DEVELOPING NEW APPLICATIONS FOR A METAL CHELATOR LIPID

TARGETED DELIVERY OF NUCLEIC ACIDS AND CYTOTOXIC DRUGS

THOMAS PATRICK HERRINGSON
JANUARY 2011

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY OF THE AUSTRALIAN NATIONAL UNIVERSITY
Developing New Applications for a Metal Chelator Linked Targeted Delivery of Anticancer Drugs and Cytotoxic Drugs

Thomás Patrick Herneon

January 2014

A Thesis Submitted for the Degree of
Doctor of Philosophy

The Australian National University
I, Thomas Patrick Herringson, declare that the work presented herein is my original research. All results presented are from my own experimental work although the data in Chapter 7, Figures 7.3 - 7.6 were obtained from work carried out in collaboration with Mr. Faham Abdus. The experimental work required to generate these figures was shared equally with Mr. Abdus.

[Signature]
ACKNOWLEDGEMENTS

Firstly I would like to thank my supervisor Dr. Joe Altin for his guidance in this project. His insight in the designing of experiments, the interpretation of data and the writing of this thesis has been invaluable. Also, I must thank Mr. Faham Abdus for his generous technical assistance during animal studies. In addition, great assistance in confocal imaging and cryosectioning was received from Ms Cathy Gillespie and Ms Anne Prins of the Microscopy and Cytometry Resource Facility (JCSMR). I would also like to thank Lipotek Pty Ltd for their provision of the NTA$_3$-DTDA lipid for use in this thesis.

To my family, particularly my parents, thank you for your love and prayers throughout this project. It has been an immense blessing to have your encouragement during the difficult times and your continual support. I would not have been able to complete this task without you.

Finally, and most importantly, I would like to thank my Lord and Saviour Jesus Christ for loving me, dying for me on the cross and redeeming my soul. It is my prayer that all who read these acknowledgments would come to the realisation that they need a Saviour, and that obtaining everlasting life simply requires an acceptance of the free gift God has provided.

“\textit{That if thou shalt confess with thy mouth the Lord Jesus, and shalt believe in thine heart that God hath raised him from the dead, thou shalt be saved.}” – Romans 10:9
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3NTA-Caelyx</td>
<td>Caelyx® incorporated with NTA₃-DTDA</td>
</tr>
<tr>
<td>ABC</td>
<td>Accelerated blood clearance</td>
</tr>
<tr>
<td>AF</td>
<td>Alexa Fluor</td>
</tr>
<tr>
<td>AF-DOPe</td>
<td>Alexa Fluor₆₄₇-DOPe</td>
</tr>
<tr>
<td>AF-siRNA</td>
<td>Alexa-Fluor₄₈₈-labelled siRNA</td>
</tr>
<tr>
<td>Ago2</td>
<td>Argonaut 2</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ANU</td>
<td>The Australian National University</td>
</tr>
<tr>
<td>AS-ODN</td>
<td>Antisense oligodeoxyribonucleotide</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>Cer-PEG₇₅₀</td>
<td>N-octanoyl-sphingosine-1-[succinyl(methoxy-(polyethylene glycol))₇₅₀</td>
</tr>
<tr>
<td>CHEMS</td>
<td>Cholesterol hemisuccinate</td>
</tr>
<tr>
<td>Chol</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>CSF-1</td>
<td>Colony-stimulating factor-1</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DODAP</td>
<td>1,2-dioleoyl-3-dimethylammonium-propane</td>
</tr>
<tr>
<td>DODMA</td>
<td>1,2-dioleoyloxy-N,N-dimethylaminopropane</td>
</tr>
<tr>
<td>DOPC</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphatidylcholine</td>
</tr>
<tr>
<td>DOPE</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphoethanolamine</td>
</tr>
<tr>
<td>DOTAP</td>
<td>1,2-dioleoyl-3-trimethylammonium-propane</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>DOSPA</td>
<td>2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate</td>
</tr>
<tr>
<td>DSPC</td>
<td>1,2-distearoyl-sn-glycero-3-phosphatidylcholine</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EGFR</td>
<td>Endothelial growth factor receptor</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced permeability and retention</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>Fab'</td>
<td>Antigen binding fragments</td>
</tr>
<tr>
<td>Fc</td>
<td>Constant region</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FDA</td>
<td>US Food and Drugs Administration</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GCV</td>
<td>Ganciclovir</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GDEPT</td>
<td>Gene-directed enzyme prodrug therapy</td>
</tr>
<tr>
<td>HEK-293</td>
<td>Human embryonic kidney 293 cells</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>H11</td>
<td>Inverted hexagonal</td>
</tr>
<tr>
<td>HIV-1</td>
<td>Human immunodeficiency virus-1</td>
</tr>
<tr>
<td>HRG</td>
<td>Histidine-rich glycoprotein</td>
</tr>
<tr>
<td>HSPC</td>
<td>Hydrogenated soy phosphatidylcholine</td>
</tr>
<tr>
<td>HSV-TK</td>
<td>Herpes simplex virus thymidine kinase</td>
</tr>
<tr>
<td>i.m.</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>IL-12</td>
<td>Interleukin-12</td>
</tr>
<tr>
<td>IMAC</td>
<td>Immobilised metal affinity chromatography</td>
</tr>
<tr>
<td>JCSMR</td>
<td>John Curtin School of Medical Research (ANU)</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug-resistance protein</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localisation signal</td>
</tr>
<tr>
<td>NRP-1</td>
<td>Neuropilin-1</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>NTA</td>
<td>Nitrilotriacetic acid</td>
</tr>
<tr>
<td>NTA$_3$-DTDA</td>
<td>3(nitrilotriacetic acid)-ditetradecylamine</td>
</tr>
<tr>
<td>OG$_{488}$-DHPE</td>
<td>Oregon Green-488-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>p-NP</td>
<td>p-nitrophenylcarboxyl</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PDGFRβ</td>
<td>Platelet-derived growth factor receptor β</td>
</tr>
<tr>
<td>pDNA</td>
<td>Plasmid DNA</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PE-PEG$_x$</td>
<td>Phosphatidylethanolamine-polyethylene glycol (MW of PEG = X)</td>
</tr>
<tr>
<td>PEC</td>
<td>Peritoneally excluded cells</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
</tr>
<tr>
<td>PGP</td>
<td>p-glycoprotein</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine</td>
</tr>
<tr>
<td>RES</td>
<td>Reticuloendothelial system</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RNase A</td>
<td>Ribonuclease A</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RSB</td>
<td>Research School of Biology (ANU)</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>SALP</td>
<td>Stabilised antisense lipid particles</td>
</tr>
<tr>
<td>scFv</td>
<td>Single chain variable fragment</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SOCS-1</td>
<td>Suppressor of cytokine signalling 1</td>
</tr>
<tr>
<td>$T_{1/2}$</td>
<td>Half-life</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Texas Red-DHPE</td>
<td>Texas Red 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine</td>
</tr>
<tr>
<td>TfR</td>
<td>Transferrin receptor</td>
</tr>
<tr>
<td>TLR5</td>
<td>Toll-like receptor 5</td>
</tr>
<tr>
<td>$T_m$</td>
<td>Phase transition temperature</td>
</tr>
<tr>
<td>TNFR2</td>
<td>Tumour necrosis factor receptor 2</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>Vascular endothelial growth factor receptor 2</td>
</tr>
</tbody>
</table>
ABSTRACT

The ability to target the delivery of nucleic acids and cytotoxic drugs to specific cells has applications for the treatment of a number of diseases including cancer. Liposomes have previously been used to incorporate a range of different therapeutic agents, however the targeting/delivery of liposomes to specific cell types requires a means to conveniently anchor targeting molecules onto the surface of the liposomes. Recently, the chelator lipid 3(nitrilotriacetic acid)-ditetradecylamine (NTA₃-DTDA) was incorporated into liposomes and membrane vesicles to enable the engraftment of histidine-tagged targeting molecules. Thus far, however, the in vivo use of targeted NTA₃-DTDA-liposomes has been restricted to the delivery of antigenic proteins/peptides. Therefore, the primary focus of this thesis was to utilise liposomes containing NTA₃-DTDA to target the delivery of nucleic acids and cytotoxic drugs to cells, and to investigate the potential application of these liposomes for cancer therapy.

Preliminary studies during Honours indicated that liposomes containing the ionisable lipid 1,2-dioleoyl-3-dimethylammonium-propane (DODAP) could incorporate small interfering RNA (siRNA) into lipoplexes together with NTA₃-DTDA. Importantly, siRNA-lipoplexes engrafted with targeting molecules were shown to bind to cells through specific receptor-ligand interactions. Initial studies for the present work focussed on the further development and characterisation of these targeted siRNA-lipoplexes as well as the preparation of targeted lipoplexes containing plasmid DNA (pDNA). The targeted siRNA-lipoplexes and pDNA-lipoplexes developed in this work are shown to be of near-neutral surface charge, approximately 250 nm in diameter, serum-stable, non-toxic and able to efficiently transfect different cell lines when engrafted with different targeting peptides. For in vivo experiments,
lipoplexes were prepared containing pDNA encoding for the immunodominant epitope (in C57BL/6 mice, H-2\textsuperscript{b}) of the model antigen ovalbumin (OVA). When these lipoplexes were engrafted with the TLR5-binding peptide p9-Flg and injected into C57BL/6 mice, this treatment induced antigen-specific immunity that protected the mice from challenge with B16-OVA melanoma cells.

Studies were also conducted to determine whether NTA\textsubscript{3}-DTDA could be incorporated into doxorubicin-containing liposomes. Experiments indicated that doxorubicin-containing liposomes incorporated with NTA\textsubscript{3}-DTDA and engrafted with the peptide p15-RGR could be targeted to NIH-3T3 cells \textit{in vitro} with enhanced binding and cytotoxicity compared to non-targeted liposomes. The engraftment of liposomes with either of the tumour vasculature homing peptides: p46-RGD or p24-NRP-1, and injection into tumour-bearing mice resulted in a significant increase in the accumulation of the liposomes in the tumour. Importantly, treatment of mice with doxorubicin-containing liposomes engrafted with these same peptides significantly decreased the rate of tumour growth, thereby demonstrating therapeutic potential.

In summary, the results presented in this thesis show that liposomes containing NTA\textsubscript{3}-DTDA can be used to deliver nucleic acids such as siRNA and pDNA as well as the cytotoxic drug doxorubicin to specific cells/tissues both \textit{in vitro} and \textit{in vivo}. The targeted delivery of pDNA to antigen-presenting cells and the targeting of doxorubicin to cells of the tumour vascular endothelium both resulted in significant anti-tumour effects. These findings, coupled with the convenience of the targeting method employed, suggest that the approaches developed could well find application in the quest for improving therapies against cancer.
# Table of Contents

Declaration.......................................................................................................................i
Acknowledgements..........................................................................................................ii
Abbreviations..................................................................................................................iii
Abstract..........................................................................................................................vii

Chapter One - Introduction............................................................................................1
  1.1 General introduction ..................................................................................................2
  1.2 Tumour biology and current cancer treatment regimes .............................................5
    1.2.1 Anthracycl ine chemotherapy ..............................................................................7
  1.3 Cancer gene therapy ..................................................................................................8
    1.3.1 The molecular tools used for gene therapy .......................................................9
    1.3.2 Cancer gene therapy strategies – gene expression .............................................12
    1.3.3 Cancer gene therapy strategies – silencing gene expression .........................15
    1.3.4 Difficulties associated with systemic cancer gene therapy ............................17
  1.4 Liposomes ...............................................................................................................18
    1.4.1 Liposomal structure ..........................................................................................18
    1.4.2 Liposomes prepared for in vivo applications ...................................................20
    1.4.3 Incorporation of therapeutic compounds into liposomes ...............................23
    1.4.4 Cell uptake mechanisms for liposomes ...........................................................30
  1.5 Active targeting of liposomes with conjugated ligands .............................................31
    1.5.1 Potential targeting molecules ..........................................................................33
    1.5.2 Strategies for targeting liposomes for cancer therapy ...................................36
    1.5.3 Attaching targeting moieties to the surface of liposomes ...............................40
    1.5.4 NTA\textsubscript{3}-DTDA – a novel chelator lipid ....................................................44
  1.6 New applications for NTA\textsubscript{3}-DTDA .................................................................45
    1.6.1 Utilising NTA\textsubscript{3}-DTDA liposomes to target the delivery of siRNA to cells 45
    1.6.2 Aims of this project ..........................................................................................50

Chapter Two – General Methodology ...........................................................................52
  2.1 Reagents ..................................................................................................................53
  2.2 Lipids ......................................................................................................................53
  2.3 Cell lines ..................................................................................................................54
  2.4 Mice .........................................................................................................................56
  2.5 Proteins ....................................................................................................................56
  2.6 Peptides ...................................................................................................................57
  2.7 Plasmids ..................................................................................................................59
  2.8 siRNA .....................................................................................................................60
  2.9 Liposome formulations .............................................................................................61
  2.10 Flow cytometry ......................................................................................................62
  2.11 Confocal microscopy ..............................................................................................62
  2.12 Radioactive analysis of murine tissue samples ......................................................63
  2.13 Particle size and charge measurements ..................................................................63
  2.14 Statistical analysis ..................................................................................................64
  2.15 Additional materials and methods ........................................................................64
Chapter Three - Convenient targeting of stealth siRNA-lipoplexes to cells with chelator lipid-anchored molecules .................................................. 65
  3.1 Introductory Comments................................................................. 66
  3.2 Abstract ....................................................................................... 67
  3.3 Introduction.................................................................................. 68
  3.4 Materials and Methods.................................................................. 70
    3.4.1 Reagents .................................................................................. 70
    3.4.2 Preparation of liposomes .............................................................. 70
    3.4.3 Formation of siRNA-lipoplexes ................................................... 71
    3.4.4 Incorporation of NTA\textsubscript{3}-DTDA and engraftment of lipoplexes .............................................................. 72
    3.4.5 Assaying binding of siRNA-lipoplexes to cells ................................ 72
    3.4.6 Confocal microscopy .................................................................. 73
    3.4.7 Nuclease protection assay .............................................................. 73
    3.4.8 Particle size and charge measurements ......................................... 74
    3.4.9 Induction of gene silencing ............................................................ 74

  3.5 Results ......................................................................................... 75
    3.5.1 Developing the use of NTA\textsubscript{3}-DTDA-liposomes for targeting siRNA .............................................................. 75
    3.5.2 Incorporating siRNA into NTA\textsubscript{3}-DTDA-liposomes - a novel method for targeting siRNAs .............................................................. 75
    3.5.3 Effect of initial pH on siRNA incorporation ...................................... 78
    3.5.4 Proportion of siRNA protected from RNase degradation .................... 80
    3.5.5 Physical characteristics of NTA\textsubscript{3}-DTDA-liposomes incorporating siRNA .............................................................. 82
    3.5.6 Internalisation of targeted siRNA-lipoplexes by cells ....................... 83
    3.5.7 Targeted siRNA-lipoplexes can induce gene silencing ..................... 85

  3.6 Discussion .................................................................................... 90

  3.7 In vivo application for the method of siRNA/nucleic acid delivery ......... 96

  3.8 Supplementary Figures ................................................................ 98

Chapter Four - Targeting of plasmid DNA-lipoplexes to cells with molecules anchored via a metal chelator lipid .................................................. 103
  4.1 Introductory Comments.................................................................. 104
  4.2 Abstract ......................................................................................... 105
  4.3 Introduction................................................................................... 106
  4.4 Materials and Methods.................................................................. 109
    4.4.1 Reagents .................................................................................. 109
    4.4.2 Preparation of liposomes .............................................................. 109
    4.4.3 Preparation of lipoplexes and transfection ....................................... 109
    4.4.4 Assaying binding of peptide-engrafted liposomes and anti-FIt-1 antibody to cells .............................................................. 111
    4.4.5 Confocal microscopy .................................................................. 111
    4.4.6 Particle size and charge measurements ......................................... 112
    4.4.7 Ethidium bromide (EtBr) intercalation assay .................................... 112

  4.5 Results ......................................................................................... 113
    4.5.1 Targeting of lipoplexes with peptides anchored via NTA\textsubscript{3}-DTDA .............................................................. 113
    4.5.2 Optimising conditions affecting targeted transfection ....................... 117
    4.5.3 Lipid composition for optimal transfection with lipoplexes containing NTA\textsubscript{3}-DTDA .............................................................. 119
    4.5.4 Localisation of lipoplexes internalised by cells .................................. 123
    4.5.5 Effect of incorporating fusogenic and DNase II inhibiting peptides .......... 125
    4.5.6 Efficient targeted transfection with lipoplexes containing NTA\textsubscript{3}-DTDA .............................................................. 128
    4.5.7 Size, charge, stability and toxicity of lipoplexes .................................. 131

  4.6 Discussion .................................................................................... 135

  4.7 Supplementary Figures ................................................................ 143
Chapter Five - Increasing the anti-tumour efficacy of doxorubicin-loaded liposomes with peptides anchored via a chelator lipid

5.1 Introductory Comments ......................................................... 148
5.2 Abstract ............................................................................. 149
5.3 Introduction ......................................................................... 150
5.4 Materials and Methods ........................................................ 151
5.4.1 Reagents .......................................................................... 152
5.4.2 Preparation of NTA$_2$-DTDA-containing liposomes ............ 153
5.4.3 Preparation of 3NTA-Caelyx ........................................... 153
5.4.4 Assaying binding of liposomes and 3NTA-Caelyx to cells ... 154
5.4.5 Cytotoxicity assay ......................................................... 155
5.4.6 Particle size measurements ............................................. 156
5.4.7 Biodistribution studies .................................................... 157
5.4.8 Tumour growth studies .................................................. 158
5.5 Results .................................................................................. 159
5.5.1 Incorporation of NTA$_2$-DTDA into Caelyx® for targeting to cells 159
5.5.2 Peptide-engrafted 3NTA-Caelyx induces targeted cytotoxicity 160
5.5.3 Particle size of 3NTA-Caelyx .......................................... 161
5.5.4 In vivo tissue distribution study ....................................... 162
5.5.5 Anti-tumour effect of targeted 3NTA-Caelyx .................... 163
5.6 Discussion ........................................................................... 164

Chapter Six - Effective tumour targeting and enhanced anti-tumour effect of liposomes engrafted with peptide anchored via a metal chelator lipid

6.1 Introductory Comments ......................................................... 173
6.2 Abstract ............................................................................. 174
6.3 Introduction ......................................................................... 175
6.4 Materials and Methods ........................................................ 176
6.4.1 Reagents .......................................................................... 177
6.4.2 Preparation of peptide-engrafted 3NTA-Caelyx ................. 178
6.4.3 Measuring liposome-associated doxorubicin ................. 179
6.4.4 Particle size measurements ............................................. 179
6.4.5 Liposome preparation ..................................................... 180
6.4.6 Biodistribution studies .................................................... 181
6.4.7 Intratumoural distribution of fluorescent liposomes ...... 182
6.4.8 Tumour growth studies .................................................. 183
6.5 Results .................................................................................. 184
6.5.1 Effect of targeting doxorubicin liposomes with different peptides 184
6.5.2 Some peptides can induce aggregation and leakage of liposomal doxorubicin 185
6.5.3 Biodistribution of peptide-engrafted liposomes after in vivo administration 186
6.5.4 Intratumoural distribution of peptide-engrafted liposomes 187
6.5.5 Anti-tumour effect of peptide-engrafted PE-PEG$_{750}$ doxorubicin-loaded liposomes 188
6.6 Discussion ........................................................................... 189

Chapter Seven - Targeting of antigen-encoding plasmid DNA to cells for tumour therapy

7.1 Introductory Comments ......................................................... 201
7.2 Abstract ............................................................................. 202
7.3 Introduction ......................................................................... 203
7.4 Materials and Methods ........................................................ 204
7.4.1 Reagents .......................................................................... 205
7.4.2 OT-I cells .......................................................................... 206
7.4.3 Preparation of liposomes ................................................ 207
Chapter 7 - Cancer Immunisation

7.4.4 Preparation of peptide-engrafted pDNA-lipoplexes ........................................ 209
7.4.5 Biodistribution of peptide-engrafted liposomes and pDNA-lipoplexes ............. 209
7.4.6 Combination treatment of mice with pDNA-lipoplexes and OT-I cells .............. 209
7.4.7 Assaying lipoplex binding to cells ................................................................. 210
7.4.8 Assaying transgene expression by cells in vivo ............................................. 210
7.4.9 Vaccination of mice with peptide-engrafted pDNA-lipoplexes; IFN-γ induction ........................................ 211
7.4.10 Vaccination of mice with peptide-engrafted pDNA-lipoplexes; B16-OVA study ........................................ 211

7.5 Results ........................................................................................................... 212
7.5.1 Targeting pDNA-lipoplexes in vivo ............................................................... 212
7.5.2 Targeting pDNA-lipoplexes to the tumour vasculature ............................... 214
7.5.3 Effect of administering OT-I cells to mice treated with p46-RGD lipoplexes ... 214
7.5.4 p9-Flg promotes binding of pDNA-lipoplexes to dendritic cells ................. 215
7.5.5 In vivo transfection of cells with p9-Flg pDNA-lipoplexes ......................... 217
7.5.6 Biodistribution of p9-Flg pDNA-lipoplexes ................................................. 217
7.5.7 Immunisation of mice with p9-Flg-lipoplexes generates antigen-responsive CD8+ T cells ........................................ 220
7.5.8 Effect of p9-Flg lipoplex vaccination on tumour growth and metastasis ........ 222

7.6 Discussion .................................................................................................... 226

Chapter Eight - Discussion............................................................................. 233

8.2 Overcoming in vivo barriers by using peptide-engrafted NTA3-DTDA liposomes ........................................................................................................ 235

8.2.1 Preparation of serum-stable liposomal carriers of nucleic acids and cytotoxic drugs ........................................ 236

8.2.2 Preparation of liposomes that promote tumour targeting and accumulation in tumours ........................................ 239

8.2.3 Intracellular barriers faced by targeted liposomes .................................. 243

8.3 Future Perspectives .................................................................................... 246

8.4 Conclusion .................................................................................................. 249

References ...................................................................................................... 251
CHAPTER ONE

INTRODUCTION
1.1 General introduction

Cancer was predicted to be the leading cause of death in 2010, with an estimated 8 million fatalities worldwide [1]. Encouragingly, however, the last two decades have seen a significant decrease in the proportion of cancer-related deaths. Specifically, in the years between 1990 and 2005, the US cancer mortality rate declined by ~1% per year [2]. This decrease in cancer mortality can be explained in part by the development of more effective cancer therapies. Approaches that can target or control the effects of drugs and other anti-cancer agents seem set to further improve treatment outcome in patients afflicted with this disease.

Chemotherapy has a major role in the current treatment of cancer and is based on the use of cytotoxic drugs that primarily affect rapidly dividing cells [3]. As a result, chemotherapeutic compounds target tumour cells to a somewhat greater degree than normal tissues, although unpleasant side effects still occur including myelosuppression, hair loss, nausea and diarrhoea [4, 5]. For the anthracycline doxorubicin, a major side effect is cardiotoxicity, which has restricted the dose that can be administered systemically and therefore has limited the ultimate efficacy of this drug [6]. The use of liposomes as lipid carriers for doxorubicin has been established as a means to reduce the side effects of doxorubicin. The liposomal-doxorubicin drug Caelyx® was approved for therapeutic use in 1995 and minimises the uptake of doxorubicin by heart myocytes while increasing tumour accumulation [7]. The number of registered liposomal drugs has increased in recent years and current research is focussed on the active targeting of drug-loaded liposomes as a means to further increase tumour accumulation and promote the uptake of chemotherapeutic compounds into tumour cells.
An alternate area of focus in cancer therapy research is the manipulation of gene expression in tumour cells. Evidence suggests that the misregulation of gene expression has a major role in the formation of tumours [8-10] and can initiate metastasis [11] and drug resistance [12]. Through research in molecular biology, nucleic acids such as plasmid DNA, small interfering RNA and antisense oligodeoxyribonucleotides have been developed as molecular tools for either inducing or suppressing the expression of specific genes in cells. If introduced into tumour cells, these nucleic acids could potentially enable the expression of wild-type tumour suppressor genes, as well as genes that encode proteins which activate pro-drugs or can stimulate an immune response against the tumour. The expression of genes in tumour cells could also be silenced; including genes involved in the uncontrolled growth of cells, the formation of the tumour vasculature and resistance to cytotoxic drugs. The major obstacles in cancer gene therapy, however, are the physical and biochemical barriers present in vivo that prevent unformulated (naked) nucleic acids from reaching tumour cells once injected intravenously [13]. As with chemotherapeutic drugs, targeted liposomes could potentially be used to deliver nucleic acids to specific cells for tumour therapy.

Due to their unique genetic makeup and growth kinetics, tumour cells express many cell surface proteins to which therapeutics can be targeted [14]. Monoclonal antibodies (mAb) such as rituximab, bevacizumab and trastuzumab are a feature of current cancer therapy and bind with high affinity to specific tumour cell markers [15]. Targeted liposomes prepared by conjugation of mAb or antibody fragments onto liposomes have shown increased intracellular uptake in vitro [16] and enhanced tumour accumulation when injected intravenously into animal models [17]. Other
potential targeting moieties for liposomes include short peptide sequences [18] and natural ligands, such as transferrin [19] and folate [20], for receptors overexpressed on tumour cells. An important component of a targeted liposome is the linker molecule used to conjugate targeting moieties onto the surface of the liposome. Typically, covalent bonds such as thioether and amide bonds are utilised for conjugation [21]. Recently a non-covalent bond strategy was devised: the chelator lipid 3(nitrilotriacetic acid)-ditetradecylamine (NTA$_3$-DTDA) was incorporated into liposomes, enabling the engraftment of histidine-tagged targeting molecules onto the surface of the liposomes [22]. Thus, by engrafting with antibody fragments against dendritic cell surface markers, NTA$_3$-DTDA-liposomes containing protein antigens were delivered specifically to dendritic cells in vivo and induced potent anti-tumour responses [23].

The aim of this thesis was to utilise the novel chelator lipid NTA$_3$-DTDA to target liposomes containing either nucleic acids or cytotoxic drugs to cells and to explore the potential of targeted liposomes for systemic tumour therapy. The following introductory chapter will begin by presenting a summary of tumour biology and modern cancer therapy. This is followed by an overview of the potential applications of gene therapy for the treatment of cancer, a description of how liposomes can be used as carriers for therapeutic compounds, and then a summary and analysis of the various strategies for treating cancer with targeted liposomes that have recently been tested in animal models and clinical trials.
1.2 Tumour biology and current cancer treatment regimes

Tumour development is a multistage process that is heavily dependent on the genetic characteristics of the cells involved [24]. Due to persistent genetic damage from cancer initiators such as chemical carcinogens and viruses, a single cell can develop mutations in its genome that result in uncontrolled cell division and consequently the formation of a tumour [25]. As a tumour begins to develop it will reach a point (typically thought to be a volume of 1-2 mm$^3$) where it cannot grow any further unless sufficient oxygen and nutrients are supplied through a functional blood supply [26]. This hypoxic state initiates the production of growth factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and matrix metalloproteinases (MMPs) by the tumour cells themselves or cells in the surrounding tissue (Figure 1.1) [27]. These factors induce angiogenesis, the growth of new blood vessels from pre-existing vasculature, and this process provides the necessary conditions for continued tumour development. As tumour cells continue to proliferate unchecked they become increasingly genetically unstable and acquire further mutations that can enable the invasion of the tumour into nearby tissues [28]. The invasion of tumour cells into lymph and blood vessels can lead to the seeding and growth of tumours in distant organs/tissues (metastasis) [29, 30].

The modern treatment scheme for cancer is primarily based on a combination of surgery, radiotherapy and chemotherapy. Surgery and radiotherapy are localised techniques and used for the treatment of a primary tumour, while chemotherapy can be systemically administered and potentially reach every organ in the body [31]. This is a crucial property of chemotherapy since 90% of all human cancer deaths are due to the
Figure 1.1 - Tumour angiogenesis and metastasis. A developing tumour will soon deplete its source of nutrients and oxygen (typically at a volume of 1-2 mm³). This hypoxic state induces production of growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) which bind to receptors expressed on neighbouring endothelial cells and pericytes, stimulating the growth of new blood vessels from the pre-existing vasculature (angiogenesis). Tumour cells can acquire genetic mutations which enable them to invade neighbouring blood and lymph vessels which can lead to metastasis.
development of metastases, which typically cannot be treated by localised therapy [32]. Most chemotherapeutic compounds are able to cause damage to or interfere with the synthesis of DNA [33]. Consequently, rapidly dividing cells such as tumour cells are the most sensitive to their effects. However, certain normal tissues also have high cell proliferation rates including the bone marrow and the gastrointestinal epithelium, resulting in non-specific toxicities in these tissues and ultimately side effects such as myelosuppression, nausea and diarrhoea [5, 34]. Another major challenge for current chemotherapy is the development of drug resistance, which can occur even when multiple drugs are administered simultaneously [35]. Several mechanisms for drug resistance have been described, including overexpression of the drug efflux pumps p-glycoprotein (PGP) and multidrug-resistance protein (MRP) [36].

1.2.1 Anthracycline chemotherapy

The anthracyclines are a class of cytotoxic drug that are amongst the most effective agents developed for chemotherapy [37]. Systemic administration of anthracyclines is a common component of current treatment schemes for a variety of solid tumours, in particular breast, prostate, stomach and liver tumours [38]. The first anthracyclines to be used in chemotherapy, daunorubicin and doxorubicin, were initially isolated from the pigment-producing bacterium Streptomyces peucetius in the 1960’s [39]. Since then more than 200 naturally occurring anthracyclines have been identified and several hundred more have been synthesised in the search for more toxic compounds to treat drug-resistant tumours [40]. In spite of this, doxorubicin remains the anthracycline most widely utilised for chemotherapy [41]. The cytotoxicity of doxorubicin occurs through a range of different mechanisms including intercalation with genomic DNA, the generation of free radicals and the inhibition of the nuclear
enzyme topoisomerase II, which results in an accumulation of single- and double-stranded breaks in genomic DNA [42]. As a small amphipathic molecule, doxorubicin freely distributes into both healthy and diseased tissues after systemic administration [43]. Consequently, despite its anti-tumour efficacy, the use of doxorubicin is dose-limited by severe side effects including bone marrow depression, gastrointestinal toxicity and cardiotoxicity [44]. Of these effects, cardiotoxicity is the most concerning with one study indicating that congestive heart failure can occur in more than a third of patients receiving a cumulative doxorubicin dose of >601 mg/m² [45].

1.3 Cancer gene therapy

The current cancer treatment scheme of surgery, radiotherapy and chemotherapy can significantly affect the patient's quality of life and some tumours are unresponsive to therapy. Therefore, since cancer is in essence a genetic disease, cancer gene therapy has been suggested as a potential fourth modality for cancer treatment [46]. Thus far, a number of strategies involving either gene expression or gene silencing have been tested in preclinical and clinical studies, with promising anti-tumour responses. While many studies have utilised recombinant viral vectors to introduce transgenes into cells, nucleic acids such as plasmid DNA (pDNA), small interfering RNA (siRNA) and antisense oligodeoxyribonucleotides (AS-ODN) can also be used be manipulate gene expression for cancer therapy, provided that mechanisms are in place to ensure effective delivery and efficient cell uptake.
1.3.1 The molecular tools used for gene therapy

(i) Viral vectors

Viral vectors consist of a viral genome, typically from a retrovirus, adenovirus or adeno-associated virus, which has been modified so that unwanted viral genes are replaced with a transgene of interest [47]. When these recombinant vectors are transfected into a packaging cell line, viral particles are produced which are replication incompetent but still capable of efficiently transferring their genetic material into cells.

(ii) Plasmid DNA

Plasmids are high molecular weight (2.6 MDa for a 4 kb plasmid), circular, double-stranded DNA molecules that occur naturally in bacteria, yeast and some higher eukaryotic cells and exist as separate entities from the cell’s chromosomal DNA [48]. Importantly, techniques for the genetic manipulation and amplification of pDNA have become widely used. Plasmids utilised for gene therapy are modified by recombinant techniques to include the desired transgene as well as promoter and enhancer sequences to increase protein production in eukaryotic cells [49]. In order to achieve transgene expression, however, plasmids must be taken up into the cell nucleus where they can employ the DNA transcription and translation apparatus of the cell to synthesise the encoded proteins [48].

(iii) siRNA

siRNAs are short duplexes (21-23 bp) of RNA which contain distinctive two nucleotide overhangs at each 3’ end. These RNA fragments are the mediators of RNA interference (RNAi), a gene silencing phenomenon that was first identified in Caenorhabditis elegans by Andrew Fire and Craig Mello in 1998 [50]. When
chemically synthesised and introduced into the cytoplasm of cells, siRNAs can silence the expression of specific genes through Watson-Crick base pairing with messenger RNA (mRNA). As shown in Figure 1.2, cytoplasmic siRNAs are incorporated into the RNA-induced silencing complex (RISC) through association with the protein Argonaut 2 (Ago2) [51]. One strand of the siRNA is removed and degraded, while the remaining strand (the guide strand) is maintained and directs binding of RISC to mRNA transcripts containing a complementary sequence. Ago2 mediates the cleavage of the bound mRNA, which is then degraded by cellular exonucleases. The siRNA-loaded RISC can then undergo multiple rounds of mRNA cleavage, resulting in minimal production of the protein that would be otherwise translated from the mRNA [52]. This knockdown in gene expression differs from gene knockout techniques in that the effect is transient (3-7 days for dividing cells) and typically incomplete, although in some cases the targeted mRNA may be reduced to levels that even highly sensitive PCR assays cannot detect [53].

(iv) AS-ODN

AS-ODNs are short fragments of single-stranded DNA (~20 nt) that can be chemically synthesised and introduced into cells for inhibition of gene expression [54]. As with siRNA, AS-ODNs suppress gene expression through sequence-specific binding of mRNA transcripts in the cytoplasm. In the case of AS-ODNs, this binding results in the formation of a DNA/RNA heteroduplex, which is either cleaved by RNase H or prevents protein synthesis from the mRNA through steric inhibition [55]. In comparison to siRNAs, AS-ODNs are generally considered to be less potent mediators of gene silencing on a molecule-to-molecule basis [54].
Figure 1.2 - The induction of RNA interference by small interfering RNA. When detected in the cytoplasm, small interfering RNA (siRNA) is bound by the protein Argonaute 2 (Ago2) and incorporated into the RNA-induced silencing complex (RISC). Through the helicase activity of RISC, the siRNA duplex is unwound and the sense strand of the siRNA removed. The remaining strand directs RISC to the target mRNA sequence, which is cleaved by RISC and subsequently degraded by cellular exonucleases.
(v) Other nucleic acids

Other nucleic acids developed for cancer therapy applications include: mRNA [56], which can be used for protein production without the need for nuclear delivery; ribozymes [57] and DNAzymes [58], which can suppress the expression of specific genes; and aptamers [59], which can directly interact with proteins and inhibit their molecular function.

1.3.2 Cancer gene therapy strategies – gene expression

(i) Expression of tumour suppressor genes

One potential approach for cancer gene therapy is to introduce vectors containing wild-type tumour suppressor genes into tumour cells. Many tumours have been shown to contain cells with mutations that have deactivated or altered the function of proteins that prevent the uncontrolled growth of cells [60]. The genes encoding these proteins are termed tumour suppressor genes and examples include **TP53, PTEN** and **RBI** [61]. Whole genome sequencing of breast and colon cancers has identified **TP53** as the most commonly mutated gene in these tumours [62] and it has been estimated that over half of all human tumours contain a mutation in **TP53** [63]. The protein encoded by **TP53** is a transcription factor (p53) that controls cellular proliferation through the regulation of the cell cycle and the induction of programmed cell death (apoptosis) [64]. The majority of mutations in **TP53** are missense mutations in the coding sequence of the DNA-binding domain and these mutations prevent p53 from binding DNA and regulating cell division [65]. Experiments where pDNA encoding wild-type p53 was injected directly into murine tumours led to significant inhibition of tumour growth compared to empty vectors [66, 67]. In addition, viral delivery of wild-type p53 has proven to be an effective cancer treatment. The first gene
therapy product approved by a government agency was Gendicine, an adenoviral vector encoding p53, and is approved for use in China for the treatment of both lung cancer and squamous cancer of the head and neck [68].

(ii) Gene-directed enzyme prodrug therapy

An alternative approach for cancer gene therapy is gene-directed enzyme prodrug therapy (GDEPT) and involves the transfection of tumour cells with vectors encoding a prodrug-activating enzyme. When a non-toxic prodrug is subsequently administered, it is converted to its active, toxic form in cells expressing the activating enzyme [69]. An important advantage of GDEPT is the bystander effect, where cells that have not been transfected with the transgene are killed by the activated drug through cell-to-cell transfer from transfected cells [31]. Examples of enzyme/prodrug combinations which have been tested in animal studies include herpes simplex virus thymidine kinase (HSV-TK) combined with ganciclovir (GCV) [70, 71]; cytosine deaminase with fluorouracil [72, 73]; and nitroreductase with 5-(azaridin-1-yl)-2,4-dinitrobenzamide [74, 75]. In an example of this approach, pDNA containing the HSV-TK gene was injected intratumourally into Balb/c mice bearing CT26 tumours, followed by in vivo electroporation, and systemic administration of GCV [76]. This treatment resulted in 90% suppression in tumour volume compared to controls by Day 16 after plasmid injection. In addition, HSV-TK/GCV treatment using adenoviral vectors has reached phase III human trials. In patients with malignant glioma, local administration of HSV-TK-encoding adenoviruses followed by i.v. administration of GCV increased the mean survival time of patients from 39 to 71 weeks [77].
(iii) Immunomodulation

A third approach for cancer therapy using transgene expression is immunomodulation, which aims to stimulate an immune response against tumour cells. Many mechanisms exist by which tumour cells can escape recognition and elimination by the immune system including failure to express sufficient levels of MHC molecules [78], co-stimulatory molecules [79] and adhesion molecules [80], which are necessary for stimulating anti-tumour T cell responses. In order to overcome this evasion of the immune system by tumours, the expression of immunostimulatory proteins (cytokines) in the tumour has been suggested [81]. In a demonstration of this strategy, intratumoural injection of pDNA encoding the cytokine IL-12 gave rise to potent anti-tumour responses in mice bearing Renca [82] and CT26 tumours [83]. In addition, Phase I clinical trials have been carried out where IL-12-pDNA was injected into metastatic melanoma lesions [84]. Two out of 19 patients treated with IL-12-pDNA showed complete regression of all lesions, while partial response was observed in eight additional patients.

DNA vaccines are an alternate means of manipulating the immune response to eliminate tumours. In this approach, vectors with transgenes encoding tumour associated antigens are used to transfec muscle cells which in turn stimulate antigen-presenting cells for activation of a cytotoxic T-cell response against tumour cells expressing the antigen [69]. Alternatively, the antigen-presenting cells themselves (typically dendritic cells) can be transfected with the antigen-encoding transgene [85]. In an example of the potential of DNA vaccination for cancer therapy, C57BL/6 mice were vaccinated with three subcutaneous injections of 100 μg of pDNA encoding the model antigen chicken ovalbumin (OVA) [86]. This elicited a protective immune
response against B16-OVA cells when mice were challenged with these cells two weeks after the final pDNA injection. Consequently, 60% of the OVA-pDNA-treated mice remained tumour-free 60 days after tumour challenge while all mice injected with a control plasmid rapidly developed tumours.

1.3.3 Cancer gene therapy strategies – silencing gene expression

(i) Silencing of oncogene expression

In addition to the delivery of transgene-encoding vectors into cells for cancer therapy, alternative approaches have been suggested where the expression of specific genes is silenced. The misappropriate expression of genes such as those involved in cell cycle regulation and apoptosis is a major cause of uninhibited cell growth and as a result, cancer [87]. The loss of control over the expression of these genes, termed oncogenes, can be the result of single nucleotide mutations or large-scale chromosomal translocations. For example, the myc protein is a transcription factor involved in regulating cell proliferation, cell growth and apoptosis [88]. The MYC gene is an oncogene and is susceptible to overexpression due to chromosomal translocations, most notably in Burkitt’s lymphoma cells where MYC expression is placed under the control of the very active promoter of IgG [89]. MYC is also overexpressed in a number of other cancers including colon cancer and a recent report using nude mice bearing colon cancer xenografts showed that a 40% reduction in MYC expression could be achieved in mice that received intratumoural injections of anti-MYC siRNA [90]. This treatment resulted in a significantly reduced rate of tumour growth; the average tumour size of treated mice was half of that observed in untreated mice 24 days after tumour inoculation.
(ii) Silencing of angiogenesis-related genes

Due to the importance of the tumour vasculature for continued tumour growth, a potential cancer gene therapy strategy is to silence the expression of growth factors involved in angiogenesis. The most prominent target in this approach is VEGF and the intratumoural injection of anti-VEGF siRNA has been shown to suppress tumour angiogenesis and tumour growth in mice bearing PC-3 tumours [91]. Macrophages also play an important role in the formation of the tumour vasculature and expression of colony-stimulating factor (CSF)-1 by tumour cells promotes production of macrophages [92]. Aharinejad and colleagues compared the anti-tumour effects of anti-CSF-1 AS-ODN and anti-CSF-1 siRNA in mice bearing human MCF-7 mammary carcinoma cell xenografts [93]. When injected intratumourally these antisense therapies both resulted in a reduction in macrophage infiltration into tumours and ~50% suppression of mammary tumour growth.

(iii) Silencing of gene expression to increase the efficacy of chemotherapy

Although suppression of oncogene and growth factor expression may slow tumour growth, there is unlikely to be any direct elimination of cancer cells as a result. Therefore a potentially more effective use of the gene silencing approach is to augment the effects of traditional cancer therapeutics [88]. One mechanism through which cancer cells can resist chemotherapy or radiation treatment is by the expression of anti-apoptotic proteins such as Bcl-2, survivin and p-glycoprotein. The bcl-2 gene is overexpressed in many cancers including renal, stomach and brain cancer and therefore is an attractive target for gene knockdown [94]. Genasense (oblimerson sodium), an AS-ODN consisting of 18 DNA bases with a sequence complementary to the first six codons of Bcl-2 mRNA has been developed by Aventis and Genta [95].
Clinical trials of Genasense have reached the Phase III stage in combination with chemotherapeutic agents such as dacarbazine, dexamethasone, fludarabine/cyclophosphamide or docetaxel but results have not yet warranted clinical approval [96-99].

1.3.4 *Difficulties associated with systemic cancer gene therapy*

While the advantages of cancer gene therapy have been well established using intratumoural injections of nucleic acids, systemic administration is necessary to reach most metastatic lesions as well as some primary tumours. Currently, safety concerns remain over the systemic administration of viruses since these particles have the potential to be toxic as well as immunogenic, and can activate oncogenes and deactivate tumour suppressor genes [47]. The use of nucleic acids such as pDNA, siRNA and AS-ODN does not raise the same safety concerns, however, upon intravenous injection of nucleic acids, physical and biochemical barriers in the circulation prevent substantial accumulation in tumour cells. Firstly, the half-life of nucleic acids in serum is severely limited by nuclease degradation [100, 101]. Furthermore, molecules smaller than 40 kDa, including siRNA and AS-ODN, are rapidly eliminated from the circulation by kidney filtration [102]. In addition, non-specific distribution and tissue barricades reduce the local concentration of nucleic acids in tumours [103]. Finally, as negatively-charged molecules, nucleic acids have difficulty in crossing the plasma membrane of cells, which are often decorated with negatively-charged glycosaminoglycans [104]. Based on a knowledge of these barriers, the development of effective cancer gene therapy will depend on the use of delivery vehicles that can protect and actively target nucleic acids to tumour cells.
1.4 Liposomes

It has been nearly 50 years since Alec Bangham and colleagues observed that the hydration of phospholipids in aqueous media leads to the spontaneous formation of tiny vesicles called liposomes [105-107]. Importantly, it was quickly realised that these vesicles could be used as carriers for a diverse range of molecules. While many potential carriers for therapeutic compounds have since been investigated, including polymers [108], micelles [109] and dendrimers [110], liposome-based carriers have progressed the furthest in clinical testing. Liposomal preparations of doxorubicin and daunorubicin have reached the market and preclinical studies have demonstrated the ability of liposomes to deliver nucleic acids to tumour cells both in vitro and in vivo.

1.4.1 Liposomal structure

Liposomes consist of one or more lipid bilayers entrapping an aqueous volume and can therefore encapsulate a range of different drug types [111]. Hydrophilic drugs that are unable to pass through the lipid bilayer can be entrapped within the aqueous core while hydrophobic drugs can be incorporated into the lipid bilayer (Figure 1.3A). The characteristic structure of liposomes is due to the amphiphilic nature of the lipids used in their preparation. In aqueous solutions amphiphilic lipids spontaneously aggregate to minimise unfavourable hydrophobic interactions [112]; the hydrophobic tails cluster to form an inner layer shielded from the water while the polar headgroups of the lipids align so that they are exposed to the aqueous environment.

The precise structure of lipid aggregates is determined by the general molecular shape of the lipids involved. Lipids with a cone-like geometry, i.e. a large headgroup area and a small hydrocarbon area, typically form micelles while lipids with a more
Figure 1.3 - Liposome structure and examples of lipids typically used to prepare liposomes.

