

Novel Gadolinium Agents for Molecular Imaging

Morag Helen Oliver

Imperial College London

A thesis submitted for the degree of

Doctor of Philosophy of the University of London

and for the

Dipolma of Membership of Imperial College London

December 2004

Acknowledgments

First of all I would like to thank my supervisors, Prof. Andrew Miller and Dr. Michael Jorgensen. I must thank Andrew for accepting me into his group and for his constant encouragement. Mike has been a source of endless support, ideas, help, and enthusiasm. He has taught me a great deal and I owe him a lot. I must also thank IC-Vec Ltd. who have provided full financial support for this work

The MRI analysis would not have been possible without the members of Dr. Jimmy Bell's research group at the Biological Imaging Centre, Hammersmith Hospital. A big thanks in particular to Jimmy and Amy, who have done a lot of the analysis. Po-Wah So was also involved in the earlier stages of the project and I wish to thank her for her help as well.

The liposome work and subsequent biological analysis would not have been possible without the help of IC-Vec Ltd., particularly Eric Perouzel, Michael Keller, Annabelle Caussin and Sebastian Spagnou. I must thank them all for their patience and guidance.

I have had a good time and learnt a lot over the past 3 years and am extremely grateful to the many friends I have made in that time. There are too many people to name individually but a special thank you to Mike J, Mike K, Eric, Ayesha and Annabelle. Thanks also to my proof readers Michael Jorgensen, Eric Perouzel, Arnaud Cheguillaume, Ayesha Ahmed, Jimmy Bell, Amy Herlihy and Steve Foreman.

Last and by no means least, is the most sincere thank you of all, to Steve. Thank you very much for your continuous support, understanding and love. Really thank you, it has meant a lot to me, you never know, I might make a domestic goddess yet!

Abstract

Molecular imaging is rapidly emerging as an innovative approach to drug discovery and development as well as a method for disease monitoring. Magnetic Resonance Imaging (MRI) and contrast agent chemistry (iron / gadolinium) are increasingly becoming valuable tools for this field. To date research in molecular imaging has generally focused on three key areas; imaging gene therapy, imaging molecular markers and imaging cell tracking. Given this, liposomes present an interesting opportunity for molecular imaging studies as they are established vectors for mediating gene therapy, can be targeted to the molecular markers of specific cell types or organs, and can easily traverse cell membranes (a key requirement for cell labelling and subsequent tracking).

To this end a series of MRI visible gadolinium lipids were designed and synthesised to enable production of MRI visible liposomes. The design of these lipids was based on clinically used contrast agents ($\text{Gd}(\text{DTPA})^{2-}$ or $\text{Gd}(\text{DOTA})^-$) conjugated to biologically available lipids (cholesterol and phospholipids). MRI analysis of the new gadolinium lipids showed them to be highly effective MRI contrast agents, reducing the relaxation of the surrounding solvents to 10-15% of their original values, comparable to clinically used agents. Incorporation of the gadolinium lipids into liposomes resulted in the production of MRI active particles.

These new gadolinium liposomes were first examined as cell labelling vehicles. Using a series of cell uptake and cytotoxicity assays, an optimised liposome formulation for cell uptake (labelling) was determined. *In vitro* MRI analysis of the labelled cells showed them to be highly MRI active, establishing this strategy as a feasible technique for cell labelling and subsequent tracking studies. Intramuscular injection of these lipids into mice resulted in good MRI contrast which persisted for up to 56 h, demonstrating the new gadolinium lipids as viable tools for *in vivo* tracking of liposomes.

In an effort to improve on this system, an alternative gadolinium liposome labelling strategy was sought. Borrowing from the principles of bioconjugate chemistry, a post-coating liposome strategy was developed. Here liposomes were made containing a novel cholesterol-based lipid designed to react chemoselectively with an isothiocyanate functionalised gadolinium chelate. Analysis showed that these new gadolinium liposomes were also highly effective contrast agents.

Table of Contents

Acknowledgments	2
Abstract	3
Table of Contents	4
List of Figures	8
List of Schemes	10
List of Tables	11
List of Equations	11
Abbreviations	12
Chapter One: Introduction	16
1.1 Molecular Imaging	17
Imaging Gene Therapy	17
Imaging Molecular Markers	21
Imaging of Cell Tracking	23
Review of Imaging Modalities	25
1.2 MRI	28
Longitudinal Relaxation (Spin-Lattice Relaxation, T_1)	30
Transverse Relaxation (Spin-Spin Relaxation, T_2)	31
Spatial Encoding	32
Pulse Sequences	33
Contrast Agents	34
Gadolinium-Based Contrast Agents	35
Chemical Mechanisms of Relaxation	36
Gadolinium-Based Contrast Agents for Molecular Imaging	38
1.3 Liposomes	40
Liposomes and Drug Delivery	41
Liposomes and Nucleic Acid Delivery	43
Labelling and Imaging Liposomes	45
1.4 Thesis Synopsis	48
Chapter Two: Synthesis and MRI Analysis of Novel Gadolinium Lipids	50
2.1 Liposomes and Molecular Imaging	51
Gadolinium Lipids	52
2.2 Synthesis of Gd.DOTA Lipids	55
Retrosynthetic Analysis of Gd.DOTA.Chol and Gd.DOTA.DSPE	55
Synthesis of Cyclen	57
Mono- <i>N</i> -Functionalisation of Cyclen	59

DOTA.NHS.Ester	61
Synthesis of DOTA	62
Synthesis of Gd.DOTA.Chol (6)	64
Synthesis of Gd.DOTA.DSPE (7)	67
2.3 Synthesis of Gd.DTPA.Lipids	70
Retrosynthesis of Gd.DTPA.Chol (8)	70
Synthesis of <i>t</i> -Butyl Selectively Protected DTPA (31)	71
Synthesis of Gd.DTPA.Chol (8)	72
2.4 MRI Analysis of Gd.Lipids	75
2.5 Solid Phase Methodologies	77
Selective <i>N</i> -Functionalisation of Azacrowns	79
Synthesis of selectively protected polyamine polyacetic acid derivatives	82
2.6 Chapter Conclusions	84
Chapter Three: Synthesis of Cationic Lipids	85
3.1 Cationic Lipids	86
3.2 Synthesis of CDAN-DOGS	91
Retrosynthetic Analysis of CDAN-DOGS	91
Synthesis of Head Group	92
Synthesis of the Lipophilic Tail	97
Synthesis of the Lipid	97
3.3 Synthesis of CDAN	99
Solid Phase Synthesis of CDAN	102
3.4 Chapter Conclusions	105
Chapter Four: Biological Analysis of Gadolinium Lipids and Liposomes. Cell Labelling and <i>In Vivo</i> Studies	106
4.1 Gadolinium-Based Cell Labelling	107
Ascertaining an Optimised Cationic Liposome Formulation	110
Cytotoxicity of Optimised Liposome Formulation	115
MRI Analysis of Labelled Cells	118
Mechanism of Cell Labelling	120
Transfection of Plasmid DNA	123
Stability of Gadolinium Liposome and LD's	125
Conclusion	127
4.2 <i>In Vivo</i> Studies	127
Intramuscular Injection of Lipids	128
Conclusion	130
Chapter Five: Gadolinium Post-Coating of Liposomes	131

5.1	Functionalising Liposomes	132
5.2	Synthesis of Gd.SCN.Bn.DTPA	137
	Retrosynthesis of Gd.SCN.Bn.DTPA (115)	137
	Synthesis of SCN.Bn.DTPA	139
	Gadolinium Complexation to SCN.Bn.DTPA	142
5.3	Synthesis of Chol.PEG ₁₁ .NH ₂	144
	Retrosynthesis of Chol.PEG ₁₁ .NH ₂	144
	Synthesis of Chol.PEG ₁₁ .NH ₂	146
5.4	Conjugation Chemistry	147
5.5	Gadolinium Post Coating of Liposomes	150
	MRI Analysis of Post-Coated Liposomes	156
5.6	Chapter Conclusions	157
Chapter Six: Conclusions and Future Directions		158
Chapter Seven: Experimental		163
7.1	General Information	164
7.2	General Procedures	165
7.3	Synthesis of First Generation Gadolinium Lipids	166
7.4	Synthesis of Selectively <i>N</i> -Functionalised Aza-Crowns	181
7.5	Synthesis of Selectively Protected Polyamine Polyacetic Acid Derivatives	184
7.6	Synthesis of Selectively Protected DTPA and EDTA Derivatives	187
7.7	Synthesis of CDAN-DOGS	191
7.8	Solid Phase Synthesis of Cholesterol-Based Polyamine Lipids	199
7.9	Synthesis of Compounds for Gadolinium Post-Coating of Liposomes	204
7.10	MRI Analysis of Gd.Lipids	215
7.11	General Liposome Preparation	215
7.12	<i>In vivo</i> Experiments	215
7.13	Cell labelling Experiments	216
7.14	Gadolinium Post coating of Liposomes	220
Chapter Eight: References		221
Chapter Nine: Appendix		230
9.1	Lipids	231
	Lipid Shape and Consequences for Packing	231
	Chain Melting Temperature (T_c)	232
	Cholesterol	233
	Phospholipids	234
9.2	Xylenol Orange	236
	Xylenol Orange Test	237

9.3	Calculating T_1 Relaxation Times	238
9.4	Synthesis of Polyamines	239
9.5	Miscellaneous Data	240
	DOTA.Chol (27)	240
	DOTA.DSPE (28)	241
	DTPA.Chol (29)	242

List of Figures

Figure 1.1: FIAU Phosphorylation by HSV-1-tk	20
Figure 1.2: Structures Selected Radionuclide Probes for Imaging Molecular Markers	22
Figure 1.3: Behaviour of protons in an applied magnetic field	29
Figure 1.4: Longitudinal Relaxation	30
Figure 1.5: Transverse Relaxation	31
Figure 1.6: Spatial Encoding of the Signals.	32
Figure 1.7: Phase Encoding Gradients	33
Figure 1.8: Spin Echo Pulse Sequences	34
Figure 1.9: Structures of $\text{Gd}(\text{DOTA})^-$ and $\text{Gd}(\text{DTPA})^{2-}$	36
Figure 1.10: Chemical Mechanisms of Relaxation	37
Figure 1.11: Gadolinium MRI Agent with Two Inner Sphere Water Molecules	37
Figure 1.12: Selected $\text{Gd}(\text{III})$ probes for molecular imaging	39
Figure 1.13: β -Galactosidase Sensitive MRI Probe	40
Figure 1.14: General Structures of Lipids and Liposomes	41
Figure 1.15: Formation of a Lipoplex.	44
Figure 1.16: Steps of Endocytosis	44
Figure 1.17: Common Techniques for Radiolabelling liposomes	46
Figure 2.1: Selected Gadolinium Lipids	52
Figure 2.2: Target Molecules	54
Figure 2.3: Methods for Mono- <i>N</i> -Functionalisation of Cyclen	61
Figure 2.4: DOTA.NHS.Ester	61
Figure 2.5: Mass Spectrum of $\text{Gd}(\text{DOTA})^-$ (25).	64
Figure 2.6: HPLC and MS of $\text{Gd}.\text{DOTA}.\text{Chol}$ (6)	66
Figure 2.7: HPLC and MS of $\text{Gd}.\text{DOTA}.\text{DSPE}$ (7)	69
Figure 2.8: HPLC and MS of $\text{Gd}.\text{DTPA}.\text{Chol}$ (8)	75
Figure 2.9: MRI Phantom Images of $\text{Gd}.$ Lipids and Control Samples	76
Figure 2.10: Selection of compounds using azacrowns and their polyacetic acid derivatives as a skeleton	78
Figure 3.1: Cationic Lipid Target Molecules	89
Figure 3.2: Product of two concurrent Michael Additions	95
Figure 3.3: HPLC and MS of CDAN-DOGS (71)	99
Figure 3.4: Selected Structures of Symmetrically Spaced Linear Polyamines	100
Figure 3.5: Selection of Polyamine Conjugates	100
Figure 3.6: HPLC and MS of CDAN (70)	104

Figure 4.1:	Selected Gadolinium Agents for Cell Labelling	109
Figure 4.2:	Schematic Representation of Liposome Uptake Assay	110
Figure 4.3:	Cellular Uptake of Liposomes	112
Figure 4.4:	Cellular Uptake of Gadolinium Lipid	113
Figure 4.5:	Cellular Uptake of Liposomes	114
Figure 4.6:	Cellular Uptake of Gadolinium Lipid	115
Figure 4.7:	LDH Release from Cells	117
Figure 4.8:	Phantom Images of Gadolinium Liposomes and Gadolinium Labelled Cells	118
Figure 4.9:	Fluorescence Microscopy Results	121
Figure 4.10:	Selected z-Slice from Confocal Microscopy Results	122
Figure 4.11:	3D Laser Scanning Micrograph Projection of Labelled Cells	123
Figure 4.12:	Transfection of LD's	124
Figure 4.13:	LDH Release from Cell upon LD Transfection	125
Figure 4.14:	Serum Stability of Gadolinium Liposomes and LD's	126
Figure 4.15:	Images of IM Injections of Gd.DOTA.Chol into Hind Leg of Mouse	129
Figure 5.1:	Post-Coating of Liposomes	132
Figure 5.2:	First Generation Gadolinium Liposome	134
Figure 5.3:	Gadolinium Post-Coating of Liposomes	135
Figure 5.4:	Gadolinium Post-Coating Liposome Target Molecules	136
Figure 5.5:	HPLC and MS of SCN.Bn.DTPA (115)	142
Figure 5.6:	HPLC and MS of Gd.SCN.Bn.DTPA (113)	144
Figure 5.7:	HPLC and MS of Chol.PEG ₁₁ .NH ₂ (122)	147
Figure 5.8:	HPLC and MS of Conjugate (129)	150
Figure 5.9:	HPLC and MS of Liposomes before Post-Coating	152
Figure 5.10:	HPLC and MS of Liposomes after Post-Coating (SCN.Bn.DTPA)	153
Figure 5.11:	HPLC and MS of Liposomes after Post-Coating (Gd.SCN.Bn.DTPA)	154
Figure 5.12:	MRI Phantom Analysis of Gadolinium Post-Coated Liposomes	156
Figure 9.1:	Chain Melting Temperature (T_c)	232
Figure 9.2:	Structure of Cholesterol	233
Figure 9.3:	Influence of Cholesterol on Membrane Rigidity	234
Figure 9.4:	General Structure of Phospholipid	234
Figure 9.5:	Xylenol Orange	236
Figure 9.6:	Absorbance Spectrum and Corresponding Images of Xylenol Orange and Gd(III) Xylenol Orange Solutions	236
Figure 9.7:	Gd(III) Xylenol Orange Titration Curve	237
Figure 9.8:	T_1 Data for Gd(DOTA) ⁻ (25) and Gd.DOTA.Chol (6)	238

Figure 9.9: HPLC and MS of DOTA.Chol (27)	241
Figure 9.10: HPLC and MS of DOTA.DSPE (28)	242
Figure 9.11: HPLC and MS of DTPA.Chol (29)	243

List of Schemes

Scheme 2.1: Retrosynthetic Analysis of Gd.DOTA.Lipids	56
Scheme 2.2: Synthesis of Cyclen (14)	57
Scheme 2.3: Macrocyclisation Reaction	58
Scheme 2.4: Alternative Retrosynthesis of Gd.DOTA.Lipids	59
Scheme 2.5: Synthesis of 2-(2-Bromo-acetylamino)-ethyl-cholesterol (21)	59
Scheme 2.6: Conversion of ROH to RBr using Carbon Tetrabromide and Triphenylphosphine	60
Scheme 2.7: Synthesis of Gd(DOTA) ⁻ (25)	62
Scheme 2.8: Synthesis of Gd.DOTA.Chol (6).	65
Scheme 2.9: Synthesis of Gd.DOTA.DSPE (7)	67
Scheme 2.10: Retrosynthesis of Gd.DTPA.Chol (8)	70
Scheme 2.11: Synthesis of Selectively <i>t</i> -Butyl Protected DTPA (31)	71
Scheme 2.12: Synthesis of Gd.DTPA.Chol (8)	73
Scheme 2.13: General Scheme for Selective <i>N</i> -Functionalisation of Azacrowns	80
Scheme 2.14: General Scheme for Synthesis of Selectively Protected Polyamine Polyacetic Acid Derivatives	82
Scheme 3.1: Retrosynthetic Analysis of CDAN-DOGS	91
Scheme 3.2: Synthesis of Selectively Protected Azide (85)	92
Scheme 3.3: Synthesis of Polar Head Group (91) of CDAN-DOGS (71)	93
Scheme 3.4: Mechanism for Reduction of Azide to Amine	94
Scheme 3.5: First Coupling of Head (89) and Tail (74) group of CDAN-DOGS (71)	96
Scheme 3.6: Synthesis of Lipophilic Tail (74) of CDAN-DOGS (71)	97
Scheme 3.7: Synthesis of CDAN-DOGS (71).	98
Scheme 3.8: Retrosynthesis of CDAN	101
Scheme 3.9: Solid Phase Synthesis of CDAN (70)	102
Scheme 4.1: Principles of LDH Assay	116
Scheme 5.1: Retrosynthetic Analysis of Gd.SCN.Bn.DTPA (113)	138
Scheme 5.2: Synthesis of SCN.Bn.DTPA (115)	139
Scheme 5.3: Mechanism of Borane Reduction of Amides	140
Scheme 5.4: Retrosynthesis of Chol.PEG ₁₁ .NH ₂ (114)	145

Scheme 5.5:	Synthesis of Chol.PEG ₁₁ .NH ₂ (114)	146
Scheme 5.6:	Model Isothiocyanate Conjugation	148
Scheme 5.7:	Bioconjugation of SCN.Bn.DTPA (115) and Chol.PEG ₁₁ .NH ₂ (114).	149
Scheme 9.1:	Synthesis of Primary Amines	239
Scheme 9.2:	Synthesis of Secondary Amines from Primary Amines	240

List of Tables

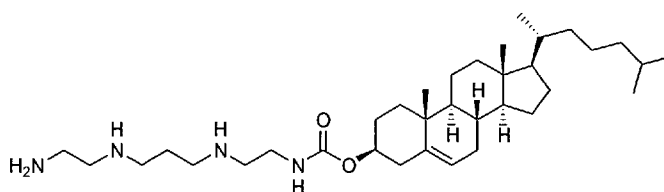
Table 1.1:	An overview of Imaging Modalities Used in Molecular Imaging	26
Table 1.2:	Selection of Liposomal Drugs	42
Table 2.1:	T ₁ Relaxation Times for Gd.Lipids and Control Samples	76
Table 2.2:	Selectively Protected Cyclic Polyamines and Azacrowns	81
Table 2.3:	Selectively Protected Polyacetate Analogues	83
Table 2.4:	Selectively protected DTPA and EDTA Derivatives	84
Table 3.1:	Selection of Commonly Used Cationic Lipids.	87
Table 3.2:	Cationic Lipids prepared <i>via</i> solid phase methodology	105
Table 4.1:	Liposome Formulations for First Round of Optimisation	111
Table 4.2:	Liposome Formulations for Second Round of Optimisation	114
Table 4.3:	T ₁ Relaxation Times for Gd.Liposomes, Gd labelled cells and appropriate controls	119
Table 4.4:	Change in Signal Intensity over Time Post IM Administration of Gd.DOTA.Chol	129
Table 5.1:	Selected and Classical Liposomal Bioconjugation Chemistries	133
Table 5.2:	T ₁ Relaxation Times for Gd-Post Coated Liposome and Appropriate Controls	156
Table 9.1:	Packing Shapes of Lipids and the Resultant Structures Formed.	231
Table 9.2:	Selected Lipid Chain Melting Temperatures	233
Table 9.3:	Properties of Phosholipids and their Effect on the Lipid Bilayer	235

List of Equations

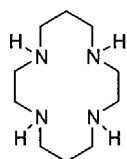
Equation 1.1:	Larmor Equation	29
Equation 9.1:	T ₁ Equation	238

Abbreviations

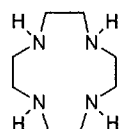
aq.	Aqueous
bp	base pair(s)
Bn	benzyl
br	broad
Boc	<i>tert</i> -butoxycarbonyl
Boc ₂ O	di- <i>tert</i> -butyl dicarbonate
cat.	catalyst/ catalytic
Cbz	benzyloxycarbonyl
CDAN	<i>N</i> ¹ -cholesteryloxycarbonyl-3,7-diaza-1,9-diaminonane



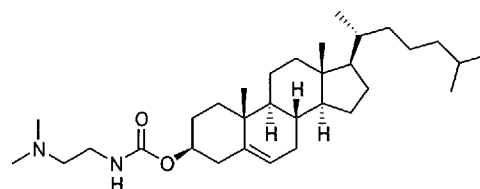
Chol	cholesterol (cholest-5- β -ol)
CI	chemical ionisation
Cyclam	1,4,8,11-tetraazacyclotetradecane



Cyclen	1,4,7,10-tetrazacyclododecane
--------	-------------------------------

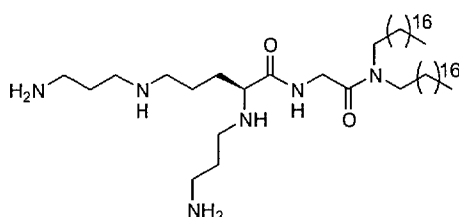


DC-Chol	3 β { <i>N</i> -(<i>N,N'</i> -dimethylaminoethane)carbamoyl]cholesterol
---------	--

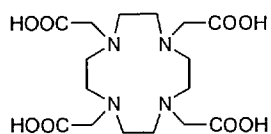


DCC	dicyclohexylcarbodiimide
d	doublet

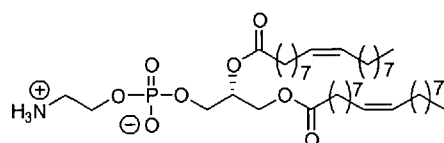
DMAP	4- <i>N,N</i> -dimethylaminopyridine
DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOGS	dioctadecylamidoglycylspermine



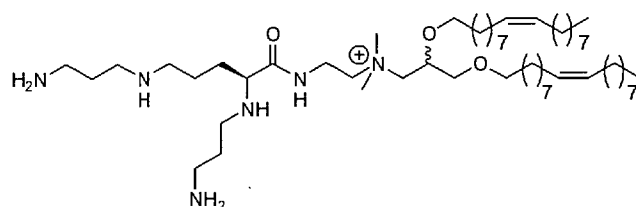
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
------	--



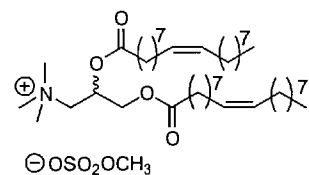
DOPE	dioleoylphosphatidylethanolamine
------	----------------------------------



DOSPA	(2,3-dioleoyl-N-[2-spermincarboxamido)ethyl]- <i>N,N</i> -dimethyl-1-propanaminium pentafluoroacetate
-------	---



DOTAP	1,2-dioleoyloxy-3-(trimethylammonio)propane
-------	---



DSPE	distearoylphosphatidylethanolamine
DTPA	diethylenetriaminepentaacetic acid
EPR	enhanced permeability and retention
equiv.	Equivalents
EI	electron ionisation
ESI	electrospray ionisation
Et	ethyl
EtOH	ethanol
EtOAc	ethyl acetate
FAB	fast atom bombardment
Fmoc(Cl)	(9-fluorenyl)methyloxycarbonyl (chloride)
h	hour(s)
H _{II}	inverted hexagonal
HBTU	O-benzytriazol-1-yl- <i>N,N,N',N'</i> -tetramethyluronium
	hexafluorophosphate
HEPES	hydroxyethylpiperazine ethane sulfonic acid
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
IR	infra-red
<i>J</i>	coupling constant
LD	Liposome:DNA
LUV	large unilamellar vesicle
m	multiplet
Me	methyl
MeOH	methanol
min	minute(s)
MLV	multi lamellar vesicle
MRI	magnetic resonance imaging
m/z	mass-to-charge ratio

NEt ₃	triethylamine
NLS	nuclear localisation signal
nm	nanometre (10 ⁻⁹ m)
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PEG	polyethylene glycol
PET	positron emission tomography
Ph	phenyl
(m)RNA	(messenger) ribonucleic acid
RLU	relative light units
RT	room temperature
s	singlet
secs	seconds
SUV	small unilamellar vesicle
SPECT	single photon emission computed tomography
t	triplet
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
Ts	tosyl, (4-methylphenyl)sulfonyl
UV	ultraviolet

CHAPTER ONE

Introduction

1.1 Molecular Imaging

Molecular Imaging can be broadly defined as the *in vivo* characterisation and measurement of biological processes at the cellular and molecular level [1]. It is rapidly emerging as an important development for the drug discovery process and disease monitoring. Compared to conventional diagnostic imaging it intends to probe the origin and mechanisms of disease rather than imaging the resulting condition itself, such as anatomical manifestations. Molecular imaging has largely been made possible by the advances in molecular and cell biology techniques such as identification of new genes and proteins, unravelling cell signalling mechanisms, combined with advances in imaging science including, increased image resolution and novel imaging techniques. However it was not until the late 1990's that concerted research efforts from a broad range of subject disciplines (chemistry, molecular modelling, molecular biology and imaging science) began to embrace key technologies and focus the field in a biological and molecular perspective, particularly that associated with disease.

Central to molecular imaging are the imaging modalities themselves, principally Nuclear imaging (Positron Emission Tomography (PET), Single Photon Emission Computed Tomography (SPECT)), Optical Imaging and Magnetic Resonance Imaging (MRI) [2]. Many of these techniques are established key tools in modern medicine and therefore it is expected that the 'fruits' of current molecular imaging research will have a direct impact on patient care within the next 5 – 15 years [1].

Molecular imaging requires two basic elements: (i) molecular probes that traverse appropriate delivery barriers and (ii) a means by which to monitor these probes [3]. Whilst there are a few examples of clinical molecular imaging experiments [4], [5], [6] much of the research is still performed at the preclinical level, mostly *in vitro* or in small animal models. The majority of research to date has focussed on three key areas; imaging gene therapy (gene delivery and gene expression), imaging of molecular markers and imaging of cell tracking.

Imaging Gene Therapy

Gene therapy is defined as the intracellular delivery of genetic material (DNA or RNA) with a vector, to generate a therapeutic effect, either by correcting an existing abnormality or by conferring new functions to the cell [7]. Since its advent in the early 1980's gene therapy has been heralded as a potential revolution in medicine, because for the first time a therapy has been directed at correcting the genetic make-up of a

disease rather than treating the resulting condition. Recently gene therapy has hit major stumbling blocks because of the manner in which genes are delivered and the lack of immediate information regarding both vector uptake and gene expression [8]. By using molecular imaging to guide injections and monitor progress of the therapy, clinicians will not only be able to improve the effectiveness and safety profile of gene therapy, but also obtain temporal definitions without sacrificing animals, reducing the overall cost of research.

Molecular imaging research for gene therapy has focussed on two key areas: (i) imaging vector delivery and (ii) imaging gene expression.

Imaging Vector Delivery

The ideal system for imaging gene therapy must be applicable to a broad range of applications. Imaging the delivery process itself could potentially provide a more universal method of following gene therapy.

Nuclear Imaging (PET and SPECT) and MRI are the principle techniques that have been used to monitor delivery and biodistribution of both viral [9], [10], [11] and non-viral vectors [12], [13], [5], [14]. The most successful examples of imaging vector delivery to date have involved their radiolabelling with ^{111}In for PET. Nichol *et al* [12], [15] labelled polyethyleneimine (PEI), a cationic polymer frequently used for non-viral gene therapy with diethylenetriaminepentaacetic acid (DTPA) and complexed it with radioactive ^{111}In . The resulting complex ^{111}In -DTPA-PEI was injected into the tail vein of rats containing a subcutaneously-implanted tumour. PET imaging revealed that the vector did not show any specificity to the tumour and was rapidly cleared through the kidneys after 24 hours post injection. The DTPA-PEI construct is also amenable for adaptation for MRI; indeed Alfke *et al* [16] developed an analogous system replacing ^{111}In with MRI sensitive gadolinium (Gd(III)). The system was shown to retain DNA binding and transfecting abilities *in vitro*, whilst conferring the Gd label to the cells making them MRI visible.

The Herpes Simplex Virus (HSV) is an attractive prospect for gene therapists as it can transport up to 50 kb of therapeutic DNA and shows a reduced immunogenic profile compared to other viruses. Schellingerhout *et al* [17] have labelled HSV with Indium-oxine (^{111}In -oxyquinoline) to monitor its biodistribution. The ^{111}In labelled HSV was administered to tumour bearing rats both *intra*-arterially and directly into the tumour itself. Administration *via* the arteries showed a low virus uptake into the tumours

(0.1%), whilst direct administration resulted in 72% of the total dose administered remaining in the tumour at 24h post injection. Recently alternative labelling strategies with more favourable radioisotopes with shorter half-lives have been developed. Labelling of Adenovirus vectors with ^{99m}Tc [10] and ^{124}I [11] have both been validated as viable techniques and interestingly a ^{99m}Tc probe for labelling plasmid DNA has been shown to be an effective label for imaging gene delivery in tumour bearing animals [14].

Whilst there are several promising vector-based imaging systems, delivery of the vector to the correct location is only the first event in any therapy. Arguably gene expression and synthesis of the protein products are the most important events. As gene therapy technology stands today imaging the site of delivery and biodistribution of the gene delivery vector does not necessarily correspond to the location of gene delivery or more importantly gene expression.

Imaging Gene Expression

A more elegant approach to imaging gene therapy is to image the gene expression event itself. To image gene expression cells need to express so-called image marker genes alongside the delivered therapeutic genes. These genes can encode for intracellular enzymes, cell surface receptors or peptides that imaging probes can successfully interact with. Although it could be feasible to design probes specifically to interact with the therapeutic gene products, this approach is much less attractive at this early stage in the field. Unlike imaging vector delivery a more diverse range of techniques have been employed to image gene expression including Nuclear Imaging, MRI, Optical imaging and Fluorescence imaging (the later two drawing from the more traditional molecular biology techniques of examining proteins, such as green fluorescent protein (GFP), with an intrinsic fluorescence).

The most frequently used 'imaging reporter gene' has been herpes simplex virus type 1 thymidine kinase (HSV-1-tk) enzyme [18]. HSV-1-tk has a broad substrate specificity and can phosphorylate nucleoside analogues that are structurally different from its own substrate, deoxythymidine. One of the most studied examples involves the use of a ^{124}I and ^{18}F labelled nucleoside analogue (FIAU) for nuclear imaging [19], [20]. Upon administration of FIAU to the subject the nucleoside can freely traverse the cell membrane *via* an active transport mechanism. When HSV-1-tk is expressed inside the cells, the substrate is phosphorylated and can no longer traverse the cell membrane

and becomes trapped inside the cell. The cellular retention of radioactivity is therefore an indicator of gene expression (see Figure 1.1).

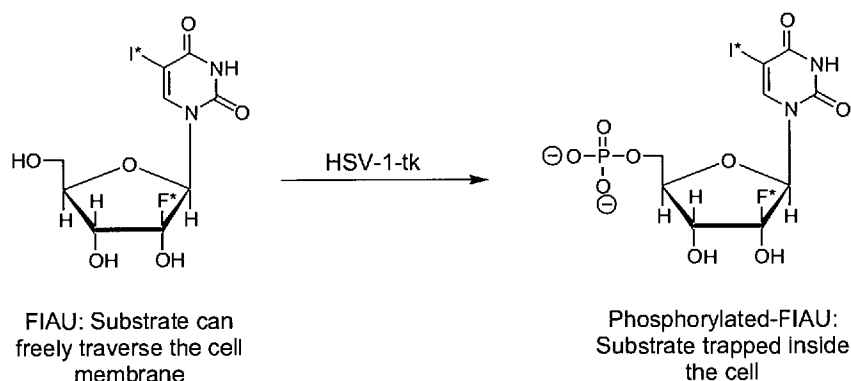


Figure 1.1: FIAU Phosphorylation by HSV-1-tk

Meade *et al* have developed an enzyme responsive system for imaging gene expression using MRI [21]. Here the gadolinium-based imaging agent is delivered in a 'silent' state, but becomes activated upon expression of β -galactosidase from the reporter gene. The system was shown to be successful in examining gene expression in *Xenopus* embryo.

The advantage of using expression of intracellular proteins is that it employs a relatively uncomplicated expression strategy and avoids recognition of the gene expression product by the host immune system. Whilst both the above examples have been proved successful they both rely on imaging probes crossing the cellular membrane, an unfavourable kinetic event [1]. An alternative strategy is to express cell surface proteins where the kinetics of probe interaction are more favourable.

The human transferrin receptor (hTfR) is overexpressed in various tumour cells and as such has been a target for transporting drugs into tumour cells. Overexpression of this cell surface receptor has been exploited to monitor gene expression using MRI [22], [23]. A synthetic MRI sensitive iron probe was used to show that receptor expression could be visualised in rat gliosarcoma cells using MRI. Another advantage of the cell surface protein approach is that it is possible to exploit the engineering of cell surface receptors to recognize already approved imaging drugs. Indeed Bogdanov *et al* [24] exploited the affinity of oxo- ^{99m}Tc technetate, an approved PET probe, for a GGC (glycine-glycine-cysteine) peptide motif. Several fusion peptides bearing GGC motif

were investigated and their localisation *in vivo* could be visualised using PET making them potential *in vivo* extracellular gene expression markers.

The use of nuclear imaging (PET, SPECT) and MRI to image gene expression can provide information on both the location and magnitude of expression. Optical imaging techniques can provide further information, as the strength of this technology is that it can image gene expression under dynamic conditions [15]. There are several examples of using optical imaging to image gene expression *in vivo* [25], [26], [27]. One of the most documented examples employs the use of the luciferase enzyme (enzyme present in luminescent organisms, which reacts with the substrate luciferin resulting in light emission) to monitor growth of a xenografted tumour over 2 weeks in rats [28]. Despite its ability to provide real time information on gene therapy events, the nature of optical imaging techniques (limited tissue penetration, use of fluorescent probes) means they are primarily limited to experimental imaging in small animal models [1].

Imaging Molecular Markers

A molecular marker is a defined protein, small signalling molecule or process, which is highly specific for a disease state or progress. Many of these molecules have long been known to medicine and are tested for in post tissue biopsy analysis. Molecular imaging of molecular markers will permit whole body non-invasive *in situ* analysis of disease state or progress [29]. This will be a great aid to diagnosis and will permit more accurate clinical decision making.

This imaging has largely been made possible by developments in the understanding of the biochemistry of specific pathways. Many of the challenges facing traditional medicinal chemistry such as design of molecules that specifically interact with a given target, with a given efficacy, are challenges facing imaging of molecular markers. Thus far, labelling of molecular markers has been generally restricted to the labelling of already defined *in vitro* assay molecules. Nuclear imaging (PET and SPECT) is the principle technique that has been used to image molecular markers. A diverse range of molecular markers are being assessed for molecular imaging including; dopamine and serotonin transporters using ^{123}I -IPT for assessing and treating Parkinson's disease [30], [31], imaging $\alpha_v\beta_3$ receptors with cyclic ^{125}I -radiolabelled RGD derived peptides for imaging angiogenesis [32], and imaging the presence of permeability glycoprotein (Pgp) and Multi drug resistance protein 1 (MRP₁) in cancer cells, using $^{99\text{m}}\text{Tc}$ labelled probes as a measure of multidrug resistance [33] (see Figure 1.2).

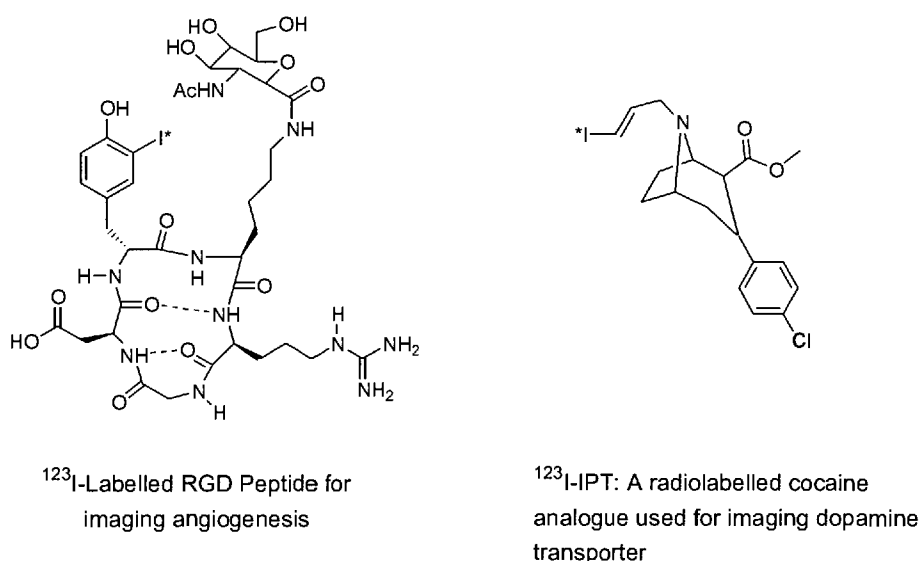


Figure 1.2: Structures Selected Radionuclide Probes for Imaging Molecular Markers

However, some of the most studied molecular markers are for imaging the process of apoptosis.

Imaging Apoptosis

Apoptosis is a form of programmed cell death, which is a result of the induction of an internal suicide program. It is used by multicellular organisms to regulate their cell numbers and to remove either unwanted or dysfunctional cells. The strict regulation of apoptosis is an essential process in the normal development and homeostasis of a healthy adult [34]. However, apoptosis is deregulated in a number of disease states and is a major determinant of cancer, cardiovascular and neurodegeneration and diseases. Additionally, induction of apoptosis is a key therapeutic requirement in treatment of tumour cells through chemotherapy and radiotherapy [34]. The mechanism of apoptosis can be initiated by a large number of stimuli, which all ultimately lead to the common cascade of cysteine aspartic acid proteases (caspases) which leads to irreversible changes in the cell morphology including cytoskeletal disruption and DNA cleavage, ultimately leading to cell death.

Upon induction of apoptosis, phosphatidylserine, an anionic phospholipid normally located on the internal surface of the cell membrane, is translocated to the outer surface of the cell membrane. In human tissues under ambient conditions annexin V, a 35kDa protein, binds to the cell surface membrane bound phosphatidylserine and can

therefore be used as a marker for apoptosis. Annexin V was initially labelled with ^{99m}Tc [35]. The radiolabelled probe has been used successfully to image apoptosis in grafts of lung [36] and liver [37] as well as arteriosclerotic plaques in small animal models [38]. The radiolabelled protein has also been used in clinical research assessing apoptosis in patients as a result of myocardial infarction [6]. Initial studies have also been performed examining the effectiveness of the probe as a marker of apoptosis after chemotherapy and radiotherapy. The study in breast tumour-bearing rats showed a marked increase in annexin V labelling of cancer cells after treatment by paclitaxel and or radiotherapy using ^{99m}Tc -annexin V [39]. More recently annexin V has been successfully labelled with ^{124}I [40] and ^{18}F [41] to obtain PET images of higher resolution. Annexin V has also been labelled with MRI sensitive iron [42], [43], [44] and $\text{Gd}(\text{DTPA})^{2-}$ [43] and Brindle *et al* have demonstrated their system to be effective both *in vitro* [43], [44] and *in vivo* [44] studying apoptosis in a tumour model treated with chemotherapeutic drugs.

An alternative method for imaging apoptosis is to target the caspase enzymes in the 'suicide' enzymatic cascade. An inhibitor of caspase activity benzyloxycarbonyl-Val-Ala-DL-Asp(O-methyl)-fluoromethyl ketone (Z-VAD-fmk) has been labelled with ^{131}I [45]. In an *in vitro* model study, cells undergoing apoptosis had a 2-fold increase in cellular uptake of Z-VAD-fmk.

Although imaging molecular markers provides a valuable insight into the disease process, knowledge of the pathways and biochemical interactions of the disease are an essential prerequisite for accurate results.

Imaging of Cell Tracking

Most of our understanding of the biological function and interaction of cells comes from a static viewpoint obtained from light and electron microscopy studies [46]. With molecular imaging it will be possible to monitor the fate and interactions of labelled cells non-invasively and repeatedly in order to obtain dynamic information about the movements and interactions of cells within the whole body. Cell tracking has become an invaluable tool for studying movement of cells in animal disease models [47], [48] but perhaps the most exciting application of cell tracking studies lies in the future; the potential to monitor stem cell therapy in patients.

In order to image cell tracking, two basic requirements must be met: (i) An effective method to label the cells with a suitable probe (long term labelling with minimal cytotoxicity) and (ii) a method by which to track these cells.

To date, the majority of cell labelling methods have involved the *ex vivo* labelling of cells. Cells have been labelled with a variety of probes including radionucleotides for PET and SPECT [49], [50], magnetic probes for MRI [51], [52] and both fluorescence [53] and bioluminescence probes [54] for optical imaging. For effective cell labelling the imaging probe must remain on/in the cells either *via* adhering to the cell surface or internalisation of the probe. Although the latter avoids any problems with recognition by the host immune system, it creates an extra delivery barrier (cell membrane) that must be traversed. For adhering to the cell surface, proteins [55] and antibodies [56] have been employed as effective tools. For cellular internalisation of an imaging probe many strategies have been employed to circumnavigate the cell membrane including conjugation of probes to cell internalising peptides or antibodies [57], [47] incorporation into liposomes [58], [59] or nanoparticles [51] and conjugation to cell receptor ligands, such as transferrin [47]. An elegant alternative to *ex vivo* cell labelling is the direct *in vivo* labelling of cells. This has been achieved using antibodies as a targeted labelling vector [56]. Despite its elegance, at this early stage in the field this strategy is heavily restrictive to sterically available cells and reliant on effective targeting of the label to the desired cells.

Whilst there are many effective methods for labelling cells, the majority of work in this area has concentrated on the labelling of cells to make them MRI visible. MRI labelling and subsequent tracking of cells is the most popular technique used as magnetic probes do not have a limited lifetime, permitting long-term studies and the resolution of images gained is more favourable than those from nuclear imaging.

The first cell labelling and tracking experiment was conducted by Jacobs *et al* [60]. Using a Gd-dextran based MRI probe, a 16-cell frog embryo was labelled and its development monitored using MRI. The experiment permitted visualisation of structures within the living embryo, usually inaccessible to light microscopy to be imaged, non-destructively over a period of days. Whilst this experiment followed embryonic cell lineages and movement, the sample size was small and the cells were not tracked throughout a whole living body. The first experiment tracking labelled cells in a living animal was conducted by Bulte *et al* [47], who successfully labelled oligodendrocyte progenitors and tracked their migration and subsequent role in

neurone remyelination in rats. Since then many cell types have been labelled and tracked including macrophages [48], neutrophils [61] and bone marrow stromal cells [62].

Tracking of Stem Cells

The main aim of many researchers [63], [51], [64], [65] remains imaging of stem cell migration and differentiation. Stem cells are undifferentiated cells that can undergo unlimited divisions and can give rise to one or several different cell types. They are essential cells in embryogenesis and formation of renewable tissues, such as blood and skin, in adults. This property of stem cells makes them an extremely attractive therapeutic prospect, particularly for treatment of degenerative diseases and repairing essential damaged tissues. One of the most interesting examples to date is the mapping of transplanted neural stem cell migration after a stroke in rats [51]. Neural stem cells were labelled *ex vivo* with a dual bimodal probe containing rhodamine for fluorescence imaging and Gd for MRI. The ischaemic lesion was located in the right hemisphere of the rat brains. The labelled stem cells were injected into the left hemisphere of rat brains. Serial *in vivo* MRI imaging of the rats showed that at 7 days post administration the stem cells had migrated from the injection tract and were found mostly in the corpus callosum (fibres connecting the two hemispheres of the brain) and at 14 days post administration the stem cells had extensively repopulated the lesion area, whilst in control rats with no stroke lesion, stem cells do not show any substantial migration from the injection tract. These results were confirmed post mortem using fluorescence microscopy. These results clearly show the value of molecular imaging in stem cell therapy development.

Molecular imaging is a fast growing research discipline. Whilst there has been a lot of progress in the field already exploiting the available technology to image biological processes, further improvements in current imaging modalities and probe design are still at the centre stage of research [1].

Review of Imaging Modalities

The principle imaging modalities used in molecular imaging are nuclear imaging (PET and SPECT), MRI and optical imaging techniques (fluorescence and bioluminescence). An overview of the imaging techniques is shown in Table 1.1.

Imaging Modality	Probes	Resolution	Tissue Depth	Time for image acquisition	Potential for clinical Molecular imaging
PET	Radioisotopes e.g. ^{124}I , ^{18}F , ^{11}C	1-2 mm	No limit	min	Yes
SPECT	Radioisotopes e.g. ^{111}In , ^{123}I , $^{99\text{m}}\text{Tc}$	1-2 mm	No limit	min	Yes
MRI	Paramagnetic Elements e.g. Gd, Fe(III), Mn	10-100 μm	No limit	Min/hours	Yes
Fluorescence	Fluorescent Molecules e.g. Rhodamine, GFP	1-2 mm	1 cm	Sec/min	No
Bioluminescence	Photoproteins e.g. Luciferin	Several mm	1–2 cm	min	No

Table 1.1: An overview of Imaging Modalities Used in Molecular Imaging [66], [18]

Nuclear Imaging Techniques (PET and SPECT) [66]

The main principle of nuclear imaging techniques is the quantitative detection of radioisotopes. With PET this involves the detection of nuclides such as ^{15}O , ^{11}C , ^{124}I and $^{94\text{m}}\text{Tc}$. When these radioisotopes begin to decay they emit two particles; a positron and a free electron. When these particles collide, two γ -rays are formed which emerge in opposite directions. It is these emerging rays that are detected by PET using an array of detectors around the subject. Whilst with SPECT this involves the direct detection of gamma emitting radionuclides such as $^{99\text{m}}\text{Tc}$, ^{123}I and ^{111}In . The radionuclide is either followed using a sodium iodide gamma camera or a solid state cadmium-zinc telluride detector. In both cases the radionuclides are usually employed to tag small molecules or cells.

Magnetic Resonance Imaging [66]

The underlying principle of MRI, parallel to NMR, is that unpaired nuclear spins (such as those of hydrogen atoms on water molecules) align themselves when placed into a magnetic field. Application of a temporary radiofrequency pulse excites these spins and their subsequent return to base line energy (relaxation) is marked by production of an

alternating current that can be measured. This relaxation can be easily manipulated (for example by application of magnetic gradients) to gain spatial information from a sample. MRI probes (paramagnetic metals) effect the relaxation process of nuclear spins in their surrounding environment and their location in a sample can be subsequently pin pointed.

Optical Imaging (Fluorescence and Bioluminescence) [66]

The fundamental principle behind optical imaging techniques is the measuring and quantitation of light emitted from a given sample. With fluorescence imaging light of a particular wavelength (excitation) is used to excite fluorescent sample, resulting in a production shifted (emission) wavelength that is normally collected by a CCD camera. Fluorescent probes can be synthetic molecules, such as rhodamine or naturally fluorescent proteins such as green fluorescent protein (GFP). Bioluminescence imaging requires the presence of a photon emitting substance. For example the enzyme luciferase, present in all luminescent organisms reacts with the substrate luciferin, this reaction results in photon/light emission which is detected and quantified within the sample using a low light photon counting camera.

Optical imaging techniques (fluorescence and bioluminescence) are low cost imaging modalities that can provide real-time information on events, such as imaging gene expression [66]. However, many fluorescence imaging probes have an excitation and emission wavelength in the range of 500 nm (such as GFP), which means that the probes have limited penetration in mammalian tissues, restricting the tissue depth of imaging [18]. Although recently a number of Near Infrared spectrum (NIR) fluoro-probes (excitation and emission wavelength in the range of 700-900 nm) have been developed to overcome the tissue penetration problem, and these are becoming more readily available [66]. For bioluminescence imaging, mammalian tissues have minimal background noise, but limited topographic information can be gained from this technique. Overall, these techniques provide excellent tools for *in vitro* and small animal analysis, but will have little use in clinical molecular imaging.

PET, SPECT and MRI have long been established as clinical diagnostic tools, making the transfer of pre-clinical research to the clinic a simple process. Nuclear techniques (PET and SPECT) have been the most used within clinical molecular imaging experiments to date. The high sensitivity of the techniques ($10^{-11} - 10^{-12}$ M of probe can be detected [18]) and the speed of data acquisition makes them extremely

attractive prospects. However, the probes have a limited lifetime (dependent on the half life of the radioisotope), which, for assessing immediate effects, is satisfactory, but increasingly many molecular imaging experiments require long term monitoring. Whilst it is possible to use radioisotopes with longer half-lives in small animals, this approach will be unacceptable for clinical molecular imaging due to safety issues concerning their use. The resolution of the image gained is also low [66], which means that nuclear techniques will have limited applicability in the increasingly prominent cellular applications.

Despite its inherent lack of sensitivity (10 – 100 μ M of probe are needed to be detected [18]), MRI is the technique that arguably holds the most promise for molecular imaging. The heavy metal probes (Gd, Fe) have been FDA approved for many years and do not have a limited lifetime. The longer image acquisition times are counterbalanced by the high image resolution gained. This makes MRI extremely attractive for all applications of molecular imaging.

To the best of all current knowledge MRI is harmless. MRI is experiencing an increasing prominence in molecular imaging experiments and developments in the chemical synthesis of probes can only further enhance this. The work in this thesis will concentrate on the use of MRI for molecular imaging.

1.2 MRI [67], [68]

Magnetic Resonance Imaging is a non-invasive imaging technique that allows investigation of opaque organisms in 3D. In order to understand how MRI can be used in molecular imaging and the role the contrast agents (probes) play in this process it is important to understand the underlying principles of MRI.

Protons have an inherent dipole moment that rises from their electrical charges and spins. MRI is founded on the manipulation of this dipole moment to generate signals that can be translated into a visual image of the sample.

When protons are placed in an external magnetic field (B_0) they align within the field either in a parallel or anti-parallel fashion. The energy level (parallel or anti-parallel) that is lowest in energy will have the highest occupancy. When aligned, the protons are

not stationary but precessing about the z-axis with a speed defined by the Larmor Frequency (see Equation 1.1).

$$F = \gamma B_0 / 2\pi$$

F = Precessional frequency,
 γ = gyromagnetic ratio of the nucleus
 B_0 = Strength of the applied magnetic field

Equation 1.1: Larmor Equation

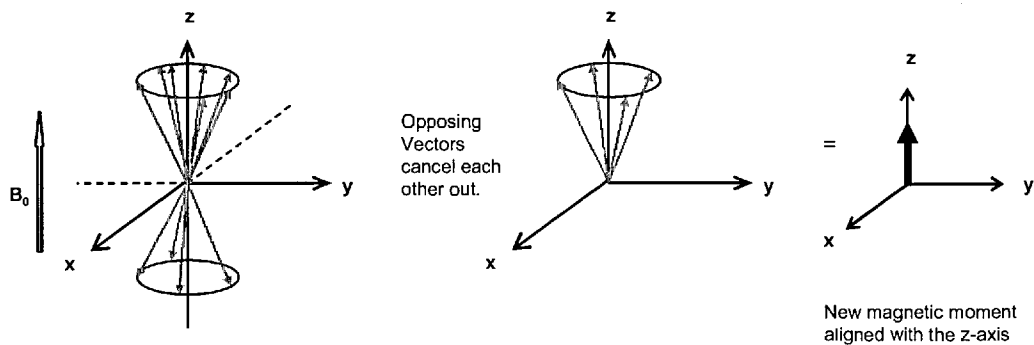


Figure 1.3: Behaviour of protons in an applied magnetic field

Proton vectors with opposing forces cancel each other out. As there are more protons aligned in the lower energy state, there is a net magnetic moment aligned with, or longitudinal to, the external field (z axis) (see Figure 1.3). It is manipulation of this new magnetic moment that gives the signals for imaging.

An applied radio-frequency pulse (RF pulse) with the same frequency as the precessing protons (F) can disturb the new magnetisation. The RF pulse transfers energy to the protons, which has two effects:

- (i) some protons shift from the lower energy state (aligned parallel) to a higher energy state (aligned anti-parallel), which decreases the new longitudinal magnetisation vector.
- (ii) protons begin to precess in synch/phase resulting in a new transverse magnetisation vector (in the xy plane). This rotating transverse magnetisation can be measured as it induces an alternating current (AC) in the receiver coil.

When the RF pulse is switched off the system returns *via* relaxation, to its original state. The longitudinal magnetisation increases and the transversal magnetisation

decreases. The decrease in the induced transverse magnetisation is called Free Induction Decay (FID). Relaxation occurs *via* two independent processes: longitudinal relaxation (T_1) and transverse relaxation (T_2).

Longitudinal Relaxation (Spin-Lattice Relaxation, T_1)

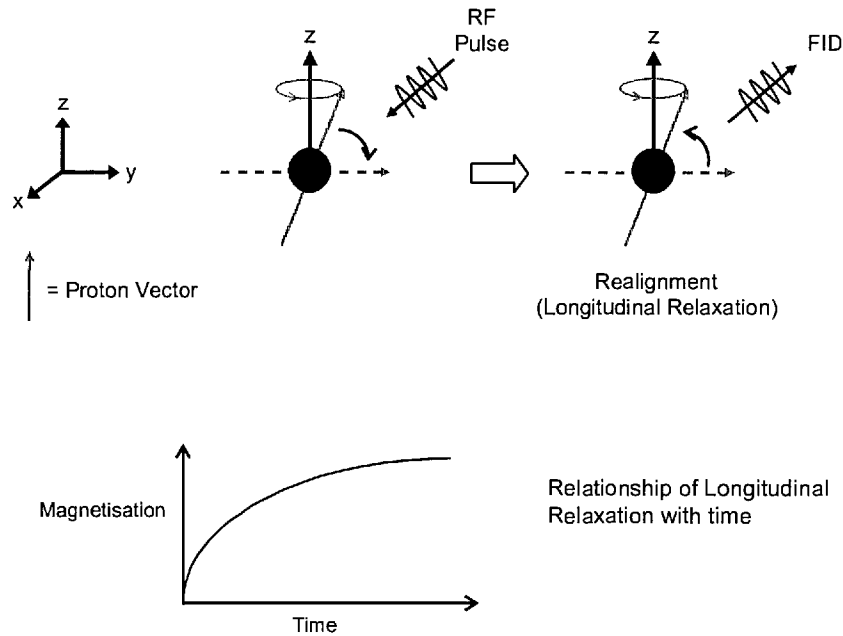


Figure 1.4: Longitudinal Relaxation

During longitudinal relaxation protons continually transfer from the higher energy state back to the lower energy state (see Figure 1.4). The thermal energy released from this process is transferred to the surroundings (lattice). The process of longitudinal relaxation is characterised by the T_1 relaxation time, a parameter used to define a sample. The T_1 time is the time required for the system to recover to 63% of its equilibrium value after a RF pulse.

Longitudinal relaxation is influenced by two factors; composition of the lattice (such as tissue composition) and the strength of the magnetic field.

Composition of the lattice: The lattice has internal magnetic fields. For effective energy transfer to occur between the protons and the lattice, fluctuations in the internal magnetic field must be near the Larmor Frequency. In more fluid samples, rapid molecule diffusion makes the process slower, increasing the T_1 relaxation time. In less

fluid samples, the rate of molecule diffusion is less making energy transfer more efficient and therefore a shorter T_1 relaxation time.

Magnetic Field Strength: A stronger external magnetic field increases the precession frequency of protons. This decreases the efficiency of the energy transfer process between the protons and the lattice, making T_1 relaxation times longer.

Transverse Relaxation (Spin-Spin Relaxation, T_2)

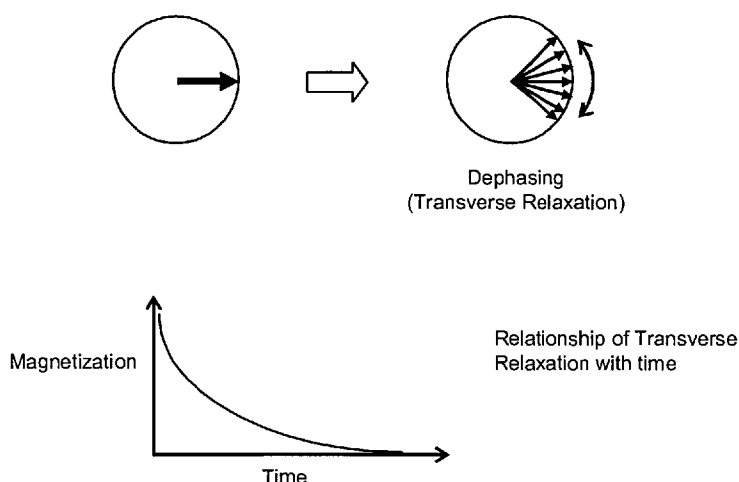


Figure 1.5: Transverse Relaxation

Transverse relaxation arises as the protons move out of phase in the xy plane (see Figure 1.5). The process of transverse relaxation is characterised by T_2 relaxation time. The T_2 relaxation time is the time it takes for de-phasing of the signal to decay to 37% of its original value. Protons de-phase when they experience an inhomogeneous magnetic field, which can arise from either the external applied magnetic field or internal magnetic fields.

External Magnetic Field Disruptions: Magnetic gradients are frequently used in an MRI experiment to permit spatial encoding of the signal (see section Spatial Encoding). These gradients disturb the external magnetic field and therefore increase rate of transverse relaxation, decreasing T_2 relaxation times.

Internal Magnetic Field Inhomogeneities: This is influenced by the lattice composition. In fluid samples, rapid diffusion of molecules means local magnetic fields effectively cancel each other out. As a result the protons stay in phase longer, and T_2 relaxation

times are longer. With less fluid samples the fluctuations in local magnetic fields will be larger. This results in the protons moving out of phase quicker and results in shorter T_2 relaxation times.

Changes in magnetic moments during and after (relaxation) the RF pulse induce an electrical current. This is the signal received by the receiver coil and used in MR imaging.

Spatial Encoding

In order to create a 3-dimensional image of the sample, signals received from the protons have to contain information about their positioning within the sample. This is achieved using slice selection, frequency encoding and phase encoding (see Figure 1.6).

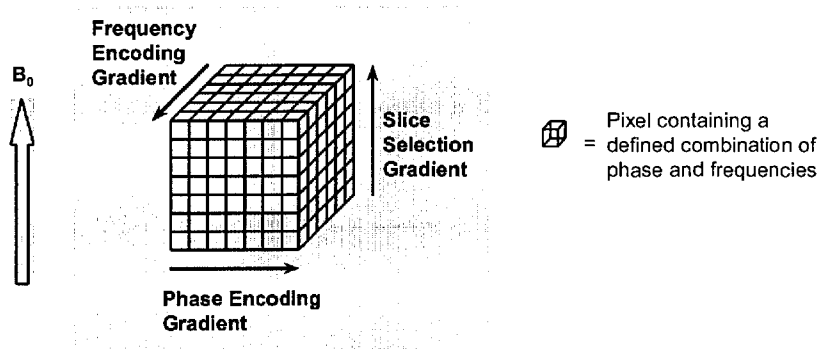


Figure 1.6: Spatial Encoding of the Signals.

Slice Selection: To select an imaging slice, a magnetic gradient is added along the main magnetic field. The frequency of precession of the protons, and hence the frequency of a RF pulse needed to excite protons will vary along the gradient. Slice selection is achieved by using an RF pulse with a narrow band of frequencies. Changing the range of frequencies in the RF pulse means a different slice of the sample can be selected.

Phase Encoding: To further differentiate signals within a slice, a phase encoding gradient is applied for a short time after the RF pulse, but before the readout of the signal. This change in the magnetic field results in a change of the frequency of the precession, so when the phase encoding gradient is switched off the phase of the precessing protons is dependent on the phase encoding gradient (see Figure 1.7).

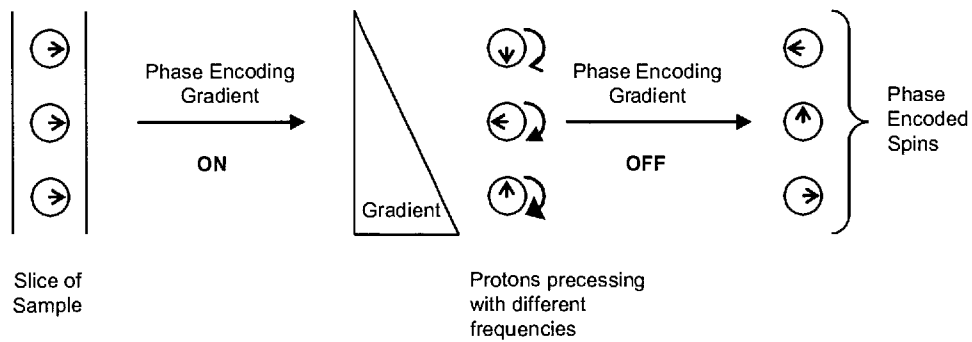


Figure 1.7: Phase Encoding Gradients

Frequency Encoding: The frequency encoding gradient further differentiates the signals within the phase encoded slice. During readout a magnetic gradient is applied within the slice (range of RF frequencies), changing the frequency of precession of the protons.

Combining the slice selection, frequency and phase encoding gradients allows creation of a 3-dimensional grid of raw data, in which each block (pixel) of the grid has a defined combination of phase and frequency (see Figure 1.6). The signals gained are analysed by means of Fourier transformation to give the resulting image.

Pulse Sequences

In order to obtain maximum information from a desired sample, changes in magnetic moments caused by an RF pulse have to be optimised. The protons in the magnetic applied magnetic field (B_0) are subjected to “pulse sequences”. A Pulse sequence is a software program used to control the MRI scanner hardware. Pulse sequences are used to manipulate the spins by changing the direction of net magnetization and utilizing differences in the rates of transverse and longitudinal relaxation to produce different contrast in the image. These consist of a variety of RF pulses and gradients which vary the information gained from the sample. The timing of the pulse sequence (TR (time to repeat) and TE (echo time (see below))) dictates the information gained from the experiments.

Spin Echo Sequences

Spin echo sequences are the most frequently used pulse sequences. The sequence consists of a 90° RF pulse, followed by a 180° RF pulse. This rephases the protons and results in production of a second signal, or echo (see Figure 1.8). By changing the

times between the RF pulses the image can be manipulated to be T_1 or T_2 weighted. This is particularly advantageous for gaining high quality images of various samples, which may have different T_1 or T_2 relaxation times.

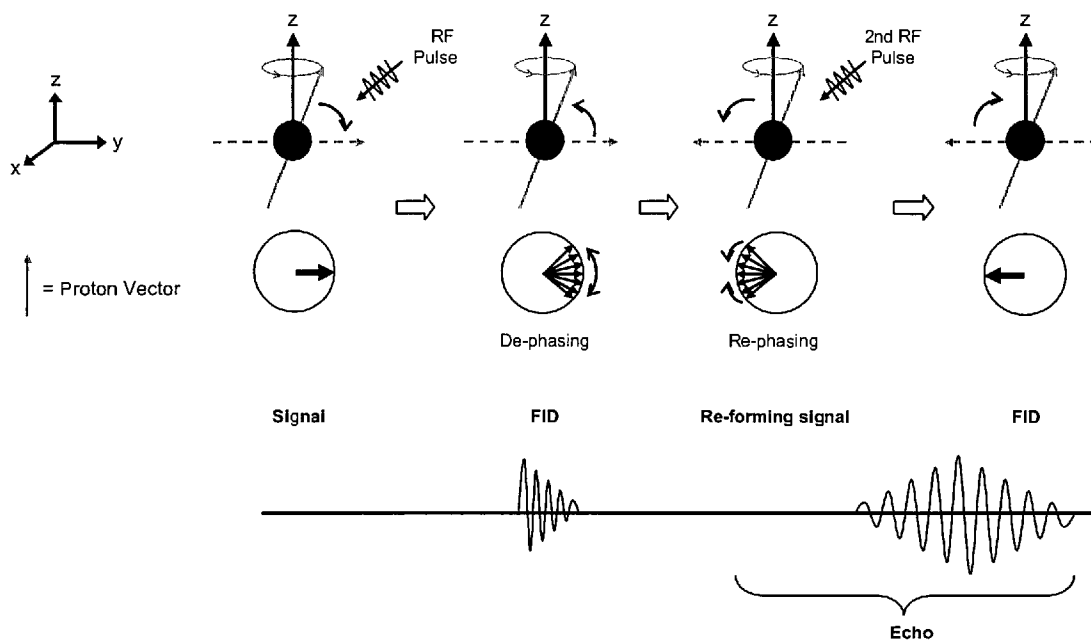


Figure 1.8: Spin Echo Pulse Sequences

Contrast Agents

Unaccompanied MRI is a powerful technique, however its diagnostic ability can be further enhanced by the use of contrast agents. Indeed 30% of all clinical MRI examinations employ contrast enhancement [69]. Contrast agents act by increasing or lowering the signal intensity of a given region on an MR image. Using pulse sequences designed to highlight these changes it is possible to pin point the exact location of the contrast agent within a given sample. This makes them of particular interest to molecular imaging studies. All MRI contrast agents are based on paramagnetic metals, such as Gd(III), Fe(III) and Mn(II). These agents act by affecting both the spin lattice (longitudinal, T_1) and spin-spin (transverse, T_2) relaxation time of the surrounding protons, which gives result to the change in signal intensity.

Contrast agents can be divided into two sub groups; T_1 contrast agents and T_2 contrast agents, depending upon the process of proton relaxation they predominantly affect. T_1 contrast agents such as gadolinium containing compounds are inherently more attractive, as they result in an increase in signal intensity (brightening of the image),

which makes them easier to detect. T_2 contrast agents (Fe(III), Mn(II)) result in a decrease in signal intensity and hence a darkening of the image. All metals used for contrast agents are paramagnetic, but the inherent properties of the metals mean that the predominant mechanisms of proton relaxation are different.

Metals in T_1 contrast agents have a larger magnetic moment, or dipole moment than those in T_2 contrast agents. The principle effect of these agents arises from interactions of the fluctuating magnetic field on the metal (as a result of the unpaired electrons) with the protons in the applied magnetic field. T_1 contrast agents effect the relaxation of protons in direct contact with the agent.

Metals in T_2 contrast agents have a larger magnetic susceptibility. The magnetic susceptibility of a substance describes its ability to become magnetised or to distort a magnetic field. Metals in T_2 contrast agents induce a mini magnetic field (inhomogeneity) in the applied magnetic field (B_0). This shortens the T_2 relaxation of protons surrounding the metal by causing them to dephase faster. The effect of these contrast agents is more disperse than T_1 contrast agent, as the magnetic susceptibility is a field and protons within that field, not solely in direct contact with the agent, show a decreased T_2 relaxation. Over the past decade a new class of T_2 contrast agents have been developed. These so called 'superparamagnetic agents' are made of crystalline iron oxide particles that have the general formula Fe_2O_3MO (M = divalent metal ion, such as iron, manganese, nickel, cobalt or magnesium) [70]. These particles can vary in size considerably, which can greatly affect their physiochemical and pharmacokinetic properties.

Gadolinium (III) based contrast agents have received the most attention for use in MRI [71] and the work in this thesis will concentrate on the use of gadolinium-based contrast agents for molecular imaging.

Gadolinium-Based Contrast Agents

For a contrast agent to be effective it must be able to efficiently relax the nuclear spins of protons, show low toxicity and good water solubility; Gadolinium (III) posses several of these favourable properties for use as a contrast agent. It has seven unpaired electrons and is therefore highly paramagnetic. This effect is further enhanced by the slow rate of relaxation of the Gd(III) electron spin, which is similar to the relaxation rate of protons [72]. In order to be an effective T_1 contrast agent a high rate of water exchange from the parent ion is needed to maximise the water protons in direct contact

with the metal. This requires a degree of coordinate flexibility from the metal ion. Gadolinium is at the centre of the change (8 to 9) in coordination ground state of the lanthanide series, which means that gadolinium displays coordinate flexibility between 8 and 9 with only a small energy change. This is reflected in the fast rate of water exchange for gadolinium, indeed $\text{Gd}(\text{H}_2\text{O})_8^{3+}$ has a water exchange rate of 10^9 s^{-1} (compare to the diffusion limit of $\sim 10^{10} \text{ s}^{-1}$) [73]. $\text{Gd}(\text{III})$ also has no redox properties. These intrinsic physical characteristics ensure that $\text{Gd}(\text{III})$ is an optimal candidate for efficient relaxation of protons.

Alone, $\text{Gd}(\text{III})$ is a highly toxic heavy metal ion, but when complexed into certain ligands it forms essentially inert complexes [72]. The anionic complexes $\text{Gd}(\text{DTPA})^{2-}$ (diethylenetriaminepentaacetic acid) and $\text{Gd}(\text{DOTA})^-$ (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) (see Figure 1.9) were the first complexes that entered into clinical practice and they represent the reference compounds for the later development and evaluation of new agents [74].

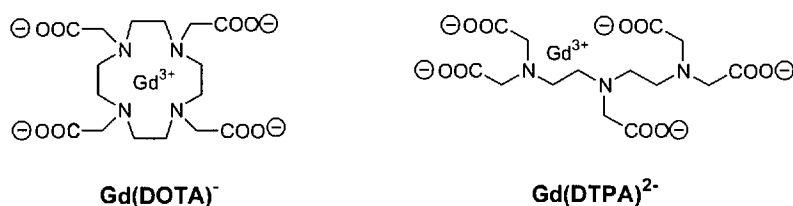


Figure 1.9: Structures of $\text{Gd}(\text{DOTA})^-$ and $\text{Gd}(\text{DTPA})^{2-}$

The highly water soluble polyaminepolyacetic acid skeletons provide the required 8 coordination sites for the gadolinium, leaving the 9th coordination site for a water molecule from the surrounding environment. These complexes have been shown to be highly stable, with thermodynamic stabilities of DOTA $\log K_{\text{Gd}} = 24.7$ and DTPA $\log K_{\text{Gd}} = 22.5$ (at 25°C and $\mu = 0.1 \text{ M}$) [74] and exhibit minimal toxicity, $\text{Gd}(\text{DTPA})^{2-}$ $\text{LD}_{50} = 8 \text{ mmol/kg}$ and $\text{Gd}(\text{DOTA})^-$ $\text{LD}_{50} = 25 \text{ mmol/kg}$ [69].

Chemical Mechanisms of Relaxation

Gadolinium contrast agents interact with the surrounding water molecules through a variety of mechanisms (see Figure 1.10). Each of these factors can influence relaxation efficacy of the gadolinium agent.

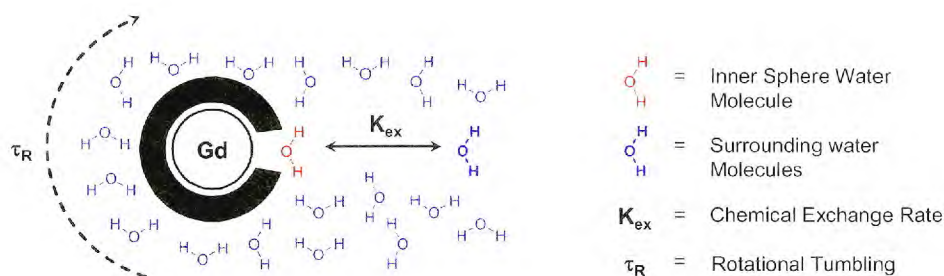


Figure 1.10: Chemical Mechanisms of Relaxation

(Gadolinium complex interacting with water molecules)

The principle mechanism of relaxation arises from the interaction of the inner-sphere water molecule with the fluctuating local magnetic field on the gadolinium ion [75]. The inner-sphere water molecule coordinates to the gadolinium ion in its first coordination sphere, occupying the 9th coordination site. The resulting relaxation is transferred to the bulk of the water *via* chemical exchange of the coordinated water molecule with another water molecule from the immediate surroundings. Efforts to increase the efficacy of gadolinium contrast agents by leaving space for more than one innersphere water molecule have been successful (see Figure 1.11). These contrast agents show an increase in relaxivity but have shown reduced water solubility [76]. These complexes have yet to be approved for use in the clinic.

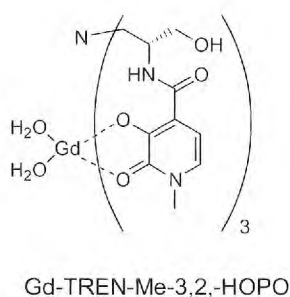


Figure 1.11: Gadolinium MRI Agent with Two Inner Sphere Water Molecules

The carboxylic acids on the ligands facilitate hydrogen bonding with the surrounding water molecules. This gives rise to the second coordination sphere of the metal. Water molecules in the second coordination sphere also experience the paramagnetic effects of the gadolinium ion, and have a shorter relaxation time, giving the so-called outer-sphere relaxation term. This effect is much less predominant than the innersphere relaxation term. The exact mechanism by which outer-sphere relaxation occurs is less

understood than that of the inner-sphere, but it is postulated that random diffusion of both water molecules and the gadolinium complex contribute to the overall effect [75]. To influence the volume of the second coordination sphere, ligands have been developed to increase the hydrogen bonding, such as replacing the carboxylic acids of DOTA and DTPA with phosphonate groups [77].

The rotational correlation time (molecular tumbling; τ_R) of the complex also strongly affects the efficacy of the agent. Indeed the proton relaxivity of small contrast agents is limited by the fast correlation times. A slow rotational correlation time increases the relaxivity of the agent. Rotational correlation times are related to molecular weight of the agent (increased molecular weight gives slower rotational correlation times), but this effect is not always predictable as internal flexibilities in some macromolecules mean that the increase in relaxivity is often less than expected. Several groups have worked on increasing the rotational correlation times of gadolinium agents in an effort to increase the overall T_1 relaxation of the agents [78], [79].

The development of high relaxivity gadolinium agents is an active research area. For the development of new ligands emphasis must be placed on control and optimisation of all parameters influencing relaxation of protons.

Gadolinium-Based Contrast Agents for Molecular Imaging

Several research groups have focussed on the development of Gd(III) based contrast agents as probes for molecular imaging. These agents have been targeted towards many aspects of molecular imaging including cell labelling and tracking [80] imaging gene expression [21], imaging gene delivery [16], and imaging of molecular markers [81]. All Gd(III) based agents for molecular imaging have to overcome the inherent insensitivity of MRI to become successful probes. Several amplification strategies have been employed to date including labelling target seeking molecules [82], amplification of the signal upon recognition of a biological trigger [21] or increasing the τ_R of small molecular weight contrast agents by either enzymatic polymerisation of Gd-monomers [78] or conjugating them to large biomolecules [79].

A selection of Gd(III) probes used for molecular imaging is shown in Figure 1.12.

