Folate Receptor-Targeting Liposomes for the Delivery of Antisense Molecules to Cancer Cells

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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FOLATE RECEPTOR-TARGETING LIPOSOMES FOR THE DELIVERY OF ANTISENSE MOLECULES TO CANCER CELLS

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by

Colin Way

Graduate Program in Microbiology and Immunology

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Abstract

RNAi (RNA interference) is emerging as a promising tool for cancer therapy. Small interfering RNA (siRNA) molecules are activated in that pathway to reduce specific tumour cell RNAs that mediate malignancy. SiRNA treatment has been primarily limited to in vitro studies: lack of efficient, preferential in vivo delivery to target cells remains a major obstacle. Many human tumours overexpress folate receptors (FR), and siRNA-mediated reduction of thymidylate synthase (TS) sensitizes tumour cells to anti-TS drugs. I developed a folate-containing cationic liposome to preferentially deliver anti-TS siRNA to FR-expressing human tumour cells. I show, in vitro, that liposome-encapsulated siRNA (but not free siRNA) is delivered to human tumour cells, and that FR-targeting liposomes preferentially deliver siRNA into FR-positive human tumour cells. However, liposome-delivered siRNA did not reduce TS mRNA, an obstacle that must be overcome before the advantage of preferential siRNA delivery can be realized in vivo.

Keywords

Cationic liposomes, folate receptor, cancer, small interfering RNA (siRNA), RNA interference, thymidylate synthase
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List of Abbreviations

ΔΔCT – delta delta cycle threshold
5-FU – 5-fluorouracil
6-FAM – 6-carboxyfluorescein
Ago2 – argonaute-2 protein
AMD – age-related macular degenerative disease
AMEM – minimum essential medium alpha medium
ASO – antisense oligodeoxynucleotides
AuNP – gold nanoparticle
BRAF – v-raf murine sarcoma viral oncogene homolog B1
BSA – bovine serum albumin
C2 – control 2
cDNA – complimentary deoxyribonucleic acid
CH$_2$-THF – N$_5$,N$_{10}$-methylenetetrahydrofolate
Chol-siRNA – cholesterol conjugates small interfering ribonucleic acid
CNT – carbon nanotube
CPP – cell penetrating peptide
Cy3 – cyanine 3 fluorophore
DDT - dichlorodiphenyltrichloroethane
diNF-7 – influenza-derived fusogenic peptide-7
DLinDMA – 1,2-dilinoleyloxy-3-dimethylaminopropane
DMEM – Dulbecco’s modified eagle’s medium
DMSO – dimethyl sulfoxide
DNA – deoxyribonucleic acid
dNTP – deoxyribonucleotide triphosphate
DOPE – dioleoylphosphatidylethanolamine
DOTAP – 1,3-dioleoyl-3-trimethylammonium propane
DPPC – 1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DSPE-PEG-2000 – 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000

dTMP – thymidylate
dUMP – deoxyuridylate
GAPDH – glyceraldehyde 3-phosphate dehydrogenase
HER-2 – human epidermal growth factor receptor 2
ECM – extracellular matrix
EDTA – ethylenediaminetetraacetic acid
EFC – extracellular folate concentration
EP - electroporation
EPR – enhanced permeability and retention effect
f-CNT – functionalized carbon nanotube
FA – folic acid
FBS – fetal bovine serum
Fol-lip – folate decorated liposome
Fol-lip-C2 – folate decorated liposome containing control 2 siRNA
Fol-lip-Cy3-C2 - folate decorated liposome containing cyanine 3 labeled control 2 siRNA
Fol-lip-Cy3-TS - folate decorated liposome containing cyanine 3 labeled thymidylate synthase siRNA
Fol-lip-TS - folate decorated liposome containing thymidylate synthase siRNA
Folate-DSPE(-PEG_{2000}) – 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[folate(polyethylene glycol)-2000]
FR-a/β – folate receptor α/β
GFP – green fluorescence protein
GPI - glycosyl phosphatidylinositol
HRP – horse radish peroxidase
IgG\textsubscript{1}-PE – phycoerythrin-conjugated anti-mouse immunoglobulin G
IgM – immunoglobulin M
LF2K – lipofectamine 2000
mAb – monoclonal antibody
M-MLV – murine leukemia virus
MAPK – mitogen-activated protein kinase
MFI – mean fluorescence intensity
MGB – minor groove binder
Mid domain – middle domain
miRNA – micro ribonucleic acid
mRNA – messenger ribonucleic acid
NIH-III mice – National Institute of Health-3 mice
Non-fol-lip – non-targeting liposome
Non-fol-lip-C2 – non-targeting liposome containing control 2 siRNA
Non-fol-lip-Cy3-C2 - non-targeting liposome containing cyanine 3 labeled control 2 siRNA
Non-fol-lip-Cy3-TS - non-targeting liposome containing cyanine 3 labeled thymidylate synthase siRNA
Non-fol-lip-TS - non-targeting liposome containing thymidylate synthase siRNA
NTC – non-treated control
PAZ – PIWI argonaute zwille
PBS – phosphate-buffered saline
PCI – photochemical internalization
PE - phycoerythrin
PEG – polyethylene glycol
PEGylated - polyethylene glycol decorated
PEI - polyethyleneimine
piRNA – PIWI-interacting ribonucleic acid
PSMA – prostate specific membrane antigen
qPCR – quantitative polymerase chain reaction
RES – reticuloendothelial system
RISC – ribonucleic acid induced silencing complex
RNAi – ribonucleic acid interference
RNase III – ribonuclease III
RPMI – Roswell Park memorial institute medium
RT – reverse-transcribed
siRNA – small interfering ribonucleic acid
SDS – sodium dodecyl sulfate
SNALP – stable nucleic acid-lipid particles
TBE-PAGE – tris/borate/EDTA-polyacrylamide
TBS-T – tris-buffered saline with tween 20
TE – tris/EDTA
TK – thymidine kinase
TRBP – human immunodeficiency virus transactivating response ribonucleic acid-binding protein
TS – thymidylate synthase
UV-Vis – ultraviolet-visible
VEGF – vascular endothelial growth factor
Chapter 1

1 Introduction

Preamble

The purpose of this introduction section will be to establish the basic principles encompassing antisense therapy with emphasis placed on the delivery challenges limiting their effective application in an \textit{in vivo} setting. Initially, I will give a brief overview on the history (Section 1.1) and mechanism (Section 1.2) of the RNA interference (RNAi) pathway and its exploitation in disease treatment (Section 1.3). Emphasis will be placed on RNAi effector molecules: small interfering RNAs (siRNAs) and their application(s) in cancer therapeutics (Section 1.4) and specifically targeting thymidylate synthase (TS), an enzyme recognized as mediating malignant characteristics and well-established as a target of multiple anticancer drugs (Section 1.5). I will then discuss the inherent challenges to antisense delivery (Section 1.6), referencing several common delivery strategies (Section 1.7.1-1.7.2) before focusing on the use of liposomes as they pertain to this project (Section 1.7.3). Finally, I will talk about the use of targeting moieties to enhance liposome-antisense delivery (Section 1.8) and specifically the benefits and challenges of decorating liposomes with folate to exploit the folate receptor on cancer cells (Section 1.9).

1.1 Discovery and history of RNA interference

The discovery of the RNA interference pathway is the culmination of the work of many scientific groups. In an attempt to circumvent problems inherent in classical genetic analysis using mutant variants, Izant and Weintraub investigated the ability of expression vectors directing production of antisense RNA \textit{(i.e., RNA complementary to mRNA encoding information essential for production of protein)} to inhibit thymidine kinase (TK) gene activity in eukaryotic cells. They discovered a 4-fold reduction of transient TK expression in TK\textsuperscript{-} mouse L cells micro-injected with expression vectors containing DNA directing production of TK mRNA and, coincidentally, RNA antisense to TK mRNA, compared to control cells injected with the same TK mRNA expression vector but a
second vector producing control RNA that was not complementary to TK mRNA. Similarly, Fire and colleagues reported that DNA fragments inserted into expression vectors in reverse orientation could produce antisense RNA molecules that interfered with gene expression in *Caenorhabditis elegans*. In plants, posttranscriptional gene silencing was achieved through nucleic acids of either foreign or endogenous origin, where a reduction of RNA molecules similar in sequence to introduced DNA was observed. Then in 1998, a significant advancement took place in the field of RNAi when Fire and Mello reported that the introduction of only a few strands of double-stranded RNA could cause potent and specific gene inhibition in *C. elegans*. The finding of the causative agent for this phenomenon, which they called RNAi, earned them a Nobel Prize in Physiology or Medicine in 2006.

### 1.2 Mechanism of action of RNAi

Although genomic sequencing may reveal more in the future, there are currently three major classes of small regulatory RNAs that have been recognized as naturally-occurring antisense molecules: short interfering RNAs (siRNAs), microRNAs (miRNAs) and PIWI-Interacting RNAs (piRNAs) (Figure 1.1). For the purposes of this thesis, only siRNAs will be discussed in detail (all three classes involved in RNAi are reviewed in detail by Jinek and Doudna). SiRNA molecules are typically characterized as being approximately 20-30 nucleotides in length and are generated from longer double-stranded RNA precursors that are processed by Dicer, an evolutionarily conserved enzyme from the RNase III family of nucleases, in the cytoplasm of cells. Studies have shown that a significant proportion of the mammalian genome is responsible for coding endogenous antisense transcripts, however, synthetic siRNA molecules have also been constructed and transfected into cells. The resultant siRNA molecules contain a characteristic 5’-monophosphate group and 3’ dinucleotide overhang, critical for loading into the RNA induced silencing complex (RISC). In the processed siRNA duplex, one strand is preferentially selected as the “guide” strand for RISC loading, while the “passenger” strand is inevitably cleaved by the argonaute-2 protein (Ago2), thus facilitating its release and RISC activation. Asymmetrical strand selection to guide RNAi depends on the thermodynamic stability of the 5’ end with the less
Figure 1.1. Three classical categories of antisense molecules and their respective precursors. A) long double stranded RNA (dsRNA) before being processed into small interfering RNA (siRNA). B) original micro RNA containing poly-A tail and loop structure before being cleaved. The bubbles denote mismatches in base pairs and the miRNA* nomenclature indicates the passenger strand. C) PIWI-interacting RNA (piRNA) cycle regenerating antisense piRNA strands from reciprocal cleavage of sense and antisense transposon transcripts.
A. small interfering RNA

Long dsRNA

↓

siRNA

B. micro RNA

pri-mRNA

↓

miRNA-miRNA* duplex

C. PIWI-interacting RNA

Sense transposon

Antisense piRNA

→

Sense piRNA

Antisense transposon

→

Regenerated antisense piRNA
thermodynamically stable 5’ strand being selected as the guide strand. In fact, it has been shown that a difference of a single hydrogen bond can direct strand selection\(^\text{14}\). In humans, RISC loading and recruitment of Ago2 recruitment (the catalytic enzyme in RISC responsible for target RNA scission) requires association of TRBP (the human immunodeficiency virus transactivating response RNA-binding protein) with siRNA bound to Dicer. This suggests that Dicer, Ago2 and TRPB are all involved in the RISC-loading complex\(^\text{15}\). Ago2 and single-stranded siRNA together alone form a minimal RISC that could accurately cut target RNAs\(^\text{16}\), while determination of the crystal structure of Argonaute protein from \textit{Pyrococcus furiosus} revealed distinct N-terminal, middle and PIWI domains with a Piwi Argonaute Zwille (PAZ) domain connected above the base via a “stalk” region. The PIWI domain resembles ribonuclease H, suggesting its function as “Slicer” responsible for target cleavage\(^\text{17}\). Further analysis of the crystal structure of \textit{Thermus thermophilus} argonaute revealed the guide strands 5’-phosphate end is anchored in the Mid domain, while the 3’ end is anchored within the PAZ domain. This orientation permits Watson-Crick base-pairing with target mRNA between nucleotides 2-6 and cleavage at the 10-11 position\(^\text{18}\). Based on sequence similarity between the siRNA molecule and target mRNA, the mRNA transcript is either enzymatically cleaved (exact base pairing) or translationally repressed (inexact base pairing), however, the mechanism has not been completely illuminated\(^\text{19,20}\). The factor determining whether target RNA is degraded, or translationally repressed without degradation, is thought to depend on the degree of sequence matching between the siRNA and its target mRNA\(^\text{21}\). Perfect matches are thought to result in mRNA degradation while imperfect matches lead to translational repression. However, this rule is not always followed\(^\text{22}\). A schematic outlining the role of siRNA role in the RNAi pathway can be seen in Figure 1.2. Additionally, it is also important to note, especially when considering the issue of the amount of siRNA potentially needed to exert a therapeutic effect when siRNA-mediated reduction in disease-potentiating genes is proposed as a treatment strategy, that RISC is able to catalyze several rounds of RNA cleavage leading to gene silencing from only a few molecules of siRNA per cell\(^\text{2,23}\).
Figure 1.2. Schematic of siRNA being processed by Dicer and repressing mRNA translation through RISC.
1.3 siRNA therapy in disease treatment

Since every cell in the body possesses the intrinsic machinery required to process endogenous RNAi molecules and exogenous molecules designed to mimic endogenous RNAi, and any RNA sequence can theoretically be targeted, there exists a plethora of diseases resulting from abnormal gene expression that could be suitable candidates for siRNA therapy. In general, antisense research has focused on areas where standard treatment options have been relatively ineffective or where improvement is desired and required. The list of diseases, infections, and pathological conditions in this category includes, but is not restricted to, viral infection, age-related macular (AMD) degenerative diseases, cancer, (discussed further below), neurodegenerative diseases, Duchenne Muscular dystrophy, and Huntington Disease. SiRNA strategies have also become powerful research tools to explore the consequences of gene silencing on disease progression. In one study, Karlas and colleagues utilized a genome-wide siRNA library composed of ~60,000 siRNAs targeting over 20,000 gene products to determine which host cell genes are critical for Influenza A virus replication. Their conclusion was that siRNA therapy has enormous potential to generate increased understanding and to be applied to treatment of many diseases. The actual implementation of siRNA strategies is easier said than done, however, since factors such as tissue accessibility and siRNA delivery remain obstacles. Those factors are addressed below.

1.4 siRNA in cancer therapy

In Hanahan and Weinberg’s prominent paper on the Hallmarks of Cancer, one of the enabling characteristics contributing to cancer development is genome instability and mutation. A central concept is that certain mutant genotypes will provide an advantage over other cells, and that those advantageous characteristics lead to their growth and dominance in the local environment. Whether it is the activation or upregulation of certain oncogenes or the downregulation of tumour suppressor genes (both are events leading to tumour progression), both are situations where siRNA strategies may be utilized to modulate abnormally-expressed genes. Conventionally, siRNA strategies are
employed to silence mRNA targets resulting in loss of gene function, however, alternatively they may also be utilized indirectly to upregulate certain genes of interest\textsuperscript{35}. This can potentially be achieved by targeting miRNAs, which normally function to regulate over 100 different mRNA transcripts\textsuperscript{36}. This may become even more critical when trying to target disease-causing gene products previously considered “non-druggable” through application of standard strategies employing small molecules, proteins or antibodies to bind to and alter the activity of target molecules\textsuperscript{37}. In some cases, treatment with siRNAs has led to the direct inhibition of cancer cells both \textit{in vitro}\textsuperscript{38} and \textit{in vivo}\textsuperscript{39}, while in other instances the use of siRNAs has re-sensitized cancer cells to chemotherapeutic drugs that have arisen during chemotherapy\textsuperscript{38,40}. This is especially valuable when trying to reduce effective drug doses of chemotherapeutic drugs to limit side effects on patients. MacKeigan and colleagues used an RNAi screen to identify over 650 human kinases and 220 phosphatases potentially implicated in apoptosis and chemo-resistance, and were then able to subsequently downregulate a selected few and show enhanced apoptosis in cancer cells treated with low doses of the chemotherapeutic drug Taxol\textsuperscript{41}. The ability to perform these robust RNAi screens using siRNA libraries is beginning to be even more critical as improvements in sequencing has revealed the highly heterogeneous nature of many cancer types and the abundance of genetic mutations they carry\textsuperscript{42}. As a result, the development of more proficient tumour sequencing technology and the ability of siRNAs to, in theory, target any gene may allow for more effective personalized treatments where conventional therapeutic strategies are not sufficiently effective and/or are accompanied by toxicities such that cancers cannot be cured or controlled to a satisfactory degree.

\textit{1.5 Targeting thymidylate synthase (TS) in cancers}

With advancements in sequencing, bioinformatics and diagnostic tools such as those described above, genome-wide siRNA screens the discovery of cancer-associated genes is ever-growing\textsuperscript{43,44}. Some of the gene targets undergo mutations prominent in specific cancer types (for example, a BRAF mutation occurring in 66\% of malignant melanomas\textsuperscript{45}), while others are more generally implicated in cancer progression (for example, those playing prominent roles in signaling pathways that control growth and
proliferation of a large fraction of tumour types). An example of a general target receiving attention is Ras, part of the mitogen-activated protein kinase (MAPK) pathway, which is mutated in approximately 15% of human cancers\textsuperscript{44}. Another popular target is vascular endothelial growth factor (VEGF), due to its role in facilitating increased blood supply for tumour growth resulting from a transition from a dormant to active vascularization state. Reductions in tumour volume have been limited with the use of antiangiogenic agents alone. However, studies utilizing combinations of antiangiogenic molecules, such as those targeting thrombospondin-1 and VEGF, appear to be more promising\textsuperscript{46}.

