
# Novel siRNA Lipoplexes: Their Targeted and Untargeted Delivery to Mammalian Cells in Culture

by

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This dissertation is dedicated to my beloved family.

## Abstract

The high gene knockdown specificity and efficiency of RNA interference (RNAi) provides a potentially viable avenue for the development of a new class of nucleic acid therapeutics for gene-based disease conditions. However, serum instability, inefficient cellular trafficking and non-specific effects of small interfering RNAs (siRNAs), one of the functional mediators of RNAi, has necessitated the development of carriers to facilitate targeted cell delivery. The decline of viral vectors in human gene therapy as a consequence of safety issues has intensified the importance of non-viral vector development. Among the non-viral vectors available for siRNA delivery, cationic liposomes have emerged as an attractive option owing to their simplicity, versatility, relatively low toxicity and potential for cell-specific targeting. Although existing cationic lipids and liposomes traditionally used for DNA delivery have also been used for siRNAs, there still exists a need to develop cationic lipids tailored towards siRNA transfection for improved gene silencing efficiency. Among the cell specific targets available for RNAi therapeutics, hepatocytes expressing the asialoglycoprotein receptor (ASGP-R) are an ideal choice due to the large number of disease targets present for treatment.

In this investigation four novel cationic liposome formulations were prepared from equi-molar quantities of either the cationic cholesterol derivative 3β [N-(N',N'-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) or 3β [N-(N', N', N'-trimethylammoniumpropyl)-carbamoyl] cholesterol iodide (Chol-Q) and DOPE, with and without the hepatotropic ligand, cholesteryl-β-D-galactopyranoside. Electrophoretic gel analysis and SYBR®green displacement assays were employed to determine siRNA binding and condensation efficiencies for all cationic liposomes; while liposome and lipoplex size measurements were made by cryoTEM. SiRNA-lipoplex stability in serum was determined by the nuclease protection assay. Cell studies performed on the ASGP-$R^+$ human hepatoma cells, HepG2 and the ASGP-$R^-$ embryonic kidney cells, HEK293, to determine lipoplex toxicity and transfection efficiencies were also undertaken.

We show that the cationic liposomes formulated for this investigation were able to bind synthetic siRNA optimally at a positive to negative charge ratio of ± 1 : 6. In addition, the cationic liposomes were able to afford siRNA duplexes substantial protection from ribonuclease digestion in serum. From the results obtained in this study, it appears that the cationic liposomes are well tolerated by both the HEK293 and HepG2 cells *in vitro*. More importantly, the results obtained demonstrated higher transfection efficiencies for the targeted lipoplexes compared with the untargeted controls, strongly supporting the notion that incorporation of the cholestryl-β-D-galactopyranoside into the liposome structure increases transfection efficiency to the targeted HepG2 cells in culture via ASGP receptor mediation. Comparative studies in the HEK293 cell line yielded low transfection effeciences in the order of 20%, with no significant difference being recorded between galactosylated and non-galactosylated lipoplexes.

# Preface

The experimental work described in this thesis was carried out in the School of Biochemistry, Genetics and Microbiology, University of Kwazulu-Natal, Westville, Durban from January 2008 to July 2011, under the supervision of Dr.Moganavelli Singh and co-supervision of Professor M. 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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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: Full length Research Paper – Appendix B

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.

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# List of Abbreviations

| | | |
|---|---|---|
| d/s | - | Double stranded |
| RNAi | - | RNA interference |
| mRNA | - | Messenger RNA |
| DNA | - | Deoxyribonucleic acid |
| RNA | - | Ribonucleic acid |
| CHS | - | Chalcone synthase |
| PTGS | - | Post transcriptional gene silencing |
| s/s | - | Single stranded |
| HEPB | - | Hepatitis B |
| siRNA | - | Small interfering RNA |
| piRNA | - | PIWI interacting RNA |
| miRNA | - | Micro RNA |
| ra-siRNA | - | Repeat associated siRNA |
| ta-siRNA | - | Trans acting siRNA |
| endo-siRNA | - | Endogenous siRNA |
| natRNA | - | Natural antisense transcript RNA |
| scnRNA | - | Scan RNA |
| tncRNA | - | Tiny non-coding RNA |
| pri-mRNA | - | Primary mRNA |
| pre-miRNA | - | Precursor miRNA |
| UTR | - | Untranslated region |
| bp(s) | - | Base pair(s) |
| RdRP | - | RNA dependant RNA polymerases |
| nts | - | Nucleotides |
| IFN | - | Interferon |
| BLAST | - | Basic local alignment search tool |
| kDa | - | Kilodalton |
| DUF | - | Domain of unknown function |
| PAZ | - | PIWI Argonaute Zwille |
| RBD | - | RNA binding domain |
| TRBP | - | TAR-RNA binding protein |
| Ago | - | Argonaute proteins |
| RISC | - | RNA induced silencing complex |
| RNase | - | Ribonucleas |
| DDH | - | D(Asp)-D(Asp)-H(His) |
| P-bodies | - | Processing bodies |
| cDNA | - | Chromosomal DNA |
| EST | - | Expressed sequence tag |
| shRNA | - | Small hairpin RNA |
| sisiRNA | - | Small internally segmented interfering RNA |
| AAV | - | Adeno associated viruses |
| CNS | - | Central nervous system |
| FATP-5 | - | Fatty acid transport protein-5 |
| Ras | - | Rat sarcoma |
| pol | - | Polymerase |

| | | |
|---|---|---|
| TG | - | Transgene |
| mGluR2 | - | Metabotropic glutamate receptor-2 |
| PEG | - | Polyethylene glycol |
| PEI | - | Polyethyleneimine |
| MPP's | - | Membrane penetrating peptides |
| DOTMA chloride | - | N-[1-(2,3-dioleyloxy)propyl]-N,N,N- trimethylammonium |
| DC-Chol | - | 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride |
| DMKE | - | O,O'-dimethylstristyl-N-lysyl aspartate |
| DOPC | - | 1,2-Dioleoyl-sn-glycero-3-phosphocholine |
| DOPE | - | 1,2 Dioleoly-sn-glycero-3-phosphoethanolamine |
| RPR209120 | - | 2-(3-[bis-(3-amino-propyl)-amino]-propylamino)-N-ditetradecylcarbomoyl-thyl-acetamide |
| TNF-α | - | Tumor necrosis factor α |
| AtuFECT01 oleylamide | - | β-L-arginyl-2,3-L-diaminopropionicacid-N-palmityl-N-triyhdrochloride |
| HBV | - | Hepatitis B Virus |
| RME | - | Receptor mediated endocytosis |
| LDL | - | Low density lipoprotein |
| HCV | - | Hepatitis C Virus |
| ASGP | - | Asialoglycoprotein |
| Chol-T | - | 3β[N-(N',N'-dimethylaminopropane) - carbamoyl] cholesterol |
| Chol-Q | - | 3β[N-(N',N',N'-trimethylammoniumpropane) - carbamoyl] cholesterol iodide |
| β-GAL | - | (β)-D-galactopyranoside |
| TEM | - | Transmission electron microscopy |
| FBS | - | Foetal bovine serum |
| ASGP-R | - | Asialoglycoprotein receptor |
| SUV | - | Small unilamellar vesicle |
| LUV | - | Large unilamellar vesicle |
| GUV | - | Giant unilamellar vesicle |
| MLV | - | Multilamellar vesicle |
| DOSPA hydroxyethyl] | - | 1,3-dioleoyloxy-N-[2-(S-oxy[ethyl-2-heptadecenyl-3 imidazolinium chloride |
| DOSPER | - | 1,3-dioleoyloxy-2-(6-carboxyspermyl)-propylamide |
| DOTAP | - | 1,2-dioleoyl-3-dimethylammoniumpropane |
| DOGS | - | Dioctadecylamidoglycylspermine |
| $T_C$ | - | Transition temperature |
| HEPES | - | 2-[4-(2-hedoxyethyl)-1-piperazinyl] ethane sulphonic acid |
| TLC | - | Thin layer chromatography |
| C | - | Cholesteryl chloroformate |
| SD-CholTC | - | Cytofectin Chol-T control liposomes |
| SD-CholTT | - | Cytofectin Chol-T targeted liposomes |
| SD-CholQC | - | Cytofectin Chol-Q control liposomes |

| | | |
|---|---|---|
| SD-CholQT | - | Cytofectin Chol-Q targeted liposomes |
| NMR | - | Nuclear magnetic resonance |
| DLS | - | Dynamic light scattering |
| EM | - | Electron microscopy |
| ODNs | - | Oligodeoxynucleotides |
| tRNA | - | Transfer RNA |
| control siRNA | - | siGenome non-targetting siRNA |
| siTOX | - | siCONTROL TOX siRNA |
| DMSO | - | Dimethylsulphoxide |
| HBS | - | HEPES buffered saline |
| Tris-HCL | - | Tris(hydroxymethyl)aminomethane |
| EDTA | - | Ethylenediaminetetraacetic acid |
| TBE | - | Tris Borate EDTA |
| UV | - | Ultraviolet |
| SDS | - | Sodium dodecyl sulphate |
| DEAE | - | Diethylaminoethyl |
| PCR | - | Polymerase chain reaction |
| MEM | - | Minimum essential medium |
| $NaHCO_3$ | - | Sodium carbonate |
| KCL | - | Potassium chloride |
| $KH_2PO_4$ | - | Potassium dihydrogen phosphate |
| $NA_2HPO_4$ | - | Disodium hydrogen phosphate |
| MTS | - | [3-(4,5-dimethylthiozol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] |

# Chapter One: Introduction

## 1.1 Gene Therapy Embraces RNA interference (RNAi)

Genes are the basic physical and functional units of heredity in living organisms. The human genome contains approximately 25 000 genes, with expression of individual genes requiring appropriate regulation to suit the function and environment of distinct cells (Sun *et al.*, 2008). Gene abnormalities such as mutations, variations or deletions may result in a gene's inability to function properly, leading to a diseased state (www.ornl.gov). The concept of gene therapy arose in the late 1960s and involves the introduction of a good copy gene to replace or compensate for one or more abnormal or missing genes (Emery, 2004; Tatum *et al.*, 1967). It offered the prospect of treatment for a wide range of acquired or inherited human diseases such as cystic fibrosis, muscular dystrophy and various cancers (Emery, 2004; Lasic, 1997; Yadava, 2007). However, decades after its initial conception, the potential of gene therapy has been limited by problems of delivery, toxicity and immunogenecity (Yadava, 2007). Recent deaths in two gene therapy clinical trials (www.ornl.gov) have also contributed to its decline in favour. Researchers subsequently expanded the scope of gene therapy, to include gene repression molecules such as ribozymes and anti-sense oligonucleotides (ODNs), but these also proved disappointing (Wilson, 2005).

The discovery of a double-stranded (d/s) RNA-driven gene silencing mechanism in the late 1990s led to a revitalization in the field of genetic therapeutics (Castanotto *et al.*, 2009; Wilson, 2005). The mechanism, termed 'RNA interference' (RNAi), was initially only viewed as a basic loss-of-function technique, but scientists soon recognised its potential as a viable and innovative therapeutic tool (Paroo *et al.*, 2007). While traditional gene therapy focused on supplying a normal copy gene, RNAi harnesses a natural biological process to 'turn off' a problematic gene through cleavage or repression of the associated messenger RNA (mRNA) transcript (Joshua-Tor, 2004; www.biologynews.net). It is being viewed as an improved system or

successor of anti-sense techniques; and is an emerging frontier of gene therapy with immense therapeutic potential (Tyagi *et al.*, 2006). Commonly referred to as 'the RNAi revolution', the discovery and development of RNAi, from basic research technique to therapeutic modality, has been prolific (Paroo *et al.*, 2007).

## 1.2 Discovery and Significance of RNAi

### 1.2.1 History

The proliferation of RNA studies coupled with the advancement of gene transfer technologies in the latter half of the twentieth century, heralded a new era in genomic science. Of great importance was the demonstration that foreign genetic material introduced into cells could induce potent and unexpected responses in a variety of organisms (Fire, 1999). The phenomenon of RNA-induced silencing was first documented in transgenic plants (petunias) by Jorgensen and colleagues in 1990 (Paroo *et al.*, 2007). They showed that the introduction of a Chalcone synthase (CHS) transgene resulted in a dramatic and unexpected reduction in expression of a homologous gene of independent transgenotes in three genetic variants (Napoli *et al.*, 1990). This phenomenon was labelled co-suppression or post-transcriptional gene silencing (PTGS) (Jana *et al.*, 2004). Shortly thereafter, evidence of homology-dependent gene silencing in other organisms, such as "quelling" in fungi, emerged (Siomi, 2009; www.sirna.com).

Nearly half a decade later, Andrew Fire and co-workers (1998) induced sequence-specific gene silencing by the experimental introduction of RNA into the nematode *Caenorhabditis elegans* (Yigit *et al.*, 2006). They presented a remarkable set of findings, namely: (i) d/s RNA proved substantially more effective at triggering silencing than either sense or anti-sense s/s RNA alone (Fire *et al.*, 1998), (ii) silencing was specific for an mRNA homologous to the inserted d/s RNA sequence (DeVincenzo, 2009) and (iii) only a few molecules of injected d/s RNA were required per affected cell, suggesting a catalytic or amplification rather than a stoichiometric

action (Fire *et al.*, 1998). This gene silencing mechanism was termed 'RNA interference' (RNAi) and the field of RNAi was initiated.

As RNAi research gained momentum, focus subsequently shifted towards demonstrating its existence in mammalian cells. Initial studies were hindered by long d/s RNAs (>30 base pairs) activation of the interferon response pathway. It was not until Tuschl and colleagues (Elbashir *et al.*, 2001 a) demonstrated or discovered that a smaller, synthetic duplex RNA molecule (± 21 nucleotides) inhibited specific mRNA targets in somatic mammalian cells (Sioud, 2004) that RNAi was catapulted from an 'interesting' cellular phenomenon to so potent a technology that its discoverers were awarded the Nobel Prize in Medicine and Physiology a mere eight years after its discovery (Lee *et al.*, 2009; www.nobelprize.org).

Today, RNA interference can broadly be defined as: "a post-transcriptional gene silencing pathway that occurs in response to the introduction of d/s RNA into a cell" (Duxbury *et al.*, 2004; Hammond, 2005). Depending on the nature of the d/s RNA trigger and biological system, it down-regulates homologous target genes through cleavage, translational repression or transcriptional inhibition of target mRNA (Joshua-Tor, 2004). RNAi is a diverse, evolutionarily conserved system in eukaryotic cells (Hutvagner, 2005) that constitutes a natural, innate defence mechanism against various types of foreign nucleic acids such as viruses, transposable elements (transposons, transgenes) and d/s RNA (Jana *et al.*, 2004; Lee *et al.*, 2009). In addition, RNAi has been implicated in proper functioning and development of organisms via roles in translational silencing, chromatin structure organisation and cellular development and differentiation (Lee *et al.*, 2009).

### 1.2.2 RNAi in Functional Genomics

Not too long ago, scientists performed gene knock-outs using anti-sense, dominant negative or other knock-out techniques which proved rather ineffective (Thakur, 2003). RNAi is generally believed to have revolutionized modern genetics by offering the ability to specifically and efficiently target any mRNA of interest. RNAi-based functional genomics, for the first time, permitted evaluation of the role of individual

gene products on a genome-wide scale in higher organisms through what is known as 'reverse' genetics (www.biomedexperts.com), whereby a known gene is sought for an unknown function. The principle of RNAi analysis of gene function is simple i.e. introduction of d/s RNA or DNA encoding for d/s RNA activates the RNAi pathway so that properties of the affected cell(s) reflect a loss of function in the corresponding gene (Thakur, 2003). The ability to selectively silence specific genes without affecting the genome itself, combined with its ease-of-use and speed means that RNAi is one of the main techniques researchers employ for isolation and analysis of single gene function and subsequent dissection of a wide range of cellular processes (DeVincenzo, 2009). It is associated with a number of advantages over previous methods employed for gene suppression (Table 1.1) (Duxbury *et al.*, 2004).

The unbounded potential of RNAi has encouraged numerous strategies for silencing of virtually all protein-encoding genes in the human genome as well as various other plant and animal species, with studies yielding valuable information on gene function and pathway analysis (Aagaard *et al*., 2007). There are numerous examples of experiments in which RNAi has been utilized to (i) ascertain the function of genes and create large-scale high throughput screens eg. in *C.elegans*, *Drosophila* and several species of plants; screening of *C. elegans* for genes involved in obesity and ageing (www.functionalgenomics.org), human kinome identification of major growth regulating kinases in acute myeloid leukaemia cells (www.ash.confex.com); as well as to (ii) assess and discover what enzymes and proteins are involved in certain metabolic pathways eg. elucidation of the Gibberellin/ Abscisic acid signalling pathway by Zentella *et al.* (2002).

**Table 1.1:** Advantages and disadvantages of various gene suppression strategies (Adapted from Duxbury *et al.*, 2004; Eggert *et al.*, 2006).

| METHOD | ADVANTAGES | DISADVANTAGES |
|---|---|---|
| **1. RNAi** | Potent<br>Specific<br>Catalytic<br>Small regulatory RNAs are readily available | Transfection dependent<br>"Knockdown" not "Knockout"<br>Off-target effects |
| **2. Anti-sense DNA oligonucleotides** | Simple<br>Inexpensive | Variable efficiency and specificity<br>Transfection dependent<br>Stoichiometric |
| **3. Small molecule inhibitors** | Ease of administration<br>High temporal and some spatial control<br>Fast (effects observed in real time) | Non-specific<br>Limited number of bioactive small molecules of known mechanism(not readily available) |
| **4. Transfection of dominant negative mutant gene** | Stable suppression<br>Ability to determine functions of discrete regions of a protein | Transfection dependent<br>Unanticipated effects |
| **5. "Knockout mouse"** | 100% gene silencing | Expensive<br>Lethal phenotypes |

### 1.2.3 Therapeutic Implications

Beyond its value for dissecting gene function, RNAi offered the prospect of target validation in drug development. Target validation determines whether a known candidate gene is responsible for a disease and whether alteration of expression of said gene can lead to a therapeutic effect. Drug target validation is a major obstacle for the bio-pharmaceutical industry (Appasani, 2005). The timeline associated with target validation, from discovery to completion of clinical trials, is roughly 14 years (Figure.1.1 a). Consequently, the financial implications are immense, approximately $800 million for research and development per novel chemical entity, with less than

1% resulting in a viable therapeutic. Due to its ability to specifically suppress gene expression, RNAi can and, to some degree, has been successfully utilized to identify and validate novel therapeutic targets in disease states, thus reducing the length and cost of drug development (Figure.1.1 b) (Appasani, 2005; Boese *et al.*, 2003).

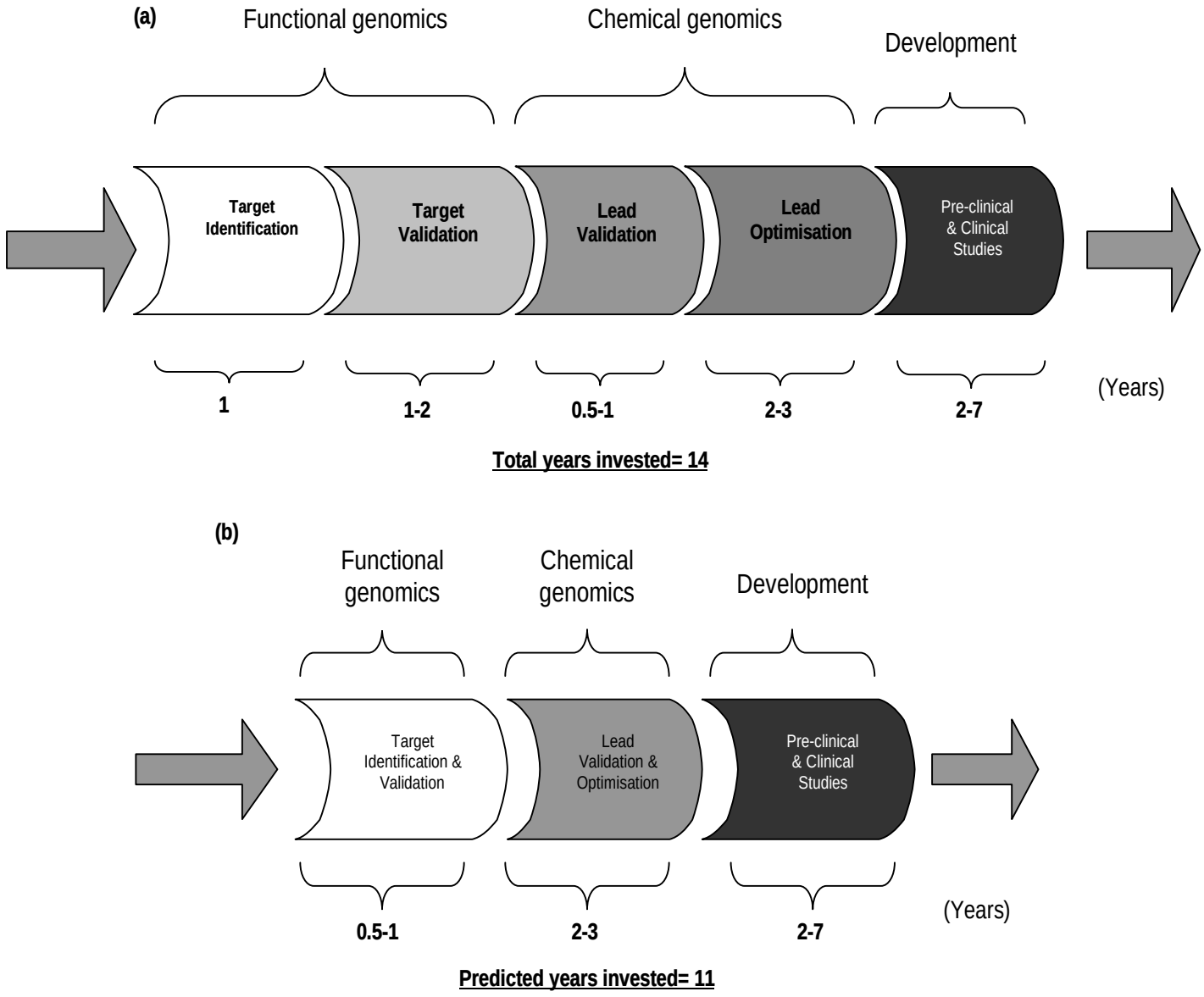


**Figure 1.1:** Estimates of drug development timeline: (a) with no implementation of RNAi and (b) with full implementation of RNAi coupled with other technologies (Adapted from Boese *et al.*, 2003)

RNAi was, moreover, a potentially useful technology for development of highly specific d/s RNA-based gene silencing therapeutics (ur-Rahman *et al.*, 2009) for treatment of various genetic disorders, infections and other disease conditions (Rieschl and Zimmer, 2009). According to the central dogma of molecular biology, protein synthesis occurs in two steps .i.e.

1. *Transcription* copies genes from d/s DNA to s/s mRNA molecules;
2. *Translation* turns mRNA into functional protein form.

Hence, there are two ways of preventing gene expression and three main time points at which a disease can be blocked i.e. transcriptional, post-transcriptional and post-translational. RNAi allows protein synthesis to be prevented at the second, translational/post-transcriptional step and offers the ability to control the development of a disease earlier on in the process, resulting in possible eradication of the disease state (www.bio.davidson.edu; Thakur, 2003).

Conventional chemical-based drugs have, traditionally, been restricted to certain classes of receptors, ion-channels and enzymes. RNAi-based 'drugs' have expanded the scope of potential druggable targets since, theoretically, any disease that can be ameliorated through endogenous or exogenous protein knockdown is a potential target for RNAi therapeutics (DeVincenzo, 2009; Oh *et al.*, 2009). RNAi offered a number of advantages over existing conventional and genetic therapies, namely: (i) RNAi utilizes cellular machinery, (ii) only requires entrance into the cytoplasm, not the nucleus, (iii) high potency allows for lower therapeutic dose concentrations and (iv) catalytic nature means that catalytic rather than stoichiometric amounts are required (Aagaard *et al.*, 2007; DeVincenzo, 2009; Zhang *et al.*, 2007). In essence, RNAi silencing of disease-related mRNAs holds many advantages and may yield patient benefits unobtainable by other therapeutic approaches (Appasani, 2007).

Scientists studied how to manipulate RNAi to silence specific genes associated with disease processes (Wilson, 2005), with the swift advancement from RNAi research discovery to clinical trials being well-documented (de Fougerolles, 2008) example:. McCaffrey and colleagues (2002) demonstrate external induction of RNAi in adult

mice infected with the Hepatitis B virus. This was rapidly followed by the first therapeutic application of RNAi, where a class of small regulatory RNAs targeted to the *Fas* mRNA resulted in protection of treated mice against liver fibrosis (Castanotto *et al.*, 2009). Clinical trials for RNAi-based treatment of human diseases commenced in 2004 and despite its infancy, the current list of diseases for which RNAi is being tested as a therapeutic modality includes: cancers, diabetes (type 2), HIV infection, hypocholesterolaemia, Lou Gehrig's disease, obesity, Parkinson's disease, respiratory disease, rheumatoid arthritis, wet age-related macular degeneration (Castanotto *et al.*, 2009). At present, there are several RNAi clinical trials that are in progress worldwide (Table 1.2.) with more planned for the coming years (Castanotto *et al.*, 2009; Siomi, 2009).

**Table 1.2:** List of clinical trials for RNAi-based therapeutics *as of 2009* (Adapted from Castanotto *et al.*, 2009; Shrey *et al.*, 2009)

| Drug | Company | Target Disease | Stage |
|---|---|---|---|
| **Bevasiranib** | Acuity Pharmaceuticals | Wet age-related macular degeneration | Phase III |
| | | Diabetic macular oedema | Phase II |
| **Sirna-027** | Merck-Sirna Therapeutics | Wet age-related macular degeneration | Phase II |
| ***RTP801i-14** | Quark Pharmaceuticals, and Silence Therapeutics | Wet age-related macular degeneration | Phase I/ IIA |
| **ALN-RSV01** | Alynlam Pharmaceuticals | Respiratory syncytial virus infection | Phase II |
| **MiR-122** | Santaris Pharma | Hepatitis C viral infections | Phase I |
| **NUC B1000** | Nucleonics | Hepatitis B | Phase I |
| **Anti-*tat/ rev* shRNA** | City of Hope National Medical Centre, and Benitec | AIDS | Pilot feasibility study |
| **CALAA-01** | Calando Pharmaceuticals | Solid tumors | Phase I |
| **TD101** | TransDerm, and the International Pachyonychia Congenita Consortium | Pachyonychia Congenita | Phase I |

** RTP801i-14 has been licensed to Pfizer (PF-4523655)*

*Other companies involved in this field, but not mentioned above, include: Avocoel, Sequitur, Cenix Biosciences, RNA TEC, Sigma-Aldrich and Invitrogen.*

The development of RNAi drugs has become an enormous and burgeoning market (Grimm, 2009), with vast resources and research directed towards elucidation of all RNAi components and parameters. An area receiving considerable attention is the isolation and identification of small RNAs and the elucidation of the RNAi pathway.

### 1.3 Role of small RNAs in RNAi

RNA interference is part of a sophisticated network of interconnected gene control pathways collectively referred to as RNA silencing (Filipowicz *et al.*, 2005; Jana *et al.*, 2004). An important feature uniting all RNA silencing pathways is the presence of d/s RNA as a trigger or intermediate molecule (Jana *et al.*, 2004). Although the core protein machinery of RNA silencing is widely conserved in eukaryotes (excluding *S. cerevisiae*), different d/s RNA sources are encountered (Aravin *et al.*, 2005). RNA silencing is induced by exogenous or endogenous long d/s RNAs or hairpin RNAs, which are processed into shorter d/s molecules, termed small, small regulatory or small silencing RNAs (Ameres *et al.*, 2007; Finnegan *et al.*, 2003; Matranga *et al.*, 2006).

These tiny RNAs, generally 21- 30 nucleotides in length, come in many guises (Table 1.3); however, defining aspects such as origin, structure, function and associated-effector proteins have led to the recognition of three dominant classes: microRNAs (miRNAs), small (short) interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs) (*usually single-strand*) (Carthew *et al.*, 2009; Finnegan *et al.*, 2003; Matrange *et al.*, 2006). Of these three classes, miRNAs and siRNAs are the principal categories of small RNAs that induce RNAi in a vast array of eukaryotic organisms including *Homo sapiens* (Pfeifer *et al.*, 2010). They function as regulators of gene expression by altering the stability or translational efficiency of mRNAs with which they share full or partial sequence complementarity (Pfiefer *et al.*, 2010; Sun *et al.*, 2008). miRNAs are regarded as regulators of endogenous genes, while siRNAs are charged with

defending genome integrity against invasive nucleic acids (Carthew *et al.*, 2009). siRNAs are the primary mediators of RNAi in mammalian cells (DeVincenzo, 2009).

| **Class** | **Size** (nucleotide) | **Functions** | **Mechanisms** | **Origin** | **Organisms found in** |
|---|---|---|---|---|---|
| **SiRNAs** | 21-25 | Regulating gene expression, restricting transposons, providing antiviral response | RNA degradation, transposon restriction | Intergenic regions, exons, introns | *C.elegans*, *D.melanogaster*, *S.pombe*, *A.thaliana*, *O.sativa* (rice) |
| **MiRNAs** | 21-25 | Regulating gene expression at the post-transcriptional level | RNA degradation, Blocking translation | Intergenic regions, introns | *C.elegans*, *D.melanogaster*, *S.pombe*, *A.thaliana*, *O.sativa*, mammals |
| **PiRNAs** | 24-31* | Regulating germline development and integrity, silencing selfish DNA | Unknown | Defective trans-poson sequences And other repeats | *C.elegans*, *D.melanogaster*, *D.rerio*, mammals |
| **endo-siRNAs** | 21-25 | Restricting transposons, regulating mRNAs and heterochromatin | RNA degradation | Transposable elements, pseudogenes | *D.melanogaster*, mammals |
| **ra-siRNAs** | 23-28 | Remodelling chromatin, transcriptional gene silencing | Unknown | Repeated sequence elements (subset of piRNAs) | *C.elegans*, *D.melanogaster*, *S.pombe*, *A.thaliana*, *D.rerio*, *T.brucei* |
| **ta-siRNAs** | 21-22 | *Trans*-acting cleavage of endogenous mRNAs | RNA degradation | Non-coding endogenous trans-cripts | *D.melanogaster*, *S.pombe*, *A.thaliana*, *O.sativa* |
| **NatRNAs** | 21-22 | Regulating gene expression at the post-transcriptional level | RNA degradation | Convergent partly overlapping transcripts | *A.thaliana* |
| **ScnRNAs** | 26-30 | Regulating chromatin structure | DNA elimination | Meiotic micronuclei | *T.thermophila*, *P.tetraurelia* |
| **TncRNAs** | 22 | Unknown | Unknown | Non-coding regions | *C.elegans* |

**Table 1.3:** Small regulatory RNAs involved in gene silencing
(Adapted from Castanotto *et al.*, 2009)

***C.elegans* piRNAs are 21-nucleotides.

**siRNAs**: small interfering RNAs, **miRNAs**: microRNAs, **piRNAs**: PIWI-interacting RNAs,

**endo-siRNAs**: endogenous siRNAs, **ra-siRNAs**: repeat-associated siRNAs, **ta-siRNAs**: *trans*-acting siRNAs, **natRNAs**: natural antisense transcript siRNAs, **scnRNAs**: scan RNAs, **tncRNAs**: tiny non-coding RNAs.

Furthermore, the majority of research to date into the use of RNAi as a therapeutic modality has focused on siRNAs as the inducer or remedial agent (de Fougerolles, 2008). Hence greater focus shall be directed towards siRNAs in RNAi, with a brief introduction into the biogenesis and function of miRNAs.

### 1.3.1 MicroRNAs (miRNA)

MicroRNAs, first discovered in 1993 by Ambros and co-workers (Lee *et al*., 1993), constitute a class of non-coding small RNAs phylogenetically widespread in plants and animals (Carthew *et al.*, 2009; Cerutti, 2003; Nelson *et al.*, 2003; Seto, 2010). These small RNAs, generally 20-23 nucleotides in length, are encoded within the eukaryotic genome, activated by transcription factors and (most) transcribed by RNA polymerase II from individual miRNA genes or introns of protein-coding genes or polycistronic clusters encoding multiple miRNAs on a single transcript (Aravin *et al.*, 2005; Cullen, 2004; Seto, 2010; Sun *et al.*, 2006). As diverse regulators of gene expression, miRNAs play important roles in cell growth, differentiation and proliferation (Pfeifer *et al.*, 2010); and have been implicated in regulation of developmental timing, synaptic plasticity, insulin secretion in pancreatic islet cells and control of cholesterol homeostasis in the liver (Carthew *et al.*, 2009; Sun *et al.*, 2008). They regulate approximately 30% of all human genes and at present more than 700 human miRNA sequences have been identified (Pfeifer *et al.*, 2010, Seto, 2010).

miRNAs are generated from long stem-loop primary transcripts termed pri-miRNAs that are then processed within the nucleus by Drosha enzyme to yield 70 to 100 nucleotide precursor miRNAs (pre-miRNAs) (Figure.1.2 a). Exportin-5 then transports these pre-miRNAs into the cytoplasm where they undergo further processing by the Dicer enzyme, yielding ~22 nucleotide mature miRNA duplexes with 5' phosphates

and 2 nucleotide 3' overhangs (Figure.1.2 b) (Cullen, 2004; Matrange *et al.*, 2007; Pfeifer *et al.*, 2010; Sun *et al.*, 2008).

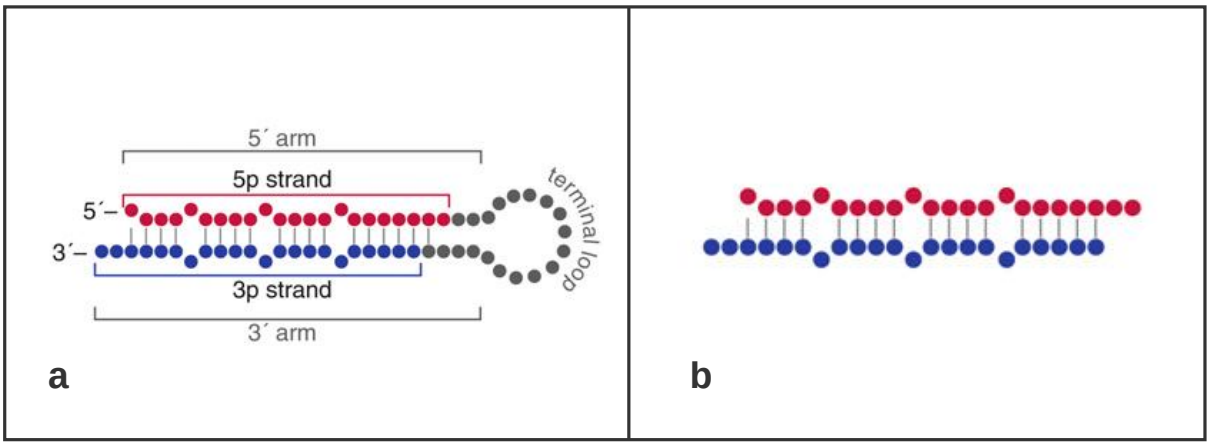


**Figure 1.2:** (a) pre-miRNA hairpin structure (b) mature miRNA; ●: anti-sense strand and ● : sense strand (Adapted from Kawamata *et al.*, 2010).

miRNAs mainly interact with target mRNAs via "seed sites" between nucleotide base pairs 2 and 7/8 of the miRNA molecule which corresponds to sequences within the 3' untranslated region (UTR) of the target transcript (Matrange *et al.*, 2007; Pfeifer *et al.*, 2010; Shrey *et al.*, 2009). Inhibition of target RNAs is achieved through one of two mechanisms as determined by the degree of complementarity between miRNAs and target mRNAs (Sun *et al.*, 2008). Full or high complementarity is associated with mRNA degradation -most common in plants (Meister *et al.*, 2004), while imperfect or partial complementarity, with translational repression, characteristic of animal miRNAs (Cullen, 2004; Pfeifer *et al.*, 2010).

### 1.3.2 small interfering RNAs (siRNAs)

siRNAs, often referred to as the function mediators, effectors or triggers of RNAi (Oliveira *et al.*, 2006; Watts *et al.*, 2008), were first detected in 1999 by Hamilton *et al* during virus- and transgene-induced silencing experiments in plants (Finnegan *et al.*, 2003; Pfeifer *et al.*, 2010). These d/s nucleic acids are generally 19 to 25 nucleotides in length, possess 38 to 46 negative charges and have a molecular

weight of approximately 13 to 15 kilodaltons (Hutvagner *et al*., 2005; Oliveira *et al.*, 2006; Zhang *et al.*, 2007; Wadhwa *et al.*, 2004). siRNAs are generated from an ATP-driven (in certain species), Dicer-mediated cleavage of long d/s RNA precursors, yielding short duplexes with 5'-phosphate and 3'-hydroxyl terminal groups, and symmetrical 2 nucleotide 3' overhangs. (Figure.1.3) (Hutvagner *et al.*, 2005; Nguyen *et al.*, 2008; Shrey *et al*., 2009 ; Sun *et al*., 2008; Wadhwa *et al.*, 2004).

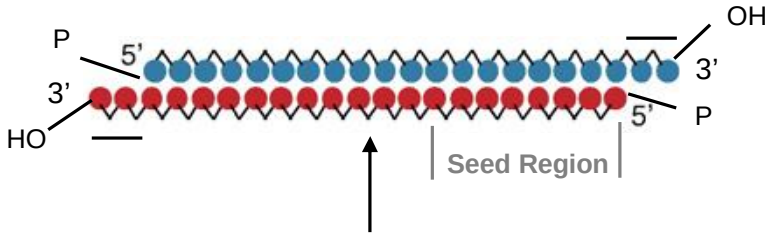


**Figure 1.3:** Structure of siRNA; siRNA is composed of two strands- a 21 bp region of duplex, with dinucleotide overhangs (—) on the 3' end of each strand, 5'-phoshates and 3'-hydroxy termini. The site at which the mRNA target is cleaved is indicated by an arrow. ● : anti-sense strand and ● :sense strand (Modified from Chen *et al.*, 2010; DeVincenzo, 2007; Shan, 2009).

The d/s precursors are primarily exogenous in origin, with endogenous sources being rare (Aravin *et al*., 2005; Carthew *et al.*, 2009). siRNA sources include: viral RNAs, hairpin RNAs, repeat-associated transcripts (centromeres, transposons), convergent transcripts and other sense/anti-sense pairs, gene/pseudogene duplexes, transgene transcripts, trans-acting RNAs and environmentally, experimentally or clinically introduced d/s RNAs ( Carthew *et al.*, 2009). Eukaryotes synthesize long d/s RNAs via 2 distinct mechanisms i.e. (i) *bi-directional transcription* of inverted transgene/ transposon repeats, and (ii) *read-through transcription* of convergent promoters. Primary or aberrant transcripts can also be utilised by organisms encoding for RNA-dependent RNA polymerases (RdRP) (Hutvagner, 2005). The length of the d/s precursor may vary, depending on species (Hutvagner *et al.*, 2005) for example, in *C.elegans* and *Drosophila*, they may be greater than 500 base pairs (bps). Exogenous d/s RNA precursors longer than 30 nts induce an interferon (IFN) response in mammalian cells (Duxbury *et al.*, 2003; Pfeifer *et al.*, 2010). Therefore, siRNAs generated for experimental or therapeutic use in mammalian systems are produced

through chemical synthesis, enzymatic cleavage or expression systems/cassettes to reduce host cell immune recognition of inserted d/s RNAs *(These methods of siRNA production shall be addressed in greater detail later on in this chapter)* (Leung *et al.*, 2005; Pfeifer *et al.*, 2010).

siRNAs function in defence, protecting the host organism against foreign nucleic acids (Finnegan *et al.*, 2003). They have been widely utilized in mammalian cells for elucidation of gene function, cellular pathway analysis, identification of disease-related genes and, most importantly, as potential therapeutic agents (Leung *et al.*, 2005). siRNAs, once generated, are assembled asymmetrically into a multi-component complex, in which they serve as a sequence recognition element, directing homology-dependent cleavage of target mRNA in the region of complementarity (Aagaard *et al.*, 2007; Boese *et al.*, 2003; Moss, 2001; Yadava, 2007). In contrast to miRNAs, siRNAs are perfectly complementary to target mRNAs (Moss, 2001; Pfeifer *et al.*, 2010); they are complementary to both the sense and anti-sense strands of the targeted mRNA and have a distinct chemical polarity that is essential for their function (Appasani, 2005). The characteristic nucleotide 3' overhangs aid in recognition by the multi-component complex (Aagaard *et al.*, 2007); while the thermodynamically less stable anti-sense strand is preferentially loaded at the 5' terminus (Shrey *et al.*, 2009; Yadava, 2007). Base composition at the 5' end can also influence which siRNA strand initiates RNAi ( Appasani, 2005).

