ELUCIDATION OF GENE FUNCTION USING RNA INTERFERENCE IN A CANCER CELL CULTURE MODEL

by

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

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ABSTRACT

RNA interference (RNAi), mediated by small interfering RNA (siRNA), has emerged as a powerful tool for elucidating gene function and also holds great potential for the treatment of acquired and inherited diseases. The delivery of siRNAs still remains a major obstacle for their therapeutic use. Cationic liposomes, a group of positively charged nanovesicles, represent a class of non-viral vectors that have shown the ability to efficiently bind and deliver siRNA.

In this study, six cationic liposome formulations containing either cationic cholesterol derivative [N-(N',N'-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) or N,N-dimethylaminopropylaminylsuccinylcholesterylformyl- hydrazide (MSO9) were prepared with the neutral lipid dioleoylphosphatidylethanolamine (DOPE). Varying amounts of distearoylphosphatidylethanolamine polyethylene glycol 2000 (DSPE-PEG_{2000}), (0, 2 and 5 mole percent) were also included in the liposomal formulations as polyethylene glycol is known to improve the lipoplex bioavailability *in vivo*.

We present evidence that siRNA may be delivered to mammalian cells, *in vitro*, using a novel cationic liposome carrier system and that siRNA binding and transfection efficiency of the cationic liposomes are affected by the degree of pegylation.

Cryoelectron microscopy revealed that the liposome vesicles were unilamellar and were in the 30 - 130 nm size range, while band shift assays confirmed the formation of complexes between the siRNA and the liposome preparations. These siRNA lipoplexes were shown to afford protection to their siRNA cargoes against serum nuclease degradation and were also shown to be relatively non-toxic to the HeLa *tat luc* cell line which stably expresses the firefly luciferase gene. Cryoelectron microscopy revealed that an inverse relationship exists between the lipoplex size and the degree of pegylation. To determine the transfection efficiency of the cationic liposome preparations in the HeLa *tat luc* cell line, complexes were prepared with anti-luciferase siRNA, which is specific for the firefly luciferase gene, and knockdown of the luciferase gene was monitored in transfected cells. The results show that liposomes containing the cytofectin

Chol-T were particularly effective, achieving up to 93.4% gene knockdown at the 30 nM siRNA concentration. The non- pegylated and pegylated cationic liposomes that have been formulated and examined in this study therefore warrant further development to facilitate *in vivo* studies.

PREFACE

The experimental work described in this dissertation was carried out in the Discipline of Biochemistry, School of Biochemistry, Genetics and Microbiology, University of KwaZulu-Natal, Durban from February 2010 to December 2011, 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.

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DECLARATION 1- PLAGIARISM

I, Aliscia Nicole Daniels , 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)*.

Publication 1: Peer Reviewed Published Abstract- Appendix.

Singh M., Daniels A.N. and Ariatti M. (2011). *'Novel cationic liposomes mediate silencing of luciferase gene in the HeLa tat luc cell line'*. Human Gene Therapy. **22(2)**: 93.

Publication 2: Related publication from BSc Honours Project. (Accepted: March 2011).

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
ATP	Adenosine-5’-triphosphate
BCA	Bicinchoninic acid
BICS	Block Ionomer complexes
Chol-T	3 β [N-(N’, N’-dimethylaminopropane)-carbamoyl] cholesterol
DCC	Dicyclohexylcarbodiimide
DC-CHOL	3β [N-(N’,N’- dimethylaminoethane)- carbamoyl] cholesterol
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DOPE	Dioleoylphosphatidylethanolamine
DOTAP	N- [1-(2,3-dioleoyloxy)propyl]- N,N,N-trimethylammonium methyl sulphate
DOTMA	N- [1-(2,3-dioleyloxy)propyl]- N,N,N-trimethylammonium chloride
DSPE-PEG	Distearoylphosphatidylethanolamine polyethylene glycol
dsRNA	Double stranded RNA
EDTA	Ethylenediaminetetraacetic acid
FBS	Foetal bovine serum
HBS	HEPES buffered saline
HEPES	2-[4-(2-hydoxyethyl)-1-piperazinyl] ethanesulphonic acid
IPEC	Interpolyelectrolyte complexes

LUV	Large unilamellar vesicles
MEM	Minimal essential medium
MLV	Multilamellar vesicles
MPS	Mononuclear phagocyte system
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mRNA	Messenger RNA
MSO9	N,N-dimethylaminopropylaminylsuccinylcholesterylformyl- hydrazide
PAZ domain	Piwi, Argonaute, Zwille domain
PBS	Phosphate buffered saline
PEI	Polyethylene imine
PEG	Polyethylene glycol
PEO	Polyethylene oxide
PHPMA	poly [(N-2-hydroxypropyl)methacrylamide]
RISC	RNA Induced Silencing Complex
RES	Reticuloendothelial system
RLU	Relative light units
RNA	Ribonucleic acid
RNAi	RNA Interference
rRNA	Ribosomal RNA
Rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate

siRNA	Small Interfering RNA
shRNA	Short hairpin RNA
ssRNA	Single stranded RNA
SUV	Small unilamellar vesicles
TEM	Transmission Electron Microscopy
tRNA	Transfer RNA
Tris	Tris(hydroxymethyl)aminomethane

CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

1.1 AN OVERVIEW OF GENE THERAPY AND RNA INTERFERENCE

The concept of gene therapy has been developed for the treatment of both inherited and acquired diseases. Many laboratories in biochemistry, medicine and pharmacy are focusing their research on gene therapy (El-Aneed, 2004). This research has now become quite broad. Gene therapy does not strictly refer to the treatment of a disorder by introducing a functional gene to replace a defective gene by gene transfer, but rather, it can now be defined by the introduction of nucleic acids, either RNA or DNA, which can be used to prevent or treat an inherited or acquired disease (El-Aneed, 2004; Robbins and Ghivizzani, 1998). Many genetic conditions, some forms of cancer and certain viral infections such as AIDS can be potentially treated with gene therapy (El-Aneed, 2004).

For many years, it was thought that RNA was a molecule that could function either as a messenger (mRNA) or form part of the translation machinery (tRNA, rRNA) (Brantl, 2002). After much research, it was found that RNA molecules are quite versatile and that they play important roles in many biological processes such as splicing, editing, protein export, among others. However, it is now well established that they also have the ability to act catalytically (Brantl, 2002).

The use of small interfering RNA (siRNA) for gene therapy has attracted attention as a new therapeutic approach for the treatment of various diseases (Kim *et al.*, 2010; Zhang *et al.*, 2010). Over the past decade, much interest has been given to RNA Interference (RNAi) for analyzing mammalian gene functions, both *in vitro* and *in vivo* and for the silencing of gene expression (Kim *et al.,* 2010; Leung and Whittaker, 2005). The phenomenon of RNAi involves a sequence-specific gene silencing brought about by small double stranded RNA (dsRNA) molecules termed siRNA (Zhang *et al.*, 2007). The siRNA consist of 21-23 nucleotides with a duplex region of 19 base pairs (Kim *et al.*, 2010). A characteristic feature of siRNA is the presence of a two nucleotide 3' overhang which is recognized by the enzymatic RNAi machinery. This results in homology dependant target mRNA cleavage (Meister and Tuschl, 2004).

The RNAi discovery occurred as a convergence of different experiments that were not related (Hammond, 2005). In the late 1980's, RNAi was first discovered by plant biologists who found gene silencing pathways in plants and fungi brought about by transgene expression or viral replication, but the exact mechanism was not fully understood (Hammond, 2005; Zhang *et al.*, 2007). The groundbreaking discovery by Andrew Fire and Craig Mello in the late 1990's showed that dsRNA could bring about gene silencing in *Caenorhabditis elegans* (Fire *et al.*, 1998). Their studies showed that RNAi is an evolutionary conserved gene silencing mechanism and that interference of the targeted gene was more effective when using dsRNA as opposed to using either individual strand (Zhang *et al.*, 2007). Since the discovery of RNAi, it is rapidly becoming the preferred method for gene function analysis. There are many advantages in using the process of RNAi over other therapeutic methods. These include a high specificity of the siRNA with low toxicity as is often observed in antisense methods and chemotherapy. Moreover, the siRNAs show a greater resistance to nuclease degradation as compared with antisense oligonucleotides therefore increasing the therapeutic effects of RNAi as compared to antisense therapy (Zhang *et al.*, 2007).

1.2 MECHANISM OF RNA INTERFERENCE (RNAi)

RNAi functions naturally as a defence mechanism against viruses which produce dsRNA in the host cell when they are active and against transposons. The specific degradation of the mRNA during the RNAi process prevents the replication of the viruses (Elbashir *et al.,* 2000). When siRNA is introduced, the RNAi process is initiated. The siRNA is produced from exogenous or endogenous long dsRNA (Kong *et al.,* 2007). In an initiation step, the long dsRNA is identified by the endonuclease DICER. This endonuclease is a specific enzyme for dsRNA and cleaves the long dsRNA into the shorter siRNAs (Zhang *et al.*, 2007) The DICER enzyme has an N-terminal RNA helicase domain, a Piwi, Argonaute, Zwille (PAZ) domain, two RNase III domains and a C-terminal dsRNA binding motif (Kim and Rossi, 2007). DICER is found complexed to the TAR-RNA binding protein (TRBP) (Aagaard and Rossi, 2007). The step that follows is referred to as the effector step. In this step, the siRNA is released from the DICER-TRBP complex and forms part of a nuclease complex called the RNA- induced silencing complex (RISC) (Zhang *et al.,* 2007; Hammond, 2005; Aagaard and Rossi, 2007). The RISC complex contains Argonaute 2 which is responsible for melting the ds siRNA and cleaves the target mRNA (Aagaard and Rossi, 2007). The cleavage of the ds siRNA results in the formation of an activated form of the RISC complex which contains a ss RNA molecule, being the anti-sense strand of the siRNA (Hammond, 2005; Aagaard and Rossi, 2007). This ‘new’ single stranded RNA (ssRNA)- RISC complex then recognizes complimentary mRNA sequences, this recognition is guided by the anti-sense RNA strand within the activated RISC complex (Ryther *et al.*, 2005; Aagaard and Rossi, 2007). This occurs by Watson-Crick base pairing in the cytoplasm (Elmén *et al.*, 2005). The target mRNA is then cleaved between bases 10 and 11 relative to the 5’ end of the antisense siRNA by Argonaute 2 and, therefore, the translation of the target mRNA is inhibited (Hammond, 2005; Aagaard and Rossi, 2007). If the siRNA/mRNA duplex contains any mismatches, the target mRNA will not be degraded (Hammond, 2005). Therefore, it can be said that gene silencing is brought about as a result of translational inhibition (Zhang *et al.*, 2007, Hammond, 2005). The RNAi pathway is illustrated in Figure 1.1

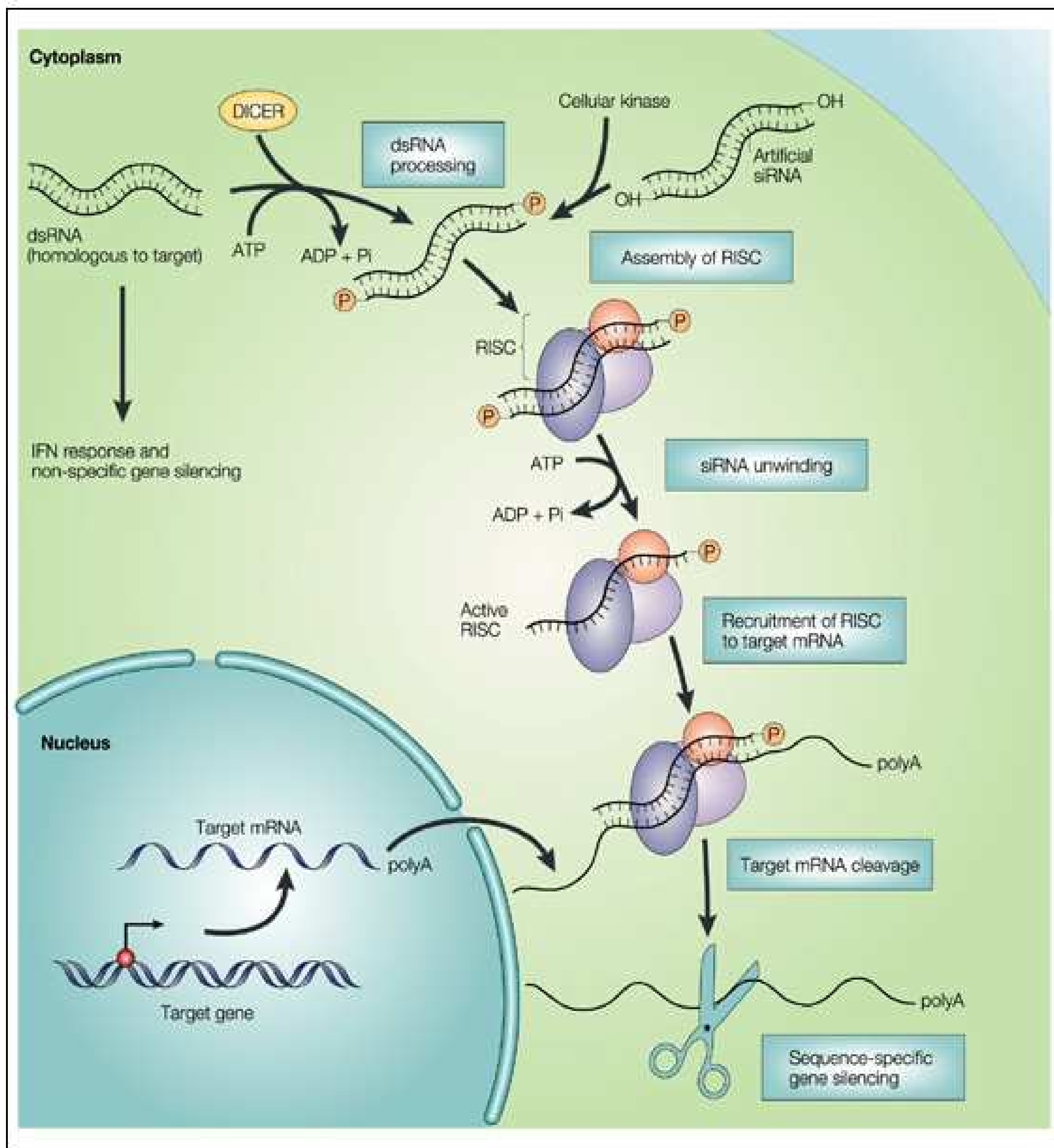


Figure 1.1 Illustration of the mechanism of the RNA Interference Pathway (Stevenson, 2003)

RNAi can be initiated by short synthetic dsRNA molecules made up of 21-23 nucleotides in mammalian cells (Figure 1.2). These short RNA duplexes will be able to guide the cleavage of the complimentary mRNA sequence and hence, the corresponding protein production is stopped. The dsRNA can be introduced into the cytoplasm through the cleavage of long dsRNA by DICER to siRNA; chemically synthesized siRNA can be introduced into the cytosol which will bypass the DICER step; plasmids or viral vectors can be used which encode either of the two RNA strands or by action of endogenous microRNAs (miRNAs). The miRNA maturation is initiated by nuclear cleavage carried out by DROSHA RNase III into a 60-70 nucleotide intermediate. In all these mechanisms the activity of RISC is activated and the expression of the mRNA is inhibited. However, the introduction of siRNAs results in the degradation of the mRNA while miRNAs repress the translation of the target mRNA by partial complementarity. This mechanism by miRNAs may be responsible for some of the 'off target' effects which are observed in some cases (Masiero *et al*., 2006). Since RNAi results in transient sequence specific interference of gene expression, a new category of molecules, that are nucleic acid based, is created and has significant medical potential for the treatment of diseases (Thomas *et al.*, 2007). It can be said that every human disease that results due to the activity of one or many genes should have the potential to be treated by RNAi based therapeutics (Aagaard and Rossi, 2007), some of these diseases are outlined in table 1.1. Therefore, it has been said that in biology, the discovery of RNAi is one of the most important in recent years (Zhang *et al.,* 2007; Hammond, 2005; Thomas *et al.*, 2007).

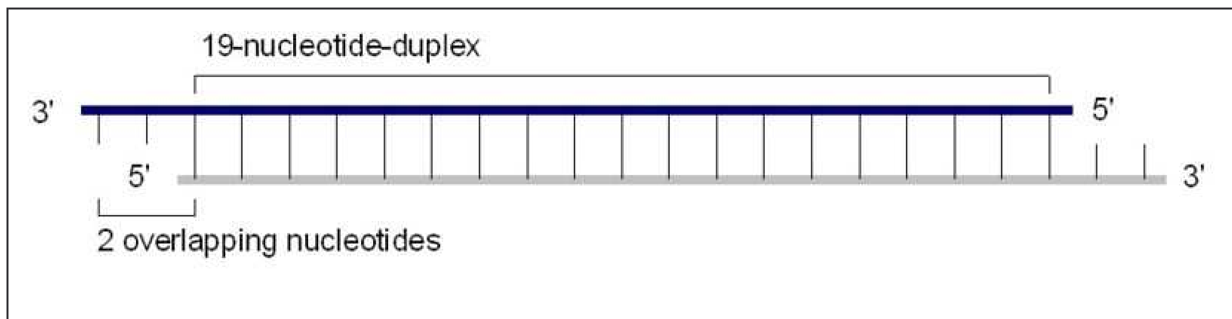


Figure 1.2: Schematic representation of a siRNA molecule (www.openwetware.org/wiki/Griffin:siRNA_transfection)

Table 1.1 Examples of human diseases with potential targets for RNAi based therapeutics (Cheng *et al.*, 2003)

DISEASE	**mRNA TARGETED**
Leukemia	BCR/ABL fusion, CREB
Neuroblastoma	N-Myc
Carcinomas	Ras
Malaria	Falcipain-1,2
HIV	p24 Gag, CCR5
Cryptococcus neoformans	CAP 59, ADE2
Influenza	Nucleoplasmid(NP), RNA transcriptase
Human Papilloma virus	E6, E7

The ability to use synthetic siRNAs to induce RNAi in mammalian cells has stimulated interest in its therapeutic potential (Lu *et al.*, 2009). If siRNA can be targeted to an oncogene, it can be used as a therapeutic agent in cancer therapy (Gao and Huang, 2008). The growing potential of RNAi for the treatment of genetically based and infectious diseases will not be able to achieve its goals without resolving the issue of gene delivery (El-Aneed, 2003). The success of gene therapy is greatly dependant on the delivery vector (Gao and Huang, 2008). One of the main objectives of gene therapy is the transfer of the genetic material to the targeted cells. The gene therapy application influences the aim of the delivery system (El-Aneed, 2003). The question of delivery still remains the biggest hurdle which has to be solved in order to avoid any setbacks during therapeutic application (Shi *et al.*, 2003).

1.3 GENE DELIVERY VEHICLES

In order for the effectiveness of RNAi to be achieved, the siRNA needs to reach the cytoplasm of the target cell. The efficient delivery of siRNA still remains a main challenge for RNAi based therapeutics. This obstacle arises due to the properties of siRNA. Firstly, the naked siRNAs have a half life time of less than an hour in human plasma (Zhang *et al.*, 2007). Due to the size of the siRNA, which is usually 21 nucleotides in length, circulating siRNAs are excreted by kidneys quite rapidly. Furthermore, the naked siRNA is highly charged and cannot penetrate the lipid membranes of cells and lastly, circulating naked siRNAs are susceptible to degradation by the enzyme RNase A and nucleases (Lu *et al.*, 2009; Tseng *et al.,* 2009). Due to these hurdles, there is a need to develop siRNA delivery systems that are capable of efficiently introducing siRNA into the cytoplasm (Han *et al.*, 2008).

Many gene delivery systems that exist to date were originally designed for the delivery of DNA and many have been adapted for the delivery of siRNA, since the barriers to delivery are similar (Lu *et al.*, 2009, Thomas *et al.*, 2007). The differences that exist between DNA and siRNA include the size and electrostatic charge of the DNA which is much larger than that of siRNA (typical molecular weights are 1600kDa and 15kDa, respectively) as well as their site of action (Lu *et al.,* 2009). Nuclear entry is required for DNA in order for it to access the transcriptional machinery whereas siRNA is a post-transcriptional process and mediates its effect in the cytoplasm of the target cells therefore, the delivery of siRNA may be more easily achieved as compared to DNA delivery in non-dividing cells (Lu *et al.*, 2009; Thomas *et al.*, 2007). These differences could affect the function and the efficiency of the gene delivery system; however, since DNA and RNA share chemical similarity, their delivery barriers are likely to be similar. Both DNA and siRNA need to cross the cellular membrane and should be able to successfully escape degradation in the lysosome (Thomas *et al.*, 2007). The success of gene therapy is dependent on the gene delivery vehicle, which is divided into two categories, namely, viral and non-viral vectors, both of which shall be discussed briefly.

1.3.1 Viral Vectors

Viral vectors are viruses that have been modified such that they are replication defective. This allows nucleic acids to be introduced into the cell via the natural mechanisms of viral cell infection (Masiero *et al.*, 2007). Viral vectors are considered as one of the most efficient gene transfection agents due to their transduction efficiency and also the ability of some viral vectors to infect non-dividing cells (Ramon *et al.*, 2008). Retroviruses, adenoviruses and adeno-associated viruses are some of the different types of viral vectors that can be used for siRNA delivery. Within many of the viral vectors, a short hairpin RNA (shRNA) will be encoded which is found between polymerase (pol) III promoter (H1 or H6) and a transcription termination site (Masiero *et al.*, 2007). Following transcription, the RNA folds which results in the formation of a stem-loop structure which is initially processed in the nucleus and thereafter within the cytoplasm. The shRNA is then converted to a small RNA molecule which is approximately 21 nucleotides in length. This small RNA molecule is capable of inducing RNAi (Ramon, *et al.,* 2008).

Retroviral vectors are based on the murine stem cell virus or Moloney murine leukemia virus (Masiero *et al.*, 2007). They are developed by replacing the viral genes with the therapeutic genes. The delivery of foreign genetic material using the retroviral vectors was first described in 1981 (El-Aneed, 2004). Retroviruses are small RNA viruses that have a DNA intermediate. They are capable of entering a variety of host cells and are capable of integrating into the host genome. This can only be done in replicating cells. A disadvantage with the use of these retroviral vectors is that accidental random integration in the host genome which may result in deleterious effects such as activation of proto-oncogenes through insertional mutagenesis (Seth, 2005). Adenoviruses are double stranded DNA viruses that are capable of infecting dividing and non-dividing cells. The adenoviral vectors are developed by replacing the viral *E1* gene with the therapeutic genetic material. Immunological responses to the adenoviral vector is a limitation which can be potentially harmful to the patients. Adeno-associated viruses are single stranded DNA viruses. Their two viral proteins, *Rep* and *Cap*, are removed which makes them defective

and allows them to be used in gene therapy. The adeno-associated viral vectors are also capable of infecting dividing and non-dividing cells. The main limitation is that the adeno-associated viral vectors require helper viruses (adenoviruses, herpes simplex virus) for adeno-associated virus production, which may result in contamination of the adeno-associated viruses during production (Seth, 2005).

Viral vectors have a high gene transfer efficiency and are still considered as a powerful method of gene transfer. However, as mentioned above, there are many limitations that exist when using viral vectors. Some of the viral vectors have a very small loading capacity, large scale production is difficult and they pose severe safety risks because of their oncogenic potential, and immunogenic and inflammatory effects. These prevent viral vectors from being administered repeatedly. In order to overcome these limitations, non-viral vectors have emerged as an attractive alternative to viral vectors (Gao and Huang, 2008)

1.3.2 Non- Viral Delivery Systems

Although the use of viral vectors has shown to result in higher transfection efficiency in most cell lines tested, non-viral vectors have attracted more attention for their use as gene delivery vehicles (Akhtar and Benter, 2007). This can be attributed to the many advantages associated with non-viral delivery vehicles. These advantages include the ease of synthesis, unrestricted size of gene material, low immune response and the potential safety benefits (Zhang *et al.*, 2007). The aim of non-viral gene therapy is to mimic viral methods to overcome the cellular barriers but minimize toxicity associated with viral approaches (Balicki and Beutler, 2002). Physical and chemical methods can efficiently introduce siRNA to mammalian cells *in vitro*, but there are a few *in vivo* delivery systems that exist. Physical methods that have been studied for siRNA delivery include hydrodynamic injection, electroporation and the gene gun method. The chemical, synthetic approaches that have been studied include the use of cationic lipids and polymers, as well as nanoparticles. These non-viral vectors shall be discussed briefly.

1.3.2.1 Physical Non-Viral Delivery Approaches

1.3.2.1.1 Hydrodynamic Injection

The hydrodynamic approach involves the rapid injection of siRNA in a large volume of physiological buffer (Gao and Huang, 2008; Davis, 2002). This quick injection results in a transient increase in the venous pressure and results in the entry of the nucleic acid primarily to the liver (Rozema and Lewis, 2003). Song *et al.* (2003), introduced siRNA by utilizing many hydrodynamic injections which resulted in 90% knockdown of expression of the endogenous *Fas* receptor in hepatocytes (Song *et al*., 2003). McCaffery *et al.* (2002), used hydrodynamic injection to transfect the luciferase gene with siRNA or shRNA in mice which resulted in reduced expression of the luciferase gene in the liver (McCaffrey *et al.*, 2002). Due to the invasive nature of this technique, as well as the generation of high pressure in the vascular system due to the injection of large volumes which could result in heart failure, the use of hydrodynamic injection is currently not viable for human clinical applications at this point (Spagnou *et al.,* 2004; Vandenbrouke *et al.,* 2008; Gao and Huang, 2008).

1.3.2.1.2. Electroporation

The electroporation technique was designed to allow for the penetration of molecules into a cell due to the application of an electric field. DNA and RNA can be effectively introduced using this method. Electroporation involves two stages: in the first stage, an electric shock creates pores in the cell membranes and in the second stage, electrophoresis is applied which allows the negatively charged molecules to enter the cytosol (Ramon *et al*., 2008). Electroporation was used by Akaneya and co-workers (2005) to deliver siRNA to the brain which resulted in inhibiton of gene expression of the *GluR2* and *cox-1* genes (Akaneya *et al.*, 2005). Targeting can be achieved by using electroporation but this technique requires electrodes to be inserted into the target area

and, therefore, for this to be achieved, invasive procedures are required to expose the target tissue for transfer, which limits the application of electroporation (Vandenbrouke *et al.*, 2008).

1.3.2.1.3. Gene Gun Method

The gene gun method involves the firing of siRNA coated gold particles. This allows for the direct delivery of nucleic acids to the tissue or cells. The gene gun approach allows for siRNA to directly penetrate the cell membrane and enter the cytoplasm which is done by bypassing the endosomal compartment (Ramon *et al*., 2008). Kim and colleagues (2005) made use of the gene gun method for the delivery of siRNA *in vivo*. This was done to enhance a vaccine's effectiveness (Kim *et al*., 2005). The gene gun approach can only be local and the tissues are not deeply penetrated by the nucleic acids using this approach (Ramon *et al.*, 2005; Niidome and Huang, 2002).

In these limited applications, it is possible for the siRNA to be delivered directly to the target sites, however, in most applications, a carrier system is necessary for the protection of the siRNA against degradation and to guide in the uptake of the siRNA by the target cell (Almofti *et al.*, 2002).

1.3.2.2 Chemical Non-Viral Delivery Approaches

The carrier systems that exist are synthetic systems that usually contain a cationic component, for example, cationic lipids, cationic polymers and cationic peptides (Almofti *et al.*, 2002). The cationic component binds to the negatively charged siRNA. A chemical non-viral delivery vector can also include specific binding to the surface of the cell, cellular entry, escape from the endosome, among others (Balicki and Beutler, 2002).

1.3.2.2.1 Polymer Nanoparticles

Polymer nanoparticles have been used for the delivery of siRNA. These nanoparticles can be synthetic from a number of different polymers, which include poly-ethyleneimine (PEI), chitosan as well as PEG-branched polymers. These polymer nanoparticles are polycations or they contain polycation block polymers which are capable of forming interpolyelectrolyte complexes (IPECs) or block ionomer complexes (BICs), respectively with the siRNA (Zhang *et al.*, 2007). IPEC systems that are being used are made up of complexed polycations for example, poly-L-lysine and PEI together with nucleic acids (DNA and RNA). Self assembly of these complexes occurs due to the strong electrostatics between the oppositely charged polyelectrolytes which results in prevention of enzymatic degradation of the incorporated nucleic acid in the blood stream (Niidome and Huang, 2002). BICs have been recently synthesized which could overcome the problems of circulation which is present in conventional systems. Poly-ethylene oxide (PEO) and poly [(N-2-hydroxypropyl)methacrylamide] (PHPMA) are usually chosen as the neutral block. They are hydrophilic and non-immunogenic. The cationic segments are synthetic amine polymers which bind to the nucleic acid (Zhang *et al.*, 2007).

(a) Polyethylene imine (PEI)

Polyethylene imine (PEI) consists of a series of synthetic polymers with a very high cationic charge. This positive charge allows PEI to form complexes with the nucleic acids and condense them. This tight compaction together with their buffering capacity in endosomes and lysosomes protect the nucleic acids against being degraded. Branched PEI (25 and 800kDa) and linear PEI (25kDa), have been utilized as transfection agents. Efficient *in vitro* and *in vivo* transfection was shown using branched PEI (800 kDa) linked to a ligand. Much attention has been given to linear PEI for the delivery of siRNA (Zhang *et al.*, 2007).

(b) Chitosan

Chitosan has gained much interest in recent years as a safe and cost effective non-viral based delivery system for gene materials. Chitosan has shown low levels of immunogenicity and toxicity, very high levels of biodegradability and a very high positive charge which, through electrostatic interactions, can form complexes with the negatively charged nucleotides (El-Aneed, 2004). There have been many studies on chitosan as a carrier for DNA delivery. The results have shown efficient expression of the reporter genes *in vitro* and *in vivo* and, therefore, have increased interest in their use as a carrier for siRNA. siRNA delivery using chitosan may have first been studied by Katas and co-workers in 2006 (Katas and Alpar, 2006). A novel chitosan mediated siRNA delivery system was developed by Howard *et al.* (2006) for RNAi *in vitro* and *in vivo*. The results showed knockdown was evident of the endogenous enhanced green fluorescent protein (EGFP) in human lung carcinoma cells and murine macrophages (72.9% and 89.3% reduction in expression, respectively) (Howard *et al.,* 2006).