A putative siRNA target that has been well implicated in many cancers is thymidylate synthase (TS). TS protein enzymatically functions to catalyze the reductive methylation of deoxyuridylate (dUMP) to produce thymidylate (dTMP) with the N\textsubscript{5},N\textsubscript{10}-methylene-tetrahydrofolate (CH\textsubscript{2}-THF) acting as the methyl group donor (Figure 1.3)\textsuperscript{47}. Both normal and cancerous cells depend upon the TS-catalyzed reaction for DNA replication and repair as it provides the only \textit{de novo} source of dTMP. With the enhanced reliance of cancer cells on TS for a source of dTMP in DNA replication during uncontrolled proliferation, TS has proven to be an attractive therapeutic anticancer target. Surprisingly, use of traditional TS protein small molecule inhibitors can actually lead to transiently elevated TS protein levels and subsequent drug resistance as depicted in several 5-fluorouracil (5-FU)-selected, drug resistant cell lines\textsuperscript{48}. This is likely due, in part, to disruption of the autoregulatory feedback loop between TS mRNA and TS protein, whereby exposure to TS protein inhibitors leads to a decrease in intracellular unbound TS, resulting in enhanced TS mRNA translation and ensuing elevated TS protein levels\textsuperscript{49}. Issues of TS protein drug resistance in many cases coupled with unwanted toxicity profiles have warranted an alternative approach to targeting TS function in cancer therapy. By targeting TS at the mRNA level problems of autoregulation and protein translation can theoretically be ablated, making an antisense approach attractive as a component of TS-targeted therapy. Initially, synthetic antisense oligodeoxynucleotides (ASOs), which facilitate mRNA degradation via ribonuclease H activation and exonuclease cleavage\textsuperscript{50}, were implemented for TS mRNA silencing due to their enhanced stability through a 2'-O-(2-methoxyethyl) backbone modification\textsuperscript{51}. These
Figure 1.3. Reductive methylation of deoxyuridylate (dUMP) to produce thymidylate (dTMP). N\textsuperscript{5},N\textsuperscript{10}-methylene tetrahydrofolate (CH\textsubscript{2}-THF) acts as the methyl group donor.
ASOs enhanced cancer cells to TS inhibitors and reduced cell proliferation when used alone in vitro\textsuperscript{52}. They were also able to reduce both TS mRNA and protein levels in tumour tissues when administered intraperitoneally\textsuperscript{52}. As an alternative to synthetic ASOs and perhaps offering a more natural and potent route\textsuperscript{53}, siRNAs have more recently been tested to reduce TS levels. In vitro, TS siRNA alone was able to reduce mRNA and protein levels over 80\% and in combination with pemetrexed significantly reduced cell proliferation\textsuperscript{28}. The effectiveness of using siRNAs targeting TS or any other gene, however, has largely been restricted to cell culture as problems arise when delivering siRNA in vivo.

1.6 Challenges associated with antisense delivery

As mentioned above, ASOs are capable of accumulating in cultured cells in the absence of delivery agent (although only at relatively high concentrations that also induce toxicity; liposomal delivery vehicles are generally required to mediate uptake of lower, non-toxic concentrations)\textsuperscript{54} and in tumour tissue in vivo, which take up ASOs relatively efficiently without the use of exogenous delivery agents\textsuperscript{55}. Alternatively, siRNAs are transfected into recipient cells in culture using a standard transfection agent such as Lipofectamine 2000. Administration of naked siRNA (no delivery agent or molecule modification) in vivo is inefficient for a number of reasons, especially when systemically administered, including: (1) limited cellular uptake across the plasma membrane due to repulsion of negative charges; (2) non-specific distribution and uptake into non-tumour tissues; (3) serum nuclease-mediated degradation; (4) reticuloendothelial system (RES) capture and renal elimination; and (5) inefficient capillary escape into tissues (extravasation)\textsuperscript{56,57}.

Consequently, to be implemented in vivo, siRNA molecules are usually administered in conjunction with some type of delivery agent. However, any systemically-delivered nanoparticle siRNA delivery agent must still overcome many hurdles: first it must evade filtration/renal excretion, phagocytosis by circulating and tissue-resident white blood cells, and degradation by plasma nucleases in the bloodstream. Thus it must be capable of movement across the vascular endothelial layer;
diffusion through the extracellular matrix (ECM); uptake by target cells; escape the endosome; and, finally, productive association with RNAi-processing complexes in the cytosol (Figure 1.4). Following systemic administration, siRNA delivery nanoparticles are preferentially taken up by the reticuloendothelial system (RES) composed primarily of macrophages and Kupffer cells present in RES organs such as the spleen and liver. This process is further enhanced by serum proteins adsorbing to the surface of nanoparticles, thus promoting opsonization (complement activation) and rapid clearance. Factors such as particle size, charge and surface properties can influence clearance and biodistribution (discussed in the section on PEGylating liposomes [Section 1.7.3]). During siRNA biodistribution, siRNA particles leave blood vessels and enter the tissue interstitium. Diffusion across capillaries results from the difference in hydrostatic and osmotic pressures on either side of the blood vessel membrane. The type of endothelium in blood vessels also comes into play as major differences occur between the three types: continuous (e.g., arteries, capillaries); fenestrated (e.g., digestive mucosa); and discontinuous (e.g., liver). One advantage when trying to target tumours with siRNAs or other therapeutic molecules is their “leaky” vasculature compared to normal tissues. Tumour microvessels can have pores ranging from 100 to 780 nm in diameter, while normal capillaries can have pores <6 nm, which is why optimizing particle sizes can become important. Leaky vasculature and lack of lymphatic drainage (characteristic of most solid tumours) has led to recognition of the Enhanced Permeability and Retention (EPR) effect, whereby systemically injected particles will tend to accumulate at the tumour site. One tradeoff, however, is that a lack of a lymphatic system actually increases interstitial fluid pressure from the centre of the tumour outward making passive diffusion of particles through the interstitium to the tumour border more difficult. Once the nanoparticle reaches the tumour cell it must cross the cell's plasma membrane into cytoplasm. This is another major advantage to using a delivery agent, as naked siRNA is not favourably taken up across anionic cell membranes due to its high molecular weight, large size, and negatively charged phosphate backbone. Functionalizing siRNA carriers with ligands or antibodies can promote specific uptake and will be explored further when discussing targeting moieties (Section 1.8). Positively charged siRNA carriers generally associate with the negative plasma membrane and are internalized in cytoplasmic lipid
Figure 1.4. Barriers associated with various siRNA delivery methods. The three major areas depicted include circulation in the bloodstream, cellular uptake and intracellular trafficking\textsuperscript{58}.
vesicles (endosomes) via endocytosis\textsuperscript{63}. Upon internalization, the endosome will typically fuse with early endosomes before maturing into late endosomes and fusing with lysosomes for degradation. It is imperative that the siRNA/nanoparticles escape the endocytic vesicle prior to lysosome processing, otherwise they will be degraded before reaching the RNAi processing complex in the cytosol. A great deal of effort has gone into understanding the principles of endosomal escape and what properties promote it. Currently, it is hypothesized that there are two modes for siRNA carriers to facilitate endosomal escape: (1) polyplexes favour the proton sponge effect and umbrella hypothesis; while (2) lipid vesicles favour membrane destabilization via ion pair formation\textsuperscript{64}. Briefly, cationic polyplexes are able to promote escape by acting as good buffering agents at pH 5-7. Normally, as early endosomes progress to late endosomes and eventually to lysosomes, the pH is lowered. However, by acting as buffers the polyplexes prevent acidification (they act as "proton sponges") in the endosomes, resulting in proton influx and osmotic swelling\textsuperscript{65}. This hypothesis has been further extended to include the umbrella hypothesis, whereby the polyplexes become protonated at a lower pH (5-6) causing a change in their conformation due to electrostatic repulsion of their recently protonated amine groups\textsuperscript{64,66}. This extension coupled with the increase in osmotic pressure due to the “proton sponge” effect is presumed to facilitate endosomal rupture and escape. Alternatively, cationic lipid vectors are thought to promote endosomal escape via interactions with the anionic phospholipids forming the endosome. Close proximity of the cationic liposome lipids and anionic endosome lipids it thought to result in destabilization and formation of cationic-anionic ion pairs\textsuperscript{67}. This new configuration presumes a cone shape and promotes the transition from a lamellar phase to an inverted hexagonal phase, which can be likened to the physical opening of a “zipper” with the siRNA content being released to the cytosol\textsuperscript{68}. Regardless of the mode of endosomal escape, this barrier remains a major obstacle to efficiently delivering siRNA to RNAi processing complexes in cells.

Currently, siRNAs in clinical trials have been limited to local administration at sites near target tissues, such as the eye. Alternatively, they have taken advantage of the natural biodistribution of nanoparticles following systemic injection (for example, in testing siRNAs for treatment of liver cancer)(see Table 1.1 for examples of various
Table 1.1. Selection of siRNA therapies using various delivery techniques that currently or recently completed clinical trials. Legend: IV – intravenous injection; IVT – intravitreal injection; VEGF – vascular endothelial growth factor; PLK1 – polo-like kinase 1; KSP – kinesin spindle protein; PKN3 – protein kinase 3; RRM2 – ribonucleotide reductase; GM-CSF – granulocyte-macrophage colony stimulating factor; EphA2 – receptor tyrosine kinase; SNALP – stable nucleic acid lipid nanoparticle; AMD – age-related macular degeneration; NAION – non-arteritic anterior ischemic optic neuropathy; term – terminated; ongo - ongoing
<table>
<thead>
<tr>
<th>Drug</th>
<th>Delivery Route</th>
<th>Target</th>
<th>Vehicle</th>
<th>Disease</th>
<th>Status</th>
</tr>
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<tbody>
<tr>
<td>Bevasiranib</td>
<td>IVT</td>
<td>VEGF</td>
<td>Naked siRNA</td>
<td>AMD</td>
<td>PhIII - term</td>
</tr>
<tr>
<td>QPI-1007</td>
<td>IVT</td>
<td>Caspase-2</td>
<td>Naked siRNA</td>
<td>NAION</td>
<td>Ph1 - ongo</td>
</tr>
<tr>
<td>TKM-PLK1</td>
<td>IV</td>
<td>PLK1</td>
<td>SNALP</td>
<td>Solid tumours</td>
<td>Ph1 - comp</td>
</tr>
<tr>
<td>ALN-VSP02</td>
<td>IV</td>
<td>KSP &amp; VEGF</td>
<td>SNALP</td>
<td>Solid tumours</td>
<td>Ph1 - ongo</td>
</tr>
<tr>
<td>Atu027</td>
<td>IV</td>
<td>PKN3</td>
<td>siRNA-lipoplex</td>
<td>Advanced solid cancer</td>
<td>Ph1 - comp</td>
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<tr>
<td>CALAA-01</td>
<td>IV</td>
<td>RRM2</td>
<td>Cyclodextrin nanoparticle</td>
<td>Solid tumours</td>
<td>Ph1 - ongo</td>
</tr>
<tr>
<td>FANG Vaccine</td>
<td>Ex vivo IV</td>
<td>Furin &amp; GM-CSF</td>
<td>Electroporation</td>
<td>Solid tumours</td>
<td>PhII - start</td>
</tr>
<tr>
<td>siRNA-EphA2-DOPC</td>
<td>IV</td>
<td>EphA2</td>
<td>Neutral liposomes</td>
<td>Solid tumours</td>
<td>Ph1 - start</td>
</tr>
</tbody>
</table>
siRNA delivery systems in clinical trials, and www.clinicaltrials.org for a complete list of siRNA therapies being tested in humans). These methods, however, are not viable for many specific cancers or other diseases, due to poor local accessibility and/or non-advantageous patterns of tissue distribution after systemic administration. After discussing the many inherent barriers involved with the systemic delivery of siRNA delivery agents it is not surprising that an ideal delivery system should possess most or all of the following attributes: (1) safety: biocompatible, biodegradable and non-immunogenic; (2) capacity to enhance tissue-specific distribution following intravenous injection; (3) deliver an effective amount of siRNA into target cells while protecting them from serum nuclease degradation and RES clearance; and (4) promote endosomal release, thus permitting the association of siRNA with RNAi machinery, and 5) be relatively simple and inexpensive to manufacture\textsuperscript{57,58}. It is important to keep these general characteristics in mind when considering the advantages and limitations to the common siRNA delivery strategies described below.

1.7 Overview of common siRNA delivery systems

SiRNA delivery systems are broadly categorized as either viral or non-viral methods. For this project, only those methods considered non-viral will be explored. The non-viral methods can be further divided into 3 categories: physical, conjugation-mediated, and carrier-mediated. The last of these three will be discussed in more depth as it relates to this project.

1.7.1 Physical delivery methods

One of the more straightforward and initial methods to deliver siRNA is via hydrodynamic injection. This method involves the intravascular injection of siRNA typically suspended in an aqueous buffer solution\textsuperscript{69}. The advantage of this method is that siRNAs can be locally administered and concentrated in specific tissues, thus avoiding many obstacles (discussed above) associated with systemic delivery strategies. Typically, in murine models, a 10% injection volume to body mass ratio is administered\textsuperscript{70}. However, and depending on the organ and injection site, this can be successfully lowered to <2%, as observed when attempting to achieve gene silencing in the liver through
hepatic portal vein injection\textsuperscript{71}. Due to its invasive nature, hydrodynamic injection is not a realistic option for treatment of many disease types. However, there are a few situations (for example, age-related macular degeneration [AMD]) where some success has been achieved. Generally, intravitreal treatment with siRNAs is well tolerated by patients and has even helped to improve visual acuity\textsuperscript{72,73}. Even though there are multiple sites surrounding the eye into which bioactive agents can be injected and from which those agents can be released into their site of action, it is still thought that the development of advanced nanocarriers can provide a more effective delivery system than ocular injections alone\textsuperscript{72}. Such nanocarriers are now receiving more attention in planning strategies to apply siRNAs in a clinical setting.

Another method, termed electroporation (EP), uses electrical pulses to facilitate the cellular uptake of material into cells\textsuperscript{74}. The pulses are believed to create transient aqueous pores in the plasmid membrane, thus making them more susceptible to transit of macromolecules such as nucleic acids\textsuperscript{75}. Its attractiveness stems from the ability to restrict delivery within a given area (electric field) with minimal side effects\textsuperscript{76}. EP may not be suitable for all situations, including treatment of tumours deep within the body. However, EP-mediated transfection of VEGF-targeting siRNA was able to suppress tumour growth by 90\% in mice\textsuperscript{77}, and dermal EP of siRNA has been optimized to reduce GFP signal by 50\% in guinea pig\textsuperscript{75}.

A final method of physical delivery worth noting is the phenomenon of mechanical massage, whereby the application of light, physical pressure to an organ enhances exogenous nucleotide uptake from intravenous circulation. Initially shown to be effective in mice, with increased renal uptake following intravenous injection and later repeated in the spleen, this method is hypothesized to physically activate certain transcription factors that increase the accumulation of nucleic acids in the cells of the massaged tissue\textsuperscript{78,79}.

\textbf{1.7.2 Conjugation-mediated delivery}

Another strategy taken to increase the delivery efficiency of siRNA is to conjugate them with a variety of moieties (\textit{e.g.}, small molecules, peptides, antibodies, or
aptamers) that improve stability and increase the time in circulation, increase the specificity of tissue accumulation, and increase cellular uptake and/or endosomal escape. For example, the small molecule cholesterol was conjugated to the 3’ end of siRNA (chol-siRNA) before being intravenously injected in mice. The modified chol-siRNA displayed enhanced serum stability, reduced plasma clearance and significant levels were detected in liver, heart, kidney, adipose and lung tissues 24 h following injection compared to the unmodified siRNA. Recently, Parmer and colleagues conjugated poly(amido amine disulfide) polymer on the 5’ end of the passenger siRNA strand to enhance endosomal lysis and escape. They also took advantage of the acidic nature of endosomes to reduce cytotoxicity by additionally conjugating an acid labile poly(ethylene glycol)(PEG) molecule to mask the lytic activity of the polymer.

Cell-penetrating peptides (CPPs) may also be complexed or conjugated with siRNA to increase their uptake across plasma membranes. They are typically amphipathic or cationic in nature, which aids them in transfer across the plasma membrane. Although still a controversial concept, it appears they are taken up via both endocytic and non-endocytic mediated pathways. Conjugation of the CPPs penetratin or transportin to siRNA showed competitive target knockdown and better sustained gene silencing over 3 days compared to commercially available lipid-mediated transfection agents (Lipofectamine) in vitro.

Association of antibodies with siRNA has been shown to enhance tissue specific targeting. Xia et al. first demonstrated in human epithelial cells an over 90% reduction in luciferase signal target following uptake of monoclonal antibody (mAb) against human insulin receptor conjugated to anti-luciferase siRNA. They expanded on this work in vivo by achieving a 69-81% reduction in luciferase gene mRNA levels in rat glial cells implanted in adult rat brains using a transferrin receptor targeting mAb conjugated to siRNA.

An emerging siRNA delivery strategy utilizes the conjugation of oligonucleotides, called aptamers, for increased delivery efficiency. Aptamers function in a fashion similar
to small molecules in that they are about the same size and therefore display similar clearance patterns. However, they also share characteristics with antibodies in that they tightly bind to targets in a specific manner and can modulate downstream pathways. Aptamers can be designed against numerous targets and have the ability to differentiate between tumorigenic and normal cells. Due to their ability to affect downstream regulators, aptamers alone are used to treat a number of diseases. However, their efficacy is believed to be improved when functionalized with other therapeutic molecules such as siRNA. The most well-studied of these aptamer-siRNA chimeric molecules are those designed to bind to cells expressing cell-surface receptor prostate specific membrane antigen (PSMA), a transmembrane protein highly expressed in human prostate cancer. “Second generation” PSMA-siRNA chimeras contain a truncated PSMA binding region that allows for large-scale synthesis while maintaining specificity; a 2-nt overhang on the siRNA 3’ end guide strand for greater accessibility to RISC; and an additional conjugation to PEG to increase serum stability for up to 5 days. These modifications resulted in significant regression of PSMA-positive tumours in athymic mice following intravenous injection. Since aptamers are composed of nucleotides they face many of the same challenges as siRNAs. It is anticipated that further modifications to both aptamers and siRNAs will lead to potent anti-cancer chimeric molecules.

