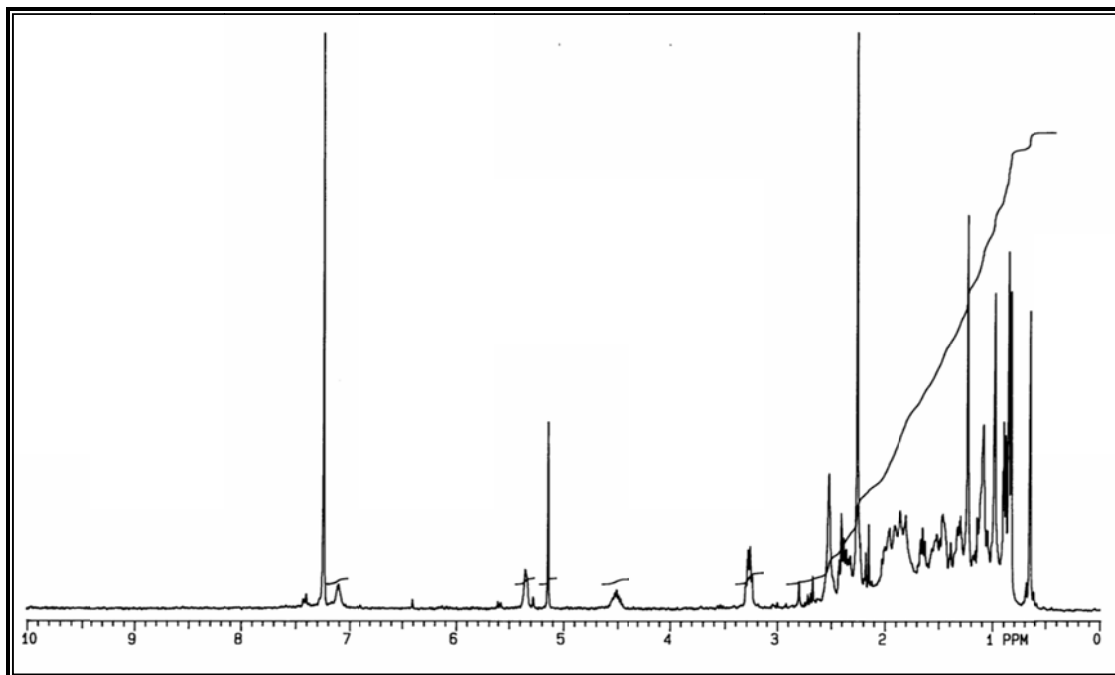
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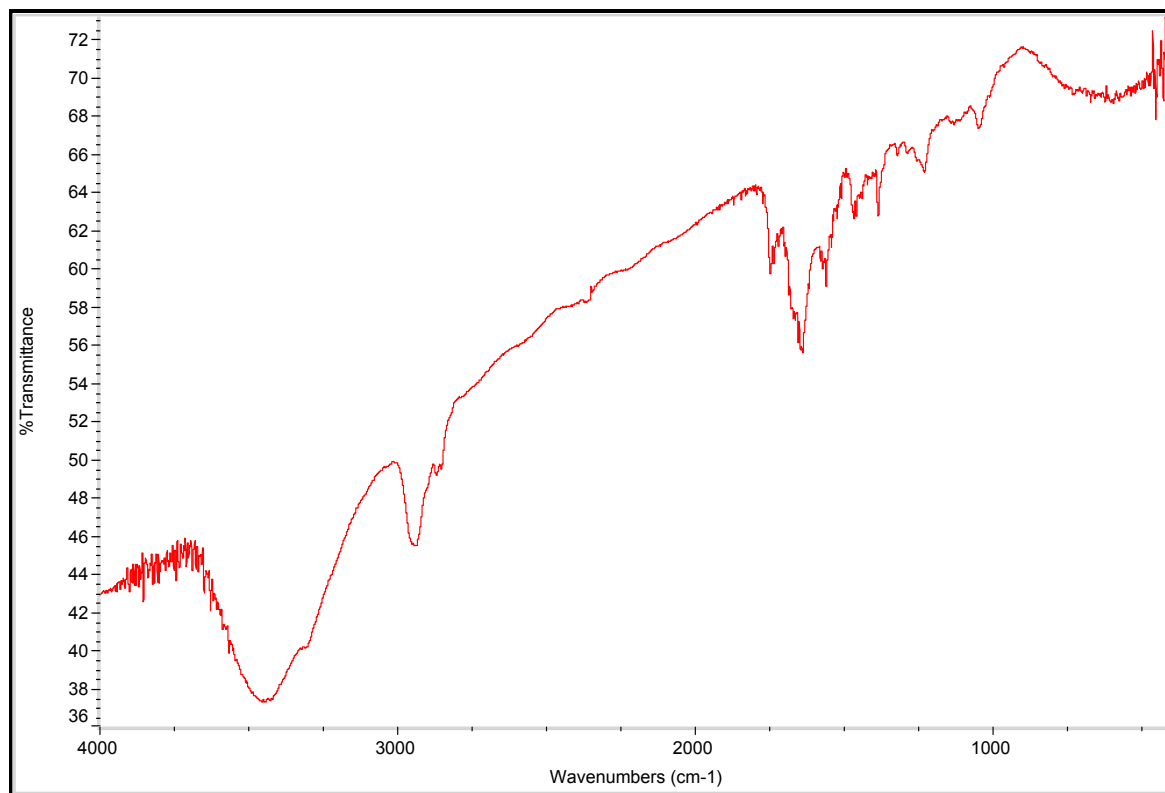
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APPENDIX-A