1.3.2.2.2 Liposomes

The dispersion of phospholipids in an aqueous medium results in the spontaneous formation of closed structures consisting of phospholipid bilayer membranes that encapsulate an aqueous cavity. This system is referred to as a liposome. Liposomes, therefore, are referred to as vesicular, spherical shaped systems which can be produced from cholesterols, sphingolipids, glycolipids, long chain fatty acids and even membrane proteins (Samad *et al.,* 2007). Bangham and co-workers first discovered liposomes approximately 40 years ago following the initial publication of their use in 1965. The practicality and the value of the use of liposomes has been since identified and improved and are at present useful tools in science (Bangham, 1980; Samad *et al.*, 2007; Balazs and Godbey, 2010). A variety of molecules including small drug molecules, nucleotides and proteins have been incorporated into liposomal systems and delivered to cells.

Many liposomal formulations have been commercialized to date namely lipofectamine, fugene, among others.

Liposomal reagents can be divided into four categories, namely, conventional or anionic liposomes, cationic liposomes, stealth liposomes and targeted liposomes (Figure 1.3). The use of an anionic liposomal system requires entrapment of the nucleic acid in the aqueous space of the liposome. Minimal use of this type of system has occurred over the years, which could be attributed to the variable encapsulation efficiency and also since the process is time consuming (Ramon *et al.,* 2008; Singh, 1998).

Liposomal targeting can be achieved by introducing targeting moieties to the liposomal composition which can recognize and bind to target cells (Immordino *et al.*, 2006). Targeting moieties include ligands, proteins, peptides, antibodies, polysaccharides, glycolipids, glycoproteins and lectins (Kelly *et al.*, 2010). A targeting system that is commonly used is the transferrin (Tf) receptor (TfR) for binding, as well as cellular entry because these receptors are over expressed in many tumour cells (Dass and Choong, 2006). A major advantage in the use of targeted liposomes is the selective targeting of specific tissues or cell types which can result in an increase in the amount of nucleic acid delivered to the target cell (Immordino *et al.*, 2006, Kim *et al.*, 2009). Cationic liposomes and stealth liposomes will be discussed below.

The unique advantages associated with the use of liposomes over other vectors include a diverse range of morphologies, composition, ability to envelope and protect many types of therapeutic molecules, low toxicity, low immunogenic response, low cost as well as different release characteristics (Balazs and Godbey, 2010).

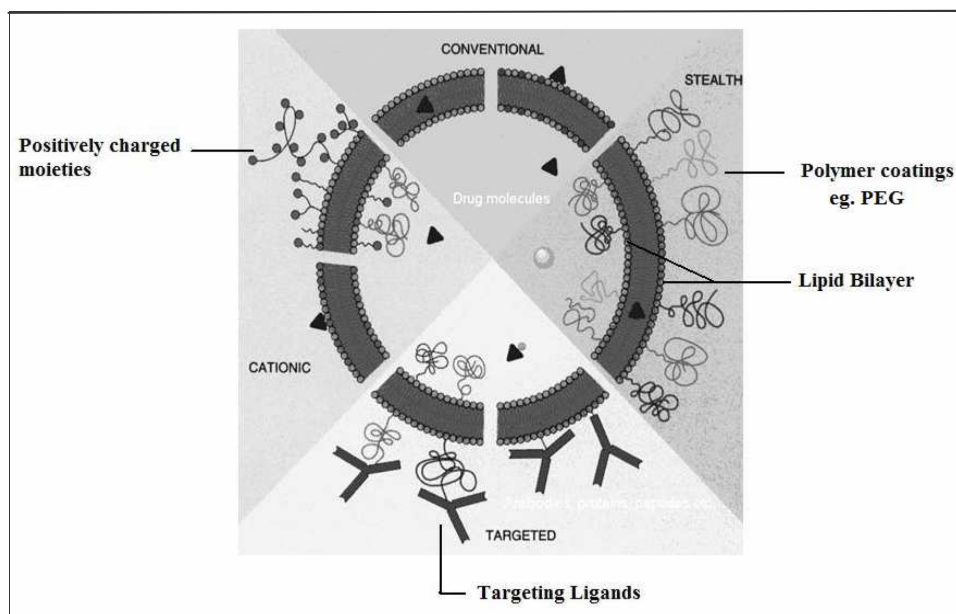


Figure 1.3: Four classes of liposomes defined by their functionality and used for gene delivery (Adapted from Lasic, 1997).

1.2.2.2.1 Cationic Liposomes

The delivery of nucleic acids using cationic liposomes was pioneered by Felgner and co- workers in the late 1980s who developed cationic liposomes consisting of a monovalent cationic lipid N-[1-(2,3-dioleyloxy) propyl]- N,N,N- trimethylammonium chloride (DOTMA) and the neutral lipid dioleoylphosphatidylethanolamine (DOPE) in a 1:1 ratio (Felgner *et al.*, 1987). Since this invention, many other cationic liposome formulations have been synthesized and are available commercially. These include DOTMA (Figure 1.4), N-[1-(2,3-dioleoyloxy) propyl]- N,N,N-trimethylammonium methyl sulphate (DOTAP) (Figure 1.5), 3β[N-(N',N'- dimethylamino-ethane)- carbamoyl] cholesterol (DC-CHOL) (Figure 1.6), among others (Miller, 1998). Cationic lipids contain the same general structure. They consist of a hydrophobic lipid anchor, a linker group for example an ester, amido or carbamate, and a positively charged head group, which in most cases is a positive amino group (Hattori *et al.*, 2008).

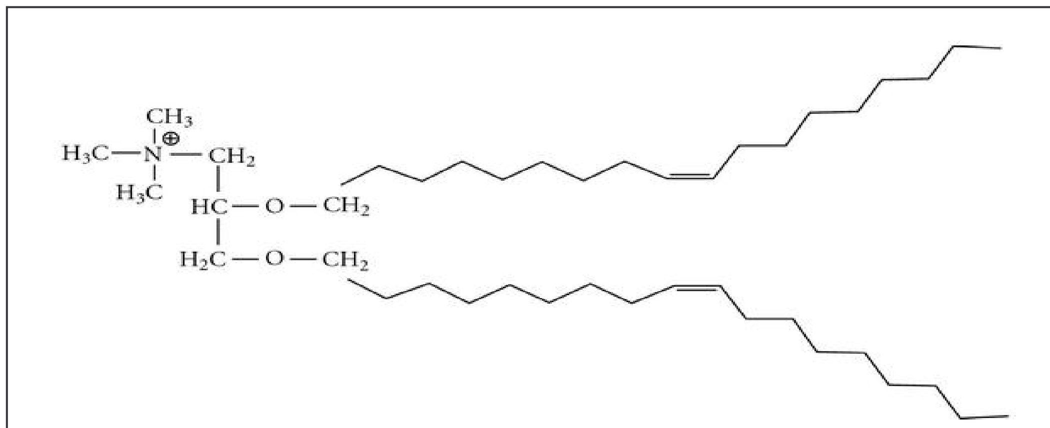


Figure 1.4: Structure of DOTMA (www.hindawi.com/journals/jdd/2011/326497)

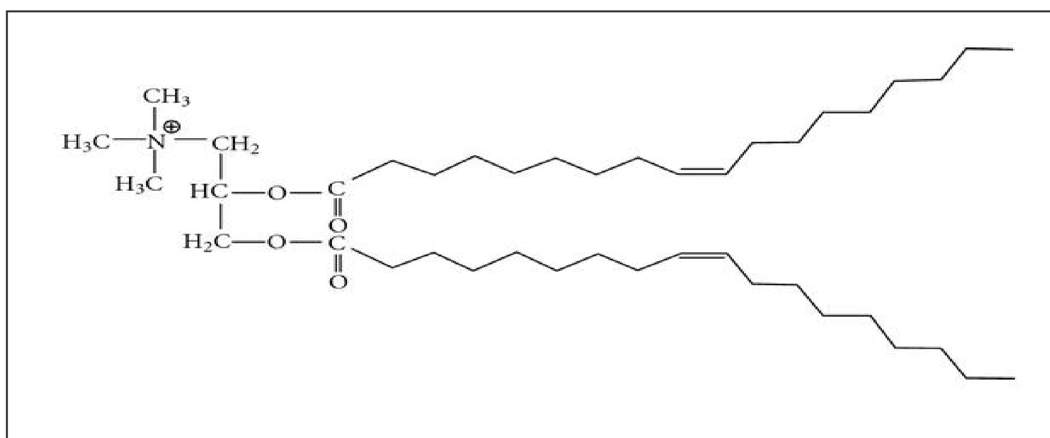


Figure 1.5: Structure of DOTAP (www.hindawi.com/journals/jdd/2011/326497)

The structure of DOTMA consists of two unsaturated oleoyl chains which are linked to a three carbon skeleton of glycerol via ether bonds and a head group consisting of a quaternary amine. In the late 1980s, DOTMA showed a higher gene transfer efficiency as compared to other gene transfer methods (Balazs and Godbey, 2010). The relative simplicity of preparing complexes with RNA or DNA together with effective gene delivery had influenced the potential of non-viral

vectors for gene therapy. The initial success with DOTMA for *in vitro* transfection resulted in the formation of other cationic liposome formulations including DOTAP and DC-CHOL (Balazs and Godbey, 2010).

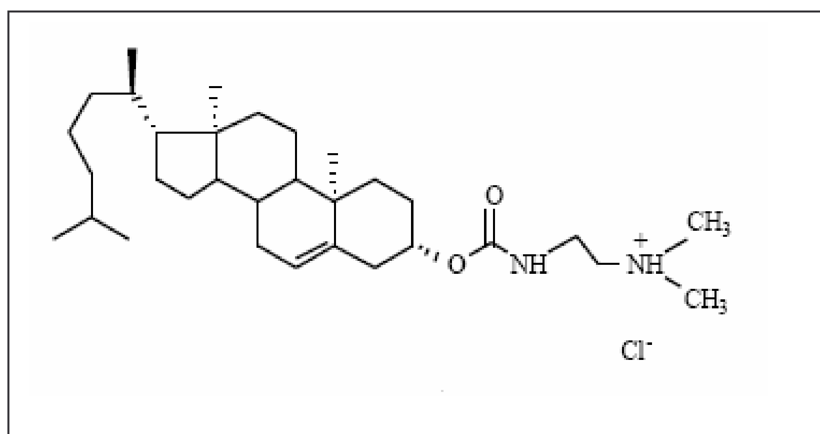


Figure 1.6: Structure of DC-CHOL (Martin *et al.,* 2005).

Leventus and Silvius synthesized DOTAP in 1990 (Leventis and Silvius, 1990). The structure of DOTAP consists of two oleoyl chains that are linked to a glycerol backbone with a quaternary amine head group. The only difference that exists between the structures of DOTAP and DOTMA is that of the linker bond, that is, the ester bond linkage between the backbone and the chains in DOTAP rather than the ether bond. Ester bonds are hydrolysable, therefore, it was hypothesized that the introduction of ester bonds could reduce cytotoxicity and make the lipid biodegradable. Studies showed that there were minimal differences between DOTAP/ DOPE and DOTMA/DOPE transfection activities and cytotoxicity. At pH 7.4, DOTAP becomes completely protonated and is usually combined with a helper lipid, which is now the case for most cationic liposome formulations (Balazs and Godbey, 2010).

DC-CHOL was synthesized in 1991 by Gao and Huang and consists of a cholesterol moiety which is linked by an ester bond to dimethylethylenediamine, which is hydrolysable (Gao and

Huang, 1991). Cholesterol was introduced due to its biocompatibility as well as the stability it imparts to the lipid membranes. This idea was supported by the increased transfection efficiency when using this liposome. The cytotoxicity associated with DC-CHOL was much lower than that of DOTMA in certain cell lines. Unlike DOTMA and DOTAP which contain fully charged quaternary amines, DC-CHOL in a 1:1 molar ratio with DOPE, has a tertiary amine function and at a pH of 7.4, is only charged on approximately 50% of the surface of the liposome. Due to this feature, the aggregation of lipoplexes with DC-CHOL is said to be reduced which, therefore, leads to higher transfection efficiencies (Balazs and Godbey, 2010).

Cationic liposomes are formulated with positively charged lipids, and, therefore, are physically associated with the nucleic acid and do not require encapsulation. These physical associations occur through electrostatic interactions between the positive charges of the cationic liposome and the negative charges of the nucleic acid which exist due to the presence of phosphate groups in the nucleic acid (Ramon *et al.*, 2008). Complexes between the cationic liposome and the nucleic acid are easily prepared by simply mixing the two components and incubating for a short period of time, which results in the nucleic acid becoming associated with the outer surface of the liposome. This method is quick, simple and does not require the separation of the liposome bound material (Lonez *et al.*, 2008). The positive charge of the head group also allows for the binding of the lipoplex to the negatively charged components that exist on the cell membrane before uptake which results in improved delivery of the nucleic acid (Lonez *et al.*, 2008).

Cationic liposomes are formulated with the cationic lipid together with the neutral lipid DOPE. DOPE is usually incorporated into the liposomal formulation due to its membrane destabilizing effects at a low pH which assists in escape from the endosome during endocytosis (Farhood *et al.*, 1995).

A major disadvantage in the use of cationic liposomes is their uptake by macrophages in the mononuclear phagocyte system (MPS) and their removal from blood circulation. This problem

can be circumvented by the introduction of poly-ethylene glycol (PEG) which coats the liposomal surface and, in doing so, increases the circulation time of the liposome.

1.2.2.2.2 Stealth Liposomes

With respect to the above mentioned hurdle, many strategies have been tested where the surface of the liposome can be coated with an inert molecule which results in the formation of a spatial barrier (Immordhino *et al.*, 2006). In initial studies, the surface of the liposome was modified by introducing monosialganglioside (G_{M1}) which increased the hydrophilicity of the liposome (Gabizon and Papahadyopoulous, 1988; Allen *et al.*, 1989). These liposomes remained in blood circulation for many hours as the MPS uptake was reduced (Immordhino *et al.*, 2006). However, there were problems associated with the availability of G_{M1} which resulted in the search for a substitute and, in turn, led to the development of the introduction of poly-ethylene glycol (PEG) into formulations (Allen, 1994).

PEG is commonly used as a polymeric steric stabilizer (Immordhino *et al.*, 2006). PEG is a linear polyether diol which is soluble in aqueous and organic media, has a low toxicity and immunogenicity, is biocompatible, and exhibits good excretion kinetics (Immordhino *et al.*, 2006). PEG can be introduced onto the liposomal surface in many ways, but the most commonly used method involves the anchoring of PEG via a cross linked lipid, that is, distearoylphosphatidylethanolamine-PEG (DSPE-PEG) into the membrane of the liposome (Allen *et al.*, 1991) (Figure 1.7). The presence of DSPE-PEG improves their stability, inhibits protein adsorption onto the liposomal surface and opsonization *in vivo* which prevents the recognition of the liposome by the reticoendothelial system (RES). This enhances the circulation time of the liposomes in the blood system (Gabizon, 2001; Garinot *et al.*, 2007).

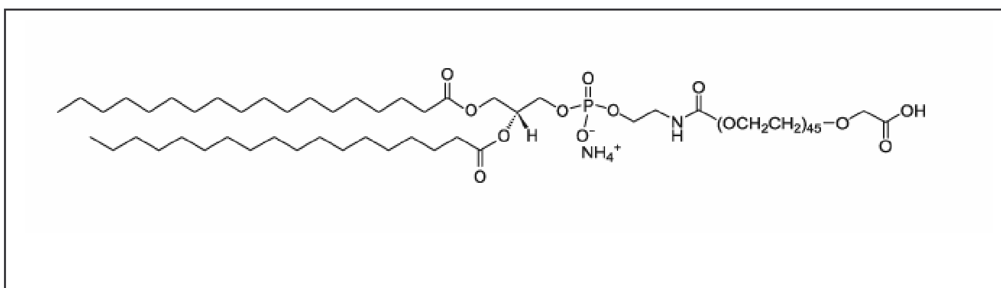


Figure 1.7: Structure of DSPE-PEG (www.avantipolarlipids.com).

There are many advantages that exist with pegylated liposomes, with the most significant being the large decrease in MPS uptake and increased circulation time in blood which results in their increased distribution. Interbilayer attraction due to the presence of Van der Waals forces is overcome due to the presence of PEG which avoids the aggregation of the liposomes, and, hence, the formulation is stabilized (Immordhino *et al.*, 2006).

The properties of the PEG molecule that is grafted onto the liposomal surface determines the behavior of the stealth liposomes. Two regimes proposed by deGennes describe the attachment of the PEG molecules onto the liposomal surface (deGennes, 1980). They are the brush and mushroom regimes, and are so named depending on the grafting density (Figure 1.8). The surface coverage and the distance between the grafting sites are determined by the graft density and the molecular mass of PEG (Immordhino *et al.*, 2006). The presence of a single elongated PEG molecule on the liposomal surface is referred to as the mushroom regime. The brush regime refers to the presence of more than one elongated PEG molecule, regardless if they exist as individual 'mushrooms' or are made up of a dense network of entangled chain. The molecular brushes are said to sterically protect the liposomes from biological interactions. This occurs by sterically stabilizing the liposomes or preventing opsonization. The mechanisms that have been suggested for this stabilization include 'repulsion' as a result of polymer chain constriction, the formation of a molecular cloud which protects the surface from opsonins or the formation of a hydrated shell that prevents the entrance of opsonins (Papisov *et al.*, 1998).

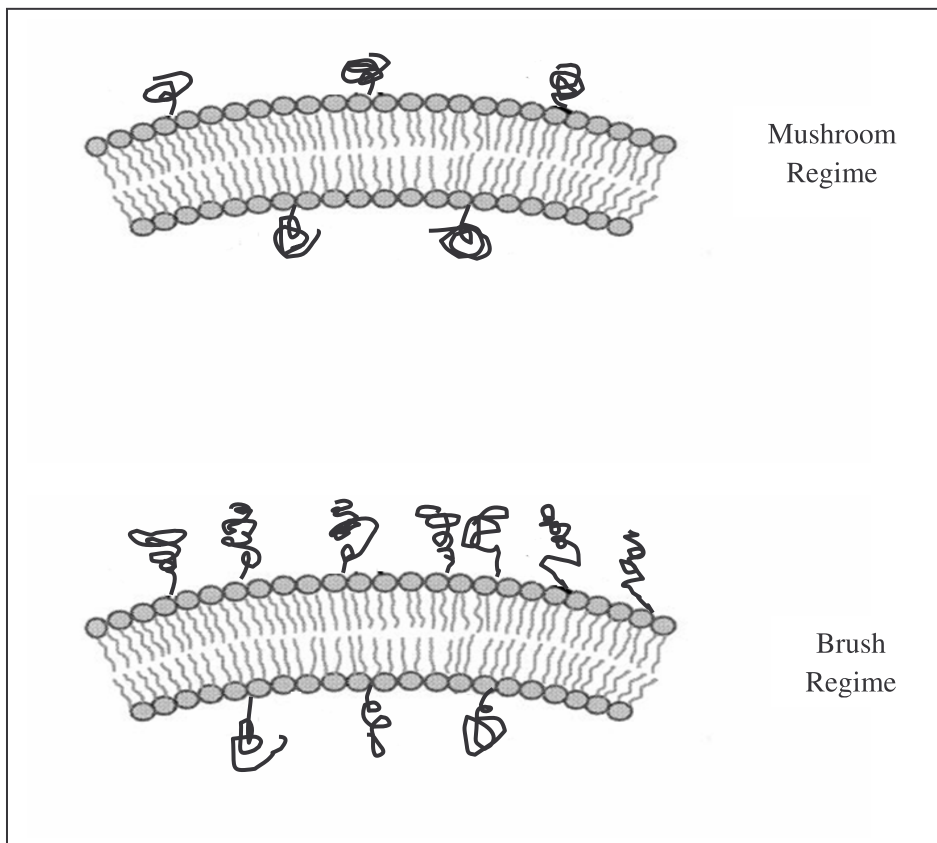


Figure 1.8: Schematic representation of the brush and mushroom regimes of PEG (Adapted from Immordhino *et al.*, 2006; Narainpersad, 2009).

A major characteristic of stealth liposomes is their increased circulation time which occurs regardless of the surface charge or the presence of a stabilizing agent. The hydrophilic shell that exists in PEG molecules has the ability to prevent aggregation between liposome particles as well as to reduce interactions between the liposome and biological fluids. This occurs not only because of the molecular mass and the uniformity of the PEG molecule, but is also due to the conformational flexibility of the polymer (Torchillin *et al.*, 1994). The circulation longevity of pegylated liposomes depends on the amount of grafted PEG as well as the length and molecular weight of the polymer (Immordhino *et al.*, 2006).

1.4 ENDOCYTOSIS

The first step in the delivery of nucleic acids by cationic liposomes is the entry of these complexes into the cells (Miller, 1998). There are three models for the interaction of cationic liposomes with mammalian cells, they are (i) fusion of the liposome with the cell or destabilization of the endosome, (ii) direct fusion of the liposome with the plasma membrane or (iii) transfer of the lipoplex across the cell membrane into the cytosol (Wrobel and Collins, 1995).

It was initially proposed that the primary mechanism for the entry of liposomes into the cell involved the direct fusion between the liposome and the cell membrane. This was based on the observation by Felgner and co-workers on the interaction between free liposomes and the plasma membrane (Felgner *et al*., 1987). However, studies have shown that the cationic liposome: nucleic acid complexes do not enter the cell by direct fusion but rather, through slow endocytosis. This was first proposed by Behr *et al.* in 1993 and was later observed by Zabner *et al.* in 1995. Electron microscopy was utilized by Zabner *et al.* to follow cell entry of the complexes (Zabner *et al*., 1995).

Once siRNA: cationic liposome complexes are prepared, the net negative charge of the siRNA is ‘hidden’ and the overall positive charge of the lipoplex facilitates its binding to the cell membrane, which is negatively charged (Figure 1.9). Once the lipoplexes are bound to the cell, the complexed siRNAs are taken up by endocytosis and are located within the endosome (Figure 1.10) (Dominska and Dykxhoorn, 2010).

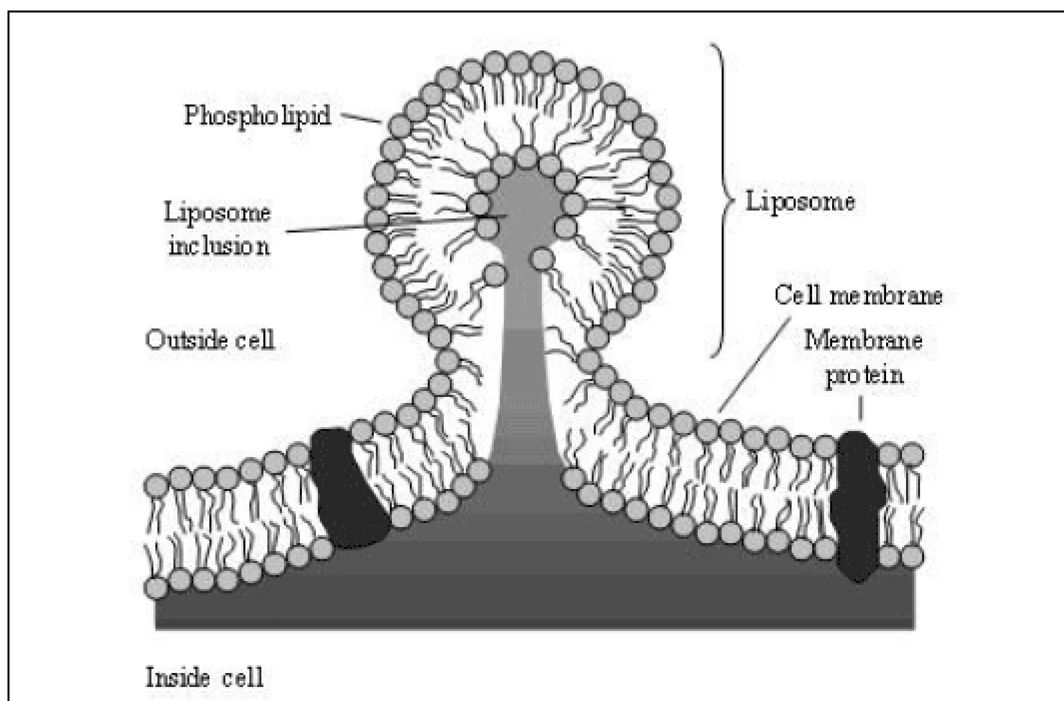


Figure 1.9: Schematic representation of the acceptance of the cationic liposome by mammalian cells (www.jpp.krakow.pl/.../articles/05_article.html)

The intracellular trafficking of the siRNA begins in the early endosome. The early endosomes fuse with sorting endosomes, which transfers their contents to late endosomes (Dominska and Dykxhoorn, 2010). The late endosomes are acidified (pH 5-6) and their content is then transferred to the lysosomes. The lysosomes are then further acidified (pH~4.5). To prevent degradation of the siRNA in the lysosomes, the siRNA needs to escape from the endosome (Dominska and Dykxhoorn, 2010). The transfection agent must have the ability to disrupt or fuse with the endosomal membrane in order to deliver the siRNA to the cytosol (Rozema and Lewis, 2003). The most common strategy used to achieve this criterium is the incorporation of DOPE into the liposomal formulation. DOPE is said to promote polymorphic changes of the lipids by promoting the formation of the reverse hexaganol phase from the lamellar liquid crystal phase (Miller, 1998). DOPE, therefore, can be used for the disruption of the endosome. Electrostatic interaction between the membranes of the endosome and the cationic liposome results in a "flip-flop" of the negatively charged lipids of the endosome that faces the cytosol which diffuses into

the complex (Miller, 1998). Here, they form neutral ion pairs with the cytofectins which results in the disruption of the lipoplex and, therefore, the nucleic acid diffuses into the cytosol (Miller, 1998). Once in the cytosol, the siRNA can associate with the RNAi machinery.

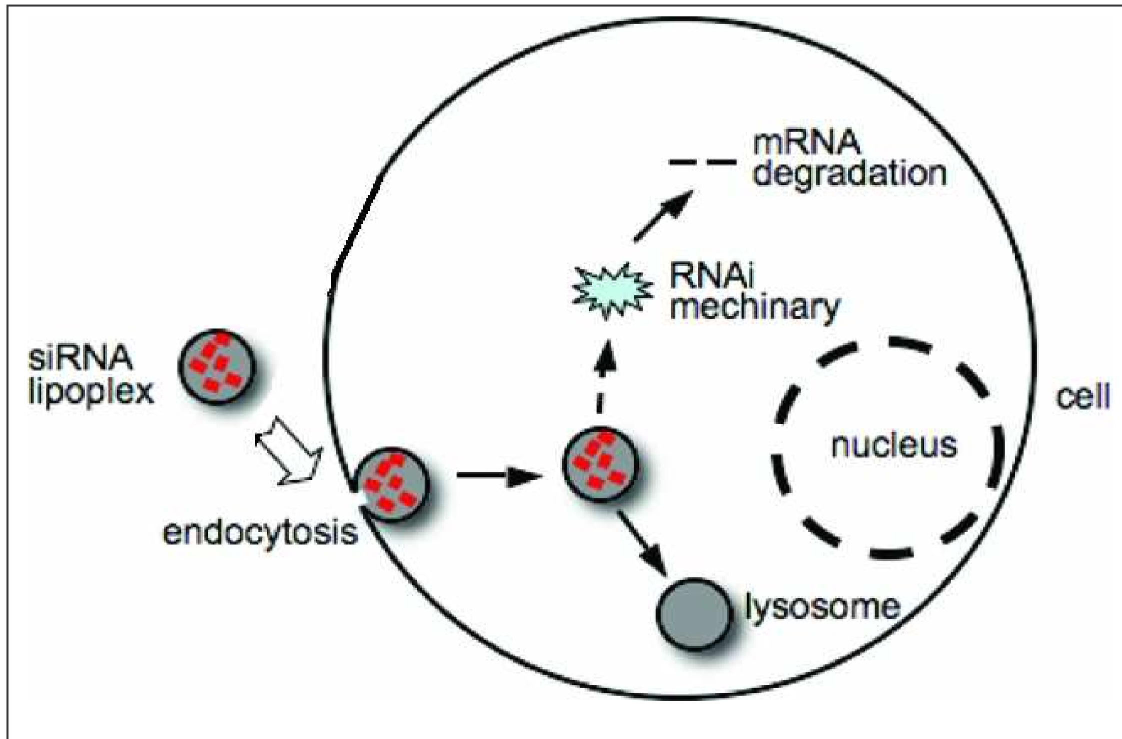


Figure 1.10: Illustration of endocytosis of siRNA: cationic liposome complex (Lu *et al.*, 2009)

1.5 OUTLINE OF THESIS

Gene therapy using siRNA has emerged as a promising approach for the treatment of various diseases including cancer and genetic disorders. However, a major challenge lies in the delivery of siRNA where intracellular and extracellular barriers need to be overcome. To date, many non-viral vectors have been studied for the delivery of siRNA (Kim *et al.*, 2010). In this thesis, the delivery of siRNA, *in vitro*, using novel cationic liposomes was investigated. Studies have

suggested that cationic liposomes generally have a poor circulation time in the blood system. The introduction of PEG is said to increase the circulation time of the cationic liposome and, in doing so, the amount of the siRNA introduced into the cell can be increased (Vandenbrouke *et al.*, 2008).

Cationic liposomes were prepared either with cytofectin Chol-T or MSO9, the neutral co-lipid DOPE and varying degrees of pegylation (0, 2, 5 mole percent). These liposomes were characterized using Transmission Electron Microscopy. Complexation of the siRNA with the cationic liposomes was determined using the gel retardation assay, and the ability of the cationic liposomes to protect the siRNA from nuclease degradation was studied with the nuclease protection assay.