(A) Liposomes consist of a lipid bilayer surrounding an aqueous core and so can be used to deliver a range of different compounds. Hydrophilic compounds can be entrapped within the aqueous core and hydrophobic compounds can be incorporated into the lipid bilayer. (B) Examples of lipids commonly used to prepare liposomes include 1,2-distearoyl-sn-glycero-3-phosphatidylcholine (DSPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPE). Each of the lipids shown consist of a hydrophilic headgroup and a hydrophobic tail separated by a glycerol linkage.
cylindrical shape form bilayers and subsequently liposomes [113]. The molecular structure of a selection of lipids commonly used in liposomes is shown in Figure 1.3B. These lipids include 1,2-distearoyl-sn-glycero-3-phosphatidylcholine (DSPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). While DSPC and POPC are representative of bilayer-forming lipids, DOPE has a distinctly different geometry – a small headgroup area and a large hydrocarbon area. Consequently, at temperatures above 10 °C, DOPE adopts the so-called inverted hexagonal (H$_{II}$) phase, which is not conducive to liposome formation [114]. However, DOPE can to used to form liposomes when combined with bilayer forming lipids.

1.4.2 *Liposomes prepared for in vivo applications*

The size and structural stability of liposomes are key parameters that must be controlled in order for liposomes to be successfully used for drug delivery. Upon hydration of a dry lipid film, a heterogeneous mixture is formed consisting of multilamellar liposomes (multiple bilayers) several microns in diameter [111]. However, evidence suggests that when particles of this size are injected intravenously they do not easily pass through capillaries and have a tendency to accumulate in the lungs [115]. This can be an advantage when this population of cells is the intended target for drug delivery, however when alternate sites are desired this becomes a major obstacle. It is important, therefore, that large multilamellar liposomes are reduced in size and lamellarity by extrusion or sonication before intravenous injection. In addition, the *in vivo* stability of liposomes depends on the nature of the lipids used in their preparation. When in bilayers, lipids undergo a temperature-dependent transition from a highly ordered solid phase to a less ordered fluid phase [116]. The temperature
at which this occurs is determined by the phase transition temperature ($T_m$) of each individual lipid component. Generally, lipids with unsaturated hydrocarbon chains have a lower phase transition temperature ($T_m$ of POPC = -7 °C) while lipids with two saturated hydrocarbon chains have a high phase transition temperature ($T_m$ of DSPC = 55 °C) [43, 117]. Addition of cholesterol into a liposome at high concentrations (>30 mol%) can eliminate phase transition [111]. Therefore, the most stable liposomes for in vivo use contain phospholipids with saturated fatty acid chains as well as high concentrations of cholesterol.

Even in formulations where size and stability have been optimised, systemically administered liposomes are vulnerable to rapid uptake by the cells of the reticuloendothelial system (RES). In the blood stream, liposomes interact with lipoproteins and opsonins that promote phagocytosis by macrophages resident in the spleen and liver [118]. Addition of lipids conjugated to the hydrophilic polymer polyethylene glycol (PEG) has been identified as an approach that reduces the rate of phagocytic uptake and therefore extends the circulation time of liposomes [43]. In one study, the proportion of DSPC/cholesterol liposomes remaining in the blood at 24 h after injection into mice was determined to be 3.1%, while the corresponding value for liposomes containing 6 mol% phosphatidylethanolamine-polyethylene glycol-1900 (PE-PEG$_{1900}$) was 19.4% [119]. Liposomes containing PEG-conjugated (PEGylated) lipids are often referred to as either stealth liposomes or long-circulating liposomes. It is thought that when PEGylated lipids are incorporated into liposomes, the PEG chains occupy the space immediately adjacent to the liposome surface resulting in the steric exclusion of macromolecules such as opsonins from this space [117] (Figure 1.4). It has also been suggested that PEGylation of liposomes could inhibit the interaction of
Figure 1.4 - The structure of a stealth liposome. Stealth liposomes contain lipids conjugated to polyethylene glycol such as phosphatidylethanolamine-polyethylene glycol-2000 (PE-PEG$_{2000}$). These lipids shield the liposome surface from interactions with opsonizing proteins and therefore slow the rate of liposomal uptake by cells of the reticuloendothelial system.
liposome-associated opsonins with receptors expressed on RES macrophages [43].

An important attribute of stealth liposomes is their ability to passively accumulate in tumours due a phenomenon known as the enhanced permeability and retention (EPR) effect. It has been estimated that the vasculature in growing tumours proliferates at a rate 50-1000-fold higher than in normal tissues, and this results in structural abnormalities that make tumour blood vessels generally more leaky than normal vessels [120, 121]. Pores of several microns in size have been observed in the tumour endothelium and these enable the extravasation of nanoparticles such as liposomes into the tumour interstitium [122] (Figure 1.5). An impaired lymphatic clearance system in tumours also ensures that any extravasated liposomes remain trapped in the tumour [123].

1.4.3 Incorporation of therapeutic compounds into liposomes

Therapeutic compounds can be incorporated into liposomes by a variety of means. The most direct and simple method is to include the compound in the aqueous solution used to prepare the liposomes. However, not all molecules can be incorporated into liposomes in this way; anthracyclines are able to diffuse through lipid bilayers and so do not readily remain entrapped and large nucleic acids are prone to shearing when sonicated. Consequently, alternative approaches for loading liposomes have developed including remote-loading for the anthracyclines and the use of cationic lipids, peptides or polymers to aid in the liposomal incorporation of nucleic acids.
Figure 1.5 - The tumour accumulation of stealth liposomes is promoted by the high permeability of the tumour vasculature. After intravenous administration, stealth liposomes circulate for extended periods and are excluded from extravasation into normal tissue by tight (<6nm) gap junctions between endothelial cells. In comparison, the tumour vasculature is disorganized and leaky with large pores that enable extravasation of stealth liposomes into the tumour interstitium.
(i) Preparation of anthracycline-loaded liposomes

Anthracycline-loaded liposomes are typically prepared using the remote-loading technique. This technique is reliant on the pH-dependent protonation of anthracyclines and the fact that protonated anthracyclines are unable to pass through lipid bilayers [124]. The amino group of the sugar moiety in doxorubicin has a pKa of 8.8, therefore at pH 8 the protonated and neutral forms of doxorubicin are in equilibrium, whilst at pH 4 almost all doxorubicin molecules are protonated [125]. Therefore, if doxorubicin is added to liposomes prepared with a mildly acidic interior and a slightly alkaline exterior, any neutral doxorubicin that diffuses through the lipid bilayer will be protonated in the acidic interior of the liposome and become trapped. This process leads to highly efficient loading of doxorubicin into liposomes and a high drug-retention if stable liposome formulations are utilised [126].

The development of this simple method for loading doxorubicin into liposomes, together with an increasing knowledge of the in vivo behaviour of liposomes led to the registration of the first liposomal drug Caelyx® (Doxil® in US) in 1995 [127]. As indicated in Table 1.1, Caelyx® consists of doxorubicin entrapped in liposomes with three components: hydrogenated soy phosphatidylcholine (HSPC), cholesterol, and PE-PEG2000. The inclusion of HSPC (Tm = 52 °C) ensures liposomal stability in vivo, particularly in combination with cholesterol. Subsequent liposomal drug formulations approved for clinical use include Myocet® (liposomal doxorubicin) and DaunoXome® (liposomal daunorubicin). Interestingly, the lipid formulations of these more recent anti-cancer drugs are considerably different to Caelyx®, in particular, neither formulation contains PEGylated lipids. Despite this, DaunoXome® (plasma half-life, T1/2 = 5.2 h) remains in the circulation significantly longer than free
daunorubicin ($T_{1/2} = 0.77$ h) [128]. It has been suggested that the lack of PEGylation in DaunoXome® is compensated somewhat by a very small particle size as well as the high lipid to drug ratio used in its preparation, which could result in the saturation of cells in the RES [125]. Both DaunoXome® and Caelyx® have been shown to have increased intertumoural accumulation in tumours compared to free drug [129-131]. While this property is not shared by Myocet®, all three liposomal-anthracycline formulations have a reduced cardiotoxicity compared to free drug since liposomes are unable to pass through the continuous (non-fenestrated) capillary system of the heart [7, 132-134].

<table>
<thead>
<tr>
<th>Drug</th>
<th>Anthracycline</th>
<th>Lipid composition (mol ratio)</th>
<th>Lipid:drug w/w</th>
<th>Size nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caelyx®/Doxil®</td>
<td>Doxorubicin</td>
<td>HSPC/Chol/PE-PEG$_{2000}$ (56:39:5)</td>
<td>8:1</td>
<td>100</td>
</tr>
<tr>
<td>DaunoXome®</td>
<td>Daunorubicin</td>
<td>DSPC/Chol (2:1)</td>
<td>12.6:1</td>
<td>45</td>
</tr>
<tr>
<td>Myocet®</td>
<td>Doxorubicin</td>
<td>EggPC/Chol (55:45)</td>
<td>4:1</td>
<td>180</td>
</tr>
</tbody>
</table>

(ii) Liposomal incorporation of nucleic acids

The incorporation of nucleic acids into liposomes requires a different approach than that developed for anthracyclines, primarily due to the inherent charge present on these molecules. The phosphate backbone of nucleic acids ensures that pDNA, siRNA and AS-ODN molecules possess a negative charge and therefore are hydrophilic and do not easily pass through lipid bilayers. In addition, the size of the typical plasmid (300-500 nm diameter for a 4 Kb vector) precludes passage through lipid bilayers [126]. The size of pDNA presents another obstacle for liposomal incorporation in that sonication would likely result in the shearing of the vector, rendering it ineffective for transgene expression [135].
The most common approach for the liposomal incorporation of nucleic acids involves the use of cationic lipids. In comparison to the zwitterionic phospholipids used in the preparation of Caelyx® and DaunoXome®, the headgroups of cationic lipids such as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) lack the negative phosphate group and therefore have an overall positive charge (Figure 1.6A). Cationic lipids and other cationic agents can interact with the negatively charged phosphate backbone of nucleic acids through electrostatic interactions [136]. In the case of pDNA, such interaction neutralises the negative charge in the DNA backbone, leading to a collapse of the pDNA structure and condensation of the plasmid. The addition of nucleic acids to cationic liposomes therefore results in the spontaneous formation of structures termed lipoplexes. When a ratio of cationic lipid to nucleic acid is chosen so that the final lipoplex retains a positive charge, this promotes association of the complex with cell membranes (which are usually negatively charged) and subsequent internalisation into the cell [47]. Many commercial in vitro transfection reagents, including Lipofectamine™ 2000 (Invitrogen) and ESCORT™ (Sigma), are essentially cationic liposomes; however, concerns exist regarding the use of cationic liposomes for gene delivery in vivo. While nucleic acids incorporated into a lipoplex would be protected from degradation by serum nucleases, it has been shown that cationic lipoplexes are reactive toward anionic proteins present in the blood [137]. This interaction results in the formation of large cross-linked aggregates that can become trapped in the lung endothelial capillaries or be taken up by the RES [13]. As a result, cationic lipoplexes typically have reduced circulation times in the blood and are not ideally suited for systemic tumour therapy.
Figure 1.6 - Lipids used for the preparation of lipoplexes. (A) DOTAP is a cationic lipid, bearing a quaternary amine in its headgroup that can interact with the negatively charged phosphate groups of nucleic acids. (B) DODAP is an ionisable lipid, bearing a tertiary amine in its headgroup that is protonated at pH <6.7. DODAP is therefore cationic at acidic pH (<6.7) but uncharged at physiological pH.
In order to avoid the negative effects associated with cationic lipoplexes, several approaches have been developed where lipoplexes are formulated so as to have an anionic or near-neutral charge before administration *in vivo*. In one such approach, pDNA is condensed with an excess of polycations such as protamine, polyethylenimine (PEI) or poly-L-lysine. To shield the positive charge of these complexes, the condensed pDNA is then mixed with negatively-charged liposomes [138, 139]. In an example of this, Heyes and colleagues developed nanoparticles consisting of a PEI-condensed pDNA core entrapped within an anionic liposome composed of a combination of neutral lipids, anionic lipids and PEGylated lipids [140]. When these complexes were injected i.v. into Neuro-2a tumour-bearing A/J mice, the authors measured >300-fold greater level of transgene expression in the tumour compared to all other tissues.

An interesting alternative to this approach is to utilise liposomes containing ionisable lipids such as 1,2-dioleoyl-3-dimethylammonium-propane (DODAP) or 1,2-dioleyloxy-N,N-dimethylaminopropane (DODMA). In comparison to cationic lipids such as DOTAP, these lipids contain tertiary rather than quaternary amines and so only exhibit cationic properties in an acidic environment. For example, the pKa of the amine group of DODAP is estimated to be between 6.6 and 7, therefore at pH levels below this the amine group will be protonated and so bear a positive charge [141] (Figure 1.6B). Semple and colleagues utilised this property of DODAP to develop a method for preparing AS-ODN lipoplexes with near-neutral surface charge [142]. Ethanolic lipid solutions were mixed and incubated with AS-ODN in acidic conditions before dialysis to remove the ethanol and neutralise the solution. Since the lipid composition used included cholesterol and PEGylated lipids in addition to DODAP,
the complexes formed by this process are referred to as stabilised antisense lipid particles (SALP). In experiments carried out by Leonetti et al., nude mice bearing NG human primary melanoma were injected i.v. with free or SALP-formulated INX-6295, an anti-MYC AS-ODN [143]. It was determined that incorporation of INX-6295 into the liposomes results in a significant increase in tumour accumulation of the AS-ODN. Importantly, this increased tumour accumulation was associated with a prolonged reduction of MYC expression, a reduced rate of tumour growth and increased survival of the tumour-bearing mice.

1.4.4 Cell uptake mechanisms for liposomes

A prerequisite for cancer therapy with both cytotoxic drugs and nucleic acids is efficient delivery to and uptake by target cells. Specifically, DNA-binding drugs such as doxorubicin, as well as pDNA vectors require delivery into the cell nucleus, while AS-ODN and siRNA require cytoplasmic delivery in order to have a therapeutic effect. While amphililic drugs can diffuse from liposomes and into cells without liposome internalisation [122], nucleic acids within lipoplexes require endocytosis of the entire complex in order to reach the necessary intracellular compartment.

Liposomes can be processed through a number of endocytic pathways including clathrin-mediated endocytosis, caveolae-mediated endocytosis, phagocytosis and micropinocytosis [144]. While large particles (~500 nm in diameter) have been shown to be taken up through the caveolae-mediated pathway, the majority of liposomes of pharmaceutically relevant size are taken up through clathrin-mediated endocytosis [145]. The first step in this process is cell binding, and this can occur through binding of cationic liposomes to negatively charged glycoproteins,
proteoglycans and glycerophosphates on the cell membrane or by liposome-conjugated ligands interacting with internalising cell surface receptors [146]. As shown in Figure 1.7, binding of liposomes to cells triggers the formation of clathrin-coated pits on the surface of the cell, which invaginate and subsequently form coated vesicles [147]. After separation from the cell membrane, the clathrin coat is removed and the internalised vesicle is delivered to an early endosome, which is slightly acidic (pH 6.0-6.5) due to the action of ATP-dependent proton pumps [145]. The early endosome eventually matures into the more acidic late endosome, which after fusion with prelysosomal vesicles becomes a lysosome. This entire process from cell binding to accumulation in the lysosome occurs rapidly, generally taking 30-35 min [148]. Due to a combination of acidic pH (pH 4.0-5.0) and enzymatic action, compounds that reach the lysosome are rapidly degraded and consequently liposomes are often prepared with endosomal-escape mechanisms. For example, many liposomal formulations contain DOPE, since the propensity of this lipid to form the non-bilayer H_{II} phase can lead to the destabilisation of endosomes [116].

1.5 Active targeting of liposomes with conjugated ligands

Anti-tumour effects associated with passive targeting of therapeutic liposomes to tumours via the EPR effect have been observed in animal models and this has led to numerous clinical trials and the subsequent approval of a number of non-targeted liposomal formulations for cancer treatment. However, there are several advantages in the use of targeted liposome formulations, where a ligand is attached to the surface of a liposome to direct its delivery to specific subsets of cells that express cell surface molecules with an affinity for the particular ligand. Firstly, not all tumour vessels are
Figure 1.7 - Clathrin-mediated endocytosis of a targeted liposome. Binding of a targeted liposome to its receptor on the cell surface triggers the formation of clathrin-coated pits, which invaginate and transfer the liposome to early endosomes. Through the action of ATPase the endosome becomes acidic and eventually progresses to a lysosome, an acidic and enzyme-rich environment, where any liposomal contents will be prone to degradation.
leaky and the intratumoural distribution of passively-targeted liposomes is variable and not ideal for consistent tumour targeting [149, 150]. Secondly, attaching a ligand to the surface of a liposome can help promote the uptake of the liposome into targeted cells through clathrin-mediated endocytosis. Typically, neutral liposomes do not extensively interact with cells without active targeting and therefore are less likely to be taken up by cells [111]. Thirdly, some targeting ligands have properties that can enhance the penetration of liposomes through in vivo barriers. For example, the RVG peptide [151] and mAbs to the transferrin and insulin receptors [152] are known to promote the transcytosis of particles through the blood brain barrier. Furthermore, the targeting of liposomes provides a means to overcome the multi-drug resistance observed in numerous cancers. The targeting of drug-loaded liposomes to the folate and transferrin receptors targeting in particular, has been shown to circumvent the p-glycoprotein efflux pump, resulting in cytotoxicity in typically drug-resistant cell lines [153, 154]. Finally, the common side effects of passively-targeted liposomes can be overcome by the use of tumour targeting ligands. With its small size and long circulation time, Caelyx® has been shown to accumulate in the skin, and thereby induce uncomfortable mucocutaneous reactions in patients such as palmar-plantar erythrodysesthesia (PPE) [155]. Recent experiments where mice were treated with tumour-targeted Caelyx® have demonstrated a reduction in mucocutaneous reactions compared with native Caelyx® due to a decreased skin accumulation of the targeted liposomes [156].

1.5.1 Potential targeting molecules

Targeting of liposomes has been carried out with different classes of targeting molecules. These include antibodies, peptides, natural receptor ligands and less commonly aptamers, and affibodies. Liposomes targeted with antibodies, folate or
transferrin have been the most extensively researched to date. However, in recent years, an increasing amount of work has focussed on the use of short peptides as targeting molecules. This has coincided with an increase in the number of publications using phage display to identify novel peptide ligands for cell surface markers.

(i) Monoclonal antibodies and fragments

Antibodies (immunoglobulins) are proteins used by the immune system to identify foreign antigen. Due to their highly specific binding properties, mAbs have been developed against proteins overexpressed on the surface of tumour cells and several mAbs have been approved by the FDA for cancer treatment including Rituximab (anti-CD20) and Bevacizumab (anti-VEGF) [15]. mAbs typically consist of two heavy and two light polypeptide chains in a Y structure [122]. The variable regions in the heavy and light chains are responsible for binding antigen while the constant (Fc) region binds to Fc receptors on macrophages and other cells [157]. Consequently, several reports have indicated that mAb-conjugated liposomes (immunoliposomes) have reduced circulation half-lives due to increased Fc receptor-mediated uptake by the RES [158, 159]. Antibody fragments such as antigen binding fragments (Fab') and single chain variable fragments (scFv) can also be used to target liposomes, and since these proteins lack the Fc region, immunoliposomes prepared with these fragments have circulation half-lives comparable to that of non-targeted liposomes [159]. scFvs in particular have many advantages over mAbs for preparing immunoliposomes such as lower production costs, an increased capacity to penetrate tumour tissue and the ability to be selected for high affinity and specificity using phage display [160]. There are concerns, however, about the stability of scFv fragments during storage (they are prone to aggregation), which need to be addressed considering
the large amounts (hundreds of grams to kilograms) that would be needed for clinical trials and clinical use [157].

(ii) Peptides

In contrast to mAbs and antibody fragments, peptide sequences can be chemically synthesised and are therefore easier and less costly to produce. Peptides are seldom immunogenic and their small size (1-2 kDa) increases the tumour penetration of peptide-conjugated liposomes compared to immunoliposomes [161]. When used independently, peptides tend to bind targets with lower affinity than antibodies. However, the multimeric binding achieved when peptides are conjugated onto liposomes typically compensates for this [150]. *In vivo* phage display has led to the identification of many novel tumour homing peptide sequences, particularly peptides that bind to receptors in the tumour vasculature [18].

(iii) Transferrin and folate

Natural ligands for cellular receptors have been widely used for the delivery of liposomes to tumour cells. Transferrin and folate are the two primary examples of this approach. The transferrin protein is an endogenous blood plasma protein with a molecular weight of approximately 80 kDa [162]. In comparison, folate is a much smaller molecule (MW 441). However despite its small size, folate-targeted particles bind to cell surface folate receptors with nanomolar affinity [123]. Both transferrin and folate are non-immunogenic and very stable, enabling easy handling and storage.
1.5.2 Strategies for targeting liposomes for cancer therapy

Table 1.2 contains an overview of targeted liposome approaches that have been utilised for cancer therapy in animal studies and have resulted in a significantly enhanced therapeutic effect compared to non-targeted liposomes. Two major approaches are apparent from this summary: the targeting of liposomes directly to tumour cells themselves and the targeting of liposomes to the tumour vasculature. A more detailed description of the prominent examples of these approaches, namely HER2, transferrin receptor and αv-integrin targeting, is given below.

(i) Targeting liposomes to tumour cells

A particularly popular target for the delivery of liposomes to tumours is the human epidermal growth factor receptor (HER2). Many cancers, particularly those of the breast and ovaries, have been shown to have an upregulated expression of the HER2 gene, resulting in an increased number of these tyrosine kinase receptors on the cell surface [163]. With high levels of HER2 expression, spontaneous receptor-receptor interactions occur, facilitating uncontrolled receptor activation without extracellular growth factors [164]. Activation of HER2 results in cell proliferation and inhibition of apoptosis [165, 166], two key attributes required for tumour formation and continued growth. Based on this knowledge, the mAb Herceptin (Trastuzumab) was developed as a means to control the growth of HER2-overexpressing cells and has been approved by the FDA for the treatment of HER2-positive metastatic breast cancer [15]. To determine the potential of Herceptin to target drug-containing liposomes to tumour cells, Park and colleagues prepared PEGylated liposomes loaded with doxorubicin and conjugated with Fab' fragments of Herceptin [167]. These anti-HER2 immunoliposomes demonstrated superior anti-tumour efficacy in mice bearing human
Table 1.2 - A selected list of targeted liposome preparations that have demonstrated increased therapeutic efficacy in murine tumour models compared to non-targeted controls.

<table>
<thead>
<tr>
<th>Cell surface target</th>
<th>Targeting agent</th>
<th>Therapeutic compound [Reference]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Targeting liposomes to tumour cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β₁-integrins</td>
<td>anti-β₁-integrin Fab’</td>
<td>Doxorubicin [168]</td>
</tr>
<tr>
<td>CD19</td>
<td>anti-CD19 mAb, Fab’ or scFv</td>
<td>Doxorubicin [159, 169, 170], vincristine [171]</td>
</tr>
<tr>
<td>CD44</td>
<td>Hyaluronan</td>
<td>Doxorubicin [172], mitomycin C [173]</td>
</tr>
<tr>
<td>disialoganglioside GD2</td>
<td>anti-GD2 Fab’</td>
<td>Doxorubicin [174], CpG-ODN [175]</td>
</tr>
<tr>
<td>EGFR</td>
<td>anti-EGFR Fab’</td>
<td>Doxorubicin [176], epirubicin [176], vinorelbine [176]</td>
</tr>
<tr>
<td>Folate receptor</td>
<td>Folate vitamin</td>
<td>Doxorubicin [177-179], paclitaxel-7-carbonyl-cholesterol [180], HSV-TK pDNA [181]</td>
</tr>
<tr>
<td>GAH antigen</td>
<td>GAH F(ab’)_2</td>
<td>Doxorubicin [182]</td>
</tr>
<tr>
<td>Ganglioside G_{M3}</td>
<td>anti-G_{M3} mAb</td>
<td>Doxorubicin [183]</td>
</tr>
<tr>
<td>HER2</td>
<td>anti-HER2 Fab’ or scFv</td>
<td>Doxorubicin [167, 184, 185], paclitaxel [186], vincristine</td>
</tr>
<tr>
<td>Nucleosomes from apoptotic cells</td>
<td>2C5 mAb</td>
<td>Doxorubicin [187, 188]</td>
</tr>
<tr>
<td>p32</td>
<td>LyP-1 peptide</td>
<td>Doxorubicin [189]</td>
</tr>
<tr>
<td>Prostate-specific membrane antigen</td>
<td>anti-PSMA mAb</td>
<td>HSV-TK pDNA [190]</td>
</tr>
<tr>
<td>Sigma receptor</td>
<td>Anisamide</td>
<td>Doxorubicin [191], anti-MYC siRNA and doxorubicin [192], anti-MDM2/MYC/VEGF siRNA [193]</td>
</tr>
<tr>
<td>Transferrin receptor</td>
<td>Transferrin</td>
<td>doxorubicin [194], anti-bcl-2 AS-ODN [195], HSV-TK pDNA [196], CD pDNA [196]</td>
</tr>
<tr>
<td>Transferrin receptor</td>
<td>anti-TfR scFv</td>
<td>Anti-HER2 [197]</td>
</tr>
<tr>
<td>Unknown</td>
<td>GC4 scFv</td>
<td>anti-MYC/MDM2/VEGF siRNA [198]</td>
</tr>
</tbody>
</table>

| **Targeting liposomes to the tumour vasculature** | | |
| α₁-integrins and neuropilin-1 | P2 peptide | Paclitaxel [206] |
| Aminopeptidase A and aminopeptidase N | CARCECS and CNGRC peptides | Doxorubicin [207] |
| Aminopeptidase N | APRPG and GNGRG peptides | Doxorubicin [208] |
| Aminopeptidase N | NGR peptide | Doxorubicin [209, 210], anti-MYC siRNA and doxorubicin [211] |
| B-fibronectin (ED-B domain) | anti-ED-B scFv | 5-FdU-NOAC [212] |
| E-selectin | anti-E-selectin mAb | Combrastatin [213] |
| FGFR | Truncated bFGF peptide | Doxorubicin [214], paclitaxel [214] |
| Laminin receptor | YIGSR peptide | Fluorouracil [215] |
| Metalloproteinase MT1-MMP | anti-MT1-MMP Fab’ | Doxorubicin [216] |
| Unknown | SP5-52 peptide | Doxorubicin [217] |
| Unknown | APRPG peptide | SU1498 [218] |
breast cancer xenografts (BT-474 cells) as compared with free doxorubicin or non-targeted doxorubicin liposomes. Similar results have been observed when alternative HER2-targeting molecules have been utilised including scFv [184, 185]. Drug delivery has not been restricted to doxorubicin either, with targeted anti-tumour effects recorded after administration of liposomes loaded with paclitaxel [186] and vincristine [219]. In addition, HER2 targeting has enabled tumour-specific targeting of nucleic acids. Intravenous injection of anti-HER2 Fab' conjugated pDNA lipoplexes into BT-474 tumour-bearing mice resulted in a >100-fold increase in transgene expression in tumour tissue compared to control liposomes [163].

The transferrin receptor (TfR) represents another major focus for targeted delivery of liposomes, and is one of the few tumour cell receptors to have been targeted with liposomes in clinical trials. While TfR is ubiquitously expressed on the surface of normal cells, its expression has been found to be elevated (2-10-fold higher) on tumour cells [123]. TfR is an iron-transporting glycoprotein [220] and it has been suggested that rapidly dividing cells upregulate TfR in order to maintain the necessary iron concentration required for DNA synthesis [122]. Targeting of liposomes to TfR has many advantages including efficient endocytosis, the overcoming of drug-resistance and the ability to cross the blood brain barrier [123]. The transferrin protein itself has been used to target doxorubicin liposomes [194] and lipoplexes containing anti-bcl-2 AS-ODN [195] to tumours in mice for enhanced therapeutic effect. MBP-426, a transferrin-conjugated liposome formulation of oxaliplatin, has been through a Phase I clinical trial involving patients with solid tumours [221]. Favourable safety results were observed in this trial and Phase IIa studies are ongoing [222]. In addition to the use of transferrin, liposomes can be targeted to TfR-expressing cells through the
use of anti-TfR antibodies. Anti-HER2 siRNA has been incorporated into PEGylated liposomes containing DOTAP and DOPE before conjugation of anti-TfR scFv [197]. Intravenous injection of these targeted siRNA-lipoplexes into mice resulted in efficient knockdown of HER2 expression in PANC-1 xenograft tumours and when combined with gemcitabine, this treatment was able to significantly inhibit tumour growth. Anti-TfR scFvs have also been used to target lipoplexes incorporating plasmid DNA encoding p53 (SGT-53) to tumours [64, 223]. The results from these studies have led to the initiation of a Phase I clinical trial into the safety and pharmacokinetics of SGT-53 in patients with advanced solid tumours [224].

(ii) Targeting liposomes to the tumour vasculature

Due to the rapid growth of blood vessels during angiogenesis, the endothelial cells of the tumour vasculature express many receptors that are either not expressed at all or expressed at low levels in quiescent endothelial cells. In one study using endothelial cells from human cancer biopsies, mRNAs for 46 out of 170 endothelial cell associated proteins were detected at significantly elevated levels compared to cells in the corresponding normal vasculature [225]. These upregulated proteins included receptors for VEGF and cell adhesion molecules such as E-selectin, CD105 and αv-integrins. This unique “vascular zip-code” for tumours has seen the development of numerous approaches for the targeting of liposomes to the tumour endothelium [226].

Peptide sequences containing the arginine-glycine-aspartate (RGD) triad have been shown to bind with high affinity and specificity to αv-integrins [227]. Consequently, a number of studies have focussed on the use of RGD-liposomes for targeted delivery to the tumour vasculature [228]. Cytotoxic drugs including
doxorubicin [199-201], paclitaxel [202], fluorouracil [203] and combretastatin [204] have been incorporated into RGD-liposomes and administration of each of these liposomal preparations to tumour-bearing mice led to a significant improvement in anti-tumour efficacy compared to non-targeted liposomes. Recently, a dual targeted approach was developed by Meng and colleagues [206]. The authors synthesised a peptide (P2) containing both the RGD motif and the ATWLPPR motif, which binds neuropilin-1, another cell marker that is overexpressed in the tumour vasculature. Liposomes loaded with paclitaxel and targeted with P2 were more effective in reducing tumour volume than liposomes targeted with either single peptide motif. RGD-containing peptides have also been utilised to target nucleic acids to the tumour vasculature. Increased tumour expression of a chloramphenicol acetyltransferase transgene was noted when pDNA was incorporated into RGD-liposomes and injected into tumour-bearing mice [229]. In a separate study, siRNA targeting the MDR gene, which encodes the p-glycoprotein drug efflux pump, was incorporated into RGD-liposomes and injected into mice prior to treatment with RGD-targeted doxorubicin-liposomes [205]. Although the authors did not attempt to distinguish between liposomal targeting of tumour cells and targeting of tumour endothelial cells, this combination approach resulted in a significant reduction in the growth of a typically drug-resistant tumour (MCF7/A).

1.5.3 Attaching targeting moieties to the surface of liposomes

The simplest method of attaching targeting moieties to liposomes involves the addition of a targeting ligand to the lipid mixture during liposome preparation. However, depending on the nature of the targeting moiety, this is typically an inefficient process that cannot control the extent of ligand incorporation nor ensure the
stable presentation of the ligand on the liposome surface [21]. Therefore, the targeting of liposomes typically requires the presence of a customised lipid with a functional group capable of reacting/binding to an alternative functionality present on the ligand. The most common approach is to attach targeting ligands onto liposomes through covalent bonding – namely amide, disulphide, thioether and carbonate bonds [230]. Ligands with thiol functional groups can be coupled to maleimide-derivatised lipids through a thioether bond. While this reaction can occur under mild conditions, many proteins have few native thiol groups and require thiolation before conjugation. This process involves treatment with chemical crosslinkers such as N-hydroxysuccinimidy 3-(2-pyridyldithio)propionate and succinimidyl-S-acetylthioacetate and is random, leading to a loss of control over the orientation of the targeting molecule relative to the surface of the liposome [21]. In order to be conjugated onto maleimide-lipids, peptides must either contain a terminal cysteine residue or be thiolated prior to use. A method developed by Torchilin and colleagues yields a carbamate bond via the reaction of p-nitrophenylcarbonyl (p-NP) with amino groups on the targeting moiety [231]. This one-step reaction can be carried out at pH 8.0 and allows conjugation of unmodified proteins or peptides to pNP-PEG-PE-containing liposomes.

Since the reaction of components to form chemical bonds can be critically dependent on the conditions used, there has been an increased interest in recent years in the development of non-covalent approaches for conjugating targeting moieties onto liposomes. Rather than reacting components together to form a chemical bond, these methods mediate liposome-ligand conjugation through combinations of proteins or small molecules that have strong affinity for each other. For example an anti-endothelial growth factor receptor (EGFR) mAb linked to folate-binding protein was
recently attached onto liposomes containing folate-PEG-cholesterol conjugates and used to deliver doxorubicin to EGFR-overexpressing U87 human glioblastoma cells [232]. In an alternate approach, streptavidin-conjugated anti-TfR antibodies were coupled onto liposomes through biotinylated linker lipids [233]. These liposomes were stable in vivo and allowed targeted delivery of daunomycin to the rat brain.

Another non-covalent approach for conjugation of targeting molecules onto liposomes is based on the affinity of polyhistidine sequences for electropositive transition metals. This interaction is well established and commonly exploited in immobilised metal affinity chromatography (IMAC), a technique used to purify recombinant proteins from cell lysates. In IMAC, a sequence of contiguous histidine residues (typically 6) at either terminus of a protein provides a tag that can bind stably to a metal chelate affinity column and thus allow purification from non-specifically bound proteins [234]. The metal chelator nitrilotriacetic acid (NTA) is often used in such purification columns and forms a coordination complex between two adjacent histidine residues and a divalent metal ion such as Ni$^{2+}$ (Figure 1.8A). Lipids with headgroups containing NTA have been developed and when incorporated into liposomes or membrane vesicles these lipids enable the engrafting of histidine-tagged molecules onto the liposomal surface [235-237]. scFvs are often expressed with a His-tag to simplify purification and are therefore useful candidates for engrafting onto Ni-NTA liposomes. In addition, peptide sequences can be easily modified during synthesis so as to contain a His-tag. A major advantage of this approach is that the His-tagged targeting molecules are anchored vectorially onto the surface of the liposome, in the orientation that can minimise steric hindrance and optimise liposome targeting.
Figure 1.8 - A non-convalent mechanism for attaching histidine tagged targeting moieties onto liposomes. (A) Nitrilotriacetic acid (NTA), a molecule commonly found in immobilized metal affinity chromatography (IMAC) columns, can chelate to two adjacent histidine residues of a protein/peptide. The divalent nickel atom is a key component of this association. (B) The chelator lipid 3(nitrilotriacetic acid)-ditetradecylamine (NTA₃-DTDA) consists of a headgroup containing three NTA groups linked to a hydrophobic tail of two 14-carbon acyl chains.
1.5.4 *NTA*$_3$-*DTDA – a novel chelator lipid*

The chelator lipid 3(nitrilotriacetic acid)-ditetradecylamine (NTA$_3$-DTDA) has been developed as a means to increase the stability of linkage between histidine-tagged targeting molecules and Ni-NTA liposomes. The headgroup of NTA$_3$-DTDA contains three NTA metal chelating groups (Figure 1.8B), which can act cooperatively to stably bind His-tagged molecules. Biosensor experiments have indicated that 6His-tagged proteins bind more strongly to NTA$_3$-DTDA-containing membranes than to membranes containing NTA-DTDA, which has a single NTA functionality in its headgroup [22]. The ability of protein-engrafted NTA$_3$-DTDA liposomes to target cells *in vivo* has previously been analysed. Histidine-tagged scFvs against the dendritic cell surface markers DEC-205 and CD11c allowed targeting of liposomes to dendritic cells (DCs) in the draining lymph node after injection into the hind footpad of mice [23]. Moreover, the vaccination of mice with DC-targeted antigens induced potent anti-tumour responses and therapeutic effects in the murine tumour model B16-OVA melanoma. In subsequent work, NTA$_3$-DTDA liposomes were targeted to T cells *in vivo* by engrafting histidine tagged proteins (B7.1-6H and CD40-6H) onto the liposomes [22]. More recently, peptide-targeted NTA$_3$-DTDA liposomes have been used to deliver proteins to antigen presenting cells *in vivo*. A targeting peptide (p9-Flg) consisting of a sequence derived from the bacterial protein flagellin was prepared with a 12His-tag and engrafted onto liposomes containing the model antigen ovalbumin [238]. When injected i.v. into mice pre-inoculated with B16-OVA cells, the targeted OVA-liposomes prevented the emergence of tumour metastases within the lung of the mice. In comparison, OVA-liposomes engrafted with a control (non-targeting) peptide had no significant effect on the number of tumour foci present in the lung. In short,
these studies demonstrate the ability of NTA$_3$-DTDA to target antigen-containing liposomes to cells in vivo.

1.6 New applications for NTA$_3$-DTDA

1.6.1 Utilising NTA$_3$-DTDA liposomes to target the delivery of siRNA to cells

As outlined earlier (see Section 1.4.3), compared with the liposomal loading of proteins such as ovalbumin, a different approach is required for the incorporation of nucleic acids into liposomes. The primary aim of my Honours project that preceded this thesis was to examine the potential of incorporating siRNA into NTA$_3$-DTDA-liposomes to enable targeting to cells. In preliminary experiments it was attempted to incorporate Alexa-Fluor$_{488}$-labelled siRNA (AF-siRNA) into liposomes composed primarily of neutral lipids such as POPC and containing 1% NTA$_3$-DTDA. However, cell binding experiments indicated that under these conditions only relatively low levels of siRNA could be incorporated/encapsulated into NTA$_3$-DTDA-liposomes for targeting to cells.

The low level of incorporation of siRNA into NTA$_3$-DTDA-liposomes suggested that the inclusion of a cationic lipid such as DOTAP might be essential to promote active siRNA incorporation. Experiments were therefore carried out where suspensions of POPC liposomes containing different proportions of DOTAP were incubated with AF-siRNA, and the lipoplexes incubated with A20 cells. The flow cytometry results (Figure 1.9) indicated that the level of siRNA incorporation was relatively low at DOTAP levels up to 25 mol%, with a 2-3-fold increase in cell fluorescence compared to a 16-fold increase in cell-fluorescence for 100 mol%
Figure 1.9 – Binding of siRNA-lipoplexes prepared by mixing AF-siRNA with liposomes containing DOTAP to cells. siRNA-lipoplexes were produced by mixing a suspension of POPC liposomes containing different amounts of DOTAP (ranging from 0-100 mol%) with Alexa Fluor_488-labelled siRNA (AF-siRNA). The siRNA-lipoplexes were then incubated with A20 cells, before washing and analyzing the cells for AF_488-fluorescence by flow cytometry. The results represent the mean fold increase in fluorescence of the cells in each condition relative to control cells. Each datum point represents the mean result from three experiments ± SEM.
DOTAP. This suggests that a relatively high density of cationic lipid is required to promote substantial incorporation of the rather small siRNA molecules under these conditions. This finding underscores an important role for cationic lipids in promoting efficient incorporation of siRNA. However, when DOTAP was included in NTA₃-DTDA-liposomes in quantities >10 mol%, the presence of the cationic lipid interfered strongly with the ability of targeting molecules to be engrafted onto lipoplexes via the NTA₃-DTDA.

To further explore the potential of NTA₃-DTDA-liposomes for targeting siRNA, lipid formulations were devised containing a low proportion of DOTAP (2%), but a relatively high proportion of the ionisable lipid DODAP [see Section 1.4.3(ii)]. Several different lipid formulation were explored and the formulation Mix-5 comprising: DODAP:DOTAP:DOPE:DOPC:Chol:Cer-PEG₇₅₀ (45:2:13:5:30:5 mol%) was found to be both highly effective for incorporating siRNA into liposomes and compatible with NTA₃-DTDA. Experiments indicated that the use of Mix-5 liposomes enabled excellent targeting to DC-SIGN-expressing HEK-293 cells when the lipoplexes were engrafted with p293, a His-tagged peptide with high affinity for DC-SIGN (Figure 1.10). Importantly, lipoplexes engrafted with the non-targeting control protein histidine-rich glycoprotein did not bind significantly to cells, indicating that the binding was based on specific receptor-ligand interactions.

The ability of targeted siRNA-lipoplexes to induce gene silencing was also examined in Honours using lipoplexes containing anti-EGFP siRNA. When targeted to DC-293 cells with p293, anti-EGFP siRNA-lipoplexes induced noticable silencing (~30% knockdown) of EGFP expression (Figure 1.11). This provided evidence that
Figure 1.10 – p293-engrafted siRNA-lipoplexes can bind efficiently to DC-293 cells. Mix-5 (DODAP:DOTAP:DOPE:DOPC:cholesterol:Cer-PEG$_{750}$; 45:2:13:5:30:5 molar ratio) was used to prepare siRNA-lipoplexes through an initial acidification step followed by incubation with AF-siRNA. siRNA-lipoplexes were then neutralised, incorporated with Ni-NTA$_3$-DTDA (added to 1 mol% of total lipid) and engrafted with either the targeting peptide p293 or histidine-rich glycoprotein (HRG) as the non-targeted control (as indicated). The engrafted lipoplexes were incubated with DC-293 cells (30 min, 4 °C), and cell-associated AF$_{488}$-fluorescence then assessed by flow cytometry. Control conditions were also included where DC-293 cells were incubated either without siRNA-lipoplexes or with non-engrafted siRNA-lipoplexes (as indicated). Each fluorescence profile is a representative obtained from two experiments performed in duplicate.
Figure 1.11 - Targeted siRNA-lipoplexes induce knockdown of EGFP expression in DC-293 cells. Mix-5 lipoplexes (1:20 siRNA:lipid ratio, w/w) containing 7.5 pmol of negative control siRNA (NC-siRNA) or anti-EGFP siRNA were engrafted with either targeting peptide p293, or histidine-rich glycoprotein (HRG, non-targeted control). The lipoplexes were then incubated for 3 h at 37 °C with human DC-SIGN-expressing HEK-293 cells suspended in growth medium with 10% FCS. The cells were then washed, cultured for 24 h (37 °C) in growth medium, before being transfected with pEGFP-N1 using Lipofectamine™ 2000. The cells were cultured for a further 24 h and then analysed for EGFP fluorescence by flow cytometry. Each result represents the mean EGFP expression of the cells, relative to cells treated with HRG NC-siRNA-lipoplexes. Each bar represents the results from three separate experiments ± SEM. *p<0.05
NTA$_3$-DTDA can be used to develop strategies for receptor-specific targeting of siRNA. However, HEK-293 cells are known to be transfected easily and are not representative of cells that could potentially be targeted \textit{in vivo} for tumour therapy. Therefore, further work is needed to determine whether the approach developed in Honours is capable of inducing targeted knockdown of gene expression in cells that are typically more resistant to lipid-mediated transfection and are derived from human/murine tumour cells, endothelial cells or immune effector cells.

\subsection*{1.6.2 Aims of this project}

The results achieved in Honours indicated that ligand-engrafted siRNA-lipoplexes prepared from Mix-5 liposomes and the chelator lipid NTA$_3$-DTDA can target the delivery of siRNA to cells \textit{in vitro} for induction of RNAi. However, further work is needed to optimise the preparation of the siRNA-lipoplexes especially for potential use for \textit{in vivo} applications. In addition, given the therapeutic potential of pDNA delivery, the possibility of utilising the same approach as developed for siRNA to target the delivery of pDNA to cells should be explored. While both siRNA and pDNA have a similar charge distribution, siRNA has a significantly lower molecular weight than pDNA and is known to interact differently with lipid carriers, most likely requiring refinement or adaptation of the technique used for encapsulation.

Targeted delivery of lipoplexes to tumours requires the use of targeting moieties that are specific for tumour structures. Although many potential ligands for the molecular markers of tumours and supporting cells have been identified, none have yet been tested for their ability to target NTA$_3$-DTDA-containing liposomes to tumours. Moreover, the identification of peptides that have potential for targeting the
delivery of liposomes containing anti-cancer agents has high priority for improving treatment outcomes in cancer. The use of NTA$_3$-DTDA-liposomes provides a convenient approach for testing different targeting moieties that may have potential for developing new therapies. For example, identification of targeting peptides that increase tumour accumulation of liposomes would allow the analysis of in vivo transfection efficiency of targeted lipoplexes. In addition, liposomes containing NTA$_3$-DTDA and loaded with doxorubicin could potentially be targeted to tumours utilising histidine-tagged targeting peptides to increase their anti-tumour efficacy and reduce unwanted side-effects.

The specific aims of this project are:

1. To develop and further optimise the potential of NTA$_3$-DTDA-liposomes to target the delivery of siRNA to cells.
2. To determine whether NTA$_3$-DTDA liposomes can be used to target the delivery of plasmid DNA.
3. To explore the use of different tumour-targeting peptides for their potential to target delivery of NTA$_3$-DTDA liposomes to tumours.
4. To investigate the application of targeted lipoplexes and doxorubicin-loaded liposomes for anti-tumour therapy.
CHAPTER TWO

GENERAL METHODOLOGY
2.1 Reagents

Heparin (sodium salt), ribonuclease A (RNase A), ribonuclease inhibitor from human placenta, DNase I and β-mercaptoethanol were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). RPMI 1640 medium, PSN antibiotic solution and L-glutamine were obtained from the Media Unit, John Curtin School of Medical Research (ANU). Genetecin, foetal calf serum (FCS), newborn bovine serum and Lipofectamine™ 2000 were purchased from Invitrogen Australia Pty Ltd (Mulgrave, VIC, Australia). Paraformaldehyde was from BDH Chemicals (Kilsyth, VIC, Australia). Caelyx® is a product of Johnson & Johnson, and is marketed in Australia by Schering-Plough Pty Ltd (Baulkham Hills, NSW, Australia). Emulsifier-Safe scintillant was purchased from Perkin-Elmer (Glen Waverley, VIC, Australia). Hoechst 33342 was kindly provided by Tom Karagiannis (Peter MacCallum Cancer Centre, Melbourne, Australia). Doxorubicin (Adriamycin) was obtained from Pfizer Australia (West Ryde, NSW, Australia). Tissue-Tek O.C.T. is manufactured by Sakura Finetek (Torrance, CA, USA) anti-CD8-PE (clone 53-6.7), anti-IFN-γ-Alexa Fluor647 (clone XMG1.2) and BD Golgistop was obtained from BD Biosciences (North Ryde, NSW, Australia). NiSO₄ was used for all of the additions of Ni²⁺ to liposome preparations. Analytical grade reagents were used in all experiments.