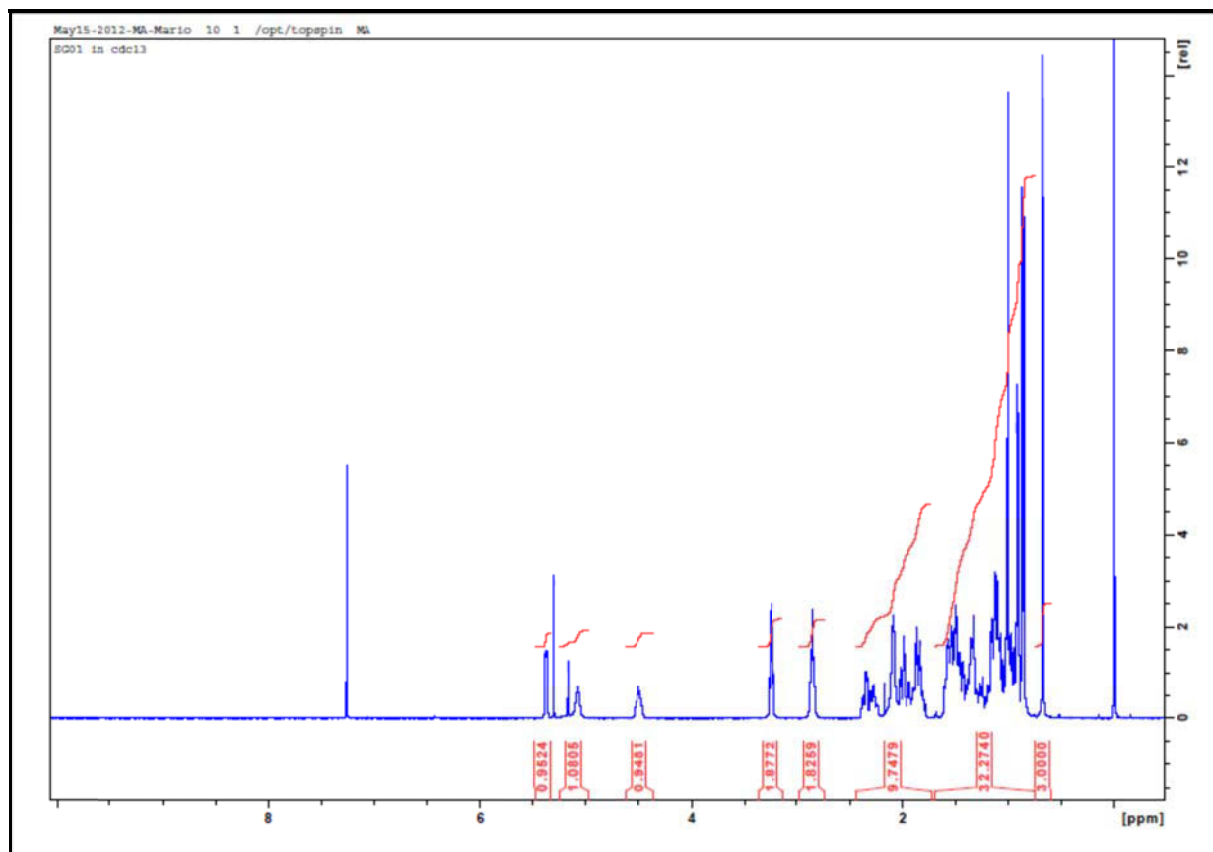
Proton NMR Spectra of MSO9



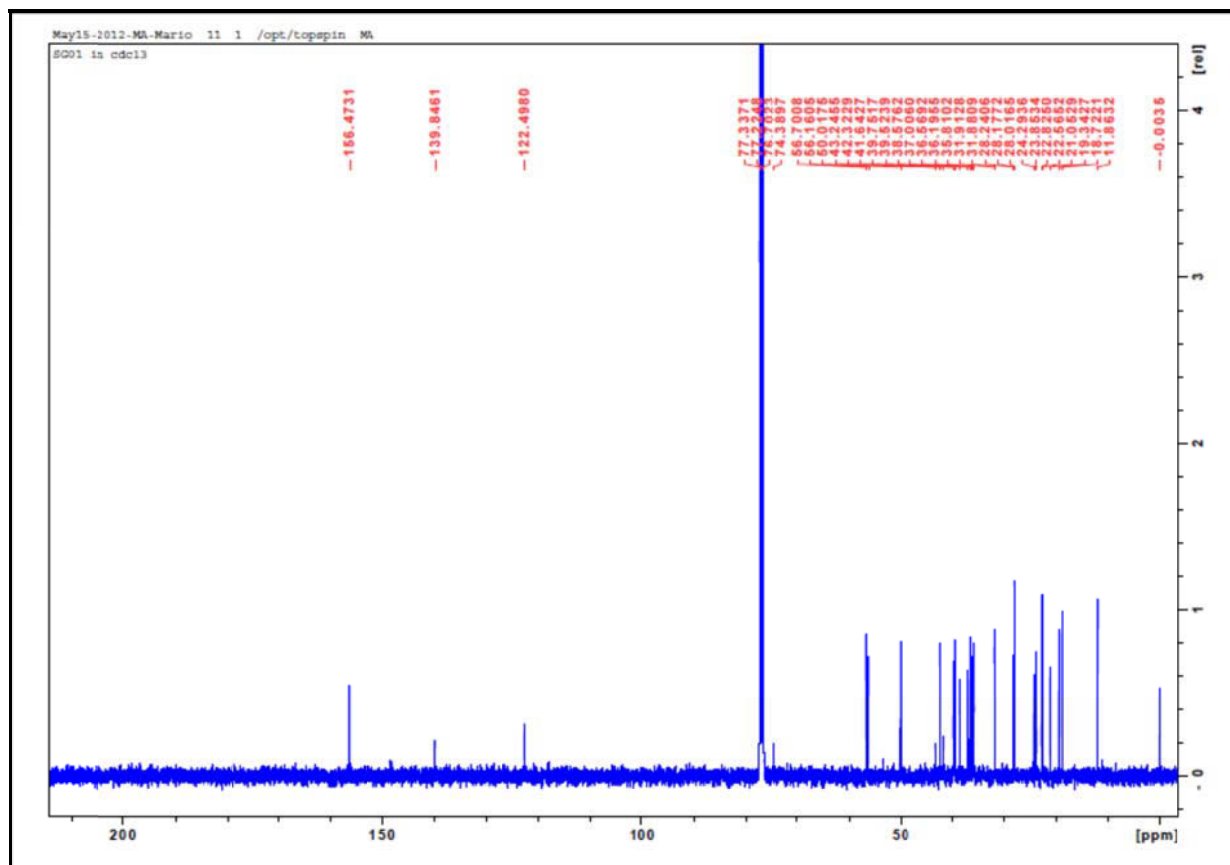
Infrared Spectra of MSO9



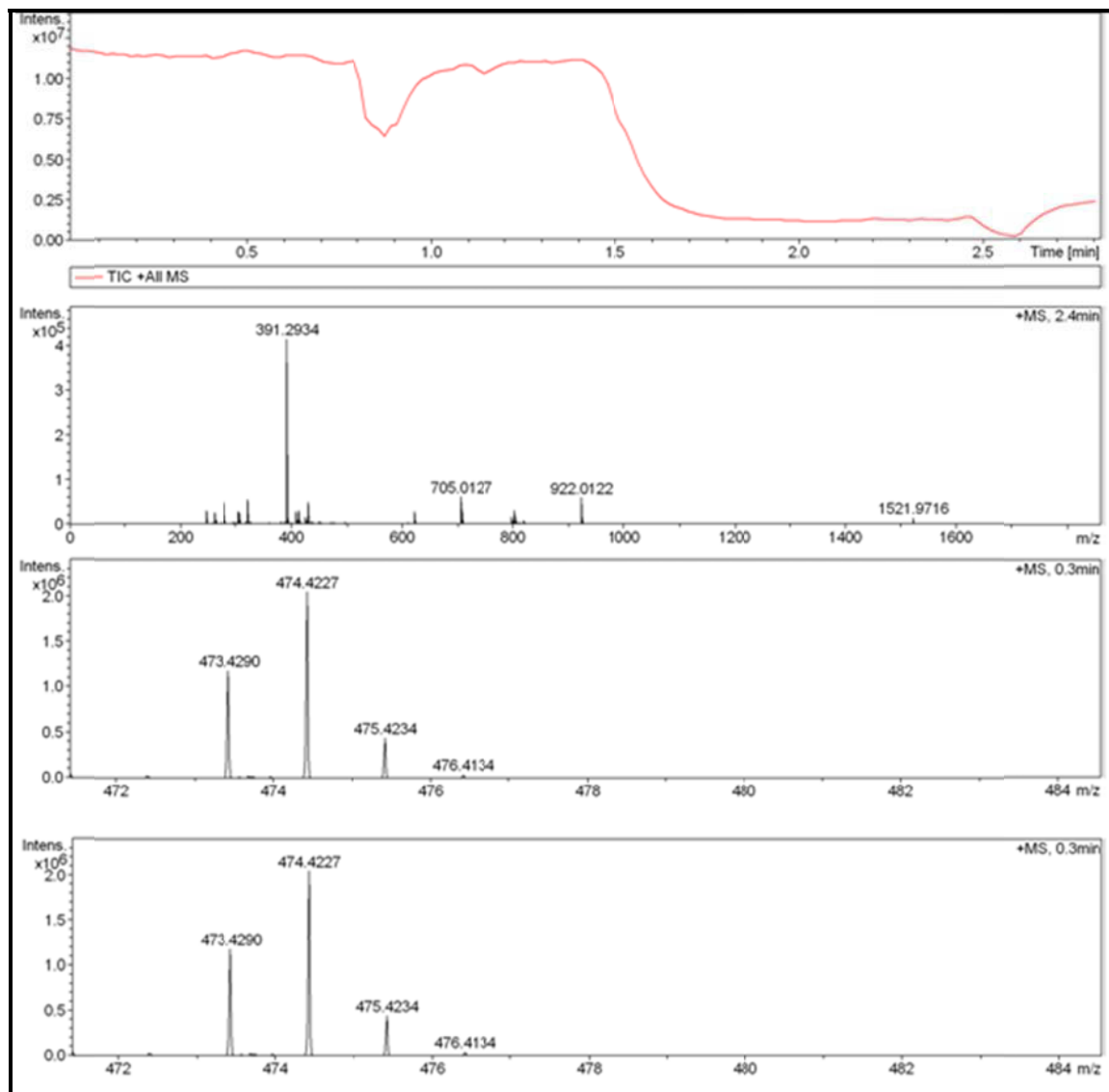
Proton NMR Spectra of SGO1



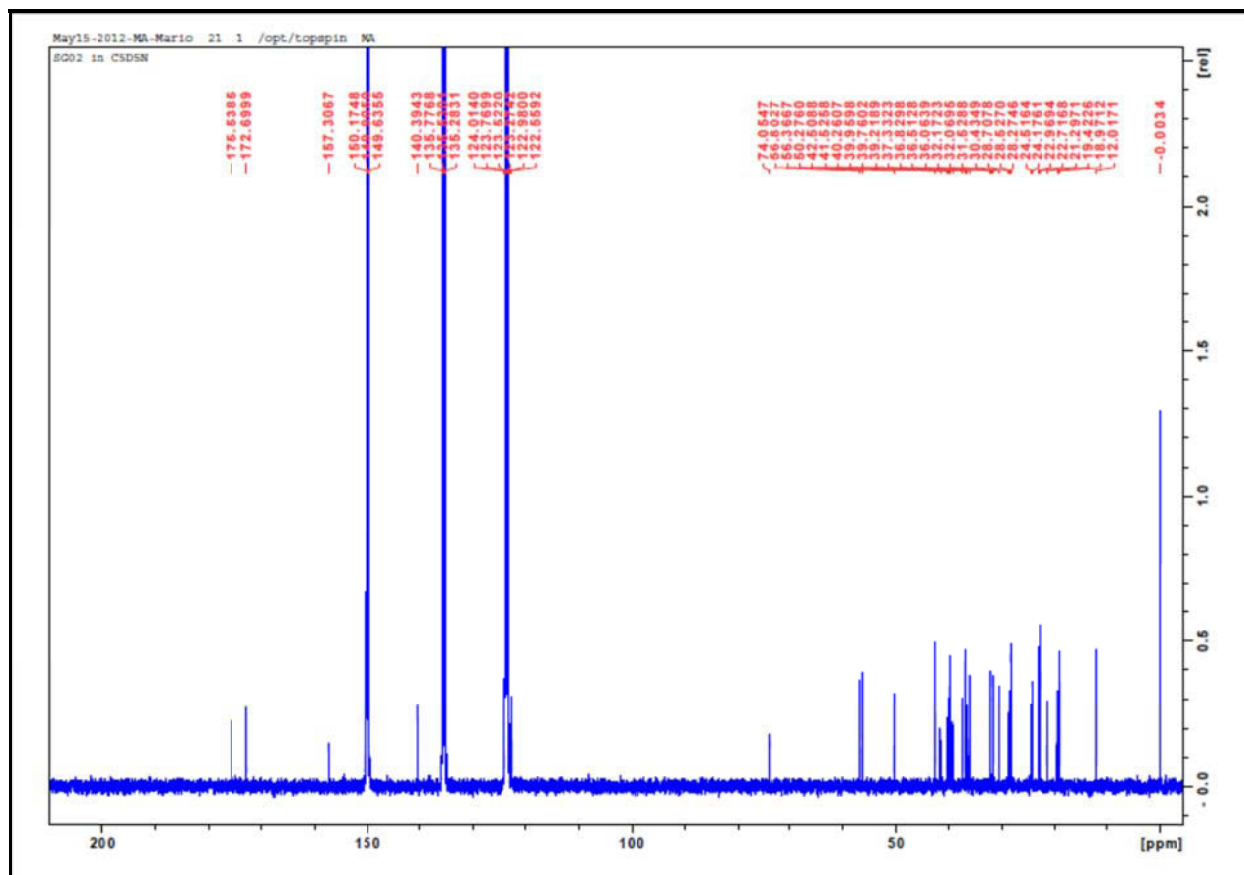
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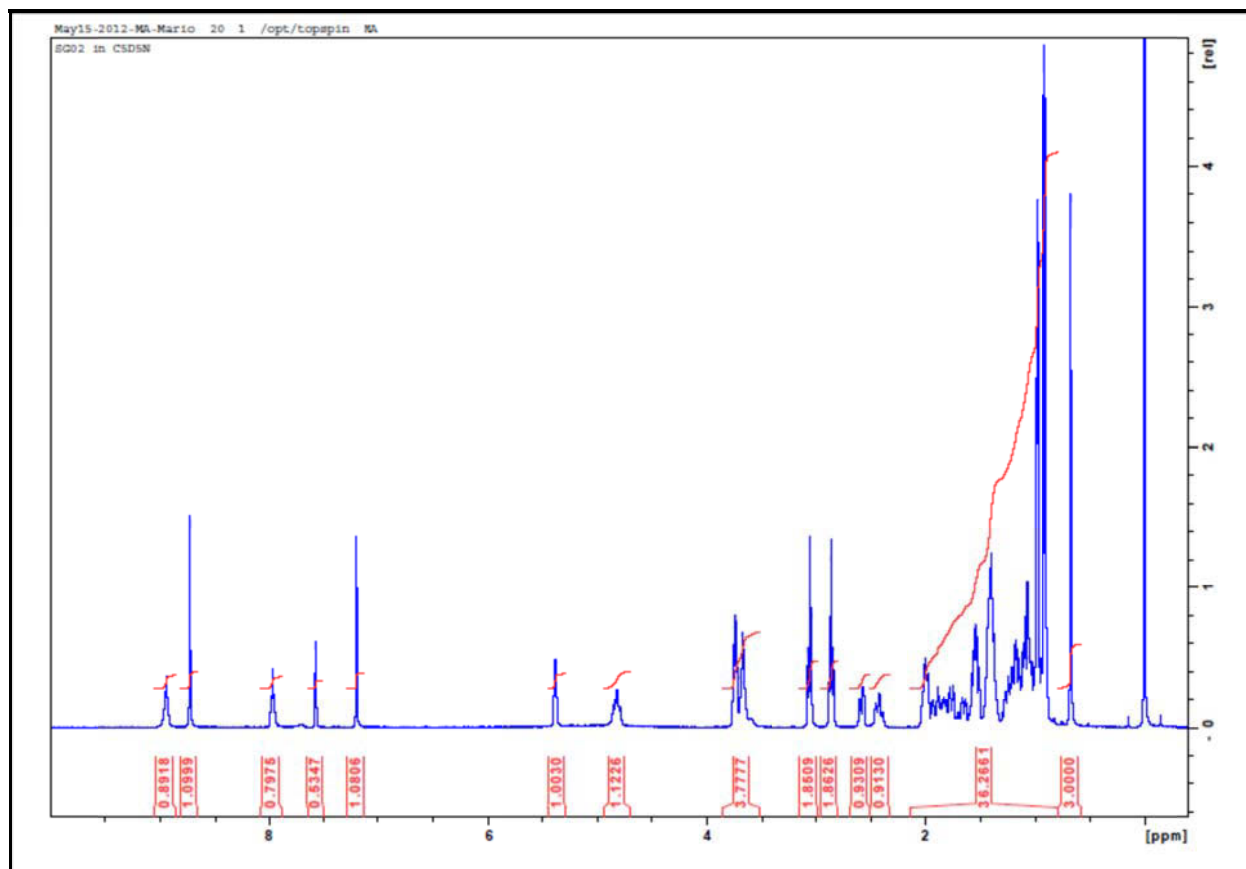
High Resolution Mass Spectra of SGO1