Cytotoxicity and transfection studies were carried out using the HeLa *tat luc* cell line, a human cervical cancer cell line that stably expresses the firefly luciferase gene. These assays are outlined in chapter 4. The efficiency of transfection was determined using the luciferase assay where luciferase gene knockdown was determined.

A further aim of this study was to investigate the effect of pegylation on the efficiency of cationic liposome mediated siRNA delivery to the HeLa *tat luc* cell line in culture. The introduction of PEG to the liposomal formulation was carried out to enhance the circulation time and prevent opsonization of the liposomes, which would result in a more efficient delivery of the siRNA. Although pegylation is known to increase the circulation times of lipoplexes under *in vivo* conditions, it is also believed to adversely affect the cellular uptake of the lipoplexes. The introduction of the PEG moiety to the liposomal surface may partially mask the cationic charges close to the liposome bilayer adversely affecting siRNA: liposome charge-charge interactions. In this study, two related cytofectins differing in spacer arm length have been compared for siRNA transfection efficiency and also, the cellular uptake of siRNA promoted by liposomes that are pegylated up to 5 mole percent is being investigated by measuring luciferase gene knockdown in

the HeLa *tat luc* cells in culture. These studies will facilitate the development of cholesteryl cytofectin PEG liposomes for *in vivo* applications.

CHAPTER TWO

LIPOSOME PREPARATION AND CHARACTERIZATION

2.1 INTRODUCTION

This chapter focuses on the synthesis of six different cationic liposomes formulated with either cytofectin Chol-T or MSO9. All liposomes were formulated with the neutral helper lipid DOPE. Different mole percentages of distearoylphosphatidylethanolamine polyethylene glycol 2000 (DSPE-PEG_{2000}), (0, 2 and 5 mole percent) were also incorporated into the formulation.

Liposomes are defined as vesicular structures that are formed as a result of self assembly of dissolved lipids. These lipids interact with each other in a manner that is energetically favourable and results in these vesicles having one or more bilayers of the amphipathic lipid which encapsulates an aqueous compartment (Riaz, 1996; MacLachlan, 2007; Balazs and Godbey, 2010).

Upon formation of the liposomes, the lipids associate such that bimolecular leaflets are formed. These leaflets are characterized by the hydrophobic tails which face each other and the hydrophilic head groups that face the aqueous solution. At this point in the assembly, the formation of the bilayer still remains energetically unfavorable. This is due to the hydrophobic component of the lipid which is still in contact with the aqueous medium. This problem is overcome when the bilayer membrane, during its formation, curves upon itself and results in the formation of a vesicle with closed edges (Figure 2.1) (Balasz and Godbey. 2010).

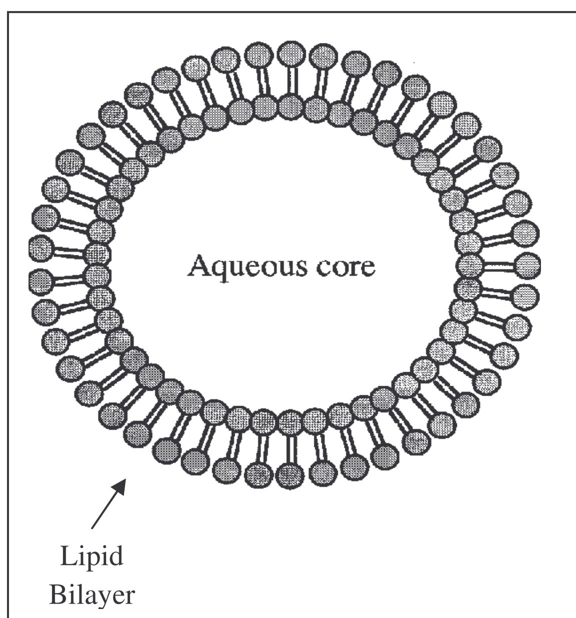


Figure 2.1: Structure of a typical liposome with the lipid bilayer and aqueous compartment (www.grin.com/object/external_document.256998/)

Liposomes are not rigid structures but are fluid entities which are versatile assemblies. Due to their dynamic preparation and the ease with which they can be manipulated, liposomes have been used for drug and gene delivery. Liposomes present unique advantages which include their range of diverse morphologies, their ability to envelope therapeutic biomolecules, among others. Therefore, they have been applied in chemical and biochemical studies, cosmetics, drug and gene delivery (Chonn and Cullis, 1995; Balazs and Godbey, 2010).

The lipid molecules that are utilized in liposomes contain a hydrophilic head group with a hydrophobic hydrocarbon tail that are linked via a backbone linker. The cationic lipids attain their positive charge generally due to the presence of amines. This positive charge allows for the binding of the cationic liposome to anionic molecules such as nucleic acids (Lonez *et al*., 2008).

The preparation of liposomes can be carried out using several methods. All these methods have a common step which involves the evaporation of the organic solvent in which the lipid molecules are dissolved (Singh, 1998). These methods can be classified as follows:

i. The original hand-shaken preparation producing multilamellar vesicles (Bangham *et al.,* 1965)
ii. Sonication to prepare small unilamellar vesicles (SUVs) (Johnson *et al.,* 1971)
iii. Extrusion through filters to form large unilamellar vesicles (LUVs) or SUVs (Lasic, 1997)
iv. Homogenisation for mass production of liposomes (Lasic, 1997)
v. Ethanol injection technique (Batzri and Korn, 1973; Campbell, 1995)
vi. Ether injection technique (Deamer and Bangham, 1976)
vii. Detergent depletion (Torchilin, 2003)
viii. Reverse phase evaporation technique (Szoka and Papahadjopoulos, 1978)
ix. Thin lipid film hydration (Gao and Huang, 1991)

The method of Gao and Huang was adapted and used for the preparation of the cationic liposomes used in this study.

The cationic liposomes are usually formulated with cationic lipids together with a neutral helper lipid which, in most cases is DOPE (Figure 2.2). DOPE is said to act as a fusogenic lipid which allows fusion of the complexed nucleic acid to the target cell as well as aiding in escape from the endosome (Farhood *et al.*, 1994).

Due to the ease of synthesis of liposomes, it is quite simple to modify the liposomal surface. One of these modifications involves the introduction of polyethylene glycol. Pegylation is said to

prevent the liposome complexed nucleic acid from aggregating in the blood as well as prolonging the circulation time of the liposome (Vandenbrouke *et al.*, 2008).

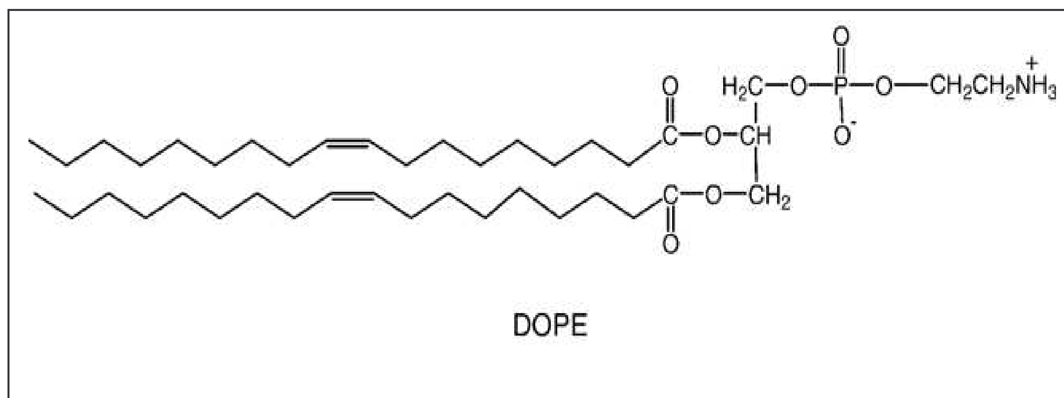


Figure 2.2: Structure of neutral helper lipid, DOPE (Lv *et al.*, 2006)

Liposomes exhibit a diverse range of morphologies that depend on the lipid mixtures in the aqueous medium and their assembly (Balazs and Godbey, 2010). Liposomes are classified on the basis of their size as well as the arrangement and number of lipid bilayers present. Multilamellar vesicles (MLV) are easily prepared by hydrating the lipid film. These vesicles are large structures that are made up of multiple concentric bilayers. The bilayers are separated by the presence of small aqueous compartments (MacLachlan, 2007). The multilamellar vesicles are hundreds of nanometers in diameter. Sonication of the multilamellar vesicles results in the formation of small unilamellar vesicles (SUV). The size range of these vesicles is between 20-200 nm in diameter. Unilamellar vesicles are analogous to the eukaryotic cell membrane and are characterized by the presence of a single bilayer membrane. This membrane encapsulates an aqueous solution which separates it from the external solution (Samad *et al.*, 2007). Large unilamellar vesicles (LUV) also exist and their sizes range from 200 nm to 1 µm in diameter.

This chapter focuses on the preparation of cationic liposomes utilizing novel cationic cholesterol derived cytofectins. These liposomes were characterized using transmission electron microscopy and their size and lamellarity were determined.

2.2 MATERIALS AND METHOD

2.2.1 MATERIALS

Cationic cholesterol derivatives Chol-T and MSO9 were prepared in the department of Biochemistry, University of KwaZulu- Natal, Westville campus. DOPE was purchased from Sigma Chemical Company (St. Louis, USA). Polyethylene $glycol_{2000}$ (DSPE-PEG_{2000}) was purchased from Avanti Polar Lipids (Alabaster, USA). 2-[4-(2-hydoxyethyl)-1–piperazinyl] ethanesulphonic acid (HEPES) was purchased from Merck (Darmstadt, Germany). Ultrapure water (Milli-Q50) was used throughout. All other chemicals were of analytical grade.

2.2.2 METHOD

2.2.2.1 Synthesis of Cationic Cholesterol Derivatives

2.2.2.1.1 Preparation of Cationic Cholesterol Derivative Chol-T

The Chol-T used in this study was previously synthesized and the synthesis shall be briefly described. The preparation is represented in Figure 2.3.

To a solution of cholesteryl chloroformate (90 mg, 0.2 µmoles) in 1 ml dichloromethane was added 3- dimethylaminopropylamine (62.8 µl, 0.11 µmoles). Following an hour at room temperature, the solvent (dichloromethane and excess 3-dimethylaminoproylamine) was

removed by rotary evaporation in a Büchii Rotavapor-R. The resultant residue was dissolved in absolute ethanol and allowed to crystallize overnight at - 4°C. The resulting product was then recrystallized and filtered under dry nitrogen gas. This was followed by further drying by rotary evaporation to yield whitish coloured crystals.

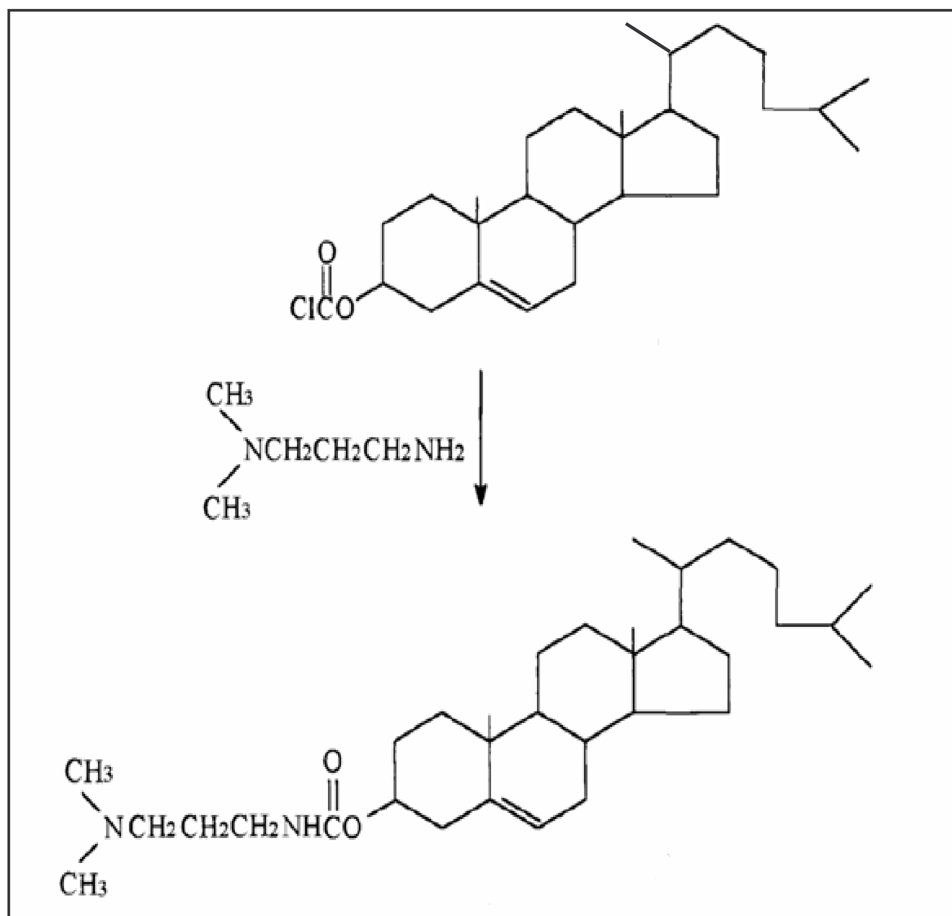


Figure 2.3: Scheme for the synthesis of cationic cholesterol derivative Chol-T (Narainpersad, 2009)

2.2.2.1.2 Preparation of Cationic Cholesterol Derivative MSO9

MSO9 was synthesized in four steps with the first being the preparation of cholesterylformylhydrazide. Each of these four steps shall be discussed briefly as described by Singh and Ariatti (2006), and is illustrated in Figure 2.4.

(a) Cholesterylformylhydrazide (MSO4)

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

(b) Cholesterylformylhydrazidehemisuccinate (MSO8)

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

(c) N- hydroxysuccinimide ester of cholesterylformylhydrazidehemisuccinate

MSO8 (82 mg, 0.15 mmole), DCC (62 mg, 0.3 mmole) and N- hydroxysuccinimide (35 mg, 0.3 mmol) were dissolved in 1 ml of DMF. The reaction was monitored by TLC (results not shown). After 48 hours, filtration was used to remove the dicyclohexylurea crystals. The solvent was removed by evaporation and the resultant crude product was dissolved in chloroform: water mixture (1:1 v/v). The water layer, containing excess N- hydroxysuccinimide, was removed. Following solvent evaporation, petroleum ether (60-80 °C) was utilized to extract the residue and remove DCC traces. The product was then obtained from absolute ethanol as white crystals.

(d) N, N-dimethylpropylamidosuccinylcholesterylformylhydrazide (MSO9)

The N- hydroxysuccinimide ester of cholesterylformylhydrazidehemisuccinate (53 mg, 0.083 mmole) and dimethylaminopropylamine (36 mg, 0.35 mmole) 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 four 60 F_{254} TL plates developed in a chloroform: methanol (95: 5 v/v) solvent system.

2.2.2.2 Preparation of Cationic Liposomes

The cationic liposomes were prepared using the method described by Gao and Huang (1991). The quantities of each lipid component of the cationic liposomes can be seen in Table 2.1. Six cationic liposomes were prepared containing either cytofectin Chol-T or MSO9.

Each liposome preparation was composed of 2 µmoles of lipid. Each lipid component, which was first dissolved in chloroform to a concentration of 10 µg/µl, was deposited into its respective quick fit tube. The organic solvent was then removed using the Buchii Rotavapor-R rotary evaporator. This resulted in a thin lipid film on the inside of the quick fit test tube. This lipid film was then further dried, under vacuum, in a drying pistol for 30 minutes. The lipid film was then hydrated with sterile hepes buffered saline (HBS) (20 mM, pH 7.5). This was followed by vortexing and the liposome preparation was stored at 4°C overnight. Thereafter, the preparation was vortexed and sonicated for 5 minutes, which affords unilamellar liposomes. The liposomes were then stably stored at 4°C.

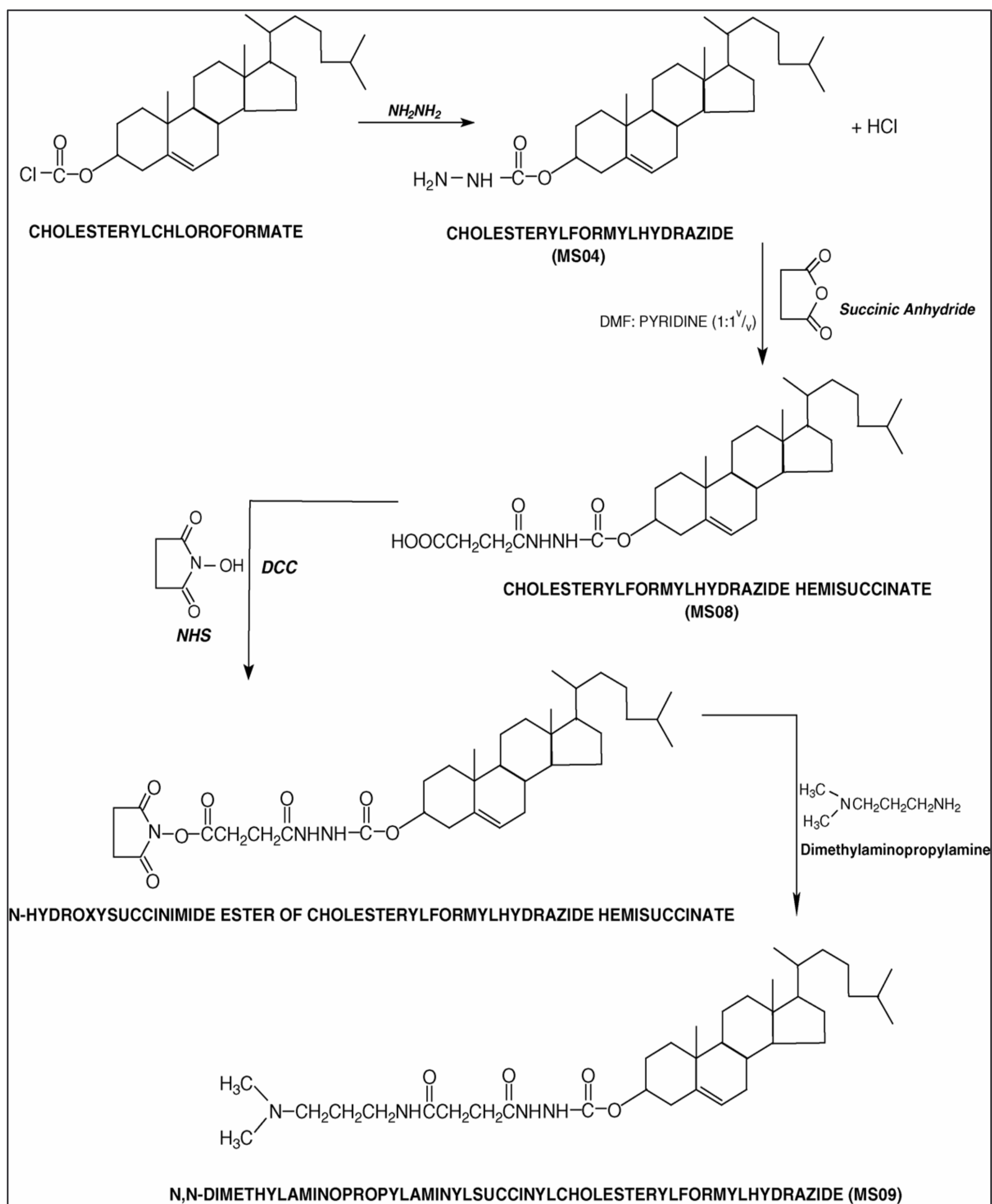


Figure 2.4: Scheme for the synthesis of cationic cholesterol derivative MSO9 (Singh and Ariatti, 2006).

Table 2.1: Components of Cationic Liposomes

Cationic Liposome Preparation	MASS (mg)				MOLAR RATIO (µmol)			
	Chol-T	**MSO9**	**DOPE**	**DSPE-PEG_{2000}**	**Chol-T**	**MSO9**	**DOPE**	**DSPE-PEG_{2000}**
Chol-T	0.52	-	0.74	-	1	-	1	-
2%PEG_{2000} Chol-T	0.52	-	0.72	0.11	1	-	0.96	0.04
5%PEG_{2000} Chol-T	0.52	-	0.67	0.28	1	-	0.9	0.1
MSO9	-	0.63	0.74	-	-	1	1	-
2%PEG_{2000} MSO9	-	0.63	0.72	0.11	-	1	0.96	0.04
5%PEG_{2000} MSO9	-	0.63	0.67	0.28	-	1	0.9	0.1

2.2.2.2 Characterization of Liposomes by Transmission Electron Microscopy (TEM)

Each liposome suspension was diluted to a 1:5 ratio with sterile HBS. The diluted liposome suspension (1 µl) was placed on a formvar coated copper grid. To this, 1 µl of 1% (w/v) uranyl acetate was added and allowed to dry for 2 minutes. Thereafter, the excess suspension was removed with filter paper and the grid was then immediately vitrified in liquid nitrogen. The vitrified samples were viewed using a GATAN cryotransfer device at -150 °C in a JEOL JEM 1010 electron microscope (Tokyo, Japan). Images were captured digitally using a MegaView III camera and SIS i-TEM software facilitated measurements of liposomes on calibrated images.

2.3 RESULTS AND DISCUSSION

2.3.1 Preparation of Cationic Liposomes

The cationic liposomes were successfully prepared using the method described in section 2.2.1. Cytofectins, Chol-T and MSO9, were both previously successfully synthesized and have the same general structural features of most cationic lipids that are utilized to date, namely, a positively charged head group, a spacer arm, linker bond and a hydrophobic lipid anchor (Figures 2.5 and 2.6). Chol-T and MSO9 have similar chemical structural properties which include a cholesterol ring anchor, a carbamoyl linker bond and a dimethylamino head group. The difference between the two lipids lies in the length of their spacer arms where MSO9 has a 12 atom spacer arm and Chol-T has a 6 atom spacer (Singh and Ariatti, 2006).

The cholesterol anchor is said to impart better stability and also acts as an intercalator within the phospholipid molecules which results in the lowering of membrane permeability. Cholesterol also aids in membrane-protein interactions (Samad *et al.*, 2007). The linker bond is a carbamoyl bond which is said to influence the biodegradability of the cationic lipid and also influences the chemical stability of the lipid (Singh and Ariatti, 2006). 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). A longer spacer arm could reduce steric hindrance between the polar head group and the cholesterol ring system which would result in a better interaction between the cationic lipid and the nucleic acid (Singh, 1998). The transfection activity and cytotoxicity of the cationic lipid may be influenced by the nature of the head group. Chol-T and MSO9 are monovalent cationic lipids and are able to condense nucleic acids less strongly as 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 which results in a low transfection efficiency (Miller, 1998).

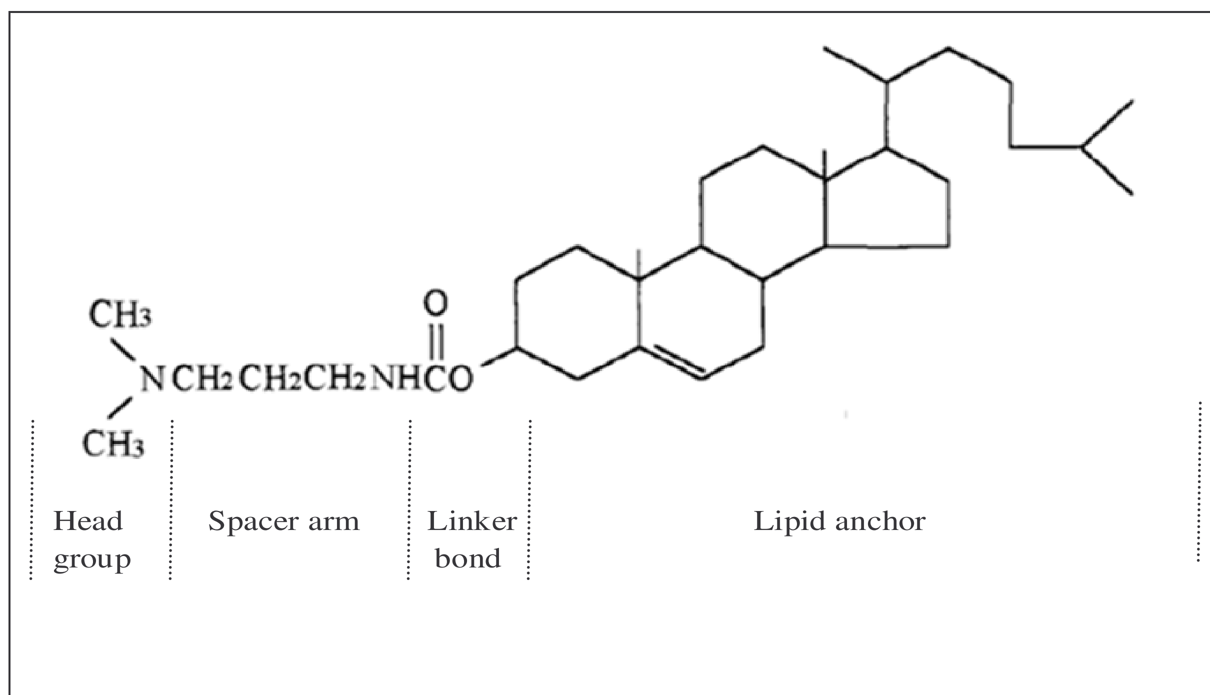


Figure 2.5: Structure of cationic lipid Chol-T showing the four basic structural components

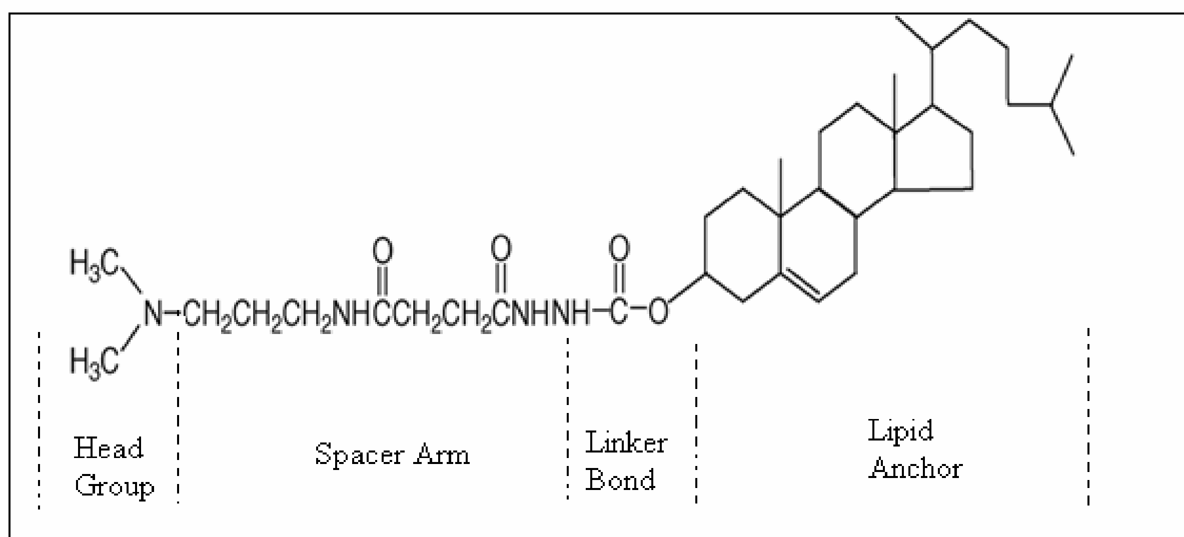


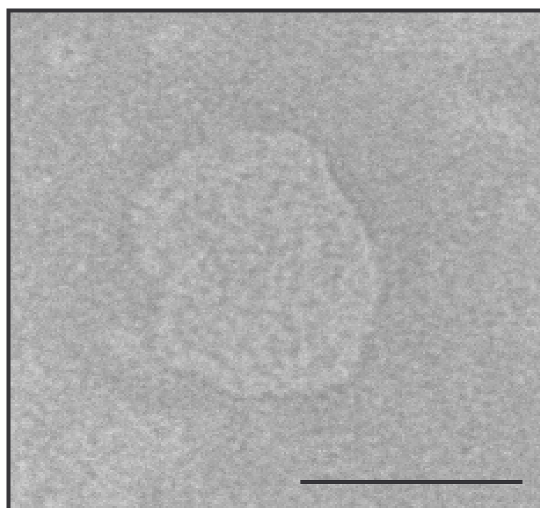
Figure 2.6: Structure of cationic lipid MSO9 showing the four basic structural components

All six cationic liposomes were prepared with the neutral lipid DOPE (Figure 2.2). DOPE is usually incorporated into cationic liposome formulations as a helper lipid as most cationic lipids form micelles and not liposomes (Singh, 1998). DOPE is said to aid in membrane fusion as well as destabilization of the cellular and endosomal membrane as this neutral lipid has the ability to adopt the reverse hexagonal H_{II} phase which is a non-bilayer phase (Dass and Choong, 2006; MacLachlan, 2007). Additionally, cationic liposome formulations are stabilized by the presence of DOPE as cationic liposomes repel each other. Liposomes formulated in the absence of DOPE are shown to have inferior rates of cell uptake and transfection (Dass and Choong, 2006). The addition of DOPE is also said to reduce the cytotoxicity of the cationic liposomes (Sternberg *et al.*, 1994).