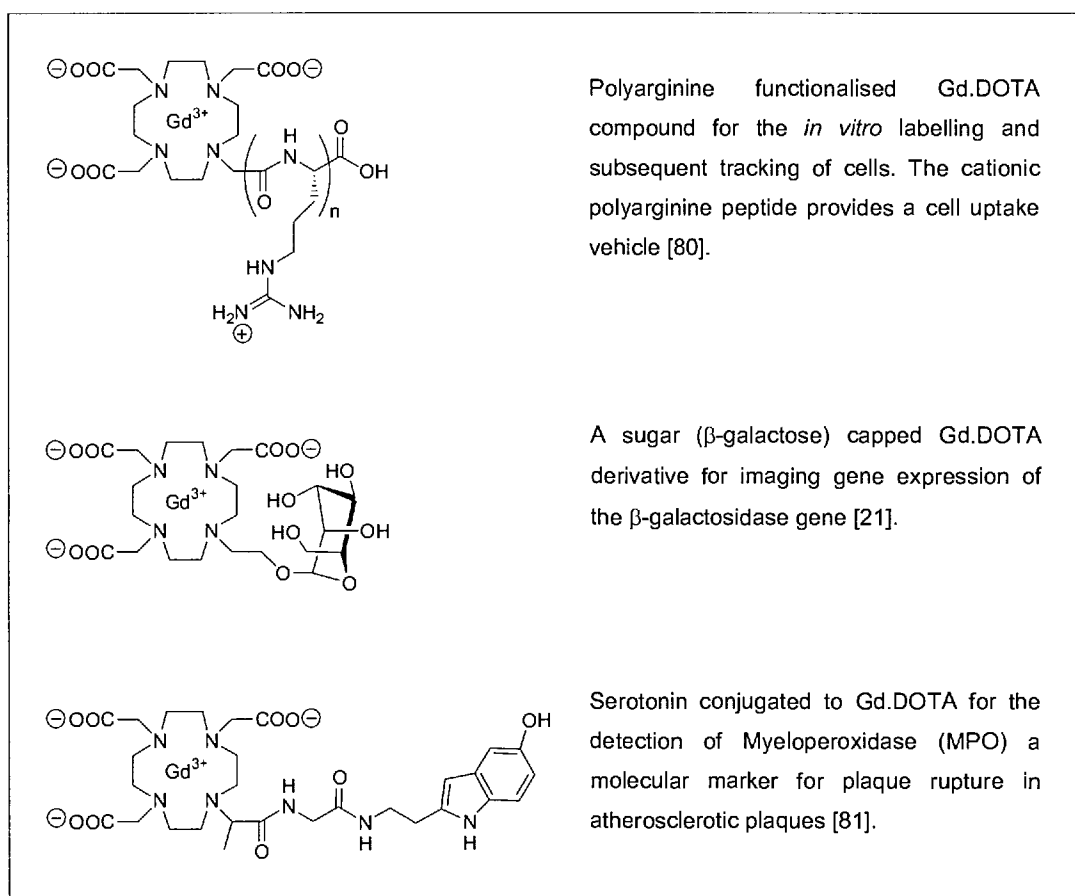


Figure 1.12: Selected Gd(III) probes for molecular imaging

One of the well documented and arguably most elegant Gd(III) probes for molecular imaging is the β -galactosidase sensitive probe for imaging gene expression (see Figure 1.13) [83]. The β -galactosidase moiety acts as a lid preventing the access of water to the 9th coordination site of the Gd ion. The action of the β -galactosidase enzyme causes cleavage of the glucosidic linkage to yield a Gd.DOTA analogue, in which the 9th coordination site is freely accessible to water.

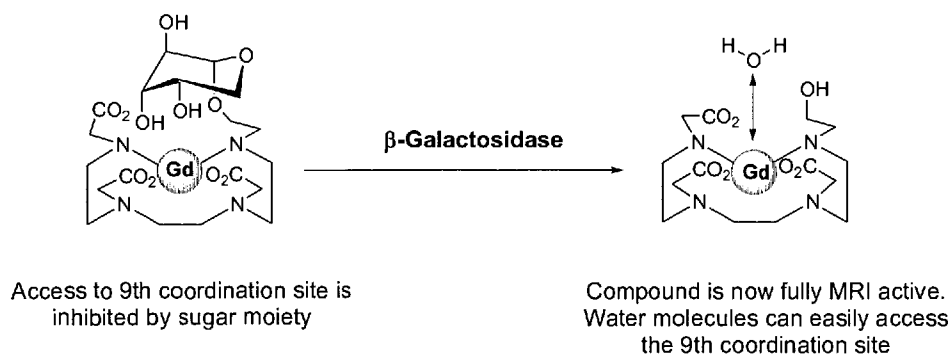


Figure 1.13: β -Galactosidase Sensitive MRI Probe

Experiments in *Xenopus Laevis* embryos co-expressing the *LacZ* gene (for β -Galactosidase) showed that the system could be used to successfully image gene expression *in vivo* [21]. Indeed, regions expressing β -galactosidase showed up to 57% signal enhancement. However, in this experiment the contrast agent was introduced to the animal system by microinjection. For such a system to progress further an alternative method of delivery into cells must be sought.

All new molecular imaging agents or probes are designed with reference to a specific target. The work in this thesis will focus on the development of Gd(III) agents for the molecular imaging of liposomes.

1.3 Liposomes

Liposomes are defined as artificially constructed spheres of lipid bilayer enclosing an aqueous compartment (see Figure 1.14). Originally conceived as models for the study of biological membranes, their intrinsic hydrophobic and hydrophilic character makes them a versatile tool for biology, biochemistry and medicine, particularly in areas related to cellular delivery.

The properties of liposomes can vary substantially with the lipid composition, size, surface charge and method of preparation. For this reason they are classified into three classes based on their size and the number of bilayers present: (i) small unilamellar vesicles (SUV), single lipid bilayer particles of 25 - 50 nm in size, (ii) large unilamellar vesicles (LUV), single lipid bilayer particles of > 50 nm and (iii) Multilamellar vesicles (MLV), particles consisting of several lipid bilayers separated from one another by a layer of aqueous solution [84]. SUV and LUV are generally the more sought after particles as they are more defined in structure and physical properties.

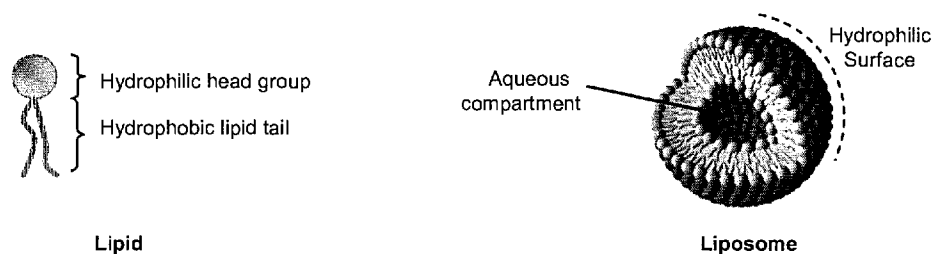


Figure 1.14: General Structures of Lipids and Liposomes

The principle component of liposomes are the lipids themselves and it is these that can dictate the overall properties of the liposome (see Figure 1.14). The surface charge and membrane rigidity of the liposome are the most frequently modified factors. Surface charge can be modified by the introduction of appropriate positively or negatively charged lipids [85]. The rigidity of the membrane depends upon the chemical nature of the lipid tails. Lipid derivatives of stearic acid (fully saturated C18 chains) confer rigid and impermeable characteristics to the membrane, whilst lipid derivatives of oleic acid (unsaturated C18 chains) result in a more permeable and less stable lipid bilayer [85].

For successful and often prolonged *in vivo* circulation of liposomes, the liposome bilayer is frequently coated with a neutral polymer, such as polyethylene glycol (PEG), resulting in production of so called 'stealth liposomes' [86]. This helps prevent opsonization (process where by foreign particles become coated with antibodies, making them more readily ingested by phagocytic cells), and halts their subsequent degradation.

The intrinsic properties of liposomes (hydrophobic and hydrophilic) along with the adjustable nature of these particles means they can be easily adapted for use *in vitro*, *ex vivo* and *in vivo*. Liposomes have numerous applications but they are most frequently used for drug and nucleic acid delivery.

Liposomes and Drug Delivery

Many of the pharmacological properties of drugs can be improved through their encapsulation into liposomes. Liposomes can entrap up to tens of thousands of drug molecules [87] and this has been exploited in the development of several liposome-encapsulated drugs that have reached the market and many more that are in advanced

clinical trials (see Table 1.2). The majority of liposomes approved for the clinic are PEG coated stealth liposomes [88].

Drug or Therapeutic agent (trade name)	Disease	Year of Approval or current status	Reference
Liposomal amphotericin B (AmBisome)	Fungal infections	1990 (Europe) 1997 (USA)	[89]
Liposomal Cytosine arabinoside (DepoCyt)	Lymphomatous meningitis	1999 (USA)	[90]
Liposomal Doxorubicin (Doxil/Caelyx)	Refractory breast/ovarian cancer	1999 2003 (Europe)	[91]
Liposomal Verteporfin (Visudyne)	Wet macular degeneration	2000 (USA)	[92]
Liposomal Paclitaxel (LEP ETU)	Advanced solid tumours	Phase I/II	[93]
Liposomal Lurtotecan (OSI-211)	Ovarian cancer, small lung cell cancer	Phase II	[94]

Table 1.2: Selection of Liposomal Drugs

One of the main advantages of liposomal drug delivery is the enhanced permeability and retention (EPR) effect or passive targeting [95]. In certain pathological conditions, such as growth of solid tumours, the tissue vasculature increases *via* the process of angiogenesis. Angiogenic blood vessels, unlike normal blood vessels, have large gaps between the adjacent endothelial cells, up to 600-800 nm [88]. This allows particles such as liposomes, which normally are excluded from healthy tissue, to access these diseased tissues and localise in the intrastitial space. In tumours this effect is further enhanced by the impaired lymphatic drainage [88]. The accumulation of liposomes and subsequent drug release results in a 10 fold higher delivery of anti-tumour drugs using liposome encapsulation compared to delivery of the 'free-drug' alone [88]. This effect has been readily exploited with the delivery of anti-tumour drugs such as doxorubicin [96] and paclitaxel [93] to solid tumours. However, it must be noted that the EPR effect does not result in a homogeneous distribution of drug within the tumour itself. The

accumulation of liposomes in the tumour is localised, and the factors that result in high concentrations of liposome/drug in one part of a tumour but not another are not well understood yet [97].

Targeting of liposomes can also be an active process. Attaching ligands to the surface of liposomes can actively traffic them to a desired tissue. Liposomes have been functionalised with antibodies [98], peptides [99] and receptor ligands such as folate [100], which have targeted specific cell types. Despite numerous investigations of improved efficacy of ligand-targeted liposomes, these formulations have been slow to reach the clinic [101].

Not all liposome-encapsulated drugs exploit the EPR or targeting effects. The hydrophobic and hydrophilic nature of liposomes makes them excellent candidates for solubilising drugs of limited solubility. Many drugs, such as chemotherapeutics, antibiotics and antifungals are very hydrophobic in nature. The hydrophobicity of these drugs often causes them to precipitate in aqueous media [88], and they are often associated with excipients (solubilising agents), such as Cremphor EL for taxol [102], which can result in increased toxicities. Liposomes can easily solubilise hydrophobic drugs with a limited toxicity compared to the free drug [88]. This is a result of the make-up of the liposome bilayers whose components are often naturally occurring lipids.

Liposomes can also dramatically alter the pharmacokinetics and biodistribution of drugs. Indeed liposomes make excellent systems for the controlled *in situ* release of drugs, a system analogous to a continuous drug infusion, but with less patient inconvenience [88]. The bioavailability of a drug can also be altered providing excellent protection for drug molecules that are rapidly degraded or cleared *in vivo*, such as the loss of activity of Camptothecins at physiological pH [103].

Liposomes and Nucleic Acid Delivery

There is also a considerable research effort to exploit the use of liposomes for the delivery of nucleic acids, either in the form of plasmid DNA for gene therapy systems, or as agents to deliver antisense oligonucleotides or small interfering RNA (siRNA) for the down regulation of specific genes. The negatively charged nucleic acids are complexed in the liposome by means of electrostatic interactions with cationic lipids, resulting in a gain of entropy and the formation of a so-called lipoplex (see Figure 1.15).

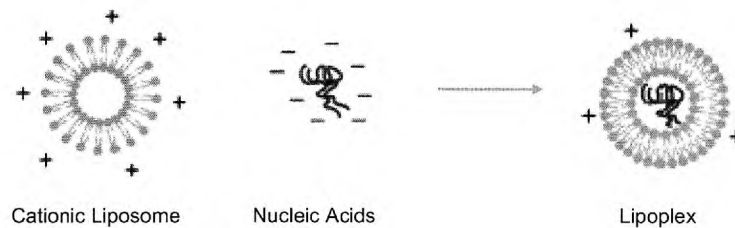


Figure 1.15: Formation of a Lipoplex.

For the nucleic acids to successfully be processed it is essential that they enter the cell. The principle mechanism by which liposomes and lipoplexes enter the cell is by the process of endocytosis (see Figure 1.16).

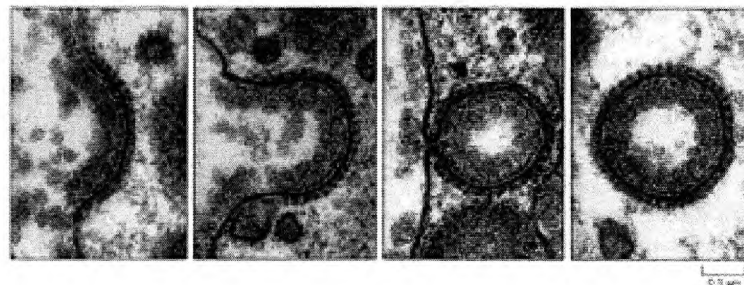


Figure 1.16: Steps of Endocytosis

The uptake of foreign material into a cell *via* endocytosis depicted using electron microscopy of clathrin uptake into cells. <http://www.sp.uconn.edu/~bi107vc/images/cell/clathrin.jpg>

Endocytosis is the mechanism by which eukaryotic cells take up material from the outside by invagination of the plasma membrane to form intracellular vesicles (endosomes) enclosing the external material. It is an essential process for cellular stabilisation, as it is responsible for uptake of cellular nutrients and many other physiological processes [104]. Endocytotic mechanisms are initiated upon contact of the lipoplex with the cell surface. An initial indentation of the plasma membrane forms, which increases in size until a vesicle is formed, which then buds off to form the endosome (see Figure 1.16). Once inside the cell the nucleic acids must escape the endosome for successful processing of the nucleic acids and therapy to occur.

An endosome has a limited lifetime in the cell cytoplasm before it fuses with a lysosome. Lysosomes are organelles that have a relatively acidic internal environment and contain digestive enzymes that will degrade the nucleic acids. It is essential that the nucleic acids escape the endosome before their destruction allowing them to

function effectively inside the cell. This process is assumed to occur by membrane fusion (lipoplex with endosome), which results in a destabilisation of the endosome membrane and eventual release of the nucleic acids [105]. Finally, DNA has to be transported into the nucleus for transcription, whilst antisense oligonucleotides and siRNA act on messenger RNA (mRNA) in the cytoplasm.

Lipoplexes are cationic in nature, which means they are easily recognised by macrophages, and therefore have a limited *in vivo* circulation time. Coating the lipoplexes with neutral polymers, such as PEG, 'masks' the cationic charge and delays recognition by the immune system. However, this has been shown to affect the efficiency of lipoplex fusion with the endosome [106] and transfection efficiency of DNA [107].

For successful delivery of nucleic acids to the cell, a lipoplex has to overcome many delivery barriers [107] and it is for this reason that there has been limited progress of these systems to *in vivo* research, particularly intravenous applications. Successful gene therapy (delivery of DNA) experiments have however been achieved with delivery of lipoplexes directly to tumours, vascular endothelial cells *via* means of catheter-mediated delivery and to lung tissue by nasal inhalation [88]. Recently there has also been progress in the development of surface neutral long circulating stealth liposomes containing antisense oligonucleotides, which resulted in antitumour effects in animal models [108].

Liposomes provide a diverse range of opportunities for molecular imaging. Immediately apparent is the imaging of drug and nucleic acid delivery, but perhaps the most exciting application lies with their ability to cross the cell membrane. The adjustability of these particles means they can easily be adapted for uptake into various cell lines. Targeting of these particles (either active or passive) could potentially provide a means to label cells *in situ*, which is particularly interesting for disease monitoring and investigation.

For effective molecular imaging of liposomes, they need to be labelled with probes to permit their visualisation. Labelling and imaging of liposomes is however not a new topic.

Labelling and Imaging Liposomes

The continued development of liposome-based agents for diagnostic and therapeutic applications requires an understanding of the pharmacokinetics and biodistribution of

these particles after administration into the body, and it is here that imaging has proved to be an invaluable tool. Historically, liposomes have been labelled with radioisotopes for nuclear imaging (such as ^{111}In [5], $^{99\text{m}}\text{Tc}$ [109], ^{67}Ga [110]) and MRI sensitive probes (such as Fe [111] and Gd [112]). To date the radiolabelling of liposomes has been the most frequently used and successful technique.

Radioisotope Labelling of Liposomes

Initially, liposomes were labelled by encapsulation of radioisotopes into either the lipid phase [113] or the aqueous phase [114] during their manufacture. However, radioisotopes frequently leaked from the liposomes and the liposome needed to be manufactured and used within a short time period (half-life of the radioisotope), which meant that this method was not a practical choice. Alternative methods have since been developed based on the labelling of liposomes after their manufacture. The most frequently used are: (i) incubating radioisotopes with liposomes containing a lipid-chelator conjugate (such as DTPA) and (ii) 'Post-Loading' of the aqueous phase with radioisotopes (see Figure 1.17).

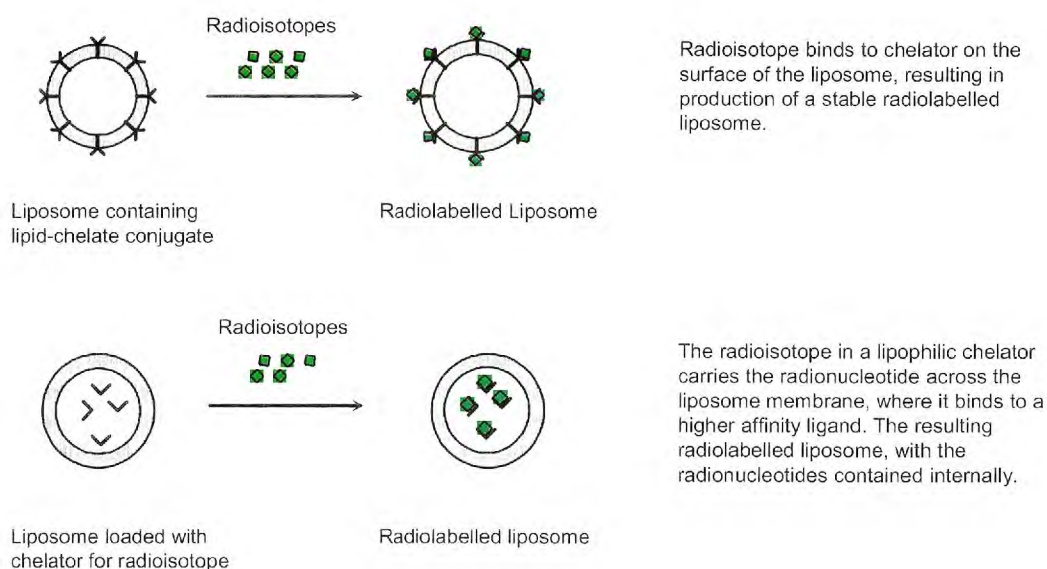


Figure 1.17: Common Techniques for Radiolabelling liposomes

Liposomes containing lipid-chelate conjugates can be effectively labelled with a range of radioisotopes giving highly stable complexes. However, these lipid chelate systems suffer from a decreased *in vivo* stability, most probably as a result of the radiolabel being present on the surface of the liposome [115]. Recently attempts have been made to counteract this using PEG stealth liposomes. These radiolabelled stealth liposomes

have shown some *in vivo* success [5]. In the so-called 'after loading' method a lipophilic chelate is used to carry the radioisotope through the lipid membrane into the aqueous phase, where the isotope interacts with a new, higher binding affinity chelate, and becomes entrapped inside the liposome membrane. These particles have a greater *in vivo* stability, as the chelator is not exposed on the surface of the liposome [115]. It is important to note that in both cases the liposome must be purified from the remaining free radionucleotides before use in order to gain successful and accurate imaging. Both techniques have been successfully applied for labelling of liposomes used in *in vivo* experiments.

Radioactive liposomes were initially used as a tool for ascertaining the biodistribution and pharmacokinetics of liposomes within the body, but more recently they have been employed for imaging the manifestations of certain diseases, such as solid tumours [5] and in the development of new liposome formulations, particularly for the delivery of anti-cancer drugs [116]. The fast image acquisition time and small quantities of probe needed has meant that radiolabelling and subsequent nuclear imaging of liposomes provides a quick and accurate method for determining liposome efficacy.

Magnetic Labelling of Liposomes

The first magnetoliposomes (iron-based) were made in 1988 to study fundamental membrane processes [117]. Since then they have found many alternative applications, particularly within MRI. Classical magnetoliposomes have the metal (iron [111] or chelated-gadolinium [112]) entrapped in the aqueous compartment of the liposomes. The metals are entrapped during the manufacture of the liposomes as unlike radioisotopes the MRI sensitive metals do not have a limited lifetime so this method of manufacture becomes both convenient and practical. Whilst the high molecular weight iron complexes show limited leakage from the liposome, the low molecular weight water-soluble gadolinium-chelates often leak from the liposome upon contact with body fluids [118]. Moreover it has also been shown that when too high concentrations of gadolinium-chelates are encapsulated in the liposomes for better image enhancement, the relaxivity of the resulting liposome is lower than that of the free gadolinium chelate [118].

To harness the favourable T_1 relaxation properties of gadolinium for imaging, alternative methods of labelling liposomes have recently been developed. These centre around covalently attaching gadolinium-chelates to the liposome. The development of a poly-gadolinium chelating polymer to coat liposomes (analogous to PEG in stealth

liposomes) has been described [119] and several research groups have synthesised gadolinium-chelate lipids for incorporation into the liposomes [58, 120, 121].

Magnetoliposomes have most commonly been used as tools to alter the pharmacokinetics and biodistribution of the normally small water-soluble contrast agents. The principal applications of magnetoliposomes have been in exploiting the inherent pharmacokinetics and biodistribution of the liposomes themselves, particularly for imaging the liver, spleen [122], [123] and lymphatic system [124]. The longevity of probe life means that magnetoliposomes have also been a valuable tool for the development and assessment of long circulating liposomes [118]. More recently, stable stealth gadolinium-based magnetoliposomes have been developed [58]. These are promising nanoparticles for the investigation of disease manifestations and processes *in vivo*.

Labelling liposomes with probes for nuclear imaging and MRI has provided a valuable system for disease diagnosis and tissue mapping, an essential tool for conventional diagnostic imaging. Whilst radiolabelling and subsequent imaging of liposomes has seen more prominence in the literature, magnetoliposomes for MRI arguably hold more promise for developments in molecular imaging. The limited life times of nuclear imaging probes means that limited long-term information can be gained, and for studies like cell tracking this is a big disadvantage. Logistically the synthesis and immediate use of radioisotopic probes is also limiting. MRI sensitive liposomes can be manufactured and stored easily, without losing sensitivity, as the metal probes do not have a limited lifetime. However, if the potential of magnetoliposomes for molecular imaging is to be realised, methods need to be developed for the controlled and facile labelling of these particles in a manner that is amenable to the established adaptability of the liposomes themselves.

1.4 Thesis Synopsis

The work in this thesis focuses on developing a novel and efficient method for the gadolinium labelling of liposomes for molecular imaging using MRI. The principle components of liposomes are the lipids themselves and it is these molecules that confer many of the essential physical properties. An efficient labelling system must be compatible with a range of liposomes (for example liposomes that complex nucleic acids, liposomes that encapsulate and deliver drugs or empty liposomes for pure molecular imaging purposes). Initially we addressed the design and synthesis of a

range of gadolinium lipid conjugates for incorporation into liposomes (Chapter 2). To complement these lipids, a range of cationic lipids were also developed which would assist in transporting the gadolinium lipids across cell membranes as part of a cationic liposome (Chapter 3). Two biological applications for our gadolinium lipids and subsequent gadolinium liposomes were explored, namely *in vivo* tracking of liposomes and cell labelling (Chapter 4). Finally an alternative gadolinium liposome labelling strategy was developed borrowing from techniques used in bioconjugate chemistry. This strategy aims to increase the labelling payload per gadolinium ion (see Chapter 5).

CHAPTER TWO

Synthesis and MRI Analysis of Novel Gadolinium Lipids

2.1 Liposomes and Molecular Imaging

Liposomes are artificially constructed spheres of lipid bilayer that enclose an aqueous compartment. The intrinsic hydrophobic yet hydrophilic nature of these particles makes them interesting candidates for transport and delivery of cargo within the body. Indeed, liposomes are frequently used in the clinic as drug delivery vehicles and are being explored as vectors for nucleic acid delivery [88]. The principle components of liposomes are the lipids themselves and it is these molecules that can confer the overall and varied physical properties (size, charge and membrane permeability) of liposomes [85]. Liposomes can be attuned to traverse cell membranes, alter the pharmacokinetics and biodistribution of their cargo and provide steric protection of the cargo from *in vivo* degradation. It is these vast functions combined with the adjustability of these particles that makes them interesting targets for molecular imaging.

For effective molecular imaging an efficient labelling strategy and imaging modality must be established. Historically liposomes have been labelled with radioisotopes for nuclear imaging [5], [109], [110]. This has provided information on the efficacy of new liposome formulations as well as a method of imaging the location and size of solid tumours [5]. However, the limited probe lifetime of radioisotopes, combined with lack of image resolution [18] from nuclear imaging means that despite its success as a tool for assessing the efficacy for liposomes, this technique cannot directly translate to all aspects of molecular imaging. Although there has been limited research of liposomes using MRI, the advantages of using MRI for a molecular imaging tool are apparent. MRI produces images of high resolution (10-100 μm) [18] and the paramagnetic metal probes used (Gd(III) or Fe(III)) do not have a limited lifetime. Combined with its increasing prominence as a research tool and prevalence as a clinical imaging modality, MRI is an interesting candidate for liposomal molecular imaging studies.

The favourable properties of Gd(III) have meant that it has received the most attention with respect to developing new contrast agents [71], and the work in this thesis aims to harness these properties to produce an efficient liposome labelling strategy. To date, gadolinium labelling of liposomes has predominantly involved the encapsulation of Gd(DTPA)^{2-} into the aqueous compartment of the liposome [112]. However, there are several restricting problems with this method: the small polar Gd(DTPA)^{2-} frequently leaks from the liposome, even with relatively impermeable liposome membranes the leakage is still significant [118] and secondly the Gd(DTPA)^{2-} occupies the space of liposome cargo which places restrictions on the overall functionality of the liposome.

One solution to this problem is to label liposomes with gadolinium lipids (gadolinium chelate lipid conjugates). The gadolinium lipids would be held in the membrane by the same electrostatic forces that govern formation and stability of the lipid bilayer. The gadolinium chelate head group will be positioned on the hydrophilic surface of the liposome with maximum access to the surrounding water, ensuring maximum efficacy of the contrast agent, whilst the hydrophobic lipid tail will anchor the chelate to the membrane. The aqueous compartment of the liposome will be left vacant to permit the liposome to transport a cargo such as a therapeutic drug or nucleic acids.

Gadolinium Lipids

Several groups have already addressed the synthesis of gadolinium lipids. Selected structures of these molecules are shown in Figure 2.1.

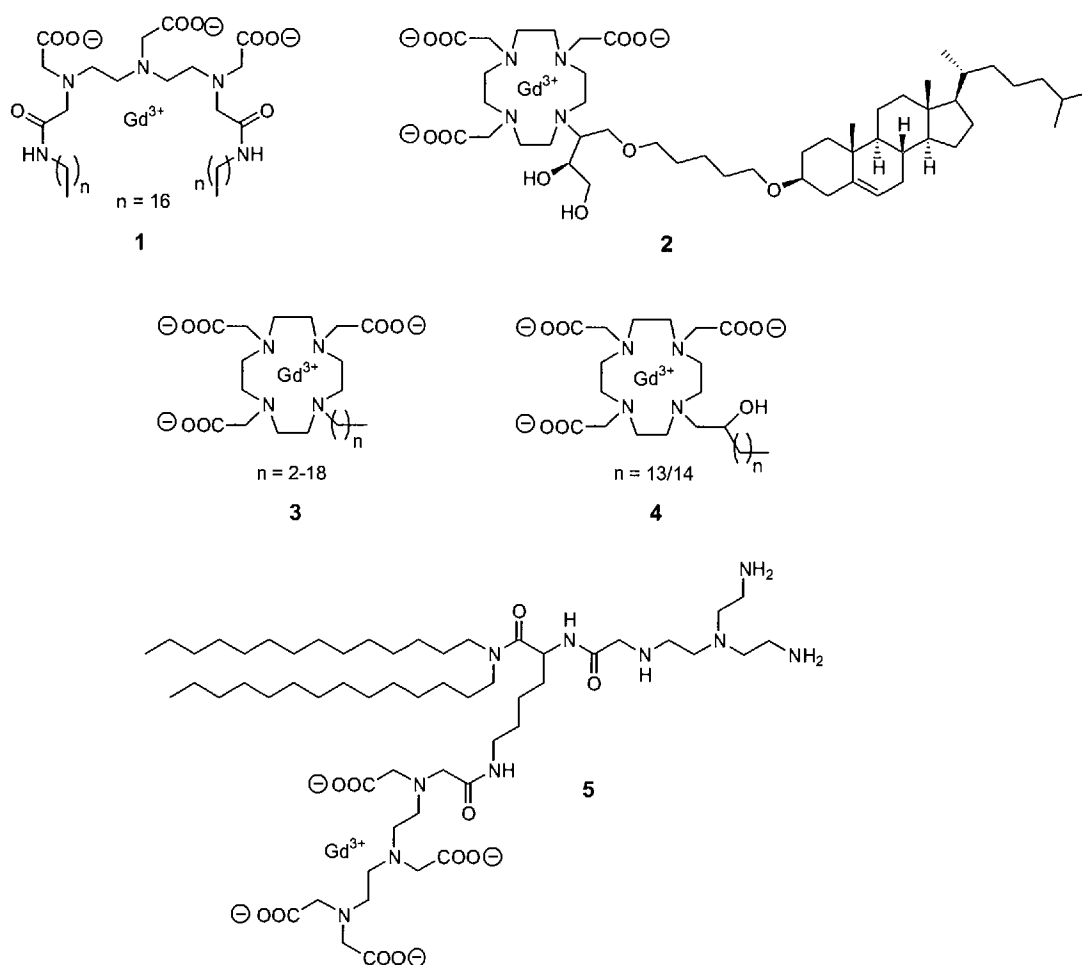


Figure 2.1: Selected Gadolinium Lipids

Originally gadolinium lipids were reported as contrast agents for conventional diagnostic imaging of the liver and spleen, **1** [125]. Addition of a lipophilic tail to the normally small water-soluble gadolinium chelate (DTPA) altered its pharmacokinetics and biodistribution resulting in clearance of the compound through, and subsequent imaging of the hepatobiliary system. These initial lipids were designed to remain solely outside cells, in their secondary environment. However, with the recent emergence of molecular imaging, several groups have begun to readdress the design and synthesis of gadolinium lipids specifically for molecular imaging. Indeed a cholesterol-based lipid has been synthesised for imaging of the adrenal gland, **2** [126] and several groups have synthesised gadolinium lipids for incorporation into liposomes (**3**, **4**, **5**) during the course of this thesis [127], [121], [128].

The structures of the first gadolinium lipids (**3**, **4**) for incorporation into liposomes use a single alkyl chain, of varying length for the hydrophobic lipid tail [121], [128]. These simple lipid structures provided proof that gadolinium lipids can successfully be incorporated into liposomes resulting in the formation of MRI active particles. However, the overall shape of these gadolinium lipids means that these compounds are more predisposed for the formation of micellar structures over the lamellar structure required for formation of a lipid bilayer. This severely limits the maximum quantity of these gadolinium lipids that can be incorporated to the liposome and also limits the choice of co-lipids that can be employed for liposome formation. Byk *et al* [127] have synthesised a cationic-based gadolinium lipid, **5**. The cationic charge, arising from the polyamine head group, permits facile formation of gadolinium labelled lipoplexes (lipid-DNA complexes). Although this adds an extra function to the lipid it conversely limits the scope of use of the gadolinium lipid for all aspects of molecular imaging with liposomes.

Despite the success of the current gadolinium lipids, these molecules are either designed for a specific liposome application or their presence in the lipid bilayer restricts the scope of liposome use. An ideal gadolinium lipid labelling system would be applicable to the broad range of liposome functions. Thus the aim of the work in this chapter is to develop an efficient gadolinium lipid-based labelling strategy for liposomes that does not impair the adaptability of these particles.

Target Molecules

The structures of the three target molecules, Gd.DOTA.Chol **6**, Gd.DOTA.DSPE **7** and Gd.DTPA.Chol **8** are shown in Figure 2.2. In each case the gadolinium chelate forms

the hydrophilic head group of the lipid and is linked *via* an amide bond to a hydrophobic lipid tail.

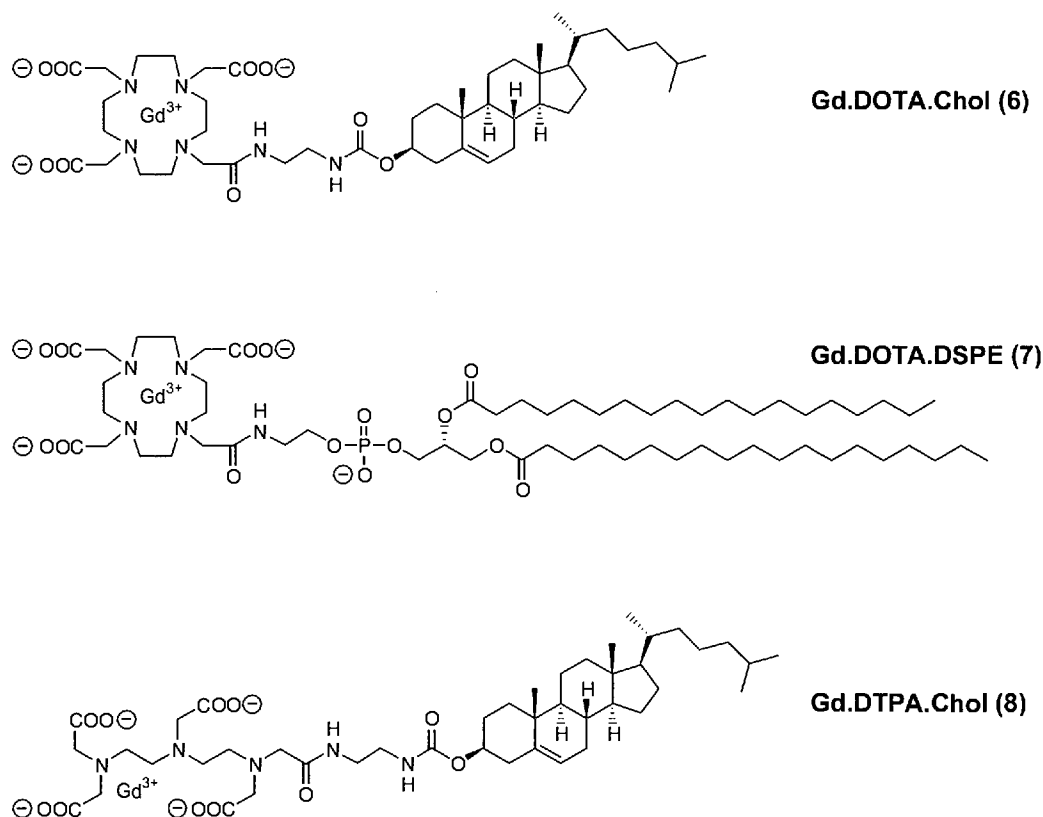


Figure 2.2: Target Molecules

In all three-target molecules the gadolinium is chelated to a DTPA or DOTA analogue (Introduction, Figure 1.9). Gd(DTPA)²⁻ and Gd(DOTA)⁻ were the first ligands to be approved for clinical use for MRI [74]. These compounds display a high *in vivo* stability and exhibit minimal toxicity [74]. As such these compounds provide an ideal skeleton for the development of new gadolinium-based agents for molecular imaging.

The lipid tails are linked to the chelate *via* an amide bond. Amide bonds are biologically stable linkages and should prevent any potential cleavage of the gadolinium from the lipid and liposome. In each case there is a spacer of at least 5 atoms between the gadolinium chelate and the lipid tail. This provides an optimal distance between the head and tail sections of the lipid, ensuring that the gadolinium chelate is on the exposed hydrophilic surface of the liposome ensuring maximum efficacy of the contrast agent.

The lipid tails consist of either cholesterol or DSPE (distearoylphosphatidylethanolamine). Cholesterol is a neutral naturally occurring sterol lipid found in abundance in animal cell membranes. Many liposome formulations employ cholesterol to alter the physical properties of the membrane. For example, in fluid lipid membranes introduction of cholesterol confers a degree of rigidity, whilst conversely in more rigid membranes, cholesterol can disrupt the intramolecular lipid interactions and therefore can introduce some fluidity (see Appendix, Section 9.1 Lipids, Cholesterol) [129]. It is for these reasons cholesterol is frequently employed in many liposome formulations. Cholesterol can also be utilised in large quantities in liposomal membranes (up to 66 mol%) without disturbing the overall structure of the lipid bilayer [130]. This is particularly advantageous when considering the insensitivity of MRI towards detecting gadolinium probes (10 – 100 μ M of probe needed) [18].

DSPE is a synthetic phospholipid, with two fully saturated C18 alkyl chain tails. DSPE confers rigidity to the liposome membrane, and is frequently used in the production of long circulating liposomes for both drug and nucleic acid delivery [100], [87]. Phospholipids themselves are the anchors of lipid membranes. They are principle components of many lipid bilayers and it is the intramolecular hydrophobic-hydrophilic interactions between the phospholipids themselves that leads to the formation and ensures stability of the lipid bilayer [131].

In summary, the structures of the target molecules combine known and biologically safe gadolinium complexes with different lipid tails, which should confer diverse properties to the membrane. We propose that the versatility of the lipid tails will ensure that these gadolinium lipids will produce an efficient labelling system for a variety of liposomes.

2.2 Synthesis of Gd.DOTA Lipids

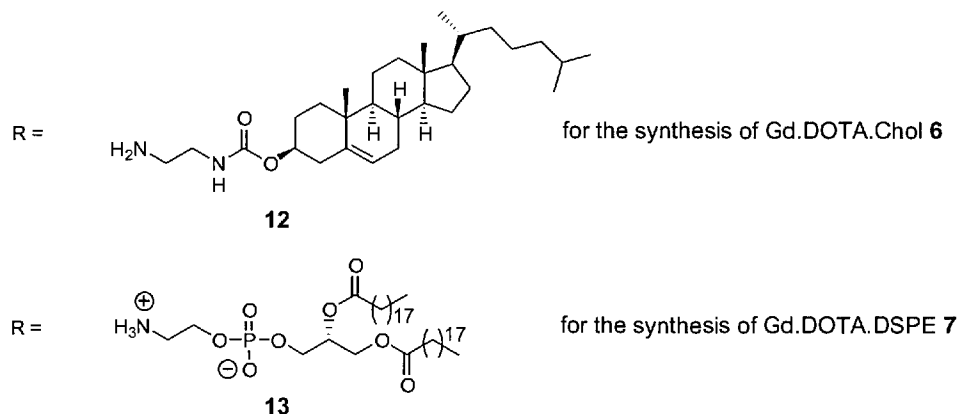
Retrosynthetic Analysis of Gd.DOTA.Chol and Gd.DOTA.DSPE

A general scheme for retrosynthetic analysis of Gd.DOTA.Chol (**6**) and Gd.DOTA.DSPE (**7**) is shown in Scheme 2.1. First, the gadolinium chelate is made from the metal free ligand **10** (DOTA.Chol or DOTA.DSPE), by addition of a suitably labile gadolinium ion source. Disconnection of the amide bond on the metal free ligand results in DOTA **11** (1, 4, 7, 10-tetrazacyclododecane-1, 4, 7, 10-tetraacetic acid) and the corresponding amine. In the case of Gd.DOTA.DSPE, this is the commercially available DSPE **13** (distearoylphosphatidylethanolamine), and for Gd.DOTA.Chol this

Chemical reaction scheme showing the synthesis of macrocyclic ligands 9, 10, 11, 14, 15, 16, and 17.

The scheme illustrates the following transformations:

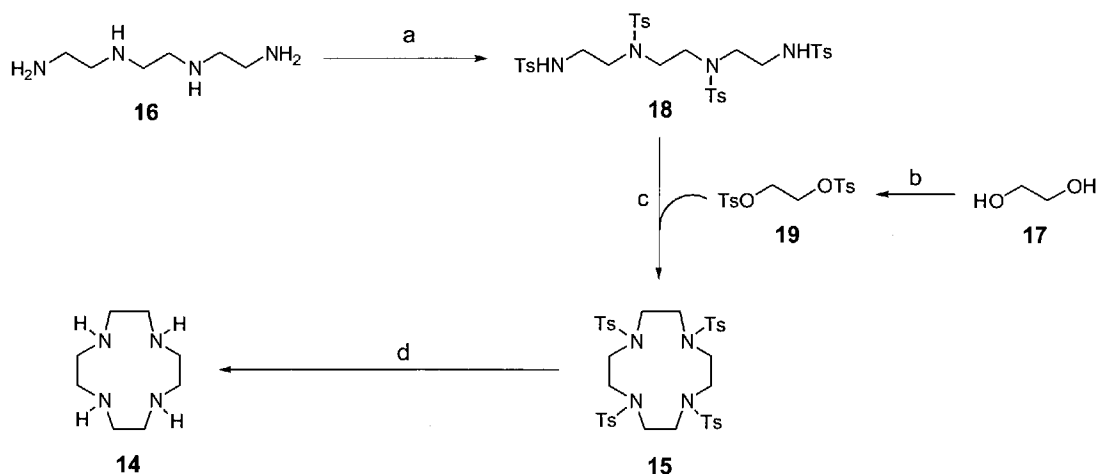
- Macrocycle **9** (with Gd^{3+}) is converted to macrocycle **10** (with HOOC groups).
- Macrocycle **10** is converted to macrocycle **11** (DOTA) and a diamine $\text{H}_2\text{N-R}$.
- Macrocycle **11** is converted to macrocycle **14** (Cyclen) and a diamine $\text{H}_2\text{N-R}$.
- Macrocycle **14** is converted to macrocycle **15** (with Ts groups) and a diamine $\text{H}_2\text{N-R}$.
- Macrocycle **15** is converted to macrocycle **16** (with H_2N groups) and a diamine $\text{H}_2\text{N-R}$.
- Macrocycle **16** is converted to macrocycle **17** (with HO groups) and a diamine $\text{H}_2\text{N-R}$.



56

Synthesis of Cyclen

Cyclen was synthesised as shown in Scheme 2.2, employing the Richman Atkins procedure [132]. There are several alternative syntheses of cyclen reported [133], [134], [135], [136], but the most robust and frequently used method is the Richman Atkins procedure. The key step in the synthesis is a metal templated macrocyclisation reaction between a fully tosyl protected polyamine **18**, and a tosyl activated diol **19**.

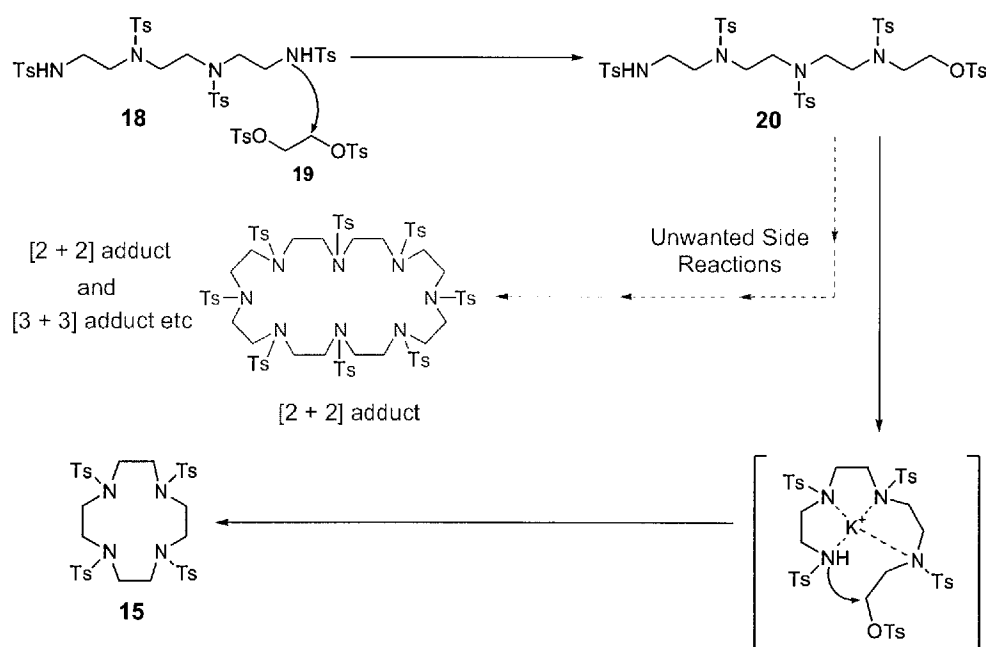


Scheme 2.2: a) *p*-TsCl, K₂CO₃, H₂O, 80 °C, 12 h, 59%; b) *p*-TsCl, anhydrous pyridine, 4°C, 12 h 71%;
c) K₂CO₃, DMF, 70°C, 18 h, 57%; d) c.H₂SO₄, 110°C, 40 h, 50%.

The first step in the synthesis involves the protection of tetraethylethylenediamine **16** with tosyl groups. Four equivalents of *p*-toluenesulfonylchloride were slowly added to the reaction mixture, ensuring the correct selectivity of the reaction. The reaction proceeds *via* a series of simple nucleophilic substitution reactions under basic conditions. The desired product **18** was isolated by precipitation and both NMR and TLC analysis indicated that no further purification was required.

The tetraamine forms the backbone of the cyclen **14** structure. The remaining two carbons are obtained from ethylene glycol di-*p*-toluenesulfonate **19**, which was easily synthesised from the corresponding alcohol **17**. The tosyl-protected alcohol **19** was isolated as a precipitate and subsequently recrystallised from ethanol to give the pure product **15** in excellent yield.

The next step in the synthesis is the pivotal macrocyclisation reaction. The mechanism for this reaction is shown in Scheme 2.3.



Scheme 2.3: Macrocyclisation Reaction

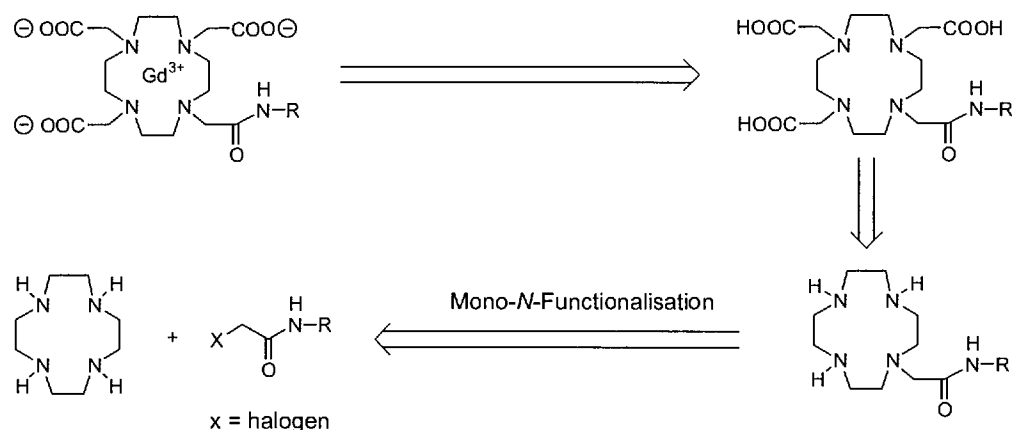
The initial reaction is an intermolecular nucleophilic attack of the protected diol **19** by a terminal amine of **18**, resulting in intermediate **20**. To obtain the correct 12 membered ring a second intramolecular nucleophilic attack is required on the terminal OTs group of **20**. However, at this stage there is a competing reaction from a second intermolecular nucleophilic attack, which ultimately results in production of unwanted [2 + 2], [3 + 3] and so on, adducts. To prevent this and ensure maximum yield of the desired product two distinct solutions are employed. Firstly, the effective concentrations of both components are reduced, by employing high dilution conditions, and secondly an ion template is used. In the case of this reaction, K_2CO_3 acts as both the base and an ion template. The potassium ion effectively 'bends' the molecules into a favourable conformation for the desired intramolecular nucleophilic attack. The fully tosylated macrocycle **15**, was isolated as a pale yellow solid, *via* crystallisation from toluene. The highly symmetrical nature of cyclen **14** and the unwanted adducts ([2 + 2], [3 + 3] and so on), makes assessment of purity by NMR difficult. However, HPLC and MS analysis showed that minimal adducts were present.

The final step in the synthesis of cyclen is the full deprotection of the tosyl-protected macrocycle **15**. This is achieved under highly acidic conditions, by heating **15** in concentrated sulphuric acid for 40 hours. Cyclen **14** was obtained after work up, as a

colourless solid in overall reasonable yield, comparable to the literature value (41% for 4 steps) [132].

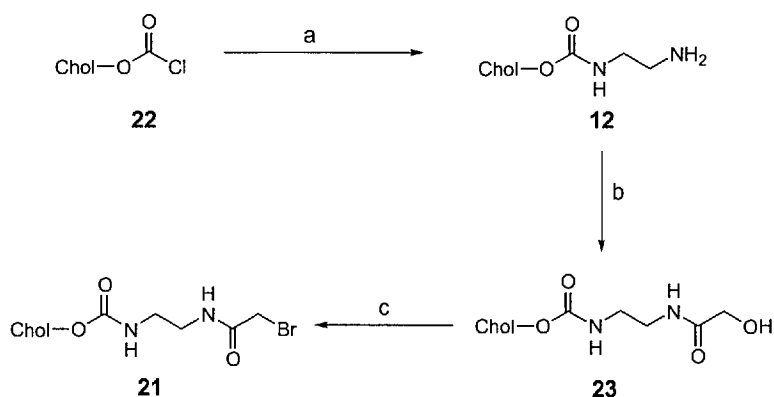
Mono-*N*-Functionalisation of Cyclen

Initially it was proposed that the synthetic routes to the target molecules, Gd.DOTA.Chol (**6**) and Gd.DOTA.DSPE (**7**) could include a mono-*N*-functionalisation of cyclen (see Scheme 2.4).



Scheme 2.4: Alternative Retrosynthesis of Gd.DOTA.Lipids

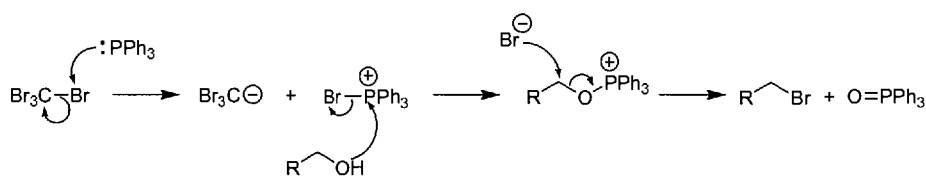
To this end the appropriate cholesterol halide-adduct **21** for the production of Gd.DOTA.Chol **6** was synthesised. The synthetic route is depicted in Scheme 2.5.



Scheme 2.5: a) Ethylene diamine, RT, 8 h, 65%; b) glycolic acid, HBTU, DMAP, CH₂Cl₂, RT, 12 h 40%; c) CBr₄, solid phase triphenylphosphine, CH₂Cl₂, RT, 15 min, 78%.

Firstly, cholesteryl chloroformate **22** is converted into cholesterol amine **12** by reaction with ethylene diamine. Cholesterol amine was isolated as a white solid after silica gel column chromatography. The next step involves addition of glycolic acid, a two-carbon unit. Despite the potential for glycolic acid to polymerise under peptide coupling conditions the desired alcohol **23** was isolated in reasonable yield after reaction with HBTU and DMAP. Attempts to increase the yield of this reaction by prior protection of the glycolic acid alcohol with a THP group were unsuccessful and it is noted in the literature that these intermediates are in fact unstable [137].

The final step in the synthesis is the conversion of the terminal primary alcohol on **23** to a bromide. This was achieved using carbon tetrabromide and solid phase triphenyl phosphine at room temperature. The reaction is marked by a significant colour change of the solution from clear to deep red/brown. The mechanism for this conversion is shown in Scheme 2.6.



Scheme 2.6: Conversion of ROH to RBr using Carbon Tetrabromide and Triphenylphosphine

The reaction conditions produce a halide nucleophile, which performs an S_N2 reaction on an activated triphenyl phosphine alcohol giving the desired bromoalkane and triphenylphosphine oxide as a by-product. Initial small-scale experiments using triphenylphosphine demonstrated that the reaction proceeded well. However, the triphenylphosphine oxide and excess triphenylphosphine proved difficult to separate from the product. Substituting triphenylphosphine for its solid phase analogue overcame these problems as the excess starting materials and unwanted by-products could be easily removed by filtration. The desired bromide **21** was isolated as a yellow oil.

Model studies showed that addition of even a simple electrophile to cyclen under a range of conditions resulted in production of a range of differently substituted cyclens (1, 2, 3 or 4 of the nitrogens functionalised). Slow addition of the electrophile and/or high dilution conditions did not solve this problem. There are however a few reports in the literature detailing methods for mono-*N*-functionalisation of cyclen. These involve

employing either metals [138], boron [139] or phosphorous [140] complexes which position themselves inside the macrocycle, coordinate and in turn reduce the nucleophilicity of three nitrogens, leaving the remaining fourth nitrogen available for nucleophilic attack (see Figure 2.3).

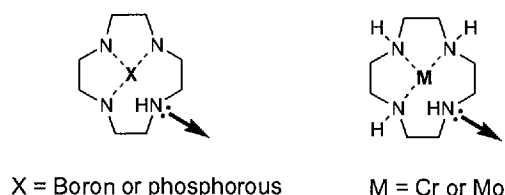


Figure 2.3: Methods for Mono-*N*-Functionalisation of Cyclen

We wished to avoid the use of metals during our synthetic pathway to prevent any adventitious metal complexations that would effect the final complexation of gadolinium. Trial reactions using selective boron protection of cyclen [139] and reactions with electrophiles including **21** unfortunately still yielded significant unwanted by-products. The highly polar nature of both the products and by-products meant that it was very difficult to achieve separation and subsequent reactions would not make purification any easier. In the interests of time and developing a more robust route an alternative strategy was sort.

DOTA.NHS.Ester

DOTA.NHS.Ester **24** (see Figure 2.4) is a mono functionalised *N*-hydroxysuccinimide ester of DOTA. Several groups have reported the use of DOTA.NHS ester as a useful intermediate in bioconjugation chemistry [141], [142] and in the synthesis of labelled peptides [143]. The NHS ester readily reacts with a range of nucleophiles, in our case this will be an amine to give the desired amide.

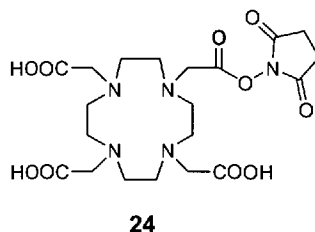
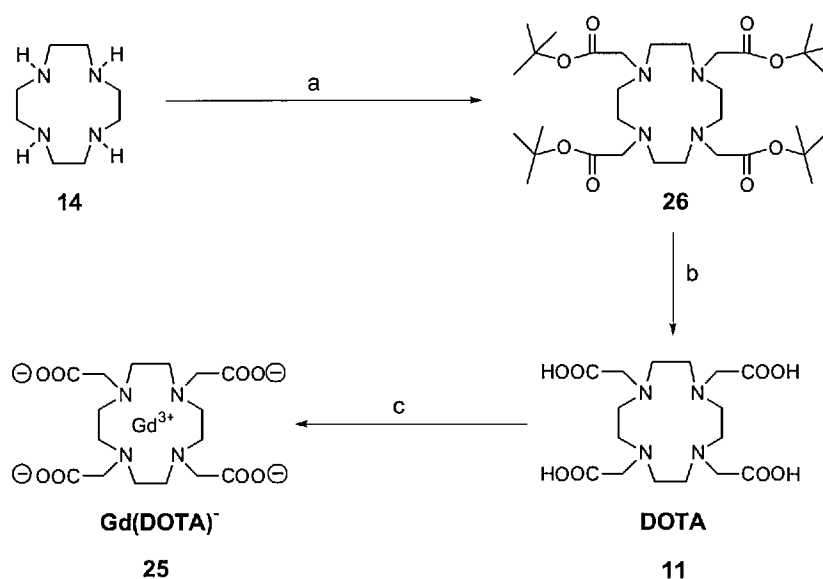


Figure 2.4: DOTA.NHS.Ester

DOTA.NHS ester is a commercially available intermediate (Macrocyclics Inc., USA), but there are reports of its synthesis from DOTA [143], [144].

Synthesis of DOTA

DOTA is the tetraacetic acid derivative of cyclen **14**, and is synthesised by appropriate alkylation of all the secondary amines of cyclen **14**. The synthetic route to DOTA **11** and $\text{Gd}(\text{DOTA})^-$ **25** is given in Scheme 2.7. $\text{Gd}(\text{DOTA})^-$ **25** is commercially available as Doterem™ in sterile solutions for *in vivo* experiments, however the solution contains sugars to enhance relaxation. As $\text{Gd}(\text{DOTA})^-$ **25** would provide an excellent bench mark of efficacy for examining our new Gd.DOTA.Lipids (**6**, **7**) the synthesis of a pure chemical sample was necessary.



Scheme 2.7: a) *t*-butyl bromoacetate, K_2CO_3 , MeCN, RT, 90 min, 98%; b) TFA, CH_2Cl_2 , triisopropylsilane, N_2 (g), 5 h, 60%; c) Gd_2O_3 , H_2O , 90°C , 12 h, 61%.

The first step in the synthesis involves the addition of protected acetic acid groups to cyclen **14** via serial $\text{S}_{\text{N}}2$ reactions in the presence of base. The desired compound **26** was obtained in excellent yield as a yellow oil, requiring no further purification. NMR analysis of **26** had to be performed at an elevated temperature (400K). The fully *t*-Butyl protected DOTA **26** is severely conformationally strained. At room temperature the signals for all the CH_2 protons on **26** are very broad and indistinguishable from 2 – 3.5 ppm. Upon increasing the temperature in increments of 10K, two distinct peaks emerge at 2.58 ppm and 3.14 ppm for the $\text{NCH}_2\text{CH}_2\text{N}$ and $\text{CH}_2\text{COOC}(\text{CH}_3)_3$ respectively. The increase in temperature transfers energy to the compound which induces rapid rotation and flipping of the molecule. These events become faster than the NMR time scale and therefore an average of signals (resulting in production of sharper peaks) is observed in place of individual signals for each environment

(resulting in a series of broad peaks). The ^{13}C NMR was similarly conducted at an elevated temperature.

Removal of the *tert*-butyl groups *via* acidolysis gave the metal free ligand DOTA **11**. Initial attempts to remove the protecting groups with TFA in dichloromethane were unsuccessful, as a mixture of products was always obtained (1, 2, 3 or 4 *t*-butyl groups remaining). Addition of a silyl scavenger and a positive flow of nitrogen gas significantly improved the reaction and DOTA **11** was isolated as a pale solid *via* precipitation from crude reaction mixture with solvent. The polyacetic acid polyamine nature of DOTA **11** means that it is difficult to solubilise the compound in a range of solvents. Indeed DOTA **11** is only soluble in water and DMSO (sparingly). NMR analysis (D_2O) had to be performed at elevated temperatures (340K). Although DOTA **11** is less conformationally strained than its *t*-butyl substituted analogue **26**, the elevated temperatures aid rapid molecular conformation flipping and subsequently production of defined and sharper NMR signals (see previous page).

Complexing of gadolinium into the DOTA **11** was achieved using gadolinium oxide (Gd_2O_3). Gadolinium oxide is insoluble in water at room temperature, however at elevated temperatures free Gd^{3+} is released. The complexing reaction occurs when the ligand is dissolved in water and stoichiometric amounts of gadolinium oxide added. The resulting solution is heated to 90°C and gadolinium complexation is marked by the solution turning clear. The resulting solution was freeze dried to give $\text{Gd}(\text{DOTA})^-$ **25** as a shiny white solid.

The paramagnetic nature of all gadolinium compounds makes analysis by NMR difficult. The purity of gadolinium complexes is therefore assessed by MS, HPLC and the xylenol orange test (a colorimetric test to assess for the presence of any free gadolinium [145]). HPLC and simultaneous MS analysis of $\text{Gd}(\text{DOTA})^-$ **25** indicated that the compound had been isolated in sufficient purity (> 98%) and the xylenol orange test indicated that there was no free gadolinium present (see Appendix, Section 9.2 Xylenol Orange). Interestingly, in the MS of gadolinium complexes it is possible to see peaks for all abundant isotopes of gadolinium. This provides a good analytical tool for monitoring gadolinium complexation in reactions and is also an excellent tool for characterisation of gadolinium containing compounds. As an example of this the MS of $\text{Gd}(\text{DOTA})^-$ **25** is shown in Figure 2.5.

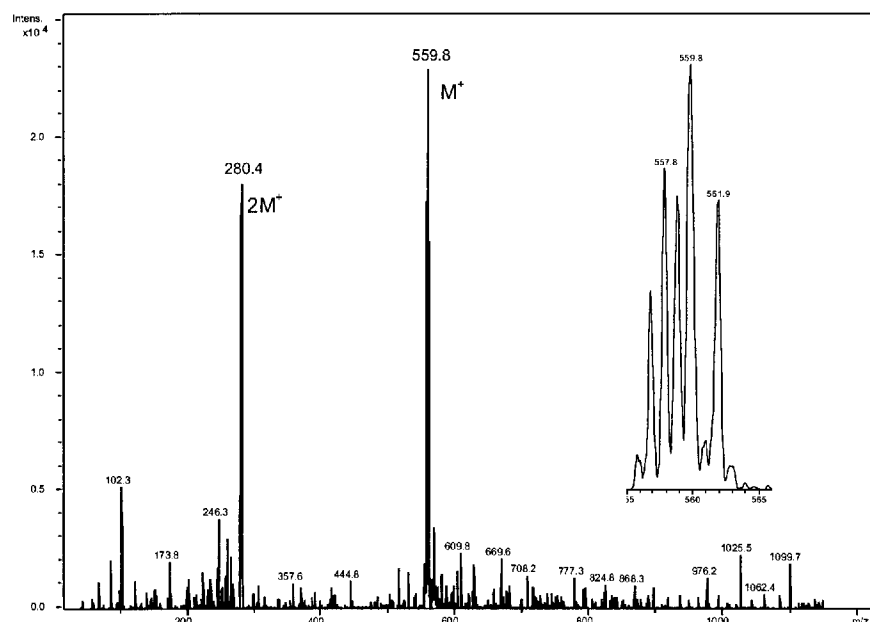


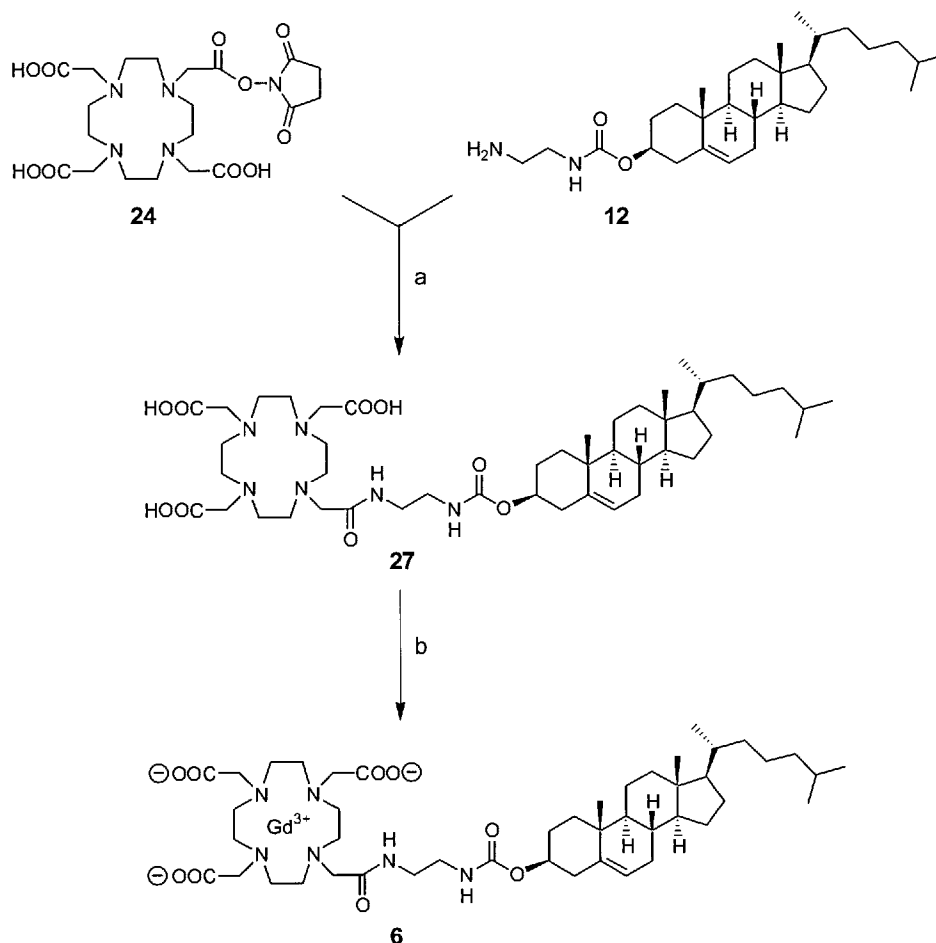
Figure 2.5: Mass Spectrum of Gd(DOTA)⁺ **25**.

(Positive Ionisation ESI. Inset shows the gadolinium isotopic distribution within the M⁺ peak)

Several attempts were made to synthesise DOTA.NHS.ester **24** from DOTA **11**. These involved using a coupling reagent (Dimethylaminopropyl-3-ethylcarbodiimide (EDCI) or Dicyclohexylcarbodiimide (DCC)) in the presence of DOTA **11** and *N*-Hydroxysuccinimide in either DMF/H₂O [143] or DMSO [144]. The highly limited solubility of DOTA **11** restricted the reactions to these unfavourable solvent systems. However, all attempts were unsuccessful in either isolating or producing an *in situ* sample of DOTA.NHS.ester in adequate purity and yield. As an alternative solution DOTA.NHS.ester **24** was used from the commercial source.

Synthesis of Gd.DOTA.Chol (**6**)

Gd.DOTA.Chol **6** was synthesised from the intermediate DOTA.NHS.ester **24**. The synthetic route to the target molecule is given in Scheme 2.8.



Scheme 2.8: a) CHCl_3 , NEt_3 , RT, 3 h, 76%; b) Gd_2O_3 , H_2O , 90°C , 12 h, 58%.

The reaction between DOTA.NHS.Ester **24** and cholesterol amine **12** occurs readily at room temperature in the presence of base. Initially the reaction was attempted with 4-(Dimethylamino)pyridine (DMAP). Although the reaction proceeded effectively, residual DMAP proved difficult to separate from the final product. Triethylamine was employed as an alternative, and this proved easier to separate from DOTA.Chol **27**. After tedious experimentation, the correct, highly basic column conditions were found. DOTA.Chol **27** was isolated as a pale water-soluble solid in good yield. HPLC analysis of the metal free ligand **27** demonstrated that it had been purified to above 98% (see Appendix, Section 9.5, DOTA.Chol).

As DOTA.Chol **27** proved to be water-soluble the gadolinium oxide method of complexation was used (same as that used for DOTA **11** to $\text{Gd}(\text{DOTA})^-$ **25**, (see Synthesis of DOTA). After the reaction had gone to completion, the solution was freeze

dried to give Gd.DOTA.Chol **6** as a white solid. The xylenol orange test indicated that no free gadolinium ions were present.

Gd.DOTA.Chol was analysed by HPLC and MS (see Figure 2.6), and was shown to be > 98% pure.

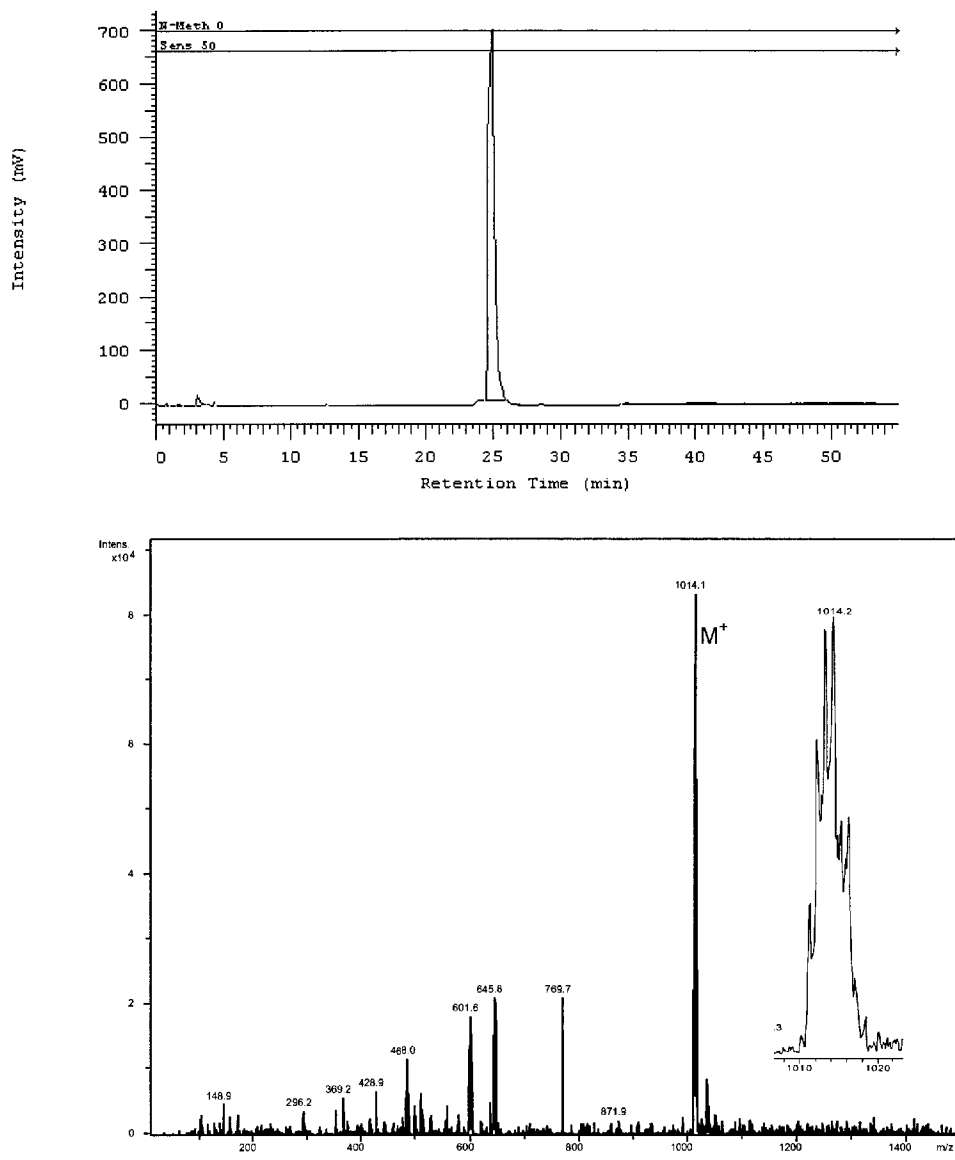
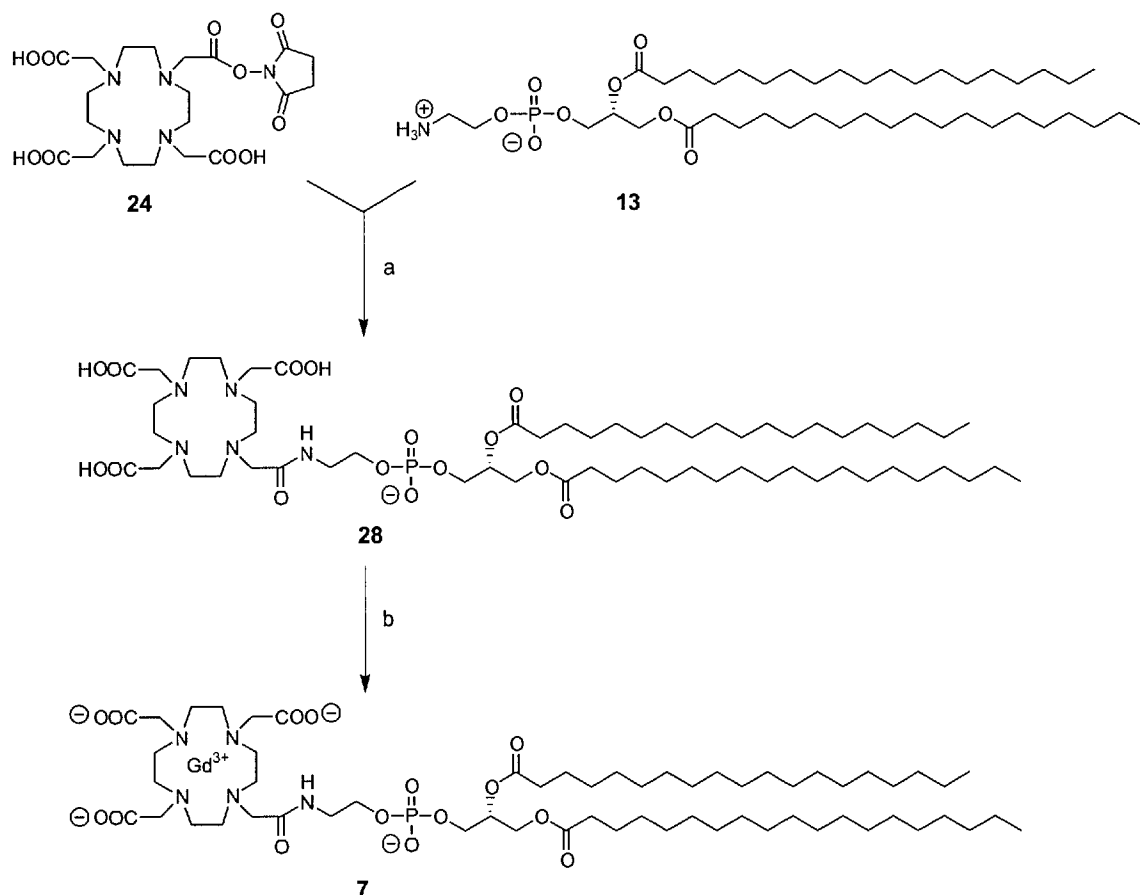


Figure 2.6: HPLC and MS of Gd.DOTA.Chol (**6**)

(HPLC conditions: See page 174. Mass Spectrum: Positive ionisation ESI, inset shows the gadolinium isotopic distribution within the M⁺ peak)

Synthesis of Gd.DOTA.DSPE (7)

Gd.DOTA.DSPE **7** was synthesised in an analogous method to Gd.DOTA.Chol **6**, via DOTA.NHS.ester **24**. The synthetic route to Gd.DOTA.DSPE is shown in Scheme 2.9.



Scheme 2.9: a) NEt₃, CHCl₃, Δ, Benzaldehyde scavenger resin, 12 h, 77%; b) GdCl₃, CHCl₃/MeOH, NH₄OH, 4 h, 88%

Although the reaction between DOTA.NHS.ester **24** and cholesterol amine **12** proceeded well at room temperature, for a reaction between DSPE **13** and DOTA.NHS.Ester **24** the reaction mixture had to be heated. This is a direct result of the chain melting phase transition temperature (T_C) of DSPE **13**. DSPE is a white powder that forms a fine suspension upon hydration with solvents (CHCl₃ and water) at room temperature. Due to interactions between the two C18 fully saturated alkyl chain tails, DSPE is in a crystalline state at room temperature. The T_C (crystalline to fluid state) of DSPE is 64°C [131]. Above this temperature DSPE 'dissolves' in solution and its terminal amine becomes chemically available. When the reaction had gone to completion the solvents were removed *in vacuo* and crude product (~75% pure,

impurity predominantly un-reacted DSPE (determined by HPLC)) was obtained by precipitation with cold water. The crude product was purified by the use of benzaldehyde scavenger resin to remove the residual DSPE. DOTA.DSPE **28** was isolated as a white powder in good yield and purity (see Appendix, Section 9.5, DOTA.DSPE).