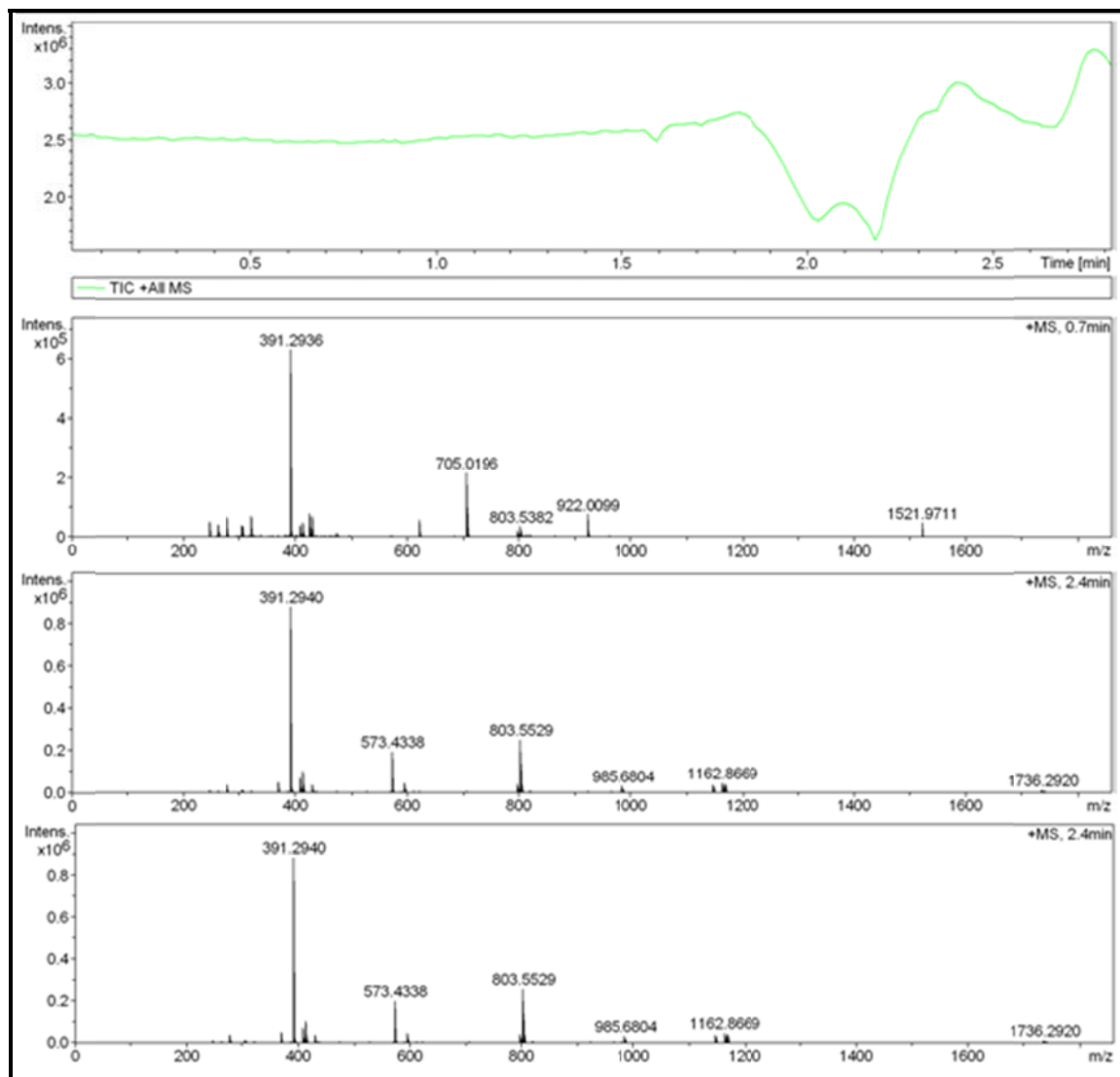
Proton NMR Spectra of SGO2



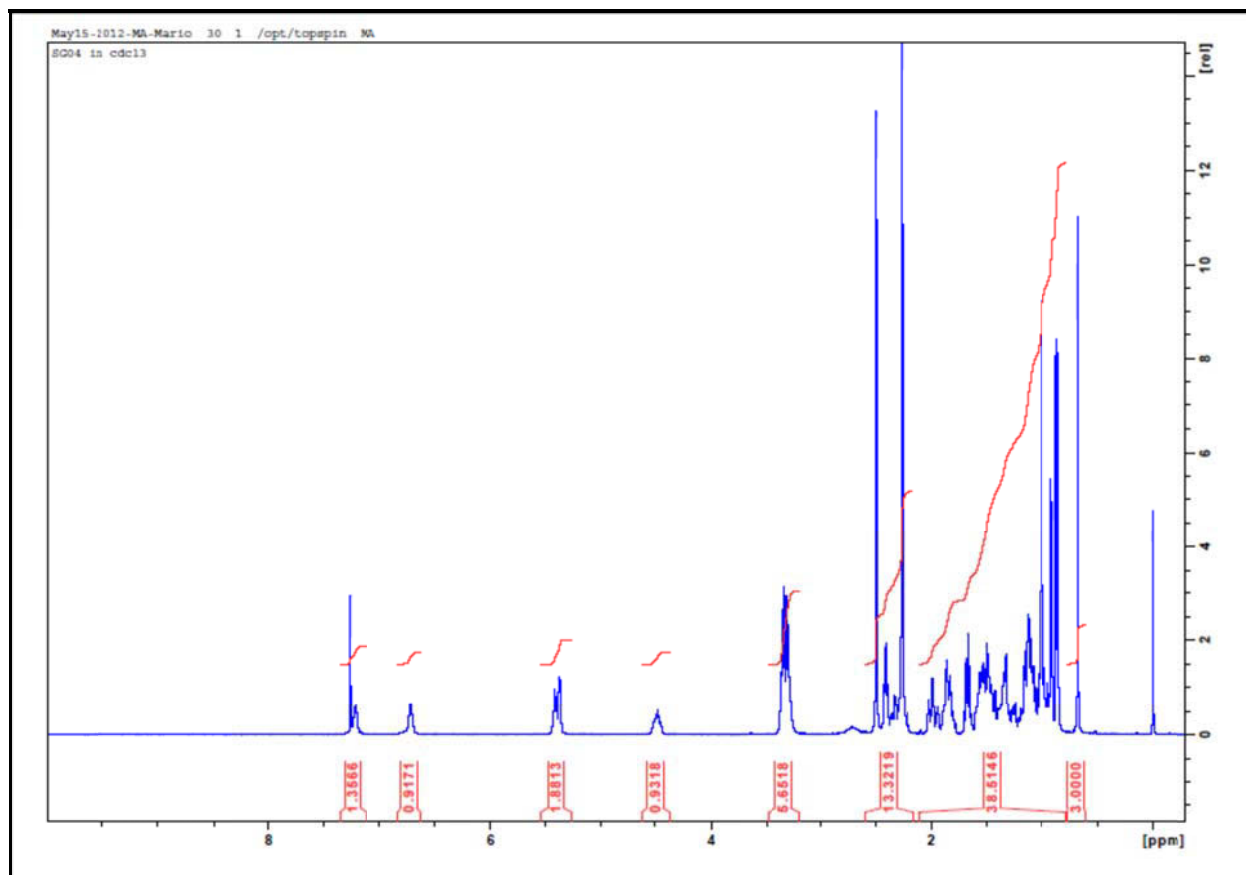
Carbon NMR Spectra of SGO2



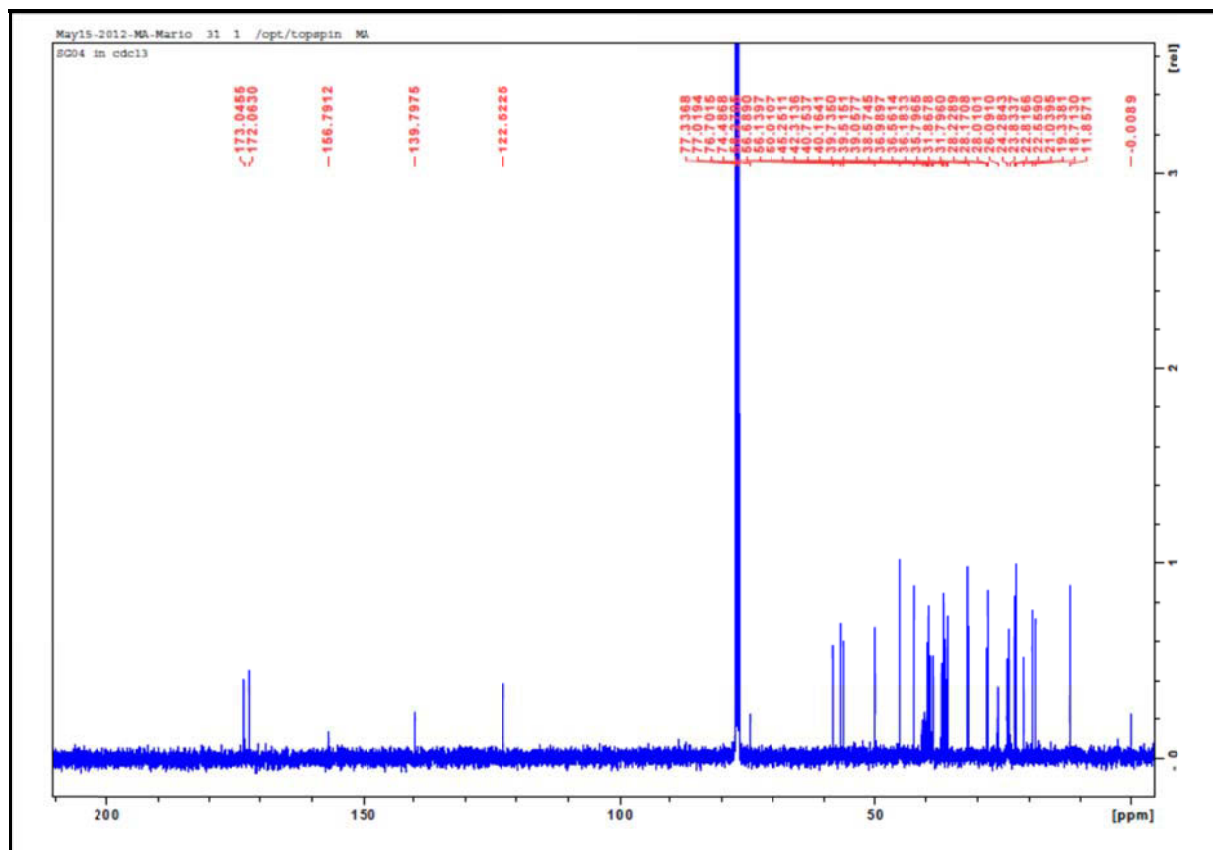
High Resolution Mass Spectra of SGO2



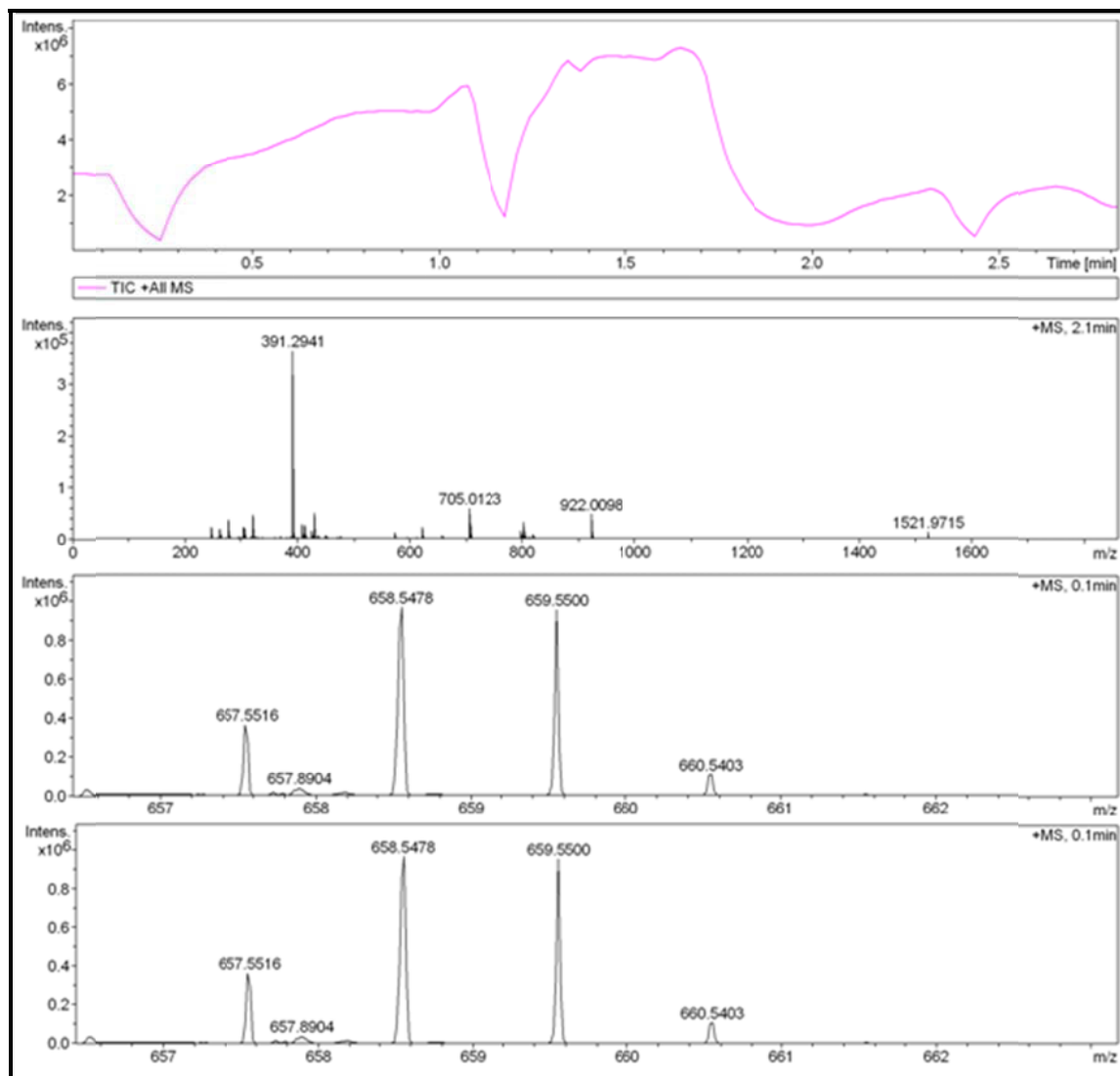
Proton NMR Spectra of SGO4



Carbon NMR Spectra of SGO4



High Resolution Mass Spectra of SGO4



APPENDIX- B

Publications

1. **S. Gorle**, M. Ariatti and M. Singh. “Novel serum-tolerant lipoplexes target the folate receptor efficiently”. *European Journal of Pharmaceutical Sciences*. 59, (2014) 83-93.
2. **S. Gorle**, M. Ariatti and M. Singh. “Folate receptor targeted gene delivery using cationic liposomes as non-viral vectors”. *International Proceedings of Chemical, Biological & Environmental Engineering*. 52, (2013) 1-4. ISSN: 2010-4618, ISBN: 978-981-07-6825-6.



Novel serum-tolerant lipoplexes target the folate receptor efficiently



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ABSTRACT

Gene transfer using non-viral vectors is a promising approach for the safe delivery of nucleic acid therapeutics. In this study, we investigate a lipid-based system for targeted gene delivery to malignant cells overexpressing the folate receptor (FR). Cationic liposomes were formulated with and without the targeting ligand folate conjugated to distearoylphosphatidyl ethanolamine polyethylene glycol 2000 (DSPE-PEG₂₀₀₀), the novel cytofectin 3β[N(N¹,N¹-dimethylaminopropylsuccinamidoethane)-carbamoyl]cholesterol (SGO4), which contains a 13 atom, 15 Å spacer element, and the helper lipid, dioleoylphosphatidylethanolamine (DOPE). Physicochemical parameters of the liposomes and lipoplexes were obtained by zeta sizing, zeta potential measurement and cryo-TEM. DNA-binding and protection capabilities of liposomes were confirmed by gel retardation assays, EtBr intercalation and nuclease protection assays. The complexes were assessed in an *in vitro* system for their effect on cell viability using the MTT assay, and gene transfection activity using the luciferase assay in three cell lines; HEK293 (FR-negative), HeLa (FR⁺-positive), KB (FR⁺⁺-positive). Low cytotoxicities were observed in all cell lines, while transgene activity promoted by folate-tagged lipoplexes in FR-positive lines was tenfold greater than that by untargeted constructs and cell entry by folate complexes was demonstrably by FR mediation. These liposome formulations have the design capacity for *in vivo* application and may therefore be promising candidates for further development.

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1. Introduction

Gene therapy is a treatment modality for many diseases with a genetic origin. Thus, the delivery of the appropriate, therapeutic gene (DNA) into the cells that will replace, repair or regulate the defective gene that causes the disease is a key step in gene therapy. DNA, however is a negatively charged polyanion and does not easily traverse the negatively charged and hydrophobic cell membrane. Consequently, gene delivery carriers (also called vectors or vehicles) have been developed (Ward and Georgiou, 2011). Antitumor drug delivery systems with nanometric dimensions have received much attention due to their unique accumulation behaviour at the tumour site. Various nanoparticulate carriers such as liposomes, polymer conjugates, polymer micelles, and nanoparticles are utilized for selective delivery of various anti-cancer drugs to tumours in a passive targeting manner. However, a more effective and active targeting system is needed to enhance the uptake of drugs using nanocarriers into cancerous cells at the tumour site (Kawano and Maitani, 2011). This may be achieved by ligand–receptor, antigen–antibody and other forms of molecular recognition for site-specific delivery (Steichen et al., 2013). The

over-expression of receptors such as those for folate and transferrin, by tumour cells, may be exploited for this purpose (Liechty and Peppas, 2012). Non-viral vectors are generally cationic in nature. They include cationic polymers such as polyethyleneimine (PEI) and poly L-lysine (PLL), cationic peptides and cationic liposomes. Recently, a liposomal preparation LPD (liposomes–protamine–DNA) has shown transfection efficiency greater than that of conventional liposome:DNA complexes (lipoplexes) (Tros de Ilarduya et al., 2010). Physical properties such as size and zeta potential play a critical role in determining their efficiency. Selected modifications to these approaches that can produce safe, efficient and targetable gene carriers are desirable. Although non-viral vectors are less efficient than their viral counterparts, they have the advantages of safety, simplicity of preparation and high gene encapsulation capability (Nasiruddin, 2007).