## 1.4 The RNA interference Pathway

The RNAi mechanism (Figure.1.4) is a multi-step process that can be divided into two phases: (i) *initiation* phase and (ii) *effector* phase. During the initiation phase, long d/s RNA or precursor miRNA are cleaved, by an RNase III endonuclease enzyme, *Dicer*, into 19-25 nt siRNAs or mature miRNAs (Duxbury *et al.*, 2004; Shrey *et al.*, 2009; Yadava, 2007). Dicer (Figure.1.5 a) is a ~200 kDa protein generally containing an N-terminal ATPase/RNA helicase, followed by a domain of unknown function (DUF 283), a Piwi/Argonaute/Zwille (PAZ) domain, two RNase III domains and a C-terminal RNA binding domain (RBD) (Filipowicz *et al*, 2005; Patel *et al*, 2006; Wadhwa *et al.*, 2004; Van den Berg *et al.*, 2008). Some organisms encode for a single Dicer gene (eg. vertebrates), whilst others have more than one Dicer homologue (eg. *Drosophila*) (Filipowicz *et al.*, 2005; Van den Berg *et al.*, 2008).

Dicer functions as a monomer with a single processing centre (Filipowicz *et al.*, 2005). First, the PAZ domain engages the d/s RNA substrate at the 3' end (recognition of ~2 nucleotide overhang), with the substrate extending approximately 2 helical turns along the protein surface prior to reaching a cleft created by intramolecular dimerization of the two RNase III domains (Figure.1.5 b). This is the processing centre which contains two independent catalytic sites, each cutting one RNA strand simultaneously and asymmetrically, generating appropriately sized siRNAs or miRNAs with characteristic 3' dinucleotide overhangs and 5' phosphates (Carthew *et al.*, 2009; Filipowicz *et al.*, 2005; Patel *et al.*, 2006). The distance between the PAZ domain and the processing centre appears to dictate the size of the generated duplex, differing among species (Carthew *et al.*, 2009). In some species, Dicer may function as part of a heterodimeric complex with a TAR-RNA binding protein (TRBP) or R2D2 or PACT (Carthew *et al.*, 2009; Siomi, 2009; Tseng *et al.*, 2009).

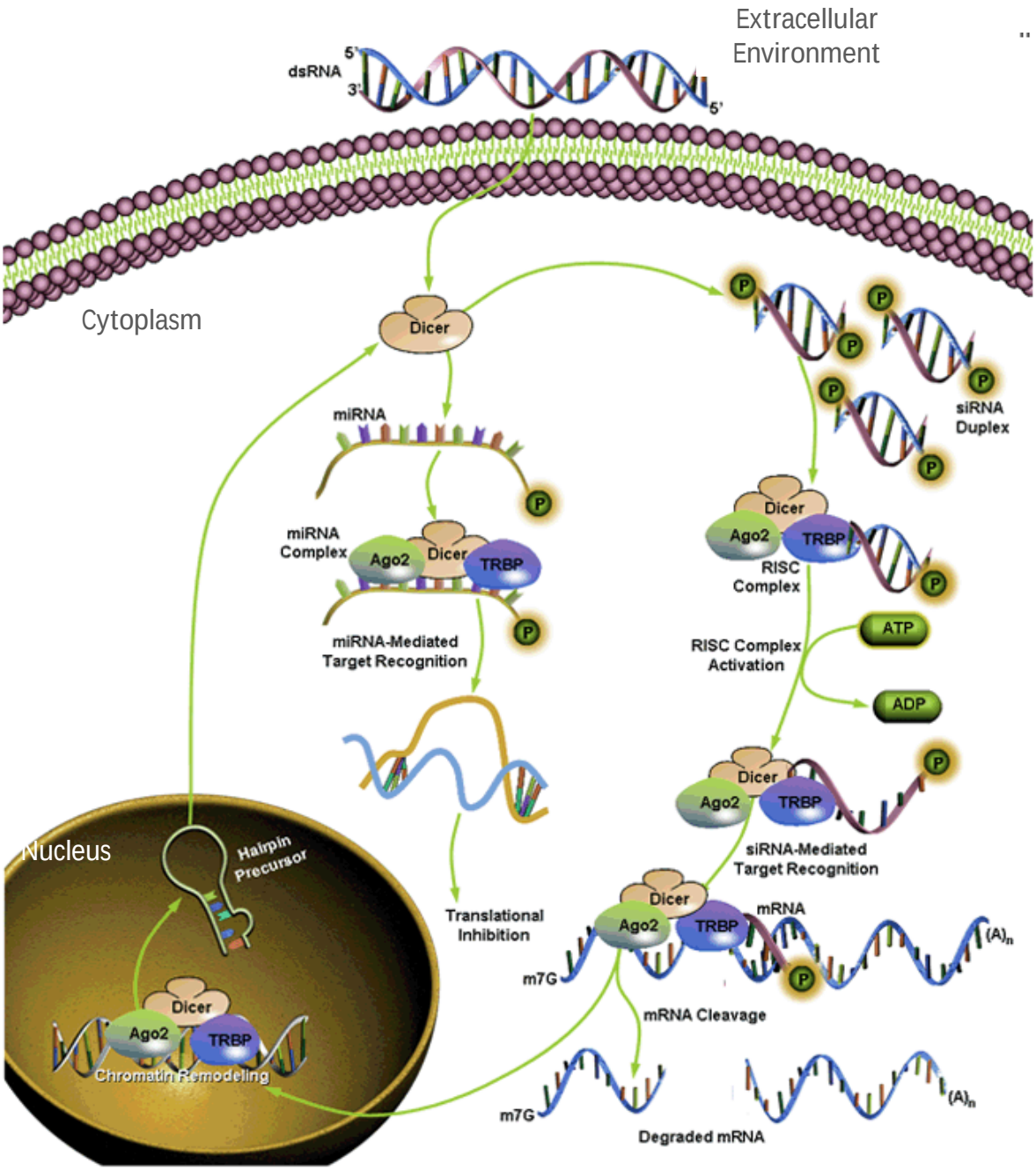
Extracellular Environment
dsRNA
5'
3'
5'
Cytoplasm
Dicer
miRNA
miRNA Complex
Ago2
Dicer
TRBP
miRNA-Mediated Target Recognition
siRNA Duplex
RISC Complex
RISC Complex Activation
ATP
ADP
siRNA-Mediated Target Recognition
Translational Inhibition
mRNA
m7G
mRNA Cleavage
Degraded mRNA
Nucleus
Hairpin Precursor
Chromatin Remodeling

**Figure 1.4:** The RNAi pathway, showing both miRNA-mediated mRNA repression and siRNA induced mRNA degradation (www.sabiosciences.com).

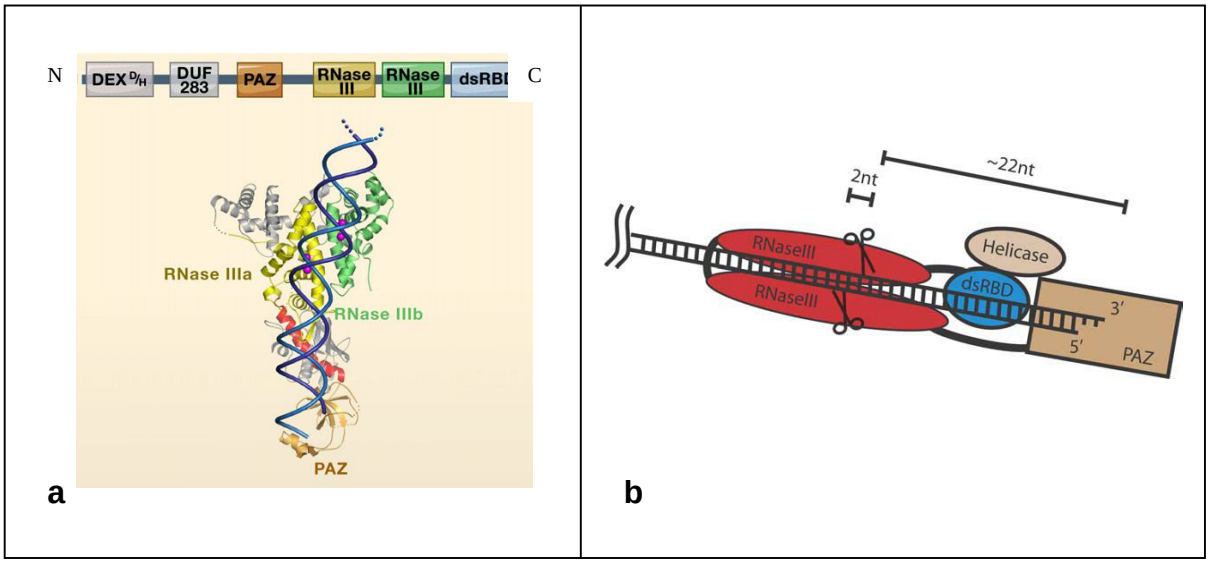


**Figure 1.5.:** (**a**) shows domain arrangement of most Dicer enzymes (top) and Dicer structure with long d/s RNA (blue), active-site metal ions (purple) and a 'ruler' helix (red). (**b**) is an illustration of Dicer binding long d/s RNA or pre-miRNA (Modified from Carthew *et al.*, 2009; Hammond, 2005).

The effector phase commences when Dicer transfers the d/s RNA substrate to the multi-protein RNA-induced Silencing Complex (RISC) (Duxbury *et al.*, 2004; Shrey *et al.*, 2009; Tseng *et al*, 2009). RISC is a 160 to 500 kilodalton (Ameres *et al.*, 2007; Yadava, 2007) complex containing Argonaute (Ago) proteins, a DEAD-box helicase (Gemin-3), a protein of unknown function (Gemin-4), Dicer and other as yet undetermined proteins (Wadhwa *et al.*, 2004; Watts *et al.*, 2008; Yadava, 2007). The Ago proteins are regarded as the core of RISC (Filipowicz *et al.*, 2005; Kawamata *et al.*, 2010; Tseng *et al.*, 2009), having 4 homologs (Ago1, 2, 3 and 4) in humans- of which only Ago2 demonstrates catalytic cleavage activity (Tseng *et al.*, 2009), and a structure composed of an N-terminal PAZ domain (± 150 residues) linked by a region of variable length (MID) to a C-terminal Piwi domain (± 400 residues) (Parker *et al.*, 2006; Tseng *et al.*, 2009). These 3 domains form a crescent-shaped base, with the centrally located PAZ domain binding the 3' end of the d/s RNA substrate (Filipowicz

*et al.*, 2005). The less thermodynamically stable 5' end is then chosen as the guide strand (i.e. anti-sense strand), the duplex is unwound and the passenger strand (i.e. sense strand) is cleaved (Kawamata *et al.*, 2010; Tseng *et al.*, 2009). The process via which unwinding (ATP-dependent) and passenger-strand degradation occurs has not yet been fully elucidated, although numerous theoretical models (eg. the helicase model) have been proposed (Hutvagner, 2005; Kawamata *et al.*, 2010).

The now functional RISC (denoted by RISC*) containing the guide-strand hybridizes to its complementary target mRNA via Watson-Crick intermolecular base pairing (Aagaard *et al.*, 2007; Sashital *et al.*, 2009). The RNase H-like Piwi domain of Ago, containing the conserved D(Asp)-D(Asp)-H(His) (DDH) motif, hydrolyzes the phosphodiester bonds between the target nucleotides base paired to guide-strand residues 10 and 11, relative to the 5' end of the siRNA (Aagaard *et al.*, 2007; Carthew *et al.*, 2009; Filipowicz *et al.*, 2005; Siomi, 2009). The resulting 5' and 3' fragments are released from RISC* and attacked by cellular exonucleases, completing the degradation process (Carthew *et al.*, 2009; Sioud, 2004). RISC* is now free to target other complementary mRNAs, allowing for multiple-rounds of RNA cleavage (Carthew *et al.*, 2009; Filipowicz *et al.*, 2005). In the case of mismatches between the siRNA and target mRNA, the siRNA behaves like miRNA in RNAi, with the 'seed region' of the anti-sense strand base pairing precisely with the target while mismatches are seen in other regions, leading to arrest in translation and sequestration of target mRNA in cytosolic P-bodies. This might eventually result in de-capping and degradation of the mRNA (Juliano *et al.*, 2008). The above mechanism may demonstrate slight variance amongst the species, but it is a good representation of the pathway as observed in most eukaryotic systems, including humans. Although RNAi can be activated by miRNAs and siRNAs, the involvement of the nucleus in miRNA biosynthesis has meant that developers of human therapeutics have largely steered clear of it, opting instead to utilize the siRNA-mediated RNAi pathway (DeVincenzo, 2009).

## 1.5 siRNA Therapeutics

### 1.5.1 Promise and Limitations of siRNAs as Remedial Agents

For therapeutic purposes, siRNAs hold several distinct advantages over other gene-based therapeutic agents or traditional pharmaceutical drugs currently on the market (Sato *et al.*, 2008). These include the fact that siRNAs are naturally utilized by cells for regulation of gene expression, making them a low toxicity or non-toxic risk (www.biologykenyon.edu; Sato *et al.*, 2007). The fact that they are part of a natural process also means that these molecules are highly effective and reliable at gene silencing (www.biology.kenyon.edu; Grimm, 2009; Ma *et al.*, 2005). siRNAs can be produced synthetically, they make use of a catalytic mechanism, operate in the cytoplasm, demonstrate high target selectivity, mediate PTGS in a wide variety of primary cells and demonstrate greater resistance to nuclease degradation than their anti-sense counter-parts (Sato *et al.*, 2007; Zhang *et al.*, 2007). The above, combined with their ease of design and synthesis, low production cost (compared with other therapies), consistent and predictable manner and ability to enter the RNAi pathway late, so as to minimize interference with endogenous miRNA gene regulation, makes siRNAs particularly attractive therapeutic agents (De Fougerolles, 2008; Leung *et al.*, 2005; Sato *et al.*, 2007).

Despite this immense attractiveness, siRNA duplexes are not optimal drug-like molecules (Watts *et al.*, 2008). Their physiochemical properties, such as anionic nature, high molecular weight and hydrophilicity mean that siRNAs do not easily cross lipophilic cell membranes (Kim *et al.*, 2010; Oliveira *et al.*, 2007; Wang *et al.*, 2009) and are rapidly eliminated by the renal glomeruli (Sato *et al.*, 2008; Watts *et al.*, 2008). They are highly vulnerable to serum exo- and endonucleases, leading to a half-life of less than 15 minutes in blood, can stimulate the immune system, induce

off-target effects and have limited tissue specificity/distribution (Nguyen *et al.*, 2008; Watts *et al.*, 2008). The inherent promise of siRNAs as therapeutic agents out-weighs their disadvantages, but these problems need to be addressed so as to produce a practical and efficient means of treating human diseases (Minchin *et al.*, 1999). The development of new approaches and adaptation of previously utilized gene delivery strategies, for siRNA therapeutics shall be discussed in detail.

### 1.6 Strategies for siRNA Design and Manufacture

The first set of empirical rules for siRNA design, based on studies performed on *Drosophila,* was compiled by Tuschl *et al* (Elbashir *et al*., 2001b) and basically stated:

1) siRNA duplexes should contain 21 nucleotide base pairs with 3' dinucleotide dTdT overhangs;
2) Nucleotides 1-19 of the sense siRNA strand should match positions 3-21 of the 23-nucleotide target mRNA;
3) Target sequence should ideally contain a 50% GC content;
4) Targeted region is selected from a given cDNA sequence, 50 to 100 nucleotides downstream of the start codon;
5) The target motif is selected in the following order of preference: i) NAR(N17)YNN, where N= is any nucleotide, R= purine and Y=pyrimidine; ii) AA(N19)TT; iii) NA(N21);
6) Selected siRNA sequences should be aligned against EST libraries to ensure target of a single gene.

Exceptions to the above were reported by Ding *et al.* (2005) (Appasani, 2007; www.sfold.wadsworth.org) and advances in our understanding of siRNAs and RNAi helped refine these rules. Today, general guidelines for siRNA design include the avoidance of intron regions, repeats, stretches of 4 or more nucleotides such as AAAA (homopolymime), and GC content <30% or >60%. It is also advisable to perform BLAST homology searches to avoid off-target effects. Rational siRNA design

can also be utilized (www.protocol-online.org). There are currently multiple software and internet search programs available to researchers for the selection of optimal siRNA sequences (Shan, 2009). An example of an siRNA sequence can be seen in Figure 1.6. The careful selection of sequences is required for minimization of off-target and non-specific effects, while still maximising gene silencing (Leung *et al.*, 2005).

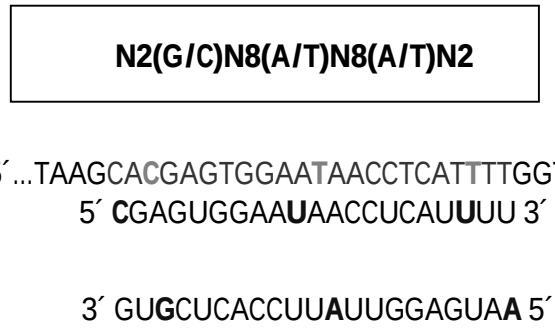


**Figure 1.6:** An example of target mRNA and corresponding siRNAs. Presented sequences are illustrative and do not agree with any mRNA and/or published siRNAs (Pei and Tuschl, 2006).

Scientists developed various approaches (Figure 1.7) for the production of synthetic siRNAs. The two main strategies currently employed are: (i) chemically synthesized or *in vitro* transcribed siRNAs and (ii) viral/plasmid vectors containing cassettes encoding short hairpin RNAs (shRNAs) transcribed with U6- or H1-like promoters and processed into siRNA by Dicer (Myers *et al.*, 2005; Shan, 2009; Wadhwa *et al.*, 2004). shRNA cassettes can be integrated into the host genome or stably retained as extra-chromosomal copies enabling long-term, persistent therapeutic effect (Lee *et al.*, 2009); however, concerns over access to the nucleus and saturation of endogenous miRNA pathways, have most impending RNAi therapeutics utilizing direct introduction of chemically synthesized siRNAs instead (Castanotto *et al.*, 2009). Although the cost of chemical synthesis is high and the effects are transient (< 2 weeks), their ease of production and administration, as well as their amenability to

chemical modifications mean that these molecules are favoured for induction of RNAi in mammalian cells (Castanotto *et al.*, 2009; Lee *et al.*, 2009; Wadhwa *et al.*, 2004).

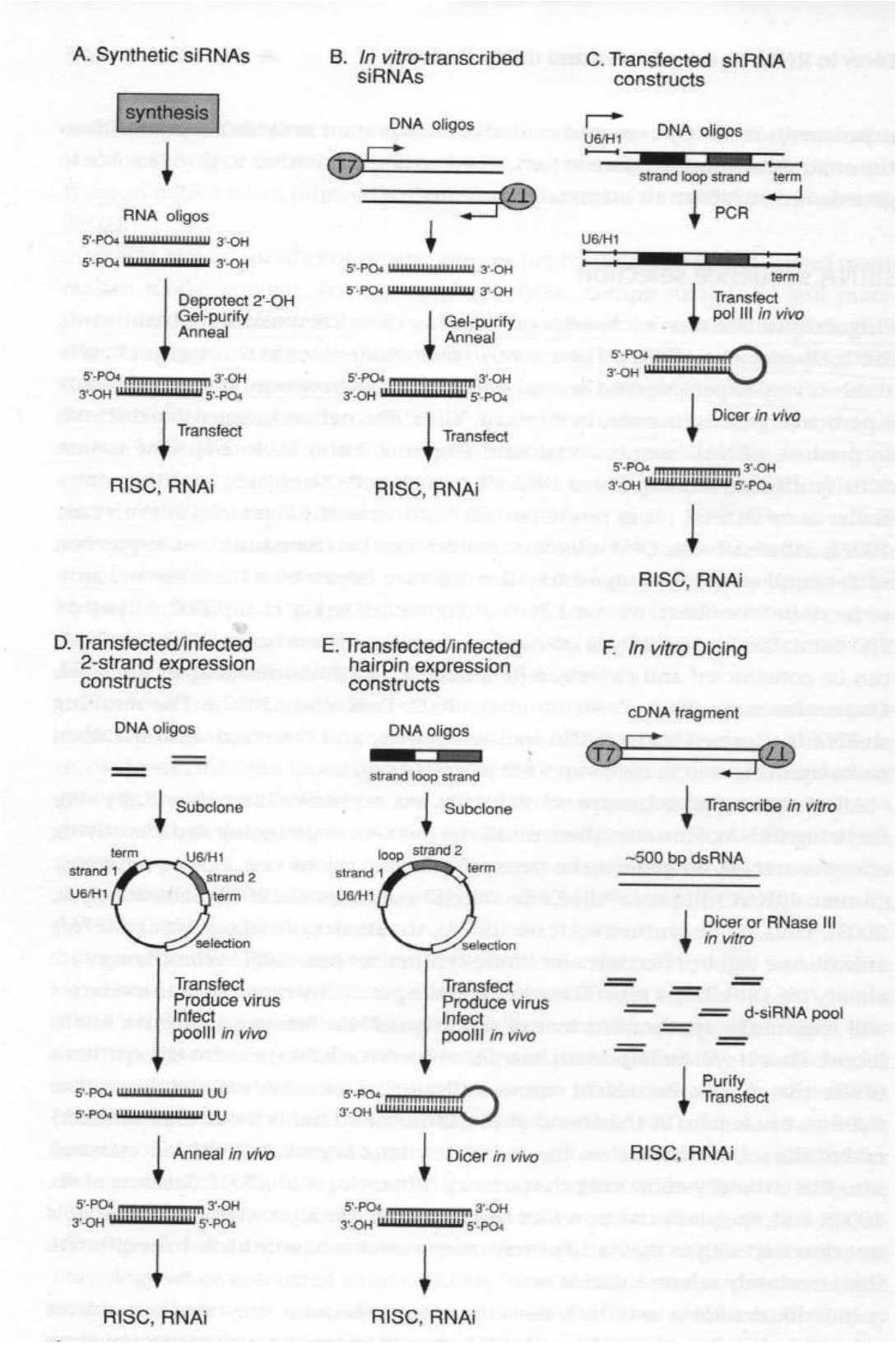

**Figure 1.7:** Strategies for siRNA production. siRNAs can be either generated *in vitro* and then introduced into cells (**A**,**B** or **F**), or produced *in vivo* from transcription of a DNA template (**C**, **D** or **E**). **C** and **E**: siRNAs derived from hairpin precursors (Appasani, 2005).

Chemical modifications (Figure 1.8.), such as incorporation of a modified sugar moiety (eg. 2'-*O*-methylation of the ribose), modifications to the duplex structure, overhangs and termini (eg. deoxy units), linkage modifications (eg. phosphorothioate bonds) and base modifications have been shown to improve siRNA stability in serum, increase potency, reduce immunostimulatory properties and diminish hybridization-dependent off-target effects (Watts *et al.*, 2008; Yadava, 2007). Conjugation to molecules, such as cholesterol have also aided in improving the overall pharmacokinetics of siRNAs for medical application (Yadava, 2007). Although chemical modifications have been shown to increase stability of siRNAs in serum, they fail to solve both the problem of poor pharmacokinetics and the lack of tissue specificity associated with these molecules (Cardoso *et al.*, 2008). Hence development of effective delivery systems or vectors is an imperative and on-going quest for successful siRNA therapy (Han *et al.*, 2008).

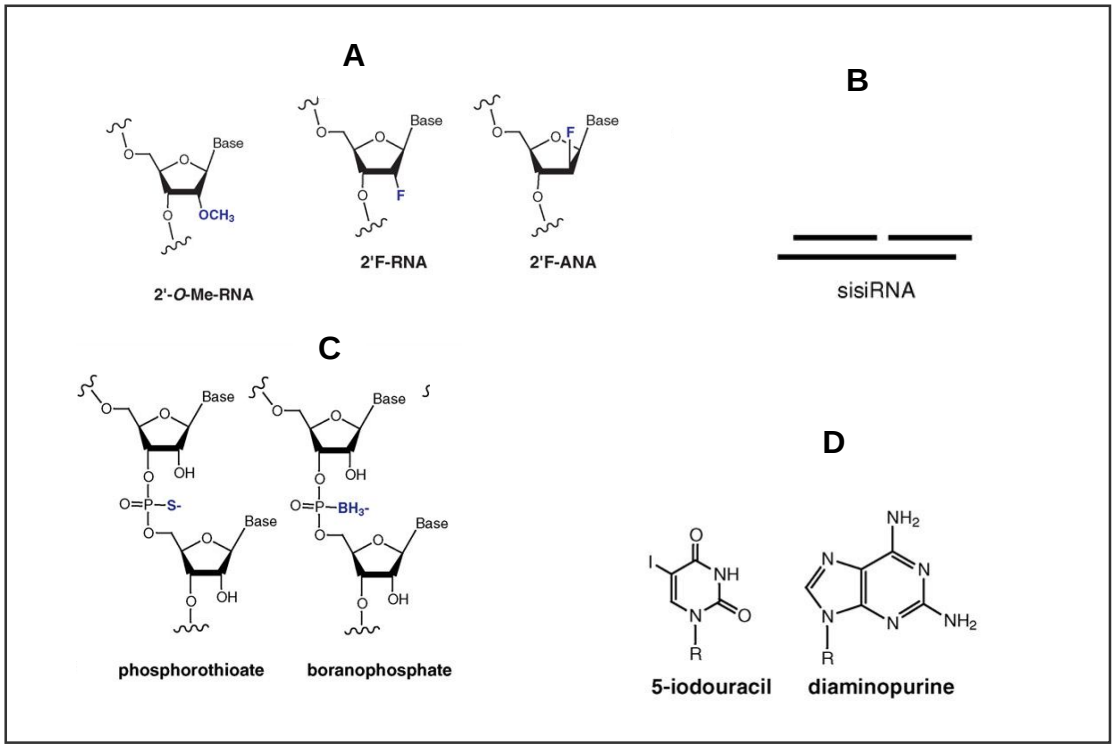

**Figure 1.8:** Examples of different types of chemical modifications applied in siRNA synthesis, where **A**: sugar modifications (2'-*O*-alkyl and other 2' modification), **B**: structural modifications (sisiRNA: small internally segmented interfering RNA), **C**: internucleotide linkages (s=sulphur, $BH_3$=borane), and **D**: nucleobase modifications (R= ribose phosphate backbone) (Modified from Watts *et al.*, 2008).

## 1.7 siRNA Delivery Systems and Vectors

The anionic nature and large size of nucleic acids necessitates involvement of delivery systems or vehicles for therapeutics (Lasic, 1997). A delivery vector refers to a 'bearer' or 'carrier', and in the context of gene delivery, is a means of transporting therapeutic nucleic acids from the site of administration to the site of action (Mēvel *et al.*, 2009). Delivery could be local (target organ), systemic (bloodstream) or targeted (systemic designed for intended target) (Seto, 2010). An ideal vector should be easy to produce and reproducible, biodegradable, non-toxic, non-immunogenic, stable (storage and administration), demonstrate efficient gene expression and possess cell-specific targeting ability (Guo *et al.*, 2010; Huang *et al.*, 1999; Li and Ma, 2001; Reischl *et al.*, 2008). Most vectors can be classified into 1 of 2 groups, either: viral or non-viral. Both have been utilized for siRNA or shRNA delivery and have intrinsic advantages and disadvantages (Mēvel *et al.*, 2009).

### 1.7.1 Viral Vectors

Viruses are naturally evolved vehicles which efficiently transfer their genes into host cells, making them attractive delivery vectors for transport of therapeutic genes into humans (Huang *et al.*, 1999; Walther, 2000; Yadava, 2007). They can be genetically engineered to be less or non-virulent, with all or part of their viral sequence replaced by therapeutic genes (Li and Ma, 2001; Singh *et al.*, 2009). Viral vectors are highly efficient gene delivery vehicles, owing to their superior cell adhesion, membrane translocation and transcriptional and translational abilities (Cristiano, 1998; Yadava, 2007). In addition, their ability to transduce both dividing and non-dividing cells, stably integrate exogenous DNA or RNA into the host genome- yielding prolonged expression of therapeutic genes, low dose requirement, amenability to pseudotyping and high specificity are among the main reasons why viral vectors are employed in

numerous gene therapy /RNAi experiments and clinical trials (Singh *et al*., 2006; Singh *et al.*, 2009).

Adenoviruses, Adeno-Associated viruses (AAV), Lentiviruses and Retroviruses are the most widely utilized viral vectors in RNAi therapeutics (Lee *et al.*, 2009; Singh *et al.*, 2009). Each virus type has its advantages and disadvantages (Table 1.4.). Viral delivery in RNAi is usually associated with shRNAs as most viruses are unable to carry synthetic chemical analogs (siRNAs) (Grimm, 2009; Oliveira *et al*., 2006; Yadava, 2007). Adenoviruses are icosahedral, non-enveloped DNA viruses (Huang *et al.*, 1999; Majhen and Ambriovic-Ristov, 2006), with the ability to transduce dividing and non-dividing cells. However, low packaging capacity (approximately 7.5 kb), no genome integration and immunogenicity have hindered their application (Singh *et al.*, 2009). AAV are non-pathogenic viruses belonging to the *Parvoviridae* class. They are regarded as the safest viral vector due to lack of associated malignancy and immune response (Huang *et al.*, 1999; Singh *et al.*, 2009). They have been extensively utilized in shRNA delivery. In 2007 Li *et al* engineered AAV-1, -2 and -5 serotypes to express shRNA against *Hec1* (cancer-associated gene) in central nervous system (CNS) tumor cells. Doege *et al* used AAV vectors for expression of shRNA against the hepatic fatty acid transport protein-5 (FATP-5). Both investigations demonstrated efficient and persistent target gene knockdown (Grimm, 2009).

Lentiviral vectors have also been successfully employed for shRNA delivery in various mammalian systems. Carlson *et al* (2008) delivered a *Smad3*-target shRNA for satellite cell regeneration (Singh *et al.*, 2009); while An *et al* knocked down rhesus-CCRS (HIV chemokine receptor) in non-human primates (Nyugen *et al.*, 2008). Down regulation of Ras oncogene in mice have also been reported (Brummelkamp *et al.*, 2002). The most widely used lentiviral vectors are HIV-based. Major safety concerns are associated with this type of vector (Singh *et al.*, 2009). Retroviruses have been used to express shRNAs by pol II/III vectors, but random integration has impeded application (Singh *et al.*, 2009). Herpes viruses, baculoviruses and virosomes (empty influenza envelops) are also being investigated as potential vectors for shRNA delivery (Nyugen *et al.*, 2008).

**Table 1.4:** Advantages and disadvantages of different types of viruses as nucleic acid delivery vectors (Modified from Mountain, 2000; Singh *et al.*, 2009).

| Viral Vector | Advantages | Disadvantages |
|---|---|---|
| **AdenoVirus** | Transfects dividing and non-dividing cells<br>High transfection efficiency (*in vitro* & *in vivo*)<br>Extensive clinical use | Transgene size limit of 7.5 kb<br>Immunogenicity<br>No genome integration i.e. short-lived gene expression<br>Production and storage difficulty |
| **Adeno-associated Virus** | Wide tissue range<br>Transfects dividing and non-dividing cells<br>No associated immune response or malignancy<br>High transfection efficiency | Transgene size limit of 4.5 kb<br>Random genome integration i.e. mutagenesis<br>Little clinical experience<br>Difficult to manufacture |
| **Lentivirus** | Transfects dividing and non-dividing cells<br>Convenient to manufacture | Limited cell range<br>Immunogenicity |
| **Retrovirus** | Host genome integration i.e. long-lasting gene expression<br>Low immunogenicity<br>High transfection efficiency (*ex vivo*)<br>Extensive clinical use | Transfects dividing cells only<br>Transgene size limit of 8 kb<br>Random genome integration i.e. mutagenesis<br>Restricted host range<br>Low transfection efficiency *in vivo* |

Despite the success and reduced risks associated with new generation viral vectors (Castanotto *et al.*, 2009; Nyugen *et al.*, 2008), possible immunogenicity, toxicity, carcinogenicity, insertional mutagenesis, aberrant gene induction and infection due to pathogenic reversion (Chaudhuri, 2002; Lasic, 1997; Walther, 2000) are still major limitations to their therapeutic application. These safety concerns have not yet been resolved for human application (Takahashi *et al.*, 2009).

### 1.7.2 Non-Viral Systems and Vectors

Non-viral delivery systems are attractive alternatives to their viral counter-parts. Ease of preparation, low cost, low toxicity, reduced risk of immunogenicity, high stability, flexible nucleic acid loading capacity, amenability to modifications and ability for cell-specific targeting are among the many reasons why they are regarded as more attractive vectors for clinical application (Han *et al.*, 2008; Huang *et al.*, 1999; Li *et al.,* 2008; Mēvel *et al.*, 2010; Oliveira *et al.*, 2006; Zhang *et al.*, 2010). They encompass a wide array of molecules and techniques, broadly divided into two categories: naked nucleic acid delivery by a physical method or complexation/precipitation with a carrier molecule (Nishikawa *et al.*, 2001). Generally, chemical or colloidal in nature (Lasic, 1997; Singh, 1998), there are 3 major classes of non-viral delivery vectors: synthetic polymers, biodegradable/natural polymers and lipids (Rao *et al.*, 2009). Low transfection efficiency and transient gene expression were features of early non-viral delivery systems, however recent advancements in vector technology have yielded molecules and techniques with transfection efficiencies comparable with those of viruses (Yadava, 2007). Currently, various siRNA delivery vectors are being developed and investigated, including polymers, peptides and liposomes (Kim *et al.*, 2010). Some non-viral delivery systems shall be discussed below.

#### 1.7.2.1 Electroporation, Nucleofection and Hydrodynamic Injection

Electroporation, first used for gene delivery by Zimmerman *et al*. (1976), involves the application of short bursts of high voltage electric pulses to cells, inducing transient membrane breakdown and allowing entrance of nucleic acids into the cytoplasm (Nishikawa *et al.*, 2001, Singh, 1998). It has been successfully employed for siRNA delivery, example: local delivery into the hippocampal region demonstrating efficient knockdown of the COX1 gene and reduced levels of metabotropic glutamate receptor-2 (mGluR2) in the brain (Akaneya *et al.*, 2005; Singh *et al.*, 2009). The simplicity and reproducibility of electroporation have been overshadowed by its lack of *in vivo* applicability i.e. cell damage at pulse-applied sites and inconsistent expression in assorted tissue-types makes large-scale implementation difficult (Nishikawa *et al.*, 2001; Oh *et al*., 2009; Singh, 1998).

Nucleofection is an advancement of electroporation. It employs less harmful electric pulses and specialized solutions for cell-specific delivery (Gresch *et al.*, 2004). Although effective, Electroporation and Nucleofection are fairly toxic to several cell types (Lee *et al.*, 2009).

Hydrodynamic injection technique involves intravascular injection of a large amount of physiological buffer containing naked siRNA. It was the first technique used to demonstrate siRNA induced RNAi in mammalian cells. Advantages include relative simplicity, low cost, relatively high efficacy and low dose requirement. Transient toxicity, rapid nucleic acid degradation and the need for organ-specific vasculature are the three major limitations for therapeutics (Lewis and Wolff, 2005; Lewis and Wolff, 2007).

#### 1.7.2.2 Polymers and Peptides

Polymers utilized for gene delivery can be either natural or synthetic and are generally cationic in nature. The complexes formed between nucleic acids and cationic polymers, via electrostatic interaction, are referred to as 'polyplexes' (Felgner *et al.*, 1997; Huang *et al.*, 1999). Examples of polymers used in *in vivo* siRNA delivery include: atellocollagen, chitosan, polyethylene glycol (PEG),

polyethyleneimine (PEI) and cyclodextrin (De Fougerolles, 2008; Singh *et al*, 2009; Zhang *et al.*, 2007). Although very efficient, toxicity (example: PEI) has been a deterrent for delivery purposes (Yadava, 2007).

In general, cationic proteins and peptides are used to form complexes with anionic nucleic acids (De Fougerolles, 2008). Membrane penetrating peptides (MPPs), characterised by presence of highly basic amino acids such as arginine and lysine, have been extensively investigated as siRNA delivery vectors. Low cost, ease of preparation and non-toxic effects are advantages (De Fougerolles, 2008; Hwang et al., 2008). Observed immunogenicity and tendency of positively charged MPPs to precipitate out are limiting factors (Watts *et al.*, 2008).

#### 1.7.2.3 Liposomes

Liposomes are vesicular colloidal particles composed of self-assembled amphiphilic molecules in an aqueous environment (Lasic, 1997). They were first described by Bangham *et al* in 1965; and owing to their resemblance to biological membranes, were originally used to model membrane systems in molecular biology studies. Recognition of their carrier potential resulted in their use in the fields of drug and gene delivery (Singh, 1998; Huang *et al*., 1999). Liposomes consist of one or more concentric lipid bilayers alternating with aqueous compartments, within which a variety of lipid- or water-soluble substances can be entrapped (Bangham *et al*.,1972; Singh *et al*., 2006). Although composition depends largely on the goals of the experiment, liposomes are generally comprised of phospholipids, charged amphiphiles and sterols (Kinsky, 1974). There are four different types of liposomes, defined on the basis of functionality (Figure 1.9.) (Huang *et al*., 1999; Lasic, 1997). Conventional or classical liposomes were the first to be synthesized. They were composed of neutral or anionic lipids such as phosphatidylethanolamine (zwitterion) or phosphatidylserine (anionic). They encapsulated nucleic acid material within their aqueous compartments. However, variable encapsulation efficiencies, relatively small packaging capacity and prolonged processing-time, meant that they were

deemed inefficient for gene delivery, particularly for *in vivo* application (Szelei and Duda, 1989; Singh, 1998).

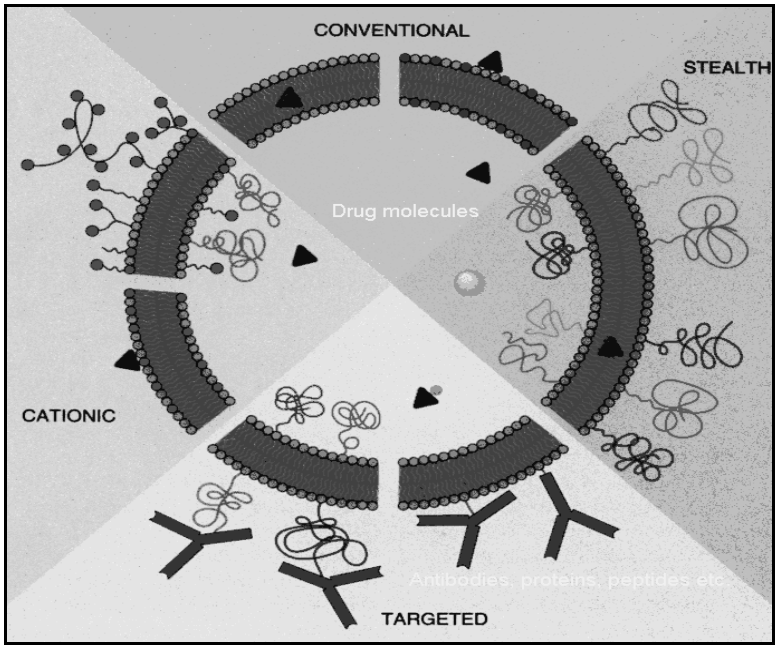


**Figure 1.9:** Illustration of the four different classes of liposomes (conventional, cationic, targeted and stealth) as defined by functionality (Lasic, 1997); where (●) refers to positively charged moieties, (**Y**) targeting ligands and (〰) polymer coatings (Lasic, 1997).