As DOTA.DSPE **28** was not soluble in water an alternative gadolinium complexation strategy was needed. GdCl_3 is a highly labile source of Gd^{3+} in protic solvents and there have been several literature reports detailing its use in the synthesis of gadolinium containing compounds [120], [127]. Although DOTA.DSPE **28** is not soluble in a protic solvent alone, it was found to be soluble in a mixture of chloroform and methanol, a system compatible with solvating GdCl_3 . Trial reactions showed that this complexation reaction required the presence of base. After much experimentation with a variety of bases (such as triethylamine, diisopropylethylamine) NH_4OH (aq) proved to be the best candidate, as the NH_4Cl salt precipitated out of solution and could thus be easily removed by filtration. Gd.DOTA.DSPE **7** was isolated as a white solid in excellent yield. The xylenol orange test showed that there were no free gadolinium ions present. Gd.DOTA.DSPE was analysed by HPLC and MS (see Figure 2.7) and shown to be >97% pure.

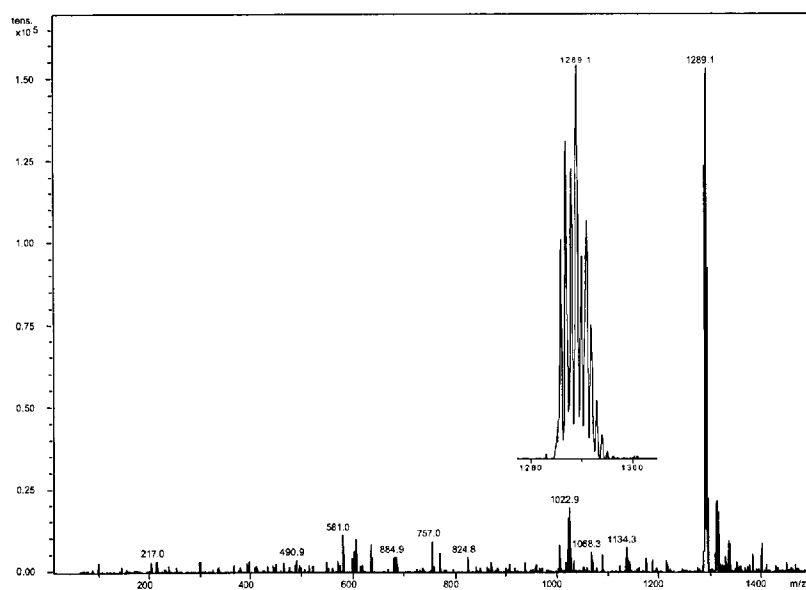
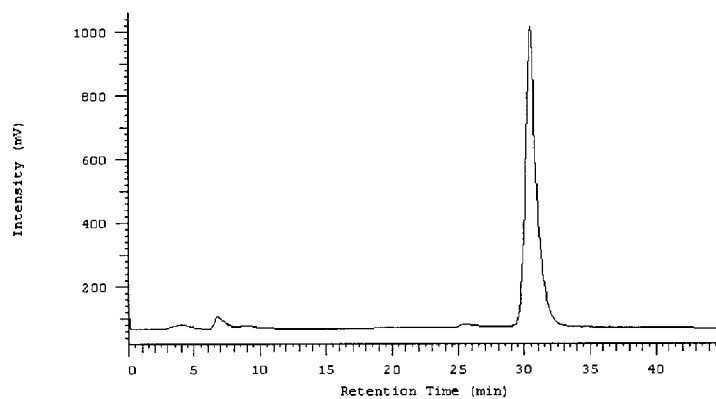


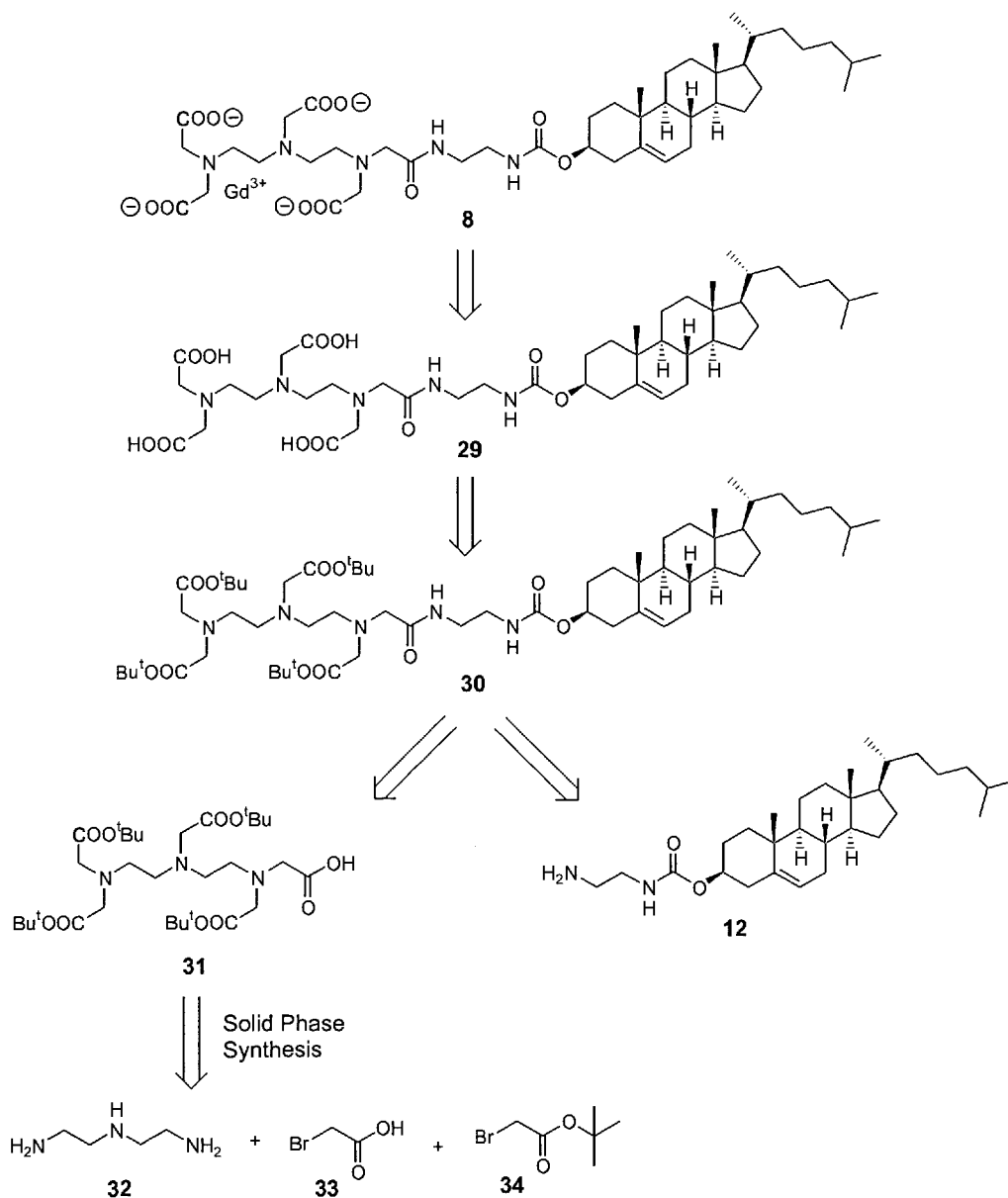
Figure 2.7: HPLC and MS of Gd.DOTA.DSPE (7)

(HPLC conditions: See page 176. Mass Spectrum: Positive ionisation ESI, inset shows the gadolinium isotopic distribution of the M^+ peak)

2.3 Synthesis of Gd.DTPA.Lipids

Retrosynthesis of Gd.DTPA.Chol (8)

The retrosynthesis of Gd.DTPA.Chol 8 is detailed in Scheme 2.10.



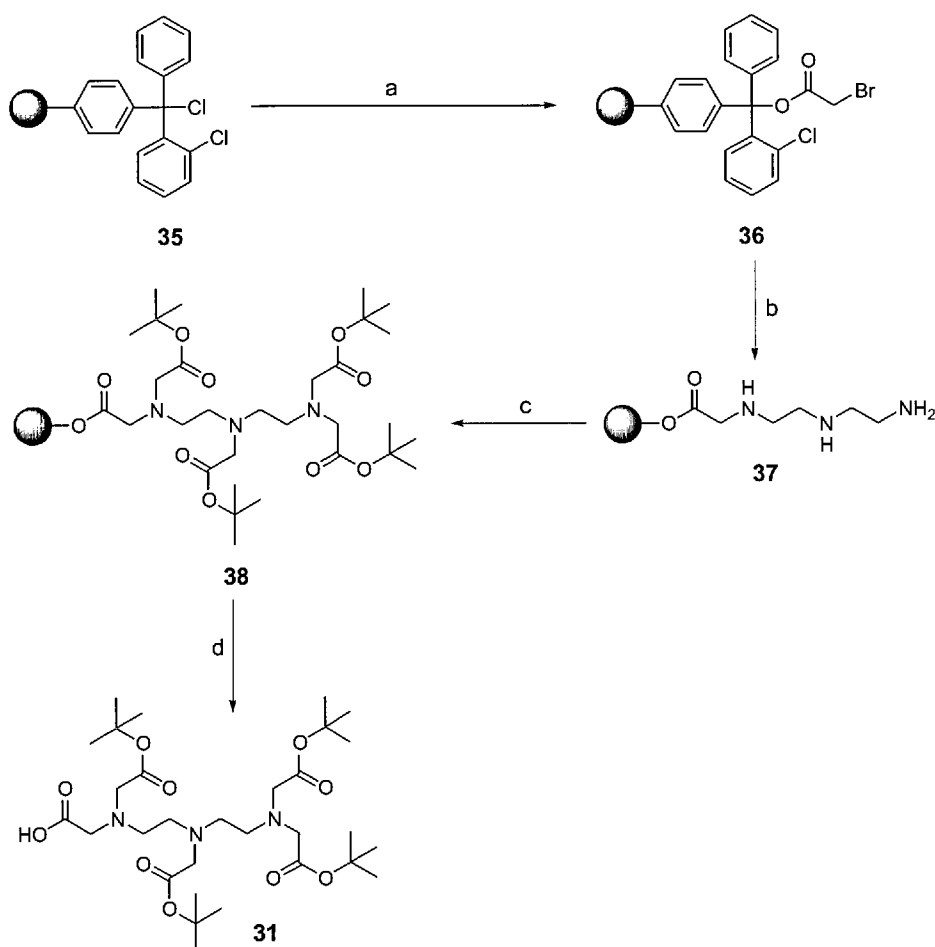
Scheme 2.10: Retrosynthesis of Gd.DTPA.Chol (**8**)

As with the retrosynthesis of Gd.DOTA.Chol **6** and Gd.DOTA.DSPE **7** the gadolinium lipid is made from the metal free ligand **29**, by addition of a suitably labile source of gadolinium ions. In an effort to overcome the previously described problems of

selective functionalisation of either the amine backbone of the ligand (previously cyclen **14**) or its polyacetic acid analogue (previously DOTA **11**), an alternative strategy was sort. To this end the metal free ligand **29** is derived from its fully *t*-butyl protected analogue **30**. A disconnection at the amide bond renders cholesterol amine **12** and a selectively *t*-butyl protected DTPA **31**. The selectively protected *t*-butyl DTPA **31** can be made using solid phase chemistry from diethylenetriamine **32**, bromoacetic acid **33** and *t*-butyl bromoacetic acid **34**, all cheap commercially available starting materials.

Synthesis of *t*-Butyl Selectively Protected DTPA (**31**)

Selectively protected *t*-butyl DTPA **31** was synthesised using a solid phase methodology adapted from Leclercq *et al* [127] (see Scheme 2.11).

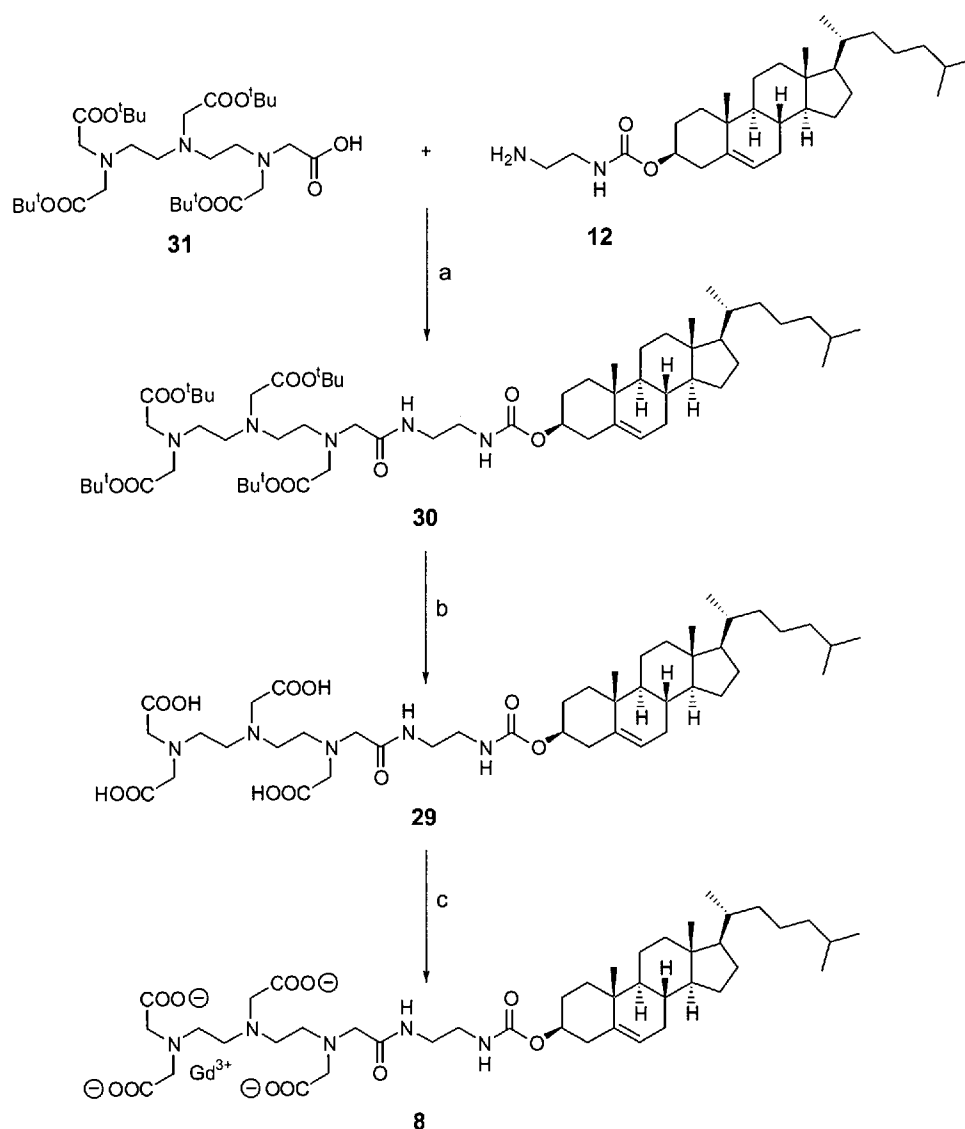


Scheme 2.11: a) Bromoacetic acid, DIPEA, CH₂Cl₂, 12 h, RT; b) Diethylenetriamine, CH₂Cl₂, 4 h, RT; c) *t*-Butyl Bromoacetate, DIPEA, CH₂Cl₂, 12 h, RT; d) Trifluoroethanol, CH₂Cl₂, 2 h, RT, 68% overall yield.

Firstly, resin (2-chlorotritylchloride resin, **35**, 0.8 mmol.g⁻¹) was loaded by treatment with excess bromoacetic acid **33** in the presence of base, to give bromoacetate 2-chlorotrityl resin **36**. To prevent any unwanted side reactions any remaining free chlorotrityl sites on the resin were capped by addition of excess methanol. The solid supported bromide **36** was then reacted with excess diethylenetriamine **32** in dichloromethane. All of the amines on the solid supported polyamine **37** were then subsequently functionalised by addition of excess *t*-butyl bromoacetate **34** in the presence of base. The Kaiser test (test for primary amines) and chloranil test (test for secondary amines) were both used (as appropriate) to check for completion of the reaction. Finally the product **31** was cleaved from the resin using mild acidic conditions (trifluoroethanol in dichloromethane), to give selectively protected *t*-butyl DTPA **31** as a yellow oil, in good yield and excellent purity (as determined by HPLC).

Synthesis of Gd.DTPA.Chol (8)

The synthetic route to Gd.DTPA.Chol **8** is depicted in Scheme 2.12.



Scheme 2.12: a) HBTU, NEt₃, CH₂Cl₂, RT, 4 h 72%; b) c.HCl, Dioxane, N₂ (g), RT, 2 h, 95%; c) GdCl₃, MeOH, RT, 24 h, 60%.

The key step in the synthesis of Gd.DTPA.Chol **8** is the coupling of the selectively *t*-butyl protected DTPA **31** and cholesterol amine **12**. HBTU was used as the coupling reagent and borrowing from previous experience gained from the synthesis of Gd.DOTA.Chol **6** and Gd.DOTA.DSPE **7**, triethylamine was used as a base. The crude product was purified using highly polar silica gel column chromatography conditions (10% MeOH in CH₂Cl₂) to give fully *t*-butyl protected DTPA.Chol **30** as a yellow oil. The *t*-butyl groups were removed by acidolysis. Initially this was attempted with TFA in dichloromethane but a mixture of products was always gained (1, 2, 3 or 4 *t*-butyl groups remaining). To prevent a difficult purification the use of a silyl scavenger, as described for the synthesis of DOTA **11** was avoided and an alternative method was

sort. The use of 4M HCl in dioxane has been documented for removal of Boc groups, in view of the nature of dioxane as a polar hydrogen bonding solvent this method seemed attractive especially considering the highly polar nature of the deprotected product **29**. However, experimentation showed that 4M HCl in dioxane was not sufficient to remove all the *t*-butyl esters. Using c.HCl in dioxane (1:1 (v/v)) and a positive flow of nitrogen over the reaction smoothly overcame this problem and DTPA.Chol **29** was isolated as a white powder in good yield and purity (as determined HPLC) (see Appendix, Section 9.5, DTPA.Chol).

The solvent compatibility of DTPA.Chol **29** meant that the gadolinium oxide method of metal complexation could not be employed. Borrowing from our experience with Gd.DOTA.DSPE **7**, the use of gadolinium chloride was explored. Both GdCl₃ and DTPA.Chol **29** were found to be soluble in methanol and the complexation reaction was found to proceed well upon stoichiometric addition of GdCl₃ to DTPA.Chol **29** at room temperature. Gd.DTPA.Chol **8** was isolated as a white powder in good yield and excellent purity (see Figure 2.8). The xylenol orange test indicated that there were no free gadolinium ions present.

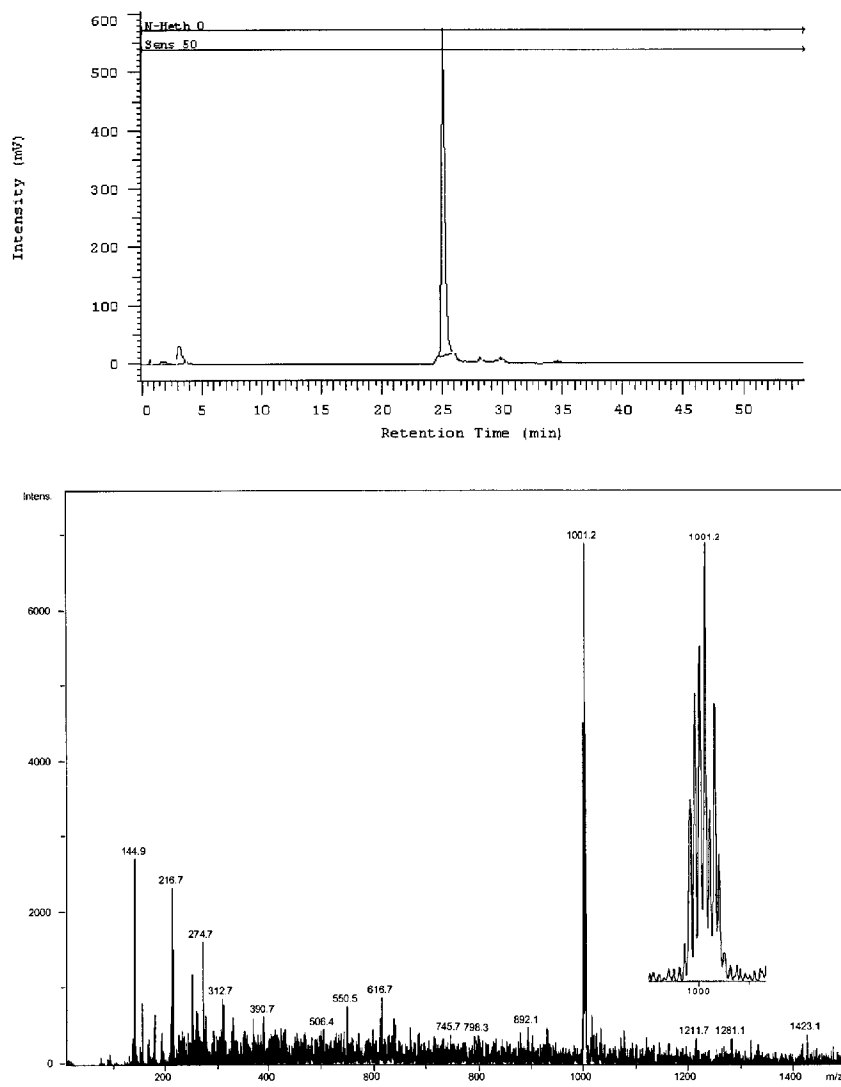


Figure 2.8: HPLC and MS of Gd.DTPA.Chol (**8**)

(HPLC conditions: See page 180. Mass Spectrum: Negative ionisation ESI, inset shows the gadolinium isotopic distribution of the M⁺ peak)

2.4 MRI Analysis of Gd.Lipids

With all three gadolinium lipids in hand, their efficacy as MRI contrast agents was assessed by measuring their T_1 relaxation times and obtaining phantom MRI images of each compound (see Table 2.1 and Figure 2.9 respectively). Phantom images and T_1 relaxation times of Gd(DOTA)⁻ **25** and Gd(DTPA)²⁻ were also measured as control compounds to give a bench mark of efficacy to compare the new gadolinium lipids against. Images and T_1 relaxation times were calculated from experiments performed at Hammersmith Hospital (Dr. A. Herlihy, Biological Imaging Centre, Hammersmith Hospital, Imperial College London).

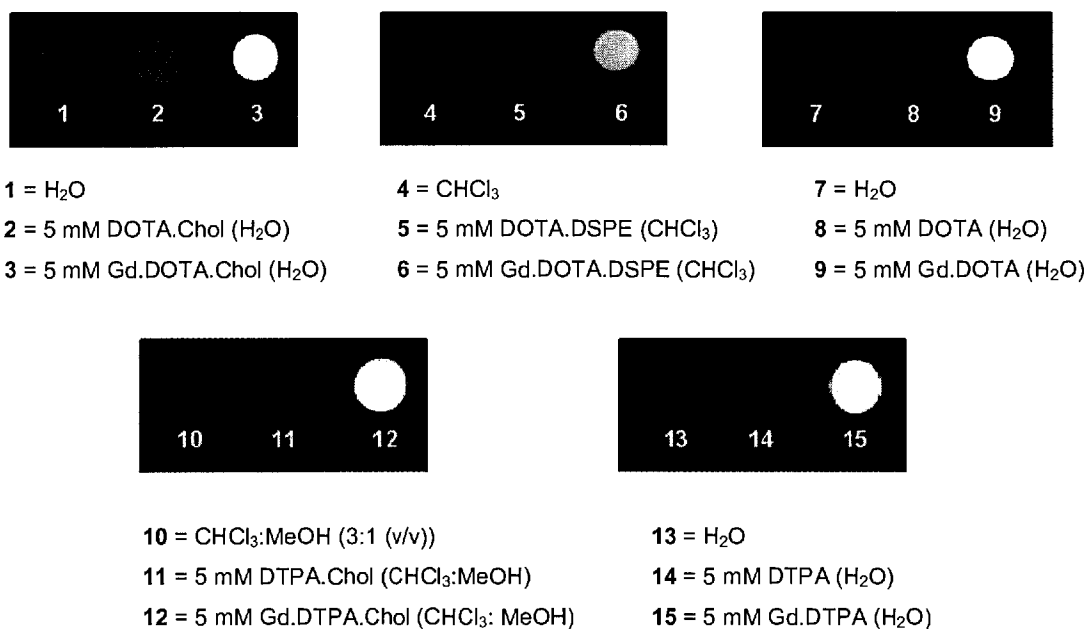


Figure 2.9: MRI Phantom Images of Gd.Lipids and Control Samples

Sample	Compound ID	T ₁ Relaxation (msec)	% Decrease in T ₁ Relaxation
Water	-	2937 ± 115	
DOTA.Chol	24	2898 ± 119	
Gd.DOTA.Chol	6	446 ± 13	85%
CHCl ₃	-	3363 ± 109	
DOTA.DSPE	26	4257 ± 63	
Gd.DOTA.DSPE	7	355 ± 10	90%
Water	-	2156 ± 28	
DOTA	22	2166 ± 27	
Gd.DOTA	23	390 ± 8	87%
MeOH	-	4408 ± 135	
DTPA.Chol	33	4024 ± 120	
Gd.DTPA.Chol	8	703 ± 81	84%
MeOH	-	2141 ± 35	
DTPA	*	2154 ± 31	
Gd.DTPA	*	430 ± 12	80%

* Commercially sourced from Sigma Aldrich.

Table 2.1: T₁ Relaxation Times for Gd.Lipids and Control Samples

MRI Phantom Images

MRI phantom images provide an excellent visual representation of the efficacy of a sample. An increase in signal intensity as a result of gadolinium enhanced relaxation of protons is manifested in a 'brightening' of the image (see Introduction, Contrast Agents). Samples of each of the gadolinium lipids and appropriate controls were prepared in glass vials. The images shown are of a horizontal slice (2 mm thick) through a vertically positioned vial containing sample excited using a spin echo sequence (see Experimental, Section 7.10, MRI Analysis of Gd.Lipids). It is clear from these results that each of the new gadolinium lipids (Gd.DOTA.Chol (**6**), Gd.DOTA.DSPE (**7**) and Gd.DTPA.Chol (**8**)) can significantly enhance the signal intensity of surrounding protons compared to that of the solvents alone. A comparison of the Gd.Lipid samples with Gd(DOTA)^- and Gd(DTPA)^{2-} shows the efficacy of our new Gd.Lipids is comparable to that of clinically used agents.

T₁ Relaxation Times

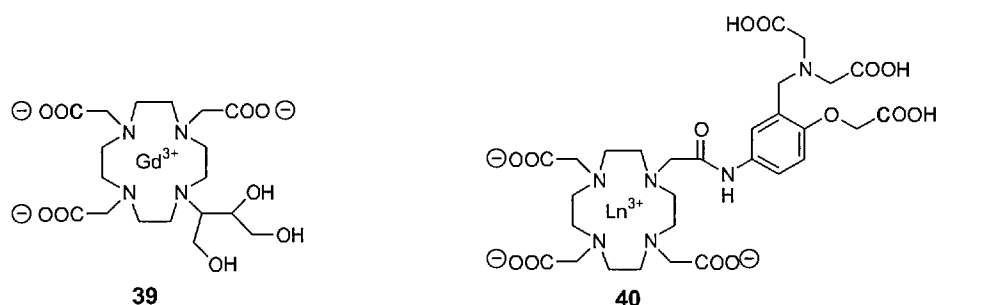
Gadolinium agents act by significantly reducing the T_1 relaxation times of surrounding protons, therefore the T_1 relaxation time (in msec) of a given sample is a good quantitative measure of the efficacy of a gadolinium contrast agent. The T_1 relaxation times of the gadolinium lipids and appropriate controls were calculated from data acquired using a spin echo sequence (see Experimental, Section 7.10, MRI Analysis of Gd.Lipids). The results show that each of the gadolinium lipids significantly decreases the T_1 relaxation time of the surrounding solvents and comparison with Gd(DOTA)^- and Gd(DTPA)^{2-} proves the decrease in relaxation times is comparable with clinically used agents.

2.5 Solid Phase Methodologies

Our efforts to selectively mono-functionalise DOTA **11** or cyclen **14** as part a synthetic strategy to the target molecules Gd.DOTA.Chol **6** and Gd.DOTA.DSPE **7**, has led us to the development of two independent solid phase methodologies for the synthesis of: (i) selectively protected cyclic polyamines and (ii) selectively protected polyamine polyacetic acid derivatives [146].

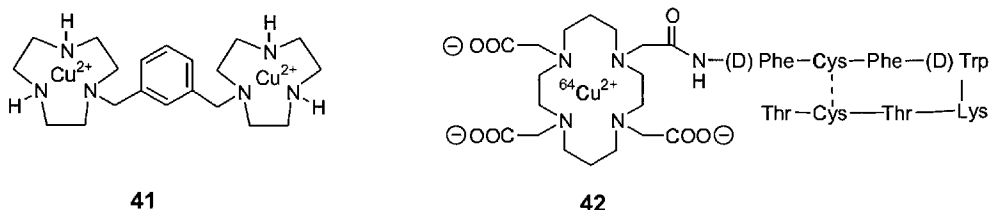
Azacrowns and their polyacetic acid derivatives are a family of unnatural compounds that display excellent metal chelating properties, especially for heavy metal cations and the lanthanides. This has made this class of compounds useful in a host of biological and biomedical applications such as MRI contrast agents **39** [72], luminescent probes

40 [147], DNA/RNA cleavers **41** [148], in radioimmunotherapies and diagnosis **42** [149] and as compounds used in anti-HIV therapies **43** [150] (see Figure 2.10).



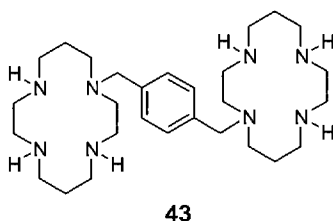
Gd(DO3A-Butrol), a neutral gadolinium based contrast agent approved for in clinical MRI [72].

Luminescent Lanthanide probe developed for detection of free zinc [147].



A dinuclear Cu(II) agent used for cleavage of mRNA [148].

A copper-64 peptide conjugate developed for imaging tumours [149].



AMD-3100 currently in phase II clinical trials for anti-HIV therapies [150]

Figure 2.10: Selection of compounds using azacrowns and their polyacetic acid derivatives as a skeleton

Many of these compounds are characterised by a selective *N*-functionalisation of the molecular backbone of either the azacrown or their polyacetic acid analogue and therefore unsurprisingly this selective functionalisation is a key step in the synthesis of such compounds. To date, approaches to this have been typically characterised by: (i) introducing the alkyl group prior to the cyclisation step, a tedious method in which the

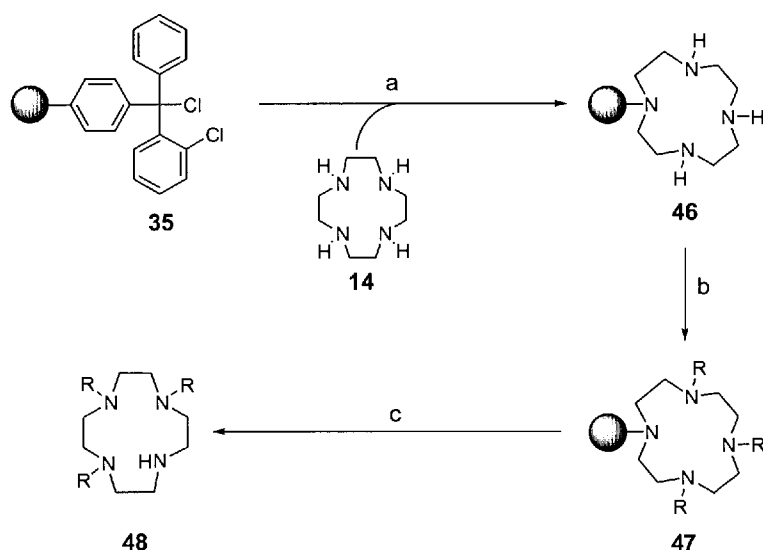
choice of alkyl group is limited [151], (ii) use of large excesses of expensive polyamines in the presence of alkylating agents [152] or (iii) employing metals to afford a temporary protection of appropriate amine groups [138] (see Figure 2.3). These methods tend to suffer from variable yields, significant amounts of di and tri-functionalised by-products and thus difficult purification steps. More recently improved boron [139] and phosphorous [140] temporary protection approaches have been developed for the functionalisation of cyclen **14** and cyclam **44** (1, 4, 8, 11-tetraazacyclotetradecane, see Table 2.2 for structure). However, these methods still suffer from production of by-products and are limited to the aforementioned tetraazacrowns.

The principle shortcoming of many of these methods is that they do not allow quick and clean functionalisation of the polyamine backbone which in turn restricts the scope of use of these invaluable chelators. The ideal methodology would permit a quick and facile *N*-functionalisation of a range of azacrowns and their polyacetic acid analogues with a range of alkylating agents permitting their use in areas such as combinatorial and bioconjugate chemistry.

Selective *N*-Functionalisation of Azacrowns

It has long been known that a range of functional groups (carboxylic acids, alcohols and amines) can be immobilised onto trityl chloride resins, affording a temporary protection of that functional group and thus permitting modification of the rest of the molecule. The resin provides excellent protection against highly nucleophilic and basic conditions, whilst being suitably acid labile enough to permit product cleavage without effecting other acid sensitive moieties. There have been several reports of the use of trityl chloride resins for the immobilisation of primary amines [153], [154], [155], but only limited reports of immobilisation of secondary amines [156], [157] (which have notably been small non-conformationally restricted compounds, such as piperazine **45**). We wanted to build on the successes in the literature and selectively load one secondary amine from an azacrown macrocycle onto the chlorotrityl resin. The steric bulk of the trityl groups should prevent a second secondary amine loading onto the resin, resulting in production of a mono-*N*-immobilised azacrown on trityl resin. This would leave the remaining secondary amines free for functionalisation with a range of electrophiles including, for example, a simple amine protecting group. The acid lability of the resin should permit cleavage of a selectively functionalised azacrown under mild conditions.

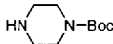
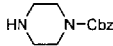
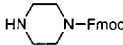
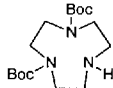
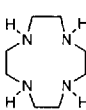
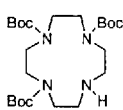
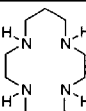
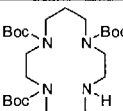
The synthetic route developed to a range of selectively protected cyclic polyamines is shown in Scheme 2.13, using the functionalisation of cyclen **14** as an example.



Scheme 2.13: a) (i) Cyclic polyamines (for example cyclen **14**) (10 equiv.), DIPEA (20 equiv.), CH_2Cl_2 , RT, 12 h, (ii) MeOH (1000 equiv.) (capping reagent), 10 min; b) protecting group (Boc_2O , CbzCl , $\text{FmocCl}^\dagger$) (5 equiv. per free amine), NEt_3 (10 equiv.), CH_2Cl_2 , RT, 4 h; c) 1% TFA in CH_2Cl_2 , 3 x 90 sec. † Pyridine was used as a base for addition of FmocCl .

First, resin (2-chlorotrityl chloride resin, **35**, 0.8 mmol.g^{-1}) was loaded by treatment with excess of a given cyclic polyamine in the presence of a tertiary amine base. The combination of the use of excess cyclic polyamine and sterics of the resin ensured that only one secondary amine from each azacrown immobilised on the resin. Initially 2-chlorotrityl chloride resin with a higher loading ($> 1.5 \text{ mmol.g}^{-1}$) was used, but this resulted in production of impure products, probably caused by high compound loading on the resin resulting in steric congestion, and subsequent inaccessibility of all reaction sites. Reducing the resin loading to below 1 mmol.g^{-1} by addition of stoichiometric amounts of methanol in the presence of base before loading the macrocycle onto the resin overcame this problem. The excess polyamine used could easily be isolated by filtration and recycled to reduce waste of the expensive cyclic polyamines. The free secondary amines of mono-*N*-immobilised cyclic polyamine **46** were then easily functionalised with a range of protecting groups (Boc, Cbz and Fmoc). After each step the chloranil test (test for secondary amines) was used to check for the presence of incomplete reaction products. If a negative test was gained the previous step was repeated, to ensure the purity of the final compound. Initially it was proposed that the product could be cleaved from the resin using trifluoroethanol in dichloromethane, but yields of the products gained were low. Increasing the acidity of the resin cleavage mixture (1% trifluoroacetic acid in dichloromethane) resulted in more efficient and

expedious release of the desired product (**48**). This methodology was found to be compatible with a range of cyclic polyamines including the frequently used cyclen **14**, cyclam **44** and 1, 4, 7-triazacyclonane **49**, giving the desired selectively functionalised macrocycles in excellent yield and purity (See Table 2.2).

Cyclic Polyamine	Protecting Group (R)	Product	% Yield	% Purity ^a
 45	Boc	 50	98	≥ 99
 45	Cbz	 51	96	≥ 98
 45	Fmoc	 52	96	≥ 98
 49	Boc	 53	88	≥ 98
 14	Boc	 54	80	≥ 97 ^b
 44	Boc	 55	77	≥ 98 ^c

^a Purity determined by HPLC and MS

^b Yields based on amount of recovered azacrown

^c A small silica gel chromatograph was performed to improve purity

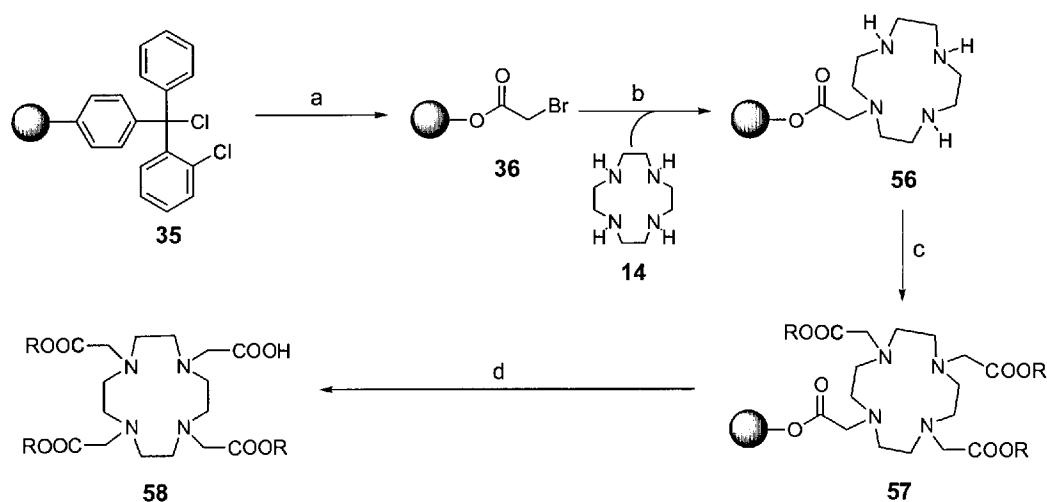
Table 2.2: Selective Protection of Cyclic polyamines and Azacrowns

Although this methodology does not directly result in the production of mono-*N*-functionalised macrocycles, the selectively functionalised products (**50**, **51**, **52**, **53**, **54** and **55**) are very useful precursors to a range of mono-*N*-functionalised compounds. These valuable intermediates can be produced in excellent yield and purity and

moreover the choice of alkylating agent is not limited by the selective functionalisation protocol (as with previous methodologies). This methodology represents a robust and expeditious alternative to current functionalisation strategies for azacrowns and is easily amenable to the synthesis of a library of compounds.

Synthesis of selectively protected polyamine polyacetic acid derivatives

Borrowing from the method used for the synthesis of selectively *t*-butyl DTPA **31** (see Scheme 2.11) we have developed a route to selectively protected polyamine polyacetic acid derivatives (see Scheme 2.14). The principle alteration to the published methodology is the exchange of the linear polyamine (previously diethylenetriamine) for a cyclic polyamine (for example cyclen **14**). Analogous to the previously discussed methodology for the synthesis of selectively protected azacrowns, the combination of the steric bulk of the trityl resin combined with the use of excess cyclic polyamine should ensure that only one secondary amine is immobilised onto the bromoacetate 2-chlorotrityl resin. Here the resin immobilised product is made by functionalising the remaining free secondary amines with suitably protected bromoacetic acid derivatives (*t*-butyl bromoacetate and 2-benzylbromoacetate). Scheme 2.14 details the synthetic route to cyclic polyamine polyacetic acid derivatives using derivatisation of cyclen **14** as an example.



Scheme 2.14: a) (i) Bromoacetic Acid (10 equiv.), DIPEA (20 equiv.), CH₂Cl₂, RT, 12 h, (ii) MeOH (1000 equiv.) (capping reagent), 10 min; b) azacrown (for example cyclen **14**) (10 equiv.), DIPEA (20 equiv.), CH₂Cl₂, RT, 4 h; c) *t*-butyl bromoacetate or 2-benzylbromoacetate (5 equiv. per free amine), NEt₃, DMF, RT, 12 h; d) Trifluoroethanol: CH₂Cl₂ (1:2 (v/v)), RT, 2 h.

Bromoacetate 2-chlorotrityl resin **36** was synthesised from 2-chlorotrityl chloride resin **35** as described previously (see Section 2.3, Scheme 2.11). The solid supported bromide was then subsequently reacted with excess cyclic polyamine (**45** and **14**) in the presence of base to give mono-substituted cyclic polyamine **56**. The remaining free secondary amines were then functionalised with either *t*-butyl bromoacetate or benzyl 2-bromoacetate to give the desired product immobilised on resin (**57**). Attempts were made to introduce methyl ester protected acetic acid groups by employing bromomethyl acetate (in place of *t*-butyl bromoacetate or benzyl 2-bromoacetate), but this resulted in significant side reactions and production of significantly impure product. The final product was cleaved from the resin using mildly acidic trifluoroethanol in dichloromethane. Attempts were made to derivatise cyclam **44** and 1, 4, 7-triazacyclonane **49**, but these were unsuccessful. The limitations of this method should however be noted: The limited solubility of cyclam **44** and high reactivity of 1, 4, 7-triazacyclonane **49** meant that the desired products could never be gained in adequate purity. Table 2.3 shows the results gained from derivatisation of cyclen **14** and piperazine **45**.

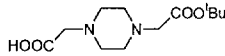
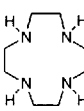
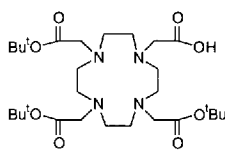
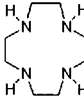
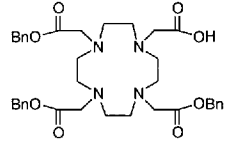
Azacrown	R	Product	% Yield	% Purity
 45	<i>t</i> -Bu	 59	82	≥ 98
 14	<i>t</i> -Bu	 60	66	≥ 98
 14	Bn	 61	69	≥ 95

Table 2.3: Selective Protection of Polyacetate Analogues

In efforts parallel to the methodology described above it was decided to test the durability of the methodology for production of selectively protected DTPA, developed

by Byk *et al* [127]. To this end, a selectively benzyl protected DTPA **63** and *t*-butyl protected EDTA **64** were synthesised using the methodology described in Scheme 2.11 (see Table 2.4).

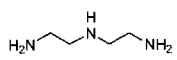
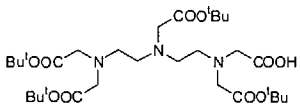
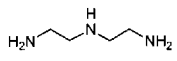
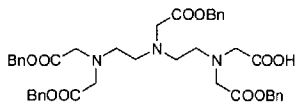
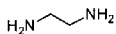
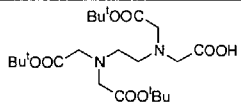
Azacrown	R	Product	% Yield	% Purity
 32	t-Bu	 31	70	≥ 98
 32	Bn	 63	74	≥ 97
 62	t-Bu	 64	73	≥ 98

Table 2.4: Selectively protected DTPA and EDTA Derivatives

2.6 Chapter Conclusions

Three novel gadolinium lipids Gd.DOTA.Chol **6**, Gd.DOTA.DSPE **7** and Gd.DTPA.Chol **8** have been successfully synthesised. These were based on the structures of known and clinically used agents. MRI analysis shows that all three gadolinium lipids are successful MRI contrast agents, with efficacies comparable to clinically used agents. The high purities of these lipids (as judged by ¹H NMR, ¹³C NMR, MS and HPLC) are important factors for their physical and future biological characterisations.

Efforts to synthesise the gadolinium lipids (**6**, **7**, **8**) has led to the development of two independent solid phase chemistry methodologies for the synthesis of: (i) selectively protected cyclic polyamines and (ii) selectively protected polyamine polyacetic acid derivatives [146]. These methodologies provide robust and attractive alternative routes to the selective functionalisation of highly symmetrical azacrowns and their polyacetic acid derivatives.

CHAPTER THREE

Synthesis of Cationic Lipids

3.1 Cationic Lipids

Cationic lipids are an essential component of liposomes designed to enter cells. The cationic charge they infer mediates initial contact of the liposome with the slightly negatively-charged cell membrane, which in turn initiates their uptake *via* the process of endocytosis. Furthermore, in liposomes transporting nucleic acids, the cationic lipids also aid the condensation and complexation of the nucleic acids into the liposome particle (see Introduction, Section 1.3, Liposomes).

The structure of cationic lipids is analogous to all other lipids; it comprises a polar head group linked to a hydrophobic lipid tail. The positive charge on cationic lipids arises from the presence of a cationic ammonium head group (either permanent (i.e. quaternary) or titratable). The development of new cationic lipids for liposomes is an active area of research, with many groups aiming to improve on the efficacy of current cationic lipids by varying the length, spacings and number of amines on a polyamine chain [158], [159], [160], [161].

There are a plethora of cationic lipids structures, and a selection of better known examples is shown in Table 3.1.

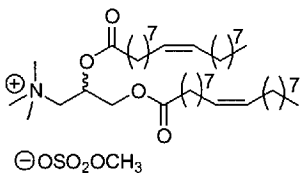
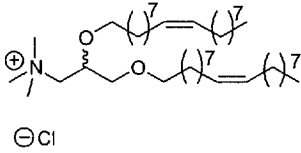
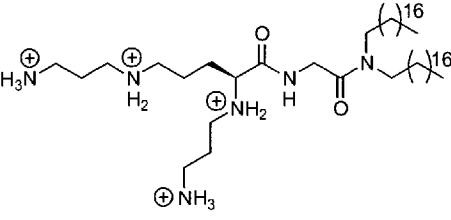
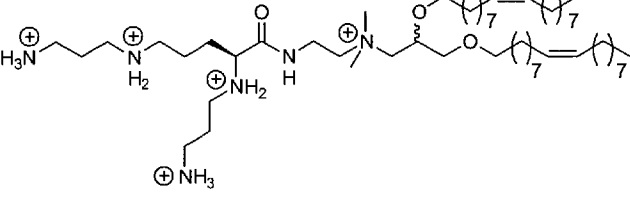
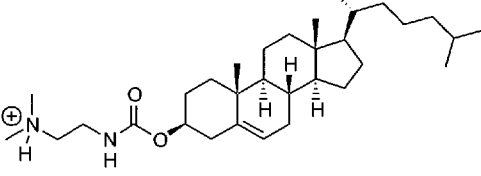
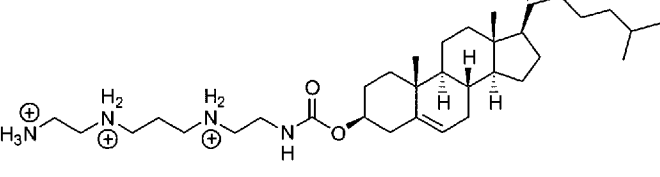
Cationic Lipid	Structure at Physiological pH	Maximum Cationic Charge
DOTAP (65)		+1
DOTMA (66)		+1
DOGS (67)		+4
DOSPA (68)		+5
DC-Chol (69)		+1
CDAN (70)		+3

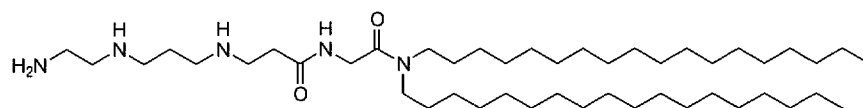
Table 3.1: Selection of Commonly Used Cationic Lipids [162].

The valency of a cationic lipid is determined by the number of charges on the head group [163]. In monovalent lipids (for example DOTAP (65), DOTMA (66), DC-Chol (69)) the head group can be a primary, secondary or tertiary amine (which become protonated at physiological pH) or a quaternary ammonium group. Multivalent cationic

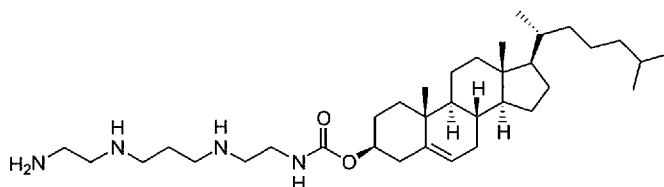
lipids consist of a number of protonable amine groups, which are either linked in a linear fashion (for example CDAN (**70**) or are separated in a branched manner (for example DOGS (**67**), DOSPA (**68**)). The pK_{aH} 's of amines on polyvalent cationic lipids vary considerably and are directly influenced by the number of amines present on the polyamine chain as well as the methylene spacing between the individual amines [160]. Indeed the pK_{aH} 's of these amines can vary from as little as 2.5 to 10.9 [160]. As a result these molecules carry a variety of charges at physiological pH (pH 7.4) and this can impart a variety of different biological activities [160]. The principle application of polyvalent cationic lipids is, however, in the formation of lipoplexes (liposome–nucleic acid complex) [164]. The multiple positive charges present on the lipid permits a more efficient complexation (compared to monovalent cationic lipids) of nucleic acids within a lipoplex. Whilst the charges on the branched cationic lipids (**67**, **68**) are more sterically available within the liposome to complex negatively charged molecules, this must be off-set by a decrease in the inclination of the lipid to form a stable lamellar structure (see Appendix, Table 9.1). Linear linked polyvalent cationic lipids (**70**) provide an alternative to this and the propensity of the lipid for the lamellar phase is not disrupted.

Cationic lipids have been made with a variety of lipophilic tail groups, including cholesterol (**69**, **70**), fully saturated alkyl chains (**67**) and partially unsaturated alkyl chains (**65**, **66**, **67**). The lipophilic tail anchors the cationic lipid to the liposome membrane but permits exposure of the amine(s) and cationic charge beyond the liposome surface. As with other lipids the hydrophobic tail can strongly influence the overall packing shape of the lipid and therefore determine the scope of use of the lipid within liposomes (see Appendix Section 9.1, Lipids).

One of the biggest challenges for molecular imaging is the labelling and subsequent tracking of cells. Despite the advantages of labelling cells with gadolinium contrast agents over iron-based agents for MRI applications, there has been little success in this area. In an effort to develop an effective gadolinium cell labelling system, two cationic lipids (one novel) were designed to complement the new gadolinium lipids (Gd.DOTA.Chol (**6**), Gd.DOTA.DSPE (**7**) and Gd.DTPA.Chol (**8**)) in a cationic liposome formulation. The subsequent gadolinium containing cationic liposome would be able to enter cells in an analogous manner to standard cationic liposomes and successfully deliver the gadolinium label to the cells. The cationic lipid target molecules are shown in Figure 3.1.



CDAN-DOGS (71)



CDAN (70)

Figure 3.1: Cationic Lipid Target Molecules

Design of Target Molecules

CDAN (*N*¹-Cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine) **70** is an invention from our laboratory [165], [161]. The cholesterol-based cationic lipid incorporates a novel un-natural 2-3-2 spacing between the nitrogen atoms on the polyamine lipid head group. CDAN **70** has been found to be an extremely successful lipid for mediating *in vitro* cell transfection (liposome uptake followed by DNA expression), even in conditions containing 10% serum. Indeed, liposomes containing CDAN **70** are up-taken into cells in greater amounts than commercially available transfection agents (for example Lipofectamine™, Invitrogen) and also display a lower cytotoxicity. The measured pK_{aH} 's of the amine groups on CDAN **70** are 9.3, 7.6 and 5.7 [160], and it is postulated that these unusual pK_{aH} 's are the basis for the success of this reagent. Considering the success of CDAN **70** we proposed that it would make an ideal cationic lipid to complement our gadolinium-lipids in the liposome membrane. The design of CDAN-DOGS (3-[3-(2-Amino-ethylamino)-propylamino]-*N*-dioctadecylcarbamoylmethyl-propionamide) **71** incorporates the novel linear 2-3-2 spaced polyamine of CDAN **70**, but the cholesterol lipid tail is replaced with two amine derived C18 (steroyl) alkyl chains, analogous to the cationic lipid DOGS (Dioctadecylaminoglycylspermine) **67**. The two alkyl chains will confer more rigidity to the liposome membrane (see Appendix, Table 9.3), whilst retaining the strong propensity for liposome uptake into cells.

Liposome membranes rarely consist of one lipid. For example, combinations of steroidal and glycerol-based phospholipids are often combined in optimised proportions

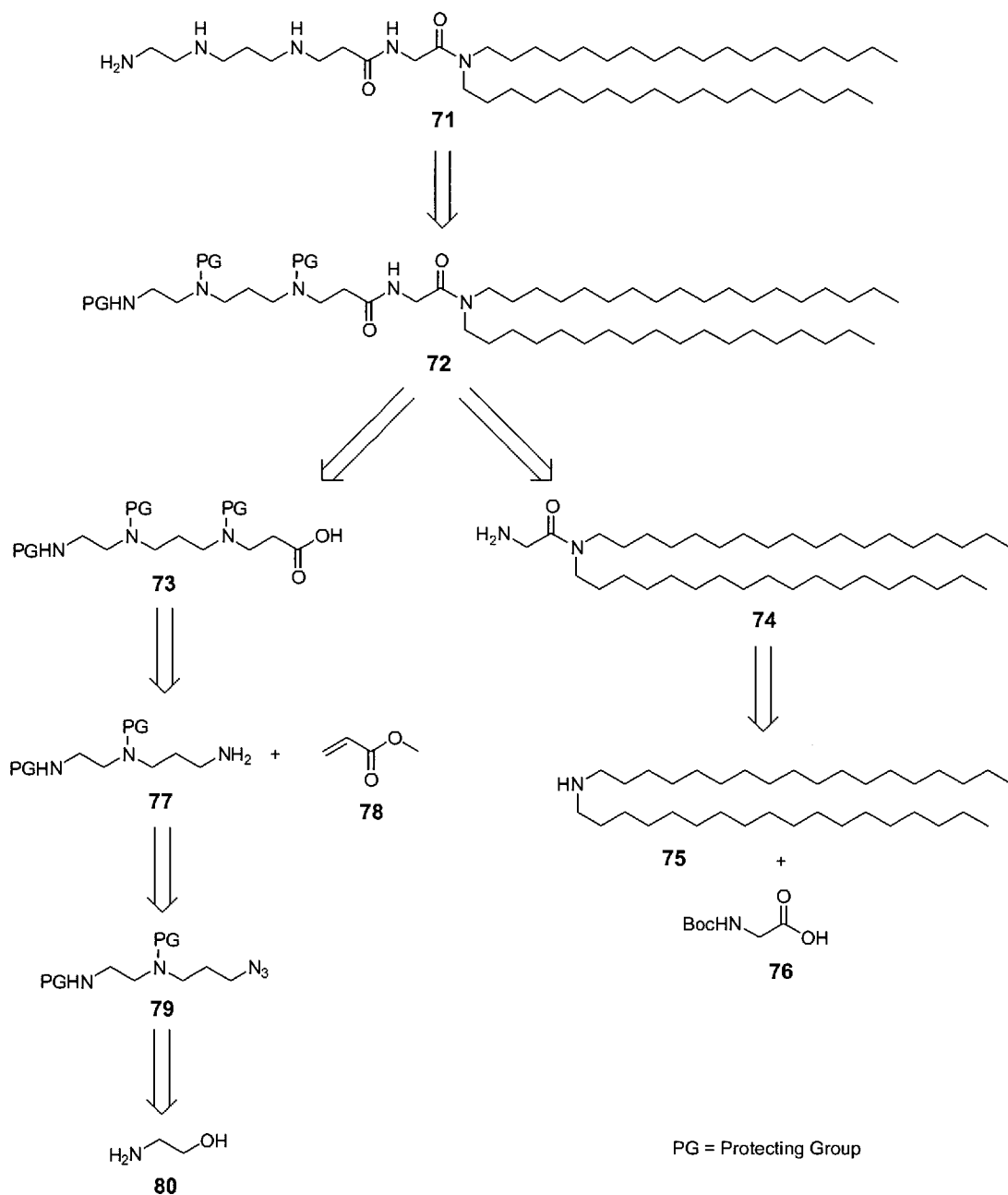
in order to generate a stable liposome formulation. Considering this, we propose that mixtures of gadolinium or cationic lipids with either cholesterol or steroyl tails should provide an effective gadolinium labelled liposome formulation capable of rapid uptake into cells.

The efficiency of polyamine synthesis relies heavily upon the effectiveness of the synthesis of amines. Despite the numerous methods used for the synthesis of primary and secondary amines (see Appendix, Section 9.4, Synthesis of Polyamines), the synthesis of linear polyamines relies on the marriage of many cumbersome, frequently low yielding conversion steps. The purification of nitrogenous intermediates and the final polyamine product can also be challenging as a result of the polar nature of the products. With this in mind the syntheses of CDAN-DOGS **71** and CDAN **70** were designed using methodologies that have been well established in our laboratory [161], [166].

3.2 Synthesis of CDAN-DOGS

Retrosynthetic Analysis of CDAN-DOGS

The retrosynthetic analysis of CDAN-DOGS is shown in Scheme 3.1.



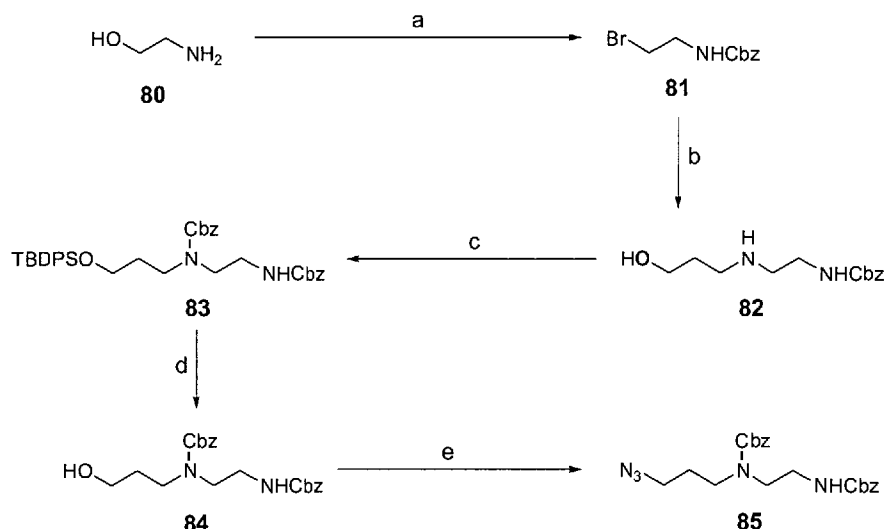
Scheme 3.1: Retrosynthetic Analysis of CDAN-DOGS

The target molecule CDAN-DOGS **71** is obtained from its fully protected analogue **72**. A disconnection at the primary amide of **72** yields two synthons, **73** and **74**, which form

the head (**73**) and tail (**74**) group of the lipid. The tail group **74** can easily be obtained from a peptide coupling reaction between dioctadecylamine **75** and a *t*-butylcarboxy (Boc) protected glycine **76**, both commercially available starting materials. The polyamine head group segment of the lipid **63** can be obtained from the reaction between the selectively protected unsymmetrical polyamine **77** with methylacrylate **78**. The methyl ester can then be hydrolysed to give the required carboxylic acid **69**. The selectively protected 2-3 spaced triamine **77** is obtained from reduction of the corresponding azide **79**. This azide **79** is a common intermediate in our laboratory and is derived in 5 steps from ethanolamine **80** [166].

Synthesis of Head Group

The synthetic route to the polyamine head group of the lipid proceeds *via* the azide **79**. The azide (Cbz protected) **85** was made by Dr. Michael Jorgensen using a previously established route as depicted in Scheme 3.2 [166].

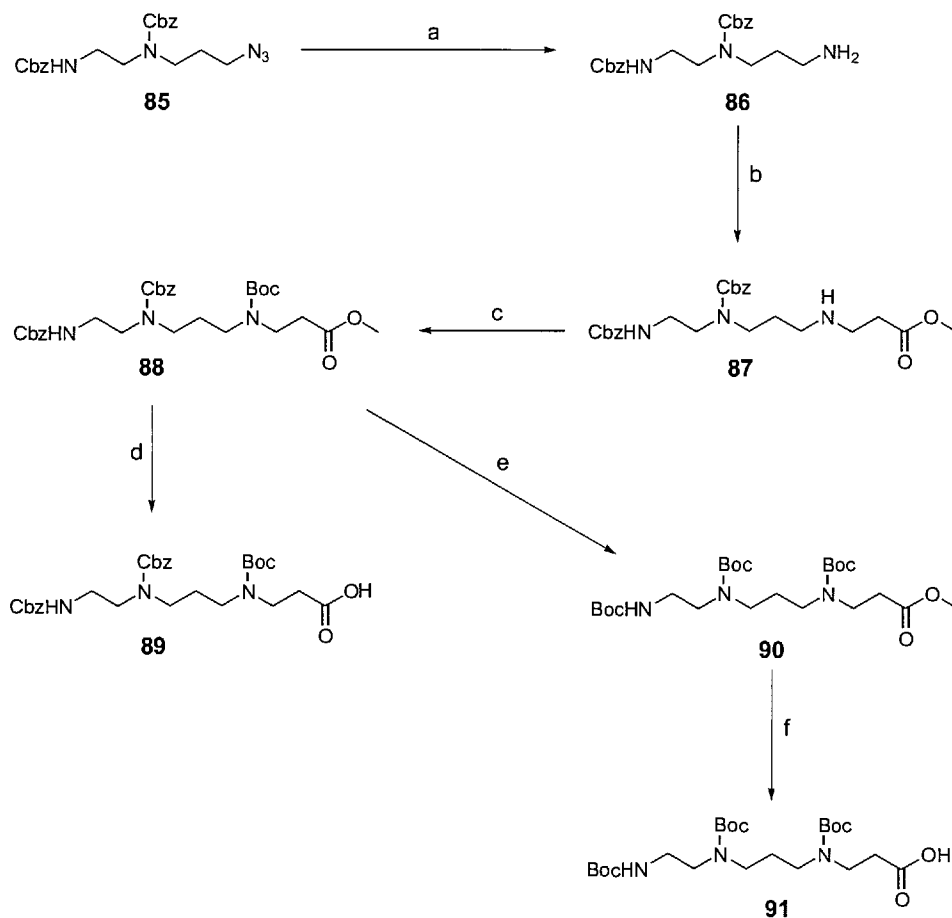


Scheme 3.2: a) (i) Cbz-Cl, NEt₃, CH₂Cl₂, RT, 4 h, 88%, (ii) PPh₃, CBr₄, CH₂Cl₂, RT, 3 h, 70%; b) 3-aminopropan-1-ol, NaI, K₂CO₃, DMF, RT, 60 h, 66%; c) (i) TBDPS-Cl, NEt₃, DMAP, CH₂Cl₂, RT, 4 h, 84%, (ii) Cbz-Cl, CH₂Cl₂, NEt₃, RT, 69%; d) TBAF, THF, RT, 3 h, 91%; e) (i) MsCl, NEt₃, CH₂Cl₂, RT, 4 h, 99%, (ii) NaN₃, NaI, DMF, 80 °C, 1.5 h, 80%.

Briefly, ethanolamine **80** was first Cbz protected and the free primary alcohol was then converted to the bromide **81**, using PPh₃ CBr₄ (as described previously, see Scheme 2.6). Next, the bromide was displaced with 3-aminopropan-1-ol, the free alcohol protected with *tert*-butyldiphenylsilyl chloride (to give **83**), and finally, the secondary

amine protected with a Cbz group, affording after chromatography pure **83** (38% overall yield). Next, the alcohol **83** was liberated with TBAF (tetrabutylammonium fluoride) and transformed to the azide **85** via the activated mesylate intermediate in 17% overall yield.

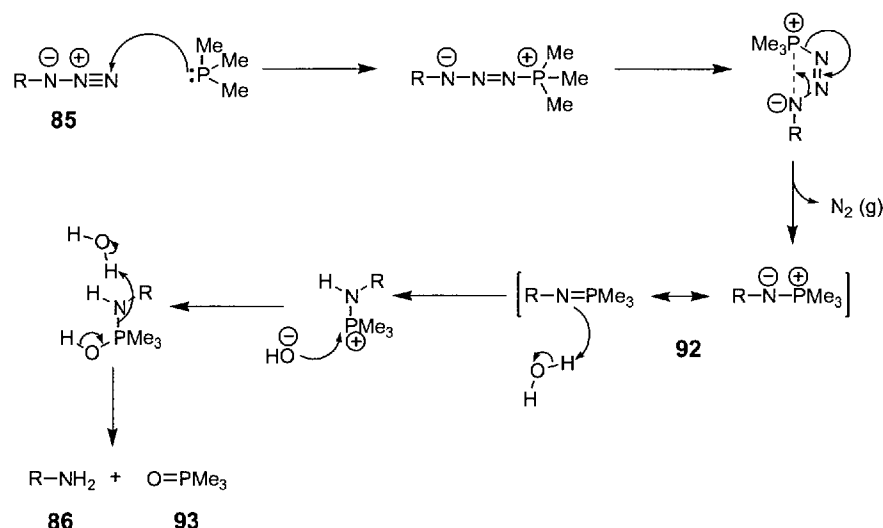
Using the azide as a key building block, the polyamine cationic head group of CDAN-DOGS **71** was assembled as shown in Scheme 3.3.



Scheme 3.3: a) PMe_3 , THF, RT, 2.5 h, H_2O , $\text{NH}_4\text{OH}_{(\text{aq})}$, 1 h, 82%; b) Methylacrylate, MeOH, RT, 12 h, 54%; c) Boc_2O , NEt_3 , CH_2Cl_2 , RT, 12 h, 70%; d) LiOH, THF, 0 °C, 12 h, 91%; e) Pd/C-10%, Boc_2O , MeOH, H_2 (g), RT, 12 h, 92%; f) LiOH, THF, 0 °C, 12 h, 97%.

Firstly, the azide **85** was reduced under mild conditions using trimethylphosphine in THF in an adaptation of the Staudinger reaction to give the corresponding amine **86**. Trimethylphosphine was chosen for the reaction over triphenylphosphine, as the larger phenyl groups sterically hinder the initial nucleophilic attack on the azide, thereby

decreasing the rate of reaction [167]. The mechanism of the reaction proceeds *via* an iminophosphorane intermediate **92** (see Scheme 3.4).



Scheme 3.4: Reduction of Azide to Amine

The iminophosphorane intermediate **92** is hydrolysed upon basic aqueous work up rendering the free amine **86**. Mechanistically this reaction is analogous to the Wittig reaction. The reaction is driven enthalpically by the formation of strong P=O and N-H bonds and entropically by the liberation of nitrogen gas. The side product of the reaction is trimethylphosphine oxide **93**, which proved difficult to separate from the desired amine **86**. The bis-protected polyamine **86** was obtained as a yellow oil in good yield, which is comparable to the reported literature yields for these reactions [161].

The next step required alkylation of the terminal amine of **86**. Initial experiments were carried out with acrylic acid, but the yields of the desired product were poor. Methylacrylate **78** was employed as a substitute, resulting in production of the methyl ester protected carboxylic acid **87** which could be hydrolysed under basic conditions to give the desired carboxylic acid. The Michael addition reaction proceeds readily at room temperature. The major by-product of the reaction (as determined after isolation by NMR) is the so-called double addition product **94** (see Figure 3.2), arising as a result of two concurrent Michael additions at the terminal amine.

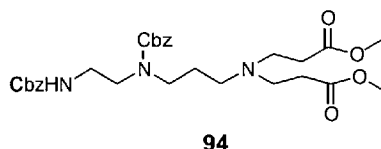


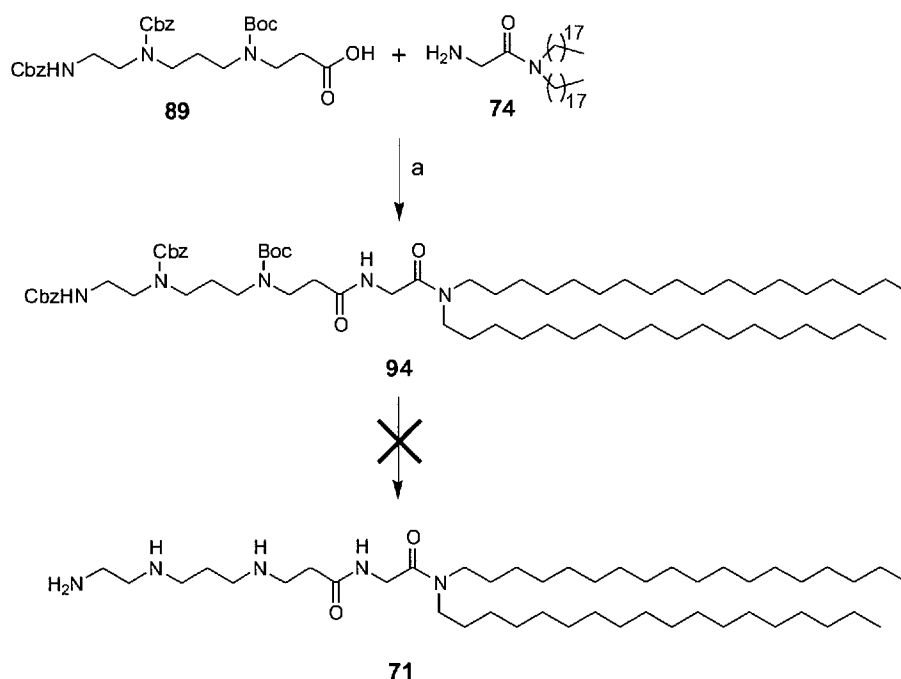
Figure 3.2: Product of two concurrent Michael Additions

Slow addition of methylacrylate decreased the yields of the by-product, which was normally obtained in yields of 15-23%. Overall the desired methylester **87** was isolated as a yellow oil in good yield (70%).

For effective coupling of the carboxylic acid **73** and amine **74**, the secondary amine of the methylester **87** must be protected, because two Cbz groups protected the amines, addition of another seemed a logical step. Deprotection would thus be a simple one step process. However, attempts to isolate the triprotected Cbz polyamine proved very difficult and subsequently yields were low. An alternative protecting group strategy resulted in the use of Boc protection and treatment with Boc_2O gave the desired protected polyamine **88** in good yield.

The final step in the synthesis was the isolation of the carboxylic acid (**89**). Ester hydrolysis under basic conditions yields the carboxylate anion followed by acidic work up to give the desired product. The ester was hydrolysed with aqueous LiOH in THF and the acidic work up was performed using citric acid (pH 2-3) to avoid any adventitious Boc-group removal. After work up, TLC indicated that the product **89** was sufficiently pure and this was confirmed by NMR analysis.

With the carboxylic acid **89** and quantities of the lipophilic tail **74** in hand, initial studies on the final coupling reaction commenced. The route is shown in Scheme 3.5.



Scheme 3.5: a) HBTU, DMAP, CHCl_3 , RT, 24 h, 58%

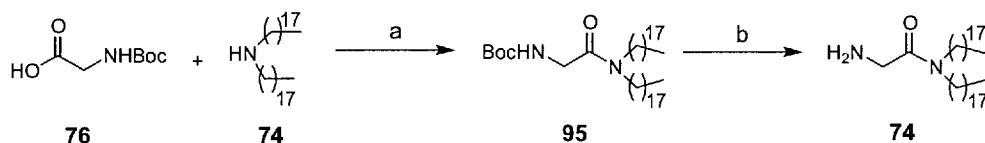
The coupling reaction proceeded in reasonable yield. Some loss of product can be generally accounted for through the work up, as handling these amphiphilic compounds is generally difficult, and frequently an emulsion forms between the aqueous and organic layer. Full spectroscopic analysis confirmed the presence of the product **94**. However, it proved impossible to deprotect this compound. A variety of hydrogenation and transfer hydrogenation conditions were employed, but they either resulted in minimal or no product, presumably through either degradation of the desired product or the product effectively chelating to the palladium catalyst. This was not satisfactory and a slight synthetic modification had to be implemented.

It is possible to exchange a Cbz protecting group for a Boc protecting group in a “one-pot” reaction. $\text{Pd/C-10\%}$, H_2 (g) in MeOH as a solvent can remove Cbz protecting groups. However, in the presence of Boc_2O the resulting free amine is immediately protected with a Boc group, resulting in protecting group exchange. The methyl ester (**89**) was dissolved in MeOH and using the methodology described, the two Cbz groups were exchanged for Boc groups, rendering a tri-Boc protected methyl ester polyamine (**90**). The reaction proceeds at room temperature and is marked by a subtle change in R_f of the product compared to the starting material. The desired ester (**90**) was isolated as a yellow oil in excellent yield. Using an identical procedure to that already employed

for ester hydrolysis, the corresponding carboxylic acid (**91**) was also isolated in good yield.

Synthesis of the Lipophilic Tail

The synthetic route to the lipophilic tail is given in Scheme 3.6.



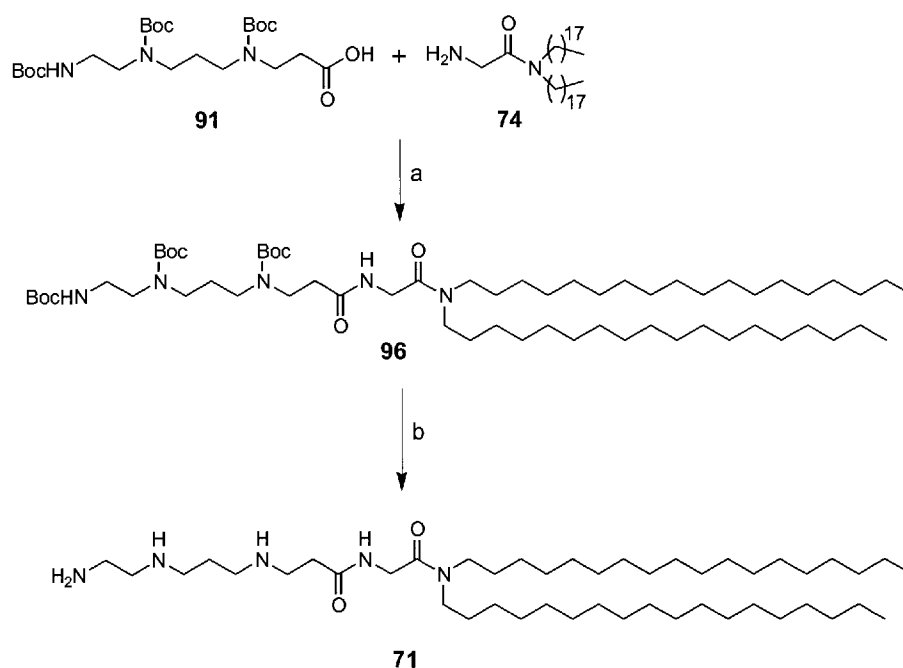
Scheme 3.6: a) HBTU, DMAP, CHCl₃, RT, 12 h, 70%; b) TFA, CH₂Cl₂, N₂ (g), RT, 2 h, 92%.

Boc-Glycine **76** was coupled to dioctadecylamine **74** under peptide coupling reaction conditions using HBTU as the carbodiimide reagent. HBTU is a single reagent that replaces the function of both DCC and HOBT (established coupling reagents). In our case DMAP was also added to the reaction as a base. The desired amide **95** was isolated in good yield after aqueous acidic work-up.

The target amine **74** was obtained after acidolysis of the Boc protected analogue **95**, as a pale solid in excellent yield. Both TLC and NMR analysis indicated that no further purification of the compound was required.

Synthesis of the Lipid

The final steps in the synthesis of CDAN-DOGS **71** involves coupling the poly-Boc protected amine **91** and the lipophilic tail **74** together and then deprotecting the resulting fully protected CDAN-DOGS **96**. Details of the reactions are given in Scheme 3.7.



Scheme 3.7: a) HBTU, DMAP, CHCl_3 , RT, 24 h, 62%; b) TFA, CH_2Cl_2 , RT, 3 h, 97%.