Although cationic liposomal vectors mediate effective gene transfer, tissue specific *in vivo* DNA delivery is still a major challenge in gene therapy (Zhang et al., 2012; Jin et al., 2014). To date, tissue-specific targeting of cationic liposomal DNA has been accomplished by two distinct techniques. The first method involves transfection of selected tissues, such as nasal epithelium, arterial endothelium, lung or tumours by locally administering the complexes within a defined region (Reddy et al., 2002; Alton, 2007). This method has proven to be a viable option for the clinical

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treatment of several diseases, including cystic fibrosis, and cancer. A second method used to enhance the specificity of gene delivery is by the coupling of cell-binding ligands, such as folic acid (FA), transferrin or carbohydrates to liposomes for the purpose of combining the intrinsic activities of lipids with the receptor-mediated uptake properties of the attached ligand (Kamaly et al., 2012). We have been interested in the use of FA as a targeting ligand to deliver attached therapeutic and imaging agents to cancer cells that over-express the folate receptors (Kukowska-Latalo et al., 2005). Because FA-linked cargoes are efficiently bound and internalized by folate receptor-expressing cells, we have explored the possibility of using FA to enhance cationic liposomal vector delivery to FR-enriched tumours (Reddy and Low, 2000). When FA is linked via a carboxyl group to virtually any molecular construct (e.g. a drug, imaging agent, proteins, virus, liposome, etc.), folate receptor binding proceeds unhindered and folate-conjugate uptake occurs via receptor-mediated endocytosis (Lu and Low, 2012). The high affinity of folate for its receptors ($\sim 10^{-10}$ M), the small size of FA, and the compatibility of FA with a variety of solvents and solution conditions also adds to the attractiveness of the targeting ligand (Reddy and Low, 2000). It has been shown that folate-combined nanoparticles concentrated in tumour cells and liver tissue over four days longer after administering than non-targeted agents (Kukowska-Latalo et al., 2005). The role of folate receptors in the cellular transport of folate is not well understood, although a potocytosis (caveolin-coated endocytosis) model has been proposed. After binding to receptors on the cell surface, folate conjugates have been shown to traffic to endosomes (Lu and Low, 2012). Recently, it has been reported that folate mediated delivery of drug loaded nanoparticles can enable binding, promote uptake, and exhibit increased cytotoxicity to cancer cells *in vitro* and *in vivo* (Zhao et al., 2010). For *in vitro* applications, lipoplexes are usually formed with excess positive charge (cationic moiety (+) to nucleotide (-)). However for gene transfer applications *in vivo*, lipoplexes formed with excess positive or excess negative charge ratios have been used (Xu et al., 1999). It has been shown that lipoplexes adsorb a 'protein corona' in serum by low affinity and competitive binding, which may promote the formation of large aggregates of intact lipoplexes (Caracciolo et al., 2010). This, in turn may affect the mode of cellular uptake *in vitro*. The possible effects on *in vivo* applications are also far reaching as the nanoparticles perceived by cells may differ considerably from the intended formulation. However, the inclusion of polyethylene glycol (PEG) in lipoplexes creates a steric hindrance, which greatly reduces protein adsorption, opsonization and elimination by macrophages (Pozzi et al., 2014).

The aim of this study was to formulate novel serum-tolerant folate-decorated stealth lipoplexes for gene delivery to tumour cells that overexpress the folate receptor. Thus a new cholesteryl cytofectin featuring a 13 atom 15 Å spacer element separating the cationic head group and the hydrophobic cholesteryl fused ring anchor element was prepared to facilitate DNA interaction with PEGylated liposomes displaying folate on the distal end of membrane tethered polyethylene glycol 2000. Interactions were characterized by cryo transmission electron microscopy (cryo-TEM), gel retardation and ethidium displacement assays. Systems were further assessed for cytotoxicity and transfection activity in folate receptor-positive (HeLa, KB) and receptor-negative (HEK293) cell lines.

2. Materials and methods

2.1. Materials

Dioleoylphosphatidylethanolamine (DOPE), folic acid and biconchonic acid (BCA) assay reagents were purchased from the

Sigma Chemical Company, St. Louis, USA. Distearoylphosphatidylethanolamine poly(ethylene glycol) 2000 (DSPE-PEG₂₀₀₀) and amino distearoylphosphatidylethanolamine poly(ethylene glycol) 2000 (DSPE-PEG₂₀₀₀NH₂) were purchased from Avanti Polar Lipids, Alabaster, USA. Cholesteryl chloroformate, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethane sulphonic acid (HEPES), ethidium bromide and silica gel 60F₂₅₄ thin layer plates were purchased from Merck, Darmstadt, Germany. Cationic lipid, 3β[N(N¹,N¹-dimethylaminopropylsuccinamidoethane)-carbamoyl]cholesterol (SGO4) was synthesized as described below. Ultrapure DNA grade agarose was purchased from Bio-Rad Laboratories, Richmond, USA. pCMV-luc DNA was purchased from Plasmid Factory, Bielefeld, Germany. HEK293 cells were supplied by the University of the Witwatersrand Medical School (South Africa). HeLa cells were obtained from Highveld Biologicals (Pty) Ltd. (Lyndhurst, South Africa). KB cells were provided by Professor S.T. Chen, Institute of Biological Chemistry, Academia Sinica (Taipei, Taiwan). Minimum Essential Medium (MEM) containing Earle's salts and L-glutamine, trypsin-versene and penicillin (5000 units/ml)/streptomycin (5000 µg/ml) were purchased from Lonza BioWhittaker, Walkersville, USA. The Luciferase Assay kit was purchased from the Promega Corporation, Madison, USA. All tissue culture plastic consumables were purchased from Corning Incorporated, New York, USA. All other reagents were of analytical grade. Milli-Q ultrapure 18 MΩ cm water was used throughout.

2.2. Chemistry

2.2.1. Preparation of 3β[N(2-aminoethyl)-carbamoyl]cholesterol (SGO1)

To a solution of ethylenediamine (2.25 g, 37.5 mmol) in dry dichloromethane (40 ml) was added a solution of 2.0 g of cholesterylchloroformate (4.45 mmol) in dry CH₂Cl₂ (40 ml) drop wise over 5 min. After 48 h at room temperature the reaction mixture was extracted with 3 × 150 ml H₂O. The CH₂Cl₂ layer was dried over anhydrous Na₂SO₄ and evaporated to a white powder. Thereafter, the product was recrystallized from cyclohexane. Next, the product was purified further by column chromatography on a silica gel 60 column (2.2 × 23 cm) equilibrated with chloroform (50 ml). Column elution was with CHCl₃:MeOH:conc.NH₄OH (95:4:1, v/v/v) (100 ml) followed by CHCl₃:MeOH:conc.NH₄OH (90:10:1, v/v/v). Product fractions were pooled and evaporated and the title compound was obtained in crystalline form from cyclohexane. Yield: 67%; Mp: 168–170 °C; ¹H NMR (400 MHz, CDCl₃): δ 0.67 (s, 3H, H-18'), 0.86 (d, 6H, J = 7 Hz, overlapping 2 Hz, H-26', H-27'), 0.91 (d, 3H, J = 6.5 Hz, H-21'), 1.00 (s, 3H, H-19'), 1.0–2.1 (m, 28H, cholesteryl), 2.35 (m, 2H, H-4'), 2.85 (m, 2H, H₂NCH₂), 3.25 (q, 2H, J = 5.7 Hz, H₂NCH₂CH₂), 4.49 (m, 1H, H-3'), 5.37 (d, 1H, J = 4.9 Hz, H-6') ppm. ¹³C NMR (100 MHz, CDCl₃): 11.9 (C-18'), 18.7 (C-21'), 19.3 (C-19'), 21.0 (C-11'), 22.6 (C-26'), 22.8 (C-27'), 23.9 (C-23'), 24.3 (C-15'), 28.0, 28.2 (C-2', C-16', C-25' overlapping), 31.9 (C-7', C-8' overlapping), 35.8 (C-20'), 36.2 (C-22'), 36.6 (C-1, C-10'), 37.0 (C-1'), 38.6 (C-24'), 39.5, 39.7 (C-4', C-12'), 42.3 (C-13'), 50.0 (C-9'), 56.2 (C-17'), 56.7 (C-14'), 74.4 (C-3'), 122.5 (C-6'), 156.5 (NHCOO), 139.8 (C-5'). HR-MS (ESI-QTOF +ve): Anal. Calcd. for C₃₀H₅₃O₂N₂: (M+H) 473.4113, Found 473.4290.

2.2.2. Preparation of 3β[N(hemisuccinamidoethane)-carbamoyl]cholesterol (SGO2)

A solution of 3β[N(2-aminoethane)-carbamoyl]cholesterol (237 mg, 0.5 mmol) in CH₂Cl₂ (5 ml) was added drop wise to a solution of succinic anhydride (60 mg, 0.6 mmol) in pyridine (1 ml). After 24 h a gel-like product was formed. A further aliquot of succinic anhydride (60 mg, 0.6 mmol) was added and 1 ml of DMF. A clear solution was obtained. After a further 24 h a

quantitative yield of product was obtained. The product was recrystallized from ethanol. Yield: 84%; Mp: 168–170 °C; ¹H NMR (400 MHz, C₅D₅N): δ 0.67 (s, 3H, H-18'), 0.90 (d, 6H, J = 6.6 Hz, H-26', H-27'), 1.0–2.1 (m, 28H, cholesteryl), 2.56 (m, 2H, H-4'), 2.85 (t, 2H, J = 6.9 Hz, H-2), 3.06 (t, 2H, J = 6.8 Hz, H-1), 3.68 (t, 2H, J = 6.5 Hz, H-5), 3.74 (t, 2H, J = 5.5 Hz, H-6), 4.82 (m, 1H, H-3'), 5.38 (bs, 1H, H-6'). ¹³C NMR (100 MHz, C₅D₅N): 12.0 (C-18'), 19.0 (C-21'), 19.4 (C-19'), 21.3 (C-11'), 22.7 (C-27'), 23.0 (C-26'), 28.2, 28.5, 28.7 (C-2', C-16', C-25'), 31.5 (C-5), 32.1, 32.2 (C-7', C-8'), 36.1 (C-22'), 36.5 (C-10'), 36.8 (C-1'), 39.2 (C-1), 39.7, 39.9 (C-4', C-12'), 42.5 (C-13'), 50.3 (C-9'), 56.4 (C-17'), 56.8 (C-14'), 74.1 (C-3'), 122.6 (C-6), 140.4 (C-5), 157.3 (NH₂COO), 172.7 (C-4), 175.5 (C-7). HR-MS (ESI-QTOF +ve): Anal. Calcd. for C₃₄H₅₇N₂O₅ (M+H) 573.4273, Found 573.4338.

2.2.3. Preparation of 3β[N(N',N')-dimethylaminopropylsuccinamidoethane]-carbonyl]cholesterol (SGO4)

To a solution of 3β[N(hemisuccinamidoethane)-carbonyl]cholesterol (114 mg, 0.2 mmol) and *N*-hydroxysuccinamide (32 mg, 0.28 mmol) in pyridine (2 ml) was added a solution of dicyclohexylcarbodiimide (55 mg, 0.26 mmol) in pyridine (0.8 ml). A catalytic amount of 4-dimethylaminopyridine (2.4 mg, 0.02 mmol) was also included in the reaction mixture. After 24 h, 3-dimethylaminopropylamine (51 mg, 0.5 mmol) was added to the mixture which was stored in the dark at room temperature for a further 24 h. Dicyclohexylurea crystals were removed by filtration. The filtrate was concentrated and the product was purified by silica gel 60 column (2.0 × 17.0 cm) chromatography. Equilibration and elution was with CHCl₃:MeOH:conc.NH₄OH (43:7:1). Yield: 61%; Mp: 216–218 °C; ¹H NMR (400 MHz, CDCl₃): δ 0.68 (s, 3H, H-18'), 0.86 (d, 6H, J = 6.7 Hz, overlapping 2 Hz, H-26', H-27'), 0.91 (d, 3H, J = 6.5 Hz, H-21'), 1.00 (s, 3H, H-19'), 1.00–2.21 (m, 27H, cholesteryl), 2.27 (s, 6H, H-13, H-14), 2.42 (t, 2H, J = 6.3 Hz, H-4), 2.50 (s, 4H, H-6, H-7), 3.30–3.36 (m, 6H, H-1, H-2, H-9), 4.48 (m, 1H, H-3'), 5.37 (d, 1H, J = 4.9 Hz, H-6') ppm. ¹³C NMR (100 MHz, CDCl₃): 11.9 (C-18'), 18.7 (C-21'), 19.3 (C-19'), 21.0 (C-11'), 22.6 (C-26'), 22.8 (C-27'), 23.8 (C-23'), 24.3 (C-15'), 28.0, 28.2 (C-2', C-16', C-25' overlapping), 31.8 (C-10), 31.9 (C-7', C-8' overlapping), 35.8 (C-5'), 36.2 (C-22'), 36.6 (C-1, C-10' overlapping), 37.0 (C-1'), 38.6 (C-24'), 39.5, 39.7 (C-4', C-12'), 42.3 (C-13', C-9 overlapping), 45.2 (C-13, C-14 overlapping), 50.0 (C-9'), 56.1 (C-17'), 56.7 (C-14'), 58.3 (C-11), 74.5 (C-3'), 122.5 (C-6'), 139.8 (C-5'), 156.8 (NH₂COO), 172.1 (C-7), 173.0 (C-4). HRMS (ESI-QTOF +ve): Anal. Calcd. for C₃₉H₆₉N₄O₄ (M+H) 657.5324, Found 657.5516.

2.3. Synthesis of DSPE-PEG₂₀₀₀FOL

DSPE-PEG₂₀₀₀FOL was synthesized as reported previously (Yoshida et al., 2006; Liu et al., 2011). Briefly, folic acid (100 mg, 0.226 mmol) was dissolved in 5 ml dimethyl sulfoxide (DMSO). To this was added an equimolar amounts of *N*-hydroxysuccinimide (NHS) (28.8 mg, 0.25 mmol) and dicyclohexylcarbodiimide (DCC) (51.5 mg, 0.25 mmol). The reaction mixture was incubated overnight at room temperature. The insoluble dicyclohexylurea (DCU) was filtered off from the reaction mixture. To a DMSO solution (44 μl) of the NHS ester of folic acid (NHS-FOL, 2 μmol) was added DSPE-PEG₂₀₀₀NH₂ (2.79 mg, 1 μmol). DMSO (40 μl) and pyridine (40 μl) were added to the mixture, whereupon a clear solution was obtained. The solution was left overnight and pyridine was removed by rotary evaporation. Water (500 μl) was added to the reaction mixture, which was then dialyzed (MW cut off 2000 Da) against water (3 × 500 ml). The dialysate was centrifuged at 12,000 rpm (SANYO MSE) for 5 min at room temperature and the supernatant was stored at –20 °C before use. An aliquot of the

dialysate (30 μl) was diluted to 1000 μl with water and the absorbance at 280 was determined (Shimadzu UV-160A). Untargeted PEGylated liposomes were used as a control. The concentration of folate was calculated ($\epsilon_{280} = 2.582 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, Kranz et al., 1995) and the loading onto DSPE-PEG₂₀₀₀NH₂ was found to be 95%. The synthesis of DSPE-PEG₂₀₀₀FOL and the absence of DSPE-PEG₂₀₀₀NH₂ (ninhydrin) was also confirmed by thin layer chromatography (TLC) on silica gel 60F₂₅₄ plates, which were developed in CHCl₃:MeOH:H₂O (75:36:6 v/v/v) (Kang et al., 2013).