The introduction of cationic lipids into liposome preparation allowed for the first truly efficient liposomal gene delivery system *(to be discussed)* (Lasic, 1997). Targeted and stealth liposomes are variations of conventional and cationic liposomes. Targeted liposomes contain targeting moieties, such as antibodies, lectins, or oligosaccharides, directed towards cognate receptors/sites on specific cells and tissues. They addressed a major limitation of gene delivery systems i.e. lack of cell specificity. Stealth liposomes carry a polymer coating that increases their circulation time by preventing liposomal cell adhesion and protein opsinization (Lasic, 1997; Singh *et al*., 2006). Liposomes exhibit many of the characteristics required of an ideal delivery vector including their ease of production, amenability to scale-up production, relatively low cost and safety (Huang *et al.*, 1999; Lasic, 1997; Percot *et al*, 2004).

Liposomal gene transfer can occur through one of four mechanisms (figure 1.10.), namely: (i) Adsorption with extracellular release, (ii) Adsorption with lipid exchange, (iii) Endocytosis, and (iv) fusion of vesicle with cell wall. The mechanism used is determined by liposome characteristics, such as size, charge, composition, targeting ligand; and cell type (Torchilin, 2003). It was originally hypothesized that liposomal gene transfer occurred primarily as a result of direct fusion between the plasma membrane and the nucleic acid-liposome complex (termed 'lipoplex') (Felgner *et al*., 1987), but that was later disputed (Zhou *et al*., 1994). Rather, it is mainly through the process of endocytosis that lipoplexes entered the cell (Pinnaduage *et al*., 1989). Escape of lipoplexes from the endosome is considered a critical factor for efficient therapeutics. Several mechanisms have been proposed (Figure 1.11.).

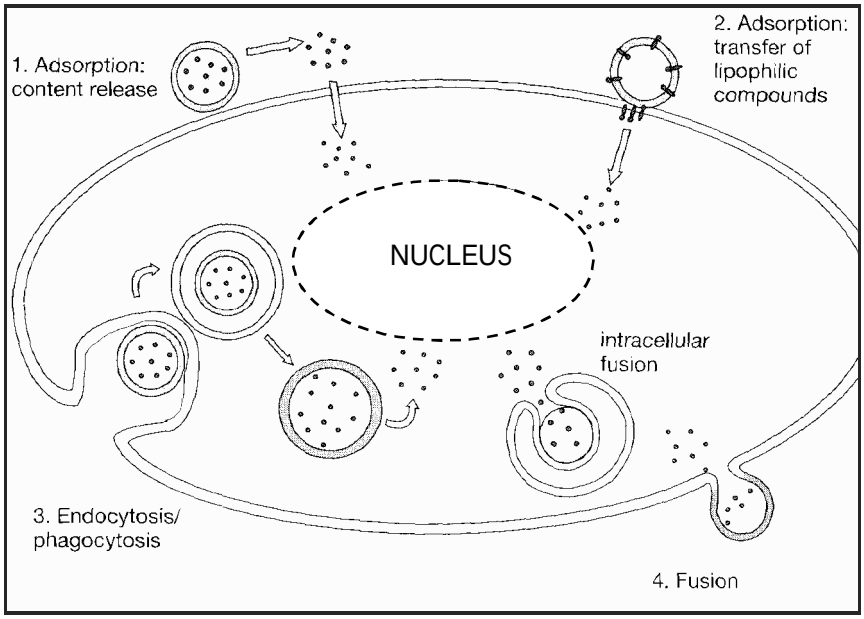


**Figure 1.10:** The four mechanisms of liposome-cell interaction by which liposomes deliver their cargo (Modified from Torchilin, 2003).

In one mechanism proposed by Xu and Szoka, cationic lipids may form ion-pairs with the anionic lipids of the endosome resulting in destabilization of the endosome membrane by exclusion of surface bound water (Tseng *et al.*, 2009). The 'proton sponge effect' is another very effective method via which siRNA escapes endosomal degradation. Here, the buffering capacity of molecules contained within the

liposome can cause the influx of $Cl^-$ molecules resulting in swelling and rupture of the endosome (Tseng *et al.*, 2009).

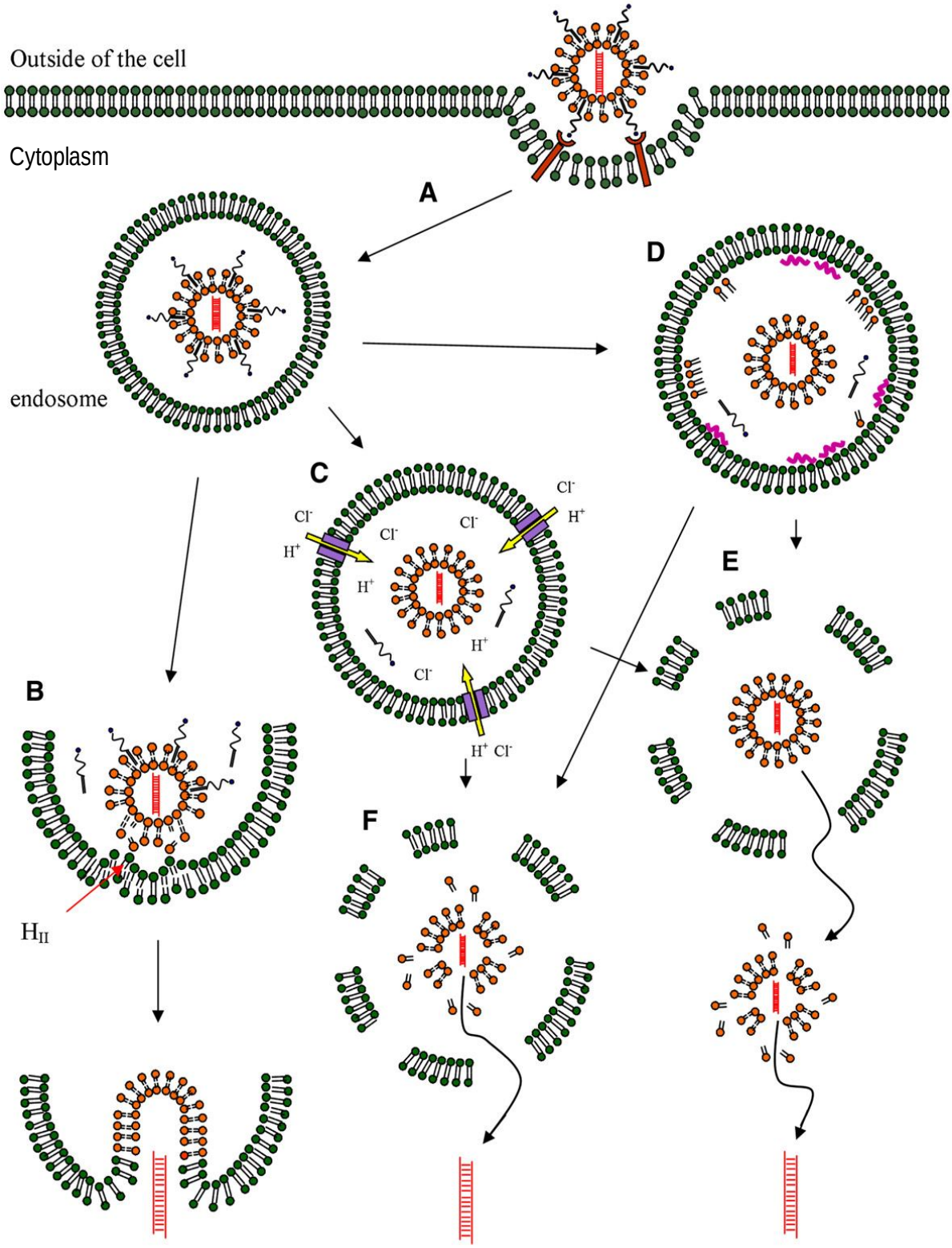


**Figure 1.11:** Endosomal escape in lipoplex-mediated siRNA delivery (Tseng *et al*, 2009). **A**: Endosome containing siRNA lipoplex; **B**: Ion-pair formation; **C:** Proton

sponge effect; **D**: Free highly positive charged molecules; **E**: Ruptured endosome and cytoplasmic de-assemblage of siRNA; and **F**: SiRNA de-assemblage in endosome prior to release from 'holes' in ruptured endosome (Tseng *et al.*, 2009).

**1.7.2.4 Cationic Liposomes**

The apparent high toxicity associated with cationic lipids limited their exploration and use in early liposomal and gene delivery research (Lasic, 1997). It was only in 1987 when Felgner *et al* synthesized a mono-cationic lipid, N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTMA), that the first evidence of their efficient transfection ability with low toxicity was demonstrated (Bhattacharya *et al.*, 1998; Lasic, 1997; Felgner *et al.*, 1987; Singh, 1998). This resulted in the development and synthesis of a plethora of other cationic lipids for use in gene delivery (Figure 1.12.) (Lasic, 1997; Singh, 1998).

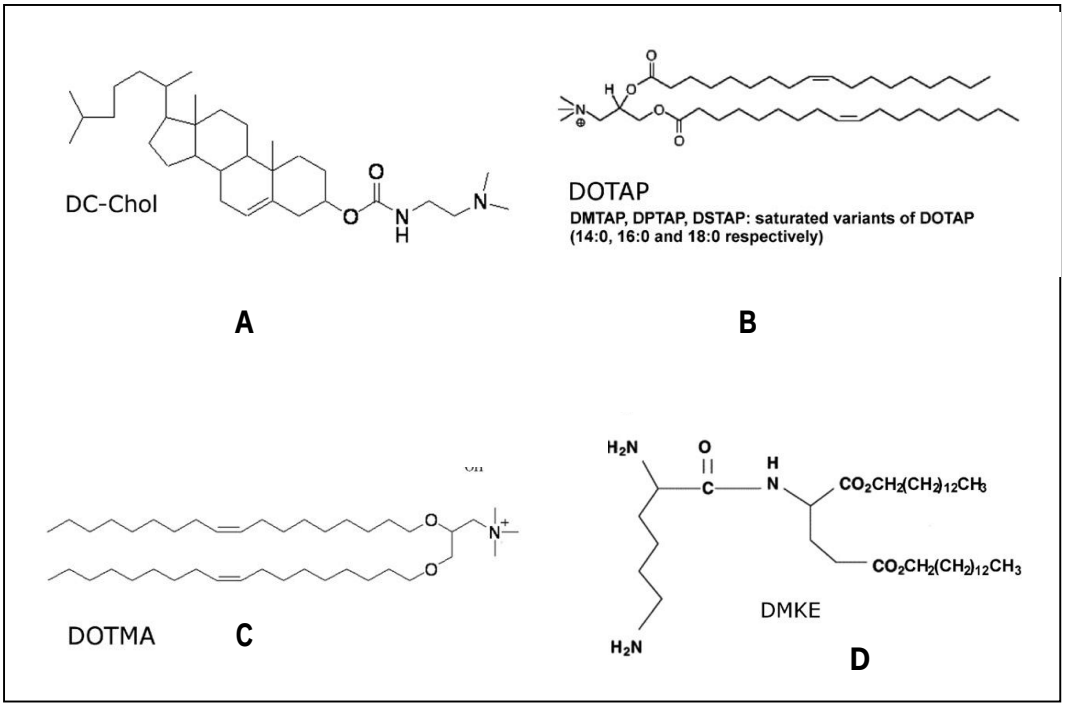


**Figure 1.12:** Structures of four common cationic lipids, **A:** 3β-[N-(N',N'-dimethylaminoethane)- carbamoyl] cholesterol hydrochoride (DC-Chol); **B:** 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) ; **C:** DOTMA and **D:** O,O'-dimystristyl-N-lysyl aspartate (DMKE) (Lonez *et al.*, 2008).

Cationic liposomes are generally composed of a cationic lipid in combination with a neutral co-lipid (Lasic, 1997; Reischl *et al.*, 2008). Commonly used co-lipids include 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-Dioleolyphophatidyl-ethanolamine (Lasic, 1997; Guo *et al.*, 2010). Cationic liposomes carry a net positive charge over their surface allowing for spontaneous complexation with anionic nucleic acids such as siRNAs, via electrostatic interaction between the negatively charged phosphate backbone of the nucleic acid and the positively charged amine head group of the cationic lipid (Behr, 1986; Reischl *et al.*, 2008). They offer a number of advantages as gene delivery vectors, including ease of preparation, limitless nucleic acid loading capacity, relatively high transfection ability due to increased binding affinity with negatively charged cell membranes, and significantly reduce therapeutic dose concentrations (Bhattacharya *et al.*, 1998; Guo *et al.*, 2010; Rao, 2009; Rhinn *et al.*, 2009).

Cationic liposome-mediated siRNA delivery has been successfully utilized by several groups for *in vitro* and *in vivo* gene knockdown (Guo *et al.*, 2010). It was demonstrated that a cationic lipid, 2-(3-[bis-(3-amino-propyl)-amino]-propylamino)-N-ditetradecylcarbomoyl-thyl-acetamide (RPR209120), combined with DOPE, efficiently delivered siRNAs to mammalian cells in culture, silencing tumor necrosis factor α (TNF-α) in collagen induced arthritis (Khoury *et al.*, 2006; Zhang *et al.*, 2007). In 2006, Santel and collegues synthesized a stealth cationic liposome composed of the cationic lipid, β-L-arginyl-2,3-L-diaminopropionicacid-N-palmityl-N-oleylamide trihydrochloride (AtuFECT01) in combination with a fusogenic lipid (1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine) and PEG. It was used to deliver siRNA to the vascular endothelium of mice, targeting CD31 and Tie2 genes. Reduction of target protein levels were observed in the vascular endothelium of the heart, lung and liver (Santel *et al.*, 2006; Oh *et al.*, 2009). Recently, a cationic lipid (DODAG 8) synthesized and complexed to anti-Hepatitis B Virus (HBV) siRNAs, demonstrated efficient delivery and partial treatment of HBV infection in transgenic mice (Mēvel *et al.*, 2010).

Although cationic lipid/liposome based systems have demonstrated their potential as siRNA delivery vectors, several hurdles remain for therapeutic application (Oh *et al.*, 2009). Toxicity, rapid clearance from circulation and lack of tissue specificity are associated problems (Lasic, 1997; Li and Ma, 2001; Singh, 1998). Researchers are aiming to solve these problems. One solution for non-specific cellular uptake involves the attachment of a targeting moiety like transferrin, directed to a cognate site on a target cell.

### 1.8 Targeted siRNA-Lipoplex Delivery

Targeted gene delivery refers to the transport of nucleic acids to pre-defined cells and tissues for a specific therapeutic response (Burkhanov *et al*., 1988). Lack of cell or tissue specificity limits the *in* vivo application of liposomes and other non-viral vectors, as intravenous injection of siRNA delivery systems commonly results in accumulation or removal by the lung or liver, respectively (Hashida *et al*., 2000; Qi *et al*., 2005, Zhu *et al*., 1993). Targeting can be either 'passive' or 'active'. Passive targeting refers to the natural localisation pattern of administered liposomes (Singh, 1998). Active targeting involves either conjugation of a targeting moiety to the therapeutic molecule or encapsulation/binding by a 'nanoparticle' that contains a targeting component. It directs and enhances specific cellular uptake of siRNAs and other molecules (Seto *et al.*, 2010; Singh, 1998). Targeted delivery generally utilizes specific and unique characteristics of particular cells to deliver therapeutic nucleic acids to the correct locale for effective genetic treatment (Singh, 1998).

Examples of early gene targeting were accomplished by Wu and co-workers, an asialoglycoprotein coupled to poly-L-lysine for hepatocyte targeting (Wu and Wu, 1988); and Wagner and colleagues, a polycationic-transferrin conjugate targeted to cancer cells (Wagner *et al.*, 1990). Among the different methods of liposome targeting, attachment of a ligand to the vector surface is the most effective method of delivery to a selective site (Huang, 1999). Commonly utilized targeting ligands include monoclonal antibodies, peptides, glycoproteins or receptor ligands (Lasic,

1997). When receptor ligands are incorporated into gene delivery systems, receptor mediated endocytosis is responsible for their cellular uptake.

### 1.8.1 Receptor Mediated Endocytosis for Targeted siRNA Delivery

Receptor mediated endocytosis (RME) is a multi-step cellular mechanism for uptake of macromolecules, such as nutrients (LDL), growth factors (epidermal growth factor) and hormone (insulin), from the extracellular environment (Smythe and Warren, 1991; Wolfe, 1995). It is an attractive mechanism for use in cell-specific gene delivery. The basic principle of receptor mediated gene delivery is to subvert RMEs efficient transport system so as to allow nucleic acids (siRNA/shRNA) complexed to ligands entrance into the target cell (Phillips, 1995). The actual RME mechanism (figure 1.13) involves binding of a ligand to specific membrane-spanning cognate receptors on the cell surface (Smythe and Warren, 1991). This leads to a conformational change in the receptor and results in clustering of receptor-ligand complexes within clathrin coated pits. Clathrin forms lattices on the plasma membrane, which undergo growth and rearrangement, resulting in invagination and scission of these pits to generate a coated endocytic vesicle. The vesicle is rapidly uncoated to form the early endosome (Phillips, 1995; Smythe and Warren, 1991; Wolfe, 1995). Within the endosome, ligands and receptors dissociate (acidic pH) and are sorted to their appropriate intracellular destinations (example: golgi apparatus or nucleus) (Smythe and Warren, 1991; Templeton, 2008). Receptors may be degraded (lysosome) or recycled back to the cell surface for further rounds of uptake. The late endosome fuses with lysosomes, leading to the degradation of ingested material, with, in some cases, release of the digested products into the extracellular fluid (Wolfe, 1995; Singh, 1998; Smythe and Warren, 1991).

Incorporation of a targeting ligand into non-viral vector constructs such as liposomes has proven an effective method for gene delivery into target cells (Singh, 1998). An important factor for successful therapeutics is the escape of siRNA or other nucleic

acids from the endosome prior to lysosome fusion, so as to avoid the degradation step (Rao, 2010).

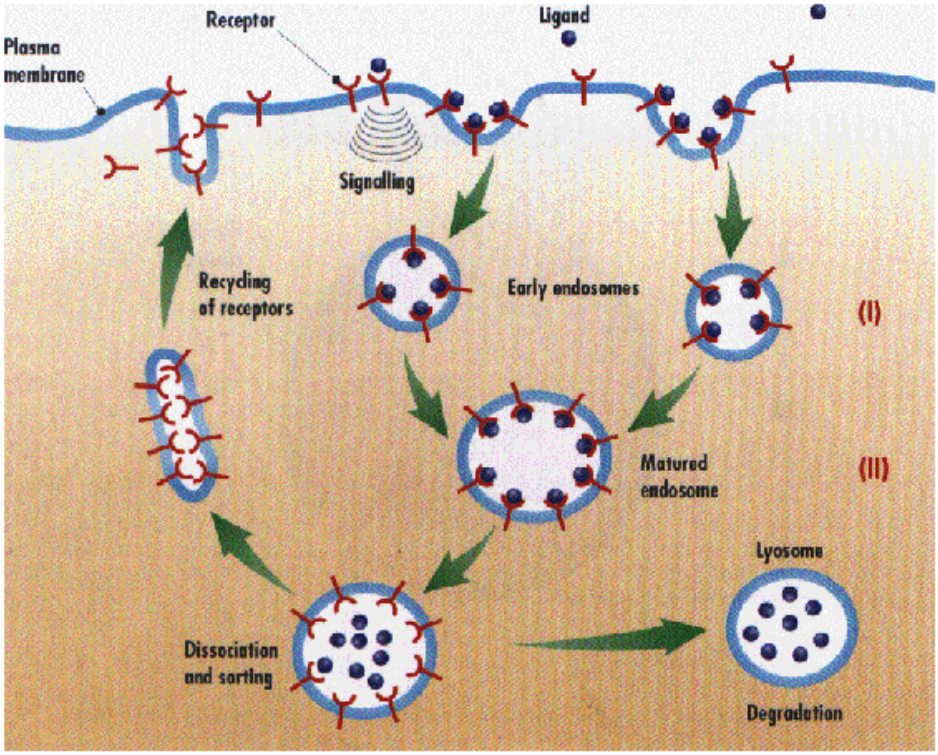


**Figure 1.13:** Mechanism of Receptor-Mediated Endocytosis (www.srs.dl.ac.uk)

### 1.8.2 Hepatocyte-Targeted siRNA Delivery

The liver is an attractive target tissue for an evolving technology such as RNAi therapeutics due to its large size, metabolic capacity, rich blood supply, active filter function and possession of large numbers of high affinity cell-surface receptors that can bind asialogylcoproteins (Kim *et al.*, 2007; Nguyen *et al.*, 2008). The asialogylcoprotein receptors on mammalian hepatocytes are known to be present at a density of 500 000 receptors per cell, exhibit high ligand affinity, rapid internalization rates, with retainment on several human hepatoma cell lines (Kim *et al.*, 2007). Hepatocytes produce a large number of serum proteins and hold great appeal for siRNA therapy to correct defects, such as metastatic hepatocarcinomas, Hepatitis C virus (HCV) and HBV infections (Wu *et al.*, 2002; Nguyen *et al.*, 2008). It

was shown *in vitro* and *in* vivo that hepatocytes, bearing on their surface asialoglycoprotein receptors, exhibited increased uptake of liposomes modified with ligands containing a terminal galactose residue (Hoekstra *et al*., 1978; Spanjer and Sherphof, 1983).

### 1.9 Outline of Thesis

siRNAs are emerging as new generation 'bio-drugs' due to their specific and potent RNAi triggering capabilities. However, siRNAs are anionic molecules that require transport across the cell membrane hence a major obstacle for their use in human therapeutics is efficient and specific cell delivery. Consequently carrier development is critical for its widespread clinical application (Oh *et* al., 2009; Singh *et al.*, 2009). In this thesis we have investigated the *in vitro* delivery of hepatocyte-targeted cationic liposomes complexed to synthetic siRNA. The liver is a particularly attractive target for RNAi therapeutics. Composed of one of the most physiologically active cells in the body (i.e. hepatocytes) and a site for synthesis of a wide range of serum proteins, the liver demonstrates a predisposition to a variety of genetic and metabolic diseases. Additionally, hepatocytes exclusively express large numbers of the high affinity asialoglycoprotein (ASGP) receptors that recognise and bind non-reducing termini of heteroglycans (Kawakami *et al*., 1998; Kim *et al.*, 2007).

Thus two previously synthesized cationic lipids, 3β [N-(N',N'-dimethylaminopropane)- carbamoyl] cholesterol (Chol-T) and 3β [N-(N',N',N'-trimethylammonium propane iodide)- carbamoyl] cholesterol (Chol-Q), were incorporated into hepatocyte-targeted cationic liposomes for investigation of their *in vitro* siRNA delivery efficacy to the hepatocyte-derived human cell line, HepG2. Targeting was achieved by incorporation of the (β)-D-galactopyranoside ligand into the structure of the cationic liposomes. Untargeted cationic liposomes were also prepared for comparison. In chapter two the preparation of the liposomal components is described. In addition, the chapter also details the manufacture and characterization of the cationic liposomes. Characterization was achieved by cryo transmission electron microscopy (TEM). Chapter three examines siRNA-liposome

interaction by means of agarose gel electrophoretic retardation and the SYBR®green displacement assay. siRNA lipoplex characterization (via microscopy) and stability in 10% foetal bovine serum (FBS) was also explored in this chapter. Chapter four focuses on the evaluation of siRNA lipoplex cytotoxicity and transfection efficiency as elucidated by delivery to transformed cells, ASGP-$R^+$ HepG2 and ASGP-$R^-$ HEK293 , in culture.

The main aim of this study was to design, synthesize and evaluate novel cationic liposomes as siRNA delivery vehicles targeted towards hepatoma cells in culture. The use of the (β)-D-galactopyranoside ligand was envisioned to provide specific uptake by hepatocytes, hence providing a more efficient siRNA delivery vehicle. This type of specific cellular targeting may have significant applications *in vivo*.

# Chapter Two : Liposome Preparation and Characterisation

## 2.1 Introduction

This chapter focuses on the preparation of four cationic liposomes, containing either the synthesized ternary cationic cholesterol derivative, Chol-T or the quaternary cationic cholesterol derivative, Chol-Q, with and without targeting elements. All liposomes were characterised by transmission electron microscopy.

Liposomes are synthetic analogues of natural membranes and can be defined as spherical particles with one or more lipid membranes (Figure 2.1) enclosing part of the aqueous environment (Lasic and Papahadjopoulos, 1998). Liposome composition depends largely on the intended use, but generally consists of a phospholipid, a sterol and charged amphiphiles in varying ratios (Kinsky, 1974). Amphiphiles are molecules that contain a hydrophilic head group and a hydrophobic tail. Upon dispersion in an aqueous solution, these molecules self-assemble into ordered structures, such as lipid bilayers, which subsequently self-close to form liposomes, due to the unfavourable energy of the hydrophobic edges located adjacent to the water (Lasic, 1997; Singh, 1998). Phospholipids available for use in liposome synthesis are usually biodegradable and reusable. These include: phospholipids from natural sources, modified natural phospholipids, phospholipids with non-natural head groups, semi-synthetic and fully synthetic phospholipids (Chapman, 1983; Singh, 1998).

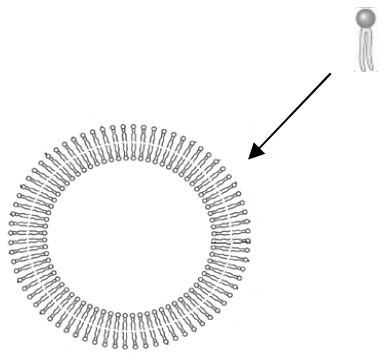

**Figure 2.1:** Schematic representation of a typical liposome lipid bilayer structure.

Liposomes are characterised by their lipid composition, particle size distribution, number of lamellae and inner/outer aqueous phases (Lasic and Papahadjopoulos, 1998). Liposomes, morphologically, range from small (S), large (L) and giant (G) unilamellar vesicles (UV) to multilamellar vesicles (MLV) (Lasic, 1997). Structurally, liposomes vary from long tubules to oval or spherical vesicles in shape (Singh, 1998). Liposome size and structure is dependent on the method of preparation employed. MLVs are the simplest liposomes to prepare. They are composed of multiple concentric lamellae separated by aqueous layers and range in diameter size from nanometers (nm) to micrometers (µm). Small unilamella vesicles (SUVs) are formed from sonication of MLVs and have a diameter range of 20-200 nm (Lasic, 1997; Lui *et al*., 2009). SUVs have previously displayed a propensity to aggregate and attach to larger structures due to their highly curved nature (Lui *et al*., 2009). Both MLVs and SUVs have low encapsulation efficiencies and are thus considered unsuitable for nucleic acid or drug delivery (Singh, 1998). LUVs range in size from 500 nm to several microns (Chapman, 1984; Lui *et al*., 2009). GUVs normally have diameters above 1 µm (Lasic, 1997). Due to their large size, GUVs are sensitive to the slightest change in osmotic pressure, resulting in undulation or rupture of the vesicle (Lui *et al*., 2009).

Most cationic liposomes consist of two lipid species, namely: a positively charged lipid and a neutral co-lipid (Lasic, 1997). Examples of commonly utilized cationic and neutral lipids can be seen in Figures 2.2 and 2.3, respectively. The majority of positive charges of cationic lipids are based on (poly)amines and quaternary ammonium salts (Lasic, 1997). All cationic lipids have four functional domains, namely: one or more positively charged head groups, a spacer, a linker bond and a hydrophobic anchor. The role of the hydrophobic anchor is to ensure assembly into a lipid bilayer vesicle upon dispersion in an aqueous medium. The hydrophobic anchor is usually a cholesterol derivative or a double hydrocarbon chain (Lonez *et al.*, 2008). The positively charged head group (s) is the nucleic acid binding moiety of the lipid (Huang *et al*., 1999; Lasic, 1997; Singh, 2005). The linker bond acts as the bridge

between the cationic head group and the hydrophobic anchor; it determines lipid stability and biodegradability. Various chemical linkers have been utilized in lipid synthesis. These include: amide, amine, carbamate, ester, ether, urea and peptide bonds (Huang *et al*., 1999; Singh, 1998).

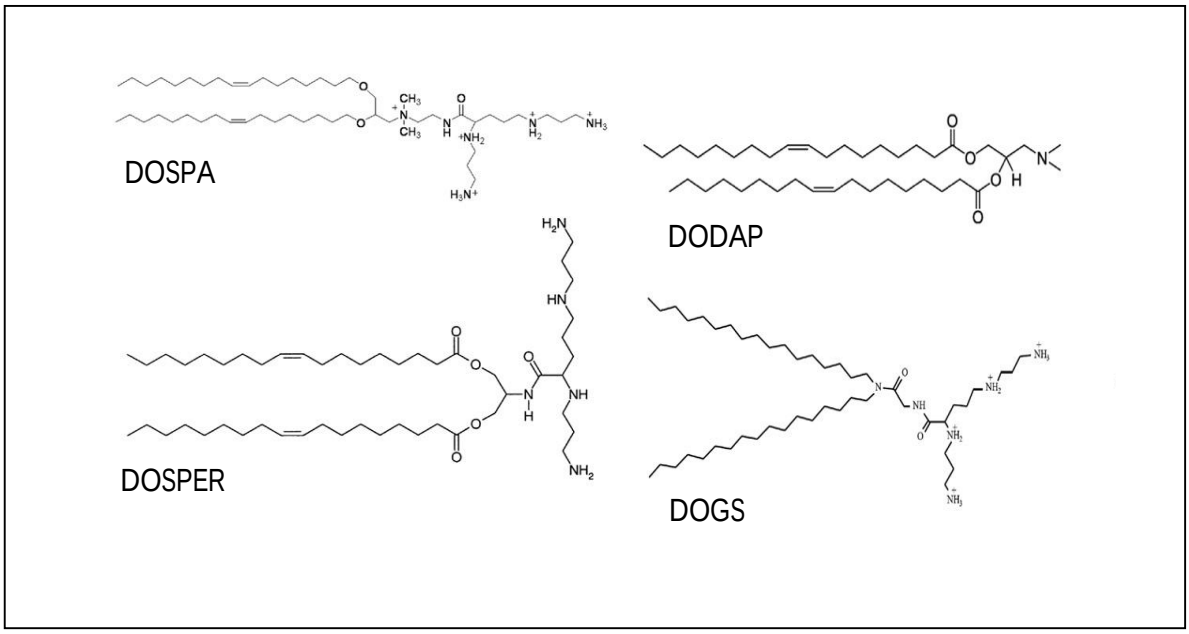


**Figure 2.2:** Structures of four cationic lipids used in liposome formulations. DOSPA: 1,3-dioleoyloxy-*N*-[2-(s-oxy[ethyl-2-heptadecenyl-3 hydroxyethyl] imidazolinium chloride; DOSPER: 1,3-dioleoyloxy-2-(6-carboxyspermyl)-propylamide; DOTAP: 1,2-dioleoyl-3-dimethylammoniumpropane; DOGS: dioctadecylamidoglycylspermine (Lasic, 1997; Lonez *et al*., 2008).

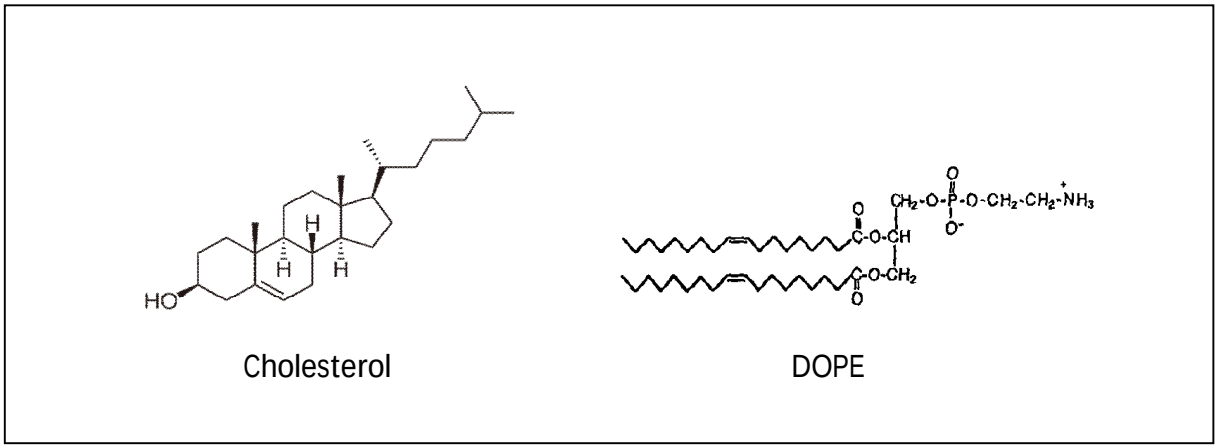

**Figure 2.3:** Examples of neutral lipids used in cationic liposome formulations. DOPE: dioleoylphosphatidylethanolamine; (Lasic, 1997; Mēvel *et al*., 2010).

An important parameter in liposome formation is the rigidity of the bilayer. Hydrated single component phospholipid bilayers are characteristically in a liquid crystalline (fluid) or gel state. Phospholipids are characterised by their phase transition temperature ($T_c$), which allows transition of membranes from a fluid to a gel state. Bilayer $T_c$ depends on the acyl chain length, polar head group and degree of saturation. Increased acyl chain lengths and saturation result in an increased $T_c$ value (Lasic, 1997; Crommelin and Schreier, 1999 ; Singh, 1998).

Liposomes can be synthesized through a variety of methods (Table 2.1.). These preparation techniques are broadly classified into several categories according to the basic mode of dispersion, example: mechanical dispersion, solvent dispersion and detergent solubilization (Gregoriadis, 2007). The simplest method for liposome formation is the shaking or film method. This is a mechanical method, originally used by Bangham *et al*, that produces liposomes by shaking hydrated thin-lipid films above the $T_c$, yielding MLVs of a heterogenous population size (Bangham *et al*., 1965). Another mechanical method employs sonication of aqueous phospholipid dispersions, yielding SUVs. It can be performed via two different approaches. The first employs a sonication probe placed directly into the liposome suspension, while the second utilizes a sonication bath. Although the latter is a slower method, it decreases contamination of the liposomes (Crommelin and Schreier, 1999; Maguire et al., 2003).

During the reverse-phase evaporation method, formation of a reverse-phase emulsion (water-in-oil dispersion) is followed by evaporation of the organic phase. This results in a gel consistency of aqueous vesicles bounded by a phospholipid monolayer. Mechanical agitation ruptures some vesicles, with the released phospholipid acting as the outer monolayer, converting other vesicles into LUVs (Kim *et al*., 1993; Philippot, 1995). Homogeneous populations and high encapsulation efficiencies are the two major advantages of this technique (Szoka *et al*., 1980).

**Table 2.1:** Various techniques for Liposome synthesis

| | METHOD | SIZE | REFERENCE |
|---|---|---|---|
| 1. | Hand-shaken preparation | MLV | Bangham *et al*., 1965 |
| 2. | Sonication | SUVs | Johnson *et al*., 1971 |
| 3. | Extrusion by Filters | SUVs/LUVs | Lasic, 1997 |
| 4. | Homogenisation | SUVs/MLVS | Lasic, 1997 |
| 5. | Ether Injection | LUVs | Deamer and Bangham, 1976 |
| 6. | Ethanol Injection | SUVs | Batzi and Korn, 1973; Campbell, 1995 |
| 7. | Detergent Depletion | SUVs | Torchilin, 2003 |
| 8. | Reverse-Phase Evaporation | LUVs | Szoka and Papahadjopoulos, 1978 |
| 9. | Thin lipid Film Hydration | SUVs/LUVs | Gao and Huang, 1991 |
| 10. | Calcium-induced Fusion | LUVs | Papahadjopoulos and Vail, 1978 |
| 11. | Micro-fluidization | MLVs/LUVs | Mayhew *et al*., 1984 |

The extrusion technique employs filters of various pore sizes (0.2 – 500 nm) through which lipid dispersions are passed. It yields the best vesicles with respect to homogenously sized populations (Lasic, 1997). It can be employed in combination with other techniques. A variation of this technique is freeze-thaw extrusion, where liposomes formed by the film method are vortexed with the solute to be entrapped until the entire film is suspended. The resulting vesicles are frozen in a dry ice/ acetone bath and thawed in warm water, prior to vortexing. This cycle is repeated

multiple times (Chapman *et al.,* 1991). The choice of liposome preparation technique depends on the purpose, availability of equipment and liposome size required. It is important to note that some of the above mentioned techniques cannot be employed for cationic liposome synthesis. These include: detergent depletion, ether and ethanol injection (Lasic, 1997).

The aim of this chapter was to present the preparation of targeted (Figure 2.4) and untargeted cationic liposomes comprised of: 3β [N-(N',N'-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) or 3β [N-(N', N', N'-trimethylammoniumpropyl)-carbamoyl] cholesterol iodide (Chol-Q) and DOPE, with and without cholesteryl β-D-galactopyranoside ligands. The liposomes, once synthesized, were subjected to transmission electron microscopy for size and lamellar characterization.

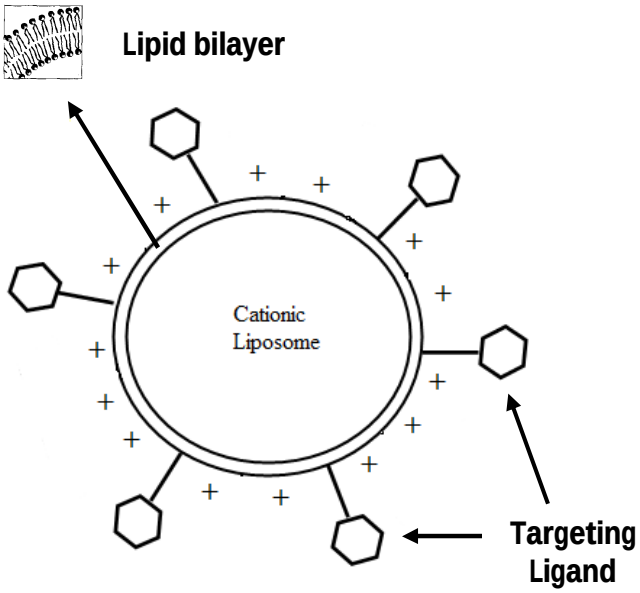


**Figure 2.4:** Illustration of the cationic liposome with targeting ligands

## 2.2 Materials

Dioleoylphosphatidylethanolamine (DOPE) was purchased from the Sigma Chemical Company, St Louis, USA. Cholesteryl chloroformate, 2-[4-(2-hedoxyethyl)-1-piperazinyl] ethanesulphonic acid (HEPES), 3-dimethylaminopropylamine, silica gel beads (60 µm) and silica gel $F_{254}$ chromatography plates were purchased from Merck, Damstadt, Germany. All other chemicals were of analytical grade.

## 2.3 Methods

### 2.3.1 Preparation of Cationic Cholesterol Derivatives

The cholesterol derivatives (Chol-T and Chol-Q) were previously synthesized in our laboratory as follows:

#### 2.3.1.1 Chol-T Synthesis

3-dimethylaminopropylamine (62.8 µl, 0.11 µmoles) was added to a solution of cholesteryl chloroformate (90 mg, 0.2 µmoles) in 1 ml dichloromethane. The synthesis reaction (Figure 2.5 a) was allowed to proceed at room temperature for one hour and was monitored by thin layer chromatography (TLC) in a chloroform : methanol (95 : 5 v/v) solvent system (A) (result not shown). The solvent (dichloromethane and excess 3-dimethylaminopropylamine) was subsequently removed by rotary evaporation in a Büchi Rotavapor-R. The resulting residue was dissolved in absolute ethanol and allowed to crystallise overnight at 4°C. The product was subsequently recrystallised, filtered under a stream of dry nitrogen gas and further dried by rotary evaporation to yield whitish coloured crystals.