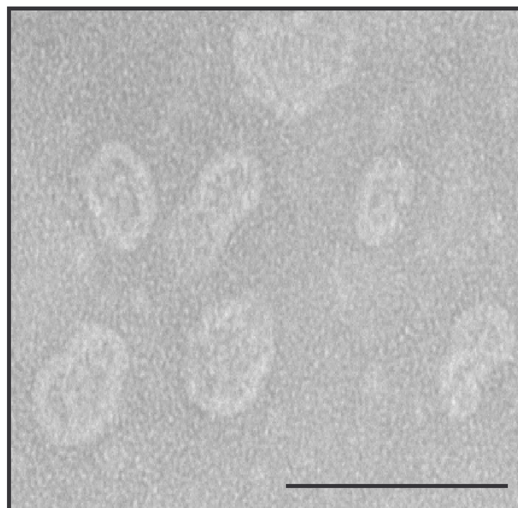
The final constituent of the liposome preparations is DSPE-PEG_{2000}. DSPE-PEG_{2000} was incorporated into the cationic liposomes at concentrations of 2 and 5 mole percentages. This was accomodated by reducing the percentage of DOPE in the respective liposomes. Distearoylphosphatidylethanolamine is present in the PEG molecule and serves as the lipid anchor which is attached to the PEG moiety via a carbamate linkage (Rejmana *et al.*, 2004). PEG is a hydrophilic polymer, which, upon incorporation into the bilayer of the liposome, protrudes from the liposomal surface and affords a steric barrier to the liposome. In doing so, any interactions between the liposome and biological molecules are reduced, the liposome is stabilized and its circulation time in the blood is increased (Hong *et al.*, 1997; Song *et al.*, 2002).

2.3.2 Transmission Electron Microscopy (TEM)

Transmission electron microscopy of all six liposome preparations revealed the unilamellar nature of the liposomes (Figures 2.7 and 2.8). All the liposomes were spherical to oval in shape whilst some also exhibited a deformable nature. Artefacts present in some of the images can be attributed to the freezing process.



(a)



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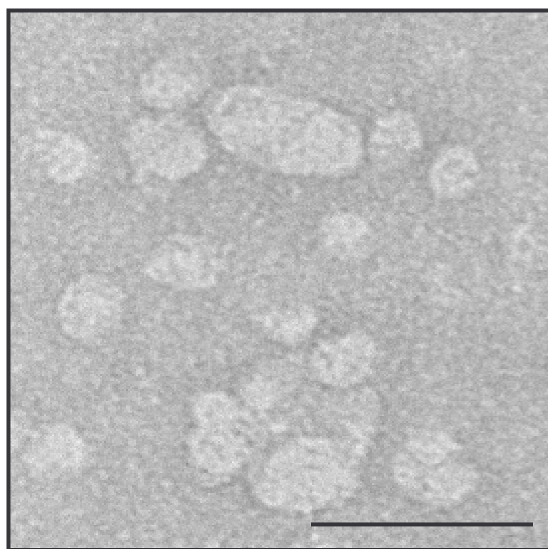
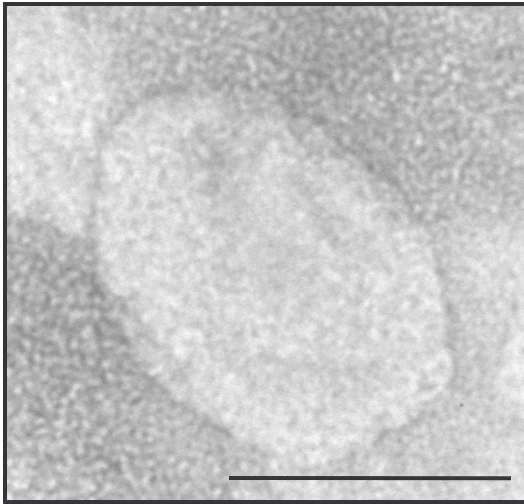
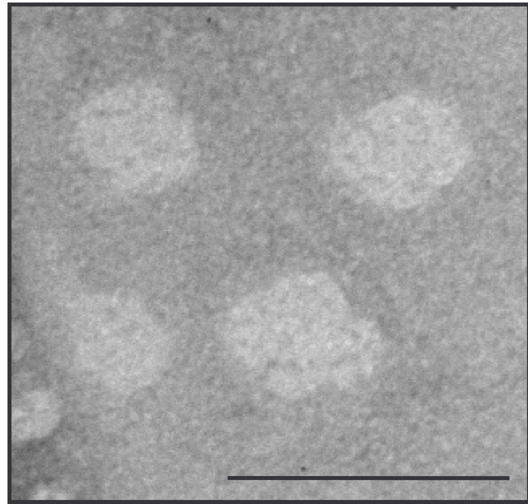


(c)

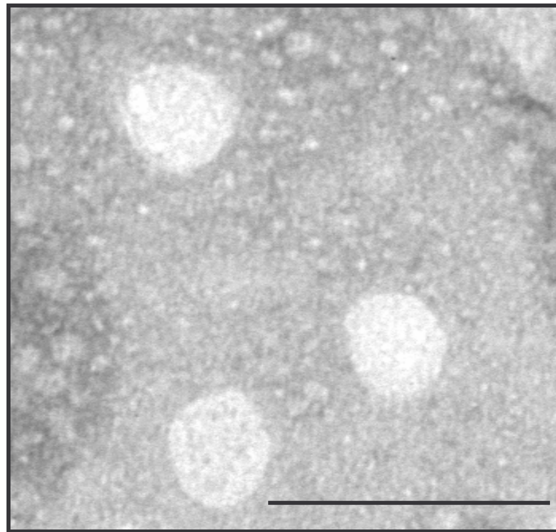
Figure 2.7: Transmission electron micrographs of (a) Chol-T, (b) 2% PEG_{2000}Chol-T and (c) 5% PEG_{2000}Chol-T liposomes. Bar = 100 nm.



(a)



(b)



(c)

Figure 2.8: Transmission electron micrographs of (a) MSO9, (b) 2% PEG_{2000}MSO9 and (c) 5% PEG_{2000}MSO9 liposomes. Bar = 100 nm.

The average size measured for Chol-T was 120.7 nm, 2% PEG_{2000}Chol-T was 37.85 nm and 5% PEG_{2000}Chol-T was 43.67 nm (Figure 2.9). Measurements obtained for MSO9 and the pegylated (2 and 5 mole percent) MSO9 liposomes were 86.5 nm, 38.23 nm and 43.98 nm, respectively (Figure 2.10). It appears that the sizes of the liposomes are reduced upon pegylation. Lee *et al.* (2005) made a similar observation during their studies and attributed this to the incorporation of the PEG molecule onto the liposomal surface. They proposed that the PEG molecules present on the liposomal surface tend to repel each other, which prevents aggregation of the liposomes during preparation and hence, leads to the formation of smaller sized vesicles (Lee *et al.*, 2005). Statistical analyses of liposome and lipoplex (Chapter 3) dimensions are presented as means ± SD. Results were examined by one way ANOVA and groups were compared by Student's t-test. P values less than 0.05 were considered to be statistically significant.

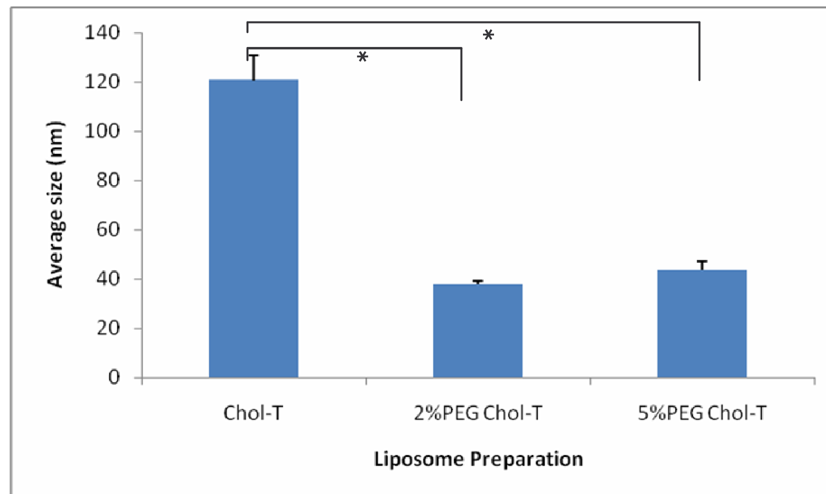


Figure 2.9: Average sizes of cationic liposome preparations Chol-T, 2% PEG_{2000}Chol-T and 5% PEG_{2000}Chol-T. Data presented as a means ± S.D (n = 5) (* $p < 0.05$).

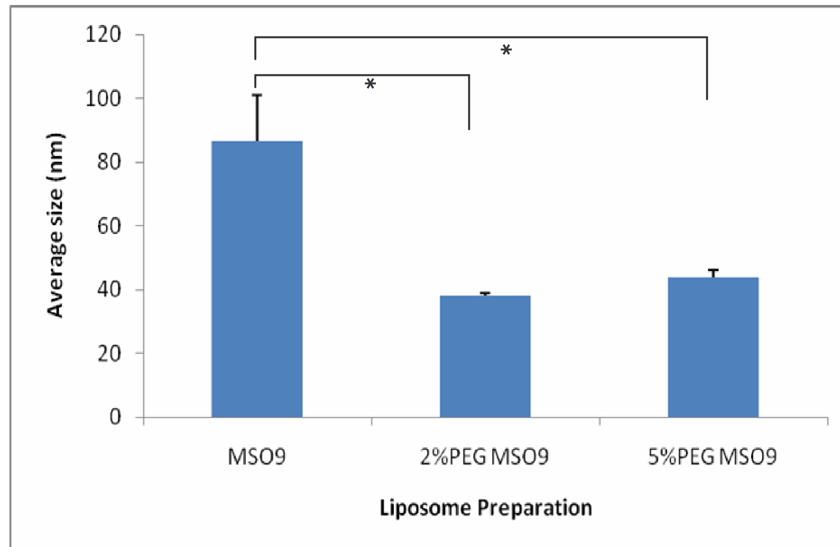


Figure 2.10: Average sizes of cationic liposome preparations MSO9, 2% PEG_{2000}MSO9 and 5% PEG_{2000}MSO9. Data presented as a means ± S.D (n = 5) (* $p < 0.05$).

The size of the liposome plays a major part in their accumulation in the body and their blood clearance rates (Lee *et al.*, 2005; Campbell *et al.*, 2001). Although the size of the liposomes was determined using TEM in this study, additional information on the distribution of the liposome population may also be determined by dynamic light scattering and zeta sizing, the relevant instrumentation was not available at UKZN. However, cryo-electron microscopy with a brief and relatively mild negative staining affords images of liposome vesicles which may be sized reliably with the aid of appropriate software (Dorasamy *et al*., 2009).

CHAPTER THREE

PREPARATION AND CHARACTERIZATION OF LIPOSOME: siRNA COMPLEXES

3.1 INTRODUCTION

Cationic liposomes are formulated with positively charged lipids that physically associate via charge interactions with negatively charged nucleic acids. These complexes that are formed are termed lipoplexes, a term that was coined by Felgner *et al.* (1997) to describe complexes formed between cationic liposomes and DNA, however, this term has now been broadened to describe the interaction between cationic liposomes and negatively charged nucleic acids (DNA and RNA) (Felgner *et* al., 1997; Thomas *et al.*, 2009). A lipoplex, therefore, is composed of the cationic liposome containing a neutral helper lipid and the nucleic acid.

Although much information is available to describe the physical structures of DNA lipoplexes, very little is known about the exact structure and properties of siRNA lipoplexes. Due to the presence of charge complementarities between the siRNA and the cationic liposomes, lipoplexes are spontaneously formed via electrostatic interactions between the positively charged head groups of the cationic liposomes and the negatively charged phosphate backbone of the siRNA (Ramon *et al.*, 2008). The lipoplexes are prepared by simply mixing the nucleic acid and the cationic liposomes and incubating for a short period of time (Guo *et al.*, 2010). It has been proposed that due to these charge interactions, the siRNA becomes physically associated with the outer surface of the liposome (Buyens *et al.*, 2009) (Figure 3.1)

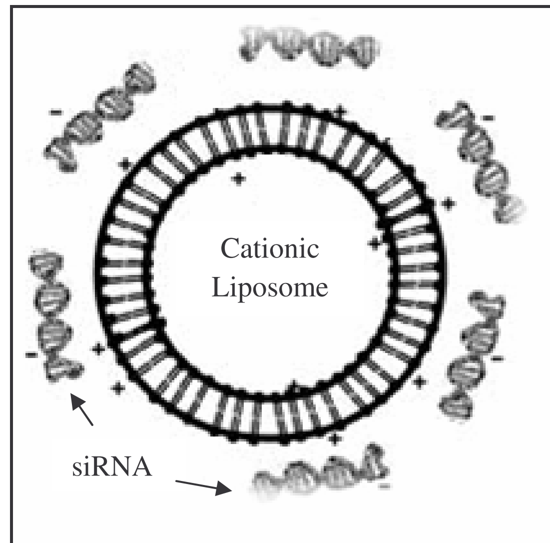


Figure 3.1: Illustration of the physical association between the siRNA and cationic liposome (siRNA lipoplex) (Santel *et al.*, 2006)

The main forces involved in the spontaneous formation of lipoplexes include electrostatic forces and elastic forces, that is, the bending and stretching forces. When the lipoplexes are formed, the electrostatic forces are lowered due to the neutralization of the positive charges of the cationic liposome by the negatively charged nucleic acids, however, this must be higher than the elastic energy of the liposome, which results in the lipoplex geometry being achieved (Zuidom *et al.*, 1999).

In this study, complexes were prepared with the non-pegylated and pegylated cationic liposomes and control, non targeting siRNA. The optimum binding ratios were determined from the gel retardation assay. This assay is based on the observation that lipoplexes do not migrate or migrate slower in an agarose gel as compared to the free nucleic acid. The assay is carried out by preparing lipoplexes and subjecting them to agarose gel electrophoresis. The gel is then analyzed to view any migration of the siRNA within the gel. The protection afforded by the cationic liposomes to the siRNA was analyzed with the nuclease protection assay using gel

electrophoresis. The ultrastructural characteristics of the siRNA lipoplexes prepared were studied using transmission electron microscopy.

3.2 MATERIALS AND METHOD

3.2.1 Materials

Control, non-targeting siRNA and 5× siRNA buffer was purchased from Thermo Scientific Dharmacon Products (Lafayette, CO, USA). Ultrapure Agarose was purchased from Invitrogen (Spain). Ethidium Bromide was obtained from Merck (Dermstadt, Germany). RNase A was purchased from Novagen, Calbiochem (USA). Ultrapure water (Milli-Q50) was utilized for all assays. All other reagents were of analytical grade.

3.2.2 Method

3.2.2.1 Resuspension of siRNA

Control siRNA (20 pmoles) was resuspended using 1 ml of 1× RNA buffer, prepared from a 5× RNA buffer stock (Dharmacon, Lafeyette, CO, USA) with 18 MOhm water. The suspension was then vortexed and inverted to ensure that the siRNA was properly resuspended. The concentration of the stock was then determined using a Thermo Biomate 3 spectrophotometer and was found to be 0.29 μg/μl.

3.2.2.2 Gel Retardation Assays

A 2% (w/v) agarose gel was prepared by dissolving 0.4 g of agarose in 18 ml of 18 MOhm water (100 °C). Once all the agarose had dissolved, 2 ml of 10× electrophoresis buffer (0.36 M Tris

HCl, 0.3 M sodium phosphate, 0.1 M EDTA, pH 7.5) and 3 µl of ethidium bromide was added to the gel to afford a final concentration of 1 µg/ml. The gel was then allowed to set for an hour. Cationic liposome: siRNA complexes of varying ratios were prepared as seen in Tables 3.1 (Chol-T), 3.2 (2%PEG_{2000} Chol-T), 3.3 (5%PEG_{2000}Chol-T), 3.4 (MSO9), 3.5 (2%PEG_{2000}-MSO9) and 3.6 (5%PEG_{2000}-MSO9). A constant concentration of siRNA (0.5 µg) was added to increasing amounts of the cationic liposomes. These complexes were then made up to 10 µl with HBS. The reaction mixtures were incubated at room temperature for 30 minutes. Gel loading buffer (40% sucrose, 0.5% Bromophenol Blue, 2 µl) was added to each reaction mixture following this incubation. The siRNA lipoplexes were then subjected to agarose gel electrophoresis for 30 minutes at 50 volts using a 2% agarose gel in a Bio-Rad electrophoresis tank which contained 1× electrophoresis buffer (36 mM Tris-HCl, 30 mM sodium phosphate, 10 mM EDTA pH 7.5). Thereafter, the gel was viewed under UV transillumination at 300 millisecond exposure time in a Vacutec Syngene G:Box gel documentation system.

Table 3.1: Chol-T cationic liposome: siRNA complexes

siRNA lipoplexes	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
siRNA (µg)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Chol-T (µg)	-	2	3	4	5	6	7	8

Table 3.2: 2% PEG_{2000}Chol-T cationic liposome: siRNA complexes

siRNA lipoplexes	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
siRNA (µg)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
2%PEG_{2000} Chol-T (µg)	-	3	4	5	6	7	8	9

Table 3.3: 5% PEG_{2000}Chol-T cationic liposome: siRNA complexes

siRNA lipoplexes	1	2	3	4	5	6	7	8
siRNA (µg)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
5%PEG_{2000} Chol-T (µg)	-	4	5	6	7	8	9	10

Table 3.4: MSO9 cationic liposome: siRNA complexes

siRNA lipoplexes	1	2	3	4	5	6	7	8
siRNA (µg)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
MSO9 (µg)	-	3	4	5	6	7	8	9

Table 3.5: 2% PEG_{2000} MSO9 cationic liposome: siRNA complexes

siRNA lipoplexes	1	2	3	4	5	6	7	8
siRNA (µg)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
2% PEG_{2000} MSO9 (µg)	-	18	19	20	21	22	23	24

Table 3.6: 5% PEG_{2000} MSO9 cationic liposome: siRNA complexes

siRNA lipoplexes	1	2	3	4	5	6	7	8
siRNA (µg)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
5% PEG_{2000} MSO9 (µg)	-	22.5	25	27.5	30	32.5	35	37.5

3.2.2.3 Nuclease Protection Assays

The siRNA lipoplex ratios used in this study are shown in Table 3.7 and represent the sub-optimum, optimum and supra-optimum binding ratios as determined in the gel retardation assays. The complexes were prepared using a constant amount of control siRNA (0.3 µg) and made up to a final volume of 10 µl with HBS. Two controls were used for this study, namely, siRNA in the absence of the cationic liposome and RNase A (negative control) and siRNA in the presence of RNase A but in the absence of the cationic liposome (positive control). The freshly prepared lipoplexes were allowed to mature at room temperature for 30 minutes. Thereafter, RNase A (1 µg/µl) was added to the siRNA lipoplexes to a concentration of 0.1 µg/µl and the mixtures were then incubated for a further 2 hours at 37 ˚C. Following this incubation period, ethylenediaminetetraacetic acid (EDTA) and sodium dodecyl sulphate (SDS) were added to the reaction mixtures to a final concentration of 10 mM and 0.5%, respectively. This was followed by a further incubation at 55˚C for 20 minutes. Thereafter, the siRNA lipoplexes were subjected to agarose gel electrophoresis on a 2% agarose gel as in 3.2.2.2. The gel was then viewed and the images were obtained using a Vacutec Syngene G:Box gel documentation system as described in 3.2.2.2..

Table 3.7: Varying amounts of the cationic liposomes for nuclease protection studies.

LIPOSOME PREPARATION	LIPOSOME AMOUNT (µg)		
Chol-T	1.8	2.4	3
2% PEG_{2000}-Chol-T	2.4	3	3.6
5% PEG_{2000}-Chol-T	3.6	4.2	4.8
MSO9	3	3.6	4.2
2%PEG_{2000}-MSO9	13.2	13.8	14.4
5%PEG_{2000}-MSO9	9	12	15

3.2.2.6 Transmission Electron Microscopy of siRNA: Cationic Liposome Complexes

Lipoplexes were prepared using the ratios at which complete retardation of 0.5 µg of control non-targeting siRNA was obtained. These complexes were allowed to incubate at room temperature for 30 minutes. The protocol outlined in section 2.2.2.2 for the transmission electron microscopy of liposomes was followed for the characterization of siRNA lipoplexes.

3.3 RESULTS AND DISCUSSION

3.3.1 Gel Retardation Assay

The results obtained from the gel retardation assay using the cationic liposomes formulated with either cytofectin Chol-T or MSO9 with different degrees of pegylation (0, 2 and 5 mole percent) can be seen in Figures 3.3 and 3.4. Each liposome shows different optimum binding ratios. These optimum binding ratios together with their corresponding charge ratios are shown in Table 3.8. The charge ratios were derived assuming that an average nucleotide molecular mass of 350 with one negative charge per nucleotide and a single positive charge per cytofectin molecule at pH 7.5.

Table 3.8: Optimum Binding Ratios and Charge Ratios of the cationic liposomes and pegylated cationic liposomes with siRNA

LIPOSOME PREPARATION	siRNA: Liposome ratio (w/w)	Charge Ratio (-ve/+ve)
Chol-T	1: 8	1: 1.9
2% PEG_{2000}-Chol-T	1: 10	1: 2.2
5% PEG_{2000}-Chol-T	1: 14	1: 2.8
MSO9	1: 12	1: 2.5
2% PEG_{2000}-MSO9	1: 46	1: 8.6
5% PEG_{2000}-MSO9	*	

*Incomplete complexing observed

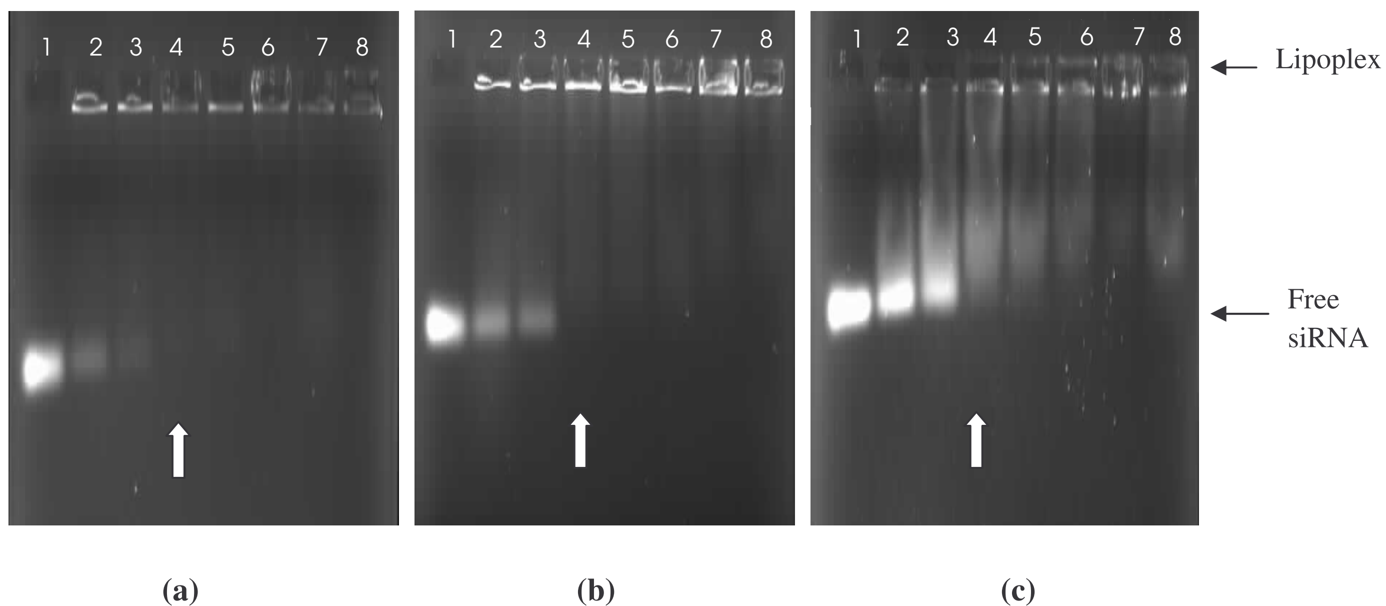


Figure 3.2: Gel Retardation analysis of binding interactions between varying amounts of cationic liposome preparations containing cytofectin Chol-T and 0-5% of DSPE-PEG_{2000} with control siRNA (0.5 µg) in a reaction volume of 10 µl.

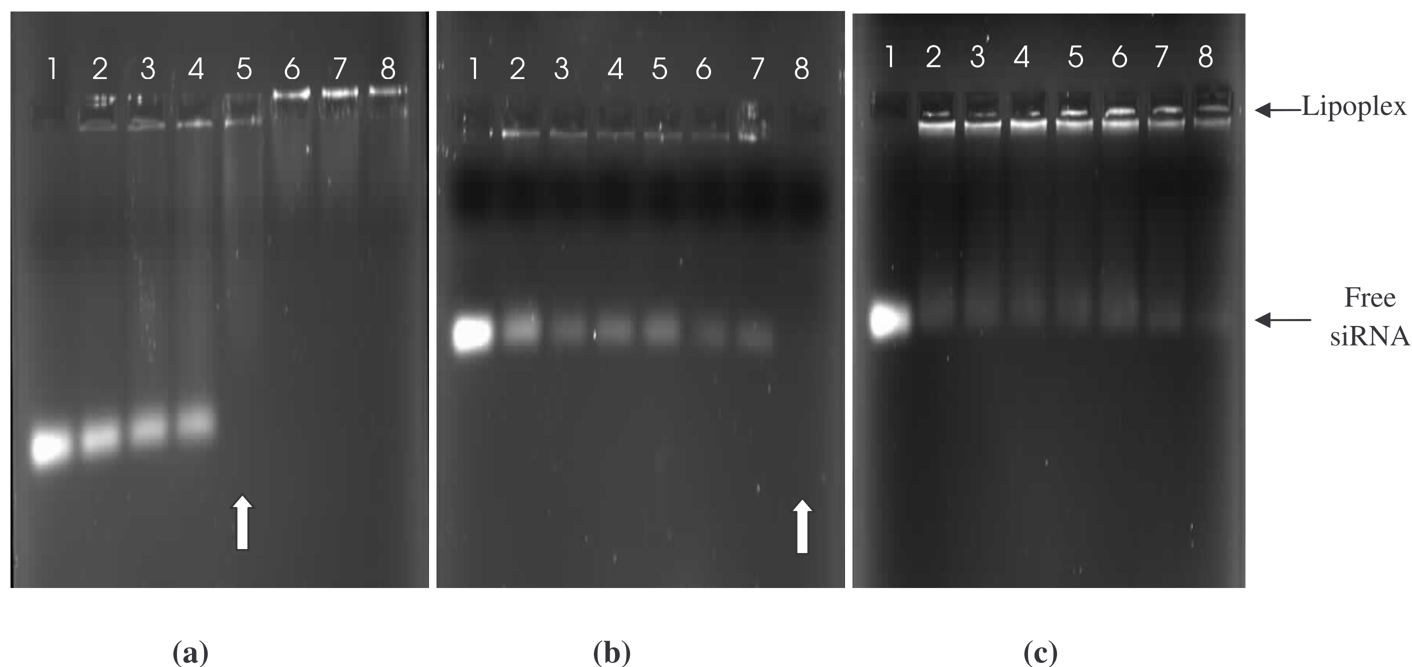
(a) Varying amounts of non-pegylated Chol-T in lanes 2-8 (2,3,4,5,6,7,8 µg)

(b) Varying amounts of 2% PEG_{2000} Chol-T in lanes 2-8 (3,4,5,6,7,8,9 µg)

(c) Varying amounts of 5% PEG_{2000} Chol-T in lanes 2-8 (4,5,6,7,8,9,10 µg)

while control siRNA was kept constant at 0.5 µg per well.

* White arrows indicate end point ratios



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

Figure 3.3: Gel Retardation analysis of binding interactions between varying amounts of cationic liposome preparations containing cytofectin MSO9 and 0-5% of DSPE-PEG_{2000} with control siRNA (0.5 μg) in a reaction volume of 10 μl.

(a) Varying amounts of non-pegylated MSO9 in lanes 2-8 (3,4,5,6,7,8,9 μg)

(b) Varying amounts of 2% PEG_{2000} MSO9 in lanes 2-8 (18, 19, 20, 21, 22, 23, 24 μg).

(c) Varying amounts of 5% PEG_{2000} MSO9 in lanes 2-8 (22.5,25,27.5, 30, 32.5, 35, 37.5 μg).

while control siRNA was kept constant at 0.5 μg per well.

*White arrows indicate endpoint ratios

The gel retardation assay was carried out using a constant amount of non-targeting control siRNA (0.5 µg) and increasing amounts of the various cationic liposome preparations. Agarose gel electrophoresis is used to show the complexation that occurs between the siRNA and the cationic liposome. In lane 1, siRNA was present in all gels in the absence of any cationic liposome. In this lane, the siRNA, which is anionic, migrates into the agarose gel towards the positively charged electrode and a bright, single band is present which is visible after ethidium bromide staining. The siRNA in this particular lane serves as a control and allows one to view the migration of naked siRNA in an agarose gel. With increasing amounts of cationic liposome, the siRNA becomes liposome bound via electrostatic interactions between the positive charges of the cationic liposome and the negative charges of the siRNA and is retained in the wells. At complete retardation, the cationic liposomes and siRNA form complexes which do not migrate through the small pores in the agarose gel during electrophoresis and remain in the wells. These complexes can be seen in the wells after ethidium bromide staining. In some cases, the electroneutral complexes may precipitate in the wells, which results in these complexes floating out of the wells (Singh, 1998). In these cases, the siRNA is not detected in the wells (Figure 3.3 c).

The 5% PEG_{2000} Chol-T liposome showed a higher liposome: siRNA binding ratio compared to the 2% PEG_{2000} Chol-T and the Chol-T liposomes. Complete retardation of 0.5 µg of siRNA was obtained using 4 µg of Chol-T (Figure 3.2 a), 5 µg of 2% PEG_{2000} Chol-T (Figure 3.2 b) and 7 µg of 5% PEG_{2000} Chol-T (Figure 3.2 c). As the concentration of each liposome increased, their binding to the siRNA also increased. These results suggest that with an increase in the degree of liposome pegylation, the ratio to obtain complete retardation of the siRNA also increases. A similar result was obtained by Zhang and co-workers (2010) who found that an increase in pegylation had weakened the binding affinity of the siRNA with the cationic liposome being studied, namely, DC-Chol/ DOPE liposome and therefore, the binding ratios to achieve complete retardation increased as the degree of pegylation increased (Zhang *et al*., 2010).