2.4. Preparation of cationic liposomes

Cationic liposomes and PEGylated cationic liposomes were prepared with or without the conjugated lipid DSPE-PEG₂₀₀₀FOL and DSPE-PEG₂₀₀₀ (Table 1) by a method adapted from Gao and Huang (1991). Briefly, SGO4 (1 μmol) and the helper lipid DOPE (1 μmol) were dissolved in chloroform (1 ml) and the solutes were deposited as a thin film on the inner wall of a test tube by rotary evaporation of the solvent *in vacuo* at 25 °C (Buchii Rotavapor-R). Residual chloroform was removed in Büchi-TO drying pistol drier (200 mTorr, 30 °C, 2 h). The dried lipid film was rehydrated overnight at 4 °C in a sterile solution containing 20 mM HEPES and 150 mM NaCl (pH 7.5, 0.5 ml). The resulting liposome dispersion (4 mM total lipid, 2.9 mg lipid/ml) was briefly vortexed and sonicated for 5 min in a Transonic 460/H bath-type sonicator (35 kHz) at 20 °C. Liposome dispersions were routinely stored at 4 °C for several months, without visible aggregation, until use.

2.5. Preparation of lipoplex

Lipoplexes were formed by the addition of pCMV-luc DNA (0.5 μg) to various amounts of cationic liposome dispersions (1–14 μg) and brought to a final volume of 10 μl with HEPES-buffered saline (HBS) to achieve specific liposome (+)/pDNA (–) charge ratios ranging from 1:1 to 7:1. Lipoplexes were incubated at room temperature for 20 min prior to use.

2.6. Gel retardation assay

The gel retardation assay was performed to confirm the binding of cationic liposome to pDNA (Duckett et al., 1996). Lipoplexes were prepared as described above to achieve (+/–) charge ratios in the range 1:1–7:1. After addition of gel loading buffer (0.05% bromophenol blue, 40% sucrose, 2 μl) to complexes samples were subjected to electrophoresis (50 V) on a 1% agarose gel containing ethidium bromide (EtBr) (1.5 μg/ml) in a buffer comprising 36 mM Tris–HCl, 30 mM sodium phosphate and 10 mM EDTA (pH 7.5) for 90 min. Thereafter gels were viewed in a Syngene G-box under transillumination at 300 nm, and images were captured with GeneSnap software following exposure for 800 ms.

2.7. Serum nuclease digestion assay

For this assay liposome:DNA complexes containing 0.5 μg pCMV-luc DNA from below to above end point retardation ratios were incubated in HBS for 20 min at room temperature and thereafter foetal bovine serum (FBS) was added to a final concentration of 10% (v/v). Samples were incubated for 4 h at 37 °C, whereupon EDTA and SDS were added to final concentrations of 10 mM and 0.5% (w/v) respectively. After a further incubation for 20 min at 55 °C, the samples were analysed by electrophoresis on 1% agarose gel containing ethidium bromide (1.5 μg/ml) and viewed as described above.

2.8. Ethidium bromide intercalation assay

Ethidium bromide (EtBr), a DNA-intercalating dye, was used to examine the association of DNA with the cationic liposomes. Initially, 2 μ l of EtBr solution (100 μ g/ml) was added to 100 μ l of HBS in wells of a black 96-well plate and the fluorescence measured at 580 nm in a Glomax multi⁺ detector system (Promega) (excitation wavelength 525 nm) was set to 0% relative fluorescence. Thereafter, 2.4 μ l (1.2 μ g) of pCMV-luc DNA was added to the solution and the fluorescence reading was set to represent 100% relative fluorescence. Cationic liposome dispersions (1 μ l) (2.9 μ g total lipid) were added stepwise to the pDNA-EtBr solution and the fluorescence intensity after each addition was recorded. Results are presented graphically as relative fluorescence versus liposome concentration.

2.9. Cryo-transmission electron microscopy

The morphology and particle size distribution of the liposomes and lipoplexes were examined using cryo transmission electron microscopy (cryo-TEM). The cationic liposome dispersions were diluted to a 1:20 ratio, and the lipoplexes diluted to 1:100 with sterile HEPES buffered saline (HBS). A 1 μ l droplet of the diluted cationic liposome/lipoplex suspension was deposited on an electron microscopy carbon coated 400-mesh copper grid. To this, was added 1 μ l of 1% (w/v) uranyl acetate and the grid was allowed to dry for 2 min. After removing the excess solution with a filter paper the grid was kept under liquid nitrogen and then transferred into a GATAN cryo-holder maintained at -170 °C, which was then introduced into the microscope for observation at -150 °C. Images were obtained under cryogenic conditions and at 100 kV using a JEOL JEM1010 electron microscope (Tokyo, Japan). The micrographs were generated by a MegaView III camera and SIS i-TEM software facilitated measurements of liposomes on calibrated images.

2.10. Size and zeta potential measurements

Liposome and lipoplex particle sizes, polydispersity and zeta potential were measured in a Malvern Zetasizer Nanoseries (Malvern Instrument). All measurements were carried out at 25 °C. The cell types chosen for Z-average measurement were DTS0012 (polystyrene disposable sizing cuvette), and DTS-1061 for zeta potential measurements. Measurements were performed in triplicate.

2.11. Cell culture

HEK293 (human embryonic kidney), HeLa (human cervical carcinoma), and KB (human nasopharyngeal carcinoma) were maintained in 25 cm² flasks at 37 °C under 5% CO₂ in Minimum Essential Medium (MEM). Cell culture medium was supplemented with 10% (v/v) heat inactivated foetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μ g/ml), and L-glutamine (4 mM) (Gibco BRL Life Technologies). Cells were split 1:3 every 4–5 days.

2.12. MTT cell viability assay

Cells were seeded at a density of 2.5×10^4 cells/well in 48-well plates, and incubated at 37 °C in a humidified 5% CO₂ incubator for 24 h before treatment. Medium was then removed and the liposome:pCMV-luc complexes (10 μ l) containing 0.5 μ g pCMV-luc plasmid DNA were added to each well in a total volume of 0.3 ml complete medium (with 10% FBS, streptomycin at 100 μ g/ml, and penicillin at 100 U/ml) and incubated for a further 48 h at 37 °C.

After incubation, the medium was discarded and equal volumes of (0.2 ml) fresh complete medium and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide solution (MTT) (5 mg/ml PBS) were added to the sample wells and incubated for an additional 4 h at 37 °C. Thereafter, the MTT containing medium was removed, and 0.2 ml of dimethylsulphoxide (DMSO) was added to dissolve the formazan crystals produced by the cells. The absorbance was measured at 575 nm using a Mindray micro plate reader (MR-96A). Cell viability was determined as the percentage relative to the untreated control (100%). Experiments were performed in triplicate and the results were calculated and expressed as the mean \pm standard deviation.

2.13. Transfection assay

HEK293, HeLa, and KB cells were seeded onto a 48-well plate at a density of 2.5×10^4 cells/well, in 300 μ l complete medium (MEM + 10% FBS + penicillin (100 units/ml), streptomycin (100 μ g/ml), and L-glutamine (4 mM) at 0.3 ml per well. After 24 h of growth in 5% CO₂ at 37 °C, the medium was replaced by 0.3 ml complete medium. Lipoplexes, which had been prepared as described in Section 2.5, were prepared separately to cover a range from below to above end point charge ratios (2:1–4:1) and contained 0.5 μ g pCMV-luc plasmid DNA in 10 μ l HBS, were added to wells. The plate was incubated for a further 48 h at 37 °C in humidified 5% CO₂. In competition experiments free folate (final concentration 200 μ M) was introduced to cells 20 min before addition of lipoplexes. Thereafter, the cells were washed with phosphate buffered saline (PBS) (2 \times 0.5 ml), followed by addition of 100 μ l of cell culture lysis buffer (Promega) to each well (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% v/v glycerol, 1% v/v Triton X-100). The level of luciferase gene expression in the lysates was evaluated by measuring luminescence using a luminometer (Glomax Multi⁺ Detector system). Protein content in lysates was measured by the BCA Protein Assay reagent (Sigma) using bovine serum albumin as the standard. The data are expressed as relative light units (RLU) per milligram of total soluble cell protein. Two controls were employed i.e. C1: untreated cells, C2: cells with free DNA, without liposome. All experiments were carried out in triplicate.

2.14. Statistics

Statistical analysis was performed using one way ANOVA and Tukey's Multiple Comparison Test to compare between groups. Differences were considered statistically significant at $p < 0.05$.

3. Results and discussion

3.1. Synthesis of the cationic cholesterol derivative SGO4

Cholesterylchloroformate was treated with ethylenediamine to afford 3 β [N(2-aminoethyl)-carbamoyl]cholesterol (SGO1), which was then succinylated (succinic anhydride/pyridine) to give 3 β [N(hemisuccinamidoethane)-carbamoyl]cholesterol (SGO2). The hemisuccinate (SGO2) was converted to 3 β [N(N¹,N¹-dimethylaminopropylsuccinamidoethane)-carbamoyl]cholesterol (SGO4) by activation of the carboxyl function to its N-hydroxy-succinimide ester followed by treatment with 3,3-dimethylamino-propylamine (Fig. 1). All the synthesized compounds gave satisfactory analyses for their proposed structures, which were confirmed on the basis of their spectral ¹H NMR, ¹³C NMR, and HR-MS spectral data (Supplementary Materials Figs. A.1a–c, A.2a–c and A.3a–c) respectively.

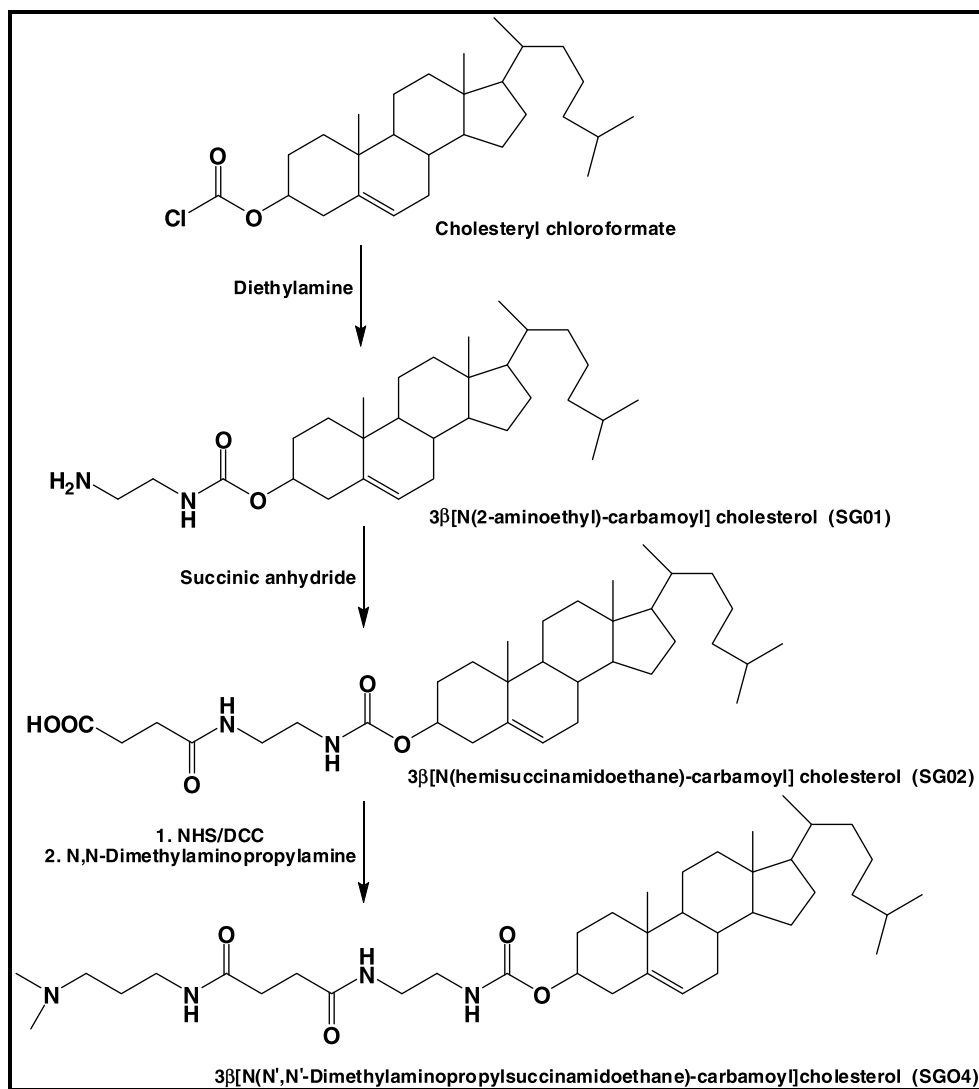


Fig. 1. Synthesis of cationic cholesterol derivative 3β[N(N',N'-dimethylaminopropylsuccinamidoethane)-carbamoyl]cholesterol (SG04).

3.2. Synthesis of DSPE-PEG₂₀₀₀FOL for folate targeted liposome

The delivery of nanoparticles to cancer cells may be facilitated by folate decoration, as many cancer cells overexpress the folate receptor, which is rapidly internalized by receptor-mediated endocytosis (Kularatne and Low, 2010; Xiong et al., 2011). Here we have linked the ligand folate to DSPE-PEG₂₀₀₀NH₂ for inclusion in a cationic liposome formulation containing a novel cytofectin. The key intermediate, NHS-folate, was prepared by activating the carboxyl group on folic acid with *N*-hydroxysuccinamide (NHS) according to previously reported methods using a dicyclohexylcarbodiimide coupling procedure (Yoshida et al., 2006; Liu et al., 2011). The product obtained is a mixture of the γ carboxylate (80%) and α carboxylate (20%) esters (Lee and Low, 1994). It is important to note that for folate to retain its receptor recognition, it must be appended via the γ carboxyl group (Bhattacharya et al., 2008). The title compound DSPE-PEG₂₀₀₀FOL was obtained by the reaction of the ester NHS-FOL with the distal amino function on DSPE-PEG₂₀₀₀NH₂ (Fig. 2). Covalent attachment of folate to PEG was deemed to be advantageous as the ligand would not only be prominently displayed, but the spacer, PEG₂₀₀₀ enjoys low cytotoxicity (Ogris et al., 1998), water solubility (Lukyanov and Torchilin, 2004) and low immunogenicity (Tian-Lu et al., 2012). Moreover,

the presence of PEG to form a shell at the outside of the carrier provides functional end groups for the attachment of the targeting ligand through a flexible tether. The attachment and stoichiometry of the folate moiety in the compound were confirmed by UV spectroscopic analysis and TLC.