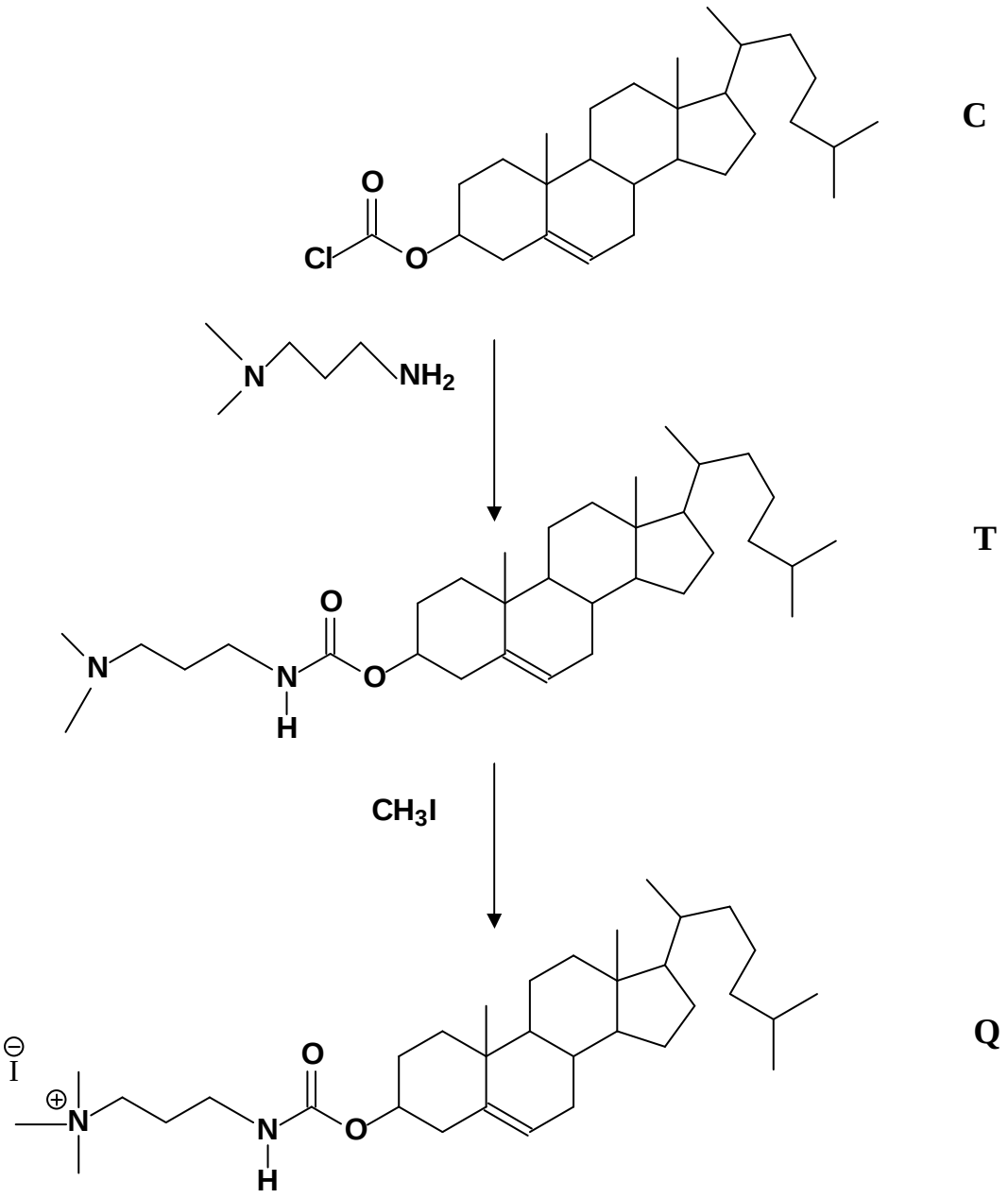
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**Figure 2.5:** Synthesis scheme for the cholesterol derivatives, Chol-T and Chol-Q, where **C=** cholesteryl chloroformate, **T=** Chol-T, 3β [N-(N',N'-dimethylaminopropane)-carbamoyl] cholesterol, and **Q=** Chol-Q, 3β [N-(N',N', N'-trimethylammonium propane iodide)-carbamoyl] cholesterol iodide.

#### 2.3.1.2 Chol-Q Synthesis

Methyl iodide (1.5 molar excess) was added to an ether solution of Chol-T (Figure 2.5 b). The reaction was then centrifuged at 3000rpm for 5 minutes in an MSE bench top centrifuge. The resulting pellet was suspended in dry ether (10 ml) and re-centrifuged as before. The pellet was then dried under vacuum at room temperature, prior to re-crystallization from hot ethanol, yielding a crystalline product. The reaction was also monitored by TLC in solvent system A (result not shown). Both Chol-T and Chol-Q were subjected to melting point determination and to 250 MHz proton spectrometry (Figure 1 and 2, Appendix A).

### 2.3.2 Isolation and De-acetylation of Per-acetylated Cholesteryl-β-D-Galactopyranoside

The isolation and de-acetylation of per-acetylated cholesteryl β-galactose was previously performed in our laboratory as follows:

The reaction mixture from Koenigs Knorr synthesis of per-acetylated cholesteryl-β-D-galactopyranoside was purified via column chromatography, yielding per-acetylated cholesteryl-β-D-galactopyranoside. The column (257 mm x 26 mm) was packed with a slurry of silica gel beads (60 nm) in a hexane : ethyl acetate (8 : 3 v/v), solvent system (B). The reaction mixture was added to the column and collected upon elution in fractions over a period of approximately 3 hours. Fraction samples were monitored by TLC in solvent system B (result not shown). Fractions containing the per-acetylated cholesteryl-β-D-galactopyranoside were pooled, prior to evaporation of the solvent using a Büchi Rotavapor-R.

The per-acetylated cholesteryl-β-D-galactopyranoside (75.8 mg, 0.105 µmoles) was then dissolved in anhydrous chloroform (7.58 ml), followed by the addition of sodium ethoxide (1.05 µmoles). The de-acetylation reaction was allowed to proceed for 24 hours at room temperature and was monitored by TLC in a formic acid : ethyl acetate : chloroform (1 : 5 : 4 v/v/v), solvent system (C) (result not shown). The product yield was quantitative.

### 2.3.3 Preparation of Cationic Liposomes

The cationic liposomes were prepared by a method modified from that employed by Goa and Huang (1991). The relative quantities of the components of the cationic liposomes were as set out in Table 2.2.

**Table 2.2:** Lipid composition of cationic liposomes, where β-GAL[1] represents cholesteryl-(β)-D-galactopyranoside

| CATIONIC LIPOSOME PREPARATION | MOLAR RATIOS (µmoles) | | | | MASS (mg) | | | |
|---|---|---|---|---|---|---|---|---|
| | CHOL-T | CHOL-Q | DOPE | β-GAL[1] | CHOL-T | CHOL-Q | DOPE | β-GAL[1] |
| SD-CholT Control (SD-CholTC) | 2 | - | 2 | - | 1.029 | - | 1.488 | - |
| SD-CholT Targeted (SD-CholTT) | 2 | - | 2 | 0.5 | 1.029 | - | 1.488 | 0.280 |
| SD-CholQ Control (SD-CholQC) | - | 2 | 2 | - | - | 1.310 | 1.488 | - |
| SD-CholQ Targeted (SD-CholQT) | - | 2 | 2 | 0.5 | - | 1.310 | 1.488 | 0.280 |

Briefly, the components of each cationic liposome preparation were deposited as a thin film on the inside of test tubes using a Büchi Rotavapor-R. The thin film was

then dried for 1 hour in a drying pistol, prior to rehydration overnight in 1 ml of sterile HEPES buffered saline (20 mM HEPES and 150 mM NaCl, pH 7.5). The mixtures were vortexed, sonicated for 1 minute and left for 24 hours at 4°C. The mixtures were subsequently sonicated for a further 5 minutes to produce unilamellar liposomes. Each liposome preparation contained a total of 4 µmoles of lipid. Liposomes were stored at 4°C and sonicated prior to use.

### 2.3.4 Characterization of Liposomes by Transmission Electron Microscopy (TEM)

Liposomes were diluted (1:5) with HEPES buffered saline. Thereafter, 1 µl of liposome solution was added to formvar (polyvinyl formal)-coated grids, followed by addition of 1 µl of 1% uranyl acetate. The grids were immediately blotted with filter paper to remove excess moisture and subsequently vitrified by plunging into liquid ethane using a Leica CPC system. Grids were then transferred to a Gatan cryotransfer system and viewed using a JEOL-1010 transmission electron microscope, without warming above -150°C.

## 2.4 Results and Discussion

### 2.4.1 Preparation of Cationic Cholesterol Derivatives

Both cationic cholesterol derivatives, Chol-T and Chol-Q, were previously successfully synthesized by Singh (1998). Chol-T had a melting range of 103-105°C, and Chol-Q had a melting range of 168-170°C when subjected to melting point determination. $^{1}$H-NMR spectroscopy of the derivatives (Figure 1 and 2, Appendix A) showed singlets at δ 2.19 and δ 3.4 which were characteristic of tertiary methyl amino and quaternary methylammonium headgroups respectively (Singh, 1998).

Chol-T: δ 2.19 (singlet, 6H, $(\underline{CH_3})_2NCH_2CH_2$);
δ 2.30 (triplet, 2H, $(CH_3)_2N\underline{CH_2}CH_2CH_2NH$);
δ 3.21 (quartet, 2H, $(CH_3)_2NCH_2CH_2\underline{CH_2}NH$);
δ 5.35 (doublet, 1H, $(CH_3)_2NCH_2CH_2CH_2N\underline{H}$)

Chol-Q: δ3.30 (quartet, 2H, $(CH_3)_2NCH_2CH_2\underline{CH_2}NH$);
δ 3.40 (singlet, 9H, $(\underline{CH_3})_3NCH_2CH_2$);
δ 3.75 (triplet, 2H, $(CH_3)_2N\underline{CH_2}CH_2CH_2NH$);
δ 5.34 (doublet, 1H, $(CH_3)_2NCH_2CH_2CH_2N\underline{H}$)

**Figure 2.6:** The $^{1}$H-NMR spectral data of Chol-T and Chol-Q are taken from Singh (1998).

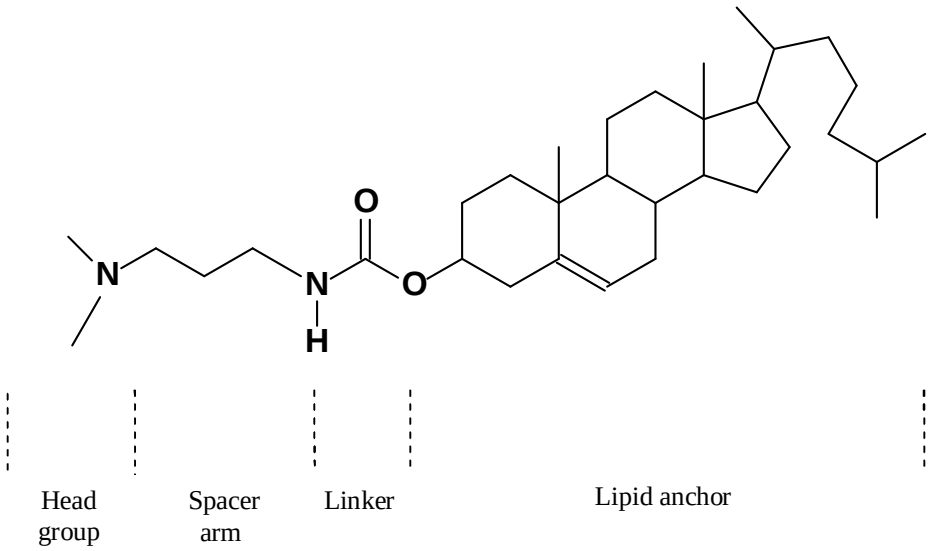
Chol-T (Figure 2.6 a) and Chol-Q (Figure 2.6 b) both have a cholesterol ring structure, a carbamoyl linker bond and a spacer arm. They differ in the head group, with Chol-T possessing a monovalent dimethylamino head group and Chol-Q, a monovalent

trimethylammonium head group. Cationic lipids can broadly be divided into two classes, namely: (i) those that have cholesterol as their lipid anchor and (ii) those that utilize diacyl chains (Huang, 1999). Cholesterol anchors generally lead to more active cytofectins than their diacyl counterparts (Lee *et al*., 1996). The cholesterol anchor provides rigidity to the liposome structure, as well as influencing membrane fluidity.

It has been reported that when a carbamoyl unit is employed as the linker bond, the lipid has increased degradability and therefore is potentially less toxic to the target cells both *in vitro* and *in vivo* (Goa and Hui, 2001). The relatively labile carbamoyl bond is more stable and not as easily hydrolysed as ester bonds, but once inside the cell it is quite easily degraded by cellular esterases (Lasic and Papahadjopoulos, 1998; Lee *et al.*, 2005). The length of the spacer arm is purported to play a role in the interaction between the charged head groups and the nucleic acid. A longer spacer arm is said to result in decreased steric hindrance between the polar head group and the hydrophobic anchor (Singh, 1998). A spacer arm consisting of between three and six carbon atoms between the amino group and the linker bond was also reported to provide the optimal distance for efficient gene delivery activity (Goa and Hui, 2001).

The positively charged head group of a cationic lipid is the most important domain in determining the overall efficiency of gene delivery characteristics for the particular cationic lipid (Goa and Hui, 2001). It also plays an important role in determining cytotoxicity of the liposome (Huang *et al*., 1999). Multivalent head groups, although more active than their monovalent counterparts such as Chol-T and Chol-Q, can render the cationic liposome more water-soluble, leading to a greater propensity to form micelles, which in turn may lead to less stable, more toxic liposomes (Huang *et al*., 1999). Lipids bearing linear amines or polyamines as the positively charged head-group have demonstrated good gene delivery activity (Goa and Hui, 2001).

**(a)**


O
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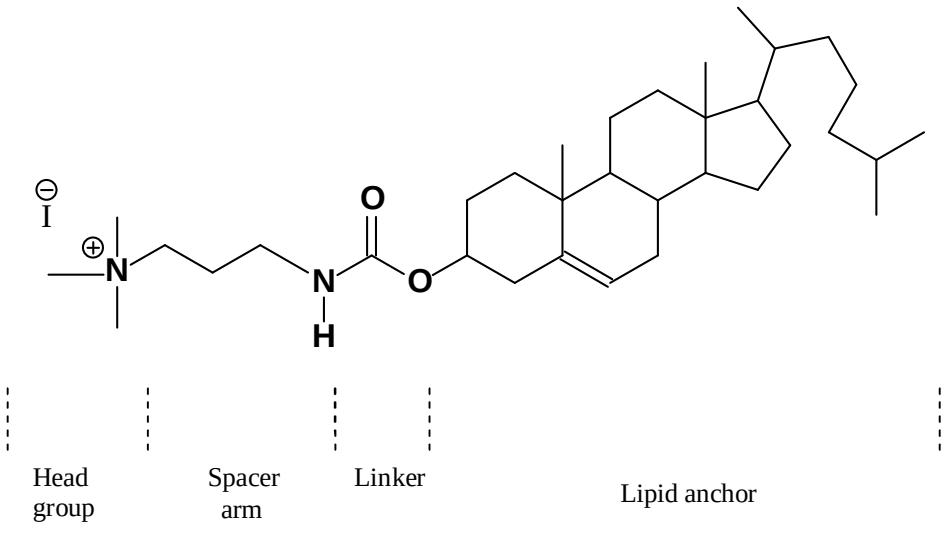
Head group
Spacer arm
Linker
Lipid anchor

**(b)**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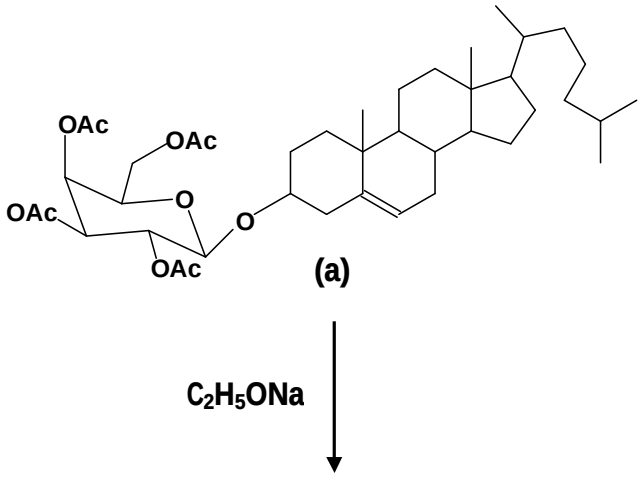
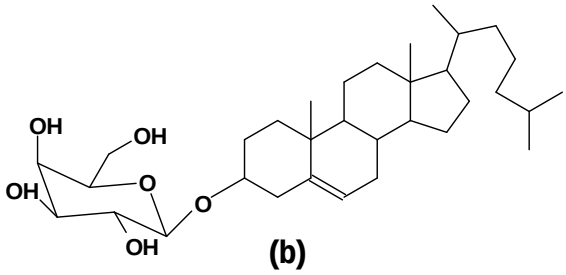
Lipid anchor

**Figure 2.7:** Structures of **(a)** Chol-T and **(b)** Chol-Q showing the four basic domains and differences in head group.

### 2.4.2 Isolation and De-acetylation of Per-acetylated Cholesteryl-β-D-Galactopyranoside

The isolation and de-acetylation of per-acetylated cholesteryl-β-D-galactopyranoside was previously successfully performed in our laboratory. Fractions collected from column chromatography on silica gel 60 were subjected to TLC, prior to pooling of all per-acetylated cholesteryl-β-D-galactopyranoside containing fractions. A confirmation test was then performed, where the per-acetylated cholesteryl (β)-D-galactopyranoside was run on a TLC plate against a standard, as well as the starting reaction mixture. All TLC plates were sprayed with 10% sulphuric acid to aid in visualization via colour development. Acid causes dehydration of sterols and free sugar resulting in a pink/purple and brown/black colour, respectively. The acetate protecting groups were then removed by treatment with an excess of sodium ethoxide, an alkoxide salt (Figure 2.8). Successful de-acetylation was demonstrated by running the reaction product against acetylated and non-acetylated standards on TLC plate with the same treatment as before.






**(b)**

**Figure 2.8:** Reaction scheme showing the de-acetylation of per-acetylated cholesteryl-β-D-galactopyranoside, where **(a)** represents the per-acetylated cholesteryl-β-D-galactopyranoside and **(b)** cholesteryl-β-D-galactopyranoside.

Different methods exist for attachment of ligands to liposomes i.e. covalent, non-covalent or incorporation via a hydrophobic anchor (Philippot, 1995). The latter method was used in this investigation. The asialoglycoprotein receptor on mammalian hepatocytes specifically recognises ligands with terminal glucose, galactose or N-acetylgalactosamine residues (Wu *et al*., 2002). Research previously performed in our laboratory by Singh *et al*. (2007), determined that the β-anomer of galactose (Figure 2.8) demonstrates superior transfection ability as compared with the α-anomer, as well as α-and β-glucose residues. Thus it was chosen as the targeting element in our liposome formulations.

### 2.4.3 Preparation of Cationic Liposomes

Cationic liposomes were successfully prepared using the method described. This was demonstrated by transmission electron microscopy (2.3.4) and positive electrostatic interaction with siRNA (chapter three). The control cationic liposomes were prepared using an equimolar amount of either Chol-T or Chol-Q and DOPE. Their targeted counterparts contained an additional 11 mole % cholesteryl-β-D-galactopyranoside. The synthesis method (lipid film hydration/sonication) used, proved simple and efficient.

The 1 : 1 molar ratio of cytofectin to co-lipid was chosen because it was previously employed with great success in our laboratory (Singh, 1998). Research done by Farhood *et al*. (1995) and Maitani *et al*. (2007) also suggested that either a 3 : 2 or

1 : 1 ratio of a similar cytofectin (DC-chol) with DOPE demonstrated high transfection activity; while Zhang *et al*. (2010) demonstrated that a DC-chol/DOPE molar ratio of 1 : 1 showed the highest transfection efficiency with regard to siRNA delivery. The control liposomes were synthesized to demonstrate that increased transfection efficiency of HepG2 cells (target cell line) occurred through receptor mediated endocytosis via the asialoorsomucoid receptor.

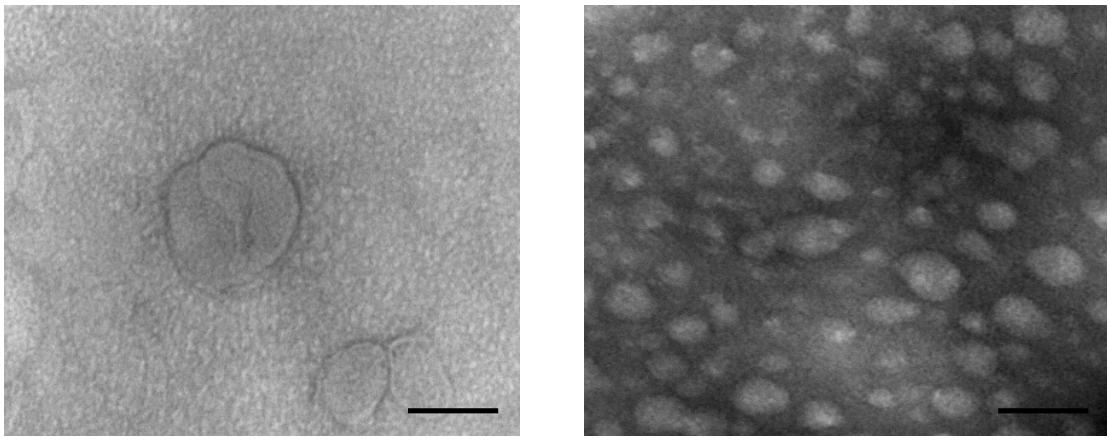
DOPE, a neutral zwitterionic phospholipid, is the most prevalent co-lipid used in liposome formulations due to its ability to increase transfection efficiency and reduced cytotoxicity of the cationic surfactant (Hoekstra *et al*., 2007; Sternberg *et al*., 1994). DOPE has an inverse-conical molecular shape that is wide at the termini of the dioleoyl fatty acid side chains and narrow at the polar head group (Mēvel *et al*., 2010) (Figure 2.3). It is this molecular shape that facilitates liposomal cellular interaction and endosomal escape. This fusogenic lipid has a tendency to form a hexagonal $H_{II}$ phase, thus moving the liposomal membrane away from lamellar order and destabilising it, which is critical for fusion of the liposomal and endosomal membranes (Hoekstra *et al*., 2007; Lasic, 1997; Mēvel *et al*., 2010; Singh, 1998; Zuhorn *et al*., 2005).

### 2.4.4 Characterization of Liposomes by Transmission Electron Microscopy (TEM)

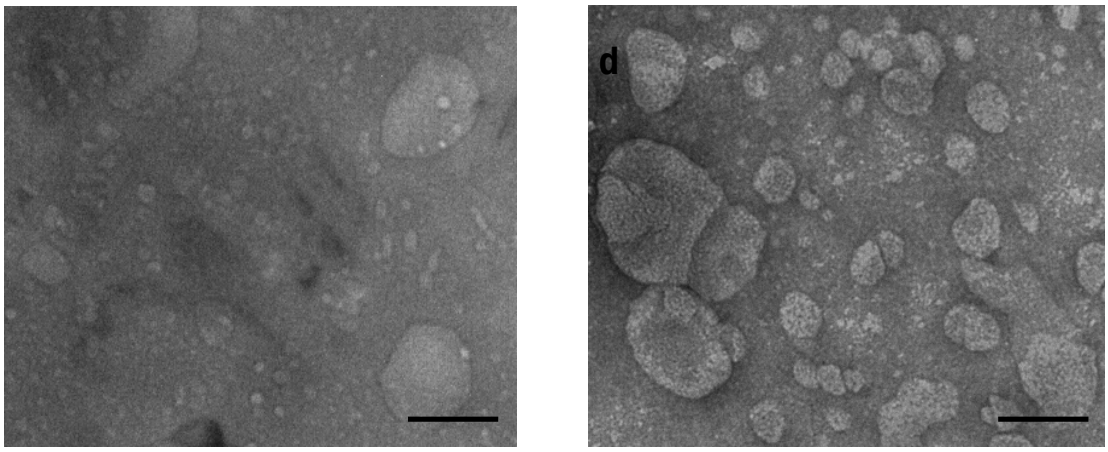
Transmission electron microscopy revealed the unilamellar nature and sizes of the different cationic liposomes (Figure 2.9). A heterogeneous population size was obtained for all liposome preparations. The majority of liposomes ranged from 80 nm to 200 nm in size, although liposomes up to ±400 nm were also observed. The untargeted liposomes containing Chol-T were, on average, slightly smaller (± 30 - 50 nm) than those containing the Chol-Q derivative. This is in accordance with results obtained by Singh *et al*. (1998) during microscopy studies of liposomes with the same molar ratios and lipid composition. No significant size difference was observed for the targeted and untargeted liposomes. All liposome preparations were generally spherical in nature, however liposomes of a deformable nature were also observed.

Note that any artefacts seen in the TEM micrographs can be attributed to the cryoTEM process.

Size is an important factor for liposome circulation time and internal accumulation (Düzgünes, 2004; Lee *et al*., 2005). Generally, smaller liposomes (<100 nm) are slowly eliminated from the blood as compared with larger liposomes, which are rapidly taken up by Kupffer cells (Düzgünes, 2004). Studies performed by Lui *et al*. (2003) showed that liposomes less than 70 nm in diameter were predominantly taken up by the liver (parenchymal cells), while those that were greater then 200 nm were preferentially taken up by the spleen. Vesicle size characterization can be performed using a variety of techniques, including: dynamic light scattering (DLS), gel exclusion chromatography, specific turbidity, sedimentation field-flow fractionation, coulter counter, light diffraction and electron microscopy (EM) (Korgel *et al*., 1998; Lasic, 1997). DLS and EM are the most widely employed of the above techniques. While EM generally provides an evaluation of liposome lamellarity and size, DLS provides better information on the size distribution of the liposomal population (Lasic, 1997).



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




**(c)** **(d)**

**Figure 2.9:** Transmission electron microscopy with negative staining, of the four liposome preparations. Bar = 100 nm; **(a)** SD-CholTC, **(b)** SD-CholTT, **(c)** SD-CholQC and **(d)** SD-CholQT.

Negative staining, freeze-fracture and cryoelectron microscopy are among the most widely employed EM techniques (Lasic, 1997). Negative staining is prone to artefacts as a result of vesicle dehydration, while the unknown plane during freeze-fracture is a hindrance. Cryoelectron microscopy, although more reliable, suffers from poor visualization due to a lack of contrast between the liposome and the background. Hence, during this investigation negative staining in combination with cyroTEM was employed. The problem of vesicle dehydration was eliminated by testing a range of negative stains at various incubation periods. Results obtained indicated that 1% uranyl acetate, with immediate blotting, demonstrated little or no dehydration of liposomal vesicles.

In this chapter, cationic lipids were successfully synthesized and formulated into liposomes containing the Cholesteryl-β-D-galactopyranoside targeting ligand. Cationic liposome characterisation by cryoTEM revealed a generally homogeneous size population in all four preparations.

# Chapter Three: siRNA-Cationic Liposome Interaction and Characterisation

## 3.1 Introduction

The understanding of siRNA-cationic liposome interaction is vital for the optimisation of transfection efficiency with the overall aim of achieving clinical application as a genetic therapy. This chapter focuses on the interaction between the cationic liposomes synthesized as described in chapter two and siRNA. It evaluates and details the characterisation, binding and protection efficiency of the resultant siRNA lipoplexes.

The term lipoplex, first coined by Felgner *et al*. (1987) was initially, exclusively used to describe the complexes formed between cationic liposomes and DNA. With the growth of gene therapy and the advent of RNAi, this concept was expanded to include Oligodeoxynucleotides (ODNs), miRNAs and siRNAs. Hence, a more concise definition of the term lipoplex is: the complexes formed between cationic liposomes

and nucleic acids (Guo *et al.*, 2010). Lipoplex formation is generally achieved by incubating the nucleic acid with the cationic liposome solution under well defined conditions of pH and salt strength (Felgner *et al*., 1987). The formation of nucleic acid–cationic liposome complexes is a highly co-operative process involving spontaneous ionic interaction between the positively charged head groups of the cationic lipid and the negatively charged phosphate backbone of the nucleic acid (De Paula *et al*., 2007; De Rosa *et al.*, 2009; Huang *et al*., 1999; Ma *et al*., 2007). The physiochemical properties of the resultant complexes are dependent on: (i) the type and relative proportions of nucleic acid and cationic lipid, (ii) liposome size, (iii) presence of neutral lipids, (iv) nature of head group of the cationic lipid, (v) the charge ratio and (vi) temperature, amongst other factors (De Paula *et al*., 2007; De Rosa *et al*., 2009; Ma *et al*., 2007; Wasungu and Hoekstra, 2006).

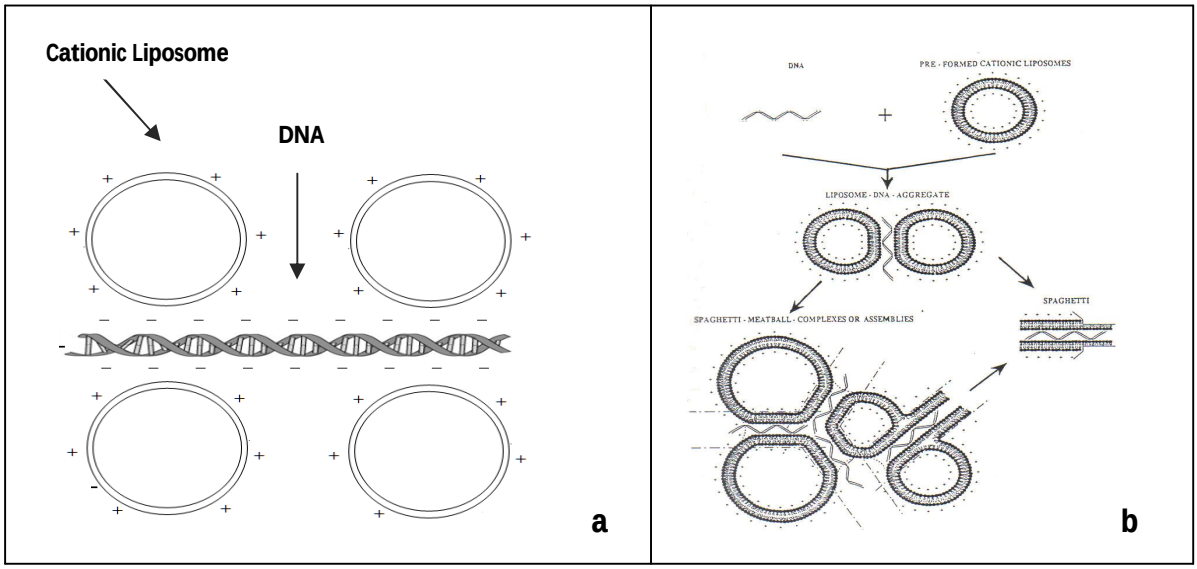



**Figure 3.1:** Illustrations of the **(a)** electrostatic model of DNA lipoplexes and **(b)** the coated electrostatic model resembling 'spaghetti and meatball-like 'DNA-liposome complexes (Singh, 1998; Sternberg *et al*., 1994).

Conventional DNA-cationic liposome complexes have been extensively characterized, with several models being proposed describing the nature of complexation, including the electrostatic and coated electrostatic models (Figure 3.1) (Lasic, 1997; Marty *et al*., 2009). However, despite considerable knowledge and investigations into DNA-

cationic liposome complexes, little is known about siRNA/RNA-cationic liposome interactions (Marty *et al*., 2009). Of critical importance is the fact that DNA and siRNA are completely different types of nucleic acids (De Paula *et al*., 2007; Lu *et al*., 2009). Although both are double-stranded with anionic phosphodiester backbones composed of the same nucleotide to negative charge ratio and they can each interact electrostatically with cationic liposomes, the size and ionic charge of siRNAs are much smaller than those of DNA. siRNAs have a molecular weight of ± 15 kDa, compared to the ± 10 000 kDa of plasmid DNA (Lasic, 1997; Schiffelers *et al*., 2005). These differences in molecular weight and topography mean that theoretically, siRNA cannot condense into particles of nanometric dimensions due to their already compact size and rigidity (De Paula *et al*., 2007; Lu *et al*., 2009; Rhinn *et al*., 2009). A recent study by Marty *et al.* (2009) demonstrated that smaller RNA molecules, in this case tRNA, preserved its folded state when complexed to cationic lipids, whereas DNA-lipid complexes induced partial B to A and B to C conformational changes within the DNA. Although tRNAs are on average four times large than siRNAs, they are still considerable smaller than DNA, providing some sort of tangible evidence that supports the theory that siRNA behaves differently when complexed to cationic liposomes compared with DNA. Bouxsien *et al*. (2009) revealed the isotropic ordering of siRNA in siRNA-cationic liposome complexes compared with the smectic ordering of DNA observed in DNA-cationic liposome complexes. They hypothesized that this difference in spatial ordering may prevent optimal siRNA packaging, resulting in decreased efficiencies as compared with DNA lipoplexes (Bouxsein *et al*., 2009). Much ambiguity still surrounds the field of siRNA delivery, however various studies have provided preliminary insight into the nature of siRNA : cationic liposome interaction.

In this investigation we utilized the gel retardation and dye displacement assays to assess siRNA lipoplex formation and elucidate binding/condensation abilities for all cationic liposome preparations. The gel retardation or band shift assay was adapted for use with cationic liposomes from the method originally employed by Fried and Crothers (1981) for studying DNA-protein interactions. It exploits the charge neutralisation experienced by nucleic acids upon addition of cationic liposomes

(Huang *et al.*, 1999). The dye displacement assay exploits the fluorescence-quenching that siRNA bound to an intercalating dye exhibits in the presence of cationic liposomes (Dorasamy *et al*., 2009). The ability of cationic liposomes to protect its cargo (i.e. siRNA) from nuclease degradation was ascertained through the nuclease protection assay. Finally, microscopy was also employed to provide visual evidence on the structure of the lipoplexes formed.

## 3.2 Materials

siGenome non-targeting siRNA (control siRNA) and siCONTROL TOX siRNA (siTOX) were purchased from Thermo Sciencific Dharmacon, Lafayette, CO. SYBR®green II nucleic acid gel stain (10 000x concentrate in DMSO) was obtained from Cambrex Bio Science Rockland Inc, Rockland, USA. Molecular biology grade agarose was purchased from Bio-Rad Laboratories, California, USA. Foetal bovine serum (FBS) was obtained from Highveld Biological (PTY) Ltd., Lyndhurst, South Africa. All other chemicals were of analytical grade.

## 3.3 Methods

### 3.3.1 Reconstitution of siRNA and Preparation of siRNA-Cationic Liposome Complexes (siRNA Lipoplexes)

siRNA was reconstituted as per the manufacture's recommendation. 1 ml of 18 ohm water was added to the lyophilized pellet, followed by 5 minutes of vortexing. The

nucleic acid solutions were subsequently aliquoted into micro-eppendorf tubes and stored at -20°C.

To a constant amount of either control siRNA or siTOX (0.5 µg), varying amounts of cationic liposome (0 to 16 µg) was added. Lipoplex samples were then made up to a final volume of 10 µl or 13 µl (nuclease assay only) with HEPES buffered saline (HBS) (20 mM HEPES, 150 mM NaCl, pH 7.5). Complexes were allowed to incubate at room temperature (25°C) for a minimum of 30 minutes prior to use.

### 3.3.2 Gel Retardation Assay

siRNA lipoplexes were prepared as described in 3.3.1., with control siRNA. 2 µl of gel loading buffer (50% glycerol, 0.05% bromophenol blue, 0.05% xylene cyanol) was added to all lipoplex samples. The samples were then subjected to electrophoresis on a 2% agarose gel in a Bio-Rad mini-sub cell electrophoresis apparatus containing 1x electrophoresis buffer (36 mM Tris-HCL, 30 mM sodium phosphate, 10 mM EDTA, pH 7.5), for 40 minutes at 50 volts. The gel was subsequently stained in a SYBR®green solution (1:10 000 dilution) in TBE buffer (89 mM Tris base, 89 mM boric acid, 1 mM EDTA, pH 8) for 20 minutes with constant shaking on a Stuart scientific shaker . The gel was then viewed under UV transillumination using the Vacutec Syngene G:Box gel documentation system with short pass filter.

### 3.3.3 SYBR®Green Displacement Assay

Fluorescence measurements were conducted on a Shimadzu RF-551 spectrofluorometric detector set at an emission wavelength of 520 nm and an excitation wavelength of 497 nm. 50 µl of a 1000x dilution of SYBR®green (10 000x concentrate) was made up to 500 µl with HBS (20 mM HEPES, 150 mM NaCl, pH 7.5). The fluorescence was measured to obtain a baseline relative fluorescence of 0. Thereafter, 5 µl (1.3 µg) of control siRNA was added to the baseline solution and the reading taken was assumed to represent 100% relative fluorescence. Subsequently, 1 µl aliquots, approximately 2.5 - 3.1 µg, of liposome preparation were added

stepwise to the solution until a plateau and/ or approximately 40 µg of liposome was added. The solution was thoroughly mixed after each addition to promote even distribution prior to measurement of fluorescence. The results were plotted relative to the 100% fluorescence value. This procedure was conducted for all liposome preparations.

### 3.3.4 Nuclease Protection Assay

SiTOX at three concentrations: 25 nM, 50 nM and 100 nM, were complexed to varying amounts of cationic liposome (Table 3.1.) as described in 3.3.1. Thereafter, FBS was added to the complexes to a final serum concentration of 10% ($^{v}/_{v}$). Negative and positive controls were also setup (at the various siTOX concentrations) containing siTOX alone and siTOX with FBS, respectively. All reaction mixtures were then incubated at 37°C for 4 hours. Thereafter, ethylenediaminetetraacetic acid (EDTA) was added to the reaction mixtures to a final concentration of 10 mM and sodium dodecyl sulphate (SDS) to a final concentration of 0.5% ($^{w}/_{v}$). Reaction mixtures were incubated for a further 20 minutes at 55°C. The samples were then subjected to electrophoresis on a 2% agarose gel (as per 3.3.2) for 60 minutes at 50 volts. The gel was subsequently stained in a SYBR®green solution (as per 3.3.2) for 20 minutes with constant shaking on a Stuart scientific shaker and viewed under UV transillumination using the Vacutec Syngene G:Box gel documentation system with short pass filter.

**Table 3.1:** The amount of liposome in lipoplex preparations used in the nuclease protection assay. Reaction mixtures contained 0.5 µg control siRNA.

| **Liposome Preparation** | Liposome Amount **(µg)** | | |
|---|---|---|---|
| 1. SD-CholTC | 8 | 9 | 10 |

| | | | |
|---|---|---|---|
| 2. SD-CholTT | 11 | 12 | 13 |
| 3. SD-CholQC | 10 | 11 | 12 |
| 4. SD-CholQT | 13 | 14 | 15 |

### 3.3.5 TEM of siRNA Lipoplexes

SiRNA lipoplexes were prepared as described in 3.3.1, with control siRNA. The lipoplexes were then diluted (1:5) with HBS. Thereafter, 1 µl of liposome solution was placed on formvar-coated grids, followed by the addition of 1 µl of 1% uranyl acetate. The grids were blotted after 3 minutes with filter paper to remove excess moisture and immediately vitrified by plunging into liquid ethane using a Leica CPC system. Grids were then transferred to a Gatan cryotransfer system and viewed using a JEOL-1010 transmission electron microscope, without warming above -150°C.

## 3.4 Results and Discussion

### 3.4.1 Preparation of siRNA Lipoplexes

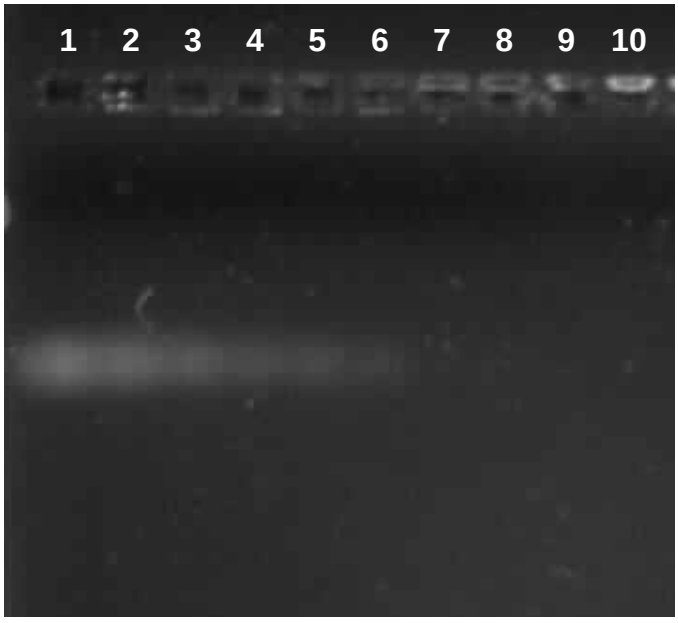
siRNA lipoplexes were successfully prepared as demonstrated by gel retardation assays (3.4.2), dye displacement studies (3.4.3) and transmission electron microscopy (3.4.5). The method employed for lipoplex formation proved to be both simple and efficient. For our investigation, two different types of siRNA were utilized. The siGENOME non-targeting siRNA (control siRNA) and siCONTROL TOX siRNA (siTOX) molecules were reconstituted as per the manufacturer's recommendation.