The same pattern was observed with the cationic liposomes formulated with the cytofectin MSO9 and different degrees of pegylation. Complete retardation of 0.5 µg siRNA was achieved with 6 µg of MSO9 (Figure 3.3 a) and 23 µg of 2% PEG_{2000} MSO9 (Figure 3.3 b). The 5% PEG_{2000} MSO9 was not able to fully complex the siRNA at a ratio of 1:75 (37.5 µg of the liposome). Therefore, three ratios, namely, 1:30, 1:40 and 1:50, were selected for further studies.

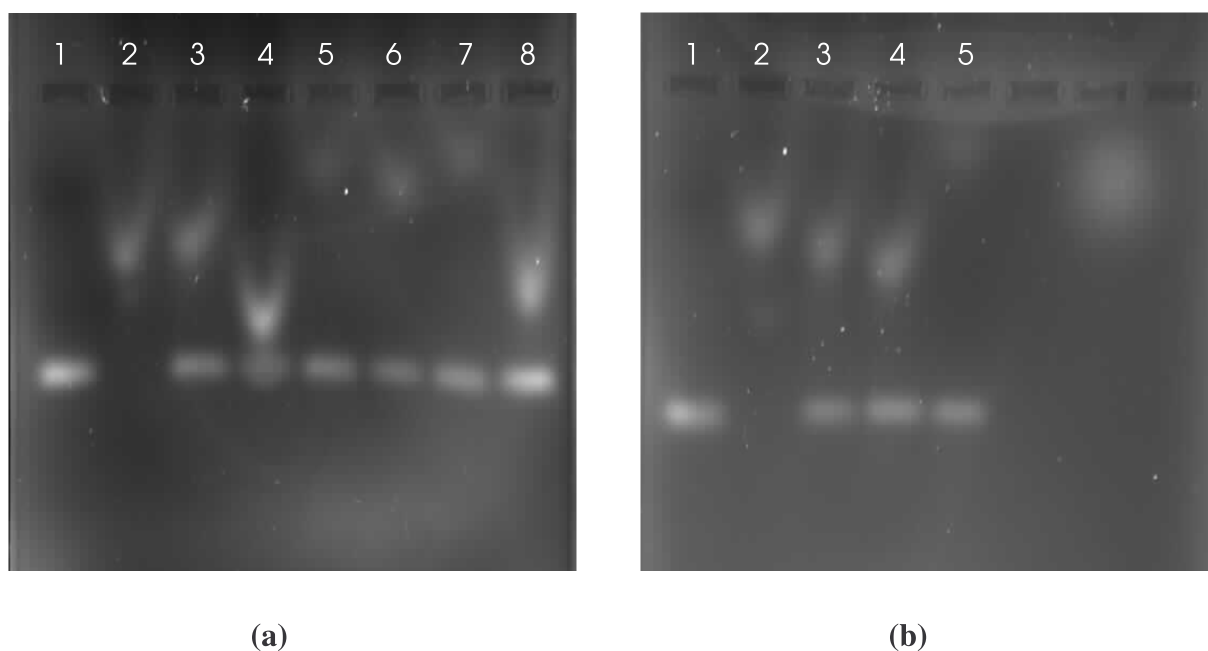
From the gel retardation studies it is observed that an increase in the degree of pegylation increases the charge ratio at the end point (Table 3.8). This could be attributed to the presence of the PEG molecule on the liposomal surface. During the formulation of the liposomes, the introduction of PEG may have resulted in the internalization of the cytofectin (Chol-T and MSO9) hence, the amount of positive charge available for interaction with the negatively charged siRNA is reduced. Furthermore, the presence of PEG may have a shielding effect on the positive charges afforded by the cytofectins, and, therefore, the interaction of the cationic liposome with the siRNA will be affected.

The results obtained for the gel retardation assay have been used in the design of transfection complexes for the transfection studies in the Hela *tat luc* cells in culture, which is described in chapter four. Hence, the sub-optimum, optimum and super optimum siRNA: liposome ratios were studied in transfection assays.

3.3.2 Nuclease Protection Studies

One of the obstacles in inducing RNAi with the introduction of siRNA is the susceptibility of the siRNA to nucleases in the blood serum, such as RNase, during circulation and in the interstitial space (Lu *et al.*, 2009). A desirable feature of any delivery vector is their ability to protect the siRNA from degradation by nucleases.

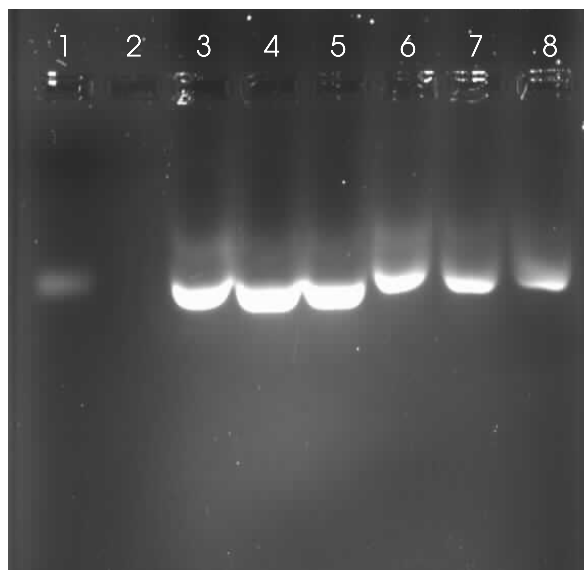
The ability of the six liposomes prepared to protect the siRNA from enzymatic degradation was studied using the nuclease protection assay. The results obtained are presented in Figure 3.4 and 3.5.



(a) **(b)**

Figure 3.4: Nuclease Protection Assay using varying amounts of pegylated and non-pegylated Chol-T cationic liposome in a 10 µl reaction mixture with 0.3 µg of non-targeting siRNA.
Lane 1: undigested siRNA (0.3 µg)
Lane 2: unprotected siRNA (0.3 µg) digested by 10% RNase A.

(a) Lanes 3-5: Varying amounts of Chol-T (1.8, 2.4, 3 µg) with siRNA and 10% RNase A.
Lanes 6-8: Varying amounts of 2% PEG_{2000} Chol-T (2.4, 3, 3.6 µg) with siRNA and 10% RNase A.
(b) Lanes 3-5: Varying amounts of 5% PEG_{2000} Chol-T (3.6, 4.2, 4.8 µg) with siRNA and 10% RNase A.



(a) **(b)**

Figure 3.5: Nuclease Protection Assay using varying amounts of pegylated and non-pegylated MSO9 cationic liposome in a 10 µl reaction mixture with 0.3 µg of non-targeting siRNA.
Lane 1: undigested siRNA (0.3 µg)
Lane 2: unprotected siRNA (0.3 µg) digested by 10% RNase A.

(a) Lanes 3-5: Varying amounts of MSO9 (3, 3.6, 4.2 µg) with siRNA and 10% RNase A.
(b) Lanes 3-5: Varying amounts of 2% PEG_{2000} MSO9 (13.2, 13.8, 14.4 µg) with siRNA and 10% RNase A.
Lanes 6-8: Varying amounts of 5% PEG_{2000} MS09 (9, 12, 15 µg) with siRNA and 10% RNase A.

EDTA is added to the complexes to stop the action of the enzyme RNase A. Sodium dodecyl sulphate (SDS) is used to release the bound siRNA from the siRNA- liposome complex. These complexes are then subjected to agarose gel electrophoresis where the unbound, negatively charged siRNA will migrate into the gel during electrophoresis.

The cationic liposome: siRNA ratios used for this study were those obtained from the gel retardation assay. The liposome bound siRNA appears to be protected from nuclease degradation at the three ratios used, and not just at the optimum binding ratio. The results obtained suggest that the non-pegylated and pegylated Chol-T liposomes afford protection of the siRNA in the siRNA lipoplex. This can be deduced when comparing the results obtained in lane 2 (Figures 3.4 a-c) to those achieved by the lipoplexes. The unprotected siRNA in lane 2 was completely degraded in the presence of 0.1 µg/µl RNase A whereas the siRNA released from the siRNA lipoplexes is intact and migrates as a single band in the gel during electrophoresis. A similar result was seen with the MSO9 and the 2% PEG_{2000}MSO9 where protection of the siRNA was evident following electrophoresis. It has been suggested in studies with DNA that the electrostatic interaction between the negatively charged DNA and the cationic liposomes results in the formation of highly organized structures where the DNA molecules are condensed and protected against enzymatic degradation (Singh, 2005). Since a similar electrostatic interaction occurs between the cationic liposomes and the polyanionic siRNA molecule, it can be suggested, that a similar condensed lipoplex could be formed and, therefore, the siRNA was protected against RNase A degradation.

The siRNA band intensities in lanes 6-8 in Figure 3.5 (b) suggest that the 5% PEG_{2000}MSO9 liposome afforded less protection to the siRNA than the MSO9 (Figure 3.5 a) and the 2% PEG_{2000}MSO9 preparations (Figure 3.5 (b) lanes 3-5). In this regard it is noteworthy that the gel retardation analysis for the 5% PEG_{2000}MSO9 did not reveal an end point, that is, an optimum binding ratio. Hence, it can be assumed that not all the siRNA was liposome bound and therefore, a proportion of siRNA was exposed to enzymatic degradation by RNase A. Partial siRNA protection was also obtained by Kim *et al.* (2010) whose liposomes had shown partial

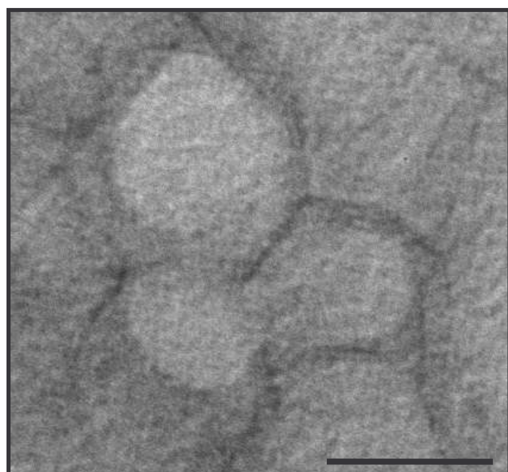
protection of the siRNA. They attributed their results to the process of the interaction between the siRNA and the cationic liposome which may result in the incomplete complexation of the siRNA molecules, and, therefore, the siRNA is exposed to potential degradation by the enzymes.

The protection afforded to the siRNA by the liposomes is promising for transfection studies *in vitro* and *in vivo*, as this increases the delivery of large amounts of intact siRNA to the target cells, which is required for efficient gene knockdown.

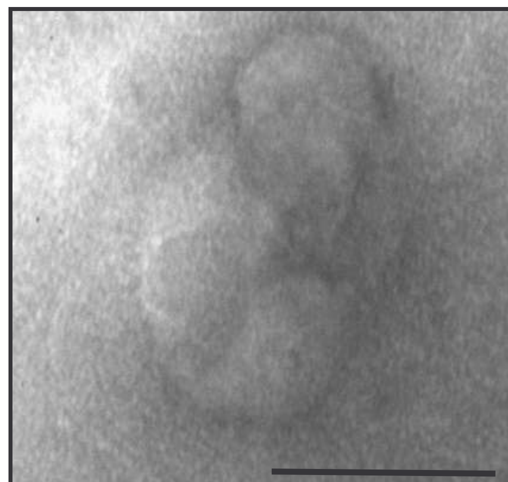
3.3.3 Transmission Electron Microscopy of siRNA Lipoplexes

Cryo-TEM was used to determine the ultrastructural characteristics and sizes of the different siRNA lipoplexes being utilized in this study. The siRNA lipoplexes were prepared using the ratios at which optimal binding was achieved.

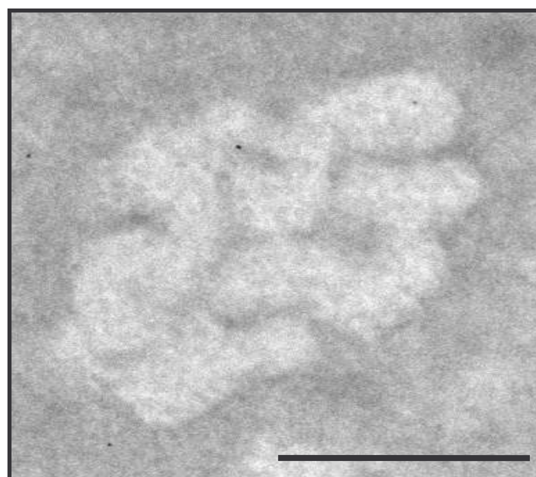
The cryo-TEM images revealed siRNA lipoplexes that appeared as spherical clusters, some of which also appeared to have a deformable nature (Figure 3.6 a-c and Figure 3.7 a-c). The sizes of the siRNA lipoplexes were also measured and are represented in Figures 3.8 and 3.9. The statistical analyses for the siRNA lipoplexes were carried out as described in chapter 2, section 2.3.2.



(a)

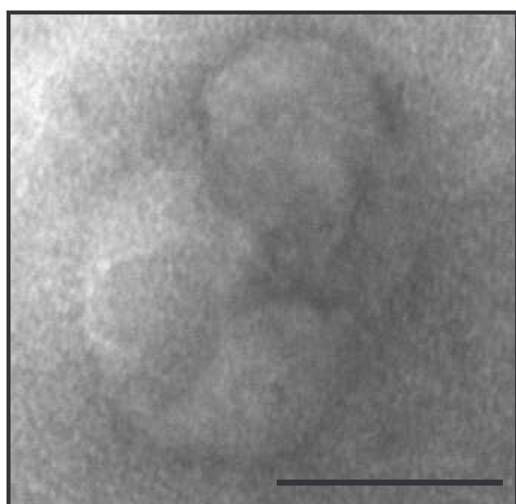


(b)

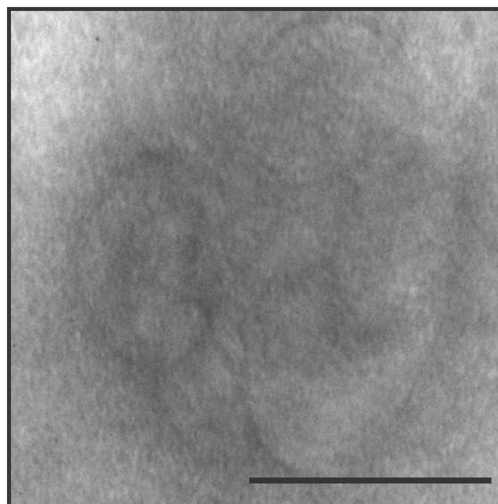


(c)

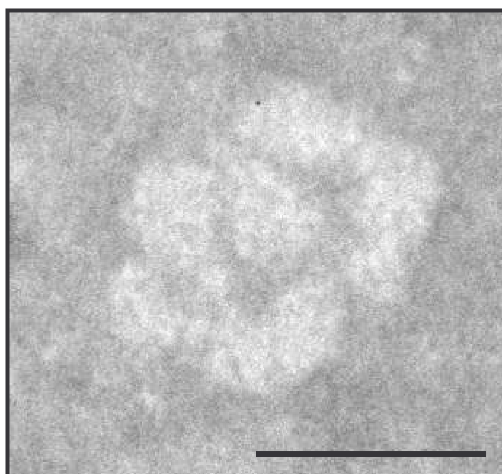
Figure 3.6: Transmission electron micrographs of (a) siRNA:Chol-T lipoplex, (b) siRNA: 2% PEG_{2000}Chol-T lipoplex and (c) siRNA: 5% PEG_{2000}Chol-T lipoplex. Bar = 100 nm except in (c) where Bar = 50 nm



(a)



(b)



(c)

Figure 3.7: Transmission electron micrographs of (a) siRNA: MSO9 lipoplex, (b) siRNA: 2% PEG_{2000}MSO9 lipoplex and (c) siRNA: 5% PEG_{2000}MSO9 lipoplex. Bar = 100 nm

It has been reported that the sizes of lipoplexes are said to range between 50 nm to 200 nm in diameter (Thomas *et al*., 2007). The images obtained following cryo-TEM are consistent with the above as the sizes of the lipoplexes were within this range. Chol-T lipoplexes had a mean diameter of 140.2 nm and 2% PEG_{2000} Chol-T lipoplexes and 5% PEG_{2000} Chol-T lipoplexes exhibited mean diameters of 134 nm and 87.65 nm, respectively. The average sizes of MSO9, 2% PEG_{2000} MSO9 and 5% PEG_{2000} MSO9 lipoplexes were 153 nm, 100 nm and 128.6 nm, respectively. The results obtained indicate that the sizes of the lipoplexes are much larger than the corresponding liposomes and pegylation appears to reduce the sizes of the lipoplexes. These results are consistent with those obtained by Zhang *et al* (2010) who suggested that a large lipoplex may form during neutralization of the lipoplex charge and they also found that pegylation could decrease the lipoplex size by inhibiting the formation of aggregates.

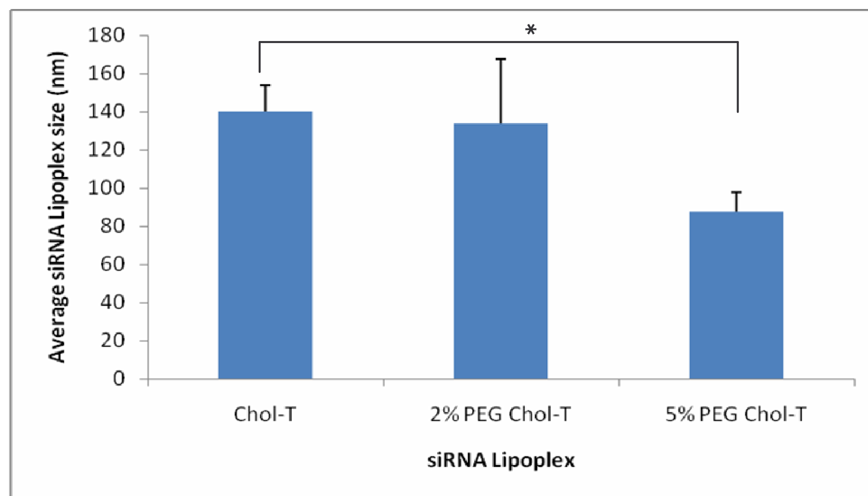


Figure 3.8: Average sizes of siRNA: Chol-T lipoplexes, siRNA: 2% PEG_{2000}Chol-T lipoplexes and siRNA: 5% PEG_{2000}Chol-T lipoplexes at optimum binding ratios. 0.5 µg of siRNA is present. Data presented as a means ± S.D (n = 5) (* $p < 0.05$).

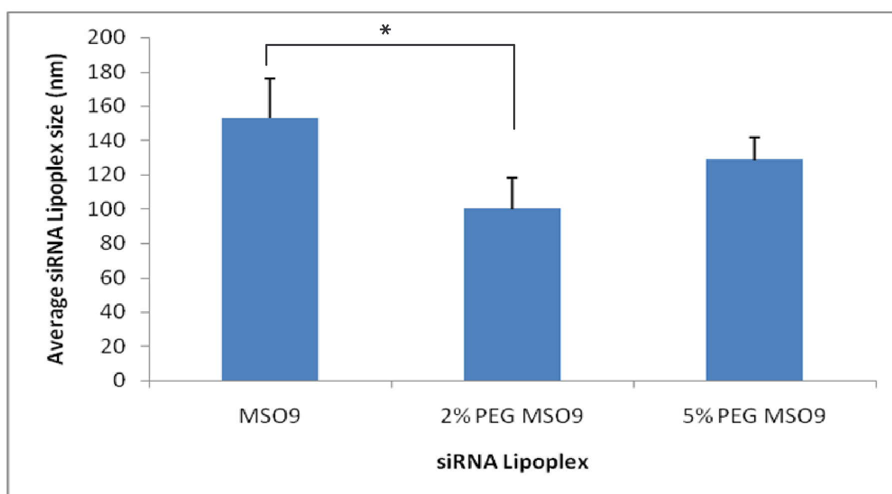


Figure 3.9: Average sizes of siRNA: MSO9 lipoplexes, siRNA: 2% PEG_{2000}MSO9 lipoplexes and siRNA: 5% PEG_{2000}MSO9 lipoplexes at optimum binding ratios. 0.5 µg of siRNA is present. Data presented as a means ± S.D (n = 5) (* $p < 0.05$)

The large standard deviations (n > 10%) suggests that there are variations that exist in the sizes of the lipoplex population and these variations in size could affect the transfection of the liposomes, however, there are conflicting reports that have been published on the relationship between lipoplex size and *in vitro* transfection efficiency. The trend is clearer for *in vivo* conditions as lipoplexes have to be small enough (generally < 150 nm) to extravasate in order to reach the target organs (Singh, 2005).

CHAPTER FOUR

CELL CULTURE AND TRANSFECTION STUDIES

4.1 INTRODUCTION

The delivery of a target gene into the appropriate cell is termed transfection and can be regarded as a powerful tool in cell biology (Weber *et al.*, 1995). More recently, there have been many advances in this field to enhance transfection by improving on the methods whereby a gene of interest is introduced into an appropriate cell.

RNA interference (RNAi) is regarded as one of the major discoveries in biology in recent years (Shan, 2009). RNAi, in mammalian cells, can be initiated by the introduction of short (21-23 nucleotides) synthetic RNA duplexes. These siRNA sequences are capable of inhibiting translation of the cognate mRNA sequence, which occurs in a sequence specific manner, and hence, the production of the corresponding protein is inhibited (Thomas *et al.*, 2009). Theoretically, a sequence of siRNA can be designed to target any given mRNA, a process which can be exploited for therapeutic use (Shan, 2009; Thomas *et al.,* 2009). An example of such a methodology involves the design of a siRNA molecule which can be used to target K-RAS transcripts that carry a valine-112 (K-RAS^{V112}) mutation. This is an oncogene mutation that activates the RAS gene which, in turn, leads to pancreatic and colon cancer. Knockdown of this K-RAS^{V112} gene may result in the degradation of the K-RAS^{V112} gene which can delay the onset of the disease (Ryther *et al.,*2005). A similar principle was applied in this study, with the gene of interest being luciferase and the efficiency of transfection of the cationic liposomes was determined by knockdown of the luciferase gene in the HeLa *tat luc* cell line, a cell line that stably expresses the luciferase gene.

The HeLa cell line is an immortal cell line isolated from a human cervical carcinoma and is one of the most popular culture cells used for biological studies (Starr and Taggart, 2004). The HeLa cells utilized in this study, as mentioned previously, stably express the Firefly luciferase gene. Luciferase is one of the most studied enzymes in both DNA and RNA studies and can catalyze light production in bioluminescent organisms (Singh, 1998). The substrates that are required for the action of luciferase include luciferin, ATP and oxygen (Singh, 2005). The Firefly luciferin structure is shown in figure 4.1.

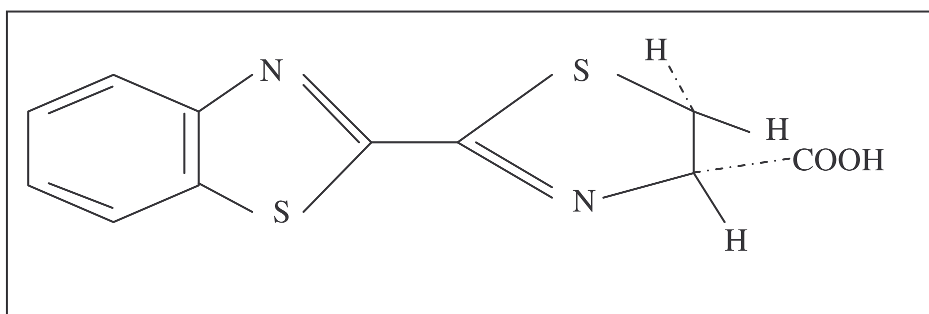


Figure 4.1: Structure of Firefly D-(-)-Luciferin (Narainpersad, 2009)

In this study, an attempt was made to develop synthetic cationic liposome based gene transfer systems that could be used for the successful delivery of siRNA. These cationic liposomes were enhanced by pegylation in an attempt to improve the circulation time of the cationic liposomes. Pegylation was achieved by introducing DSPE-PEG_{2000} into the liposomal formulation. Lipoplexes were prepared with the cationic liposomes together with the anti-luciferase siRNA and a reduction in the luciferase activity (gene knockdown) in the HeLa *tat luc* cells after exposure of cells to complexes was measured to indicate the degree of transfection.

4.2 MATERIALS AND METHOD

4.2.1 MATERIALS

HeLa *tat luc* cell line was provided by the Department of Physiology, University of KwaZulu-Natal, Westville Campus. Anti- luciferase siRNA was purchased from Thermo Scientific Dharmacon Products (Lafayette, CO, USA). Minimal Essential Medium (MEM) and foetal calf serum (FCS) was purchased from GIBCO, Life Technologies Ltd (Inchinnan, Scotland). Trypsin-EDTA and penicillin-streptomycin mixtures were purchased from Lonza BioWhittaker (Walkersville, USA). All tissue culture plastic wear was purchased from Corning Incorporated (New York, USA). The Luciferase Assay Kit (Luciferase Assay reagent and Cell Culture Lysis reagent) was purchased from Promega Corporation (Madison, USA). The Bicinchoninic acid (BCA) assay reagents were purchased from Sigma-Aldrich Co (St. Louis, USA). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) salt was purchased from Merck (Darmstadt, Germany). All other reagents were of analytical grade.

4.2.2 METHOD

4.2.2.1 Growth and Maintenance of HeLa *tat luc* Cell Line

4.2.2.1.1 Reconstitution of HeLa *tat luc* Cells

The HeLa *tat luc* cells which were cryopreserved were obtained in an ampoule and were immediately placed in a 37°C water bath to thaw. The cell suspension was then transferred, aseptically, to a centrifuge tube and centrifuged at 1000 rpm for 5 minutes using a MSE bench top centrifuge. This resulted in a pellet of cells. The supernatant was then discarded and the pellet of cells were resuspended with 1 ml of complete medium (MEM + 1% Antibiotic + 10%

FCS). The cell suspension was then transferred to a 25 cm^3 tissue culture flask containing 4 ml of complete medium. The flask was then placed in a 37°C incubator and checked regularly.

4.2.2.1.2 Propagation of HeLa *tat luc* Cell line

Once the cells had reached confluency or semi-confluency, they were trypsinized. This was done by discarding the growth medium and washing the cells with 5 ml of phosphate buffered saline (PBS) (150 mM NaCl, 2.7 mM KCl, 1 mM KH_2PO_4, 6 mM Na_2HPO_4; pH 7.5). The PBS was then decanted and 1ml of trypsin-EDTA was then added to the cells for trypsinization. A Nikon TMS inverted microscope was used to observe the trypsinization of the cells. The trypsinization time for the HeLa *tat luc* cells was approximately 2 minutes. Once the cells had rounded off, 2 ml of complete medium was added to the cells and the flask was tapped gently on the palm of the hand. The cells were then split to a desired ratio into 25 cm^2 tissue culture flasks or multiwell plates as required. Each flask contained 5ml of complete medium and the cells were incubated at 37°C. The cells were checked at regular intervals and the medium was changed when necessary. Once the cells had reached confluency, they were trypsinized again and split to required ratios or they were frozen and stored at -80°C in a NUAIRE biofreezer (4.2.2.1.3).

4.2.2.1.3 Cryopreservation of HeLa *tat luc* Cells

Cells that had reached confluency were washed with PBS and trypsinized following the procedure outlined in section 4.2.2.1.2. Thereafter, the cells were pelleted by centrifugation using a MSE bench top centrifuge for 3 minutes at 1000 rpm. The medium was decanted and 0.9 ml of complete medium and 0.1 ml of dimethylsulphoxide (DMSO) was added to the pelleted cells which were then resuspended. The cells were then transferred to a cryogenic ampoule and frozen using one of two methods. The first method involves the use of a cold probe. Here the cells were frozen to -70°C at a drop rate of 1°C per minute from room temperature. The cells were then

stored at -80°C in a NUAIRE biofreezer. In the second method, the cryogenic ampoule was transferred to a NALGENE Cryo 1°C Freezing Container which contains isopropanol. This freezing container is placed directly into the NUAIRE biofreezer at -80°C. The ampoules were then removed from the container after 4 hours and stored in the biofreezer at -80°C.

4.2.2.2 siRNA duplexes

SiGENOME non-targeting siRNA (D-001210-01) and Anti-Luc siRNA-1 (D-002050-01) were obtained from Thermo Scientific Dharmacon Products (Lafayette CO). The anti-luciferase siRNA is specific for the firefly luciferase gene and HeLa *tat luc* cells that are successfully transfected with the anti-luciferase siRNA lipoplexes will exhibit a decrease in luciferase expression. The target sequence of the anti-luciferase siRNA is 5'-GAU UAU GUC CGG UUA UGU A(UU)-3'. The non-targeting siRNA duplex contains at least four mismatches with all known human genes and is used in the transfection studies to assess non-sequence specific effects of the siRNA lipoplexes on the HeLa *tat luc* cells *in vitro*. The target sequence of this siRNA duplex is 5'-UAG CGA CUA AAC ACA UCA A-3'. The siRNA duplexes were resuspended as outlined in section 3.2.2.1.

4.2.2.3 Cytotoxicity Studies

The HeLa *tat luc* cells were trypsinized and seeded into 48 well plates at different seeding densities. For the toxicity studies using Chol-T, 2% PEG_{2000}Chol-T and 5% PEG_{2000}Chol-T, the cells were seeded at 2.2×10^4 cells per well and for the MSO9, 2% PEG_{2000}-MSO9 and 5% PEG_{2000}-MSO9 cationic liposomes the cells were seeded at 2.1×10^4 cells per well. The cells were incubated at 37°C and allowed to attach overnight. The siRNA lipoplexes were prepared as in table 4.1. Three concentrations of anti-luciferase siRNA were used, namely, 20 nM (0.067 µg),

30 nM (0.1 μg) and 50 nM (0.17 μg). All the complexes were made up to a final volume of 10 μl with HBS and incubated at room temperature for 30 minutes.