2.2 Lipids

The phospholipids 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycerol-3-phosphatidylcholine (DOPC), 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) and 1,2-distearoyl-sn-glycerol-3-phosphatidylcholine
(DSPC) as well as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), cholesterol and cholesterol hemisuccinate (CHEMS) were all obtained from Sigma-Aldrich. The ionisable aminolipid 1,2-dioleoyl-3-dimethylammonium-propane (DODAP) as well as N-octanoyl-sphingosine-1-[succinyl(methoxy-(polyethylene glycol)750] (Cer-PEG<sub>750</sub>), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol)-2000] (PE-PEG<sub>2000</sub>) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol)-750] (PE-PEG<sub>750</sub>) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The fluorescent lipids Texas Red 1,2-dihexadecanoyl-sn-glycero-3- phosphoethanolamine (Texas Red-DHPE) and Oregon Green-488-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (OG<sub>488</sub>-DHPE) were purchased from Invitrogen. [4-<sup>14</sup>C]-Cholesterol was purchased from Perkin Elmer (Glen Waverley, VIC, Australia). The chelator-lipid 3(nitrilotriacetic acid)-ditetradecylamine (NTA<sub>3</sub>-DTDA) was produced in the Research School of Chemistry (ANU) as described [237].

Alexa Fluor<sub>647</sub>-DOPE (AF-DOPE) was produced by reacting DOPE [5 mM suspension in phosphate-buffered saline (PBS)] with an equimolar amount of Alexa Fluor<sub>647</sub> carboxylic acid succinimidyl ester (Invitrogen) for 2 h at 60 °C, and then purifying the AF-DOPE liposomes by size-exclusion using a Nick G-50 Sephadex column (Amersham Biosciences AB, Uppsala, Sweden); it was estimated that approximately 20% of the DOPE was AF<sub>647</sub>-conjugated.

2.3 Cell lines

Murine A20 B cells (Balb/c, I-A<sup>d</sup>) were obtained from Dr. A. Gautum (JCSMR, ANU). Human embryonic kidney 293 cells (HEK-293) were obtained from
Dr. Hilary Warren (Royal Canberra Hospital, Canberra, Australia). HEK-293 cells expressing human DC-SIGN (DC-293) were generated by transfection with pcDNA3-DC-SIGN using Lipofectamine™ 2000. The murine dendritic cell line DC2.4 was provided by Dr. David Tscharke (RSB, ANU), and DC2.4 cells expressing EGFP (DC2.4-EGFP) were generated by transfection with pEGFP-N1 using Lipofectamine 2000. The murine macrophage cell line RAW264.7 was obtained from Mr. Duncan Sutherland (JCSMR, ANU). The human hepatocarcinoma cell line HepG2 and the murine melanoma cell line B16-F1 were obtained from Professor Chris Parish (JCSMR, ANU). The murine fibroblast cell line NIH-3T3 was kindly provided by Associate Professor Ruth Ganns (Western Australian Institute for Medical Research, University of Western Australia). The human T cell leukemia cell line Jurkat was provided by Professor Ian Young (JCSMR, ANU). The highly metastatic murine melanoma B16-OVA [C57BL/6 (H-2b)], an OVA-secreting tumour cell line, was from Dr. Mark Hulett (JCSMR, ANU).

The transformed cells were positively selected by growing in the presence of 0.4 mg/mL Geneticin. Sorting by flow cytometry enabled further positive selection of cells expressing high levels of the desired protein. All cells were cultured in RPMI 1640 medium supplemented with 5% FCS plus 5% newborn bovine serum, 50 µM β-mercaptoethanol, 0.1 mM L-glutamine and 0.1% PSN antibiotics solution in a 37 °C incubator with atmosphere 5% CO₂. Cells of between 50% and 80% confluency were routinely used for experiments; they were harvested by gentle pipetting and then incubated in suspension with the liposomes/lipoplexes for use in experiments.
2.4 Mice

C57BL/6 mice of various ages were obtained from the Animal Resource Facility (Perth, WA, Australia). All animal experiments were carried out in accordance with protocols approved by the ANU Animal Experimentation Ethics Committee.

2.5 Proteins

A recombinant form of the extracellular region of murine CD4 with a 6His tag at the carboxy terminal (hereafter referred to as CD4) was previously produced in the Altin lab using the baculovirus expression system. Briefly, recombinant viruses containing the CD4 gene were amplified in SF9 insect cells, with the supernatant subsequently used to infect High-5 insect cells. The High-5 cells were cultured at 28 °C for 4 days in Express 5 culture medium containing 5% SF900 and 1.5 mM L-glutamine. Recombinant CD4 expressed by the virally infected cells was purified from the supernatant by IMAC followed by size-exclusion gel filtration on fast pressure liquid chromatography. Purity of the eluate was confirmed by SDS-PAGE analysis and was typically judged at being >95%. Proteins were stabilised by the addition of urea (160 mM) and a serine protease inhibitor, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (0.5 mg/mL). This treatment enables the proteins to be stored at 4 °C without any appreciable loss in binding capacity.

Similar to the method described in Section 2.2, fluorescein isothiocyanate (FITC)-conjugated anti-Flt-1 antibody was produced by reacting affinity purified goat anti-hVEGF R1(Flt-1) immunoglobulin (Ig)G antibody from R&D Systems
(Minneapolis, MN, USA) with a 4-fold molar excess of 6-[fluorescein-5-(and-6)-
carboxamido]-hexanoic acid succinimidyl ester (Invitrogen) for 1 h at 37 °C followed
by purification by size-exclusion.

2.6 Peptides

Targeting peptides for engraftment onto NTA₃-DTDA liposomes/lipoplexes
were produced to contain a His-tag (to enable engraftment onto Ni-NTA₃-DTDA), an
inert spacer sequence (to reduce steric hindrance), followed by a targeting amino acid
sequence reported to bind to specific receptors on cells (see Table 2.1). Typically
targeting peptides were synthesised with the following structure:

![Peptide Structure Diagram]

In contrast to the other targeting peptides described in this work, peptide p24-
NRP-1 was produced with the His-tag at the carboxy-terminal end of the peptide to
maintain the same anchoring orientation as used previously [239]. Also, p24-NRP-1
was produced in a different batch and the His-tag contained four HHG repeats. This
change was made to facilitate synthesis and was not considered to significantly affect
the activity of either the NRP-1 targeting moiety or the His-tag.
<table>
<thead>
<tr>
<th>Peptide</th>
<th>His-tag</th>
<th>Spacer</th>
<th>Targeting motif</th>
<th>Putative target</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>p9-Flg</td>
<td>HHHHHHHHHHHHH</td>
<td>GSGSG</td>
<td>INNLQVRVELAVQSANSTNSQSDLDS</td>
<td>TLR5</td>
<td>[238]</td>
</tr>
<tr>
<td>p12-TNFR2</td>
<td>HHHHHHHHHHHHH</td>
<td>GSGSGS</td>
<td>CYTYQGKLC</td>
<td>TNFR2</td>
<td>[240]</td>
</tr>
<tr>
<td>p15-RGR</td>
<td>HHHHHHHHHHHHHH</td>
<td>GSAGSAGSAGSAGSAGSA</td>
<td>GCRGRRST</td>
<td>PDGFRβ</td>
<td>[241]</td>
</tr>
<tr>
<td>p24-NRP-1</td>
<td>HHHHHHHHHHHHHH</td>
<td>GASGASGASGASGAS</td>
<td>GATWLPPRG</td>
<td>NRP-1</td>
<td>[242]</td>
</tr>
<tr>
<td>p25-Flt-1</td>
<td>HHHHHHHHHHHHHH</td>
<td>GSGSG</td>
<td>GWHSDMEWWYLLG</td>
<td>VEGFR2 (Flt-1)</td>
<td>[243]</td>
</tr>
<tr>
<td>p28-TLR2</td>
<td>HHHHHHHHHHHHHH</td>
<td>GSGSG</td>
<td>CDPDSDPGDSPGC</td>
<td>TLR2</td>
<td>[244]</td>
</tr>
<tr>
<td>p39-Flt-1</td>
<td>HHHHHHHHHHHHHH</td>
<td>GSGSGSAGSAGSAGS</td>
<td>GWHSDMEWWYLLG</td>
<td>VEGFR2 (Flt-1)</td>
<td>[243]</td>
</tr>
<tr>
<td>p46-RGD</td>
<td>HHHHHHHHHHHHHH</td>
<td>GSAGSAGSAGSAGSAGS</td>
<td>GARYCRGDCFDG</td>
<td>αv-integrin</td>
<td>[199]</td>
</tr>
<tr>
<td>p47-LyP-1</td>
<td>HHHHHHHHHHHHHH</td>
<td>GSAGSAGSAGSAGSAGSAGS</td>
<td>CNKRTRGC</td>
<td>p32</td>
<td>[245]</td>
</tr>
<tr>
<td>p49-Control</td>
<td>HHHHHHHHHHHHHH</td>
<td>GSAGSAGSAGSAGSAGSAGS</td>
<td>-</td>
<td>-</td>
<td>[246]</td>
</tr>
<tr>
<td>T2</td>
<td>HHHHHHHHHHHHHH</td>
<td>GASGAGSASGAS</td>
<td>YGRKRRQSRAPQPC</td>
<td>GAGs</td>
<td>[247]</td>
</tr>
</tbody>
</table>

TLR5, toll-like receptor 5; TNFR2, tumour necrosis factor receptor 2; PDGFRβ, platelet-derived growth factor receptor β; NRP-1, neuropilin-1; VEGFR2, vascular endothelial growth factor receptor 2; TLR2, toll-like receptor 2; GAGs, glycosaminoglycans.
In addition to p49-Control, the L2 peptide (a 10 amino acid peptide of sequence GHHPHGHHPH derived from human histidine-rich glycoprotein) and a peptide designated 12His (HHHHHHHHHHHH) were used as non-targeted (control) peptides since they are both likely to bind strongly to Ni-NTA₃-DTDA and thus reduce non-specific binding of Ni-NTA₃-DTDA-containing liposomes/lipoplexes to cells. A His-tagged (10His) form of a peptide that can target DC-SIGN expressing HEK-293 cells (p293) was kindly provided by Lipotek (ANU). For commercial reasons this peptide cannot be described in further detail. The fusogenic peptide diINF-7 was synthesised as a monomer (GLFEAIEGFIENGWEIDGHWYGCG) and dimerised through disulphide bond formation between terminal cysteine residues as previously described [248]. A DNase II inhibitor peptide identified by phage display [249] of amino sequence CSLRLQWFLWAC was produced containing HHHHHHHGGG at the N-terminus (6His-ID2). The peptide SIINFKEKL consisting of the immunodominant epitope of OVA (aa 257-264) was also produced.

All peptides were synthesised and HPLC purified by the Biomolecular Resource Facility, JCSMR (ANU). Peptides p46-RGD and p47-LyP-1 were both cyclised through disulphide bond formation between the cysteine residues present in each peptide. Stock solutions of peptides were made in ddH₂O and stored at -20 °C.

2.7 Plasmids

The vector pEGFP-N1 was obtained from Becton Dickinson as a glycerol stock of transformed DH5α E. coli. pcDNA3-DC-SIGN was likewise obtained from NIH AIDS Research and Reference Reagent Program (Rockville, MD, USA). The vector
pcDNA3.1D/V5-His-TOPO (pSFKL) with an insert encoding NP-SIMNFEKL-eGFP was kindly provided by Dr. David Tscharke (RSB, ANU). pKUNMpt (pKUN), a Kunjin virus based vector encoding for different CTL epitopes, one of which is SIINFEKL, was provided by Prof. Alexander Khromykh (School of Chemistry and Molecular Biosciences, University of Queensland, Australia). pSFKL and pKUN were transformed into DH5α E. coli. Plasmids were amplified in the bacteria and purified using the PureYield Plasmid Maxiprep System from Promega (Sydney, NSW, Australia). Purified plasmid DNA was eluted in nuclease-free water and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.8 siRNA

Silencer GFP (eGFP) siRNA (anti-EGFP-siRNA) was obtained from Ambion (Applied Biosystems, Scoresby, VIC, Australia). All other siRNAs were obtained from Invitrogen. Alexa Fluor488-labelled siRNA (AF-siRNA) was designed based on a published sequence [250] capable of reducing the expression of the EGFP gene expressed from BD Clontech vectors. The sequence of this siRNA was 5’ Alexa Fluor488 – GGC UAC GUC CAG GAG CGC ACC 3’ (sense) and 5’ Alexa Fluor488 – GGU GCG CUC CUG GAC GUA GCC 3’ (antisense). Negative control siRNA (NC-siRNA) previously reported to have no gene silencing activity [251] was: 5’ AAU UCU CCG AAC GUG UCA CGU TT 3’ (sense) and 5’ ACG UGA CAC GUU CGG AGA AUU TT 3’ (antisense).
Table 2.2 - Lipid compositions of liposome formulations utilised in this work.

<table>
<thead>
<tr>
<th>Name</th>
<th>Lipid/peptide composition</th>
<th>Molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mix-1</td>
<td>DODAP : DOPE : DOPC : Cer-PEG_{750}</td>
<td>30 : 30 : 30 : 10</td>
</tr>
<tr>
<td>Mix-6</td>
<td>DODAP : DOTAP : DOPE : Chol : Cer-PEG_{750} : diINF-7</td>
<td>45 : 2 : 20 : 30 : 2 : 1</td>
</tr>
<tr>
<td>Mix-7</td>
<td>DODAP : DOTAP : DOPC : Chol : Cer-PEG_{750} : Ni-NTA_{3}-DTDA</td>
<td>56 : 2 : 5 : 30 : 5 : 2</td>
</tr>
<tr>
<td>Mix-8</td>
<td>DOTAP : DOPE : DOPC : Chol : Cer-PEG_{750} : Ni-NTA_{3}-DTDA</td>
<td>2 : 56 : 5 : 30 : 5 : 2</td>
</tr>
<tr>
<td>Mix-10</td>
<td>DODAP : DOTAP : DOPE : Chol : Cer-PEG_{750} : Ni-NTA_{3}-DTDA</td>
<td>47 : 2 : 14 : 30 : 5 : 2</td>
</tr>
<tr>
<td>Mix-12</td>
<td>DODAP : DOTAP : DOPE : DOPC : CHEMS : Cer-PEG_{750} : Ni-NTA_{3}-DTDA</td>
<td>44 : 2 : 12 : 5 : 30 : 5 : 2</td>
</tr>
<tr>
<td>Mix-13</td>
<td>DODAP : DOTAP : DOPE : Chol : Cer-PEG_{750} : Ni-NTA_{3}-DTDA</td>
<td>44 : 2 : 20 : 30 : 2 : 2</td>
</tr>
</tbody>
</table>

When Mix-5 was prepared for experiments in Chapter 4, 2 mol% NTAs-DTDA was included in the lipid formulation. Other liposome formulations that were not given specific names are described in the results chapters. DODAP, 1,2-dioleoyl-3-dimethylammonium-propane; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine; Cer-PEG_{750}, N-octanoyl-sphingosine-1-[succinyl(methoxy-(polyethylene glycol)750]; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; Chol, cholesterol; diINF-7, an endosmotic peptide; Ni-NTA_{3}-DTDA, nickel 3(nitrotriacetic acid)-ditetradecylamine; CHEMS, cholesterol hemisuccinate; PE-PEG_{2000}, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol)-2000].

2.9 Liposome formulations
2.10 Flow cytometry

Flow cytometric analyses were performed using a BD LSR II flow cytometer (Becton Dickinson) equipped with a 20 mW coherent sapphire laser. Cells were analysed on the basis of forward light scatter (FSC), side light scatter (SSC), FITC-fluorescence, PE-fluorescence and APC-fluorescence (where applicable). Data for 10,000 cells were collected for each condition, and the data processed using CellQuest software (Becton Dickinson). Data were analysed by gating only live cells, as judged by FSC versus SSC dot plots, and plotting the fluorescence profile as a histogram or dot plot.

2.11 Confocal microscopy

After staining and fixation in 2% paraformaldehyde, cells were suspended in embedding medium (2% propyl gallate in 70% glycerol) and deposited into 0.05 mm deep chambers on glass microscope slides formed using perforated Scotch 465 adhesive transfer tape, and the chambers were then sealed with glass cover slips. The localisation of cell-associated fluorescence was observed using a BioRad Radiance-2000 Confocal Microscope (488ex, 520em) for experiments in Chapter 3 or alternatively using a Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) (543 nm excitation/615 nm emission for Texas Red, 488 nm excitation/520 nm emission for EGFP and 355 nm excitation/465 nm emission for Hoechst 33342) for the experiments in Chapter 4.
2.12 Radioactive analysis of murine tissue samples

The tissues (spleen, liver, kidney, lung, heart, hind leg muscle, tumour) from mice euthanized via CO₂ asphyxiation were harvested, weighed and solubilised in 1 mL of a solution containing 0.5 M sodium hydroxide, 150 mM sodium chloride and 2% (v/v) SDS. Solubilisation was assisted by heating at 60 °C for 24 h. Blood samples (100 μL) were removed using cardiac puncture and solubilised with the same solution as above, with heating at 60 °C for 24 h. The blood samples were cooled to room temperature and EDTA added for a final concentration of 25 mM. 400 μL H₂O₂ was then added dropwise to the blood sample, followed by a 1-h incubation at 60 °C. Solubilised tissue and blood samples (0.3 mL) were cooled to room temperature and added to 3 mL Emulsifier-Safe scintillant. Radioactivity (dpm) was measured using a LS6500 Multipurpose Scintillation Counter (Beckman Coulter, USA). The results were expressed as the percentage of the injected dose per gram of tissue (%ID/g). To ensure valid results, control (blank) tissue samples were prepared and spiked with a known amount of radioactive cholesterol. Radiometric readings of these controls indicated that the colour present in each tissue type tested did not significantly interfere with quantitation of ¹⁴C in samples.

2.13 Particle size and charge measurements

The particle size and zeta-potential of liposomes and lipoplexes were measured with a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Size measurements were taken by diluting liposomes/lipoplexes by approximately 20-fold to 500 μl in filtered PBS (pH 6.0 or 7.4) or ddH₂O and transferring to a disposable 1-mL
cuvette for analysis. Sample were measured at 25 °C with a chosen dispersant viscosity value of 1.0200 cP (PBS). The particle size (Z-average diameter) was determined by the manufacturer’s software based on the mean intensity of scattered light produced by the Brownian motion of the particles. One run consisted of five individual measurements (10 s in duration) and the results presented are the mean values of three runs. Importantly, particle size measurements were observed to be independent of the concentration of liposomes/lipoplexes in the cuvette. Zeta-potential measurements were also taken by diluting liposomes/lipoplexes to 1 mL in filtered PBS (pH 7.4) and transferring to a folded capillary cell for analysis. Samples were measured at the same temperature and viscosity as for size measurements. The zeta-potential was determined based on the electrophoretic mobility of the particles in the dispersant (monomodal mode). Automatic settings were used to select the number of measurements per run (typically 25–30 measurements per run).

2.14 Statistical analysis

For statistical analysis, a two-tailed Student’s t-test was used. p<0.05 was considered statistically significant. The results are presented as the mean ± standard error of the mean (SEM).

2.15 Additional materials and methods

Additional materials and methods relating to experiments for work carried out for each chapter are included in the Materials and Methods sections for each chapter.
Chapter Three

Convenient Targeting of Stealth siRNA-Lipoplexes to Cells with Chelator Lipid Anchored Molecules
3.1 Introductory Comments

Studies during Honours had indicated that complexes of siRNA and liposomes (siRNA-lipoplexes) could be prepared to contain NTA$_3$-DTDA, thereby enabling the engraftment of His-tagged targeting molecules onto the surface of the lipoplexes for targeting to specific receptors expressed on cells. In this chapter, the method of preparing targeted siRNA-lipoplexes is further optimised and developed, and a thorough characterisation of siRNA-lipoplex properties is undertaken including analysis of particle size, surface charge and sensitivity to serum. In addition, several different targeting molecules, predominantly short peptides, are assessed for their ability to deliver siRNA-lipoplexes to the surface of specific cells \textit{in vitro}. The ability of these targeting molecules to promote intracellular uptake of the bound lipoplexes is also investigated. Finally, functional studies are carried out utilising anti-\textit{EGFP} siRNA to determine if knockdown of gene expression can be induced. Work for these experiments resulted in the development of a new liposome formulation (\textit{Mix}-6) optimised for \textit{in vitro} transfection delivery. Much of the work presented in this chapter was conducted in parallel to experiments described in Chapter 4 and consequently the \textit{Mix}-6 formulation described herein was developed also from the experiments with pDNA in Chapter 4.

This chapter represents data published in the paper: "Convenient targeting of stealth siRNA-lipoplexes to cells with chelator lipid-anchored molecules" by T.P. Herringson and J.G. Altin. Journal of Controlled Release, 2009, 193(3) pp 229-238.
3.2 Abstract

A major obstacle for the use of siRNAs as novel therapeutics is the requirement for functional delivery to specific cells in vivo. siRNA delivery by cationic agents is generally non-specific and a convenient targeting strategy has been lacking. This work explored the potential for using the chelator lipid NTA$_3$-DTDA with neutral stealth liposomes to target siRNA to cells. A novel method for encapsulating siRNAs into liposomes was developed which utilised helper lipids and the ionisable lipid DODAP. This approach results in an efficient (>50%) incorporation of siRNA into lipoplexes, which when incorporated with Ni-NTA$_3$-DTDA and engrafted with a His-tagged form of murine CD4 can target siRNA to murine A20 B cells, in vitro. Also, siRNA-lipoplexes engrafted with His-tagged peptides that target receptors on HEK-293 cells, or the receptor for tumour necrosis factor alpha expressed on the murine dendritic cell line DC2.4, could target siRNA and silence the expression of EGFP. siRNA-lipoplexes produced by this method are ~240 nm in diameter, exhibit low zeta-potential (-1 mV), and target cells in serum-containing media. The results show that NTA$_3$-DTDA can be used to target siRNA-lipoplexes to cells, and could provide a convenient approach for targeting siRNA to cells in vivo for therapeutic applications.
3.3 Introduction

RNA interference (RNAi) has been hailed as one of the most exciting discoveries in functional genomics of the past decade, having enormous potential as a tool for analysing gene function, and for developing novel therapeutics based on gene silencing [252, 253]. The finding that the introduction of siRNA duplexes (19-23 nucleotides long) into cells can induce a sequence-specific degradation and inhibition in the expression of the targeted mRNA, seems set to revolutionise the potential for effective use of RNAi for both research and therapeutic applications [254, 255]. siRNAs for use in mammalian cells can be produced as synthetic molecules, and pre-designed synthetic siRNAs optimised for maximum potency and specificity using effective and extensively tested algorithms, have become commercially available for >99% of all human, mouse and rat genes. siRNAs act catalytically to mediate cleavage of the targeted mRNA, and are very potent [256]. Systemic delivery of siRNAs can achieve effective knockdown of certain genes [257, 258], but this approach lacks efficiency and tissue specificity, and increases the likelihood of unwanted off-target effects. A convenient strategy for targeting siRNAs to specific cell types is required to better exploit their therapeutic potential [259-262].

An attractive approach for delivery of siRNA to cells is the use of liposomes as targeted delivery vehicles [116]. Cationic lipids can form complexes with nucleic acids, and are widely used as components of liposomal reagents used for the transfection of cells with DNA and siRNAs in vitro [263-265]. Such liposomes often contain a large proportion of one or more cationic lipids (e.g. DOTAP), as well as the zwitterionic helper lipid DOPE. Cationic lipids interact efficiently with nucleic acids, forming lipid/nucleic acid complexes (lipoplexes). Unfortunately, whilst cationic
liposomes can facilitate transfection of cells *in vitro*, their propensity to aggregate and to interact non-specifically with negative charges on the surface of cells makes their targeted delivery to specific cells *in vivo* difficult [266, 267].

As an alternative to using cationic liposomes, the encapsulation of siRNA into neutral stealth liposomes seems an excellent strategy for delivering siRNAs to cells *in vivo*. Such liposomes are generally non-toxic, avoid non-specific interactions with blood components [268, 269], and the lipid barrier protects the encapsulated siRNA cargo from rapid degradation by serum nucleases. Despite progress towards the efficient encapsulation of drugs and nucleic acids into neutral stealth liposomes [116, 141], a convenient method of targeting has been lacking [255, 261, 270]. Using the metal chelator lipid NTA3-DTDA [237], a method for engrafting targeting molecules onto liposomes for targeting to specific cells was recently developed in the Altin lab [22, 23]. Thus, the incorporation of NTA3-DTDA into stealth liposomes containing antigen and cytokine, allowed stable engraftment (by Ni2+-chelating linkage) of Histagged forms of B7.1, CD40 and scFvs enabling the targeting of the liposomes to T cells and dendritic cells *in vitro* and *in vivo* [22, 23]. In this chapter, the potential of the NTA3-DTDA technology for targeting siRNA encapsulated within stealth liposomes was explored. The results show that a formulation of NTA3-DTDA-containing liposomes can be designed to enable convenient receptor-mediated delivery of lipoplex- incorporates siRNAs to cells for the induction of gene silencing. The potential of this approach for targeting siRNA delivery in therapeutic applications is discussed.
3.4 Materials and Methods

3.4.1 Reagents

All reagents, lipids, cell lines, proteins, peptides, plasmids and siRNA used in the experiments described in this chapter are listed in Chapter 2, Sections 2.1, 2.2, 2.3, 2.5, 2.6, 2.7 and 2.8.

3.4.2 Preparation of liposomes

Stock solutions of lipids in ethanol were stored at −80 °C. For production of liposomes, lipids were mixed, dried under a stream of nitrogen gas, and liposomes produced by suspending the lipids in PBS by sonicating for 45 s (two bursts) at maximum amplitude using a TOSCO 100W ultrasonic disintegrator (Measuring and Scientific Ltd, London, UK). Where appropriate, diINF-7 was incorporated into liposomes by addition at 1 mol% to the dried lipids before addition of PBS and sonication. For some experiments the Ni-NTA₃-DTDA was included in the liposome lipid mixtures. Previous studies in the Altin lab (unpublished) showed that NTA₃-DTDA has detergent-like properties and is completely miscible with other lipids and phospholipids such as DSPC. NTA₃-DTDA is not readily suspended in water, but readily forms a clear dispersion in PBS, suggesting it enters into solution or forms micelle-like structures. Where indicated, to incorporate Ni-NTA₃-DTDA into lipoplexes a stock suspension of 10 μM NTA₃-DTDA containing 30 μM NiSO₄ in PBS was used. All stock suspensions of liposomes were stored for up to 1 week at 4 °C, but storage for longer time was at −20 °C; liposomes were briefly re-sonicated prior to use in experiments.
3.4.3 Formation of siRNA-lipoplexes

Previous use of the ionisable lipid DODAP with oligonucleotides has been described [142, 271, 272]. To employ DODAP with siRNA and NTA₃-DTDA liposomes, two approaches were used. In the first, a 4-μL aliquot of an ethanolic solution (1 mM total lipid) of Mix-1 (see Table 3.1), was mixed with 20 pmol acidified AF-siRNA (acidified by adding 100 mM tri-sodium citrate buffer (pH 5) to a final 10 mM citrate), and incubated for 30 min at room temperature. In the second approach, 1 μL of Mix-2, Mix-5 or Mix-6 (Table 3.1, prepared as 4 mM total lipid stocks in PBS), was acidified by adding 1 μL glycine (100 mM, pH 3.0), before adding 3 μL of AF-siRNA (5 μM). After 5 min of incubation, ethanol (4 μL) was added, and the mixture vortexed and incubated for 30 min at room temperature. The resulting siRNA-lipoplexes contained 15 pmol siRNA with siRNA:lipid ratio of 1:10 (w/w); except where indicated this siRNA:lipid ratio 1:10 (w/w) was used in all experiments. As indicated, for some experiments the ethanol was replaced with an equal volume of ddH₂O; also 1 μL tri-sodium citrate (25 mM) buffer of different pH was used instead of glycine.

| Table 3.1 - Composition of the different liposome formulations tested for siRNA-lipoplex formations and targeting of siRNA to cells. |
|---|---|---|
| Formulation | Lipid/peptide composition | Molar ratio |
| Mix-1 | DODAP:DOPE:DOPC:Cer-PEG₇₅₀ | 30 : 30 : 30 : 10 |

Each mixture was incubated with siRNA as described in the text, before incorporation of Ni-NTA₃-DTDA and engraftment of His-tagged protein/peptide for targeting of the siRNA-lipoplex to cells. Two exploratory lipid mixtures Mix-3 and Mix-4 are not discussed in this work and are not shown for clarity.
3.4.4 Incorporation of NTA<sub>3</sub>-DTDA and engraftment of lipoplexes

siRNA-lipoplexes produced as in Section 3.4.3, were incorporated with NTA<sub>3</sub>-DTDA by first neutralising with 4 μL of 25 mM Na<sub>2</sub>HPO<sub>4</sub> (pH 8.0), and then adding 4 μL of Ni-NTA<sub>3</sub>-DTDA (10 μM), mixing and incubating for 30 min at room temperature. Since these siRNA-lipoplexes generally contained 1 mol% of NTA<sub>3</sub>-DTDA, they were engrafted by adding a targeting protein or peptide as indicated, and incubating for 30 min at room temperature with occasional vortexing. For each experimental condition the amount of targeting peptide used for engraftment was based on a theoretical calculation of the amount of peptide required to be equi-molar with anchoring moiety NTA<sub>3</sub>-DTDA and an empirical titration to give the greatest binding of lipoplexes to cells known to express the receptor being targeted. Since the siRNA-lipoplexes were typically produced in relatively small volumes (<50 μL), used minimal amounts of targeting molecule that give optimum binding of lipoplexes to cells, and the cells were washed after incubation with the targeted lipoplexes (which removes excess lipoplexes and any free siRNA/peptide), we did not purify any free peptide/siRNA from the lipoplexes (say by dialysis or gel filtration) for each experimental condition. However, the presence of free siRNA/peptide in the lipoplex mixtures used did not significantly affect the results since in silencing experiments non-targeted L2-engrafted lipoplexes failed to induce any significant silencing under the same conditions.

3.4.5 Assaying binding of siRNA-lipoplexes to cells

Cultured cells were pelleted and suspended in medium consisting of a mixture of 44% PBS, 45% RPMI-1640, 10% FCS plus 1% 1 M HEPES buffer (pH 7.4). To assay binding, 1 × 10<sup>3</sup> cells were added to wells in a V-bottom plate containing the
engrafted liposomes/lipoplexes (typical final volume 30 μL), and the plate incubated on ice for 30 min with occasional gentle vortexing. Unbound or loosely bound siRNA-lipoplexes were then removed by washing the cells three times with PBS; and the cells were then fixed with 2% paraformaldehyde and analysed for AF₄₈₈-fluorescence by flow cytometry.

3.4.6 Confocal microscopy

Cells were incubated with targeted AF-siRNA-lipoplexes for 30 min at 4 °C as described for flow cytometric analysis. These cells were subsequently fixed immediately in 2% paraformaldehyde, or incubated for 24 h at 37 °C before being fixed. All samples were mounted on glass slides and cell-associated fluorescence observed as described in Chapter 2, Section 2.11.

3.4.7 Nuclease protection assay

This assay was based on a method used for oligonucleotide–liposome complexes [142]. siRNA-lipoplexes prepared using Mix-5 with or without 40% ethanol as indicated were diluted, and ethanol added to some samples to equalise the ethanol content, before adding RNase A (1 μg) and incubating for 1 h at 37 °C. Triton X-100 (1%) also was added to some samples during RNase treatment. All samples were desiccated in a Savant SpeedVac (Thermo Fisher Scientific Inc, MA, USA) then reconstituted in 25 mM Tris–HCl (pH 8.0), 37.5 mM KCl, 1.5 mM MgCl₂, 5 mM DTT, 0.5 mM EDTA and 5 U/10 μL ribonuclease inhibitor and incubated for 30 min at 37 °C. siRNA-lipoplexes were treated in 1% Triton X-100 and loaded for analysis by agarose gel electrophoresis [using 1% agarose with trace ethidium bromide in Tris-Acetate EDTA (TAE) buffer]. Nucleic acid bands were visualised by UV illumination;
and densitometric analysis was performed with ImageJ 1.37 software (National Institutes of Health, MA, USA).

3.4.8 Particle size and charge measurements

Particle size and charge measurements were performed using a Zetasizer Nano ZS as described in Chapter 2, Section 2.1.3.

3.4.9 Induction of gene silencing

Gene silencing was assessed in DC-293 cells and DC2.4-EGFP cells targeted with NC-siRNA or anti-EGFP-siRNA. Lipoplexes were prepared using Mix-5 or Mix-6 and engrafted with either L2, p293 or p12-TNFR2, and incubated in the wells of a V-bottom plate with $1 \times 10^4$ cells in 150 µL RPMI 1640 with 10% FCS and 20 mM HEPES (pH 7.4). The cells were mixed by slow rotation for 2 h (37 °C), before pelleting, removing the medium, and transferring to a 96-well flat-bottom plate and incubating in fresh growth medium containing 0.2% PSN antibiotics. After 24 h, DC-293 cells were transfected with pEGFP-N1 using Lipofectamine™ 2000, cultured for a further 24 h and then analysed for EGFP fluorescence by flow cytometry. DC2.4-EGFP cells were cultured for 72 h before flow cytometric analysis. Relative EGFP expression was calculated by comparing the weighted mean fluorescence of EGFP-siRNA-treated cells with the geometric mean fluorescence of NC-siRNA-treated cells, after adjusting each for background; this assumes equal number of pEGFP-N1-transfected cells in all samples.
3.5 Results

3.5.1 Developing the use of NTA₃-DTDA-liposomes for targeting siRNA

In preliminary studies we used NTA₃-DTDA liposomes engrafted with murine CD4 to see if they could be used to encapsulate and deliver siRNA to A20 cells, through the well-established interaction of CD4 with cell surface MHC class II [273]. Initial experiments explored whether Alexa-Fluor₄₈₈-labelled siRNA (AF-siRNA) could be incorporated into neutral liposomes composed primarily of POPC or DOPC, each containing 1 mol% NTA₃-DTDA. However, an engraftment of these siRNA-lipoplexes with CD4 (to target A20 cells) followed by an incubation with A20 cells, indicated that only very small increases in cell fluorescence could be observed when the cells were analysed by flow cytometry (data not shown). This indicated that only relatively low levels of siRNA could be incorporated/encapsulated into NTA₃-DTDA-liposomes under these conditions. As expected, the inclusion of cationic lipids such as DOTAP (at molar ratios >20%) in the lipid mixtures was found to actively promote siRNA incorporation. However, previous observations in the Altin lab have indicated that the inclusion of >10 mol% DOTAP in NTA₃-DTDA-liposomes interferes strongly with the ability of His-tagged molecules to be engrafted onto the liposomes via the NTA₃-DTDA.

3.5.2 Incorporating siRNA into NTA₃-DTDA-liposomes - a novel method for targeting siRNAs

To further explore the potential of NTA₃-DTDA-liposomes for targeting siRNA, we devised lipid formulations either not containing or containing a low proportion of DOTAP (2%), but a relatively high proportion of DODAP, a titratable
lipid reported to promote liposome encapsulation of oligonucleotides [142]. DODAP carries a titratable hydrogen ion on its polar headgroup [141], and at pH <6.7 liposomes containing DODAP can be expected to bind siRNA, promoting siRNA incorporation/encapsulation. At physiological pH the DODAP is neutral, and should allow engraftment of targeting proteins, and exhibit low non-specific binding to cells. Based on these considerations and knowledge of the interaction between lipids with nucleic acids [116, 264, 274], three different lipid formulations containing DOTAP, DODAP, NTA3-DTDA and carrier lipids were developed for testing (see Table 3.1). siRNA-lipoplexes were prepared by acidifying aliquots of each lipid mixture before addition of AF-siRNA. Since it is reported that the presence of ethanol can increase lipid fluidity and promote lipoplex formation [142], the addition of siRNA to lipid was carried out in the presence of 40% ethanol. The lipoplexes formed as a result of this incubation were neutralised, incorporated with NTA3-DTDA and engrafted with CD4, before incubating with A20 cells to assess siRNA-incorporation and targeting (as above). Fluorescence profiles for A20 cells incubated (30 min, 4 °C) with siRNA-lipoplexes produced by incubating AF-siRNA with the different formulations are shown in Figure 3.1. It can be seen that the increase in A20 cell fluorescence (each relative to its respective non-targeted control) was ~10-fold for Mix-1 (Figure 3.1A), 30-fold for Mix-2 (Figure 3.1B), and ~100-fold for Mix-5 (Figure 3.1C). This identifies Mix-5, with composition: DODAP:DOTAP:DOPC:DOPC:Chol:Cer-PEG750 (45:2:13:5:30:5 mol %) with incorporated NTA3-DTDA (1 mol%), as the most effective for incorporation and delivery of siRNA.

Importantly, the delivery which Mix-5 demonstrates is a reflection of targeted or receptor-mediated binding of the engrafted siRNA-lipoplexes to these cells. This is
Figure 3.1 - DODAP promotes formation of siRNA-lipoplexes that can be incorporated with NTA₃-DTDA for targeting to cells. (A) An ethanolic solution of lipids with the formulation Mix-1 (DODAP:DOPE:DOPC:Cer-PEG₇₅₀; 30:30:30:10 molar ratio) was incubated with an acidic solution containing 10 pmol AF-siRNA. Alternatively, the lipid mixtures (B) Mix-2 (DODAP:DOTAP:DOPE:DOPC:cholesterol:Cer-PEG₇₅₀; 30:2:20:14:30:5 molar ratio), or (C) Mix-5 (DODAP:DOTAP:DOPE:DOPC:cholesterol:Cer-PEG₇₅₀; 45:2:13:5:30:5 molar ratio) were used to prepare siRNA-lipoplexes through an initial acidification step followed by incubation with 7.5 pmol AF-siRNA and 40% ethanol (v/v). siRNA-lipoplexes were then neutralised, incorporated with Ni-NTA₃-DTDA (added to 1 mol% of total lipid), and engrafted with either the targeting protein CD4 or with L2 as the non-targeted control (as indicated). The engrafted siRNA-lipoplexes were incubated with A20 cells (30 min, 4 °C), and cell-associated AF₄₈₈-fluorescence then assessed by flow cytometry. Similar experiments using Mix-5 siRNA-lipoplexes were carried out in (D) however in these experiments the lipoplexes were incubated with DC2.4 cells. In each instance, the background fluorescence (shaded histogram) is that of A20 or DC2.4 cells incubated without lipoplexes. Each fluorescence profile is a representative from two experiments performed in duplicate.
suggested by the fact that control A20 cells incubated with L2-engrafted (i.e. non-targeted) AF-siRNA-lipoplexes exhibit near-background fluorescence (Figure 3.1C); and a murine dendritic cell line DC2.4 (which expresses the CD4 ligand MHC class II, but to a much lower level than on A20 cells) exhibit correspondingly much less binding of CD4-engrafted siRNA-lipoplexes [the increase in fluorescence of the DC2.4 cells was 1-2 orders of magnitude less than for A20 cells (compare Figure 3.1C and D)]. The binding specificity of engrafted siRNA-lipoplexes to several different cell lines used in this study are also shown (Supplementary Figure 3.1). Lipid formulations can be devised, therefore, for efficient incorporation of siRNA into lipoplexes containing NTA3-DTDA, for receptor-mediated targeting of siRNA to cells.

3.5.3 Effect of initial pH on siRNA incorporation

Since DODAP is cationic at pH <6.7, the driving force for siRNA incorporation into DODAP-containing liposomes is likely to be pH-dependent. The incorporation of AF-siRNA into lipoplexes therefore was examined with acidification buffers of different pH. Flow cytometric analyses indicated that at pH 8, the fluorescence of A20 cells was ~30-fold above background, whereas at pH 6 and pH 4 the fluorescence increased to ~65-fold and 110-fold above background, respectively (Figure 3.2A). The results thus show that an approximately linear relationship was obtained between pH and cell fluorescence, with the highest increase in cell fluorescence (~120-fold) occurring at the lowest pH tested (pH 3) (see Figure 3.2B). Whilst a sigmoidal relationship between pH and cell fluorescence could have been expected, this response would have been more likely over a wider range of pH. The range of pH for the experiments was chosen so as to promote siRNA incorporation, yet minimise the likely hydrolysis of siRNA at low pH; thus pH < 3 and pH > 8 were
Figure 3.2 - Effect of initial pH on the incorporation of siRNA and targeting of siRNA-lipoplexes to cells. siRNA-lipoplexes were produced by the addition of 25 mM citrate of different pH (from pH 3.0 to pH 8.0, as indicated) to an aliquot of Mix-5, and then incubated with 7.5 pmol AF-siRNA and 40% ethanol (v/v). The lipoplex mixture was then neutralised, incorporated with NTA3-DTDA, engrafted with CD4, and incubated with A20 cells, to assess cell binding by flow cytometry. The profiles in (A) show binding of lipoplexes at the pH values: pH 4.0, pH 6.0 and pH 8.0 (as indicated). The results in (B) show the mean-fold increase in cell fluorescence for each condition relative to untreated cells with acidification buffer at pH ranging from 3.0 to 8.0. Each profile is a representative from three experiments; and each datum point is the mean ± SEM from three experiments.
not investigated. Also, it should be noted that Figure 3.2B represents data obtained using a complex system where the measured fluorescence is dependent both on the incorporation of siRNA into lipoplexes, and on the multimeric binding of these lipoplexes to cells. The relationship between pH and cell fluorescence under these conditions therefore need not necessarily follow a sigmoidal pattern.

3.5.4 Proportion of siRNA protected from RNase degradation

The efficiency of siRNA incorporation may depend on the siRNA:lipid ratio, and lipoplex-incorporated siRNA should be protected from RNase degradation. To better estimate siRNA incorporation efficiency, siRNA-lipoplexes were prepared, with some samples then being subjected to RNase A treatment before their analysis by agarose gel electrophoresis (see Figure 3.3A). Densitometric analysis of the siRNA bands showed that, compared to the control siRNA sample, the siRNA subjected to the encapsulation procedure resulted in at least 50% of the siRNA being protected from RNase degradation (Figure 3.3B). Interestingly, a similar protection was seen in the absence or presence of ethanol during siRNA-lipoplex formation, indicating that ethanol does not promote siRNA incorporation under these conditions (Figure 3.3A and B). Whilst these experiments were performed at a siRNA:lipid ratio of 1:10 (w/w), experiments with lipoplexes produced at 1:20 and 1:50 siRNA:lipid ratio (w/w) showed that only a slight increase in incorporation efficiency could be obtained (not shown); a 1:10 siRNA:lipid ratio was therefore used in all subsequent experiments. Additional experiments showed that a pre-treatment of CD4-engrafted AF-siRNA-lipoplexes with RNase A before an incubation with A20 cells does not affect the amount of AF-siRNA being targeted, since control A20 cells incubated with AF-siRNA-lipoplexes not treated with RNase give the same level of fluorescence (see
Figure 3.3 - Estimation of siRNA encapsulation by RNase protection assay. siRNA-lipoplexes were prepared using *Mix-5* and 30 pmol siRNA. Some samples (as indicated) were then incubated with RNase A and analysed for intact siRNA by agarose gel electrophoresis. Triton X-100 (1%) was added to release siRNA from lipoplexes after RNase treatment. An RNase control was included where the RNase A was added to siRNA-lipoplexes in the presence of Triton X-100 (indicated by *). A reverse contrast image of the resultant gel is shown in (A). The siRNA bands in each condition containing Triton X-100 were analysed to give a relative measure of the amount of protected siRNA with the results shown in (B). Each bar represents the results from three experiments ± SEM. The results in (C) show fluorescence profiles of A20 cells incubated with AF-siRNA-lipoplexes that had been either not treated or treated with RNase A (as indicated), then engrafted with CD4 and used in binding studies with A20 cells. siRNA-lipoplexes produced as above also were incubated with RNase in the presence of Triton X-100 before analysis of residual siRNA using agarose gel electrophoresis. An image of a representative gel is shown in the inset of panel C.
Figure 3.3C). These findings also are consistent with a substantial amount of the siRNA being incorporated within the lipoplexes.