3.3. Zeta sizing

Particle size of non-viral vectors for gene delivery is a key parameter to be considered in the development of new systems with the capacity for *in vivo* application (Pathak et al., 2009; Nishikawa and Huang, 2001). The extent of nanoparticle uptake *in vivo* is variable and also dependent on the size, charge, rigidity, and other physicochemical properties of the particles (Aranda et al., 2013). Not only may particle size influence the mode of cellular uptake, it also determines if vectors may successfully extravasate and successfully negotiate the extracellular matrix to access tumour masses. Particle sizes of complexes were measured by dynamic light scattering in the absence and presence of the folate ligand. Results presented in Table 1 show that for all three formulations, liposomes were small and fell within a narrow size range (78–89 nm) and were monodisperse (PDI < 0.21). Lipoplexes at end point ratios, however varied in size. Targeted PEGylated

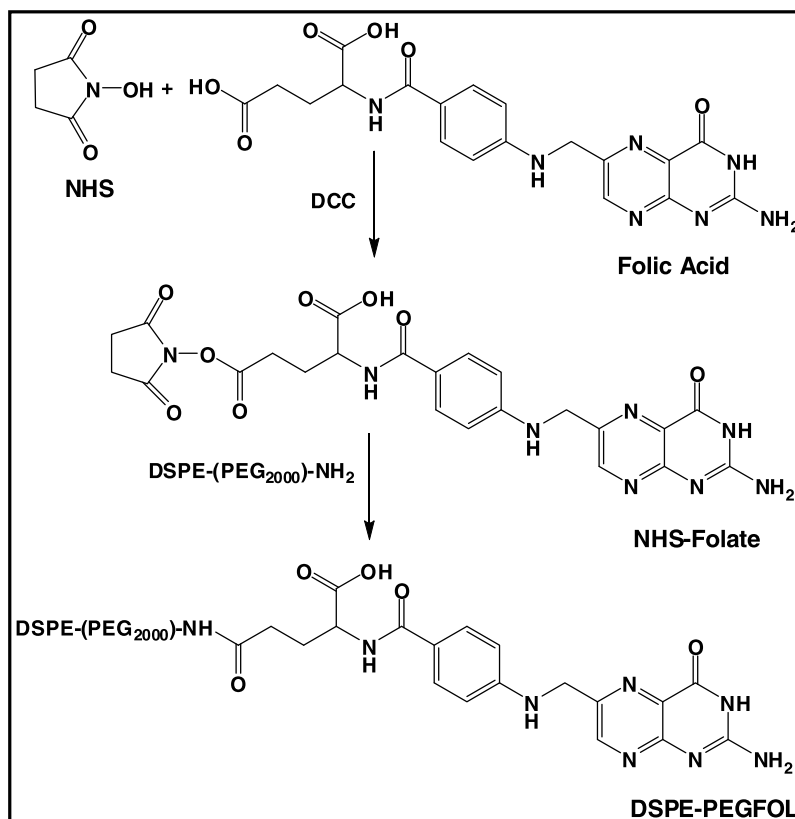


Fig. 2. Synthesis of DSPE-PEG₂₀₀₀FOL.

lipoplexes were the smallest (147 nm) with a low PDI value (0.15) indicating a monodisperse population. Untargeted PEGylated lipoplexes were somewhat larger (195 nm) and more heterogeneous in size distribution (PDI = 0.33). By contrast, untargeted, unPEGylated lipoplexes were very large (1055 nm) and polydisperse (PDI = 0.64). From the above it may be concluded that the steric repulsion imparted by PEG moieties prevents lipoplex aggregation, resulting in smaller, more uniform dispersions, while unPEGylated lipoplexes, with low ζ potential, aggregate to form ill-defined larger assemblies (Table 1). Similar trends have been reported by Zhang et al. (2010) with lipoplexes containing the cholesterol cytofectin DC-Chol. Targeted lipoplexes prepared in this study with a mean diameter <200 nm are therefore able to exploit the enhanced permeation and retention (EPR) effect for delivery of therapeutic molecules to cancer cells (Malhi et al., 2012). While it has been shown *in vitro* that an increase in lipoplex size to >1000 nm is associated with improved transfection activity (Ross and Hui, 1999), particles with diameters >200 nm are more readily cleared by the reticuloendothelial system *in vivo* (Alexis et al., 2008). The 'stealth' aspect afforded by PEGylation and the associated size reduction leads to improved pharmacokinetic properties of lipoplexes (Kesharwani et al., 2012). However the polymer reduces fusion of lipoplexes with endosomal membranes thereby negatively affecting release of cargo nucleic acid from endosomes.

3.4. Zeta potential

Zeta potential is an indicator of the electric potential in the interfacial double layer of nanoparticle:DNA complexes and is affected by the charge density of the cationic moiety at the surface of the liposomes (Nie et al., 2011). The interaction of cationic liposomes with pDNA is dominated by the electrostatic interaction of the polyanionic DNA and positively charged liposomal cytofectins

and hydrophobic interaction (Zuidam and Barenholz, 1997). The positive charges on the surface of the complexes promote tight binding of the nanoparticles to the negatively charged cellular membrane, thereby facilitating their entry into the cells by endocytosis (Morille et al., 2008). The charge neutralization point of the anionic DNA:cationic lipid complex is considered as one of the most fundamental parameters of a lipoplex, being defined as the cationic lipid:DNA ratio at which the charge ratio of the lipoplex equals 1. At this point, positive charges of liposomes neutralize the negative charges of the DNA stoichiometrically (Cuomo et al., 2012). It has been shown that positively charged lipoplexes may also be taken up by cells using a temperature-dependent transport mechanism, while negatively charged lipoplexes favour an energy independent transport, which could be driven by lipid mixing (Resina et al., 2009). The zeta potential of the unPEGylated untargeted liposomes in this study reflects a clear positive value, while that of PEGylated liposomes, whether untargeted or targeted, was close to zero (Table 1). This may be attributed, in part, to some shielding of the cationic centres by PEG and the contribution of negative charges on the appended folate moieties, which are located closer to the slipping plane than the cationic centres (Weijun et al., 2004). From the results obtained it may be concluded that the small negative zeta potential values point to a population of cationic liposomes whose charge is substantially adumbrated by membrane-embedded polyethylene glycol, which has caused a shift of the slipping plane further away from the liposome bilayer thus reducing the zeta potential (Kim et al., 2009). While the surface potential on cationic liposomes is positive, the zeta potential may be somewhat different as is seen in the PEGylated liposomes prepared in this investigation. Indeed PEGylation has been shown to reduce zeta potential of cationic liposomes by as much as 30 mV (Meyer et al., 1998; Zhang et al., 2010). The outward extension of PEG₂₀₀₀ from the liposome bilayer substantially

increases drag, thereby reducing the mobility of lipoplexes during measurement of the electrokinetic (zeta) potential (Kim et al., 2009), resulting in low readings. In a related study, the introduction of the folate ligand onto chitosan was shown to be accompanied by a reduction in particle size and a decrease in zeta potential (Fernandes et al., 2012).

3.5. Gel retardation assay

The ability of the liposomes to bind to DNA was assessed using a gel retardation assay. The electrostatic interaction between the cationic liposomes and plasmid DNA neutralizes the negative charge of the phosphate groups on the DNA backbone, thus retarding the DNA mobility in an electric field. To transport pDNA to cells, the cationic liposome vectors should be able to complex pDNA through electrostatic interaction. The partial or complete charge neutralization of pDNA by the vector results in complete retardation of pDNA and no migration toward the anode (Simoes et al., 1999). Incomplete pDNA binding was noted at (+/–) ratios of 1:1, 2:1 (Fig. 3A–C, lanes 2 and 3). At lipoplex (+/–) ratios >2, migration of pDNA was completely prevented, indicating tight complex formation between the liposome and pDNA. These results are consistent with previous reports that employed agarose gel electrophoresis to examine cationic lipid–pDNA binding (Simoes et al., 1999) and lipopolylysine:DNA complexes (Eastman et al., 1997).

3.6. Nuclease protection assay

Folate receptor-targeted liposomes in this study have been formulated with PEG₂₀₀₀, anchored to the liposomal bilayer, by attachment to DSPE. It has been established that PEGylation affords lipoplexes protection from opsonization and elimination by the reticuloendothelial system (RES) (Kuai et al., 2011). However, there remains the possibility that the cargo DNA may not be sufficiently protected from degradation by serum nucleases during its extended period in circulation. Therefore, a serum nuclease digestion assay was performed on the lipoplexes to examine the integrity of liposome-bound pDNA after exposure to foetal calf serum (10% v/v) for 4 h at 37 °C. Results presented in (Fig. 4A) confirm that the DNA underwent some nicking, as evidenced by the increase in the closed circular form of the plasmid and associated decline of the super helical species. The naked pDNA in lane 2 was however extensively degraded under the same incubation conditions. These results demonstrate that the cationic liposomes in this study have the potential to provide pDNA a high degree of stability in a nuclease rich environment and therefore may be used

as non-viral vectors for transporting integral pDNA into the cells (Schatzlein, 2001; Merdan et al., 2002).

3.7. Transmission electron microscopy

The liposome morphology was analyzed by cryo-TEM. At end point charge ratios established from gel retardation assays, the liposomes in complexes appeared more compacted than those in the absence of pDNA (Fig. 5). The observed vesicle fusion, twisting and wrapping on one another was due to the presence of nucleic acid molecules that induced deformation of the original liposomes. These findings are in accordance with those of Laura et al. (2007). A few large aggregates observed in the liposome:DNA ratios investigated, were probably due to aggregation caused during sample preparation. Liposomes, which appeared as unilamellar vesicles showed spherical, unimodal, relatively narrow size distributions in the range of 50–100 nm, whilst lipoplexes ranged from 100 to 200 nm except for the untargeted-unPEGylated lipoplex which had a size of approximately 1055 nm in diameter. Otherwise no significant differences in sizes between targeted and untargeted liposomes were seen in micrographs.

3.8. EtBr intercalation assay

The ethidium bromide assay was used to evaluate the extent to which lipoplexes were able to condense and compact the DNA. Ethidium bromide is a monovalent DNA-intercalating agent whose fluorescence is dramatically enhanced upon binding to DNA and quenched when displaced by higher affinity compounds or by condensation of the DNA structure (Duarte et al., 2011). Initially charge neutralization is believed to occur, which is followed by condensation of the nucleic acid (Geall and Blagbrough, 2000). It is noted, in Fig. 6, that EtBr fluorescence decreased with increasing (+/–) charge ratio of lipoplexes, indicating that an increase in the amount of cationic liposomes led to a higher degree of DNA condensation. Results presented in (Fig. 6) also show ethidium displacement to be the greatest with untargeted, unPEGylated liposomes indicating a higher degree of DNA condensation in the corresponding lipoplexes than in the PEGylated-untargeted lipoplexes, whilst DNA in targeted-PEGylated complexes was least condensed and more tenuously bound (least ethidium displacement). At low grafting density, as is the case in this study, the PEG chains assume a ‘mushroom’ regime, in which the the polymer chains protrude 30–50 Å from the liposomal bilayer and do not interact laterally (Barenholz, 2001). Furthermore the PEG polymer chains provide ‘steric stabilization’ to liposomes by attracting a water shell around them (Tirosh et al., 1998). This arrangement may lead to a reduction in DNA compaction and more tenuous

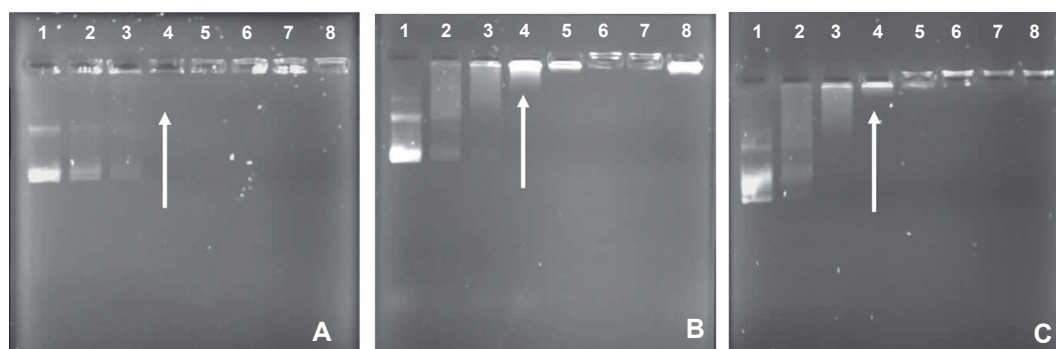


Fig. 3. Gel retardation assays. (A) untargeted, (B) untargeted PEGylated, and (C) targeted PEGylated liposomes. Cationic liposomes were complexed with pCMV-luc plasmid DNA (0.5 µg) at various weight ratios to achieve the following (+/–) charge ratios: 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1 (lanes 2, 3, 4, 5, 6, 7 and 8, respectively). Lane 1 contained 0.5 µg pDNA alone. White arrows indicate endpoint at which the pDNA was completely bound to cationic liposomes.