The control siRNA was employed for the majority of characterization studies with the cationic liposomes; as well as growth inhibition studies (chapter four). It has a comparable GC content to that of functional siRNA, but lacks homology with known target genes in that it has four mismatches with all identified human, rat and mouse genes. Conversely, transfection (chapter four) and nuclease protection studies made

use of siTOX. It has a comparable molecular weight to that of the control siRNA, however it is a functional siRNA molecule that upon entering the cell induces programmed cell death (www.dharmacon.com). Hence it has been extensively utilized for analysis of transfection efficiency, especially as an initial indicator of vector or delivery efficacy in siRNA knock-down/out investigations (Yadava, 2007; www.dharmacon.com). Once generated, the siRNA lipoplexes were subjected to various assays for characterization and determination of binding efficacy.

### 3.4.2 Gel Retardation Assay

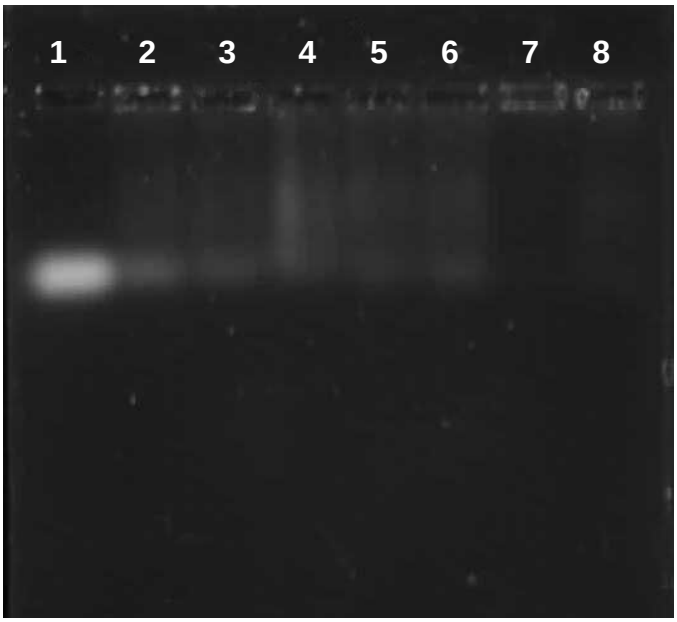
The results of the agarose gel electrophoresis of the siRNA-CholT and siRNA-CholQ lipoplexes can be seen in Figures 3.2 and 3.3, respectively.



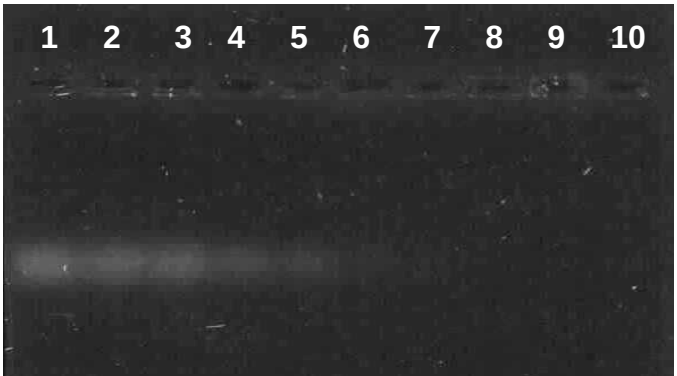

**(a)**

**(b)**

**Figure 3.2:** Gel retardation study of the binding interaction between control siRNA (0.5 µg) and increasing amounts of cationic liposomes containing Chol-T **(a)** SD-CholTC and **(b)** SD-CholTT , where: **Lane 1:** control siRNA alone (0.5 µg) and **Lane 2 – 10:** siRNA (0.5 µg) with varying amounts of cationic liposome (5, 6, 7, 8, 9, 10, 11, 12, 13 µg).




**(a)**



**(b)**

**Figure 3.3:** Gel retardation study of the binding interaction between control siRNA (0.5 µg) and increasing amounts of cationic liposomes containing Chol-Q. **(a)** SD-CholQC and **(b)** SD-CholQT , where: **Lane 1:** control siRNA alone (0.5 µg); **(a) Lanes 2 – 8:** siRNA (0.5 µg) with varying amounts of cationic liposome (6, 7, 8, 9, 10, 11, 12 µg); **(b) Lanes 2 – 10:** siRNA (0.5 µg) with varying amounts of cationic liposome (8, 9, 10, 11, 12, 13, 14, 15, 16 µg).

The results demonstrate that the cationic liposomes are able to bind siRNA for all liposome preparations tested. Lane 1 of each gel contained 0.5 µg control siRNA, which was shown to produce a single band within the agarose gel upon electrophoresis. As the liposome concentration of each preparation increased, more siRNA was bound to the liposomes and hence less siRNA was free to enter the gel. This is demonstrated by the gradual decrease in fluorescence of the bands within the gels. Complete retardation of the siRNA occurred at various siRNA : liposome ratios for the different liposome preparations as set out in Table 3.2. Complete retardation is seen by the lack of a band within the gel. Agarose gel electrophoresis is used to demonstrate complex formation between the negatively charged siRNA and the positively charged liposomes. Gel electrophoresis is a standard method used to separate, identify and purify charged molecules, such as nucleic acids, through application of an electric field (Guilliatt, 2002). Naked siRNA subjected to electrophoresis migrates through the gel matrix. However, upon binding to the cationic liposomes, the negative charges of the siRNA are neutralised or titrated by the positive charges of the liposome through electrostatic interaction between the

two. Therefore, the neutral or slightly positively charged lipoplexes are 'retarded' and remain within the wells of the gel. Complete retardation suggests that all the negative charges of the siRNA are completely titrated by the positive charges of the cationic liposomes.

Although small molecules (eg. RNA) are generally subjected to polyacrylamide gel electrophoresis, while larger molecules (eg. plasmid DNA) are analysed on agarose gels (Guilliatt, 2002), studies performed by Read *et al.* (2005) and Han *et al.* (2008) demonstrated successful use of agarose gels for siRNA retardation experiments. The above, combined with its availability and ease of preparation and use meant that agarose gels were used in this investigation. Various $^{w}/_{v}$ percentages of agarose (i.e. 1%, 2% and 3%) were tested to determine the best concentration for our purposes. The 2% agarose gel demonstrated adequate resolution and slower siRNA gel migration rates and was consequently selected for use. As shown by the results (Figures 3.2 and 3.3) all cationic liposome preparations successfully bound the siRNA at varying ratios.

**Table 3.2:** siRNA-liposome ratios at which complete retardation/complex formation is achieved.

| **Liposome Preparation** | **Liposome Amount (µg)** | **siRNA : Liposome Ratio ($^{w}/_{w}$)** |
|---|---|---|
| 1. SD-CholTC | 9 | 1 : 18 |
| 2. SD-CholTT | 12 | 1 : 24 |
| 3. SD-CholQC | 11 | 1 : 22 |
| 4. SD-CholQT | 14 | 1 : 28 |

It is interesting to note that the overall results of the binding assay demonstrate that a significant increase in liposome quantity is required to bind the siRNA compared

with conventional DNA-liposome binding interaction. This result is in accordance with studies performed by Bouxsein *et al.* (2007), Watanabe *et al.* (2007) and Rhinn *et al*. (2009), all of whom demonstrated the need for a substantial increase in cationic liposome mass to form complexes with siRNA. These results seem to support the theory that siRNA are unable to condense into smaller structures during complex formation with cationic liposomes due to their already small size.

From the results obtained it can be seen that more of the SD-CholQ liposomes were required to bind the same amount of siRNA as compared with the SD-CholT liposomes. This maybe attributed to the large molecular weight difference between Chol-T and Chol-Q iodide, which affects formulation. However when considering the positive (+)/negative (-) charge ratios of lipoplexes, differences between the two liposome species are small (Table 3.3). It must be remembered however that the monocationic head groups differ in methyl group substitution and the resultant difference in spatial structure may contribute to observe differences in optimum binding ratio. A similar result was observed by Kawakami *et al.* (2000b) who showed that a greater amount of liposome composed of the quaternary cationic lipid, DOTMA was required to bind a given amount of nucleic acid (DNA) compared with liposome containing the ternary cationic lipid, DC-Chol.

The gel retardation results also show that cationic liposomes containing the galactose targeting ligand (SD-CholTT and SD-CholQT) seem to be less positively charged than cationic liposome preparations lacking this targeting ligand (SD-CholTC and SD-CholQC). A plausible reason for this observed trend could be due to the steric shielding effect of the targeting ligand. The β-galactose pyranoside ligand contains a lipid anchor which allows for assembly into the lipid bilayer of the liposome resulting in protrusion of the head group, which could possibly shield or block the positive charges of the liposome thus reducing availability of these positive charges and thereby interfering with the binding of the liposome to the siRNA. Another possible reason for the observed disparity in targeted versus untargeted liposome binding efficiency could be that the incorporation of the β-galactose residue caused more of the cationic lipid, Chol-T or Chol-Q, to be internalized during liposome formation

affording less positively charged cationic liposomes. Hence more of the liposome was required to bind the negatively charged siRNA.

**Table 3.3:** The end point charge ratios of siRNA to liposome for each of the cationic liposome preparations.

| Liposome Preparation | Charge Ratio (siRNA : Cationic liposome) (-ve : +ve) |
|---|---|
| 1. SD-CholTC | 1 : 4.7 |
| 2. SD-CholTT | 1: 5.7 |
| 3. SD-CholQC | 1 : 5.1 |
| 4. SD-CholQT | 1 : 6 |

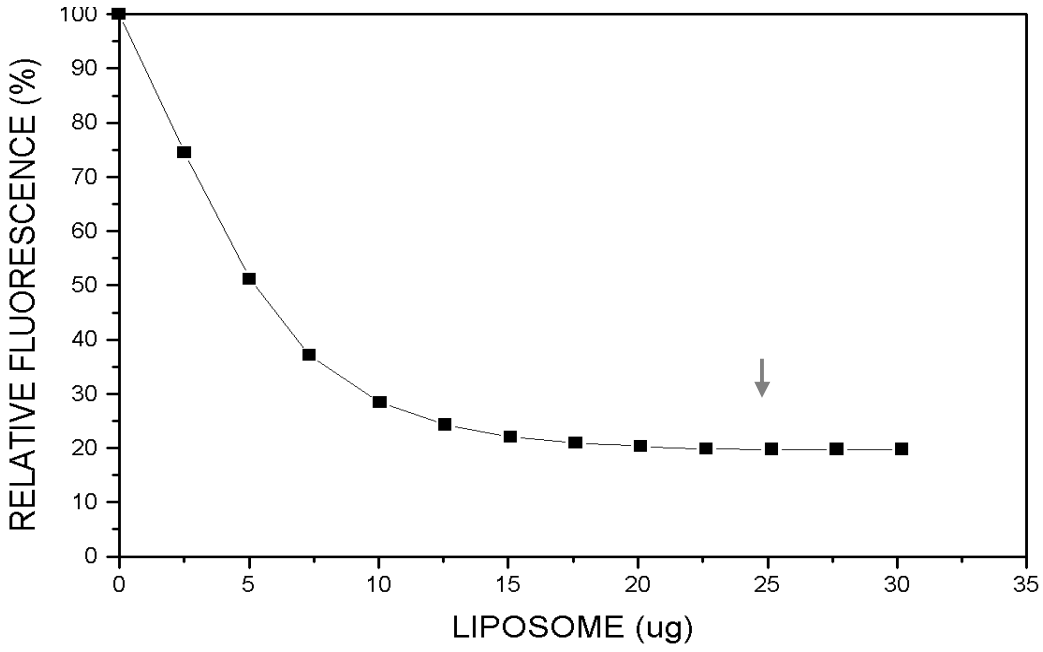
The information obtained from the retardation studies was utilized for optimising transfection studies of HepG2 cells *in vitro* (chapter four). Lipoplexes containing liposomes ranging from above to below optimum binding ratio (end points) were investigated.

### 3.4.3 SYBR®Green Displacement Assay

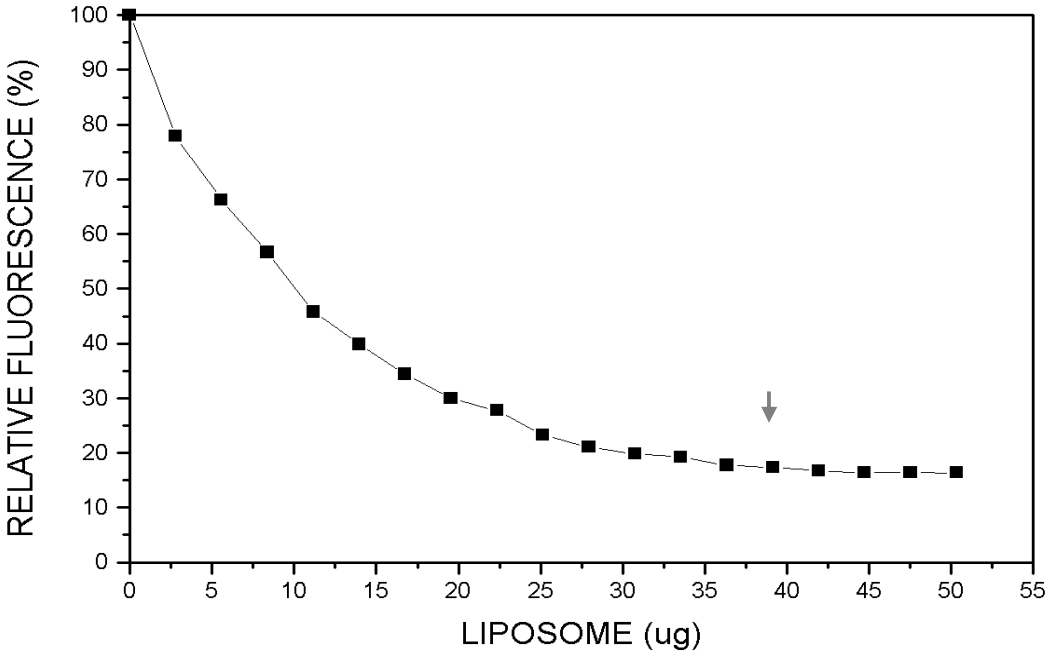
Traditionally, the dye displacement or intercalation assay demonstrates displacement of DNA-associated ethidium bromide by cationic liposomes (Geall *et al.*, 2000; Xu *et al.*, 1996). Ethidium bromide is a cationic dye that intercalates between the base pairs of both double-stranded DNA and RNA. It exhibits weak fluorescence in solution, but upon 'binding' to nucleic acids it fluoresces strongly. It was Sorbell *et al.* (1977) who first proposed that ethidium bromide requires flexibility within the structure of the nucleic acid for intercalation. They also suggested that ethidium bromide exists in equilibrium between the intercalated sites on the nucleic acid and free in solution. Thus a loss of flexibility through DNA condensation would result in a subsequent shift in the binding equilibrium into solution, with a resultant decrease in

fluorescence (Geall *et al*., 2000). This premise has been extensively utilised to demonstrate cationic liposome induced DNA condensation and binding (Hsieh *et al.,* 1994; *Xu et a.l*, 1996; Singh *et al.*, 2006).

In this investigation the ethidium bromide intercalation assay was adapted for use with siRNA. SYBR®green II nucleic acid dye was substituted for ethidium bromide. SYBR®green is also an intercalating dye from the cyanine family of dyes. The mechanism of action of this minor-groove binding dye has not been fully elucidated, however it does exhibit a higher quantum yield when bound to RNA compared with DNA and is significantly more sensitive than ethidium bromide i.e. it can detect as little as 500 pg RNA and exhibits several times greater affinity for RNA than does ethidium bromide. In addition, it is non-mutagenic making it a safer alternative than ethidium bromide (Kirsanov *et al.*, 2010; www.Lonza.com). For these reasons SYBR®green II dye was used in the dye displacement assays. The results of the assay (Figure 3.4) revealed that all cationic liposome preparations were able to successfully displace SYBR®green from the control siRNA. This was demonstrated by a continuous decrease in SYBR®green fluorescence upon stepwise addition of the cationic liposomes into the reaction mixture.




**(a)**


RELATIVE FLUORESCENCE (%)
LIPOSOME (ug)
0
10
20
30
40
50
60
70
80
90
100
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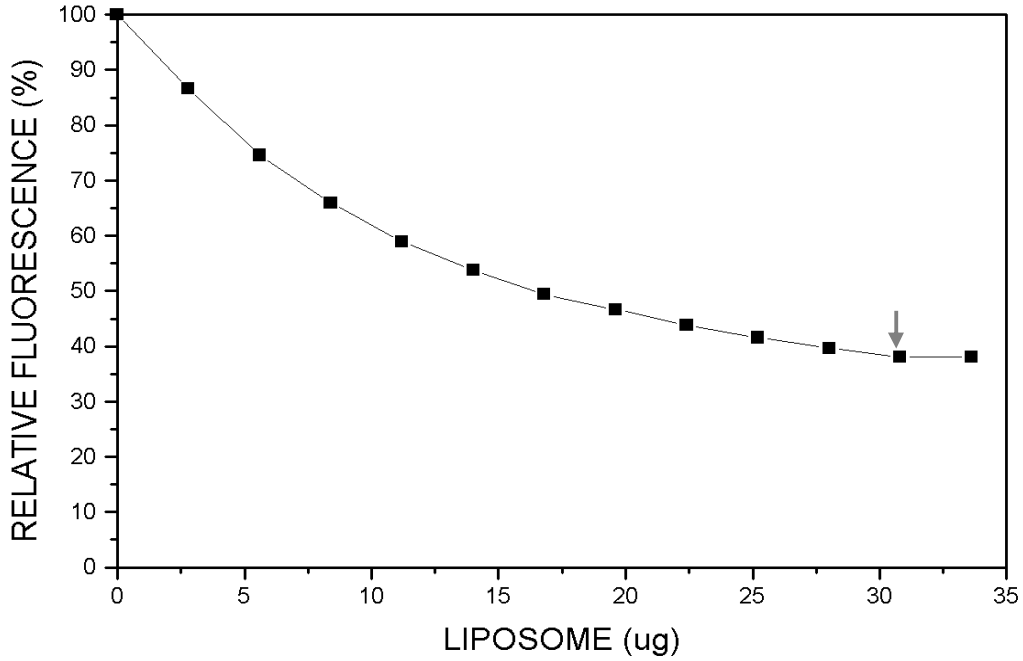
5
10
15
20
25
30
35
40
45
50
55

**(b)**


RELATIVE FLUORESCENCE (%)
LIPOSOME (ug)
0
10
20
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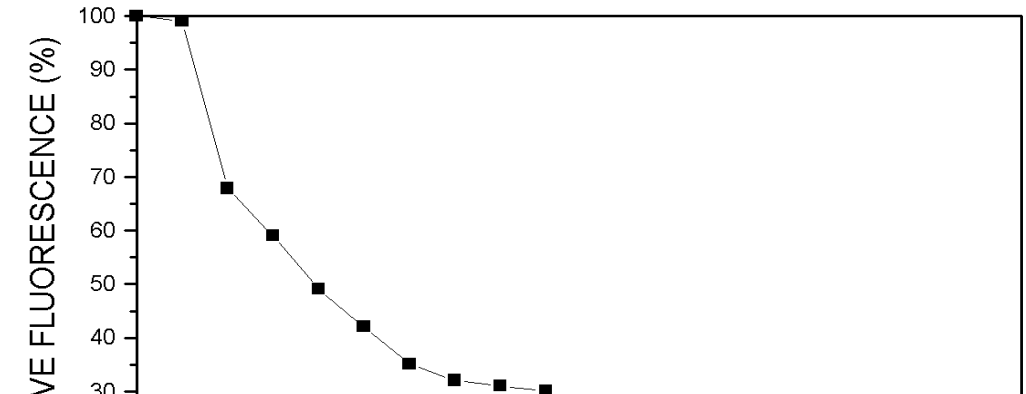
**(c)**


VE FLUORESCENCE (%)
30
40
50
60
70
80
90
100

**(d)**

**Figure 3.4:** The dye (SYBR green) displacement assay for cationic liposomes in 500 µl total reaction mixture containing 1.3 µg control siRNA and increasing amounts of liposome preparation (1 ml aliquots ≈ ± 3 µg). **(a)** SD-CholTC; **(b)** SD-CholTT; **(c)** SD-CholQC and **(d)** SD-CholQT. (↓)Point of inflection.

Varying amounts of cationic liposome (± 40 µg) were added to the reaction mixture for each preparation, until fluorescence measurements reached a constant value or plateau. The point at which this plateau occurred is referred to as the point of inflection and represents the siRNA : cationic liposome ratio at which maximum displacement of SYBR green or complete binding/condensation of siRNA occurred. Approximately 25 µg of SD-CholTC liposome was required to reach this point, equivalent to siRNA : liposome ratio of 1 : 19. SD-CholTT required 33.5 µg at a ratio of 1 : 26, SD-CholQC required 31 µg at a ratio of 1 : 24, while SD-CholQT needed 40 µg at a ratio of 1 : 31. The siRNA : liposome ratios obtained for this assay seem to correlate well with those obtained for the gel retardation assay (Table 3.2). This would suggest that siRNA binding and compaction occurs at a similar point. The relatively small difference observed between the ratios could be attributed to one of two reasons, namely: the fact that the dye displacement assay is based on nucleic acid condensation while the gel retardation assay is based on charge neutralisation (i.e. the point at which the negative charges of the nucleic acid are completely titrated by the positive charges of the liposome), and the relatively high sensitivity of this assay compared with the gel retardation assay.

From the results obtained, it can be seen that both the SD-CholT and SD-CholQT liposomes exhibited a higher degree of siRNA compaction as compared with the SD-CholQC liposome. SD-CholTC and SD-CholTT yielded a 78% and 80% reduction in fluorescence, respectively, while, SD-CholQC and SD-CholQT demonstrated a fluorescence decrease of 60% and 80%, respectively. This percentage of fluorescence reduction is said to correlate with the degree of nucleic acid condensation or compaction. Thus, although all four liposome preparations demonstrated siRNA condensation capabilities, three of the preparations demonstrated superior compaction ability. From the results obtained it can also be seen that the SD-CholT liposomes were able to displace SYBR®green more efficiently than the SD-CholQ liposomes. This is seen by the 23.5% average reduction in fluorescence observed after initial addition of the SD-CholT liposomes compared with the 7.5% average reduction observed after addition of the SD-CholQ liposomes. This supports the results obtained for the gel retardation studies which showed that greater quantities of the SD-CholQ liposomes were required to form complexes with the siRNA as compared with the SD-CholT liposomes. This difference in SYBR®green displacement could be attributed to steric hindrance of the larger trimethyl head group as opposed to the smaller dimethyl head group. Also noteworthy is the fact that the SD-CholQC liposome demonstrated a slightly poorer ability to condense the siRNA when compared with its targeted counterpart. This is a somewhat surprising result, as one would theoretically expect the galactose residues on the SD-CholQT liposome to interfere with compaction due to shielding. A possible reason for this difference in condensation ability could be attributed to charge repulsion between the trimethyl amino head groups, which may affect SD-CholQC's ability to condense siRNA. The presence of the galactose residues may reduce this charge repulsion, thus affording better compaction ability. Further study is required, perhaps x-ray diffraction to provide additional data on the nature and structure of the lipoplexes. In accordance with work done by Kunath *et al.* (2003), who showed that the incorporation of a targeting ligand affects nucleic acid binding, the findings of this assay also show that a greater amount of the SD-CholTT and SD-CholQT liposomes was required to reach the point of inflection. The results of this assay suggest that the presence of the galactose ligand did not influence the degree of compaction achieved but rather the

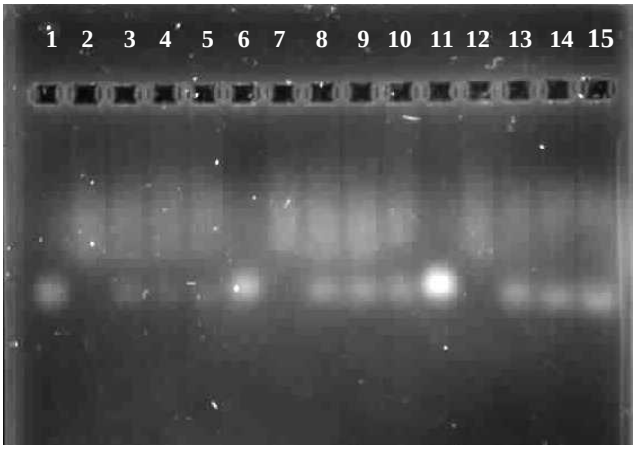
quantity required to condense the siRNA. Once again, steric shielding could be a possible reason for the observed result. Martin *et al.* (2005) noted that head group size may cause steric repulsion between neighbouring head groups affecting binding/condensation ability.

Overall, the results from this assay provide further evidence that the cationic liposomes described in chapter two are able to successfully bind the siRNA. It also provides preliminary proof that cationic liposomes are able to cause condensation or compaction of siRNA.

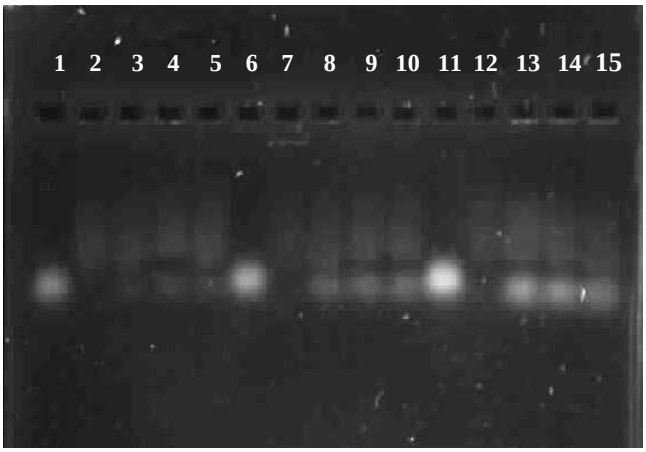
### 3.4.4 Nuclease Protection Assay

Enzymatic degradation of nucleic acids is a major obstacle facing successful genetic therapeutics (Roa, 2010). Various nucleases found in blood serum attack siRNA resulting in its disintegration and inability to bring about a therapeutic effect (Zhang *et al*., 2006). Naked siRNA has been shown to be stable in serum for several minutes to about an hour, depending on serum concentration (Reischl and Zimmer, 2008). In addition to nucleases, serum also contains a number of components such as lipids (eg. phospholipids), polysaccharides and proteins (e.g. albumin, lipoproteins, and macroglobulins) that could interfere with interaction between (i) lipoplex components and (ii) lipoplexes and cells (Rhinn *et al*., 2009; Ryhänen, 2006; Sato *et al.,* 2007). Thus the effect of serum components on lipoplexes is of particular interest as inefficient protection of the nucleic acid or unstable complexes are adverse features of any delivery system (Oku *et al*., 2000). The protection or shielding ability afforded to siRNA by the cationic liposomes described in chapter two was determined by the nuclease protection assay. In addition, this assay allowed for the assessment of lipoplex stability when exposed to serum.

The results of the nuclease protection assay post-electrophoresis can be seen in Figure 3.5. Three concentrations of siTOX (25 nM, 50 nM and 100 nM) were examined. Lanes 1, 6 and 11 contained siTOX alone at the various concentrations, and were used as positive controls. Lanes 2, 7 and 12 contained siTOX (at the various concentrations) exposed to 10% FBS for four hours at 37°C. They served as the negative controls. The remaining lanes (3-5, 8-10 and 13-15) contained lipoplexes after exposure to 10% FBS. Lipoplexes were composed of siTOX at 25 nM, 50 nM and 100 nM and liposomes at below, optimum and above siRNA : cationic liposome optimum binding ratios as elucidated by gel retardation studies. From the result obtained, it can be seen that siTOX was completely degraded when subjected to the FBS. This was demonstrated by the lack of a band within the gel. From the results it can be deduced that all cationic liposomes afforded some degree of protection to the siTOX. Protection efficiency increased with an increase in siTOX concentration. This is seen by the streaking and weak intensity of bands within the gel.




**(a)**

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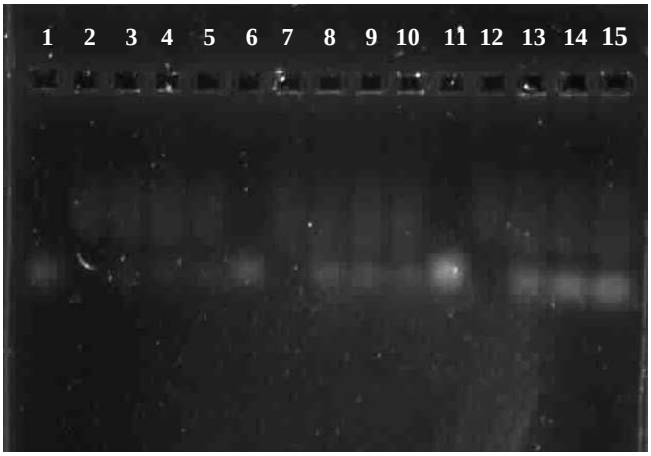


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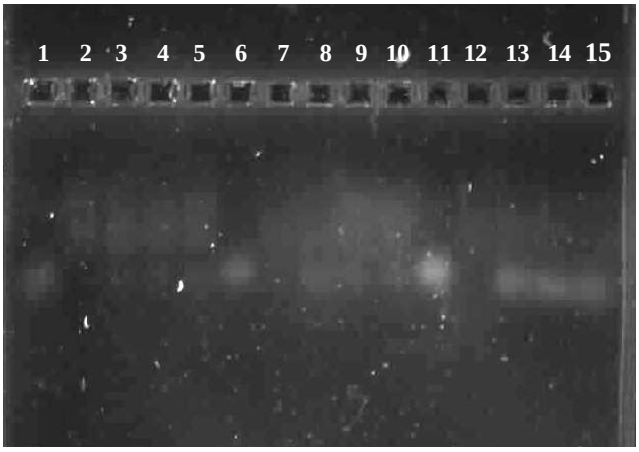



**(d)**

**Figure 3.5:** Nuclease protection assay of siTOX-cationic liposome complexes of **(a)** SD-CholTC, **(b)** SD-CholTT, **(c)** SD-CholQC and **(d)** SD-CholQT in 13 µl reaction mixture with varying concentrations of siTOX, where:

Lane 1, 6, 11: Naked siTOX at 25 nM, 50 nM and 100 nM concentrations, respectively.

Lane 2, 7, 12: siTOX at 25 nM, 50 nM and 100 nM, respectively, with 10% FBS.

(a) Lane 3-5: 25 nM siTOX with varying amounts of SD-CholTC (8, 9 and 10 µg) and 10% FBS.
Lane 8–10: 50 nM siTOX with varying amounts of SD-CholTC (8, 9 and 10 µg) and 10% FBS.
Lane 13–15: 100 nM siTOX with varying amounts of SD-CholTC (8, 9 and 10 µg) and 10% FBS.

(b) Lane 3-5: 25 nM siTOX with varying amounts of SD-CholTT (11 , 12 and 13 µg) and 10% FBS.
Lane 8–10: 50 nM siTOX with varying amounts of SD-CholTT (11, 12 and 13 µg) and 10% FBS.
Lane 13–15: 100 nM siTOX with varying amounts of SD-CholTT (11, 12 and 13 µg) and 10% FBS.

(c) Lane 3-5: 25 nM siTOX with varying amounts of SD-CholQC (10, 11 and 12 µg) and 10% FBS.
Lane 8–10: 50 nM siTOX with varying amounts of SD-CholQC (10, 11 and 12 µg) and 10% FBS.
Lane 13–15: 100 nM siTOX with varying amounts of SD-CholQC (10, 11 and 12 µg) and 10% FBS.

(d) Lane 3-5: 25 nM siTOX with varying amounts of SD-CholQT (13, 14 and 15 µg) and 10% FBS.
Lane 8–10: 50 nM siTOX with varying amounts of SD-CholQT (13, 14 and 15 µg) and 10% FBS.
Lane 13–15: 100 nM siTOX with varying amounts of SD-CholQT (13, 14 and 15 µg) and 10% FBS.

It was also observed that SD-CholT liposomes afforded slightly better protection to siTOX as compared with the SD-CholQ liposomes, suggesting that the complexes formed between the SD-CholQ liposomes and siTOX may be less stable than the SD-CholT lipoplexes. It is also noteworthy that an increase in molar concentration resulted in an observed increase in protection efficiency. This is probably an erroneous conclusion and a more plausible explanation would be that an increase in siTOX concentration resulted in a subsequent increase in the amount of intact siTOX remaining after exposure to the FBS.

The three concentrations of siTOX (25 nM, 50 nM and 100 nM) used were those tested for transfection studies of HepG2 cells *in vitro* (chapter four). Several studies

and various siRNA manufacturers (including Dharmacon) have suggested investigating a range of siRNA concentrations for optimisation of transfection in different/specific cell lines (Yadava, 2004; www.dharmacon.com). Typically, siRNA concentrations ranging from 20 nM to 200 nM have been utilized for transfection of mammalian cells (Yadava, 2007). However, due to low transfection efficacy and non-specific effects at the lower (<20 nM) and upper ranges (>100 nM), respectively (Oh *et al.,* 2009; Persengiev *et al*., 2004; Yadava, 2007) concentration range of 25 nM to 100 nM was chosen for investigation. For this assay, EDTA and SDS were added to reaction mixtures after nuclease digestion. EDTA is a chelating agent which was used to halt nuclease activity by removing necessary divalent ions from solution, while SDS, an anionic surfactant, was used to release the siTOX RNA (nucleic acid) from the lipoplexes.

Overall, the results of this assay demonstrated that all cationic liposome preparations provided some degree of protection against nuclease digestion to siTOX in the presence of 10% FBS for a period of four hours at 37°C. Increased siRNA concentration resulted in a greater amount of intact siRNA remaining after serum exposure.

### 3.4.5 TEM of siRNA Lipoplexes

Transmission electron microscopy (Figure 3.6) revealed a generally, heterogenous population size amongst the various siRNA lipoplexes examined. The majority of lipoplexes ranged, on average, from 300 nm to 600 nm in size. Visual examination of the various lipoplexes showed that the SD-CholT lipoplexes appeared as tightly compacted clusters or aggregates. In contrast, the SD-CholQ lipoplex micrographs showed very loosely bound structures. These findings support results obtained from the gel retardation, dye displacement and nuclease protection assays, which noted the difference in binding and compaction ability observed for the different cationic liposomes. This result also adds credence to the theory that the SD-CholQ liposomes

form less stable complexes with the siRNA. This may be a result of weak electrostatic interaction between the trimethyl head group of the SD-CholQ liposomes and the phosphodiester backbone of the siRNA. Dispersion in a dilute environment may have led to rapid disintegration of the lipoplexes as a result of this weak interaction. Please note that any artefacts seen in the TEM micrographs can be attributed to the cryoTEM process.

Lipoplex size is a critical factor for transfection efficiency (Roa *et al.*, 2010). Appropriate lipoplex size has not yet been clearly defined, with conflicting results from various studies creating ambiguity within the field. Ross *et al*. (1999) demonstrated that efficient transfection could be achieved with lipoplexes sized between 200 and 2000 nm, while Zhang *et al*. (2003) indicated lipoplexes smaller than 200 nm were more efficient transfectors of cells. An important parameter in determining the size of lipoplexes is the charge density of its components (Roa *et al*., 2010). In accordance with this, all lipoplexes assembled were of a similar siRNA : cationic liposome charge ratio hence lipoplexes were of a similar size range, if not structure.

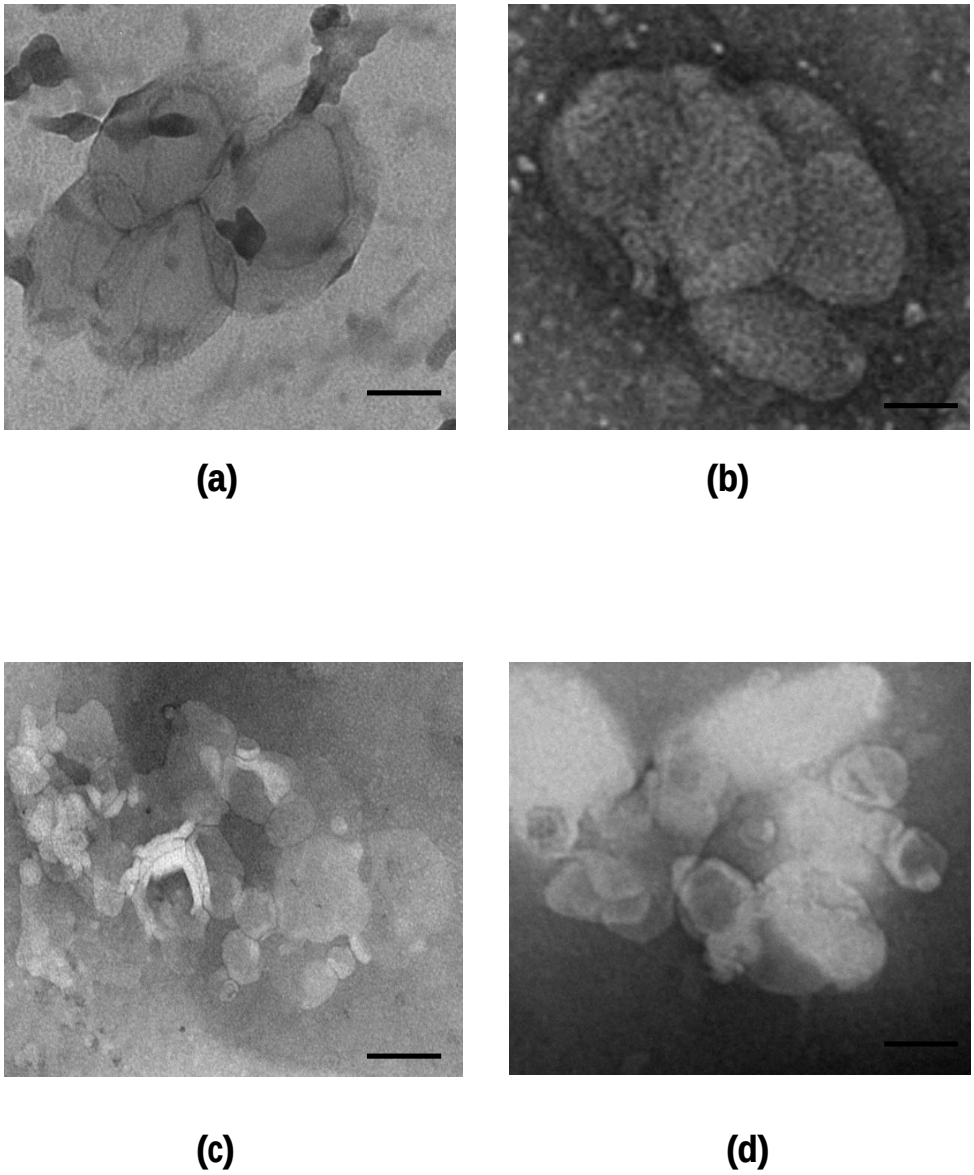



**Figure 3.6:** Transmission electron microscopy with negative staining, of the four siRNA lipoplexes. Bar = 100 nm; **(a)** SD-CholTC, **(b)** SD-CholTT, **(c)** SD-CholQC and **(d)** SD-CholQT

In summary, this chapter has yielded information on the binding and condensation ability of the cationic liposomes described in chapter two. Successful siRNA : cationic liposome binding and elucidation of optimum binding ratios was demonstrated. Compaction or condensation of siRNA was also achieved. In addition, the ability of the cationic liposomes to protect siRNA from nuclease attack was established. From

the results, it can be seen that the interaction between the Chol-Q containing liposomes and the siRNA was weak, as suggested by the band shift and nuclease protection assays. This apparent weak interaction is confirmed in the dye displacement assay, where the SD-CholQC liposome, in particular displaced less dye than the other three liposomes. Although the Chol-Q : siRNA interaction appears weaker than the Chol-T : siRNA association, this maybe a desirable feature in transfection studies as the siRNA cargo might be released more readily from the lipoplex. Overall, these findings demonstrate the interaction between the siRNA and the SD-CholT and SD-CholQ liposomes.