3.5.5 Physical characteristics of NTA<sub>3</sub>-DTDA-liposomes incorporating siRNA

The physical characteristics of siRNA-lipoplexes containing NTA<sub>3</sub>-DTDA, namely particle size and surface charge, were determined by light scattering techniques employing a Zetasizer Nano ZS. siRNA-lipoplexes containing NTA<sub>3</sub>-DTDA were prepared at 1:10 siRNA:lipid ratio (w/w), and at different stages of preparation samples were diluted to permit size analysis. These measurements indicated that the particles formed were of uniform size distribution, with a mean diameter of 243 nm (see Table 3.2). Lipoplexes of the same size were produced regardless of the presence or absence of ethanol during addition of siRNA to lipids. Measurement of the zeta-potential indicated these liposomes exhibited a surface charge of −11.5 mV; this was further reduced to −1.5 mV after engraftment of a His-tagged protein (Table 3.2).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Diameter (nm)</th>
<th>Polydispersity</th>
<th>Zeta-potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mix-5</td>
<td>131.5 ± 4.5</td>
<td>0.20 ± 0.03</td>
<td>−7.53</td>
</tr>
<tr>
<td>+ Glycine + siRNA + EtOH</td>
<td>168.5 ± 13.5</td>
<td>0.29 ± 0.04</td>
<td>−3.38</td>
</tr>
<tr>
<td>+ Na&lt;sub&gt;2&lt;/sub&gt;HPO&lt;sub&gt;4&lt;/sub&gt; + NTA&lt;sub&gt;3&lt;/sub&gt;-DTDA</td>
<td>190.5 ± 19.2</td>
<td>0.19 ± 0.04</td>
<td>−5.44</td>
</tr>
<tr>
<td>Engrafted siRNA-lipoplex (+ CD4)</td>
<td>243 ± 6</td>
<td>0.29 ± 0.04</td>
<td>−1.30 ± 1.9</td>
</tr>
<tr>
<td>Targeted siRNA-lipoplex (−EtOH)</td>
<td>244 ± 9.8</td>
<td>0.26 ± 0.03</td>
<td>−1.48 ± 0.2</td>
</tr>
</tbody>
</table>

Targeted siRNA-lipoplexes were produced by incubating Mix-5 with (glycine pH 3.0 + siRNA + EtOH) for 20 min, then adding (Na<sub>2</sub>HPO<sub>4</sub> + Ni-NTA<sub>3</sub>-DTDA) and incubating for 30 min, before addition of targeting protein (CD4) and incubating for a further 30 min. Samples at these different stages of preparation were analysed for particle size, polydispersity and zeta-potential using a Zetasizer Nano ZS. All size measurements were carried out with PBS as the dispersant. The size of siRNA-lipoplexes prepared without the use of ethanol (−EtOH) also is shown for comparison. Zeta-potential measurements were carried out using PBS or water as the dispersant as indicated. Results represent the mean of readings from 3 separate preparations ± SEM.
No direct measurement of particle stability was carried out. However, studies in which AF-siRNA-lipoplexes were neutralised, engrafted with targeting molecule, and then pre-incubated for either 4 weeks in PBS (at 4 °C), or for 4 days in PBS containing 50% FCS (at 37 °C) indicated that there was no significant decrease in the ability of the AF-siRNA-lipoplexes to bind to target cells (see Supplementary Figure 3.5). This suggests that the lipoplexes produced are stable and that no significant siRNA leakage from the lipoplexes occurs under these conditions. Presumably, therefore, the small proportion (2%) of cationic DOTAP included in the lipid mixture is sufficient to prevent significant loss of lipoplex-incorporated siRNA.

3.5.6 Internalisation of targeted siRNA-lipoplexes by cells

The internalisation of targeted siRNA-lipoplexes by cells is important to elicit the functional effects of siRNA. To establish whether targeted siRNA-lipoplexes could be internalised, AF-siRNA-lipoplexes engrafted with CD4 were bound to A20 cells at 4 °C, and the cells then incubated in growth medium for either 0 h (control) or 24 h at 37 °C, before fixing and examining the cells for localisation of AF488-fluorescence by confocal microscopy. These experiments showed the majority of AF-siRNA fluorescence is distributed relatively uniformly at the A20 cell surface after targeting with the CD4-engrafted AF-siRNA lipoplexes at 4 °C (Figure 3.4A). Surprisingly, however, although some clustering of the fluorescence could be observed, A20 cells exhibited little if any uptake of the CD4-engrafted AF-siRNA-lipoplexes with the majority of the fluorescence being localised either at, or near the cell membrane, even after a 24 h incubation at 37 °C, (Figure 3.4B). This suggests that under these conditions A20 cells internalise poorly. Consistent with a low level of internalisation, the transfection of A20 cells with pEGFP-N1 using Lipofectamine™ 2000, also
Figure 3.4 - Analysis of internalisation of AF-siRNA-lipoplexes by A20 and DC2.4 cells.
CD4- and p12-TNFR2-engrafted M1x-5 lipoplexes containing 7.5 pmol AF-siRNA were incubated with A20 and DC2.4 cells, respectively, for 30 min at 4 °C. Aliquots of cells were then either fixed immediately (time 0), or fixed after incubating for 24 h at 37 °C. The fixed cells were then mounted on glass coverslips and analysed for AF_{488}-fluorescence by confocal microscopy.
resulted in a very poor level of transfection, with typically <1% of A20 cells expressing EGFP after 48 h (data not shown). By contrast, the targeting of AF-siRNA-lipoplexes to the TNFR2 on DC2.4 cells by engrafting the peptide p12-TNFR2, showed that whereas staining was exclusive to the cell surface at time 0 (Figure 3.4C), after 24 h culture a large proportion of the cells exhibited fluorescence that was clearly intracellular (Figure 3.4D).

Similarly, as shown in Supplementary Figure 3.2 (see also binding data Supplementary Figure 3.1), substantial levels of AF-siRNA was seen to be internalised by human DC-SIGN-expressing HEK-293 cells targeted with AF-siRNA-lipoplexes engrafted with p293 known to bind DC-SIGN; the human hepatocellular carcinoma cell line HepG2 targeted with AF-siRNA-lipoplexes engrafted with p25-Flt-1 reported to bind to the receptor Flt-1 [243]; and the murine monocytic-macrophage cell line RAW-264.7 targeted with AF-siRNA-lipoplexes engrafted with p28-TLR2 reported to bind TLR2 [244]. Notwithstanding differences between different cell types, the data show that targeted siRNA-lipoplexes can be actively internalised when targeted to cells.

3.5.7 Targeted siRNA-lipoplexes can induce gene silencing

The ability of targeted siRNA-lipoplexes to induce gene silencing was examined using lipoplexes containing siRNA for silencing the gene for enhanced green fluorescence protein (anti-EGFP siRNA). The use of co-transfection is a valid approach to establish silencing with specific siRNAs [275]. Initial silencing experiments were aimed at silencing EGFP in co-transfections with DC-SIGN-expressing HEK-293 cells (DC-293). We compared the EGFP fluorescence of DC-293
cells after incubation with different amounts of siRNA-lipoplexes (prepared using Mix-5) that had been engrafted with either L2 (non-targeted) or with targeting peptide p293, and that contained either negative control siRNA (NC-siRNA) or anti-EGFP siRNA. While the targeting of anti-EGFP siRNA via L2 (non-targeted control) or of targeted NC-siRNA each had either little or no effect on EGFP expression, anti-EGFP siRNA lipoplexes targeted to DC-293 cells with p293 induced marked silencing (~60-80% knockdown) of EGFP expression (Figure 3.5).

In other silencing experiments we compared the fluorescence of EGFP-expressing DC2.4 cells that had been incubated with either L2 or targeted p12-TNFR2-engrafted siRNA-lipoplexes, containing either NC-siRNA or anti-EGFP siRNA. Since EGFP remains in the cytoplasm for several days, an accurate analysis of gene-silencing due to siRNA can be made only after any existing EGFP present at transfection has turned over. In these experiments EGFP expression was therefore analysed at 72 h post-transfection. Interestingly, these latter results showed that when Mix-5 is used to prepare siRNA-lipoplexes only a relatively small reduction (~10%) in EGFP expression in the DC2.4 cells is observed under these conditions (Figure 3.6A).

In view of the only slight effect seen in silencing EGFP in DC2.4 cells, additional studies (see Chapter 4) were carried out to attempt to modify the Mix-5 formulation to promote better release of the siRNA from the endosomal pathway; this involved slight changes in the lipid composition, and the inclusion of fusogenic peptides such as diINF-7, a known promoter of cytoplasmic release [248]. The lipid mixture defined as Mix-6 was developed to contain (compared to Mix-5) an increased proportion of the helper lipid DOPE, a decreased proportion of Cer-PEG750, plus the
Figure 3.5 - Targeted siRNA-lipoplexes induce substantial knockdown of EGFP expression in DC-293 cells. Human DC-SIGN-expressing HEK-293 cells suspended in growth medium with 10% FCS were incubated for 2 h with different amounts of Mix-5 lipoplexes (1:10 siRNA:lipid ratio, w/w) to give 7.5–30 pmol of negative control siRNA (NC-siRNA) in lipoplexes engrafted with targeting peptide p293, or anti-EGFP siRNA in lipoplexes engrafted with either non-targeting peptide L2 or targeting peptide p293 (as indicated). The cells were then washed, cultured for 24 h (37 °C) in growth medium, before being transfected with pEGFP-N1 using Lipofectamine™ 2000. The cells were cultured for a further 24 h and then analysed for EGFP fluorescence by FACS. Each result represents the mean EGFP expression of the cells, relative to cells treated with p293 NC-siRNA-lipoplexes. Each bar represents the results from three separate experiments ± SEM. *p<0.05, ***p<0.001
Figure 3.6 - Targeted siRNA-lipoplexes induce substantial knockdown of EGFP expression in DC2.4 cells. In (A) EGFP-expressing DC2.4 cells suspended in growth medium with 10% FCS were incubated for 2 h with different amounts of Mix-5 lipoplexes (1:10 siRNA:lipid ratio, w/w) to give 10–30 pmol of negative control siRNA (NC-siRNA) in lipoplexes engrafted with targeting peptide p12-TNFR2, or anti-EGFP siRNA in lipoplexes engrafted with either non-targeting peptide L2 or targeting peptide p12-TNFR2 (as indicated) The cells were then transferred into fresh growth medium and incubated for a further 72 h at 37 °C before measuring the relative EGFP fluorescence by flow cytometry. (B) Shows results obtained using DC2.4 cells in experiments identical to those (A), but which used the lipid formulation Mix-6 instead of Mix-5. Each result represents the mean EGFP expression of the cells, relative to cells treated with p12-TNFR2 NC-siRNA-lipoplexes. Each bar represents the results from three separate experiments ± SEM. *p<0.05
inclusion of 1 mol% of the fusogenic peptide diINF-7 (Table 3.1) Zetasizer measurements indicated that the Mix-6 formulation resulted in the formation of siRNA-lipoplexes of size and surface charge similar to those observed for Mix-5. Importantly, experiments with siRNA-lipoplexes prepared using the Mix-6 formulation showed that whereas the targeting of anti-EGFP siRNA via L2 had either little or no effect on EGFP expression, the targeting of anti-EGFP siRNA-lipoplexes to TNFR2 on DC2.4 cells induced marked silencing (~40-50% knockdown) of EGFP expression when used at 30 pmol siRNA/well (Figure 3.6B). Interestingly, the use of Lipofectamine™ 2000 (following the manufacturer’s protocol) to introduce the anti-EGFP siRNA into the cells (non-targeted approach) could only achieve a similar level of silencing in this system (not shown). The results demonstrate, that the approach developed can target siRNA to cells in serum-containing media for the induction of effective gene silencing.
3.6 Discussion

The emergence of siRNAs as potential mediators of gene silencing has opened the door to providing novel therapeutics to combat a wide range of diseases, including genetic disorders and cancer [276-278]. A major obstacle in the use of siRNAs as therapeutics, however, is the requirement for functional delivery to specific target cells. The present work explored the potential of employing NTA$_3$-DTDA to enable siRNA-lipoplexes to be targeted to cells following engraftment of a targeting protein by metal-chelating linkage.

The results demonstrate that the method developed (Figure 3.7) results in a substantial proportion of the added siRNA (typically ~50% at 1:10 siRNA: lipid w/w ratio), becoming resistant to RNase degradation, and thus presumably entrapped within the liposomes/lipoplexes (Figure 3.3). Also, the relatively small size and low surface charge of these lipoplexes (Table 3.2) are characteristics compatible with low non-specific binding and good stability in the blood circulation [116, 268]. Experiments in which the siRNA-lipoplexes incorporated with Ni-NTA$_3$-DTDA and engrafted targeting molecule, were incubated with cells in vitro, demonstrate excellent targeting of CD4-engrafted siRNA-lipoplexes to A20 cells, and p12-TNFR2-engrafted siRNA-lipoplexes to DC2.4 cells (Figure 3.1 and 3.4).

An important consideration is whether the targeted siRNA-lipoplexes are internalised, and whether the siRNA payload is subsequently released into the cytoplasm [146]. Different cell types differ widely in their ability to internalise targeted siRNA-lipoplexes, with the present work showing that A20 cells exhibit a
Figure 3.7 – Preparation of targeted siRNA-lipoplexes. The simplified diagram shows how siRNA can be incorporated with stealth lipoplexes for targeting to cells with proteins engrafted via NTA$_3$-DTDA. The siRNA in aqueous media is mixed with an acidified suspension of a DODAP-containing lipid mixture (e.g. Mix-5 or Mix-6, Table 3.1) and then incubated to allow formation of siRNA-lipid complexes. Ethanol (40%) can be added to promote siRNA incorporation, and entrapped siRNA then stabilised by dilution or removal of the ethanol. The siRNA-lipoplexes are then neutralised, (where it is expected that any exteriorly bound siRNA would be dissociated from the complex) and incubated with Ni-NTA$_3$-DTDA to allow its incorporation. The siRNA-lipoplexes are then engrafted with a His-tagged targeting protein (i.e. specific for a receptor on the target cell), to enable targeted delivery of the siRNA cargo specifically to target cells.
poor uptake of siRNA-lipoplexes compared to DC2.4 cells, even after 24 h incubation (Figure 3.4). The degree of silencing that can be achieved through targeting siRNA-lipoplexes will clearly depend on the rate and/or extent of uptake of the lipoplexes by the cells. A study of the reasons for the poor internalisation by A20 cells was beyond the scope of this work, but it is likely to depend on the nature of both the cells and the cell surface receptor being targeted [279].

Of especial interest was our observation that despite being able to induce intracellular uptake of siRNA, p12-TNFR2-lipoplexes prepared using Mix-5 were unable to induce significant knockdown of EGFP expression in DC2.4-EGFP cells. It has been reported that cytoplasmic release is particularly difficult to achieve in DC2.4 cells, most likely due to their specialised antigen-processing ability, a feature shared by other dendritic cells [280]. We did not directly examine the release of the siRNA into the cytoplasm, nor the mechanism or cellular location at which the siRNA becomes dissociated from lipoplexes after their internalisation by cells. However, kinetic studies of the AF488-fluorescence of DC2.4 cells containing bound AF-siRNA-lipoplexes indicated that AF488-fluorescence quenching was substantially reduced with the cells already exhibiting a marked increase in fluorescence after just 1 h incubation at 37 °C to normalise their metabolic activity, indicating that the siRNA was being dissociated from the lipoplexes (see Supplementary Figure 3.4). The release process presumably involves charge neutralisation of protonated DODAP by anionic lipids in acidic endosomes, as has been proposed for oligonucleotides [281]. Importantly, consistent with this we found that a lipid mixture designed to enhance cytoplasmic release substantially increased the RNAi effect in these cells, such that a ~50% silencing of EGFP could be induced after a single 2 h exposure of the cells to the
lipoplexes (Figure 3.6B). Since the siRNA-lipoplexes produced using this method exhibit good stability in serum (Figure 3.3), it is likely that after administering these lipoplexes \textit{in vivo} target cells will be exposed to the lipoplexes for a much longer period of time - potentially increasing silencing efficiency. Also, we envisage that a more potent silencing effect could be achieved by multiple administrations of the siRNA-lipoplexes, and when targeting more easily transfectable cells, such as liver, nerve tissue and certain tumours.

While the increased DOPE and decreased Cer-PEG\textsubscript{750} in the Mix-6 formulation may have contributed to the increased knockdown observed (compare Figure 3.6A and B), additional experiments (data not shown) indicated that the major contributor was the inclusion of the fusogenic peptide diINF-7 (known to induce endosomolysis), but which was otherwise lacking in the other formulations tested. This dimeric peptide, resembling the N-terminal domain of the hemagglutinin subunit HA-2 on influenza virus, adopts an altered conformation in the acidic pH of the late endosome, promoting fusion of diINF-7-containing liposomes with the endosomal membrane and resulting in cytoplasmic release of liposomal contents [248, 282]. The diINF-7 was incorporated into the liposomes by mixing with the dried lipids before addition of PBS and sonication to produce the liposomes. No detailed characterisation of diINF-7 containing liposomes/lipoplexes was carried out. However, the inclusion of diINF-7 up to 1 mol\% appeared not to affect the integrity/stability of the siRNA-lipoplexes, as judged by the fact that its inclusion affected neither the non-specific binding of L2-engrafted AF-siRNA-lipoplexes to cells, nor the ability of targeting peptide-engrafted AF-siRNA-lipoplexes to bind to target cells (not shown). The ability of peptide-engrafted-siRNA-lipoplexes containing diINF-7 lipoplexes to target cells also was
unaffected by a 24 h pre-incubation of the lipoplexes in RPMI with 50% FCS at 37 °C (Supplementary Figure 3.5), suggesting these lipoplexes exhibit good stability.

The gene silencing effect of siRNA\textsubscript{s} introduced into the cytoplasm is usually transient; with the greatest knock-down in target protein levels usually occurring 72-96 h after its introduction in rapidly dividing cells, and the effect often lasting several weeks in cells that are not dividing or have a low rate of proliferation [283]. Plasmid vectors (pDNA) that allow a continuous production of siRNA\textsubscript{s} within cells, and potentially a long-lasting effect on gene silencing, also have been developed. However, the generation of siRNA\textsubscript{s} within cells, is complicated by the fact that pDNA first has to be delivered to and then efficiently expressed by the cells. The use of synthetic siRNA\textsubscript{s} requires only their introduction into the cytoplasm, but pDNA additionally requires transport to the nucleus to allow transcription of short hairpin-RNA\textsubscript{s} for production of siRNA\textsubscript{s} [283]. Utilising the method developed in Chapter 4, there is a possibility of using the NTA-technology for delivering pDNA that encodes for shRNA\textsubscript{s}.

Many human diseases including cancer as well as metabolic and neurodegenerative diseases have an underlying genetic basis or can be remedied by silencing specific genes. The systemic administration of siRNA is costly and may result in unwanted off-target effects, highlighting the importance of being able to target siRNA\textsubscript{s} to specific cells. The present work shows that the incorporation of NTA\textsubscript{3}-DTDA and engraftment of a suitable His-tagged targeting protein onto lipoplexes provides a convenient approach for targeting functional siRNA\textsubscript{s}. Since our studies were carried out in serum-containing media, we envisage that the same
approach can be applied for receptor-ligand mediated targeting to specific cells for therapeutic gene silencing \textit{in vivo}. Potential applications of this technology, therefore, include the development of gene therapy to correct or modify cell function in genetic and other disorders such as cancer. Also, an ability to target siRNAs to immune cells such as dendritic cells \textit{in vivo}, could have enormous potential for manipulating immune function and the development of more effective vaccines and cancer immunotherapies. For example, the targeting of siRNAs to DCs could be useful in strategies to overcome tumour-induced immunosuppression [284], or for altering immunity as a treatment for autoimmune diseases [285, 286]. The approach for targeting siRNA delivery presented herein could thus potentially be developed for use in many therapeutic applications.

In addition to demonstrating the potential of the chelator lipid NTA$_3$-DTDA for targeting siRNA-lipoplexes to cells, to our knowledge this is the first report on the use of the pH-sensitivity of DODAP for encapsulation and delivery of siRNA. The approach is convenient, as different His-tagged targeting molecules or peptides can readily be produced and grafted onto the siRNA-lipoplexes for targeting to different cell types, thus providing a platform technology for targeting delivery of siRNAs for therapeutic applications. The present work demonstrates that NTA$_3$-DTDA can be used to target siRNA-lipoplexes \textit{in vitro}; the next step is to show that the approach also is effective for targeting siRNA delivery and gene silencing \textit{in vivo}.
3.7 *In vivo* application for the method of siRNA/nucleic acid delivery

The method for producing siRNA-lipoplexes described in the present chapter has not yet been tested *in vivo*. However, development of this method has led to a collaboration with colleagues at the Western Australian Institute for Medical Research resulted in the following publication:


In this paper, liposomes of similar composition to that developed for siRNA encapsulation were employed to incorporate CpG-ODN into lipoplexes following the same procedure as for siRNA. Thus, liposomes with a lipid formulation consisting of DODAP:DOTAP:DOPE:cholesterol:PE-PEG_{2000} (45:2:13:30:10 molar ratio) were mixed with CpG-ODN to produce CpG-ODN lipoplexes, which were then incorporated with Ni-NTA_{3}-DTDA, enabling the engraftment of a histidine-tagged peptide (RGR) with known affinity for PDGFRβ expressed on pericytes in the tumour vasculature. Targeted CpG-ODN lipoplexes prepared in the Altin lab via this approach were injected (i.v.) into Rip1-Tag5 mice and were shown to home to the vasculature of the pancreatic tumours that spontaneously form in these mice. Furthermore, due to the immunostimulatory effects of CpG-ODN, Rip1-Tag5 mice treated with RGR-engrafted lipoplexes were shown to have increased survival probability compared to
mice treated with control lipoplexes. These results demonstrate that with minor modification (10 mol% PE-PEG\textsubscript{2000} in the liposomes used rather than the 5 mol% Cer-PEG\textsubscript{750} in \textit{Mix-5}), the method for preparing targeted siRNA-lipoplexes in the present chapter can be used to deliver CpG-ODN to the tumour vasculature \textit{in vivo}. Consequently, there is potential for utilising targeted nucleic acid lipoplexes \textit{in vivo} for therapeutic effect. More details on this approach are presented in Chapters 4 and 7.
3.8 Supplementary Figures

Supplementary Figure 3.1 - Specific targeting of siRNA-lipoplexes to cell lines *in vitro*. AF-siRNA-lipoplexes prepared using *Mix*-5 were engrafted with control peptide L2, p293, p25-Flt-1 or p28-TLR2 (as indicated). The lipoplexes were subsequently incubated (4 °C, 30 min) in RPMI medium containing 10% FCS with (A) DC-293 cells, (B) HepG2 cells or (C) RAW264.7 cells. The cells were then washed and their AF<sub>488</sub>-fluorescence (a measure of AF-siRNA-lipoplex binding) was assessed by flow cytometry; each profile shown is a representative from two experiments. For each cell line only one His-tagged peptide (when engrafted onto siRNA-lipoplexes) showed substantial binding above that observed for L2; and each peptide only bound to one cell line, indicating binding specificity.
Supplementary Figure 3.2 - Targeted AF-siRNA-lipoplexes are internalised by DC-293, HepG2 and RAW264.7 cells. Mix-5 siRNA-lipoplexes containing 7.5 pmol AF-siRNA were incubated with the different cell lines for 30 min at 4 °C. After washing, the cells were cultured for 24 h, before then fixing, mounting on glass coverslips and analysing for AF_{488}-fluorescence by confocal microscopy. The cell images shown were captured under excitation at 488 nm and are: (A) DC-293 (C), HepG2 and (E) RAW264.7; the corresponding image overlaid with the bright field image of the same cells is also shown in (B), (D) and (F). In (A) and (B), DC-293 cells were incubated with p293-engrafted lipoplexes. In (C) and (D), HepG2 cells were incubated with p25-Flt-1-engrafted lipoplexes. In (E) and (F) RAW264.7 cells were incubated with p28-TLR2-engrafted lipoplexes. For each cell type only cell surface-associated AF_{488}-fluorescence could be observed after the incubation at 4 °C (time 0, not shown); but after 24 h culture at 37 °C substantial intracellular localisation and hence uptake of targeted AF-siRNA can be seen for each cell type.
Supplementary Figure 3.3 – Effect of incubation with siRNA-lipoplexes on cell viability: a comparison with Lipofectamine™ 2000. siRNA-lipoplexes containing NC-siRNA were prepared using Mix-5, Mix-6 or Lipofectamine™ 2000 (following the manufacturer’s instructions). Lipoplexes containing NTA$_3$-DTDA were subsequently engrafted with either control peptide L2 or p12-TNFR2 and incubated with EGFP-DC2.4 cells as described for knockdown experiments (see Section 3.4.9). The cells were then washed to remove unbound complexes and cultured for 72 h at 37 °C, before measuring the relative number of viable cells in each condition by using the Cell Titer 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega). Microscopic examination of the cells revealed no significant difference between the numbers of dead cells in each condition (each <5% dead cells, not shown). The percentage of viable cells in each condition shown is relative to that for untreated cells. Each datum point is the mean ± SEM from three experiments.
Supplementary Figure 3.4 – Fluorescence quenching of lipoplex-associated AF-siRNA, and monitoring AF-siRNA release. (A) Solutions of free AF-siRNA and suspensions of AF-siRNA-lipoplexes (prepared using Mix-6) were analysed in a FLUOstar Optima Microplate Reader (BMG Labtech) at 490ex, 520em. All wells contained 37.5 pmol of lipoplexed AF-siRNA. In the condition indicated 10% Triton X-100 was added to solubilise the lipids and release the siRNA; and this resulted in an increase in AF_{488}-fluorescence due to a relief in AF_{488}-fluorescence quenching. The mean Relative Fluorescence Unit (RFU) measured for each condition (± SEM) from three experiments is shown. (B) AF-siRNA-lipoplexes prepared using Mix-6 were engrafted with p12-TNFR2 and incubated with DC2.4 cells (4 °C, 30 min) in RPMI containing 10% FCS. The cells were washed, suspended in growth medium and incubated at 37 °C for time periods ranging from 0–24 h. After each time period cells were fixed and analysed for AF_{488}-fluorescence by flow cytometry. The mean-fold increase in cell AF_{488}-fluorescence (a measure of the relief in AF_{488}-fluorescence quenching and hence AF-siRNA-release from the lipoplexes), for each time period relative to untreated cells is shown. Each bar is the mean ± SEM from three experiments.
Supplementary Figure 3.5 - Stability of p25-Flt-1-engrafted siRNA-lipoplexes. AF-siRNA-lipoplexes prepared using either Mix-5 or Mix-6 were engrafted with either control peptide L2 or p25-Flt-1. Lipoplexes were incubated in RPMI containing 50% FCS (v/v) for different times between 0–96 h at 37 °C before incubation with HepG2 cells (37 °C, 1 h). The AF_{488} fluorescence of the cells (a measure of AF-siRNA-lipoplex binding) was assessed by flow cytometry. The geometric mean AF_{488}-fluorescence of cells for each condition relative to untreated cells was determined. The result represented by each bar is the mean ± SEM from three experiments. Similar experiments in which AF-siRNA-lipoplexes engrafted with p25-Flt-1 were pre-incubated for 4 weeks in PBS at (4 °C), before the incubation with HepG2 cells, indicated no significant decrease in the ability of the lipoplexes to bind these cells (not shown).
CHAPTER FOUR

TARGETING OF PLASMID DNA-LIPOPLEXES TO CELLS WITH MOLECULES ANCHORED VIA A METAL CHELATOR LIPID
4.1 Introductory Comments

The results in Chapter 3 described the use of lipid formulations (*Mix*-5 and *Mix*-6) to incorporate siRNA together with the chelator lipid NTA₃-DTDA into lipoplexes in order to target siRNA to specific cells *in vitro*. However, the ability to induce gene expression using a targeted approach also has many potential therapeutic applications. The experiments in the present chapter therefore examine the use of *Mix*-5 liposomes for their ability to incorporate pDNA into lipoplexes. In order to simplify the method of lipoplex preparation, in this chapter, Ni-NTA₃-DTDA is introduced into *Mix*-5 through sonication prior to addition of pDNA encoding for EGFP. Initial experiments involve the transfection of HEK-293 cells with lipoplexes engrafted with the TAT-related peptide T2. Subsequently, this chapter undertakes an analysis of the impact of liposome formulation on the transfection efficiency of lipoplexes, and sets out to characterise the effect of an endosomolytic peptide and a DNase II inhibitor on the efficiency of transfection. In addition, the ability of a VEGFR2-binding peptide (p25-Flt-1) to target pDNA to HepG2 cells *in vitro* is determined, hence establishing the basis for *in vivo* studies to target engrafted liposomes and lipoplexes to VEGFR2 on the vasculature of tumours.

4.2 Abstract

The ability to deliver pDNA to specific cells in vivo is crucial for achieving efficient targeted transfection with nonviral vectors. We previously used stealth liposomes containing the chelator lipid NTA<sub>3</sub>-DTDA to target delivery of antigen and cytokines to immune cells in vivo. In the present study, we utilised liposomes containing NTA<sub>3</sub>-DTDA and the ionisable aminolipid DODAP to incorporate pDNA into complexes for targeting to cells. Liposomes containing DODAP, NTA<sub>3</sub>-DTDA and helper lipids were acidified (pH 5.5) and mixed with pDNA to form complexes. These lipoplexes were neutralised and engrafted with His-tagged molecules for targeting to extracellular receptors. Targeted transfection efficiency was assessed using the EGFP reporter gene. Initial transfections of HEK-293 cells using a His-tagged peptide (T2) related to the Arg-rich motif of HIV-1 TAT protein resulted in a low transfection efficiency (<2.5%). Optimisation of the lipid formulation and use of an endosome-destabilising peptide and inhibitor of DNase II, increased transfection approximately 20-fold. These lipoplexes are approximately 250 nm in diameter, and transfection efficiencies were approximately 50% for HEK-293 cells targeted with lipoplexes containing pEGFP-N1 and engrafted with T2, and 30–40% for HepG2 cells targeted with lipoplexes engrafted with a peptide specific for the VEGF receptor Flt-1. The results show that DODAP-containing lipoplexes incorporating NTA<sub>3</sub>-DTDA enable the engraftment of targeting molecules and the effective targeting of pDNA to cells in serum-containing media, resulting in efficient transgene expression. The strategy may provide a convenient approach for targeting pDNA to cells in vivo in therapeutic applications.
4.3 Introduction

The ability to deliver genes to specific cell types \textit{in vivo} has enormous potential for the development of novel treatments for many diseases, including genetic/metabolic diseases and cancer. The use of retroviral, lentiviral and adenoviral vectors can be highly efficient at introducing genes into target cells \textit{in vivo}, although these approaches can be associated with complications as a result of insertional mutagenesis by the viral DNA and the generation of unwanted immune responses to the viral vectors themselves [287]. An attractive alternative to viral vectors is the use of lipid vehicles such as targeted liposomes or lipid-DNA-complexes to target the delivery of DNA to cells because these are considered safe and can be produced from synthetic components [138, 288]. The use of lipid vehicles for DNA delivery, however, can be associated with low transfection efficiencies and difficulty in anchoring suitable targeting molecules onto the lipid–DNA complexes [289].

Knowledge of the use of lipids for gene delivery has emanated largely from studies that employ cationic lipids to transfer pDNA into cells \textit{in vitro}. Commonly used transfection reagents generally contain cationic lipids [e.g. DOTAP and 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate] (DOSPA) and/or mixtures of cationic lipids and helper lipids such as DOPE [290, 291]. Transfection reagents can also contain cationic DNA binding proteins like protamine [292, 293], and branched cationic polymers such as polyethylenimine [288, 294]. There is evidence indicating that cationic lipids and other cationic agents promote DNA transfection into cells by at least two mechanisms. First, they neutralise the negative charge on the DNA backbone, promoting condensation of the relatively large pDNA to form pDNA-complexes (hereafter referred to as
lipoplexes). Such lipoplexes can protect the DNA from degradation by serum and intracellular nucleases, with the smaller size reducing nonspecific uptake by the reticuloendothelial system for *in vivo* delivery [295]. Second, depending on the charge ratio (i.e. cationic agent:DNA), cationic lipid-containing complexes can bind negative charges on the surface of cells, hence facilitating transfection by promoting lipoplex binding and uptake by the cells [296]. Such interactions with cells, however, are generally nonspecific. Moreover, cationic complexes have a propensity to aggregate in serum, often making them unsuitable for targeted DNA delivery *in vivo* [297].

The available evidence suggests that targeted delivery *in vivo* is facilitated by structures that bear either an anionic or a near neutral charge [298]. In particular, lipoplexes containing helper lipids such as DOPE (or other pH-sensitive agents) can destabilise upon endocytosis and acidification in endosomes, promoting the intracellular release of the complexed DNA before degradation in lysosomes [299]. Strategies to enhance transfection by promoting nuclear uptake of the DNA released into the cytoplasm have been reported [300, 301]. With this knowledge, approaches using functionalised lipids to anchor targeting moieties for the delivery of DNA-containing lipoplexes to tumour cells that over-express receptors for transferrin and/or folic acid have been developed [302, 303]. Despite such progress, a straightforward method of packaging pDNA into non-cationic lipoplexes for convenient targeting to cells is lacking. In the present study, we explored the potential of using the chelator lipid NTA₃-DTDA, previously used to anchor His-tagged proteins for targeting the delivery of antigen-containing liposomes and plasma membrane vesicles to cells *in vivo* [22], to target the delivery of lipoplexes to cells. The effect of different lipid combinations and transfection-promoting peptides on the efficiency of targeted
transfection in this system was examined using pDNA encoding for the reporter gene EGFP. The results obtained show that a formulation composed of mainly neutral and/or ionisable lipids can be designed to achieve excellent targeting and receptor-mediated transfection of cells.
4.4 Materials and Methods

4.4.1 Reagents

All reagents, lipids, cell lines, proteins, peptides and plasmids used in the experiments described in this chapter are listed in Chapter 2, Sections 2.1, 2.2, 2.3, 2.5, 2.6, and 2.7.

4.4.2 Preparation of liposomes

Stock solutions of all lipids were prepared in ethanol, and stored at −80 °C. For the production of liposomes, lipids were mixed, dried under a stream of nitrogen gas, and liposomes produced by suspending the lipids in PBS or ddH2O by sonicating for 45 s (two bursts) at maximum amplitude using a TOSCO 100W ultrasonic disintegrator (Measuring and Scientific Ltd, London, UK). Under the conditions tested, no significant difference to the transfection results could be observed when the lipids were additionally subjected to extensive drying under vacuum before use in experiments. Liposomes containing diINF-7 or 6His-ID2 were prepared by addition of the peptides (typically at 1 mol% of the total lipid, or as indicated) to the dried lipids before sonication. All stock suspensions of liposomes were stored for up to 1 week at 4 °C, but storage for a longer time was at −20 °C; liposomes were briefly re-sonicated prior to use in experiments. There was no detectable difference in the transfection achieved when using liposomes stored and processed in this way compared to using freshly prepared liposomes.

4.4.3 Preparation of lipoplexes and transfection

Lipoplexes were prepared by adding 1 μL of citrate buffer (50 mM, pH 4.5) to 5 μL of liposomes (4 mM total lipid); where indicated, 30 μL of ddH2O was added.
This mixture was incubated for 5 min before the addition of 625 ng of pEGFP-N1 (20:1 lipid:pDNA, w/w). Once mixed by pipetting, lipoplexes were allowed to form over 30 min at room temperature. The pH of these incubations was neutralised by the addition of 4 µL of 50 mM Na₂HPO₄ and 5 µL of 10× PBS. The appropriate His-tagged peptides were added and incubated for 30 min at room temperature. The amount of peptide used for the engraftment of targeted lipoplexes was based on a theoretical calculation of the amount of peptide required to be equimolar with anchoring moiety NTA₃-DTDA, followed by an empirical titration of the concentration to give the greatest binding of the engrafted lipoplexes to cells that are known to express the receptor being targeted.

Cells (1 × 10⁵) were incubated with lipoplexes in sterile 500-µl polymerase chain reaction tubes in volumes of 500 µl of RPMI supplemented with 10% FCS and 20 mM HEPES (pH 7.5). The tubes were placed on a rotator and rotated slowly (six revolutions per min) for 2 h in a incubator at 37 °C. The cells were then pelleted by centrifugation, washed in sterile PBS, and then suspended in 500 µL of complete RPMI medium for seeding in a 24-well plate. Cells were incubated for 48 h at 37 °C before the analysis of EGFP-fluorescence by flow cytometry. Cells were deemed to express EGFP if their EGFP-fluorescence intensity was above a threshold set to exclude > 98% of cells in a condition where no pEGFP-N1 was added.

In experiments when 0.5 mol% Texas Red-DHPE was included in the liposome formulation, binding of lipoplexes to cells was measured after the initial 2-h incubation with cells. In these experiments, after washing, approximately half of the cells were removed and fixed with 2% paraformaldehyde for analysis of Texas Red-fluorescence
by flow cytometry. The remaining cells were seeded in a 24-well plate and cultured for 48 h, before the analysis of EGFP expression.

4.4.4 Assaying binding of peptide-engrafted liposomes and anti-Flt-1 antibody to cells

p25-Flt-1- and 12His-engrafted liposomes were prepared by incubating (30 min) equal volumes of a suspension of liposomes composed of POPC:Cer-PEG<sub>750</sub>:AF-DOPE:NTA<sub>3</sub>-DTDA (molar ratio 93:5:5:1:1, total lipid concentration 0.33 mM) and peptide (10 μM). HepG2 cells were suspended in PBS medium supplemented with 50% FCS. To assay binding, approximately 1 × 10<sup>5</sup> cells were added to wells in a polypropylene 96-well V-bottom plate (Corning Inc., New York, NY, USA) containing the engrafted liposomes, and the plate incubated on ice for 30 min with occasional gentle vortexing. Unbound or loosely bound liposomes were then removed by washing the cells twice with PBS, and the cells were then pre-incubated for 10 min with goat IgG, before incubating (40 min at 4 °C) with FITC-conjugated goat anti-Flt-1 antibody. Cells were washed a further three times in PBS before being fixed with 2% paraformaldehyde and analysed for AF<sub>647</sub>- and FITC-fluorescence by flow cytometry.

4.4.5 Confocal microscopy

Lipoplexes were prepared using *Mix-13* and pEGFP-N1 (20:1 lipid:pDNA, w/w) and engrafted with T2 before incubating with 1 × 10<sup>5</sup> HEK-293 cells (2 h at 37 °C). The cells were subsequently washed and incubated for a further 72 h at 37 °C before being fixed in 2% paraformaldehyde. Cells were then treated with 1 μM Hoechst 33342 in PBS for 15 min before mounting on glass slides observing cell-associated fluorescence as described in Chapter 2, Section 2.11.
4.4.6 Particle size and charge measurements

Particle size and charge measurements were performed using a Zetasizer Nano ZS as described in Chapter 2, Section 2.1.3.

4.4.7 Ethidium bromide (EtBr) intercalation assay

The proportion of lipoplex-associated pDNA accessible to EtBr was evaluated based on a previously described method [304]. Lipoplexes prepared using pEGFP-N1 and different liposome formulations (20:1 lipid:pDNA, w/w) were treated with EtBr for 5 min at 37 °C before the fluorescence was measured using a FLUOstar Optima Microplate Reader (BMG Labtech, Offenburg, Germany). EtBr-fluorescence was read at excitation and emission wavelengths of 544 and 612 nm, respectively. The initial fluorescence of EtBr (2 μL of a 2 mM solution was added to each well of a 96-well plate containing 200 μL of PBS) was recorded as well as the fluorescence obtained upon addition of 625 ng pEGFP-N1 (this was used as the complete EtBr access control). The proportion of pDNA accessible to EtBr intercalation was calculated by subtracting the fluorescence value of EtBr from each sample and is expressed as the percentage of the control.
4.5 Results

4.5.1 Targeting of lipoplexes with peptides anchored via NTA$_3$-DTDA

The use of liposomes to deliver pDNA has seen the development of lipid formulations that promote DNA condensation, as well as DNA uptake and expression by cells. Initial attempts at utilising a number of these, however, indicated that a high content of cationic lipids, such as DOTAP and cholesteryl 3β-N-(dimethylaminoethyl)carbamate hydrochloride (DC-Chol), strongly interfere with the ability of NTA$_3$-DTDA-containing lipoplexes to be targeted because engraftment of a targeting moiety did not promote their binding to cells expressing the target receptor (not shown). Therefore, the use of alternative formulations was explored. Several lipid mixtures containing predominantly neutral lipids were tested for targeting with NTA$_3$-DTDA, but the resulting lipoplexes gave poor transfection efficiencies (cells transfected <1%), presumably as a result of poor condensation of the pDNA. One innovative approach reported recently comprises the use of the ionisable amino-lipid DODAP to promote condensation of pDNA and its incorporation into liposomes [141]. We reasoned therefore that lipid formulations containing DODAP, which is cationic at low pH but charge-neutral at neutral pH, might provide a compatible system for targeting of pDNA with NTA$_3$-DTDA. This led us to develop Mix-5 (see formulation Mix-5; Table 4.1) which was designed to contain a high proportion of DODAP plus two neutral helper lipids often used as components of cationic transfection reagents, namely DOPE and cholesterol. In addition to these major components, some DOTAP (to promote lipoplex stability at neutral pH), DOPC (a lipid that promotes the formation of lamellar structures), sterically stabilising lipid Cer-PEG$_{750}$ (to promote lipoplex stability) and chelator lipid NTA$_3$-DTDA (used to anchor His-tagged targeting
Table 4.1 - Composition of the different liposome formulations tested for lipoplex formation and targeting of pDNA to cells.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Lipid/peptide composition</th>
<th>Molar ratio</th>
<th>Charge ratio (+ve : −ve)</th>
<th>EtBr access (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mix-7</td>
<td>DODAP : DOTAP : DOPE : Chol : Cer-PEG&lt;sub&gt;750&lt;/sub&gt; : Ni-NTA&lt;sub&gt;3&lt;/sub&gt;-DTDA</td>
<td>56 : 2 : 5 : 30 : 5 : 2</td>
<td>5.8 : 1</td>
<td>74.5 ± 2.0</td>
</tr>
<tr>
<td>Mix-8</td>
<td>DOTAP : DOPE : DOPC : Chol : Cer-PEG&lt;sub&gt;750&lt;/sub&gt; : Ni-NTA&lt;sub&gt;3&lt;/sub&gt;-DTDA</td>
<td>2 : 56 : 5 : 30 : 5 : 2</td>
<td>1 : 5</td>
<td>98.0 ± 1.7</td>
</tr>
<tr>
<td>Mix-12</td>
<td>DODAP : DOTAP : DOPE : CHEMS : Cer-PEG&lt;sub&gt;750&lt;/sub&gt; : Ni-NTA&lt;sub&gt;3&lt;/sub&gt;-DTDA</td>
<td>44 : 2 : 12 : 5 : 30 : 5 : 2</td>
<td>4.6 : 1</td>
<td>37.6 ± 5.4</td>
</tr>
</tbody>
</table>

Each lipid mixture was suspended in aqueous media and incubated with pEGFP-N1 as described in the text. The charge ratio (+ve amine on cationic lipids : −ve PO₄ on pDNA) for the resulting lipoplexes are shown at a pH well below 6.6 (pKₐ of DODAP) at which all the amines on DODAP can be assumed to be protonated. Each lipoplex preparation was incubated with EtBr and the proportion of pDNA accessible to EtBr intercalation determined by comparing the fluorescence of each sample with that of free pDNA.
moieties) were included as components of *Mix-5*, for testing in transfections.

To determine whether pDNA could be incorporated into NTA₃-DTDA containing liposomes, the lipids of formulation *Mix-5* were suspended in PBS to produce liposomes, and lipoplexes were produced and assessed for their ability to mediate transfections. Briefly, the vector pEGFP-N1 was incubated with a suspension of formulation *Mix-5* liposomes (20:1 lipid:pDNA, w/w) under acidic conditions for 30 min to promote formation of DNA complexes. The lipoplexes were then neutralised, and engrafted with a histidine-tagged targeting molecule before incubating with cells to assay targeted transfection.

Preliminary experiments, utilised the peptide T2 as a convenient targeting molecule to study the delivery of lipoplexes containing NTA₃-DTDA to HEK-293 cells. T2 is a His-tagged form of a basic peptide derived from the arginine-rich motif of the HIV-1 TAT protein, which we found to promote strong heparin-inhibitable binding of NTA₃-DTDA-liposomes to several cell lines including HEK-293 (Supplementary Figure 4.1). The incubation of HEK-293 cells (2 h at 37 °C) with lipoplexes containing NTA₃-DTDA (plus 0.5 mol% Texas Red-DHPE as tracer) increased the Texas Red-fluorescence of the cells by 30-fold (Figure 4.1A). Importantly, lipoplexes engrafted with control peptide L2 showed markedly less binding to HEK-293 cells. When the HEK-293 cells were further incubated for 48 h at 37 °C, a significant proportion of the cells exhibited strong EGFP fluorescence reflecting the expression of EGFP. Compared to HEK-293 cells incubated with lipoplexes engrafted with L2 (<0.1% cells expressing EGFP, Figure 4.1B), it can be seen that the incubation of cells with T2-engrafted lipoplexes resulted in a higher
Figure 4.1 - T2-engrafted NTA<sub>3</sub>-DTDA-containing lipoplexes bind strongly to HEK-293 cells and induce targeted transfection. Lipoplexes were prepared by rehydrating desiccated lipids (Mix-5 formulation + 0.5 mol% Texas Red-DHPE) in 100% PBS to form liposomes which were subsequently acidified and mixed with pEGFP-N1 (20:1 lipid:DNA, w/w). The resulting lipoplexes were engrafted with either L2 or T2 peptide and used in transfections by incubating with HEK-293 cells for 2 h at 37 °C. After incubation, half of the cells were immediately analysed for Texas Red-fluorescence by flow cytometry, whereas the remainder were cultured and analysed for EGFP expression by flow cytometry 48 h post-transfection. The fluorescence profiles shown in (A) reflect binding of L2-engrafted or T2-engrafted (as indicated) Mix-5 lipoplexes to the cells. Each fluorescent profile is a representative obtained from three experiments, with the shaded histogram representing the background fluorescence of cells. Representative dot plots are included to show the EGFP-fluorescence of HEK-293 cells treated with either the L2- (B) or T2- (C) engrafted lipoplexes.
proportion (approximately 2%) of cells expressing EGFP (Figure 4.1C), and hence
being transfected by the procedure. Although the percentage of EGFP positive cells
was small (approximately 2%), the geo. mean fluorescence intensity of the EGFP
positive cells was approximately 20-fold higher than that of cells either not expressing
EGFP or that had been incubated with L2-engrafted lipoplexes. This represents a
substantial increase in fluorescence in a small but significant proportion of these cells.