1.7.3 Carrier-mediated delivery

Carrier-mediated delivery vehicles are similar to conjugated ones in that they facilitate siRNA uptake into target tissues, with the difference that the siRNA and carriers do not form covalent complexes with the siRNA as the conjugate vehicles do. There are many types of nanoparticles that could be considered carriers for siRNA. For the purposes of this project, only carbon nanotubes (CNTs), polymer carriers, and lipid-derived carriers will be discussed.

Carbon nanotubes (CNTs) are highly ordered, hollow carbon graphite nanomaterials shaped in a nano-needle structure giving them a large surface area for interactions. Their shape would suggest they facilitate cellular uptake through physical penetration of the plasma membrane. However, recent studies indicate they enter via
endocytosis. CNTs are capable of forming stable complexes with siRNA molecules through a mechanism driven by Van der Waals interaction whereby the siRNA duplex partially “unzips” and wraps around the CNT wall. Alone, CNTs display poor dispersion and solubility in aqueous solutions: functionalized CNTs (f-CNTs) have been synthesized to solve these problems. An example of an f-CNT is the addition of PEG to the outer wall, which improves overall stability and hydrophilicity. F-CNTS coated with PEG and an acid-labile disulfide group to enhance endosomal escape showed a higher silencing efficiency then the standard commercial transfection agent Lipofectamine. Much more work is needed with CNTs before they can become viable siRNA delivery vehicles in vivo.

Cationic polymer delivery agents form strong electrostatic interactions with negatively charged siRNAs, thus forming polyplexes where the siRNA becomes condensed and protected from nuclease degradation. Cationic polymers can be categorized as either natural or synthetic. Natural polymers have the inherent advantage of being biocompatible, biodegradable, and minimally toxic, while synthetic polymers, although designed to achieve high endosomal release and low enzymatic degradation, often exert undesirable toxicity. Atelocollagen, an example of a natural polymer, when complexed with siRNAs targeting candidate genes of prostate cancer and administered systemically, induced selective and efficient inhibition of tumour growth in mice. The atelocollagen/siRNA complexes also showed great stability, remaining intact for over 3 days while incurring no significant side effects or interferon response. Poly-L-lysine, or polyethyleneimine (PEI), is the most studied synthetic polymer for delivering antisense molecules. As a cationic polymer with a high charge density, PEI takes advantage of the “proton sponge effect” for efficient endosomal escape. PEIs have been synthesized with various degrees of branching and molecular weights. Generally, a branched structure results in a higher transfection efficiency, while low molecular weight structures confer lower toxicity. Urban-Klein and associates were able to reduce subcutaneous tumour growth in mice using a low molecular weight PEI complexed with siRNA targeting c-erbB2/neu (HER-2) receptor administered intravenously. The PEI/siRNA complexes were able to shield the siRNA from serum nucleases and did not induce any apparent toxicity to animals. Systemic administration of a low molecular weight/siRNA
complex targeting VEGF did not induce liver damage or induce cytokines, but did efficiently deliver siRNA to subcutaneous tumours of pancreatic and prostate origin. Recent strategies to further advance the efficiency and safety of cationic polymers include: combining them with liposomes (lipopolyplexes); functionalizing them with PEG; and coating them with gold nanoparticles (AuNPs).

Liposomes are spheroid vesicles composed of a phospholipid bilayer and aqueous core. Due to their inherent amphipathic nature and well-studied pharmaceutical properties, liposomes have became attractive carriers for siRNA and other antisense molecules. While anionic, neutral and cationic liposomes have all been synthesized for siRNA delivery and have resulted in some successes (including initiation of a clinical trial using neutral liposomes), the general emphasis has been on cationic liposomes because of their efficient delivery of siRNA to cells. This is because stable complexes can naturally form from the electrostatic interaction of the positive lipids and negative siRNA molecules; the interaction of the positive lipids and negative plasma membrane during cellular uptake; and the mechanism by which the positive lipids facilitate endosomal escape once inside the cell. The addition of negatively charged siRNA to cationic lipids spontaneously forms stable multilamellar structures that protect siRNA from serum nucleases and facilitates their uptake in cells. There are numerous cationic lipids available that vary in the number of amines in their head group capable of being protonated (an event that affects siRNA binding and overall surface charge); the orientation between the head group and backbone; and the overall nature of the hydrophobic backbone itself (length and saturation). The addition of a helper neutral lipid such as dioleoylphosphatidylethanolamine (DOPE) or cholesterol helps by allowing the complex to adopt a nonbilayer structure: this is thought to be critical for transitioning to an inverted hexagonal phase and to ion pairing formation with the endosome, both of which facilitate siRNA release (previously described in Section 1.6). The size of the liposome particles is also an important factor. Considering the leaky vasculature of the tumour environment that results from the EPR effect, and the restrictions on siRNA carrier size imposed by serum clearance and RES entrapment, there is still a debate on the acceptable size limit of particles. While many believe 200 nm is appropriate as an upper limit, one study using synthetic 3D models to mimic the interstitial milieu suggested that
particles <100 nm were required to effectively and rapidly diffuse through membranes and tissues to reach target cells\textsuperscript{104}. The size can largely be controlled through the synthesis process, which can range from the classic lipoplex protocol, to passive encapsulation or ethanol dilution (see Figure 1.5)\textsuperscript{104}.

While cationic liposomes can enhance cellular uptake and endosomal release, their positive charge unfortunately enhances aggregation and opsonization in the bloodstream leading to RES uptake and decreased serum half lives\textsuperscript{105}. This is what led to the advancements in the functionalization of liposomes such as the addition of PEG. These PEGylated liposomes have been shown to increase the overall transfection efficiency to prostate tumour xenografts in vivo by preventing systemic clearance and serum opsonization (see Figure 1.4)\textsuperscript{105}. However, the incorporation of PEG was not without limitations. Repeated injections appeared to induce anti-PEG IgM, leading to accelerated blood clearance\textsuperscript{106}. The generation of anti-PEG IgM did seem to become attenuated when siRNA was encapsulated in the core of PEGylated liposomes (\textit{i.e.}, passive encapsulation method) versus complexation of siRNA to the outside of the liposomes (\textit{i.e.}, lipoplex method)\textsuperscript{107}. The presence of PEG on the surface of cationic liposomes has also been shown to inhibit cellular uptake and endosomal escape as it partially shields the positive charge. In fact, a 5 mol\% solution of 1,2-distearoyl-\textit{sn}-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000 (DSPE-PEG-2000) completely abolished gene silencing and was not reinstated until the fraction was lowered to 1-2 mol\%\textsuperscript{60}. These issues have led to recent advancements in particle design such as the creation of stable nucleic acid-lipid particles (SNALPs), which contain a mixture of cationic and fusogenic lipids initially stabilized by a PEG coating. Following intravenous administration, the PEG coating is shed and the cationic interior remains, leading to increased transfection efficiency\textsuperscript{108}. Tekmira Pharmaceuticals has further advanced SNALP technology by synthesizing the ionizable cationic lipid 1,2-dilinoleyleoxy-3-dimethylaminopropane (DLinDMA). A derivative termed DLin-KC2-DMA has now been developed, and shows heightened endosomal escape via a strong propensity for undergoing an inverse hexagonal transition at acidic pHs\textsuperscript{109}. This technology has been implemented in a phase 1 clinical trial to treat primary and secondary liver. However, results are not yet published (see www.clinicaltrials.org for study details). Another
Figure 1.5. Illustration of common liposome synthesis techniques. The figure illustrates the fact that a higher percentage of siRNA molecules become encapsulated in the liposome when using the passive encapsulation method compared to the lipoplex method. Legend: SUV – small unilamellar vesicle
strategy to further enhance liposome/siRNA delivery, especially with regard to specificity (discussed above) is the inclusion of a targeting ligand capable of binding specifically to a tissue/tumour specific marker or receptor. This is discussed in the following section.

1.8 Ligand targeting nanoparticles for enhanced uptake

Similar to the methodology underlying targeting conjugated delivery vehicles, liposomes can also be functionalized with different ligands to enhance cellular uptake in specific tissues. For example, while non-targeting PEGylated liposomes resist aggregation and have long half-lives they still end up in normal tissues as well as tumours: less than 5% of the initial dose is taken up by the tumour\textsuperscript{110}. That is why, in the case of Tekmira Pharmaceutical’s SNALPs, those agents are currently only in development to target liver cancer because, physiologically, that is where the majority of nanoparticles will naturally accumulate\textsuperscript{60}. Davis and colleagues, with the backing of Calando Pharmaceuticals, has a cyclodextrin polymer-based drug (CALAA-01) currently in phase 1 clinical trials: this is the first siRNA therapy systemically administered and targeted to transferrin-expressing solid tumours\textsuperscript{111}. It is important to keep in mind that the targeting ligands to not direct the particles to specific tissues. However, they do enhance the uptake in the targeted tissues once they get there through receptor-mediated endocytosis. This was shown when both transferrin receptor targeted and non-targeted particles accumulated in the same amounts at the tumour site, but intracellular delivery is seen only in the targeted vesicles\textsuperscript{112}. Many other ligands have been assessed to target cancer-related markers including: fibroblast growth factor receptors\textsuperscript{113}; HER2\textsuperscript{114}; and the folate receptor (FR)\textsuperscript{115}. Folate receptors are the focus of the following section.

1.9 Targeting liposomes via the folate receptor (FR)

Good cancer target candidates should have the following properties: homogenous expression on all target cells; binding with their ligand should facilitate internalization (endocytosis); a low degree of "shedding" of target molecules (\textit{i.e.}, release from cells); and they should play an important role in tumour cell viability such that downregulation (to avoid targeting) would lead to decreased survival and malignancy\textsuperscript{116}. Many of these qualities are what make exploiting the FR on cancer cells an attractive strategy. Folic acid
(FA), an essential vitamin, is required for one-carbon reactions and the synthesis of nucleotide bases. Cells normally take up physiological levels of FA via the reduced folate carrier\textsuperscript{117} or, in some cases, the proton-coupled folate transporter\textsuperscript{118}. Fortunately, neither of these transporters have an affinity for folate conjugates, so folate-functionalized particles are left to interact with FRs expressed on a select few cell types. Depending on the source of FR-expressing cells, 4 or 5 different FRs have been described. For the purposes of this study only FR-\(\alpha\) (FOLR1) will be explored, although it is noteworthy that FR-\(\beta\) can be found on the surfaces of macrophages and hematopoietic malignancies\textsuperscript{119}. FR-\(\alpha\) is 38-44 kDa glycosyl phosphatidylinositol-(GPI-)anchored membrane protein normally found on the apical surfaces of many epithelial cells, where it would be inaccessible to intravenously administered folate therapies. It is constitutively overexpressed in many human cancers, especially those of ovary, cervix, endometrium, lung, kidney, breast, colon and brain origin\textsuperscript{120}. In normal cells, proliferation is inversely correlated with FR expression and positively correlated with extracellular folate concentrations (EFC), since the EFC inversely regulates FR levels\textsuperscript{121}. Therefore, it is believed that FR expression in many cancer types has arisen to allow for greater competition for the minimal folate concentrations (\(\sim 2 \times 10^{-8}\) M) found in extracellular fluids\textsuperscript{122}. It is difficult to directly assess the exact number of FR’s per cell, however, studies using radiolabeled folic acid derivatives indicate binding can range from 1-4 pmol/10\(^6\) cells within the first 30 min depending on the cell type\textsuperscript{123,124}. Upon binding with FA, the FR becomes internalized and brings FA or any FA-conjugated particles along with it, which is a good trait for a target. There does seem to be a debate on the fate of the folate conjugates, as monovalent folate particles (internalized via a single FR) are believed to cycle back to the plasma membrane after forming early endosomes, while multivalent folate conjugates (internalized via multiple FRs; \textit{i.e.}, liposomes, nanoparticles) are trafficked to late endosomes and lysosomes for degradation right away\textsuperscript{125}. This needs to be considered when using FR-targeting liposome particles, but also offers an advantage as the late endosome pathway has a pH of around 5.0 that may be exploited by pH-triggered molecules\textsuperscript{126}. Another advantage of using the FR is that its ligand folate is a small, non-toxic molecule with low immunogenicity that binds to the FR with a high
affinity ($K_d = 0.1$ nM) even after being modified at its carboxyl end to permit lipid anchoring, making it a suitable conjugate for many particles$^{127}$. 
Objectives and Hypothesis

The purpose of this project is to test the capacity of a folate receptor (FR)-targeting liposome to deliver thymidylate synthase (TS) siRNA to the cytosol of FR-positive cancer cells where it can activate the RNAi machinery. We first hypothesize that non-targeting liposomes will mediate siRNA uptake into cells more efficiently than naked siRNA alone. We further hypothesize that decoration of siRNA/liposome complexes with folate will enhance their capacity to deliver siRNA to FR-positive human tumour cells both in vitro and in vivo, as a strategy to increase antisense effectiveness. We test these hypotheses through three objectives. Firstly, we characterize the liposomes based on their siRNA encapsulation yield, particle stability and size, and ability to protect against serum nucleases. Next, we determine transfection efficiencies (cellular uptake) in vitro, and organ/tissue distribution of siRNA/liposome complexes following systemic administration in vivo. Finally, we assess the ability of the siRNA/liposomes to knock down their gene targets both in vitro and in vivo.
Chapter 2

2 Materials and Methods

2.1 Cell culture

Cell lines shown to have various levels of folate receptor expression were chosen for experimentation. KB (human HeLa cell variant, cervical adenocarcinoma), A549 (human adenocarcinoma), HeLa (human cervical carcinoma), OVCAR-3 (human ovarian adenocarcinoma), B16-F10 (mouse melanoma) cell lines were acquired from the American Type Culture Collection (ATTC). KB, HeLa and B16-F10 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM)(GIBCO, Carlsbad, CA, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS). A549 cells were maintained in Minimum Essential Medium Alpha Medium (AMEM) (GIBCO) supplemented with 10% (v/v) FBS. OVCAR-3 cells were cultured in Roswell Park Memorial Institute medium (RPMI) (GIBCO) supplemented with 20% (v/v) FBS. All cell lines were incubated at 37°C in 5% CO₂. Cells were typically passaged 1:10 by trypsonization 1-2 times a week and regularly frozen in 1:10 DMSO:FBS at -80°C to maintain low passage numbers.

2.2 siRNA sequence design

A 19 nucleotide sequence targeting human TS mRNA (TS siRNA)(coding region 526-544) and a 19 nucleotide control sequence (C2 siRNA with no known target were synthesized by Dharmacon, Inc. (Lafayette, CO, USA). TS siRNA and C2 siRNA were labeled with Cyanine 3 (Cy3) on the 5’ end of their sense strands, yielding Cy3-TS siRNA and Cy3-C2 siRNA, respectively. The sequence constructs were: TS siRNA: 5’-GGACUUGGGCCAGUUUAU-3’ (sense) and 5’-AUAAACUGGGCCCAAGUCC-3’ (antisense); C2 siRNA: 5’-UGGUUUACUGUUGUGUGA-3’ (sense) and 5’-UCACACAACAUGUAAACCA-3’ (antisense).

2.3 Materials and preparation of liposome particles

1,3-Dioleoyl-3-trimethylammonium propane (DOTAP), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol, 1,2-distearoyl-sn-glycero-3-
phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-PEG\textsubscript{2000}), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[folate(polyethylene glycol)-2000] (Folate-DSPE-PEG\textsubscript{2000}) were purchased from Avanti Lipids, Inc. (Alabaster, AL). All lipids were dissolved and stored at -20°C in a 9:1 chloroform/methanol solution.

2.3.1 Passive encapsulation protocol

The “passive encapsulation” protocol was characterized by the incubation of siRNA to the lipid film prior to the extrusion of the liposome particles and for the purposes of our project was the primary mode of particle synthesis. This differed from the less-used “lipoplex” protocol where siRNA was added to the fully formed liposomes following extrusion (discussed below). Using the “passive encapsulation” protocol, folate receptor-targeting liposomes were constructed using one of two separate methods: the “folate before” method or the “folate after” method. In the “folate before” method DOTAP, DPPC, cholesterol and Folate-DSPE-PEG\textsubscript{2000} (at lowest concentration) were mixed in a 1:3.5:3.5:0.018 molar ratio and evaporated to complete dryness under vacuum, forming a lipid film. The lipid film was hydrated using a TS (fol-lip-TS siRNA) or control (fol-lip-C2 siRNA) siRNA/protamine mixture at a molar ratio of 30:0.0037:1 DOTAP:protamine:siRNA and incubated at 50-60°C for 10 min to form large multilamellar liposome-siRNA/protamine complexes in solution. The multilamellar liposome solution was then rigorously vortexed and sonicated for 2-5 m at 50-60°C in a bath sonicator. The sonicated solution was extruded sequentially through polycarbonate membranes of decreasing pore size (0.2 and 0.1 mm diameter pores). The resultant small, unilamellar liposome solution was kept at 50-60°C until it could be transfected into cells. The “folate after” method followed the same protocol as above with the exception that Folate-DSPE-PEG\textsubscript{2000}, in the same molar ratio, was added to the extruded unilamellar liposome solution following extrusion instead of to the lipid film. The liposomes and Folate-DSPE-PEG\textsubscript{2000} were allowed to incubate for 1-2 h to allow folate integration. To construct the non-targeting (non-fol-lip-C2 siRNA or non-fol-lip-TS siRNA) liposomes, the above protocol was followed aside from the incorporation of Folate-DSPE-PEG\textsubscript{2000} to the liposome solution. When an experiment called for PEGylated liposomes, DSPE-PEG\textsubscript{2000} was added to the extruded liposome solution in a molar ratio of 1:0.22
DOTAP:PEG for folate and 1:0.23 DOTAP:PEG for non-folated liposomes, respectively.