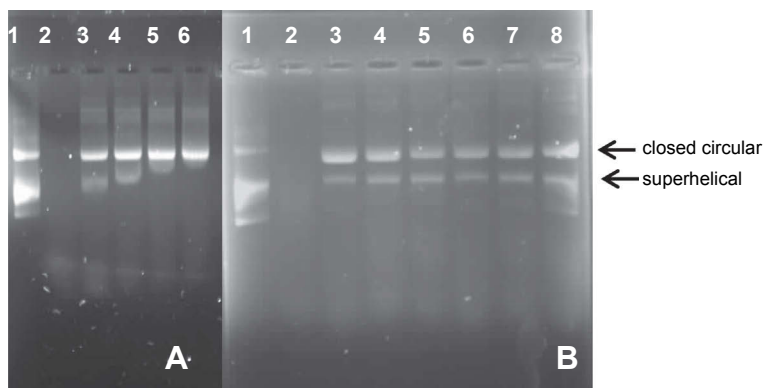


Fig. 4. Serum nuclease protection assay of SGO4 liposomes. Reaction mixtures (10 μ l) contained pCMV-luc DNA lipoplex suspensions from below to above end point ratios (2:1, 3:1, 4:1). Lane 1: naked plasmid DNA; lane 2: plasmid DNA with serum. (A) Lanes 3–6: untargeted-unPEGylated liposomes (4, 6, 8, 10 μ g). (B) Lanes 3–5: untargeted-PEGylated liposomes (4, 6, 8 μ g); lanes 6–8: targeted-PEGylated liposomes (4, 6, 8 μ g). All lanes contained 0.5 μ g plasmid DNA.

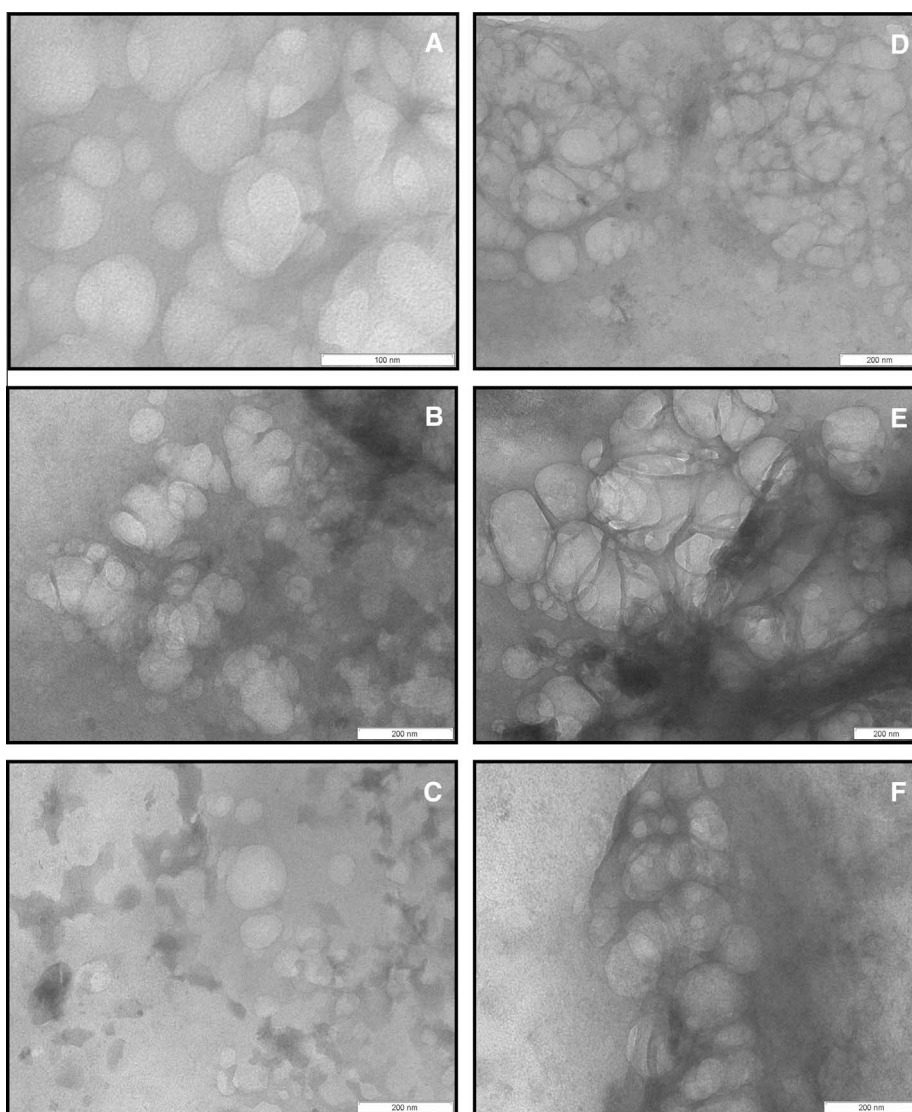


Fig. 5. Transmission electron micrographs of liposomes and lipoplexes. (A) Untargeted-unPEGylated, (B) untargeted-PEGylated, (C) targeted-PEGylated liposomes and (D–F): their respective lipoplexes. The scale bar indicates 200 nm except for (A) (100 nm).

binding of the nucleic acid (Narainpersad et al., 2012). Liposome:DNA complex end point (+/–) ratios determined for the

unPEGylated, PEGylated and targeted liposomes were 2.7:1, 2.3:1 and 2.4:1 respectively. Although a slight difference was noted

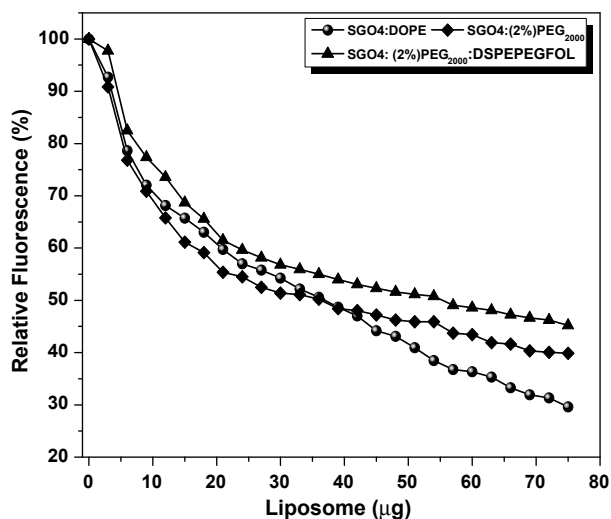


Fig. 6. Ethidium bromide intercalation assay. Incubation mixtures in HBS (100 μ l) contained pCMV-luc plasmid DNA (1.2 μ g) and increasing amounts of liposome dispersion in 1 μ l aliquots.

between these ratios and those obtained in retardation studies, it can be said that the assays are in good agreement with each other and corroborative.

3.9. Cell viability assay

Cell viability assays were performed using the MTT assay. All lipoplexes were generally well tolerated in the HEK293, HeLa and KB cell lines under transfection conditions. Good cell viability was obtained, with higher than 75% viability noted (Fig. 7), independent of the liposome formulation (targeted or untargeted) and the cell line used.

3.10. Transfection assay

A reporter gene expression assay was used for evaluating the *in vitro* gene delivery efficiency of SG04 lipoplexes in three human transformed epithelial cell lines (HEK293, HeLa, KB) (Fig. 8A–C), in the presence of 10% FBS, using the pCMV-luc plasmid vector encoding the luciferase gene. The incorporation of DOPE into liposomes improves the endosomal release of lipoplexes by promoting

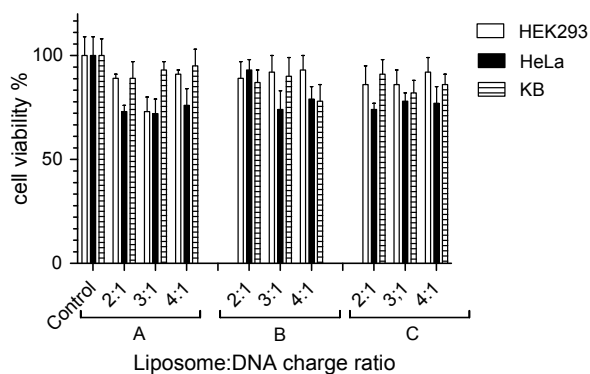


Fig. 7. Cell viability studies on liposome-pCMV-luc plasmid DNA complexes in HEK293, HeLa and KB cell lines. Incubation mixtures in complete medium (300 μ l) contained plasmid DNA (0.5 μ g) complexed to liposomes at different (+/–) charge ratios as indicated. Control: untreated cells. Data are presented as means \pm SD ($n = 3$). (A) Untargeted unPEGylated; (B) untargeted PEGylated; (C) targeted PEGylated.

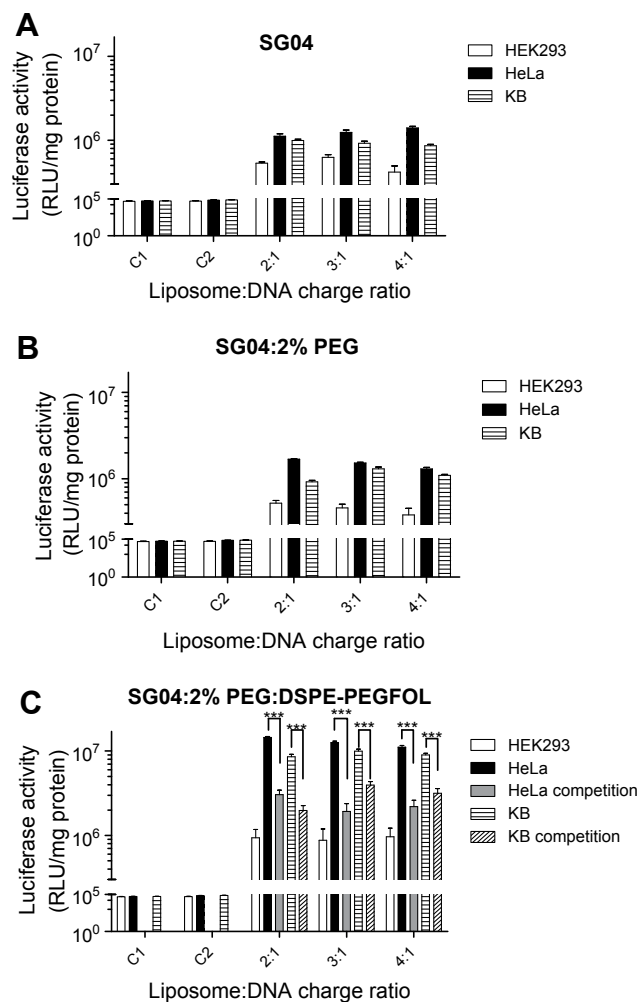


Fig. 8. Transfection studies of SG04 cationic liposome:DNA complexes in HEK293, HeLa and KB cells. Lipoplex mixtures contained 0.5 μ g of plasmid DNA and varying amounts of liposome dispersions to achieve (+/–) ratios from below to above end point ratios were incubated with cells in complete medium (300 μ l). Competition experiments with HeLa and KB cells included 200 μ M folate. Luciferase activity is expressed as RLU/mg protein. Control 1: untreated cells; Control 2: cells with naked DNA. (A) Untargeted unPEGylated lipoplexes, (B) untargeted PEGylated lipoplexes, (C) targeted PEGylated lipoplexes. Data are presented as means \pm SD ($n = 3$). A significant difference was noted in the presence of the competitor (***) $p < 0.001$.

the conversion of the lipoplex lamellar phase to an inverted hexagonal micellar arrangement (H_{II}) at endosomal pH, which exposes hydrophobic acyl chains that interfere with and destabilize endosomal bilayers. This, in turn, may contribute to efficient endosomal lysis and intracellular trafficking of plasmid DNA (Morille et al., 2008). After endosomal escape within target cells DNA complexes are believed to fuse to the nuclear membrane followed by the release of DNA in the nucleus (Kamiya et al., 2002). For the unPEGylated liposomes, a linear increase in transfection activity was obtained in HeLa cells with increasing (+/–) ratio (2:1–4:1), while a linear decrease in transfection levels was observed in KB cells over the same range (Fig. 8A). By contrast the untargeted, PEGylated liposomes gave the highest transfection activity in HeLa cells at a (+/–) ratio of 2:1 (Fig. 8B), whereas in KB cells peak activity was achieved at a ratio of 3:1. In a related study by Kim et al. (2007), it was reported that PEGylated lipoplexes promoted higher transfection efficiencies than their unPEGylated counterparts in the presence of serum. In addition, PEGylated lipoplexes display improved stabilities and longer circulation times in the blood. It is thought that the PEG forms a steric barrier around the lipoplexes,

Table 1
Liposome and lipoplex composition, size, and zeta potential.

Formulation	Liposome							Lipoplex				
	Composition ($\mu\text{mol}/0.5\text{ ml}$)							Charge ratio (+/-) ^a	Size ^b (nm)	PDI	Zeta potential ^b (mV)	
	SGO4	DOPE	DSPE- PEG ₂₀₀₀	DSPE- PEGFOL	Size ^b (nm)	PDI	Zeta potential ^b (mV)					
SGO4:DOPE	1	1	–	–	89 \pm 0.84	0.19	36.07 \pm 1.91	3:1	1055 \pm 66.2	0.64	1.91 \pm 10.58	
SGO4:DOPE:2%PEG ₂₀₀₀	1	0.96	0.04	–	77 \pm 0.58	0.17	–1.03 \pm 0.35	3:1	195 \pm 3.0	0.33	–1.79 \pm 1.02	
SGO4:DOPE:2%PEG ₂₀₀₀ :DSPE- PEGFOL	1	0.96	0.04	0.01	78 \pm 0.83	0.21	–0.38 \pm 0.03	3:1	147 \pm 0.6	0.15	–2.09 \pm 0.85	

^a Charge ratios were calculated assuming that SGO4 carries a positive charge at physiological pH and that the average MW of pDNA nucleotides is 330 Da with each carrying one negative charge.

^b The results are given as the mean \pm SD ($n = 3$).

which stifles clearance due to reduced macrophage uptake (Daniel and Godbey, 2011). PEGylation may also reduce the tendency of lipoplexes to aggregate through steric repulsion between the PEG chains. Gene delivery mediated by the folate receptor can be demonstrated by comparing the transfection efficiency between FR-positive and FR-negative cell lines (Elnakat and Ratnam, 2004; Hattori and Maitani, 2005). As can be seen in Fig. 8C, transfection levels attained by targeted-PEGylated lipoplexes for all charge ratios in HeLa and KB cells in the presence of 10% (v/v) FBS were an order of magnitude greater than those achieved by either unPEGylated or PEGylated untargeted lipoplexes ($p < 0.01$). Notably, transfection levels obtained by all three lipoplex formulations in the folate receptor-negative line HEK293 were low. Lipoplexes formulated for this study have been assembled at (+/–) ratios at, and close to, the ratios that ensure complete binding of DNA by the respective cationic liposome preparations (Fig. 3A–C). Consequently ζ potentials of lipoplexes are close to zero (Table 1) and electrostatic interaction with the negatively charged plasma membrane is therefore minimized. Under these conditions ligand-receptor interaction was believed to be the dominant lipoplex–cell interaction mechanism. During the course of this investigation Urbiola et al. (2013) have independently shown that lipopolyplexes, ornamented non-covalently with folate, transfected HeLa and B16-F10 cells efficiently in the presence of high levels of FBS. It has been shown that the transfection potential of the multi-cationic cytofectin DOSPA, found in lipofectamine, is greatly reduced in the presence of serum as there is significant competition between anionic proteins and DNA. By contrast, rigid cholesteryl-based monocationic cytofectins are less affected by the presence of serum (Ghosh et al., 2000).