HBTU coupling conditions provided poly-Boc protected CDAN-DOGS **96** as a yellow oil, in reasonable yield. The Boc groups of **96** were removed with TFA in CH_2Cl_2 . The resulting solid was freeze-dried from aqueous acetonitrile, rendering the desired compound as a fluffy white powder.

CDAN-DOGS **71** was analysed by NMR, MS and HPLC and shown to be of adequate purity to proceed with biological characterisation (see Figure 3.3).

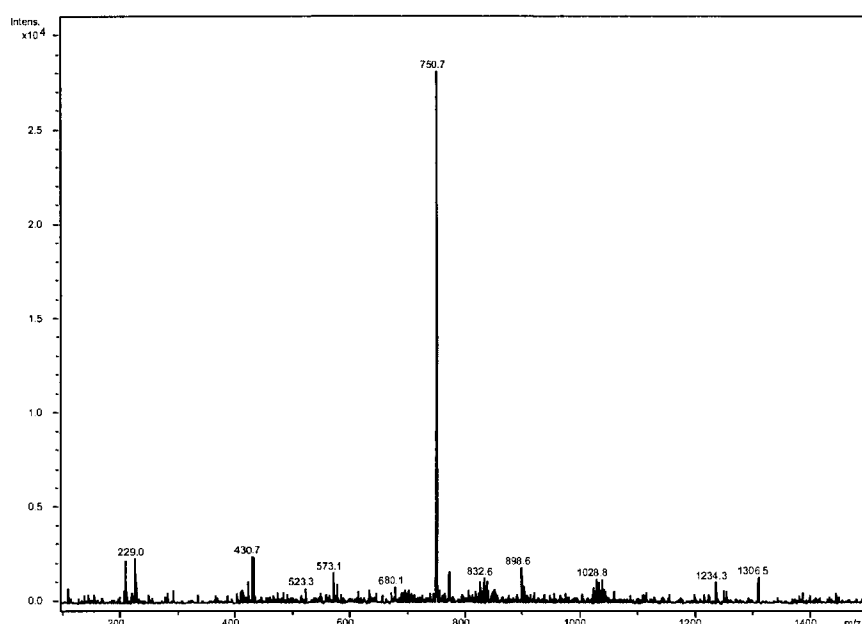
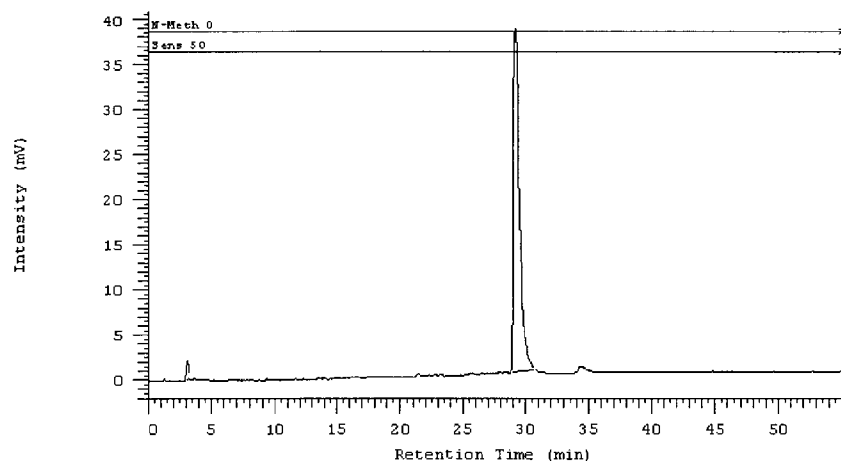


Figure 3.3: HPLC and MS of CDAN-DOGS (71)

(HPLC conditions: See page 197. Mass Spectrum: Positive ionisation ESI)

3.3 Synthesis of CDAN

Despite the increasing interest in cationic lipids [161], [159], [158], [160] and polyamine conjugates [168], the synthetic routes to these compounds are frequently tedious and time consuming. In an effort to overcome these previous problems an alternative strategy for the synthesis of CDAN **70** was sought.

Many cationic lipids and polyamine conjugates are based on the structures of symmetrically spaced linear polyamines, such as the naturally occurring spermine, homospermine and norspermine (see Figure 3.4 and Figure 3.5).

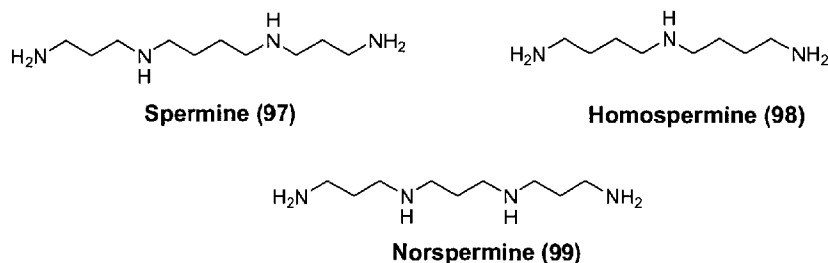
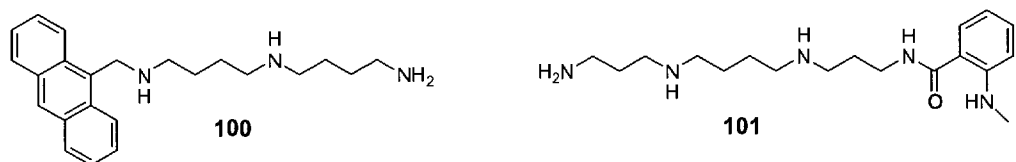
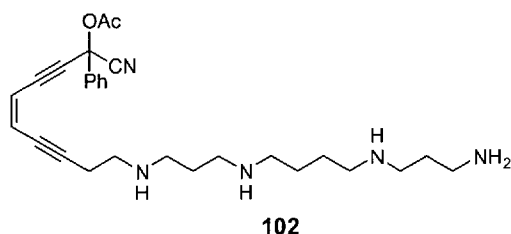


Figure 3.4: Selected Structures of Symmetrically Spaced Linear Polyamines

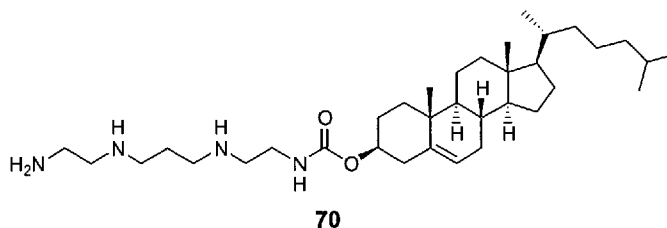


An Anthracen-9-ylmethyl norspermine conjugate for investigating polyamine transporters in cells [169]

Fluorescently labelled *N*-methylantranilic acid conjugate of spermine for examining life cycle of malaria in human erythrocyte [170]



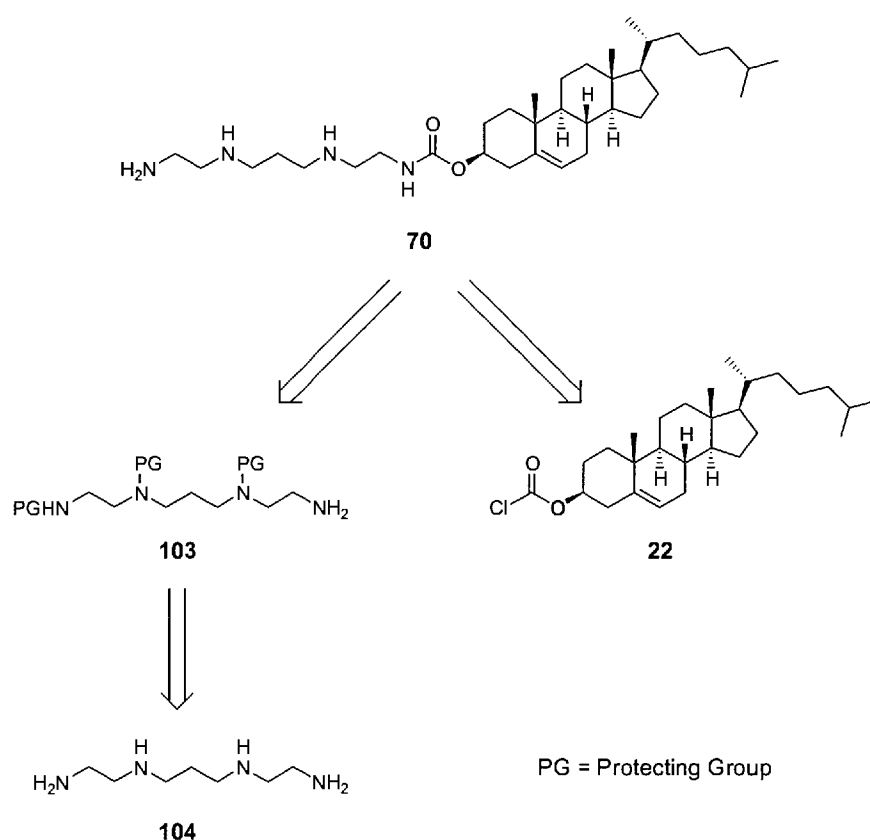
Eneidyne-polyamine (spermine) conjugate as a potential triggerable DNA damaging drug [171]



CDAN, a polyamine cholesterol lipid conjugate for mediating non-viral liposome mediated gene therapy [165], [161].

Figure 3.5: Selection of Polyamine Conjugates

Using solid phase chemistry it has been shown that it is possible to regioselectively immobilise and functionalise symmetrical polyamines [172], [173], [153]. Drawing from our previous experience gained with the use of 2-chlorotrityl chloride resin for the synthesis of selectively protected azacrowns (see Section 2.5, Solid Phase Methodologies), an alternative retrosynthesis of CDAN **70** was devised [174], with the principal disconnection separating the symmetrical polyamine from the added chemical functionality (see Scheme 3.8). This route would be applicable to the development of a range of polyamine conjugates including those shown in Figure 3.5.



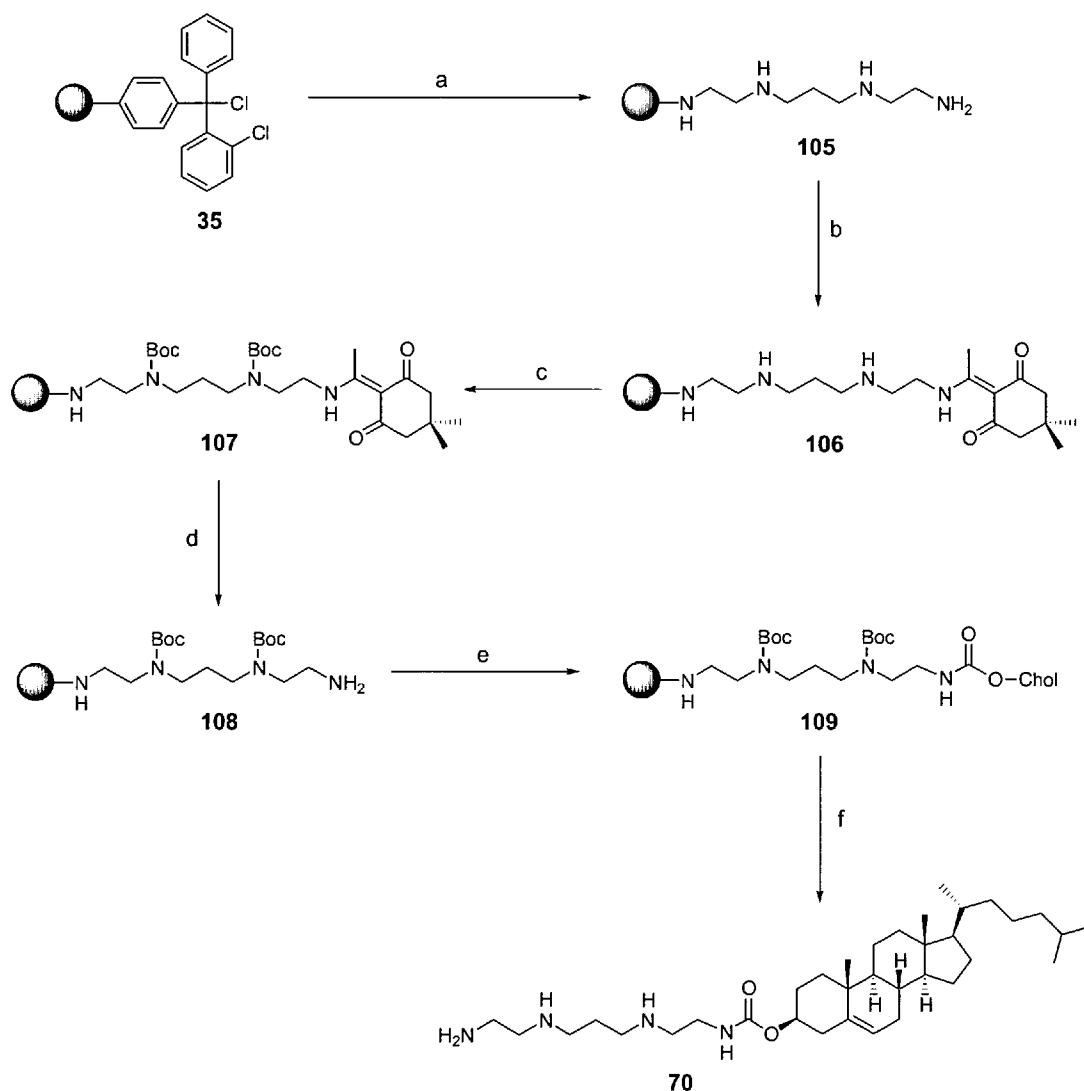
Scheme 3.8: Retrosynthesis of CDAN

A simple disconnection renders cholesterol chloroformate (commercially available) **22** and a selectively protected *N*, *N'*-bis(2-aminoethyl)-1,3-propane diamine (**103**). The selectively protected polyamine **103** can be assembled and immobilised on a solid phase resin using a Dde (2-acetyldimmedone) protecting group strategy from commercially available *N'*-bis(2-aminoethyl)-1,3-propane diamine **104**. The solid phase

immobilised selectively protected polyamine can subsequently be reacted with cholesterol chloroformate **22** on solid phase. CDAN **70** will be liberated upon cleavage of the product from the resin.

Solid Phase Synthesis of CDAN

The synthetic route to CDAN is depicted in Scheme 3.9.



Scheme 3.9: a) (i) N, N'-bis(2-aminoethyl)-1,3-propane diamine (10 equiv.), CH₂Cl₂, RT, 2 h, (ii) MeOH (capping reagent), RT, 10 min; b) Dde-OH (10 equiv.), DMF, RT, 12 h; c) Boc₂O (5 equiv. per free amine), NEt₃ (2 equiv. per free amine), CH₂Cl₂, RT, 4 h; d) 2% Hydrazine hydrate in DMF, RT, 10 min (repeat step x 2); e) Cholesterol chloroformate (10 equiv.), NEt₃ (3 equiv.), RT, 4 h; f) 50% TFA in CH₂Cl₂, RT, 1 h, (93% overall yield).

Firstly, resin (2-chlorotrityl chloride, 0.4 mmol g^{-1} , **35**) was loaded by treatment with excess polyamine (*N,N'*-bis(2-aminoethyl)-1,3-propane diamine **104**). Any un-reacted 2-chlorotrityl chloride groups were quenched by addition of excess methanol to the reaction mixture to prevent subsequent side reactions. Excess polyamine was isolated by filtration and easily recycled. The steric bulk of the chlorotrityl groups and excess quantities of polyamine used ensure the correct regioselectivity (binding of a single terminal primary amine) of polyamine immobilisation. The free primary amine sites of the immobilised polyamine **105** were then subsequently reacted with excess 2-acetyldimedone (Dde-OH), a primary amine selective protecting group. This selectivity arises as a result of the formation of a strong intramolecular hydrogen bond stabilising the resulting enamine **106**, which is not possible with secondary amines. The presence of the protected primary amine was confirmed by a negative Kaiser test. Free secondary amines were then protected with Boc groups to give **107**, and the Dde group was selectively removed upon treatment of the resin with 2% hydrazine in DMF, rendering **108** (a resin immobilised selectively protected polyamine). The solid phase assembly of CDAN **70** was completed by treating the liberated primary amine of **108** with cholesterol chloroformate **22**. CDAN **70** was cleaved from the resin after treatment with 50% TFA in dichloromethane. The filtrate was collected, concentrated *in vacuo* and the resulting oil lyophilysed to give CDAN **70** as a pale solid in excellent yield and purity (see Figure 3.6).

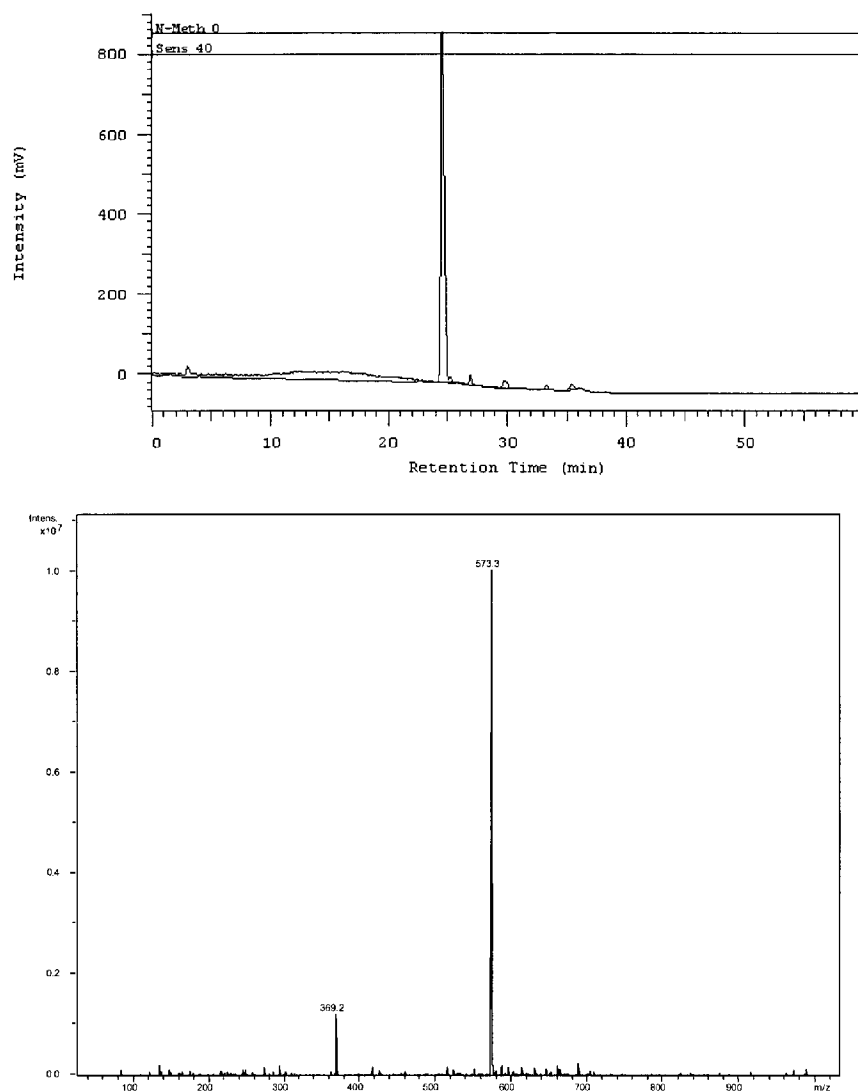
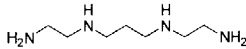
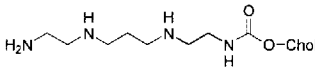
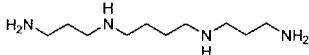
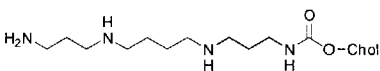
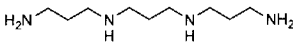
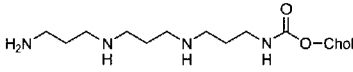
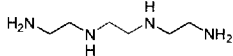
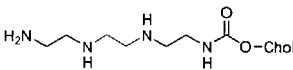


Figure 3.6: HPLC and MS of CDAN

(HPLC conditions: See page 199. Mass Spectrum: Positive ionisation ESI)

To test the scope of this methodology for the synthesis of cationic lipids, *N,N'*-bis(2-aminoethyl)-1,3-propane diamine **104** was replaced with spermine **97**, norspermine **99** and triethylene tetramine **16**, the results of which are shown in Table 3.2.

Polyamine	Product	% Yield (% Purity ^a)	Previous Literature % Yield
 104	 70	93 (96)	16 [160] 10 [161]
 97	 110	87 (95)	14 [160]
 99	 111	93 (96)	24 [160]
 16	 112	92 (95)	NA

^a As determined by HPLC and NMR

Table 3.2: Cationic Lipids prepared *via* solid phase methodology

The overall yields for the synthesis of the cationic lipids compares favourably to those gained from previous synthetic routes to these target molecules. This method permits the expeditious synthesis of a range of cationic lipids, giving the target molecules in excellent yield and purity. This establishes this methodology as a viable alternative to the traditional synthetic routes to polyamine conjugates [174].

3.4 Chapter Conclusions

Two cationic lipids have been successfully synthesised for incorporation into gadolinium liposomes. These cationic lipids will complement the previously developed gadolinium lipids (see Chapter 2) in the liposome membrane and permit the development of an MRI visible cationic liposome.

Efforts to synthesise these cationic lipids led to the development of a new solid phase synthetic route for the expeditious synthesis of cationic lipids. This route could also be adapted for the synthesis of other polyamine conjugates.

CHAPTER FOUR

Biological Analysis of Gadolinium

Lipids and Liposomes.

Cell Labelling and In Vivo Studies

Despite the many potential molecular imaging applications of our new gadolinium lipids, two principle applications will be explored; (i) the use of gadolinium lipids for cell labelling (and subsequent tracking) (see section 4.1) and (ii) the application of gadolinium labelled liposomes for their *in vivo* tracking (see section 4.2).

4.1 Gadolinium-Based Cell Labelling

Cell labelling and subsequent real time *in vivo* tracking is one of the most up-and-coming areas of molecular imaging. Since the first cell tracking experiment in 1994 [60] many research groups have focussed on labelling and subsequent tracking of cells. Indeed, imaging of cell tracking has proved to be an excellent tool for investigative biology, particularly in the area of disease mechanisms and analysis [47], [48]. However, a large amount of current research is focussed on potential future applications relating to stem cells, particularly the ability to monitor stem cell therapy in patients [63], [51], [64], [65].

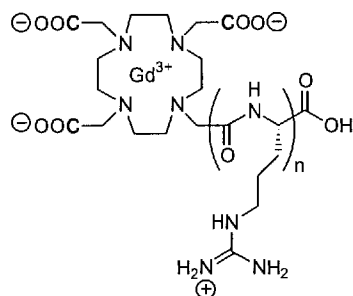
In order to image cell tracking, two basic requirements must be met: (i) an effective method to label the cells with a suitable probe, and (ii) a method by which to track these cells. The high resolution (10-100 μm) [66] and long term safety of MRI, combined with the unlimited probe life-time makes MRI the most interesting and viable candidate for cell labelling and tracking studies.

For cells to become MRI visible they must be labelled with an MRI visible probe such as gadolinium or iron. The commercial availability of iron-based agents for cell labelling (for example superparamagnetic iron oxide (SPIO) particles (Endorem™, Amersham) has meant that the majority of cell labelling experiments to date have been performed with iron. There are however, many disadvantages to this approach; iron predominantly decreases the T_2 relaxation of protons resulting in a decrease in signal intensity and subsequent darkening of the image. This makes these probes harder to detect compared to those that give rise to an increase in signal intensity (T_1 relaxation agents). Also, the presence of iron in a sample induces a mini-magnetic field (inhomogeneity) in the applied magnetic field. This shortens the relaxation time of protons not only in physical contact with the metal, but also all protons positioned within the mini-magnetic field, which is manifested in the so-called susceptibility effect. The effect of an iron contrast agent is therefore experienced well beyond the boundaries of a single cell. Although this effect can serve to counteract detection insensitivities, it

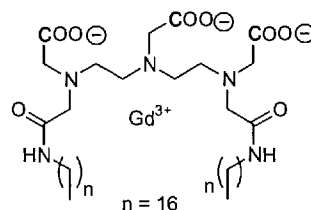
offsets the excellent resolution that can be achieved using MRI, a factor that is particularly important for cell tracking.

The principle advantage of the use of gadolinium agents arises as a result of their mechanism of inducing proton relaxation; T_1 relaxation (see Introduction, Section 1.2, MRI, Contrast Agents). As a consequence of this, gadolinium agents result in an increase in signal intensity (brightening of the image) and they do not harbour a susceptibility effect. To date, gadolinium contrast agents are only commercially available as blood pool agents, such as the small ionic $\text{Gd}(\text{DTPA})^{2-}$ and $\text{Gd}(\text{DOTA})^-$ complexes. Alone these agents do not provide an efficient method for labelling cells as the small ionic complexes are not actively taken across the cell membrane [175]. In efforts to harness the MRI favourable and biologically safe properties of these complexes to label cells, two approaches have so far been adopted; (i) incorporation of gadolinium contrast agents into nanoparticles such as liposomes or dextrans and (ii) conjugation of gadolinium contrast agent to cationic peptides (see Figure 4.1).

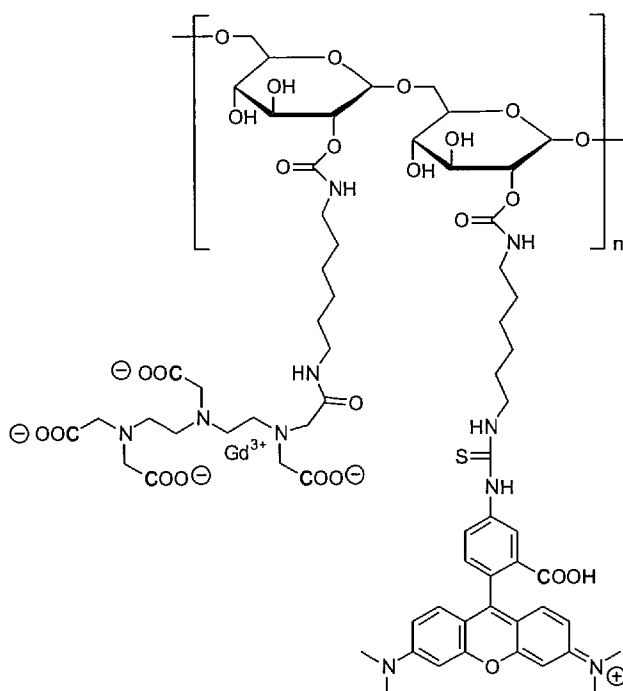
Peptides containing membrane translocation signals (MTS) [176], [177], [178] and dextrans [179], [180] are well established vectors for the delivery of cargo into cells and provided ideal starting point for the development of a gadolinium cell labelling strategy. The structures of these compounds combines these established cell delivery technologies with either $\text{Gd}(\text{DOTA})^-$ or $\text{Gd}(\text{DTPA})^{2-}$, both clinically approved contrast agents. The so-called GRID (Gadolinium Rhodamine Dextran conjugate) has been successfully applied for *in vivo* cell tracking experiments [181], [51] and the data obtained was verified using fluorescence imaging (as a result of the rhodamine tag) to confirm the results.



Gadolinium Polyarginine conjugate. The cationic polyarginine peptide contains a membrane translocation signal (MTS) and acts as a vector to transport the Gd(DOTA)⁺ across the cell membrane [80].



Gadolinium DTPA based lipid for incorporation into cationic liposomes. The cationic liposomes will act as a vector to transport the gadolinium into the cell [58].



Gadolinium Rhodamine Dextran (GRID) conjugate as a dual modality probe for cell labelling. The dextran forms the skeleton of the nanoparticle for transport into the cell [181], [51].

Figure 4.1: Selected Gadolinium Agents for Cell Labelling

Despite the success of the above agents for labelling cells, the use of gadolinium lipids in cationic liposomes as vectors for gadolinium labelling of cells can offer many advantages. Liposomes are easily adjustable particles and the lipid composition can be attuned for uptake into a vast range of cell lines. Furthermore a large amount of current liposome research is focussed on developing a targeted *in vivo* delivery system [98], [99], [100] and an effective liposome labelling strategy could be easily combined with this technology to produce a selective *in vivo* cell labelling strategy. However, one of

the most interesting properties of liposomes lies with their ability to transport drugs and nucleic acids into cells, which could be combined with delivery of a label. Using gadolinium lipid labelled liposomes as cell labelling vehicles should not impair the functional ability of the liposome, and so potentially the liposome could act as a multi-functional vector delivering a therapy and a MRI sensitive probe.

Building on previous laboratory experience with cationic liposomes, we aimed to combine the gadolinium lipids made in Chapter 2 with the cationic lipids made in Chapter 3 to produce an efficient gadolinium cell labelling based on the uptake of cationic liposomes. The good solubility of Gd.DOTA.Chol in both water and methanol at room temperature, combined with its efficiency as a T_1 contrast agent, led us to use this compound as the principle lipid for initial biological studies.

Ascertaining an Optimised Cationic Liposome Formulation

In order to obtain a suitable liposome formulation to mediate gadolinium labelling of cells a broad range of cationic liposomes (comprised of various ratios of cationic lipid, gadolinium lipid and helper lipids) were analysed using a liposome uptake assay (see Figure 4.2).

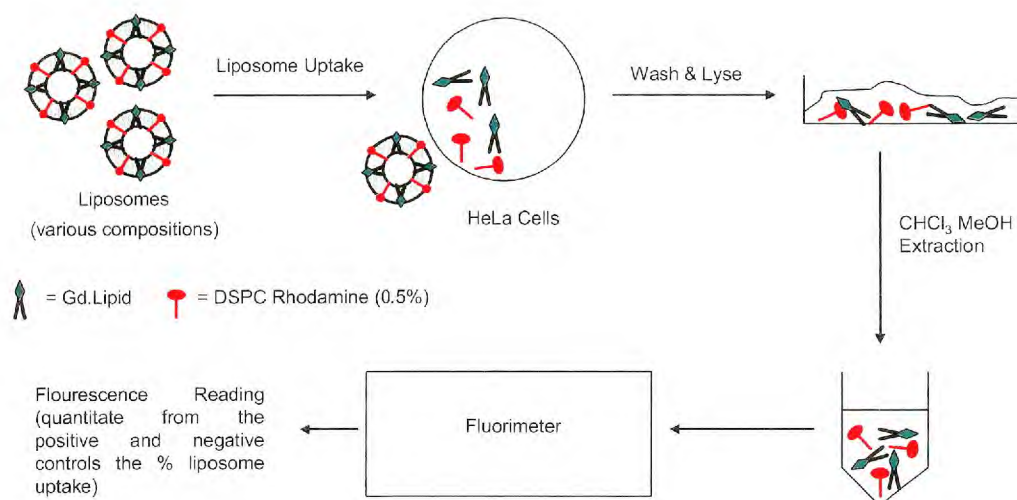


Figure 4.2: Schematic Representation of Liposome Uptake Assay

Briefly, all liposome formulations contained a constant amount of DSPE-Rhodamine (0.5 mol%). The liposomes were incubated with cultured cells for a defined time, in which time some of the liposomes will be taken into the cells *via* endocytosis. After

incubation the cells were thoroughly washed (to remove any excess liposomes) and lysed (broken up). The lipids taken into the cells, specifically the DSPE-Rhodamine, were extracted from the cell lysate using a chloroform-methanol mix. The organic extracts were then analysed by fluorescence spectroscopy to quantitate the amount of DSPE-Rhodamine, and therefore the amount of liposomes taken into the cells.

Nine cationic liposome formulations were initially analysed (see Table 4.1) for uptake in to HeLa cells. HeLa cells are a robust cell line, derived from human ovarian carcinoma, and provided the ideal starting point for our labelling experiments. The liposome formulations contained a variety of different ratios of cationic lipid, principally CDAN (to mediate efficient uptake) and gadolinium lipid (to label cells), as it was postulated that the optimal liposome formulation would contain a balance between the two types of lipids. The liposomes were prepared with identical total lipid concentrations (1.2 mg/ml) as described in Experimental, Section 7.11, General Liposome Preparation, ensuring that each formulation was made to a comparable size and pH.

Liposome	Formulation (mol %)				Size (nm)	pH
	CDAN	DOPE	Gd.DOTA.Chol	DSPE-Rhodamine		
1	20	29.5	50	0.5	130	7.3
2	20	39.5	40	0.5	121	7.2
3	40	29.5	30	0.5	101	7.3
4	35	39.5	25	0.5	158	7.1
5	30	49.5	20	0.5	127	7.1
6	60	29.5	10	0.5	115	6.9
7	50	39.5	10	0.5	106	6.9

	Formulation (mol%)			Size (nm)	pH
	DOTAP	Gd.DOTA.Chol	DSPE-Rhodamine		
8	50	50	0.5	130	7.3
9	60	40	0.5	121	7.2

Table 4.1: Liposome Formulations for First Round of Optimisation

Cellular Uptake of Liposomes and Gadolinium Lipid

Liposomes (6 μg or 12 μg of total lipid) were incubated with cultured HeLa cells (~80,000 cells (1 well, 48 well plate, 80% confluent)) for 4 hours, after which time the cells were washed and lysed. The cell lysate was analysed using the liposome uptake assay. The results of liposome uptake and the calculated mass of gadolinium lipid taken into cells are shown in Figure 4.3 and Figure 4.4 respectively.

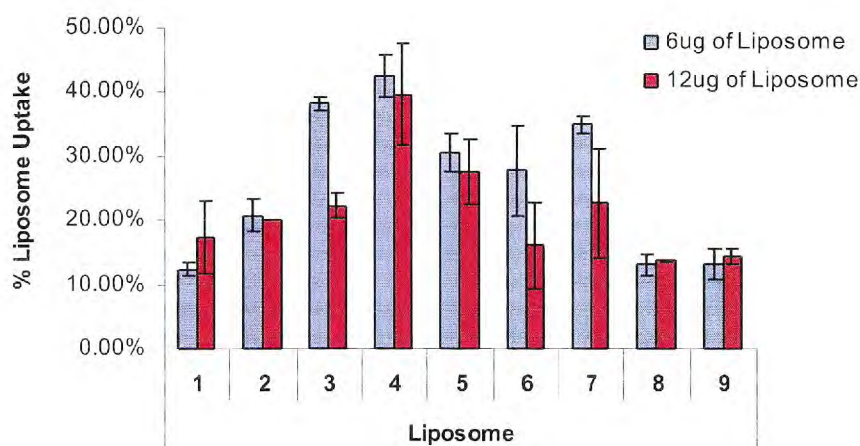


Figure 4.3: Cellular Uptake of Liposomes
(Liposome numbering corresponds to Table 4.1)

The results show that all the liposome formulations were taken up into the cells (see Figure 4.3). In general the higher the percentage of cationic lipid present in the liposome, the higher percentage of liposome uptake. CDAN containing liposomes (liposomes 1-7) appear to be more effective vectors than DOTAP liposomes (liposomes 8 and 9). It is interesting to note that with the CDAN containing liposomes the percentage of DOPE (a helper lipid) also influences the degree of liposome uptake. Indeed liposome 4 contains less CDAN but more DOPE than liposome 3, but has a higher degree of cellular uptake. It is postulated that the presence of DOPE in the liposome membrane aids their endosomal escape after uptake *via* endocytosis and therefore results in an overall more efficient uptake [182].

To ascertain the most efficient gadolinium labelling vehicle these results must be standardised against the total amount of gadolinium lipid present in each liposome (see Figure 4.4).

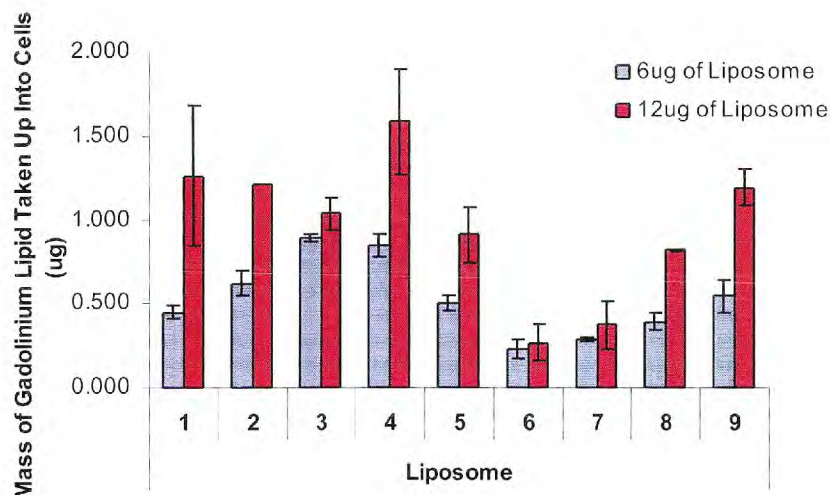


Figure 4.4: Cellular Uptake of Gadolinium Lipid
(Liposome numbering corresponds to Table 4.1)

Comparison between the overall percentage of liposome uptake (see Figure 4.3) with the amount of gadolinium lipid taken into cells (see Figure 4.4) confirms that the ideal cell labelling vector must contain a balance between the total amount of cationic and gadolinium lipid present in the liposome membrane. Liposomes containing low percentage amounts of gadolinium lipid (liposomes 6 and 7, 10 mol%) result in minimal gadolinium cell labelling, despite the high liposome uptake resulting from large amounts of cationic lipid present in the membrane. Conversely, liposomes with high ratios of gadolinium lipid (liposome 1, 50 mol%) to cationic lipid are also less effective. Despite the low percentage uptake of DOTAP liposomes (liposomes 8 and 9) into cells (see Figure 4.4), these liposomes deliver comparable, but still lower amounts of gadolinium lipid to cells than CDAN containing liposomes. With all liposomes, doubling the amount of liposomes added to the cells increases the uptake of gadolinium lipid into the cells.

With these results in hand a second, more extensive round of liposome optimisation was performed with liposomes containing 20-40 mol% cationic lipid (CDAN), 25-40 mol% gadolinium lipid and 30-40% helper lipid (DOPE). Four further liposome formulations were analysed (see Table 4.2) for liposome uptake in HeLa cells, using the same uptake assay (see Figure 4.2). The liposomes were again prepared with identical total lipid concentrations (1.2 mg/ml) as described in Experimental, Section 7.11, General Liposome Preparation, ensuring that each formulation was made to a comparable size and pH.

Liposome	Formulation (mol %)				Size (nm)	pH
	CDAN	DOPE	Gd.DOTA.Chol	DSPE-Rhodamine		
1	20	39.5	40	0.5	120	6.9
2	40	29.5	30	0.5	151	7.0
3	35	39.5	25	0.5	163	6.8
4	30	29.5	40	0.5	140	7.3

Table 4.2: Liposome Formulations for Second Round of Optimisation

Considering the observed marked increase in gadolinium lipid uptake upon increasing the dose of liposomes added to the cells, three doses of liposomes (6 μ g, 12 μ g or 18 μ g of total lipid) were incubated with cultured HeLa cells (~80,000 (1 well, 48 well plate, 80% confluent)) for either 4, 8 or 24 hours. After liposome incubation the cells were thoroughly washed and lysed. The results of liposome uptake and the calculated mass of gadolinium lipid taken into cells are shown in Figure 4.5 and Figure 4.6 respectively.

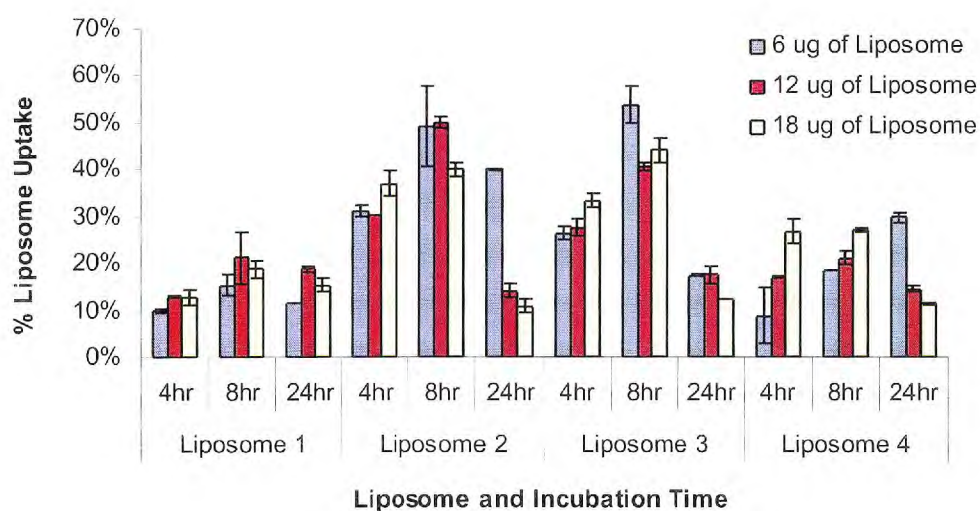


Figure 4.5: Cellular Uptake of Liposomes
(Liposome numbering corresponds to Table 4.2)

All liposome formulations show a high percentage uptake into cells (see Figure 4.5). Increasing the liposome incubation time from 4 hours to 8 hours generally increases

the amount of liposomes taken into cells. At 24 hours incubation the liposome uptake tends to decrease, particularly at higher liposome doses (a trend also observed with the amount of gadolinium lipid taken into cells). This could be a result of either cell death from cytotoxicity (a brief visual assessment of the cells showed a decrease in cell number) or cellular destruction of the fluorescent rhodamine probe. The results of standardising this data against the total amount of gadolinium lipid present in each liposome formulation are shown in Figure 4.6.

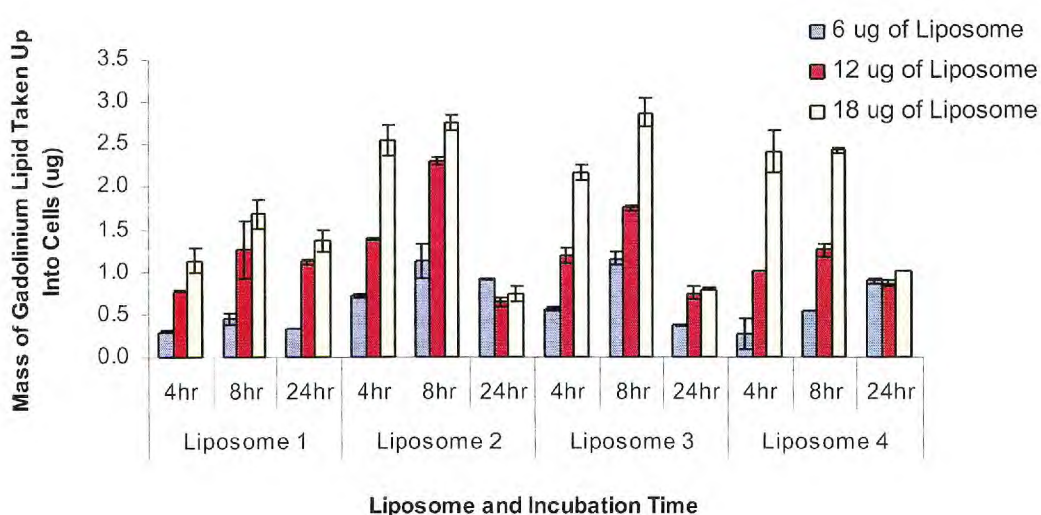


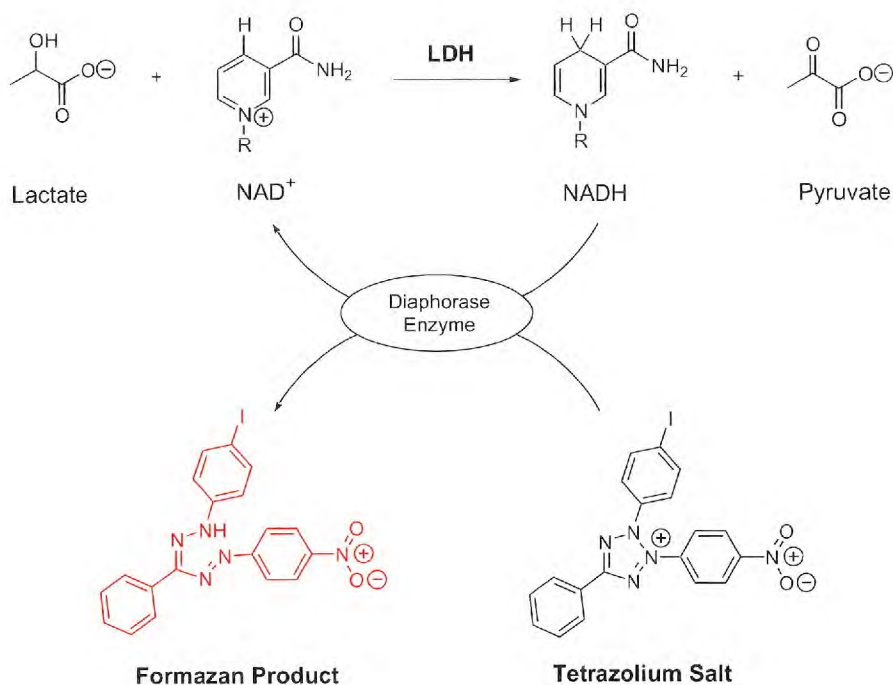
Figure 4.6: Cellular Uptake of Gadolinium Lipid
(Liposome numbering corresponds to Table 4.2)

Liposome 2 (CDAN (40 mol%) :DOPE (30 mol%) :Gd.DOTA.Chol (30 mol%)) shows the most consistently high uptake of gadolinium lipid into cells. Even at intermediate liposome doses, the uptake of gadolinium lipid into cells is high. In an effort to avoid the longer, seemingly cytotoxic incubations, whilst retaining maximum gadolinium lipid uptake, liposome 2 was chosen as the optimised candidate for further investigation.

Cytotoxicity of Optimised Liposome Formulation

If the optimised liposome formulation is to form an effective gadolinium cell labelling vehicle, it must deliver the gadolinium label whilst conferring minimal toxicity. The cytotoxicity of this liposome formulation was assessed using the CytoTox-96 assay (Promega). This assay evaluates cytotoxicity by assessing for the presence of LDH (Lactate Dehydrogenase) in cell culture media. LDH is a classic marker for cytotoxicity as it is released from the cytoplasm of cells upon membrane damage and subsequent

cell death. The LDH is detected in cell media using a coupled enzymatic assay (see Scheme 4.1).



Scheme 4.1: Principles of LDH Assay

The key to the assay is the reduction and subsequent oxidation of NAD(H). Pyruvate, NAD⁺, a diaphorase enzyme and a tetrazolium salt are all added to the cell media as part of the assay. Briefly, LDH from membrane damaged or dead cells catalyses the oxidation of lactate to pyruvate and the concomitant reduction of NAD⁺ to NADH. The diaphorase enzyme then re-oxidises the NADH to NAD⁺ and simultaneously converts the tetrazolium salt (yellow in colour) to its formazan product (red in colour). By using a series of time controlled additions and incubations the deep red colour from the formazan product (measured by fluorescence spectroscopy, λ_{ex} 560 nm λ_{em} 573 nm) can be quantitated. The colour formed is proportional to the amount of LDH present in a given sample and is subsequently a measure of cytotoxicity.

The optimised liposome (CDAN (40 mol%) :DOPE (30 mol%) :Gd.DOTA.Chol (30 mol%)) and control liposomes with no gadolinium lipid (CDAN (40 mol%) :DOPE (30 mol%) : Cholesterol (30 mol%)) were made to a concentration of 1.2 mg/ml, ensuring a reasonable size and pH. In order to obtain a full cytotoxicity profile for the optimised liposome, three doses of liposomes (gadolinium and control liposomes, (6 μg , 12 μg or

18 µg of total lipid)) were incubated with cultured HeLa cells (~80,000 cells (1 well, 48 well plate, 80% confluent)) for 4, 8, 24 hours. Two further conditions were also analysed, a 4 and 8 hour incubation followed by a period of 24 hours cell growth. The range of incubation conditions allows for the measurement of not only immediate toxicity but any delayed toxic effects as a result of the liposomes. To offset LDH release arising from naturally occurring cell death and membrane damage, a series of control experiments were run in parallel to the liposome incubations. Here the cells were treated in an identical manner to their counterparts but without the addition of liposomes.

Using the assay, the LDH released from the cultured HeLa cells (as a result of membrane damage or cell death) was calculated as a percentage of the total LDH present in the cells. The results of which are shown in Figure 4.7.

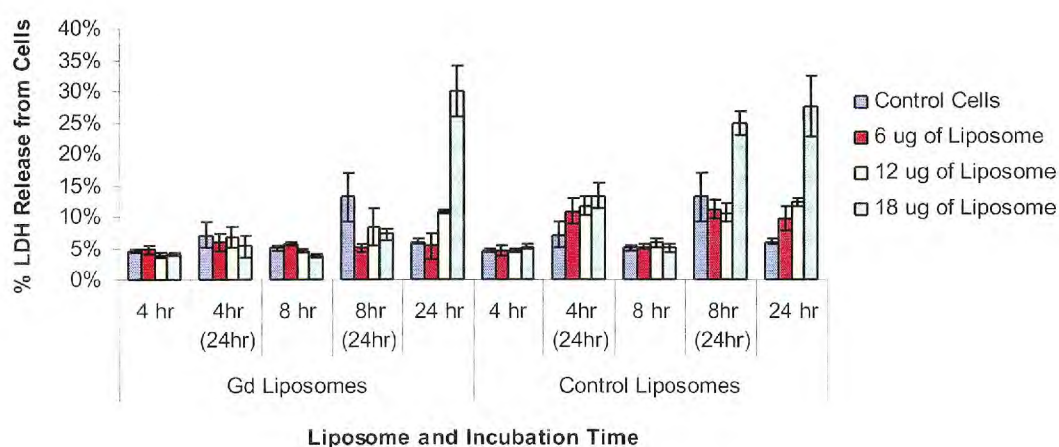


Figure 4.7: LDH Release from Cells

(Gadolinium Liposomes refers to the optimised gadolinium liposome (CDAN (40 mol%): DOPE (30 mol%): Gd.DOTA.Chol (30 mol%)); Control Liposomes refers to the gadolinium free liposomes (CDAN (40 mol%): DOPE (30 mol%): Cholesterol (30 mol%)); Incubations which were followed by a 24 hour cell growth period are marked by (24 hr))

The data shows that there is little difference in LDH release, and therefore cytotoxicity, between incubation with the gadolinium liposomes and the control liposomes at all incubation times. The 4, 8, 4(24) and 8(24) hour incubations with both liposomes show little difference in LDH release to that of the control cells, even at high doses (18 µg) of liposomes. However, the 24 hour incubations show a sharp increase in LDH release compared to the control cells upon increasing the dose of liposomes (to 18 µg).

Overall these results show that the optimised gadolinium liposome formulation does not confer significant cytotoxic effects upon HeLa cells, even with high doses (up to 18 μg) of liposome and long incubation times (8 hours). The cells also appear to be healthy 24 hours post incubation, with little or no increase in the LDH released compared to the control cells. The only significant cytotoxic effects are observed with 24 hour incubation times and high doses (18 μg) of liposomes. Considering the liposome uptake and cytotoxicity data, the optimal dose of liposome chosen for cell labelling studies was 12 μg of liposome per $\sim 80,000$ cells with 8 hour incubation.

MRI Analysis of Labelled Cells

In order to assess the efficacy of the optimised liposome formulation as a gadolinium cell labelling vector, a series of cell labelling experiments were undertaken. For initial *in vitro* studies, the most convenient method to visualise the cells using MRI is as part of a cell pellet ($\sim 10^6$ cells).

The cell labelling conditions (as deduced previously) were scaled up for labelling 10^6 cells. Cells were incubated with either gadolinium liposomes (CDAN (40 mol%) :DOPE (30 mol%) :Gd.DOTA.Chol (30 mol%)) or control liposomes with no gadolinium (CDAN (40 mol%) :DOPE (30 mol%) : Cholesterol (30 mol%)) for 8 hours, after which time they were removed from the cell culture flask, the solution centrifuged and the resulting pellet transferred to an eppendorf tube. The resulting cell pellets and liposomes (gadolinium and control) were examined by MRI using a spin echo sequence (see Experimental, Section 7.13, Cell Labelling Experiments). A sample of control cells (cells treated with no liposomes) was also analysed. Figure 4.8 shows the images gained from these experiments and Table 4.3 shows the T_1 analysis data for each sample.

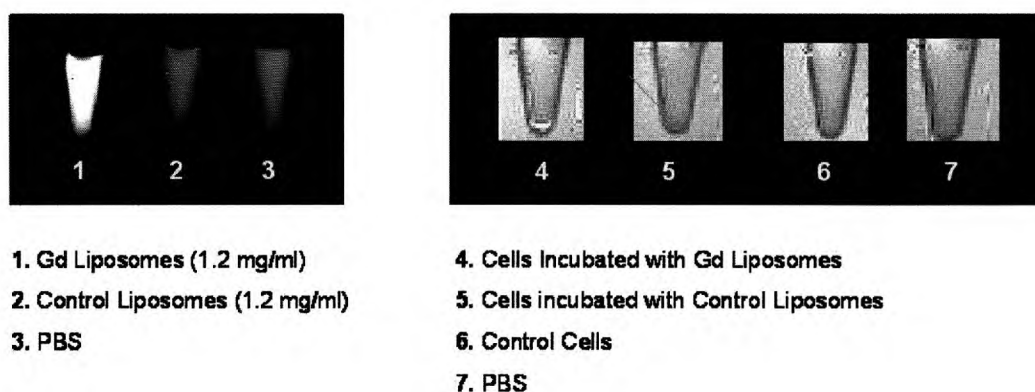


Figure 4.8: Phantom Images of Gadolinium Liposomes and Gadolinium Labelled Cells

	Sample	T ₁ Relaxation (msec)	% Decrease in T ₁ Relaxation
1	Gd.Liposomes	445 ± 13	84%
2	Control Liposomes	2763 ± 166	-
3	PBS	2826 ± 174	-
4	Cells Incubated with Gd.Liposomes	1572 ± 180	45%
5	Cells Incubated with Control Liposomes	2624 ± 354	-
6	Cells Only	2761 ± 704	-
7	PBS	2826 ± 174	-

Table 4.3: T₁ Relaxation Times for Gd.Liposomes, Gd labelled cells and appropriate controls

The optimised gadolinium liposomes are an effective contrast agent (see Figure 4.8). There is a clear increase in signal intensity (brightening of the image) with the gadolinium liposomes compared to the control liposomes, which appear dark. Analysis of the T₁ relaxation times for these samples confirms this, with the gadolinium lipids reducing the T₁ relaxation time to 84% of its original value, compared to the control liposomes and PBS alone. Indeed, the decrease in T₁ relaxation is comparable to that achieved with the gadolinium lipid alone (see Table 2.1). The cells incubated with gadolinium liposomes are clearly MRI visible at the bottom of the eppendorf tube. The presence of gadolinium in the cells gives them a characteristic increase in signal intensity and makes the cells 'bright' in appearance. The control cells and the cells incubated with control liposomes are not MRI visible. T₁ relaxation time analysis shows that the gadolinium labelled cells have a clear decrease in the T₁ relaxation time compared to the control sample. The decrease in T₁ relaxation time is however less than that observed for the gadolinium lipids and liposomes alone. This can be accounted for by a lower effective concentration of the gadolinium lipid inside the cells compared to the liposomes alone.

In summary, the optimised gadolinium liposome formulation and incubation time forms an effective gadolinium cell labelling vector and the results obtained are comparable to other published results [80] (gadolinium labelled cells T₁ relaxation time = 1640 ± 30 ms, unlabelled cells T₁ relaxation time = 2530 ± 210 ms). Further rounds of optimisation could be undertaken to increase the labelling efficiency or for uptake into a different cell line.

Mechanism of Cell Labelling

The gadolinium labelling of cells could potentially be occurring *via* two mechanisms: (i) the liposomes are entering the cells (endocytosis) or (ii) the liposomes are adhering to the cell surface. To ascertain the principle mechanism of labelling a series of microscopy experiments were undertaken.

Optimised gadolinium liposomes containing 0.5% DSPE-Rhodamine as a fluorescent probe (CDAN (40 mol%) :DOPE (29.5 mol%) :Gd.DOTA.Chol (30 mol%) :DSPE-Rhodamine (0.5 mol%)) were incubated with IGROV-1 cells. IGROV-1 cells are derived from human ovarian carcinoma and provide ideal cells for microscopy as they easily adhere to the glass slides. Cells were grown on glass slides and incubated with liposomes for either, 0 minutes (liposomes were removed immediately after their addition), 30 minutes 4 hours or 24 hours. The liposome concentration during incubation was identical to the conditions developed for cell labelling. After liposome incubation the cells were thoroughly washed to remove excess liposomes and fixed for microscopy. Initial microscopy studies were performed on a fluorescence microscope (see Figure 4.9).

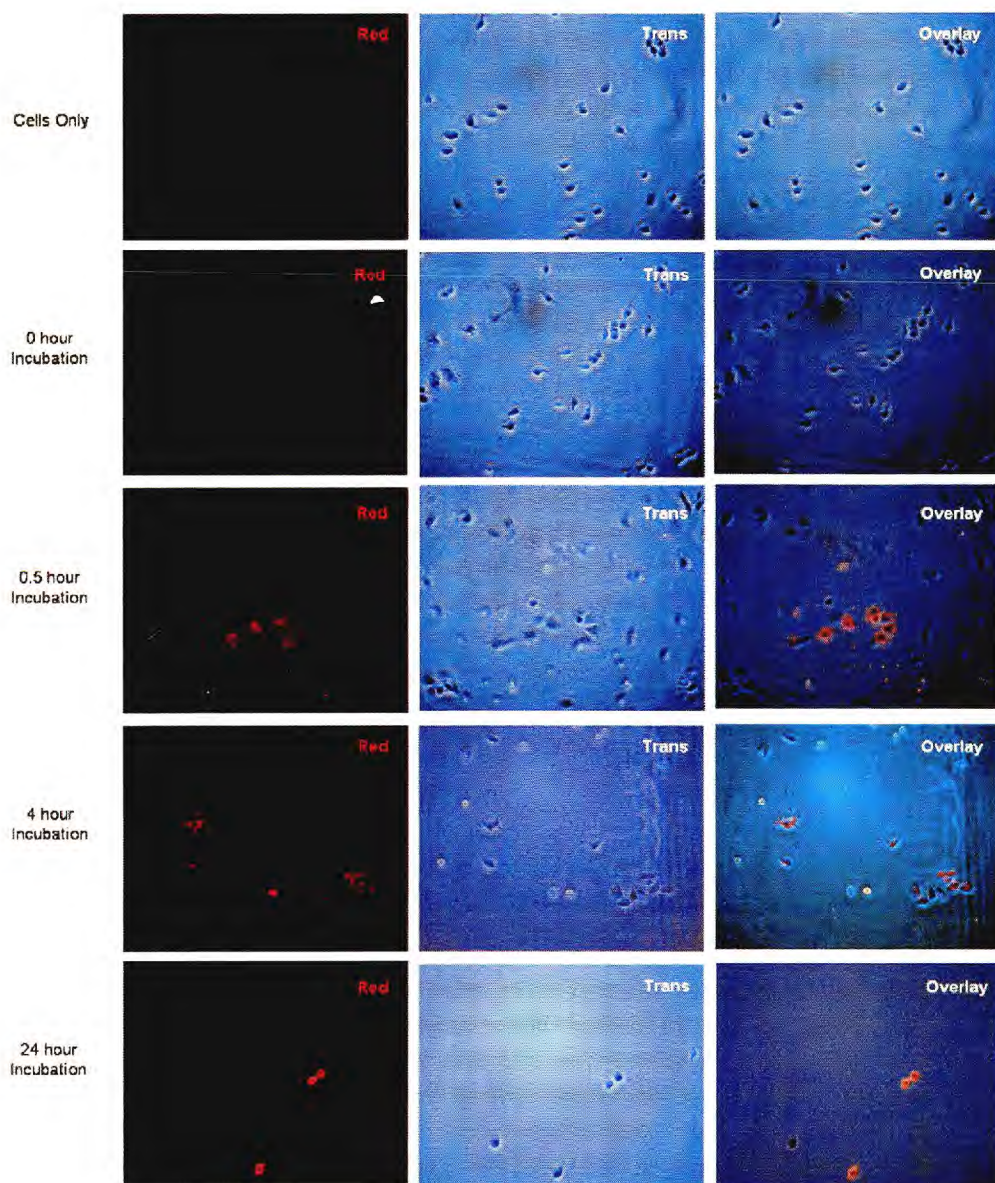


Figure 4.9: Fluorescence Microscopy Results (40 x magnification)

Analogous to a conventional microscope, fluorescence microscopy examines the cells and gives images in 2-dimensions (xy plane). The images denoted 'Red' were gained through visualisation using a green filter. The red colour observed is a result of rhodamine fluorescence from the labelled liposomes. The 'Trans' images are those gained without any light filters and are essentially normal microscope images. The images marked 'Overlay', are the result of the 'Red' and 'Trans' images being superimposed on each other, which permits visualisation of the location of liposome fluorescence.

The results show that the gadolinium liposomes immediately associate with the cells upon incubation. There appears to be a small amount of liposome uptake even after 0 hour incubation and the uptake of liposomes is clearly visible from 0.5 hour incubation onwards. The intensity of the fluorescence increases as the liposome incubation time increases, which correlates with the results gained in the previous rounds of liposome optimisation, showing increasing incubation time significantly increases the amount of liposome taken into cells. After the 24 hour incubation, the number of cells present on the microscopy slides decreased rapidly, presumably as a result of cell death, but the intensity of fluorescence (as a result of liposome uptake) from the remaining cells is at its highest.

To confirm the mechanism of uptake the cells from the 4 hour liposome incubation were examined using confocal laser scanning microscopy. Confocal laser scanning microscopy permits visualisation of a sample in 3-dimensions. Images of slices through the cells (z-plane) can be obtained in addition to the 2-dimensional microscopy images (xy plane). Figure 4.10 shows a selected z slice and Figure 4.11 shows a 3D projection of the same cells.

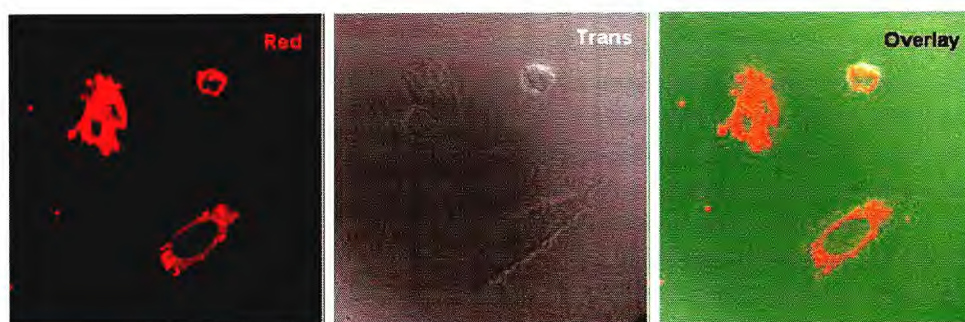


Figure 4.10: Selected z-Slice from Confocal Microscopy Results (100 x magnification)

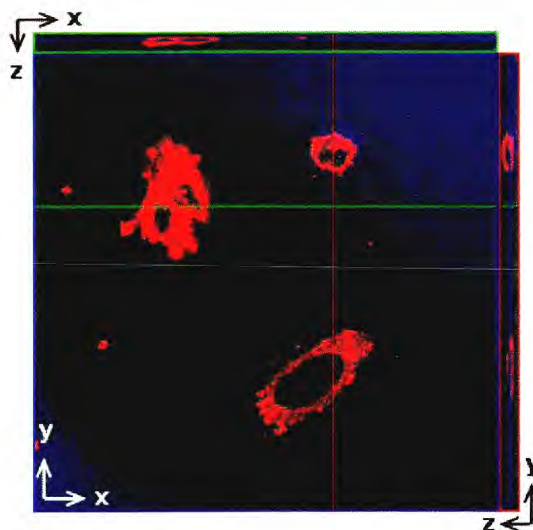


Figure 4.11: 3D Laser Scanning Micrograph Projection of Labelled Cells

The images show that the liposomes are ubiquitously present inside the cytoplasm of the cell, but do not appear to be in the nucleus. The mechanism of gadolinium cell labelling must therefore involve the cellular invagination of the gadolinium containing liposomes. The results obtained are in agreement to work done by Keller *et al* [183], which demonstrated cellular uptake of LD complexes.

Although these results confirm cellular uptake of liposome particles over time, the exact cellular location of the gadolinium lipid cannot be extrapolated from this data. To ascertain this, a fluorescently labelled gadolinium lipid would need to be developed and used in analogous experiments.

Transfection of Plasmid DNA

One of the principle advantages of using liposomes as a cell labelling vehicle is the potential for delivery of a multi-functional vector into cells. To test the scope of the new optimised gadolinium liposome developed for cell labelling, a series of cell transfections (delivery of plasmid DNA) were undertaken using gadolinium liposome: plasmid DNA complexes (termed Gd LD's).

The optimised gadolinium liposome (CDAN (40 mol%) :DOPE (30 mol%) :Gd.DOTA.Chol (30 mol%)), control liposomes (CDAN (40 mol%) :DOPE (30 mol%) :Cholesterol (30 mol%)) and Trojene™ liposomes (CDAN (50 mol%) :DOPE (50 mol%)) were prepared as described in Experimental, Section 7.11. Next, the liposomes were

complexed with the DNA in a weight ratio of 12:1 (liposome:DNA), generating the LD (liposome :DNA complexes) transfection particles. The reporter plasmid DNA (pNGVL1-nt-beta-gal; 7.53 kbp) contained the β -galactosidase gene. Cellular transfections were performed in HeLa cells, and the data gained (see Figure 4.12) is reported as β -galactosidase activity, ascertained using a chemoluminescent assay, in relative light units (RLU) per μ g of cellular protein. Trojene™ LD's provide the positive control and Trojene™ liposomes (no DNA) provide the negative control for the experiment, ensuring that the cells were transfecting normally. The toxicity of the LD particles was assessed in parallel to the transfection experiments using the LDH assay as described previously. The results gained are shown in Figure 4.12 (LD transfections) and Figure 4.13 (LD cytotoxicity).

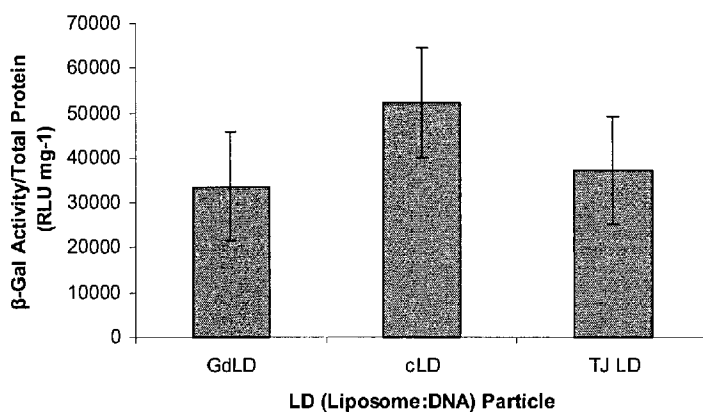


Figure 4.12: Transfection of LD's

(GdLD refers to Gadolinium liposome (CDAN (40 mol%); DOPE (30 mol%); Gd.DOTA.Chol (30 mol%)) LD complexes; cLD refers to Control liposome (CDAN (40 mol%); DOPE (30 mol%); Cholesterol (30 mol%)) LD complexes; TJ LD refers to Trojene™ (CDAN (50 mol%); DOPE (50 mol%)) LD complexes. All LD's were formulated with 12:1 Liposome DNA ratios (w/w)).

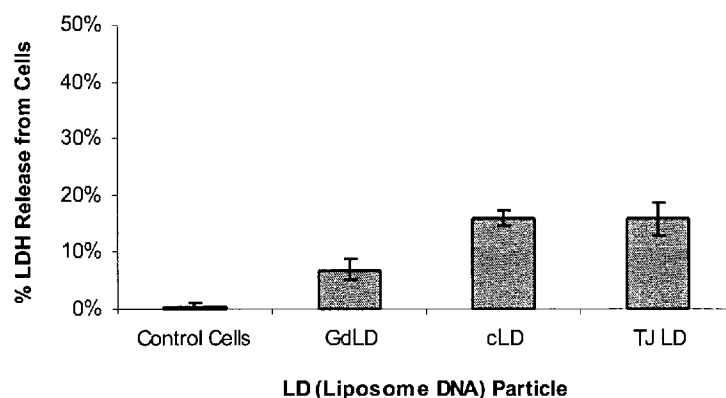


Figure 4.13: LDH Release from Cell upon LD Transfection

(GdLD refers to Gadolinium liposome (CDAN (40 mol%); DOPE (30 mol%); Gd.DOTA.Chol (30 mol%)) LD complexes; cLD refers to Control liposome (CDAN (40 mol%); DOPE (30 mol%); Cholesterol (30 mol%)) LD complexes; TJ LD refers to Trojene™ (CDAN (50 mol%); DOPE (50 mol%)) LD complexes. All LD's were formulated with 12:1 Liposome DNA ratios (w/w)).

All three LD particles display comparable transfection efficiencies. The transfer and expression of pDNA in gadolinium containing liposomes is therefore not significantly affected by the presence of a gadolinium metal chelate. The cytotoxicity of all three LD particles is also comparable. These preliminary results show that the optimised gadolinium cell labelling particle can also mediate transfer of pDNA with no enhancement in cytotoxicity.

Stability of Gadolinium Liposome and LD's

The stability of the optimised gadolinium liposome and corresponding LD particle were assessed in serum. Serum, the fluid component of blood, contains a plethora of proteins and ions. The stability of a particle (liposome or LD) in serum is an indication of the potential stability of the particle *in vivo*. Indeed, binding of serum proteins to liposomes or LD's facilitates their recognition by the host immune system and subsequent destruction [86]. Serum stable particles remain a good size over time, whilst unstable particles associate with serum proteins and coagulate together which results in a marked increase in particle size.

The stability of the optimised gadolinium liposome and corresponding LD's was measured against that for control liposomes and LD's (no gadolinium present). Solutions containing the liposomes (1.2 mg/ml) and LD's (1.2 mg/ml, 12:1 (w/w)

Lipid:DNA) were diluted 1:1 with foetal calf serum. The resulting solutions were incubated at 37°C over 30 minutes and the size of the particles was measured at defined intervals (see Figure 4.14).

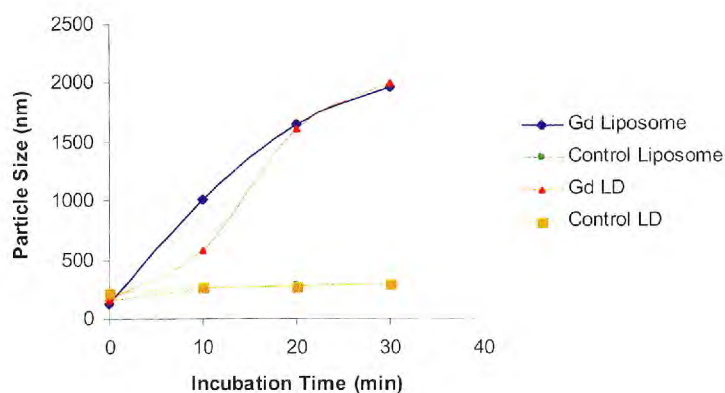


Figure 4.14: Serum Stability of Gadolinium Liposomes and LD's

(Gd Liposome refers to the optimised Gadolinium liposome formulation (CDAN (40 mol%): DOPE (30 mol%): Gd.DOTA.Chol (30 mol%)); Control Liposome refers to the gadolinium free optimised liposome formulation (CDAN (40 mol%): DOPE (30 mol%): Cholesterol (30 mol%)); GdLD refers to Gadolinium liposome LD complexes; Control LD refers to Control liposome LD complexes; All LD's were formulated with 12:1 Liposome DNA ratios (w/w)).

The gadolinium liposomes and LD's show a marked and immediate increase in particle size upon incubation with serum, compared to the control particles. This would infer that the gadolinium containing liposomes and LD's are less stable than their corresponding gadolinium free particles. One of the principle contributions to liposome and LD stability is the surface charge of the particle. Particles with a higher surface charge are easily recognised by the host immune system as a consequence of their rapid association with serum proteins. To enhance particle stability and prolong *in vivo* circulation, liposomes and LD's intended for *in vivo* use are frequently coated with a neutral polymer (such as PEG). These stealth particles are effectively surface neutralised and consequently have a higher serum and *in vivo* stability. The head group and thus surface exposed section of the gadolinium lipid (Gd.DOTA.Chol) on the liposomes/LD's is the small, polar Gd.DOTA moiety. The rapid decrease in gadolinium particle stability compared to that of the control particles, is likely to be a result of this increase in surface charge. For *in vitro* experiments, this apparent decrease in particle stability does not affect liposome or LD uptake into cells. However, for effective *in vivo* use the formulation of the gadolinium liposomes and LD's must be modified to incorporate a stealth coating to enhance particle stability.

Conclusion

It has been demonstrated that gadolinium lipids developed previously (chapter 2) can be combined with cationic lipids (chapter 3) to form an effective gadolinium cell labelling vector. This particle can also function as a dual modality vector as shown by the transfection of pDNA. Although the gadolinium liposomes and LD's are effective *in vitro* vectors, for use *in vivo* they would have to be modified further.

4.2 *In Vivo* Studies

One of the most important and apparent functions of gadolinium liposomes involves their application in real-time *in vivo* tracking of liposomes. The ability to monitor both the immediate and long term biodistribution and pharmacokinetics of liposomes is an important development for liposome technology. Currently the majority of *in vivo* studies on the biodistribution and pharmacokinetics of liposomes are undertaken using nuclear imaging techniques [116]. The liposomes are labelled with radionuclides and their distribution monitored using either PET or SPECT. Despite the high sensitivity of these techniques and their low image acquisition times [66] there are many disadvantages to this approach, principally low image resolution and the lack of potential for long term *in vivo* studies, as a result of limited probe life time. The use of MRI sensitive liposomes to study biodistribution and pharmacokinetics offers many advantages: high image resolution [66], longevity of probe life-time [66], safety of probes [74], and the logistics of MRI studies are simpler, as any MRI sensitive liposome could potentially be made in advance and stored for long-term use.

Gadolinium labelled liposomes were originally developed in the early 1990's [123], [122] for imaging the liver and spleen, the principle path of liposome excretion. Gadolinium labelling was achieved by either encapsulation of $\text{Gd}(\text{DTPA})^{2-}$ or by incorporation of a simple steroyl-based gadolinium lipid. The liposome was used as a tool to alter the pharmacokinetics and biodistribution of the normally small and water soluble $\text{Gd}(\text{DTPA})^{2-}$ and $\text{Gd}(\text{DOTA})^-$, which are excreted through the kidneys. Although both these systems provided methods for effective imaging of the hepatobiliary system, incorporation of a gadolinium lipid proved to be more successful than encapsulation of $\text{Gd}(\text{DTPA})^{2-}$ as it avoided problems of leakage of the gadolinium label from the liposome [118].

Building on this success of imaging gadolinium-based liposomes, groups that have developed gadolinium-based lipids for molecular imaging purposes (analogous to the

work in this thesis) have set about to develop this technology towards a molecular imaging bias. Leclercq *et al* [127] have successfully demonstrated *in vivo* imaging of a gadolinium lipid labelled LD particle. The labelled LD was delivered to a subcutaneously murine implanted tumour *via* direct injection to the tumour. A clear increase in signal intensity (brightening of the image) was observed in the tumour injected with labelled LD's. This signal intensity persisted, but decreased over 10 days post injection as a result of diffusion of the gadolinium lipid from the tumour into healthy tissue before its excretion. These results demonstrate the viability of gadolinium lipid labelled liposomes for particle (liposome or LD) tracking and in *in vivo* molecular imaging roles. More recently, a stable stealth gadolinium-based liposome has been developed using a *bis*-steroyl-based gadolinium lipid [58]. This DSPC/Cholesterol based system has a potentially longer *in vivo* circulation time and borrows from the successful formulations used in drug delivery. To date there is no *in vivo* data available concerning the use of this liposome formulation.