4.5.2 Optimising conditions affecting targeted transfection

A number of factors known to affect DNA condensation were examined for
their effect on transfection efficiency. These include the absolute concentration of the
lipids and pDNA prior to mixing [295] and the fluidity of the liposomes used [141].
Interestingly, cells transfected (as above) in experiments in which lipoplexes were
prepared with liposomes suspended predominantly in water (instead of PBS) showed a
more than 2-fold increase in EGFP expression (Figure 4.2). Also, experiments were
carried out in which the liposomes were diluted in ddH₂O (4× dilution) before addition
of the pDNA, or ethanol was added (to a final concentration 40% v/v) during lipoplex
formation (i.e. after the addition of pEGFP-N1). As shown in Figure 4.2, a significant
increase in transfection was observed when the liposomes (initial lipid concentration 4
mM) were diluted in ddH₂O before complex formation. By contrast, a decrease in
transfection was seen when ethanol was added to promote lipid fluidity (Figure 4.2).
All subsequent NTA₃-DTDA-liposomes therefore included a dilution with ddH₂O
before use in transfections.
Figure 4.2 - Method of preparation affects the transfection efficiency of NTA3-DTDA-containing lipoplexes. Lipoplexes were prepared by rehydrating desiccated lipids (Mix-5 formulation) in either 100% PBS or 4% PBS to form liposomes which were subsequently acidified and mixed with pEGFP-N1 (20:1 lipid:DNA, w/w). Similar experiments for which liposomes were pre-diluted 1/4 with ddH$_2$O, or pre-mixed with ethanol (40% final) before adding pEGFP-N1 and formation of lipoplexes also were carried out. The resulting lipoplexes were engrafted with either L2 or T2 peptide and used in transfections by incubating with HEK-293 cells for 2 h at 37 °C, before washing and incubating at 37 °C. After 48 h, the cells were analysed for EGFP expression by flow cytometry. For these conditions the percentage of cells expressing EGFP (± SEM) (n = 3) after incubation of the cells with lipoplexes engrafted with either L2 or T2 (as indicated) are shown. *p<0.05
4.5.3 *Lipid composition for optimal transfection with lipoplexes containing NTA$_3$-DTDA*

It is reported that carrier lipids such as DOPC can interfere with the ability of DOPE to destabilise endosomal membranes, thus potentially limiting the release of nucleic acids into the cytoplasm [305]. Similarly, although PEG-lipids can reduce lipoplex aggregation and nonspecific binding to cells, they may also interfere with the binding of targeting molecules to receptors [270], and reduce interactions that promote the fusion of liposomes/lipoplexes with cellular membranes [306]. With this knowledge, several different lipid formulations were produced (Table 4.1), then engrafted with T2 peptide, and tested for their ability to mediate transfection of HEK-293 cells. As shown in Figure 4.3, three of the seven new formulations were found to give levels of transfection that were significantly higher than the original *(Mix-5).* Thus, formulation *Mix-7* (with higher DODAP but lower DOPE content), gave a 1.5-fold increase in transfection, whereas *Mix-10* (in which DOPC is omitted) gave more than a 2-fold increase compared to *Mix-5*; this suggested that the inclusion of DOPC has a negative effect on transfection. The greatest increase in transfection, namely a 3.4-fold increase (compared to *Mix-5*), was achieved when the concentration of Cer-PEG$_{750}$ was reduced from 5 mol% to 2 mol% *(Mix-11).* In addition, we noted that higher levels of Cer-PEG$_{750}$ significantly interfered with the targeting of engrafted lipoplexes to cells (Supplementary Figure 4.2). Interestingly, other modifications, such as a reduction in the amount of DODAP, and the substitution of cholesterol with the anionic cholesterol derivative CHEMS, all resulted in a significantly lower level of transfection.
Figure 4.3 - Transfection efficiency of lipoplexes prepared using different NTA<sub>3</sub>-DTDA-containing liposome formulations. Liposomes were prepared by separately rehydrating the different lipid mixtures (each denoted as a different Mix; Table 4.1) in 4% PBS; and each different Mix then subsequently diluted in ddH<sub>2</sub>O, acidified and mixed with pEGFP-N1 (20 : 1 lipid : DNA, w/w) to form lipoplexes. The lipoplexes resulting from the use of each Mix were engrafted with peptide before separately using in transfections with HEK-293 cells as described in Figure 2. The EGFP fluorescence of the cells were assessed by flow cytometry after 48 h of culture. The results shown represents the mean of the percentage of cells expressing EGFP (± SEM) (n = 3), as analysed by flow cytometry 48 h after incubation with lipoplexes engrafted with either L2 or T2 (as indicated). *p<0.05 and **p<0.01 versus cells incubated with T2-engrafted Mix-5 lipoplexes.
To more fully understand the physical-chemical consequences of these modifications, the proportion of lipoplex-associated pDNA accessible to EtBr intercalation for each formulation was determined. The fluorescence of EtBr is substantially increased upon binding to DNA, but quenched by condensation of the nucleic acid [304]. Consequently, EtBr has previously been used as a means to determine the proportion of pDNA incorporated and condensed into lipoplexes [304, 307]. Table 4.1 shows that the proportion of accessible pDNA was most affected by changes in the DODAP content of each formulation (see Mix-5 compared to Mix-7 and Mix-8). Interestingly, both Mix-11 and Mix-12 protected the pDNA significantly more than Mix-5, whereas only the former significantly increased transfection.

On the basis of these transfection and EtBr intercalation results, a formulation designated Mix-13 [and containing DODAP, DOTAP, DOPE, cholesterol, Cer-PEG$_{750}$ and Ni-NTA$_3$-DTDA (44:2:20:30:2:2 molar ratio; Table 4.1] was designed to combine the beneficial effects of the formulations tested (specifically Mix-10 and Mix-11). The results obtained from experiments using Mix-13 in targeted transfections are shown in Figure 4.4. Of note, the incubation of HEK-293 cells with lipoplexes produced with Mix-13 liposomes (but containing 0.5 mol% Texas Red-DHPE as tracer) and engrafted with T2, induced an approximately 50-fold increase in the Texas Red-fluorescence compared to control cells incubated with L2-lipoplexes, indicating that T2-lipoplexes bind strongly to cells (Figure 4.4A). As expected, Mix-13 gave very good levels of transfection; approximately 37% of the cells expressed EGFP at 48 h post-transfection (Figure 4.4C). Although these results demonstrate substantial improvements in the efficiency of transfection compared to Mix-5, it was noted that, despite the high level of T2-lipoplex binding by the majority of the cells (approximately 80%; Figure 4.4A),
Figure 4.4 - Cell binding and transfection efficiency of Mix-13 lipoplexes. Mix-13 lipoplexes were prepared by rehydrating desiccated lipids (including 0.5% Texas Red-DHPE) in 4% PBS to form liposomes, which were subsequently diluted 1:4 with ddH₂O, acidified and mixed with pEGFP-N1 (20:1 lipid:DNA, w/w). The resulting lipoplexes were engrafted with targeting peptide before incubating with HEK-293 cells for 2 h at 37 °C. After incubation, half of the cells were immediately analysed for Texas Red-fluorescence by flow cytometry, whereas the remainder were cultured and analysed for EGFP expression by flow cytometry 48 h post-transfection. The fluorescence profiles shown in (A) reflect binding of L2-engrafted or T2-engrafted (as indicated) Mix-13 lipoplexes to the cells. Each fluorescent profile is a representative obtained from three experiments, with the shaded histogram representing the background fluorescence of cells. Representative dot plots are included to show the EGFP-fluorescence of HEK-293 cells treated with either the L2- (B) or T2- (C) engrafted lipoplexes.
a large proportion of cells that bind the T2-lipoplexes fail to be transfected (Figure 4.4C).

To further explore the relationship between the binding of lipoplexes to cells and eventual EGFP expression by targeted cells, we also analysed 48-h transfected HEK-293 cells (targeted with T2 lipoplexes) by two-colour analysis using flow cytometry. Compared to control (untreated) cells (Figure 4.5), the majority of treated cells exhibited Texas Red-fluorescence after the initial 2-h incubation at 37 °C (Figure 5B). After incubation for 48 h at 37 °C, the mean Texas Red-fluorescence of the total cell population significantly decreased (Figure 4.5C), and a significant proportion (approximately 35%) of the cells then also exhibited strong EGFP-fluorescence indicative of EGFP protein expression (Figure 4.5C). Interestingly, however, approximately 65% of the EGFP-expressing cells at this time exhibited little if any Texas Red-fluorescence, with approximately 35% exhibiting substantial levels of both Texas Red- and EGFP-fluorescence. Notably, a substantial proportion of the cells (approximately 18%) also exhibited strong Texas Red-fluorescence, but no detectable EGFP fluorescence.

4.5.4 Localisation of lipoplexes internalised by cells

Because the use of flow cytometry does not reveal whether the lipoplex-associated Texas Red-fluorescence is intra- or extracellular, it is possible that the difference between the proportion of cells that bind lipoplexes, and those that express EGFP is a result of the inefficient intracellular uptake of the targeted lipoplexed pDNA. To investigate this, fluorescence confocal microscopy was used to examine the HEK-293 cells after incubation with T2-engrafted lipoplexes containing Texas Red-
Figure 4.5 - Analysis of lipoplex binding and EGFP expression by treated cells, and intracellular localisation of internalised lipoplexes. Lipoplexes containing pEGFP-N1 were prepared using formulation Mix-I3 (including 0.5% Texas Red-DHPE) and engrafted with T2 peptide before incubating (2 h at 37 °C) with HEK-293 cells. After incubation, the cells were washed, and half of the cells were immediately analysed for Texas Red- and EGFP-fluorescence by flow cytometry, whereas the remaining cells were incubated for 48 h at 37 °C before further analysis by flow cytometry. Two-colour dot plots shown above reflect fluorescence of (A) untreated HEK-293 cells (B) cells immediately after the initial 2 h of incubation with lipoplexes and (C) cells 48 h post-transfection. Similar transfection experiments also were carried out in which the fluorescence of the cells at 72 h post-transfection was examined by fluorescence confocal microscopy to determine EGFP expression and the localisation of the lipoplex-associated Texas Red-DHPE. Hoeschst 33 342 treatment after cell fixation allowed localisation of nuclei. Cross-sectional images through the centre of four cells are shown in (D) to (G). Texas Red-fluorescence is shown in (D), EGFP fluorescence in (E), Hoeschst 33342 fluorescence in (F), and the merged image in (G)
DHPE as tracer. After a 2-h incubation at 37 °C, the Texas Red-fluorescence associated with treated cells was almost entirely intracellular (not shown). Importantly, even after 72 h of culture, when many cells exhibited high EGFP fluorescence, a significant proportion of the cells that exhibited strong intracellular Texas Red-fluorescence (indicative of good lipoplex uptake, Figure 4.5D) failed to express EGFP (Figure 4.5E). Interestingly, the Texas Red-fluorescence in these cells was not localised in the perinuclear region (as demonstrated by nuclear staining with Hoechst 33342, Figure 4.5F), but was distributed rather uniformly in vesicular-like structures in the cytoplasm (Figures 4.5D and 4.5G). The generally lower Texas Red-fluorescence of EGFP-expressing cells (compare Figures 4.5D and 4.5E) suggests that, in these cells, the Texas Red-containing lipoplexes were more effectively disrupted with the tracer becoming lost and/or metabolised more efficiently.

4.5.5 Effect of incorporating fusogenic and DNase II inhibiting peptides

Because the studies using confocal microscopy indicated that many cells that had internalised lipoplexes failed to express EGFP, it seemed likely that increases in transfection could be achieved by using strategies to increase the amount of viable pDNA that is available for entering the nucleus. One approach for increasing the amount of pDNA available for nuclear uptake is to promote the release of DNA from internalised lipoplexes by incorporating fusogenic agents such as the virally derived peptide diINF-7 [282]. The effect of diINF-7 on the ability of engrafted NTA<sub>3</sub>-DTDA containing liposomes to deliver DNA and mediate transfection therefore was explored. To determine the optimal amount of diINF-7 to incorporate into DODAP liposomes, different quantities of the peptide were sonicated into the liposomes (Mtx- 13) for use in transfection experiments. As shown in Figure 4.6A, the inclusion of diINF-7
Figure 4.6 - Effect of incorporating fusogenic and DNase II inhibiting peptides on the transfection efficiency of NTA₂-DTDA-containing lipoplexes. Liposomes were prepared by suspending desiccated lipids of Mix-13 formulation with different amounts of (A) diINF-7 or (B) 6His-ID2 in 4% PBS. The different liposome preparations were subsequently diluted 1 : 4 with ddH₂O, acidified, and mixed with pEGFP-N1 (20:1 lipid:DNA, w/w) to form lipoplexes. The resulting lipoplexes were engrafted with peptide before incubating with HEK-293 cells for targeted transfections and analysis by flow cytometry, as described in Figure 4.2. Each datum point represents the mean of the percentage of cells expressing EGFP (± SEM) (n = 3), obtained from a flow cytometric analysis of the cells 48 h after incubation with the indicated lipoplexes engrafted with either L2 or T2 (as indicated). *p<0.05 versus cells incubated with lipoplexes containing 0% diINF-7 and 6His-ID2.
significantly increased the transfection efficiency of HEK-293 cells incubated with T2-engrafted lipoplexes, reaching a maximum effect (approximately 2-fold increase relative to 0% dilINF-7) when included in the liposomes at 1 mol%. Notably, the transfection efficiency decreased when the amount of dilINF-7 was >1 mol%; cell binding assays indicated that this decrease was the result of an interference of the binding of lipoplexes to cells (not shown).

Another approach for increasing the amount of viable pDNA available for nuclear uptake is to inhibit the activity of DNase II because this enzyme is present in lysosomes, a major site for degradation of intracellular DNA [308]. Interestingly, recent studies have identified a 6His tagged peptide sequence (6His-ID2) that can potently inhibit DNase II [249]. To determine whether this peptide could be incorporated into NTA3-DTDA liposomes to enhance transfection, 6His-ID2 was produced and included into Mix-13 before sonication to produce the liposomes. The results of transfections obtained using lipoplexes (engrafted with either L2 or T2) prepared using 6His-ID2-containing liposomes are shown in Figure 4.6B. The inclusion of 6His-ID2 at 1 mol% significantly increased the proportion of transfected HEK-cells by 1.5-fold relative to cells that had been transfected with lipoplexes without the 6His-ID2.

The effect of including both dilINF-7 and 6His-ID2 on the transfection efficiency was also explored. Mix-13 lipoplexes were prepared incorporating 1 mol% dilINF-7 and 1 mol% 6His-ID2. The formation of lipoplexes with these preparations resulted in a substantial increase in transfection efficiency for T2-engrafted lipoplexes, which resulted in approximately 51% of the HEK-293 cells becoming transfected.
under these conditions (Figure 4.7). Importantly, the inclusion of the diINF-7 and 6His-ID2 did not substantially increase transfection of L2-engrafted lipoplexes, indicating that the transfection was dependent on the binding mediated by the engrafted targeting peptide.

4.5.6 Efficient targeted transfection with lipoplexes containing NTA$_3$-DTDA

To establish that the transfections observed above were indeed receptor-specific, native HEK-293 cells and DC-293 cells (HEK-293 cells expressing human DC-SIGN) were used in transfections with Mix-13 lipoplexes engrafted with peptide p293 known to bind human DC-SIGN. As shown in Figure 4.8A, p293-engrafted lipoplexes with or without incorporated 1 mol% diINF-7 and 6His-ID2 exhibited substantial binding to DC-293 cells, with the fluorescence increasing by 10- to 20-fold compared to control HEK-293 cells not expressing DC-SIGN. This shows that the targeting of these lipoplexes is receptor-specific. Consistent with this, transfection of DC-293 cells with p293-engrafted lipoplexes resulted in approximately 8% of the DC-293 cells becoming transfected; and transfection of these cells with p293-engrafted lipoplexes that also contained 1 mol% diINF-7 and 6His-ID2, resulted in approximately 50% being transfected, at 48 h post-transfection (Figure 4.8B). This indicates that, for these cells, the transfection is both receptor-specific and strongly dependent on the presence of the fusogenic and DNase II inhibiting peptides.

To further demonstrate the flexibility of the NTA$_3$-DTDA targeting system, additional transfection studies were carried out using the human hepatocarcinoma cell line HepG2. Through peptide screening assays, we identified that a His-tagged form of a peptide previously shown to bind the VEGF receptor Flt-1 [243] promotes strong
Figure 4.7 - Comparison of the transfection efficiency of HEK-293 cells targeted with lipoplexes incorporating both fusogenic and DNase II inhibiting peptides. Liposomes were prepared by suspending dessicated lipids of Mix-13 formulation in 4% PBS that either contained or did not contain 1 mol% of diINF-7 and 1 mol% 6His-ID2 (indicated by +/-). The different liposome preparations were then used to prepare lipoplexes containing pEGFP-N1; the lipoplexes were engrafted with peptide before incubating with HEK-293 cells for targeted transfections and analysis by flow cytometry as described in Figure 2. Each bar represents the mean of the percentage of cells expressing EGFP (± SEM) (n = 3), obtained from a flow cytometric analysis of the cells 48 h after incubation with the indicated lipoplexes engrafted with either L2 or T2 (as indicated). *p<0.05
Figure 4.8 - Efficient transfection of DC-293 cells targeted with p293-engrafted lipoplexes.

Lipoplexes containing pEGFP-N1 were prepared using formulation Mix-13 (including 0.5% Texas Red-DHPE) with or without 1 mol% of diINF-7 and 1 mol% 6His-ID2 (indicated by +/−). Lipoplexes were engrafted with p293 peptide before incubating (2 h at 37 °C) separately with HEK-293 and DC-293 cells. After incubation, cells were washed, and half of the cells were immediately analysed for Texas Red-fluorescence by flow cytometry, whereas the remaining cells were incubated for 48 h at 37 °C before analysis of EGFP expression by flow cytometry. Fluorescence profiles shown in (A) reflect binding of p293-engrafted lipoplexes (without diINF-7 and 6His-ID2) to HEK-293 cells (not expressing DC-SIGN, thin line) and DC-293 cells (expressing DC-SIGN, bold line). Also shown is binding of lipoplexes containing 1% diINF-7 and 6His-ID2 to control HEK-293 (dotted line) cells and DC-293 cells (dashed line). Each fluorescence profile is representative of three experiments. The transfection results in (B) indicate the mean of the percentage of cells expressing EGFP (± SEM) (n = 3) as analysed for EGFP fluorescence by flow cytometry after 48 h of incubation. HEK-293 cells and DC-293 cells (as indicated) are shown. **p<0.01
binding of liposomes to HepG2 cells, with this binding correlating strongly with the expression of Flt-1 on these cells (Figures 4.9A to 4.9C). Therefore, this peptide (termed p25-Flt-1) was engrafted onto lipoplexes that had been prepared using Mix-13 (with or without diINF-7 and 6His-ID2), and the resulting complexes used in transfections with HepG2 cells. As indicated in Figure 4.9D, approximately 30% of HepG2 cells expressed EGFP after 48 h of incubation with p25-Flt-1-engrafted lipoplexes. Notably, this transfection efficiency did not increase, but, in some experiments, was decreased upon inclusion of diINF-7 and 6His-ID2 in the lipoplexes. In all cases, the transfection efficiency of L2-engrafted lipoplexes was below 2%, demonstrating that the transfection was specific for the targeting peptide p25-Flt-1.

4.5.7 Size, charge, stability and toxicity of lipoplexes

Physical size is an important parameter in determining the accessibility of lipoplexes to tissues, as well as their stability and half-life in vivo [297]. Particle size measurements at different stages of lipoplex production with Mix-5, Mix-13 and the commonly used cationic transfection reagent DOTAP:DOPE (at 1:1 mol ratio, and included for comparison) are shown in Table 4.2. It can be seen that the size of the initial liposomes were all in the range 111–133 nm in diameter and, upon addition of pEGFP-N1 and targeting molecule, the size of the complexes was approximately 260 nm diameter (or less). Also, measurement of zeta-potential was carried out to determine the surface charge of the initial liposomes and final lipoplexes produced. As shown in Table 4.2, these measurements indicate that Mix-5 and Mix-13 liposomes have a zeta-potential of −2 and −7 mV, respectively; with these values being reduced to −22 and −18 mV, respectively, upon addition of pDNA (pEGFP-N1). The zeta-potential was not substantially changed by engraftment of targeting moiety and,
Figure 4.9 Efficient targeting and transfection of HepG2 cells with p25Ft1-engrafted lipoplexes. HepG2 cells suspended in PBS containing 50% FCS were incubated (30 min at 4 °C) with liposomes of Mix-13 formulation (but also containing 1 mol% of lipid tracer AF-DPPE) engrafted with peptide. After incubation, cells were washed twice with PBS and pre-incubated for 10 min with goat IgG, before incubating (40 min at 4 °C) with FITC-conjugated goat anti-Ft1 antibody, and then analysing the cells for two-colour fluorescence (AF647 and FITC) by flow cytometry. The two-colour dot plots show staining of HepG2 cells incubated with liposomes engrafted with control 12His peptide (A) and p25-Ft1 (B, C), with the cells in (C) also being stained with anti-Ft1 monoclonal antibody. The results in (D) show the percentage of cells expressing EGFP after transfection of HEK-293 and DC-293 cells (as indicated) with p293-engrafted lipoplexes containing pEGFP-N1 in transfection experiments with lipoplexes with or without 1 mol% of diINF-7 and 1 mol% 6His-ID2 (indicated by +/−). Other details are as described in the legend to Figure 4.8. Each result is the mean of three experiments (± SEM).
importantly, the inclusion of diINF-7 and 6His-ID2 to Mix-13 did not result in any increase in lipoplex size or zeta-potential (Table 4.2).

Table 4.2 - Size, polydispersity and zeta-potential of lipoplexes at different stages of preparation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Diameter (nm)</th>
<th>Polydispersity (mV)</th>
<th>Zeta-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mix-5</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liposomes alone</td>
<td>111 ± 2</td>
<td>0.24 ± 0.03</td>
<td>−2.2 ± 0.3</td>
</tr>
<tr>
<td>Lipos + pEGFP-N1</td>
<td>133 ± 2</td>
<td>0.22 ± 0.05</td>
<td>−21.8 ± 0.2</td>
</tr>
<tr>
<td>Lipos + pEGFP-N1 + p293</td>
<td>148 ± 4</td>
<td>0.07 ± 0.18</td>
<td>−18.7 ± 1.1</td>
</tr>
<tr>
<td><strong>Mix-13</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liposomes alone</td>
<td>133 ± 3</td>
<td>0.23 ± 0.01</td>
<td>7.2 ± 0.3</td>
</tr>
<tr>
<td>Lipos + pEGFP-N1</td>
<td>164 ± 1</td>
<td>0.22 ± 0.02</td>
<td>−18.0 ± 2.1</td>
</tr>
<tr>
<td>Lipos + pEGFP-N1 + p293</td>
<td>255 ± 12</td>
<td>0.56 ± 0.03</td>
<td>−19.2 ± 3.2</td>
</tr>
<tr>
<td><strong>Mix-13 + 1% diINF-7 + 1% 6His-ID2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liposomes alone</td>
<td>133 ± 1</td>
<td>0.24 ± 0.01</td>
<td>−7.8 ± 1.8</td>
</tr>
<tr>
<td>Lipos + pEGFP-N1</td>
<td>167 ± 3</td>
<td>0.27 ± 0.01</td>
<td>−24.6 ± 0.4</td>
</tr>
<tr>
<td>Lipos + pEGFP-N1 + p293</td>
<td>213 ± 7</td>
<td>0.32 ± 0.02</td>
<td>−26.4 ± 1.3</td>
</tr>
<tr>
<td><strong>DOTAP/DOPE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liposomes alone</td>
<td>112 ± 17</td>
<td>0.29 ± 0.04</td>
<td>33.6 ± 1.0</td>
</tr>
<tr>
<td>Lipos + pEGFP-N1</td>
<td>267 ± 17</td>
<td>0.47 ± 0.06</td>
<td>30.7 ± 1.2</td>
</tr>
</tbody>
</table>

Lipoplexes prepared using pEGFP-N1 and different liposome suspensions were engrafted with p293 (except for DOTAP/DOPE). Particle size, polydispersity and zeta-potential of liposomes/lipoplexes were measured both before and after addition of pDNA to liposomes and upon completion of targeted lipoplex preparation (addition of p293). Results represent the mean of readings from three separate preparations ±SEM.

Other experiments showed that the pDNA in lipoplexes produced using Mix-13 was substantially protected from degradation (approximately 45% protection) following DNase I treatment (Supplementary Figure 4.3A), and that the lipoplexes had near maximal activity in transfections of HEK-293 cells, even when the engrafted lipoplexes were used after a 2- to 3-day incubation at 37 °C in the presence of 50% FCS (Supplementary Figure 4.3B). Lipoplexes containing fusogenic/DNase II inhibiting peptides, however, showed substantially less activity under these conditions.
(Supplementary Figure 4.3B). This was reinforced by the DNase protection assay (Supplementary Figure 4.3A), which indicates that Mix-13 lipoplexes protect 40-50% of the added DNA, whereas, upon inclusion of dilNF-7 and 6His-ID2 in the lipoplexes, protection is reduced to approximately 20%. Importantly, targeted lipoplexes showed no significant toxicity even at the highest dose tested (Supplementary Figure 4.4).
4.6 Discussion

The present study provides a novel approach for targeting pDNA to cells, utilising stealth liposomes containing DODAP and NTA$_3$-DTDA, to permit pDNA encapsulation and receptor-mediated targeted transfection of cells in serum-containing media. pDNA is a relatively large structure requiring condensation before it can be encapsulated into liposomes [309]. By contrast to DOTAP, which contains a non-ionisable tertiary amine in its cationic headgroup, DODAP has an ionisable secondary amine which is cationic at pH $<$6.6, but is uncharged at neutral pH [142]. This attribute confers the ability of DODAP-containing liposomes to incorporate/condense DNA at acidic pH (mimicking DOTAP), yet avoid nonspecific interactions at physiological pH.

We therefore developed lipid mixtures containing DODAP (20-50 mol%) plus NTA$_3$-DTDA, DOPE, cholesterol, DOTAP and Cer-PEG$_{750}$, for producing liposomes for use in transfections. The liposomes were first acidified (final pH $\sim$5.5) by the addition of citrate buffer, before adding pDNA and allowing lipoplex formation by incubating for 30 min at room temperature. The resulting lipoplexes are then neutralised and engrafted with a His-tagged targeting molecule. Using this approach (Figure 4.10), our studies demonstrate that pDNA can be efficiently incorporated into NTA$_3$-DTDA-containing lipoplexes and effectively targeted to receptors on cells in the presence of serum. Direct comparisons with reported studies targeting lipoplexes to the transferrin or folic acid receptors was not possible because, as yet, no His-tagged moieties for targeting to these receptors are available. However, the present approach utilising the chelator lipid NTA$_3$-DTDA is more convenient (simply incubate the lipoplexes with the His-tagged targeting moiety) and versatile (can be used to target
Figure 4.10 – Preparation of targeted pDNA-lipoplexes. A schematic diagram showing how pDNA can be incorporated into NTA<sub>3</sub>-DTDA-containing stealth lipoplexes, which can be engrafted with molecules for targeting to cells. The pDNA in aqueous media is mixed with an acidified suspension of a lipid mixture containing DODAP and Ni-NTA<sub>3</sub>-DTDA (e.g. Mix-13; Table 4.1) and then incubated to allow formation of pDNA-lipid complexes or lipoplexes. The lipoplexes are then neutralised and engrafted with a His-tagged targeting protein or peptide to enable targeted delivery of the lipoplexes specifically to cells that express and bind the targeting moiety. Although the diagram above shows liposomes encapsulating condensed pDNA for illustrative purposes, the lipoplexes most likely comprise irregular structures of condensed pDNA complexed in lipid.
any receptor on any cell), provided that a suitable His-tagged targeting moiety (protein or peptide molecule) is available.

Experiments in which T2-engrafted lipoplexes were incubated with HEK-293 cells clearly indicated that the TAT-related peptide is effective at promoting lipoplex binding to and transfection of cells (Figure 4.1), establishing that engraftment of T2 could be used to assay the ability of different NTA$_3$-DTDA-liposome formulations to promote effective pDNA-lipoplex formation and transfection. The results obtained in the present study show that the ability of T2-engrafted lipoplexes to mediate transfection can be influenced by several factors during the stage of lipoplex formation. These include the total concentration of lipid/pDNA, the pH and presence of salt in the mixture, and the lipid composition of the liposomes (particularly the proportion of DODAP, DOPC and Cer-PEG$_{750}$). Because several of these factors can influence the interaction between lipids and DNA [295], their negative effect on transfection efficiency can be attributed to an interfering effect on pDNA condensation and/or the poorer quality of the lipoplexes formed. Thus, the increase in transfection observed with lower concentrations of lipid/pDNA is likely to be caused by the formation of lipoplexes that are more conducive to efficient transfection. Whether this is a result of more tightly packed lipoplexes or a looser association between the pDNA and lipid is unclear from the present study. Also, the presence of PBS during lipoplex formation has previously been reported to interfere with pDNA complexation and the formation of lipoplexes that can mediate efficient transfection [288]. Consistent with this, our data show that transfection efficiency is increased when lipoplexes are formed in buffer of lower ionic strength (Figure 4.2). Interestingly, although ethanol has been previously reported to increase membrane fluidity and
hence liposomal incorporation of DNA [142], our experiments showed no increase in
the transfection efficiency when 40% ethanol was present during lipoplex formation. A
likely explanation for this is that, for the present study, only small volumes (<50 μl) of
lipoplexes were produced for each condition and so lipoplexes were not extruded
through sizing membranes, and the ethanol was subsequently only partially removed
by evaporation and dilution, instead of extensive dialysis.

Optimisation experiments also showed that the presence of DOPC and Cer-
PEG$_{750}$ had a negative effect on transfection efficiency (Figure 4.3). This may be
explained by a reduced level of lipoplex binding to the cells and/or a less efficient
release of the lipoplex-associated pDNA within the targeted cells. Because the binding
of the lipoplexes to cells is decreased as the lipoplex content of Cer-PEG$_{750}$ is
increased (Supplementary Figure 4.2), the negative effect of the higher Cer-PEG$_{750}$
content on transfection efficiency can be explained, at least in part, by the Cer-PEG$_{750}$
sterically hindering the interaction of the p25-Flt-1 targeting peptide with receptors on
the cell surface. Our findings thus support previous studies indicating that targeting
molecules should be anchored onto the distal ends of PEG chains of sterically
stabilising lipids to maximise targeting efficiency [270, 272, 310], which is a factor
likely to depend not only on the nature of the targeting molecule (especially when
using short peptides as the targeting moiety), but also on the particular receptor being
targeted.

By contrast to the effect of Cer-PEG$_{750}$, the presence of DOPC did not
significantly alter the binding of targeted lipoplexes to cells (not shown), suggesting its
negative effect on transfection is affected by a different mechanism. Because the effect
of the endosmolytic agents was much more pronounced when using Mix-5 than when using Mix-13 (not shown), our findings are consistent with other studies [306] indicating that lipids with a PC headgroup can reduce the fusogenicity of membranes and the ability of lipoplexed DNA to escape from endosomal compartments. Reports on the internalisation of TAT-related molecules are not definitive [311-313]. However, experiments in which HEK-293 cells pre-incubated with T2-engrafted lipoplexes (prepared using Mix-5) were subsequently treated with endosmolytic agents such as Ca\(^{2+}\) (6 mM) and sucrose (0.5 M), showed significant increases in transfection (Supplementary Figure 4.5). This is consistent with the major pathway of T2-lipoplex uptake by cells being that of receptor-mediated endocytosis, and points to the release of internalised pDNA from the endosomal pathway, as a major limiting step to efficient transfection in this system. This conclusion is also supported by confocal microscopy studies showing that, in a substantial proportion of cells not expressing the EGFP transgene, the lipoplexes remain in cytoplasmic endosomal compartments even after 72 h of incubation at 37 °C (Figures 4.5D to 4.5G).

The presence of sterically stabilising lipids in the lipoplexes can also be expected to prolong their stability and half-life \textit{in vivo} [297]. Lipoplexes produced using the most active formulation developed in the present study (Mix-13) showed excellent stability after 48 h incubation at 37 °C in the presence of 50% FCS (Supplementary Figure 4.3B). Similar lipoplexes containing fusogenic/DNase II inhibiting peptides, however, showed substantially less activity under these conditions, implying a reduced stability presumably as a result of the destabilising effect of the fusogenic peptide. These findings, when coupled with the fact that the lipoplexes developed are relatively small in size (approximately 260 nm), and are not cationic but
instead carry a negative charge (which reduces nonspecific binding to negatively charged glycosaminoglycans on cell surfaces), suggest that lipoplexes prepared in this way could be useful for targeting the delivery of genes/pDNA to specific cells in vivo in therapeutic applications.

Experiments using liposomes made to include the peptides diINF-7 and 6His-ID2 also showed a substantial increase in transfection efficiency when used in transfections with HEK-293 cells (Figure 4.6). The dimeric peptide diINF-7, derived from the influenza virus haemagglutinin subunit HA-2, is reported to change its conformation upon acidification in early endosomes, promoting fusion of diINF-7-containing liposomes with endosomal membranes [248]. The inclusion of diINF-7 has been reported to promote release of the liposome contents into the cytoplasm [248], and to increase the level of gene knockdown with siRNA-lipoplexes [282]. Interestingly, inhibition of DNase II, an activity found in the lysosomal compartment, has also been reported to increase transfection efficiency [314]. Moreover, using phage display Sperinde et al. [249] recently identified a DNase II inhibitor peptide (ID2) that, when produced with a His-tag (to promote solubility) and pre-incubated with cells for 1 h, could increase the transfection of cells treated with polyethylenimine-polyplexes by as much as 270-fold.

The respective effects of diINF-7 and the DNase inhibitor 6His-ID2 therefore were explored for their ability to enhance targeted transfections. Our experiments indicated that the transfection efficiency of HEK-293-derived cells incubated with either T2 or p293 engrafted lipoplexes prepared using Mix-13 could be significantly increased by inclusion of diINF-7, 6His-ID2 or a combination of the two peptides
(Figures 4.6, 4.7 and 4.8), despite the peptides reducing condensation/protection of the DNA from DNase degradation under these conditions (Supplementary Figure 4.3). Interestingly, the inclusion of diINF-7 plus 6His-ID2 did not increase the transfection efficiency of HepG2 cells incubated with p25-Flt1-engrafted lipoplexes (Figure 4.9D). This finding suggests that the release of pDNA from internalised lipoplexes in endosomes can be more of a barrier to transfection for some cells compared to others.

It should be noted that the mechanism by which the fusogenic peptide diINF-7 and DNase II inhibitor peptide 6His-ID2 induce their effects in the present system is unclear, and that their use in the present study was based on previous reports on the ability of the diINF-7 peptide to increase transfection efficiency by promoting membrane fusion [282], and on the ability of 6His-ID2 to inhibit DNase II activity [249]. A determination of the exact localisation of these peptides in lipoplexes was considered to be beyond the scope of the present study. Previous studies utilising diINF-7 assumed that the peptide was encapsulated in the aqueous core of liposomes upon sonication [248]. In addition, because the His-tagged peptide 6His-ID2 is likely to bind the chelating lipid NTA$_3$-DTDA, after sonication it will become evenly distributed on both sides of the bilayer.

Despite the ineffect of the diINF-7 and 6His-ID2 peptides on the transfection of HepG2 cells, the EGFP expression achieved in these cells with p25-Flt-1-engrafted lipoplexes was substantial (approximately 30% of the cells showed high levels of EGFP expression), suggesting that this approach may have potential for development of novel clinical treatments based on gene delivery. We note that the cells HepG2 are hepatocellular carcinoma, a cancer that is currently the third leading cause of cancer
death worldwide with very poor prognosis for patients [315]. The results obtained in
the present study show that HepG2 cells express the VEGFR Flt-1 (Figure 4.9), a
receptor thought to play an important role in the vascularisation of growing tumours.
Peng et al. [316] recently reported the use of the Apoptin gene to inhibit
hepatocarcinoma growth in nude mice. By targeting Apoptin to the asiaglycoprotein
receptor expressed in normal hepatocytes but at higher levels in hepatocarcinoma,
significant regression of the tumour was achieved. It seems feasible therefore that
NTA3-DTDA-lipoplexes produced to contain Apoptin pDNA and targeted to
hepatocytes (for example by use of the p25-Flt-1 peptide) could also comprise a useful
approach for inducing regression of hepatic tumours.

In conclusion, we have developed an approach for incorporating pDNA into
stealth liposomes containing NTA3-DTDA. This method utilises the ionisable
aminolipid DODAP to form lipoplexes at acidic pH and, when neutralised and
engrafted with a suitable His-tagged targeting molecule, can be targeted to cells. After
optimisation, engrafted lipoplexes could elicit efficient peptide-specific transfection of
HEK-293 cells and HepG2 cells, in the presence of serum. Importantly, lipoplexes
engrafted with a non-targeted control peptide induced only minimal transfection in
both cell lines, indicating that the transfection is mediated through peptide-ligand
specific interactions. Because different peptides can conveniently be engrafted onto
lipoplexes for targeting to different cells, we envisage that this approach has the
potential for developing effective non-viral pDNA delivery systems in therapeutic
applications. The present study demonstrates that NTA3-DTDA can be used to target
lipoplexes to cells in the presence of serum in vitro; the next step is to show that the
approach is also effective for targeting pDNA delivery in vivo.
4.7 Supplementary Figures

Supplementary Figure 4.1 - T2-engrafted NTA$_3$-DTDA-containing liposomes bind strongly to HEK-293 cells. Liposomes composed of POPC:Cer-PEG$_{790}$:Texas-Red-DHPE:NTA$_3$-DTDA (93.5:5:0.5:1 mol%) were produced, engrafted with peptide, and incubated with HEK-293 cells for 30 min at 4 °C. The cells were then washed and assessed for liposome binding by analysis of their Texas Red-fluorescence using flow cytometry. The fluorescence profiles represent cells incubated with liposomes engrafted with L2 as control and T2 peptide (as indicated). The fluorescence profiles of cells incubated with liposomes engrafted with L2 and T2 in the presence of 50 µg/ml heparin also are shown (as indicated); these profiles are indistinguishable from background, indicating that liposome binding is inhibited by heparin. The background fluorescence (shaded histogram) is that of HEK-293 cells incubated without liposomes. Each fluorescence profile is representative of two experiments performed in duplicate.
Supplementary Figure 4.2 - Effect of liposomal Cer-PEG$_{750}$ content on binding and transfection of HepG2 cells. Lipoplexes were prepared using pEGFP-N1 and Mix-13 liposomes (containing 2% Cer-PEG$_{750}$) or modified Mix-13 liposomes containing 5% or 10% Cer-PEG$_{750}$. All liposomes used contained 0.5 mol% Texas Red-DHPE. Lipoplexes were engrafted with p25-Flt-1 before use in transfections with HepG2 cells. After the initial 2-h incubation, approximately half of the cells were immediately analysed for Texas Red-fluorescence using flow cytometry (indicated by $\Diamond$), whereas the remaining cells were incubated at 37 °C and analysed for EGFP expression using flow cytometry at 48 h post-transfection (results represented by the bar graph). The results show the mean of the percentage of cells expressing EGFP as well as the mean-fold increase in cell Texas-Red (TR) fluorescence for each condition relative to untreated cells. Each result is the mean ± SEM of three experiments.
Supplementary Figure 4.3 - DNase protection and stability of T2-engrafted lipoplexes.
Lipoplexes containing pEGFP-N1 were prepared using Mix-13 without or with 1 mol% of
diINF-7 and 1 mol% 6His-ID2. Some samples were then incubated with 5 μg/ml DNase I (1 h at
37 °C,) before inactivating the enzyme, and analysing for residual pDNA by agarose gel
electrophoresis. A DNase control was included where the DNase I was added to lipoplexes in the
presence of 1% Triton X-100. A reverse contrast image of the resultant gel is shown in (A).
Alternatively, lipoplexes were engrafted with T2, diluted 1:2 with 100% FCS (50% final FCS)
and incubated for 0–72 h at 37 °C, before using in targeted transfections with HEK-293 cells.
Each result shown in (B) represents the proportion of cells expressing EGFP (± SEM) (n = 3)
relative to the number of cells expressing EGFP after transfection with freshly prepared
lipoplexes that had not been pre-treated with 50% FCS. EGFP expression was analysed by flow
cytometry at 48 h post-transfection.
Supplementary Figure 4.4 - Effect of targeted lipoplexes on cell viability. Lipoplexes containing pEGFP-N1 were prepared using Mix-13 without or with 1 mol% of diINF-7 and 1 mol% 6His-ID2. The resulting lipoplexes were engrafted with peptide and used in transfections by adding different amounts of the engrafted lipoplexes (312.5, 625 or 1250 ng pEGFP-N1, as indicated) to HEK-293 cells, and incubating the cells for 2 h at 37 °C. The cells were then washed, seeded at $1 \times 10^4$ cells in separate wells of a 96-well plate, and incubated for 48 h at 37 °C. Alamar Blue was then added to each well (10 μL/100 μL media) and the fluorescence intensity (excitation 544 nm, emission 590 nm) was measured 16 h later to obtain a cell count. The results show the percentage of cells in each condition relative to that of untreated cells used as a control (defined as 100%). Each result is the mean ± SEM (n = 3).
Supplementary Figure 4.5 - Effect of treatment with endosomolytic agents on cells transfected with lipoplexes. Lipoplexes prepared using Mix-5 and pEGFP-N1 were engrafted with T2 and incubated with HEK-293 cells for 2 h at 37 °C. The cells were then washed and incubated (1 h at 37 °C) with RPMI medium supplemented with 100 μM chloroquine, 6 mM CaCl$_2$ or 0.5 M sucrose, as indicated. After changing the media, the cells were cultured for 48 h before analysis of EGFP expression by flow cytometry. Each result represents the mean of the percentage of cells expressing EGFP (± SEM) (n = 3) as analysed by flow cytometry. *p<0.05 versus cells not treated with endosomolytic agents.
CHAPTER FIVE

INCREASING THE ANTI-TUMOUR EFFICACY OF DOXORUBICIN-LOADED LIPOSOMES WITH PEPTIDES ANCHORED VIA A CHELTOR LIPID
5.1 Introductory Comments

The results in Chapter 3 and Chapter 4 showed that nucleic acids such as siRNA and pDNA could be incorporated into NTA3-DTDA liposomes for targeting to cells. Importantly, targeted transfection was observed in different cell lines in vitro. In order to progress with the use of these targeted lipoplexes, however, in vivo studies are necessary, which in turn require the identification of targeting molecules capable of homing in on specific cells in vivo. Many potential tumour-targeting peptides have been described in the literature that have the ability to home in on tumour cells themselves, or alternatively the cells of the tumour vasculature endothelium. In order to test whether some of these peptides can target NTA3-DTDA-containing liposomes to tumours, it was considered that a convenient assay would be to engraft histidine-tagged targeting peptides onto doxorubicin-containing liposomes, and determine whether this induces an anti-tumour effect when injected into tumour-bearing mice.

The results in the present chapter examine the ability of NTA3-DTDA to incorporate into doxorubicin-loaded liposomes, specifically this employs the commercial formulation Caelyx® that is currently utilised in cancer treatment. Initial experiments are aimed at determining whether Caelyx® can be incorporated with NTA3-DTDA for engraftment of targeting peptides to promote targeted binding and cytotoxicity to target cells in vitro. Subsequent experiments are aimed at determining the effect of treatment with peptide-engrafted 3NTA-Caelyx on tumour growth in mice.

5.2 Abstract

The therapeutic efficacy of anti-cancer drugs like doxorubicin can be significantly increased by their incorporation into liposomes; but an ability to actively target the drug-containing liposomes to tumours could well provide an even greater curative effect. In this work, a commercial preparation of doxorubicin-loaded liposomes (Caelyx®) was modified by incorporation of the metal chelator lipid NTA3-DTDA to enable engraftment of histidine-tagged targeting molecules. Our results show that when engrafted with p15-RGR, a His-tagged peptide containing a sequence purported to bind platelet-derived growth factor receptor β (PDGFRβ), NTA3-DTDA-containing Caelyx® (3NTA-Caelyx) can be targeted to NIH-3T3 cells in vitro, leading to increased cytotoxicity compared to non-targeted 3NTA-Caelyx. PDGFRβ is known to be expressed on pericytes in the tumour vasculature; however, when radiolabelled p15-RGR liposomes were administered to mice bearing subcutaneous B16-F1 tumours, minimal accumulation into tumours was observed. In contrast, an alternative targeting peptide, p46-RGD, was found to actively direct liposomes to tumours (4.7 %ID/g). Importantly, when injected into tumour-bearing mice, p46-RGD-engrafted 3NTA-Caelyx significantly decreased the tumour growth rate compared to controls. These results indicate that the incorporation of NTA3-DTDA into liposomal drugs could represent a simple modification to the drug to allow engraftment of targeting molecules and to increase its efficacy.
5.3 Introduction

The enduring dilemma of cancer treatment regimes is to balance high antitumour efficacy with low toxicity. For example, the anthracycline doxorubicin has been widely used in the treatment of solid tumours, but its use has been associated with significant side effects including cardiotoxicity [317]. Consequently, liposomal formulations of doxorubicin have been developed to increase tumour accumulation of the drug and are currently being used for the treatment of AIDS-related Kaposi’s sarcoma, ovarian cancer and multiple myeloma [318-320]. Clinically approved preparations of doxorubicin-loaded liposomes such as Caelyx® contain sterically stabilising lipids such as phosphatidylethanolamine conjugated to polyethylene glycol (PE-PEG\textsubscript{2000}), which confer an extended plasma half-life (50-60 h) [321] and the ability to avoid uptake by mononuclear phagocytes of the reticular endothelial system (RES) [117]. Such an extended half-life for Caelyx® facilitates higher passive delivery of doxorubicin to tumours that have a highly permeable or “leaky” vasculature compared to normal tissue [322]. Importantly, this is thought to provide the basis for the higher efficacy observed with liposome-encapsulated drug, compared to “free” drug.