Once the reaction mixtures were prepared, the growth medium was removed and replaced with 0.25 ml of serum free medium (MEM + 1% Antibiotics). The complexes were then added to the wells containing the cells. All the assays were done in triplicate. The multiwell plates were then incubated at 37°C for 4 hours. Thereafter, the serum free medium was removed and replaced with 0.25 ml of complete medium. The cells were then incubated at 37 °C for 36 hours. After this time, the growth medium was removed and replaced with 0.2 ml of MTT (5 mg/ml in PBS) and 0.2 ml of complete medium. The cells were then incubated at 37°C for four hours. Thereafter, the MTT and the medium were removed and replaced with 0.2 ml of dimethylsulphoxide (DMSO). The absorbances were then measured at 540 nm using a Vacutec MR-96A microplate reader.

Table 4.1: siRNA: cationic liposome ratios used for cytotoxicity studies and transfection studies

LIPOSOME PREPARATION	**siRNA: CATIONIC LIPOSOME RATIOS (w/w)**		
CHOL-T	1:6	1:8	1:10
2% PEG_{2000}CHOL-T	1:8	1:10	1:12
5% PEG_{2000}CHOL-T	1:12	1:14	1:16
MSO9	1:10	1:12	1:14
2% PEG_{2000}MSO9	1:44	1:46	1:48
5% PEG_{2000}MSO9	1:30	1:40	1:50

4.2.2.4 Transfection Studies

4.2.2.4.1 Transfection Studies with Positive Control (Anti-Luciferase siRNA)

The HeLa *tat luc* cells were seeded in 48 well plates at a cell density of 2.0×10^4 cells per well. The cells were incubated at 37°C overnight to allow for the attachment of the cells. The reaction mixtures of the siRNA and cationic liposomes were prepared according to the ratios in table 4.1 and as outlined in section 4.2.2.3. The cells were prepared by removing the growth medium and replacing it with 0.25 ml serum free medium. The complexes were then introduced to the cells, each assay being done in triplicate. For this study, two controls were present. The first being the HeLa *tat luc* cells only and the second being the cells with naked anti-luciferase siRNA. All three concentrations of the anti-luciferase siRNA were studied. The 48 well plates were then incubated at 37°C for 4 hours. After this time, the medium was removed and replaced with complete medium. The multiwell plates were then incubated at 37°C for 36 hours. After this incubation, the luciferase activity of the cells was determined.

4.2.2.4.2 Luciferase Assay

The luciferase assay was carried out using the Promega Luciferase Assay kit. The cell culture lysis reagent (5x) (25 mM Tris-phosphate, pH 7.8; 2 mM dithiothreiotol, 2 mM 1,2-diaminocyclohexane – N, N, N'N'- tetra-acetic acid, 10% ($^v/_v$) glycerol, 1% ($^v/_v$) triton X-100), was diluted to a 1× stock with distilled water. The luciferase assay reagent and the 1× cell culture lysis reagent were allowed to equilibrate to room temperature.

The cells were prepared by removing the growth medium and washing twice with PBS. Eighty µl of 1× cell culture lysis reagent was added to the cells. The multiwell plates were then placed on a

Stuart Scientific Platform Rocker for 15 minutes at 30 rev/ min. Thereafter, the lysed cells were dislodged from the multiwell plates by scraping the wells. The cell suspension was then transferred to microcentrifuge tubes and pelleted by centrifugation at 12000 × g for 20 seconds in an Eppendorf Microcentrifuge. The supernatant, being the cell free extract, was then retained for the determination of luciferase activity. This was done by adding 100 µl of the luciferase reagent to 20 µl of the cell free extract at room temperature. This was immediately mixed and placed in a Lumac Biocounter 1500 luminometer. The light produced was then measured for a period of 10 seconds. The protein determination of the cell free extracts was then carried out using the BCA assay.

4.2.2.4.3 Transfection Studies with Negative Control (Non- targeting Control siRNA)

HeLa *tat luc* cells were seeded at a cell density of 1.8×10^4 cells per well in 48 well plates. The cells were allowed to attach overnight at 37°C. The complexes were prepared as outlined in section 4.2.2.3 as per Table 4.1. In this study, the non-targeting control siRNA was used when preparing the siRNA lipoplexes at the three concentrations, namely 20 nM, 30 nM and 50 nM. The same protocol was followed as outlined in section 4.2.2.4.1. The luciferase activity was determined as outlined in section 4.2.2.4.2. The protein determination of the cell free extracts was carried out using the BCA assay.

4.2.2.5 Statistical Analysis

Cell numbers and percentage of luciferase expression are presented as means ± SD. Results were examined by one way ANOVA and groups were compared by Student's t-test. P values less than 0.05 were considered to be statistically significant.

4.3 RESULTS AND DISCUSSION

4.3.1 Growth and Maintenance of HeLa *tat luc* cells

The HeLa *tat luc* cell line was grown and maintained in MEM + 1% Antibiotics + 10% New Born Calf Serum. Rapid cell growth was observed with the HeLa *tat luc* cells, which is a characteristic of this cell line. Confluency of the HeLa *tat luc* cells was reached within 3 to 4 days. The HeLa *tat luc* cells were trypsinized upon reaching confluency and subdivided into ratios of 1:3 or 1:4.

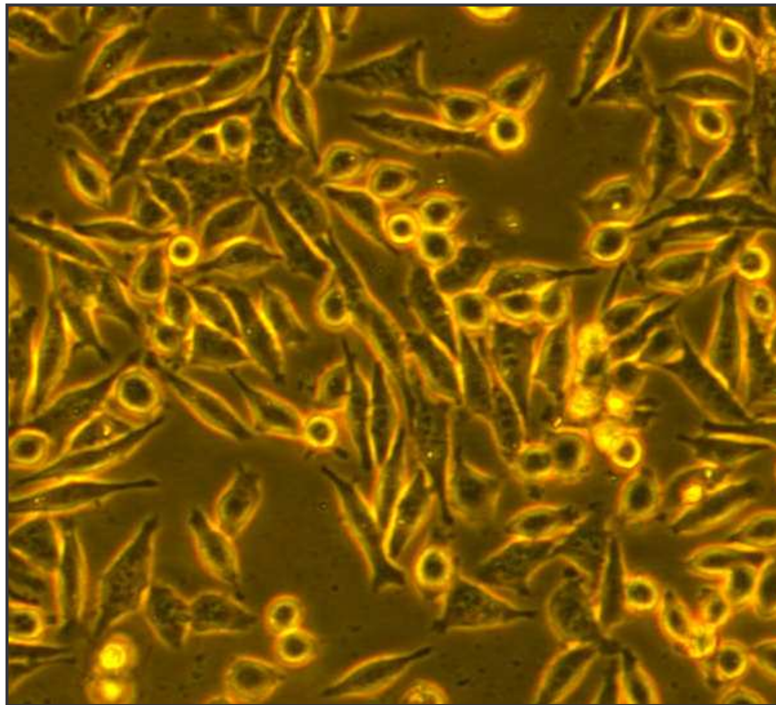


Figure 4.2: Monolayer of HeLa *tat luc* cells at semi- confluency viewed under an Olympus fluorescence microscope (100×)

4.3.2 Cytotoxicity Studies

An ideal characteristic of non-viral vectors is safety and minimal toxicity, therefore, cell toxicity studies of the delivery vehicle, which is sometimes overlooked, is very important. It has been suggested that cationic lipids may have a toxic effect to cells *in vitro*. The toxicity of the cationic liposomes is due to the presence of the positive charges on the head group of the liposome (Lv *et al.*, 2006). The cationic liposomes may interact with biological components that may induce aggregation or the germicidal action of the positive surfactants against bacteria, fungi, viruses and invertebrates (Lasic, 1997).

In this study, the MTT cell proliferation assay was used to determine the toxicity of the cationic liposomes. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) is a yellow tetrazolium salt which is water soluble and is converted to an insoluble formazon product (Fotakis *et al.*, 2006). This conversion is carried out by the mitochondrial enzyme, succinate dehydrogenase, which cleaves the tetrazolium ring. This reduction will only take place when the enzymes are active and, therefore, can be directly related to cell viability (Vellanen *et al.*, 2004). Following solubilization of the formazon crystals by the addition of the solubilization agent, which was dimethylsulphoxide (DMSO), the absorbance of the resulting purple solution was then determined.

All six lipoplex preparations were well tolerated by the HeLa *tat luc* cell line over the three different siRNA concentrations (20 nM, 30 nM and 50 nM). The control for this study was the untreated cells. Since these cells were not exposed to any siRNA lipoplexes, their absorbance readings were represented as 100% cell survival. The results for the cytotoxicity studies are represented in Figures 4.3 (a-c) and 4.4 (a-c).

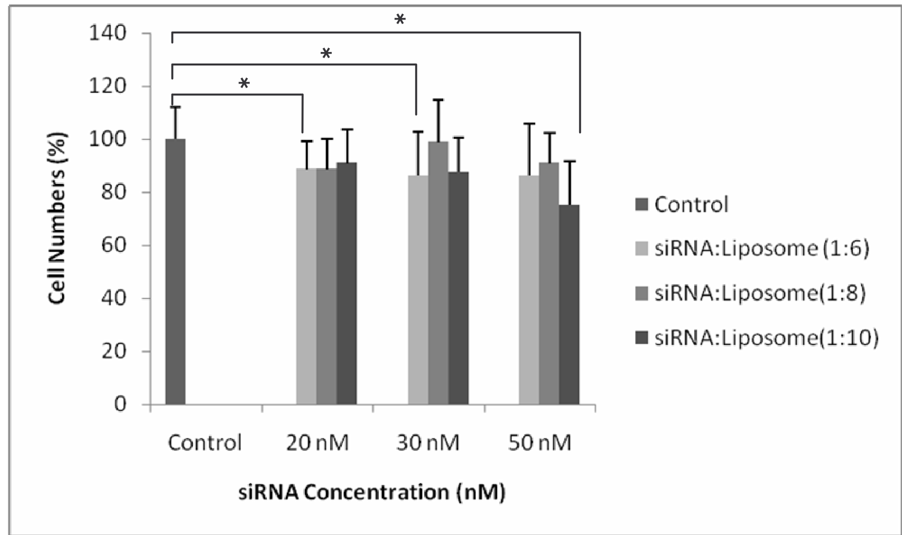


Figure 4.3 (a)

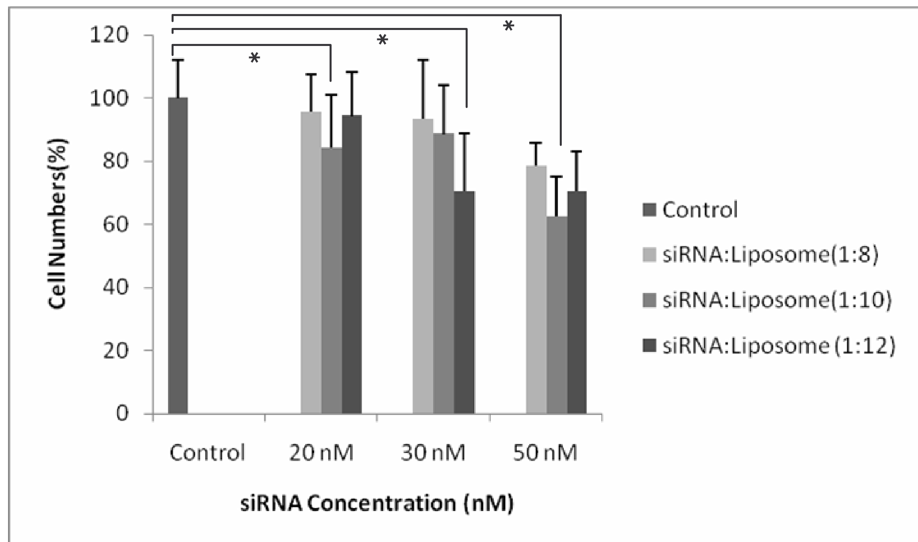


Figure 4.3 (b)

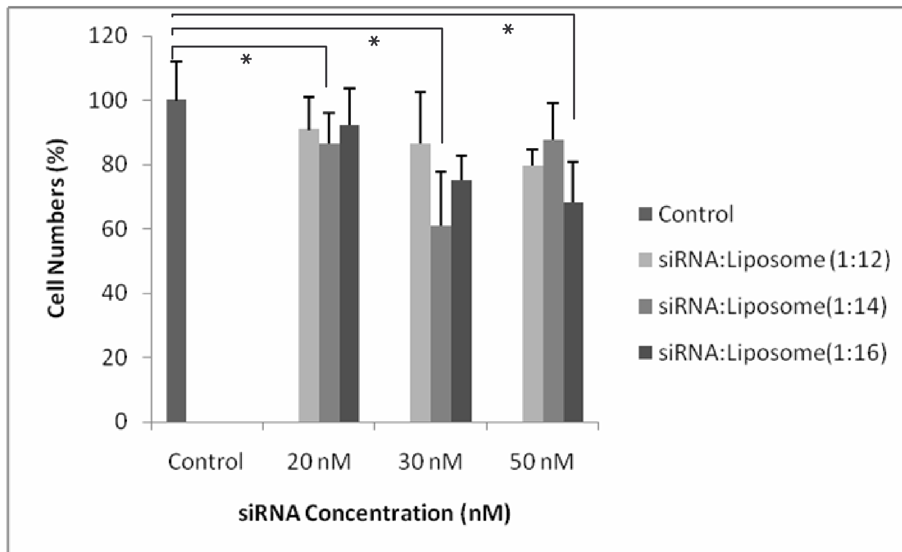


Figure 4.3 (c)

Figure 4.3: Cytotoxicity of cationic liposome: siRNA complexes in HeLa *tat luc* cells *in vitro.* siRNA concentrations utilized were 20 nM (0.067 µg), 30 nM (0.1 µg) and 50 nM (0.168 µg),

(a) Varying concentrations of Chol-T (0.4 µg, 0.54 µg, 0.67 µg/ 10 µl), (0.6 µg, 0.8 µg, 1 µg/10 µl), (1.01 µg, 1.34 µg, 1.68 µg/10 µl) for the 20 nM, 30 nM and 50 nM concentrations, respectively,

(b) Varying concentrations of 2% PEG_{2000}Chol-T (0.54 µg, 0.67 µg, 0.8 µg/ 10 µl), (0.8 µg, 1 µg, 1.2 µg/10 µl), (1.34 µg, 1.68 µg, 2.02 µg/10 µl) for the 20 nM, 30 nM and 50 nM concentrations, respectively,

(c) Varying concentrations of 5% PEG_{2000}Chol-T (0.8 µg, 0.94 µg, 1.07 µg/ 10 µl), (1.2 µg, 1.4 µg, 1.6 µg/10 µl), (2.02 µg, 2.35 µg, 2.69 µg/10 µl) for the 20 nM, 30 nM and 50 nM concentrations, respectively,

in a total volume of 0.25 ml medium (MEM). A control sample (no liposome), contained only HeLa *tat luc* cells was assumed to have 100% survival. Data presented as means ± S.D (n = 3) (* $p < 0.05$).

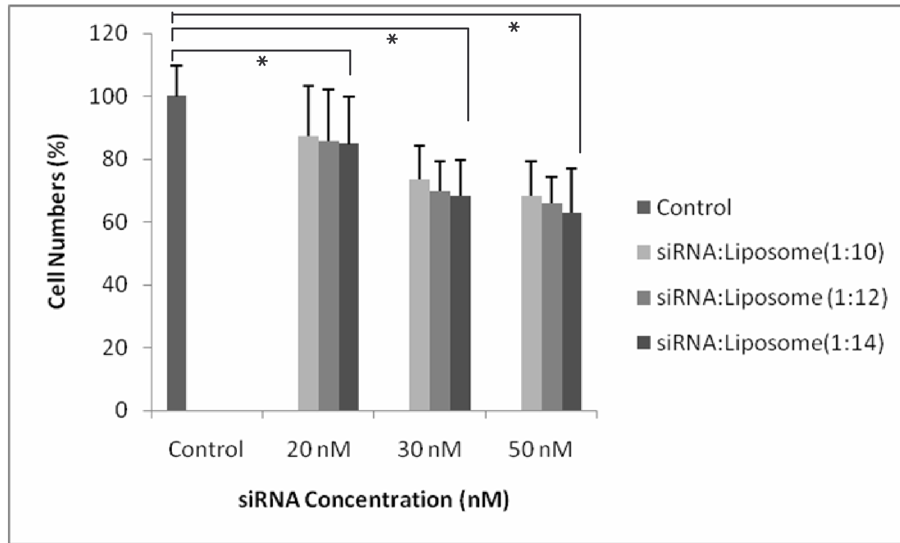


Figure 4.4 (a)

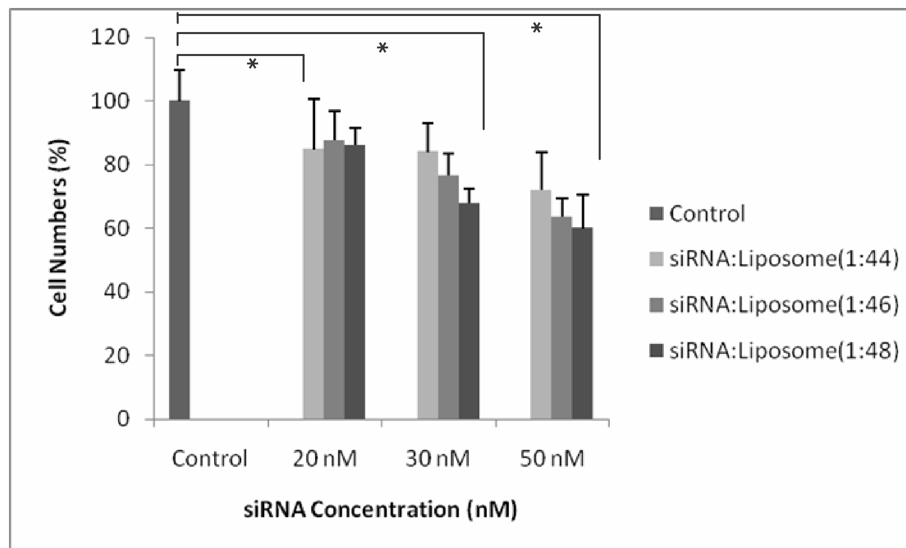


Figure 4.4 (b)

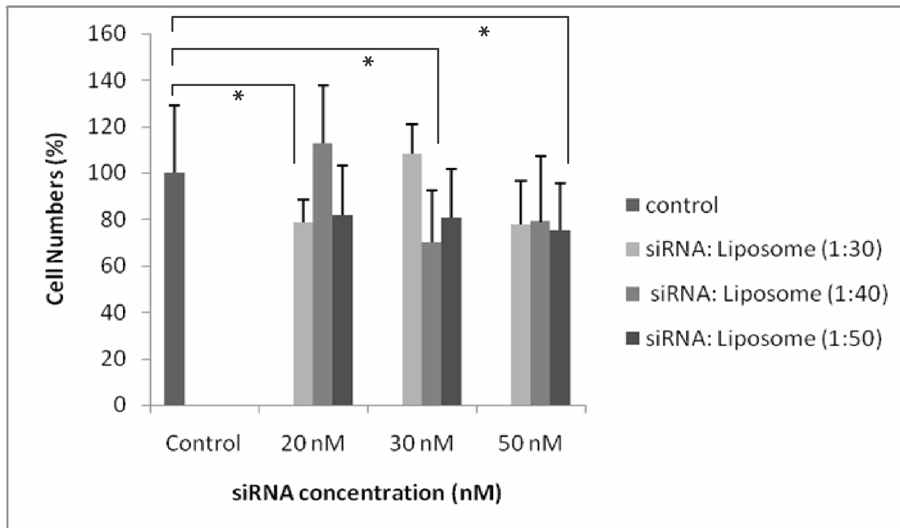


Figure 4.4 (c)

Figure 4.4: Cytotoxicity of cationic liposome: siRNA complexes in HeLa *tat luc* cells *in vitro*. siRNA concentrations utilized were 20 nM (0.067 µg), 30 nM (0.1 µg) and 50 nM (0.168 µg).

(a) Varying concentrations of MSO9 (0.67 µg, 0.8 µg, 0.94 µg/10 µl), (1 µg, 1.2 µg, 1.4µg/10µl), (1.68 µg, 2.02 µg, 2.4 µg/10 µl) for the 20 nM, 30 nM and 50 nM concentrations, respectively,

(b) Varying concentrations of 2% PEG_{2000}MSO9 (2.95 µg, 3.08 µg, 3.22 µg/10 µl), (4.4 µg, 4.6 µg, 4.8µg/10 µl), (7.4 µg, 7.7 µg, 8.06 µg/10 µl) for the 20 nM, 30 nM and 50 nM concentrations, respectively,

(c) Varying concentrations of 5% PEG_{2000}MSO9 (2 µg, 2.68 µg, 3.35 µg/10 µl), (3 µg, 4 µg, 5 µg/10 µl), (5.04 µg, 6.72 µg, 8.4 µg/10 µl) for the 20 nM, 30 nM and 50 nM concentrations, respectively,

in a total volume of 0.25 ml medium (MEM). A control sample (no liposome), contained only HeLa *tat luc* cells was assumed to have 100% survival. Data presented as means ± S.D (n=3) (* $p < 0.05$).

Maximum cell death recorded for the Chol-T lipoplexes was 25% at the 50 nM siRNA concentration, 37% for 2% PEG_{2000}Chol-T and 39% for 5% PEG_{2000}Chol-T at the 50 nM and 30 nM siRNA concentrations, respectively (Figure 4.3 a-c). Cells treated with MSO9 showed maximal cell growth inhibition of 33% at the 50 nM siRNA concentration, and those cells exposed to 2% PEG_{2000}MSO9 and 5% PEG_{2000}MSO9 showed maximum cell death of 36% and 33% at the 50 nM and 30 nM siRNA concentrations, respectively (Figure 4.4 a-c).

The results obtained suggest that the pegylated cationic liposomes exhibit a marginally higher cytotoxicity levels as compared to the non-pegylated counterparts. This is consistent with the results obtained by Dadashzadeh *et al.* (2008), who showed that their pegylated cationic liposomes had a higher toxicity when compared to the non-pegylated cationic liposome.

Another observation from the results is that for most of the liposomes, maximum growth inhibition of the cells occurred at the siRNA concentration of 50 nM. This could be attributed to the presence of a higher concentration of the cationic liposome that was exposed to the cells. This higher cationic liposome concentration was required since a higher siRNA concentration was used and the optimum binding ratios for each liposome was studied. A similar result was obtained by Zhang *et al.* (2010), who attributed an increase in the cytotoxicity of their liposomes to the presence of an increased amount of the cationic liposome being present.

Overall, the relatively low toxicity of these lipoplexes is significant for their further development as siRNA carriers.

4.3.3 Transfection Studies

The HeLa *tat luc* cell line was utilized for this study. This cell line is a human cervical cancer cell line which stably expresses the firefly luciferase gene. The anti- luciferase siRNA, which is specific for the firefly luciferase gene, was used to determine the efficiency of transfection of the cationic liposomes as a measure of gene knockdown. The results are presented in Figures 4.5 (a-c) and 4.6 (a-c).

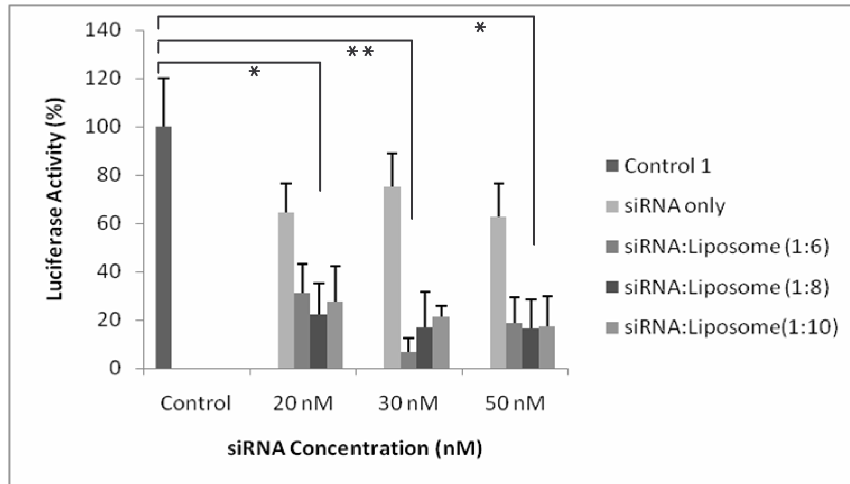


Figure 4.5 (a)

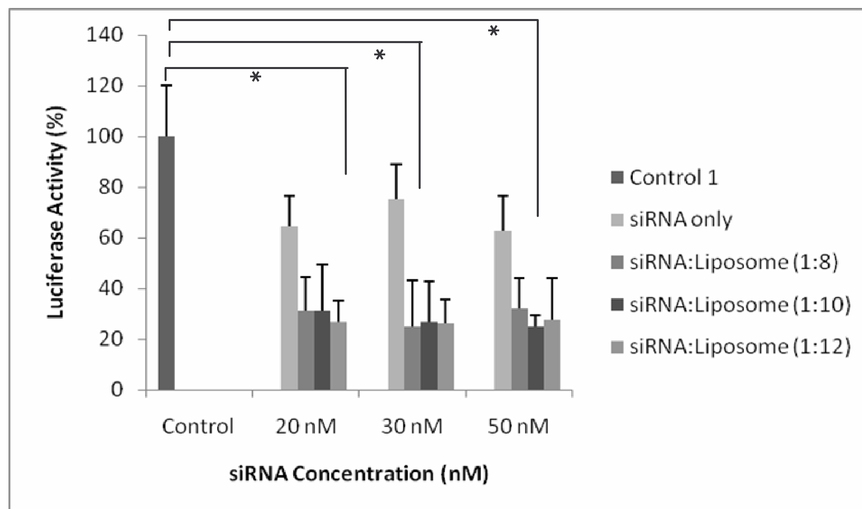


Figure 4.5 (b)

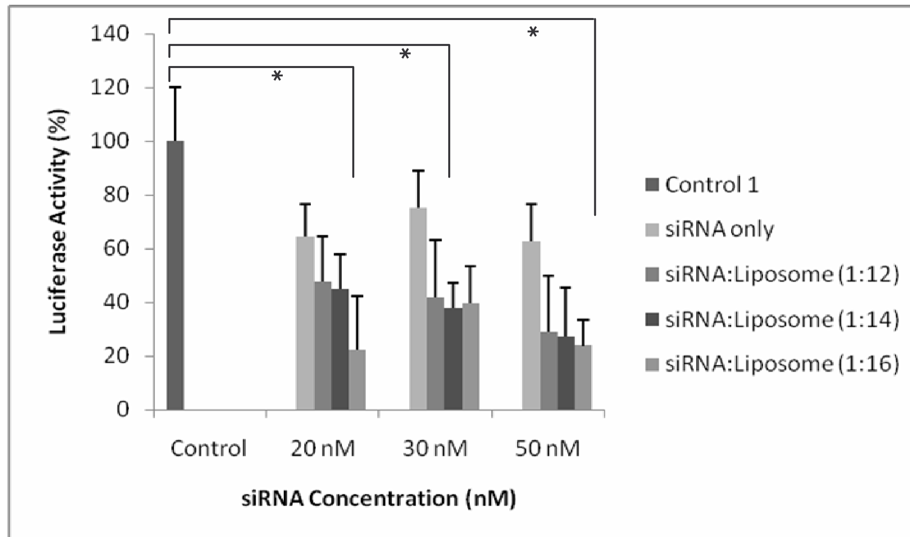


Figure 4.5 (c)

Figure 4.5: Transfection studies of cationic liposome: anti-luciferase siRNA complexes in HeLa *tat luc* cells *in vitro*. siRNA concentrations utilized were 20 nM, 30 nM and 50 nM.

(a) Varying concentrations of Chol-T (0.4 µg, 0.54 µg, 0.67 µg/10 µl), (0.6 µg, 0.8 µg, 1 µg/10 µl), (1.01 µg, 1.34 µg, 1.68 µg/10 µl) for the 20 nM, 30 nM and 50 nM concentration, respectively,

(b) Varying concentrations of 2% PEG_{2000}Chol –T (0.54 µg, 0.67 µg, 0.8 µg/10 µl), (0.8 µg, 1 µg, 1.2 µg/10 µl), (1.34 µg, 1.68 µg, 2.02 µg/10 µl) for the 20 nM, 30 nM and 50 nM concentration, respectively,

(c) Varying concentrations of 5% PEG_{2000}Chol -T (0.8 µg, 0.94 µg, 1.07 µg/10µl), (1.2 µg, 1.4 µg, 1.6 µg/10 µl), (2.02 µg, 2.35 µg, 2.69 µg/10 µl) for the 20 nM, 30 nM and 50 nM concentration, respectively,

in a total volume of 0.25 ml medium (MEM). Control 1 contained HeLa *tat luc* cells only and Control 2 contained HeLa *tat luc* cells and siRNA only. Data presented as a means ± S.D (n= 3) (* $p < 0.05$) (** $p < 0.01$).