It is postulated that the diverse structures of our gadolinium lipids developed in Chapter 2 should form a set of versatile gadolinium labels for incorporation into a range of liposomes, specifically for *in vivo* use. Cholesterol and phospholipids are frequently used as the principle component of many long circulating liposomes and as such our gadolinium lipids could be used as effective substitutes in the lipid bi-layer without significantly altering the biophysical or biological properties of the liposome membrane. Having successfully shown that incorporation of a gadolinium lipid (Gd.DOTA.Chol) into a liposome results in production of highly effective MRI active liposomes (see Figure 4.8), we wished to develop a formulation for *in vivo* visualisation of liposomes. To avoid the problems of short term *in vivo* particle stability a neutral stealth liposome formulation (as compared to cationic liposome developed for cell labelling) would need to be employed for this purpose. The aim of this work for this thesis was to establish the preliminary viability of our gadolinium lipids for *in vivo* work.

Samples of the lipids were prepared in house and all animal work was performed by Dr. Jimmy Bell (Biological Imaging Centre, Imperial College London, Hammersmith Hospital).

Intramuscular Injection of Lipids

Initially the *in vivo* viability of the gadolinium lipids had to be assessed. A sample containing Gd.DOTA.Chol (in saline) was injected into the muscle of the hind leg of a mouse. The remaining leg was used as a control and a blank sample (saline only) was

injected into the muscle. The mouse was immediately imaged using a spin echo sequence (see Experimental, Section 7.12, *In Vivo* Experiments) and then re-examined 24, 36, 48 and 56 hour post injection. Selected slices of the images obtained are shown in Figure 4.15.

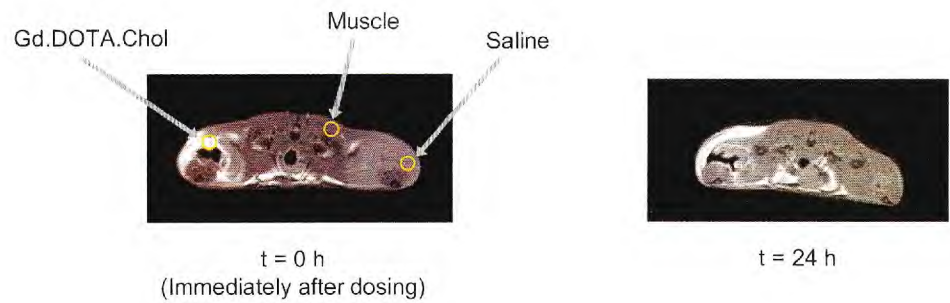


Figure 4.15: Images of IM Injections of Gd.DOTA.Chol into Hind Leg of Mouse

There is a clear enhancement of signal intensity in the leg injected with gadolinium lipid compared to the control leg. The region of muscle injected with gadolinium lipid appears bright around the injection site, a direct result of enhanced T_1 relaxation of the surrounding protons. To quantify the visual effect the signal intensity of the Gd.DOTA.Chol administered leg compared to the control leg and control muscle was measured. The signal intensity enhancement is still clear at $t = 24$ hour post injection and persisted for up to 56 hour post injection (see Table 4.4). This itself is an interesting result as it suggests that the lipids could be incorporating themselves into cellular membranes and not immediately being cleared from the injection site.

Time Post IM Injection (hr)	% Increase in Signal Intensity from Gd.DOTA.Chol vs Control Leg
0	221
6	193
24	139
56	77
648	5

Table 4.4: Change in Signal Intensity over Time Post IM Administration of Gd.DOTA.Chol

(Signal Intensity was measured with TR = 2200 msec and TE = 20 msec)

The observed increase in signal intensity shows that the gadolinium lipid is an effective *in vivo* contrast agent. These results compare well to those of Leclercq *et al* [127] who demonstrated their gadolinium lipid contrast agent to be effective *in vivo* after direct injection into a murine tumour.

Conclusion

Both the gadolinium lipids and the gadolinium liposomes have demonstrated to be effective contrast agents in initial *in vivo* studies. These preliminary results form a promising start to a more comprehensive liposome formulation and *in vivo* development programme.

CHAPTER FIVE

Gadolinium Post-Coating of Liposomes

5.1 Functionalising Liposomes

To further enhance the functionality of liposomes many research groups derivatise these particles with a range of ligands. Indeed, liposomes have been successfully functionalised with proteins [184], peptides [185], antibodies [186], sugars [187] and antigens [188]. This additional functionality can serve many purposes, such as an active targeting ligand, stealth protection or to yield a specific immune response. For the additional ligand to successfully exert its desired effect, it must however be positioned on the exposed outer-surface of the liposome. To ensure the integrity of the liposome bilayer and the correct positioning of the ligand, methods for functionalising liposomes often rely on bioconjugation of the ligand to an already formed liposome particle; in other words the liposome is post-coated with the desired ligand (see Figure 5.1).

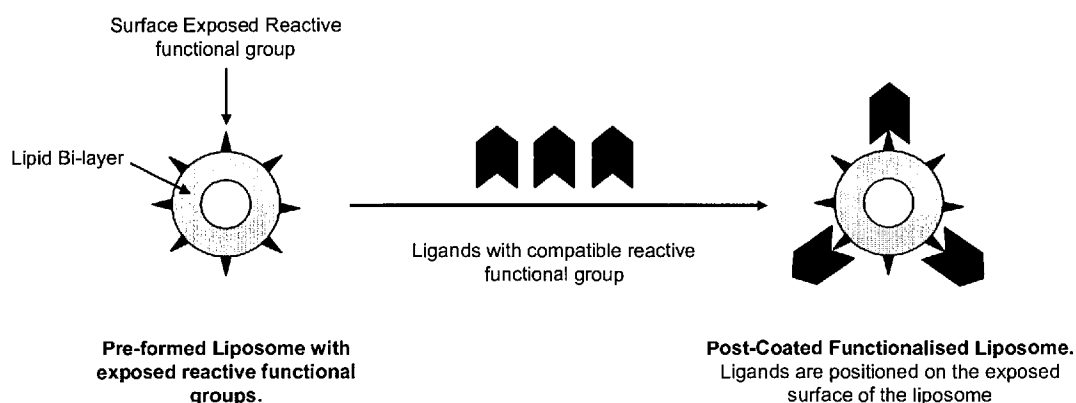


Figure 5.1: Post-Coating of Liposomes

Post-coating of liposomes relies strongly on the principles of bioconjugate chemistry. The key principle of bioconjugate chemistry involves the linking of two or more molecules together (frequently of biological origin) to form a novel complex having the combined properties of its individual components [189]. In all cases a covalent bond is formed between the two or more moieties *via* reaction of their respective functional groups with another. The chemistries behind this linkage utilise common organic chemistry reactions that can be performed in biologically compatible conditions (that is in aqueous media and at approximately physiological pH) [189]. A key requirement for a successful bioconjugation is that the reaction must be chemo-selective for the required functional groups.

Post-coating and bioconjugation with liposomes generally involves reaction of a functionally reactive lipid positioned within the liposome bi-layer with a naturally occurring or synthetically modified, compatible functional group on the ligand. A diverse range of conjugation chemistries have been employed for functionalising liposomes, and some of the most frequently used are shown in Table 5.1.

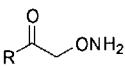
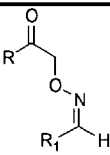
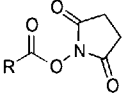
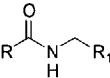
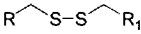
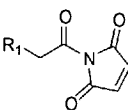
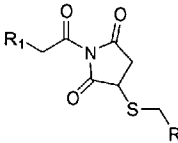
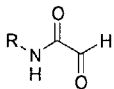
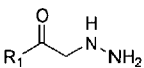
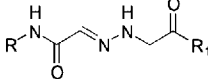
Functional Group on Lipid/Liposome	Functional Group on Ligand	Conjugate	Example
 Aminoxy	 Aldehyde	 Oxime	Coating liposomes with sugars [187].
 NHS-Ester	 Amine	 Amide	Conjugating antibodies to liposomes.
 Thiol	 Thiol	 Disulfide Bond	A classical bioconjugation linkage. Has been employed for functionalising liposomes with proteins [189].
 Thiol	 Maleimide	 Thioether	Conjugation of antibodies to liposomes [186].
 α -oxo aldehyde	 Hydrazine	 Hydrazone	Functionalising liposomes with peptides [185].

Table 5.1: Selected and Classical Liposomal Bioconjugation Chemistries

In an effort to develop a more efficacious gadolinium labelled liposome an alternative gadolinium labelling strategy was sort; post-coating the liposome with a gadolinium chelate.

In our previous, first generation gadolinium liposomes, the gadolinium lipid and hence the gadolinium chelate is held within the lipid bilayer, resulting in the gadolinium chelates being positioned on both the outer and inner surface of the liposome (see Figure 5.2). The principle mechanism of gadolinium induced proton relaxation requires direct contact of the gadolinium ion with water molecules positioned within its immediate environment (see Introduction, Figure 1.10). Therefore, for a gadolinium chelate to be an effective contrast agent it must have access to a maximum of surrounding water molecules. Whilst the gadolinium chelates positioned on the outer surface of the liposome have contact with the bulk of the aqueous environment, those chelates positioned on the internal surface have a restricted access to potential surrounding water molecules. It is hypothesised that this reduces the overall efficacy of the gadolinium liposome as a contrast agent. Also, the gadolinium chelates positioned on the outer surface of the liposome are being partially shielded and may even be partially buried in the liposome bilayer. This will restrict access to the surrounding water molecules, further reducing the efficacy of the gadolinium liposome.

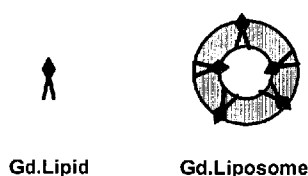


Figure 5.2: First Generation Gadolinium Liposome

(Gadolinium chelates are positioned on both the outer and inner surface of the liposome, reducing the overall efficacy of the liposome as a contrast agent. Moreover, gadolinium chelates positioned in the outer surface of the liposome could be partially buried within the bilayer, reducing their exposure to the surrounding water molecules.)

To improve on this, a post coating strategy was devised in which the gadolinium chelate is positioned solely on the outer surface of the liposome and is separated from the lipid bilayer by a stealth coating hydrophilic polymer, such as PEG (see Figure 5.3).

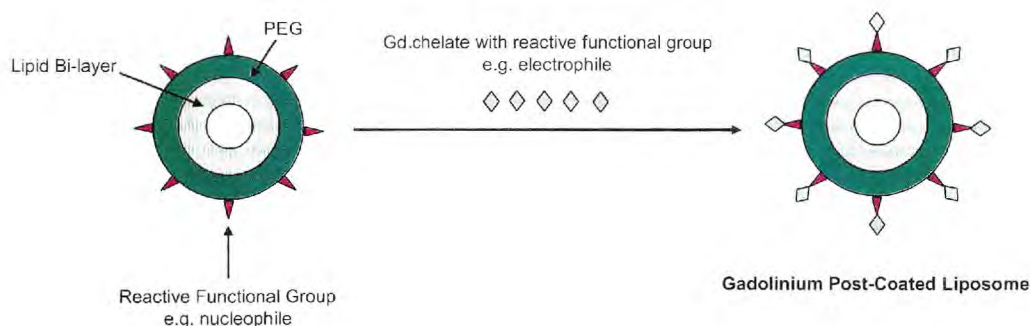


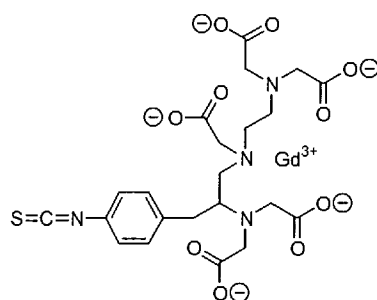
Figure 5.3: Gadolinium Post-Coating of Liposomes

(Gd.chelate is attached selectively on the outside of the bi-layer. Further more a PEG linker separates it from the lipid surface, increasing surface hydration of the liposome and thus allowing maximum gadolinium water exchange.)

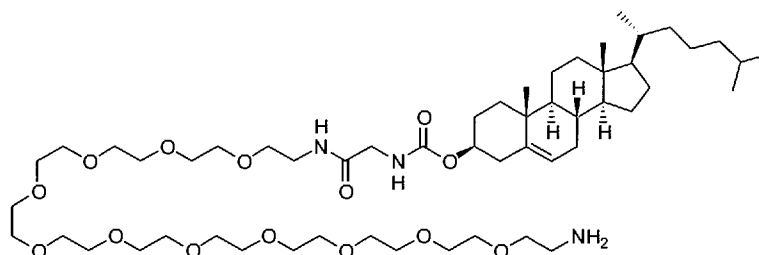
In this second generation gadolinium liposome, the gadolinium chelate will be added after formation of the liposome. This will ensure that the gadolinium is positioned solely on the outer water-exposed surface of the liposome, ensuring maximum efficacy of the all the gadolinium chelates on the liposome. To further enhance the efficacy of the liposome as a contrast agent, the gadolinium chelates are separated from the lipid bilayer by a hydrophilic PEG polymer. PEG is a well established polymer for coating liposomes and has been shown to increase the surface hydration of the liposome [85]. The increased water available to the gadolinium chelate combined with their protrusion from the liposome membrane should further enhance the efficacy of the liposome as a contrast agent.

Target Molecules

Two target molecules have been designed for the gadolinium post-coating liposome strategy: Gd.SCN.Bn.DTPA **113** and Chol.PEG₁₁.NH₂ **114** (see Figure 5.4).



Gd.SCN.Bn.DTPA (113)



Chol.PEG₁₁.NH₂ (114)

Figure 5.4: Gadolinium Post-Coating Liposome Target Molecules

SCN.Bn.DTPA (gadolinium free **113**) is a well known bioconjugate chelator for binding to antibodies and chelating radionuclides for targeted radiotherapy. Although there have been no reports of its chelation with gadolinium ions, the core DTPA skeleton is identical to that of many gadolinium containing contrast agents. The isothiocyanate group provides the key bioconjugate linker; Isothiocyanates are the sulphur containing analogues of isocyanates. Although both functional groups react with primary amines, isocyanate groups are a lot more reactive and subsequently less stable than isothiocyanates (they decompose upon contact with moisture). To avoid any unnecessary complications the isothiocyanate functionality was chosen in favour of the isocyanate. The target compound also contains an aromatic linker between the isothiocyanate and the chelator. This will ensure the isothiocyanate protrudes from the chelator and is sterically available for reaction.

Chol.PEG₁₁.NH₂ **114** is a cholesterol-based lipid with eleven repeating glycol units and a terminal primary amine. Cholesterol is an ideal lipid for incorporation into the liposome membrane as it can confer both rigidity and flexibility on the membrane (see Appendix, Section 9.1, Lipids, Cholesterol). The PEG units will extend from the liposome membrane and create a hydrophilic stealth coat. The terminal amine provides the compatible reactive functional group for the isothiocyanate group.

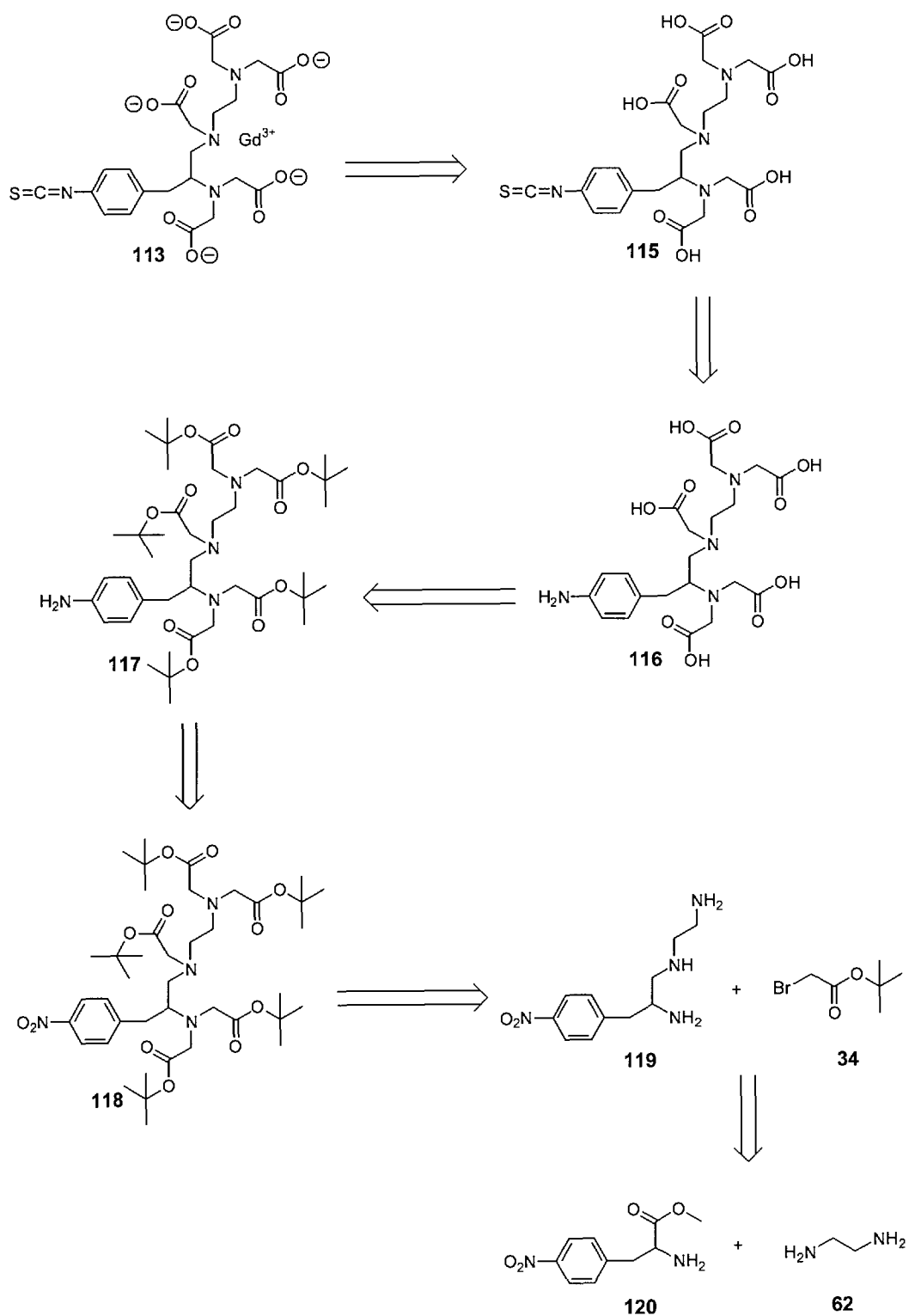
The bioconjugation will result from a reaction between the terminal primary amine of Chol.PEG₁₁.NH₂ **114** and the isothiocyanate group of Gd.SCN.Bn.DTPA **113**. The resulting thiourea bond formed will create a gadolinium chelate-lipid conjugate in which the gadolinium ion protrudes from the core of the liposome particle, to create the post-coated gadolinium liposome.

5.2 Synthesis of Gd.SCN.Bn.DTPA

Retrosynthesis of Gd.SCN.Bn.DTPA (**115**)

The retrosynthetic analysis of Gd.SCN.Bn.DTPA is shown in Scheme 5.1.

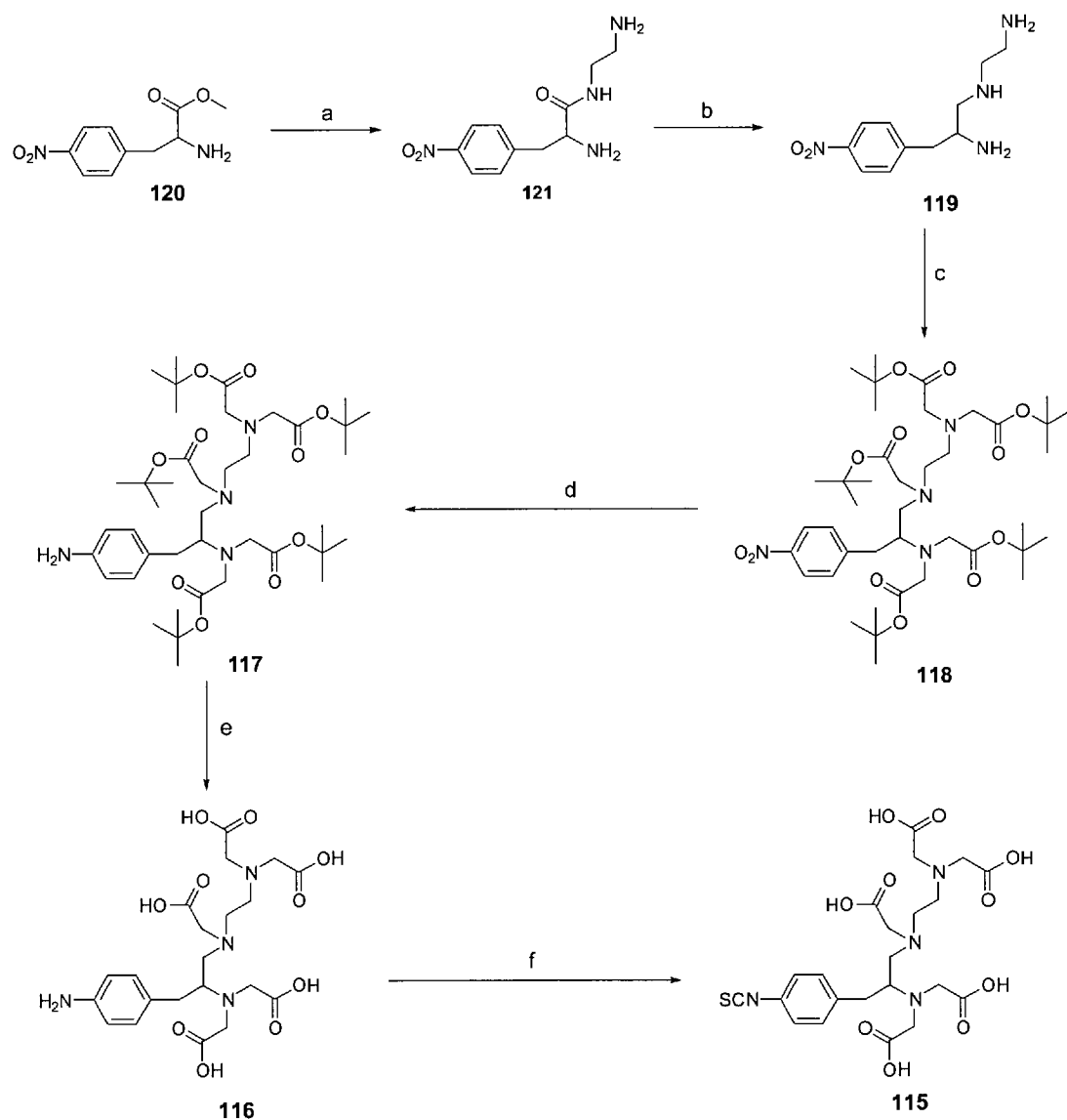
First, Gd.SCN.Bn.DTPA **113** will be derived from the metal free complex SCN.Bn.DTPA **115**. Isothiocyanate groups are highly reactive functional groups and so this functionality will be added just prior to the metal complexation. The isothiocyanate **115** could be easily made from reaction of an aromatic amine **116** with thiophosgene. Considering the polyamine basis of the target molecule and to avoid any adventitious side reactions during assembly of the DTPA chelator, the aromatic amine **116** will be made from the corresponding aromatic nitro compound **6**, just prior to its conversion to the isothiocyanate **115**. The fully *t*-butyl protected aromatic nitro compound **118**, is easily derived from the corresponding amino skeleton **119** by reaction with *t*-butyl bromoacetic acid **34**. The amine skeleton can be made by a reductive amination of *p*-nitrophenyl alanine methyl ester **120** and ethylene diamine **62**, both cheap commercially available starting materials.



Scheme 5.1: Retrosynthetic Analysis of Gd.SCN.Bn.DTPA

Synthesis of SCN.Bn.DTPA

The synthetic route to the metal free ligand is shown in Scheme 5.2.

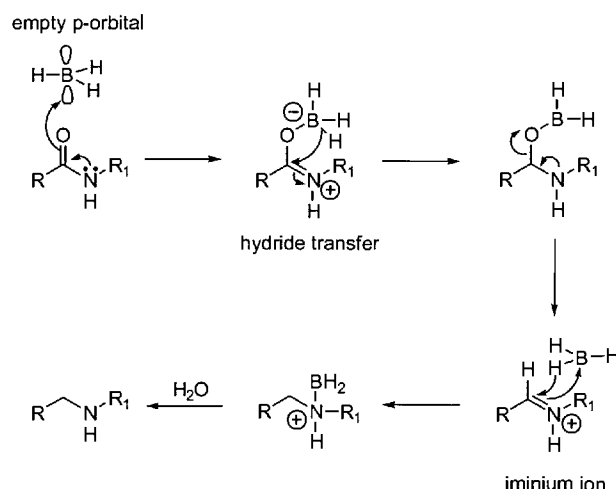


Scheme 5.2: a) Ethylenediamine, RT, 12 h, 71%; b) (i) $\text{BH}_3\cdot\text{THF}$, THF, 5°C , 15 h, (ii) HCl (6 M), 24 h, 89%; c) *t*-butyl bromoacetate, KI, DIPEA, DMF, 81%; d) Pd/C (10 mol%), H_2 (g), MeOH, 98%; e) c.HCl, Dioxane, N_2 (g), 4 h, 97%; f) HCl (3 M), CCl_4 , thiophosgene, 6 h, 98%.

First, *p*-nitrophenyl alanine methyl ester **120** was converted to the aminoethylamide derivative **121** by reaction with excess ethylenediamine (12 equiv.). The methyl ester was added gradually (in six equal aliquots over 2 hours) to the excess neat ethylene diamine. The large excess of ethylenediamine was used to prevent formation of any dimers, trimers etc. The reaction is marked by a distinct colour change upon addition of

the methyl ester to the neat ethylenediamine, with the reaction solution initially turning green and then deep red. The desired amide **121** was isolated after purification through a short silica gel column as a yellow oil in excellent yield. Spectral analysis (NMR and MS) confirmed that no dimer or trimer adduct products were present.

The amide **121** was reduced to the corresponding polyamine **119** upon reaction with a borane-THF complex. The mechanism of the reaction is shown in Scheme 5.3.



Scheme 5.3: Borane Reduction of Amides

Lewis acidic borane easily forms complexes and reduces Lewis basic electron rich carbonyl compounds. Indeed, the reactivity of borane is dominated by its desire to accept an electron pair into its empty p-orbital. After Lewis-complex formation, hydride transfer occurs from the anionic borane to the electrophilic carbonyl carbon. The resulting tetrahedral intermediate then collapses to form an iminium ion, which is subsequently reduced by the borane. The final borane complex is destroyed upon addition of water to the reaction mixture resulting in production of the desired amine. The polyamine **119** was isolated as a yellow oil in excellent yield (89%) after acidic work-up.

Alkylation of the triamine **119** was achieved using conditions developed by Corson *et al* [190] employing KI, *t*-butyl bromoacetate and base in a one pot reaction. The *in situ* Finkelstein reaction serves to make the more reactive *t*-butyl iodoacetate, which subsequently reacts with the triamine. The reaction proceeded well at room temperature and the desired penta-alkylated product **118** was isolated after silica gel column chromatography as a dark yellow oil in excellent yield and purity.

The nitrobenzene group of **118** was subsequently reduced to the corresponding aromatic amine **117** using palladium catalysed hydrogenation conditions. Upon reaction completion (TLC) the catalyst was removed *via* filtration over celite and the filtrate concentrated *in vacuo* to give the desired product **117** as a yellow oil in excellent yield. Spectroscopic analysis indicated that no further purification was required.

To prevent complexation of any adventitious metals over the remaining two synthetic steps, all glass wear was thoroughly washed with mixed acids (c.H₂SO₄ and c.HNO₃) before usage. The *t*-butyl groups of **117** were removed by acidolysis using identical conditions to those previously employed (c.HCl in dioxane and a positive flow of nitrogen). The penta-acetic acid product **116** was isolated, after lypholisation, in excellent yield as a pale solid.

The isothiocyanate **115** was prepared from the corresponding aromatic amine **116** using the standard bi-phasic methodology. The amine **116** was dissolved in 3 M HCl and thiophosgene dissolved in CCl₄. The organic phase was combined with the acidic aqueous phase and then vigorously stirred to mix the two phases. The reaction was monitored using a ninhydrin spot test (on filter paper), which analysed for the disappearance of the aromatic amine. After the reaction had gone to completion the solvents were removed *in vacuo*. All the bi-products of the reaction are volatile and were therefore removed during this process to give SCN.Bn.DTPA **115** as a pale crusty solid. Spectral analysis indicated that no further purification was required (see Figure 5.5).

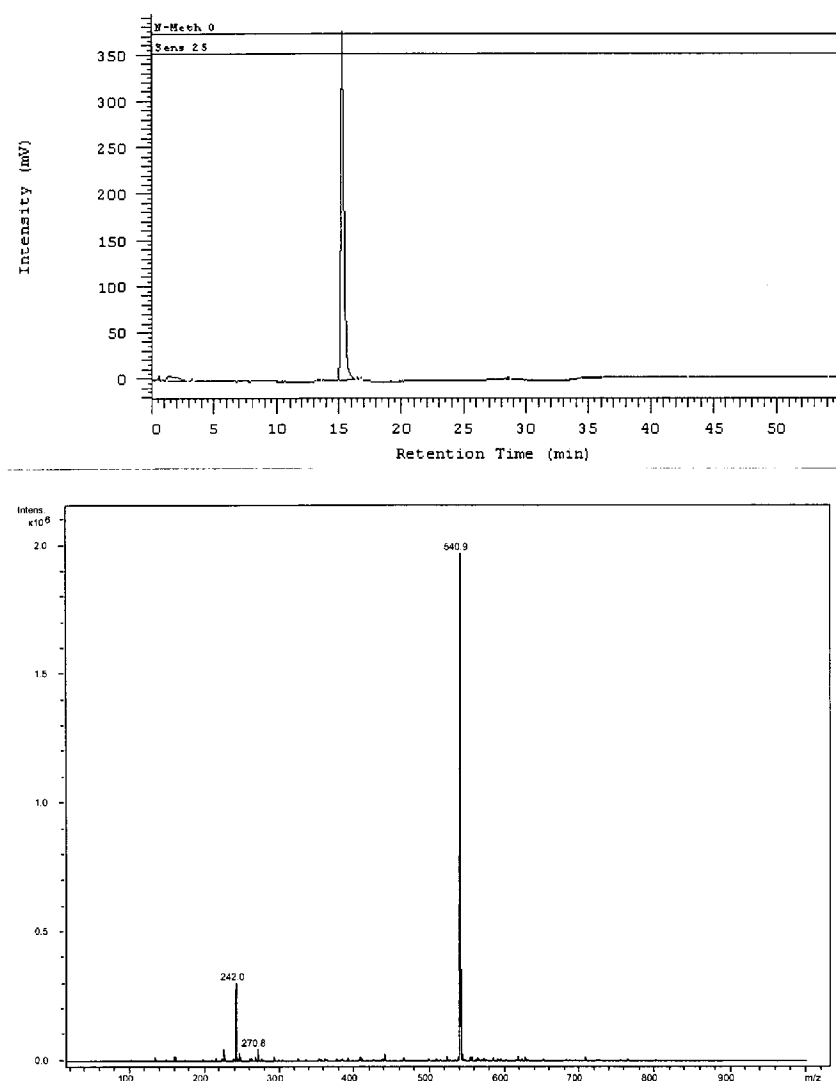


Figure 5.5: HPLC and MS of SCN.Bn.DTPA
(HPLC conditions: See page 209. Mass Spectrum: Positive ionisation ESI)

Gadolinium Complexation to SCN.Bn.DTPA

The final step in the synthesis of the Gd.SCN.Bn.DTPA **113** is complexation of gadolinium to the metal free compound **115**. Considering the results of our previous work complexing gadolinium to a DTPA-based ligand, the use of GdCl₃ seemed an ideal starting point. SCN.Bn.DTPA **115** is soluble in both MeOH and water. A series of trial reactions were set up in both solvents, with either neutral, basic (addition of NEt₃) or acidic (pH 5, by addition of 1 M HCl) conditions, and gadolinium complexation was observed by mass spectrometry. Analysis of the reactions showed that gadolinium complexation required the addition of either base or acid.

Although the basic conditions resulted in efficient metal complexation, the isothiocyanate group appeared to react with the base, resulting in production of unwanted bi-products. Complexation using acidic conditions was slow, but the isothiocyanate group was unaffected and therefore these conditions appeared most favourable. At very acidic pH's (< 2) a gadolinium ion can decomplex from its corresponding chelator [191], therefore it was imperative to monitor the pH of the reaction during metal complexation. Although employing a buffer would have provided this pH control, the excess salt present would have meant that another purification step was necessary. The optimal gadolinium complexation reaction conditions were found to be with water as a solvent (pH 5). After the complexation reaction had gone to completion the product was isolated after lyophilisation as a white solid. The xylenol orange test indicated that there were no free gadolinium ions present. Gd.SCN.Bn.DTPA **113** was analysed by HPLC and MS (see Figure 5.6).

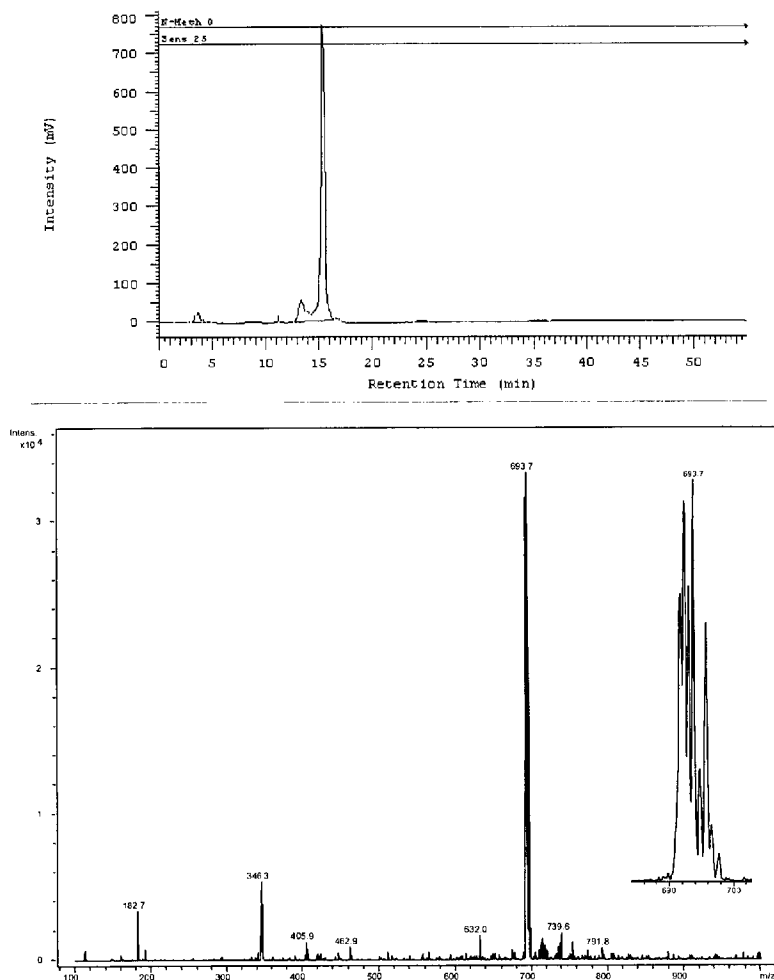


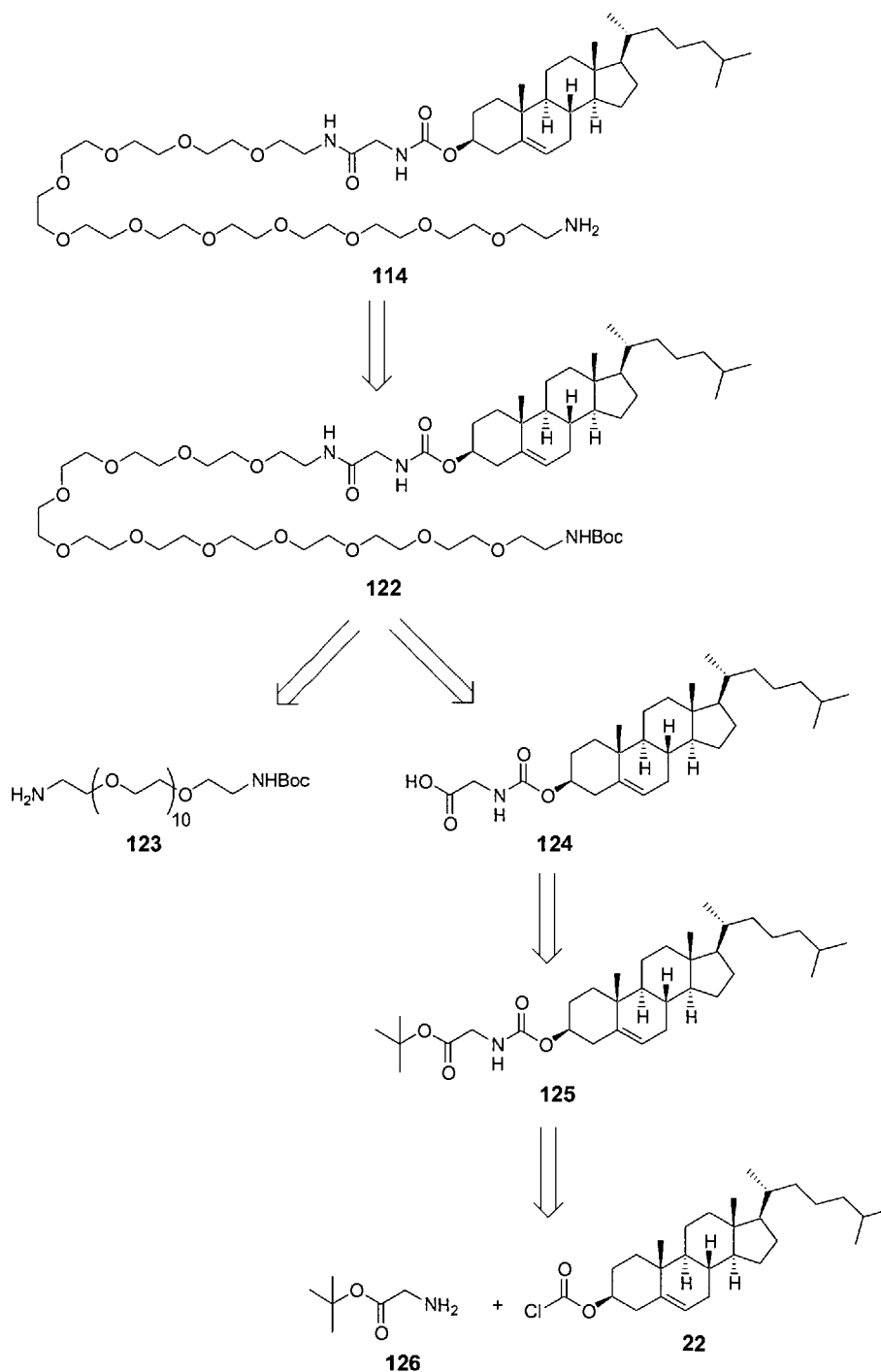
Figure 5.6: HPLC and MS of Gd.SCN.Bn.DTPA

(HPLC conditions: See page 210. Mass Spectrum: Positive ionisation ESI, inset shows the gadolinium isotopic distribution of the M^+ peak)

5.3 Synthesis of Chol.PEG₁₁.NH₂

Retrosynthesis of Chol.PEG₁₁.NH₂

The retrosynthetic analysis of Chol.PEG₁₁.NH₂ is shown in Scheme 5.4.



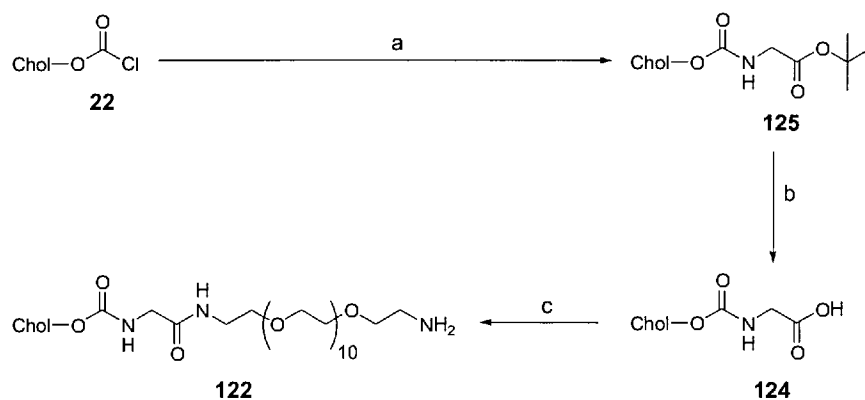
Scheme 5.4: Retrosynthesis of Chol.PEG₁₁.NH₂

Firstly the target molecule **114** is derived from its Boc protected analogue **122**. Disconnection at the amide bond on **122** renders the commercially available O-(2-aminoethyl)-O-[2-(Boc-amino)ethyl]decaethylene glycol **123** and a cholesterol glycine adduct **124**. The cholesterol glycine **124** can be obtained from its *t*-butyl ester analogue

125, which can be obtained by reaction of cholesterol chloroformate **22** and *t*-butyl protected glycine **126**, both commercially available starting materials.

Synthesis of Chol.PEG₁₁.NH₂

Scheme 5.5 details the synthetic route to Chol.PEG₁₁.NH₂.



Scheme 5.5: a) *t*-Butyl Glycine, NEt₃, CH₂Cl₂, 87%; b) TFA, CH₂Cl₂, N₂ (g), 97%; c) (i) O-(2-aminoethyl)-O-[2-(Boc-amino)ethyl]decaethylene glycol, HBTU, NEt₃, CH₂Cl₂, (ii) TFA, CH₂Cl₂, N₂ (g) 69%.

Cholesterol chloroformate **22** readily reacts with *t*-butyl protected glycine **126** under basic conditions. The desired product **125** was obtained in good yield after silica gel column chromatography as a white powder. The *t*-butyl ester of **125** was subsequently removed *via* acidolysis using TFA in dichloromethane over a positive flow of nitrogen. Cholesterol glycine adduct **124** was isolated as a white solid after lyophilisation. Spectral analysis concluded that no further purification was required. The free carboxylic acid of **124** was coupled to the free amine of O-(2-aminoethyl)-O-[2-(Boc-amino)ethyl]decaethylene glycol **123** under peptide coupling conditions using HBTU as the carbodiimide reagent. The Boc group on the resulting crude product **122** was immediately deprotected using TFA in dichloromethane. Overall, the target molecule **122** was isolated in good yield and purity after silica gel column chromatography as a yellow oil (see Figure 5.7).

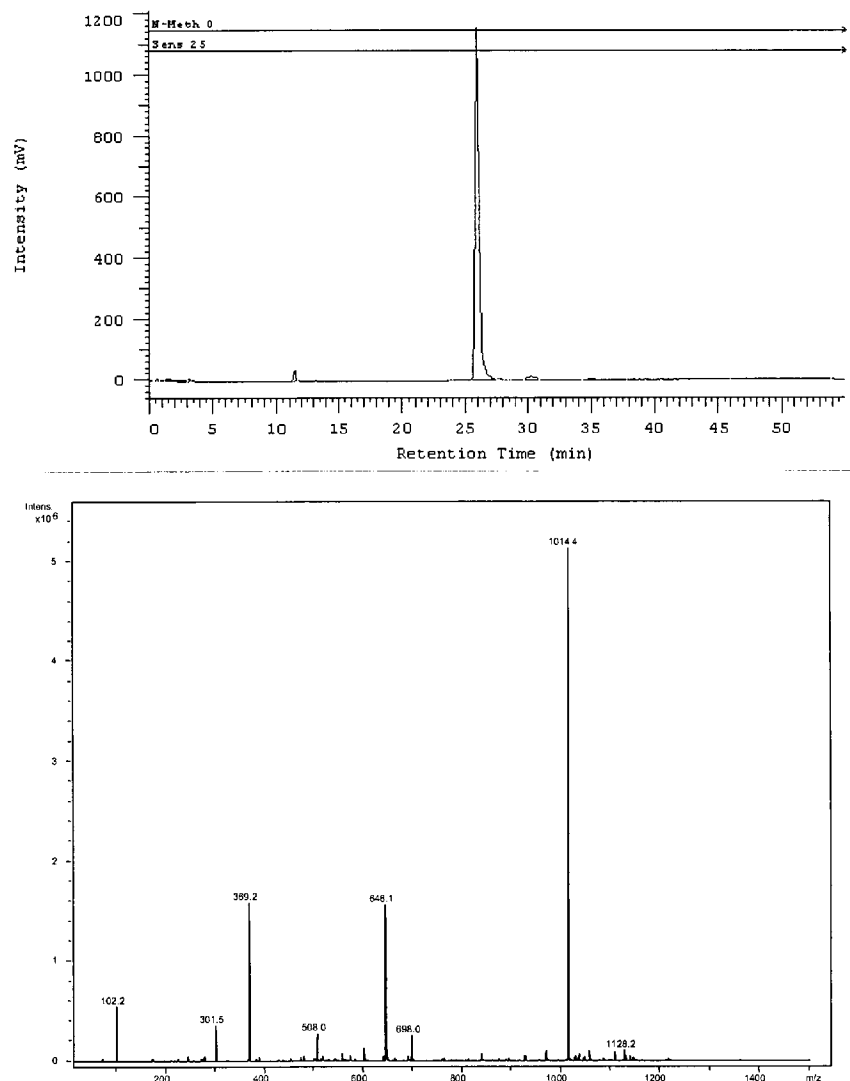


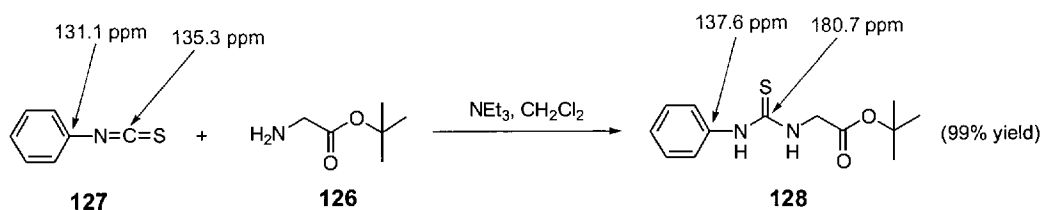
Figure 5.7: HPLC and MS of Chol.PEG₁₁.NH₂
(HPLC conditions: See page 212. Mass Spectrum: Positive ionisation ESI)

5.4 Conjugation Chemistry

To fully characterise the reaction between Gd.SCN.Bn.DTPA **113** and Chol.PEG₁₁.NH₂ **114**, a chemical sample of the resulting conjugate had to be synthesised. To enable full NMR characterisation of the conjugate product the coupling was performed between the gadolinium free chelate, SCN.Bn.DTPA **115** and Chol.PEG₁₁.NH₂ **114**. It is well known within the literature that isothiocyanate compounds react best at alkaline pH values, where the primary amine exists in its deprotonated state. For conjugation reactions in the organic phase this will be achieved by addition of a hindered base

(such as triethylamine) and for those in the aqueous phase this will be achieved by use of carbonate buffer (pH 9.6).

Prior to the key conjugation, a simple model isothiocyanate conjugation study was undertaken using readily available starting materials (see Scheme 5.6). The results from this will provide a good template for the reaction and product characterisation.

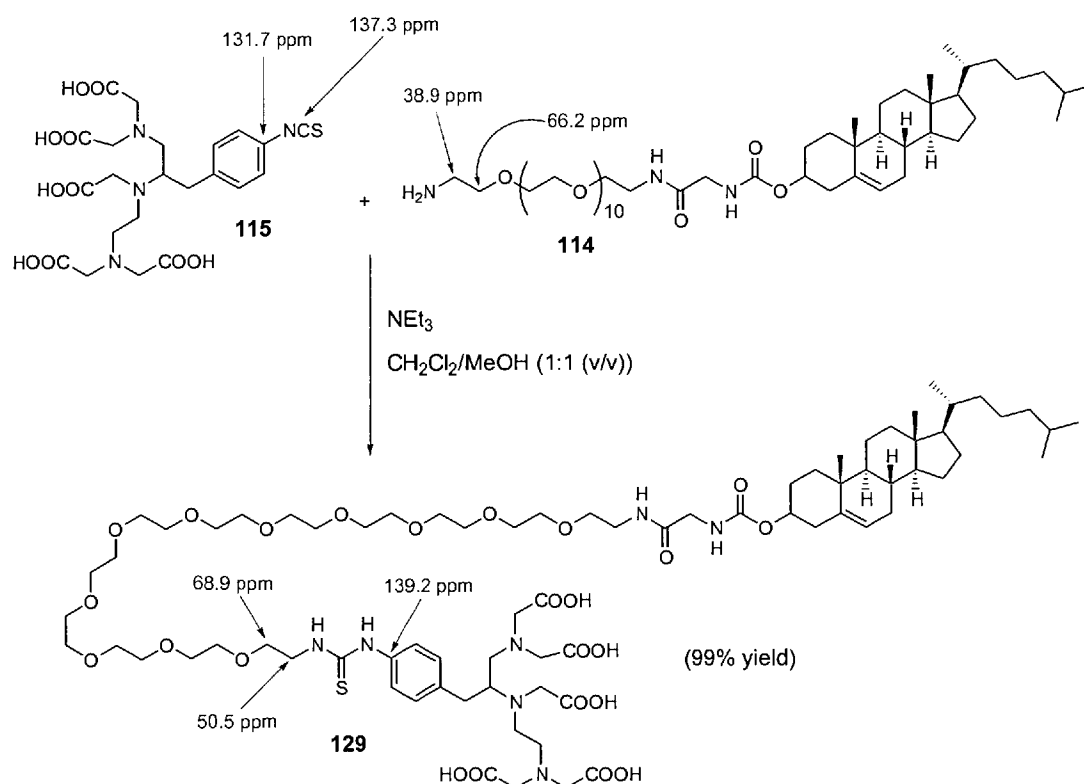


Scheme 5.6: Model Isothiocyanate Conjugation

(Key ^{13}C NMR shifts are shown in red)

The formation of the thiourea bond is characterised by downfield shifts in the chemical shifts of the *ipso* aromatic carbon and the quaternary carbon (both highlighted in Scheme 5.6). In both cases the intensity of the peaks in the phenyl isothiocyanate starting material is weak, but sharply increases in the thiourea product. Both peaks provide the ideal marker for the formation of thiourea bond and subsequent bioconjugation.

Scheme 5.7 shows the conjugation reaction between SCN.Bn.DTPA **115** and Chol.PEG₁₁.NH₂ **114**.



Scheme 5.7: Bioconjugation of SCN.Bn.DTPA and Chol.PEG₁₁.NH₂.

(Key ¹³C NMR Shifts are shown in red)

The conjugation proceeds readily at room temperature in a mixture of dichloromethane and methanol, in the presence of base. The reaction between the isothiocyanate and the primary amine is highly chemoselective and proceeds without production of by-products. Therefore, the desired conjugate **129** was obtained in excellent yield and purity (see Figure 5.8). The structure of the conjugate was confirmed by ¹³C NMR and MS.

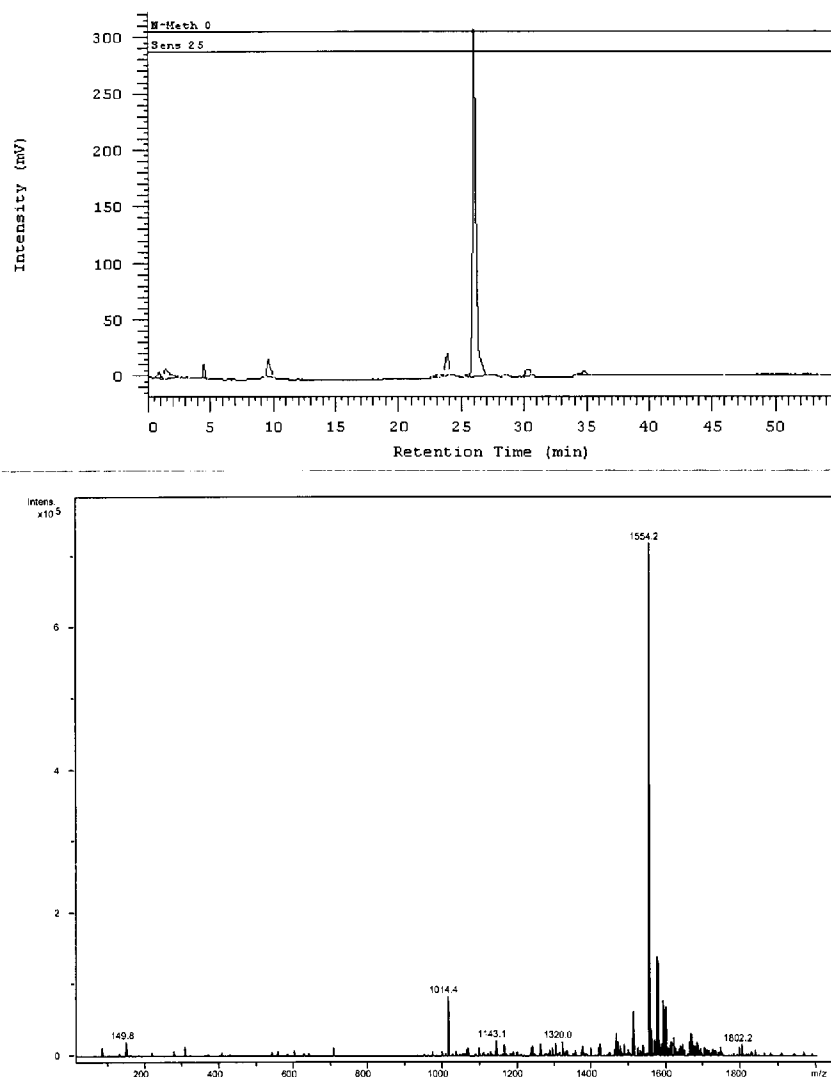


Figure 5.8: HPLC and MS of Conjugate

(HPLC conditions: See page 214. Mass Spectrum: Positive ionisation ESI)

The HPLC retention time of the conjugate ($t_R = 26.5$ minutes) is slightly different to that of the to Chol.PEG₁₁.NH₂ ($t_R = 26$ minutes) and markedly different from SCN.Bn.DTPA ($t_R = 15.5$ minutes), which means that HPLC will provide an excellent tool for assessing gadolinium post-coating of liposomes.

5.5 Gadolinium Post Coating of Liposomes

To ascertain the viability of the gadolinium liposome post-coating strategy as a method for gadolinium labelling liposomes, a series of experiments were set up using DOTAP-based liposomes. DOTAP (**65**) is a cationic lipid with a tertiary amine head group. Although the primary amine from Chol.PEG₁₁.NH₂ **114** should protrude from the

liposome bi-layer and thus react preferentially to any other amines present in the liposome, tertiary amines do not react with isothiocyanates which will prevent any adventitious side reactions and therefore provide an ideal model assessing the post-coating strategy. Liposomes containing DOTAP (65 mol%) and Chol.PEG₁₁.NH₂ (35 mol%) (6 mg/ml total lipid) were made as described in Experimental, Section 7.11. All liposomes were made to a good size and reasonable pH. Liposomes were then diluted 1:1 (v/v) with carbonate-bicarbonate buffer (to give a final lipid concentration of 3 mg/ml) containing a stoichiometric amount of either Gd.SCN.Bn.DTPA **113** or SCN.Bn.DTPA **115** (as a control) and incubated at 37°C for 12 hours. The coupling was monitored by both MS and HPLC (see Figure 5.9 (liposomes before post-coating), Figure 5.10 (liposomes post coated with SCN.Bn.DTPA **115**) and Figure 5.11 (liposomes post-coated with Gd.SCN.Bn.DTPA **113**)).

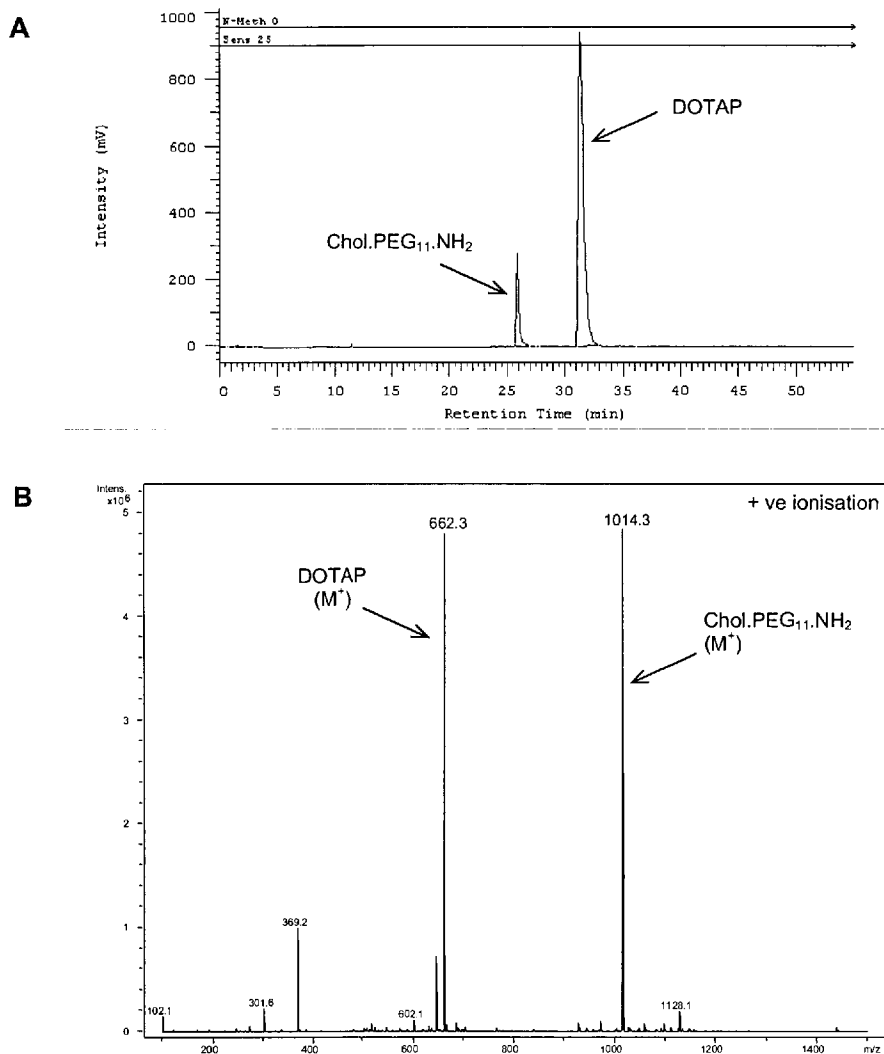


Figure 5.9: HPLC and MS of Liposomes before Post-Coating

Panel A is a HPLC trace of the liposomes before post-coating (see page 220 for conditions); Panel B is a positive ionisation mass spectrum of the liposomes before post-coating.

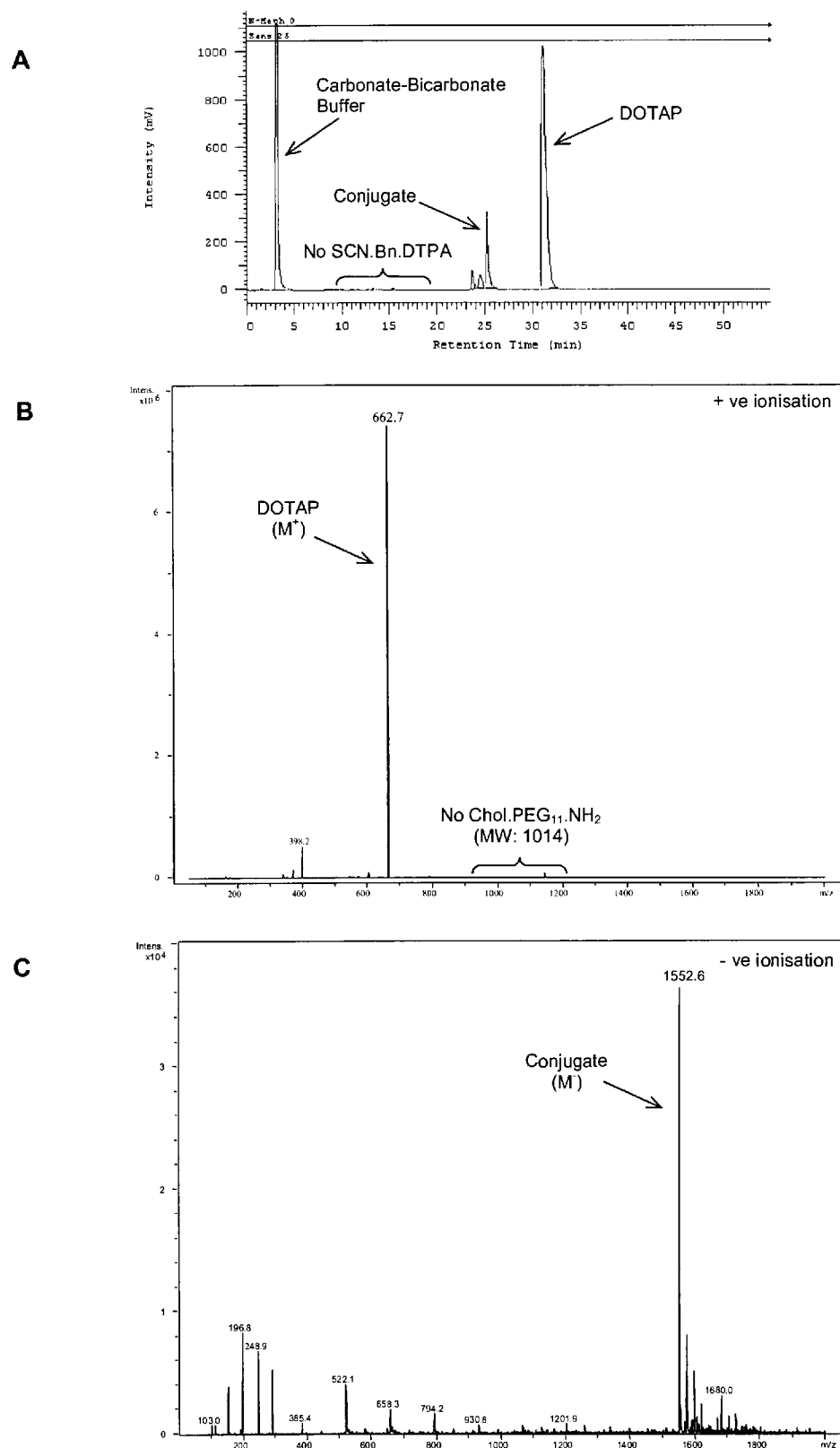


Figure 5.10: HPLC and MS of Liposomes after Post-Coating (SCN.Bn.DTPA)

Panel A is a HPLC trace of the post-coated liposomes (see page 220 for conditions); Panel B is a positive ionisation mass spectrum of the liposomes; Panel C is negative ionisation mass spectrum of the liposome.

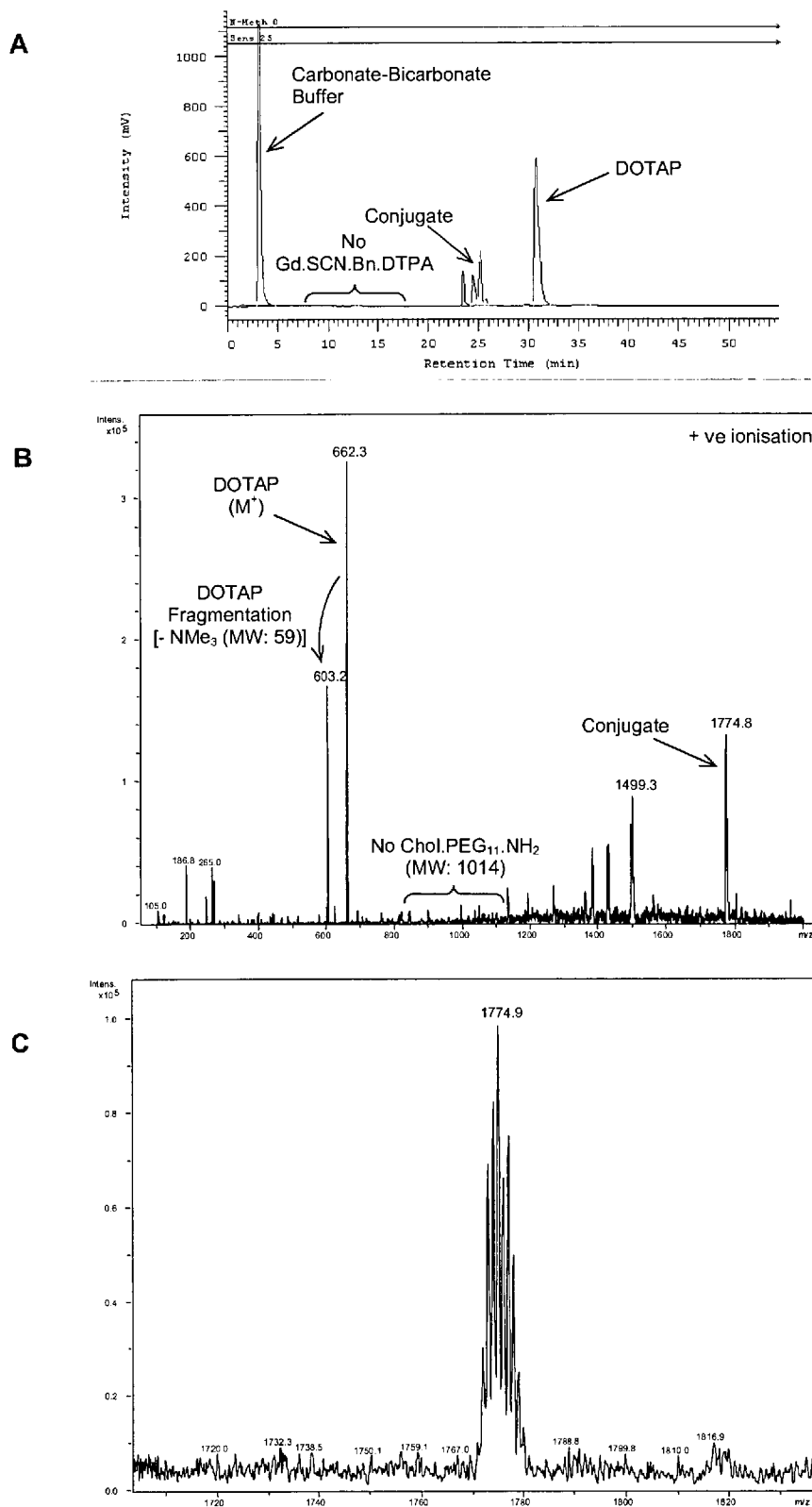


Figure 5.11: HPLC and MS of Liposomes after Post-Coating (Gd.SCN.Bn.DTPA)

Panel A is a HPLC trace of the post-coated liposomes (see page 220 for conditions); Panel B is a positive ionisation mass spectrum of the liposomes; Panel C is zoom in on the conjugate peak, illustrating the isotopes of Gd.

Analysis of the pre-formed liposome particles (see Figure 5.9) shows that there are two HPLC peaks of different intensity, which correspond to the two lipids in the liposome and at their respective molar ratios (DOTAP (65 mol%) and Chol.PEG₁₁.NH₂ (35 mol%)). Both lipids are also detectable on positive ionisation ESI-MS.

After incubation with SCN.Bn.DTPA **115** (see Figure 5.10) and Gd.SCN.Bn.DTPA **113** (see Figure 5.11) the HPLC peak for Chol.PEG₁₁.NH₂ (t_R = 26 minutes) disappears and is replaced with a peak corresponding to the formation of the conjugate (t_R = 26.5 minutes). This result is confirmed by MS analysis of each liposome. In both cases the strong peak for Chol.PEG₁₁.NH₂ (MW: 1014) disappears and a peak corresponding to the mass of the conjugate appears (Gd.Conjugate MW: 1744, Gd Free Conjugate MW: 1553). In the case of liposome post-coating with SCN.Bn.DTPA **115** the conjugate is only visible on negative ion ESI-MS. This is a direct result of the liposomes being present in alkaline buffer, which deprotonates the five free carboxylic acids present on the conjugate **129** (for structure see Scheme 5.7), giving the molecule a -5 charge, which in turn makes it easier to detect in negative ion mass spectrometry. In the case of the liposome post-coating with Gd.SCN.Bn.DTPA **113** the peak for the conjugate displays all the abundant isotopes of gadolinium (see Figure 5.11, Panel C), proving that the gadolinium ion is still chelated after liposome post-coating. This was confirmed by the xylenol orange test which indicated that there were no free gadolinium ions present in the liposomes. Although the conjugation chemistry is clean, the HPLC traces for both coupling with SCN.Bn.DTPA **115** and Gd.SCN.Bn.DTPA **113** show production of impurities. Investigation revealed that these are the result of hydrolysis of DOTAP in the carbonate-bicarbonate buffer. In both cases the size of the liposomes did not significantly change after post-coating.

Overall the results show that the isothiocyanate-amine conjugation chemistry is an effective tool for the post-coating liposomes. Curiously in both cases all of the Chol.PEG₁₁.NH₂ reacts with the available isothiocyanate compound. This phenomenon could arise as a result of either: (a) diffusion of the small metal chelator (SCN.Bn.DTPA **115** or Gd.SCN.Bn.DTPA **113**) across the liposome membrane and its subsequent reaction with internalised free amines or (b) through a process of membrane flip-flop where the internalised free amines become exposed on the outer surface of the liposome. Although we only anticipated that the surface exposed amines would react, this result is in full agreement with those of Keller *et al* [192].

MRI Analysis of Post-Coated Liposomes

To ascertain if the post-coupling methodology is an efficacious technique for gadolinium liposome labelling a series of liposomes were assessed using MRI. Phantom images (see Figure 5.12) and T_1 relaxation times (see Table 5.2) were obtained for the liposomes and appropriate controls. The images and T_1 relaxation times were extracted from experiments performed at Hammersmith hospital (Dr. A. Herlihy, Biological Imaging Centre, Imperial College London, Hammersmith Hospital).

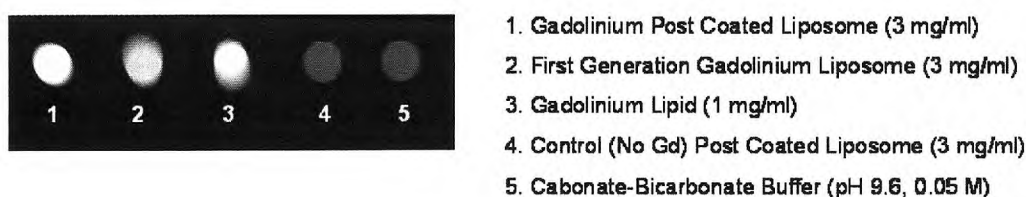


Figure 5.12: MRI Phantom Analysis of Gadolinium Post-Coated Liposomes

	Sample	T_1 (msec)	% Decrease in T_1 Relaxation
1	Gadolinium Post-Coated Liposome (3mg/ml)	231.4 ± 3.0	92%
2	First Generation Gadolinium Liposome (3 mg/ml)	222.5 ± 9.9	92%
3	Gadolinium Lipid (1 mg/ml)	221.8 ± 9.8	92%
4	Control (No Gd) Post-Coated Liposome (3 mg/ml)	2695 ± 75.4	-
5	Carbonate-Bicarbonate Buffer (pH 9.6, 0.05 M)	2842 ± 64.1	-

Table 5.2: T_1 Relaxation Times for Gd-Post Coated Liposome and Appropriate Controls

The results show that the gadolinium post-coating of liposomes is an effective labelling strategy. There is a clear enhancement of signal intensity in the gadolinium post-coated liposome sample (sample 1) compared to the control post-coated liposomes (sample 4) and buffer (sample 5). Indeed, the T_1 relaxation of the surrounding solvent is reduced to 92% of its original value, which is identical to the T_1 relaxation of a solution of gadolinium lipid alone (sample 3). However, examination of the first generation liposomes (sample 2) reveals that there is no additional enhancement of signal intensity or T_1 relaxation times with the new gadolinium post-coating methodology. Although this result was unexpected the signal enhancement and T_1 relaxation times

for the first generation liposomes is already at a high level and it might be difficult to significantly improve on this.

Overall the results show that gadolinium post-coating of liposomes successfully results in the production of MRI active liposomes. The MRI efficacy of these liposomes is comparable to the previous first generation gadolinium liposomes which demonstrates that the new post-coating strategy provides a viable alternative to the previous liposome labelling methodologies.

5.6 Chapter Conclusions

An alternative and novel gadolinium liposome labelling strategy was successfully devised, based on the principles of bioconjugate chemistry (specifically isothiocyanate-amine chemistry). A novel functionally reactive cholesterol-PEG lipid and compatible metal chelator were synthesised and were demonstrated to be effective tools for post-coating liposomes. MRI analysis of the resulting liposomes reveals that the new gadolinium post-coating strategy is a successful method for labelling liposomes, providing a viable alternative to our previous labelling methodology.

CHAPTER SIX

*Conclusions and
Future Directions*

Molecular imaging is defined as the *in vivo* characterisation of biological processes at a molecular or cellular level. It is rapidly emerging as an innovative approach to drug discovery and development, as well as a method for disease monitoring. Central to molecular imaging are the imaging modalities themselves, principally Positron Emission Tomography (PET), Fluorescence Imaging and Magnetic Resonance Imaging (MRI). With recent developments in MRI technology and its increasing prominence as a research tool, MRI is proving to be an invaluable diagnostic modality for this field. Research in molecular imaging has generally focussed on three key areas; imaging gene therapy (gene delivery and expression), imaging molecular markers and imaging cell tracking. Given this, liposomes present an interesting opportunity for molecular imaging studies as they are established vectors for mediating gene therapy, can be targeted to the molecular markers of specific cell types or organs and can easily traverse cell membranes (a fundamental requirement for cell labelling and subsequent tracking).

The key components of liposomes are the lipids themselves and it is the particular assembly of different varieties of lipids that determine the overall properties of the liposome. The structure of all lipids comprises a polar head group conjugated to a lipidic tail and many research groups have worked on diversifying the properties of lipids and liposomes by chemically modifying the lipid structure. Borrowing from well established gadolinium-based contrast agent chemistry, a series of gadolinium based lipids were synthesised for incorporation into liposomes to enable production of MRI active liposomes (Chapter 2). The design of these lipids was based on known and clinically used MRI contrast agents ($\text{Gd}(\text{DOTA})^-$ and $\text{Gd}(\text{DTPA})^{2-}$) combined with biologically available lipids (cholesterol and phospholipids). Mixtures of solution- and solid-phase chemistry techniques were employed to reach the final targets. In each case the key step was mono-functionalisation of the highly symmetrical polyamino acetate DTPA or DOTA moiety, producing the final compound in good yield. Phantom MRI analysis of the new gadolinium lipids revealed them to be extremely effective contrast agents, reducing the relaxation of surrounding solvents to 10 – 15% of their original value, comparable to clinically used agents.

Cationic liposomes are often utilised as vectors (gene carriers) for non-viral gene therapy and are extremely effective vehicles for mediating cell uptake *in vitro*. To enable efficient production of gadolinium-based cationic liposomes for molecular imaging, two new cationic lipids were synthesised (Chapter 3). Both lipids contained the novel un-natural 2-3-2 methylene spaced linear polyamine as a cationic head

group, but possessed different lipid tails (either cholesterol or saturated alkyl chains), which allowed for overall variation in the liposome constituents and properties.