# Chapter Four: Cell Interaction Studies

## 4.1 Introduction

Transfection involves the non-viral introduction or 'transfer' of foreign genetic material into eukaryotic cells (Gopalakrishnan and Wolff, 2009). It is an important technique in cellular biology research allowing for the study of gene function and treatment of gene-related diseases (Gopalakrishnan and Wolff, 2009). There are a variety of physical or chemical methods that can be employed for the introduction of nucleic acids into recipient cells (Hodgson, 1995). Some earlier transfection methods involved the use of electroporation, micro-injection, particle bombardment and complexation of nucleic acid to calcium phosphate or diethylaminoethyl (DEAE) dextran (Sambrook and Russel, 2001; Szelei and Duda, 1989). Liposome-mediated gene transfer offered a promising alternative to other transfection methods that proved either unsafe or unsuitable, especially for *in vivo* gene delivery. However, invariable entrapment/binding and transfection efficiencies and high toxicity meant that conventional and cationic liposomes did not initially gain widespread use as gene delivery vectors. It wasn't until Felgner and co-workers, in 1987 synthesized the monovalent cationic lipid N-[1-(2,3-dioleoyl) propyl-N,N,N-trimethylammonium chloride] (DOTMA), that cationic liposome-mediated transfection gained support (Felgner *et al*., 1987; Lasic, 1997; Szelei and Duda, 1989).

Gene transfer can be conducted *in vitro* or *in vivo*. *In vitro* gene delivery (DNA and RNA) has achieved much success using a diverse range of vectors including cationic liposomes. However, *in vivo* transfection with cationic liposomes has met with limited success due to problems associated with binding to polyanionic glycans or proteins present in the extracellular matrix, rapid elimination by the reticuloendothelial system, instability in serum and serum-associated degradation of nucleic acid (Gad, 2007; Zanta *et al*., 1997). Traditionally, two basic types of transfection can occur, namely: (i) transient, and, (ii) stable transfection. Transient transfection refers to the introduction of foreign nucleic acids into a cell without change to the chromosomal DNA, while stable transfection refers to the incorporation of foreign DNA into the cell genome. Expression efficiency is generally an order of magnitude higher in transient transfections as compared with stable transfections (Sambrook and Russel, 2001). The use of synthetic siRNA in transfection is classified as transient in nature (Doran and Helliwell, 2008). All

transfection experiments should include positive and negative controls to compare or test the validity of results. An important consideration in transfection efficiency is the selection of cell line to be used. It has been demonstrated that a transfection agent that works well in one type of cell may be ineffective in another cell line (Sambrook and Russel, 2001).

The cationic lipoplex system has formed the basis for development of numerous ligand-targeted gene delivery systems over the years. Emerging studies have shown the adaptation of these systems for siRNA delivery (Huang *et al*., 2005). In this investigation, we designed and developed a synthetic siRNA transfer system based on cationic liposome-mediated transfection that could be targeted to a specific cell type. The cell line chosen for specific targeting was the HepG2 cell line, one of two human cell lines that were isolated from liver biopsies of hepatocellular carcinomas and hepatoblastomas (Aden *et al*., 1979), which exhibit the same capabilities of normal liver parenchymal cells (Knowles *et al*., 1980). Targeting was achieved by incorporation of galactose-terminating residues into the cationic liposome structure during synthesis. The targeted siRNA delivery system was subsequently prepared by incubation of the various cationic liposome preparations (SD-CholTC, SD-CholTT, SD-CholQC and SD-CholQT) with the control siRNA or the siTOX molecules.

Hepatocytes are attractive targets for gene delivery as they produce a large number of serum proteins (such as albumin, transferrin, fibrinogen, ceruloplasmin and α-fetoprotein), allowing for study and treatment of a wide range of genetic defects. In addition, hepatocytes contain a number of receptors that can be targeted for gene delivery, including the asialoglycoprotein (ASGP) receptor. Also referred to as the hepatic lectin, this 41 kD transmembrane heteroligomer glycoprotein composed of two subunits, H1 and H2 in human hepatocytes, is predominantly expressed on the sinusoidal surface and is responsible for the clearance of glycoproteins with desialylated acetylglucosamine or galactose residues from circulation via receptor-mediated endocytosis (Wu *et al*., 2002). ASGP receptor recognition and internalization is both highly specific and rapid, making it an extremely effective means of achieving hepatocyte targeting (Kawakami *et al*., 2000; Qi *et al*., 2005). The

proposed receptor-ligand recognition of β-D-galactopyranoside containing siRNA lipoplexes directed towards HepG2 cells can be seen in Figure 4.1.

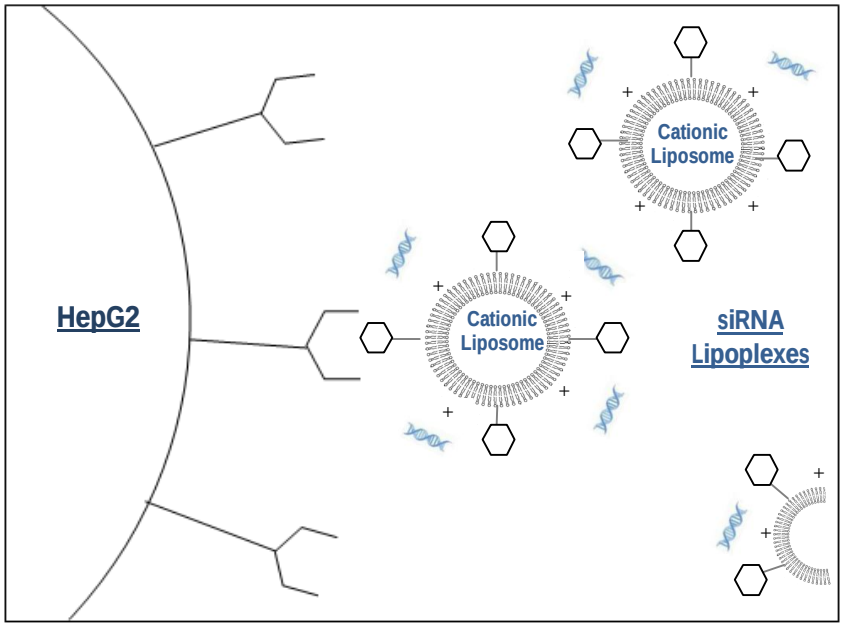



**Figure 4.1:** An illustration of the interaction between the galactose-containing siRNA lipoplexes and the HepG2 cell where: represents the siRNA molecule and represents the (β)-D-galatopyranoside residue (Diagram not drawn to scale).

siRNA knockdown can be achieved and assessed using a variety of methods, such as: reporter genes, visual inspection, conventional viability assays and assaying mRNA (example: Real time-PCR) or protein levels (example: western blot analysis), depending on the siRNA source being utilized (refer to chapter 1) i.e. DNA-based RNAi or synthetic siRNAs (www.promega.com). Like most DNA or plasmid transfections, there are numerous factors that can affect efficiency of siRNA delivery into mammalian cells *in vitro*. These include: lipoplex formation, particle size, surface charge, cell density, incubation and assay time and siRNA concentration (Kim *et al*., 2010; Vestin *et al*., 2008; www.dharmacon.com). Hence transfection conditions require optimization to obtain the best knockdown of gene expression (Engelke and Rossi, 2005).

## 4.2 Materials

siGenome non-targeting siRNA (control siRNA) and siCONTROL TOX siRNA (siTOX) were purchased from Thermo Sciencific Dharmacon, Lafayette, CO. HEK293 cells, HepG2 cells and irradiated foetal bovine serum (FBS) were obtained from Highveld Biological (PTY) Ltd., Lyndhurst, South Africa. Minimum Essential Medium (MEM) containing Earle's salt and L-glutamine, trypsin/versene and penicillin/streptomycin (5000 units penicillin/5000 µg streptomycin/ml) were purchased from Lonza BioWhittaker, Walkersville, USA. The Cell Titre 96® aqueous one solution cell proliferation assay kit was obtained from the Promega Corporation, Madison, USA. All tissue culture plastic consumables were purchased from Corning Incorporated, New York, USA. All other chemical reagents used were of analytical grade.

## 4.3 Method

### 4.3.1 Maintenance of HEK293 and HepG2 Cells

#### 4.3.1.1 Preparation of Culture Medium

MEM powder was dissolved in 900 ml of 18 Mohm water. Thereafter, 10 mM $NaHCO_3$, 20 mM HEPES and 5 000 units (10 ml) penicillin/streptomycin antibiotic was added to the medium and the pH of the solution was adjusted to between 7.3 and 7.4. The medium was then made-up to 1 litre with 18 Mohm water and subsequently filter sterilized using a Millipore 0.22 µm bell filter and Cole-Palmer Masterflex (7017-12) peristaltic pump. The sterilized MEM was aliquoted into 250 ml autoclaved Schott bottles and left for 24 hours at room temperature (23-25°C) to assess sterility. The MEM was subsequently stored at 4°C. Prior to use, the MEM was warmed to 37°C in a water bath and supplemented with 10% FBS (complete medium).

#### 4.3.1.2 Reconstitution of Cells

A vial of cryopreserved HEK293 or HepG2 cells was removed from the biofreezer (-80°C) and placed in a 37°C water bath to thaw (± 2 minutes). The vial was subsequently removed from the water bath and subjected to centrifugation at 1000 rpm for 3-5 minutes to pellet cells. The vial was then wiped with ethanol and opened aseptically in a laminar flow cabinet. The supernatant was discarded and the cell pellet was resuspended in 1 ml of fresh complete medium (MEM with 10% FBS). This cell suspension was vortexed and then transferred to a cell culture flask containing 5 ml complete medium. The flask containing the cells was placed in a 37°C incubator. Cells were visualized using a Nikon TMS light microscope. Medium was changed every two to three days when cells were at or near confluency.

#### 4.3.1.3 Propagation of Cells

Cells near or at confluency were subjected to trypsinisation. This process involved discarding spent medium from the culture flask into a sterile waste bottle and then washing cells with 5 ml sterile phosphate buffered saline (PBS) (150 mM NaCl, 2.7 mM KCl, 1 mM $KH_2PO_4$, 6 mM $Na_2HPO_4$; pH 7.5). Thereafter 1 ml of trypsin/versene solution was added to the cells. Trypsinisation was allowed to proceed for approximately 5 minutes at room temperature and observed with a Nikon TMS light microscope. Subsequently, 2 ml of complete medium (MEM with 10% FBS) was added to the flask and then trypsinised cells were dislodged from the inner flask surface by firmly tapping the flask against the palm. The resultant cell suspension was split at a desired ratio (eg. 1 : 3) into flasks containing 5 ml complete medium each. Flasks were then incubated at 37°C and medium was changed when necessary. Confluent cells were either re-trypsinised or cryopreserved when neccessary.

#### 4.3.1.4 Cyropresevation of Cells

Confluent or semi-confluent cells were trypsinised as outlined in 4.3.1.3. The cell suspension was then subjected to centrifugation at 1000 rpm for 3 – 5 minutes. The resultant supernatant was discarded and the cell pellet was resuspended in 0.9 ml

complete medium (MEM with 10% FBS) and 0.1 ml dimethoxysulfoxide (DMSO). The cell suspension was vortexed and then aliquoted into cryogenic vials which were vacuum sealed in plastic jackets and frozen at a drop rate of 1°C per minute to -50°C using a cold probe. The frozen cells were then stored in a NUAIRE biofreezer at -80°C.

### 4.3.2 Preparation of siRNA Lipoplexes

Varying amounts of cationic liposome were added to various concentrations of either control siRNA or siTOX (25 nm, 50 nm and 100 nm) at ratios set out in Table 4.1. Lipoplex reaction mixtures were then made up to a final volume of 13 µl with HBS. Complexes were allowed to incubate at room temperature (25°C) for a minimum of 30 minutes prior to introduction into cells.

**Table 4.1:** The siRNA : cationic liposome ratios used for cell growth inhibition and transfection studies.

| **Liposome Preparation** | **siRNA :Liposome ratio (w/w) *** | | |
|---|---|---|---|
| 1. SD-CholTC | 1 : 16 | 1 : 18 | 1 : 20 |
| 2. SD-CholTT | 1 : 22 | 1 : 24 | 1 : 26 |
| 3. SD-CholQC | 1 : 20 | 1 : 22 | 1 : 24 |
| 4. SD-CholQT | 1 : 26 | 1 : 28 | 1 : 30 |

***End-point ratio as determined by gel retardation assay

### 4.3.3 Growth Inhibition Studies

HEK293 or HepG2 cells at or near confluency were trypsinised and seeded evenly into 48-well plates at a seeding density of approximately 2.8 x $10^6$ cells/unit volume for all plates. The plates containing the cells were then incubated at 37°C for 24

hours to allow for cell adherence. siRNA lipoplexes containing control siRNA were prepared as described in 4.3.1. Cells were prepared by first removing spent medium and replacing with 0.25 ml of serum-free medium (MEM without 10% FBS). The siRNA lipoplexes were then added to the wells containing the cells. Control wells containing cells alone, cells with siRNA at the 3 weight ratios and liposome alone at optimum binding amount were also set-up. The assays were carried out in triplicate. Cells were then incubated for 4 hours at 37°C, after which the medium was replenished with complete medium. The cells were then incubated for a further 48 hours at 37°C. After the 48 hour incubation period, 50 µl of Cell Titre 96® aqueous one solution cell proliferation reagent was added to all wells. Cells were then incubated for 4 hours at 37°C. A blank sample containing complete medium and the cell proliferation reagent was also prepared. Absorbance values for all samples were read on a Thermo Electron Corporation Biomate 3 UV/visible spectrophotometer at a wavelength of 490 nm.

### 4.3.4 Transfection Studies

siRNA lipoplexes containing siTOX at three concentrations were prepared as outlined in 4.3.1. As in 4.3.3, HEK293 or HepG2 cells near or at confluency were trypsinised and seeded evenly into 48-well plates at a seeding density of approximately 2.8 x $10^6$ cells/unit volume. Cells were then incubated at 37°C for 24 hours, prior to medium change and introduction of siRNA lipoplexes into wells. After a 4 hour incubation at 37°C, serum-free medium was replaced with complete medium (MEM with 10% FBS) and cells were incubated for a further 48 hours at 37°C. Following incubation, 50 µl of Cell Titre 96® aqueous one solution cell proliferation reagent was added to all wells. Cells were then incubated for 4 hours at 37°C. Absorbance values for all samples were then read as in 4.3.3.

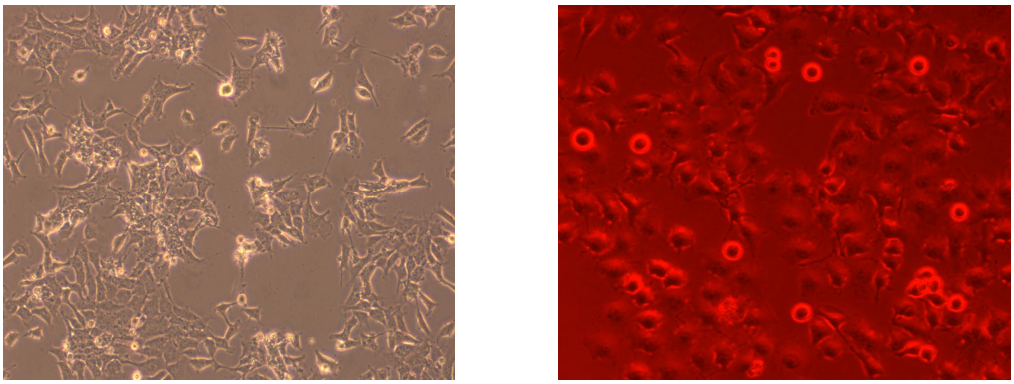
## 4.4 Results and Discussion

### 4.4.1 Maintenance of Cells

The HEK293 and HepG2 cells (Figure 4.2a and b, respectively) were successfully reconstituted, maintained and propagated during the course of this investigation. HEK293 cells are derived from human embryonic kidney cells. The cell line was initiated by transformation of HEK cells with sheared adenovirus 5 DNA. Some of the receptors expressed on this cell include: corticotrophin, muscarinic acetylcholine, sphingosine-1-phosphate and transient potential receptors, but not the ASGP receptor (www.hek293.com). As previously mentioned, HepG2 is a liver cell line derived from human hepatoblastoma (Javitt, 1990). It expresses the asialoglycoprotein (ASGP) receptor on the extracellular surface of the plasma membrane, which recognizes and binds ligands with terminal D-glucose or galactose residues (Watanabe *et al*., 2007). A single HepG2 cell contains approximately 225 000 ASGP receptors, with 80% located on the plasma membrane (Schwartz *et al*., 1982). Thus the HEK293 cells which do not express the ASGP-R were utilized as the receptor negative cell line and the HepG2 cells were the receptor positive cell line in our investigation.

Viability of cells post-cryopreservation was assessed by reconstituting 1 vial of a batch of frozen cells every 3-4 weeks. Successful reconstitution and growth of frozen cells was used as an indicator of successful cryopresevation. Both the HEK293 and HepG2 cells were grown in minimium essential medium (MEM) with 10% foetal bovine serum. The cells demonstrated a slight difference in growth pattern, as the HEK293 cells attached and multiplied at an initial rate that was faster than that of the HepG2 cells. The HepG2 cells initially exhibited slow growth. This is an observed characteristic of HepG2 cells after a prolonged period of cryopreservation. The HEK293 cells reached confluency 3 to 4 days after reconstitution and required trypsinization. The HepG2 cells reached confluency 4 to 5 days following reconstitution and subsequently required trypsinization every 2 to 3 days. The increase in HepG2 growth rate can be attributed to the increase in growth factors present in the medium over a period of time, a direct result of increased cell growth. It can be deduced that the HepG2 cells seemed to require a longer time to settle, but

once settled they grew at a faster rate than the HEK293 cells which maintained a relatively constant growth rate during the study period.



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

**Figure 4.2:** Monolayer of **(a)** HEK293 cells and **(b)** HepG2 cells at semi-confluency viewed with an Olympus microscope (at 10x and 20x magnification, repectively).

### 4.4.2 siRNA Lipoplex Formation

siRNA/siTOX lipoplexes were successfully prepared as demonstrated by cryoTEM, gel retardation, serum nuclease assay (chapter three) and transfection studies (4.3.4). A 30 minute incubation of the siRNA/siTOX with the various cationic liposome preparations (SD-CholTC, SD-CholTT, SD-CholQC and SD-CholQT) was sufficient to form the lipoplexes. As previously mentioned (3.4.1), the control siRNA was employed to monitor non sequence-specific toxicity, while siTOX was used for transfection studies.

### 4.4.3 Growth Inhibition Studies

All cationic liposome preparations exhibited low toxicity and were well tolerated by both the HEK293 and HepG2 cells, as shown in Figures 4.4 and 4.5, respectively.

Cytotoxicity is an important factor in non-viral gene therapy that is often ignored. (Liu, 2006). Naked siRNA is essentially inert when administered, however when complexed to cationic lipids it has been shown to induce an interferon response (type I and II) and STAT 1 activation (Ma *et al*., 2005). First generation cationic lipids were largely toxic to most insect and mammalian cells. Later generations, although considerably less toxic than their predecessors have been shown to reduce cell viability at higher doses (Sambrook and Russel, 2001; Zhdanov *et al*., 2001). Cationic lipids and liposomes can cause two types of toxicity in biological systems that can result in apoptosis of cells, namely: (i) aggregation, flocculation and thrombosis of blood components and (ii) solubilization, hemolysis and poration of the plasma membrane (Lasic, 1997). Cell shrinking, vacuolization of the cytoplasm, creation of transmembrane pores and reduction in mitoses cycles have been observed as toxic effects of cationic lipids (Lappalainen *et al*., 1994; Lasic, 1997). Lipoplex cytotoxicity has been attributed to cationic lipids binding to intracellular anionic lipids with a resultant compromise of cellular metabolic pathways (De Rosa *et al*., 2009). Cationic lipid toxicity is generally closely associated with the charge ratio between nucleic acid and liposome, dose of lipoplex and number of positive charges on the lipid (Dass, 2002; Lasic, 1997).

Toxicity of drugs and delivery vectors can be determined by performing thrombosis, hemolysis and cytotoxicity assays (Lasic, 1997). In our investigation we utilized the Cell Titre 96® aqueous one solution cell proliferation assay containing the [3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (MTS) reagent, which is bioreduced by viable cells into a coloured formazan product (Figure 4.3) that is soluble in tissue culture medium. The quantity of formazan produced is directly proportional to the number of living cells as measured by the absorbance at 490 nm (www.promega.com). Thus cytotoxicity and transfection efficiencies were determined by comparing cell survival rates after cell exposure to siRNA/siTOX lipoplexes versus cells with non-exposure.

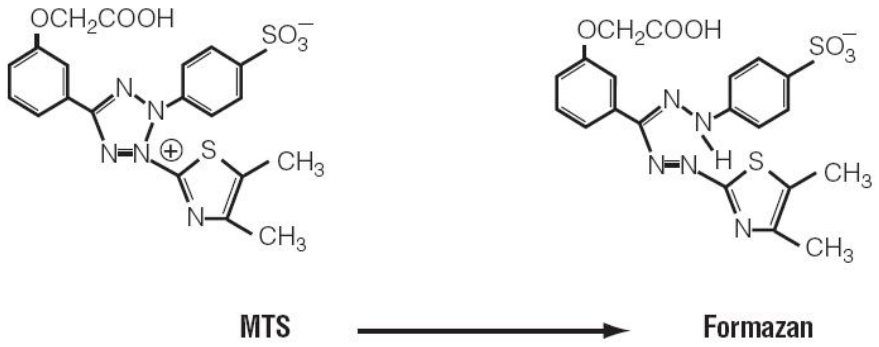
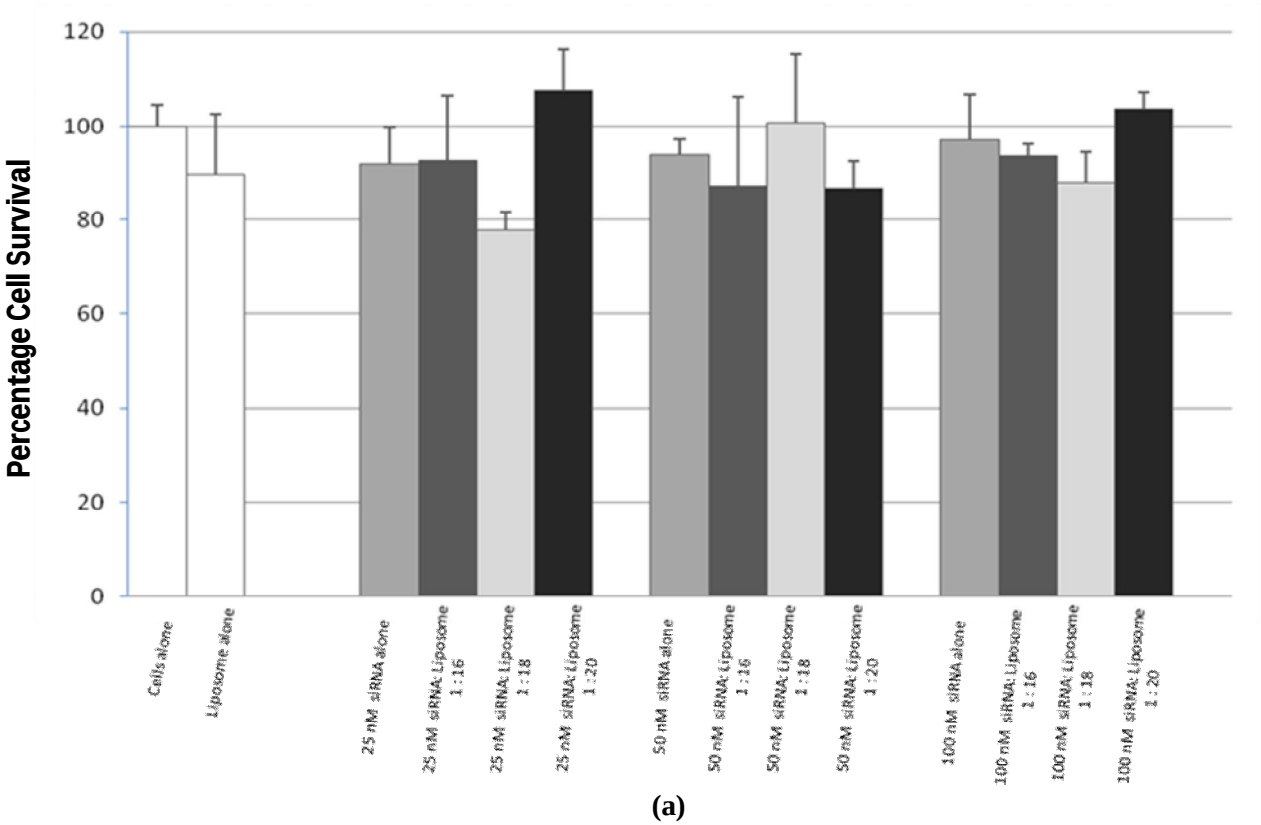
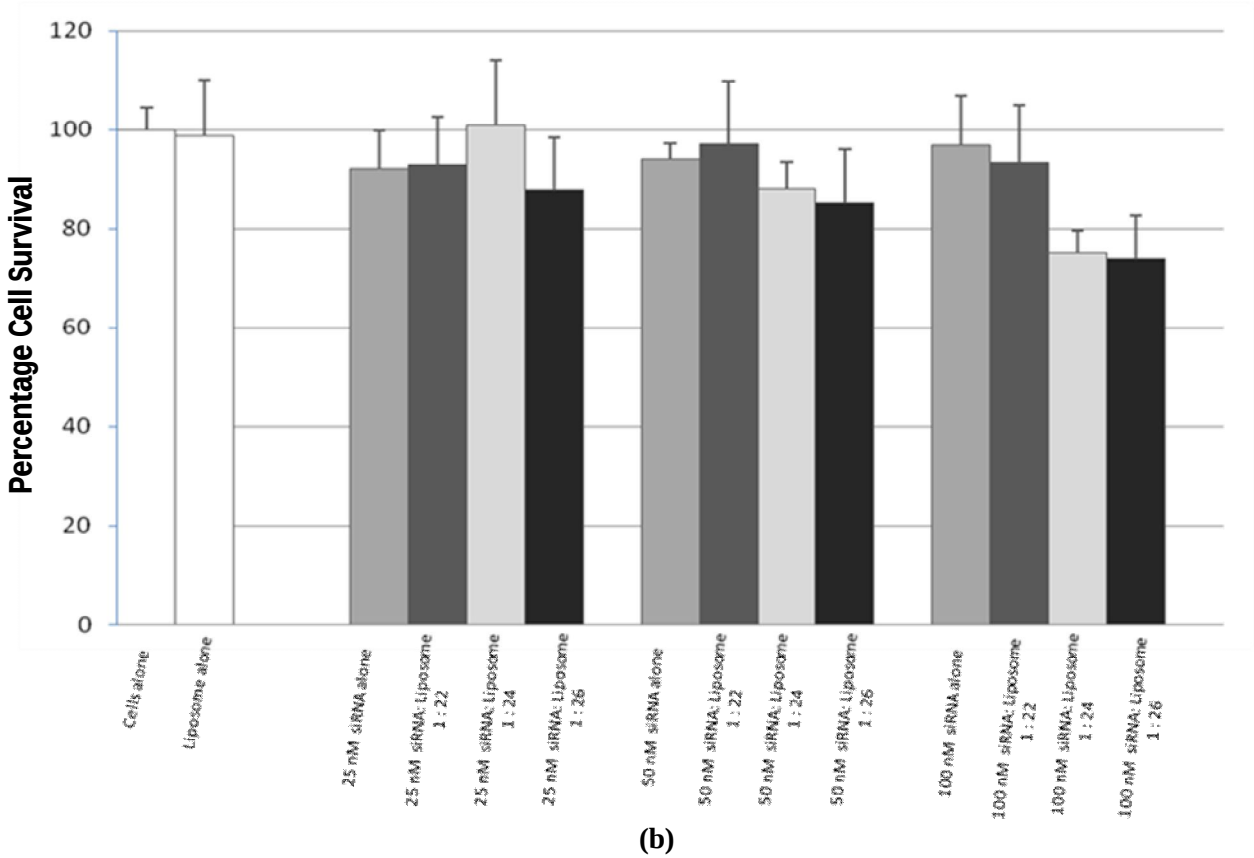



**Figure 4.3:** Structure of MTS tetrazolium reagent and its bioreduced formazan product (www.promega.com or Promega Technical Manual- Cell Titre 96® aqueous one solution cell proliferation assay).

From results presented in Figures 4.4 and 4.5 it can be seen that all lipoplexes were well tolerated by the HEK293 and HepG2 cells at the various siRNA concentrations and siRNA : cationic liposome ratios tested. Control siRNA was used in this assay to determine sequence independent cytotoxicity of functional siRNA (i.e. siTOX). There were five controls set up, i.e. untreated cells, liposome treated cells (amount at optimum binding ratio), and siRNA treated cells at three concentrations (25 nM, 50 nM and 100 nM). On average, the HEK293 cells observed an 88% cell survival when exposed to the four liposomes formulations. The HepG2 cells also observed a high average cell survival rate of 87%. The low toxicity observed is in accordance with studies by Wen *et al*. (2004), who also observed that a DC-Chol/DOPE galactosylated liposome exhibited low toxicity in HepG2 cells *in vitro*. There was little or no appreciable difference in cell mortality rates observed between the non-targeted and the targeted liposomes. This is also in agreement with the work performed by Wen *et al*. (2004). An average maximum growth inhibition of 18% in HEK293 cells (Figure 4.4 c) and 21% in HepG2 cells (Figure 4.5 c) was observed for the SD-Chol QC liposome at a siRNA : liposome ratio of 1 : 22 and 1 : 24, respectively, complexed to the 25 nM siRNA.


Percentage Cell Survival
120
100
80
60
40
20
0
Cells alone
Liposome alone
25 nM siRNA alone
25 nM siRNA: Liposome 1 : 16
25 nM siRNA: Liposome 1 : 18
25 nM siRNA: Liposome 1 : 20
50 nM siRNA alone
50 nM siRNA: Liposome 1 : 16
50 nM siRNA: Liposome 1 : 18
50 nM siRNA: Liposome 1 : 20
100 nM siRNA alone
100 nM siRNA: Liposome 1 : 16
100 nM siRNA: Liposome 1 : 18
100 nM siRNA: Liposome 1 : 20

**(a)**


Percentage Cell Survival
120
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20
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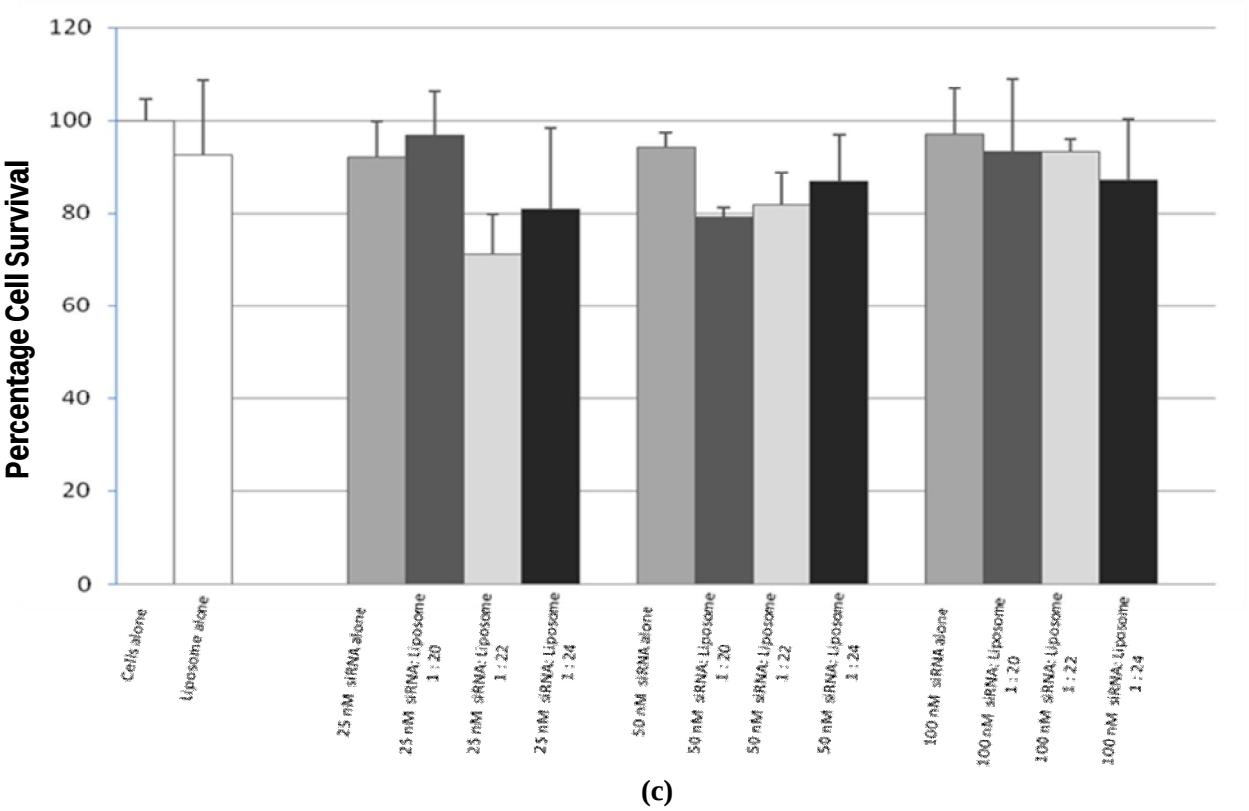
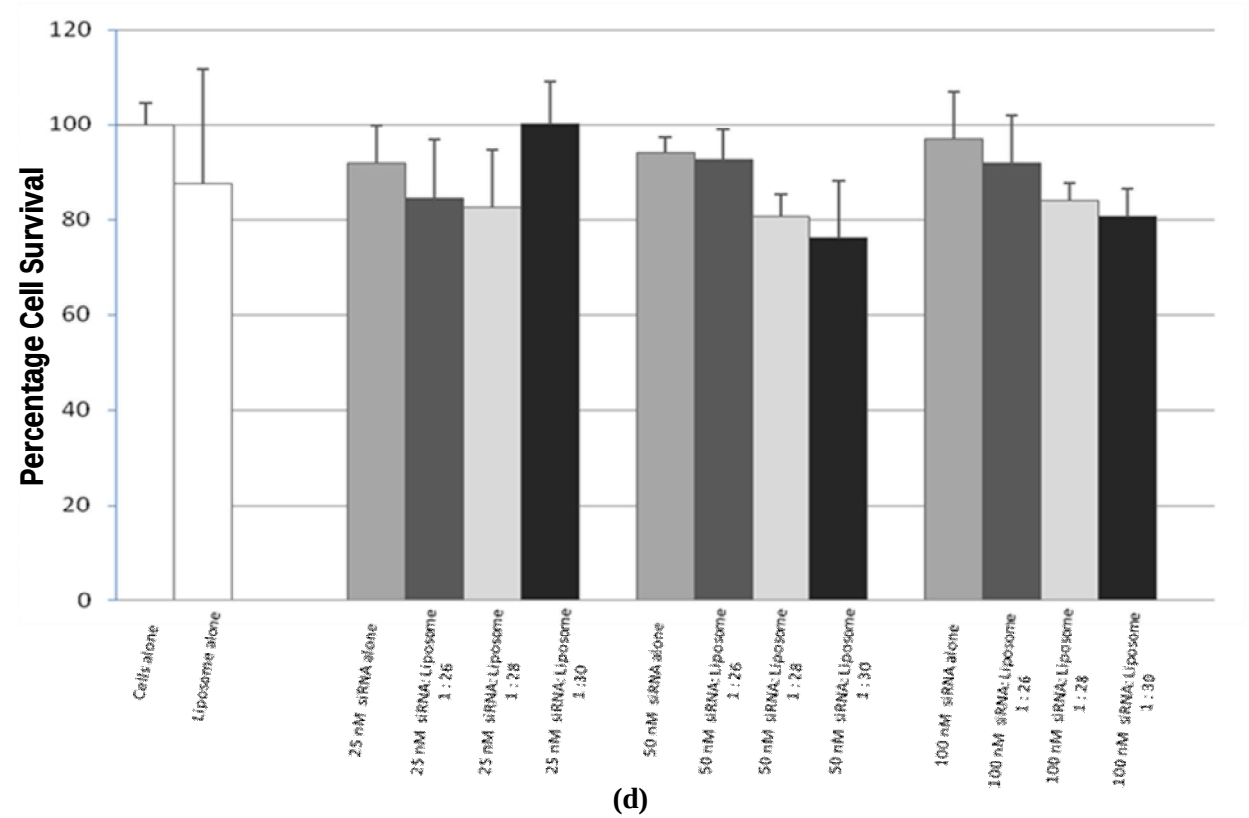
Cells alone
Liposome alone
25 nM siRNA alone
25 nM siRNA: Liposome 1 : 22
25 nM siRNA: Liposome 1 : 24
25 nM siRNA: Liposome 1 : 26
50 nM siRNA alone
50 nM siRNA: Liposome 1 : 22
50 nM siRNA: Liposome 1 : 24
50 nM siRNA: Liposome 1 : 26
100 nM siRNA alone
100 nM siRNA: Liposome 1 : 22
100 nM siRNA: Liposome 1 : 24
100 nM siRNA: Liposome 1 : 26

**(b)**

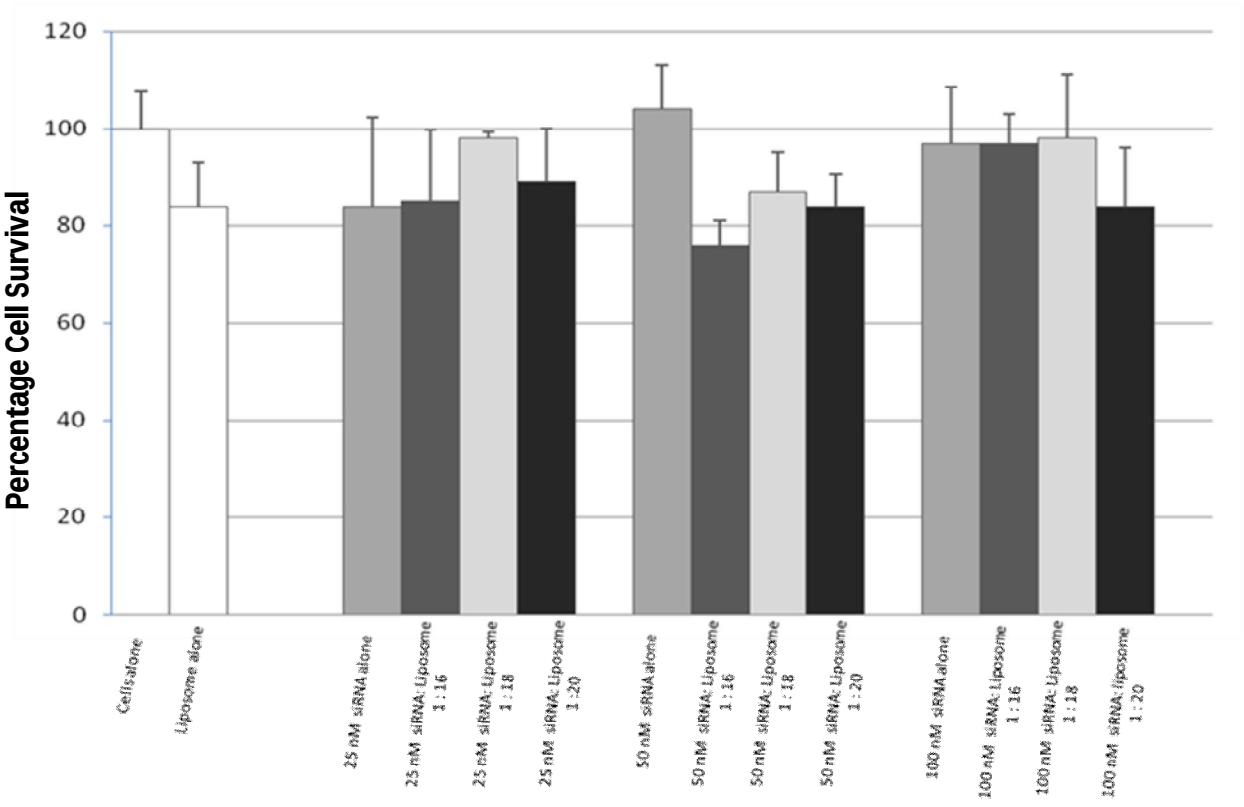



**(c)**




**(d)**

**Figure 4.4:** Growth inhibition studies of cationic liposomes in HEK293 cells *in vitro*. Control siRNA at 25 nM, 50 nM and 100 nM concentrations were complexed to cationic liposomes at varying ratios. **(a)** SD-CholTC, **(b)** SD-CholTT, **(c)** SD-CholQC and **(d)** SD-CholQT. Data is represented as a means ±SD (n=3).