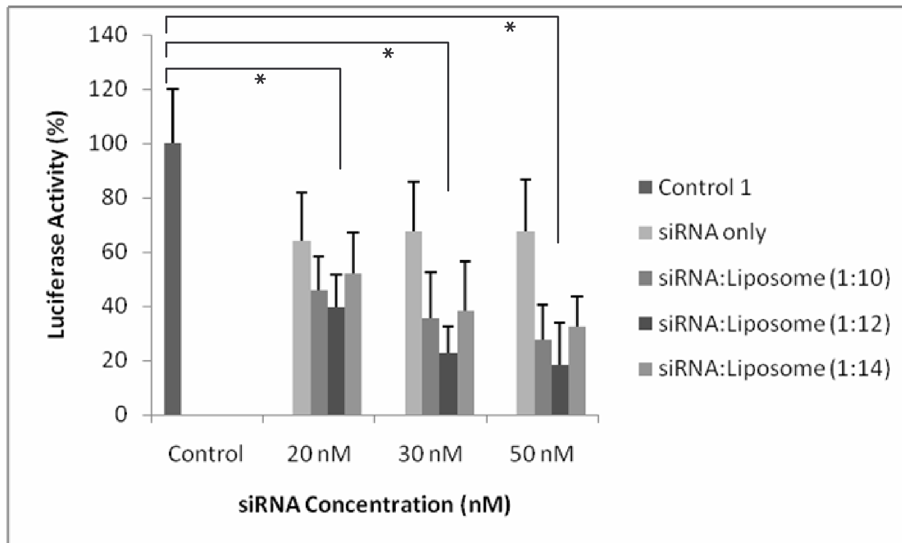


Figure 4.6 (a)

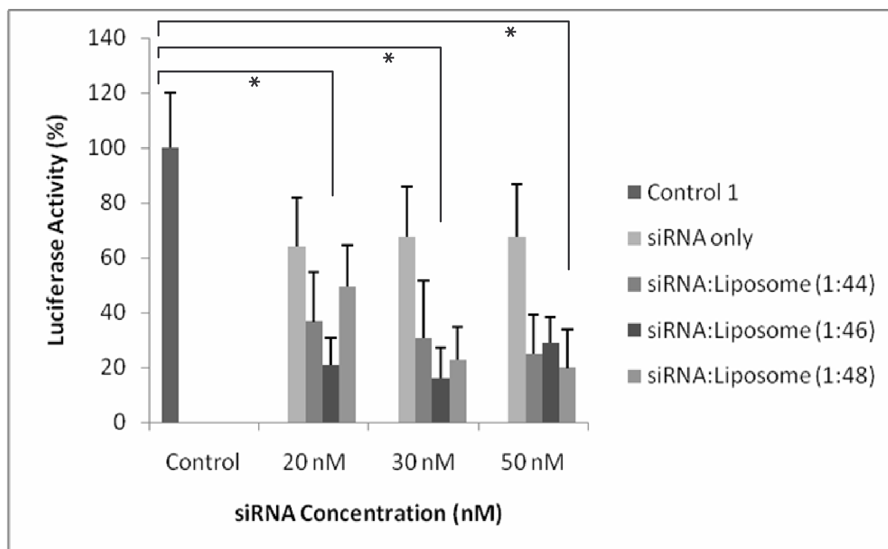


Figure 4.6 (b)

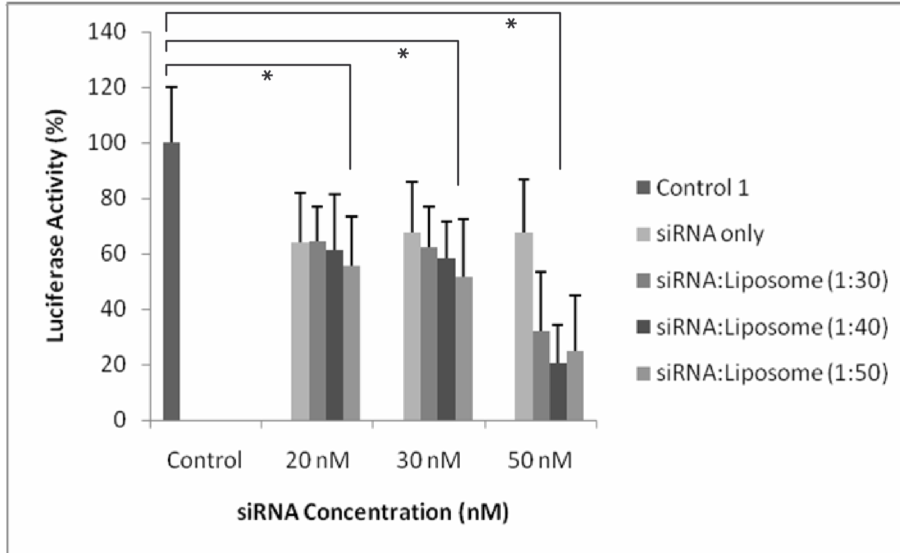


Figure 4.6 (c)

Figure 4.6: Transfection studies of cationic liposome: anti-luciferase siRNA complexes in HeLa *tat luc* cells *in vitro*. siRNA concentrations utilized were 20 nM, 30 nM and 50 nM.

(a) Varying concentrations of MSO9 (0.67 µg, 0.8 µg, 0.94 µg/10 µl), (1 µg, 1.2 µg, 1.4µg/10 µl), (1.68 µg, 2.02 µg, 2.4 µg/10 µl) for the 20 nM, 30 nM and 50 nM concentrations, respectively,

(b) Varying concentrations of 2% PEG_{2000} MSO9 (2.95 µg, 3.08 µg, 3.22 µg/10 µl), (4.4 µg, 4.6 µg, 4.8µg/10 µl), (7.4 µg, 7.7 µg, 8.06 µg/10 µl) for the 20 nM, 30 nM and 50 nM concentrations, respectively,

(c) Varying concentrations of 5% PEG_{2000} MSO9 (2 µg, 2.68 µg, 3.35 µg/10 µl), (3 µg, 4 µg, 5 µg/10 µl), (5.04 µg, 6.72 µg, 8.4 µg/10 µl) for the 20 nM, 30 nM and 50 nM concentrations, respectively,

in a total volume of 0.25 ml medium (MEM). Control 1 contained HeLa *tat luc* cells only and Control 2 contained HeLa *tat luc* cells and siRNA only. Data presented as a means ± S.D (n= 3) (* $p < 0.05$).

The luciferase activity was measured as relative light units/mg protein (RLU/mg protein) and expressed as a percentage relative to a control (Control 1). Hence, in this assay, control 1 represents untreated HeLa *tat luc* cells and, therefore, the RLU/mg protein was expressed as a 100% as one could assume that the luciferase expression would be the highest in this control.

A second control (Control 2) for this study represents cells that were treated with anti-luciferase siRNA only. Here, at all three siRNA concentrations used (20 nM, 30 nM and 50 nM), little gene knockdown was evident. When comparing all three siRNA concentrations, the maximum gene knockdown evident was 47.3% at 50 nM siRNA concentration. The siRNA, in this case, may have been taken up into the cell by processes that were not intended during the transfection experimient carried out. An example of such a mechanism can involve the attachment of the siRNA to lipoproteins on the cell surface. This may result in the cellular uptake of the siRNA which, in turn, can result in marginal gene knockdown.

The results obtained with the cells treated with the cationic liposomes Chol-T and MSO9 showed efficient knockdown of the luciferase gene when compared to the controls (Figure 4.5 a-c and Figure 4.6 a-c). The results show that there was a significant difference in the luciferase expression of the control cells and those cells that were treated with the anti-luciferase siRNA lipoplexes ($p < 0.05$). Cells treated with Chol-T showed maximum gene knockdown at 30 nM siRNA concentration of 93.4% at a siRNA: Chol-T ratio of 1:6 and charge ratio of 1:1.3 (-/+) (Figure 4.5 a). The 2% PEG_{2000} Chol-T presented a maximum gene knockdown at a siRNA concentration of 50 nM of 75% at a siRNA : liposome ratio of 1:10 [charge ratio 1:2.2 (-/+)] (Figure 4.5 b) and 5% PEG_{2000} Chol-T was capable of 77.8% gene knockdown at the 20 nM siRNA concentration at a siRNA: liposome ratio of 1:16 [charge ratio of 1:3.1 (-/+)] (Figure 4.5 c).

The cells transfected with the MSO9 liposomes exhibited maximum gene knockdown of 84% at the siRNA concentration of 50 nM. This occurred at a siRNA: MSO9 ratio of 1:12 at a charge

ratio of 1:2.5 (-/+) (Figure 4.6 a). Maximum gene knockdown of 81.6% and 79.4% was evident for 2% PEG_{2000} MSO9 (Figure 4.6 b) and 5% PEG_{2000} MSO9 (Figure 4.6 c), respectively. This gene knockdown occurred at siRNA: liposome ratios of 1:46 (charge ratio of 1:8.6 (-/+)) and 1:40 (charge ratio of 1:7.2 (-/+)) for the 2% PEG_{2000} MSO9 and 5% PEG_{2000} MSO9, respectively.

The transfection results achieved suggest that the length of the spacer arm has a minimal effect on the siRNA transfection, in this study. This is evident when comparing the results achieved by MSO9 (with a 12 atom spacer) and Chol-T (6 atom spacer), where both the liposomes exhibited similar levels of transfection when the luciferase activity was measured.

Maximum gene knockdown for the non-pegylated and pegylated MSO9 liposomes was evident at the optimum binding ratios for each liposome. This trend was not observed with the pegylated and non-pegylated Chol-T liposomes as optimum transfection for Chol-T was at the sub-optimum binding ratio, for 2% PEG_{2000} Chol-T was at the optimum binding ratio and for the 5% PEG_{2000} Chol-T maximum gene knockdown occurred at the supra-optimum binding ratio. These results could be attributed to the differences in the sizes of the lipoplexes as well as the charge ratios which may have affected the transfection results (Higuchi *et al.,* 2006; Torchillin *et al.*, 2003). Studies have shown that a relationship exists between the net charge of the lipoplexes and their effectiveness in transfection where, in most cases, a small net positive charge is required for *in vitro* transfection. Studies conducted have reported the use of charge ratios between 1:1 to 1:10 (-/+). This resultant positive charge can enhance the interaction with cellular membranes which are known to have a net negative charge (Torchillin *et al*., 2003).

The transfection results achieved with the siRNA complexed to the cationic liposomes showed higher levels of gene knockdown as compared to the corresponding concentration of the naked siRNA. This suggests that the complexation of the siRNA with the cationic liposomes is able to overcome cellular barriers as well as protect the siRNA from nuclease degradation and hence, a

greater amount of the siRNA became available to the cells which resulted in a significant gene knockdown.

The use of negative controls is also important in RNAi studies. An accepted negative control for siRNA is the use of a control siRNA duplex with a scrambled sequence, which does not contain a perfect match in the genome (Shan *et al*., 2009). This negative control was applied in this study, in addition, the results were compared to luciferase activity in untreated cells. The transfection results achieved for the negative control are represented in Figures 4.7 (a-c) and 4.8 (a-c).

To ensure that knockdown of the luciferase gene was achieved due to the introduction of the anti-luciferase siRNA, the transfection experiment was repeated, however, the siGENOME non-targeting siRNA was used as opposed to the anti-luciferase siRNA. The results obtained for this transfection assay showed that gene knockdown did not occur. The results for this assay show that the luciferase expression was very high and, in some instances, the cells treated with the siRNA lipoplexes exhibited higher levels of luciferase activity as compared to the control. Since non-targeting siRNA was used for this transfection study, the results obtained support those that were achieved for the luciferase assay where anti-luciferase siRNA was used and, therefore, it can be deduced that knockdown of the luciferase gene occurred as a result of the delivery of the anti-luciferase siRNA, which is specific for the firefly luciferase gene.

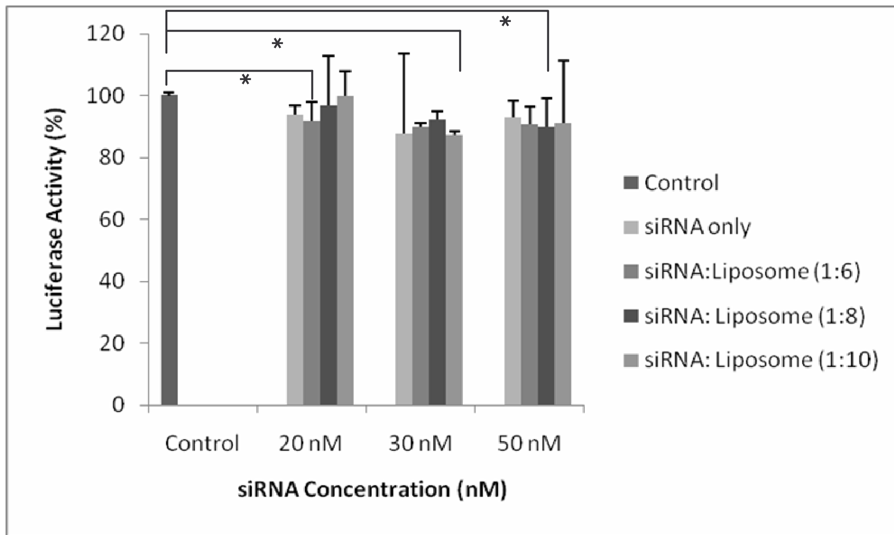


Figure 4.7 (a)

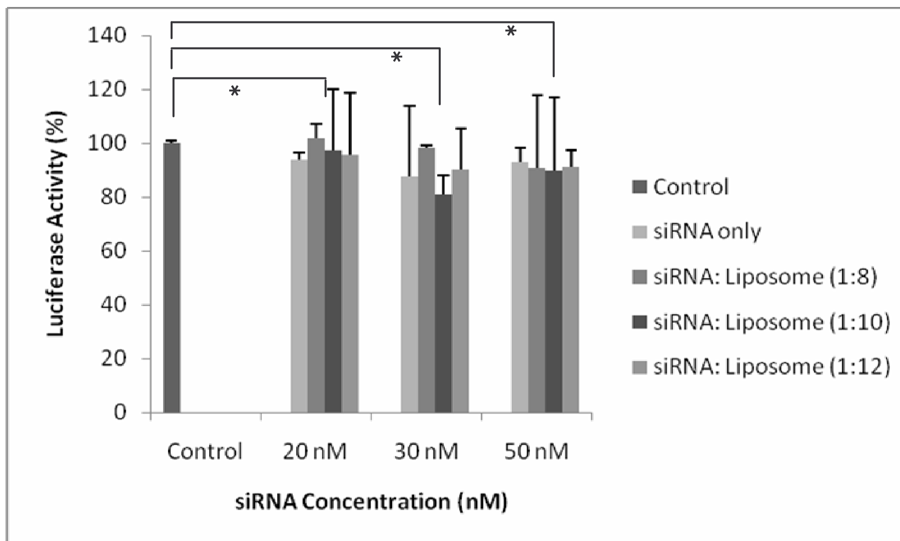


Figure 4.7 (b)

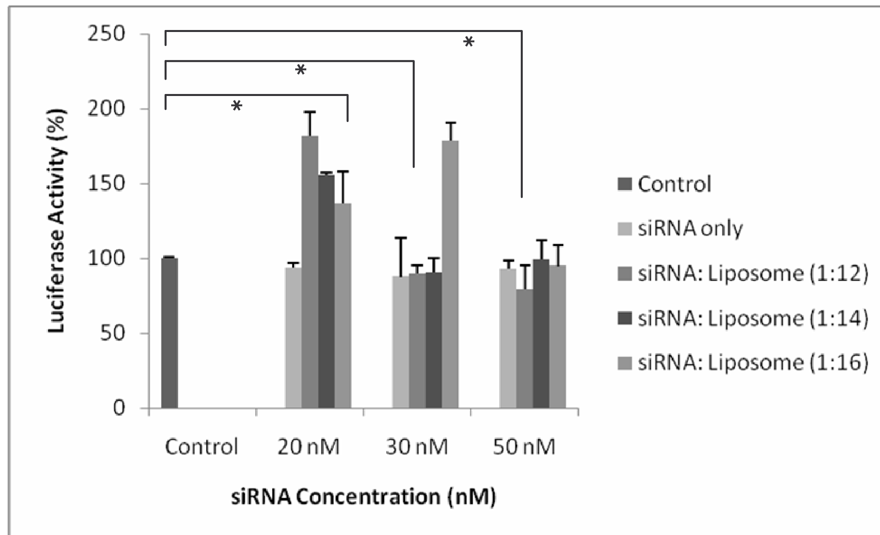


Figure 4.7 (c)

Figure 4.7: Transfection studies of cationic liposome: non-targeting siRNA complexes in HeLa *tat luc* cells *in vitro*. siRNA concentrations utilized were 20 nM, 30 nM and 50 nM.

(a) Varying concentrations of Chol-T (0.4 µg, 0.54 µg, 0.67 µg/10 µl), (0.6 µg, 0.8 µg, 1 µg/10 µl), (1.01 µg, 1.34µg, 1.68 µg/10 µl) for the 20 nM, 30 nM and 50 nM concentrations, respectively,

(b) Varying concentrations of 2% PEG_{2000}Chol –T (0.54 µg, 0.67 µg, 0.8 µg/10 µl), (0.8 µg, 1 µg, 1.2 µg/10 µl), (1.34 µg, 1.68 µg, 2.02 µg/10 µl) for the 20 nM, 30 nM and 50 nM concentrations, respectively,

(c) Varying concentrations of 5% PEG_{2000}Chol -T (0.8 µg, 0.94 µg, 1.07 µg/10µl), (1.2 µg, 1.4 µg, 1.6 µg/10 µl), (2.02 µg, 2.35 µg, 2.69 µg/10 µl) for the 20 nM, 30 nM and 50 nM concentrations, respectively,

in a total volume of 0.25 ml medium (MEM). Control 1 contained HeLa *tat luc* cells only and Control 2 contained HeLa *tat luc* cells and siRNA only. Data presented as a means ± S.D (n= 3) (* $p < 0.05$).

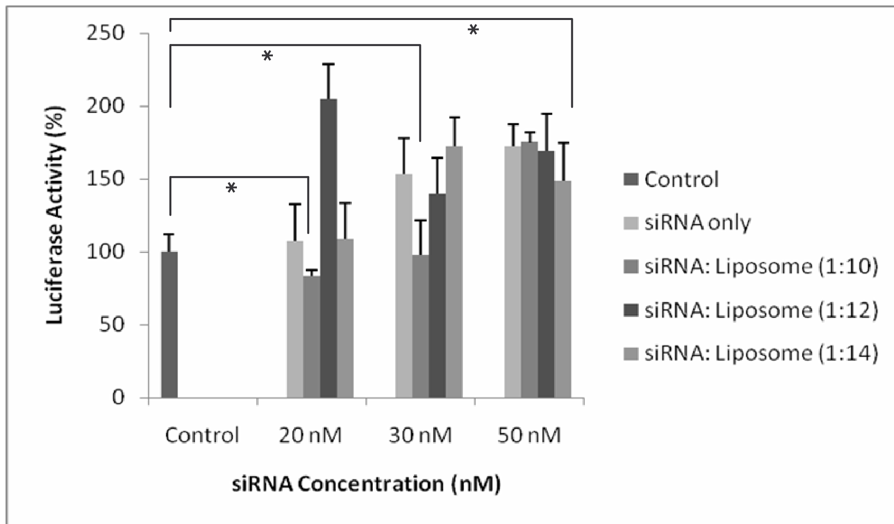


Figure 4.8 (a)

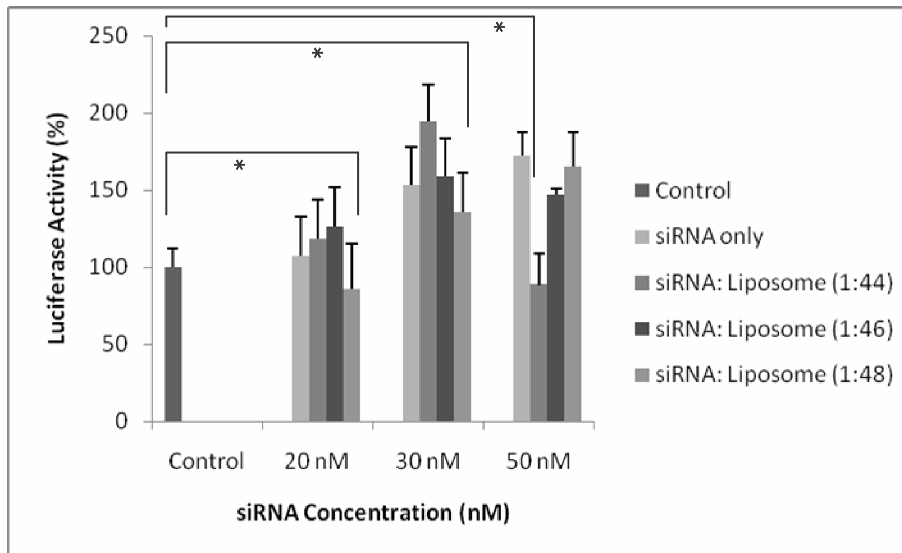


Figure 4.8 (b)

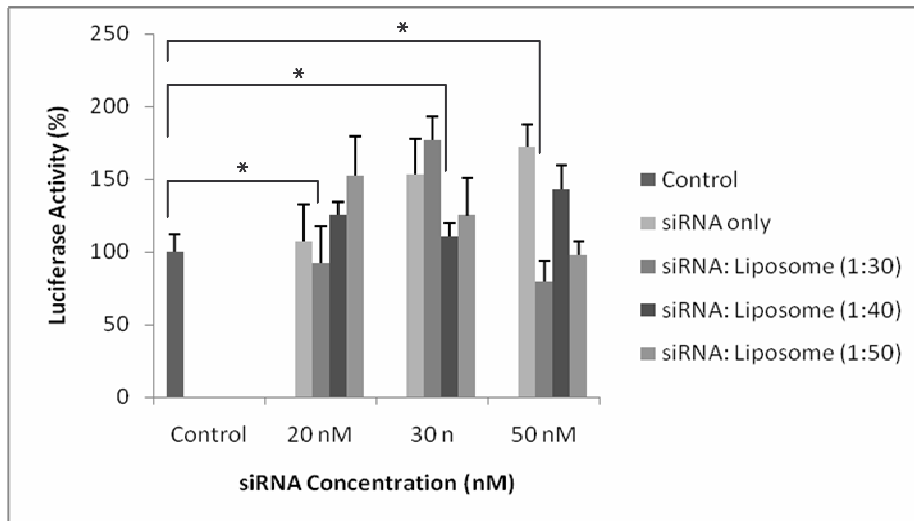


Figure 4.8 (c)

Figure 4.8: Transfection studies of cationic liposome: non-targeting siRNA complexes in HeLa *tat luc* cells *in vitro*. siRNA concentrations utilized were 20 nM, 30 nM and 50 nM.

(a) Varying concentrations of MSO9 (0.67 µg, 0.8 µg, 0.94 µg/10 µl), (1 µg, 1.2 µg, 1.4µg/10 µl), (1.68 µg, 2.02 µg, 2.4 µg/10 µl) for the 20 nM, 30 nM and 50 nM concentrations, respectively,

(b) Varying concentrations of 2% PEG_{2000} MSO9 (2.95 µg, 3.08 µg, 3.22 µg/10 µl), (4.4 µg, 4.6 µg, 4.8µg/10 µl), (7.4 µg, 7.7 µg, 8.06 µg/10 µl) for the 20 nM, 30 nM and 50 nM concentrations, respectively,

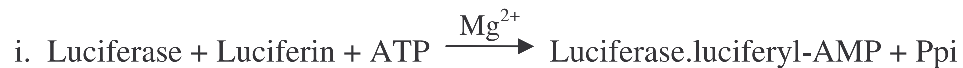
(c) Varying concentrations of 5% PEG_{2000} MSO9 (2 µg, 2.68 µg, 3.35 µg/10 µl), (3 µg, 4 µg, 5 µg/10 µl), (5.04 µg, 6.72 µg, 8.4 µg/10 µl) for the 20 nM, 30 nM and 50 nM concentrations, respectively,

in a total volume of 0.25 ml medium (MEM). Control 1 contained HeLa *tat luc* cells only and Control 2 contained HeLa *tat luc* cells and siRNA only. Data presented as a means ± S.D (n= 3) (* $p < 0.05$).

A trend that was evident was that as the degree of pegylation increased, there was a marginal but measurable decrease in the transfection efficiency of the cationic liposomes. This occurred with the liposomes prepared with both cytofectins, Chol-T and MSO9. This is consistent with the observations made by Vandenbrouke *et al* (2008), Zhang *et al* (2010) and Yang *et al* (2009). This negative effect of pegylation has been studied intensively for plasmid DNA delivery and it has been suggested by Desphande *et al* (2004) that the presence of PEG in lipoplexes results in reduced cellular binding and uptake. Yang *et al* proposed that this result may be attributed to the shielded positive charges of the liposome due to the incorporation of the PEG moiety and also the long PEG chain may produce a steric hindrance effect (Yang *et al*., 2009). It has also been said that the endosomal release of the nucleic acids into the cytoplasm may be inhibited by pegylation (Vandenbrouke *et al*., 2008). Song *et al* (2002) showed that pegylation of lipoplexes did not inhibit cellular uptake and, therefore, it is possible that this decrease in transfection efficiency is located at the endosomal escape step (Kim *et al*., 2010).

It has been reported that serum impairs the transfection efficiency of cationic liposomes; therefore, serum free medium is often used for the first few hours. Studies have shown that this inhibition may arise due to the presence of negatively charged proteins in the serum. These proteins can become adsorbed onto the surface of the lipoplex which results in their aggregation and precipitation or the lipoplex may destabilize which results in pre-mature release of nucleic acids and, therefore, transfection may fail (Li *et al*., 2011). Once the serum free medium is removed, the lipoplex is not displaced from the cell surface, but remains tightly bound and is continuously internalized into the cell (Singh, 1998).

The detection of luciferase gene knockdown in the HeLa *tat luc* cell line was determined using the luciferase assay. Luciferase is a 62 kD monomeric protein and post translational processing is not required for enzymatic activity, therefore, it has the ability to function as a reporter gene (Singh, 1998). The reaction involves the ATP-dependant oxidative decarboxylation of beetle luciferin which produces light at a wavelength of 562 nm (Roche Practical Manual). The reaction is catalyzed by firefly luciferase and can be divided into two stages:



The photon emmission deteriorates following the mixing of the enzyme and substrate. The light intensity is dependent on temperature, the optimum being room temperature (20-25 °C) therefore; all reagents are required to equilibrate to room temperature before proceeding with the assay.

The results obtained for the liposomal formulations in this study warrant their further development for *in vivo* studies where a gene to be targeted can be expressed in rat models and, in doing so, the therapeutic potential of these formulations can be determined.

CONCLUSION

The RNAi phenomenon refers to the process of post transcriptional gene silencing mediated by siRNA. Theoretically, the process of RNAi can be used for the knockdown of any mRNA expression and the therapeutic siRNA can be designed, provided that the sequence of the target gene is known. Hence, siRNA mediated RNAi has emerged not only as a useful biological tool for the determination of gene functions but also has therapeutic potential for the treatment of various human diseases (Guo *et al*., 2010). The numerous potential applications of these siRNAs have not yet been fully exploited due to the lack of a delivery vehicle that can be administered safely, efficiently and repeatedly (Sioud and Sørenson, 2003). Cationic liposomes represent delivery vehicles that can meet these requirements. Efficient gene delivery can be achieved with cationic liposomes under *in vitro* conditions, however, under *in vivo* conditions they have been shown to exhibit poor circulation time in the blood system, a problem which can be circumvented by the introduction of PEG. PEG is said to enhance the circulation time of the liposome *in vivo*, by preventing the binding of plasma proteins to the liposomal surface and, in doing so, RES uptake is reduced.

Pegylated and non-pegylated cationic liposomes were prepared with either cytofectin Chol-T or MSO9. All liposomal formulations contained DOPE and varying amounts of DSPE-PEG_{2000} was introduced into the pegylated liposomal formulations. The liposomes were prepared using the thin film hydration- sonication technique. All the liposome preparations were characterized using cryo-TEM which revealed the unilamellar nature of the liposomes. siRNA binding to the cationic liposome was demonstrated using the band shift assay and these siRNA lipoplexes were characterized using cryo-TEM. The siRNA lipoplexes appeared to exist as spherical clusters, some of which also exhibited a deformable nature. The liposomes were also shown to offer protection to the siRNA in the siRNA lipoplexes against enzymatic degradation.

The ratios of the siRNA: cationic liposome complexes utilized in transfection studies were determined from the binding studies. These complexes showed minimal toxicity to the HeLa *tat luc* cell line. Transfection studies were carried out using the luciferase assay and the anti-luciferase siRNA was utilized for this study. It appears from the results that the length of the spacer arm did not affect the transfection efficiency as MSO9, which has a 12 atom spacer, exhibited similar levels of transfection when compared to Chol-T, whose spacer arm consists of 6 atoms. All the cationic liposome: siRNA complexes showed efficient knockdown of the luciferase gene with pegylated cationic liposomes showing marginally lower levels of gene knockdown as compared to their non-pegylated counterparts. The exact mechanism responsible for this reduction in transfection requires further investigation. However, the drawbacks associated with pegylated cationic liposomes can be overcome by the introduction of PEG-lipids such as PEG-ceramides which are capable of diffusing out of the liposome or PEG-lipids that are acid labile. Here, the PEG moieties of the liposome will become detached at a lower pH, much like the pH that exists in the endosomal vesicle. These types of modifications can result in the controlled removal of the PEG moiety and, therefore, the biological activity of the pegylated liposome can be improved (Buyens *et al*., 2009).

To further enhance the efficiency of the cationic liposomes in this study, targeting ligands can be introduced into the liposomal formulation which will increase the specificity of the liposome to a particular tissue, for example, by introducing asialoglycoproteins to the liposomal formulation, hepatocytes can be specifically targeted.

The findings in this study suggest that these liposomal formulations have the potential for clinical gene delivery and hence, should be further evaluated to determine their therapeutic potential under *in vivo* conditions. Furtermore, it has shown that RNAi is a useful tool for the downregulation of gene expression. One of the main objectives in the development of the non-viral delivery system for siRNA delivery is the use of these systems for the treatment of diseases in the future and hence, the targeting of a specific gene linked to the disease. However, there are many concerns that need to be overcome before RNAi can be applied as a therapeutic modality

as there is great importance in understanding the mechanism fully before it can be effective in clinical applications. Nevertheless, due to the speed at which new discoveries are being made, it is anticipated that an ideal vector that is safe, effective and stable will be prepared allowing RNAi to become a major therapeutic modality in years to come (Aargaard and Rossi, 2009).

REFERENCES

Aargaard L. and Rossi J.J. (2007). *'RNAi therapeutics: Principles, prospects and challenges'*. Advanced Drug Delivery Reviews. **59**: 75-86.

Agami R. (2002). *'RNAi and related mechanisms and their potential use for therapy'*. Current Opinion in Chemical Biology. **6**: 829-834.