In addition to facilitating the passive delivery of liposome-encapsulated drug into tumours, liposomes provide the potential for receptor-mediated targeting. Evidence suggests that, compared to untargeted liposomes, doxorubicin-loaded liposomes coupled to tumour targeting molecules such as monoclonal antibodies [167], peptides [214, 323] and transferrin [194] can have increased anti-tumour efficacy when tested in animal models. Recently, folate was conjugated to the distal end of the PEG
chains in PE-PEG$_{2000}$ and incorporated into Doxil® (the US-equivalent of Caelyx®) using a “post-insertion” approach for targeting to folate receptor-expressing tumour cells [179]. Of especial interest also is the fact that the use of phage display techniques has identified the sequence of peptides that can target tumours by binding to the vascular endothelial cells and other supportive cells such as pericytes, surrounding the tumour endothelium [241]. One such peptide is the RGR peptide (CRGRRST), the putative receptor of which is the platelet derived growth factor receptor β (PDGFRβ) expressed on pericytes [18]. In another example, peptides containing the central motif RGD have been shown to bind αv integrins and therefore have the potential to deliver cytotoxic drugs to αv-integrin-expressing endothelial cells of the tumour vasculature [228]. The identification of small molecules such as peptides that can interact with specific receptors has thus provided a significant advance towards effective drug targeting; but a simple approach for anchoring targeting moieties onto liposomes has been lacking.

In previous work we utilised the chelator lipid 3(nitrilotriacetic acid)-ditetradecylamine (NTA$_3$-DTDA) to engraft histidine-tagged targeting moieties onto liposomes to enable the targeting of antigens [324] and nucleic acids (see Chapter 3 and Chapter 4) to cells both in vitro and in vivo. In the present study, we explored the potential of incorporating NTA$_3$-DTDA into Caelyx® as a means to allow the anchoring or engraftment onto the liposomal drug of synthetic His-tagged peptides containing targeting moieties such as RGD and RGR. The results show that the incorporation of NTA$_3$-DTDA permits convenient engraftment of tumour-specific targeting peptides onto the Caelyx®, enabling the drug to be targeted; and resulting in increased targeted cytotoxicity in vitro, and a greater anti-tumour effect in vivo.
5.4 Materials and Methods

5.4.1 Reagents

All reagents, lipids, cell lines, mice, and peptides used in the experiments described in this chapter are listed in Chapter 2, Sections 2.1, 2.2, 2.3, 2.4, and 2.6.

5.4.2 Preparation of NTA$_3$-DTDA-containing liposomes

Liposomes were prepared with the lipid composition: DSPC/cholesterol/PE-PEG$_{2000}$/NTA$_3$-DTDA (55:39:5:1 molar ratio). For cell-binding assays OG$_{488}$-DHPE was also included at 1 mol%. The lipids were dried under a stream of nitrogen gas, and liposomes then produced by suspending the lipids in PBS (pH 7.4; hereafter referred to as PBS except where indicated) containing the appropriate amount of Ni-NTA$_3$-DTDA and sonicating for 45 s (two bursts) at maximum amplitude using a TOSCO 100W ultrasonic disintegrator (Measuring and Scientific Ltd, London, UK). Histidine-tagged peptides were engrafted onto liposomes containing NTA$_3$-DTDA by incubating for 30 min at room temperature.

5.4.3 Preparation of 3NTA-Caelyx

Liposomal Caelyx$^\circledR$ stock (2 mg/mL doxorubicin) was subjected to buffer-exchange by loading onto a NICK column containing Sephadex G-50 (Amersham Biosciences AB, Uppsala, Sweden) equilibrated in PBS and eluting in PBS. Ni-NTA$_3$-DTDA was added (typically at 1 mol% of total lipid) and was incorporated with the Caelyx$^\circledR$ by incubating at either 4 or 37 °C for up to 24 h in initial experiments. This method was later standardised to a 2-h incubation at 37 °C. Size-exclusion chromatography of 3NTA-Caelyx indicated that the incorporation of NTA$_3$-DTDA
into Caelyx® under these conditions did not result in any significant leaching or release of the drug from the liposomal Caelyx®. 3NTA-Caelyx was engrafted with peptides as described above for liposomes.

5.4.4 Assaying binding of liposomes and 3NTA-Caelyx to cells

Cultured NIH-3T3 cells were harvested, pelleted and resuspended in 300 µL of RPMI 1640 medium containing 50% FCS buffered with 20 mM HEPES (pH 7.4). The cells (1 × 10^5) were incubated with peptide-engrafted liposomes or 3NTA-Caelyx (20 µg/mL doxorubicin) in the wells of a polypropylene 96-well V-bottom plate (Corning Inc., New York, NY). The plate was then aseptically sealed with supported parafilm and clamps, and the cells were mixed by slow rotation of the plate for 2 h (37 °C), before pelleting and removing the medium. Unbound or loosely bound liposomes were then removed by washing the three times with PBS; the cells were then fixed with 2% paraformaldehyde and analysed for fluorescence by flow cytometry (see Chapter 2, Section 2.10).

5.4.5 Cytotoxicity assay

NIH-3T3 cells or Jurkat cells were incubated with doxorubicin alone or peptide-engrafted 3NTA-Caelyx as described for binding assays. However, instead of being fixed with paraformaldehyde, a proportion of the cells (approx. 10^4 cells) were transferred to a 24-well flat bottom plate in fresh growth medium and cultured for 6 days. After incubation, the growth medium was removed and replaced with fresh medium containing 10% (v/v) Alamar Blue (Astral Scientific, Caringbah, NSW, Australia). The cells were then incubated for 4 h at 37 °C, before measuring the absorbance of each well at 570 nm and 600 nm (μQuant Microplate Reader, BioTek
Intruments Inc, Winooski, VT, USA). To determine cell viability, the $A_{570} - A_{600}$ of each condition was compared to that of untreated cells. IC$_{50}$ values were determined by interpolation from the dose-response curve.

### 5.4.6 Particle size measurements

The particle size of 3NTA-Caelyx liposomes was measured with a Zetasizer Nano ZS as described in Chapter 2, Section 2.1.3.

### 5.4.7 Biodistribution studies

Liposomes with the formulation DSPC/cholesterol/PE-PEG$_{2000}$/NTA$_3$-DTDA (55:39:5:1 molar ratio) were prepared in PBS and [4-$^{14}$C]-Cholesterol was added as tracer and incorporated into liposomes (5 min) using a Model 450 sonifier (Branson Ultrasonics Corporation, Danbury, CT, USA). C57BL/6 mice were inoculated subcutaneously (into the back of the mice) with $1 \times 10^6$ B16-F1 melanoma cells in a volume of 50 $\mu$L of PBS. Biodistribution studies were performed when the tumour diameter reached 5-10 mm (7-12 days after tumour inoculation). The mice were injected i.v. (via the tail vein) with 200 $\mu$L of peptide-engrafted liposomes (2 mM lipid, 0.8 $\mu$Ci $^{14}$C-cholesterol). At 24 h post-injection, mice were killed by CO$_2$ asphyxiation. Tissue samples were harvested from mice and analysed for $^{14}$C-content as described in Chapter 2, Section 2.12.

### 5.4.8 Tumour growth studies

C57BL/6 mice were inoculated subcutaneously with $5 \times 10^5$ B16-F1 cells in the back of the mice. 3NTA-Caelyx containing 1 mol% NTA$_3$-DTDA was engrafted with targeting peptides and injected i.v. 10 days after tumour inoculation when the
majority of the mice had visible tumours of approx. 3-4 mm diameter. The dose of
doxorubicin injected as 3NTA-Caelyx was 2 mg/kg. Mice were injected every 4 days
for a total of 3 injections. Tumour size was measured every 2-3 days with callipers;
and any mouse for which the diameter of the tumour reached 15 mm was euthanized.
Tumour volume was calculated by using the formula: \( \text{volume} = 0.5(\text{length} \times \text{width}^2) \) [188].
5.5 Results

5.5.1 Incorporation of NTA₃-DTDA into Caelyx® for targeting to cells

In Chapter 3 it was shown that the metal chelator lipid NTA₃-DTDA can be incorporated into preformed siRNA-lipoplexes to enable the engrafting of histidine-tagged targeting molecules. It was hypothesised therefore that a similar approach could be used to incorporate NTA₃-DTDA into the lipid bilayer of doxorubicin-loaded liposomes to facilitate anchorage of targeting molecules. To test this hypothesis we initially utilised the \( \alpha_v \)-integrin-binding peptide p46-RGD. However, we found that when engrafted onto liposomes p46-RGD promoted only weak binding of liposome to the different cell lines tested (B16-F1 melanoma, NIH-3T3 fibroblasts and human HEK-293 cells), most likely because these cells express low surface levels of \( \alpha_v \)-integrin. We therefore chose to utilise the peptide p15-RGR for \textit{in vitro} binding assays. p15-RGR contains the RGR targeting motif that purportedly interacts with PDGFR\( \beta \) [241] and can target liposomes to pancreatic tumours in Rip-Tag mice [325]. The engraftment of p15-RGR promoted strong binding of p15-RGR-engrafted liposomes to PDGFR\( \beta \)-expressing NIH-3T3 cells. Thus, while the incubation of NIH-3T3 cells with liposomes engrafted with the control peptide p49-Control increased cell fluorescence by 3-fold relative to background, incubation of the cells with p15-RGR liposomes resulted in a 55-fold increase in cell fluorescence (Figure 5.1A).

To promote the incorporation of the NTA₃-DTDA into Caelyx®, we initially incubated the Caelyx® with a suspension of Ni-NTA₃-DTDA (1 mol% of total lipid) for 2 h at 37 °C. The resulting 3NTA-Caelyx liposomes were then incubated with either p15-RGR or the control peptide p49-Control to permit engraftment. The peptide-
Figure 5.1 - Liposomes and 3NTA-Caelyx can be engrafted with peptides for receptor-mediated targeting to cells. In (A) fluorescent liposomes were prepared containing NTA$_3$-DTDA plus 1 mol% OG$_{488}$-DHPE as tracer and engrafted with either the non-targeted control peptide p49-Control or the targeting peptide p15-RGR (as indicated). Alternatively, Caelyx was incubated with either 0 mol% (B) or 1 mol% NTA$_3$-DTDA (C) for 2 h at 37 °C before addition of targeting peptides. The peptide-engrafted liposomes were incubated with cultured NIH-3T3 cells for 2 h (37 °C), before washing and assessing the cell-associated fluorescence by flow cytometry. The shaded histogram represents the background fluorescence of NIH-3T3 cells incubated without liposomes. Each fluorescence profile is a representative from three experiments.
engrafted Caelyx® was incubated with NIH-3T3 cells, and the extent of cell-bound Caelyx® was assessed by analysing the cells by flow cytometry, utilising the innate fluorescence of doxorubicin as a marker for detection. As shown in Figure 5.1B, the incubation of NIH-3T3 cells with either p49-Control or p15-RGR and Caelyx® (without the incorporation of NTA₃-DTDA) resulted in a 3.3-fold increase in the fluorescence of cells relative to background. A similar increase in cell fluorescence occurred when Caelyx® was incorporated with NTA₃-DTDA and engrafted with p49-Control (Figure 5.1C). However, the incubation of NIH-3T3 cells with p15-RGR-engrafted 3NTA-Caelyx resulted in a 9-fold increase in cell fluorescence relative to background, thus representing a 2.7-fold increase in cell fluorescence between the non-targeted and targeted conditions.

The concentration of NTA₃-DTDA and the duration and temperature of the incubation are likely to be key variables affecting the incorporation of NTA₃-DTDA into Caelyx®. To explore the relative importance of these parameters, the 3NTA-Caelyx was prepared under different conditions and engrafted with either p49-Control or p15-RGR, and then assessed for its ability to bind to cells by flow cytometry. The shift in cell fluorescence between the non-targeted and targeted conditions was measured and the results presented in bar graphs (Figure 5.2). It can be seen that the incubation of Caelyx® with either 0.25 mol% or 0.5 mol% of NTA₃-DTDA resulted in a 1.5-fold increase in cell fluorescence (Figure 5.2A). A greater than 2-fold increase in cell fluorescence was seen when the Caelyx® was incubated with 1 mol% NTA₃-DTDA. At each concentration of NTA₃-DTDA tested the increase in cell fluorescence was the same irrespective of whether the incubation was carried out at 4 °C or 37 °C. Higher concentrations of NTA₃-DTDA (2 and 5 mol%) also were used for
Figure 5.2 - The effect of temperature, concentration of NTA₃-DTDA and duration of incubation on the incorporation of NTA₃-DTDA into Caelyx. Different amounts of NTA₃-DTDA (0/0.25/0.5/1 mol%) were incubated with Caelyx for 2 h at either 4 °C (empty columns) or 37 °C (solid columns). The 3NTA-Caelyx for each condition was then engrafted with targeting peptides and incubated with NIH-3T3 cells to assess cell binding by measurement of cell fluorescence using flow cytometry. The results in (A) show the mean-fold increase in cell fluorescence for each 3NTA-Caelyx preparation engrafted with p15-RGR relative to cells incubated with p49-Control-engrafted 3NTA-Caelyx. For the results in (B), 1 mol% NTA₃-DTDA was incubated with Caelyx for different periods of time (as indicated) at either 4 °C (empty) or 37 °C (solid) before incubation with NIH-3T3 cells and analysing the cells for fluorescence to assess cell binding. Each bar represents the results from three experiments ± SEM. **p<0.01
incorporation into Caelyx®; however at these concentrations, when the Caelyx® was engrafted with p15-RGR and incubated with NIH-3T3 cells, high levels of cell death were observed (data not shown).

To determine the importance of the duration of the incubation of the Caelyx® with NTA₃-DTDA, we varied the incubation time from 0 to 24 h. Interestingly, our results show that a 15 min incubation at 4 °C was sufficient to produce a near-maximal 1.9-fold increase in cell fluorescence between the non-targeted and targeted conditions; and a higher temperature and/or a longer incubation time had only small effects on the results (Figure 5.2B). Based on these results, all subsequent incorporations of NTA₃-DTDA into Caelyx® (see below) were carried out using 1 mol% NTA₃-DTDA and incubation for 2 h at 37 °C.

5.5.2 Peptide-engrafted 3NTA-Caelyx induces targeted cytotoxicity

Peptide-engrafted 3NTA-Caelyx was assessed for cytotoxicity by incubating with an equivalent amount of free doxorubicin, p49-Control-Caelyx and p15-RGR-Caelyx with NIH-3T3 cells. Experiments were carried out in which the cells were incubated with the doxorubicin preparations at different effective concentrations of doxorubicin for 2 h, and the cells then washed and maintained in culture for 6 days before determining the number of viable cells that remained in culture for each condition. The results in Figure 5.3A indicate that at doxorubicin concentrations ≥5 μg/mL, the proportion of viable cells remaining after treatment with either free doxorubicin or p15-RGR-Caelyx was significantly reduced compared to cells treated with p49-Control-Caelyx. Interestingly, the free drug was more effective in reducing the number of viable cells than p15-RGR-Caelyx at these same concentrations of
Figure 5.3 - Peptide-engrafted 3NTA-Caelyx mediates targeted killing of NIH-3T3 cells *in vitro*. 3NTA-Caelyx containing 1 mol% NTA₃-DTDA was engrafted with either the non-targeted control peptide p49-Control or the targeting peptide p15-RGR. Different amounts of peptide-engrafted 3NTA-Caelyx (0.1/0.5/1/5/10/20 µg/mL doxorubicin) were incubated with either (A) NIH-3T3 cells or (B) Jurkat cells, for 2 h at 37 °C. All cells were then pelleted, washed and incubated in fresh medium for 6 days before assaying for cell viability using the Alamar Blue assay. For comparison, conditions where cells were incubated with free doxorubicin are also included. Each datum point represents the results from three experiments ± SEM. *p<0.05, **p<0.001, ***p<0.001 compared with the equivalent concentration of p49-Control-Caelyx.
doxorubicin. The calculated IC$_{50}$ values were 2.4 ± 0.5 µg/mL for free doxorubicin, 11.7 ± 5.7 µg/mL for p15-RGR-Caelyx and >20 µg/mL for p49-Control-Caelyx.

To establish whether the cytotoxic effects of the peptide-engrafted Caelyx® were dependent on the cells expressing receptors for the engrafted peptide, we conducted similar experiments with Jurkat cells, which are PDGFRβ-negative. In these experiments there was no significant difference in the viability of Jurkat cells incubated with p49-Control-Caelyx and p15-RGR-Caelyx at any of the concentrations tested (Figure 5.3B). After treating with either engrafted Caelyx® preparation, the proportion of viable cells remained high (85-100%) with increasing concentrations of drug, until it was noticeably reduced only at 20 µg/mL doxorubicin.

5.5.3 Particle size of 3NTA-Caelyx

Physical size is an important parameter in determining the accessibility of liposomes to tumours [117]. Particle size measurements indicated that there was no significant change in size after incorporation of the NTA$_3$-DTDA into Caelyx® (Table 5.1). Thus, both Caelyx® and 3NTA-Caelyx liposomes measured approximately 90 nm in diameter. After engraftment of peptides however, the particle size of 3NTA-Caelyx increased to 127 ± 8 nm for p49-Control, and 124 ± 3 nm for p15-RGR.
Table 5.1 – Particle size of 3NTA-Caelyx liposomes.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Diameter</th>
<th>Polydispersity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nm)</td>
<td></td>
</tr>
<tr>
<td>Caelyx®</td>
<td>90 ± 3</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>3NTA-Caelyx</td>
<td>93 ± 1</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>+ p49-Control</td>
<td>127 ± 8</td>
<td>0.13 ± 0.05</td>
</tr>
<tr>
<td>+ p15-RGR</td>
<td>124 ± 3</td>
<td>0.28 ± 0.09</td>
</tr>
</tbody>
</table>

Particle size and polydispersity of Caelyx® was measured with a Zetasizer before and after addition of NTA₃-DTDA, and after engraftment of indicated peptides. The results represent the mean ± SEM of readings from three separate preparations.

5.5.4 *In vivo tissue distribution study*

To determine whether targeting peptides can direct accumulation of NTA₃-DTDA-containing liposomes into tumours, we prepared liposomes with a formulation comparable to that of 3NTA-Caelyx (DSPC/cholesterol/PE-PEG₂₀₀₀/NTA₃-DTDA, 55:39:5:1 molar ratio) plus a small proportion of ¹⁴C-cholesterol as tracer. Liposomes were engrafted with peptides: p49-Control, p15-RGR, or p46-RGD. The peptide-engrafted liposomes were then injected i.v. into C57BL/6 mice bearing subcutaneous B16-F1 tumours and 24 h after injection the proportion of ¹⁴C-liposomes recovered in the different tissues was determined. Experiments in which tissues were assessed at three different times points, namely at 4 h, 24 h and 48 h after injection indicated that the highest level of radiolabel in the tumour was recoverable at 24 h post-injection (not shown). The results in Figure 5.4 show that regardless of engrafted peptide, the primary reservoirs of these liposomes are the liver (9.5-15 %ID/g), spleen (10-13 %ID/g) and lungs (10.5-13 %ID/g). By comparison, only a small percentage of the injected dose was detected in the tumour when using liposomes engrafted with p49-Control (2.0 ± 0.03 %ID/g), and similarly with liposomes engrafted with p15-RGR (2.5 ± 0.4 %ID/g). However, a significant increase in the proportion of liposomes in the tumour was found when liposomes were engrafted with p46-RGD (4.7 ± 0.4
Figure 5.4 - Tissue distribution of peptide-engrafted liposomes after i.v. injection. Liposomes containing a lipid composition similar to that of Caelyx: DSPC/cholesterol/PE-PEG2000/NTA₃-DTDA (mol ratio 55:39:5:1) plus the radiolabel [4⁻¹⁴C]-cholesterol as tracer, were engrafted with p49-Control, p15-RGR or p46-RGD (as indicated), and the engrafted liposomes injected i.v. into different groups of C57BL/6 mice bearing subcutaneous B16-F1 tumours. After 24 h, tissues were harvested from the mice, the amount of radioactivity associated with each tissue was measured, and the result expressed as a proportion of the injected radiolabel per gram of tissue. The results represent the mean ± SEM from three mice per condition. *p<0.05
%ID/g). Also, the distribution of liposomes to all other organs was not significantly affected by the nature of the peptide engrafted onto the liposomes. Importantly, muscle tissue collected in close proximity to the B16-F1 tumour showed a low level of liposome accumulation (~1 %ID/g) for all peptide-targeted formulations tested (Figure 5.4).

5.5.5 Anti-tumour effect of targeted 3NTA-Caelyx

Peptide-targeted 3NTA-Caelyx was examined for its anti-tumour effects in B16-F1 tumour-bearing mice. Beginning on Day 10 after tumour inoculation, when the subcutaneous tumours were 3-4 mm in diameter, the mice were injected i.v. three times with either PBS or 3NTA-Caelyx engrafted with p49-Control, p15-RGR or p46-RGD (2 mg/kg doxorubicin per injection). The effect of these treatments on tumour growth is shown in Figure 5.5. It can be seen that compared to the control (PBS treated) group, mice that had been treated with the 3NTA-Caelyx preparations showed a reduced rate of tumour growth. By Day 21 all mice that had been treated with PBS had to be culled because of their tumour size. Importantly, however, by Day 25 there was a significant difference in the mean volume of the tumours in mice that had been treated with p49-Control-Caelyx (1233 ± 73 mm³) and p15-RGR-Caelyx (1143 ± 108 mm³), compared to that in mice that had been treated with p46-RGD-Caelyx (880 ± 123 mm³) (Figure 5.5).
Figure 5.5 - The effect of in vivo administration of peptide-engrastered 3NTA-Caelyx on B16-F1 tumour growth. 3NTA-Caelyx containing 1 mol% NTA₂-DTDA was engrafted with p49-Control, p15-RGR or p46-RGD. Groups of C57BL/6 mice (n = 6) bearing subcutaneous B16-F1 tumours were injected i.v. with PBS or peptide-engrastered 3NTA-Caelyx (2 mg/kg doxorubicin) on Day 10, 14 and 18 after tumour inoculation. Tumour size was measured every 2-3 days and the mean tumour size at each time point is shown. Error bars indicate the SEM. *p<0.05.
5.6 Discussion

Targeted delivery of anti-cancer therapeutics provides a means to minimise side effects while maximising efficacy. In this work, we sought to develop a convenient method for engrafting tumour-homing peptides onto the surface of Caelyx®, a liposomal formulation of the anti-cancer drug doxorubicin. We found that the metal chelator lipid NTA₃-DTDA can be incorporated with Caelyx®, presumably into the lipid bilayer, providing an anchor for the convenient engraftment of histidine-tagged targeting molecules such as peptides.

Our initial experiments showed that a simple incubation for 15 min to 2 h of Caelyx® with NTA₃-DTDA at either 4 °C or 37 °C is sufficient to promote effective incorporation of the NTA₃-DTDA with the Caelyx®, as judged by the ability of the resulting 3NTA-Caelyx to be useful for targeting to cells (Figure 5.2). This is in contrast to previously published work where longer incubation times and/or incubations at higher temperatures [8 h at 4 °C [326], 2 h at 45 °C [179] or 1 h at 60 °C [327]] were used for insertion of PE-PEG₂₀₀₀ conjugates for targeting Caelyx®/Doxil®. Importantly, the present work also shows that Caelyx® with incorporated NTA₃-DTDA can be engrafted with the peptide p15-RGR to promote binding of the 3NTA-Caelyx to receptors on NIH-3T3 cells in vitro. This increased binding of p15-RGR-Caelyx to NIH-3T3 cells resulted in a significantly increased cytotoxicity compared to cells incubated with 3NTA-Caelyx engrafted with control non-targeting peptide (Figure 5.3). As expected, both targeted and non-targeted Caelyx® preparations were less cytotoxic to cells compared to an equivalent concentration of “free” doxorubicin. This can be explained by the fact that in its uncharged state, free doxorubicin can readily
diffuse through the plasma membrane of cells [328], resulting in an increased rate of cell uptake compared to liposomal doxorubicin. Once bound to cell surface receptors, liposomal doxorubicin can be taken up by receptor-mediated endocytosis for subsequent intracellular release. Free drug can also potentially diffuse into cells after being released from cell-bound doxorubicin liposomes.

Despite the ability of p15-RGR-Caelyx to target NIH-3T3 cells in vitro, biodistribution studies indicated that, compared with non-targeted liposomes, the i.v. administration of p15-RGR-liposomes did not result in any significant accumulation in subcutaneous B16-F1 tumours in C57BL/6 mice (Figure 5.4). Consistent with this, p15-RGR-Caelyx had no detectable effect on tumour growth compared to p49-Control-Caelyx (Figure 5.5). The targeting motif contained within p15-RGR (CRGRRST) was identified by phage display studies in the Rip1-Tag2 model of pancreatic cancer [241], and could be used to target liposomes to these pancreatic tumours in vivo [325]. To our knowledge, the present work represents the first study utilising this targeting motif in a different cancer model, namely B16 melanoma. While it is likely that the pericytes that provide structural support to the tumour vasculature are present in most tumours, the level of PDGFRβ expression and accessibility of blood-borne nanoparticles to these cells is likely to differ between tumour types. Indeed, nanoparticles circulating in the vasculature of Rip1-Tag2 mice are purported to have easy access to the perivascular environment compared to other tumour models [241]. Evidence also suggests that there is a high heterogeneity among tumours [329] and targeting molecules that work well in one model of cancer may not work in another [245]. This is likely to be particularly evident when progressing from animal
models to human subjects; highlighting the need for an easily adaptable drug delivery system for effective drug targeting to tumours.

In contrast to p15-RGR, peptide p46-RGD contains the triad RGD established to target the tumour vasculature in a number of different tumour models [330, 331]. Our data show that whilst the intravenous administration of all peptide-engrafted liposomes tested results in major sequestration by the spleen, liver and lungs, the administration of p46-RGD-liposomes results in a significant increase of liposomal accumulation in subcutaneous B16-F1 tumours (Figure 5.4). The proportion of NTA₃-DTDA-liposomes engrafted with p46-RGD that accumulated in tumours after i.v. administration (4.7 %ID/g) was comparable to that observed by others using radiolabelled nanoparticles targeted with RGD-motifs [332, 333]. Importantly, our findings show that the administration of p46-RGD-Caelyx results in a small but significant therapeutic advantage and anti-tumour effect compared to the administration of the non-targeted formulation (Figure 5.5), consistent with the observed increased tumour accumulation of the drug-containing p46-RGD liposomes. We envisage that the identification and use of alternative targeting molecules with a greater ability to direct tumour accumulation of drug-containing NTA₃-DTDA-liposomes could well lead to a more compelling anti-tumour effect. Also, use of the 3NTA-Caelyx system developed in this work could provide an ideal approach for screening different histidine-tagged targeting molecules for their ability to inhibit the growth of different tumours.

The potential for using liposomal delivery to reduce the dose-limiting cardiotoxicity of the free drug, yet take advantage of its anti-tumour properties has
been an area of investigation for some 30 years. Numerous reports have been published detailing the effectiveness of doxorubicin-containing liposomes in a wide range of animal tumour models [129, 334, 335]. Previous studies where Caelyx®/Doxil® was modified to increase tumour targeting [179, 326, 327], have typically involved the chemical conjugation of ligands with PEG-lipids and then inserting these into Caelyx®/Doxil® (post-insertion). The present work, utilising NTA3-DTDA to target Caelyx® to cells, employs an analogous approach. However, instead of attaching histidine-tagged peptides to NTA3-DTDA and then incorporating this with Caelyx®, the NTA3-DTDA is first incorporated with the Caelyx® and the resulting 3NTA-Caelyx is then grafted with targeting peptide. Thus, the advantage of the approach presented herein is that the 3NTA-Caelyx can be prepared in a large batch for future engraftment of different His-tagged targeting peptides. This has clear benefits when it may be considered preferable to target the drug to different receptors that may be expressed at different levels in different tumour types.

A major implication of the present work is that the same approach for incorporating NTA3-DTDA can potentially be used to target any drug-containing nanoparticle comprising of a lipid bilayer. In addition to Caelyx®, a number of other liposomal products are in clinical use as anti-cancer drugs, antibiotics and analgesics; these include Myocet® (non-PEGylated liposomal doxorubicin), DaunoXome® (liposomal daunorubicin), Ambisome® (liposomal amphotericin B), DepoCyt® (liposomal cytarabine) and DepoDur® (liposomal morphine sulfate) [117]. Using an approach analogous to that detailed herein for Caelyx®, we see no reason why the NTA3-DTDA lipid cannot be incorporated also with these agents, to enable the attachment of any His-tagged targeting molecule/peptide for targeting to specific
receptors on cells. This simple modification to enable targeting has the potential to markedly reduce drug toxicity, while increasing efficacy.

In conclusion, this work has presented a unique and convenient method for targeting commercially available Caelyx® liposomes with synthetic histidine-tagged peptides. A simple incubation of the NTA3-DTDA with the Caelyx® results in its incorporation, enabling the 3NTA-Caelyx to be grafted with His-tagged tumour-homing moieties. This results in a significant increase in efficacy as judged by the reduced rate of tumour growth in B16-F1 tumour-bearing mice. The approach could thus lead to the development of novel applications of other liposomal drugs as targeted therapeutics for cancer and other diseases.
CHAPTER SIX

EFFECTIVE TUMOUR TARGETING AND ENHANCED ANTI-TUMOUR EFFECT OF LIPOSOMES ENGRAFTED WITH PEPTIDE ANCHORED VIA A METAL CHELATOR LIPID
6.1 Introductory Comments

The results in Chapter 5 indicated that the chelator lipid NTA₃-DTDA can be incorporated into the liposomal anti-cancer drug Caelyx® for the targeted delivery of doxorubicin to cells *in vitro* and *in vivo*. While significant anti-tumour effects were detected in mice treated with p46-RGD-engrafted 3NTA-Caelyx, it is possible that alternative tumour-targeting peptides could also induce anti-tumour effects when engrafted onto doxorubicin-containing liposomes. The results presented in this chapter describe the screening of three different potential tumour-targeting peptides, p24-NRP-1, p39-Flt-1 and p47-Lyp-1, in order to determine their ability to target NTA₃-DTDA containing liposomes to tumours. Initial experiments involve the engraftment of these peptides onto 3NTA-Caelyx and injection into mice bearing subcutaneous tumours. Subsequent experiments employ liposomes containing PE-PEG₇₅₀ rather than the PE-PEG₂₀₀₀ in Caelyx® in an effort to investigate the effect of liposome PEGylation on the peptide-mediated targeting of NTA₃-DTDA liposomes.

6.2 Abstract

The use of liposomes to target drugs to tumours represents an attractive therapeutic strategy, especially when used with convenient targeting moieties such as peptides. Here we explored several peptides for their ability to target liposomes to tumours. The metal chelator lipid NTA$_3$-DTDA was incorporated into liposomes to enable the engraftment of His-tagged peptides containing targeting motifs specific for the tumour vasculature markers VEGFR-1 (p39-Flt-1) and neuropilin-1 (p24-NRP-1), or a motif known to accumulate in hypoxic areas of tumours (p47-LyP-1). Peptide-engrafted liposomes were examined for their biodistribution and anti-tumour effects after i.v. administration. Our results show that radionlabelled liposomes engrafted with either p24-NRP-1 or p47-LyP-1 and then injected into mice bearing subcutaneous B16-F1 tumours, show increased accumulation in the tumour. For p24-NRP-1-liposomes, tumour targeting was significantly increased when the stabilising lipid phosphatidylethanolamine polyethylene glycol-750 (PE-PEG$_{750}$) was used instead of PE-PEG$_{2000}$ in the liposome lipid mixture. Importantly, compared to the controls, p24-NRP-1 liposomes containing 10 mol% PE-PEG$_{750}$ and loaded with doxorubicin significantly inhibited the rate of tumour growth of the tumour-bearing mice. Our findings demonstrate that the use of drug-containing liposomes incorporating NTA$_3$-DTDA and engrafted with NRP-1 targeting peptide is a convenient strategy to enhance the therapeutic effect of non-targeted doxorubicin.
6.3 Introduction

The ability to target liposomal drugs to tumours represents an attractive strategy for developing more effective cancer therapies. Adequate targeting of liposomes to tumours has been difficult to achieve, however, often due to the poor tumour penetrability of liposomes, and difficulties associated with the attachment of suitable liposome-targeting moieties. The endothelial cells that form the walls of tumour blood vessels present a significant barrier to blood-borne therapeutics, yet these cells also represent an attractive target for anti-tumour therapy [336, 337]. Thus, it is reported that the tumour vasculature plays a major role in supporting tumour growth, and that destruction of tumour blood vessels can lead to tumour regression by blocking the supply of oxygen and nutrients needed for growth [338]. Endothelial cells associated with the tumour are directly accessible to systemically administered therapeutics, and are generally more genetically stable and therefore less prone to drug resistance compared to cells of the tumour [339]. The ability to target liposomal drugs to the tumour vasculature could thus provide a powerful approach for cancer therapy.

New blood vessel growth within tumours occurs primarily through the process of angiogenesis [340]. A key factor promoting angiogenesis is vascular endothelial growth factor (VEGF), which can be secreted by tumour cells, and binds to receptors expressed on endothelial cells [341]. Recently, phage display has led to the identification of the peptide WHSDMEWWYLLG as an antagonist for VEGFR-1 (Flt-1) [21]; while in a separate study the peptide ATWLPPR was found to bind specifically to neuropilin-1, a VEGFR-2 co-receptor [342]. Interestingly, when injected into tumour-bearing mice, ATWLPPR-photosensitiser conjugates accumulate in the tumour vasculature [343]. Tumours also contain lymphatic vessels that are
morphologically distinct from normal lymphatics. Perhaps the first demonstration of this distinction was provided by the finding that the peptide LyP-1 (CGNRKTRGC) preferentially targets tumour lymphatics [245]. Subsequent studies also showed that LyP-1 binds the mitochondrial protein p32 (gC1qR), which is aberrantly expressed on the surface of cells in the lymphatics and hypoxic regions within tumours [344]. In addition to VEGFR-binding peptides, the LyP-1 peptide could thus also have potential for targeting anti-cancer drugs to tumours.

In previous chapters it was shown that the incorporation of the chelator lipid NTA₃-DTDA into liposomes and lipoplexes enables the engraftment of histidine-tagged targeting peptides (see Chapter 3 and Chapter 4). Also, in Chapter 5 it was shown that the liposomal drug Caelyx®, when incorporated with NTA₃-DTDA (3NTA-Caelyx) and engrafted with the tumour vasculature-specific peptide p46-RGD, exhibits increased anti-tumour efficacy compared to Caelyx® engrafted with a control peptide. In the present work we sought to determine whether other tumour-targeting peptides can also be used to increase the therapeutic efficacy of liposomal doxorubicin. We prepared histidine-tagged peptides containing the targeting motifs WHSDMEWWYLLG (p39-Flt-1), ATWLPPR (p24-NRP-1) and CGNKRTYRGC (p47-LyP-1), and engrafted these onto doxorubicin-containing liposomes, before i.v. injection to study their effects in tumour-bearing mice. The results show that doxorubicin-containing NTA₃-DTDA liposomes engrafted with targeting peptide (p24-NRP-1) is a convenient approach to enhance the therapeutic effect of non-targeted drug.
6.4 Materials and Methods

6.4.1 Reagents

All reagents, lipids, mice, and peptides used in the experiments described in this chapter are listed in Chapter 2, Sections 2.1, 2.2, 2.4, and 2.6.

6.4.2 Preparation of peptide-engrafted 3NTA-Caelyx

Liposomal Caelyx® stock (2 mg/mL doxorubicin) was subjected to buffer-exchange by loading onto a NICK column containing Sephadex G-50 (Amersham Biosciences AB, Uppsala, Sweden) equilibrated in PBS and eluting in PBS. Ni-NTA3-DTDA was added (1 mol% total lipid) and incorporated with the Caelyx® by incubating at 37 °C for 2 h. For engraftment with targeting peptide, the indicated histidine-tagged peptide dissolved in H₂O was added to the 3NTA-Caelyx and incubated for 30 min at room temperature with occasional vortexing. The amount of peptide used for engraftment was based on a theoretical calculation of the amount of peptide required to be equimolar with anchoring moiety NTA3-DTDA, and an empirical titration to give optimal binding of the engrafted Caelyx® to target cells in vitro.

6.4.3 Measuring liposome-associated doxorubicin

To determine the amount of doxorubicin contained in liposomes, samples of the liposomal drug were subjected to size-exclusion chromatography by loading onto a NICK column containing Sephadex G-50 equilibrated in PBS and eluting in PBS. After the first 400 μL wash, liposomes containing entrapped doxorubicin were eluted in the following 400 μL while free doxorubicin was eluted in a subsequent volume of 3
mL. The doxorubicin concentration in the eluant was determined by measurement of the absorbance at 485 nm (\(\mu\)Quant Microplate Reader, BioTek Instruments Inc, Winooski, VT, USA) and comparing the reading to that from a standard curve of liposomal doxorubicin.

6.4.4 Particle size measurements

The particle size of liposomes were measured with a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) as described in Chapter 2, Section 2.13.

6.4.5 Liposome preparation

Liposomes were prepared with one of two lipid compositions: DSPC/cholesterol/PE-PEG\(_{2000}\)/NTA\(_3\)-DTDA (55:39:5:1 molar ratio) or DSPC/cholesterol/PE-PEG\(_{750}\)/NTA\(_3\)-DTDA (50:39:10:1 molar ratio). For fluorescence studies Texas Red-DHPE also was included at 1 mol%. Lipids were dried under a stream of nitrogen gas, and liposomes produced by suspending the lipids in PBS containing the appropriate amount of Ni-NTA\(_3\)-DTDA and sonicating for 45 s (two bursts) at maximum amplitude using a TOSCO 100W ultrasonic disintegrator (Measuring and Scientific Ltd, London, UK). For radiolabelled liposomes, [4-\(^{14}\)C]Cholesterol was added to the lipid mixture and incorporated into liposomes (5 min) using a Model 450 sonifier (Branson Ultrasonics Corporation, Danbury, CT, USA). For loading of doxorubicin, liposomes were prepared in 250 mM ammonium sulphate (pH 5.5) and then subjected to buffer-exchange by loading onto a NICK column containing Sephadex G-50 equilibrated in PBS (pH 8) and eluting in PBS. Doxorubicin was mixed with the liposomes (100 \(\mu\)g drug per \(\mu\)mol lipid) and the mixture incubated at 65 °C for 1.5 h. Doxorubicin-liposome samples were then
purified through size-exclusion chromatography (NICK column). All liposomes were
engrafted with targeting peptides as described for 3NTA-Caelyx (Section 6.4.2).

6.4.6 Biodistribution studies

C57BL/6 mice were inoculated subcutaneously (into the back of mice) with 1 x
10^6 B16-F1 cells in a volume of 50 µL of PBS. Biodistribution studies were performed
when the tumour diameter reached 5-10 mm (7-12 days after tumour inoculation). The
mice were injected i.v. with peptide-engrafted ^14C-cholesterol liposomes (0.4 µmol
total lipid, 0.8 µCi total radioactivity). At 24 h post-injection, mice were killed by CO₂
asphyxiation. Tissue samples were harvested from mice and analysed for ^14C-content
as described in Chapter 2, Section 2.12.

6.4.7 Intratumoural distribution of fluorescent liposomes.

B16-F1 tumour-bearing C57BL/6 mice (inoculated as Section 6.4.8) were
injected i.v. with 200 µL of peptide-engrafted Texas Red liposomes (2 mM lipid). To
allow visualisation of the functional vasculature, mice were injected with 20 µL of
Hoescht 33342 (10 mM) 4 h after liposome injection. After 1-2 min the mice were
euthanized and the tumour tissue was harvested, embedded in Tissue-Tek O.C.T.
compound and snap frozen in a slurry of dry ice and ethanol. Using a cryostat (Bright
Instruments, Huntingdon, UK) set to a temperature of -25 °C, tissue sections of 10 µm
were obtained and mounted on glass slides. Sections were viewed on a Leica DMIRE2
fluorescence microscope (Leica Microsystems, Wetzlar, Germany) using 590 nm
(Texas Red) or 460 nm (Hoescht 33342) filters.
6.4.8 Tumour growth studies

C57BL/6 mice were inoculated subcutaneously with $5 \times 10^5$ B16-F1 cells in the back of the mice. 3NTA-Caelyx or PE-PEG$_{750}$ liposomes containing 1 mol% NTA$_3$-DTDA and loaded with doxorubicin (approx. 100 μg doxorubicin per μmol lipid) were engrafted with targeting peptides and injected i.v. (via the tail vein) 10 days after tumour inoculation when the majority of the mice had visible tumours of approx. 3-4 mm diameter. The dose of drug injected as doxorubicin liposomes was 2 mg/kg. Mice were injected every 4 days for a total of 3 injections. Tumour size was measured every 2-3 days with callipers; and any mouse for which the diameter of the tumour reached 15 mm was euthanized. Tumour volume was calculated by using the formula: volume = 0.5(length × width$^2$) [188].
6.5 Results

6.5.1 Effect of targeting doxorubicin liposomes with different peptides

To target doxorubicin-loaded liposomes containing NTA$_3$-DTDA to the tumour vasculature, targeting peptides were produced to contain a His-tag, a spacer sequence and a targeting motif with purported affinity for either VEGFR-1 (p39-Flt-1) or neuropilin-1 (p24-NRP-1). Similarly, the targeting peptide p47-LyP-1 was produced to contain the targeting motif LyP-1, previously shown to bind the tumour lymphatic marker p32. The peptides were engrafted onto Caelyx$^\circledR$ that had been incorporated with NTA$_3$-DTDA, a procedure we showed to promote binding of the Caelyx$^\circledR$ to cells that express receptors for liposome-anchored targeting moieties (Chapter 5). Engrafted Caelyx$^\circledR$ was injected i.v. (3 separate injections of 2 mg/kg doxorubicin) into C57BL/6 mice bearing subcutaneous B16-F1 tumours. 3NTA-Caelyx engrafted with the non-targeted peptide p49-Control also was administered separately in mice as a negative control.

The effect of treatment with 3NTA-Caelyx engrafted with p49-Control, p24-KDR-1 or p39-Flt-1 on tumour growth is shown in Figure 6.1A, where it can be seen that treatment with each Caelyx$^\circledR$ preparation resulted in a slightly slower rate of tumour growth (survival prolonged 2-4 days). By Day 24 after the injection of tumour cells, however, no significant difference ($p = 0.71$) in the mean tumour volume in mice treated with p49-Control-Caelyx ($779 \pm 137$ mm$^3$) or p24-NRP-1-Caelyx ($709 \pm 121$ mm$^3$) could be observed. Tumours in mice treated with p39-Flt-1-Caelyx grew more rapidly; and by Day 22 these mice had to be culled due to the size of their tumour.
Figure 6.1 - The effect of \textit{in vivo} administration of peptide-engrafted 3NTA-Caelyx on B16-F1 tumour growth. In (A) 3NTA-Caelyx was engrafted with p49-Control, p24-NRP-1 or p39-Flt-1, while in (B) 3NTA-Caelyx was engrafted with either p49-Control or p47-LyP-1. C57BL/6 mice (n = 6-8) bearing subcutaneous B16-F1 tumours were injected i.v. with PBS or peptide-engrafted 3NTA-Caelyx (2 mg/kg doxorubicin) on Day 10, 14 and 18 after tumour inoculation. Tumour size was measured every 2-3 days and the mean tumour size at each time point is shown. Error bars indicate the SEM.
Analogous experiments also were carried out with 3NTA-Caelyx engrafted with p47-LyP-1. The results in Figure 6.1B indicate that for these experiments the B16-F1 tumours in the control group grew rapidly, reaching a volume of more than 950 mm$^3$ within 18 days of tumour cell inoculation. Treatment with peptide-engrafted Caelyx® resulted in a slight (but not statistically significant) delay in tumour growth. Importantly, at the end of the experiment (Day 25), there was only a small difference in the mean tumour volumes of mice treated with either p49-Control-Caelyx (1233 ± 73 mm$^3$) or p47-LyP-1-Caelyx (1026 ± 101 mm$^3$) (Figure 6.1B). These initial studies therefore indicated that the targeting of Caelyx® with p24-NRP-1, p39-Flt-1 and p47-LyP-1 exhibits no significant therapeutic advantage over the non-targeted control.