Percentage Cell Survival
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Cells alone
Liposome alone
25 nM siRNA alone
25 nM siRNA: Liposome 1 : 16
25 nM siRNA: Liposome 1 : 18
25 nM siRNA: Liposome 1 :20
50 nM siRNA alone
50 nM siRNA: Liposome 1 : 16
50 nM siRNA: Liposome 1 : 18
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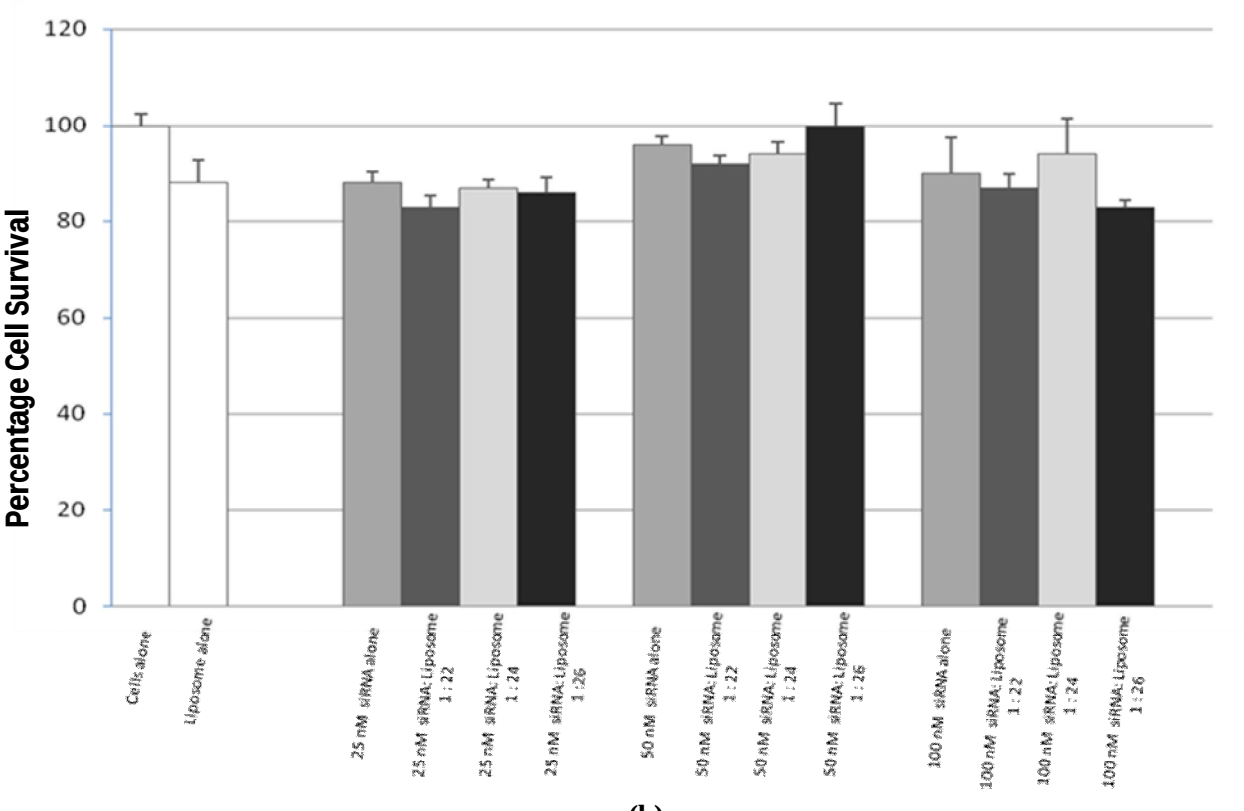
100 nM siRNA alone
100 nM siRNA: Liposome 1 : 16
100 nM siRNA: Liposome 1 : 18
100 nM siRNA: liposome 1 : 20

**(a)**


Percentage Cell Survival
120
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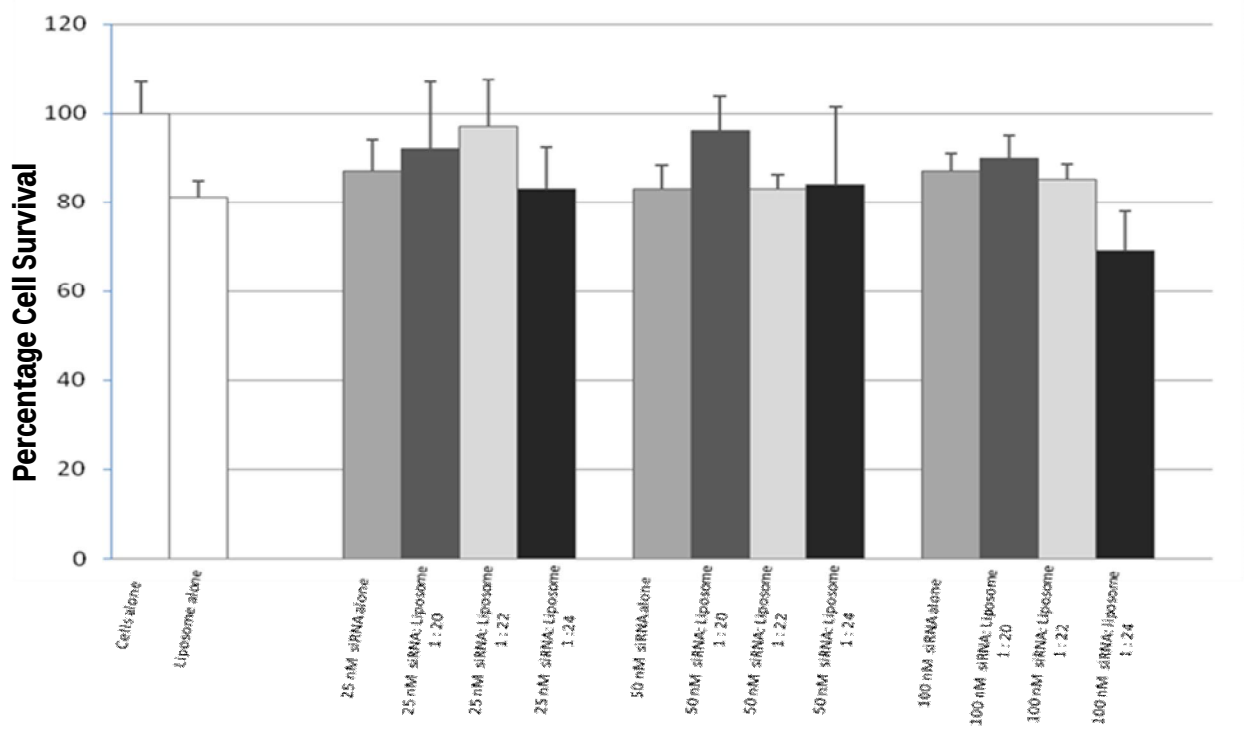
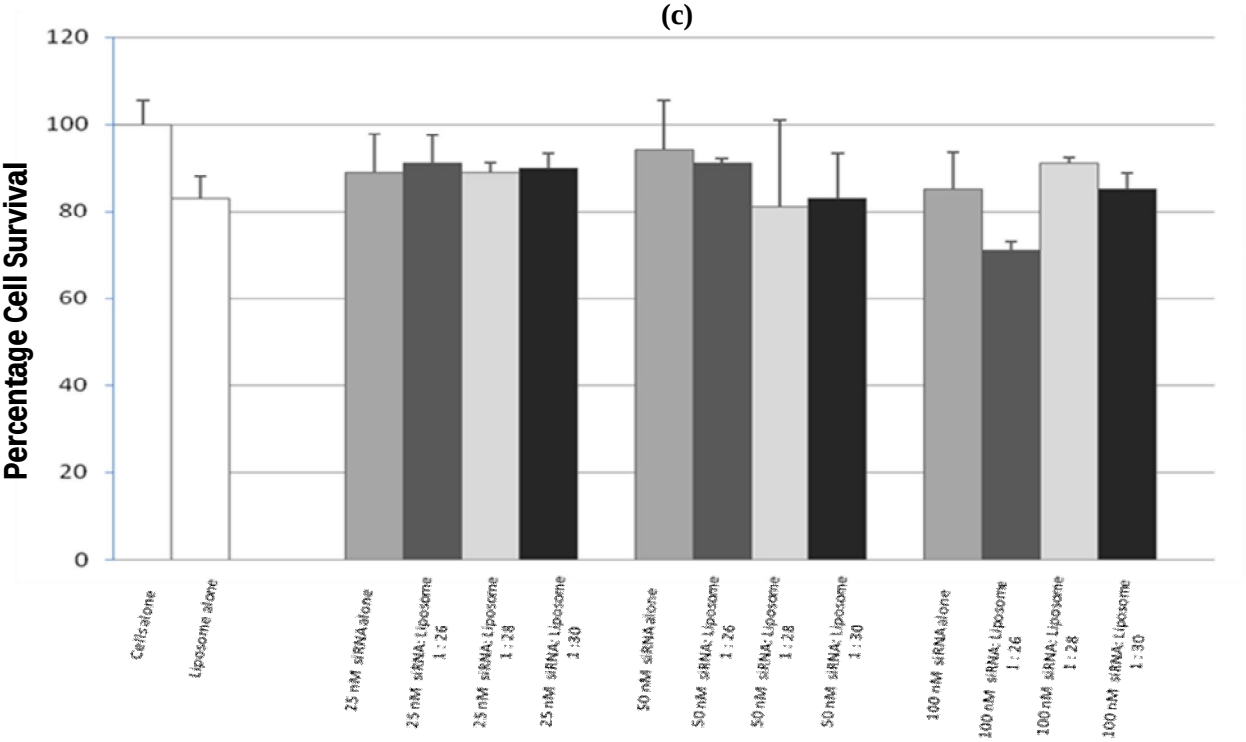
Cells alone
Liposome alone
25 nM siRNA alone
25 nM siRNA: Liposome 1 : 22
25 nM siRNA: Liposome 1 : 24
25 nM siRNA: Liposome 1 :26
50 nM siRNA alone
50 nM siRNA: Liposome 1 : 22
50 nM siRNA: Liposome 1 : 24
50 nM siRNA: Liposome 1 : 26
100 nM siRNA alone
100 nM siRNA: Liposome 1 : 22
100 nM siRNA: Liposome 1 : 24
100 nM siRNA: Liposome 1 : 26

**(b)**




**(c)**




**(d)**

**Figure 4.5:** Growth inhibition studies of cationic liposomes in HepG2 cells *in vitro*. Control siRNA at 25 nM, 50 nM and 100 nM concentrations were complexed to cationic liposomes at varying ratios. **(a)** SD-CholTC, **(b)** SD-CholTT, **(c)** SD-CholQC and **(d)** SD-CholQT. Data is represented as a means ±SD (n=3).

It is also interesting to note that the cationic liposomes containing the Chol-Q derivative exhibited a slightly higher cell mortality rate as compared with their Chol-T counterparts. This result is in agreement with studies by Bottega and Epand (1992), who showed that a quaternary ammonium head group was more toxic than a tertiary head group. An increase in liposome quantity did not result in a decrease in cell viability, suggesting that even at higher doses the cationic liposomes proved relatively non-toxic to the cells. A possible contributing factor to the observed low toxicity of the cationic liposomes could be attributed to their carbamoyl linker bond. Biodegradable bonds such as ester and carbomoyl are reported to be less cytotxic than more stable bonds (Huang, 1999). Galactosylation has also been shown to reduce membrane damaging effects typical of polycations, by affecting electrostatic interaction between cationic complexes and anionic cell surfaces (Kunuth *et al*., 2003). $IC_{50}$ (i.e. 50% cell mortality) was not seen for any of the liposome preparations at the tested ratios.

Toxicity studies are important determinants of the safety and thus viability of non-viral gene delivery vectors for future *in vivo* studies. The results of the growth inhibition assay demonstrated the relative lack of toxicity of the cationic liposomes described in chapter two.

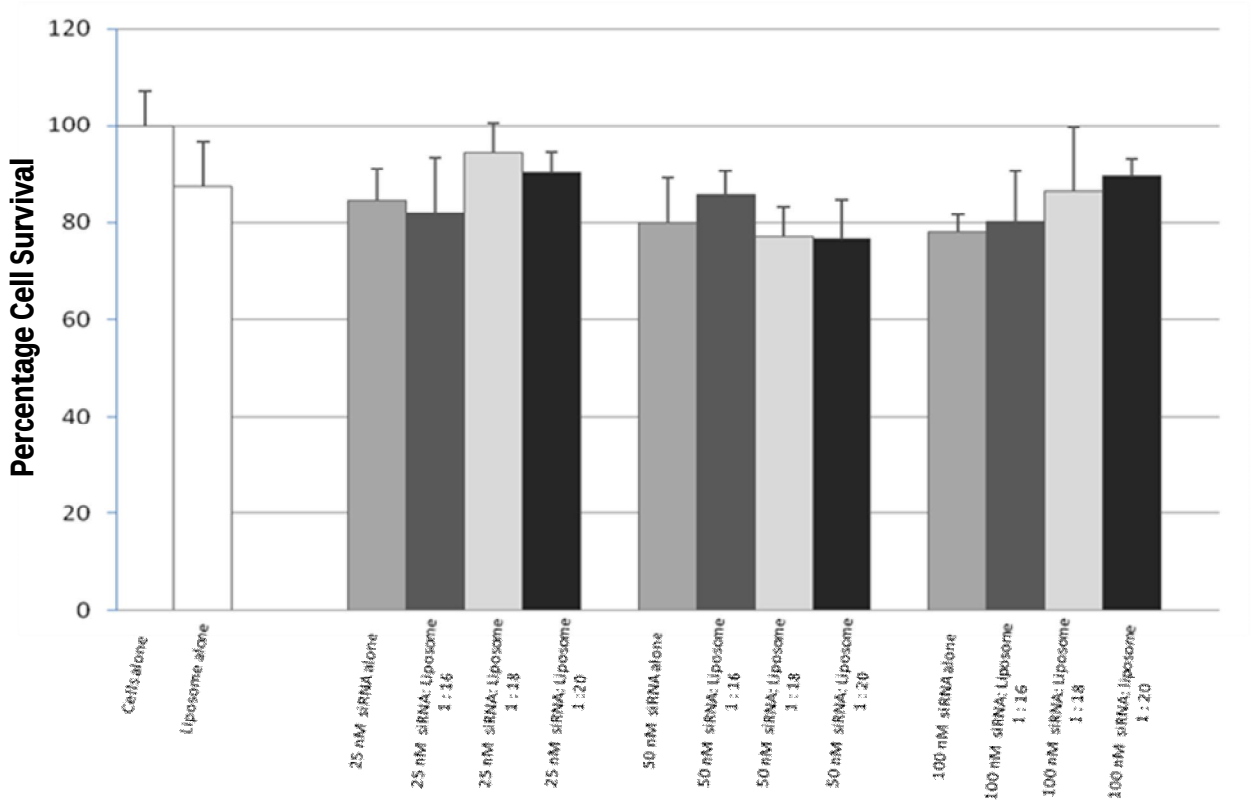
### 4.4.4 Transfection Studies

All cationic liposome preparations (SD-CholTC, SD-CholTT, SD-CholQC and SD-CholQT) demonstrated varying degrees of transfection activity as assessed by the MTS cell proliferation assay (Figures 4.6 and 4.7).

Transfection experiments were performed on HepG2 hepatocytes expressing the asialoglycoprotein receptor and on HEK293 embryonic kidney cells lacking this receptor. siRNA lipoplex transfection efficiency was evaluated by the MTS assay (4.3.3). Functional siRNA (i.e. siTOX) was substituted for the control siRNA. As

previously mentioned, Dharmacon's siTOX is toxic only when delivered to cells, with apoptosis and cell death observed within 24 – 48 hours post successful transfection. For transfection studies five controls were set up as per the cell inhibition assay. In our investigation we were unable to test mRNA down-regulation as the target mRNA was not known, however siTOX is an effective system for measurement of transfection efficacy and suitable controls (listed above, section 4.3.3) were included in transfection studies to demonstrate that a decrease in cell viability was due to successful transfection of cells by the siRNA lipoplexes. Transfection efficiency was expressed as a 'percentage cell viability' with respect to the cells alone control (100%). Yadava (2007) used a similar approach in siRNA knockdown experiments. Significance values (p) were calculated using the two tailed student's t-test for the comparison of two groups. Transfection experiments were performed in the absence of serum as it is believed that polyvalent negatively charged molecules present in serum may compete in charge interaction or coat complexes with serum components, thus hindering and reducing cellular uptake (Harrison *et al*., 1995; Madeira *et al*., 2008; Zhang *et al*., 2010).

From the results obtained it can be seen that the cells exposed to the controls demonstrated a higher cell survival than those exposed to the siTOX lipoplexes. Thus the increased cell mortality rates could be attributed to successful transfection by the cationic liposomes. The 100 nM siTOX alone demonstrated the best knockdown capability as compared with its 20 and 50 nM counterparts. However this transfection/knockdown rate was significantly lower compared with siRNA lipoplex knockdown levels. It was also observed that, on average, the siRNA lipoplexes demonstrated an increased ability (11%) to transfect the HepG2 cells as compared with the HEK293 cells. Transfection using the targeted liposomes demonstrated the highest cell knockdown in both the HEK293 (18%) and HepG2 cells (33%), although transfection efficacy was higher in the HepG2 cells. Transfection of HepG2 cells using the two untargeted liposomes, SD-CholTC and SD-CholQC, demonstrated the lowest cell knockdown activity of all liposome preparations tested (Figure 4.7, a and c).


Percentage Cell Survival
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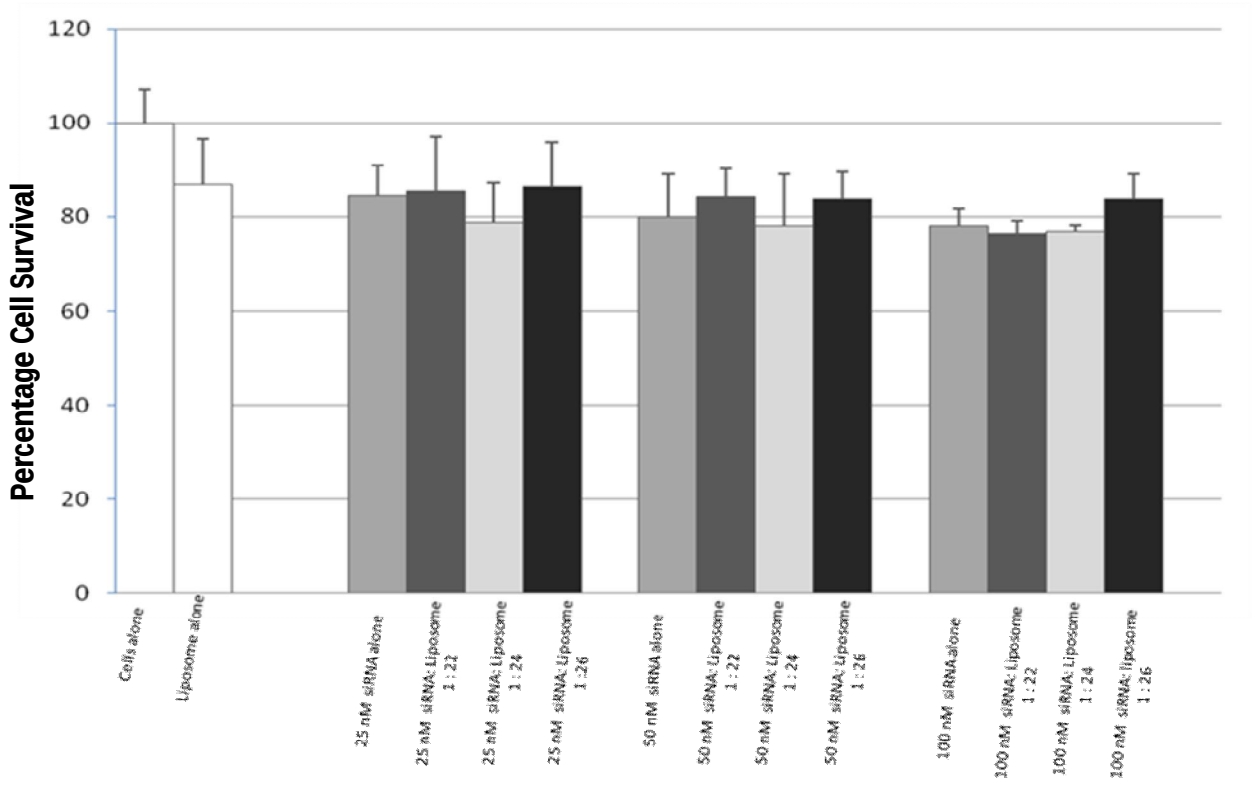
Cells alone
Liposome alone
25 nM siRNA alone
25 nM siRNA: Liposome 1 : 16
25 nM siRNA: Liposome 1 : 18
25 nM siRNA: Liposome 1 :20
50 nM siRNA alone
50 nM siRNA: Liposome 1 : 16
50 nM siRNA: Liposome 1 : 18
50 nM siRNA: Liposome 1 : 20
100 nM siRNA alone
100 nM siRNA: Liposome 1 : 16
100 nM siRNA: Liposome 1 : 18
100 nM siRNA: liposome 1 : 20

**(a)**


Percentage Cell Survival
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Cells alone
Liposome alone
25 nM siRNA alone
25 nM siRNA: Liposome 1 : 22
25 nM siRNA: Liposome 1 : 24
25 nM siRNA: Liposome 1 :26
50 nM siRNA alone
50 nM siRNA: Liposome 1 : 22
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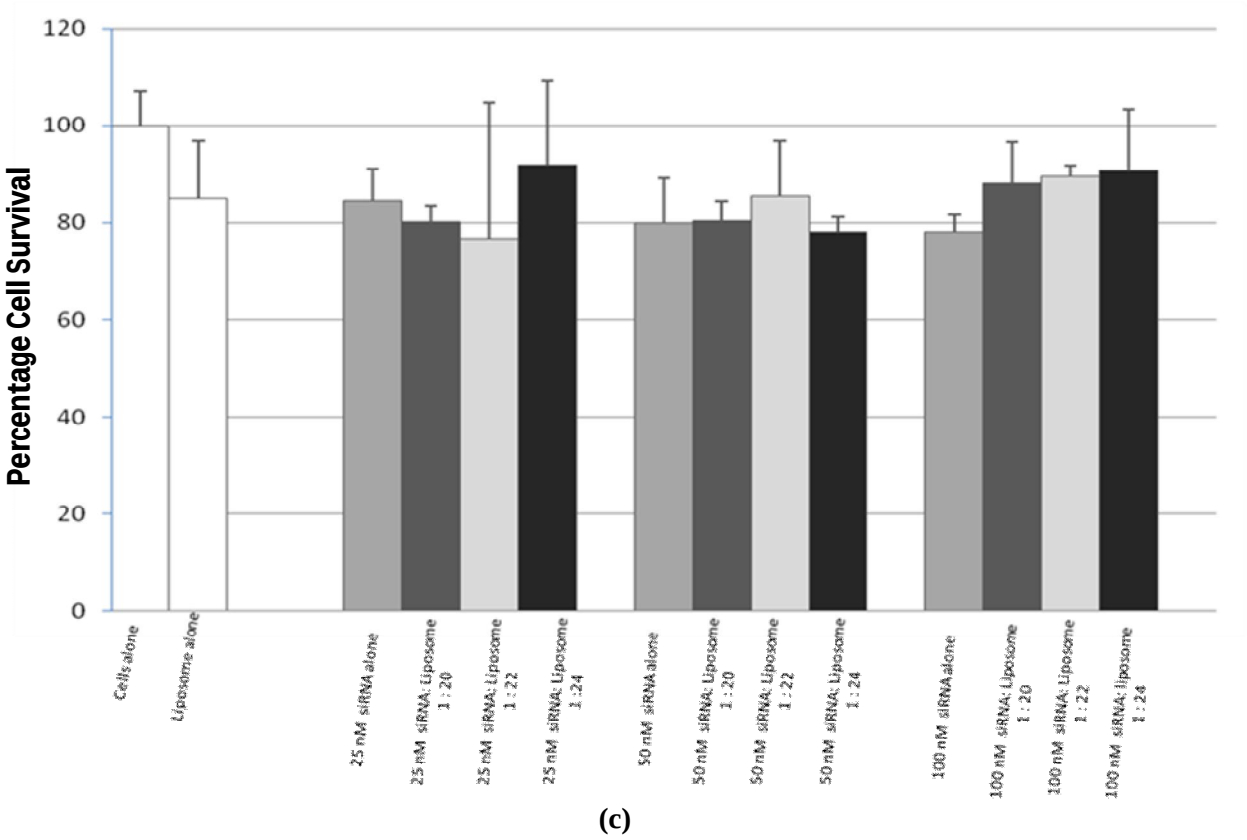
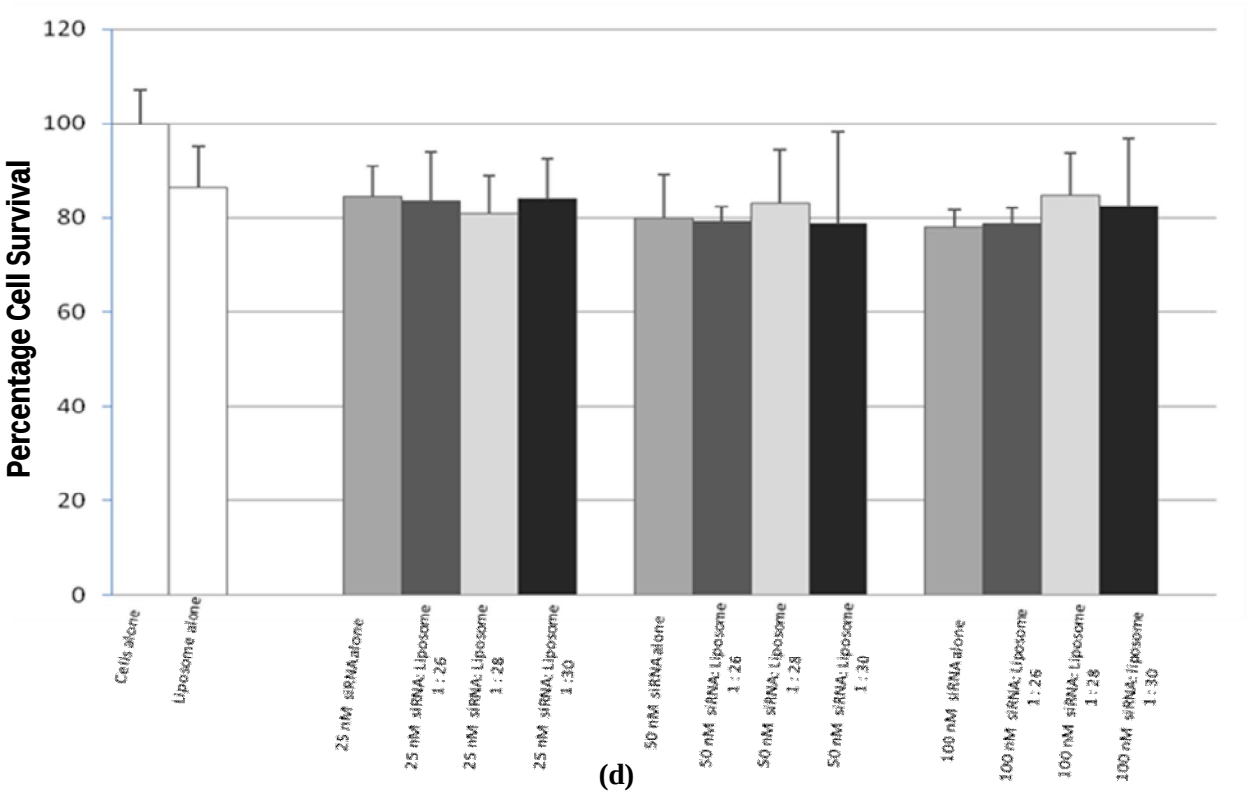
100 nM siRNA alone
100 nM siRNA: Liposome 1 : 22
100 nM siRNA: Liposome 1 : 24
100 nM siRNA: liposome 1 : 26

**(b)**

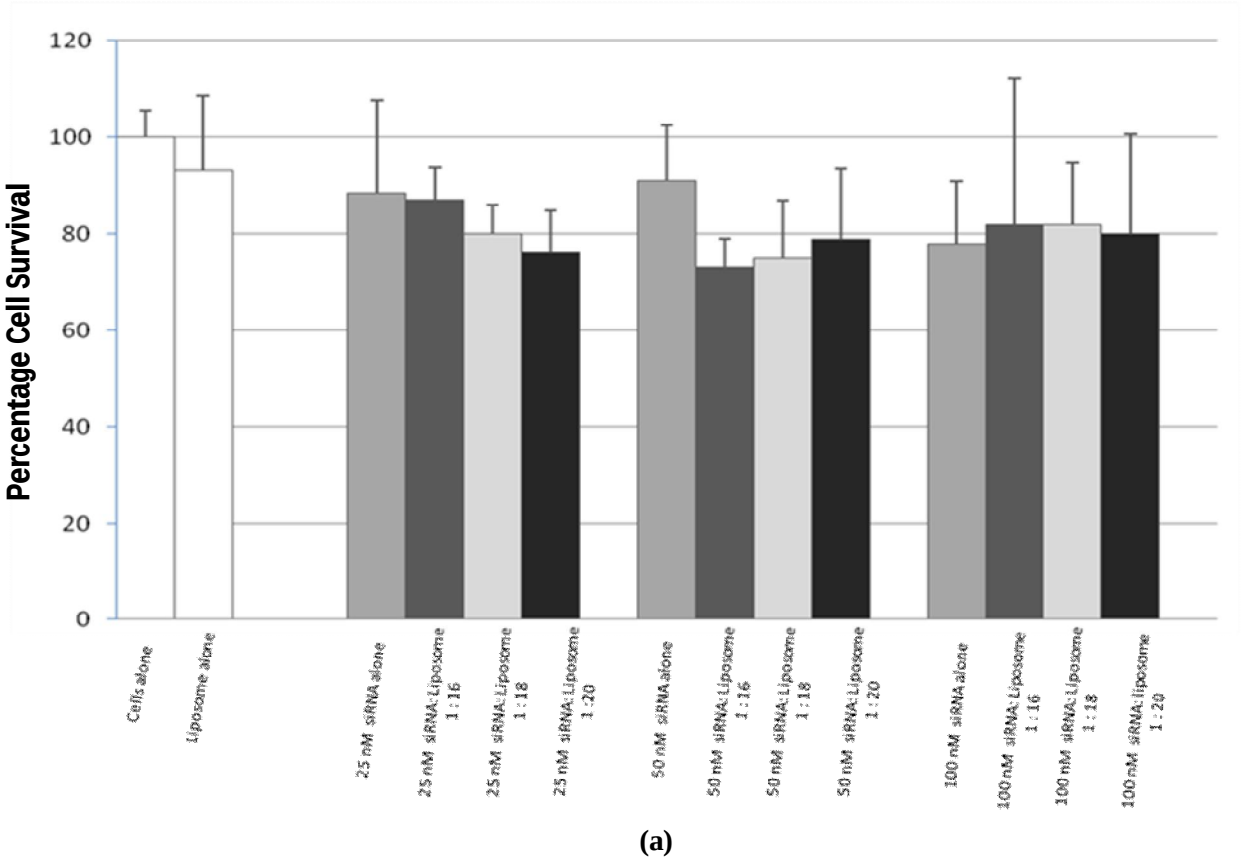



**(c)**

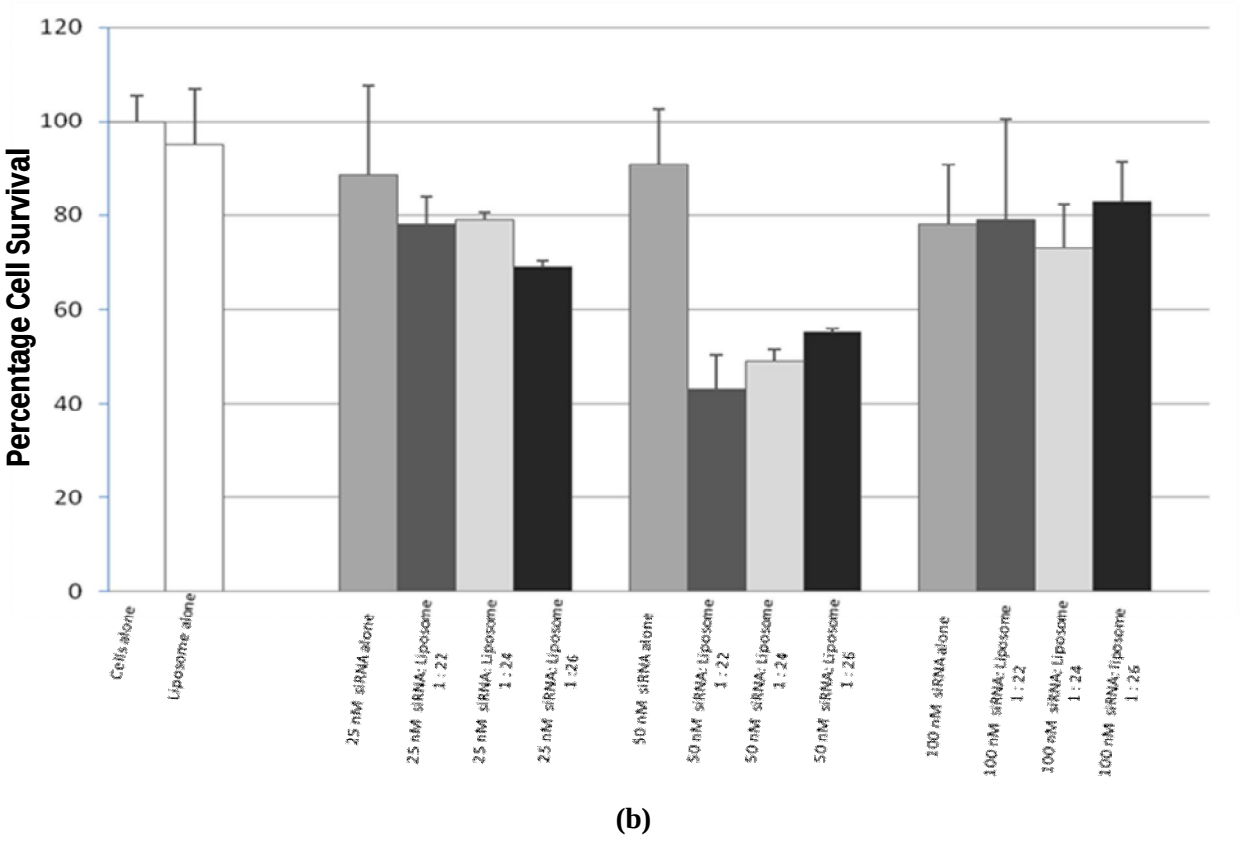



**(d)**

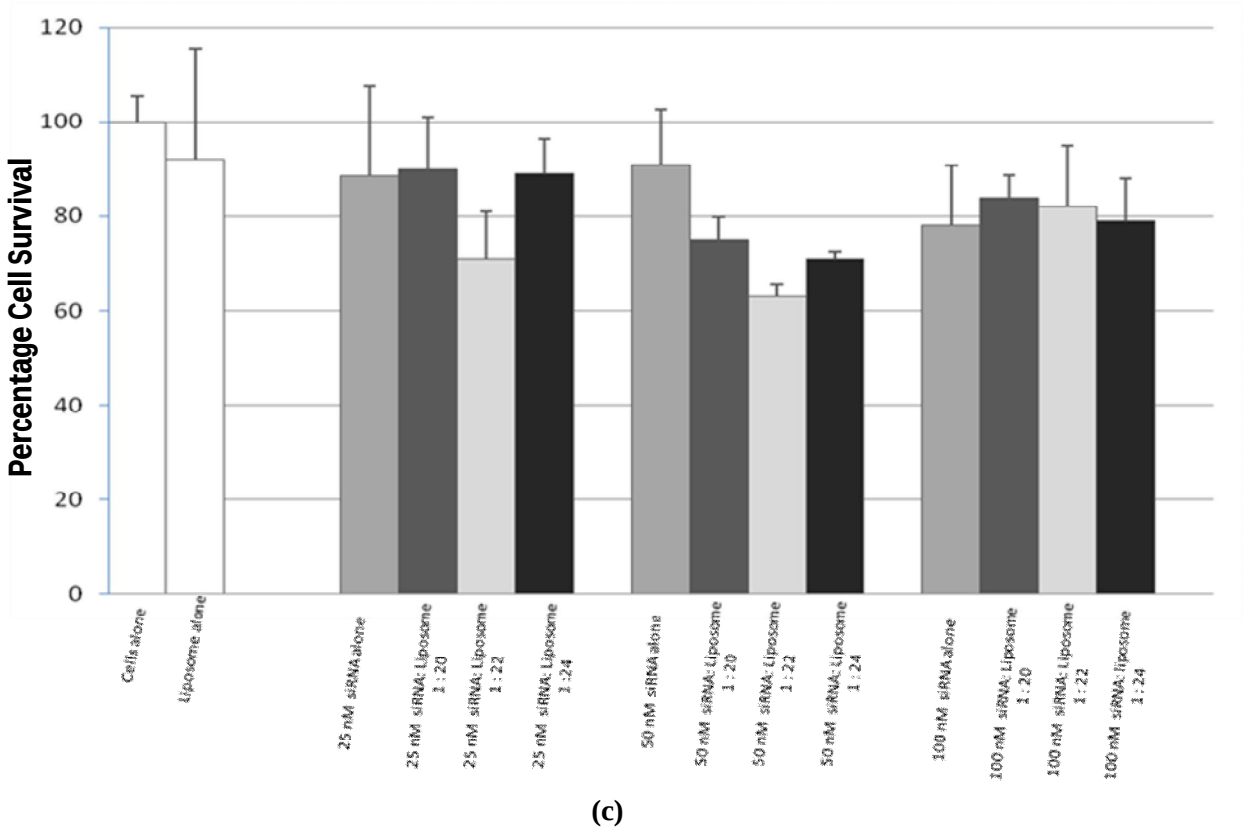
**Figure 4.6:** Transfection studies of cationic liposomes in HEK293 cells *in vitro*. siTOX at 25 nM, 50 nM and 100 nM concentrations were complexed to cationic liposomes at varying ratios. **(a)** SD-CholTC, **(b)** SD-CholTT, **(c)** SD-CholQC and **(d)** SD-CholQT. Data is represented as a means ±SD (n=3).