Aigner A. (2006). *'Gene silencing through RNA interference (RNAi) in vivo: Strategies based on the direct application of siRNAs'*. Journal of Biotechnology. **124**: 12-25.

Akaneya Y., Jiang B. and Tsumoto T. (2005). *'RNA interference induced gene silencing by local electroporation in targeting brain region'*. Journal of Neurophysiology. **93(1)**: 594-602.

Akhtar S. and Benter I. (2007). *'Toxicogenomics of non-viral drug delivery systems for RNA interference: Potential impact on small interfering RNA mediated gene silencing activity and specificity'*. Advanced Drug Delivery Reviews. **59**: 164-182.

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

Allen T.M., Hansen C., Martin F., Redemann C. and Yau-Young A. (1991). *'Liposomes containing synthetic lipid derivatives of poly (ethyleneglycol) show prolonged circulation half lives in vivo'*. Biochimica et Biophysica Acta. **1066**: 29-36.

Allen T.M., Hansen C. and Rutledge J. (1989). *'Liposomes with prolonged circulation times: Factors affecting uptake by reticuloendothelial and other tissues'*. Biochimica et Biophysica Acta. **981**: 27-35.

Almofti M.R., Harashima H., Shinohara Y., Almofti A., Baba Y. and Kiwada H. (2003). *'Cationic liposome-mediated gene delivery: Biophysical study and mechanism of internalization'*. Archives of Biochemistry and Biophysics. **410**: 246-253.

Angelini G., Chiarini M., DeMaria P., Fontana A., Gasbarri C., Sioni G. and Velluto D. (2011). *'Characterization of cationic liposomes. Influence of the bilayer composition on the kinetics of the liposome breakdown'*. Chemistry and Physics of Lipids. doi: 10.1016/j.chemphyslip.2011.07.002.

Bailey A.L. and Cullis P.R. (1997). *'Membrane fusion with cationic liposomes: Effects of target membrane lipid composition'*. Biochemistry. **36**: 1628-1634.

Balazs D.A. and Godbey W.T. (2010). *'Liposomes for use in gene delivery'*. Journal of Drug Delivery. **2011**: 1-12

Balicki D. and Beutler E. (2002). *'Gene therapy of human disease'*. Medicine. **81(1)**: 69-86.

Bangham A.D. (1980). *'Liposomes in biological systems'*. John Wiley and Sons, Chichester.

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

Barichello J.M., Kizuki S., Tagami T., Asai T., Ishida T., Kikuchi H., Oku N. and Kiwado H. (2011). *'Agitation during lipoplex formation improves gene knockdown effect of siRNA'*. Journal of Pharmaceutics. **410**: 153-160.

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

Behr J-P. (1993). *'Synthetic gene transfer vectors'*. Accounts of Chemical Research. **26**: 274-278.

Bonicelli M.G., Giansanti L., Ierino M. and Mancini G. (2011). *'Interaction of cationic liposomes with cell membrane models'*. Journal of Colloid and Interface Science. **355**: 1-8.

Brantl S. (2002). *'Antisense-RNA regulation and RNA interference'*. Biochimica et Biophysica Acta. **1575**: 15-25.

Buyens K., Demeester J., DeSmedt S.S. and Sanders N.N. (2009). *'Elucidating the encapsulation of short interfering RNA in pegylated cationic liposomes'*. Langmuir. **25(9)**: 4886-4891.

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

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

Caplen N.J. (2004). *'Gene Therapy progress and prospects. Downregulating gene expression: The impact of RNA interference'*. Gene Therapy. **11**: 1241-1248.

Caplen N.J., Fleenor J., Fire A. and Morgan R.A. (2000). *'dsRNA- mediated gene silencing in cultured Drosophilia cells: A tissue culture model for the analysis of RNA interference'*. Gene. **252**: 95-105.

Carthew R.W. (2001). *'Gene silencing by double stranded RNA'*. Current Opinion in Cell Biology. **13**: 244-248.

Cerutti H. (2003). *'RNA interference: Travelling through the cell and gaining functions?'*. TRENDS in Genetics. **19(1)**: 39-46.

Cheng J.C., Moore T.B. and Sakamoto K.M. (2003). *'RNA interference and human disease'*. Molecular Genetics and Metabolism. **80**: 121-128.

Chiu Y-L., Ali A., Chu C-Y., Cao H. and Rona T.M. (2004). *'Visualizing correlation between siRNA localization, cellular uptake, and RNAi in living cells'*. Chemistry and Biology. **11**: 1165-1175.

Chonn A. and Cullis P.R. (1995). *'Recent advances in liposomal drug delivery systems'*. Current Opinion in Biotechnology. **6**: 698-708.

Chung H.J., Hong C.A., Lee S.H., Jo S.D. and Park T.G. (2010). *'Reducible siRNA dimeric conjugates for efficient cellular uptake and gene silencing'*. Bioconjugate Chemistry. doi: 10.1021/bc.100438m.

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

Dass C.R. and Choong P.F.M. (2006). *'Targeting of small molecule anti cancer drugs to the tumour and its vasculature using cationic liposomes: Lessons from gene therapy'*. Cancer Cell International. **6(17)**: 1-9.

David S., Pitard B., Benoît J-P. and Passiran C. (2010). *'Non-viral nanosystems for systemic siRNA delivery'*. Pharmacological Research. **62**: 100-114.

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

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

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

De Rosa G., De Stefano D. and Galeone A. (2010). *'Oligonucleotide delivery in cancer therapy'*. Expert Opinion Drug Delivery. **17(11)**: 1263-1278.

De Smedt S.C., Demeester J. and Hennink W.E. (2000). *'Cationic polymer based gene delivery systems'*. Pharmaceutical Research. **17(2)**: 113-126.

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

Dominska M. and Dykxhoorn D.M. (2010). *'Breaking down the barriers: siRNA delivery and endosome escape'*. Journal of Cell Science. **123**: 1183-1189.

Dorasamy S., Singh M. and Ariatti M. (2009). *'Rapid and sensitive fluorometric analysis of novel galactosylated cationic liposome interaction with siRNA'*. African Journal of Pharmacy and Pharmacology. **3(12)**: 632-635.

El-Aneed A. (2004). *'An overview of current delivery systems in cancer gene therapy'*. Journal of Controlled Release. **94**: 1-14.

Elbashir S.M., Lendeckel W. and Tuschl T. (2001). *'RNA interference is mediated by 21-22 nucleotide RNAs'*. Genes and Development. **15**: 188-200.

Elmén J., Thonberg H., Ljungberg K., Frieden M., Westergaard M., Xu Y., Wahren B., Liang Z., Øram H., Koch T. and Wahlestedt C. (2005). *'Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality'*. Nucleic Acids Research. **33(1)**: 439-447.

Farhood H., Serbina N. and Huang L. (1995). *'The role of dioleoylphosphatidylethanolamine in cationic liposome mediated gene transfer'*. Biochimica et Biophysica Acta. **1235**: 289-295.

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

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

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

Filipowicz W., Jaskiewicz L., Kolb F.A. and Pillai R.S. (2005). *'Post-transcriptional gene silencing by siRNAs and miRNAs'*. Current Opinion in Structural Biology. **15**: 331-341.

Fire A., Xu S., Montgomery M.K., Kostas S.A., Driver S.E. and Mello C.C. (1998). *'Potent and specific genetic interference in Caenorhabditis elegans'*. Proceedings of the National Academy of Sciences, USA. **95**: 15502-15507.

Fischer S.E.J. (2010). *'Small interfering RNA –mediated silencing pathways in C.elegans'*. Journal of Biochemistry and Cell Biology. **42(8)**: 1306-1315.

Fotakis G. and Timbroll I.A. (2006). *'In vitro cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride'*. Toxicology Letters. **160**: 171-177.

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

Gabizon A. and Papahadjopoulos D. (1988). *'Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumours'*. Proceedings of the National Academy of Sciences, USA. **85**: 6949-6953.

Gao K. and Huang L. (2008). *'Non-viral methods for siRNA delivery'*. Molecular Pharmaceutics. **6(3)**: 651-658.

Gao X. and Huang L. (1991). *'A novel cationic liposome reagent for efficient transfection of mammalian cells'*. Biochimica et Biophysica Acta. **179**:280-285.

Gao X., Kim K-S. and Liu D. (2007). *Non-viral gene delivery: What we know and what is next'*. The AAPS Journal. **9(1)**: E92-E104.

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

Guo P., Coban O., Snead N., Trebley J., Hoeprich S., Guo S. and Shu Y. (2010). *'Engineering RNA for targeted siRNA delivery and medical application'*. Advanced Drug Delivery Reviews. **62(6)**: 650-666.

Hammond S.M. (2005). *'Dicing and slicing, the core machinery of RNA interference pathway'*. FEBS Letters. **579**: 5822-5829.

Hammond S.M., Coudy A.A. and Hannon G.J. (2001). *'Post transcriptional gene silencing by double stranded RNA'*. Nature Reviews:Genetics. **2**: 110-119.

Han S-E., Kong H., Shim G.Y., Suh M.S., Kim S.J., Kim J.S. and Oh Y-K. (2008). *'Novel cationic cholesterol derivative-based liposomes for serum enhanced delivery of siRNA'*. International Journal of Pharmaceutics. **353**: 260-269.

Handy C.R., Krudy C. and Boulis N. (2011). *'Gene therapy: A potential approach for cancer pain'*. Pain Research and Treatment. doi:10.1155/987597.

Hasegawa S., Hirashima N. and Nakanishi M. (2002). *'Comparative study of transfection efficiency of cationic cholesterols mediated by liposomes-based gene delivery'*. Bioorganic and Medicinal Chemistry Letters. **12**: 1299-1302.

Hattori Y., Hagiwana A., Ding W. and Maitani Y. (2008). *'NaCl improves siRNA delivery mediated by nanoparticles of hydroxyethylated cationic cholesterol with amido-linker'*. Bioorganic and Medicinal Chemistry Letters. **18**: 5228-5232.

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

Hirsch-Lerner D., Zhang M., Eliyanu H., Ferrari M.E., Wheeler C.J. and Barenholz Y. (2005). *'Effect of " helper lipid" on lipoplex electrostatics'*. Biochimica et Biophysica Acta. **1714**: 71-84.

Hirsch M., Ziroli V., Helm M. and Massing U. (2009). *'Preparation of small amounts of sterile siRNA- liposomes with high entrapping efficiency by dual asymmetric centrifugation (DAC)'*. Journal of Controlled Release. **135**: 80-88.

Hohjoh H. (2002). *'RNA interference (RNAi) induction with various types of synthetic oligonucleotide duplexes in cultured human cells'*. FEBS Letters. **521**: 195-199.

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

Howard K.A., Rahbek U.L., Liu X.D., Damgaard C.K., Glud S.Z., Anderson M.O., Hougaard M.B., Schmidtz A., Nyengaard J.R., Besenbacher F. and Kjerns J. (2006). *'RNA interference in vitro and in vivo using a chitosan/ siRNA nanoparticle system'*. Journal Biological Chemistry. **14**: 476-484.

Hutvagner G. (2005). *'Small RNA asymmetry in RNA interference: Function in RISC assembly and gene regulation'*. FEBS Letters. **579**: 5850-5857.

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

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

Katas H.and Alpar H.O. (2006). *'Development and characterization of chitosan nanoparticles for siRNA delivery'*. Journal of Controlled Release. **115**: 216-225.

Kelly C., Jefferies C. and Cryan S-A. (2010). *'Targeted liposomal drug delivery to monocytes and macrophages'*. Journal of Drug Delivery. **2011**: 1-11.

Khaliq S., Khaliq S.A., Zahur M., Ijaz B., Jahon S., Ansar M., Riazuddin S. and Hassan S. (2010). *RNAi as a new therapeutic strategy against HCV'*. Biotechnology Advances. **28**: 27-34.

Khierolomoom A. and Ferrara K.W. (2007). *'Cholesterol transport from liposomal delivery vehicles'*. Biomaterials. **28**: 4311-4320.

Kim D.H. and Rossi J.J. (2007). *'Strategies for silencing human disease using RNA interference'*. Nature Reviews. **8**: 173-184.

Kim H-K., Davaa E., Myung C-S. and Park J-S. (2010). *'Enhanced siRNA delivery of cationic liposomes by using new polyarginine-conjugated PEG-lipid'*. International Journal of Pharmaceutics. **392(1-2)**: 141-147.

Kim S-S., Garg H., Joshi A. and Manjunath N. (2009). *'Strategies for targeted non-viral delivery of siRNAs in vivo'*. Trends in Molecular Medicine. **15(11)**: 491-500.

Kim T.W., Lee J.H., He L., Boyd D.A., Hardwick J.M., Hung C.F. and Wu T.C. (2005). *'Modification of professional antigen- preventing cells with small interfering RNA in vivo to enhance cancer vaccine potency'*. Cancer Research. **65(1)**: 309-316.

Kim W.J. and Kim S.W. (2009). *'Efficient siRNA delivery with non-viral polymeric vehicles'*. Pharmaceutical Research. **26(3)**: 657-666.

Kong Y., Ruan L., Ma L., Cui Y., Wong J.M. and Le Y. (2007). *'RNA interference as a novel and powerful tool in immunopharmacological research'*. International Immunopharmacology. **7**: 417-426.

Lasic D.D. (1997). *'Liposomes in gene delivery'*. CRC Press LLC, Boca Raton , Florida.

Lee S. and Kumar P. (2009). *'Conditional RNAi: Towards a silent gene therapy'*. Advanced Drug Delivery Reviews. **61**: 650-664.

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

Leung R.K.M. and Whittaker P.A. (2005). *'RNA interference: From gene silencing to gene specific therapeutics'*. Pharmacology and Therapeutics. **107**: 222-239.

Leventis R. and Silvius J.R. (1990). *'Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles'*. Biochimica et Biophysica Acta. **1023**: 124-132.

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

Lieberman J., Song E., Lee S-K. and Shankar P. (2003). *'Interfering with disease: Opportunities and roadblocks to harnessing RNA interference'*. TRENDS in Molecular Medicine. **9(9)**: 397-403.

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

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

Lu J.J., Langer R. and Chen J. (2009). *'A novel mechanism is involved in cationic lipid mediated functional siRNA delivery'*. Molecular Pharmaceutics. **6(3)**: 763-771.

Lv H., Zhang S., Wang B., Cui S. and Yan J. (2006). *'Toxicity of cationic lipids and cationic polymers in gene delivery'*. Journal of Controlled Release. **114**: 100-109.

MacLachlan I. (2007). *'Liposomal formulations for nucleic acid delivery'*. Antisense Drug Technologies. **2**: 237-262.

Maghimi S.M. and Szebeni J. (2003). *'Stealth liposomes and long circulating nanoparticles: Critical issues on pharmacokinetics, opsonization and protein binding*'. Progress in Lipid Research. **42**: 463-478.

Managhan M. and Pandit A. (2011). *'RNA interference therapy via functionalized scaffolds'*. Advanced Drug Delivery Reviews. **63**: 197-208.

Manoharan M. (2004). *'RNA interference and chemically modified small interfering RNAs'*. Current Opinion in Chemical Biology. **8**: 570-579.

Marques J.T. and Williams B.R.G. (2005). *'Activation of the mammalian immune system by siRNAs'*. Nature Biotechnology. **23(11)**: 1399-1405.

Masiero M., Nardo G., Indracoolo S. and Favaro E. (2007). *‘RNA interference: Implications for cancer treatment’*. Molecular Aspects of Medicine. **28**: 143-166.

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

McCaffrey A.P., Meuse L., Pham T.T., Conklin D.S., Hannon G.J. and Kay M.A. (2002). *‘RNA interference in adult mice’*. Nature. **418**: 38-39.

Meade B.R. and Dowdy S.F. (2007). *‘Exogenous siRNA delivery using peptide transduction domains/ cell penetrating peptides’*. Advanced Drug Delivery Reviews. **59**: 134-140.

Meister G. and Tuschl T. (2004). *‘Mechanisms of gene silencing by double stranded RNA’*. Nature. **431**: 343-349.

Mello C.C. and Canter Jr D. (2004). *‘Revealing the world of RNA interference’*. Nature. **431**: 338-342.

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

Miller A.D. (1998). *‘Cationic liposomes for gene therapy’*. Angewandte Chemie International Edition. **37**: 1768-1785.

Miller C.R., Bandurant B., McLean K.A. and O’Brien D.F. (1998). *‘Liposome- cell interactions in vitro: Effect of liposome surface charge on the binding and endocytosis of conventional and sterically stabilized liposomes’*. Biochemistry. **37**: 12875-12883.

Mocellin S. and Provenzono M. (2004). *‘RNA Interference: Learning gene knockdown from cell physiology’*. Journal of Translational Medicine. **2(39)**: 1-6.

Narainpersad N. (2009). *'Cationic liposome mediated targeted gene delivery with and without pegylated accessories'*. MSc Thesis. University of KwaZulu-Natal, Westville Campus.

Niidome T. and Huang L. (2002). *'Gene therapy progress and prospectsof non-viral vectors'*. Gene Therapy. **9**: 1647-1652.

Nothisen M., Kotera M., Voiren E., Remy J-S. and Behr J-P. (2009). *'Cationic small interfering RNAs provide carrier- free gene silencing in animal cells'*. Journal American Chemical Society. **131**: 17730-17731.

Oh Y-K. and Park T.G. (2009). *'siRNA delivery systems for cancer treatment'*. Advanced Drug Delivery Reviews. **61**: 850-862.

Pagano R.E and Weinstein J.W. (1978). *'Interactions of liposomes with mammalian cells'*. Annual Reviews Biophys. Bioeng. **7**: 435-468.

Papisov M.I. (1998). *'Theoretical considerations of RES- avoiding liposomes: Molecular mechanics and chemistry of liposome interactions'*. Advanced Drug Delivery Reviews. **32**: 119-138.

Pirollo K.F. and Chang E.H. (2008). *'Targeted delivery of small interfering RNA: Approaching effective cancer therapies'*. Cancer Research. **68(5)**: 1247-1250.

Promega. *'Electrophoretic mobility shift assay'*. Promega Protein Interaction Guide.

Ramezani M., Khashhamdam M., Dehshahri A. and Malaekeh- Nikouei B. (2009). *'The influence of size, lipid composition and bilayer fluidity of cationic liposomes on the transfection efficiency of nanolipoplexes'*. Colloids and Surfaces B: Biointerfaces. **75**: 1-5.

Ramon A-L., Bertrand J-R. and Malvy C. (2008). *'Delivery of small interfering RNA: A review and an example of application to a junction oncogene'*. Tumori. **94**: 254-263.

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

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

Riaz M. (1996). *'Liposomes preparation methods'*. Pakistan Journal of Pharmaceutical Sciences. **19(1)**: 65-77.

Robbins P.D. and Ghivizzani S.C (1998). *'Viral vectors for gene therapy'*. Pharmacological Therapy. **80(1)**: 35-47.

Roche. (2007). *'Luciferase reporter gene assay, high sensitivity, chemiluminescent assay for the quantitative determination of firefly luciferase activity in transfected cells'*. Roche Instruction Manual.

Ross P.C. and Hui S.N. (1999). *'Lipoplex size is a major determinant of in vitro lipofection efficiency'*. Gene Therapy. **6**: 651-659.

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

Rozema D.B. and Lewis D.L. (2003). *'siRNA delivery technologies for mammalian systems'*. TARGETS. **2(6)**: 253-260.

Ryther R.C.C., Flynt A.S., Phillips III J.A. and Patton J.G. (2005). *'Small interfering RNA therapeutics: Big potential from small RNAs'*. Gene Therapy. **12**: 5-11.

Samad A., Sultana Y. and Aqil M. (2007). *Liposomal drug delivery systems: An update review'*. Current Drug Delivery. **4**: 297-305.

Santel A., Aleku M., Keil O., Endruschat J., Esche V., Fisch G., Dames S., Löffler K., Fechtner M., Arnold W., Giese K., Klippel A. and Kaufmann J. (2006). *'A novel siRNA-lipoplex technology for RNA interference in the mouse vascular endothelium'*. Gene Therapy. **13**: 1222-1234.

Sato A., Takagi M., Shimamoto A., Kawakami S. and Hashida M. (2007). *'Small interfering RNA delivery to the liver by intravenous administration of galactosylated cationic liposomes in mice'*. Biomaterials. **28**: 1434-1442.

Seth P. (2005). *'Vector mediated cancer gene therapy: An overview'*. Cancer Biology and Therapy. **4(5)**: 512-517.

Shan G. (2009). *'RNA Interference as a gene knockdown technique'*. International Journal of Biochemistry and Cell Biology. **42(8)**: 1243-1251.

Shannon A.J., Tyson T., Dix I., Boyd J and Burnell A.M. (2008). *'Systemic RNA interference mediated gene silencing in the anhydrobiotic nematode Panagrolaimus superbus'*. BMC Molecular Biology. **9(58)**: 1-12.

Sharma S., Mishra L., Grover I., Guptar A. and Kaur K. (2010). *'Liposomes: Vesicular system an overview'*. International Journal of Pharmacy and Pharmaceutical Sciences. **2(4)**: 11-17.

Shen C., Buck A.K., Liu X., Winkler M. and Reske S.N. (2003). *'Gene silencing by adenovirus-delivered siRNA'*. FEBS Letters. **539**: 111-114.

Shi Y. (2003). *'Mammalian RNA interference for the masses'*. TRENDS in Genetics. **19(1)**: 9-12.

Shuey D.J. McCallus D.E. and Giordono T. (2002). *'RNAi: Gene silencing in therapeutic intervention'*. Drug Discovery Today. **7(20)**: 1040-1046.

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

Singh M (2005). *'Targeted gene transfer to mammalian systems using liposome constructs containing cholesterol components with or without biotinylated molecular accessories'*. Phd Thesis, University of KwaZulu Natal, Westville Campus.

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

Singh M., Borain J., Noor-Mahomed N. and Ariatti M. (2011). *'The effect of pegylation on the transfection activity of two homologous cationic cholesteryl cytofectins'*. African Journal of Biotechnology. **10(8)**: 1400-1407.

Siomi M. (2009). *'Short interfering RNA mediated gene silencing; towards successful applications in human patients'*. Advanced Drug Delivery Reviews. **61**: 668-671.

Sioud M. and Sørenson D.R. (2003). *'Cationic liposome-mediated delivery of siRNAs in adult mice'*. Biochimica et Biophysica Acta. **312**: 1220-1225.

Song E., Lee S-K., Wang J., Ince N., Ouyang N., Min J., Chen J., Shankar P. and Lieberman J. (2003). *'RNAi targeting Fas protects mice from fulminant hepatitis'*. Nature Medicine. **9**: 347-351.

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

Spagnou S., Miller A.D. and Keller M. (2004). *'Lipidic carriers of siRNA: Differences in formulation, cellular uptake and delivery with plasmid DNA'*. Biochemistry. **43(42)**: 13348-13356.

Starr C. and Taggart R. (2004). *'Biology: The unity and diversity of life'*. **10th edition.** Brookes/Cole- Thomson Learning, Belmont, CA, USA.

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

Stevenson M. (2003). *'Dissecting HIV-1 through RNA interference'*. Nature Reviews Immunology. **3**: 851-858.

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

Takahashi Y., Nishikawa M. and Takakura Y. (2009). *'Non-viral vector mediated RNA interference: Its gene silencing characteristics and important factors to achieve RNAi- based gene therapy'*. Advanced Drug Delivery Reviews. **61**: 760-766.

Takano S., Aramaiki Y. and Tsuchiya S. (2003). *'Physicochemical properties of liposomes affecting apoptosis induced by cationic liposomes in macrophages'*. Pharmamceutical Research. **20(7)**: 962-968.

Takeshita D., Zenno S., Lee W.C., Nagata K., Saigo K. and Tanokura M. (2007). *'Homodimeric structure of double stranded cleavage activity of the C-terminal RNase III domain of human dicer'*. Journal of Molecular Biology. **374**: 106-120.

Thomas M., Lu J.J., Chen J. and Klibanov A.M. (2007). *'Non-viral siRNA delivery to the lung'*. Advanced Drug Delivery Reviews. **59**: 124-133.

Torchillin V. (2003). *'Liposomes: A practical approach'* **2nd edition**. Oxford University Press, New York.

Torchillin V.P., Omelyanenko V.G., Papisov M.I., Bogdhanov Jr A.A., Trubetskoy V.S. Herron J.N. and Gentry C.A. (1994). *'Poly(ethyleneglycol) on the liposome surface: On the mechanism of polymer- coated liposomes longevity'*. Biochimica et Biophysica Acta. **1195**: 11-20.

Tseng Y-C., Mozumdar S. and Huang L. (2009). *'Lipid-based systemic delivery of small interfering RNA'*. Advanced Drug Delivery Reviews. **61**: 721-731.

Turner J.J., Jones S., Fabani M.M., Ivanova G., Arzumanov A.A. and Gait M.J. (2007). *'RNA targeting with peptide conjugates of oligonucleotides, siRNA and PNA'*. Blood Cells, Molecules and Diseases. **38**: 1-7.

Ui-Tei K., Zenno S., Miyata Y. and Saigo K. (2000). *'Sensitive assay of RNA interference in Drosiphila and chinese hamster cultured cells using firefly luciferase gene as target'*. FEBS Letters. **479**: 79-82.

Uprichard S.L. (2005). *'The therapeutic potential of RNA interference'*. FEBS Letters. **579**: 5996-6007.

Van Es H.H.G. and Arts G.J. (2005). *'Biology calls the targets: Combining RNA interference and disease biology'*. Drug Discovery Today. **10(20)**: 1385-1391.

Vandenbroucke R.E., Lentacker I., Demeester J., De Smedt S.C. and Sanders N.N. (2008). *'Ultrasound assisted siRNA delivery using PEG- siPlex loaded microbubbles'*. Journal of Controlled Release. **126**: 265-273.

Vellanen K., Honkakoski P. and Urtti A. (2004). *'Substrates and inhibitors of efflux proteins interfere with the MTT assay in cells and may lead to underestimation of drug toxicity'*. European Journal of Pharmaceutical Sciences. **23**: 181-188.

Weber M., Möller K., Welzek M. and Schorr J. (1995). *'Effects of lipopolysaccharide on transfection efficiency in eukaryotic cells'*. BioTechniques. **19(6)**: 930-939.

Wiesen J.L. and Tomasi T.B. (2009). *'Dicer is regulated by cellular stresses and interferons'*. Molecular Immunology. **46**: 1222-1228.

Woodle M.C. (1995). *'Sterically stabilized liposome therapeutics'*. Advanced Drug Delivery Reviews. **16**: 249-265.

Wrobel I. and Collins D. (1995). *'Fusion of cationic liposomes with mammalian cells occurs after endocytosis'*. Biochimica et Biophysica Acta. **1235**: 296-304.

Xia H., Mao Q., Paulson H.L. and Davidson B.L. (2002). *'siRNA mediated gene silencing in vitro and in vivo'*. Nature Biotechnology. **20**: 1006-1010.

Yadava P., Roura D. and Hughes J.A. (2007). *'Evaluation of two cationic delivery systems for siRNA'*. Oligonucleotides. **17**: 213-221.

Yang L-J., Yang T., Jiong J., Xu Z-Z., Wong J-C. and Zhang Q. (2009). *'Effects of pegylated cationic liposomes on siRNA transfection'*. Journal of Chinese Pharmaceutical Sciences. **18**: 267-272.

Yu W., Pirollo K.F., Rait A., Yu B., Xiang L.M., Huang W.Q., Zhou Q., Ertem G. and Chang E.H. (2004). *'A sterically stabilized immunolipoplex for systemic administration of a therapeutic gene'*. Gene Therapy. **11**: 1434-1440.

Zabner J., Fasbender J., Moninger T., Poellinger K.A. and Welsh M.J. (1995). *'Molecular barriers to genen transfer by a cationic lipid'*. Biol. Chem. **270**: 18997-19007.

Zalipsky S., Brandeis E., Newman M.S and Woodle M.C. (1994). *'Long circulating cationic liposomes containing amino-PEG-phosphatidylethanolamine'*. FEBS Letters. **353**: 71-74.

Zamore P.D., Tuschl T., Sharp P.A. and Bartel D.P. (2000). *'RNAi: Double stranded RNA directs the ATP- dependant cleavage of mRNA at 21- 23 nucleotide intervals'*. Cell. **101**: 25-33.

Zelphati O., Uyechi L.S., Barron L.G. and Szoka Jr F. (1998). *'Effect of serum components on the physico-chemical properties of cationic lipid/ oligonucleotide complexes and on their interactions with cells'*. Biochimica et Biophysica Acta. **1390**: 119-133.

Zhang C., Tang N., Liu X., Liang W., Xu W. and Torchillin V.P. (2006). *'siRNA containing liposomes modified with polyarginine effectively silence the targeted gene'*. Journal of Controlled Release. **112**: 229-239.

Zhang S., Zhao B., Jiang H., Wang B. and Ma B. (2007). *'Cationic lipids and polymers mediated vectors for delivery of siRNA'*. Journal of Controlled Release. **123**: 1-10.

Zhang Y., Li H., Sun J., Gao J., Liu W., Li B., Guo Y. and Chen J. (2010). '*DC-CHOL/DOPE cationic liposomes: A comparative study of the influence factors on plasmid pDNA and siRNA gene delivery'*. International Journal of Pharmaceutics. **390(2)**: 198-207.

Zuidom N.J., Hirsch-Lorner D., Marguilies S. and Borenholz Y. (1999). *'Lamellarity of cationic liposomes and mode of preparation of lipoplexes affect transfection efficiency'*. Biochimica et Biophysica Acta. **1419**: 207-220.

Zuhorn I.S., Engberts J.B.F.N. and Hoekstra D. (2007). *'Gene delivery by cationic lipid vectors: Overcoming cellular barriers'*. Eur. Biophys. Journal. **36**: 349-362.

www.avantipolarlipids.com

www.grin.com/object/external_document.256998/

www.hindawi.com/journals/jdd/2011/326497

www.hindawi.com/journals/jdd/2011/326497

www.jpp.krakow.pl/.../articles/05_article.html

www.openwetware.org/wiki/Griffin:siRNA_transfection

APPENDIX A