Investigations into viable synthetic routes for both the gadolinium and cationic lipids led to the development of two novel synthetic solid phase methodologies; one for the selective functionalisation of cyclic polyamines and their polyacetic acid derivatives [146] (Chapter 2), and a second for the synthesis of cholesterol-based polyamine lipids [174] (Chapter 3). Polyamines and their derivatives are key components of many interesting compounds, and these molecules have proved to be synthetically challenging targets for which only a few synthetic routes exist (see Appendix, Section 9.4, Synthesis of Polyamines). These new methodologies permit rapid and clean functionalisation of appropriate polyamines, giving the desired compounds in excellent yield and purity.

Biological analysis of the gadolinium lipids and resultant liposomes focussed on two aims: (i) developing an *in vitro* gadolinium cell labelling system and (ii) studies towards *in vivo* tracking of liposomes (Chapter 4).

Effective cell labelling is a fundamental requirement for cell tracking investigations and the production of long term MRI active cells is a highly desirable aim. Combining the propensity of cationic liposomes for cell uptake, with the gadolinium lipids developed in Chapter 2 and the cationic lipids developed in Chapter 3, a gadolinium liposome cell labelling strategy was devised. Using a fluorescence-based liposome uptake assay an optimised gadolinium labelled cationic liposome was determined. This liposome formulation was found to confer minimal cytotoxicity upon cultured cells and MRI analysis of these cells after liposome uptake revealed them to be highly MRI active. Indeed, the T_1 relaxation value of the cells was reduced to 50% of their original value. Microscopy investigations, using a rhodamine tag for the liposomes, revealed that the gadolinium liposomes were being taken-up into the cells and were ubiquitously distributed over the cytoplasm, but did not enter the cell nucleus. Further experiments demonstrated that the gadolinium labelled cationic liposomes were also able to effectively transfect pDNA to cultured cells. Overall the success of these preliminary cell labelling studies demonstrates the new gadolinium lipids / liposomes to be highly effective tools for cell labelling and their subsequent *in vivo* tracking. Indeed, the efficacy of the gadolinium liposome is comparable to a peptide-based gadolinium delivery system very recently developed by Allen *et al* [80].

Further development of this system, would require determination of the efficacy of the gadolinium liposome to label other cell lines (for example stem cells). This may require adjustment of the ascertained liposome formulation to incorporate different lipid varieties, but the assays previously developed to assess liposome uptake and cytotoxicity would be key in these developments. Very recently, Mulder *et al* [58] developed an analogous liposomal cell labelling system using a *bis*-steroyl DTPA lipid. Comparison between their labelling system and the system developed in this work would act as a bench mark of efficacy for the further development of liposomes.

In vivo tracking of liposomes is a valuable tool for both drug development and monitoring liposome based therapies. To assure the viability of the new gadolinium lipids for *in vivo* studies, a solution containing a gadolinium lipid was administered into the hind leg of a mouse, and then subsequently imaged by MRI. The *in vivo* location of the gadolinium lipid was easily detectable and interestingly this effect persisted for over 56 hours. The results indicate that the gadolinium lipids are viable tools for future *in vivo* studies and may possess some interesting pharmacokinetic properties.

Building upon this preliminary data, a series of *in vivo* competent liposomes would need to be developed and tested to ascertain the viability of *in vivo* tracking of liposomes using MRI. Initial efficacy of the new liposomes could be assessed in an analogous manner with IM (intramuscular) injections and promising candidates could then be assessed post IV (intravenous) injection of liposomes. One of the most interesting applications of the new gadolinium lipids / liposomes would be built on the back of targeted liposomes. For effective imaging of molecular markers the liposomes must be targeted to a specific cell type or organ, and several research groups have demonstrated effective *in vivo* targeting of liposomes by further functionalising them with proteins, antibodies, and peptides. The combination of this technology with an effective gadolinium label will provide an exciting molecular imaging tool with the potential to image tumours, inflammation sites and specific regions in the brain.

The efficacy of gadolinium contrast agents is heavily dependent upon the rate of water exchange. In the previous gadolinium lipid / liposome system the gadolinium chelate may be partially buried at the liposome surface, which in turn restricts the water exchange and efficacy of the gadolinium chelate. In order to investigate this hypothesis a new gadolinium labelled liposome offering an alternative labelling strategy was devised. Borrowing from the principles of bioconjugate chemistry and techniques used

to functionalise liposomes, a strategy to post-coat liposomes with gadolinium chelates was developed.

Using the well established isothiocyanate - primary amine chemistry, two new target molecules were designed and synthesised, a cholesterol-based lipid displaying a primary amine projected away from the liposome surface and a isothiocyanate-functionalised Gd(DTPA)^{2-} compound. In order to allow the gadolinium chelate to protrude from the liposome membrane the activated cholesterol lipid incorporates a short PEG unit (eleven repeats) between the amine and cholesterol moieties. PEG has also been demonstrated to increase the surface hydration of the lipid bi-layer and would therefore potentially further optimise water exchange [85]. Post-coating of liposomes was successfully monitored by HPLC and ESI-MS. MRI analysis of the resulting gadolinium post-coated liposomes demonstrated that although they are highly effective contrast agents, the efficacy of these new liposomes was comparable to those devised using the original strategy. This approach provides an interesting and viable alternative to gadolinium labelling liposomes.

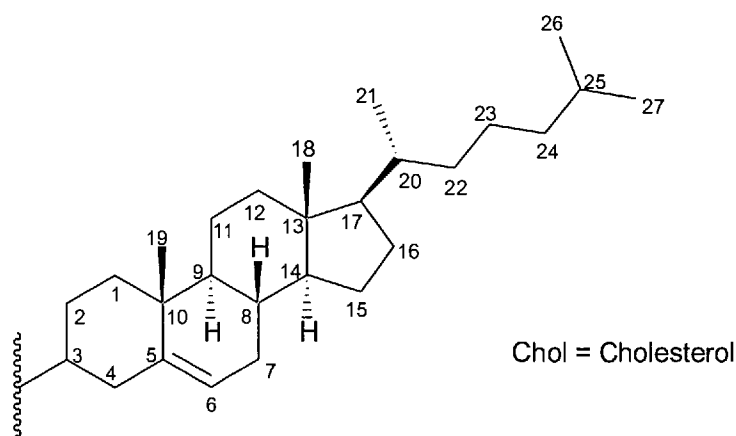
CHAPTER SEVEN

Experimental

7.1 General Information

All reactions were carried out in an atmosphere of nitrogen or argon, in oven-dried glassware. Silica gel column chromatography was performed with Merck silica gel 60 (230 – 240 mesh). TLC refers to thin layer chromatography performed on pre-coated Merck silica gel 60 F₂₅₄ aluminium backed plates and visualised with a UV lamp (254 and 366 nm) and / or stained with acidic ammonium molybdate (IV), basic potassium manganate (VII; KMnO₄), iodine, ninhydrin or Rhodamine B. CH₂Cl₂ was distilled over P₂O₅, and other solvents were bought in and pre-dried as required. All chemicals were purchased from Sigma-Aldrich, Lancaster, Merck Biosciences, Macrocyclics and Strem. ¹H and ¹³C NMR spectra were recorded on a Bruker Avance 400, using residual isotopic solvent (CDCl₃, δ_{H} = 7.27 ppm, δ_{C} = 77.0 ppm; CD₃OD, δ_{H} = 3.84 ppm, δ_{C} = 49.05 ppm; (CD₃)₂SO, δ_{H} = 2.50 ppm, δ_{C} = 39.43 ppm; D₂O, δ_{H} = 4.82 ppm, δ_{C} = 0.0 ppm (sodium trimethylsilanolate)) as internal reference. Data are reported as follows: (integration; br = broad, s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, sex = sextet, m = multiplet; coupling constant(s) in Hz; assignment). Mass spectra were performed using VG-070B, Joel SX-102 or Bruker Esquire 3000 ESI instruments. IRs were obtained on a JASCO FT/IR-620 intra-red spectrometer. UV spectroscopy was conducted on a Pharmacia Biotech UI-trospec 4000 spectrometer at defined wavelengths. Analytical HPLC was conducted on a Hitachi-LaChrom L-7150 pump system equipped with a Polymer laboratories PL-ELS 1000 evaporative light scattering detector. MRI experiments were conducted on a 4.7 T Magnex magnet using a varian unity Inova console.

The following numbering system has been employed for the assignment of cholesterol.



7.2 General Procedures

Solid Phase Chemistry: Reducing resin loading

Resin loading was altered to the desired value by appropriate addition MeOH in the presence of excess base (*i*Pr₂NEt) and CH₂Cl₂ to the resin. The solution was agitated at room temperature for 4 h. The solvents were removed by filtration and the resin was washed with CH₂Cl₂ (× 3).

Colorimetric Tests

(i) Kaiser test

To 1-5 mg of resin add 3 drops of each of the following solutions a) 80 g phenol in 20 mL EtOH; b) 2 mL KCN (0.001 M, aqueous) in 98 mL of pyridine and c) 5 g ninhydrin in 100 mL EtOH. Heat the resulting solution at 80 °C (in hot sand) for 2 minutes. Blue stained beads indicates a free primary amine, transparent colour indicates a protected amine.

NB. Ninhydrin should always be added last.

NB. Proline and secondary amines do not react with ninhydrin.

(ii) Chloranil test

To 1-5 mg of resin add one drop of 2 % acetaldehyde in DMF followed by one drop of 2 % *p*-chloranil in DMF. Allow to stand at room temperature for 5 min. Blue stained beads indicate presence of secondary amines.

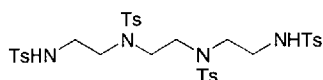
Xylenol Orange Test

The presence of free gadolinium in the gadolinium compounds was determined by measuring the A₅₇₃ of a mixture of xylenol orange solution (990 μL) (0.5 mM in sodium acetate buffer (0.1 M, pH 5.2)) and test solution (in an appropriate solvent) containing the gadolinium compound (10 μL). Extinction coefficient was $\epsilon = 20,700 \text{ L mol}^{-1} \text{ cm}^{-1}$.

$$[\text{Free Gd}] = A_{573} / \epsilon$$

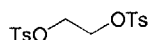
7.3 Synthesis of First Generation Gadolinium Lipids

*Tetra-*p*-tosyl triethylenetetraamine (Acyclic Tetratosylamide (18))*



Potassium Carbonate (14.6 g, 308 mmol) and triethylenetetraamine (10 g, 68.4 mmol) was dissolved in water (400 mL) at room temperature. The solution was heated to 80°C whilst stirring vigorously. *p*-toluenesulfonyl chloride (57.5 g, 302 mmol) was added to the solution in small portions over 3 hours (3.194 g every 10 minutes). The solution was then heated at 80°C and stirred vigorously for a further 12 hours. The reaction was removed from the heat and allowed to cool to room temperature. Upon cooling a colourless precipitate became evident. The precipitate was isolated by filtration. The solids were washed in turn with water (3 x 50 mL), methanol (3 x 50 mL), diethyl ether (3 x 50 mL) and dried under high vacuum, to yield the acyclic tetratosylamide (31.35 g, 59%) as a white solid (mp 210-212°C [lit., 211-212°C]); FTIR (nujol mull) $\nu_{\max}$ 3273, 2985, 2483, 1339, 1159 cm^{-1} ; ^1H NMR (d_6 -DMSO) δ 2.5 (12 H, s, 4 x CH_3), 2.75-2.9 (4 H, m, $(\text{Ts})\text{NCH}_2\text{CH}_2(\text{Ts})$), 3.0-3.1 (4 H, m, 2 x $\text{CH}_2\text{CH}_2\text{NHTs}$), 3.3-3.45 (4 H, m, CH_2NHTs), 7.32-7.72 (16 H, m, aromatic); ^{13}C NMR (d_6 -DMSO) δ 22.0 (4 x CH_3), 42.5 $((\text{Ts})\text{NCH}_2\text{CH}_2\text{N}(\text{Ts}))$, 48.0 (2 x $\text{CH}_2\text{CH}_2\text{NHTs}$), 49.0 (2 x CH_2NHTs), 127.0-131.0 (16 x aromatic CH), 136.0 (CH_3C on 2° amine), 137.5 (CH_3C on 3° amine), 143.0 ($-\text{SO}_2\text{C}$ on 2° amine), 144.0 ($-\text{SO}_2\text{C}$ on 3° amine); m/z (ESI +ve) 763 ($\text{M}+\text{H}$), 785 ($\text{M}+\text{Na}$); FAB-MS m/z calculated for $\text{C}_{34}\text{H}_{43}\text{N}_4\text{O}_8\text{S}_4$ ($\text{M}+\text{H}$) 763.1960, found 763.1964.

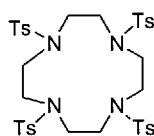
*Ethylene glycol di-*p*-toluenesulfonate (19)*



A mixture of anhydrous ethylene glycol (6.2 g, 0.1 mol) and dry pyridine (64 mL) was cooled to 0°C. *p*-toluenesulfonyl chloride (38 g, 0.2 mol) was added portionwise to the reaction over 2 hours. The reaction temperature was maintained at 0°C. The resulting slurry was stored at 4°C for 12 hours. The reaction mixture was poured into an ice water mixture (400 mL). Upon addition a colourless precipitate became evident. The precipitate was isolated by filtration with a sintered filter funnel (pore size 3), and washed with cold water (3 x 200 mL). The solids were recrystallised from ethanol and

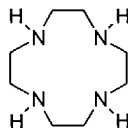
dried under high vacuum to render ethylene glycol di-*p*-toluenesulfonate (26.32 g, 71%) as a white solid (mp 127-129°C [lit. 128-130°C]); FTIR (nujol mull) $\nu_{\max}$ 2977, 2877, 2828, 2284, 1592, 1467, 1378, 1296, 1173, 1038 cm^{-1} ; ^1H NMR (CDCl_3) δ 2.45 (s, 6 H, 2 x CH_3), 4.20 (s, 4 H, CH_2CH_2), 7.30-7.40 (m, 4 H, aromatic protons), 7.7-7.8 (m, 4 H, aromatic protons); ^{13}C NMR (CDCl_3) δ 22.0 (2 x CH_3), 67.5 (CH_2CH_2), 128.0 (2 x $\text{CHC}(\text{CH}_3)$), 131.0 (2 x $\text{CHC}(\text{SO}_2)$), 133.0 (CCH_3), 146.0 (CSO_2). m/z (ESI +ve) 371 (M+H); FAB-MS m/z for $\text{C}_{16}\text{H}_{19}\text{O}_6\text{S}_2$ (M+H) calculated 371.0615, found 371.0623.

1,4,7,10-Tetra-*p*-tosyl-1,4,7,10-tetraazacyclododecane (Cyclic Tetratosylamide (15**))**



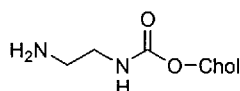
Potassium carbonate (16.7 g, 121 mmol) and acyclic tetratosylamine **18** (44.4 g, 58.2 mmol) was dissolved in dry DMF (700 mL). The resulting solution was vigorously stirred at room temperature under nitrogen. A solution of ethylene glycol di-*p*-toluenesulfonate **19** (21.8 g, 59.3 mmol) in dry DMF (100 mL) was added to the reaction mixture slowly over 6 hours. The resulting solution was vigorously stirred at room temperature for a further 12 hours. The reaction mixture was then heated to 70°C for 3 hours. The DMF was removed *in vacuo* (bath temperature 70°C). The residue was taken up in DCM (1000 mL) and water (1000 mL). Insoluble solids were removed by filtration over celite. The organic layer was separated and the aqueous layers washed with DCM (3 x 250 mL). The combined organic extracts were dried with K_2CO_3 . The solvent was removed *in vacuo* to render a brown/orange solid. The resulting solid was taken up in hot toluene (1000 mL). Insoluble solids were removed by filtration using a sintered funnel (pore size 3). The resulting clear orange solution was concentrated *in vacuo* to ~500 mL and allowed to cool to room temperature slowly. A white precipitate was deposited. The solid was collected by filtration, washed with cold toluene (2 x 150 mL) and dried under high vacuum to render the cyclic tetratosylamide (26.00 g, 57%) as a white solid (mp >260°C [lit. >260°C]); FTIR (nujol mull) $\nu_{\max}$ 2979, 2828, 1467, 1335, 1160 cm^{-1} ; ^1H NMR (CDCl_3) δ 2.25 (12 H, s, 4 x CH_3), 3.10-3.37 (16 H, m, 8 x CH_2), 7.07-7.25 (8 H, m, aromatic), 7.42-7.62 (8 H, m, aromatic); ^{13}C NMR (CDCl_3) δ 23.0 (4 x CH_3), 55.0 (8 x CH_2), 128.0 (8 x $\text{CHC}(\text{CH}_3)$), 130.0 (8 x $\text{CHC}(\text{SO}_2)$), 134.0 (4 x CCH_3), 145.0 (CSO_2); m/z (ESI +ve) 789 (M^+); FAB-MS m/z for $\text{C}_{36}\text{H}_{45}\text{N}_4\text{O}_8\text{S}_4$ (M+H) calculated 789.2165, found 789.2120.

1, 4, 7, 10- Tetraaza-cyclododecane (Cyclen, 14)



Cyclic tetratosylamide **15** (15.6 g, 19.8 mmol) was dissolved in concentrated sulphuric acid (25 mL) and the resulting solution was heated to 110°C under nitrogen for 40 hours. The reaction was removed from the heat and cooled in an ice water bath. Water (20 mL) was added dropwise slowly. The pH of the solution was further raised to 13 by addition of KOH (aq). Ethanol (150 mL) was added to the solution. The solid salt present was removed by filtration and was further washed with ethanol (2 x 20 mL). The combined aqueous/alcohol residues were reduced *in vacuo* rendering a yellow solid residue. The residue was taken up in HCl (0.1 M, 25 mL). DCM (30 mL) was added to the solution. The aqueous layer was separated and further extracted with DCM (2 x 30 mL). The pH of the combined aqueous layers was raised to 13 with KOH pellets. The aqueous layer was then extracted with chloroform (5 x 50 mL). The organic extracts were combined and dried (K₂CO₃). The solvents were removed *in vacuo* rendering 1, 4, 7, 10-Tetraaza-cyclododecane (1.7 g, 50%) as a colourless solid (mp 113-114°C [lit. 113-114°C]); FTIR (nujol mull) $\nu_{\max}$ 3273, 2984, 2811, 2237, 1913, 1690, 1478, 1426, 1381, 1384, 1248 cm⁻¹; ¹H NMR (CDCl₃) δ 1.55 (4 H, br, s, 4 x NH), 2.09 (16 H, br, s, 8 x CH₂); ¹³C NMR (CDCl₃) δ 46.0 (8 x CH₂); *m/z* (FAB +ve) 173 (M+H), 345 (2M+H); FAB-MS *m/z* for C₈H₂₁N₄ (M+H) calculated 173.1759, found 173.1766.

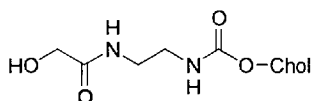
***N*¹-Cholesteryloxy-3-carbonyl-1,2-diaminoethane (Cholesterol amine, 12)**



Cholesterol chloroformate (10 g, 22.2 mmol) was dissolved in ethylene diamine (300 mL, 267 g, 445.4 mmol) and stirred at room temperature under argon. The progress of the reaction was monitored by TLC. Upon completion (~8 h) the reaction mixture was neutralised by slow addition of water (200 mL). CH₂Cl₂ (100 mL) was added and the layers separated. The organic layer was extracted with water (3 x 150 mL). The combined aqueous extracts were back extracted with CH₂Cl₂ (3 x 150 mL) and CHCl₃:

MeOH (2:1 (v/v)) (2 x 150 mL). The organic layers were combined, dried (MgSO₄) and concentrated *in vacuo*. The resulting solid was purified by silica gel column chromatography [CH₂Cl₂ : MeOH : NH₃ 192 : 7 : 1 → CH₂Cl₂ : MeOH : NH₃ 92 : 7 : 1 (v/v)] giving the title compound (6.82g, 65%) as a white solid (mp 175-177°C): FTIR (nujol mull) $\nu_{\max}$ 3338, 2977, 2830, 1692, 1552, 1468, 1247 cm⁻¹; ¹H NMR (CDCl₃) δ 0.66 (3 H, s, 18-CH₃), 0.84-0.85, 0.84-0.86 (2 x 3 H, d, *J* = 6.4 Hz, overlapping 1.6 Hz, 27-CH₃, 26-CH₃), 0.89 - 0.91 (3 H, d, *J* = 6.4 Hz, 21-CH₃), 0.92 (3 H, s, 19-CH₃), 1.02-1.63 (21 H, m, 1-CH₂, 9-CH, 11-CH₂, 12-CH₂, 14-CH, 15-CH₂, 16-CH₂, 17-CH, 20-CH, 22-CH₂, 23-CH₂, 24-CH₂, 25-CH), 1.76-2.1 (5 H, m, 2-CH₂, 7-CH₂, 8-CH), 2.22-2.36 (2 H, m, 4-CH₂), 2.79-2.81 (2 H, m, H₂NCH₂), 3.19-3.21 (2 H, m, H₂NCH₂CH₂), 4.52 (1 H, m, 3-CH), 5.31 (1 H, s, 6-CH); ¹³C NMR (CDCl₃) δ 11.8 (18-CH₃), 18.6 (21-CH₃), 19.3 (19-CH₃), 20.9 (11-CH₂), 22.5 (26-CH₃), 22.8 (27-CH₃), 23.7 (23-CH₂), 24.2 (15-CH₂), 27.9 (25-CH), 28.1 (2-CH₂), 28.2 (16-CH₂), 31.7 (8-CH), 31.8 (7-CH₂), 35.7 (20-CH), 36.1 (22-CH₂), 36.5 (10-C), 36.9 (1-CH₂), 38.5 (24-CH₂), 39.4 (4-CH₂), 39.6 (12-CH₂), 42.2 (13-C), 41.7 (H₂NCH₂CH₂), 43.6 (H₂NCH₂), 49.9 (9-CH), 56.0 (17-CH), 56.6 (14-CH), 74.2 (3-CH), 122.4 (6-CH), 139.7 (5-C), 156.4 (C=O); *m/z* (ESI +ve) 473 (M+H); FAB-MS *m/z* for calculated for C₃₀H₅₃N₂O₂ (M+H) 473.4119 found, 473.4107.

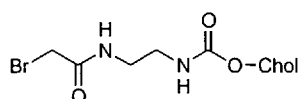
2-(2-Hydroxy-acetyl-amino)-ethyl-Cholesterol (**23**)



Cholesterol amine **12** (5.0 g, 10.6 mmol, 1 eq.) and glycolic acid (806 mg, 10.6 mmol, 1 eq.) were dissolved in dry CH₂Cl₂ (150 mL) under anhydrous conditions. To the solution was added DMAP (3.91 g, 31.8 mmol, 3 eq.) and HBTU (4.02 g, 10.6 mmol, 1 eq.). The resulting reaction mixture was stirred at room temperature under argon until TLC indicated completion (~12 hrs). Water (150 mL) was added to the reaction mixture and the layers separated. The organic layer was extracted with water (3 x 100 mL), the combined aqueous layers were back extracted with CH₂Cl₂ (3 x 100 mL) and 2 CHCl₃ : 1 MeOH (2 x 10 0mL). The organic layers were combined, dried (MgSO₄) and concentrated *in vacuo*. The resulting oil was purified by silica gel column chromatography [CH₂Cl₂ : MeOH : NH₃ 192 : 7 : 1 → CH₂Cl₂ : MeOH : NH₃ 92 : 7 : 1 (v/v)] rendering the desired alcohol (2.3 g, 41%) as a pale solid; FTIR (nujol mull) $\nu_{\max}$ 3373 (alcohol), 2948 (alkyl), 1641 (amide C=O) cm⁻¹; ¹H NMR (CDCl₃) δ 0.66 (3 H, s, H-18), 0.838-0.854 (3 H, d, H-27 (*J* = 6.4 Hz)), 0.842-0.858 (3 H, d, H-26 (*J* = 6.4 Hz)),

0.890-0.906 (3 H, d, H-21 ($J = 6.4$ Hz)), 0.922 (3 H, s, H-19), 1.02-1.63 (21 H, m, H-1, H-9, H-11, H-12, H-14, H-15, H-16, H-17, H-20, H-22, H-23, H-24, H-25), 1.76-2.1 (5 H, m, H-2, H-7, H-8), 2.22-2.36 (2 H, m, H-4), 3.2-3.32 (2 H, m, HOCH₂CONHCH₂CH₂NH), 3.35-3.51 (2 H, m, HOCH₂CONHCH₂CH₂NH), 4.05 (2 H, s, HOCH₂), 4.33-4.58 (1 H, m, H-3), 5.35 (1 H, s, H-6); ¹³C NMR (CDCl₃) δ 11.80 (C-18), 18.64 (C-21), 19.26 (C-19), 20.96 (C-11), 22.49 (C-26), 22.75 (C-27), 23.83 (C-23), 24.20 (C-15), 27.94 (C-25), 28.09 (C-2), 28.16 (C-16), 31.82 (C-8), 31.81 (C-7), 35.72 (C-20), 36.09 (C-22) 36.46 (C-10), 36.91 (C-1), 38.50 (C-24), 39.43 (C-4), 39.64 (C-12), 42.2 (C-13), 40.58 (OHCH₂CONHCH₂CH₂NH), 49.91 (C-9), 56.12 (C-17), 56.59 (C-14), 62.05 (HOCH₂), 74.7 (C-3), 122.58 (C-6), 156.97 (COO-Chol), 173.36 (HOCH₂CO); MS (ESI +ve) 530 (M⁺).

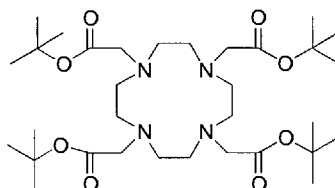
2-(2-Bromo-acetylamino)-ethyl-Cholesterol (**21**)



2-(2-Hydroxy-acetylamino)-ethyl-Cholesterol **23** (150 mg, 0.282 mmol, 1 eq.) was dissolved in dry CH₂Cl₂ (15 mL). Carbon tetrabromide (187 mg, 0.565 mmol, 2 eq.) and solid phase triphenylphosphine (1 g, 1 mmol, 4 eq. (Resin Loading: 1 mmolg⁻¹) were added to the solution. The reaction was marked by a vivid colour change from clear to deep red/brown. TLC was used to monitor the progress of the reaction. Upon completion (~15 minutes), the resin was removed by filtration and repeatedly washed with MeOH and CH₂Cl₂. The filtrate was concentrated *in vacuo* and the resulting oil was purified by silica gel column chromatography [CH₂Cl₂ : MeOH : NH₃ 392 : 7 : 1 → CH₂Cl₂ : MeOH : NH₃ 192 : 7 : 1] giving the title compound (130 mg, 78%) as a yellow oil: FTIR $\nu_{\max}$ 2935 (alkyl), 1644 (amide NH) cm⁻¹; ¹H NMR (CDCl₃) δ 0.66 (3 H, s, H-18), 0.838-0.854 (3 H, d, H-27 ($J = 6.4$ Hz)), 0.842-0.858 (3 H, d, H-26 ($J = 6.4$ Hz)), 0.890-0.906 (3 H, d, H-21 ($J = 6.4$ Hz)), 0.922 (3 H, s, H-19), 1.02-1.63 (21 H, m, H-1, H-9, H-11, H-12, H-14, H-15, H-16, H-17, H-20, H-22, H-23, H-24, H-25), 1.76-2.1 (5 H, m, H-2, H-7, H-8), 2.22-2.36 (2 H, m, H-4), 3.31-3.38 (2 H, m, BrCH₂CONHCH₂CH₂NH), 3.39-3.45 (2 H, m, BrCH₂CONHCH₂CH₂NH), 3.47 (2 H, s, BrCH₂), 4.33-4.58 (1 H, m, H-3), 5.35 (1 H, s, H-6); ¹³C NMR (CDCl₃) δ 11.80 (C-18), 18.64 (C-21), 19.26 (C-19), 20.96 (C-11), 22.49 (C-26), 22.75 (C-27), 23.83 (C-23), 24.20 (C-15), 27.94 (C-25), 28.09 (C-2), 28.16 (C-16), 31.82 (C-8), 31.81 (C-7), 35.72 (C-20), 36.09 (C-22) 36.46 (C-10), 36.91 (C-1), 38.50 (C-24), 39.43 (C-4), 39.64 (C-

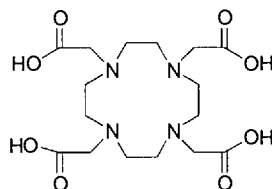
12), 42.2 (C-13), 40.131 (BrCH₂CONHCH₂CH₂NH), 41.131 (BrCH₂CONHCH₂CH₂NH), 49.91 (C-9), 53.39 (HOCH₂), 56.12 (C-17), 56.59 (C-14), 74.7 (C-3), 122.58 (C-6), 156.97 (COO-Chol), 166.47 (BrCH₂CO).

1,4,7,10-Tris-tert-butyloxycarbonylmethyl-1,4,7,10-tetraaza-cyclododec-1-yl)acetic acid tert-butyl ester (t-Butylester DOTA, 26)



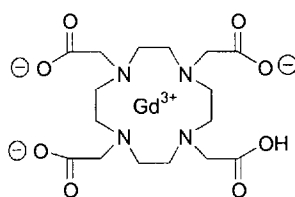
Cyclen **14** (100 mg, 0.58 mmol, 1 eq.) was dissolved in acetonitrile (10 mL). Anhydrous K₂CO₃ (802 mg, 5.8 mmol, 10 eq.) was added. The resulting suspension was stirred at room temperature under argon. *Tert*-butyl bromoacetate (453 mg, 374 μL, 2.32 mmol, 4 eq.) was dissolved in acetonitrile (0.5 mL) and added to the reaction mixture dropwise over 5 minutes. The reaction was stirred at room temperature under argon until TLC indicated completion (~90 minutes). K₂CO₃ was removed by filtration and the solvents from the reaction were concentrated *in vacuo*. To remove residual K₂CO₃, the resulting oil was redissolved in chloroform (10 mL), filtered and the filtrate was reduced *in vacuo* giving the title compound (357 mg, 98%) as light oily solid: FTIR (nujol mull) ν_{max} 3370, 2983, 2820, 1724, 1468, 1426, 1381, 1264, 1167 cm⁻¹; ¹H NMR (d₆-DMSO, 400 K) δ 1.45 (36 H, s, 4 x C(CH₃)₃), 2.58 (16 H, s, br, 4 x NCH₂CH₂N), 3.14 (8 H, s, br, 4 x CH₂COOC(CH₃)₃); ¹³C NMR (CDCl₃, 300 K) δ 27.6 (4 x C(CH₃)₃), 49.1 (br, 4 x NCH₂CH₂N), 51.9 (br, 4 x NCH₂CH₂N), 56.8 (4 x CH₂COOC(CH₃)₃), 81.7 (4 x C(CH₃)₃), 171.9 (4 x C=O); *m/z* (ESI +ve) 629 (M+H), 667 (M+K); FAB-MS *m/z* C₃₂H₆₀N₄O₈Na (M+Na) calculated for 651.4326, found 651.4301.

1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA, 11)



t-Butyl-DOTA **26** (100 mg, 0.158 mmol) was dissolved in dry dichloromethane (30 mL). Trifluoroacetic acid (10 mL) and triisopropylsilane (250 mg, 320 μ L, 1.58 mmol) were added to the solution. The reaction was stirred at room temperature under a positive flow of nitrogen for 5 hours. Solvents were removed *in vacuo* rendering a white solid, which was subsequently dissolved in CH_2Cl_2 (20 mL) and concentrated *in vacuo* repeatedly (x 10). The solid was dissolved in CH_2Cl_2 (15 mL). Addition of cold ether resulted precipitation of the desired compound, which was isolated by filtration, giving title compound (38 mg, 60%) as a white solid (mp 262-265°C [lit., 265-267°C]): FTIR (nujol mull) ν_{max} 3370, 2879, 2878, 2026, 1700, 1690, 1629, 1468, 1380, 1224 cm^{-1} ; ^1H NMR (D_2O , 340 K) 3.71 (16 H, s, 4 x $\text{NCH}_2\text{CH}_2\text{N}$), 4.18 (8 H, s, 4 x CH_2COOH); ^{13}C NMR (D_2O , 340 K) 50.7 (4 x $\text{NCH}_2\text{CH}_2\text{N}$), 55.9 (4 x CH_2COOH), 172.8 (4 x COOH); m/z (FAB +ve) 427 ($\text{M}+\text{Na}$); FAB-MS m/z for $\text{C}_{16}\text{H}_{29}\text{N}_4\text{O}_8$ ($\text{M}+\text{H}$) calculated 405.2001, found 405.1986.

Gadolinium (III) 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (Gd-DOTA, 25)

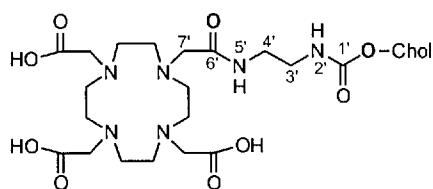


DOTA **11** (50 mg, 0.123 mmol) was dissolved in water (10 mL). Gadolinium oxide (22 mg, 0.0618 mmol, 0.5 eq. (1 eq. per Gd)) was added to the solution, forming a cloudy suspension. The reaction mixture was heated to 90°C for 12 hours, upon which the solution was clear. The xylenol orange test showed there was no free gadolinium in solution. The reaction was cooled to room temperature and to it were added cation-exchange resin (Amberlite IR 12/H⁺-form) and anion-exchange resin (Amberlite IRA 67/OH⁻-form). After stirring for 60 minutes at ambient temperature, the resin was collected by filtration through 0.22 μm filters. The filtrate was removed by freeze-drying

rendering the title compound (42 mg, 61%) as a white shiny powder (mp >260°C). The xylenol orange test showed that there was no free gadolinium present: FTIR (nujol mull) ν_{max} 3401, 2976, 2876, 2828, 1658, 1630, 1551, 1468, 1409 cm^{-1} ; m/z (ESI +ve) 558 (M⁺), 279 (2M⁺); HPLC analysis t_R = 3.5 min (Mobile phases: (a) Mix A (CHCl_3 :MeOH:H₂O:NH₃ [300:170:27.7:2.5]), (b) Mix B (CHCl_3 :MeOH:NH₃) [400:95:2.5], (c) MeOH; Gradient: Mix A / Mix B / MeOH, 0 min [80/20/0], 5 min [80/20/0], 12 min [100/0/0], 20 min [100/0/0], 30 min [90/0/10], 35 min [90/0/10], 38 min [80/20/0], 50 min [80/20/0]).

NB. Mass spectrum shows the complex with all abundant isomers of gadolinium bound.

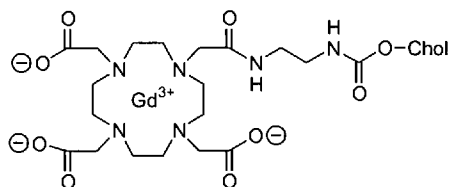
DOTA.Chol (**27**)



DOTA-NHS-ester (75 mg, 0.15 mmol) was dissolved in dry chloroform (40 mL). Cholesterol amine **12** (70.7 mg, 0.15 mmol) and triethylamine (415 μL , 302 mg, 2.99 mmol) were added to the solution. The reaction was stirred at room temperature under argon until TLC indicated completion (~3 h). The solvents were removed in vacuo and the resulting residue purified by silica gel column chromatography [CH_2Cl_2 : MeOH : NH₃ 92 : 17 : 1 $\rightarrow$ CH_2Cl_2 : MeOH : NH₃ 75 : 32 : 3], giving the title compound (97 mg, 76%) as a pale yellow solid. FTIR (nujol) ν_{max} 3139, 2919, 2725, 1708, 1589, 1459, 1405, 1376, 1154, 1092, 1013 cm^{-1} ; ^1H NMR (MeOD) 0.71 (3 H, s, 18-CH₃), 0.86 - 0.87, 0.86 - 0.88 (2 x 3 H, d, J = 6.4 Hz, overlapping 1.2 Hz, 27-CH₃, 26-CH₃), 0.93 - 0.94 (3 H, d, J = 6.4 Hz, 21-CH₃), 1.02 (3 H, s, 19-CH₃), 1.04-1.64 (21 H, m, 1-CH₂, 9-CH, 11-CH₂, 12-CH₂, 14-CH, 15-CH₂, 16-CH₂, 17-CH, 20-CH, 22-CH₂, 23-CH₂, 24-CH₂, 25-CH), 1.81 - 2.09 (5 H, m, 2-CH₂, 7-CH₂, 8-CH), 2.27-2.34 (2 H, m, 4-CH₂), 2.50 - 3.10 (22 H, m, 3'-CH₂, 4'-CH₂, 7'-CH₂, 4 x NCH₂CH₂N), 3.10 - 3.56 (8 H, m, 3 x CH₂COOH), 4.34 - 4.39 (1 H, m, 3-CH), 5.33 - 5.39 (1 H, m, 6-CH); ^{13}C NMR (MeOD) 12.4 (18-CH₃), 19.3 (21-CH₃), 19.8 (19-CH₃), 22.2 (11-CH₂), 23.0 (27-CH₃), 23.2 (26-CH₃), 25.0 (23-CH₂), 25.4 (15-CH₂), 26.4 (16-CH₂), 29.2 (25-CH), 29.4 (2-CH₂), 33.1 (8-CH), 33.3 (7-CH₂), 37.1 (20-CH), 37.4 (22-CH₂), 37.8 (10-C), 38.3 (1-CH₂), 39.8 (24-CH₂), 40.6 (4'-CH₂), 40.7 (4-CH₂), 41.1 (3'-CH₂), 41.2 (12-CH₂), 43.5 (13-C), 51.7 (9-CH), 54.22 (br, NCH₂CH₂N x 4), 57.6 (17-CH), 58.1 (7'-CH₂), 58.2 (14-CH), 60.2 (2 x CH₂COOH), 60.4 (1 x CH₂COOH), 75.7 (3-CH), 123.5 (6-CH), 141.3 (5-C), 158.8 (1'-

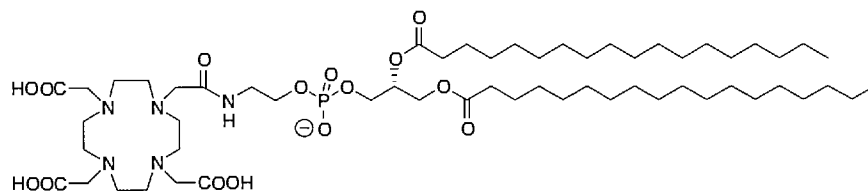
CO), 175.1 (6'-CO), 179.8 ($2 \times \text{CH}_2\text{COOH}$), 179.9 ($1 \times \text{CH}_2\text{COOH}$); m/z (FAB +ve) 859 (M+H); FAB-MS m/z for $\text{C}_{46}\text{H}_{78}\text{N}_6\text{O}_9\text{K}$ (M + K) calculated 897.5467, found 897.5487; HPLC analysis: t_R = 24.5 min, column Vydac C-4 peptide: gradient H_2O (0.1% TFA)/MeCN (0.1% TFA)/MeOH, 0 min [100/0/0], 1-15 min [0/100/0], 25 min [0/100/0], 25.1 min [0/0/100], 45 min [0/0/100], 45.1 min [100/0/0], 55 min [100/0/0]; flow 1 mL/min.

Gd.DOTA.Chol (6)



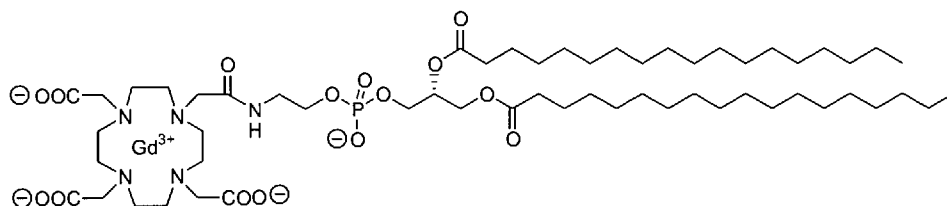
DOTA-Cholesterol **27** (50 mg, 0.058 mmol) was dissolved in water (10 mL). Gadolinium oxide (8 mg, 0.029 mmol (1 eq. per Gd)) was added to the solution, forming a cloudy suspension. The reaction mixture was heated to 90°C for 12 hours. The solution was cooled to room temperature and to it were added cation-exchange resin (Amberlite IR 12/ H^+ -form) and anion-exchange resin (Amberlite IRA 67/ OH^- form). After stirring for 60 minutes at ambient temperature, the resin was collected by filtration through 0.22 μm filters. The filtrate was removed by freeze-drying rendering the title compound (35 mg, 58%) as a pale yellow solid. The xylenol orange test showed an absence of free gadolinium ions. IR (nujol) ν_{max} 3137, 2918, 1599, 1458, 1403, 1376, 1319, 1244, 1156, 1084, 1002 cm^{-1} ; m/z (ESI +ve) 1014 (M^+); HPLC analysis: t_R = 25.5 min, column Vydac C-4 peptide: gradient H_2O (0.1% TFA)/MeCN (0.1% TFA)/MeOH, 0 min [100/0/0], 1-15 min [0/100/0], 25 min [0/100/0], 25.1 min [0/0/100], 45 min [0/0/100], 45.1 min [100/0/0], 55 min [100/0/0]; flow 1 mL/min. NB. Mass spectrum shows the complex with all abundant isomers of gadolinium bound.

DOTA.DSPE (28)



DOTA-NHS-ester (300 mg, 0.59 mmol), DSPE (447 mg, 0.59 mmol), NEt_3 (906 mg, 1.25 mL, 8.9 mmol) were dissolved in anhydrous chloroform (100 mL). The resulting suspension was gently warmed and the reaction mixture became clear. The reaction was stirred at room temperature under argon for 12 h. TLC indicated the reaction had gone to completion. The solvents were removed *in vacuo* and crude product (DSPE and DOTA.DSPE) was obtained by precipitation with water. The precipitate was isolated and dissolved in CHCl_3 (20 mL). Benzaldehyde scavenger resin (5 equiv) and NEt_3 (10 equiv) were added to the solution, which was stirred at room temperature until TLC indicated all the free DSPE had been successfully scavenged. The filtrate was isolated and solvents removed *in vacuo*. The resulting oil was lyophilised to give DOTA.DSPE as a white powder (514 mg, 77%). FTIR (nujol) ν_{max} 3376, 2919, 2853, 2724, 1733, 1632, 1462, 1376, 1216, 1178, 1060 cm^{-1} ; ^1H NMR (CDCl_3 : MeOD (1:1 (v/v)), 320 K) δ 0.65 – 0.69 (6 H, m, 2 x CH_3), 1.00 – 1.22 (56 H, m, 28 x CH_2 alkyl chains), 1.34 – 1.44 (4 H, m, 2 x $\text{CH}_2\text{CH}_2\text{CO}_2$), 2.11 (4 H, q, 2 x $\text{CH}_2\text{CH}_2\text{CO}_2$, $J = 7.2$ Hz), 2.75 – 3.05 (16 H, m, 4 x $\text{NCH}_2\text{CH}_2\text{N}$), 3.13 (2H, m, $\text{NHCH}_2\text{CH}_2\text{O}$), 3.19 – 3.50 (8 H, m, 4 x CH_2COOH), 3.71 – 3.73 (2H, m, $\text{NHCH}_2\text{CH}_2\text{O}$), 3.90 – 3.99 (3 H, m, glycerol-C2- CH_2 and glycerol-C1- CH_a), 4.17 (1H, dd, $J = 9.6, 12.1$ Hz glycerol-C1- H_b), 4.95 – 5.05 (1 H, m, glycerol-C1- H); ^{13}C NMR (CDCl_3 : MeOD (1:1 (v/v)), 320 K) δ 13.4 (2 x CH_3), 22.2, 24.5, 25.0, 28.7, 28.9, 29.1, 29.2, 31.5 (28 x CH_2 , alkyl chains), 33.7 ($\text{COCH}_2(\text{CH}_2)_{15}\text{CH}_3$), 33.9 ($\text{COCH}_2(\text{CH}_2)_{15}\text{CH}_3$), 45.9 ($\text{CH}_2\text{CH}_2\text{OP}(\text{O})_2\text{O}$), 49.9 (br, $\text{NCH}_2\text{CH}_2\text{N} \times 2$), 50.7 (br, $\text{NCH}_2\text{CH}_2\text{N} \times 2$), 54.6 (CH_2CONH), 55.3 (2 x CH_2COOH), 56.1 (1 x CH_2COOH), 62.3 (m, $\text{CH}_2\text{CH}_2\text{OP}(\text{O})\text{O}$), 63.2 (m, $\text{CH}_2\text{OCO}(\text{CH}_2)_{16}\text{CH}_3$), 63.5 (br, $\text{OP}(\text{O})_2\text{OCH}_2$), 70.2 ($\text{CHOCO}(\text{CH}_2)_{16}\text{CH}_3$, d, $J_{\text{CP}} = 7$ Hz), 172.6 (CONH), 173.0 ($\text{CO}(\text{CH}_2)_{16}\text{CH}_3$), 173.4 ($\text{CO}(\text{CH}_2)_{16}\text{CH}_3$). m/z (FAB + ve) 1134 ($\text{M} + \text{H}^+$); HPLC analysis: $t_R = 23.5$ min, column: Kromasil 60-5SIL 25 cm x 4.6 mm, mobile phases: A) CHCl_3 80%: MeOH 19.5%: NH_4OH 0.5%, B) CHCl_3 60%: MeOH 34%: H_2O 5.5%, NH_4OH 0.5%, gradient: [A/B], 0 min [50/50], 14 min [0/100], 25 min [0/100], 30 min [50/50], 45 min [50/50], flow 1 mL/min.

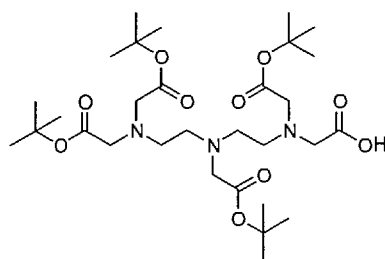
Gd.DOTA.DSPE (7)



DOTA.DSPE **28** (200 mg, 0.18 mmol) was suspended in anhydrous chloroform (30 mL). The suspension was gently warmed until the solution became clear. GdCl₃ (65 mg, 0.18 mmol) was dissolved in MeOH (15 mL) and the resulting solution was added to drop wise to the reaction mixture. NH₄OH (25 mg) was added drop wise to the solution and the reaction was left stirring at room temperature under argon (4 h). The NH₄Cl precipitate was removed by filtration and the filtrate was reduced *in vacuo*. The resulting solid was lyophilised to give the title compound as a white powder (210 mg, 88%). The xylenol orange test showed there to be no free gadolinium. FTIR (nujol) ν_{max} 3375, 2918, 1728, 1597, 1463, 1376, 1320, 1215, 1170, 1084, 1003 cm⁻¹; *m/z* (FAB + ve) 1289 (M + H⁺); HPLC analysis: *t_R* = 31.0 min, column: Kromasil 60-5SIL 25 cm × 4.6 mm, mobile phases: A) CHCl₃ 80%: MeOH 19.5%: NH₄OH 0.5%, B) CHCl₃ 60%: MeOH 34%: H₂O 5.5%, NH₄OH 0.5%, gradient [A/B], 0 min [50/50], 14 min [0/100], 25 min [0/100], 30 min [50/50], 45 min [50/50], flow 1 mL/min.

NB. Mass spectrum shows the complex with all abundant isomers of gadolinium bound.

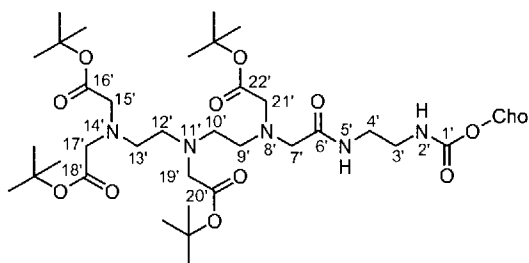
Selectively *t*-Butyl protected DTPA (**31**)



2-Chlorotrityl chloride resin (0.8 mmol.g⁻¹, 1.6 g, 1.28 mmol) was placed in a solid phase synthesis flask, and swelled by addition CH₂Cl₂. Bromoacetic acid (1.76 g, 12.8 mmol) and *i*Pr₂NEt (820 mg, 1.13 mL, 6.4 mmol) was added to the resin. The flask was then placed onto a motor shaker and shaken overnight at room temperature. MeOH (20.48 g, 25 mL, 640 mmol) was added as a capping reagent, and the reaction was left

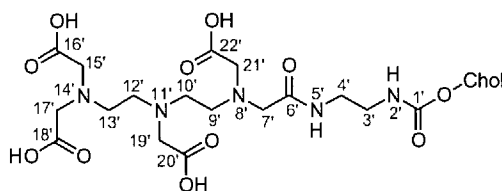
for 10 additional minutes; the solution was filtered and the resin was washed alternatively with CH_2Cl_2 and $^i\text{PrOH}$ ($\times 3$) and finally MeOH ($\times 2$) and then dried. Diethylenetriamine (1.32 g, 12.8 mmol) was dissolved in CH_2Cl_2 and added to the flask containing the bromoacetyl resin, and agitated for 4 h. The solvent was removed by filtration and the resin was washed by alternating between CH_2Cl_2 and $^i\text{PrOH}$ ($\times 3$), CH_2Cl_2 and MeOH ($\times 3$), distilled H_2O ($\times 2$) and finally CH_2Cl_2 and $^i\text{PrOH}$ ($\times 3$). The Kaiser test was positive. The resin was then washed with DMF ($\times 2$). *tert*-Butyl bromoacetate (5 g, 4.2 mL 25.6 mmol), NEt_3 (3 equiv. per free amine) were dissolved in DMF and added the flask containing the resin. The reaction was left shaking for a further 12 h. at room temperature. The solvent was removed by filtration and the resin was washed with $^i\text{PrOH}$ and distilled H_2O ($\times 3$), CH_2Cl_2 and $^i\text{PrOH}$ ($\times 3$), MeOH ($\times 2$), toluene ($\times 2$) and finally diethylether ($\times 2$). The kaiser test was negative. The resin was then transferred to a round-bottomed flask equipped with a magnetic stirrer. A solution of CH_2Cl_2 (2): Trifluoroethanol (1) (v/v) was added to the resin and the resulting solution was stirred at room temperature for 2 h. The resin was removed by filtration and washed with CH_2Cl_2 ($\times 3$). The filtrate was collected and reduced *in vacuo* to give the title compound as a yellow oil (550 mg, 68%). FTIR (film) ν_{max} 2980, 2908, 1725, 1455, 1394, 1370, 1246, 1156, 1094 cm^{-1} ; ^1H NMR (CDCl_3) δ 1.42-1.49 (36 H, m, $\text{C}(\text{CH}_3)_3 \times 4$), 2.84-3.03 (8 H, m, $\text{NCH}_2\text{CH}_2\text{NCH}_2\text{CH}_2\text{N}$), 3.44 (4 H, s, $\text{NCH}_2\text{COOtBu} \times 2$), 3.48 (2 H, s, $\text{NCH}_2\text{COOtBu}$), 3.51 (2 H, s, $\text{NCH}_2\text{COOtBu}$), 3.55 (2 H, s, NCH_2COOH), 8.22 (1 H, br, s, COOH); ^{13}C NMR (CDCl_3) δ 28.0 ($1 \times \text{C}(\text{CH}_3)_3$), 28.1 ($3 \times \text{C}(\text{CH}_3)_3$), 51.0 ($\text{NCH}_2\text{CH}_2\text{N}$), 51.1 ($\text{NCH}_2\text{CH}_2\text{N}$), 51.5 ($\text{NCH}_2\text{CH}_2\text{N}$), 52.1 ($\text{NCH}_2\text{CH}_2\text{N}$), 54.4 (CH_2COOtBu), 55.8 (CH_2COOtBu), 56.8 (CH_2COOtBu), 57.1 (CH_2COOH), 81.2 ($2 \times \text{C}(\text{CH}_3)_3$), 81.9 ($\text{C}(\text{CH}_3)_3$), 82.1 ($\text{C}(\text{CH}_3)_3$), 169.2 (COOtBu), 170.4 ($2 \times \text{COOtBu}$), 173.5 (COOtBu); m/z (ESI +ve) 618 ($\text{M} + \text{H}^+$), 640 ($\text{M} + \text{Na}^+$); FAB-MS m/z for $\text{C}_{30}\text{H}_{56}\text{N}_3\text{O}_{10}$ ($\text{M} + \text{H}^+$) calculated 618.3966, found 618.3983. HPLC analysis: t_R = 29.1 min, column Vydac C-4 peptide: gradient H_2O (0.1% TFA)/MeCN (0.1% TFA)/MeOH, 0 min [100/0], 1-20 min [0/100], 25 min [0/100], 25.1 min [100/0], 40 min [100/0]; flow 1 mL/min.

t-Butyl Protected DTPA.Chol (**30**)



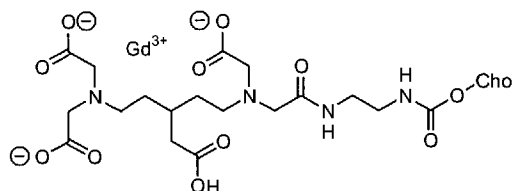
Selectively *t*-Butyl protected DTPA **31** (444 mg, 0.72 mmol) was dissolved in anhydrous chloroform (60 mL) and stirred at room temperature under argon. Cholesterol amine **12** (340 mg, 0.72 mmol), HBTU (272 mg, 0.72 mmol) and NEt₃ (218 mg, 299 μ L, 2.16 mmol) was added to the reaction. The solution became cloudy at first and then cleared overtime (~ 4 h). The reaction progress was monitored by TLC. When the reaction had gone to completion, the solvents were removed *in vacuo*. The resulting oil was purified by silica gel column chromatography (CH₂Cl₂ $\rightarrow$ 10% MeOH in CH₂Cl₂) to give the title compound as a yellow oil (556 mg, 72%). ¹H NMR (CDCl₃) 0.65 (3 H, s, 18-CH₃), 0.83-0.84, 0.83 – 0.85 (2 x 3 H, d, *J* = 6.8 Hz, overlapping 1.6 Hz, 27-CH₃, 26-CH₃), 0.88 - 0.90 (3 H, d, *J* = 6.4 Hz, 21-CH₃), 0.97 (3 H, s, 19-CH₃), 1.02-1.40 (21 H, m, 1-CH₂, 9-CH, 11-CH₂, 12-CH₂, 14-CH, 15-CH₂, 16-CH₂, 17-CH, 20-CH, 22-CH₂, 23-CH₂, 24-CH₂, 25-CH), 1.41 – 1.45 (36 H, s, br, C(CH₃)₃ x 4), 1.76-2.03 (5 H, m, 2-CH₂, 7-CH₂, 8-CH), 2.22-2.38 (2 H, m, 4-CH₂), 2.65 – 2.87 (8 H, m, 9'-CH₂, 10'-CH₂, 12'-CH₂, 13'-CH₂), 3.24 – 3.34 (6 H, m, 3'-CH₂, 4'-CH₂, 7'-CH₂), 3.36 – 3.47 (8 H, m, 15'-CH₂, 17'-CH₂, 19'-CH₂, 21'-CH₂), 4.42 – 4.52 (1 H, m, 3-CH), 5.33 – 5.34 (1 H, m, 6-CH); ¹³C NMR (CDCl₃) 11.8 (18-CH₃), 18.6 (21-CH₃), 19.2 (19-CH₃), 20.9 (11-CH₂), 22.5 (27-CH₃), 22.7 (26-CH₃), 23.7 (23-CH₂), 24.2 (15-CH₂), 27.9, 28.0 (2-CH₂, 25-CH, 16-CH₂, 4 x C(CH₃)₃, overlapping), 31.8 (7-CH₂, 8-CH overlapping), 35.7 (20-CH), 36.1 (22-CH₂), 36.5 (10-C), 36.9 (1-CH₂), 38.5 (24-CH₂), 39.4 (4-CH₂), 39.5 (4'-CH₂), 39.7 (12-CH₂), 41.3 (3'-CH₂), 42.2 (13-C), 49.9 (9-CH), 51.4 (13'-CH₂), 51.8 (12'-CH₂), 52.5 (10'-CH₂), 53.3 (9'-CH₂), 55.6 (19'-CH₂), 55.8 (15'-CH₂ and 17'-CH₂), 56.1 (17-CH), 56.6 (14-CH), 57.0 (21'-CH₂), 58.7 (7'-CH₂), 73.9 (3-CH), 80.94 (2 x C(CH₃)₃), 81.0 (1 x C(CH₃)₃), 81.5 (1 x C(CH₃)₃), 122.3 (6-CH), 139.8 (5-C), 156.3 (1'-CO), 170.5 (16'-CO and 18'-CO, overlapping), 170.7 (20'-CO and 22'-CO, overlapping), 172.5 (6'-CO); *m/z* (FAB +ve) 1073 (M + H⁺), 1095 (M + Na⁺); FAB-MS *m/z* for C₆₀H₁₀₆N₅O₁₁ (M + H⁺) calculated 1072.7889, found 1072.7885.

DTPA.Chol (**29**)



t-Butyl protected DTPA.Chol **30** (400 mg, 0.37 mmol) was dissolved in dioxane (10 mL). c.HCl (10 mL) was added to the solution and the resulting mixture was stirred at room temperature under a positive flow of nitrogen. When the reaction had gone to completion (~ 2 h) the solvents were removed *in vacuo* and the resulting solid was dissolved in water and freeze dried, to give the title compound as a white powder (298 mg, 95%). FTIR (nujol) $\nu_{\max}$ 3347, 2919, 2854, 2724, 1728, 1679, 1536, 1459, 1376, 1264, 1155, 1031 cm^{-1} ; ^1H NMR (MeOD) 0.71 (3 H, s, 18-CH₃), 0.86 - 0.88, 0.87 - 0.89 (2 x 3 H, d, J = 6.4 Hz, overlapping 1.2 Hz, 27-CH₃, 26-CH₃), 0.93 - 0.95 (3 H, d, J = 6.4 Hz, 21-CH₃), 1.03 (3 H, s, 19-CH₃), 1.04-1.64 (21 H, m, 1-CH₂, 9-CH, 11-CH₂, 12-CH₂, 14-CH, 15-CH₂, 16-CH₂, 17-CH, 20-CH, 22-CH₂, 23-CH₂, 24-CH₂, 25-CH), 1.80-2.10 (5 H, m, 2-CH₂, 7-CH₂, 8-CH), 2.23-2.37 (2 H, m, 4-CH₂), 3.40 - 4.22 (14 H, m, 3'-CH₂, 4'-CH₂, 7'-CH₂, 9'-CH₂, 10'-CH₂, 12'-CH₂, 13'-CH₂), 4.22 - 4.34 (8 H, m, 15'-CH₂, 17'-CH₂, 19'-CH₂, 21'-CH₂), 4.34 - 4.43 (1 H, m, 3-CH), 5.35 - 5.40 (1 H, m, 6-CH); ^{13}C NMR (MeOD) 12.4 (18-CH₃), 19.3 (21-CH₃), 19.8 (19-CH₃), 22.2 (11-CH₂), 23.0 (27-CH₃), 23.2 (26-CH₃), 25.0 (23-CH₂), 25.4 (15-CH₂), 29.2 (25-CH) 29.3 (2-CH₂) 29.4 (16-CH₂), 33.1 (8-CH) 33.3 (7-CH₂), 37.2 (20-CH), 37.4 (22-CH₂), 37.8 (10-C), 38.3 (1-CH₂), 39.7 (24-CH₂), 40.7 (4-CH₂ and 4'-CH₂ overlapping), 41.1 (3'-CH₂), 41.2 (12-CH₂), 43.5 (13-C), 50.8 (13'-CH₂), 51.4 (12'-CH₂), 51.7 (9-CH), 54.4 (10'-CH₂), 54.6 (9'-CH₂), 54.9 (21'-CH₂) 55.9 (19'-CH₂), 56.3 (15'-CH₂ and 17'-CH₂ overlapping), 57.5 (7'-CH₂) 57.6 (17-CH), 58.2 (14-CH), 75.8 (3-CH), 122.5 (6-CH), 141.3 (5-C), 158.9 (1'-CO), 166.9 (22'-CO), 167.4 (20'-CO), 169.4 (16'-CO and 18'-CO overlapping), 173.5 (6'-CO); m/z (FAB - ve) 846 ($M - H$); HPLC analysis: t_R = 25.0 min, column Vydac C-4 peptide: gradient H₂O (0.1% TFA)/MeCN (0.1% TFA)/MeOH, 0 min [100/0/0], 1-15 min [0/100/0], 25 min [0/100/0], 25.1 min [0/0/100], 45 min [0/0/100], 45.1 min [100/0/0], 55 min [100/0/0]; flow 1 mL/min.

Gd.DTPA.Chol (**8**)



DTPA.Chol (90 mg, 0.11 mmol) was dissolved in MeOH (10 mL). GdCl_3 (39 mg, 0.11 mmol) was added to the solution and the reaction was stirred at room temperature for 24 h. The solvents were removed *in vacuo* and the resulting solid was lyophilised to give the title compound as a white powder (60 mg, 60%). The xylenol orange test showed there to be no free gadolinium. FTIR (nujol) ν_{max} 3345, 2918, 2724, 1633, 1538, 1459, 1376, 1264, 1153, 1092, 1031 cm^{-1} ; m/z (FAB - ve) 1001; HPLC analysis: t_R = 25.0 min, column Vydac C-4 peptide: gradient [H_2O (0.1% TFA)/MeCN (0.1% TFA)/MeOH], 0 min [100/0/0], 1-15 min [0/100/0], 25 min [0/100/0], 25.1 min [0/0/100], 45 min [0/0/100], 45.1 min [100/0/0], 55 min [100/0/0]; flow 1 mL/min.

NB. Mass spectrum shows the complex with all abundant isomers of gadolinium bound.

7.4 Synthesis of Selectively *N*-Functionalised Aza-Crowns

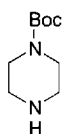
General Method

2-Chlorotrityl chloride resin (0.8 mmol.g^{-1}) was placed in a solid phase synthesis flask, and swelled by addition CH_2Cl_2 . Cyclic Polyamine (10 equiv.) was then added to the solution. The flask was then placed onto a motor shaker and shaken overnight at room temperature. MeOH (1000 equiv.) was added as a capping reagent, and the reaction was left for 10 additional minutes; the solution was filtered and the resin was washed alternatively with CH_2Cl_2 and $i\text{PrOH}$ ($\times 3$) and finally MeOH ($\times 2$) and then dried.

The resin was suspended in a solution of CH_2Cl_2 and NEt_3 (3 equiv. per free amine) and Boc_2O , CbzCl or FmocCl (5 equiv. per free amine) were added. The resulting solution was agitated at room temperature for 4 h. The solvent was removed by filtration and the resin was washed by alternating between CH_2Cl_2 and $i\text{PrOH}$ ($\times 3$), CH_2Cl_2 and MeOH ($\times 3$), distilled H_2O ($\times 2$) toluene ($\times 2$) and finally diethylether ($\times 2$). The chloranil test was negative. The resin was treated with 1 % TFA in CH_2Cl_2 for 90 sec ($\times 3$). The filtrate was collected and reduced *in vacuo* rendering the desired products.

^ξ Excess pyridine (250 equiv.) was used as a base for addition of Fmoc

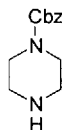
Boc-Piperazine (50)



Following general method for synthesis of selectively *N*-functionalised azacrowns (see section 7.4): 2-Chlorotrityl chloride resin (240 mg, 0.192 mmol), piperazine (165 mg, 1.92 mmol), MeOH (6.14 g, 7.7 mL, 192 mmol), NEt_3 (58 mg, 80 μL , 0.576 mmol), Boc_2O (209 mg, 0.96 mmol), gave the desired compound as a white solid (35.7 mg, 97%). FTIR (CDCl_3) ν_{max} 3431, 2982, 2757, 2253, 1676, 1422, 1203, 1155 cm^{-1} ; ^1H NMR (CDCl_3) δ 1.47 (9 H, s, $\text{C}(\text{CH}_3)_3$), 3.71 (4H, br, m, $\text{BocNCH}_2 \times 2$), 3.18 (4 H, br, m, $\text{HNCH}_2 \times 2$); ^{13}C NMR 28.2 ($\text{C}(\text{CH}_3)_3$), 10.5 ($\text{HNCH}_2 \times 2$), 43.2 ($\text{BocNCH}_2 \times 2$), 81.4 ($\text{C}(\text{CH}_3)_3$), 153.8 ($\text{COO}t\text{Bu}$); m/z (ESI +ve) 186 (M^+); FAB-MS m/z for $\text{C}_9\text{H}_{19}\text{N}_2\text{O}_2$ ($\text{M} + \text{H}^+$) calculated 187.1441, found 187.1446. HPLC analysis t_R = 12.0 min, column C-4

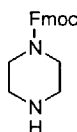
peptide: gradient H₂O/MeCN, 0 min [100/0], 1-15 min [0/100], 25 min [0/100], 25.1 min [100/0], 45 min [100/0]; flow 1 mL/min.

Cbz-Piperazine (51)



Following general method for synthesis of selectively *N*-functionalised azacrowns (see section 7.4): 2-Chlorotrityl chloride resin (230 mg, 0.184 mmol), piperazine (158 mg, 1.84 mmol), MeOH (5.88 g, 7.44 mL, 184 mmol), NEt₃ (55.7 mg, 77 μ L, 0.552 mmol), CbzCl (156.4 mg, 131 μ L, 0.92 mmol) diluted 1:1 (v/v) with CH₂Cl₂ and added drop wise to the reaction mixture, gave the desired compound as a yellow oil (39 mg, 96%). FTIR (CDCl₃) $\nu_{\max}$ 3422, 2997, 2749, 2253, 1794, 1677, 1431, 1281, 1255, 1203, 1141 cm⁻¹; ¹H NMR (CDCl₃) δ 3.11-3.22 (4 H, m, br, HNCH₂), 3.75-3.82 (4 H, t, BocNCH₂ $\times$ 2 (J = 4.8 Hz)), 5.12 (2 H, s, CH₂Ph), 7.31-7.41 (5 H, m, aromatic); ¹³C NMR (CDCl₃) δ 40.6 (HNCH₂ $\times$ 2), 43.3 (BocNCH₂ $\times$ 2), 68.1 (CH₂Ph), 127.8, 128.5, 128.6 (5 $\times$ C aromatic), 135.7 (aromatic C), 154.6 (COOCH₂Ph); m/z (ESI +ve) (M^+) 220; FAB-MS m/z for C₁₂H₁₇N₂O₂ ($M + H^+$) calculated 221.1289, found 221.1290. HPLC analysis t_R = 13.75 min, column C-4 peptide: gradient H₂O/MeCN, 0 min [100/0], 1-15 min [0/100], 25 min [0/100], 25.1 min [100/0], 45 min [100/0]; flow 1 mL/min.

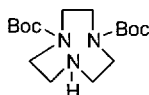
Fmoc-Piperazine (52)



Following general method for synthesis of selectively *N*-functionalised azacrowns (see section 7.4): 2-Chlorotrityl-chloride resin (230 mg, 0.184 mmol), piperazine (158 mg, 1.84 mmol), MeOH (5.88 g, 7.44 mL, 184 mmol), pyridine (3.63 g, 46 mmol), FmocCl (237 mg, 0.92 mmol), gave the desired compound as a white solid (53 mg, 94%). FTIR (CDCl₃) $\nu_{\max}$ 3408, 3154, 2983, 2253, 1793, 1676, 1469, 1431, 1380, 1252, 1202 cm⁻¹; ¹H NMR (CDCl₃) δ 2.82-3.13 (4H, m, br, HNCH₂ $\times$ 2), 3.44-3.78 (4H, m, br, FmocNCH₂ $\times$ 2), 4.63-4.65 (2H, d, (J = 5.6 Hz), COOCH₂), 4.21-4.26 (1H, t, (J = 5.6 Hz), COOCH₂CH), 7.31-7.46 (4H, m, aromatic), 7.52-7.75 (4H, m, aromatic); ¹³C NMR (CDCl₃) δ 40.6 (HNCH₂ $\times$ 2), 42.8 (FmocNCH₂ $\times$ 2), 47.4 (COOCH₂C), 67.3 (COOCH₂),

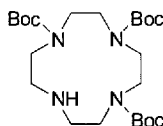
120.0, 124.7, 127.2, 127.9, 141.4, 143.5 (12 $\underline{\text{C}}$ aromatic), 154.4 ($\underline{\text{C}}\text{OOCH}_2$); m/z (FAB +ve) ($\text{M} + \text{H}^+$) 309; FAB-MS m/z for $\text{C}_{19}\text{H}_{21}\text{N}_2\text{O}_2$ ($\text{M} + \text{H}^+$) calculated 309.1621, found 309.1603. HPLC analysis t_R = 15.0 min, column C-4 peptide: gradient $\text{H}_2\text{O}/\text{MeCN}$, 0 min [100/0], 1-15 min [0/100], 25 min [0/100], 25.1 min [100/0], 45 min [100/0]; flow 1 mL/min.

Di-Boc 1,4,7-triazacyclonane (53)



Following general method for synthesis of selectively *N*-functionalised azacrowns (see section 7.4): 2-Chlorotrityl-chloride resin (225 mg, 0.180 mmol), 1,4,7-triazacyclonane (232 mg, 1.80 mmol), MeOH (5.76 g, 7.28 mL, 180 mmol), NEt_3 (109 mg, 150 μL , 1.08 mmol), Boc_2O (461 mg, 1.80 mmol), gave the desired compound as a yellow oil (53 mg, 88%). FTIR (CDCl_3) ν_{max} 3401, 3154, 2980, 2934, 2253, 1686, 1463, 1409, 1370, 1247, 1202 cm^{-1} ; ^1H NMR (CDCl_3) δ 1.48 (18 H, s, br, $\text{C}(\underline{\text{CH}}_3)_3 \times 2$), 3.33-3.41 (4 H, m, br, $\text{HNCH}_2 \times 2$), 3.42-3.54 (4 H, m, br, $\text{HNCH}_2\text{CH}_2 \times 2$), 3.57-3.70 (4 H, m, br, $\text{BocNCH}_2 \times 2$); ^{13}C NMR (CDCl_3) δ 28.2 (br, $\text{C}(\underline{\text{CH}}_3)_3 \times 2$), 46.7 ($\text{NHCH}_2 \times 2$), 49.2 (br, $\text{NHCH}_2\text{CH}_2 \times 2$), 50.3 (br, $\text{BocNCH}_2 \times 2$), 82.8 (br, $\underline{\text{C}}(\text{CH}_3)_3 \times 2$), 154.8 ($\underline{\text{C}}\text{OOtBu}$); m/z (ESI +ve) ($\text{M} + \text{H}^+$) 330; FAB-MS m/z for $\text{C}_{16}\text{H}_{32}\text{N}_3\text{O}_4$ ($\text{M} + \text{H}^+$) calculated 330.2390, found 330.2392. HPLC analysis t_R = 18.0 min, column C-4 peptide: gradient $\text{H}_2\text{O}/\text{MeCN}$, 0 min [100/0], 1-15 min [0/100], 25 min [0/100], 25.1 min [100/0], 45 min [100/0]; flow 1 mL/min.

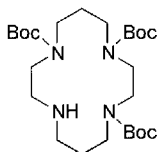
Tri-Boc Cyclen (54)



Following general method for synthesis of selectively *N*-functionalised azacrowns (see section 7.4): 2-Chlorotrityl-chloride resin (680 mg, 0.551 mmol), cyclen **14** (947 mg, 5.51 mmol) (861 mg of cyclen recovered), MeOH (17.63 g, 22.3 mL, 551 mmol), NEt_3 (501 mg, 632 μL , 4.96 mmol), Boc_2O (2.12 g, 8.27 mmol), gave the desired compound as a yellow oil (208 mg, 80%). FTIR (film) ν_{max} 3417, 2980, 2936, 2709, 2493, 1768, 1712, 1681, 1470, 1415, 1370, 1251, 1149 cm^{-1} . ^1H NMR (CDCl_3) 1.45 ($\text{C}(\underline{\text{CH}}_3)_3 \times 2$), 1.46 ($\text{C}(\underline{\text{CH}}_3)_3$), 3.25-3.34 (4 H, m, $\text{CH}_2 \times 2$), 3.39-3.46 (8 H, m, $\text{CH}_2 \times 4$), 3.54-3.59

(4H, m, CH₂ × 2). ¹³C NMR (CDCl₃) 28.1 (C(CH₃)₃), 28.2 (C(CH₃)₃ × 2), 46.8 (CH₂ × 2), 47.6 (CH₂ × 2), 50.7 (CH₂ × 2), 52.7 (CH₂ × 2), 82.0 (C(CH₃)₃), 82.2 (C(CH₃)₃ × 2), 156.8 (COOt-Bu × 2), 157.8 (COOt-Bu); *m/z* (ESI +ve) 473 (M+H); FAB-MS *m/z* for C₂₃H₄₅N₄O₆ (M + H⁺) calculated 473.3316 found 473.3339. HPLC analysis *t_R* = 24.0 min, column C-4 peptide: gradient H₂O/MeCN, 0 min [100/0], 1-15 min [0/100], 25 min [0/100], 25.1 min [100/0], 45 min [100/0]; flow 1 mL/min.

Tri-Boc Cyclam (55)



Following general method for synthesis of selectively *N*-functionalised azacrowns (see section 7.4): 2-Chlorotrityl-chloride resin (200 mg (0.4 mmol.g⁻¹), 0.08 mmol), cyclam (160 mg, 0.8 mmol) (152 mg of cyclam recovered), MeOH (2.56 g, 3.23 mL, 80 mmol), NEt₃ (72.7 mg, 92 μL, 0.72 mmol), Boc₂O (307 mg, 1.2 mmol), gave crude product (19 mg, ~92% purity), which was purified by a short silica gel chromatograph giving the desired compound as a yellow oil (15 mg, 69%). FTIR (CDCl₃) $\nu_{\max}$ 3420, 3154, 2980, 2929, 2855, 2253, 1793, 1680, 1468, 1418, 1369, 1201 cm⁻¹; ¹H NMR (CDCl₃) δ 1.43-1.49 (27 H, m, C(CH₃)₃ × 3), 1.60-1.86 (4 H, m, CH₂CH₂CH₂ × 2), 2.75-2.83 (2 H, m, br, HNCH₂), 2.92-3.07 (2 H, m, br, HNCH₂), 3.25-3.57 (12 H, m, CH₂ × 6); *m/z* (ESI +ve): *m/z* 501 (M+H). FAB-MS *m/z* for C₂₄H₄₉N₄O₆ (M + H⁺) calculated 501.3652 found 501.3629. HPLC analysis *t_R* = 20.5 min, column C-4 peptide: gradient H₂O/MeCN/MeOH, 0 min [100/0/0], 1-15 min [0/100/0], 25 min [0/100/0], 25.1 min [0/0/100], 45 min [0/0/100], 45.1 min [100/0/0], 60 min [100/0/0]; flow 1 mL/min.

7.5 Synthesis of Selectively Protected Polyamine Polyacetic Acid Derivatives

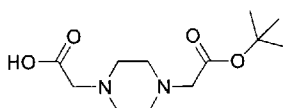
General Method

2-Chlorotrityl chloride resin (0.8 mmol.g⁻¹) was placed in a solid phase synthesis flask, and swelled by addition CH₂Cl₂. Bromoacetic acid (10 equiv.) and *i*Pr₂NEt (5 equiv.) was added to the resin. The flask was then placed onto a motor shaker and shaken overnight at room temperature. MeOH (1000 equiv.) was added as a capping reagent, and the reaction was left for 10 additional minutes; the solution was filtered and the

resin was washed alternatively with CH_2Cl_2 and ${}^i\text{PrOH}$ ($\times 3$) and finally MeOH ($\times 2$) and then dried.