3.11. Competition assay

A competition assay was carried out to validate folate receptor-mediated transfection of FR-targeted liposomes using the folate receptor positive HeLa and KB cell lines. The results (Fig. 8C) demonstrate that pre-treatment with free folate (200 μM) as a competitor, essentially prevented folate-tagged lipoplexes from binding to, and being transported into HeLa, KB cells ($p < 0.001$). More particularly, transfection activities were reduced by 85% and 70% respectively.

4. Conclusion

In summary, FA-tagged cationic liposomes containing the novel cholesteryl cytofectin SGO4, which features a monocationic centre, a 15 Å spacer and biodegradable amide and carbamate linkages, displayed very favourable physicochemical and FR-targeting properties when assembled in DNA lipoplexes. The relatively hydrophilic spacer element in SGO4 is expected to provide improved,

more prominent, display of the cationic centre, which in PEGylated liposomes is an important consideration due to the shielding effect of the polymer. All lipoplex formulations exhibited low cytotoxicity in the cell lines selected for this study. These findings suggest that FR-targeted liposomes, which display the ligand folic acid at the distal end of liposome-anchored PEG₂₀₀₀ are potentially useful for delivery of DNA therapeutics. PEGylated lipoplex particles were <200 nm in diameter, a characteristic which is necessary for whole organism intravenous applications requiring extravasation of complexes to reach target cells. In addition, we have shown that the FR-targeted lipoplexes achieved high transfection levels in the presence of serum and were specifically taken up by FR over-expressing cells, overwhelmingly by FR-mediation. The results obtained from this study suggest that, FR-targeted liposomes containing SGO4 might constitute a suitable candidate for future clinical development of gene/drug delivery vectors. The results obtained from this study may therefore provide a simple and promising strategy for the design of efficient lipid-based delivery systems for practical *in vivo* application.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ejps.2014.04.012>.

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Folate Receptor Targeted Gene Delivery Using Cationic Liposomes as Nonviral Vectors

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Abstract. Gene therapy is a promising strategy for the treatment of human diseases rooted in genetic disorders such as cancer. The success of gene therapy however depends on the efficient delivery of therapeutic genes into target cells in vitro and in vivo. Liposomes have shown the potential to be ligand-conjugated and receptor targeted. Our aim is to develop and test a lipid-based system for efficient targeted gene delivery to the folate receptors that are overexpressed in a broad spectrum of malignant tumors viz. Hence it represents an attractive target for selective delivery of anticancer agents to folate receptor expressing tumors. Novel cationic liposomes were formulated with and without the targeting ligand, folate. Folate conjugated liposomes were prepared using the cationic cholesterol derivative *N,N*-dimethylaminopropylamidodisuccinylcholesterylformyl-hydrazide (MSO9), the helper-lipid, dioleoylphosphatidylethanolamine (DOPE), and distearoylphosphatidylethanolamine (DSPEPEG₂₀₀₀) which was conjugated to folate. DNA-binding and protection abilities of all liposomes have been confirmed by band shift assays, dye displacement assays, and nuclease protection assays. The complexes were evaluated in an in vitro system for cytotoxicity using the MTT assay, and finally gene regulation using the luciferase reporter gene assay. Relatively low cytotoxicities were observed and encouraging gene expression levels were noted. This research will have a significant impact in the targeting of genes or drugs to cancer cells in vivo.

Keywords: Gene Therapy; Non-viral Vectors; Lipid Nanoparticles; Folate Receptor.

1. Introduction

Gene therapy involves the delivery of a specific gene (DNA) to the targeted cells thus combating the disease at the level of its origin. Successful Gene therapy relies on devising methods for efficient transport of nucleic acids through the cell membrane into the nucleus [1]. Targeted gene delivery systems have been used to increase the efficiency of drug/gene delivery to specific tissues as well as to optimize the minimum effective dose of the drug and its side effects. Cationic liposomes are good non-viral vectors, since they readily form complexes with DNA via electrostatic interactions [2]. Folic acid is involved in essential one carbon transfer reactions that are important in DNA synthesis and replication, cell division, growth and survival, particularly for rapidly dividing cells. Conjugates of folic acid can be taken up by cancer cells via receptor-mediated endocytosis, thus providing a mechanism for targeted delivery to FR⁺ cells [3].

2. Methods

Cationic liposomes were prepared using the method [4], with or without the conjugated lipid DSPE-PEG-FA. Briefly, MSO9 and the helper lipid DOPE were dissolved in CHCl₃ and deposited as a thin film on the inner wall of the test tube by evaporation of the solvent in vacuo. The dried lipid film was rehydrated overnight at 4°C in a solution containing 20 mM HEPES and 150 mM NaCl (pH 7.5). The resulting liposome suspension was briefly vortexed and sonicated. Size and structure of cationic liposomes and lipoplexes were established by Zetasizing, and cryoTEM. Lipoplex formation and DNA protection abilities were studied by band shift, nuclease protection and ethidium bromide assays. Growth inhibition studies of the complexes were determined using the MTT assay and luciferase gene expression levels were assayed using the Luciferase Reporter gene assays (Promega) and expressed as RLU/mg protein.

3. Results and Discussion

3.1. Gel retardation assay

Agarose gel electrophoresis of cationic lipid:DNA complexes was used to assess the relative amounts of DNA that were free or incorporated into the complex as a function of the lipid:DNA ratio. DNA in a lipid complex did not migrate out of the well. This was most likely the result of the charge neutralization. The Fig.1 shows that the amount of uncomplexed, or free DNA decreased as the ratio of lipid:DNA was increased.

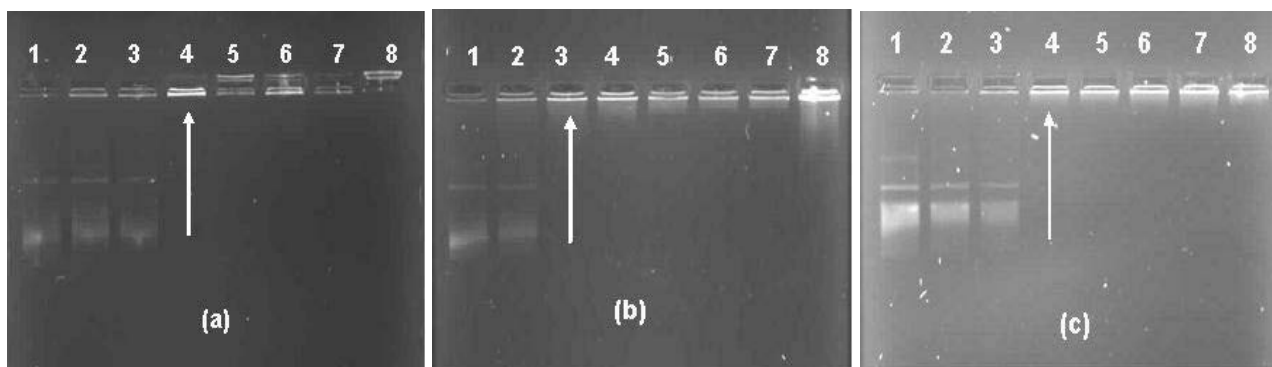


Fig 1: Gel retardation assays. Cationic liposomes were complexed with pDNA at various weight ratios. The weight ratio of cationic lipid/pDNA (a, b, c) was 1:1, 2:1, 3:1, 4:1, 5:1, 6:1,7:1 (lanes 2, 3, 4, 5, 6, 7 and 8, respectively). Lane 1, 0.5 μ g plasmid DNA only.

3.2. Cytotoxicity assay

For in vitro toxicity study cells (HeLa, HEK293) were incubated with three formulations (plain and PEG coated and folic acid conjugated liposomes) for 48 h in 48-well microtitre plates. Control cells were taken without formulations and incubated with medium. Cell viability assay was performed using MTT assay. Percent cell viability was determined using control as 100%. Results obtained were, the pegylated liposomes were slightly toxic to the cells as compared to plain and FA-targeted liposomes (see Fig.2).

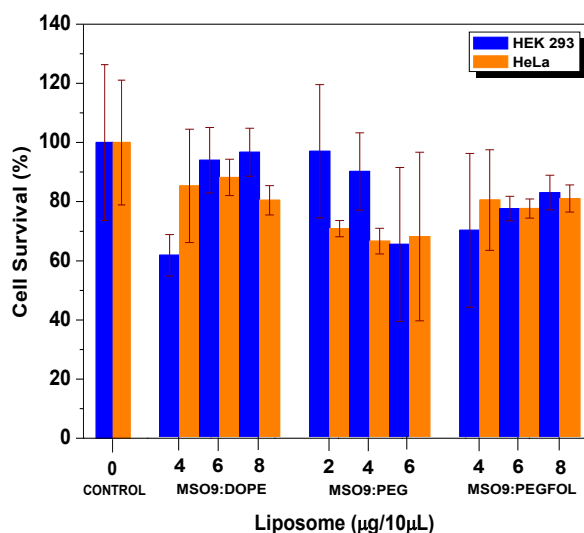


Fig 2: In vitro Growth inhibition studies of liposome:pCMV-luc DNA complexes in HEK293, HeLa cell lines. Incubation mixtures (10 μ L) contained 0.5 μ g of plasmid DNA. Varying amounts of liposome from its suboptimal to supraoptimal ratios were assayed. Control: untreated cells. Data are presented as means \pm SD (n= 3).

3.3. Zeta sizing

The relevance of the parameter 'particle size' in gene delivery by non viral vectors is known. The particle size of the vector influences the internalization pathway of particles through the cell membrane. The preferred particle size would be 100-200 nm, in theory. This point is specially required for in vivo gene delivery in order to be small enough to allow systematic delivery [5]. Particle sizes of complexes were

measured by dynamic light scattering in the absence or presence of folate ligand and the folate modification did change the sizes of liposomes. The average particle sizes of cationic liposomes (MSO9) used in this study were 196 nm (untargeted), 121 nm (untargeted, pegylated), 168 nm (pegylated, targeted) (see Table 1). The reason for the smaller pegylated liposomes could be the repulsion feature of PEG molecules that prevent the liposomal aggregation.

Table 1: Particle sizes of liposomes and lipoplexes at their end point retardation ratios.

Formulation	Lipid/DNA charge ratio	Liposome		Lipoplex	
		Size (nm)	PDI	Size (nm)	PDI
MSO9:DOPE	3:1	196	0.22	695	0.57
MSO9:DOPE:DSPEPEG 2000	2:1	121	0.23	106	0.21
MSO9:DOPE:DSPEPEG 2000:DSPEPEGFOL	3:1	168	0.33	191	0.47

3.4. Transfection assay

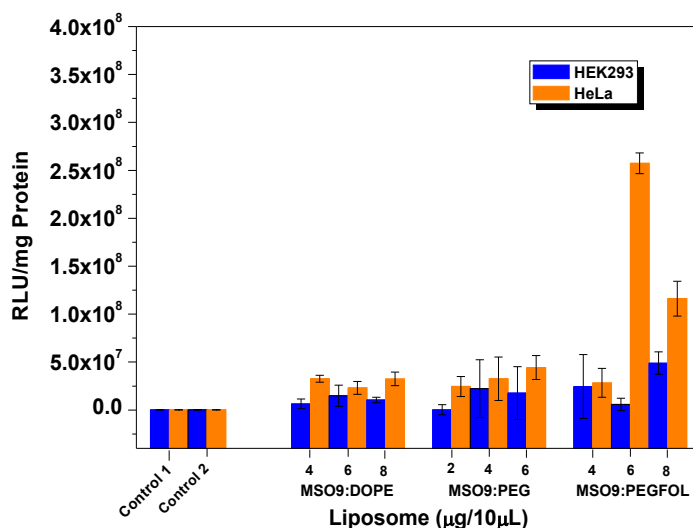


Fig.3: Transfection studies of liposome-plasmid DNA complexes in HEK293 and HeLa cells in vitro. Incubation mixtures (10µL) contained 0.5µg of plasmid DNA. Varying amounts of liposome from slightly below to slightly above end point ratios were assayed. Luciferase activity is expressed as RLU/mg soluble protein. Control 1: untreated cells; Control 2: plasmid DNA alone. Data are presented as means ± SD (n= 3).

In Fig.3, the folate:liposome:DNA complexes showed a two-fold increase in transfection activity compared to plain (without PEG or FA) liposomes and pegylated liposomes. This suggests that FA presence in liposome;DNA complexes facilitates the uptake of the FA-liposome:pDNA into the HeLa cells via receptor mediation. Low transfection levels were achieved for HEK293 cells (receptor negative cells). Significant transfection levels for HeLa cells for FR-targeted liposomes were seen at their 3:1 (+/-) ratio or (6 µg/0.5µg). These findings also support the notion that the lipoplexes with the sizes range from 100-200 nm are suitable to traverse the cell membrane to reach the nucleus. Lipoplexes achieved high transfection levels falls in this range.

4. Conclusions

FR-targeted liposomes, synthesized using F-PEG-DSPE has been shown previously to effectively target FR-expressing tumor cells. It is further shown in this study that FR-targeted lipoplexes had poor cytotoxicity and this indicates that FR-targeted liposomes are potentially useful for delivery of therapeutic agents. In addition, FR- targeted lipoplexes showed poor cytotoxicity, high transfection levels, and can be specifically

taken up by FR over expressing cells. In summary, the cationic liposome (MSO9) containing FA ligand had good physical chemical and FR targeting properties. The results obtained from this study suggest that, FR-targeted liposomes may constitute a better candidate for future clinical development of gene/drug in vivo.

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6. References

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