Percentage Cell Survival
120
100
80
60
40
20
0
Cells alone
Liposome alone
25 nM siRNA alone
25 nM siRNA:Liposome 1 : 16
25 nM siRNA:Liposome 1 : 18
25 nM siRNA:Liposome 1 :20
50 nM siRNA alone
50 nM siRNA:Liposome 1 : 16
50 nM siRNA:Liposome 1 : 18
50 nM siRNA:Liposome 1 : 20
100 nM siRNA alone
100 nM siRNA:Liposome 1 : 16
100 nM siRNA:Liposome 1 : 18
100 nM siRNA:liposome 1 : 20

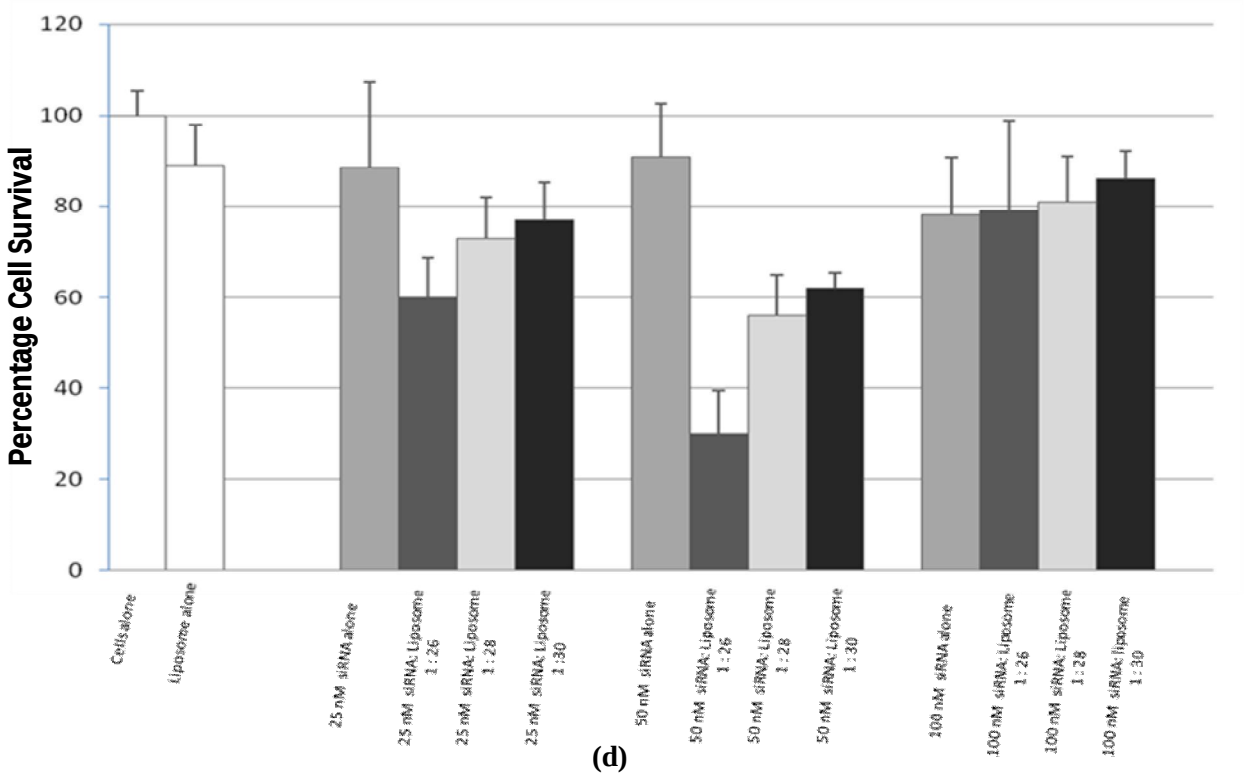
**(a)**


Percentage Cell Survival
120
100
80
60
40
20
0
Cells alone
Liposome alone
25 nM siRNA alone
25 nM siRNA:Liposome 1 : 22
25 nM siRNA: Liposome 1 : 24
25 nM siRNA:Liposome 1 :26
50 nM siRNA alone
50 nM siRNA:Liposome 1 : 22
50 nM siRNA: Liposome 1 : 24
50 nM siRNA:Liposome 1 : 26
100 nM siRNA alone
100 nM siRNA:Liposome 1 : 22
100 nM siRNA:Liposome 1 : 24
100 nM siRNA: liposome 1 : 26

**(b)**




**(c)**




**(d)**

**Figure 4.7:** Transfection studies of cationic liposomes in HepG2 cells *in vitro*. siTOX at 25 nM, 50 nM and 100 nM concentrations were complexed to cationic liposomes at varying ratios. **(a)** SD-CholTC, **(b)** SD-CholTT, **(c)** SD-CholQC and **(d)** SD-CholQT. Data is represented as a means ±SD (n=3).

SD-CholTC (Figure 4.7 a) recorded the best transfection result, a 27% decrease in cell survival in the HepG2 cells with the 50 nM siTOX at a siTOX : liposome ratio of 1 : 16. SD-CholTT (Figure 4.7 b), a 57% reduction in HepG2 cells with the same molar concentration of siTOX at a siTOX : liposome ratio of 1 : 22. A 37% decrease in HepG2 cells at a ratio of 1 : 22 was observed for the SD-CholQC (Figure 4.7 c) preparation at a siTOX concentration of 50 nM. The SD-CholQT liposome (Figure 4.7 d) demonstrated superior transfection ability, showing a 70% reduction in HepG2 cells with 50 nM siTOX at a ratio of 1 : 26.

From the results (Figure 4.8) it can be deduced that the increase in transfection efficiency observed for the targeted liposomes can be attributed to recognition and uptake by the ASGP receptor of the HepG2 cells. Moreover, overall transfection efficiencies achieved by all four siTOX lipoplexes in the receptor negative HEK293 cells were low (± 22%). An alternate experiment referred to as a competition assay may also be performed to demonstrate that receptor-mediated endocytosis enhances cellular uptake of lipoplexes. The basic premise of this assay is that a decrease in liposomal cellular uptake in the presence of increasing amounts of free galactose demonstrates the effectiveness of receptor targeting (Kunuth *et al*., 2003). ASGP receptors are known to exhibit higher affinity for the galactopyranosyl moiety with specific interaction (in part) between the sugar's 3-OH and 4-OH groups and specific carboxylate and amide groups of the receptor, with an equatorial 3-OH and axial 4-OH conformation nessessary for binding (Meier *et al*., 2000). Lee (1982) demonstrated that the equatorial 4-OH (as found in glucose) displays a weaker binding interaction than the axial 4-OH (as found in galactose). Additionally, previous work performed in our laboratory demonstrated increased transfection efficiency for the beta (β) versus alpha (α) conformation of the galactose residue. This suggests that the ASGP receptor was able to discern the C-4 epimeric and anomeric features for the hexose in direct glycosidic link to the cholesterol anchor (Singh *et al*., 2007). Thus we utilized ligands terminating in β-galactose rather than glucose residues in our investigation.

From the results, an observed trend showed that transfection efficiency is optimal using an siRNA : liposome ratio below optimum binding (as determined by gel retardation studies, chapter 3), as three of the four lipoplexes gave their highest cell knockdown rates at that particular ratio. From the results, it can also be seen that the 50 nM concentration of the siTOX demonstrated superior overall knockdown of cell viability when complexed to the cationic liposome as compared with transfection using the 20 nM and 100 nM siTOX lipoplexes. A similar result was obtained by Cardosa *et al*. (2008) who observed that an siRNA concentration of 50 nM was ideal for efficient (±50%) gene knockdown in neuronal cells with low toxicity and low non-specific effects as compared with 100 nM siRNA transfection. It is somewhat surprising to note that the 100 nM siTOX demonstrated the poorest overall knockdown ability when complexed to the cationic liposomes. A possible explanation for the observed result could be that an overly strong siRNA-cationic liposome interaction may prevent spatially appropriate release of the siRNA into the cytoplasm. Another possible reason could be that at the higher concentration, the siRNA 'competes' with itself to bind to the positive charges on the liposome, hence insufficient siRNA is bound to the liposome resulting in low transfection rates. Further study is required in this area.

The overall results (Figure 4.9) showed that the targeted SD-CholQ liposomes were more effective transfectors of the HepG2 cell as compared with the targeted SD-CholT liposomes with a 75% and 58% cell knockdown, respectively. This increased transfection efficiency could be attributed to the weaker binding interaction observed for SD-CholQ liposomes during electrophoretic gel analysis and SYBR®green displacement studies (chapter three). This weaker binding could have resulted in an easier disassembly of siRNA lipoplexes and release of siTOX into the cytoplasm for knockdown of target mRNA. This is in agreement with studies performed by Harvie *et al*. (1998) who demonstrated that weak binding interactions promoted disassembly of DNA from phosphatidylethanolamine containing lipid-based formulations.

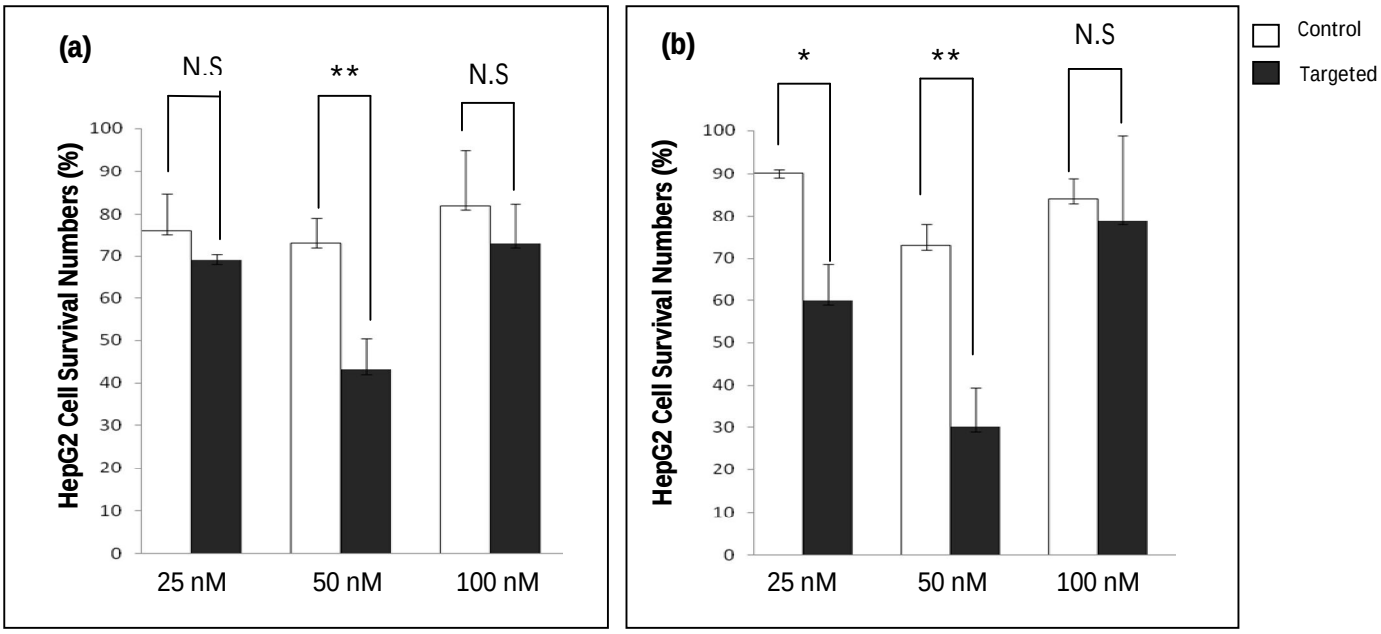



**Figure 4.8**: Summary of optimum transfection results of control versus targeted lipoplexes at the various siTOX concentrations: **(a)** Chol-T and **(b)** Chol-Q. Data is represented as a mean ±SD (n=3). Key: (N.S.) Not significant, (*) p<0.002, (**) p<0.001.

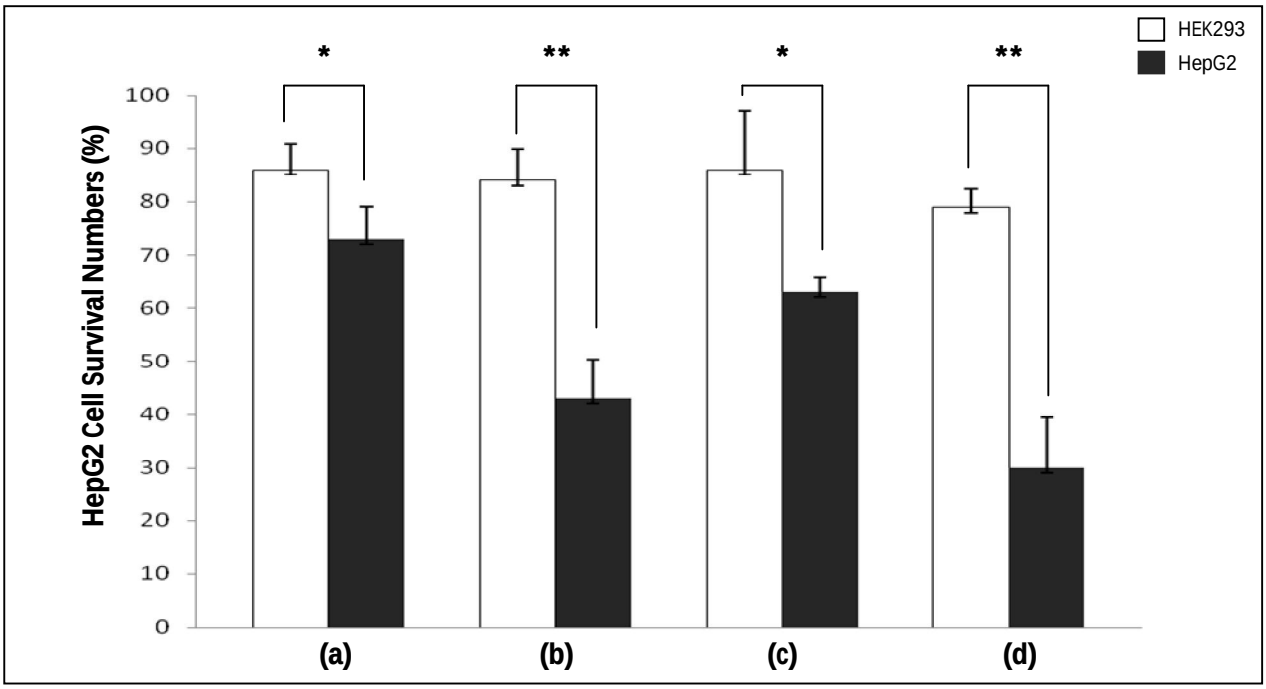



**Figure 4.9**: Summary of optimum transfection results with 50 nM siTOX complexed to: **(a)** SD-CholTC (1 : 16), **(b)** SD-CholTT (1 : 22), **(c)** SD-CholQC (1 : 22) and **(d)** SD-CholQT (1 : 26). Data is represented as a mean ±SD (n=3). Key: (*****) p<0.05, (******) p<0.001.

As previously mentioned, there are a variety of factors that can affect transfection efficiency. Three such factors are (i) cell density, (ii) assaying time and (iii) lipoplex size. (i) It has been established that low cell density may adversely affect cell recovery after transfection, while a too high cell density may inhibit cell growth and metabolism, reducing transfection rates (Dalby *et al*., 2004). Although results obtained in this study demonstrated efficient knockdown of the HepG2 cells, especially for the targeted liposomes, overall knockdown could be improved. Thus the 2.8 x $10^6$ cells/unit volume employed during this transfection study may have been too high for optimum transfection of the HepG2 cells by the cationic liposome described in chapter two; (ii) Assay results are generally assessed 24 to 48 hours following lipoplex treatment (Dalby *et al*., 2004). Kim et al. (2010) observed that treatment of Cos7 (African green monkey fibroblasts) cells with arginine/siRNA complexes gave maximum inhibition of luciferase activity after 24 hours with a return to normal luciferase levels after 48 hours. The manufacturer of the control siRNA and siTOX, Dharmacon, suggested a guideline assaying time of between 24 to 72 hours after treatment with the delivery system (siRNA technical handbook), and (iii) The effect of lipoplex size on transfection efficiency remains ambiguous. siRNA lipoplexes or complexes ranging in size from 60 nm to 600 nm have displayed efficient transfection ability with little or no difference observed between the smaller and larger aggregates (Spagnou *et al*., 2004). However, lipoplex size is still a consideration for efficient transfection. Hence further study exploring optimization of these parameters may lead to improvement in transfection activity.

In summary, the results of the transfections studies demonstrated the superior transfecting ability of the targeted lipoplexes to HepG2 cells *in vitro*. The SD-CholQ liposomes demonstrated a better overall transfection efficacy as compared with the SD-CholT liposomes. It was also ascertained that an siRNA : cationic liposome ratio below optimum binding combined with a 50 nM concentration of siTOX proved most favourable for transfection of the HepG2 cells.

# Conclusion

The potent and specific ability of siRNAs to bind to and promote degradation of target mRNAs through the endogenous biological pathway, RNAi, has immense therapeutic potential as an approach for treatment of a variety of diseases arising from aberrant protein expression (Leung, 2005; Takahashi *et al*., 2009). The efficiency of RNAi technology as a therapeutic modality hinges on a number of factors, however the crucial challenge for successful transition into clinics remains the issue of delivery (Shrey *et al*., 2007; Sioud, 2004; Takahashi *et al*., 2009). Amongst the carriers available for siRNA delivery, cationic liposomes have emerged as an attractive option owing to their versatility, relatively low toxicity and ability for cell-specific targeting. In the case of the latter, the coupling of liposomes with ligands which bind to cognate receptors on the surface of targeted cells is one of the most promising avenues for cell-specific drug and gene delivery. The ASGP receptor found on mammalian hepatocytes preferentially binds to glycoproteins that possess carbohydrate chains terminating in glucose or galactose residues, providing a unique means for the development of liver-specific siRNA delivery systems (Torchilin *et al*., 1988; Wu *et al*., 2002). Hashida *et al*. (2005) incorporated ligands with terminal galactose residues into liposomal membranes and succeeded in increasing liposomal accumulation in the liver. Although numerous cationic lipids (formulated into liposomes) commonly used for conventional DNA transfection have been employed for siRNA transport, an apparent need still exists for development of new cationic lipids for improved siRNA delivery (Desigaux *et al*., 2007).

In this investigation, novel hepatotrophic liposomes containing the cytofectins Chol-T or Chol-Q, and DOPE with and without cholesteryl-β-D-galactopyranoside were prepared by the thin film hydration technique. These cationic liposomes proved simple and quick to prepare. The liposomes were subsequently characterised by cryoTEM, revealing a relatively uniform size range of 80-150 nm and the presence of deformable vesicles for all four preparations. Gel retardation and SYBR®green

displacement studies demonstrated the ability of the liposomes to form lipoplexes with the synthetic siRNA at a negative (-ve) : positive (+ve) charge ratio of 1 : 5 to 1 : 6. Characterisation by cryoTEM revealed the relatively homogeneous nature of the siRNA lipoplex clusters at end-point ratios with a size range of ±500 nm. The ability of the liposomes to condense siRNA was also demonstrated in the dye displacement study. Moreover liposomes were shown to offer siRNA a degree of protection from serum nuclease digestion. Growth inhibition studies demonstrated that all siRNA lipoplexes exhibited low to moderate toxicity in the concentration range selected for transfection studies in HEK293 and HepG2 cells *in vitro*. Transfection studies then revealed that the targeted SD-CholQ liposomes achieved superior cellular knockdown (75%) compared with the targeted SD-CholT liposomes (58%). These studies also clearly showed that the galactosylated cationic liposomes promoted cell knockdown almost an order of magnitude greater than the non-galactosylated controls. Highest transfection activity was achieved by the SD-CholQT liposome complexed to siTOX at the 50 nM level. Transfection efficiency was shown to increase with lipoplexes at a binding ratio below optimum. This was a trend observed for most of the liposome preparations tested.

The findings of this study suggest that the galactosylated liposomes, SD-CholTT and SD-CholQT are highly promising and potentially viable vehicles for the delivery of siRNA to hepatocytes. The siRNA lipoplexes should be further evaluated with the aim of ultimately achieving *in vivo* application. Further study should concentrate on optimization of parameters influencing transfection efficiency, such as cell density, lipoplex size and galactose density. Specific gene knockdown of a known mRNA target sequence should also be undertaken. Liposomal formulation development is a major focus of RNAi delivery. This rapidly evolving and exciting field of research is expected to find clinical application in the near future.

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www.promega.com/.../cell.../cell.../celltiter-96-aqueous-one-solution-cell-proliferation-assay-_mts_/
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# APPENDIX A

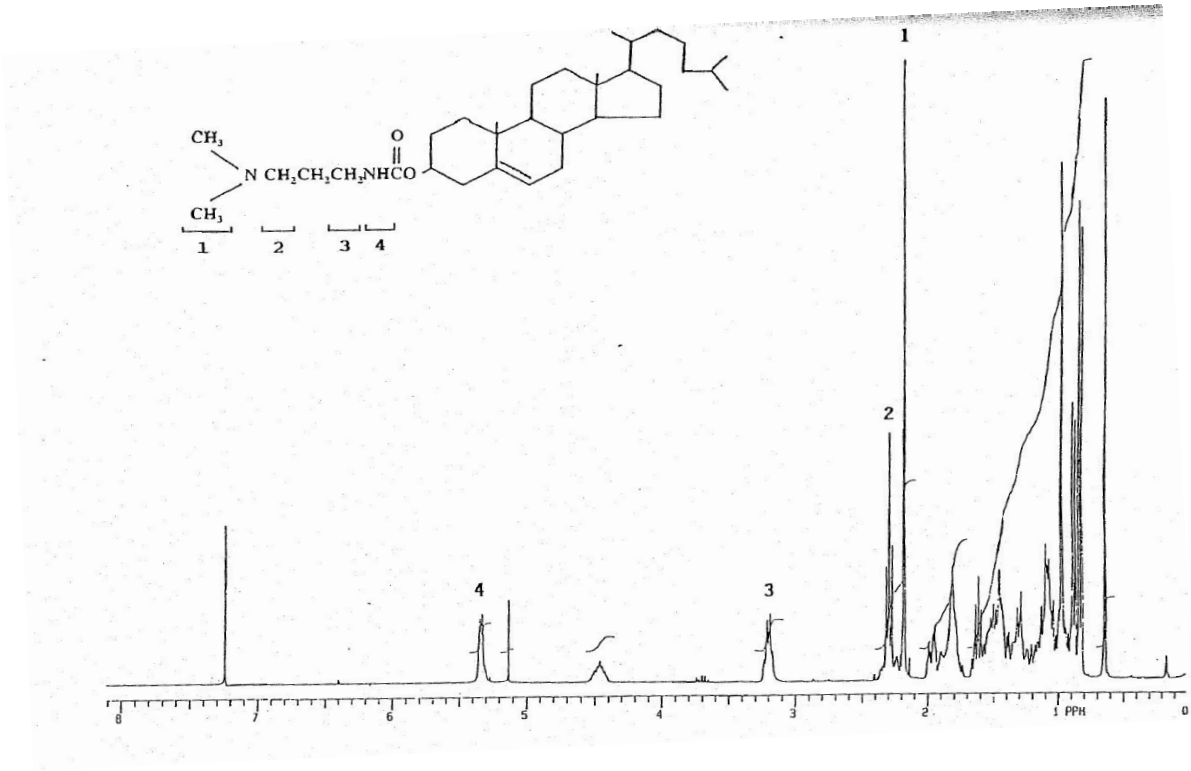


**Figure 1:** $^{1}$H NMR spectra of 3β [N-(N',N'-dimethylaminopropane)- carbamoyl] cholesterol (Chol-T).

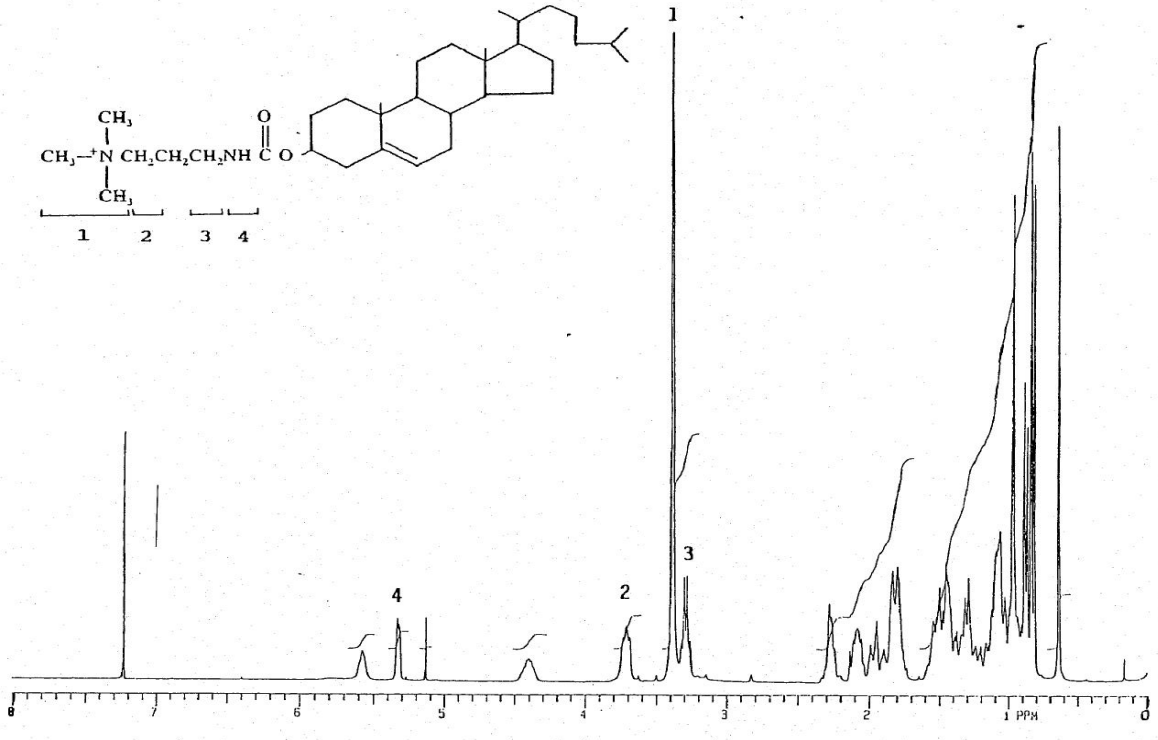


**Figure 2:** $^{1}$H NMR spectra of3β [N-(N',N',N'-trimethylammonium propane iodide)-carbamoyl] cholesterol (Chol-Q).

# APPENDIX B

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*Full Length Research Paper*

## Rapid and sensitive fluorometric analysis of novel galactosylated cationic liposome interaction with siRNA

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**RNA interference (RNAi) is being harnessed for application in the gene knockdown approach to the treatment of disease conditions. Targeted delivery of small interfering RNA (siRNA) is however an important consideration for application of this technology. We report here on the preparation of two new hepatocyte-directed liposomes designed for this purpose, containing the cholesteryl cytofectins 3β[N-(N', N'-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) and 3β[N-(N', N', N'-trimethylammonium propane)-carbamoyl] cholesterol iodide (Chol-Q). Further we describe a simple yet sensitive fluorometric assay based on the displacement of siRNA-bound SYBR Green, to monitor the association of the duplex RNA with cationic liposomes under the conditions of high dilution encountered in transfection experiments.**

**Key words:** siRNA, cationic liposomes, SYBR green, displacement assay.

### INTRODUCTION

The discovery of the RNA interference (RNAi) pathway, in which small double stranded RNA molecules named small interfering RNAs (siRNAs) are incorporated into RNA-induced silencing complexes (RISC) that bind to cognate sequences in targeted mRNA molecules destined for degradation, has revolutionized the field of nucleic acid therapeutics. Although the efficacy of siRNA far exceeds that of their classical single-stranded antisense oligodeoxynucleotide counterparts, the polyanionic nature of the molecules renders them poorly permeable to the plasma cell membrane (Watts et al., 2008). Indeed, cellular uptake is an important factor in siRNA pharmacology (Fisher et al., 2007). Liposome vehiculation, which has been successfully employed to deliver vector DNA to mammalian cells, is being actively developed and adapted to improve cellular uptake of siRNAs (Keller, 2009). Furthermore, siRNA-liposome complexes (siRNA lipoplexes) have the design potential to be engineered for cell- or tissue-specific delivery by inclusion of an appropriate ligand into the liposome bilayer (Sato et al., 2007). Here we describe the formulation of two novel liver hepatocyte-targeted cationic liposomes. Formulations include the proven hepatotropic cholesteryl-β-D-galactopyranoside (CholβGal) (Singh et al., 2007) and the cholesteryl cytofectins 3β[N-(N',N'-dimethylaminopropane)-carbamoyl] cholesterol (Chol-T) and 3β[N-(N',N',N'-trimethylammonium propane)-carbamoyl] cholesterol iodide (Chol-Q) with dioleoylphosphatidyl ethanolamine (DOPE) as co-lipid. The β-D-galactopyranosyl moiety is recognized by the asialoorosomucoid receptor expressed on the plasma membrane of liver parenchymal cells (Bilder et al., 1995).

The assembly of defined siRNA lipoplexes from naked siRNA and cationic liposomes may be followed by an electrophoretic band shift assay using ethidium bromide or SYBR Green staining to locate siRNA on gels (Zhang et al., 2006; Buyens et al., 2008). We report here a sensitive fluorometric assay in which liposome binding of siRNA may be monitored directly in solution by siRNA-bound SYBR Green displacement. Furthermore we show a close correlation with results obtained by band shift assays on 2% agarose gels.

### MATERIALS

SYBR Green II RNA gel stain (10 000x concentrate in DMSO) was from Cambrex Bio Science Rockland Inc. (Rockland, ME). Agarose (ultrapure, DNA grade) was purchased from BioRad (Richmond CA). Dioleoy-L-α-phosphatidyl ethanolamine was from SIGMA

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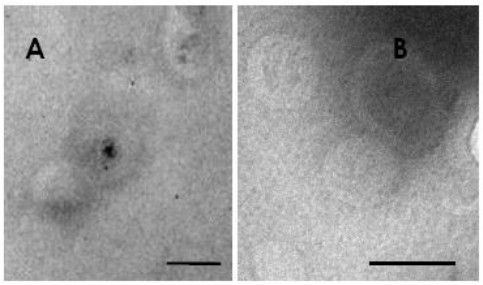


**Figure 1.** Transmission electron micrographs of targeted cationic liposomes. DOPE (2 µmole), CholβGal (0.5 µmole) and Chol-T (2 µmole) or Chol-Q (2 µmole) were deposited as a thin film in a test tube by rotary evaporation from chloroform: pyridine solution (12:1, v/v, 1.3 ml). Residual solvent was removed by overnight evacuation in a drying pistol. The film was rehydrated at 4°C for 14 h in a solution containing 20 mM HEPES, 150 mM NaCl (pH 7.5). The suspension was vortexed and sonicated in an Elma Transsonic bath-type sonicator for 5 min at 21°C. Lipo-T and Lipo-Q liposome preparations were routinely stored at 4°C and remained stable for several weeks. Liposome suspensions (1 µl) were applied to Formvar coated copper grids. After removal of excess liquid by blotting, grids were plunged into liquid ethane at -183°C. The vitrified samples were then viewed in a JEOL JEM-1010 transmission electron microscope at 100 kV. A Lipo-T (bar = 50 nm); B Lipo-Q (bar = 100 nm).

Chemical (St Louis, MI) and siGENOME non-targeting siRNA was obtained from Thermo Scientific Dharmacon Products (Lafayette, CO). 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) was supplied by Merck (Darmstadt, Germany). Chol-T was prepared from cholesteryl chloroformate as described elsewhere (Singh et al., 2001) while Chol-Q was synthesized by methylation of Chol-T (Kisoon et al., 2002).

## RESULTS AND DISCUSSION

### Liposome preparation and characterization

Much interest is being expressed in the synthesis of novel cationic cholesteryl cytofectins for the manufacture of liposomes with the design capacity for in vivo delivery of siRNA (Islam et al., 2009; Carmona et al., 2009; Han et al., 2009). This class of molecule forms stable liposomes, when formulated with equimolar or near equimolar amounts of the neutral lipid DOPE. The Chol-T cytofectin contains a cholesteryl hydrophobic anchor, three carbon spacer and a dimethylamino head group which is protonated and therefore cationic at pH 7.5. The Chol-Q bears a close resemblance to Chol-T although importantly, it carries a quaternary trimethylammonium head group which is not pH sensitive. The DOPE co-lipid assumes an inverted hexagonal phase in an aqueous environment and when incorporated into membrane bilayers cause a degree of destabilization. This is a favourable property for nucleic acid delivery by liposomes as it promotes disruption of endosomal membranes following cellular uptake of lipoplexes by endocytosis. This permits the escape of the siRNA into the cytoplasm, thus avoiding lysosomal nuclease digestion. Furthermore, liposomes may be given a targeting aspect by attachment of cell or tissue-specific ligands (Shigeta et al., 2007). Here we investigate targeted liposomes formulated at a DOPE: cholesteryl cytofectin: cholesteryl-β-D-galactopyranosyl mole ratio of 4:4:1 and prepared by a method described elsewhere for the manufacture of N, N-dimethylaminopropylaminosuccinyl cholesterylformylhydrazide (MS09) containing liposomes (Singh et al., 2007). The hepatocyte-targeting component in the liposome formulation has been fixed at 11 % on a molar basis. In related studies Hashida and co-workers have incorporated into liposomes their Gal-C4-Chol hepatotropic ligand at a 5% level (Kawakami et al., 2001) while Maitani et al. (2001) have included a sterylglucoside at a 10% level. Thus relatively low levels are required to achieve the desired targeting without causing undue interference between the siRNA and the liposomal membrane cationic centres. Size distribution of the two new liposome vesicles Lipo-T and Lipo-Q, in HEPES buffered saline was found to be in the 80 - 120 nm range by TEM (Figure 1A and B). This is well within the estimated 200 nm diameter of liver sinusoidal fenestrations which must be entered for access to the hepatocytes.Images confirm that vesicles are essentially non-deformable and spherical in shape.

### siRNA lipoplex formation and charge ratios

If synthetic siRNA molecules are to be successfully packaged into siRNA lipoplexes for in vivo cell specific delivery, the correct ratio of nucleic acid to cationic carrier must be chosen to ensure maximum protection of the siRNA from serum nuclease digestion. Currently this ratio is determined in a band shift assay. In essence, fixed amounts of siRNA are separately exposed to increasing amounts of the cationic liposomes. After brief incubation, reaction mixtures are subjected to electrophoresis on agarose or polyacrylamide gels. The liposomes are too large to enter the gel and remain in application wells. Liposome-associated siRNA would therefore also remain in the well. In this 'band shift' manner the minimum amount of liposomes required to fully bind a known amount of siRNA may be established. Figure 2A shows that at a siRNA: Lipo-T ratio of 1:24 (w/w) all the siRNA is liposome-bound and remains in the well after electrophoresis on 2% agarose, while this is achieved at a ratio of 1:28 for Lipo-Q (Figure 2B). This corresponds to a siRNA (-ve): cholesteryl cytofectin (+ve), charge ratio of 1:6 in both cases. Reaction mixtures are necessarily more concentrated than those prepared for cell culture and *in vivo* experiments and may therefore afford misleading results. We report here a rapid and sensitive method of establishing the required amount of liposome

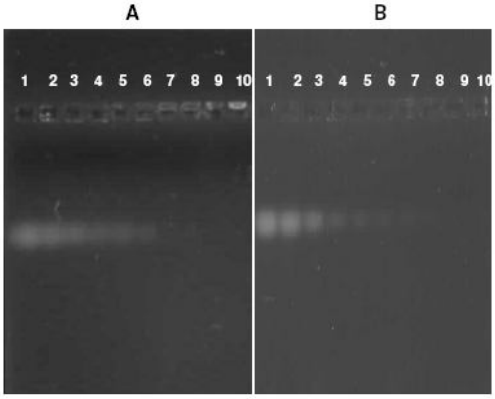


**Figure 2**. Gel retardation study of siRNA lipoplexes. Incubation mixtures (10 µl) in 20 mM HEPES, 150 mM NaCl containing siRNA (0.5 µg, 37 pmole) and increasing amounts of galactosylated cationic liposomes up to 16 µg were incubated for 30 minutes at 20°C before addition of gel loading buffer (50% glycerol, 0.5% bromophenol blue, 0.5% xylene cyanol in 2x gel buffer, 2 µl). Samples (8 µl) were subjected to electrophoresis at 50 V on 2% agarose gels in a buffer containing 36 mM Tris-HCl, 30 mM sodium phosphate, and 10 mM EDTA (pH 7.5) for 40 minutes. Gels were stained with 10 000x diluted SYBR Green stain and viewed under transillumination in a SYNGENE G-Box gel documentation and analysis system (Cambridge, UK). Lanes 2-10 contained liposomes in 1 µg increments (A Lipo-T, 7 - 15 µg; B Lipo-Q, 8 - 16 µg). Lane 1 in A and B contained siRNA alone.

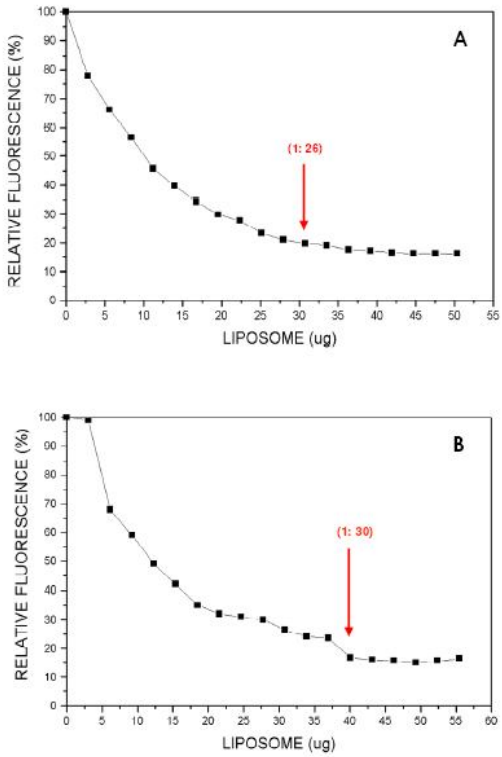


**Figure 3**. SYBR Green displacement assay. The fluorescence intensity of SYBR Green (10 000x diluted) in 20 mM HEPES, 150 mM NaCl, pH 7.5 (0.5 ml) was measured at an excitation wavelength of 497 nm and emission wavelength of 520 nm in a Shimadzu RF-551 spectrofluorometer (Japan). This was set at 0% relative fluorescence, whereupon 100 pmole siGENOME non-targeting siRNA (1.3 µg) was added and the relative fluorescence fixed at 100%. Targeted liposomes were then added in 2.5 µg aliquots in a stepwise manner to the SYBR Green-siRNA solution to a total of 55 µg and the relative fluorescence recorded at each stage.

under the more dilute incubation conditions employed in transfection experiments. The assay is based on SYBR Green displacement from as little as 100 pmole siRNA by increasing amounts of targeted cationic liposomes. The attendant reduction in SYBR Green fluorescence following dissociation from the nucleic acid is followed in a spectrofluorometer during the stepwise addition of the liposomes. Figure 3 clearly reveals that points are reached beyond which further additions of liposomes do not lead to further loss of fluorescence. These mark the points of maximum SYBR Green displacement, and complete liposome association of the siRNA. The end point ratios for Lipo-T and Lipo-Q were found to be 1:26 and 1:30 (w/w) respectively (Figure 3A and B), which is in good agreement with the values obtained by the band shift assays. Ethidium displacement assays devised to study plasmid DNA association with cationic liposomes, by comparison, employ DNA concentrations of 10 µg per ml or more (Ramezani et al., 2009; Singh et al., 2007). This is at least a 5 times higher nucleic acid concentration (on a w/w basis) than is being reported here for siRNA. The results show an excellent correlation between the band shift assay and the SYBR Green displacement assay. Moreover, the graphic presentation of dye displacement provides a clearer, unambiguous indication of the amount of liposome required to fully complex a siRNA cargo. Band shift assays typically require 8 or more separate incubation mixture in preparation for electrophoresis whereas the band shift assay reported here requires a fraction of the siRNA material, less SYBR Green and yields the desired result in a single reaction vessel in less than one hour.

## ACKNOWLEDGEMENTS

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communication is dedicated to Professor AO Hawtrey on the occasion of his 80th birthday.

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