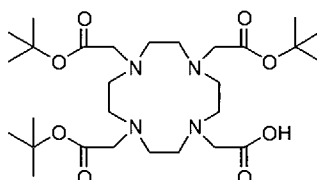
Azacrowns (10 equiv.) were dissolved in CH_2Cl_2 and added to the flask containing the bromoacetyl resin, and agitated for 4 h. The solvent was removed by filtration and the resin was washed by alternating between CH_2Cl_2 and ${}^i\text{PrOH}$ ($\times 3$), CH_2Cl_2 and MeOH ($\times 3$), distilled H_2O ($\times 2$) and finally CH_2Cl_2 and ${}^i\text{PrOH}$ ($\times 3$). The chloranil test was positive. The resin was then washed with DMF ($\times 2$). *tert*-Butyl bromoacetate or 2-benzylbromoacetate (5 equiv. per free amine), NEt_3 (3 equiv. per free amine) were dissolved in DMF and added the flask containing the resin. The reaction was left shaking for a further 12 h. at room temperature. The solvent was removed by filtration and the resin was washed with ${}^i\text{PrOH}$ and distilled H_2O ($\times 3$), CH_2Cl_2 and ${}^i\text{PrOH}$ ($\times 3$), MeOH ($\times 2$), toluene ($\times 2$) and finally diethylether ($\times 2$). The chloranil test was negative. The resin was then transferred to a round-bottomed flask equipped with a magnetic stirrer. A solution of CH_2Cl_2 (2): Trifluoroethanol (1) (v/v) was added to the resin and the resulting solution was stirred at room temperature for 2 h. The resin was removed by filtration and washed with CH_2Cl_2 ($\times 3$). The filtrate was collected and reduced *in vacuo* to give the desired products.

Selectively t-Butyl protected piperazine bis acetic acid (59)



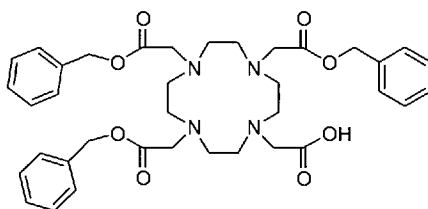
Following general method for synthesis of selectively protected polyamine polyacetic acid derivatives (see section 7.5): 2-Chlorotrityl chloride resin (200 mg, 0.16 mmol), bromoacetic acid (220 mg, 1.6 mmol), DIPEA (102 mg, 0.8 mmol), MeOH (5.12 g, 6.4 mL, 160 mmol), piperazine (137 mg, 1.6 mmol), *tert*-Butyl bromoacetate (156 mg, 0.8 mmol), NEt_3 (48 mg, 66 μL , 0.48 mmol), gave the title compound as a yellow oil (34 mg, 82%). FTIR (film) ν_{max} 3439, 2982, 2831, 2253, 1735, 1631, 1455, 1395, 1370, 1293, 1227, 1154 cm^{-1} . ${}^1\text{H}$ NMR (CDCl_3) δ 1.44 (9 H, s, $(\text{CH}_3)_3$), 2.87 (4 H, m, br, $\text{CH}_2 \times 2$), 3.16 (2 H, s, CH_2COOtBu), 3.39 (4 H, m, br, $\text{CH}_2 \times 2$), 3.49 (2 H, s, CH_2COOH). ${}^{13}\text{C}$ NMR δ 28.1 ($(\text{CH}_3)_3$), 50.0 ($\text{CH}_2 \times 4$), 52.5 (CH_2COOtBu), 58.9 (CH_2COOH), 81.6 ($\text{C}(\text{CH}_3)_3$), 168.7 (COOtBu), 169.5 (COOH). m/z (ESI -ve) 257 ($\text{M} - \text{H}$). FAB-MS m/z for $\text{C}_{12}\text{H}_{23}\text{N}_2\text{O}_4$ ($\text{M} + \text{H}^+$) calculated 259.1659, found 259.1662.

Selectively *t*-Butyl protected DOTA (**60**)



Following general method for synthesis of selectively protected polyamine polyacetic acid derivatives (see section 7.5): 2-Chlorotrityl chloride resin (400 mg, 0.32 mmol), bromoacetic acid (440 mg, 3.2 mmol), DIPEA (206 mg, 1.6 mmol), MeOH (10.24 g, 12.94 mL, 320 mmol), cyclen **14** (550 mg, 3.2 mmol), *tert*-Butyl bromoacetate (936 mg, 4.8 mmol), NEt₃ (291 mg, 400 μ L, 2.88 mmol), gave the title compound as a yellow oil (121 mg, 66%). FTIR (film) ν_{max} 3541, 3262, 3094, 2957, 2933, 2847, 1744, 1632, 1454, 1370, 1225, 1183 cm^{-1} . ¹H NMR (CDCl₃) δ 1.42 (27 H, s, C(CH₃)₃ $\times$ 3), 2.71-2.82 (4H, m, CH₂ $\times$ 2), 2.97-3.12 (8H, m, CH₂ $\times$ 4), 3.26-3.31 (4H, m, CH₂ $\times$ 2), 3.37 (2H, s, CH₂COOt-Bu), 3.36 (2H, s, br, CH₂COOH), 3.70 (4H, s, CH₂COOt-Bu $\times$ 2). ¹³C NMR (CDCl₃) δ 28.0 (C(CH₃)₃ $\times$ 3), 48.3 (CH₂ $\times$ 2), 50.1 (CH₂ $\times$ 2), 53.2 (CH₂ $\times$ 2), 53.3 (CH₂), 53.4 (CH₂), 55.6 (CH₂COOt-Bu), 55.9 (CH₂COOt-Bu $\times$ 2), 56.7 (CH₂COOH), 81.6 (C(CH₃)₃), 81.7 (C(CH₃)₃ $\times$ 2), 167.4 (COOt-Bu), 169.8 (COOt-Bu $\times$ 2), 170.5 (COOH); *m/z* (FAB +ve): *m/z* 573 (M + H⁺). FAB-MS *m/z* for C₂₈H₅₃N₄O₈ (M + H⁺) calculated 573.3863, found 573.3885. HPLC analysis *t*_R = 22.0 min, column C-4 peptide: gradient H₂O/MeCN, 0 min [100/0], 1-20 min [0/100], 25 min [0/100], 25.1 min [100/0], 40 min [100/0]; flow 1 mL/min.

Selectively protected benzyl DOTA (**61**)



Following general method for synthesis of selectively protected polyamine polyacetic acid derivatives (see section 7.5): 2-Chlorotrityl chloride resin (200 mg, 0.16 mmol), bromoacetic acid (220 mg, 1.6 mmol), DIPEA (102 mg, 0.8 mmol), MeOH (5.12 g, 6.4 mL, 160 mmol), cyclen **14** (275 mg, 1.6 mmol), 2-benzylbromoacetate (55mg, 38 μ L,

2.4 mmol), NEt₃ (24 mg, 33 μ L, 0.24 mmol), gave the title compound as a yellow oil (74 mg, 69%). FTIR (CDCl₃) $\nu_{\max}$ 3419, 2962, 2253, 1738, 1456, 1389, 1214 cm⁻¹; ¹H NMR (CDCl₃) 2.73-3.26 (16 H, m, br, NCH₂CH₂N $\times$ 4), 3.32-3.78 (8 H, m, br, CH₂COO $\times$ 4), 5.04 (2 H, s, CH₂Bz), 5.10 (4 H, s, CH₂Bz $\times$ 2), 7.22-7.39 (15 H, m, aromatic). ¹³C NMR (CDCl₃) 48.7 (CH₂ $\times$ 2), 49.2 (CH₂ $\times$ 2), 50.9 (CH₂ $\times$ 2), 52.3 (CH₂ $\times$ 2), 54.3 (CH₂COOBn), 55.6 (CH₂COOBn $\times$ 2), 60.3 (CH₂COOH), 66.7 (CH₂Bz $\times$ 2), 67.2 (CH₂Bz), 128.1, 128.3, 128.4, 128.5, 128.7, 134.9, 135.3 (aromatic). *m/z* (FAB +ve): 675 (M + H⁺); FAB-MS *m/z* for C₃₇H₄₇N₄O₈ (M + H⁺) calculated 675.3415, found 675.3394. HPLC analysis *t*_R = 21.0 min, column C-4 peptide: gradient H₂O/MeCN, 0 min [100/0], 1-15 min [0/100], 25 min [0/100], 25.1 min [100/0], 45 min [100/0]; flow 1 mL/min.

7.6 Synthesis of Selectively Protected DTPA and EDTA Derivatives

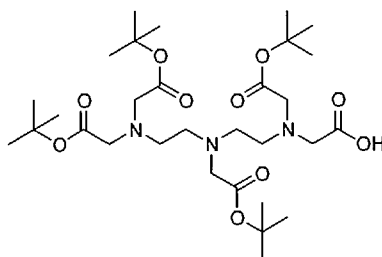
General Method

2-Chlorotriptyl chloride resin (0.8 mmol.g⁻¹) was placed in a solid phase synthesis flask, and swelled by addition CH₂Cl₂. Bromoacetic acid (10 equiv.) and *i*Pr₂NEt (5 equiv.) was added to the resin. The flask was then placed onto a motor shaker and shaken overnight at room temperature. MeOH (1000 equiv.) was added as a capping reagent, and the reaction was left for 10 additional minutes; the solution was filtered and the resin was washed alternatively with CH₂Cl₂ and ¹PrOH ($\times$ 3) and finally MeOH ($\times$ 2) and then dried.

Polyamine (10 equiv.) was dissolved in CH₂Cl₂ and added to the flask containing the bromoacetyl resin, and agitated for 4 h. The solvent was removed by filtration and the resin was washed by alternating between CH₂Cl₂ and ¹PrOH ($\times$ 3), CH₂Cl₂ and MeOH ($\times$ 3), distilled H₂O ($\times$ 2) and finally CH₂Cl₂ and ¹PrOH ($\times$ 3). The Kaiser test was positive. The resin was then washed with DMF ($\times$ 2). *tert*-Butyl bromoacetate or 2-benzylbromoacetate (5 equiv. per free amine), NEt₃ (3 equiv. per free amine) were dissolved in DMF and added the flask containing the resin. The reaction was left shaking for a further 12 h. at room temperature. The solvent was removed by filtration and the resin was washed with ¹PrOH and distilled H₂O ($\times$ 3), CH₂Cl₂ and ¹PrOH ($\times$ 3), MeOH ($\times$ 2), toluene ($\times$ 2) and finally diethylether ($\times$ 2). The kaiser test was negative. The resin was then transferred to a round-bottomed flask equipped with a magnetic

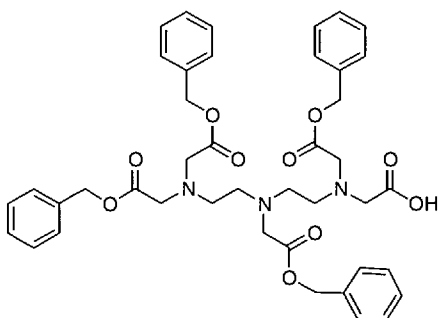
stirrer. A solution of CH₂Cl₂ (2): Trifluoroethanol (1) (v/v) was added to the resin and the resulting solution was stirred at room temperature for 2 h. The resin was removed by filtration and washed with CH₂Cl₂ (× 3). The filtrate was collected and reduced *in vacuo* to give the desired products.

Selectively *t*-Butyl protected DTPA (31)



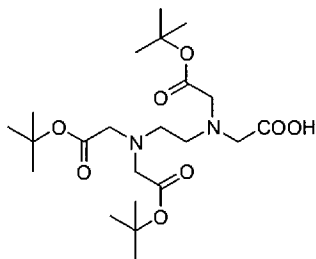
Following general method for synthesis of selectively protected DTPA and EDTA derivatives (see section 7.6): 2-chlorotriethyl chloride resin (800 mg, 0.64 mmol), bromoacetic acid (883 mg, 6.4 mmol), DIPEA (410 mg, 564 μ L, 3.2 mmol), MeOH (20.48 g, 25 mL, 640 mmol), diethylenetriamine (659 mg, 6.4 mmol), *tert*-Butyl bromoacetate (2.5 g, 2.07 mL 12.8 mmol), gave the title compound as a yellow oil (276 mg, 70%). FTIR (film) ν_{max} 2980, 2908, 1725, 1455, 1394, 1370, 1246, 1156, 1094 cm⁻¹; ¹H NMR (CDCl₃) δ 1.42-1.49 (36 H, m, C(CH₃)₃ × 4), 2.84-3.03 (8 H, m, NCH₂CH₂NCH₂CH₂N), 3.44 (4 H, s, NCH₂COOtBu × 2), 3.48 (2 H, s, NCH₂COOtBu), 3.51 (2 H, s, NCH₂COOtBu), 3.55 (2 H, s, NCH₂COOtBu), 8.22 (1 H, br, s, COOH); ¹³C NMR (CDCl₃) δ 28.0 (1 × C(CH₃)₃), 28.1 (3 × C(CH₃)₃), 51.0 (NCH₂CH₂N), 51.1 (NCH₂CH₂N), 51.5 (NCH₂CH₂N), 52.1 (NCH₂CH₂N), 54.4 (CH₂COOtBu), 55.8 (CH₂COOtBu), 56.8 (CH₂COOtBu), 57.1 (CH₂COOH), 81.2 (2 × C(CH₃)₃), 81.9 (C(CH₃)₃), 82.1 (C(CH₃)₃), 169.2 (COOtBu), 170.4 (2 × COOtBu), 173.5 (COOtBu); *m/z* (ESI +ve) 618 (M + H⁺), 640 (M + Na⁺); FAB-MS *m/z* for C₃₀H₅₆N₃O₁₀ (M + H⁺) calculated 618.3966, found 618.3983. HPLC analysis *t*_R = 29.1 min, column C-4 peptide: gradient H₂O/MeCN, 0 min [100/0], 1-20 min [0/100], 25 min [0/100], 25.1 min [100/0], 40 min [100/0]; flow 1 mL/min.

Selectively Benzyl protected DTPA (64)



Following general method for synthesis of selectively protected DTPA and EDTA derivatives (see section 7.6): 2-chlorotriptyl-chloride resin (100 mg, 0.08 mmol), bromoacetic acid (110 mg, 0.8 mmol), DIPEA (52 mg, 70 μ L, 0.4 mmol), MeOH (2.56 g, 3.2 mL, 80 mmol), diethylene triamine (82 mg, 0.8 mmol), 2-benzylbromoacetate (363 mg, 251 μ L, 1.6 mmol), gave the title compound as a yellow oil (45 mg, 74%). FTIR (film) ν_{max} 3341, 3033, 2955, 2249, 1740, 1497, 1455, 1190 cm^{-1} ; ^1H NMR (CDCl_3) δ 2.79-3.02 (8H, m, $\text{NCH}_2\text{CH}_2\text{N} \times 2$), 3.46-3.65 (8 H, m, $\text{CH}_2\text{COOBn} \times 4$), 3.77 (2 H, s, CH_2COOH), 5.03 (4 H, s, $\text{CH}_2\text{Bz} \times 2$), 5.06 (4 H, s, $\text{CH}_2\text{Bz} \times 2$), 7.21-7.39 (20 H, m, aromatic), 10.21 (1H, s, br, COOH); ^{13}C NMR (CDCl_3) δ 50.8 (CH_2), 50.9 (CH_2), 51.4 (CH_2), 51.7 (CH_2), 53.2 (CH_2COOBn), 54.6 ($\text{CH}_2\text{COOBn} \times 2$), 55.6 (CH_2COOBn), 56.9 (CH_2COOH), 66.2 ($\text{CH}_2\text{Bz} \times 2$), 66.8 ($\text{CH}_2\text{Bz} \times 2$), 127.9, 128.1, 128.3, 128.6, 128.7, 128.9, 134.8, 134.9, 135.2 (aromatic), 169.1 (COOBn), 170.0 (COOBn), 170.6 ($\text{COOBn} \times 2$), 173.5 (COOH). m/z (ESI +ve) 754 ($\text{M} + \text{H}^+$); FAB-MS m/z for $\text{C}_{42}\text{H}_{48}\text{N}_3\text{O}_{10}$ ($\text{M} + \text{H}^+$) calculated 754.3346, found 754.3340. HPLC analysis t_R = 29.25 min, column C-4 peptide: gradient $\text{H}_2\text{O}/\text{MeCN}$, 0 min [100/0], 1-20 min [0/100], 25 min [0/100], 25.1 min [100/0], 40 min [100/0]; flow 1 mL/min.

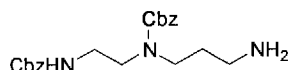
Selectively t-Butyl protected EDTA (64)



Following general method for synthesis of selectively protected DTPA and EDTA derivatives (see section 7.6): 2-chlorotrityl chloride resin (100 mg, 0.08 mmol), bromoacetic acid (110 mg, 0.8 mmol), DIPEA (52 mg, 70 μ L, 0.4 mmol), MeOH (2.56 g, 3.2 mL, 80 mmol), ethylene diamine (48 mg, 53 μ L, 0.8 mmol), *tert*-Butyl bromoacetate (234 mg, 194 μ L, 1.2 mmol), gave the title compound as a yellow oil (27 mg, 73%). ^1H NMR (CDCl_3) δ 1.47 (18 H, s, $\text{C}(\text{CH}_3)_3 \times 2$), 1.49 (9 H, s, $\text{C}(\text{CH}_3)_3$), 2.96-3.02 (2H, m, br, $\text{NCH}_2\text{CH}_2\text{N}$), 3.05-3.11 (2 H, m, br, $\text{NCH}_2\text{CH}_2\text{N}$), 3.54 (4 H, s, $\text{CH}_2\text{COO}^t\text{Bu} \times 2$), 3.67 (2 H, s, $\text{CH}_2\text{COO}^t\text{Bu}$), 3.73 (2 H, s, CH_2COOH); ^{13}C NMR (CDCl_3) δ 28.0 (1 $\times$ $\text{C}(\text{CH}_3)_3$), 28.1 (2 $\times$ $\text{C}(\text{CH}_3)_3$), 51.0 (CH_2CH_2), 52.8 (CH_2CH_2), 55.5 (2 $\times$ $\text{CH}_2\text{COO}^t\text{Bu}$), 56.6 ($\text{CH}_2\text{COO}^t\text{Bu}$), 56.8 (CH_2COOH), 82.1 (2 $\times$ $\text{C}(\text{CH}_3)_3$), 82.8 ($\text{C}(\text{CH}_3)_3$), 169.7 (2 $\times$ COO^tBu), 171.8 (COO^tBu). m/z (FAB +ve) 461 ($\text{M} + \text{H}^+$); FAB-MS m/z for $\text{C}_{22}\text{H}_{41}\text{N}_2\text{O}_8$ ($\text{M} + \text{H}^+$) calculated 461.2863, found 461.2873.

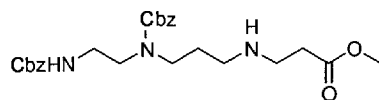
7.7 Synthesis of CDAN-DOGS

(3-Amino-propyl)-(2-benzyloxycarbonylamino-ethyl)-carbamic acid benzyl ester (**86**)



Trimethylphosphine in THF (1 M, 3 mL, 3 mmol) was added to a solution of (3-Azido-propyl)-(2-benzyloxycarbonylamino-ethyl)-carbamic acid benzyl ester (950 mg, 2.3 mmol) in THF (28 mL) under argon. The reaction was stirred at room temperature and monitored by TLC, until completion (~2.5 h). A solution of water (4.2 mL) and NH₃ (aq) (4.2 mL) was added to the reaction mixture. The reaction was stirred at room temperature for a further 1 hour. The solvents were removed *in vacuo* to render a yellow oil. This was purified by silica gel column chromatography [NH₃ : MeOH : CH₂Cl₂ 0.6 : 4.2 : 95.2 to 1 : 7 : 92 (v/v)]. Evaporation of the solvents afforded the desired product (721 mg, 83%) as a yellow oil; FTIR (film) ν_{max} 3355, 2931, 2093, 1651, 1524, 1455, 1247, 1139 cm⁻¹; ¹H NMR (CDCl₃) δ 1.45 - 1.51 (2 H, m, CH₂CH₂NH₂), 1.52 - 1.61 (2 H, m, CH₂CH₂NH₂), 2.65 - 2.45 (2 H, m, NH₂), 3.1 - 3.35 (6 H, m, CH₂CH₂N(Cbz)CH₂), 5.05 (2 H, s, PhCH₂O), 5.2 (2 H, s, PhCH₂O), 5.35 - 5.7 (1 H, m, NH amide), 7.10 - 7.35 (10 H, m, aromatic); ¹³C NMR (CDCl₃) δ 33.4 (CH₂CH₂CH₂), 39.6 (CH₂NH₂), 40.5 (NCH₂CH₂N), 42.1 (NCH₂CH₂CH₂), 46.1 (NCH₂CH₂N), 66.3 (PhCH₂O), 67.7 (PhCH₂O), 126.2-127.3 (10 x CH, aromatic), 137.6 (2 x C), 157.1 (2 x C=O); *m/z* (ESI +ve) 386 (M + H⁺), 342 (CbzHN(CH₂)₂N(Cbz)-); FAB-MS *m/z* calculated for C₂₁H₂₈N₃N₄ (M + H⁺) 386.2092, found 386.2079.

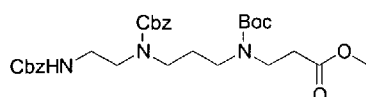
3-{3-[Benzyloxycarbonyl-(2-benzyloxycarbonylamino-ethyl)-amino]-propylamino}-propionic acid methyl ester (**87**)



(3-Amino-propyl)-(2-benzyloxycarbonylamino-ethyl)-carbamic acid benzyl ester **86** (144.6 mg, 0.38 mmol) was dissolved in methanol (7 mL) and stirred at room temperature under argon. Methylacrylate (37.2 μ L, 0.41 mmol) was added to the reaction and the resulting solution was stirred at room temperature. The progress of the reaction was monitored by TLC. When the reaction had gone to completion (~12 h) the solvent and excess methylacrylate were removed *in vacuo* to render a dark yellow oil.

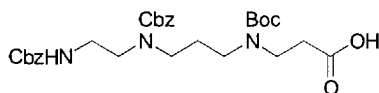
This was purified by silica gel column chromatography [MeOH : Ethylacetate : CH₂Cl₂ 2:3 : 15 to 4 : 3 : 13 (v/v)]. Evaporation of the solvents afforded the desired product (91.5 mg, 54%) as a yellow oil; ¹H NMR (CDCl₃) δ 1.7 - 1.85 (2 H, m, CH₂CH₂CH₂), 2.3-2.55 (4 H, m, CH₂NHCH₂), 2.65 - 2.8 (2 H, m, CH₂COOCH₃), 3.1 - 3.35 (6 H, m, CH₂CH₂N(Cbz)CH₂), 3.60 (3 H, s, OCH₃), 4.95 (1 H, s, PhCH₂O), 5.05 (1 H, s, PhCH₂O), 5.15-5.45 (1 H, m, NH amide), 7.15-7.32 (10 H, m, aromatic); ¹³C NMR (CDCl₃) δ 34.7 (CH₂CH₂CH₂), 40.3 (CH₂COOCH₃), 45.3 - 47.5 (CH₂CH₂N(Cbz)CH₂CH₂CH₂N(Cbz)CH₂), 52.0 (OCH₃), 67.0 (OCH₂Ph), 67.7 (OCH₂Ph), 128.9 - 128.3 (10 x CH, aromatic), 136.91 (2 x C), 156.89 (2 x COOCH₂Ph), 173.59 (COOMe); *m/z* (ESI +ve) 472 (M + H⁺).

3-({3-[Benzyloxycarbonyl-(2-benzyloxycarbonylamino-ethyl)-amino]-propyl}-tert butoxycarbonyl-amino)-propionic acid methyl ester (88)



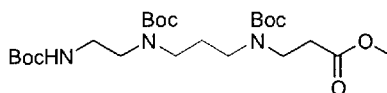
To a solution of 3-{3-[Benzyloxycarbonyl-(2-benzyloxycarbonylamino-ethyl)-amino]-propylamino}-propionic acid methyl ester **87** (150 mg, 0.32 mmol) and Boc₂O (69.5 mg, 0.32 mmol) in dry CH₂Cl₂ (80 mL) was added triethylamine (133 μL, 0.96 mmol). TLC monitored the progress of the reaction. When the reaction had reached completion (~12 h), the reaction mixture was poured onto saturated NaHCO₃ solution (20 mL). This was then poured into H₂O (50 mL). The organic layer was separated and the aqueous layer washed with CH₂Cl₂ (3 x 100 mL). The combined organic layers were dried (MgSO₄). The solvents were removed *in vacuo* to render a yellow oil. The oil was purified by silica gel column chromatography [Petroleum ether : Diethylether 8 : 2 to 2 : 8 (v/v)]. Evaporation of solvents afforded the desired product (45 mg, 25%) as a yellow oil; ¹H NMR (CDCl₃) δ 1.40 (9 H, s, (CH₃)₃), 1.53-1.72, (2 H, m, CH₂CH₂CH₂), 2.33 - 2.52 (2 H, m, CH₂COOCH₃), 2.91-2.52 (10 H, m, NHCH₂CH₂N(Cbz)CH₂CH₂CH₂N(Cbz)CH₂), 3.60 (3 H, s, OCH₃), 4.9 - 5.05 (2 H, m, 2 x PhCH₂O), 5.10 - 5.57 (1 H, m, NH amide), 7.2 - 7.35 (10 H, m, aromatic); ¹³C NMR (CDCl₃) δ 28.8 (C(CH₃)₃), 30.1 (CH₂CH₂CH₂), 34.1 (CH₂COOCH₃), 40.5 - 46.1 (CH₂CH₂N(Cbz)CH₂CH₂CH₂N(Cbz)CH₂), 52.1 (OCH₃), 66.9 (2 x OCH₂Ph), 67.7 (C(CH₃)₃), 128.3 - 128.9 (10 x CH, aromatic), 136.9 (2 x C, aromatic), 155.6 (NHCO), 157.0 (COBoc, COCbz), 172.0 (COOMe); *m/z* (ESI +ve) 572 (M + H⁺).

3-({3-[Benzyloxycarbonyl-(2-benzyloxycarbonylamino-ethyl)-amino]-propyl}-tert-butoxycarbonyl-amino)-propionic acid (**89**)



Methylester **88** (15 mg, 0.03 mmol) was dissolved in THF (3 mL) and LiOH solution 10% (w/v) (3 mL) was added. The resulting solution was stirred at 4°C until TLC indicated the reaction had gone to completion (~12 h). The reaction mixture was poured into 10% citric acid solution (w/v) (3 mL), and subsequently extracted with CH₂Cl₂ (3 x 10 mL) and 2 CHCl₃ : 1 MeOH (2 x 10 mL). The combined organic layers were back extracted with water (3 x 10 mL). The organic layers were combined, dried (MgSO₄) and concentrated *in vacuo*, rendering the title compound (13 mg, 91%) as a yellow oil; ¹H NMR (CDCl₃) δ 1.43 (9 H, s, C(CH₃)₃), 1.57 - 1.73 (2 H, m, CH₂CH₂CH₂), 2.4 - 2.62 (2 H, m, CH₂CO₂H), 3.01 - 3.52 (10 H, m, CH₂CH₂N(Cbz)CH₂CH₂CH₂N(Boc)CH₂), 4.93 - 5.17 (4 H, m, 2 x OCH₂Ph), 7.20 - 7.56 (10 H, m, aromatic); *m/z* (ESI +ve) 579 (M + Na⁺).

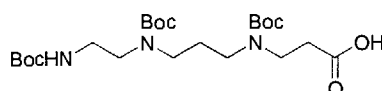
3-(tert-Butoxycarbonyl-{3-tert-butoxycarbonylamino-ethyl)-propyl}-amino)-propionic acid methyl ester (**90**)



Cbz/Boc polyamine **88** (113 mg, 0.198 mmol) was dissolved in MeOH (10 mL). Pd/C – 10% (22.6 mg, 20% eq. w/w) and Boc₂O (107.8 mg, 0.245 mmol) were added to the solution. The reaction mixture was stirred at room temperature under H₂ (g), until TLC indicated the reaction had gone to completion (~12 h). Pd/C –10% was removed by filtration over celite, and the filtrate was concentrated *in vacuo*. The resulting residue was dissolved in CH₂Cl₂ (40 mL) and extracted with water (3 x 40 mL). The combined aqueous extracts were back extracted with CH₂Cl₂ (3 x 20 mL) and 2 CHCl₃ : 1 MeOH (2 x 40 mL). The organic extracts were combined, dried (MgSO₄) and concentrated. The resulting oil was purified by silica gel column chromatography [Petroleum ether : Diethylether 1 : 1 → Petroleum ether : Diethylether 1 : 3], giving the title compound (91.5 mg, 92%) as a yellow oil: FTIR $\nu_{\max}$ 3358, 3024, 2975, 2925, 1739, 1695, 1597, 1518, 1479, 1452, 1416, 1390, 1366, 1249, 1166, 1071 cm⁻¹; ¹H NMR (CDCl₃) δ 1.23 -

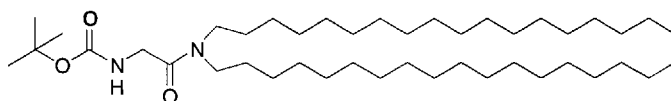
1.59 (27 H, m, Boc CH₃'s), 1.61 - 1.79 (2 H, m, CH₂CH₂CH₂), 2.43 - 2.59 (2 H, m, CH₂CO₂H), 3.02 - 3.30 (8 H, m, CH₂N(Boc)CH₂CH₂CH₂N(Boc)CH₂), 3.31 - 3.42 (2 H, m, BocNHCH₂), 3.60 (3 H, s, OCH₃); *m/z* (ESI +ve) 504 (M + H⁺).

3-(tert-Butoxycarbonyl-{3-tert-butoxycarbonylamino-ethyl)-propyl}-amino)-propionic acid (91)



Methylester **90** (93 mg, 0.185 mmol) was dissolved in THF (3 mL) and LiOH solution 10% (w/v) (3 mL) was added. The resulting solution was stirred at 4°C until TLC indicated the reaction had gone to completion (~12 h). The reaction mixture was poured into 10% citric acid solution (w/v) (30 mL), and subsequently extracted with CH₂Cl₂ (3 x 30 mL) and 2 CHCl₃ : 1 MeOH (2 x 30 mL). The combined organic layers were back extracted with water (3 x 30 mL). The organic layers were combined, dried (MgSO₄) and concentrated *in vacuo*, rendering the title compound (88 mg, 97%) as a yellow oil; ¹H NMR (CDCl₃) δ 1.31 - 1.57 (27 H, m, Boc CH₃), 1.63 - 1.75 (2 H, m, CH₂CH₂CH₂), 2.45-2.57 (2 H, m, CH₂CO₂H), 3.08-3.29 (8 H, m, CH₂N(Boc)CH₂CH₂CH₂N(Boc)CH₂), 3.39 - 3.41 (2 H, m, BocNHCH₂); ¹³C NMR (CDCl₃) δ 27.2 (9 C 3 x Boc C(CH₃)₃), 32.7 (CH₂CH₂CH₂), 38.2 (CH₂COOH), 46.5 - 42.7 (CH₂CH₂N(Boc)CH₂CH₂CH₂N(Boc)CH₂), 78.3 (2 x NCOOC(CH₃)₃), 78.7 (NCOOC(CH₃)₃), 154.2 (NCOOC(CH₃)₃), 154.9 (NCOOC(CH₃)₃), 155.3 (NCOOC(CH₃)₃), 174.2 (COOH); *m/z* (ESI +ve) 490 (M + H⁺).

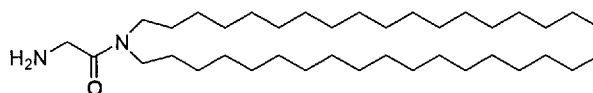
Diocadecylcarbamoylmethyl-carbamic acid tert-butyl ester (95)



Boc-glycine (307 mg, 1.75 mmol) and dioctadecylamine (915 mg, 1.75 mmol) was dissolved in dry chloroform (30 mL) under anhydrous conditions. HBTU (797.6 mg, 2.10 mmol) and DMAP (642.3 mg, 5.26 mmol) were added to the solution. The reaction was stirred at room temperature under argon until TLC indicated the reaction had gone to completion (~ 12 h). Solvents were removed *in vacuo*. The residue dissolved in CH₂Cl₂ (50 mL) and extracted with water (3 x 50 mL). The combined aqueous extracts

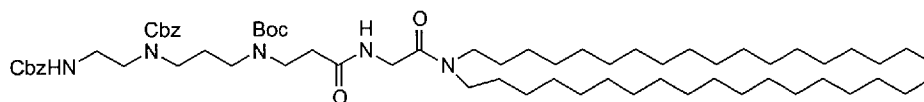
were back extracted with CH_2Cl_2 (3 x 50 mL) and 2 CHCl_3 : 1 MeOH (2 x 50 mL). The organic extracts were combined, dried (MgSO_4) and concentrated. The resulting yellow oil was purified by silica gel column chromatography [Petroleum → Petroleum : Diethylether 8 : 2 (v/v)], rendering the title compound (801 mg, 70%) as a yellow oil; FTIR (film) ν_{max} 3422, 2923, 2855, 2250, 1704, 1647, 1498, 1465, 1368, 1248, 1170, 1098 cm^{-1} ; ^1H NMR (CDCl_3) δ 0.78 - 0.91 (6 H, m, 2 x CH_3), 1.1 - 1.34 (60 H, m, alkylchain CH_2 's), 1.35 - 1.60 (13 H, m, $\text{C}(\text{CH}_3)_3$ and $\text{N}(\text{CH}_2\text{CH}_2\text{-alkylchain})_2$), 3.01 - 3.12 (2 H, m, NCH_2), 3.23 - 3.41 (2 H, m, NCH_2), 3.85 - 3.96 (2 H, m, NHCH_2CO), 5.50 - 5.59 (1 H, br, s, amide NH); ^{13}C NMR (CDCl_3) δ 14.5 (2 x CH_3), 23.1 ($\text{N}(\text{CH}_2\text{CH}_2\text{-alkylchain})_2$), 27.3 - 32.3 (30 CH_2 , alkylchain), 42.5 (NHCH_2CO), 46.5 ($\text{N}(\text{CH}_2\text{CH}_2\text{-alkylchain})_2$), 47.3 ($\text{N}(\text{CH}_2\text{CH}_2\text{-alkylchain})_2$), 79.8 ($\text{C}(\text{CH}_3)_3$), 156.2 ($\text{C}(\text{CH}_3)_3\text{COCO}$), 167.9 (CON-alkylchains); m/z (ESI +ve) 679 ($\text{M} + \text{H}^+$).

2-amino-N,N-dioctadecyl-acetamide (74)



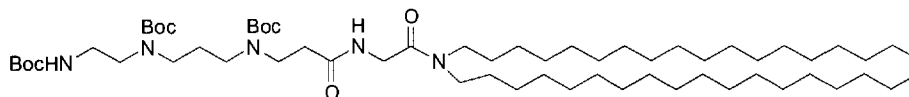
Boc-Amine **95** (260 mg, 0.38 mmol) was dissolved in dry CH_2Cl_2 (5 mL) under anhydrous conditions. Trifluoroacetic acid (3 mL) was added to the solution cautiously. The reaction was stirred at room temperature under a positive flow of nitrogen until TLC indicated the reaction had gone to completion (~2 h). The solvents were removed *in vacuo* rendering the desired compound (200 mg, 92%) as an off-white solid: FTIR (nujol mull) ν_{max} 2914, 1732, 1667, 1589, 1463, 1376, 1318, 1275, 1209, 1181, 1125 cm^{-1} ; ^1H NMR (CDCl_3) δ 0.85 - 0.92 (6 H, t, 2 x CH_3), 1.17 - 1.43 (60 H, m, 30 CH_2 's alkylchain), 1.45 - 1.60 (4 H, m, $\text{N}(\text{CH}_2\text{CH}_2\text{-alkylchain})_2$), 3.01 - 3.11 (2 H, m, $\text{NCH}_2\text{CH}_2\text{-alkylchain}$), 3.21 - 3.3 (2 H, m, $\text{N}(\text{CH}_2\text{CH}_2\text{-alkylchain})$), 3.85 - 3.95 (2 H, m, CH_2NH_2); ^{13}C NMR (CDCl_3) δ 14.5 (2 x CH_3), 23.1 (2 x CH_2CH_3), 27.1 - 32.4 (30 CH_2 , alkylchain), 40.5 (CH_2NH_2), 46.9 ($\text{N}(\text{CH}_2\text{CH}_2\text{-alkylchain})$), 47.6 ($\text{N}(\text{CH}_2\text{CH}_2\text{-alkylchain})$), 165.5 (C=O); m/z (FAB +ve) 579 (M^+); FAB MS calculated for $\text{C}_{38}\text{H}_{79}\text{N}_2\text{O}$ ($\text{M} + \text{H}^+$) 579.6194, found 579.6192.

2-[Benzyloxycarbonyl-(3{tert-butoxycarbonyl-[2-(dioctadecylcarbamoylmethylcarbamoyl)-ethyl]amino}-propyl)-amino]-ethyl]-carbamic acid benzyl ester (**94**)



Carboxylic acid **89** (20.8 mg, 0.0373 mmol) and amine **74** (21.59 mg, 0.037 mmol) was dissolved in dry chloroform (3 mL) under anhydrous conditions. DMAP (15.9 mg, 0.131 mmol) and HBTU (18.4 mg, 0.049 mmol) were added to the solution. The reaction was stirred at room temperature under argon and progress was monitored by TLC. After 24 hours the reaction had reached completion. The solvents were removed *in vacuo* and the remaining residue was dissolved in CH₂Cl₂ (10 mL) and extracted with water (3 x 10 mL). The combined aqueous extracts were back extracted with CH₂Cl₂ (3 x 10 mL) and 2 CHCl₃ : 1 MeOH (2 x 10 mL). The organic layers were combined, dried (MgSO₄) and concentrated. The resulting yellow oil was purified by silica gel column chromatography [Diethylether → Diethylether : ethyl acetate 9 : 1 (v/v)], affording the title compound (24 mg, 58%) as a yellow oil; ¹H NMR (CDCl₃) δ 0.79 - 0.83 (6 H, t, 2 x CH₃ (*J* = 6.6 Hz)), 1.14 - 1.31 (60 H, m, 30 x CH₂ alkylchains), 1.34 - 1.44 (13 H, m, Boc C(CH₃)₃ and N(CH₂CH₂-alkylchain)₂), 1.63 - 1.69 (2 H, m, CH₂CH₂CH₂), 2.33 - 2.47 (2 H, m, CH₂CH₂CONH), 3.01 - 3.51 (14 H, m, (Cbz)NHCH₂CH₂N(Cbz)CH₂CH₂CH₂N(Boc)CH₂), 3.91 - 3.99 (2 H, s, br, NHCH₂CON-alkylchains), 4.95 - 5.10 (4 H, m, 2 x OCH₂Ph), 7.29 - 7.42 (10 H, m, aromatic CH's); *m/z* (ESI +ve) 1118 (M + H⁺), 1140 (M + Na⁺).

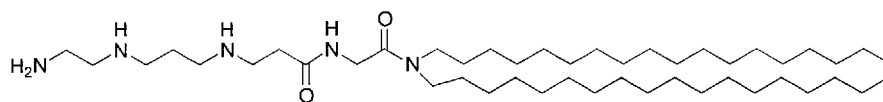
{2-[tert-Butoxycarbonyl-(3-{tert-butoxycarbonyl-[2-(dioctadecylcarbamoylmethylcarbamoyl)-ethyl]-amino}-propyl)-amino]-ethyl]-carbamic acid tert-butyl ester (**96**)



Carboxylic acid **91** (88 mg, 0.18 mmol) and amine **74** (104 mg, 0.18 mmol) was dissolved in dry chloroform (10 mL) under anhydrous conditions. DMAP (77 mg, 0.63 mmol) and HBTU (88.6 mg, 0.23 mmol) were added to the solution. The reaction was stirred at room temperature under argon and progress was monitored by TLC. After 24 hours the reaction had reached completion. The solvents were removed *in vacuo* and

the remaining residue was dissolved in CH₂Cl₂ (50 mL) and extracted with water (3 x 50 mL). The combined aqueous extracts were back extracted with CH₂Cl₂ (3 x 50 mL) and 2 CHCl₃ : 1 MeOH (2 x 50 mL). The organic layers were combined, dried (MgSO₄) and concentrated. The resulting yellow oil was purified by silica gel column chromatography [Petroleum ether : Diethylether 3 : 7 → Diethylether : ethyl acetate 9 : 1 (v/v)], affording the title compound (110 mg, 62%) as a yellow oil: FTIR (film) $\nu_{\max}$ 3398, 2923, 2854, 2251, 1771, 1684, 1642, 1508, 1467, 1392, 1367, 1249, 1164, 1072 cm⁻¹; ¹H NMR (CDCl₃) δ 0.79 - 0.83 (6 H, t, 2 x CH₃ (*J* = 6.7 Hz)), 1.14 - 1.33 (60 H, m, Alkylchain CH₂'s), 1.36 - 1.49 (31 H, m, Boc C(CH₃)₃'s and N(CH₂CH₂-alkyl chain)₂), 1.63 - 1.69 (2 H, m, CH₂CH₂CH₂), 2.42 - 2.46 (2 H, m, CH₂CH₂CONH), 3.07 - 3.40 (14 H, m, BocNHCH₂CH₂N(Boc)CH₂CH₂CH₂N(Boc)CH₂ and N(CH₂CH₂-alkyl chain)₂), 3.41 - 3.49 (2 H, m, BocNHCH₂CH₂), 3.93 - 3.97 (2 H, m, CH₂CONAlkylchain); ¹³C NMR (CDCl₃) δ 13.0 (2 x CH₃), 21.6 (2 x CH₂CH₃), 25.8 (CH₂CH₂CH₃), 25.9 (CH₂CH₂CH₃), 26.5 ((Boc)NCH₂CH₂CH₂N(Boc)), 27.0 - 29.2 (26 CH₂'s Alkyl chains and 3 x C(CH₃)₃), 30.8 ((Boc)NCH₂CH₂CONH), 33.1 ((Boc)NCH₂CH₂CONH), 34.7 (N(CH₂CH₂-alkyl chain)₂), 38.5 (BocNHCH₂), 40.1 (NHCH₂N-alkyl chains), 42.6 ((Boc)NCH₂CH₂CH₂N(Boc)), 44.2, 44.7 ((N(CH₂-alkyl chain)₂), 45.1 ((Boc)NCH₂CH₂CH₂N(Boc)), 45.8 ((Boc)NHCH₂CH₂), 77.9 (NHCOOC(CH₃)₃), 78.60, 78.7 (NCOOC(CH₃)₃), 154.2 (NHCOOC(CH₃)₃), 155.0 (2 x NCOOC(CH₃)₃), 166.0 (CONHCH₂), 169.8 (CON(Alkylchains)); *m/z* (FAB +ve) 1051 (*M* + *H*⁺); FAB-MS *m/z* calculated for C₆₁H₁₁₉N₅O₈ (*M* + *H*⁺) 1050.9124, found 1050.9136.

3-[3-(2-Amino-ethylamino)-propylamino]-*N*-dioctadecylcarbamoylethyl-methyl-propionamide (CDAN-DOGS, 71)



Poly-Boc protected lipid **96** (100 mg, 0.095 mmol) was dissolved in dry CH₂Cl₂ (20 mL) under anhydrous conditions. Trifluoroacetic acid (7 mL) was added to the solution cautiously. The reaction was stirred under a positive flow of nitrogen until TLC indicated that the reaction had gone to completion (~3 h). The solvents were removed *in vacuo* rendering a pale solid. This was re-dissolved in CH₂Cl₂ (20 mL) and concentrated *in vacuo* repeatedly (x 8). The resulting solid was then freeze-dried from acetonitrile: water 1 : 1, giving the title compound (70 mg, 97%) as a white fluffy powder: FTIR

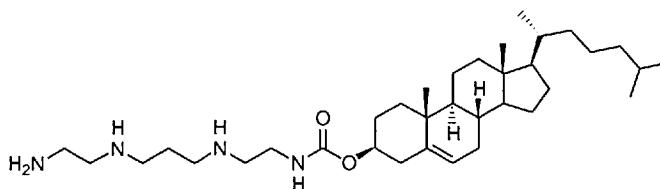
(nujol mull) $\nu_{\max}$ 3289, 2920, 2852, 1669, 1463, 1376, 1198, 1169, 1137 cm^{-1} ; ^1H NMR (CD_3OD) δ 0.80 (6 H, t, 2 x CH_3 ($J = 7.2$ Hz)), 1.14 – 1.31 (60 H, m, 30 x CH_2 -alkyl chains), 1.40 - 1.47 (2 H, m, $\text{N}(\text{CH}_2\text{CH}_2\text{-alkylchain})$), 1.48 - 1.56 (4 H, m, $\text{N}(\text{CH}_2\text{CH}_2\text{-alkylchain})$), 2.02 - 2.10 (2 H, quin, $\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$ ($J = 7.7$ Hz)), 2.63 - 2.66 (2 H, t, $\text{NHCH}_2\text{CH}_2\text{CONH}$ ($J = 6.2$ Hz)), 3.06 - 3.12 (4 H, m, $\text{N}(\text{CH}_2\text{CH}_2\text{-alkylchain})_2$), 3.18 - 3.27 (10 H, m, $\text{H}_2\text{NCH}_2\text{CH}_2\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NHCH}_2$), 3.99 (2 H, s, br, NHCH_2CO); ^{13}C NMR (CD_3OD) δ 14.9 (2 x CH_3), 24.2 (2 x CH_2CH_3), 24.7 (2 x $\text{CH}_2\text{CH}_2\text{CH}_3$), 29.0 - 31.2 (26 CH_2 's Alkyl chains), 32.3 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 33.5 (H_2NCH_2), 37.5 ($\text{NHCH}_2\text{CH}_2\text{CO}$), 42.3 ($\text{N}(\text{CH}_2\text{CH}_2\text{-alkyl chain})_2$), 45.5 ($\text{N}(\text{CH}_2\text{CH}_2\text{-alkyl chain})_2$), 46.3 ($\text{NHCH}_2\text{CH}_2\text{CO}$), 46.4 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 46.8 ($\text{NHCH}_2\text{CH}_2\text{CH}_2\text{NH}$), 48.1 (NHCH_2CO), 48.8 ($\text{H}_2\text{NCH}_2\text{CH}_2$), 170.5 (CONH), 172.8 (CON-alkyl chains); m/z (FAB +ve) 750 (M^+); FAB-MS m/z calculated for $\text{C}_{46}\text{H}_{95}\text{N}_5\text{O}_2$ (M^+) 750.7549, found 750.7564.

7.8 Solid Phase Synthesis of Cholesterol-Based Polyamine Lipids

General Method

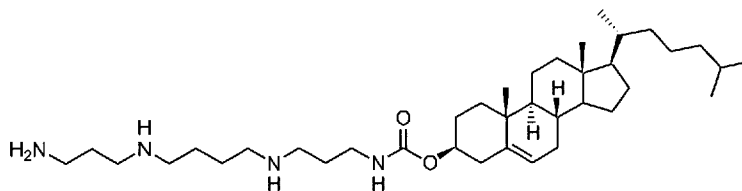
2-chlorotrityl chloride resin (0.4 mmol.g^{-1}) was placed in a solid phase synthesis flask, and swelled by addition CH_2Cl_2 . Polyamine (10 equiv.) was added to the resin in CH_2Cl_2 . The resin was agitated at room temperature for 2 hours. MeOH (1000 equiv.) was added as a capping reagent, and the reaction was left for 10 additional minutes; the solution was filtered and the resin was washed alternatively with CH_2Cl_2 and *i*PrOH ($\times 3$) and finally MeOH ($\times 2$) and dried. 2-Acetyldimedone (Dde-OH) (10 equiv.) was dissolved in DMF. The resulting solution was added to the resin and shaken for 12 hrs. The solution was filtered and the resin washed alternatively with DMF, CH_2Cl_2 and *i*PrOH ($\times 3$), CH_2Cl_2 and MeOH ($\times 3$) and finally CH_2Cl_2 and *i*PrOH ($\times 3$). The kaiser test was negative. The resin was suspended in CH_2Cl_2 . Boc_2O (5 equiv. per free amine) and NEt_3 (2 equiv. per free amine) was added to the suspension and the resulting mixture was shaken at room temperature for 4 hours. The solution was filtered and the resin washed alternatively with CH_2Cl_2 and *i*PrOH ($\times 3$), CH_2Cl_2 and MeOH ($\times 3$) and finally CH_2Cl_2 and *i*PrOH ($\times 3$). The chloranil test was negative. The Dde group was selectively removed by treatment of the resin with 2 % hydrazine in DMF (10 min $\times 2$). The resin was washed with DMF, CH_2Cl_2 and *i*PrOH ($\times 3$). The Kaiser test was positive and the chloranil test was negative. Cholesterol chloroformate (10 equiv.) and NEt_3 (3 equiv.) was dissolved in CH_2Cl_2 and the solution was added to the resin. The resulting suspension was shaken for 12 hours at room temperature. The solution was filtered and the resin washed alternatively with CH_2Cl_2 and *i*PrOH ($\times 3$), CH_2Cl_2 and MeOH ($\times 3$), MeOH and H_2O ($\times 3$), CH_2Cl_2 and *i*PrOH ($\times 3$) and finally ether ($\times 2$). The Kaiser test was negative. The resin was treated with 50% TFA in CH_2Cl_2 for 1 h. The filtrate was collected and reduced *in vacuo* rendering the desired product.

*N*¹-Cholesteryloxycarbonyl-3-7-diazanonane-1,9-diamine (CDAN, 70)



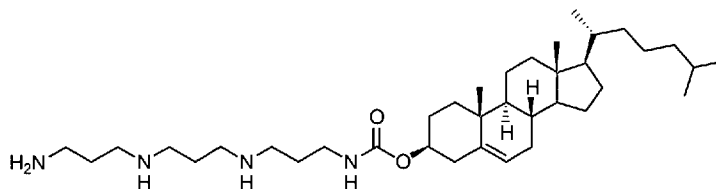
Following general method for synthesis of cholesterol-based polyamine lipids (see section 7.8): 2-Chlorotriyl chloride resin (150 mg, 0.06mmol), N,N'-Bis(3-aminopropyl)-1,3-propanediamine (96 mg, 104 μ L, 0.6 mmol), MeOH (2.4 mL, 1.92 g, 60 mmol), Dde-OH (109 mg, 0.6 mmol), Boc₂O (131 mg, 0.6 mmol), NEt₃ (24.2 mg, 32 μ L, 0.24 mmol), cholesterol chloroformate (268 mg, 0.6 mmol), NEt₃ (18.1 mg, 25 μ L, 0.18 mmol), gave the title compound as a white powder (TFA salt) (51 mg, 96%). FTIR (CH₂Cl₂) ν_{max} 3584-3245, 2937, 2868, 1695, 1538, 1469, 1379, 1251, 1133 and 1014 cm⁻¹. ¹H NMR (d₆-DMSO) δ 0.65 (3 H, s, 18-CH₃), 0.83 - 0.85, 0.83 - 0.85 (2 $\times$ 3 H, d, *J* = 6.4 Hz, overlapping 1.6 Hz, 27-CH₃, 26-CH₃), 0.89 - 0.90 (3 H, d, *J* = 6.4 Hz, 21-CH₃), 0.97 (3 H, s, 19-CH₃), 0.97 - 2.03 (28 H, m, 1-CH₂, 2-CH₂, 4-CH₂, 7-CH₂, 8-CH, 9-CH, 11-CH₂, 12-CH₂, 14-CH, 15-CH₂, 16-CH₂, 17-CH, 20-CH, 22-CH₂, 23-CH₂, 25-CH, 5'-CH₂), 2.17 - 2.34 (2 H, m, 24-CH₂), 2.92 - 3.32 (12 H, m, 1'-CH₂, 2'-CH₂, 4'-CH₂, 6'-CH₂, 8'-CH₂, 9'-CH₂), 4.27 - 4.39 (1 H, m, 3-CH), 5.30-5.37 (1 H, m, 6-CH). ¹³C NMR (400 MHz, d₆-DMSO) δ 11.6 (18-CH₃), 18.5 (21-CH₃), 18.9 (19-CH₃), 20.50 (11-CH₂), 22.3 (26-CH₃), 22.3 (5'-CH₂), 22.6 (27-CH₃), 23.1 (23-CH₂), 23.8 (15-CH₂), 27.3 (25-CH), 27.7 (2-CH₂, 16-CH₂ overlapping), 31.2 (7-CH₂), 31.3 (8-CH), 35.1 (20-CH, 1'-CH₂ overlapping), 35.6 (22-CH₂), 35.9 (10-C), 36.5 (1-CH₂), 36.6 (9'-CH₂), 38.2 (24-CH₂), 38.8 (4-CH₂), 39.1 (12-CH₂), 41.8 (13-C), 43.9 (4'-CH₂), 44.1 (6'-CH₂), 46.4 (2'-CH₂, 8'-CH₂ overlapping), 49.4 (9-CH), 55.5 (17-CH), 56.1 (14-CH), 73.4 (3-CH), 121.9 (6-CH), 139.6 (5-C), 155.8 (NCOO); *m/z* (FAB +ve) 573 (M + H⁺); FAB-MS *m/z* for C₃₅H₆₅N₄O₂ (M + H⁺) calculated 573.5108, found 573.5103. HPLC analysis *t*_R = 22.4 min, column C-4 peptide: gradient H₂O/MeCN/MeOH, 0 min [100/0/0], 1-15 min [0/100/0], 25 min [0/100/0], 25.1 min [0/0/100], 45 min [0/0/100], 45.1 min [100/0/0], 60 min [100/0/0]; flow 1 mL/min.

*N*¹-Cholesteryloxy-3-carbonyl-1,12-diamino-4,9-diazadodecane (**110**)



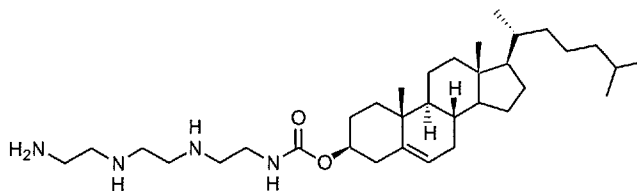
Following general method for synthesis of cholesterol-based polyamine lipids (see section 7.8): 2-Chlorotriyl chloride resin (150 mg, 0.06mmol), spermine (121 mg, 0.6 mmol), MeOH (2.4 mL, 1.92 g, 60 mmol), Dde-OH (109 mg, 0.6 mmol), Boc₂O (131 mg, 0.6 mmol), NEt₃ (24.2 mg, 32 μ L, 0.24 mmol), cholesterol chloroformate (268 mg, 0.6 mmol), NEt₃ (18.1 mg, 25 μ L, 0.18 mmol), gave the title compound as a white powder (TFA salt) (39 mg, 87%). FTIR (CH₂Cl₂) ν_{max} 3584-3245, 2937, 2868, 1695, 1538, 1469, 1379, 1251, 1133 and 1014 cm⁻¹. ¹H NMR (d₆-DMSO) δ 0.65 (3 H, s, 18-CH₃), 0.83, 0.84 (6 H, d, 27-CH₃, 26-CH₃ overlapping), 0.89 – 0.90 (3 H, d, *J* = 6.4 Hz, 21-CH₃), 0.96 (3 H, s, 19-CH₃), 0.95 - 2.00 (34 H, m, 1-CH₂, 2-CH₂, 4-CH₂, 7-CH₂, 8-CH, 9-CH, 11-CH₂, 12-CH₂, 14-CH, 15-CH₂, 16-CH₂, 17-CH, 20-CH, 22-CH₂, 23-CH₂, 25-CH, 2'-CH₂, 6'-CH₂, 7'-CH₂, 11'-CH₂), 2.15 – 2.32 (2 H, m, 24-CH₂), 2.83 – 3.08 (12 H, m, 1'-CH₂, 3'-CH₂, 5'-CH₂, 8'-CH₂, 10'-CH₂, 12'-CH₂), 4.26 - 4.36 (1 H, m, 3-CH), 5.30-5.35 (1 H, m, 6-CH). ¹³C NMR (d₆-DMSO) δ 11.6 (18-CH₃), 18.5 (21-CH₃), 18.9 (19-CH₃), 20.50 (11-CH₂), 22.3 (26-CH₃), 22.5 (6'-CH₂, 7'-CH₂), 22.6 (27-CH₃), 23.1 (23-CH₂), 23.7 (2'-CH₂), 23.8 (15-CH₂), 26.11 (11'-CH₂), 27.3 (25-CH), 27.7 (2-CH₂), 27.8 (16-CH₂), 31.2 (7-CH₂), 31.3 (8-CH), 35.1 (20-CH), 35.6 (22-CH₂), 35.9 (10-C), 36.1 (1'-CH₂), 36.5 (1-CH₂), 37.3 (12'-CH₂), 38.2 (24-CH₂), 38.9 (4-CH₂), 39.2 (12-CH₂), 41.8 (13-C), 43.8 (3'-CH₂), 44.5 (10'-CH₂), 46.0 (5'-CH₂, 8'-CH₂ overlapping), 49.4 (9-CH), 55.5 (17-CH), 56.1 (14-CH), 73.0 (3-CH), 121.8 (6-CH), 139.7 (5-C), 155.7 (NCOO); *m/z* (FAB +ve) 615 (*M* + H⁺); FAB-MS *m/z* for C₃₈H₇₁N₄O₂ (*M* + H⁺) calculated 615.5577, found 615.5596. HPLC analysis *t*_R = 22.7 min, column C-4 peptide: gradient H₂O/MeCN/MeOH, 0 min [100/0/0], 1-15 min [0/100/0], 25 min [0/100/0], 25.1 min [0/0/100], 45 min [0/0/100], 45.1 min [100/0/0], 60 min [100/0/0]; flow 1 mL/min.

*N*¹-Cholesteryloxy-3-carbonyl-1,11-diamino-4,8-diazaundecane (**111**)



Following general method for synthesis of cholesterol-based polyamine lipids (see section 7.8): 2-Chlorotriyl chloride resin (150 mg, 0.06 mmol), N,N'-Bis(2-aminoethyl)-1,3-propanediamine (112 mg, 116 μ L, 0.6 mmol), MeOH (2.4 mL, 1.92 g, 60 mmol), Dde-OH (109 mg, 0.6 mmol), Boc₂O (131 mg, 0.6 mmol), NEt₃ (24.2 mg, 32 μ L, 0.24 mmol), cholesterol chloroformate (268 mg, 0.6 mmol), NEt₃ (18.1 mg, 25 μ L, 0.18 mmol), gave the title compound as a white powder (TFA salt) (52 mg, 92%). FTIR (CH₂Cl₂) ν_{max} 3584, 3245, 2937, 2868, 1695, 1538, 1469, 1379, 1251, 1133 and 1014 cm⁻¹. ¹H NMR (d₆-DMSO) δ 0.65 (3 H, s, 18-CH₃), 0.83-0.84, 0.83-0.85 (2 $\times$ 3 H, d, *J* = 6.4 Hz, overlapping 1.6 Hz, 27-CH₃, 26-CH₃), 0.89 – 0.90 (3 H, d, *J* = 6.4 Hz, 21-CH₃), 0.96 (3 H, s, 19-CH₃), 0.97 – 2.00 (32 H, m, 1-CH₂, 2-CH₂, 4-CH₂, 7-CH₂, 8-CH, 9-CH, 11-CH₂, 12-CH₂, 14-CH, 15-CH₂, 16-CH₂, 17-CH, 20-CH, 22-CH₂, 23-CH₂, 25-CH, 2'-CH₂, 6'-CH₂, 10'-CH₂), 2.15 – 2.34 (2 H, m, 24-CH₂), 2.82 – 3.10 (12 H, m, 1'-CH₂, 3'-CH₂, 5'-CH₂, 7'-CH₂, 9'-CH₂, 11'-CH₂), 4.25 – 4.36 (1 H, m, 3-CH), 5.30-5.35 (1 H, m, 6-CH). ¹³C NMR (d₆-DMSO) δ 11.6 (18-CH₃), 18.5 (21-CH₃), 18.9 (19-CH₃), 20.50 (11-CH₂), 22.3 (26-CH₃), 22.5 (27-CH₃), 23.1 (23-CH₂), 23.7, 23.8 (2'-CH₂, 6'-CH₂, 15-CH₂ overlapping), 26.11 (10'-CH₂), 27.3 (25-CH), 27.7 (2-CH₂) 27.8 (16-CH₂), 31.2 (7-CH₂), 31.3 (8-CH), 35.1 (20-CH), 35.6 (22-CH₂), 35.9 (1'-CH₂) 36.0 (10-C), 36.5 (1-CH₂), 37.3 (11'-CH₂), 38.2 (24-CH₂), 38.9 (4-CH₂), 39.1 (12-CH₂), 41.8 (13-C), 43.8, 43.9 (3'-CH₂, 9'-CH₂ overlapping), 44.7 (5'-CH₂, 7'-CH₂ overlapping), 49.4 (9-CH), 55.5 (17-CH), 56.1 (14-CH), 73.0 (3-CH), 121.8 (6-CH), 139.6 (5-C), 155.7 (NCOO); *m/z* (FAB +ve) 601 (*M* + H⁺); FAB-MS *m/z* for C₃₇H₆₉N₄O₂ (*M* + H⁺) calculated 601.5421, found 601.5419. HPLC analysis *t*_R = 22.5 min, column C-4 peptide: gradient H₂O/MeCN/MeOH, 0 min [100/0/0], 1-15 min [0/100/0], 25 min [0/100/0], 25.1 min [0/0/100], 45 min [0/0/100], 45.1 min [100/0/0], 60 min [100/0/0]; flow 1 mL/min.

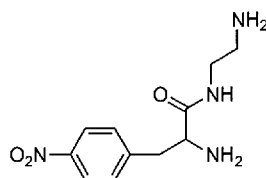
*N*¹-Cholesteryloxy-3-carbonyl-1,9-diamino-3,6-diazanonane (**112**)



Following general method for synthesis of cholesterol-based polyamine lipids (see section 7.8): 2-Chlorotriyl chloride resin (150 mg, 0.06 mmol), triethylenetetraamine (88 mg, 0.6 mmol), MeOH (2.4 mL, 1.92 g, 60 mmol), Dde-OH (109 mg, 0.6 mmol), Boc₂O (131 mg, 0.6 mmol), NEt₃ (24.2 mg, 32 μ L, 0.24 mmol), cholesterol chloroformate (268 mg, 0.6 mmol), NEt₃ (18.1 mg, 25 μ L, 0.18 mmol), gave the title compound as a white powder (TFA salt) (40 mg, 96%). FTIR (CH₂Cl₂) ν_{max} 3584-3245, 2937, 2868, 1695, 1538, 1469, 1379, 1251, 1133 and 1014 cm⁻¹. ¹H NMR (d₆-DMSO) δ 0.65 (3 H, s, 18-CH₃), 0.83-0.84, 0.83-0.85 (2 $\times$ 3 H, d, J = 6.4 Hz, overlapping 1.6 Hz, 27-CH₃, 26-CH₃), 0.89 – 0.90 (3 H, d, J = 6.4 Hz, 21-CH₃), 0.97 (3 H, s, 19-CH₃), 0.97 - 2.02 (26 H, m, 1-CH₂, 2-CH₂, 4-CH₂, 7-CH₂, 8-CH, 9-CH, 11-CH₂, 12-CH₂, 14-CH, 15-CH₂, 16-CH₂, 17-CH, 20-CH, 22-CH₂, 23-CH₂, 25-CH), 2.15 – 2.34 (2 H, m, 24-CH₂), 3.00 – 3.34 (12 H, m, 1'-CH₂, 2'-CH₂, 4'-CH₂, 5'-CH₂, 7'-CH₂, 8'-CH₂), 4.27 - 4.38 (1 H, m, 3-CH), 5.30-5.35 (1 H, m, 6-CH). ¹³C NMR (d₆-DMSO) δ 11.6 (18-CH₃), 18.4 (21-CH₃), 18.9 (19-CH₃), 20.50 (11-CH₂), 22.3 (26-CH₃), 22.5 (27-CH₃), 23.1 (23-CH₂), 23.8 (15-CH₂), 27.3 (25-CH), 27.7 (2-CH₂, 16-CH₂ overlapping), 31.2 (7-CH₂), 31.3 (8-CH), 35.1 (20-CH), 35.4 (22-CH₂), 35.6 (1'-CH₂), 36.0 (10-C), 36.5 (1-CH₂), 36.7 (8'-CH₂), 38.1 (24-CH₂), 38.9 (4-CH₂), 39.9 (12-CH₂), 41.8 (13-C), 44.1, 44.2 (4'-CH₂, 5'-CH₂ overlapping), 46.6 (2'-CH₂, 7'-CH₂ overlapping), 49.4 (9-CH), 55.5 (17-CH), 56.1 (14-CH), 73.4 (3-CH), 121.8 (6-CH), 139.6 (5-C), 155.9 (NCOO); m/z (FAB +ve) 559 (M + H⁺); FAB-MS m/z for C₃₄H₆₂N₄O₂ (M + H⁺) calculated 559.4951, found 559.4947. HPLC analysis t_R = 22.3 min, column C-4 peptide: gradient H₂O/MeCN/MeOH, 0 min [100/0/0], 1-15 min [0/100/0], 25 min [0/100/0], 25.1 min [0/0/100], 45 min [0/0/100], 45.1 min [100/0/0], 60 min [100/0/0]; flow 1 mL/min.

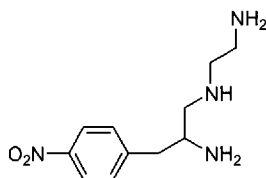
7.9 Synthesis of Compounds for Gadolinium Post-Coating of Liposomes

(S)-p-Nitrophenylalanine N-(2-aminoethyl) Amide (**121**)



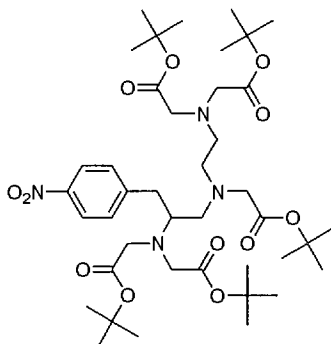
Ethylene diamine (16.2 g, 18 mL, 269 mmol) was placed in a dry round bottom flask and stirred at room temperature under argon. *p*-Nitrophenylalanine methylester.HCl (5.9 g, 22.6 mmol) was added to the flask in equal portions over 2 h. Upon addition of the ester the solution initially turned green and then deep red. The reaction mixture was stirred at room temperature until TLC indicated the reaction had gone to completion (~ 12 h). The reaction was concentrated *in vacuo* rendering dark brown oil. NH₃ (aq) (40 mL) was added to this oil, and the pH of the resulting solution adjusted from 10.5 to 11.5 by addition of 50% NaOH solution. The aqueous phase was extracted with CH₂Cl₂ (10 x 100 mL). The organic extracts were combined, dried with Na₂SO₄, filtered and concentrated *in vacuo*. The resulting oil was purified using silica gel column chromatography [CH₂Cl₂ : MeOH: NH₃ (85 : 13.2 : 1.8) (v/v)] rendering the title compound as a yellow oil (4 g, 71%). FTIR (film) $\nu_{\max}$ 3299, 3074, 3025, 2924, 2865, 1659, 1599, 1517, 1441, 1347, 1182 cm⁻¹; ¹H NMR (CDCl₃) 1.69 (4H, s, br, 2 x NH₂), 2.74 (2 H, t, *J* = 6.0 Hz, CH₂NH₂), 2.86 (2 H, dd, *J* = 8.8, 5.2 Hz, ArCHH), 3.22 – 3.27 (3 H, m, CH₂CH₂NH₂ and ArCHH), 3.61 (1 H, dd, *J* = 4.4, 8.4 Hz, CHNH₂), 7.34 (2 H, d, *J* = 8.8 Hz, 2 x CH aromatic), 7.51 (1 H, m, br, NH amide), 8.08 (1 H, m, 2 x CH aromatic); ¹³C NMR (CDCl₃) 40.9 (ArCH₂), 41.3 (CH₂NH₂), 41.8 (CH₂CH₂NH₂), 56.2 (CHNH₂), 123.5 (2 x CH, aromatic), 130.3 (2 x CH, aromatic), 146.0 (CH aromatic), 146.9 (CH aromatic), 173.9 (CO); *m/z* (FAB +ve) 253 (M + H⁺); FAB-MS *m/z* for C₁₁H₁₇N₄O₃ (M + H⁺) calculated 253.1301, found 253.1301.

1-(S)-(p-Nitrobenzyl)diethylenetriamine (**119**)



(S)-p-Nitrophenylalanine N-(2-aminoethyl) Amide **121** (2.3 g, 9.1 mmol) was placed in a dry two neck flask equipped with a stirrer bar, condenser (closed with septum) and septum. Anhydrous THF (80 mL) was added to the flask. The amide only partially dissolved in THF. The solution was cooled to 5 °C. $\text{BH}_3\cdot\text{THF}$ (1 M soln., 80 mL, 80 mmol), was added dropwise to the reaction mixture. After addition was complete the reaction was gradually warmed and brought to reflux for 15 h. The solution was then cooled to 9 °C. The septum from the top of the condenser was removed and the excess borane was quenched by the dropwise addition of H_2O until the evolution of gas ceased. The reaction was then concentrated *in situ* under reduced pressure rendering a crusty yellow solid. HCl (6 M, 56 mL) was added to the residue and the resulting solution was refluxed for 3 h followed by stirring at room temperature for 24 h. The solution was concentrated to dryness under reduced pressure. The residue was dissolved in H_2O (40 mL) and concentrated NH_4OH (40 mL). The pH of the solution was adjusted to 11.5 with 5 M KOH . The aqueous solution was extracted with CHCl_3 (10 x 80 mL). The organic extracts were combined, dried (Na_2SO_4), filtered and concentrated *in vacuo* rendering the desired compound as a yellow oil (1.8 g, 89%). FTIR (film) ν_{max} 3360, 3290, 3074, 2926, 2848, 1598, 1514, 1345, 1109 cm^{-1} ; ^1H NMR (CDCl_3) 1.45 (5 H, s, br, 2 x NH_2 and 1 x NH), 2.44 (1 H, dd, $J = 8.4, 12$ Hz, CHHNH), 2.55 – 2.69 (4 H, m, $\text{CH}_2\text{CH}_2\text{NH}_2$, CHHNH and ArCHH), 2.71 – 2.77 (2 H, m, CH_2NH_2), 2.85 (1 H, dd, $J = 5.2, 13.6$ Hz, ArCHH), 3.03 – 3.12 (1 H, m, H_2NCH), 7.33 (2 H, d, $J = 8.8$ Hz, 2 x CH , aromatic), 8.10 (2 H, d, $J = 8.8$ Hz, 2 x CH , aromatic); ^{13}C NMR (CDCl_3) 41.8 (ArCH_2), 42.6 (CH_2NH_2), 52.3 ($\text{CH}_2\text{CH}_2\text{NH}_2$), 52.4 (H_2NCH), 56.3 (CH_2NH), 123.6 (2 x CH , aromatic), 130.1 (2 x CH , aromatic), 146.6 (CH aromatic), 147.5 (CH aromatic); m/z (CI +ve) 239 ($\text{M} + \text{H}^+$); FAB-MS m/z for $\text{C}_{11}\text{H}_{19}\text{N}_4\text{O}_2$ ($\text{M} + \text{H}^+$) calculated 239.1508, found 239.1503.

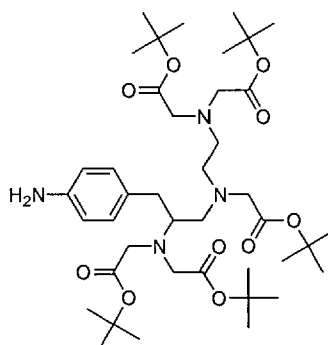
1-(S)-(p-Nitrobenzyl)diethylenetriaminepentaacetic acid penta-*t*-butyl ester (**118**)



1-(S)-(p-Nitrobenzyl)diethylenetriamine **119** (1.73 g, 7.27 mmol) was dissolved in anhydrous DMF (58 mL) and added to a flask under an argon atmosphere. DIPEA (11.3 g, 15.2 mL, 87 mmol) and *t*-Butyl bromoacetate (12.8 g, 9.67 mL, 65.5 mmol) was added to the solution in one portion. The resulting solution was golden brown. KI (1.32 g, 7.95 mmol) was added to the reaction. The solution immediately turned yellow and cloudy and became warm to touch. The reaction was stirred at room temperature under an argon atmosphere until TLC indicated the absence of starting material. The solution was reduced in vacuo using a water bath temperature no higher than 35 °C, rendering a pale solid. Et₂O (90 mL) and H₂O (45 mL) were added to the reaction residue and swirled until everything dissolved. The aqueous layer was removed and the ether layer washed with H₂O (45 mL). The organic layer was then washed with sat. NaCl (aq) (23 mL). The organic layer was dried (Na₂SO₄), filtered and concentrated *in vacuo*. The resulting yellow brown oil was purified by silica gel column chromatography [hexane: ethylacetate, 3:1] giving the title compound as a yellow oil (4.75 g, 81%). FTIR (film) ν_{max} 3447, 3201, 2978, 2933, 1731, 1679, 1599, 1519, 1456 cm⁻¹; ¹H NMR (CDCl₃) 1.41 (27 H, s, 3 x C(CH₃)₃), 1.43 (18 H, s, 2 x C(CH₃)₃), 2.45 (1 H, dd, *J* = 8.4, 12.8 Hz, CHCHH), 2.70 – 2.78 (4 H, m, CH₂CH₂NH₂), 2.82 (1H, dd, *J* = 5.18, 13.9 Hz, ArCHH), 2.94 (1 H, dd, *J* = 4.9, 12.8 Hz, CHCHH), 2.98 (1 H, dd, *J* = 7.9, 13.9 Hz, ArCHH), 3.29 (2 H, s, 1 x CH₂COO^{*t*}Bu), 3.39 (2 H, s, 1 x CH₂COO^{*t*}Bu), 3.40 (2 H, s, 1 x CH₂COO^{*t*}Bu), 3.41 (4 H, s, 2 x CH₂COO^{*t*}Bu), 7.47 (2 H, d, *J* = 8.8 Hz, 2 x CH, aromatic), 8.08 (2 H, d, *J* = 8.8 Hz, 2 x CH, aromatic); ¹³C NMR (CDCl₃) 27.9 (2 x C(CH₃)₃), 28.2 (3 x C(CH₃)₃), 37.4 (ArCH₂), 52.3 (NCH₂CH₂N), 53.2 (NCH₂CH₂N), 53.4 (2 x CH₂COO^{*t*}Bu), 56.0 (NCHCH₂N and 1 x CH₂COO^{*t*}Bu, overlapping) 56.3 (1 x CH₂COO^{*t*}Bu), 56.4 (1 x CH₂COO^{*t*}Bu), 62.9 (CH), 80.7 (2 x C(CH₃)₃), 80.9 (3 x C(CH₃)₃), 123.3 (2 x CH, aromatic), 130.3 (2 x CH, aromatic), 146.3 (CH, aromatic), 149.3 (CH, aromatic), 170.6

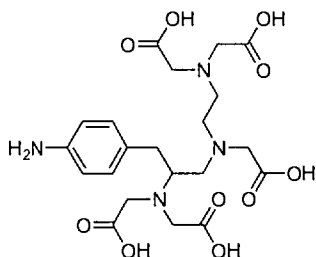
(2 x COO^tBu), 171.1 (3 x COO^tBu); m/z (FAB +ve) 809 ($M + \text{NH}$); FAB-MS m/z for $\text{C}_{41}\text{H}_{69}\text{N}_4\text{O}_{12}$ ($M + \text{H}^+$) calculated 809.4912, found 809.4924.