### 2.3.2 Lipoplex protocol

Folate decorated (fol-lip) and non-targeting (non-fol-lip) particles were synthesized in a similar fashion according to the “passive encapsulation” protocol (above), with the difference that the lipid film layer was hydrated with RNase-free water alone prior to extrusion. Following extrusion, the small unilamellar particles were lyophilized (freeze-dried) and stored at -20°C. This allowed for stable storage of the liposomes until needed. Upon use, the liposomes were re-hydrated with RNAse free water. A selected amount of siRNA was then added to the re-hydrated liposomes and incubated for 20 m to allow for the negatively charged siRNA to electrostatically complex with the positive liposomes.

### 2.4 In vitro assessment of liposome-Cy3 siRNA uptake using flow cytometry

Cells (KB, A549, HeLa, OVCAR-3 or B16 F10) were seeded in either 6 or 12 well Falcon tissue grade plates at concentrations ranging from 1-2 x 10^5 cells/well depending on the experiment. Two h prior to transfection the media was replaced with either folate-free RPMI or 1 mM folate RPMI depending on the experiment. Twenty four h after seeding the cells were with incubated with either non-fol-lip-Cy3-C2 siRNA or fol-lip-Cy3-C2 siRNA to assess the difference in uptake based on FR targeting. Initially, both non-fol-lip-C2 siRNA and fol-lip-C2 siRNA were used as controls. However, they did not yield significant differences in background Cy3-positivity from a non-treated control (NTC). In general, media was aspirated and cells washed with phosphate-buffered saline (PBS) 4 h after addition of liposomal preparations (and at different times under circumstances where incubation times were varied) before being replaced with fresh media. The cells were then trypsinized and resuspended in 250-300 μl of PBS prior to analysis by flow cytometry.

### 2.5 Confirmation of liposome-Cy3 siRNA uptake in vitro by fluorescent microscopy

Cells were seeded in plates according to the same protocol described above (section 2.4). Twenty four h after seeding the cells were incubated with either non-fol-lip-Cy3-C2 siRNA or fol-lip-Cy3-C2 siRNA. Twenty four h following addition of liposomial
preparations, media was removed and cells were rinsed (2x) with PBS and replaced with fresh media to remove any excess liposome particles in the media. The cells were then imaged using by fluorescent microscopy.

2.6 Assessment of TS silencing in vitro using lip-Cy3-TS siRNA

2.6.1 RNA extraction

Cells (KB and A549) were seeded in plates according to the same protocol described above (section 2.4). Twenty four h after seeding the cells were incubated with TS targeting non-fol-lip-Cy3-TS siRNA or fol-lip-Cy3-TS siRNA. Non-fol-lip-Cy3-C2 siRNA and fol-lip-Cy3-C2 siRNA were used as negative controls. Twenty four or 48 h following transfection, the cells were washed twice with PBS and harvested using TRIzol (Invitrogen, Carlsbad, CA, USA) for RNA isolation. Chloroform (Bioshop, Burlington, ON, CA) was added to the cell-TRIzol solution and phase separation through centrifugation allowed for RNA purification and extraction in the top aqueous layer. RNA was then precipitated out of solution using isopropanyl (Bioshop). The RNA-isopropanyl solution was centrifuged to form a RNA pellet before the isopropanyl was decanted. The RNA pellet was then suspended (2x) in 75% ethanol to remove any impurities (excess salts and proteins). The 75% ethanol solution was removed and the RNA pellets were dried for 30 m. Any remaining liquid was removed using an autoclaved Q-tip. The remaining RNA pellet was re-suspended in 20 ul of RNAsase free water. RNA was quantified using Nanodrop’s ND-1000 Spectrophotometer (Wilmington, DE, USA)). RNA quality (degradation) was assessed on a 1% agarose gel run at 50V for 20-40 m and imaged using Bio-rad’s Gel Doc (Mississauga, ON, CA).

2.6.2 Reverse Transcription

RNA (1 ug per reaction vessel) was then reverse-transcribed (RT) using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) in the presence of dNTPs, random primers and DDT. The RT reaction was carried out under the following conditions:
Table 2.1. Reverse Transcription reaction cycle parameters

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Duration (h)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>00:10:00</td>
<td>Primer attachment</td>
</tr>
<tr>
<td>37</td>
<td>1:00:00</td>
<td>Polymerization</td>
</tr>
<tr>
<td>4</td>
<td>00:05:00 (+ HOLD)</td>
<td>Prevent degradation</td>
</tr>
</tbody>
</table>

The synthesized complimentary DNA (cDNA) was stored at -20°C until it was needed for quantitative PCR (qPCR).

2.6.3 Quantitative PCR

cDNA (1 μl) was used per reaction vessel (50 ng cDNA assuming 100% RT reaction). qPCR reactions used TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA) in an Applied Biosystem’s ViiA7 qPCR machine. The TS specific forward and reverse primers were purchased from Applied Biosystems and used at a final concentration of 600 nM. The primer sequences for human TS were: 5’-GGCCTCGGTGTGCTTT-3’ (forward) and 5’-GATGTGCGCAATCATGTACGT-3’ (reverse). A TaqMan MGB probe labeled with 6-FAM (Applied Biosystems) was used at a concentration of 200 nM. It had a sequence of 5’-6-FAM-AACATCGCCAGCTACGCCTGCMGBNFQ-3’. A pre-designed human GAPDH labeled with FAM and MGB probe (Applied Biosystems) was used as an endogenous control. All individual samples/controls were run in triplicate in a 384 well plate. Standard curves (when applicable) were constructed from one of the unknown samples using a dilution series ranging from 1000 to 15.6. The parameters of the qPCR run are shown below:
Table 2.2. Quantitative PCR cycle parameters

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Duration (m)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>02:00</td>
<td>Optional</td>
</tr>
<tr>
<td>95</td>
<td>10:00</td>
<td>Initial denaturation/polymerase activation</td>
</tr>
<tr>
<td>95</td>
<td>00:15 (40 cycles)</td>
<td>Denaturation</td>
</tr>
<tr>
<td>60</td>
<td>01:00 (40 cycles)</td>
<td>Primer alignment and polymerization</td>
</tr>
</tbody>
</table>

qPCR results were analyzed in the Life systems software using one of two methods: \( \Delta \Delta CT \) method or the standard curve method. The \( \Delta \Delta CT \) method was primarily used when comparing several treatment groups to the same control, thus yielding relative quantities, while the standard curve method helped to verify amplification and overall qPCR reaction efficiencies and assess statistical significance between groups (further explored in the Discussion section\(^{128}\)).

2.7 Liposome particle size and zeta potential

Folate decorated (fol-lip-Cy3-C2 siRNA) and non-targeting (non-fol-lip-Cy3-C2 siRNA) liposomes were prepared according to our protocol. The particles were diluted in RNAse-free water and mean particle diameter and surface charge (zeta potential) was assessed using a Zetasizer Nano Series instrument (Malvern Instruments Ltd., UK) according to the manufacturer’s protocol. The instrument counts approximately 200,000 particles/second until a stable reading is obtained. Particle size was measured over several time points to investigate particle aggregation and overall stability over time.

2.8 Liposome:siRNA binding capacity assay

Lyophilized aliquots of PEGylated liposomes synthesized according to the “lipoplex” protocol were re-hydrated in 200 μl of RNAse-free water. Varying siRNA
quantities were added to the re-hydrated liposomes yielding liposome:siRNA molar weight ratios of 35, 30, 25, 20, 15, 10. Following a 20 m incubation, the PEGylated “lipoplex” lip-Cy3-C2 siRNA mixtures were run on a 1% agarose gel at 50 V for 30-40 m and imaged using a Biorad imaging system.

2.9 Nuclease degradation assay

Folate decorated (fol-lip-Cy3-C2 siRNA) and non-targeting (non-fol-lip-Cy3-C2 siRNA) PEGylated liposomes were synthesized according to the “passive encapsulation” protocol. Liposomes were aliquoted into 1.5 mL centrifuge tubes yielding approximately 0.71 nM of siRNA (before extrusion) per timepoint (0, 1, 2, 4, 8, 24, 48 h). The liposome aliquots were then incubated in a 1:1 (v/v) ratio with FBS (contains serum nucleases). Samples were incubated at 37°C (0 h) and transferred to -80°C at their specific timepoint to halt plasma nuclease activity. Free Cy3-C2 siRNA in mouse plasma was used as a positive control. Following the final timepoint, samples were thawed at room temperature and siRNA was extracted according to the TRIzol RNA extraction protocol described previously. Following suspension in RNAse free water, siRNA quantities were measured using the ND-1000 Spectrophotometer (Nanodrop). Equal amounts of siRNA were mixed with 6 x loading buffer (Table 2.3) and run on a 15% acrylamide TBE-PAGE gel at 80V for 1.5 h. The gels were incubated in ethidium bromide (50 μg/mL) for 8-10 m and imaged using Bio-rad’s image doc.

Table 2.3. Recipe for 6x loading buffer used in assessing siRNA degradation

<table>
<thead>
<tr>
<th>Amount</th>
<th>Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 mL</td>
<td>100% Glycerol</td>
</tr>
<tr>
<td>3 mL</td>
<td>0.5 M EDTA</td>
</tr>
<tr>
<td>3 mg</td>
<td>Bromophenol blue</td>
</tr>
<tr>
<td>3 mg</td>
<td>Xylene cyanol</td>
</tr>
<tr>
<td>4 mL</td>
<td>Sterile water</td>
</tr>
</tbody>
</table>
2.10 Ribogreen siRNA quantification assay

siRNA concentration in liposomes was quantified using the Quant-iT RiboGreen RNA kit (Invitrogen) according to their protocol. A high-range standard curve (assay points: 0 ng/ml, 20 ng/ml, 100 ng/ml, 500 ng/ml and 1 μg/ml) was constructed from a dilution series of 2 μg/ml siRNA stock solution. Liposomes were constructed according to the “passive encapsulation” and “lipoplex” protocols above, with the difference that siRNA was not incubated with protamine prior to addition to the lipid film layer as it prevented RiboGreen dye binding to siRNA. Liposome samples were diluted with TE buffer (10mM Tris-HCl, 1 mM EDTA, pH 7.5) over a range of 0.1 to 0.01 of the original sample concentration to fall within the standard curve. The diluted samples were incubated in a 1:1 (v/v) ratio with Quant-iT RiboGreen working solution for 10 m and read in a fluorescent microplate reader (490 nm excitation, 525 nm emission).

2.11 Folic Acid inclusion assay

According to the “passive encapsulation” protocol, folate-decorated liposomes were synthesized by both the “folate before” and “folate after” methods. No siRNA/protamine mixture was added during synthesis as it interfered with the absorbance readings of folate ligand. A standard curve was created using a dilution series of free Folate-DSPE-PEG\textsubscript{2000} in RNAse free water. Absorbance readings were taken at 285 nm using a UV-Vis spectrophotometer (Beckman Coulter, Mississauga, Ontario)

2.12 Mouse Models

NIH-III nude mice (6-8 week old females) were purchased from Charles River Laboratories International, Inc. (Wilmington, MA). Mice were maintained under a pathogen-free environment in the Victoria Hospital barrier facility. All experiments were conducted in accordance with the standard operating procedures set forth by the Canadian Council on Animal Care and Western University Animal Use Subcommittee.

2.13 FR expression of cultured cells using flow cytometry

KB, A549, HeLa, and OVCAR-3 cells were plated at a concentration of 2 x 10\textsuperscript{5}
cells/T25 flask. Twenty four h after plating, media was aspirated and 1 ml of 10 mM EDTA was added to each flask. They were then incubated at 37°C for 10-15 m or until most of the cells could be seen detached under the microscope. Four ml of fresh media was added to each flask and the contents were dispensed into appropriate flow cytometry vials. Samples were centrifuged at 12,000 rpm for 8 m and washed with fresh PBS. Following re-centrifugation the PBS was decanted and primary monoclonal mouse IgG1 FOLR1 or isotype antibody (R & D Systems, Inc., Minneapolis, MN) was added to each sample (2.5 ug/10⁶ cells). Cells were incubated in primary antibody (1º ab) for 30-40 m at 4°C. Samples were then centrifuged and washed again in PBS to remove any unbound antibody. Eight μl of phycoerythrin-conjugated anti-mouse IgG secondary antibody (IgG-PE 2º ab)(R & D Systems) was added to each sample and incubated in the dark for 30 m at 4°C. Cells were then washed again with PBS and resuspended in 300 μl of PBS for analysis by flow cytometry.

2.14 FR expression in vivo and in vitro, assessed by immunoblot

2.14.1 Tissue harvesting/protein extraction

NIH-III mice were subcutaneously inoculated with 2 x 10⁶ KB cells. Tumours were measured twice per week with hand calipers (Mitutoyo, Kawasaki, Japan) and mice were sacrificed when tumour volumes reached approximately 600 mm³. Tumour tissue was extracted, placed on dry ice and stored at -80°C until needed. A small (<0.5 g) sample of tumour was excised and suspended in 150 ul of cell lysis buffer (see Table 2.4 for recipe). The solid tumour tissue was disrupted using a hand homogenizer until it was evenly distributed throughout the lysis buffer. To process cells grown in vitro for immunoblot, cells were plated at 2 x 10⁵ cells/T25 flask and removed from the flask mechanically using a cell scraper 24 h after seeding. One hundred and fifty μl of cell lysis buffer was added to each cell pellet. Both in vivo and in vitro samples were processed following the same protocol from this point forward. Samples were placed on ice for 20 m with vortexing every 5 m before being centrifuged at 12,000 g for 10 m (4°C). The protein containing supernatant was collected and kept on ice.
Table 2.4. Sample lysis buffer for immunoblot

<table>
<thead>
<tr>
<th>Amount</th>
<th>Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.88 g</td>
<td>NaCl</td>
</tr>
<tr>
<td>2 mL</td>
<td>1M Tris-HCl (pH 7.6)</td>
</tr>
<tr>
<td>1 mL</td>
<td>10% SDS</td>
</tr>
<tr>
<td>1 mL</td>
<td>Triton X-100</td>
</tr>
<tr>
<td>500 ul</td>
<td>0.2M EDTA</td>
</tr>
<tr>
<td>100 mL</td>
<td>Deionized water</td>
</tr>
</tbody>
</table>

2.14.2 Protein quantification

Total protein content was quantified using a Bradford Assay. A standard curve was constructed using a dilution series from a 10 mg/mL working solution of bovine serum albumin (BSA). Samples were diluted in PBS (1:350) and diluted (6:4 reagent:PBS) Bradford reagent was added to each sample. Each sample was then distributed in 3 wells of a 96 well plate and read on a fluorescent microplate reader. Protein quantities were determined from the standard curve.

2.14.3 Gel electrophoresis and protein transfer

Separate gels were required to detect FOLR1 and endogenous control protein actin due to their similar size and the FOLR1 antibody requiring non-reducing conditions. Samples were mixed 1:1 (v/v) in 2x sample loading buffer (see Table 2.5 for recipe) and actin samples (reducing conditions) were heated at 90°C for 5 m prior to gel loading. A 20% acrylamide gel was used as it yielded the cleanest protein bands. Ten μg and 25 μg of protein suspended in a total volume of 35 ul were loaded into each well for FOLR1 and actin detection, respectively. Following sample loading the gels were run at 120 V for 1 h 40 m in a standard Bio-rad gel electrophoresis apparatus. After the allotted time, the gels were removed and the area containing the assessed proteins were excised from
the gel and placed in transfer buffer along with the nitrocellulose film. A “transfer sandwich” was constructed and the transfer apparatus was run on 100 V for 1 h at 4°C.

**Table 2.5. Recipe for 2x sample loading buffer**

<table>
<thead>
<tr>
<th>Amount</th>
<th>Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.05 mL</td>
<td>Deionized water</td>
</tr>
<tr>
<td>1.25 mL</td>
<td>0.5 M Tris-HCl (pH 6.8)</td>
</tr>
<tr>
<td>3 mL</td>
<td>100% Glycerol</td>
</tr>
<tr>
<td>2 mL</td>
<td>10% SDS</td>
</tr>
<tr>
<td>0.2 mL</td>
<td>0.5% Bromophenol blue</td>
</tr>
</tbody>
</table>

Store in 950 ul aliquots at -20°C

50 ul (added to 950 ul aliquot before use) 2-Mercaptoethanol

**2.14.4 Blocking, antibody labeling and imaging**

The nitrocellulose membrane was removed from the “transfer sandwich” and blocked with 5% skim milk for 1 h. The paper was then washed several times in TBS-T (15 m, 5 m, 5 m) before being incubated with either primary monoclonal mouse IgG<sub>1</sub> anti-FOLR1 antibody(1:1000)(R & D Systems) or polyclonal rabbit IgG<sub>1</sub> anti-β actin antibody(1:2000)(Santa Cruz Biotechnology, Dallas, Texas) in 1% skim milk for 1 h. The nitrocellulose film was washed in TBS-T several more times (15 m, 10 m, 10 m) to remove unbound primary antibody. The nitrocellulose film was then incubated with secondary antibody (when detecting FOLR1, 1:2000 anti-mouse-horse radish peroxidase [HRP],GE Healthcare UK Limited, Little Chalfont Buckinghamshire, UK) and, when detecting β actin, 1:10,000 anti-rabbit-HRP (GE Healthcare UK Limited) in 1% skim milk for 45 m. The nitrocellulose film was then washed again several times in TBS-T (15 m, 5 m, 5 m) before being blotted with filter paper. Approximately 1 ml of HRP substrate (GE Healthcare UK Limited) was added to each nitrocellulose film fragment for 1-2 m,
and then blotted away using filter paper. The proteins to be detected were then imaged using a 860 Storm scanner (Molecular Dynamics, Baie d’Urfe, Quebec).