6.5.2 Some peptides can induce aggregation and leakage of liposomal doxorubicin

During preparation of the targeted 3NTA-Caelyx formulations for the above experiment we observed that in contrast to the other peptides tested, p39-Flt-1 affected the appearance of the liposome suspension. The addition of p39-Flt-1 to 3NTA-Caelyx resulted in the formation of large aggregates of peptide and liposomes (Figure 6.2A). Aggregation was noticeable 5 min after addition of peptide to the 3NTA-Caelyx, and if left undisturbed the aggregates grew in size, separating from the suspension. The aggregates could be dissociated by vigorous mixing (e.g. before injection into mice); but even immediately after mixing, particle size measurements with Zetasizer indicated that the diameter of p39-Flt-1-Caelyx was 356 ± 53 nm (polydispersity: 0.44 ± 0.06), compared to 93 ± 1 nm (polydispersity: 0.13 ± 0.02) for non-engrafted 3NTA-Caelyx. By subjecting the Caelyx® to size-exclusion chromatography after incubation at room temperature for 30 min we found that the addition of peptide was accompanied
Figure 6.2 - p39-Flt-1 induces liposomal aggregation and drug leakage. In (A) p39-Flt-1 was added to a suspension of 3NTA-Caelyx and photographed after different periods of time (as indicated). A condition where 3NTA-Caelyx was incubated for 4 h also is included for comparison. In (B) different targeting peptides were added to 3NTA-Caelyx and incubated for 30 min at room temperature. The peptide-engrafted liposomes were then subjected to size-exclusion chromatography to determine the proportion of liposome-incorporated doxorubicin. Each bar represents the mean from three experiments ± SEM. ***p<0.001 compared to the condition p49-Control.
by a release of doxorubicin from the liposomes (Figure 6.2B). The results show that while the incorporation of NTA₃-DTDA into Caelyx® and the engraftment of p49-Control, p24-NRP-1 or p47-LyP-1 did not cause any significant leakage of doxorubicin, the addition of p39-Flt-1 to the 3NTA-Caelyx led to a substantial release (~65%) of the encapsulated doxorubicin under otherwise identical conditions.

6.5.3 Biodistribution of peptide-engrafted liposomes after in vivo administration

Since neither p24-NRP-1 nor p47-LyP-1 induced aggregation or significant release of doxorubicin from the 3NTA-Caelyx, we sought to determine the reason for the somewhat weak ability of these peptides to enhance the anti-tumour efficacy of 3NTA-Caelyx. Biodistribution experiments were carried out to assess the level of liposome accumulation in tumours using liposomes engrafted with the different peptides. Liposomes composed of a lipid formulation comparable to that of 3NTA-Caelyx (DSPC/Chol/PE-PEG₂₀₀₀/NTA₃-DTDA, 55:39:5:1 molar ratio) were produced to contain a trace amount of ¹⁴C-cholesterol. The liposomes were engrafted with p49-Control, p24-NRP-1, p39-Flt-1 or p47-LyP-1 and then injected i.v. into B16-F1 tumour-bearing mice. Liposomes engrafted with p46-RGD also were included in these studies for comparison. Experiments in which tissues were assessed at three different times points, namely at 4 h, 24 h and 48 h (not shown) after injection indicated that the highest level of radiolabel in the tumour was recoverable at 24 h post-injection. The proportion of ¹⁴C-liposomes (per gram of tissue, %ID/g) measured in the tumours of mice 24 h post-injection is shown in Figure 6.3. It can be seen that the proportion of liposomes detected in the tumours of mice injected with p39-Flt-1 liposomes (2.2 ± 0.2 %ID/g) did not differ greatly from tumours in mice injected with p49-Control liposomes (2.0 ± 0.03 %ID/g). However, tumours from mice injected with liposomes
Figure 6.3 - Tumour accumulation of peptide-engrafted liposomes after injection in mice. Liposomes were prepared with NTA₃-DTDA-containing lipid mixtures with either PE-PEG₉₀₀₀ or PE-PEG₇₅₀ (as indicated), and incorporated with the radiolabel [4-¹⁴C]-cholesterol as tracer. The liposomes were then engrafted with the targeting peptide indicated, and injected i.v. into different groups of C57BL/6 mice bearing subcutaneous B16-F1 tumours. After 24 h, the tumour tissue from each mouse was harvested, the amount of radioactivity associated with each tumour was measured, and the result expressed as a proportion of the injected radiolabel per gram of tissue. The results represent the mean ± SEM from three mice per condition. *p<0.05, **p<0.01, ***p<0.001 relative to the respective control (p49-Control) for the indicated experiment.
engrafted with p24-NRP-1, p46-RGD or p47-LyP-1, all showed significant increase in liposome accumulation in tumours: being 3.5 ± 0.2 %ID/g, 4.7 ± 0.4 %ID/g, 4.8 ± 1.0 %ID/g, respectively (Figure 6.3).

Lipids conjugated to the hydrophilic polymer (PEG) are a critical lipid component of Caelyx®, (which contains 5 mol% PE-PEG2000), since these lipids promote liposome stability and prolong half-life in the blood circulation [117]. However, due to steric effects, PEG-conjugated lipids also have the potential to interfere with interactions between peptides attached directly to the liposome surface and receptors on the surface of the cells being targeted [345]. To determine whether the steric effects of the PEG2000 were interfering with the targeting of peptide-engrafted liposomes, we carried out biodistribution experiments with liposomes containing PE-PEG750 instead of PE-PEG2000. We used 10 mol% (instead of 5 mol%) PEG750 to compensate for the higher non-specific liposome binding due to the lower shielding effect of the shorter PEG750 chains. The results in Figure 6.3 show that liposomes containing PE-PEG750 and engrafted with p49-Control, p24-NRP-1 or p39-Flt-1 accumulated in tumours to a significantly greater extent (~2-fold more) compared to liposomes containing PE-PEG2000 and engrafted with the same peptides. This effect was not observed for liposomes engrafted with p47-LyP-1. Interestingly, in one mouse injected with p46-RGD PEG750 liposomes, 11.1 %ID/g was detected in the tumour; but the average that accumulated in tumours did not differ significantly between the two liposome formulations. When compared to p49-Control PEG750 liposomes (3.4 ± 0.1 %ID/g), both p24-NRP-1 and p47-LyP-1 liposomes accumulated to a significantly greater extent in the tumour: being 7.0 ± 1.2 %ID/g and 5.0 ± 0.2 %ID/g, respectively (Figure 6.3).
The proportion of the injected dose recovered in other tissues such as the spleen, liver, lungs, heart, kidney and muscle is shown in Figure 6.4A and B. From the results it can be seen that regardless of the targeting peptide or liposome composition, the greatest accumulation of radiolabel occurred in the spleen (10-16 %ID/g), liver (10-16 %ID/g) and lungs (8-16 %ID/g). Although the liposomal accumulation in each tissue type did vary somewhat depending on the targeting peptide used, typically there was no significant difference in accumulation when compared with p49-Control liposomes. Interestingly, a comparison of the distribution data obtained using liposomes containing the two different PEG-lipids (Figure 6.4A & B) indicated that PEG\textsubscript{750} liposomes accumulated to a significantly higher level in the lungs compared to PEG\textsubscript{2000} liposomes, when the liposomes were engrafted either with p49-Control (PEG\textsubscript{2000}: 11.1 ± 0.2 %ID/g; PEG\textsubscript{750}: 13.2 ± 0.6 %ID/g) or with p24-NRP-1 (PEG\textsubscript{2000}: 9.7 ± 2.0 %ID/g; PEG\textsubscript{750}: 15.6 ± 0.5 %ID/g) peptide. Importantly, a comparison of the results in Figure 6.3 and Figure 6.4 revealed that, apart from the p49-Control PEG\textsubscript{2000} condition, all peptide-engrafted liposomes accumulated >2-fold more in the tumour, than in muscle. The largest tumour:muscle ratio (4.4:1) occurred for p47-LyP-1 PEG\textsubscript{2000} liposomes.

6.5.4 Intratumoural distribution of peptide-engrafted liposomes

Since both p24-NRP-1 and p47-LyP-1 liposomes accumulated into tumours to a greater extent compared to p49-Control liposomes, experiments were carried out to determine the localisation of the liposomes within the tumour. We prepared PEG\textsubscript{750} liposomes containing NTA\textsubscript{3}-DTDA as well as a fluorescent lipid marker (Texas Red-DHPE), engrafted these liposomes with targeting peptides, and then injected them into mice bearing subcutaneous B16-F1 tumours. After 4 h, the tumour tissue was
Figure 6.4 - Tissue biodistribution of peptide-engrafted liposomes after injection in mice.

The results represent the $^{14}$C radioactivity detected in different tissues of the mice described in Figure 6.3. The results in (A) represent the proportion of the injected dose (per gram of tissue) detected (24 h post-injection) in mice injected with $^{14}$C-labelled liposomes containing PE-PEG$_{2000}$, while the results in (B) represent the equivalent data from mice injected with PE-PEG$_{750}$ liposomes.
harvested and sections were prepared for analysis by fluorescence microscopy. While little or no liposomal (red) fluorescence was seen in tumours from mice injected with p49-Control liposomes, areas of strong fluorescence could be seen in the tumours of mice injected with p24-NRP-1 liposomes (Figure 6.5). Importantly, this liposome-associated fluorescence overlapped with areas of the tumour that were directly accessible to the blood flow, as reflected by the blue fluorescence due to the injected stain Hoescht 33342. For tumours from mice injected with p24-NRP-1 liposomes, there were no detectable areas where strong liposomal fluorescence occurred in the absence of Hoescht 33342 fluorescence. Similar results also were obtained when liposomes engrafted with p46-RGD were injected into mice (Figure 6.5). The results in Figure 6.6 show the intratumoural distribution of p47-LyP-1 liposomes after injection into B16-F1 tumour-bearing mice. Although there were areas where liposomal fluorescence and Hoescht 33342 fluorescence could clearly be seen to overlap, distinct regions (indicated with arrows) where liposomes accumulated away from the functional vasculature of the tumour also could be observed (Figure 6.6).

6.5.5 Anti-tumour effect of peptide-engrafted PE-PEG750 doxorubicin-loaded liposomes

Since biodistribution studies indicated that peptide-engrafted liposomes containing PE-PEG750 accumulate in tumours to an equal or sometimes greater extent compared to liposomes containing PE-PEG2000, experiments were carried out to determine whether peptide-engrafted PEG750 liposomes loaded with doxorubicin could induce a targeted anti-tumour effect. Doxorubicin-loaded PEG750 liposomes were engrafted with p49-Control, p24-NRP-1 or p47-LyP-1 i.v. before injection into C57BL/6 mice bearing subcutaneous B16-F1 tumours. The same dose schedule of
Figure 6.5 - Intratumoural distribution of peptide-engrafted liposomes. Liposomes with the lipid composition DSPC/cholesterol/PE-PEG_{750}/NTA_{3}-DTDA/Texas Red-DHPE (mol ratio 49:39:10:1:1) were engrafted with targeting peptides and then injected i.v. into different groups of C57BL/6 mice bearing subcutaneous B16-F1 tumours. After 4 h, mice were injected i.v. with Hoescht 33342 and 1-2 min later the tumour tissue from each mouse was harvested, snap frozen and sectioned. The images shown were captured via fluorescence microscopy and are representative of the fluorescence distribution seen across several tumour sections in two or more independent experiments. Scale bar = 100 μm.
Figure 6.6 - Intratumoural distribution of p47-LyP-1-engrafted liposomes. Liposomes with the lipid composition DSPC/cholesterol/PE-PEG$_{400}$/NTA$_3$-DTDA/Texas Red-DHPE (mol ratio 49:39:10:1:1) were engrafted with p47-LyP-1 and then injected i.v. into C57BL/6 mice bearing subcutaneous B16-F1 tumours. Images of fluorescent areas of the tumour were taken as described in the legend to Figure 6.5. Scale bar = 100 μm.
treatment was followed as for experiments with 3NTA-Caelyx (Figure 6.1). As shown in Figure 6.7, when compared to liposomes engrafted with p49-Control, treatment with p24-NRP-1 liposomes caused a significant inhibition in the rate of tumour growth, so that by Day 23 the mean tumour size of the p24-NRP-1 group (918 ± 58 mm³) was only 68% of the size of that of the p49-Control group (1350 ± 138 mm³). The administration of p47-LyP-1 liposomes containing doxorubicin, however, did not significantly affect the rate of tumour growth when compared to mice treated with non-targeted liposomes (Figure 6.7).
Figure 6.7 - Effect of doxorubicin-loaded liposomes containing PE-PEG$_{750}$ on B16-F1 tumour growth. Doxorubicin-loaded liposomes were prepared with the lipid composition DSPC/cholesterol/PE-PEG$_{750}$/NTA$_3$-DTDA (mol ratio 50:39:10:1). Liposomes were engrafted with p49-Control, p24-NRP-1 or p47-LyP-1. C57BL/6 mice (n = 6) bearing subcutaneous B16-F1 tumours were injected i.v. with PBS or peptide-engrafted doxorubicin-loaded liposomes (2 mg/kg doxorubicin) on Day 10, 14 and 18 after tumour inoculation. Tumour size was measured every 2-3 days and the mean tumour size at each time point is shown. Error bars indicate the SEM. *p<0.05.
6.6 Discussion

In this work three different targeting peptides containing sequences purported to bind cell markers expressed in the tumour vasculature (p24-NRP-1 and p39-Flt-1) [21, 342] and tumour lymphatics (p47-LyP-1) [245], were examined for their ability to target NTA<sub>3</sub>-DTDA-containing liposomes to subcutaneous B16-F1 tumours. Importantly, using this approach a significant anti-tumour effect was seen after administration of doxorubicin-loaded PEG<sub>750</sub> liposomes engrafted with p24-NRP-1.

Our initial studies showed that the peptide p39-Flt-1, despite containing a targeting motif that was previously demonstrated to bind VEGFR-1 [21], did not promote any significant accumulation of radiolabelled liposomes in tumours (Figure 6.3). Consistent with this observation, minimal anti-tumour effect was seen after i.v. administration of p39-Flt-1-engrafted 3NTA-Caelyx (Figure 6.1). Whilst the reason for the inability of p39-Flt-1 liposomes to target tumours was not explored, it was noted that the addition of p39-Flt-1 to a suspension of 3NTA-Caelyx increased particle size, promoted aggregation, and induced substantial leakage of free doxorubicin from the Caelyx (Figure 6.2). The mechanism for these effects is unclear, but most likely involves the highly hydrophobic nature of the p39-Flt-1 targeting motif (which contains three tryptophans, two leucines and a methionine). It is likely that p39-Flt-1 aggregates/disrupts liposomes by the His-tag binding to NTA<sub>3</sub>-DTDA on one liposome, while the hydrophobic targeting motif simultaneously interacts with or perturbs the bilayer of adjacent liposomes.

In contrast to the effects seen with p39-Flt-1, no significant aggregation or drug leakage from the liposomes was observed when p24-NRP-1 was added to 3NTA-
Caelyx, suggesting that under these conditions the somewhat lower hydrophobicity of the targeting moiety of p24-NRP-1 (which contains five hydrophobic residues) does not cause significant liposome aggregation/disruption. Interestingly, the conjugation of antibodies onto liposomes also has been reported to induce aggregation and subsequent rupture of liposomes [346]. Such antibody-mediated liposome aggregation, could readily be overcome, however, by the inclusion of PEG-conjugated lipids [347].

PEG-conjugated lipids are known to create a steric barrier around liposomes, which reduces their ability to aggregate and interact with plasma components, and extends the lifetime of the liposomes in the blood circulation [230]. However, the direct conjugation of targeting moieties onto the surface of liposomes can result in steric hindrance and a reduction in targeting efficiency when using PEGylated lipids [348, 349]. This effect is highlighted in the present work using NTA₃-DTDA-liposomes, where the presence of PE-PEG₂₀₀₀ in the Caelyx® clearly limited its ability to be targeted by p24-NRP-1 to the tumour vasculature. Although radiolabelled PEG₂₀₀₀ liposomes engrafted with p24-NRP-1 did exhibit increased tumour accumulation (3.5 %ID/g compared to 2 %ID/g for p49-Control liposomes, Figure 6.3), it appears that the amount of p24-NRP-1-engrafted Caelyx® that reached the tumour under these conditions elicited only a small if any inhibition on tumour growth (Figure 6.1). Importantly, however, the proportion of p24-NRP-1 liposomes that accumulated in tumours increased by 2-fold (compared to the non-targeted control) when liposomes were prepared to contain PE-PEG₇₅₀ instead of PE-PEG₂₀₀₀ (Figure 6.3). Consequently, compared to mice treated with non-targeted liposomes, there was a clear inhibition in the rate of tumour growth in mice treated with doxorubicin-loaded PEG₇₅₀ liposomes engrafted with p24-NRP-1 (Figure 6.7).
Interestingly, the proportion of p47-LyP-1 liposomes that accumulated in the tumour (~5 %ID/g, Figure 6.3) was not affected by whether the liposomes contained PE-PEG\textsubscript{2000} or PE-PEG\textsubscript{750}. It is unclear therefore whether both types of sterically stabilising lipid interfered with the interaction of p47-LyP-1 with p32. To reduce the possibility of the PEG chains interfering with peptide-receptor interactions, targeting peptides are often attached to the distal end of the PEG chains of PEG-lipids, rather than directly onto the liposomal surface [350, 351]. Our approach using the chelator lipid NTA\textsubscript{3}-DTDA, nonetheless, is different. We envisage that the combination of a molecular spacer between the membrane anchor and 3NTA headgroup of this lipid, and a spacer/linker region between the His-tag and targeting moiety of the peptide [237], would enable sufficient projection of the engrafted peptide to minimise steric effects due to the PEG\textsubscript{750} on the liposomes. Interestingly, a recent study where the LyP-1 sequence was conjugated to liposomal doxorubicin via maleimide-derivatised PE-PEG\textsubscript{2000}, also reports no substantial benefit or anti-tumour efficacy compared to non-targeted liposomes [189]. It would appear, therefore, that factors other than steric hindrance, such as a lower level of expression of LyP-1 receptors (p32) on B16-F1 tumours, are likely to play a role in negating the therapeutic potential of p47-LyP-1-Caelyx\textsuperscript{®} in this system.

The difference between the anti-tumour effect observed herein for p47-LyP-1-Caelyx (Figure 6.1) and that for p46-RGD-Caelyx (Figure 5.5) could reflect differences between the drug-delivery strategies employed; that is, the targeting of tumour cells directly, versus targeting of the tumour vasculature. It can be expected that cytotoxic drugs targeted specifically to tumour cells will kill only the cells that they come into direct contact with. In contrast, since tumour growth is dependent on a
functional blood supply, the death of one tumour endothelial cell could well result in
the killing of up to 100 tumour cells [242]. For similar therapeutic effect, therefore,
liposomes targeted directly to tumour cells with p47-Lyp-1 would require a greater
tumour accumulation compared to vasculature-targeted liposomes. However, the
proportion of administered p47-LyP-1 liposomes that accumulated in tumours was
similar that of the vasculature-targeted p49-RGD liposomes (approx. 5 %ID/g, Figure
6.3). Interestingly, a recent report has shown that the application of tumour-specific
hyperthermia can increase the tumour accumulation of LyP-1-targeted liposomal
doxorubicin, leading to complete tumour regression [189]. Clearly, the use of
hyperthermia or other approaches that increase the accumulation of liposomes in
tumours could also yield a similar outcome when combined with treatment with
3NTA-Caelyx engrafted with p47-LyP-1.

The present work also analysed the intratumoural distribution of peptide-
engrafted liposomes after in vivo administration. It is reported that i.v. injection of the
dye Hoescht 33342 results in a rapid uptake by cells without diffusion across cell
layers; this provides a useful tool for identifying functional vasculature [352].
Consistent with the purported ability of the targeting motif of p24-NRP-1 to target
delivery to the tumour vasculature, an examination of the fluorescence of tumour
sections revealed that p24-NRP-1 liposomes localised primarily in areas that
overlapped with areas of strong Hoescht 33342 fluorescence (Figure 6.5). Interestingly, our results also show that p47-LyP-1 liposomes accumulate in cells
surrounding the tumour vasculature as well as in extravascular regions of the tumour
(Figure 6.6). These findings contrast those of others using LyP-1 targeted
nanoparticles including micelles, baculovirus particles and inorganic azido-
nanoparticles, where no accumulation was demonstrated in tumour blood vessels [353-355]. While these differences may be due to the nature of the particles being used, they also may reflect tumour-specific differences between the murine B16-F1 melanoma used herein, versus the human MDA-MB-435 tumours used by others [353-355].

This work has shown that liposomes incorporated with the chelator lipid NTA₃-DTDA provide a convenient way to anchor peptide targeting moieties to target liposomes/liposomal drugs to tumours. Thus, NTA₃-DTDA can be incorporated into liposomes loaded with the anti-cancer drug doxorubicin, allowing convenient engraftment of histidine-tagged targeting peptides. Three peptides p39-Flt-1, p24-NRP-1 and p47-LyP-1 purported to contain moieties that can target tumours, were examined for their ability to target liposomal doxorubicin. Our results show that the use of some peptides (e.g. p39-Flt-1) can be problematic by promoting liposome aggregation and/or leakage of the encapsulated liposomal drug. Importantly, however, by careful selection of the lipid formulation in respect to the PEG-lipid used, peptides can readily be screened to identify those suitable for therapeutic targeting of liposomes to tumours. This was demonstrated by our finding of a significant anti-tumour response in mice bearing subcutaneous B16-F1 tumours treated with p24-NRP-1-Caelyx. We envisage that this same approach has potential for targeting and enhancing the efficacy of liposomal drugs used to treat cancer in humans.
CHAPTER SEVEN

TARGETING OF ANTIGEN-ENCODING PLASMID DNA TO CELLS FOR TUMOUR THERAPY
7.1 Introductory Comments

In Chapter 4 it was shown that pDNA can be incorporated into liposomes containing NTA$_3$-DTDA, and that when the lipoplexes were engrafted with His-tagged targeting peptides they could induce receptor-specific transfection of cells *in vitro*. Moreover, the results presented in Chapter 5 showed that NTA$_3$-DTDA liposomes engrafted with the $\alpha_v$-integrin binding peptide p46-RGD and administered i.v. to mice, accumulated in tumours to a greater extent than non-targeted liposomes. This increased accumulation resulted in a significant anti-tumour effect when doxorubicin-loaded liposomes were employed. It is conceivable, therefore, that lipoplexes engrafted with p46-RGD could be used to target pDNA specifically to the tumour vasculature. Initial experiments in the present chapter examine whether this is the case, with lipoplexes prepared containing pDNA that encodes the immunodominant epitope (SIINFEKL and a related sequence SIMNFEKL) of the model antigen ovalbumin (OVA). SIINFEKL-specific CD$^8^+$ T cells would be expected to kill SIINFEKL-expressing cells. Therefore, tumour-bearing mice are treated with p46-RGD pDNA-lipoplexes followed by i.v. administration of SIINFEKL-specific CD$^8^+$ T cells (OT-1 cells).

Recently, work carried out by a colleague in the Altin lab has identified the peptide p9-Flg as a means to target the delivery of NTA$_3$-DTDA-liposomes to antigen-presenting cells, and the i.v. injection of mice with OVA-liposomes resulted in protection from challenge with OVA-expressing tumour cells. In experiments in the present chapter, pDNA encoding for SIINFEKL (or SIMNFEKL) is incorporated into NTA$_3$-DTDA liposomes, and the lipoplexes are engrafted with p9-Flg for targeting to antigen-presenting cells *in vivo*. Efficacy of treatment is determined by measuring of
the induction of antigen-specific IFN-γ production by CD8⁺ splenocytes, and
monitoring the effect of vaccination with the lipoplexes on the tumour growth and
metastasis in the lungs of mice challenged with the B16-OVA melanoma.
7.2 Abstract

The ability to target pDNA-lipoplexes to specific cells \textit{in vivo} could enable the development of novel cancer therapies. In this chapter, two targeting strategies, targeted delivery to the tumour vasculature and targeted delivery to antigen-presenting cells (APCs), are employed to determine the \textit{in vivo} efficacy of NTA$_3$-DTDA-containing lipoplexes. The tumour vasculature is targeted by the $\alpha_{v}$-integrin-binding peptide p46-RGD, whereas APCs are targeted by the flagellin-related peptide p9-Flg, which interacts with TLR5. To compare these strategies, lipoplexes were prepared from pDNA constructs (pSFKL and pKUN) encoding the MHC Class I immunodominant epitope (C57BL/6 H-2$^b$) in the model antigen ovalbumin (OVA).

pDNA-lipoplexes grafted with p46-RGD and injected into C57BL/6 mice bearing B16-F1 tumours, showed increased accumulation in tumours compared to control lipoplexes. This treatment would be expected to lead to an increase in the expression of the antigen encoded by the transgene by the vascular endothelial cells of the tumour, thereby making these cells susceptible to attack by antigen-specific T cells. However, pre-treatment of mice with p46-RGD lipoplexes containing pSFKL or pKUN did not induce significant anti-tumour effects upon adoptive transfer of transgenic OT-1 cells.

In other experiments, pDNA-lipoplexes were targeted to APCs through the use of the TLR5-binding peptide p9-Flg. pDNA-lipoplexes grafted with p9-Flg exhibited increased binding to the murine dendritic cell line DC2.4 and were able to successfully transfec\textit{t} peritoneal cells \textit{in vivo} when injected i.p. Furthermore, compared
to controls, systemically administered p9-Flg-lipoplexes demonstrated an increased accumulation in APC-rich tissues such as the spleen and liver as well as the lungs. Other studies showed that the administration of p9-Flg-lipoplexes containing pSFKL or pKUN led to an increase in the number of CD8⁺ T cells that produce IFN-γ in response to OVA antigen. Importantly, the vaccination of C57BL/6 mice with these lipoplexes potently inhibited tumour growth/metastasis in mice challenged with B16-OVA melanoma cells. These results indicate that pDNA-lipoplexes prepared from liposomes containing NTA₃-DTDA can be used to target the delivery of transgenes to antigen-presenting cells in vivo for induction of an immunotherapeutic effect.
7.3 Introduction

The immune system plays an important role in the regulation of tumour growth, as indicated by the prevalence of tumours in immunosuppressed mice and humans [356]. The immunorejection of tumours is thought to involve the induction of a strong CD8$^+$ cytotoxic T lymphocyte (CTL) response [357]. The recognition of antigen presented on MHC Class I molecules on tumour cells by the T cell receptor (TCR) can initiate the release of perforin/granzyme and the upregulation of Fas/FasL on CD8$^+$ T cells, leading to the direct killing or apoptosis of the tumour cell [358]. The importance of T cells in anti-tumour immunity is demonstrated by fact that the adoptive transfer of tumour-antigen specific CD8$^+$ T cells can sometimes elicit an effective immunotherapeutic response in cancer patients. Related to this, it is reported that clinical trials have been conducted where tumour-infiltrating lymphocytes are isolated and expanded in vitro before reinjection into patients [359]. Similarly, therapeutic advantage can be seen following the adoptive transfer of peripheral blood T cells that have been isolated and genetically modified to express a TCR capable of interacting with MHC Class I-bound tumour antigen [360].

As an alternative to adoptive transfer, anti-tumour immunity can be induced by vaccinating with tumour-associated antigens, either in protein form or as a transgene, as in the form of a DNA vaccine. A number of DNA vaccination studies have shown that the injection of naked pDNA encoding tumour antigen into mouse skeletal muscle can result in the induction of a tumour-specific CTL response, leading to anti-tumour immunity [361-363]. However, this process is thought to depend on the interaction (or cross priming) of transfected muscle cells with APCs such as macrophages and
dendritic cells, which in turn lead to the activation of CD8$^+$ T cells [364]. This process is generally considered to be slow, with at least 1 month being required for a measurable degree of immunity to be achieved after first administering the pDNA [365]. An alternative and potentially more efficient means of inducing anti-tumour immunity would be to target antigen-encoding pDNA-lipoplexes directly to APCs by systemic administration.

In Chapter 4, a method was described by which lipoplexes containing NTA$_3$-DTDA can be prepared for targeting the delivery of pDNA to specific receptors on cells. The work presented in the present chapter examines the anti-tumour effects of the in vivo administration of pDNA-lipoplexes targeted either to the tumour vasculature or to APCs. The $\alpha_v$-integrin-binding peptide p46-RGD is used to target pDNA to tumour vascular endothelial cells, while p9-Flg (a peptide recently used to deliver liposomal antigens and plasma membrane vesicles to TLR5-expressing cells [238]), is used to target pDNA to dendritic cells and other APCs. The results indicate that the accumulation of pDNA-lipoplexes in target tissues can be significantly increased by the engraftment of His-tagged targeting peptides. Furthermore, the results show that the i.v. vaccination of mice with p9-Flg-engrafted pDNA-lipoplexes can prime CD8$^+$ T cells, and inhibit tumour growth and metastasis in the murine tumour model B16-OVA melanoma.
7.4 Materials and Methods

7.4.1 Reagents

All reagents, lipids, cell lines, mice, peptides and plasmids used in the experiments described in this chapter are listed in Chapter 2, Sections 2.1, 2.2, 2.3, 2.4, 2.6 and 2.7.

7.4.2 OT-I cells

CD8+ T cells isolated from the spleens of OT-I mice (SIINFEKL TCR transgenic mouse on a C57BL/6 background) and activated in vitro were obtained from Dr. Zuopeng Wu (Department of Immunology, JCSMR, ANU).

7.4.3 Preparation of liposomes

Liposomes were prepared with one of two lipid compositions: DODAP/DOTAP/DOPE/Chol/PE-PEG2000/NTA3-DTDA (Mix-14, 44:2:17:30:5:2 molar ratio) and DSPC/Chol/PE-PEG2000/NTA3-DTDA (55:39:5:1 molar ratio). For fluorescence studies OG488-DHPE was also included at 1 mol%. Lipid stocks in ethanol were mixed, dried under a stream of nitrogen gas, and liposomes produced by suspending the lipids in PBS or ddH2O (for Mix-14) containing the appropriate amount of NTA3-DTDA and sonicating for 45 s (two bursts) at maximum amplitude using a TOSCO 100W ultrasonic disintegrator (Measuring and Scientific Ltd, London, UK). For radiolabelled liposomes, [4-14C]Cholesterol was added to the lipid mixture and incorporated into liposomes (5 min) using a Model 450 sonifier (Branson Ultrasonics Corporation, Danbury, CT, USA).
7.4.4 Preparation of peptide-engrafted pDNA-lipoplexes

pDNA-lipoplexes were prepared using a modification of the protocol established in Chapter 4. Plasmid DNA was added (10:1 lipid:pDNA, w/w) to an acidic solution of liposomes (4 mM lipid in 8.3 mM citrate buffer pH 4.5). Once mixed by pipetting, lipoplexes were allowed to form over 30 min at room temperature. The pH of these incubations was neutralised by the addition of Na₂HPO₄ (final concentration 8 mM) and a 1/10 dilution of 10× PBS. Engrafted pDNA-lipoplexes were produced by adding the appropriate His-tagged peptide and incubating for 30 min at room temperature.

7.4.5 Biodistribution of peptide-engrafted liposomes and pDNA-lipoplexes

Biodistribution studies were carried out in both native and tumour-bearing C57BL/6 mice (8-9 weeks of age). For experiments with tumour-bearing mice, C57BL/6 mice were inoculated s.c. with $1 \times 10^6$ B16-F1 cells in a volume of 50 μL PBS and biodistribution studies were performed when the tumour diameter reached 5-10 mm (7-12 days after tumour inoculation). Mice were injected i.v. with peptide-engrafted liposomes/lipoplexes containing [4-¹⁴C]Cholesterol (0.4 μmol total lipid, 0.8 μCi total radioactivity). Mice injected with pDNA-lipoplexes received a dose of 20 μg pDNA (pSFKL). At 24 h post-injection, mice were killed by CO₂ asphyxiation. Tissue and blood samples were taken from mice and analysed for ¹⁴C-content as described in Chapter 2, Section 2.12.

7.4.6 Combination treatment of mice with pDNA-lipoplexes and OT-I cells

C57BL/6 mice (8-9 weeks of age) were inoculated s.c. with $5 \times 10^5$ B16-F1 cells in the back of the mice. pDNA-lipoplexes containing pSFKL or pKUN (30 μg
pDNA) were engrafted with targeting peptides and injected i.v. into mice 5 days after tumour inoculation, and the injections were repeated on Day 6 and 7. On Day 9 and 13, mice were injected i.v. with $20 \times 10^6$ OT-I cells and tumour size was subsequently monitored every 2-3 days with callipers, and any mouse for which the diameter of the tumour reached 15 mm was euthanized. Tumour volume was calculated by using the formula: volume = $0.5 \times \text{length} \times \text{width}^2$ [188].

7.4.7 Assaying lipoplex binding to cells

pDNA-lipoplexes containing pSFKL were prepared from Mix-14 containing trace amounts of OG$_{488}$-DHPE. Lipoplexes were engrafted with p9-Flg or p49-Control and used for incubation with DC2.4 cells ($1 \times 10^5$ cells/condition) suspended in 500 µL of RPMI supplemented with 10% FCS and 20 mM HEPES (pH 7.5). The cells were incubated with lipoplexes in 500 µL eppendorf tubes for 1 h at 37 °C with continuous slow rotation. After the incubation the cells were washed three times in PBS, fixed with 2% paraformaldehyde in PBS, and analysed for cell-bound OG$_{488}$-DHPE fluorescence by flow cytometry (see Chapter 2, Section 2.10).

7.4.8 Assaying transgene expression by cells in vivo

C57BL/6 mice (8-10 weeks old) were injected i.p. with pSFKL-lipoplexes engrafted with either p49-Control or p9-Flg. Mice were injected with two doses of 100 µg pDNA, 18 h apart. Mice were sacrificed 6 h after the second injection and the peritoneal cavity was washed with 1 mL PBS (3 times) to collect peritoneally excluded cells (PECs). The cells were then pelleted by centrifugation, the supernatant was removed and red blood cells were lysed by incubation with 5 mL of red blood cell lysis buffer (150 mM NH$_4$Cl, 10 mM NaHCO$_3$, 0.1 mM EDTA) for 4 min at 4 °C. The
reaction was stopped by adding 15 mL PBS. The cells were again pelleted and subsequently analysed for EGFP-fluorescence by flow cytometry as described in Chapter 2, Section 2.10.

7.4.9 Vaccination of mice with peptide-engrafted pDNA-lipoplexes; IFN-\(\gamma\) induction

C57BL/6 mice (6-8 weeks old) were vaccinated with free or lipoplex-associated pDNA (pSFKL, pKUN or pEGFP-N1) by tail vein injection (200 \(\mu\)L) or i.m. injection into the right flank (100 \(\mu\)L). Mice were injected on Day 0, 5 and 14 (30 \(\mu\)g pDNA per injection). Mice were sacrificed on Day 21 and a total of \(1 \times 10^6\) splenocytes from each mouse were stimulated for 2 h with 1 \(\mu\)g/mL SIINFEKL peptide (or PBS); and a 1 in 1000 dilution of BD GolgiStop (containing monensin) was then added and the cells incubated for another 3 h. The cells were then stained with anti-CD8-PE before being washed, fixed and permeabilised with 1% paraformaldehyde in the presence of 0.5% saponin, and then stained intracellularly with anti-IFN-\(\gamma\)-AF\(_{647}\). Cells were analysed for PE and AF\(_{647}\)-fluorescence by flow cytometry (see Chapter 2, Section 2.10) and CD8\(^+\) cells were gated from live cells to give the IFN-\(\gamma\)^+ CD8\(^+\) cells in a two-colour dot plot.

7.4.10 Vaccination of mice with peptide-engrafted pDNA-lipoplexes; B16-OVA study

C57BL/6 mice (6-8 weeks of age) were inoculated i.v. with a single cell suspension of B16-OVA cells (\(2 \times 10^5\) cells) in RPMI medium (without serum). pDNA-lipoplexes containing pSFKL or pKUN (20 \(\mu\)g pDNA) were engrafted with targeting peptides and injected i.v. or i.m. into mice 2 days after tumour inoculation, and the injections were repeated on Day 14 with a lower dose of 5 \(\mu\)g pDNA. Lung metastases of B16-OVA tumour cells were quantified by sacrificing the mice at Day 21, removing the lungs and counting the number of tumour foci in the lungs.
7.5 Results

7.5.1 Targeting pDNA-lipoplexes in vivo

The liposome formulations developed for targeting the transfection of pDNA as described in Chapter 4 were developed for use under *in vitro* conditions. Therefore, parameters such as the extent of liposome PEGylation were varied and optimised to increase the level of transgene expression *in vitro*. However, when utilising pDNA-lipoplexes for *in vivo* studies, factors such as lipoplex stability and clearance by phagocytes of the RES must also be taken into account. As a consequence, in this chapter a modified liposome formulation (*Mix-14*) was employed for the *in vivo* targeted delivery of pDNA. By comparison with *Mix-13*, which contains 2 mol% Cer-PEG$_{750}$, *Mix-14* was made to contain 5 mol% PE-PEG$_{2000}$. Thus, to compensate for the increase in PEG-lipid content, the proportion of DODAP and DOPE in the liposomes was also reduced to 44 mol% and 17 mol%, respectively (see Chapter 2, Table 2.2).

An important *in vivo* attribute of a targeted liposome formulation is an extended circulation in the bloodstream. To determine the proportion of pDNA-lipoplexes that remain in the blood after injection, lipoplexes were prepared with *Mix-14* liposomes containing trace amounts of $^{14}$C-cholesterol, and the lipoplexes were engrafted with non-targeting peptide p49-Control and injected i.v. into C57BL/6 mice. After 24 h, the mice were euthanized and blood samples were collected for analysis of $^{14}$C content. Radioactivity measurements of blood samples indicated that 16.0 $\pm$ 1.4% of radioactivity associated with the injected dose was still in the blood circulation at by 24 h post-injection (Figure 7.1A). Similarly, experiments utilising DSPC/cholesterol/
Figure 7.1 - Biodistribution of peptide-engrafted pDNA-lipoplexes after injection in B16-F1 tumour-bearing mice. One group of C57BL/6 mice bearing s.c. B16-F1 tumours was injected i.v. with p49-Control-engrafted liposomes of lipid composition: DSPC/cholesterol/PE-PEG_{2000}/NTA₃-DTDA (mol ratio 55:39:5:1) plus the radiolabel [14C]-cholesterol as tracer. Another group of tumour-bearing mice was injected i.v. with pDNA-lipoplexes prepared from 14C-labelled Mix-I4 and were engrafted with p49-Control or p46-RGD. After 24 h, blood and tissue samples were collected from the mice in each group and the amount of radioactivity associated with each sample was measured. The bars in (A) represent the proportion of the injected radiolabel detected in blood samples from mice injected with p49-Control liposomes or with p49-Control pDNA-lipoplexes. The results in (B) represent the proportion of injected radiolabel detected in the different tissues (as indicated) of mice injected with peptide-engrafted pDNA-lipoplexes; with the results being expressed as the proportion of the injected radiolabel per gram of tissue. The results represent the mean ± SEM from three mice per condition. *p<0.05
PE-PEG_{2000}/NTA_{3}-DTDA liposomes, indicated that 13.8 ± 0.8\% of the injected dose was in the blood 24 h post-injection.

7.5.2 Targeting pDNA-lipoplexes to the tumour vasculature

Lipoplexes engrafted with the peptide p46-RGD (containing a moiety that binds \(\alpha_v\)-integrins) can be expected to target \(\alpha_v\)-integrin-expressing endothelial cells in the tumour vasculature. To determine whether pDNA-lipoplexes prepared from Mix-14 could target the tumour vasculature, C57BL/6 mice bearing s.c. B16-F1 tumours were injected i.v. with \(^{14}\)C-cholesterol-containing lipoplexes engrafted with either p49-Control or p46-RGD. The proportion of radiolabel recovered in the different tissues of these mice 24 h after injection is shown in Figure 7.1B. It can be seen that only a relatively small percentage of the injected radiolabel could be detected in the tumour after injection of pDNA-lipoplexes engrafted with p49-Control (1.4 ± 0.2 \%ID/g). However, the proportion of radiolabel that accumulated in the tumour was significantly increased (2.8 ± 0.4 \%ID/g) after injecting pDNA-lipoplexes engrafted with p46-RGD. Among the other tissues analysed, only the lungs appeared to exhibit greater lipoplex accumulation for the targeted (p46-RGD) versus the p49-Control condition; however this increase was not statistically significant (p = 0.18).

7.5.3 Effect of administering OT-I cells to mice treated with p46-RGD lipoplexes

CD8\(^+\) T cells derived from OT-I mice (OT-I cells) express a transgenic TCR specific for the immunodominant Class I epitope in OVA (SIINFEKL). Therefore, cells successfully transfected with and presenting the OVA peptide antigen would be expected to be killed by activated OT-I cells leading to tumour regression. To explore this possibility, B16-F1 tumour-bearing C57BL/6 mice were injected i.v. with p46-
RGD-engrafted lipoplexes containing either pSFKL or pKUN pDNA constructs, before subsequently administering OT-I cells i.v. 2-4 days later. The effect of this treatment is shown in Figure 7.2, where it can be seen that compared to mice that received OT-I cells alone, the mice pre-treated with the different lipoplex preparations and administered with OT-I cells failed to exhibit any significant decrease in the rate of tumour growth. The data presented in Figure 7.2 are from preliminary experiments that utilised only 3 mice per condition. However, since there was no evidence to suggest that this approach would lead to any significant tumour regression, no large scale experiment to further explore this approach was planned or subsequently carried out.

7.5.4 p9-Flg promotes binding of pDNA-lipoplexes to dendritic cells

The administration of pSFKL and pKUN lipoplexes targeted to antigen-presenting cells could potentially be used to induce immunity to the encoded antigen and thus elicit anti-tumour effects. Previous studies in the Altin lab have indicated that the administration of OVA-containing liposomes engrafted with p9-Flg (which targets the liposomal antigen to TLR5 on APCs) potently inhibited tumour growth and metastasis in mice pre-inoculated with B16-OVA tumour cells [238]. It was hypothesised therefore that the targeted delivery of pSFKL and pKUN to APCs using p9-Flg could also elicit an anti-tumour effect.

To determine whether p9-Flg-lipoplexes could be used to target pDNA to APCs, in vitro binding experiments were carried out using pDNA-lipoplexes prepared from Mix-14 and containing 1 mol% OG$_{488}$-DHPE (as tracer). The tracer-containing lipoplexes were engrafted with either p49-Control or p9-Flg and then incubated with
Figure 7.2 – Tumour growth after targeting pDNA-lipoplexes to the tumour vasculature and injection of OT-I cells. pDNA-lipoplexes prepared using Mix-14 and containing pSFKL or pKUN were engrafted with p49-Control or p46-RGD. Different groups of C57BL/6 mice (n = 3) bearing subcutaneous B16-F1 tumours were injected i.v. with either PBS or the peptide-engrafted pDNA-lipoplexes (30 μg pDNA) on Day 5, 6 and 7 after tumour inoculation. For the groups indicated, mice were then injected i.v. with activated OT-I cells (20 × 10^6 cells) on Day 9 and 13. Tumour size was subsequently measured every 2-3 days, and the mean tumour volume at each time point for the different groups of mice is shown. Error bars indicate the SEM.
DC2.4 cells for 1 h at 37°C, before washing the cells and analysing them for OG₄₈₈ fluorescence by flow cytometry to assess lipoplex binding. As shown in Figure 7.3, the incubation of cells with p49-Control lipoplexes exhibited a ~13-fold increase in the fluorescence of cells relative to background. In contrast, the fluorescence of DC2.4 cells incubated with p9-Flg-lipoplexes was increased by ~35-fold relative to background. This result represents a ~2.5-fold increase in cell fluorescence between the TLR5-targeted (p9-Flg) and non-targeted (p49-Control) conditions, demonstrating that the engraftment of pDNA-lipoplexes with p9-Flg can promote their binding of the to DC2.4 cells.

7.5.5  *In vivo transfection of cells with p9-Flg pDNA-lipoplexes*

The peritoneal cavity contains a large population of APCs including macrophages and dendritic cells and i.p. injection of lipoplexes can be used to achieve *in vivo* transfection of a cell population without systemic administration. Therefore, C57BL/6 mice were injected i.p. with pSFKL-lipoplexes engrafted with either p49-Control or p9-Flg and EGFP expression in PECs determined by flow cytometry at 24 h post-injection. As shown in Figure 7.4, while only 0.18 ± 0.13% of PECs isolated from mice injected with p49-Control-lipoplexes were observed to express EGFP, a significantly greater proportion of cells expressed EGFP after injection of mice with p9-Flg-lipoplexes (4.04 ± 0.80%). Therefore, p9-Flg lipoplexes can be used to induce transgene expression in cells *in vivo*.