2.15 In vivo distribution of Cy3 siRNA using folate receptor-targeting liposomes

Non-targeting (non-fol-lip-Cy3-C2 siRNA) and folate decorated (fol-lip-Cy3-C2 siRNA) liposomes were synthesized according to the “passive encapsulation” protocol. Non-targeting or FR-targeting liposomes were injected into tail veins of NIH-III mice bearing two KB xenografts (one in each flank) of approximately 600 mm³ each. The amount of Cy3 C2 siRNA used was 50 μg/mouse prior to extrusion. There were 3 mice per treatment group. Mice were sacrificed 6 h following injection and various tissues (tumour, heart, skin, kidney, liver, muscle, connective tissue and intestine) were collected and placed in cryostat holders (Tissue-Tek, Torrance, CA, USA) and suspended in OCT (Tissue-Tek) and stored at -80°C. The cryostat samples were then sectioned in 10 um sections using a cryostat (Leica Biosystems, Concord, Ontario) and mounted on glass slides for fluorescent microscopy.

2.16 In vivo silencing of TS using folate receptor-targeting liposomes

Non-targeting (non-fol-lip-Cy3-TS siRNA) and folate decorated (fol-lip-Cy3-TS siRNA) liposomes were synthesized according to the “passive encapsulation” protocol. Folate decorated liposomes containing C2 siRNA (fol-lip-Cy3-C2 siRNA) were used as a negative control. NIH-III mice were inoculated subcutaneously in two areas on their backs with 2 x 10⁵ KB cells/inoculation. Liposomes were injected into tail veins of NIH-III mice (50 μg/injection before extrusion) 2 days following KB tumour inoculation and thereafter once per week for a total of 4 injections. There were 6 mice per treatment group. Mouse body mass was monitored twice a week and tumour size was evaluated twice a week using calipers. Twenty four h following the final treatment, the mice were euthanized and tumour tissue was harvested and stored at -80°C.

Several days later, the tumour tissue was thawed and a small amount (<0.2 g) was analyzed by qPCR for specific RNA levels. Tumour tissue was placed in TRIzol (Invitrogen) for RNA isolation and completely disrupted using a hand homogenizer.
From this point the protocol used for analyzing *in vitro* target mRNA reduction was followed (above, section 2.6.1).

### 2.17 Statistical analysis

Data is presented typically as means ± standard deviations. Differences between experimental groups were analyzed using a Student’s *t* test and differences with *p* values less than 0.05 (confidence limit selected *a priori*) were considered to be significant.
Chapter 3

3 Results

3.1 Characterization of the liposome particles

3.1.1 Liposome surface charge (zeta potential) and size as determinants of particle stability

A common indicator to determine a nanoparticle's overall stability in solution is to measure its zeta potential. Nanoparticles with an overall net charge will attract oppositely charged ions to its surface, forming a Stern layer. As the particle moves in solution an electrostatic potential is created between the two layers, which is called the zeta potential and is directly related to the surface charge of the particle. The zeta potential of both non-targeting and folate-decorated liposomes with folate added either before (“folate-before”) or after (“folate-after”) the extrusion process was measured either alone or in the presence of siRNA (Table 3.1). An example of a typical readout of zeta potential can also be seen in Figure 3.1. As expected, the zeta potentials are all positive since the liposomes contain cationic lipids. The zeta potential increased in the non-targeting liposomes when no siRNA was present, since siRNA carries a net negative charge from its phosphodiester backbone. Surprisingly, the same was not observed with the “folate before”-decorated liposome. This may have resulted from a lack of siRNA entrapment by the liposome particle or differences in the amount of folate-DSPE that integrated into the liposomes (discussed below). The folate-DSPE molecule carries a net charge between -1 and -2 depending on the aqueous environment. In an attempt to balance the surface charges of the non-targeting and folate decorated liposomes, PEG, which contains an overall net charge of -1, was added to the non-targeting liposomes. It is difficult to be exact, however, due to the protonating nature of the folate-DSPE molecule. Unexpectedly, the "folate after" liposomes had a slightly higher zeta potential, which is likely influenced by the small sample size and amount of siRNA that had been included following the extrusion process. Regardless, a zeta potential around +30 or greater is considered strongly cationic and lead to relative stability.
Table 3.1. Zeta potentials of various liposome particle mixtures. Non-targeting (non-fol) and FR-targeting (fol) liposomes with folate inserted either prior to (fol before) or following the extrusion process (fol after) were synthesized in the presence (+siRNA) or absence (-siRNA) of siRNA. Samples were diluted 1:10 in water and analyzed for size and charge using the Malvern Zetasizer. Legend: N – is number of individual experiments; n – number of individual samples.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Zeta Potential (mV)</th>
<th>Sample size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-fol-Cy3-C2RNA (+siRNA)</td>
<td>29.42 ± 2.53</td>
<td>N = 3; n = 9</td>
</tr>
<tr>
<td>Non-fol-Cy3-C2RNA (-siRNA)</td>
<td>42.8 ± 3.88</td>
<td>N = 2; n = 6</td>
</tr>
<tr>
<td>Fol-Cy3-C2RNA (fol before)(+siRNA)</td>
<td>26.7 ± 5.72</td>
<td>N = 2; n = 6</td>
</tr>
<tr>
<td>Fol-Cy3-C2RNA (fol before)(-siRNA)</td>
<td>27.07 ± 1.34</td>
<td>N = 1; n = 3</td>
</tr>
<tr>
<td>Fol-Cy3-C2RNA (fol after)(+siRNA)</td>
<td>36.7 ± 1.55</td>
<td>N = 1; n = 3</td>
</tr>
</tbody>
</table>
Figure 3.1. Example of zeta potential readouts for liposome samples. Liposomes were synthesized according to the “passive encapsulation” protocol and kept at ~55°C until the measurements were performed soon after synthesis. Samples were diluted 1:10 in water and analyzed for size and charge using the Malvern Zetasizer. A) Zeta potential profile for non-fol-Cy3-C2RNA; B) zeta potential profile for fol-Cy3-C2RNA.
Another important attribute of a nanoparticles is size (diameter). As described above, smaller sizes (< 200 nm) are preferred for effective exit from the blood stream and entry into extracellular space, and enhanced uptake especially through the interstitial tissue in vivo\textsuperscript{104}. A measure of particle size change over time is also an indicator of a particle’s overall stability. Zeta potential is the primary indicator of stability. However, changes in particle size can show how long the particles last in solution before becoming unstable and aggregating to form larger particles with less capacity to be taken up by cells. Examples of stable and aggregating particles as well as particle stability over time can be seen in Figure 2. Looking at the particle size readouts, a single normal distribution can be seen up until the 6 h time point indicating good particle stability (Figure 3.2A). At the 12 h time point, some aggregation has taken place as a distinct peak can also be seen at a size >1000 nm (Figure 3.2B). A particle stability >6 h is important because in some cases it could be several hours from the time of synthesis before the particles are transfected into cells or injected in mice, where a relatively homogenous liposome mixture is ideal. The sizes of the non-targeting and folate-decorated liposomes are similar, which is an important aspect to consider when comparing the two groups in vitro (sedimentation effect) and in vivo (RES entrapment and EPR effect).

### 3.1.2 Folic acid decoration on liposome

A primary goal was to assess differences in cellular uptake between non-targeting and folate-decorated liposomes in FR-positive and FR-negative cell lines. Therefore, the actual amount of folate-DSPE that was incorporated into liposomes was considered. Folate was either added to the liposomes according to the “folate before” or “folate after” protocols. Absorbance readings at 285 nm indicated that 3.92-9.16% and 9.56 ± 0.34% (N=2) of folate-DSPE successfully integrated into the liposomes using the “folate before” and “folate after” synthesis protocols. This was not as high as reported in the literature (see Discussion section) and may have had an impact on experiments described below.
Figure 3.2. Liposome particle size and stability over time. Liposomes were synthesized according to the “passive encapsulation method” and assessed for size and charge using the Malvern Zetasizer. Liposome solutions were kept at ~ 55°C until the specific time point. A) Size distribution profile of fol-Cy3-C2RNA (+PEG) liposomes immediately after synthesis; B) size distribution profile of fol-Cy3-C2RNA (+PEG) liposomes 12 h following synthesis; C) liposome particle stability over a 12 h period with vertical bars displaying the size range.
Particle stability over time

Size (diameter, nm)

Time after extrusion (hours)

- non-fol-Cy3-C2RNA (-PEG)
- non-fol-Cy3-C2RNA (+PEG)
- fol-Cy3-C2RNA (-PEG)
- fol-Cy3-C2RNA (+PEG)
3.1.3 siRNA binding/encapsulation yield

Another important property of liposome particles is their capacity to interact with siRNA. This would include siRNA electrostatically interacting with the cationic lipids or being encapsulated during the extrusion process. Liposome particles were synthesized using one of two methods: the “passive encapsulation” protocol (see Materials and Methods, Section 2.3.1) and the “lipoplex” protocol (see Materials and Methods, Section 2.3.2).

In the “lipoplex” protocol, liposomes were first synthesized alone and then siRNA was added after. In this case it was valuable to know how much siRNA a given amount of lipid was capable of binding. To assess this, liposome:siRNA complexes with different mass ratios (i.e., different amounts of siRNA associated with liposomes) were incubated and separated by agarose by gel electrophoresis (Figure 3.3). SiRNA disassociated from liposomes has high electrophoretic mobility, and visualization of that highly mobile siRNA was a measure of the capacity of liposomes to encapsulate the nucleic acids. Wells containing various amounts of free siRNA were included for reference. This is an indirect measure of binding ability since the bright white bands depict unbound siRNA. Liposome-bound siRNA remains in the wells (at the top of the photograph). It appears that a residual amount of free siRNA remained unbound regardless of the amount added. This could be due to the presence of negatively charged PEG molecules present on the surface of the liposomes. The amount of unbound siRNA increased at liposome:siRNA molecular mass ratios of 15 to 10 and this set the upper limit of the liposome-siRNA binding capacity at 12:7.2 DOTAP:siRNA molar mass using the “lipoplex” method. Therefore, during the course of experimentation, values below this threshold were used.

The above method was used to qualitatively determine the amount of siRNA the liposomes could bind. However, a quantitative method would be more useful, especially one that could differentiate siRNA on the inside or outside of the liposomes. Therefore, RiboGreen dye was used to detect siRNA. RiboGreen fluoresces when bound to free siRNA, with the advantage that it differentiates between siRNA bound to the interior or exterior of the liposome since the lipid bilayer is impermeable to the dye, which can
Figure 3.3. Qualitative measurement of the siRNA binding capacity of liposomes synthesized using the “lipoplex” method. Liposome solutions were aliquoted into uniform amounts and then incubated for 20 m with various amounts of siRNA creating the following liposome:siRNA molar mass ratios of 35, 30, 25, 20, 15 and 10. The samples were then run on a 1% agarose gel. The white bands indicate unbound siRNA. Wells containing siRNA only (0.34, 0.48 and 1.20 μg) were included for reference.
Lipoplex binding assay

| siRNA alone (0.34 µg) | 35 | 30 | siRNA alone (0.48 µg) | 25 | 20 | 15 | 10 | siRNA alone (1.20 µg) |

- Lip:siRNA molar mass ratio
- Bound siRNA
- Unbound siRNA
interact with interior siRNA only when the liposome:siRNA complex is treated with a
surfactant (*i.e.*, Triton X-100) to release interior nucleic acid. Liposomes were
synthesized according to the “passive encapsulation” and “lipoplex” protocols and
incubated with RiboGreen working solution in the presence or absence of Triton X-100.
RiboGreen fluorescence measurements were used to determine the percentage of total
siRNA entrapped by the liposomes (bound on either interior or exterior) and percentage
of total siRNA encapsulated by the liposomes (interior only)(Table 3.2). Several
amendments to our protocol were necessary, as we found that RiboGreen dye could not
bind siRNA complexed with protamine (*data not shown*). This made quantification of
siRNA encapsulation possible only in material produced by a protocol that approximated,
but did not exactly reproduce the “passive encapsulation” synthesis protocol.
Furthermore, it appears that RiboGreen cannot bind siRNA well when it electrostatically
interacts with an intact liposome (Table 3.2). This became evident as the percentage of
siRNA encapsulated following extrusion was 80 and 86% for the “lipoplex” and “passive
encapsulation” protocol, respectively, when theoretically the maximum amount of siRNA
that should occur on the interior from the passive encapsulation protocol is 50% and, in
the lipoplex method, much less because the siRNA is added to the exterior of preformed
liposomes. Therefore, it is hypothesized that the RiboGreen assay can accurately quantify
only the total amount of siRNA entrapped by the liposomes, determined following lysis
of the liposome:siRNA complex.

3.1.4 Ability of liposomes to protect siRNA from serum nucleases

Ultimately, liposomes must protect and deliver siRNA to target organs and cells
*in vivo*. As mentioned previously, this includes protecting the siRNA payload against
serum nucleases that can degrade the siRNA and destroy its antisense function. To test
the ability of both non-targeting and folate-decorated liposomes to protect siRNA against
serum nucleases, aliquots of liposome-siRNA synthesized using the “passive
encapsulation” protocol were incubated in FBS in a 1: ratio (v/v) and separated using a
15% acrylamide TBE-PAGE gel (Figure 4). The presence of bands indicates intact
siRNA. Degraded siRNA was removed during siRNA purification; was of high
electrophoretic mobility (due to small size of degradation products) and did not
Table 3.2. Quantification of liposome-siRNA interaction determined by the RiboGreen assay. Liposome-siRNA solutions were either treated with RiboGreen working solution alone (intact liposomes) or in the presence of 10% Triton X-100 (lysed liposomes). Lysed readings were used to determine the amount of siRNA on the interior of the liposomes and the combination of intact and lysed readings determined the total amount of siRNA entrapped.
<table>
<thead>
<tr>
<th>Metric</th>
<th>Lipoplex method</th>
<th>Passive encapsulation method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of total siRNA entrapped</td>
<td>94.2 ± 9.5</td>
<td>49.4 ± 3.42</td>
</tr>
<tr>
<td>Percentage of total siRNA encapsulated</td>
<td>76.0 ± 8.1</td>
<td>43.7 ± 2.96</td>
</tr>
<tr>
<td>Percentage of siRNA encapsulated after extrusion</td>
<td>80.68 ± 2.90</td>
<td>86.05 ± 3.03</td>
</tr>
</tbody>
</table>
appear in the size range of intact siRNA; or was undetectable by ethidium bromide staining due to small size. Figure 3.4 indicates that both the non-targeting liposomes and the folate-decorated liposomes can protect siRNA up to 48 hours, while the naked siRNA begins to degrade at 4 h. This is a critical consideration to allow sufficient time for liposome:siRNA complexes to accumulate at tumour sites.

3.2 Assessing folate-FR-mediated uptake in FR-expressing cell lines, and tissue/organ distribution following systemic administration in mice

3.2.1 FR expression in various cell lines and xenografts

To determine whether decorating liposomes with folate to target FR on cells enhances cellular uptake over non-targeting particles, the FR 1 (folate receptor 1) level on cells was first determined. Multiple cell lines were tested by incubating cells with a FOLR1 monoclonal antibody and subsequent secondary antibody conjugated to phycoerythrin (PE), which could be detected by flow cytometry (Figure 3.5). These data indicate that the KB cell line possessed the highest level of FR expression with >95% of cells showing FR positivity and a mean fluorescence intensity (MFI), a measure of the average fluorescent on a per cell basis, over fourfold higher than the cell line with the next highest FR MRI (HeLa). Both HeLa and OVCAR-3 cells displayed moderate FR levels, with approximately 50% of cells being FR-positive. A549 cells had the lowest FR level, with less than 5% cells of cells positive for FR and a negligible MFI. This wide spectrum of FR expression across the various cell lines provided a powerful diagnostic system to investigate the impact that targeting the FR had on cellular uptake. The high FR-expressing cell line KB (FR++)(Figure 3.5) was selected to investigate folate-FR mediated uptake. Because of the very low FR levels in A549 (FR-) cells (Figure 3.5), they were selected as a control for comparison to assess the capacity of FR status to predict differences in folate-mediated uptake of liposomally-encapsulated siRNA between cell lines, and provided a suitable negative control to account for factors independent of FR. The cell lines were evaluated for siRNA uptake in the presence of either folate-complete media or folate-depleted media (the latter induced by replacing folate-complete medium 3 hours prior to assessment, and the former maintained by
Figure 3.4. siRNA integrity following serum nuclease incubation. Non-targeting and folate-decorated liposome:siRNA complexes synthesized using the “passive encapsulation” method, or naked siRNA without liposomes, was incubated in a 1:1 (v/v) ratio for 1, 4, 8, 24, or 48 h. siRNA was then extracted from samples using the standard RNA extraction method and separated by electrophoresis in a 15% acrylamide TBE-PAGE gel. The presence of bands indicates intact siRNA.
Nuclease-siRNA degradation assay
**Figure 3.5. Folate receptor levels in various cell lines (determined by flow cytometry).** Cells were cultured in 6 well plates for 24 hrs and then exposed to either folate-complete or folate-depleted media for 3 h prior to harvesting and incubation with FOLR1 monoclonal antibody. Next, the cells were incubated with PE-conjugated secondary antibody and analyzed for PE levels by flow cytometer.

A) Histogram showing representative gating for all cell lines treated with FR primary antibody and KB cells treated with isotype (control) antibody

B) Percentage of cells positive for FOLR1

C) Mean fluorescence intensity (MFI) of cells (arbitrary value relating to average fluorescence on a per cell basis).
A

Cy3 fluorescence

KB cells in folate depleted media
  treated with isotype antibody
KB cells in folate depleted media
  treated with FR antibody
A549 cells in folate depleted media
  treated with FR antibody
HeLa cells in folate depleted media
  treated with FR antibody
OVCAR3 cells in folate depleted media
  treated with FR antibody

B

Folate Receptor expression
in various cell lines

Percentage of cells positive
for FR expression

(+) Folic Acid
(-) Folic Acid

Cell Lines
KB  A549  HeLa  OVCAR-3
replacing medium with fresh medium 3 hours prior to assessment) to determine whether conditions where the amount of folate unassociated with liposomes would affect FR levels in target cells. The assessment was predicated on the possibility that high unassociated folate in medium would reduce FR levels due to reduced cellular requirement for folate, and that low medium folate would result in increased cellular production of FR in response to increased requirement for folate. However, there was no difference in FR level in cells induced by altering medium folate.

To test the tissue/organ distribution and silencing ability of FR-targeting liposomes in vivo, KB cells were inoculated into nude mice and grown as subcutaneous xenografts. Since cells are likely to have different characteristics in vivo than in vitro, FR level in KB xenografts was confirmed by immunoblot (Figure 3.6). In the data shown in that figure, KB and A549 cells grown in vitro were included as positive and negative FR expressers, respectively. KB xenografts and KB cells grown in vitro had similar levels of FR protein, while the A549 cells grown in vitro did not.

3.2.2 Liposomes enhance siRNA uptake compared to naked siRNA and folate decoration further enhanced uptake in FR positive cells.

Non-targeting siRNA-loaded liposomes (i.e., no folate) were tested to determine if they facilitated cellular uptake of control, non-targeting siRNA (C2) compared to naked C2 siRNA alone (Figure 3.7). For in vivo purposes, it has been reported that PEGylating liposomes greatly enhances siRNA delivery by inhibiting RES entrapment\textsuperscript{105}, so the effect of coating liposomes with PEG either directly in the lipid cake prior to extrusion (+PEG before) or after extrusion (+PEG after) was tested (Figure 3.7). The non-targeting liposomes showed a significant increase in uptake compared to naked siRNA (p<0.05) and the inclusion of PEG either before or after had no significant effect on uptake.

The effect of decorating liposomes with folate to enhance cellular uptake in FR-expressing cells was assessed (Figure 3.8). At first glance, and looking at only the FR-positive KB cells (black bars), it would appear that folate decoration did enhance cellular uptake by 11.6%. However, there was an unexpected but similar increase (10.3%) in
Figure 3.6. FR protein expression of KB xenografts. KB cells were grown in culture and then inoculated in NIH-III nude mice. Upon reaching an average size of 600 mm$^3$, mice were sacrificed and KB tumours were harvested for immunoblot. Cultured KB and A549 cells were also included as a reference. Actin protein levels were used as an internal control and run on a separate gel due to the non-reducing conditions required for FOLR1 antibody binding.
Figure 3.7. Percentage of cells positive for Cy3-siRNA after transfection with PEGylated liposomes or naked siRNA. Non-targeting liposomes encapsulating control, non-targeting siRNA (C2) were synthesized according to the “passive encapsulation” protocol and PEG was added either prior to or following extrusion. Liposomes were transfected in HeLa cells for 4 h before cells were harvested and assessed for siRNA uptake by flow cytometry.

A) Histogram showing gating of HeLa cells treated with non-fol-Cy3-C2RNA (+PEG before). 94.6% HeLa cells positive for Cy3-siRNA uptake, 5.44% negative.

B) Percentage of HeLa cells positive for Cy3 siRNA uptake using various delivery agents.

* Naked siRNA – siRNA transfected alone with no delivery agent

* non-fol-Cy3-C2RNA (-PEG) – non-targeting liposomes containing no PEG

* non-fol-Cy3-C2RNA (+PEG before) – PEG added directly to the lipid cake prior to extrusion

* non-fol-Cy3-C2RNA (+PEG after) - PEG added after extrusion.

One experiment, n=3; *p<0.05 compared to naked siRNA.
HeLa cells (control, no treatment with liposomes)

HeLa cells (treated with non-targeting liposomes encapsulating Cy3-labeled siRNA (non-fol-Cy3-C2RNA) for 4 h)

Cy3 fluorescence

**Effect of PEGylation on uptake in HeLa cells**

<table>
<thead>
<tr>
<th>Delivery Method</th>
<th>Percentage of cells positive for Cy3-siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naked siRNA</td>
<td>*</td>
</tr>
<tr>
<td>non-fol-Cy3-C2RNA (+PEG before)</td>
<td>*</td>
</tr>
<tr>
<td>non-fol-Cy3-C2RNA (+PEG after)</td>
<td>*</td>
</tr>
</tbody>
</table>
Figure 3.8. Effect of liposome folate decoration on the cellular uptake of Cy3-siRNA. Non-targeting (no folate) and folate-decorated liposomes encapsulating Cy3-labeled control, non-targeting siRNA (C2) were synthesized according to the “passive encapsulation” protocol. Folate was added directly to the lipid film layer prior to extrusion. FR-positive (KB) and FR negative (A549) cell lines were grown in 6 well plates for 24 hours and treated with transfected for 4 h before being harvested for flow cytometry.

A) Histogram showing gating of KB cells treated with fol-Cy3-C2RNA. 61.4% KB cells positive for Cy3-siRNA uptake, 38.6% negative.

B) Percentage of cells positive for Cy3-siRNA uptake using various delivery agents. non-fol-Cy3-C2RNA – non-targeting liposomes

fol-Cy3-C2RNA – folate decorated liposomes.

One experiment, n = 3; *p<0.05 compared to naked siRNA in KB cells; &p<0.05 compared to naked siRNA in A549 cells
A

KB cells (control, no treatment with liposomes)

KB cells (treated with folated liposomes encapsulating Cy3-labeled siRNA (fol-Cy3-C2RNA))

Cy3 fluorescence

B

Effect of folate decoration on cellular uptake

Percentage of cells positive for Cy3-siRNA

Delivery Method

KB (FR++)
A549 (FR-)

Naked
non-fol-Cy3-C2RNA
fol-Cy3-C2RNA

11.6% *
10.3% σ
&
siRNA uptake mediated by folate decoration in FR-negative A549 cells (*white bars*). These data suggest that the increased uptake of siRNA encapsulated in folate-decorated liposomes was due to an FR-independent event (possibly mediated by differences in particle surface charge or the amount of siRNA that was incorporated following synthesis, since differences can occur among batches).

The next step was to increase the amount of folate added to the liposomes to determine whether increasing targeting moieties revealed differences in uptake between cells with low and high FR. Non-targeting, folate-decorated liposomes with the original amount of folate added (0.81 nmol) and folate decorated liposomes with 3 times more folate added to the lipid layer (2.42 nmol) were used to treat KB and A549 cells (Figure 3.9). In the KB cell line, the presence of folate on liposomes decreased the fraction of cells positive for siRNA uptake. In addition, increasing the amount of folate used to decorate siRNA-containing liposomes further decreased the fraction of cells positive for siRNA uptake after treatment with those liposomes (Figure 3.9B). The decreased siRNA uptake may be at least partly attributable to decreased encapsulation of siRNA in complexes treated with folate: Cy3 siRNA is red in colour, and folate-decorated liposome:siRNA complexes were visibly less intensely coloured in solution than liposome:siRNA complexes without folate (*data not shown*), suggesting that less Cy3-siRNA was entrapped in folated liposomes. This was theoretically possible, since negative charge would be increased in DSPE molecules associated with folate and that negative charge might repel similarly negatively-charged siRNA. Regardless of the delivery agent, A549 cells all had >98% of cells positive for Cy3-siRNA uptake, suggesting that too high a concentration of liposomes was being used to allow identification of differences in uptake mediated by FR (Figure 3.9B, *white bars*). This was further exemplified when treatment with either non-targeting (*i.e.*, no folate) or folate-decorated liposomes resulted in >80% and >95% of cells being Cy3-siRNA positive in KB and A549 cells, respectively (Figure 3.10). The high level of siRNA uptake, as seen is the large peak shift in Figure 10A, was unusual. When DOTAP sources were investigated it was apparent that the DOTAP used to generate the liposomes was concentrated to a degree much higher than manufacturer specifications and, therefore, had increased the overall positivity of the liposomes. The increased positivity would have
Figure 3.9. Effect of increasing liposome folate decoration on the cellular uptake of Cy3-siRNA. Non-folated and folate-decorated liposomes were synthesized according to the “passive encapsulation” protocol. Folate was added directly to the lipid film layer prior to extrusion. FR positive (KB) and FR negative (A549) cell lines were grown in 6 well plates for 24 hours and transfected for 4 h before being harvested for flow cytometry.

A) Histogram showing gating of KB cells treated with fol(3X)-Cy3-C2RNA. 74.2% KB cells positive for Cy3-siRNA uptake, 25.8% negative.

B) Percentage of cells positive for Cy3-siRNA uptake using various delivery agents.

non-fol-Cy3-C2RNA – non-targeting liposomes

fol(1X)-Cy3-C2RNA – folate decorated liposomes

fol(3X)-Cy3-C2RNA – folate decorated liposomes with 3X as much folate added

One experiment, n = 3.
A

KB cells (control, no treatment with liposomes)

KB cells (treated with folated liposomes encapsulating Cy3-labeled siRNA (fol(3X)-Cy3-C2RNA))

Cy3 fluorescence

B

Effect of increasing folate on cellular uptake

KB (FR++)

A549 (FR-)

Percentage of cells positive for Cy3-siRNA

Relative amount of folate on liposome
Figure 3.10. Effect of high DOTAP concentration on the cellular uptake of Cy3-siRNA. Non-folated and folate-decorated liposomes were synthesized according to the “passive encapsulation” protocol. Folate was added directly to the lipid film layer prior to extrusion. FR positive (KB) and FR negative (A549) cell lines were grown in 6 well plates for 24 hours and transfected for 4 h before being harvested for flow cytometry.

A) Histogram showing gating of an individual A549 sample treated with fol(1X)-Cy3-C2RNA. 99.9% A549 cells positive for Cy3-siRNA uptake, 0.113% negative.

B) Percentage of cells positive for Cy3-siRNA uptake using various delivery agents.

*non-fol*-Cy3-C2RNA – non-folated, non-targeting liposomes

*fol*(1X)-Cy3-C2RNA – folate-decorated liposomes

*fol*(3X)-Cy3-C2RNA – folate-decorated liposomes with 3 times as much folate added as to *fol*(1X)-Cy3-C2RNA.

One representative experiment of several, n = 3.
A549 cells (control, no treatment with liposomes)

A549 cells (treated with folated liposomes encapsulating Cy3-labeled siRNA (fol(1X)-Cy3-C2RNA)

Cy3 fluorescence

Effect of increased DOTAP on cellular uptake

% of Max

100
80
60
40
20
0
0
10^0
10^1
10^2
10^3
10^4

KB (FR++)
A549 (FR-)

Treatment Group

Percentage of cells positive for Cy3-siRNA

Naked
non-fol-Cy3-C2RNA
fol(1X)-Cy3-C2RNA
fol(3X)-Cy3-C2RNA
been expected to increase non-specific uptake of all liposomes, regardless of folate status, due to strong interaction with negatively-charged plasma membranes. A fresh batch of DOTAP was purchased and used in future experiments.

In addition to the issue of DOTAP concentration, there remained the observation that no difference in cellular uptake of liposome-encapsulated siRNA between non-folated and folate-decorated liposomes in the FR-positive KB cell line (Figure 3.8). Literature reports indicate that the majority of folate added to medium surrounding FR-expressing cells is taken up within the first hour of addition and then plateaus\textsuperscript{130,131}. Therefore, to assess whether addition of folate to liposomes increased uptake into FR-positive cells in a time-dependent fashion, uptake at earlier times was tested (Figure 3.11). In accord with published reports\textsuperscript{130,131}, folate-decorated liposomes, especially when folate was added after the extrusion process, were taken up by FR-positive KB cells as soon as 30 m after addition and continued to be taken up by cells up to 2 h after addition. Non-folated liposomes delivered siRNA to less than 10% of KB cells up to the 1 h after addition, although approximately 30% of cells received siRNA by 2 h post-addition. This suggests that the greatest difference in uptake between non-targeting and folate-decorated liposomes might be achieved within the first hour of addition of liposomes to cells. All subsequent experiments were performed with a transfection time of 30 m. In addition, increasing the amount of folate by addition of folate directly to the lipid film layer before extrusion reduced Cy3 siRNA uptake (Figure 3.9B). On the other hand, increasing the amount of folate after extrusion increased Cy3 siRNA uptake (Figure 3.11B). These data suggest that post-extrusion incubation with folate is desirable as a folate-decoration strategy.

To further explore addition of folate after liposome extrusion as a strategy to increase uptake of liposome-encapsulated siRNA, a partial repeat of Figure 3.11B was carried out with the desired treatment groups. Non-folated liposomes and liposomes prepared using two concentrations of folate added after liposome extrusion were generated. They were then added to FR-positive KB cells for 30 minutes and siRNA uptake into those cells was assessed (Figure 3.12). The addition of folate following the
Figure 3.11. Effect of incubation time on the cellular uptake of liposome:Cy3-siRNA complexes into FR-positive KB cells. Non-folated and folate-decorated liposomes were synthesized according to the “passive encapsulation” protocol. FR-positive KB cells were grown in 6 well plates for 24 hours and incubated with liposome:siRNA complexes (C2: non-targeting scrambled control siRNA) for 30 m, 1 h, or 2 h before being harvested for flow cytometry.

A) Histogram showing gating of an individual KB sample treated with fol(1X)-Cy3-C2RNA (after) at 30 m. 70.2% KB cells positive for Cy3-siRNA uptake, 29.8% negative.

B) Percentage of cells positive for Cy3-siRNA uptake using various delivery agents.

non-fol-Cy3-C2RNA – non-targeting liposomes

fol(1X)-Cy3-C2RNA (before) – folate added to lipid film layer prior to extrusion

fol(1X)-Cy3-C2RNA (after)– folate added following the extrusion process

fol(3X)-Cy3-C2RNA (after)–3X the amount of folate added following the extrusion process.

One experiment, n = 3.
A

KB cells (control, no treatment with liposomes)

KB cells (treated with folated liposomes encapsulating Cy3-labeled siRNA (fol(1X)-Cy3-C2RNA) for 30 min)

Cy3 fluorescence

B

Transfection time on cellular uptake

- □ non-fol-Cy3-C2RNA
- ▲ fol(1X)-Cy3-C2RNA (before)
- ▲ fol(1X)-Cy3-C2RNA (after)
- ▼ fol(3X)-Cy3-C2RNA (after)

Percentage of cells positive for Cy3-siRNA

Transfection time (hours)
Figure 3.12. Effect of increasing liposome folate decoration following extrusion on the cellular uptake of Cy3-siRNA. Non-folated and folate-decorated liposome:siRNA complexes were synthesized according to the “passive encapsulation” protocol. Folate was incubated with liposomes for >1 h following extrusion. FR-positive (KB) cells were grown in 6 well plates for 24 hours and transfected for 30 m before being harvested for flow cytometric analysis of Cy3-labeled siRNA uptake.

A) Histogram showing gating of an individual KB sample treated with fol(3X)-Cy3-C2RNA. 72.9% KB cells positive for Cy3-siRNA uptake, 27.1% negative.

B) Percentage of cells positive for Cy3 siRNA using various delivery agents

non-fol-Cy3-C2RNA – non-targeting liposomes

fol(1X)-Cy3-C2RNA – folate decorated liposomes

fol(3X)-Cy3-C2RNA – folate decorated liposomes with 3X as much folate added

Two experiments, n = 6

*p<0.05 compared to non-fol-Cy3-C2RNA

**p<0.05 compared to fol(1X)-Cy3-C2RNA
A

KB cells (control, no treatment with liposomes)

KB cells (treated with folated liposomes encapsulating Cy3-labeled siRNA (fol(3X)-Cy3-C2RNA) for 30 min

Cy3 fluorescence

B

Effect of increasing amount of folate following extrusion

Treatment Group

non-fol-Cy3-C2RNA
fol(1X)Cy3-C2RNA
fol(3X)Cy3-C2RNA
extrusion process increased the percentage of KB cells positive for Cy3 siRNA by ~30% and increasing the amount of folate added to the liposomes by 3X further enhanced Cy3-siRNA positivity by another ~20%. To take the analysis further, and to assess whether liposomes prepared with folate added after extrusion would target siRNA delivery to FR-positive cells more effectively than to FR-negative cells, a wider range of folate concentrations were added to liposome aliquots following extrusion and the resulting folated liposome:siRNA complexes added to both FR-positive KB and FR-negative A549 cells (Figure 3.13). Figures 13A and 13B are different representations of the same data for ease of comparison either within the same cell line or between the two, respectively. Decorating the liposomes with folate significantly increased Cy3 siRNA uptake in both KB and A549 cells (Figure 3.13A). However, the increase was greater in the FR positive KB cell line at all folate concentrations (Figure 3.13B). Furthermore, increasing the amount of folate added to the liposomes up to 6 times more than the initial level of added folate enhanced Cy3 siRNA uptake in a folate concentration-dependent manner, while increasing the amount of folate from 1X to 6X did not increase siRNA uptake in the FR-negative A549 cell line (Figure 3.13A).

3.2.3 Non-targeting and folate decorated liposome tissue/organ distribution following systemic administration in nude mice.

To complement the in vitro uptake data, the biodistribution of the liposome particles was assessed after tail vein (systemic) administration. An amount of 0.035:50 molar mass DOTAP:Cy3-C2RNA liposome/siRNA mixture (5 nmol siRNA per injection prior to extrusion) was injected into nude mice and, after 6 h, mice were sacrificed for tissue collection. The KB tumours (Figure 3.14a) and liver, spleen, connective tissue (surrounding tumour), kidney, and muscle (Figure 3.14b) were removed and placed in plastic cassettes containing O.C.T for cryosectioning. The samples were then cut into 10 μm thick sections and photographed immediately using a fluorescence microscope. There was limited fluorescence in both the non-folated and folate-decorated liposome treated KB xenografts. Subjectively, KB tumours treated with the non-targeting liposome may have a higher Cy3 siRNA fluorescence than tumours treated with the folate-decorated liposomes (Figure 3.14A). However, this cannot be quantified due to a lack of overall
Figure 3.13. Effect of increasing folate concentrations used to functionalize liposomes after extrusion on cellular uptake of Cy3-siRNA. Non-folated and folate-decorated liposomes were synthesized according to the “passive encapsulation” protocol. Folate was incubated with liposomes for >1 h following extrusion. FR-positive (KB) and FR-negative (A549) cells were grown in 6 well plates for 24 hours and treated with siRNA:liposomal preparations for 30 min before being harvested for flow cytometric analysis of siRNA uptake.