7.5.6  *Biodistribution of p9-Flg pDNA-lipoplexes*

While the i.p. injection of lipoplexes has previously been used to induce antigen-specific immunity [366], the APCs of the spleen and liver are more easily
Figure 7.3 - Binding of p9-Flg pDNA-lipoplexes to DC2.4 cells. pDNA-lipoplexes containing pSFKL and prepared using Mix-14 containing 1 mol% OG_{488}^+DHPE as tracer were produced for use in a binding assay with the cultured DC2.4 cells. DC2.4 cells suspended in 50% FCS in PBS were incubated with lipoplexes engrafted with either the peptide p49-Control or p9-Flg (as indicated) and incubated for 1 h at 37 °C. After the incubation, the cells were washed and assessed for lipoplex binding by analysis of cell-associated OG_{488}^+-fluorescence by flow cytometry. The shaded histogram represents the background fluorescence of DC2.4 cells incubated without lipoplexes. The fluorescence profiles are a representative set from three independent experiments.
Figure 7.4 – Intraperitoneal injection of p9-Flg lipoplexes leads to transgene expression. pDNA-lipoplexes prepared using Mix-I4 and containing pSFKL were engrafted with p49-Control or p46-RGD. Different groups of native C57BL/6 mice (n = 3) were injected i.p. with lipoplexes (2 injections of 100 μg pDNA) and peritoneally excluded cells isolated 6 h after the second injection. Isolated cells were treated with red blood cell lysis buffer and then analysed for EGFP fluorescence by flow cytometry. The results shown represents the mean of the percentage of cells expressing EGFP (± SEM). *p<0.05.
accessible to i.v. administrated lipoplexes. To determine the biodistribution of p49-Control- and p9-Flg-pDNA-lipoplexes after i.v. administration, lipoplexes were prepared to contain $^{14}$C-cholesterol as tracer and after engraftment with peptide were administered i.v. into C57BL/6 mice. The distribution of the radiolabel in the different tissues 24 h after injection of the lipoplexes was determined and the results are shown in Figure 7.5. It can be seen that the accumulation of lipoplexes in the kidney and heart was not significantly affected by which peptide was engrafted onto the lipoplexes. In addition, for both peptide-targeted formulations tested, lipoplexes exhibited only a low level of accumulation ($\sim$1 %ID/g) in muscle tissue (Figure 7.5). However, the proportion of p9-Flg-lipoplexes detected in the spleen (15.2 ± 0.6 %ID/g), liver (15.9 ± 0.9 %ID/g) and lungs (20.5 ± 1.0 %ID/g) was significantly greater than that detected in the corresponding tissue when mice were injected with p49-Control lipoplexes.

### 7.5.7 Immunisation of mice with p9-Flg-lipoplexes generates antigen-responsive CD8$^+$ T cells

Lipoplexes containing pSFKL or pKUN and engrafted with p9-Flg peptide were used to determine if targeted lipoplexes can induce antigen-specific immunity. Different groups of C57BL/6 mice were vaccinated (on Day 0, 5 and 14) with PBS (i.v.), naked pDNA (either i.v. or i.m.), or with lipoplexes engrafted with p49-Control or p9-Flg (i.v.). At Day 21, splenocytes from the different groups of vaccinated mice were isolated and used to assess IFN-γ production in response to SIINFEKL peptide as antigen. After stimulation with SIINFEKL antigen for 5 h (in the presence of monensin for the final 3 h), the splenocytes were surface stained with fluorochrome-conjugated mAb to CD8 and then fixed, permeabilised and stained intracellularly with
Figure 7.5  - Biodistribution of peptide-engrafted pDNA-lipoplexes after *in vivo* administration. pDNA-lipoplexes prepared using *Mix-14* and containing the radiolabel [4-*¹⁴C*]-cholesterol as tracer, were engrafted with p49-Control or p9-Flg (as indicated), and the engrafted lipoplexes injected i.v. into different groups of C57BL/6 mice. After 24 h, tissue samples from different organs (as indicated) were taken from the mice and the amount of radioactivity associated with each sample was measured. The bar graph shows the results expressed as a proportion of the injected radiolabel per gram of tissue. Each result represents the mean ± SEM of six mice per condition. *p < 0.05.*
fluorochrome-conjugated mAb to IFN-γ. The cells were then analysed for two-colour fluorescence by flow cytometry, with the results in Figure 7.6 indicating the percentage of CD8⁺ and IFN-γ-producing cells among the total splenocyte population. It can be seen that no significant IFN-γ response was induced in splenocytes from mice injected with PBS, naked pDNA or pDNA-lipoplexes engrafted with p49-Control (<0.2% of CD8⁺ T cells produced IFN-γ). Similar results were seen in a condition where the p9-Flg-lipoplexes contained the plasmid pEGFP-N1 (which does not encode SIINFEKL antigen) instead of pSFKL or pKUN. In contrast to these controls, splenocytes from mice vaccinated i.v. with p9-Flg-lipoplexes containing either pSFKL or pKUN, showed a significant increase in the percentage of CD8⁺ T cells that produced IFN-γ in response to antigen stimulation; which was increased to ~0.5% and ~1.3%, respectively.

7.5.8 Effect of p9-Flg lipoplex vaccination on tumour growth and metastasis

To test the potential for vaccination with p9-Flg-lipoplexes to be useful for the treatment or immunotherapy of disease conditions, the effects of vaccination with p9-Flg-lipoplexes were assessed in the B16-OVA tumour model. B16-OVA is a highly metastatic tumour which when injected i.v. into C57BL/6 mice initially metastasises to the lung before spreading to other major organs. C57BL/6 mice were challenged with B16-OVA cells, and at Day 2 and 14 after tumour challenge, different groups of these mice were then vaccinated via either the i.m or i.v. route, with lipoplexes containing either pSFKL or pKUN, and engrafted with p49-Control or p9-Flg. At Day 21, the lungs of vaccinated mice were removed and the number of tumour foci in the lungs of the mice in the groups for each condition were counted. As shown in Figure 7.7, the lungs of mice injected i.v. with non-targeted lipoplexes exhibited a substantial number
Figure 7.6 - Vaccination with p9-Flg-lipoplexes primes antigen-specific CD8+ T cells.
Separate groups of C57BL/6 mice (n = 3) were injected on Day 0, 5 and 14 with PBS (i.v.), naked pDNA, either i.v. or i.m. (as indicated), and with pDNA-lipoplexes engrafted with either p49-Control or p9-Flg (i.v.). On Day 21, splenocytes (1 × 10^6) from each mouse were isolated and incubated in the absence or presence of SIINFEKL (1 µg/ml) for 5 hours, with monensin being added after the first two hours of incubation. The cells were first stained with PE-labelled mAb to CD8, and then stained intracellularly with APC-labelled mAb to IFN-γ, before analysing the fluorescence of the cells by flow cytometry. The percentage of CD8+ cells producing IFN-γ was determined by analysis of the two-colour dot plots for each SIINFEKL- and PBS-stimulated condition. The results in the bar graph represent the difference in IFN-γ response between SIINFEKL and PBS-stimulated cells; each result represents the mean ± SEM. *p<0.05, ***p<0.001
Figure 7.7 - Vaccination with p9-Flg-lipoplexes inhibits tumour growth and metastasis. C57BL/6 mice were inoculated i.v. with $2 \times 10^5$ B16-OVA cells at Day 0. Different groups of tumour-inoculated mice were then vaccinated at Day 2 and 14 with pDNA-lipoplexes engrafted with either p49-Control or p9-Flg, i.m. or i.v. (as indicated). At Day 21, the lungs were removed from the mice and the number of tumour foci in the lungs were counted. Each result in the bar graph represents the mean number of tumour foci ± SEM in the lungs for each group of mice. *p<0.05, **p<0.01.
of tumour foci (~35), while the lungs of mice vaccinated with p9-Flg-lipoplexes containing either pSFKL or pKUN contained a much lower number of tumours; with the average being 3 ± 1 and 1 ± 1 tumour foci per mouse, respectively. Importantly, compared to the lungs of mice injected i.v. with lipoplex preparations, the lungs of mice vaccinated i.m. with p9-Flg-lipoplexes contained a significantly higher number of tumour foci; being 20 ± 4 for pSFKL, and 9 ± 2 for pKUN.
7.6 Discussion

The results presented in this chapter describe the *in vivo* use of pDNA-lipoplexes prepared from liposomes containing NTA$_3$-DTDA. The results show that, when administered *in vivo*, pDNA-lipoplexes engrafted with targeting peptides can accumulate in specific tissues to an extent, compared to non-targeted lipoplexes. Importantly, when lipoplexes are targeted to APCs by engraftment of the TLR5-binding peptide p9-Flg, potent antigen-specific immunity can be induced, resulting in the immunological rejection of B16-OVA tumour cells.

The lipid formulation of the liposomes used to prepare lipoplexes is key to determining whether successful transfection will occur *in vivo*. The formulation Mix-14 employed in this chapter was developed as a modification of the formulation Mix-13 established in Chapter 4. Mix-13 contains 2 mol% Cer-PEG$_{750}$ and it was considered that lipoplexes containing only 2 mol% PEG-lipid would be unsuitable for *in vivo* applications, particularly since all *in vivo* experiments described in Chapters 5 and 6 employed liposomes containing 5-10 mol% PEG-lipid. Accordingly, when pDNA-lipoplexes were prepared from radiolabelled Mix-14 and injected into mice, the amount of radiolabel detected in the blood 24 h post-injection was comparable to that detected after injection of radiolabelled DSPC/Chol/PE-PEG$_{2000}$/NTA$_3$-DTDA liposomes (Figure 7.1A). The results therefore indicate that the inclusion of pDNA and lipids with unsaturated acyl chains such as DODAP and DOPE does not significantly affect the long-circulating properties of the liposomes. However, it does seem out of place that liposomal formulations that are supposed to be long-circulating are only present in the blood at levels of less than 20 %ID at 24 h post-injection. One
possibility for this is that the injected dose of liposomes was quite low. Evidence suggests that the proportion of injected liposomes present in the blood at any time is dependent on how much was initially injected [43].

Another point of interest is that the accumulation of pDNA-lipoplexes engrafted with p46-RGD (2.8 %ID/g) in B16-F1 tumours occurred to a lesser extent compared to DSPC/Chol/PE-PEG2000/NTA3-DTDA liposomes engrafted with p46-RGD (4.7 % ID/g, see Chapter 5, Figure 5.4). This difference may be related to several factors. Firstly, the increased accumulation of pDNA-lipoplexes in the lung, when compared to liposomes, may have resulted in a lower lipoplex availability, and therefore reduced tumour accumulation. Since accumulation of particles in the lung is closely correlated with particle size [367], this may be an indication that pDNA-lipoplexes engrafted with p46-RGD are significantly larger than liposomes engrafted with the same peptide. Secondly, during the course of this project it has been a general observation during experiments with pDNA-lipoplexes in this project that peptide-engrafted pDNA-lipoplexes exhibit a lower degree of binding to cells in vitro compared to the same targeted liposome formulations without pDNA (not shown). As these in vitro studies were carried out with lipoplexes/liposomes containing a fluorochrome as tracer, it is possible that the presence of pDNA could have resulted in increased attenuation or quenching of the lipoplex-associated fluorescence signal that is bound to cells. However, another possibility is that pDNA that is liposome-associated but not encapsulated reduced the binding of targeted lipoplexes to cells, perhaps through steric interference or electrostatic repulsion from anionic cell surface proteins. Future experiments in which peptide-engrafted lipoplexes are treated with
DNase prior to injection \textit{in vivo} should enable us to determine whether the pDNA interferes with binding of lipoplexes to cells \textit{in vivo}. 

While previous chapters have focussed on the targeting of liposomes to tumours, this chapter presents an alternative approach where lipoplexes are delivered to APCs using the peptide p9-Flg. NTA$_3$-DTDA liposomes engrafted with p9-Flg, a targeting peptide containing a stretch of amino acids related to bacterial flagellin, have previously been shown to bind preferentially to cells expressing TLR5 [238]. Evidence suggests that TLR5 is expressed on the surface of a variety of different cells including monocytes, immature dendritic cells and epithelial cells [368]. Thus, p9-Flg-liposomes injected i.v. have been shown to bind to a higher proportion of APCs in the spleen (compared to control liposomes) [238]. However, no studies had previously been undertaken to determine the tissues in which p9-Flg liposomes preferentially accumulate. The present work shows that mice injected with $^{14}$C-labelled lipoplexes exhibit a greater than 10 %ID/g accumulation in the spleen, liver and lung 24 h post-injection; and this occurs regardless of whether the lipoplexes were engrafted with p49-Control or p9-Flg (Figure 7.4). Importantly, however, the extent of lipoplex accumulation in each of these three tissues was substantially greater with lipoplexes engrafted with p9-Flg rather than the control peptide. While the liver and spleen have been identified as APC-containing organs [369, 370], it was interesting to see increased accumulation of p9-Flg-lipoplexes in the lung. Sebastiani \textit{et al.} determined that murine TLR5 is highly expressed in the lung [371]. It is likely, therefore, that the increased accumulation of p9-Flg-lipoplexes in the lung was due to the binding of lipoplexes to TLR5-expressing endothelial cells in the lung. However, this mechanism
for the increased accumulation of lipoplexes in the lung was not confirmed in the present work.

The ability of both p46-RGD and p9-Flg lipoplexes to induce anti-tumour effects \textit{in vivo} was assessed using C57BL/6 mice bearing B16 melanoma cells. For these studies, p46-RGD-engrafted lipoplexes containing either pSFKL or pKUN were first injected into mice bearing s.c. B16-F1 tumours, before subsequently injecting the mice with activated OVA-specific OT-I cells. The results revealed no significant difference in the growth rate of tumours in mice treated with p49-Control-lipoplexes, p46-RGD-lipoplexes or PBS prior to OT-I cell injection (Figure 7.2). As the presence of a functional tumour vasculature is critical for tumour growth [372], and it is expected that successfully transfected cells expressing antigen would have been killed by the OT-I cells, the lack of an observable anti-tumour effect suggests that there was poor transgene expression in the target cells of the tumour vascular endothelium. Clearly, it remains to be established whether our treatment leads to effective transgene expression in the tumour vasculature.

In contrast to these initial experiments, an anti-tumour effect was indicated in the experiments where p9-Flg lipoplexes were injected into mice that had been challenged (i.v.) with B16-OVA tumours. The results in Figure 7.6. indicate that mice treated with p9-Flg-lipoplexes (i.v.) exhibit a dramatic reduction in the number of lung metastases, compared to both mice injected with p49-Control-lipoplexes, and also to mice injected i.m with p9-Flg lipoplexes. To the best of our knowledge, this work represents the first demonstration that potent anti-tumour immunity can be induced in mice by the i.v. administration of TLR5-targeted pDNA-lipoplexes. Previously,
mannosylated cationic lipoplexes have been shown to have the ability to target the delivery of antigen-encoding pDNA to APCs in mice, leading to high levels of IFN-γ production by isolated splenocytes after in vitro stimulation with antigen [373]. The intraperitoneal injection of Man-lipoplexes containing OVA-encoding pDNA also has been shown to significantly increase the survival of mice inoculated with E.G7-OVA tumour cells [366]. However, no measureable CTL or anti-tumour response was observed following i.v. injection of these lipoplexes, suggesting the lipoplexes were not targeted under these conditions [373].

The substantial difference in detectable anti-tumour effect between the two transgene delivery strategies employed in this chapter can be attributed in part to the large differences in peptide-engrafted lipoplex accumulation in target tissues. Only 2.8 %ID/g of p46-RGD lipoplexes was detected in the tumours of injected mice at 24 h post-injection (Figure 7.1) compared to a total of >30 %ID/g of p9-Flg-lipoplexes in the spleen and liver of injected mice (Figure 7.4). In addition, the targeting of antigen-encoding transgenes to APCs requires only a few cells to be transfected in order to initiate a significant anti-tumour immune response. Upon reaching the APC-rich spleen and liver, p9-Flg-lipoplexes could be expected to selectively transfecet resident APCs through binding to TLR5. Previous studies have indicated that the binding of p9-Flg liposomes to TLR5 results in the maturation of dendritic cells in the spleen, an event considered to be crucial for the initiation of a CTL response [238]. It is likely that the unmethylated CpG dinucleotides present in the bacterially amplified pDNA used in the present studies could also enhance the maturation of dendritic cells [374]. The presentation of plasmid-derived antigen by mature APCs (and in particular dendritic cells) to naïve T cells can be expected to lead to the generation of a large
population of activated CD8$^+$ T cells. The presence of primed CD8$^+$ T cells in the spleen was confirmed for p9-Flg-lipoplexes in IFN-γ production studies (Figure 7.5). Activated OVA-antigen-specific CTLs reaching the lung would thus be expected to induce killing of any resident B16-OVA tumour cells.

The majority of the experiments in this chapter compare the effects of targeting plasmids pSFKL and pKUN. Interestingly, when targeted to APCs with p9-Flg lipoplexes, these two plasmids were equally effective at preventing the growth of B16-OVA metastases in the lungs of treated mice. However, the results appear to provide some indication that transfection of APCs with pKUN is more effective at inducing antigen-specific immunity. For example, the results show a significant increase in the number of IFN-γ-producing splenocytes in mice vaccinated with pKUN lipoplexes, compared to mice treated with pSFKL lipoplexes (Figure 7.5). In addition, the lungs of mice vaccinated i.m. with p9-Flg pKUN-lipoplexes had significantly fewer tumour foci compared to those from mice vaccinated with p9-Flg pSFKL-lipoplexes (Figure 7.6). A contributing factor to these results that must be considered is the different antigenic sequences encoded by the plasmids: SIMNFEKL (in pSFKL) and SIINFEKL (in pKUN). While SIINFEKL is the immunodominant CTL epitope of OVA, the single amino acid change in SIMNFEKL may have resulted in the generation of CTLs whose TCRs exhibited slightly lower affinity for free SIINFEKL peptide or SIINFEKL antigen on the MHC Class I of B16-OVA cells. The fact that both isoleucine and methionine are large hydrophobic amino acids, however suggests that this is unlikely. Clearly, this is another area that requires further investigation.
Perhaps a more likely explanation for the differing results observed for pSFKL and pKUN is that the Kunjin virus sequences incorporated into pKUN initiate the amplification of transgene transcribed mRNA in the cytoplasm of transfected cells [375]. This mRNA amplification would be expected to lead to an increased production of protein and therefore an increased presentation of SIINFEKL on MHC Class I in pKUN transfected cells. The use of pKUN also has advantages over pSFKL in terms of future applications of p9-Flg-lipoplexes. pKUN contains a polyepitope transgene that, in addition to SIINFEKL, encodes peptides capable of inducing CTL responses against several viruses (including the influenza virus) as well as the parasite P. berghei [376, 377]. Therefore, p9-Flg-lipoplexes containing pKUN could potentially be used to vaccinate against multiple pathogens.

In conclusion, this chapter presents results on the anti-tumour effect of targeting the delivery of antigen-encoding pDNA lipoplexes to specific cells in vivo, utilising targeting peptides grafted on to the lipoplexes via incorporated NTA₃-DTDA. While delivery of pDNA to the tumour vasculature did not result in the inhibition of tumour growth after injection of activated OT-I cells, the targeting of pDNA to APCs resulted in a potent inhibition of B16-OVA tumour growth and metastasis in the lungs of mice. This finding indicates that lipoplexes containing NTA₃-DTDA and grafted with peptide are not only useful for targeted transfection in in vitro applications, but also exhibit properties required to elicit transfection/expression of the pDNA in specific cells in vivo.
Chapter Eight

Discussion
8.1 Introduction

Liposomal formulations of chemotherapeutic compounds have previously been developed in order to minimise drug accumulation in sensitive tissues such as the heart and bone marrow while maintaining the anti-tumour efficacy of the free drug. One of the advantages of liposomes is their ability to incorporate a wide range of different compounds, and liposomes containing nucleic acids such as siRNA and pDNA have the potential to be used for cancer gene therapy. Evidence suggests that the active targeting of liposomes to tumour cells or tumour-associated cells can increase the anti-tumour efficacy of liposomes [123]. In order to attach a targeting ligand onto the surface of a liposome, however, a linker molecule is needed that is stable and able to conjugate a range of different ligands.

The chelator lipid NTA₃-DTDA has previously enabled the targeting of liposome-encapsulated antigens to T cells and dendritic cells in vivo by providing an anchor for the attachment of His-tagged targeting moieties onto the surface of liposomes [22, 23]. The work presented in this thesis explores some alternate uses for NTA₃-DTDA, specifically to target the delivery of liposomes containing either nucleic acids or the cytotoxic drug doxorubicin to cells. Methods are developed for the targeted delivery of siRNA and pDNA based on liposome formulations consisting of NTA₃-DTDA, the ionisable lipid DODAP and additional carrier lipids. When incubated with cells in vitro, increased gene knockdown (for siRNA-lipoplexes) or higher transgene expression (for pDNA-lipoplexes) are observed for targeted formulations compared with non-targeted controls. A novel approach for preparing targeted doxorubicin liposomes is also described in this work, where NTA₃-DTDA is
incorporated into commercially available doxorubicin-liposomes (Caelyx). When targeted to the tumour vasculature with the peptide p46-RGD, 3NTA-Caelyx is shown to have an improved anti-tumour efficacy compared to controls. Finally, the in vivo use of targeted pDNA-lipoplexes is tested and the results indicate that the targeted delivery of OVA epitope-encoding transgenes to APCs can lead to the immunological rejection of B16-OVA tumour cells. Based on these results, the chelator lipid NTA3-DTDA is shown to be an effective means to engraft targeting peptides onto liposomes for targeted delivery of therapeutic compounds to cells for potential cancer therapy.

8.2 Overcoming in vivo barriers by using peptide-engrafted NTA3-DTDA liposomes

In order to reach the specific cell type of interest, systemically administered liposomes must overcome several physiological and anatomical barriers. These barriers include destabilisation by serum proteins, elimination from the bloodstream by phagocytes in the spleen and liver, and non-specific distribution [103]. Upon reaching the target cell, the liposomal cargo must then cross a series of cellular membranes, in particular the plasma membrane, the endosomal/lysosomal membrane and in some cases the nuclear membrane as well. In this thesis, liposomes were prepared with these barriers in mind, resulting in the production of serum-stable nanoparticles that exhibit increased accumulation in specific sites of interest when injected in vivo (Chapters 5-7). In addition, in vitro experiments indicated that, depending on the targeting peptide used, NTA3-DTDA liposomes can be actively internalised by cells (see Figure 3.4, Supplementary Figure 3.2, Figure 4.5). In certain cells, internalisation of targeted lipoplexes led to high levels of transgene expression or potent induction of RNAi,
particularly when the lipid and peptide components of the lipoplexes were optimised in order to promote endosomal release of internalised nucleic acids (see Figure 3.5 and Figures 4.7-4.9).

8.2.1 Preparation of serum-stable liposomal carriers of nucleic acids and cytotoxic drugs

Directly after intravenous administration, liposomes are exposed to an environment that is not conducive to efficient delivery of therapeutic compounds. Instability caused by physiological temperature (37 °C) and a high concentration of proteins in the blood can prevent targeting of liposomes to the cells of interest. Three components of a targeted liposome are particularly sensitive to in vivo conditions: the therapeutic cargo, the lipids that make up the liposome and the linker between the liposome and the targeting moiety.

The ability to protect incorporated nucleic acids from nuclease degradation is a key requirement of lipoplexes prepared for in vivo applications. In Chapter 3, siRNA-lipoplexes prepared from Mix-5 were incubated with RNase A to assess the nuclease protection afforded by liposomal incorporation of siRNA. As shown in Figure 3.3, approximately 50% of the siRNA added to a solution of Mix-5 remained intact after extensive RNase A treatment. Comparable results were observed upon DNase treatment of pDNA-lipoplexes prepared from Mix-13 (Supplementary Figure 4.3). The mechanism for this protection is based on the ionisable nature of the DODAP lipids present in Mix-5 and Mix-13 (Table 2.2). Thus, when the liposomal formulations are acidified they become cationic and are able to complex with negatively charged nucleic acids. In Chapter 4, this complex formation is shown to interfere with the
intercalation of ethidium bromide with pDNA (Table 4.1), therefore the DODAP-containing liposomes used are able to shield nucleic acids from extraliposomal components. While cationic lipids such as DOTAP are also able to protect nucleic acids from nucleases, the use of DODAP in the targeted lipoplexes developed in this work enables the final complexes to be of overall near-neutral electric charge (see Table 3.2 and Table 4.2).

Liposome stability in serum is primarily determined by the nature of the lipids utilised in the preparation of the liposomes, in particular the phase transition temperature (T_m) of the lipids [43]. Commercial liposome preparations such as Caelyx® and DaunoXome® typically contain high amounts of lipids such as HSPC and DSPC which have a T_m well above 37 °C, and so form a rigid bilayer at body temperature, particularly in combination with cholesterol. In comparison, the liposome formulations Mix-5, Mix-6, Mix-13 and Mix-14 (see Table 2.2), are primarily composed of lipids that have phase transition temperatures below 37 °C. However, despite this, the results presented in Chapters 3 and 4 indicate that both siRNA-lipoplexes and pDNA-lipoplexes are largely unaffected in their capacity to deliver nucleic acids to cells by a 48 h incubation in 50% serum (see Supplementary Figure 3.5 and Supplementary Figure 4.3). That said, the addition of the fusogenic peptide diINF-7 and the DNase I inhibitor 6His-ID2 to Mix-13 did lead to a significant reduction in the transfection efficiency of the resulting pDNA-lipoplexes when incubated in serum for ≥24 h (Supplementary Figure 4.3). This reduction in serum stability due to peptide incorporation was the primary reason that diINF-7 and 6His-ID2 were not included in the pDNA-lipoplexes prepared for the in vivo experiments in Chapter 7.
For targeted liposomes, the stability of the linkage between liposome and targeting moiety is also critical and the in vivo stability of the chelation linkage between Ni-NTA and His-tagged molecules has been queried in several papers. Recently, work carried out by Platt et al. indicated that liposomes containing mono- and tri-NTA lipids are vulnerable to the loss of engrafted 6His-proteins when injected in vivo [378]. The authors noted a rapid release of engrafted proteins from NTA-liposomes upon i.v. administration, leading to similar circulation half-lives for both liposome-engrafted protein and free protein. The authors therefore concluded that metal chelation technology in its current form is not useful for in vivo applications. It is clear from the work presented in this thesis, however, that this is not the case as histidine-tagged targeting peptides could increase the accumulation of NTA₃-DTDA liposomes in specific tissues (see Figure 5.4, Figure 6.3, Figure 7.1 and Figure 7.4) leading to enhanced therapeutic effects over non-targeted liposomes (see Figure 5.5, Figure 6.7 and Figure 7.6). While the association of His-tagged targeting peptides to NTA₃-DTDA liposomes under in vivo conditions was not directly assessed in the present work, it is known that the stability of the chelating linkage is dependent on the size of the 3D structure of the His-tagged protein, especially for large proteins with a 6His-tag [379]. The present work employed His-tagged peptides which are \( \sim 50 \times \) smaller and therefore less likely to interfere sterically with the formation of the chelation linkage compared to larger His-tagged proteins. It is most likely, therefore, that the inclusion of 12His-tags rather than 6His-tags in the targeting peptides used in the present work promoted an increased in vivo stability of the chelation linkage between the associated or engrafted peptide and the NTA₃-DTDA. This can be confirmed in future work by comparing the blood retention of radiolabelled 12His-tagged peptides with or without engraftment onto NTA₃-DTDA liposomes.
8.2.2 Preparation of liposomes that promote tumour targeting and accumulation in tumours

In order to reach tumour cells, liposomes must traverse from the site of injection through first-pass organs such as the lungs, spleen and liver [380]. These organs, particularly the phagocytic cells in the liver and spleen, are the main obstacles to the tumour accumulation of liposomes. However, the rate at which phagocytic uptake occurs can be reduced through the use of PEGylated liposomes, and each liposomal formulation in this thesis contains PEG-conjugated lipids for this precise reason (see Table 2.2). However, the negative influence of PEGylation on the targeting ability of NTA₃-DTDA liposomes is a recurring theme in this work. In Chapter 4, the transfection efficiency of both HEK-293 cells (Figure 4.3) and HepG2 cells (Supplementary Figure 4.2) were seen to be inversely proportional to the PEG-lipid content of pDNA-lipoplexes incubated with the cells. In addition, in Chapter 6 the tumour accumulation of p24-KDR liposomes was significantly less for liposomes containing 5 mol% PE-PEG₂₀₀₀ compared to liposomes containing 10 mol% PE-PEG₇₅₀ (Figure 6.3). Despite this potential for PEG-lipids to interfere with the cell binding ability of liposome-engrafted peptides, both in vitro and in vivo targeting of PEGylated liposomes to cells is observed in this work. This is somewhat surprising since nearly all targeted liposome formulations published in recent years involve the attachment of a targeting ligand to the distal end of the PEG chains in PEG-lipids in order to avoid steric inhibition. The work in this thesis, particularly the in vivo anti-tumour effects seen in Chapters 5 and 7, indicate that at least for some peptides it is not necessary to avoid attaching targeting ligands directly onto the surface of liposomes. It could well be that having an extension or spacer between the hydrophilic region and 3NTA headgroup of the chelator lipid [237], coupled with a spacer between the His-
could well be that having an extension or spacer between the hydrophilic region and 3NTA headgroup of the chelator lipid [237], coupled with a spacer between the His-tag and targeting moiety in the targeting peptides used in the present work (Table 2.1), is sufficient to overcome steric barriers for the binding of engrafted liposomes to receptors on target cells. Thus, due to the identified disadvantages of PEGylated liposomes, it may in fact be preferable to attach targeting ligands onto liposomes independently of PEG-lipids when using this approach for targeting liposomes.

Although not investigated in this thesis, a major difficulty associated with the \textit{in vivo} use of PEGylated liposomes is the rapid clearance of a second dose of liposomes, also known as the accelerated blood clearance (ABC) phenomenon. While an initial dose of PEGylated liposomes can remain in the circulation for several days, it has been observed that a subsequent dose 4-6 days later is almost completely eliminated from the circulation after only 4 h [381]. This effect is likely to be mediated by IgM, which is secreted by B cells resident in the spleen following contact with PEGylated liposomes [382]. If present in high amounts in the serum, IgM will bind to the PEG chains associated with administering a second dose of liposomes, thereby activating the complement system, and leading to opsonisation and phagocytosis by Kupffer cells in the liver. Typically, the ABC phenomenon is lipid dose dependent, with higher doses (>1 \mu mol lipid/kg) resulting in less obvious clearance of liposomes [383]. However, this has been reported not to be the case for PEGylated lipoplexes, where no decline in clearance is observed upon increasing the dose, particularly if pDNA that contains CpG motifs is used [384]. Most of the results in Chapter 7 were obtained using multiple injections of pDNA-lipoplexes and the efficacy of the later injections in these experiments could well have been comprised by the ABC
phenomenon. Studies by Judge et al. indicated that liposomes containing PEG-lipids that dissociate more readily from the lipid bilayer in vivo are less likely to induce IgM production [385]. Future experiments should therefore compare the therapeutic effectiveness of pDNA-lipoplexes prepared from Mix-13 and Mix-14, so as to contrast the use of lipoplexes containing Cer-PEG\textsubscript{750} and PE-PEG\textsubscript{2000}.

A large portion of the work in this thesis has been focussed on the active targeting of liposomes for tumour therapy. As reviewed in Chapter 1, many examples in the literature exist where the targeting of drug-loaded liposomes to tumour cells or tumour vasculature cells has resulted in a reduction of tumour growth in mice as well as an increase in the probability of survival. However, there remains some conjecture over the exact mechanism behind this increased therapeutic efficacy of targeted liposomes. Some reports indicate that targeting can increase the tumour accumulation of liposomes [188, 194, 214], while other reports suggest that both non-targeted and targeted liposomes accumulate in tumours to the same extent but differ in the extent to which they are taken up intracellularly [176, 386, 387]. The results in Chapters 5-7 indicate that targeting peptides can increase the accumulation of liposomes in tumours, and while this is not always accompanied by an increased anti-tumour effect, no examples were observed in this thesis where a tumour-targeted liposome formulation was more therapeutically effective than a non-targeted formulation in the absence of an increased tumour accumulation. It is likely that in addition to increasing the accumulation of liposomes in tumours, the targeting peptides used in this work also increase cellular uptake of liposomes in vivo, as was observed when using some peptides in in vitro experiments (Figure 3.4, Supplementary Figure 3.2, Figure 4.5). However, this phenomenon was not explored or measured directly.
While the results in Chapters 5-7 indicate that peptide-engrafted liposomes can accumulate in tumours to a greater extent than non-targeted liposomes, on average only 5-6 %ID/g was detected in tumours 24 h after i.v. injection of tumour-targeted liposomes. In contrast, liposomes with altered lipid compositions have been shown by others to have dramatically different biodistribution patterns. Notably, liposomes containing 20 mol% PE-PEG\textsubscript{2000}, have been shown to accumulate to extraordinary extents in the tumour (70-80 %ID/g) albeit with little difference between targeted and non-targeted formulations [388]. This level of tumour accumulation resulted in a significant knockdown of gene expression in the tumour when anti-luciferase siRNA was loaded into the liposomes [389], and a significantly reduced number of lung metastases when a combination of anti-\textit{MDM2}/\textit{MYC}/\textit{VEGF} siRNA was used [193]. Interestingly such siRNA-lipoplexes had a diameter of approx. 120-150 nm and so are significantly smaller than the likely size of the pDNA-lipoplexes prepared in Chapter 7 (~255 nm, see Table 4.2), but of similar size to the peptide-engrafted liposomes administered in Chapters 5-7 (see Table 5.1). Therefore small particle size is not the primary reason for the elevated tumour accumulation of these highly PEGylated liposomes/lipoplexes. Further investigation of highly PEGylated liposomes is clearly required. However, based on the results presented in this thesis, is likely that incorporation of 20 mol% PE-PEG\textsubscript{2000} would result in minimal cell uptake and interference with targeting via peptide not anchored on the distal end of PEG chains. It is also likely that extensive elimination of secondary doses would occur due to the ABC effect.

With the relatively low level of site-specific tumour accumulation of targeted liposomes achieved in this thesis (see Figure 5.4, Figure 6.3 and Figure 7.1) the choice
of cells that are targeted plays a critical role in determining the anti-tumour efficacy of administered liposomes. For tumour therapy, target cells can vary in terms of accessibility, vulnerability to treatment and the ability to amplify a targeted effect. The three populations of cells investigated in this thesis for targeted tumour therapy are the tumour cells themselves (with p47-LyP-1), the cells of the tumour vasculature (with p46-RGD, p15-RGR, p24-NRP-1, and p39-Flt-1) and antigen-presenting cells (APCs) (with p9-Flg). Of these approaches, the results indicate that targeting to APCs is the most likely to give a significant anti-tumour effect (compare Figure 7.2 and Figure 7.6). Although these cells are typically difficult to transfect, APCs play a crucial role in the regulation of the immune system and so the manipulation of only a few cells with nucleic acids can have an amplified therapeutic impact. Coupled with their high accessibility in the spleen and liver, potent anti-tumour responses can therefore be achieved with low total doses of formulations targeted to APCs (25 μg pDNA per mouse), as indicated in Chapter 7.

8.2.3 Intracellular barriers faced by targeted liposomes

Upon reaching a cell of interest, targeted liposomes must be able to make their incorporated compounds therapeutically available to the cell, which invariably involves the uptake of the liposome into the cell. However, not all ligand-receptor interactions result in internalisation, so therefore the choice of cell epitope to which liposomes are targeted is crucial. This was demonstrated in Chapter 3, where A20 cells, a cell line that displayed very high binding capacity for CD4-engrafted siRNA-lipoplexes (see Figure 3.1), did not appear to internalise surface-bound lipoplexes (Figure 3.4). In contrast, internalisation of lipoplexes was observed in DC2.4 cells (with p12-TNFR), HepG2 cells (with p25-Flt-1), RAW264.7 cells (with p28-TLR2),
DC-293 cells (with p293) and HEK-293 cells (with T2) (see Figure 3.1, Supplementary Figure 3.2 and Figure 4.5).

Once a targeted liposome binds to an internalising cell receptor this can trigger endocytosis of the liposome, most likely through the clathrin-mediated endocytosis pathway [147]. However, the endpoint of this pathway is degradation in the lysosome, therefore compounds formulated in liposomes must be able to escape from endosomes prior to their maturation into lysosomes in order to avoid degradation. While some molecules (e.g. siRNA) are active in the cytoplasm, other molecules such as doxorubicin and pDNA must also cross the nuclear envelope in order to have an effect on target cells. Of the therapeutic compounds utilised in this thesis, doxorubicin appears to be the most adept at overcoming these intracellular barriers. This was indicated by the high levels of cell death observed after incubation of p15-RGR-Caelyx with NIH-3T3 cells (Figure 5.3). This cytotoxicity occurred despite the lack of any components in the doxorubicin liposomes that actively promote the release of doxorubicin into the cytoplasm. The reason for this is most probably related to the fact that doxorubicin is a relatively small molecule (543 Da) which greatly facilitates its release from the endosome and transport across the nuclear membrane. Also, it has been suggested that doxorubicin may be resistant to lysosomal degradation [390], thus allowing enough time for passive diffusion of the drug from the lysosome into the cytoplasm. Furthermore, the nuclear transport of cytoplasmic doxorubicin has been proposed to occur through the formation of a complex with proteasomes [391]. These attributes/characteristics of liposomal doxorubicin contrast those of lipoplexes containing pDNA. Thus the much larger size and structure of pDNA (2.6 MDa for a 4
kb plasmid), and even condensed exhibiting a particle size of ~30 nm [392], is not readily conducive to efficient release from endosomes for transport into the nucleus.

The results in Chapters 3 and 4 indicate that successful transfection of nucleic acids using targeted lipoplexes is dependent on the nature/composition of the liposomal formulation used, the particular nucleic acid delivered, and the nature of the target cells. For example, siRNA-lipoplexes prepared from Mix-5 were able to induce targeted RNAi in DC-293 cells but not DC2.4 cells, despite efficient internalisation of siRNA in both cell lines (compare Figure 3.4 and Supplementary Figure 3.2 with Figures 3.5 and 3.6). Furthermore, pDNA-lipoplexes prepared from Mix-5 were only able to induce low levels of transgene expression in HEK-293 cells (approx. 12% cells expressing EGFP after 48 h) when targeted with the T2 peptide (Figure 4.3). However, the transfection efficiency of both siRNA- and pDNA-lipoplexes were significantly increased by optimisation of the liposome formulation and through the inclusion of peptide components such as the fusogenic peptide diINF-7 and the DNase II inhibitor 6His-ID2 (see Figure 3.6 and Figures 4.6 and 4.7). It is likely that these modifications increased the amount of internalised siRNA or pDNA that was able to escape from the endosomal pathway into the cytoplasm.

It is important to note that the highest in vitro transfection efficiency observed after incubation of cells with targeted pDNA-lipoplexes was approximately 50% (Figure 4.7 and Figure 4.8). This indicates that a major barrier remains in the transfer of pDNA into the nucleus of these cells and the literature suggests that this barrier is likely to be the nuclear membrane [393, 394]. While this may be dependent on particle size (see above), it is clear that for the cells that were successfully transfected with
pEGFP-N1 in Chapter 5, since the nuclear membrane disassembles during the M phase of cell division [395], it is likely that nuclear entry of pDNA occurred during cell division. However, this process is inefficient; it has previously been reported that only 0.1% of pDNA reaches the nucleus following microinjection of naked pDNA into the cytoplasm of cells [396]. Plasmid entry into the nucleus is limited even further in post-mitotic and quiescent cells, which represent the majority of cells in vivo [103]. There is scope, therefore, for further improvement of the pDNA-lipoplexes prepared in this thesis by increasing the nuclear uptake of delivered pDNA. A proven method for increasing nuclear uptake is through the complexation of pDNA with peptides containing nuclear localisation signals (NLS) [397-399]. These stretches of amino acids bind to proteins such as importins and facilitate transfer of peptide-bound pDNA through the nuclear pore. In future studies, NLS peptides could potentially be synthesised and complexed with pDNA prior to mixing with NTA₃-DTDA liposomes for targeted delivery to the nucleus.

### 8.3 Future Perspectives

Despite the numerous preclinical studies that have demonstrated the advantages of targeting drug-loaded liposomes for cancer therapy, as of yet no targeted liposome preparations have successfully emerged from clinical trials. In this thesis, three separate liposome preparations, targeted by engrafting His-tagged peptides onto the liposomal surface, were shown through in vivo studies to have the potential to be developed as cancer treatments. Firstly, 3NTA-Caelyx, prepared by incubation of NTA₃-DTDA with the commercial doxorubicin-liposome drug Caelyx®, was shown to significantly reduce the rate of growth of subcutaneous tumours in mice when targeted
with the δv-integrin-binding peptide p46-RGD (Figure 5.5). Similar results were observed when doxorubicin liposomes were prepared so as to contain PE-PEG750 instead of the PE-PEG2000 in Caelyx®, and the NTA3-DTDA-containing liposomes engrafted with the neuropilin-1-binding peptide p24-NRP-1 (Figure 6.7). Finally, administration of NTA3-DTDA-containing lipoplexes, prepared from pDNA encoding the OVA epitope SIINFEKL and targeted with the TLR5-binding peptide p9-Flg, prevented the establishment of B16-OVA tumour metastases in the lungs of treated mice (Figure 7.6). In each of these liposome preparations, the engraftment of the targeting peptide significantly increased the anti-tumour efficacy of the treatment, compared to the non-targeted controls.

The approach described in Chapter 5 for the preparation of 3NTA-Caelyx is perhaps the most easily translated to clinical use. As Caelyx® has been registered for treatment of Kaposi's sarcoma, ovarian cancer and multiple myeloma, its production is already suited to the large-scale good manufacturing practises required for human clinical trials. While the pharmacokinetics of peptide-engrafted 3NTA-Caelyx in humans is likely to be different from that of Caelyx®, the clinical testing of a modified version of a drug that has already been approved for therapeutic use is likely to progress more easily through approval processes, compared to a completely new preparation. An important factor that must be addressed before progression to clinical trials is the ligand used to target 3NTA-Caelyx to tumours. The treatment of C57BL/6 mice with p46-RGD-Caelyx, while clearly more effective in comparison to non-targeted 3NTA-Caelyx, resulted in only a slowing of tumour growth and not complete tumour regression (Figure 5.5). This modest therapeutic effect is likely to be a reflection on the targeting peptide used rather than the liposomal formulation but the
possibility of the ABC phenomenon needs to be investigated. In recent years, many novel tumour-targeting peptides have been identified through the use of phage display techniques, and these could potentially be used to target 3NTA-Caelyx for enhanced therapeutic effect. Some of these peptide sequences (p24-NRP-1, p39-Flt-1 and p47-LyP-1) were assessed in Chapter 6, but none of these, when engrafted onto 3NTA-Caelyx, improved on the anti-tumour effect of 3NTA-Caelyx (Figure 6.1). While most in vivo phage display work has been carried out in mice, a report by Arap et al. described the first in vivo screening of a random peptide library in a human patient [400]. More human-specific work such as this could well enable the development of a peptide-engrafted 3NTA-Caelyx preparation for potent and targeted cancer therapy.

In contrast to 3NTA-Caelyx, the use of p9-Flg pDNA-lipoplexes as outlined in Chapter 7 would require more extensive development for clinical applications. While the administration of p9-Flg pDNA-lipoplexes prevented the growth of B16-OVA cells, OVA is a model antigen derived from chickens and is not expressed in human tumours. Any future application of this particular approach for cancer therapy would require the use of transgenes encoding actual tumour associated antigens such as HER2 in breast cancer [401] or PSA in prostate cancer [402]. Alternatively, the Mix-14 liposome formulation used to prepare pDNA-lipoplexes in Chapter 7 could be utilised to prepare siRNA-lipoplexes. Given the inherent difficulty in achieving targeted expression of transgenes in vivo, it would seem logical to remove the barrier of nuclear delivery and utilise siRNA to knockdown gene expression for enhanced tumour therapy. In one potential application of p9-Flg siRNA-lipoplexes, anti-SOCS-1 siRNA could be used to enhance immunorecognition of tumours, since evidence
suggests that the \textit{SOCS-1} gene can restrict the ability of APCs to respond to antigens expressed on cancer cells [403].

Preclinical and clinical studies indicate that combination treatments involving targeted agents and standard chemotherapy are more likely to give better anti-tumour responses than single therapies alone [404]. For example, the anti-VEGF mAb Bevacizumab, developed as an inhibitor of angiogenesis, has only modest effects on patient survival on its own, but can improve the anti-tumour efficacy of other cytotoxic agents when used in combination therapies [405]. Consequently, a potential application of the targeted doxorubicin-liposomes and lipoplexes developed in this work could be in combination therapy. For example, the targeting of 3NTA-Caelyx to tumour cells with p47-LyP-1 could be combined with vasculature-targeted p46-RGD-Caelyx for dual therapy. Alternatively, both 3NTA-Caelyx and anti-\textit{bcl2} siRNA-lipoplexes could be targeted with the same tumour-specific ligand in order to enhance the cytotoxic effects of doxorubicin by knocking down the expression of the anti-apoptosis gene \textit{bcl2}.

\section*{8.4 Conclusion}

This thesis explored the use of the chelator lipid NTA\textsubscript{3}-DTDA to prepare liposomes with the capacity to target nucleic acids and cytotoxic drugs to cells for tumour therapy. The results demonstrate that lipoplexes containing NTA\textsubscript{3}-DTDA and siRNA or pDNA can be prepared that are serum-stable, of near neutral surface charge and approx. 200 nm in diameter. These lipoplexes can be engrafted with His-tagged targeting peptides, allowing the lipoplexes to interact specifically with receptors
expressed on the surface of target cells. Similarly, NTA₃-DTDA was shown to be
easily incorporated into doxorubicin-loaded liposomes to enable targeting to cells.
Importantly, NTA₃-DTDA liposomes can be used to deliver doxorubicin and pDNA to
cells in vivo, inducing potent anti-tumour effects when targeted to either the tumour
vasculature or antigen-presenting cells. The approach of using chelator lipids for
peptide engraftment onto liposomes represents a simple and adaptable alternative to
standard covalent bonding of targeting ligands to liposomes. It is envisaged that the
liposomal formulations established in this thesis could be developed for therapeutic
use, particularly if targeted with ligands with enhanced specificity for target tissues
such as tumours or key cells involved in regulating immune function.
REFERENCES


[345] V.P. Torchilin, R. Rammohan, V. Weissig, T.S. Levchenko, TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low