A) Histogram showing gating of an individual KB sample treated with fol(10X)-Cy3-C2RNA. 75.9% KB cells positive for Cy3-siRNA uptake, 24.1% negative.

B) Bar graph of percentage of cells positive for Cy3 siRNA using range of folate concentrations added after extrusion to compare within same cell lines

C) Line graph showing percentage of cells positive for Cy3 siRNA after treatment with liposomes prepared using a range of folate concentrations added after extrusion to compare between cell lines (different representation of same data in B)

- non-fol-Cy3-C2RNA – non-targeting liposomes
- fol(1X)-Cy3-C2RNA – folate decorated liposomes
- fol(3X)-Cy3-C2RNA – folate decorated liposomes with 3X as much folate added
- fol(6X)-Cy3-C2RNA – folate decorated liposomes with 6X as much folate added
- fol(10X)-Cy3-C2RNA – folate decorated liposomes with 10X as much folate added.

Two experiments, n = 6

A: p<0.05 compared to non-fol-Cy3-C2RNA in KB cells
B: p<0.05 compared to fol(1X)-Cy3-C2RNA in KB cells
C: p<0.05 compared to fol(3X)-Cy3-C2RNA in KB cells
D: p<0.05 compared to non-fol-Cy3-C2RNA in A549 cells

*: p<0.05 compared to corresponding folate concentrations in A549 cells
KB cells (control, no treatment with liposomes)

KB cells (treated with folated liposomes encapsulating Cy3-labeled siRNA (fol(10X)-Cy3-C2RNA) for 30 min

Effect of increasing folate following extrusion on cellular uptake

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>KB (FR++)</th>
<th>A549 (FR-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-fol-Cy3-C2RNA</td>
<td>A</td>
<td>D</td>
</tr>
<tr>
<td>fol(1X)-Cy3-C2RNA</td>
<td>A</td>
<td>D</td>
</tr>
<tr>
<td>fol(3X)-Cy3-C2RNA</td>
<td>A</td>
<td>D</td>
</tr>
<tr>
<td>fol(6X)-Cy3-C2RNA</td>
<td>A,B,C</td>
<td>D</td>
</tr>
<tr>
<td>fol(10X)-Cy3-C2RNA</td>
<td>A,B,C</td>
<td>D</td>
</tr>
</tbody>
</table>
C

Percentage of cells positive for Cy3-siRNA

Concentration of folate-DSPE added after extrusion (pmol/ul)

- KB (FR++)
- A549 (FR-)

* indicates significant difference
Figure 3.14. Tissue/organ distribution of non-targeting and folate decorated liposomes containing Cy3 siRNA. Mice were inoculated with 2 million KB cells (Section 2.16) and euthanized on day 27. Six h prior to euthanasia, mice were systemically (tail vein) injected with 0.035:50 molar mass DOTAP:Cy3-C2RNA liposome/siRNA mixture (5 nmol siRNA per injection prior to extrusion). KB xenografts and tissues were harvested from similar areas on each mouse and placed in a plastic cassette containing OCT (freezing media). The samples were then frozen at -80°C and cut in 10 μm sections for fluorescence microscopy to visualize fluorescent Cy3 C2 siRNA accumulated in tissues.

A) Representative images of KB xenograft tumours in 3 of the mice per treatment group

B) Representative images of selected tissues from the organs of a selected mouse (only one used as fluorescence patterns were similar across mice)

6 mice per treatment group.

Magnification 100X
<table>
<thead>
<tr>
<th></th>
<th>Untreated Control</th>
<th>Non-fol-Cy3-C2RNA</th>
<th>Fol-Cy3-C2RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><img src="image1" alt="Untreated Control" /></td>
<td><img src="image2" alt="Non-fol-Cy3-C2RNA" /></td>
<td><img src="image3" alt="Fol-Cy3-C2RNA" /></td>
</tr>
<tr>
<td>Mouse #1</td>
<td><img src="image4" alt="Mouse #1 Untreated Control" /></td>
<td><img src="image5" alt="Mouse #1 Non-fol-Cy3-C2RNA" /></td>
<td><img src="image6" alt="Mouse #1 Fol-Cy3-C2RNA" /></td>
</tr>
<tr>
<td>Mouse #2</td>
<td><img src="image7" alt="Mouse #2 Untreated Control" /></td>
<td><img src="image8" alt="Mouse #2 Non-fol-Cy3-C2RNA" /></td>
<td><img src="image9" alt="Mouse #2 Fol-Cy3-C2RNA" /></td>
</tr>
</tbody>
</table>
Mouse #3

B  
Untreated Control  Non-fol-Cy3-C2RNA  Fol-Cy3-C2RNA

Liver
Connective tissue

Spleen
Striated muscle
fluorescence observed within individual images and variability between imaged sections (some sections displayed no fluorescence). The other organs with the highest fluorescence were the liver and spleen, which is expected because organs in the reticuloendothelial system preferentially accumulate systemically injected nanoparticles. Unexpectedly, fluorescence was only observed in muscle treated with non-targeting liposomes, which may have only been an artifact of the non-targeting treatment group containing a higher initial concentration of Cy3-siRNA. Images were also taken of the kidney, where cationic liposomes have been known to accumulate. However, due to autofluorescence from the tissue itself, no fluorescence attributable to Cy3 siRNA accumulation could be confirmed (data not shown).

3.3 Ability of liposome:targeting siRNA complexes to knock down a gene target

3.3.1 Antisense effect of liposomes delivering TS siRNA payload in vitro

Before testing the liposome formulations for their ability to effectively downregulate TS transcript levels, the functionality of the TS siRNA (TSRNA) in KB and A549 cells (both of which express TS) was verified using a proven commercial liposomal delivery system (Lipofectamine 2000). As reported previously using this anti-TS siRNA in A549 and other cell lines, TS transcript levels were reduced by ~80%. TS knockdown in KB cells is shown for the first time.

Measurement of TS mRNA before and after treatment with liposome:siRNA complexes indicated that the liposomes were not able to downregulate TS mRNA levels 24 hours after addition of complexes (results not shown and Figure 3.16). The use of PEG in liposomes, although desirable to increase liposome:siRNA time in circulation time and to inhibit RES entrapment, might also abrogate siRNA-mediated mRNA degradation by preventing escape of internalized siRNAs from endosomes. To assess possible PEG inhibition of siRNA activity, both PEGylated and non-PEGylated liposomes were formulated and tested for their silencing ability in KB and A549 cell lines at 24 h post-addition (Figure 3.16). Neither PEGylated nor non-PEGylated liposomes were able to downregulate TS mRNA levels in either of the cell lines. To confirm that the liposome:siRNA complexes were capable of entering cells, fluorescent images were
Figure 3.15. Relative quantity of TS mRNA transcripts following treatment with TS siRNA delivered using Lipofectamine 2000 (LF2K). KB and A549 cells were grown in 6 well plates for 24 h. Cells were then transfected with TS siRNA for 4 h using LF2K. Fresh media was added to the wells and 24 h later the cells were harvested for measurement of TS mRNA (GAPDH used as endogenous control) by qPCR.

*LF2K C2RNA* – transfected with LF2K containing 10 nM control siRNA

*LF2K TSRNA* – transfected with LF2K containing 5 nM control siRNA and 5 nM TS RNA

One experiment, n = 3.
TS downregulation using commercial Lipofectamine 2000 vehicle

Relative quantity of TS mRNA
(fold change)

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>LF2K C2RNA</th>
<th>LF2K TSRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB</td>
<td>1.2</td>
<td>0.3</td>
</tr>
<tr>
<td>A549</td>
<td>1.0</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Figure 3.16. TS mRNA transcript levels following treatment with PEGylated and non-PEGylated liposomes. Non-folated and folate-decorated liposome:siRNA complexes were synthesized according to the “passive encapsulation” protocol. The PEGylated liposomes were then coated with DSPE-PEG. KB and A549 cells were grown in 6 well plates for 24 h. Cells were then treated with treated with complexes for 4 h. 24 h later the cells were harvested for real-time PCR.

A) TS mRNA levels in KB cells

B) TS mRNA levels in A549 cells

non-fol-Cy3-C2RNA (+PEG) – non-targeting liposomes containing control siRNA and coated with PEG

non-fol-Cy3-C2RNA (+PEG) – non-targeting liposomes containing control siRNA and no PEG on surface

non-fol-Cy3-TSRNA (+PEG) – non-targeting liposomes containing TS siRNA and coated with PEG

non-fol-Cy3-TSRNA (+PEG) – non-targeting liposomes containing TS siRNA and no PEG on surface

fol-Cy3-C2RNA (+PEG) – folate decorated liposomes containing control siRNA and coated with PEG

fol-Cy3-C2RNA (+PEG) – folate decorated liposomes containing control siRNA and no PEG on surface

fol-Cy3-TSRNA (+PEG) – folate decorated liposomes containing TS siRNA and coated with PEG

fol-Cy3-TSRNA (+PEG) – folate decorated liposomes containing TS siRNA and no PEG on surface

One experiment, n = 2.
A

Real-time PCR of TS transcript levels in KB cells

B

Real-time PCR of TS transcript levels in A549 cells
taken 24 h following transfection (Figure 3.17). The liposomes used were non-PEGylated in order to maximize uptake and increase potential to downregulate TS. From the images, it is evident that the cells took up a significant amount of Cy3-TS siRNA. To confirm that siRNA was not confined to target cell surfaces without entry into cell interior, several samples were analyzed by confocal microscopy through several planes. Confocal images confirmed that siRNAs were present in cytoplasm (data not shown).

Knowing that the siRNA delivered using liposomes entered target cells, it was possible that endocytosed liposome:Cy3 siRNA particles required longer than 24 h to escape the endosomal compartment and enter cytoplasm and interact productively with RISC complexes. Therefore, cells were harvested 48 h after siRNA: liposome complex addition and assessed for TS mRNA knockdown by qPCR (Figure 3.18b). Additionally, and in order to promote endosomal escape, the liposomes were synthesized with higher degrees of DOTAP (increased positivity) and the fusogenic lipid DOPE was added (see end of Section 2.1 in Introduction for discussion of factors affecting endosomal escape). The presence of protamine was also investigated to determine whether the stable complex it formed with the siRNA might interfere with siRNA activity (Figure 3.18). Only non-targeted liposomes were synthesized in an attempt to generate TS downregulation of any degree before using reagents to make targeted liposomes. No significant reduction in TS mRNA levels was observed after treatment with any of the liposome formulations at either 24 or 48 hours. Although treatment with TS siRNA:liposome formulations containing 4X DOTAP and DOPE resulted in TS mRNA levels that trended to decrease, that did not induce significant differences. It is possible that increasing the positivity and amount of DOPE used to generate liposomes might be a fruitful strategy to lead to target mRNA knockdown.

3.3.2 Antisense effect of liposomes delivering TS siRNA payload in vivo

The formulated liposomes were unable to downregulate TS mRNA levels in vitro. However, circumstances in vivo are different. Therefore, and while assessing the biodistribution of liposomes in vivo, TS mRNA levels in KB tumour xenografts were analyzed following a 4 week treatment course where liposome:Cy3 siRNA complexes
Figure 3.17. Fluorescent images of KB cells transfected with liposome/Cy3-siRNA. Non-folated and folate-decorated liposome:sirNA complexes were synthesized according to the “passive encapsulation” protocol. KB cells were cultured for 24 h in 6 well plates and then transfected for 4 h to maximize uptake. 24 h later the cells were washed 2X with PBS to remove any excess liposome:sirNA. The cells and siRNA uptake were then imaged by fluorescence microscopy. The blue background in the overlay images is an artifact generated by the computer program software.

Magnification 200X
Untreated Control  Non-fol-Cy3-C2RNA  Fol-Cy3-C2RNA

Cy3 siRNA

Bright field

Overlay
Figure 3.18. TS mRNA transcript levels 24 and 48 h after treatment with various liposomal formulations. Non-folated liposome:siRNA complexes were synthesized according to the “passive encapsulation” protocol. KB cells were grown in 6 well plates for 24 h. Cells were then treated with complexes for 4 h. 24 or 48 h later the cells were harvested for real-time PCR.

A) TS mRNA levels in KB cells 24 h after transfection

B) TS mRNA levels in KB cells 48 h after transfection

*DOT 2X C2RNA (+prot)* – liposomes with 2X the amount of DOTAP included carrying control siRNA complexed with protamine

*DOT 2X TSRNA (+prot)* – liposomes with 2X the amount of DOTAP included carrying TS siRNA complexed with protamine

*DOT 2X C2RNA (-prot)* – liposomes with 2X the amount of DOTAP included carrying control siRNA with no protamine

*DOT 2X TSRNA (-prot)* – liposomes with 2X the amount of DOTAP included carrying TS siRNA with no protamine

*DOT 2X, DOPE C2RNA (+prot)* – liposomes with 2X the amount of DOTAP and DOPE included carrying control siRNA complexed with protamine

*DOT 2X TSRNA (+prot)* – liposomes with 2X the amount of DOTAP and DOPE included carrying TS siRNA complexed with protamine

*DOT 4X C2RNA (+prot)* – liposomes with 4X the amount of DOTAP included carrying control siRNA complexed with protamine

*DOT 4X TSRNA (+prot)* – liposomes with 4X the amount of DOTAP included carrying TS siRNA complexed with protamine

*LF2K C2RNA* – transfected with LF2K containing 10 nM control siRNA

*LF2K TSRNA* – transfected with LF2K containing 5 nM control siRNA and 5 nM TS RNA.

One experiment, n = 2.
A

**TS mRNA levels 24 hours after transfection**

![Graph showing mRNA levels 24 hours after transfection.](image)

**Treatment Group**

B

**TS mRNA levels 48 hours after transfection**

![Graph showing mRNA levels 48 hours after transfection.](image)

**Treatment group**
were injected twice per week in mouse tail veins. The mice were then sacrificed on day 27 and the KB xenografts were harvested for RNA isolation and qPCR (Figure 3.19). Similar to the results seen in vitro, no TS downregulation was observed in any of the treatment groups in vivo. The lack of downregulation would have likely been further compounded by the limited uptake in tumour tissue as observed in the biodistribution study (Figure 3.14A).
Figure 3.19. **TS mRNA levels in KB xenografts from nude mice following systemic liposome:Cy3 TS siRNA administration.** Mice were inoculated at 2 sites with 2 million KB cells and sacrificed on day 27. Mice were systemically injected (tail vein) with 0.035:50 molar mass DOTAP:Cy3-TSRNA liposome:siRNA complexes once per week for 4 weeks. KB xenografts were harvested from each mouse and frozen at -80°C. Sections of tumour were thawed and RNA was isolated for qPCR analysis of TS mRNA levels (GAPDH used as endogenous control).

*non-fol-Cy3-TSRNA* – non-targeting liposomes containing TS siRNA

*fol-Cy3-C2RNA* – folate decorated liposomes containing TS siRNA

*fol-Cy3-TSRNA* – folate decorated liposomes containing TS siRNA.

6 mice per treatment group, 2 tumours per mouse.
TS mRNA levels in KB xenografts

Relative quantity of TS mRNA (fold change)

- non-treated cells
- fol-Cy3-TSRNA
- fol-Cy3-C2RNA
- fol-Cy3-TSRNA

Treatment group
Chapter 4

4 Discussion

The purpose of this work was to assess the ability of folate functionalized liposomes to deliver TS siRNA to the RNAi machinery of FR-positive cancer cells to activate its antisense effect. We initially hypothesized that our non-targeting liposomes will facilitate siRNA uptake into cells more efficiently than naked siRNA alone. Subsequently, decoration of liposomes with folate ligand will enhance their ability to deliver siRNA to FR-positive human tumour cells both in vitro and in vivo as a method to increase antisense activity over non-targeting vehicles. In this section I will discuss implications of the physical attributes of the liposome particles [Section 4.1: particle charge and size (Section 4.1.1), levels of folate decoration (Section 4.1.2), siRNA encapsulation yields (Section 4.1.3), serum nuclease protection (Section 4.1.4)]; analysis of cellular uptake and biodistribution studies [Section 4.2: choice of cells lines (Section 4.2.1), troubleshooting lack of FR-mediated enhancement of uptake (Section 4.2.2)]; and challenges faced with TS mRNA transcript knockdown (Section 4.3).

4.1 Implications of liposome physical characteristics

4.1.1 Measurements of particle charge and size

The non-targeting and folate decorated liposomes were determined to have zeta potentials in the range of 30-35 mV and diameters spanning from 130-150 nm. These values have varying implications depending on whether in vitro or in vivo liposome uptake and cargo delivery is being optimized. For example, in vitro, a higher positive zeta
potential or surface charge usually equates with higher cellular uptake\textsuperscript{133,134} and greater toxicities\textsuperscript{135} due to interactions with the negatively-charged plasma membrane on cells. In an \textit{in vivo} setting, liposomes with an increased positive charge have been reported to correlate with enhanced accumulation in the liver: a consequence that may be undesirable, depending on the where delivery is desired (in this case, undesirable because delivery to tumour and not liver is the goal). However, and even with increased liver uptake, increased positive charge on liposomes has been shown to enhance uptake by tumour vasculature compared to neutral or anionic liposomes\textsuperscript{132,136}. This correlates with the \textit{in vivo} biodistribution of positively-charged liposomes I observed, because the highest amount of Cy3 siRNA fluorescence could be seen in the liver tissue (Figure 3.14B). Particles with zeta potentials greater than 30 mV are considered strongly cationic and this strength of electrostatic repulsion has been linked to greater stability over time and resistance to the tendency of particles to aggregate over time and increase in size\textsuperscript{137}. In fact, the data showing liposome stability over time is positive and encouraging. The liposomes remained stable up to 6 h and had begun to aggregate only 12 h after synthesis (Figure 3.2C). Remaining stable in solution for longer than a few hours was critical to ensuring that aggregation did not taken place from the time of synthesis to the time of addition to cells in culture or tail vein injection into whole animals: an interval likely to be several hours under normal circumstances. On the other hand, if the particles are too stable it can prevent siRNA release into the cytosol once taken up by the cell\textsuperscript{138}.

There is some debate as to the size of liposomes for optimal delivery to tumour tissue. Some researchers believe particles should be less than 100 nm\textsuperscript{104}, while others would argue a diameter less than 200 nm is sufficient for effective \textit{in vivo} delivery\textsuperscript{133,139}. 
Sizes over 200 nm have been shown to switch from a clathrin-dependent mechanism of endocystosis to a caveolae-dependent uptake mechanism, and this could influence cellular uptake depending on mechanisms available in target cell populations\textsuperscript{140}. In addition, the maximum size of particles capable of escaping leaky tumour vasculature varies depending on tumour type, but typically falls somewhere in the range of 200-600 nm\textsuperscript{141,142}. Particles larger than this size would have a difficult time taking advantage of the EPR effect to accumulate at the tumour environment (see Section 1.6 for further explanation of EPR effect and challenges of delivery). Therefore, the non-targeting and folate-decorated liposomes fall within a size range that should allow for effective use both in vitro and in vivo. However, it will be important to conduct an in vivo study comparing the biodistribution of current liposomes versus those of smaller size resulting from further extrusion using 50 nm pores.

4.1.2 Low folate insertion in liposomes

It was surprising that less than 10\% of the DSPE-PEG-folate that was added to the liposomes either before extrusion or after was inserted in the liposomes (Section 3.1.2). Some research groups have reported between 60-90\% folate insertion with their post-insertion techniques\textsuperscript{143-145}. If 100\% of the DSPE-PEG-folate was inserted into liposomes, then the folate-decorated liposomes would have an overall folate mol\% of 0.23, meaning the actual folate mol\% < 0.023\% for the liposomes containing the standard amount of folate (fol(1X)) since less than 10\% insertion was observed (Section 3.1.2). This amount of inserted DSPE-folate-PEG is considerably lower than typically used. However, one study reported optimal cellular uptake with liposomes bearing as low as 0.03\% fol mol\%\textsuperscript{146}. The low amount of incorporated folate may be one reason that folate-decoration
of liposomes did not enhance uptake in FR-positive KB cells compared to the FR-negative A549 cells (Figure 3.8) unless the amount of folate added following liposome extrusion was increased (Figures 3.12 & 3.13). Regardless, from data reported in the literature\textsuperscript{144,147} and experience gained in the course of these studies, inserting the folate following extrusion allows for better control and folate decoration exclusively on the outside of the liposome (rather than both in the interior and on the exterior), compared to adding it directly to the lipid layer. Direct addition of folate to the lipid prior to exclusion results in folate incorporation roughly equally on the exterior and in the interior of the lipid bilayer, thus reducing the amount of exterior folate available for binding to target cell folate receptors\textsuperscript{147}.

4.1.3 Assessing siRNA encapsulation yield

Using the passive encapsulation protocol for liposome synthesis, generally 3-40% of antisense molecules will become encapsulated depending on the lipid constituents and overall charge of the particles\textsuperscript{148-150}. Incorporation of 100% of siRNA in the interior would be unexpected because, theoretically, 50% of siRNA molecules should attach to the interior of the liposome as it forms and 50% should interact with the exterior. In addition, the extrusion process is relatively inefficient at associating siRNA with liposomes, as evidenced by pink colouring on filters (due to the pink colour of Cy3 siRNA visible to the naked eye) after extrusion (subjective observation, data not shown). Therefore, a roughly 44% siRNA encapsulation percentage as indicated by the RiboGreen assay (Table 3.2) is relatively successful in terms of siRNA:liposome association. However, due to certain limitations of the RiboGreen assay (discussed above) the degree of association may be overestimated. Regardless, a higher percentage
of siRNA in liposomes achieved using this method, compared to other passive encapsulation methods, is not unexpected as the liposomes have a strong cationic charge. That charge would facilitate electrostatic interactions with the negatively charged siRNA.

**4.1.4 Prolonged protection of siRNA against serum nucleases**

The ability of both the non-targeting and folate decorated liposomes to protect siRNA from nuclease degradation up to 48 h was significant for two reasons. First, systemically-injected liposomes must pass through circulation before reaching their target tissues, so siRNA degradation soon after exposure to serum would render the therapy ineffective. Second, in many cases it can take anywhere from 12-24 h for liposome particles to reach and accumulate in the tumour microenvironment, and siRNA protection from nucleases remains essential during the period of time after arrival at target tissues and uptake into target cells\textsuperscript{151,152}.

**4.2 Folate decoration on uptake and biodistribution studies**

**4.2.1 Choice of cell lines**

It has become common practice in testing the efficacy of folate-ligated particles to use the FR-positive KB cell line as well as a low FR-expressing cell line, such as A549\textsuperscript{145,153,154}. The presence of the low FR expressing cell line, A549, helped to determine whether cell-specific, FR-independent differences in uptake between the targeting and folate-decorated liposomes are important considerations that would otherwise have been overlooked if only the FR-positive KB cells were used to determine differences in uptake between folate-positive and folate-negative liposomes. FR
expression was also measured in cells exposed to folate-depleted media for 3 h prior to exposure to siRNA:liposome preparations, to ensure that their FR levels were not upregulated by lack of folate (a possible cellular response to low folate). Upregulation would not have been expected during this very short time, but an increase in FR levels of over tenfold has been reported within 24 hours of folate depletion155.

4.2.2 Troubleshooting enhancement of FR-mediated uptake

One of the two greatest challenges with this project was achieving an enhanced uptake in the FR-positive KB cells when exposed to folate-decorated liposomes, compared to non-folated non-targeting liposomes. The first major breakthrough on this front came when the liposome exposure time was reduced. The literature indicated that FR-mediated endocytosis occurs primarily within the first hour of exposure to liposomes130,131, while the non-specific uptake of cationic liposomes still increases past 4 hours156. This is likely due to FR-mediated uptake being an active process versus passive general endocytosis. Since reducing the time to 30 minutes enhanced the difference in uptake between the folate-decorated and non-folated non-targeting liposomes (Figure 3.11) it is hypothesized that part of the lack of enhancement seen previously (Figures 3.8 & 3.9) may have been due to the non-targeting liposomes and/or both non-targeting and targeting liposomes reaching maximal uptake at some point during a 4 hour period of exposure to cells (i.e., a "plateau" effect obscuring differences that would have been evident before plateaus are reached). Furthermore, it was encouraging to see that increasing the amount of folate added to the liposomes increased cellular uptake in the KB cells, but not in the A549 cells (Figure 3.13), similar to reports of others154,157. Additionally, decorating the liposomes with any amount of folate caused a greater
increase in uptake in the KB (~40%) cells than compared to the A549 cells (~10%). The observed 10% increased in uptake of the folate decorated liposomes in FR-negative A549 cells further reinforces the usefulness of the strategy of including a negative control in the experiment, as it is likely that folate decoration also enhances uptake to some degree via a non-FR-mediated interaction (e.g., steric hindrance, surface charge).

As mentioned previously, cationic liposomes have been shown to preferentially accumulate in the liver\textsuperscript{132,158}. It was unsurprising, then, that the biodistribution study revealed an abundance of Cy3 siRNA fluorescence in the biopsied liver tissue (Figure 3.14B). Cationic liposomes also have a propensity to localize in the spleen, especially after saturating the liver, which is expected as these are the major players of the RES system\textsuperscript{132,159}. Indeed, the results reported here are consistent with that. Unfortunately, there did not appear to be a higher fluorescence of Cy3 siRNA delivered by folate-decorated liposomes to the FR-positive KB tumours compared to delivery by non-targeting liposomes. In fact, the tumours in animals treated with siRNA-containing but non-folated (non-targeting) liposomes may have accumulated a larger amount of siRNA (with subjectively-assessed higher fluorescence overall)(Figure 3.14A). However, if true, this could also be due to slightly higher amounts of Cy3 siRNA loaded into the non-folated liposomes (compared to folated liposomes), although they were prepared side by side and under identical conditions. Another possible factor contributing to lack of differences in accumulation of non-folated and folate-decorated liposomes in tumours could be the amount of folate ligand exposed on the liposomes and available for binding to receptors. The data from the \textit{in vitro} uptake studies showed that uptake increased in FR-positive KB cells when folate decoration was increased post extrusion (Figures 3.12
& 3.13). On the other hand, the *in vivo* biodistribution study was performed with liposomes where folate was inserted directly into the lipid film layer prior to extrusion, meaning that less folate would have accumulated on the outside of the liposome and been available for binding. Several studies indicate that decorating particles with a targeting ligand does not increase accumulation of liposomal cargo molecules in the tumour environment, compared to similar non-targeting particles. However, targeting does increase active uptake in tumour cells themselves, which helps to overcome inhibition of non-specific endocytosis due to the EPR effect. As a result, one would expect to see non-targeting liposomes accumulate in the interstitium and connective tissue surrounding the tumour and more targeted liposomes penetrating the actual tumour. Therefore, assuming that folate decoration was low on the targeting liposomes, it is not surprising to see the non-targeting liposomes accumulate in tumour tissues to the same extent as their targeting counterparts (Figure 3.14A). At the same time it was disappointing that no fluorescence was observed in the connective tissue in close proximity to the tumour, which likely has more to do with a low overall amount of liposome/Cy3 siRNA that reached the tumour area.

4.3 **Challenges of achieving an antisense effect (transcript knockdown) in cells**

The second major obstacle faced in this project was the inability of the non-targeting or folate decorated liposomes to achieve TS transcript downregulation both *in vitro* and *in vivo*. Initially, the thought was that a new batch of Cy3 TS siRNA obtained from Dharmacon might have an incorrect, non-complementary sequence. However, testing with a commercially-available transfection reagent (Lipofectamine 2000) indicated that the siRNA was able to downregulate TS mRNA in A549 cells when not
encapsulated in the liposomes tested in this study (Figure 3.15). Another possibility was that temperature fluctuations between 50-70°C during the extrusion process could have degraded the siRNA and rendered it ineffective, but non-modified siRNA has been shown to maintain its integrity and function after exposure to temperatures reaching 95°C and after temperature cycling from low to high\textsuperscript{161}. As mentioned previously, the incorporation of PEG, while useful, can also impede endosomal escape\textsuperscript{60}. Therefore, non-PEGylated liposomes were synthesized in an attempt to maximize the potential for TS mRNA downregulation. The absence of PEG, however, did not result in a reduction in TS transcript levels in cells treated with siRNA:liposomes. We also varied the time between exposure of cells to targeting siRNA:liposome preparations and harvesting the cells for qPCR, based on the hypothesis that a longer time for liposomes to achieve endosomal escape to release siRNA payload into cytoplasm, but this modification did not result in siRNA effectiveness. It appears, from fluorescent microscope images, as though a great deal of siRNA had been taken up by the cell and, in some cases, the fluorescence pattern was punctate, suggesting containment in endosomes\textsuperscript{162-164} (Figure 3.17). A punctate pattern is not completely diagnostic of endosomal entrapment, however, and cannot be considered sufficiently strong evidence that this is the actual cause for lack of transcript downregulation. While not statistically significant, an increase in the amount of positive DOTAP used and the inclusion of the fusogenic lipid DOPE appeared to result in a trend to decreased TS mRNA levels (Figure 3.18A) suggesting that increasing their use could potentially further decrease target mRNA levels. This would not be surprising, as an increase in DOTAP has been shown to increase liposomal uptake efficiency\textsuperscript{165}. As utilized in other studies a way to test whether the issue is, in fact, a lack of endosomal
release, may be by incubation with influenza-derived fusogenic peptide diNF-7\textsuperscript{166} or exposure of siRNA:liposome preparations to cells in conjunction with photochemical internalization (PCI)\textsuperscript{163}, both of which are proven methods to facilitate endosomal release. diNF-7 undergoes a conformational change at lower pHs due to protonation and the resulting complex fuses with endosomes, thus facilitating their destabilization. PCI relies on photosensitizer molecules that accumulate in endosomes and become activated upon exposure to blue light, whereby they produce singlet oxygen species that bind the membrane and increase its permeability\textsuperscript{167}. If, however, these have no effect, then the problem may arise from other causes, such as siRNA degradation once following cell internalization or a lack of liposome-siRNA release preventing proper RISC incorporation even if they are able to escape the endosome. The first problem could be tested by first conjugating siRNA with biotin and then following a 24 h transfection isolating the biotin-siRNA using streptavidin beads\textsuperscript{168} and re-transfect them using a proven commercial agent (Lipofectamine) to determine if they are still functional. The second potential issue could be investigated indirectly by incubating liposome-siRNA complexes in serum or various pH buffered solutions and measuring changes in free siRNA, by RiboGreen, for example\textsuperscript{169}. If no siRNA is being released, perhaps the liposomes are too stable and unable to efficiently release their payload.

The non-targeting and folate-decorated liposomes were characterized according to their surface charge, size, stability and ability to protect siRNA against nuclease degradation. Quantification of the amount of siRNA encapsulated is insufficiently precise and continues to require refinement. Some strategies to achieve more accurate readings could include adding a highly anionic molecule (i.e. heparin) to outcompete siRNA for
lipid/protamine binding so it can freely bind the RiboGreen dye, or, modify RNA isolation kits that utilize filter/ultracentrifuge techniques to concentrate siRNA. Enhanced uptake of folate-decorated liposomes compared to their non-targeting counterparts in FR positive human cells was achieved by increasing the amount of folate inserted into their outer leaflet over a shortened time of exposure of siRNA:liposomal constructs to target cells. Unfortunately, downregulation of TS mRNA levels using TS siRNA:liposome preparations was not achieved in spite of preferential uptake by FR-positive cells. However, the data suggest possible causes of lack of effectiveness of siRNA delivered using liposomal methods and areas for future study.

This work required a great deal of troubleshooting especially in the early stages to achieve enhanced delivery of folate decorated liposomes to FR positive cancer cells compared with non-targeting particles. Keeping this in consideration, the enhanced uptake with our folate decorated liposome may not have been as great as reported in some of the literature, however, I would caution fellow researchers to consider the physiological relevance of the cell cultures commonly used. The gold standard in the literature appears to be KB or cells synthetically transfected with high FR expressing vectors grown and maintained in folate depleted media, which promotes FR upregulation and may indeed have rendered these cells far more addictive to folate than the physiological folate levels exposed to normal or malignant FR expressing cells. Therefore, I think this work can be used to help bring the study of FR-mediated uptake to other FR expressing cancer types and the probable realistic gains that can be achieved.
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September 2011 – 2013

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- (NSERC CGS-M) - *National level external award*
September 2011 – 2012

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- *Institutional level award based on highest graduating average*
April 2011

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May 2008 – August 2008

MacLulich Medal in Biology
- *Departmental level award based on highest graduating average*
May 2011

WLU Scholarship for High Academics
- *Institutional level award for GPA above 11.0 (out of 12)*
September 2010 – April 2011

WLU Dean’s List
- *Institutional level award for cumulative GPA above 10.0 (out of 12)*
September 2009 – April 2010

September 2010 – April 2011
Tau Sigma Transfer Student Award  
- *Awarded to top 20% (GPA) of transfer students in USA*  
August 2008

Frank Santarossa Scholarship  
- *Awarded to 5 players in Midwestern Jr. B Hockey League with high academics*  
September 2007

Undergraduate Entrance Scholarship  
- *Above 90% entrance average*  
September 2006 – 2007

**Publications**


Di Cresce, C., Way, C., Rytelewski, M., Maleki Vareki, S., Nilam, S., Vincent, M.D., Koropatnick, J., Ferguson, P.J. 2012. Antisense Technology: From Unique Laboratory Tool to Novel Anticancer Treatments (Chapter 12). From Nucleic Acids Sequences to Molecular Medicine, RNA Technologies; Springer-Verlag Berlin Heidelberg. (*Published; Secondary Author*)

**Presentations**


**Relevant Work and Volunteer Experience**

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<th>Period</th>
<th>Position</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>May 2013 – Present</td>
<td><strong>Volunteer at Thames Valley Children’s Centre, London</strong></td>
<td>Work with special needs children.</td>
</tr>
<tr>
<td>September 2011 – Present</td>
<td><strong>Let’s Talk Science Volunteer</strong></td>
<td>Help generate interest in science at elementary schools and other children outreach programs</td>
</tr>
<tr>
<td>April 2012 – December 2012</td>
<td><strong>LAFS event representative</strong></td>
<td>Attend meetings and generate awareness of hospital events that LAFS is organizing.</td>
</tr>
<tr>
<td>May 2008 – August 2011</td>
<td><strong>Undergraduate Research Assistant</strong></td>
<td>Worked summers in Dr. Lee’s lab at WLU</td>
</tr>
<tr>
<td>September 2009 – May 2011</td>
<td><strong>Volunteer at St. Mary’s Hospital, Kitchener</strong></td>
<td>Worked in OR/ER helping facilitate patients stay</td>
</tr>
<tr>
<td>September 2010 – April 2011</td>
<td><strong>Mentor for First Year Undergraduate Students</strong></td>
<td>Shared experiences and helped first year university students handle their studies and work on facilitating good study habits</td>
</tr>
<tr>
<td>September 2010 – January 2011</td>
<td><strong>Volunteer at Kidsability, Waterloo</strong></td>
<td>Worked with special needs children.</td>
</tr>
</tbody>
</table>