1-(S)-(p-aminobenzyl)diethylenetriaminepentaacetic acid penta-*t*-butyl ester (117**)**



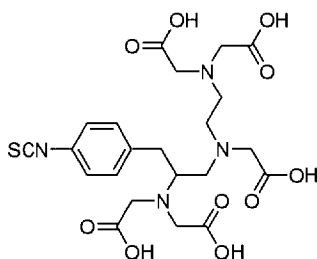
All glassware was washed with mixed acids (1:1 mixture of concentrated H_2SO_4 and concentrated HNO_3) for a minimum of 12 h and rinsed with distilled H_2O . 1-(S)-(p-Nitrobenzyl)diethylenetriaminepentaacetic acid penta-*t*-butyl ester **118** (2.5 g, 3.1 mmol) was dissolved in MeOH (100mL). Pd/C (10 mol%, 125 mg) was added to the reaction mixture. The resulting slurry was cooled to 0 °C in an ice bath. H_2 (g) was bubbled through the reaction continuously for 1 h and then the reaction was left under a hydrogen atmosphere. The ice bath was allowed to melt and the reaction gradually warmed to room temperature. After 24 h, TLC showed that the reaction had gone to completion. Celite (400 mg) was added to the reaction mixture and stirred for 15 min. The reaction solution was filtered over a celite. The celite plug was rinsed with MeOH (5 x 20 mL). The clear filtrate was concentrated *in vacuo* rendering the desired compound as a yellow oil (2.39 g, 98%). FTIR (film) ν_{max} 3456, 3374, 3225, 2977, 2932, 1737, 1625, 1517, 1455 cm^{-1} ; ^1H NMR (CDCl_3) 1.38 (27 H, s, 3 x $\text{C}(\text{CH}_3)_3$), 1.41 (18 H, s, 2 x $\text{C}(\text{CH}_3)_3$), 2.42 – 2.57 (2 H, m, CHCH_2N), 2.64 – 2.87 (6 H, m, $\text{NCH}_2\text{CH}_2\text{N}$ and ArCH_2), 2.97 – 3.07 (1 H, m, NCH_2), 3.30 – 3.52 (10 H, m, 5 x $\text{CH}_2\text{COO}^t\text{Bu}$), 6.55 (2 H, d, $J = 8.0$ Hz, 2 x CH, aromatic), 6.96 (2 H, d, $J = 8.0$ Hz, 2 x CH, aromatic); ^{13}C NMR (CDCl_3) 28.1 (2 x $\text{C}(\text{CH}_3)_3$), 28.2 (3 x $\text{C}(\text{CH}_3)_3$), 36.2 (ArCH_2), 52.5 ($\text{NCH}_2\text{CH}_2\text{N}$), 52.9 ($\text{NCH}_2\text{CH}_2\text{N}$), 53.6 (2 x $\text{CH}_2\text{COO}^t\text{Bu}$), 55.7 (1 x $\text{CH}_2\text{COO}^t\text{Bu}$), 55.9 ($\text{NCH}_2\text{CH}_2\text{N}$ and 1 x $\text{CH}_2\text{COO}^t\text{Bu}$ overlapping), 56.2 (1 x $\text{CH}_2\text{COO}^t\text{Bu}$), 80.5 (2 x $\text{C}(\text{CH}_3)_3$), 80.7 (3 x $\text{C}(\text{CH}_3)_3$), 113.8 (2 x CH, aromatic), 130.0 (2 x CH, aromatic), 130.3 (CH, aromatic), 144.3 (CH, aromatic), 170.7 (2 x COO^tBu), 171.4 (3 x COO^tBu); m/z (FAB +ve) 779 ($M + \text{H}^+$); FAB-MS m/z for $\text{C}_{41}\text{H}_{71}\text{N}_4\text{O}_{10}$ ($M + \text{H}^+$) calculated 779.5170, found 779.5134.

1-(S)-(p-aminobenzyl)diethylenetriaminepentaacetic acid (116)



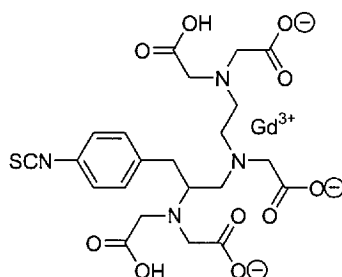
To remove any adventitious metals, all glassware was washed with mixed acids (1:1 mixture of concentrated H_2SO_4 and concentrated HNO_3) for a minimum of 12 h and rinsed with distilled H_2O . 1-(S)-(p-aminobenzyl)diethylenetriaminepentaacetic acid penta-*t*-butyl ester **117** (710 mg, 0.91 mmol) was dissolved in dioxane (5 mL). c.HCl (5 mL) was added to the solution and the resulting reaction mixture was stirred at room temperature under a positive flow of nitrogen for 4 h. The solvents were removed *in vacuo* and the resulting residue was dissolved in water and freeze dried to give the title compound as a pale solid (440 mg, 97%). FTIR (nujol) ν_{max} 2921, 2724, 1729, 1462, 1376, 1169 cm^{-1} ; ^1H NMR (D_2O) δ 2.71 – 2.77 (2 H, dd, $J = 8.8$ and 13.6 Hz, NCHCH_2), 3.07 – 3.54 (6 H, m, $\text{NCH}_2\text{CH}_2\text{N}$ and ArCH_2), 3.54 – 3.65 (1 H, m, NCHCH_2), 3.67 – 3.90 (10 H, m, $5 \times \text{CH}_2\text{COOH}$), 7.29 (2 H, d, $J = 8.4$ Hz, $2 \times \text{CH}$ aromatic), 7.36 (2H, d, $J = 8.4$ Hz, $2 \times \text{CH}$ aromatic); ^{13}C NMR (D_2O) δ 32.3 (ArCH_2), 49.8 ($\text{NCH}_2\text{CH}_2\text{N}$), 51.5 ($\text{NCH}_2\text{CH}_2\text{N}$), 52.9 ($2 \times \text{CH}_2\text{COOH}$), 53.9 ($1 \times \text{CH}_2\text{COOH}$), 55.4 ($1 \times \text{CH}_2\text{COOH}$ and NCHCH_2N overlapping), 55.6 ($1 \times \text{CH}_2\text{COOH}$), 62.2 (NCHCH_2N), 123.8 ($2 \times \text{CH}$ aromatic), 129.0 ($1 \times \text{CH}$ aromatic), 131.0 ($2 \times \text{CH}$ aromatic), 137.4 ($1 \times \text{CH}$ aromatic), 169.4 ($2 \times \text{COOH}$), 170.7 ($1 \times \text{COOH}$), 173.1 ($2 \times \text{COOH}$); m/z (FAB +ve) 499 ($\text{M} + \text{H}^+$); FAB-MS m/z for $\text{C}_{21}\text{H}_{31}\text{N}_4\text{O}_{10}$ ($\text{M} + \text{H}^+$) calculated 499.2040, found 499.2054; HPLC analysis: $t_R = 4.0$ min, column Vydac C-4 peptide: gradient H_2O (0.1% TFA)/MeCN (0.1% TFA)/MeOH, 0 min [100/0/0], 1-15 min [0/100/0], 25 min [0/100/0], 25.1 min [0/0/100], 45 min [0/0/100], 45.1 min [100/0/0], 55 min [100/0/0]; flow 1 mL/min.

1-(S)-(p-isothiocyanatobenzyl)diethylenetriaminepentaacetic acid (SCN.Bn.DTPA, 115)



All glassware was washed with mixed acids (1:1 mixture of concentrated H_2SO_4 and concentrated HNO_3) for a minimum of 12 h and rinsed with distilled H_2O . 1-(S)-(p-aminobenzyl)diethylenetriaminepentaacetic acid **116** (200 mg, 0.4 mmol) was dissolved in HCl (3 M, 1.35 mL). CCl_4 (798 μL) and thiophosgene (375 μL) were added to the solution. The reaction flask was protected from light (wrapped flask in Al foil) and the mixture was stirred at room temperature for 6 h. The solvents were removed *in vacuo* to give the title compound as a pale yellow solid (212 mg, 98%). FTIR (nujol) ν_{max} 2921, 2724, 1729, 1462, 1376, 1169 cm^{-1} ; ^1H NMR (MeOD) δ 2.74 – 2.80 (2 H, dd, $J = 9.2$ and 13.6 Hz, NCHCH_2), 3.13 – 3.64 (7 H, m, $\text{NCH}_2\text{CH}_2\text{N}$, ArCH_2 and NCHCH_2), 3.70 – 4.20 (10 H, m, $5 \times \text{CH}_2\text{COOH}$), 7.25 (2 H, d, $J = 8.4$ Hz, $2 \times \text{CH}$ aromatic), 7.38 (2H, d, $J = 8.4$ Hz, $2 \times \text{CH}$ aromatic); ^{13}C NMR (MeOD) δ 33.9 (ArCH_2), 51.3 ($\text{NCH}_2\text{CH}_2\text{N}$), 52.7 ($\text{NCH}_2\text{CH}_2\text{N}$), 53.5 ($2 \times \text{CH}_2\text{COOH}$), 54.8 ($1 \times \text{CH}_2\text{COOH}$), 56.1 ($1 \times \text{CH}_2\text{COOH}$ and NCHCH_2N overlapping), 57.1 ($1 \times \text{CH}_2\text{COOH}$), 63.2 (NCHCH_2N), 127.2 ($2 \times \text{CH}$ aromatic), 131.7 ($1 \times \text{CH}$ aromatic), 132.0 ($2 \times \text{CH}$ aromatic), 137.3 (NCS), 137.6 ($1 \times \text{CH}$ aromatic), 170.3 ($2 \times \text{COOH}$), 170.5 ($1 \times \text{COOH}$), 173.8 ($2 \times \text{COOH}$); m/z (FAB +ve) 541 ($\text{M} + \text{H}^+$); FAB-MS m/z for $\text{C}_{22}\text{H}_{29}\text{N}_4\text{O}_{10}\text{S}$ ($\text{M} + \text{H}^+$) calculated 541.1604, found 541.1621; HPLC analysis: $t_R = 15.5$ min, column Vydac C-4 peptide: gradient H_2O (0.1% TFA)/MeCN (0.1% TFA)/MeOH, 0 min [100/0/0], 1-15 min [0/100/0], 25 min [0/100/0], 25.1 min [0/0/100], 45 min [0/0/100], 45.1 min [100/0/0], 55 min [100/0/0]; flow 1 mL/min.

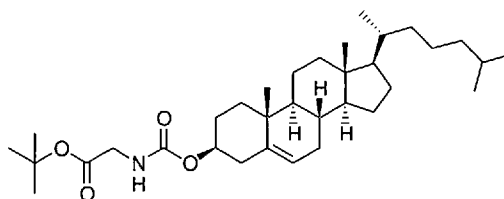
Gadolinium (III) 1-(S)-(p-isothiocyanatobenzyl)diethylenetriaminepentaacetic acid (Gd.SCN.Bn.DTPA, **113**)



SCN.Bn.DTPA **115** (20 mg, 0.04 mmol) and GdCl_3 (13 mg, 0.04 mmol) was dissolved in water (7 mL). The pH of the solution was adjusted to pH 5 by the dropwise addition of 1 M HCl. The resulting solution was stirred at room temperature until the reaction had gone to completion (~ 3 h). The reaction mixture was lyophilised to give Gd.SCN.Bn.DTPA as a white solid (24 mg, 90%). The xylenol orange test showed there to be no free gadolinium. m/z (FAB -ve) 693 (M^-); HPLC analysis: t_R = 15.5 min, column Vydac C-4 peptide: gradient H_2O (0.1% TFA)/MeCN (0.1% TFA)/MeOH, 0 min [100/0/0], 1-15 min [0/100/0], 25 min [0/100/0], 25.1 min [0/0/100], 45 min [0/0/100], 45.1 min [100/0/0], 55 min [100/0/0]; flow 1 mL/min.

NB. Mass spectrum shows the complex with all abundant isomers of gadolinium bound.

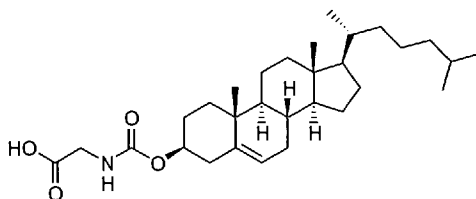
Cholesterol-*N*-Glycine-*tert* butyl ester (**125**)



Cholesterol chloroformate (3 g, 6.7 mmol) and NEt_3 (2 g, 2.75 mL, 20 mmol) were dissolved in dry CH_2Cl_2 (150 mL) under an argon atmosphere. The resulting solution was cooled to 0 °C. *Tert*-butyl glycine (1.12 g, 6.69 mmol) was added in equal portions to the reaction mixture over 20 minutes. The reaction mixture was slowly warmed to room temperature and stirred at room temperature for 12 h. The reaction mixture was concentrated *in vacuo* and the resulting pale solid was purified by silica gel column chromatography [hexane: ethyl acetate, 95:5], giving the title compound as a white solid (3.15 g, 87%). FTIR (nujol) ν_{max} 3444, 2922, 1723, 1458, 1375, 1155, 1047 cm^{-1} . ^1H NMR (CDCl_3) δ 0.67 (3 H, s, 18- CH_3), 0.84 - 0.87, 0.85 - 0.88 (2×3 H, d, J = 6.4 Hz,

overlapping 1.6 Hz, 27-CH₃, 26-CH₃), 0.90 – 0.92 (3 H, d, J = 6.4 Hz, 21-CH₃), 1.00 (3 H, s, 19-CH₃), 0.97 – 1.63 (21 H, m, 1-CH₂, 9-CH, 11-CH₂, 12-CH₂, 14-CH, 15-CH₂, 16-CH₂, 17-CH, 20-CH, 22-CH₂, 23-CH₂, 25-CH), 1.46 (9 H, s, (CH₃)₃), 1.76-2.40 (7 H, m, 2-CH₂, 4-CH₂, 7-CH₂, 8-CH), 3.83 (2 H, d, J = 5.2 Hz, CH₂COO^tBu), 4.45 - 4.57 (1 H, m, 3-CH), 5.05 - 5.16 (1 H, s, br, NH), 5.33 - 5.39 (1 H, m, 6-CH). ¹³C NMR (CDCl₃) δ 11.8 (18-CH₃), 18.7 (21-CH₃), 19.3 (19-CH₃), 21.0 (11-CH₂), 22.6 (26-CH₃), 22.8 (27-CH₃), 23.8 (23-CH₂), 24.2 (15-CH₂), 27.9 (25-CH), 28.0 (C(CH₃)₃), 28.1 (2-CH₂), 28.2 (16-CH₂), 31.8 (7-CH₂), 31.9 (8-CH), 35.7 (20-CH), 36.1 (22-CH₂), 36.5 (10-C), 36.9 (1-CH₂), 38.4 (24-CH₂), 39.5 (4-CH₂), 39.7 (12-CH₂), 42.3 (13-C), 43.3 (CH₂COOH), 49.9 (9-CH), 56.1 (17-CH), 56.7 (14-CH), 74.7 (3-CH), 81.9 (C(CH₃)₃), 122.5 (6-CH), 139.7 (5-C), 155.9 (NCOO); m/z (FAB +ve) 566 (M + Na⁺), 369 (Chol); FAB-MS m/z for C₃₄H₅₈N₁O₄ (M + H⁺) calculated 544.4370, found 544.4366.

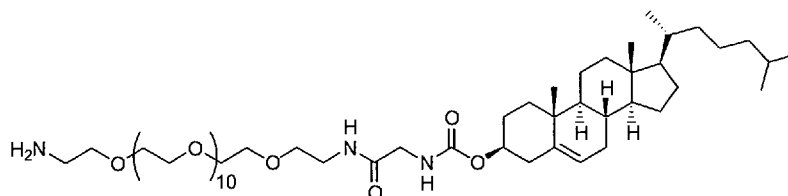
N-Cholesteryloxycarbonyl amino acetic acid (Cholesterol-*N*-Glycine) (**124**)



Cholesterol-*N*-Glycine-*tert* butyl ester **125** (2 g, 3.67 mmol) was dissolved in CH₂Cl₂ (25 mL). TFA (25 mL) was added to the reaction mixture and the resulting solution was stirred at room temperature under a positive flow of nitrogen. Once TLC had indicated the reaction had gone to completion (~ 4 h) the solvents were removed *in vacuo* and the residues were freeze dried giving the title compound as a white solid (1.73 g, 97%). FTIR (nujol) $\nu_{\max}$ 3329, 3024, 2925, 2654, 1755, 1667, 1572 cm⁻¹. ¹H NMR (CDCl₃) δ 0.67 (3 H, s, 18-CH₃), 0.84 - 0.87, 0.85 - 0.88 (2 $\times$ 3 H, d, J = 6.4 Hz, overlapping 1.6 Hz, 27-CH₃, 26-CH₃), 0.90 – 0.92 (3 H, d, J = 6.4 Hz, 21-CH₃), 1.00 (3 H, s, 19-CH₃), 0.97 – 1.63 (21 H, m, 1-CH₂, 9-CH, 11-CH₂, 12-CH₂, 14-CH, 15-CH₂, 16-CH₂, 17-CH, 20-CH, 22-CH₂, 23-CH₂, 25-CH), 1.46 (9 H, s, (CH₃)₃), 1.76-2.40 (7 H, m, 2-CH₂, 4-CH₂, 7-CH₂, 8-CH), 3.83 (2 H, d, J = 5.2 Hz, CH₂COO^tBu), 4.45 - 4.57 (1 H, m, 3-CH), 5.05 - 5.16 (1 H, s, br, NH), 5.33 - 5.39 (1 H, m, 6-CH). ¹³C NMR (CDCl₃) δ 11.8 (18-CH₃), 18.7 (21-CH₃), 19.3 (19-CH₃), 21.0 (11-CH₂), 22.6 (26-CH₃), 22.8 (27-CH₃), 23.8 (23-CH₂), 24.2 (15-CH₂), 27.9 (25-CH), 28.0 (C(CH₃)₃), 28.1 (2-CH₂), 28.2 (16-CH₂), 31.8 (7-CH₂), 31.9 (8-CH), 35.7 (20-CH), 36.1 (22-CH₂), 36.5 (10-C), 36.9 (1-CH₂), 38.4 (24-CH₂), 39.5 (4-CH₂), 39.7 (12-CH₂), 42.3 (13-C), 43.3 (CH₂COO^tBu), 49.9 (9-CH), 56.1 (17-CH), 56.7 (14-CH), 74.7 (3-CH), 81.9 (C(CH₃)₃), 122.5 (6-CH), 139.7 (5-C),

155.9 (NCOO); m/z (FAB +ve) 510 ($M + Na^+$), 369 (Chol); FAB-MS m/z for $C_{30}H_{49}N_1O_4Na$ ($M + Na^+$) calculated 510.3559, found 510.3553.

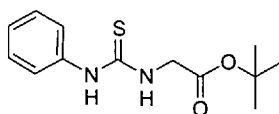
Cholesterol-Glycine-PEG₁₁-NH₂ (122)



Cholesterol-Glycine **124** (134 mg, 0.27 mmol) was dissolved in dry CH_2Cl_2 (25 mL). O-(2-aminoethyl)-O-[2-(Boc-amino)ethyl]decaethylene glycol (174 mg, 0.27 mmol), HBTU (113 mg, 0.29 mmol) and NEt_3 (82 mL, 113 μ L, 0.81 mmol) were added to the solution and the reaction was stirred at room temperature under argon. The progress of the reaction was monitored by TLC. Once the reaction had gone to completion citric acid (10 mL, 7% solution) was added. The organic phase was extracted and subsequently re-extracted with citric acid (10 mL, 7% solution). The organic phases were combined, dried ($MgSO_4$) and reduced *in vacuo*. The resulting oil was dissolved in CH_2Cl_2 (5 mL). TFA (5 mL) was added to the solution and the reaction was stirred at room temperature under a positive flow of nitrogen. When the reaction had gone to completion the solvents were removed *in vacuo* to give a yellow oil. The crude product was purified by silica gel column chromatography to give the title compound as a yellow oil (189 mg, 69%). FTIR (film) ν_{max} 3423, 2934, 2511, 1679, 1537, 1467, 1350, 1298, 1250, 1202, 1178, 1131 cm^{-1} ; ^1H NMR ($CDCl_3$: MeOD (2:1 v/v) δ 0.44 (3 H, s, 18- CH_3), 0.60 - 0.61, 0.60 - 0.62 (2 $\times$ 3 H, d, J = 6.8 Hz, overlapping 1.6 Hz, 27- CH_3 , 26- CH_3), 0.66 - 0.67 (3 H, d, J = 6.4 Hz, 21- CH_3), 0.77 (3 H, s, 19- CH_3), 0.83 - 1.40 (21 H, m, 1- CH_2 , 9-CH, 11- CH_2 , 12- CH_2 , 14-CH, 15- CH_2 , 16- CH_2 , 17-CH, 20-CH, 22- CH_2 , 23- CH_2 , 25-CH), 1.52 - 1.81 (5 H, m, 2- CH_2 , 7- CH_2 , 8-CH), 2.02 - 2.12 (2 H, m, 4- CH_2), 3.16 (2 H, t, H_2NCH_2 J = 5.2 Hz), 3.31 (2 H, t, OCH_2CH_2NHCO , J = 5.2 Hz), 3.34 - 3.48 (40 H, m, 20 $\times$ CH_2 PEG), 3.49 - 3.57 (6 H, m, $H_2NCH_2CH_2$, OCH_2CH_2NHCO and CH_2CONH), 4.15 - 4.25 (1 H, m, 3-CH), 5.08 - 5.13 (1 H, m, 6-CH); ^{13}C NMR ($CDCl_3$: MeOD (2: 1 v/v) δ 11.2 (18- CH_3), 18.1 (21- CH_3), 18.6 (19- CH_3), 21.5 (11- CH_2), 21.8 (26- CH_3), 22.1 (27- CH_3), 23.3 (23- CH_2), 23.7 (15- CH_2), 27.4 (25-CH), 27.6 (2- CH_2), 27.7 (16- CH_2), 31.3 (7- CH_2 and 8-CH overlapping), 35.3 (20-CH), 35.7 (22- CH_2), 36.1 (10-C), 36.5 (1- CH_2), 38.0 (24- CH_2), 38.6 (12- CH_2), 38.9 (H_2NCH_2 and OCH_2CH_2NHCO overlapping), 39.3 (4- CH_2), 41.8 (13-C), 43.4 (CH_2CONH), 49.6 (9-CH), 55.7 (17-CH), 56.2 (14-CH),

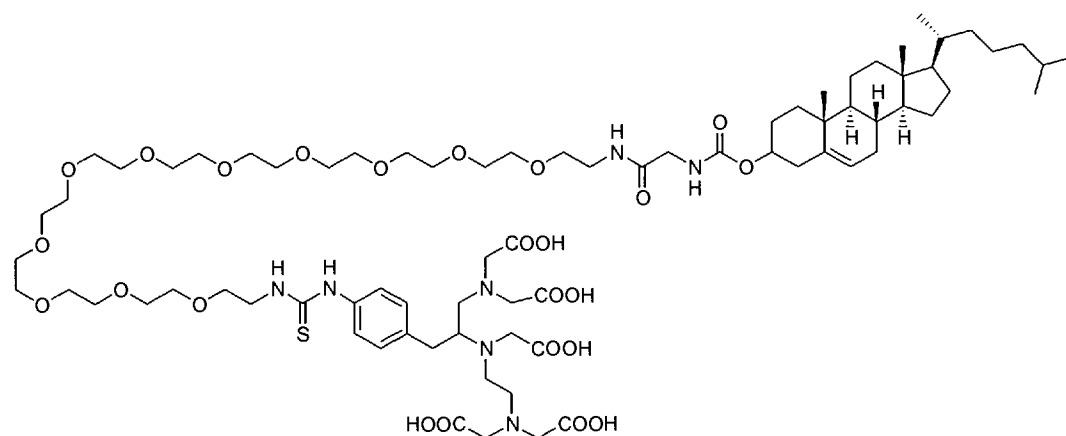
66.2 ($\text{H}_2\text{NCH}_2\text{CH}_2$ and $\text{OCH}_2\text{CH}_2\text{NHCO}$ overlapping), 68.9, 69.1, 69.2, 69.3, 69.4, 69.5, 69.6, 69.7, 69.8, 69.9 ($20 \times \text{CH}_2$ PEG), 74.5 (3-CH), 122.1 (6-CH), 139.2 (5-C), 156.5 (NCOO), 170.1 (NHCO); m/z (ESI +ve) 1014 ($\text{M} + \text{H}^+$); HPLC analysis: $t_R = 26.0$ min, column Vydac C-4 peptide: gradient H_2O (0.1% TFA)/MeCN (0.1% TFA)/MeOH, 0 min [100/0/0], 1-15 min [0/100/0], 25 min [0/100/0], 25.1 min [0/0/100], 45 min [0/0/100], 45.1 min [100/0/0], 55 min [100/0/0]; flow 1 mL/min.

3-Phenyl-thioureido-acetic acid tert-butyl ester (128)



Phenylisothiocyanate (100 mg, 91 μL , 0.84 mmol), t-butyl glycine (110 mg, 0.84 mmol) and NEt_3 (254 mg, 351 μL , 2.52 mmol) were dissolved in CH_2Cl_2 . The resulting solution was stirred at room temperature for 4 hours. The solvents and excess base were removed in vacuo to give the title compound as a pale oil (220 mg, 99%); ^1H NMR (CDCl_3) 1.37 (9 H, s, $\text{C}(\text{CH}_3)_3$), 4.23 (2 H, s, CH_2), 7.0 – 7.1 (1 H, m, CH aromatic), 7.2 – 7.3 (2 H, m, 2 x CH aromatic), 7.3 – 7.4 (2 H, m, 2 x CH aromatic); ^{13}C NMR (CDCl_3) 27.5 ($\text{C}(\text{CH}_3)_3$), 46.6 (CH_2), 81.6 ($\text{C}(\text{CH}_3)_3$), 123.6 (2 x CH aromatic), 125.3 (1 x CH aromatic), 128.8 (2 x CH aromatic), 137.6 (1 x CH aromatic), 168.4 (COO^tBu), 180.7 ($\text{NHC}(=\text{S})\text{NH}$); m/z (FAB +ve) 267 ($\text{M} + \text{H}^+$); FAB MS m/z calculated for $\text{C}_{13}\text{H}_{19}\text{N}_2\text{O}_2\text{S}$ ($\text{M} + \text{H}$) 267.1167, found 267.1167.

Chol.Gly.PEG₁₁NHC(S)NH.Bn.DTPA (129)



Cholesterol.Gly.PEG₁₁.NH₂ **125** (60 mg, 0.04 mmol), SCN.Bn.DTPA **115** (21 mg, 0.04 mmol) and NEt₃ (40 mg, 56 μ L, 0.4 mmol) were dissolved in CH₂Cl₂ and MeOH (1:1 v/v, 10 mL). The resulting solution was stirred at room temperature for 4 h. The solvents and excess base were removed *in vacuo* to give the title compound as a yellow oil (60 mg, 99%). FTIR (CDCl₃) ν_{max} 3374, 2930, 2498, 2249, 2071, 1632, 1469, 1399, 1121 cm⁻¹; ¹³C NMR (CDCl₃: MeOD (2:1 v/v)) 11.2 (18-CH₃), 18.1 (21-CH₃), 18.7 (19-CH₃), 20.2 (11-CH₂), 21.9 (27-CH₃), 22.1 (26-CH₃), 23.3 (23-CH₂), 23.7 (15-CH₂), 27.5 (25-CH), 27.7 (16-CH₂), 27.6 (2-CH₂), 31.4 (7-CH₂ and 8-CH overlapping), 32.5 (ArCH₂), 35.3 (20-CH), 35.7 (22-CH₂), 36.1 (10-C), 36.5 (1-CH₂), 38.0 (24-CH₂), 38.7 (4-CH₂), 39.0 (CH₂NHC(S)NH and CH₂NHCOCH₂NH overlapping), 39.3 (12-CH₂), 41.8 (13-C), 43.8 (NHCOCH₂NH and NCH₂CH₂N overlapping) 49.6 (9-CH), 50.5 (CH₂CH₂NHC(S)NH), 53.7 (NCH₂CH₂N), 54.5 (2 x CH₂COOH), 56.4 (2 x CH₂COOH and NCHCH₂N), 55.7 (17-CH), 56.2 (14-CH), 61.9 (ArCH₂CH), 68.9 (CH₂CH₂NHC(S)NH and CH₂CH₂NHCOCH₂NH overlapping), 69.6 – 69.9 (20 x CH₂ PEG), 74.6 (3-CH), 122.2 (6-CH), 123.4 (2 x CH aromatic), 124.0 (2 x CH aromatic), 129.1 (C aromatic), 139.2 (C aromatic), 141.3 (5-C), 156.3 (NHC(O)O), 169.9 (2 x COOH), 170.9 (1 x COOH), 173.7 (2 x COOH and NHCOCH₂); *m/z* (FAB +ve) 1554 (M+H); HPLC analysis: *t_R* = 26.5 min, column Vydac C-4 peptide: gradient H₂O (0.1% TFA)/MeCN (0.1% TFA)/MeOH, 0 min [100/0/0], 1-15 min [0/100/0], 25 min [0/100/0], 25.1 min [0/0/100], 45 min [0/0/100], 45.1 min [100/0/0], 55 min [100/0/0]; flow 1 mL/min.

7.10 MRI Analysis of Gd.Lipids

Solutions of Gd.DOTA.Chol, Gd.DOTA.DSPE, Gd.DTPA.Chol, Gd.DOTA and Gd.DTPA (and controls of the metal free compounds) were dissolved in appropriate solvents to give a final concentration of 0.5 mM. The solutions (200 μ L) were placed in PCR tubes and imaged (T_1 weighted MRI, spin echo sequence: TR = 50, 100, 200, 300, 500, 700, 1200, 3000, 5000, 7000 ms, TE = 15 ms, number of signals averages = 2, 1 slice coronal (2 mm thick). Field of view 70 cm², collected into a matrix of 256 x 128). To ensure a fair comparison of the efficacy of each compound a blank tube containing the solvent each compound was dissolved in was also imaged.

7.11 General Liposome Preparation

All lipids were stored as stock solutions in anhydrous organic solvents (CHCl₃, MeOH or mixture of both), at – 20 °C under argon. Liposomes were made with a variety of defined molar ratios of individual lipids to give a variety of defined total lipid concentration in various defined buffers.

Appropriate volumes of each lipid stock were placed in a round bottom flask (typically 10 mL) containing distilled CH₂Cl₂ (1 mL) and swirled to ensure thorough mixing of the lipids. The solvent was removed slowly *in vacuo* to ensure production of an even lipid film. The film was re-hydrated with buffer (defined volume). The resulting solution was sonicated for 1 - 10 min, in order for the liposomes to form. The pH of the liposomal suspension was checked by pH Boy (Camlab Ltd., Over, Cambridgeshire, UK) and if appropriate adjusted by addition of aqueous solutions of NaOH and HCl. For each preparation, the size distribution of liposomes was measured by photon correlation spectroscopy (PCS). Fluorescent liposomes always contained 0.5 mol% DSPE-Rhodamine as a fluorophore.

7.12 *In vivo* Experiments

Intramuscular Injections

Female C57BL mice (20 g, Harlan UK) were anaesthetised with isoflourane/oxygen mix. The mice were imaged (T_1 weighted MRI, spin echo sequence: TR = 850 ms, TE = 20 ms, number of signals averages = 8.34 serial transverse MRI slices (2 mm thick). Field of view 50 cm², collected into a matrix of 256 x 128) before and after (t = 0, 24 56

and 648 h) intramuscular injection of Gd.DOTA.Chol (6 mg, 0.006 mmol) in a saline vehicle (200 μ L) into the left hind leg. A control vehicle of saline only was injected into the right leg.

7.13 Cell labelling Experiments

In Vitro Uptake Experiments

Twenty four hours prior to liposome uptake, adherent HeLa cells were grown in Dulbecco's-Modified Eagle culture medium (DMEM) with 10% Fetal calf serum (FCS, sigma) and 1% penicillin-streptomycin (sigma), in either 48-well (3.5×10^4 cells per well, 250 μ L of complete medium) or 6-well (2.5×10^5 cells per well, 3 mL of complete medium) plates, in a wet (37 °C) 10% CO₂ / 90% air atmosphere. The cells were grown until 80% confluent. The media was then removed and replaced with fresh media (same volume as previous). Fluorescent liposomes (6 μ g (5 μ L of 1.2 mgml⁻¹ liposomes), 12 μ g (10 μ L of 1.2 mgml⁻¹ liposomes) or 18 μ g (15 μ L of 1.2 mgml⁻¹ liposomes) doses for 48-well plate, 173 μ g (144 μ L of 1.2 mgml⁻¹ liposomes) dose for 6-well plate) were then added to each well, swirled to ensure even dispersion and then incubated at in a wet (37 °C) 10 % CO₂ / 90% air atmosphere for either 4, 8 or 24 hr.

After liposome uptake the cells were washed with PBS (2 x 200 μ L) and then treated with lysis buffer (100 μ L per well). Cell lysate (50 μ L) was diluted with MeOH (50 μ L) and CHCl₃ (300 μ L). The resulting solution was vigorously vortexed and then allowed to separate into 2 phases (organic and aqueous). The organic layer was isolated and its fluorescence was recorded on a RF-5301PC Shimadzu spectrofluorophotometer (λ_{ex} 560 nm, λ_{em} 573nm). As a positive control liposomes (6, 12 or 18 μ g for 48 well plate, 173 μ g for 6-well plate) were added to the lysate of untreated cells, providing a 100% liposome uptake result. As a negative control liposomes (6, 12 or 18 μ g for 48 well plate, 173 μ g for 6-well plate) were added to wells containing no cells. These should be eliminated during the PBS washing step, providing a baseline liposome uptake result.

MRI of cells

Twenty four hours prior to liposome uptake, adherent HeLa cells were grown in Dulbecco's-Modified Eagle culture medium (DMEM) with 10% Fetal calf serum (FCS, sigma) and 1% penicillin-streptomycin (sigma), in a 6-well plate (2.5×10^5 cells per well, 3 mL of complete medium) plates, in a wet (37 °C) 10% CO₂ / 90% air

atmosphere. The cells were grown until 80% confluent. The media was then removed and replaced with fresh media (same volume as previous). Gd.Liposomes (173 μg dose (144 μL of 1.2 mgml^{-1} liposomes)) were then added to each well, swirled to ensure even dispersion and then incubated at in a wet (37 °C) 10 % CO_2 / 90% air atmosphere for 8 hr.

After liposome uptake the cells were washed with PBS (2 x 2 mL) and then treated with trypsin-EDTA (200 μL , 0.25% trypsin, 1mM EDTA) for 1 min at 37 °C. DMEM (complete, 2 mL) was added to neutralise the trypsin. The cells were counted and 1×10^6 were washed with PBS and then spun down in PBS buffer (1 mL) into a pellet in an eppendorf tube.

The resulting pellets were imaged at 4.7 T (T_1 weighted MRI, spin echo sequence: (gradient TE = 15 ms TR = 50, 100, 200, 300, 500, 700, 1200, 3000, 5000, 7000 ms number of signals averages = 4, Single sagittal slice 10 mm thick, Field of view 602 mm x 60mm,, collected into a matrix of 256 x 128). As negative controls; cells treated with liposomes containing no Gd.Lipid (substituted for cholesterol), untreated cells and an eppendorf containing PBS buffer were imaged.

LD (Liposome:DNA complex) Formation

Plasmid DNA containing the β -galactosidase gene (pNGVL1-nt-beat-gal 7.53 kbp) was stored as frozen aliquots at -80°C, at a concentration of 1.2 mg/ml. LD complexes were made by addition of DNA to a continuously vortexing solution of cationic liposomes (1.2 mg/ml total lipid concentration) to give a final ratio of 12:1 (w/w) liposome to DNA. The size distribution was measured by PCS.

LD Transfection

Twenty four hours prior to transfection, adherent HeLa cells were grown in Dulbecco's-Modified Eagle culture medium (DMEM) with 10% Fetal calf serum (FCS, sigma) and 1% penicillin-streptomycin (sigma), in 48-well (3.5×10^4 cells per well, 250 μL of complete medium) plates, in a wet (37 °C) 10% CO_2 / 90% air atmosphere. The cells were grown until 80% confluent. The media was then removed and replaced with fresh media (same volume as previous).

LD complexes (10 μ L, (12.5 μ g of pDNA)) were added to each well, swirled to ensure even distribution, and then incubated in a wet (37 °C) 10 % CO₂ / 90% air atmosphere for 3 hr. The media was removed, the cells washed with PBS and then replaced with fresh media (250 μ L). The cells were then incubated for a further 24 hr. Before β -galactosidase activity was measured the cells were washed with PBS and harvested with lysis buffer (150 μ L).

β -Galactosidase and Total Protein Assays

After lysis, equal amounts, 50 μ L and 20 μ L, of each cell suspension were used for determination of β -galactosidase activity and total cellular protein (to normalise results), respectively.

In the β -galactosidase assay, 100 μ L of the substrate reagent was added to 50 μ L of the cell suspension in a white 96 well plate (corning costar). The plate was incubated for 30min at room temperature. Automatic initiation (enhancement of enzymatic activity produced upon addition substrate reagent) was performed by a microplate luminometer (Anthos Lucy 1, Labtech International Ltd., Rigmer, East Sussex, UK) which injected 50 μ L initiation reagent and enzymatic activity was measured over the subsequent 30 s.

The amount of cellular protein was quantified in a BCA assay (Pierce, Rockford, IL, USA) using 20 μ L of the cell lysate or bovine serum albumin as internal calibration standard and adding 200 μ L of BCA reagent (according to suppliers protocol). Following a period of 30 min at room temperature, the colorimetric measurement was performed at 570 nm by means of a microplate reader (Anthos Lucy 1). β -Galactosidase activity was normalised by the results of the total protein determination assays, and expressed as RLU (relative light units) / μ g of protein.

Lactate Dehydrogenase Toxicity Assay

The CytoTox-96 assay (Promega, Madison, WI, USA) evaluates cellular cytotoxicity by assessing the release of lactate dehydrogenase (LDH) into culture medium as a consequence of damaged cell membranes compared to total LDH present in the cells.

Twenty four hours prior to the assay, adherent HeLa cells were grown in Dulbecco's-Modified Eagle culture medium (DMEM) with 10% Fetal calf serum (FCS, sigma) and

1% penicillin-streptomycin (sigma), in a 48-well (3.5×10^4 cells per well, 250 μL of complete medium) plates, in a wet (37 °C) 10% CO_2 / 90% air atmosphere. The cells were grown until 80% confluent. The media was then removed and replaced with fresh media (same volume as previous). Liposomes (6, 12 or 18 μL doses) or LD's (10 μL (12.5 μg of DNA) dose) were then added to each well, swirled to ensure even dispersion and then incubated at in a wet (37 °C) 10 % CO_2 / 90% air atmosphere for either a) Liposomes: 4 hr, 4 (24) hr, 8, 8 (24) hr or 24 hr; b) LD's: 3 (24) hr.

Measuring LDH Release

Growth media (200 μL) from each well was transferred into a 96 well plate and centrifuged (3600 rpm for 10 minute). The resulting supernatant (50 μL) was transferred to a white 96 well plate (corning costar). Reconstituted substrate mix (50 μL) (made as directed in manufacturers protocol) was added to each well. The plate was placed in an opaque box and incubated for 30 min at room temperature. Stop solution (50 μL) was added to each well and the absorbance at 490 nm was measured by means of a microplate reader (Anthos Lucy 1).

Measuring Cell Internal LDH

Growth media was removed from each well and the cells were washed with PBS. Lysis buffer (250 μL) was added to each well. Cell lysate (50 μL) was transferred to a white 96 well plate (corning costar). Reconstituted substrate mix (50 μL) (made as directed in manufacturers protocol) was added to each well. The plate was placed in an opaque box and incubated for 30 min at room temperature. Stop solution (50 μL) was added to each well and the absorbance at 490 nm was measured by means of a microplate reader (Anthos Lucy 1). Cytotoxicity was calculated as a ratio LDH released versus total LDH content.

Stability Studies

Liposomes (1.2 mg/ml) or LD particles (1.2 mg/ml, 12:1 (w/w) Lipid:DNA ratio) were placed in Fetal calf serum (1:1 v/v) and incubated at 37 °C over 90min. Size distribution of the particles (as measured by PCS) was used as a measure of stability and was measured at defined time points over the experiment ($t = 0, 10, 20, 30, 60, 90$ min).

Microscopy Experiments

Twenty four hours prior to liposome uptake, adherent IGROV-1 cells were grown in Dulbecco's-Modified Eagle culture medium (DMEM) with 10% Fetal calf serum (FCS, sigma) and 1% penicillin-streptomycin (sigma), in 6-well (2.5×10^5 cells per well, 3 mL of complete medium) plates fitted with glass slides, in a wet (37 °C) 10% CO₂ / 90% air atmosphere. The cells were grown until 80% confluent. The media was then removed and replaced with fresh media (same volume as previous). Fluorescent liposomes (173 µg (144 µL of 1.2 mgml⁻¹ liposomes) per well) were then added to each well, swirled to ensure even dispersion and then incubated at in a wet (37 °C) 10 % CO₂ / 90% air atmosphere for either 0, 0.5, 4 or 24 hr.

After liposome uptake the media was removed and the cells were washed with PBS (x 2), Heparin (x 1 (20 mg/ml)), PBS (x 2), PFA (x 1 (20 min at 37 °C)), PBS (x 2), Glycine (x 1 (20 mM, 20 min at 37 °C)), PBS (x 2). The glass slides were removed from the wells and fixed to microscopy slides. All slides were examined by epi fluorescent microscopy and the 4 hr slide was also examined by confocal microscopy.

7.14 Gadolinium Post coating of Liposomes

Liposomes containing Chol.Gly.PEG₁₁.NH₂ (35 mol%) were made following the protocol detailed in general liposome formulation to give a total lipid concentration of 6 mg/ml in water. The liposomes were diluted (1:1 (v/v)) with 0.1 M carbonate-bicarbonate buffer (pH 9.6) containing a stiochiometric amount of Gd.SCN.Bn.DTPA. The resulting solution was incubated at 37 °C for 12 h. The post coating of the liposomes was monitored by HPLC (column Vydac C-4 peptide: gradient H₂O (0.1% TFA)/MeCN (0.1% TFA)/MeOH, 0 min [100/0/0], 1-15 min [0/100/0], 25 min [0/100/0], 25.1 min [0/0/100], 45 min [0/0/100], 45.1 min [100/0/0], 55 min [100/0/0]; flow 1 mL/min.) and ESI-MS.

The resulting liposomes were imaged (T₁ weighted MRI, spin echo sequence: (gradient TE = 15 ms TR = 100, 200, 300, 500, 700, 1200, 3000, 5000, 7000 ms number of signals averages = 4, Single sagittal slice 10 mm thick, Field of view 70 mm x 70mm., collected into a matrix of 256 x 128) against control samples of liposomes made with Gd.DOTA.Chol (35 mol%).

CHAPTER EIGHT

References

1. Weissleder, R. & Mahmood, U. (2001) *Radiology* **219**, 316-333.
2. Herschman, H. R. (2004) *Journal of Nuclear Cardiology* **11**, 210-214.
3. Herschman, H. R. (2003) *Science* **302**, 605-608.
4. Jacobs, A., et al. (2001) *Lancet* **358**, 727-729.
5. Harrington, K. J., et al. (2001) *Clinical Cancer Research* **7**, 243-254.
6. Hofstra, L., et al. (2000) *Lancet* **356**, 209-212.
7. Anderson, W. F. (1984) *Science* **226**, 401-409.
8. Larkin, M. (2003) *Lancet Oncology* **4**, 455-455.
9. Zinn, K. R., et al. (1998) *Gene Therapy* **5**, 798-808.
10. Murugesan S, S. G., Gambhir S (2003) *Molecular Imaging* **2**, 288.
11. Sundaresan G, M. S., Gambhir S (2003) *Molecular Imaging* **2**, 302.
12. Nichol, C. A., et al. (1999) *Drug Delivery* **6**, 187-194.
13. de Marco, G., et al. (1998) *Radiology* **208**, 65-71.
14. Bogdanov, A., et al. (2001) *Gene Therapy* **8**, 515-522.
15. Nichol, C. & Kim, E. E. (2001) *Journal of Nuclear Medicine* **42**, 1368-1374.
16. Alfke H, B. M., Heverhagen J T, Wagner E (2002) *Molecular Imaging* **1**, 233.
17. Schellingerhout, D., et al. (2000) *Gene Therapy* **7**, 1648-1655.
18. Min, J. J. & Gambhir, S. S. (2004) *Gene Therapy* **11**, 115-125.
19. Tjuvajev, J. G., et al. (1999) *Cancer Research* **59**, 5186-5193.
20. Tjuvajev, J. G., et al. (2002) *Journal of Nuclear Medicine* **43**, 1072-1083.
21. Louie, A. Y., et al. (2000) *Nature Biotechnology* **18**, 321-325.
22. Moore, A., et al. (1998) *Biochimica Et Biophysica Acta-Molecular Cell Research* **1402**, 239-249.
23. Hogemann, D., et al. (2000) *Bioconjugate Chemistry* **11**, 941-946.
24. Bogdanov, A., et al. (1997) *Nuclear Medicine and Biology* **24**, 739-742.
25. Pfeifer, A., et al. (2001) *Molecular Therapy* **3**, 319-322.
26. Contag, C. H. & Ross, B. D. (2002) *Journal of Magnetic Resonance Imaging* **16**, 378-387.
27. Yang, M., et al. (2000) *Proceedings of the National Academy of Sciences of the United States of America* **97**, 12278-12282.
28. Contag, P. R., et al. (1998) *Nature Medicine* **4**, 245-247.
29. Van de Wiele, C. (2004) *European Journal of Nuclear Medicine and Molecular Imaging* **31**, 151-154.
30. Tatsch, K., et al. (1997) *European Journal of Nuclear Medicine* **24**, 415-421.
31. Popperl, G., et al. (2004) *Journal of Neural Transmission* **111**, 1041-1052.
32. Haubner, R., et al. (2001) *Journal of Nuclear Medicine* **42**, 326-336.
33. Van de Wiele, C., et al. (2003) *Nuclear Medicine Communications* **24**, 945-950.

34. Narula, J., Kietselaer, B. & Hofstra, L. (2004) *Journal of Nuclear Cardiology* **11**, 349-357.
35. Blankenberg, F. G., et al. (1998) *Proceedings of the National Academy of Sciences of the United States of America* **95**, 6349-6354.
36. Blankenberg, F. G., et al. (2000) *Chest* **117**, 834-840.
37. Ogura, Y., et al. (2000) *Radiology* **214**, 795-800.
38. Britz-Cunningham, S. H. & Adelstein, S. J. (2003) *Journal of Nuclear Medicine* **44**, 1945-1961.
39. Yang, D. J., et al. (2001) *Cancer Biotherapy and Radiopharmaceuticals* **16**, 73-83.
40. Russell, J., et al. (2002) *Journal of Nuclear Medicine* **43**, 671-677.
41. Murakami, Y., et al. (2004) *European Journal of Nuclear Medicine and Molecular Imaging* **31**, 469-474.
42. Schellenberger, E. A., et al. (2002) *Academic Radiology* **9**, S310-S311.
43. Jung, H. I., et al. (2004) *Bioconjugate Chemistry* **15**, 983-987.
44. Zhao, M., et al. (2001) *Nature Medicine* **7**, 1241-1244.
45. Haberkorn, U., et al. (2001) *Nuclear Medicine and Biology* **28**, 793-798.
46. Asan, E. (2002) *Histochemistry and Cell Biology* **118**, 507-525.
47. Bulte, J. W. M., et al. (1999) *Proceedings of the National Academy of Sciences of the United States of America* **96**, 15256-15261.
48. Bendszus, M. & Stoll, G. (2003) *Journal of Neuroscience* **23**, 10892-10896.
49. Adonai, N., et al. (2002) *Proceedings of the National Academy of Sciences of the United States of America* **99**, 3030-3035.
50. Dubey, P., et al. (2003) *Proceedings of the National Academy of Sciences of the United States of America* **100**, 1232-1237.
51. Modo, M., et al. (2004) *Neuroimage* **21**, 311-317.
52. Zelivyanskaya, M. L., et al. (2003) *Journal of Neuroscience Research* **73**, 284-295.
53. Weninger, W., Manjunath, N. & von Andrian, U. H. (2002) *Immunological Reviews* **186**, 221-233.
54. Hardy, J., et al. (2001) *Experimental Hematology* **29**, 1353-1360.
55. Gupta, A. K. & Curtis, A. S. G. (2004) *Biomaterials* **25**, 3029-3040.
56. Suwa, T., et al. (1998) *International Journal of Cancer* **75**, 626-634.
57. Zhao, M., et al. (2002) *Bioconjugate Chemistry* **13**, 840-844.
58. Mulder, W. J. M., et al. (2004) *Bioconjugate Chemistry* **15**, 799-806.
59. Bulte, J. W. M. a. C. d. M. (2003) *Methods in Enzymology* **373**, 175 -198.
60. Jacobs, R. E. & Fraser, S. E. (1994) *Science* **263**, 681-684.

61. Hotee, S., Changani, K., White, A., Bhakoo, K., Bell, J. (2004) *Proceedings of the International Society Magnetic Resonance Imaging* **11**, 1739.
62. Schichinohe, H., Kuroda, S., Lee, J-B., Nishimura, G., Yano, S., Seki, T., Ikeda, J., Tamura, M., Iwasaki, Y. (2004) *Brain Research Protocols* **13**, 166-175.
63. Kraitchman, D. L., *et al.* (2003) *Circulation* **108**, 3175.
64. Walter, G. A., *et al.* (2004) *Magnetic Resonance in Medicine* **51**, 273-277.
65. Hoehn, M., *et al.* (2002) *Proceedings of the National Academy of Sciences of the United States of America* **99**, 16267-16272.
66. Weissleder, R. (2002) *Nature Reviews Cancer* **2**, 11-18.
67. Schild, P. D. H. H. (1990) *MRI made easy* (Schering AG, Berlin/Bergkamen).
68. van Geuns, R. J. M., *et al.* (1999) *Progress in Cardiovascular Diseases* **42**, 149-156.
69. Gries, H. (2002) in *Contrast Agents I*, Vol. 221, pp. 1-24.
70. Wang, Y. X. J., Hussain, S. M. & Krestin, G. P. (2001) *European Radiology* **11**, 2319-2331.
71. Schwert, D. D., Davies, J. A. & Richardson, N. (2002) in *Contrast Agents I*, Vol. 221, pp. 165-199.
72. Caravan, P., *et al.* (1999) *Chemical Reviews* **99**, 2293-2352.
73. Helm, L. & Merbach, A. E. (1999) *Coordination Chemistry Reviews* **187**, 151-181.
74. Aime, S., *et al.* (1998) *Chemical Society Reviews* **27**, 19-29.
75. Toth, E., Helm, L. & Merbach, A. E. (2002) in *Contrast Agents I*, Vol. 221, pp. 61-101.
76. Hajela, S., *et al.* (2000) *Journal of the American Chemical Society* **122**, 11228-11229.
77. Aime, S., *et al.* (1993) *Magnetic Resonance in Medicine* **30**, 583-591.
78. Bogdanov, A., Matuszewski, L., Bremer, C., Petrovsky, A., Weissleder, R. (2002) *Molecular Imaging* **1**, 16-23.
79. Curtet, C., *et al.* (1998) *Investigative Radiology* **33**, 752-761.
80. Allen, M. J., *et al.* (2004) *Chemistry & Biology* **11**, 301-307.
81. Chen, J., Weissleder, R., Bogdanov, A.A. (2004) in *The Society of Molecular Imaging, Third Annual Meeting*, St. Louis, MO).
82. Kayyem, J. F., *et al.* (1995) *Chemistry & Biology* **2**, 615-620.
83. Moats, R. A., Fraser, S. E. & Meade, T. J. (1997) *Angewandte Chemie-International Edition in English* **36**, 726-728.
84. Sahoo, S. K. & Labhasetwar, V. (2003) *Drug Discovery Today* **8**, 1112-1120.
85. Lian, T. & Ho, R. J. Y. (2001) *Journal of Pharmaceutical Sciences* **90**, 667-680.

86. Moghimi, S. M. & Szebeni, J. (2003) *Progress in Lipid Research* **42**, 463-478.
87. Huwyler, J., Wu, D. F. & Pardridge, W. M. (1996) *Proceedings of the National Academy of Sciences of the United States of America* **93**, 14164-14169.
88. Allen, T. M. & Cullis, P. R. (2004) *Science* **303**, 1818-1822.
89. Gonzalez, G. M., et al. (2004) *Antimicrobial Agents and Chemotherapy* **48**, 2140-2143.
90. Glantz, M. J., et al. (1999) *Clinical Cancer Research* **5**, 3394-3402.
91. Uster, P. S., Working, P. K. & Vaage, J. (1998) *International Journal of Pharmaceutics* **162**, 77-86.
92. Bressler, N. M. (2002) *American Journal of Ophthalmology* **133**, 857-859.
93. Streith, S., Eichhorn, M.E., Sauer, B., Schulze, B., Teifel, M., Michaelis, U., Dellian, M. (2004) *International Journal of Cancer* **110**, 117-124.
94. Tomkinson, B., et al. (2003) *Leukemia Research* **27**, 1039-1050.
95. Maeda, H., et al. (2000) *Journal of Controlled Release* **65**, 271-284.
96. Harris, L., et al. (2002) *Cancer* **94**, 25-36.
97. Netti, P. A., et al. (1996) *Microvascular Research* **52**, 27-46.
98. de Menezes, D. E. L., et al. (2000) *Biochimica Et Biophysica Acta-Biomembranes* **1466**, 205-220.
99. Moreira, J. N., Gaspar, R. & Allen, T. M. (2001) *Biochimica Et Biophysica Acta-Biomembranes* **1515**, 167-176.
100. Pan, X. Q., et al. (2002) *Blood* **100**, 594-602.
101. Sapra, P. & Allen, T. M. (2003) *Progress in Lipid Research* **42**, 439-462.
102. Rowinsky, E. K., et al. (1993) *Seminars in Oncology* **20**, 1-15.
103. Pizzolato, J. F. & Saltz, L. B. (2003) *Lancet* **361**, 2235-2242.
104. Mukherjee, S., Ghosh, R. N. & Maxfield, F. R. (1997) *Physiological Reviews* **77**, 759-803.
105. Szoka, F. C., Xu, Y. H. & Zelphati, O. (1997) *Advanced Drug Delivery Reviews* **24**, 291-291.
106. Holland, J. W., Cullis, P. R. & Madden, T. D. (1996) *Biochemistry* **35**, 2610-2617.
107. Bally, M. B., et al. (1999) *Advanced Drug Delivery Reviews* **38**, 291-315.
108. Pastorino, F., et al. (2003) *Clinical Cancer Research* **9**, 4595-4605.
109. Dams, E. T. M., et al. (2000) *Journal of Nuclear Medicine* **41**, 622-630.
110. Ogiharaumeda, I. & Kojima, S. (1988) *Journal of Nuclear Medicine* **29**, 516-523.
111. Bulte, J. W. M., et al. (1999) *Journal of Magnetism and Magnetic Materials* **194**, 204-209.
112. Tilcock, C., et al. (1989) *Radiology* **171**, 77-80.

113. Hnatowich, D. J., *et al.* (1981) *Journal of Nuclear Medicine* **22**, 810-814.
114. Adzank, I. K., Seltzer, S.E., Slifkin, M., Blau, M., Adams, D.F. (1990) *Investigative Radiology* **25**, 1217.
115. Phillips, W. T. (1999) *Advanced Drug Delivery Reviews* **37**, 13-32.
116. Goins, B. A. & Phillips, W. T. (2001) *Progress in Lipid Research* **40**, 95-123.
117. Decuyper, M. & Joniau, M. (1988) *European Biophysics Journal with Biophysics Letters* **15**, 311-319.
118. Torchilin, V. P. (1998) in *Medical Applications of Liposomes*, ed. Lasic., P. (Elsevier Science, pp. 515-543.
119. Torchilin, V. P. (1997) *Advanced Drug Delivery Reviews* **24**, 301-313.
120. Lattuada, L. & Lux, G. (2003) *Tetrahedron Letters* **44**, 3893-3895.
121. Glogard, C., *et al.* (2002) *International Journal of Pharmaceutics* **233**, 131-140.
122. Unger, E. C., *et al.* (1989) *Radiology* **171**, 81-85.
123. Kabalka, G. W., *et al.* (1991) *Magnetic Resonance in Medicine* **19**, 406-415.
124. Trubetskoy, V. S., *et al.* (1995) *Magnetic Resonance Imaging* **13**, 31-37.
125. Kabalka, G., *et al.* (1987) *Radiology* **163**, 255-258.
126. Jacques, V. & Desreux, J. F. (2002) in *Contrast Agents I*, Vol. 221, pp. 123-164.
127. Leclercq, F., *et al.* (2003) *Bioconjugate Chemistry* **14**, 112-119.
128. Baker, W. C., *et al.* (1999) *Journal of Organic Chemistry* **64**, 2683-2689.
129. Bhattacharya, S. & Haldar, S. (2000) *Biochimica Et Biophysica Acta-Biomembranes* **1467**, 39-53.
130. Huang, J. Y., Buboltz, J. T. & Feigenson, G. W. (1999) *Biochimica Et Biophysica Acta-Biomembranes* **1417**, 89-100.
131. Israelachvili, J. N. (1992) *Intermolecular and Surface Forces* (Academic Press.
132. Richman, J. E., Atkins, T.J. (1974) *Journal of the American Chemical Society* **96**, 2268.
133. Weisman, G. R. & Reed, D. P. (1996) *Journal of Organic Chemistry* **61**, 5186-5187.
134. Herve, G., *et al.* (1999) *Tetrahedron Letters* **40**, 2517-2520.
135. Ferrari, M., *et al.* (2000) *Synthetic Communications* **30**, 15-21.
136. Athey, P. S. & Kiefer, G. E. (2002) *Journal of Organic Chemistry* **67**, 4081-4085.
137. Glomb, M. A. & Pfahler, C. (2001) *Journal of Biological Chemistry* **276**, 41638-41647.
138. Patinec, V., *et al.* (1995) *Tetrahedron Letters* **36**, 79-82.
139. Chuburu, F., Le Baccon, M. & Handel, H. (2001) *Tetrahedron* **57**, 2385-2390.
140. Guillaume, D. & Marshall, G. R. (1998) *Synthetic Communications* **28**, 2903-2906.

141. Tsai, S. W., *et al.* (2001) *Bioconjugate Chemistry* **12**, 264-270.
142. Lewis, M. R., *et al.* (2001) *Bioconjugate Chemistry* **12**, 320-324.
143. Schottelius, M., Schwaiger, M. & Wester, H. J. (2003) *Tetrahedron Letters* **44**, 2393-2396.
144. Mease, R. C., Mausner, L.F., Srivastava, S.C. (1995) (Associated Universities, Inc, Washington DC, USA).
145. Flaschka, H. A., Barnard, A.J. Jnr. (1967) *Chelates in Analytical Chemistry* (Marcel Dekker.
146. Oliver, M., Jorgensen, M. R. & Miller, A. D. (2004) *Synlett*, 453-456.
147. Reany, O., Gunnlaugsson, T. & Parker, D. (2000) *Chemical Communications*, 473-474.
148. McCue, K. P. & Morrow, J. R. (1999) *Inorganic Chemistry* **38**, 6136-6142.
149. Anderson, C. J. & Welch, M. J. (1999) *Chemical Reviews* **99**, 2219-2234.
150. Bridger, G. J., *et al.* (1995) *Journal of Medicinal Chemistry* **38**, 366-378.
151. Alcock, N. W., *et al.* (1984) *Journal of the Chemical Society-Dalton Transactions*, 1937-1943.
152. Helps, I. M., *et al.* (1989) *Tetrahedron* **45**, 219-226.
153. Nash, I. A., Bycroft, B. W. & Chan, W. C. (1996) *Tetrahedron Letters* **37**, 2625-2628.
154. Youngman, M. A. & Dax, S. L. (1997) *Tetrahedron Letters* **38**, 6347-6350.
155. Park, J. G., *et al.* (2004) *Journal of Combinatorial Chemistry* **6**, 407-413.
156. McNally, J. J., Youngman, M. A. & Dax, S. L. (1998) *Tetrahedron Letters* **39**, 967-970.
157. Hoekstra, W. J., *et al.* (1997) *Tetrahedron Letters* **38**, 2629-2632.
158. Lee, Y., *et al.* (2004) *Bioorganic & Medicinal Chemistry Letters* **14**, 2637-2641.
159. Kim, H. S., *et al.* (2004) *Bioconjugate Chemistry* **15**, 1095-1101.
160. Geall, A. J., *et al.* (2000) *Bioconjugate Chemistry* **11**, 314-326.
161. Cooper, R. G., *et al.* (1998) *Chemistry-a European Journal* **4**, 137-151.
162. Miller, A. D. (2003) *Current Medicinal Chemistry* **10**, 1195-1211.
163. Chesnoy, S. & Huang, L. (2000) *Annual Review of Biophysics and Biomolecular Structure* **29**, 27-47.
164. Zhang, S., Xu, Y., Wang, B., Qiao, W., Dongliang, L., Li, Z. (2004) *Journal of Controlled Release* **100**, 165-180.
165. Miller, A. D., Cooper, R.G., Etheridge, C.J. (1997), UK).
166. Keller, M., *et al.* (2003) *Biochemistry* **42**, 6067-6077.
167. Valentine, D. H. & Hillhouse, J. H. (2003) *Synthesis-Stuttgart*, 317-334.

168. Karigiannis, G. & Papaioannou, D. (2000) *European Journal of Organic Chemistry*, 1841-1863.
169. Gardner, R. A., Delcros, J-G., Konate, F., Breitbeil, F., Martin, B., Sigman, M., Huang, M., Phanstiel, O. (2004) *Journal of Medicinal Chemistry* **47**, 6055-6096.
170. Geall, A. J., Baugh, J.A., Loyevsky, M., Gordeuk, V.R., Al-Abed, Y., Bucala, R. (2004) *Bioconjugate Chemistry* **15**, 1161-1165.
171. Suzuki, I., *et al.* (2004) *Tetrahedron Letters* **45**, 1955-1959.
172. Bycroft, B. W., *et al.* (1993) *Journal of the Chemical Society-Chemical Communications*, 778-779.
173. Bycroft, B. W., *et al.* (1993) *Journal of the Chemical Society-Chemical Communications*, 776-777.
174. Oliver, M., Jorgensen, M. R. & Miller, A. (2004) *Tetrahedron Letters* **45**, 3105-3107.
175. Daldrop-Link, H. E., *et al.* (2003) *Radiology* **228**, 760-767.
176. Singh, D., Bisland, S.K., Kawamura, K., Gariepy, J. (1999) *Bioconjugate Chemistry* **10**, 745-754.
177. Antopolsky, M., Azhayeva, E., Tengvall, U., Auriola, S., Jaaskelainen, I., Ronkko, S., Honkakoski, P., Urtti, A., Lonnberg, H., Azhayev, A. (1999) *Bioconjugate Chemistry* **10**, 598-606.
178. Bayley, H. (1999) *Nature Biotechnology* **17**, 1066-1067.
179. Azzam, T., *et al.* (2002) *Macromolecules* **35**, 9947-9953.
180. Mehvar, R. (2000) *Journal of Controlled Release* **69**, 1-25.
181. Modo, M., *et al.* (2002) *Neuroimage* **17**, 803-811.
182. Felgner, J. H., *et al.* (1994) *Journal of Biological Chemistry* **269**, 2550-2561.
183. Keller, M., *et al.* (2003) *Chembiochem* **4**, 286-298.
184. Loughrey, H. C., *et al.* (1990) *Journal of Immunological Methods* **132**, 25-35.
185. Bourel-Bonnet, L., Gras-Masse, H. & Melnyk, O. (2001) *Tetrahedron Letters* **42**, 6851-6853.
186. Allen, T. M., *et al.* (1995) *Biochimica Et Biophysica Acta-Biomembranes* **1237**, 99-108.
187. Perouzel, E., *et al.* (2003) *Bioconjugate Chemistry* **14**, 884-898.
188. Harokopakis, E., *et al.* (1995) *Journal of Immunological Methods* **185**, 31-42.
189. Hermanson, G. T. (1996) *Bioconjugate Techniques* (Academic Press).
190. Corson, D. T. & Meares, C. F. (2000) *Bioconjugate Chemistry* **11**, 292-299.
191. Brucher, E. (2002) in *Contrast Agents I*, Vol. 221, pp. 103-122.
192. Keller, M. (2004).

193. Wood, M. R., Kim, J. Y. & Books, K. M. (2002) *Tetrahedron Letters* **43**, 3887-3890.
194. Tsunoda, T., *et al.* (1996) *Tetrahedron Letters* **37**, 2457-2458.
195. Henry, J. R., *et al.* (1989) *Tetrahedron Letters* **30**, 5709-5712.
196. Rolla, F. (1982) *Journal of Organic Chemistry* **47**, 4327-4329.
197. Gibson, M. S., Bradshaw, R.W. (1968) *Angewandte Chemie-International Edition in English* **7**, 919-930.
198. Larock, R. C. (1999) *Comprehensive Organic Transformations* (John Wiley and Sons).
199. Salvatore, R. N., Yoon, C. H. & Jung, K. W. (2001) *Tetrahedron* **57**, 7785-7811.

APPENDIX

9.1 Lipids

Lipid Shape and Consequences for Packing

When lipids are dispersed in aqueous media at concentrations above the Critical micelle concentration (CMC) they self assemble and form aggregates. The principle driving force for this aggregation is the hydrophobic effect. However the overall structure of aggregates formed depends upon the geometric packing constraints of the individual lipids (lipid shape). Table 9.1 gives an overview of lipid shape and aggregate structure formed.

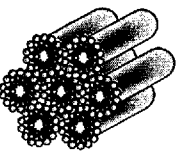
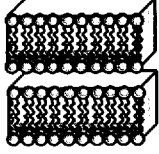
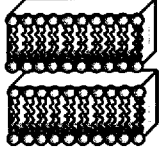
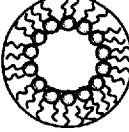
Lipid	Lipid Shape	Aggregate formed	Macroscopic assembly of aggregates
Single chained lipids with large polar head group	Cone 	Spherical Micelles 	Micelles 
Single chained lipids with small polar head group	Truncated Cone 	Cylindrical Micelles 	Hexagonal 
Double chained lipids with large polar head group	Truncated Cone 	Flexible bilayers, vesicles 	Lamellar 
Double chain lipids with small polar head group	Cylinder 	Planar bilayers 	Lamellar 
Double chained lipids with small polar head group area	Inverted Truncated Cone or Wedge 	Inverted Micelles 	Inverted Micelles 

Table 9.1: Packing Shapes of Lipids and the Resultant Structures Formed.

The structure of the aggregates formed in solution influences the lipids ability to form liposomes. The bilayer structure of liposomes means that a lamellar packing structure is favoured over the hexagonal structure. Therefore lipids predisposed to form lamellar aggregates are more inclined to form stable liposomes.

Chain Melting Temperature (T_C)

At room temperature micelle forming single-chained surfactants, as well as bilayer forming double-chained lipids having less than 14 carbons per chain are in the fluid state. However, many of the commonly used lipids for liposomes ($> C14$ chain length) have a chain melting temperature (T_C) above room temperature and consequently exist in a crystalline state, or L_β phase (see Figure 9.1). At elevated temperatures (that is above the T_C) the lipids shift to a fluid phase L_α phase.

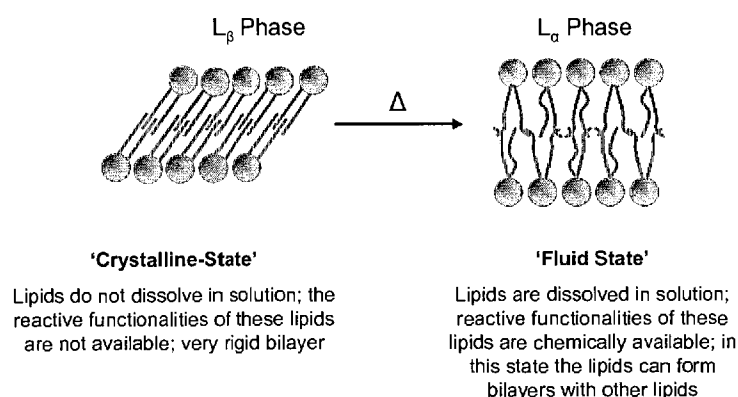


Figure 9.1: Chain Melting Temperature (T_C)

Lipid (giving the number of carbons per chain)	Head Group type and chain melting temperature (°C)	
	PC	PE
<i>Saturated</i>		
Dilauroyl (12)	-2	30
Dimyristoyl (14)	23	49
Dipalmitoyl (16)	41	64
Disteroyl (18)	55	74
<i>Unsaturated</i>		
Dioleoyl (18)	-22	-16

Table 9.2: Selected Lipid Chain Melting Temperatures

A direct consequence of this is the requirement to heat solutions containing lipids with a T_c above room temperature when forming liposomes or performing chemical reactions with these lipids.

Cholesterol

Cholesterol is a neutral naturally occurring sterol lipid found in abundance in animal cell membranes (see Figure 9.2).

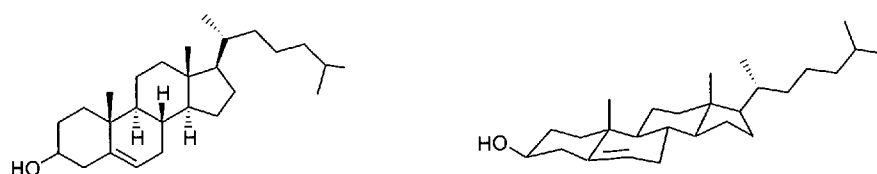


Figure 9.2: Structure of Cholesterol

Cholesterol can reduce serum induced instability caused by the binding of serum protein to the liposome membrane (a direct result of the neutral charge) and also modulate rigidity of a membrane (see Figure 9.3) [85].

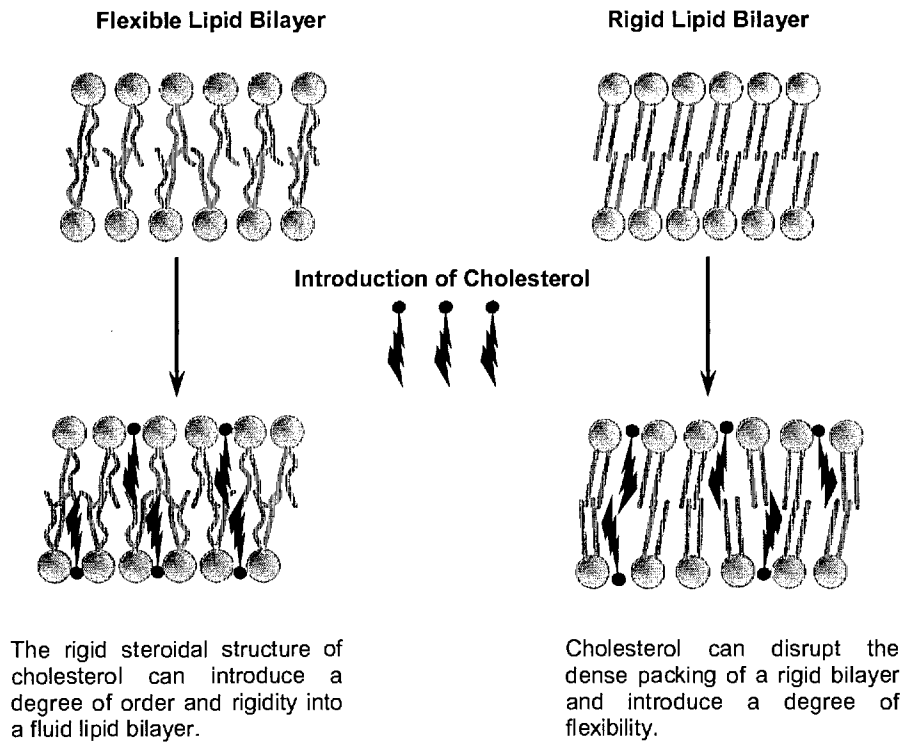


Figure 9.3: Influence of Cholesterol on Membrane Rigidity

Phospholipids

Phospholipids are the principle components of lipid membranes, and it is the intramolecular hydrophobic-hydrophilic interactions between the phospholipids themselves that leads to the formation and ensures stability of the lipid bilayer. The chemical structure of phospholipids can confer different overall properties to a lipid bilayer.

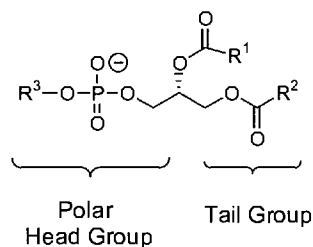


Figure 9.4: General Structure of Phospholipid

Domain	Effect on Liposome Membrane	Functional Attribute on Lipid Bilayer
Tall group-Fatty acyl chains: R_1 and R_2 (C14-18 in length)		
Increase the degree of saturation	Increase rigidity; decrease fluidity	Elevate T_c
Increase chain length of R_1 and R_2	Increase thickness of bilayer	Elevate T_c
Varying degree of saturation and chain length on R_1 and R_2	Decrease order of membrane packing	Lower T_c , (compared to phospholipid with two identical fatty acyl tails)
Head Group: R_3		
Choline ($\text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)_3^+$)	Some surface hydration	Neutral charge
Ethanolamine ($\text{CH}_2\text{CH}_2\text{NH}_3^+$)	Minimum degree of surface hydration	Neutral charge
PEG ethanolamine ($\text{CH}_2\text{CH}_2\text{NH-PEG}$)	Enhanced surface hydration and steric effect	Negative charge

Table 9.3: Properties of Phosholipids and their Effect on the Lipid Bilayer [85]

9.2 Xylenol Orange

Xylenol Orange, a derivative of cresol red, is a chemical dye that has significant photometric titration applications for the detection of metals, including Gd(III). The structure of xylenol orange includes 4 tertiary amine derived acetic acid groups, analogous to DTPA and EDTA chelators and Gd(III) is assumed to bind in an parallel manner.

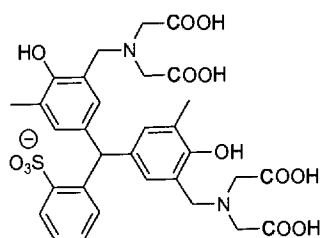


Figure 9.5: Xylenol Orange

An aqueous solution of xylenol orange is yellow orange in colour ($\lambda_{\text{max}} = 440 \text{ nm}$). Upon complexing Gd(III) the complex undergoes a bathochromic shift ($\lambda_{\text{max}} = 573 \text{ nm}$) and a significant colour change to a deep purple solution (see Figure 9.6). It is this bathochromic shift and accompanying colour change that makes xylenol orange an excellent free Gd(III) indicator.

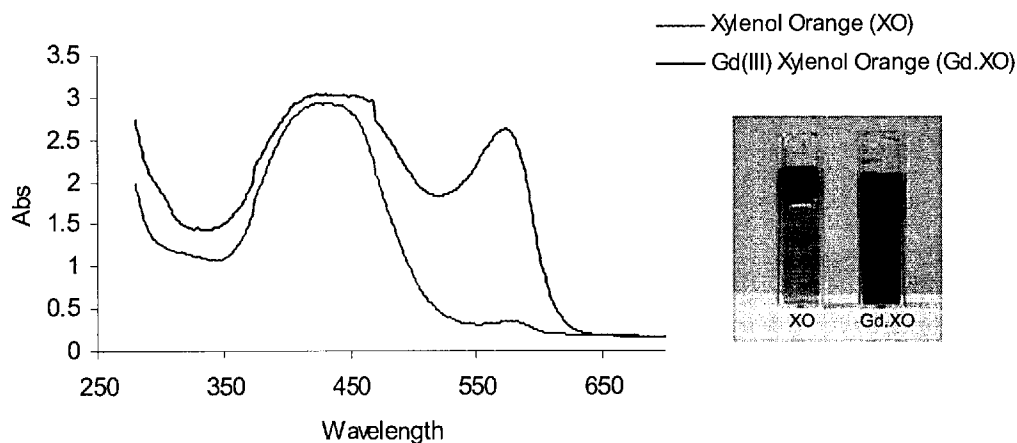


Figure 9.6: Absorbance Spectrum and Corresponding Images of Xylenol Orange and Gd(III) Xylenol Orange Solutions

Xylenol Orange Test

In order to use xylenol orange as an effective quantitative indicator to test for the presence of free Gd(III) ions in sample solutions, a titration curve for xylenol orange and free Gd(III) ions was established.

The absorbance of a solution of xylenol orange (1 mL, 0.5 mM, in 0.1 M sodium acetate buffer (pH 5.2)) with varying amounts of free Gd(III) (0 mM – 50 mM) was measured at 573 nm. The resulting titration curve is shown in Figure 9.7.

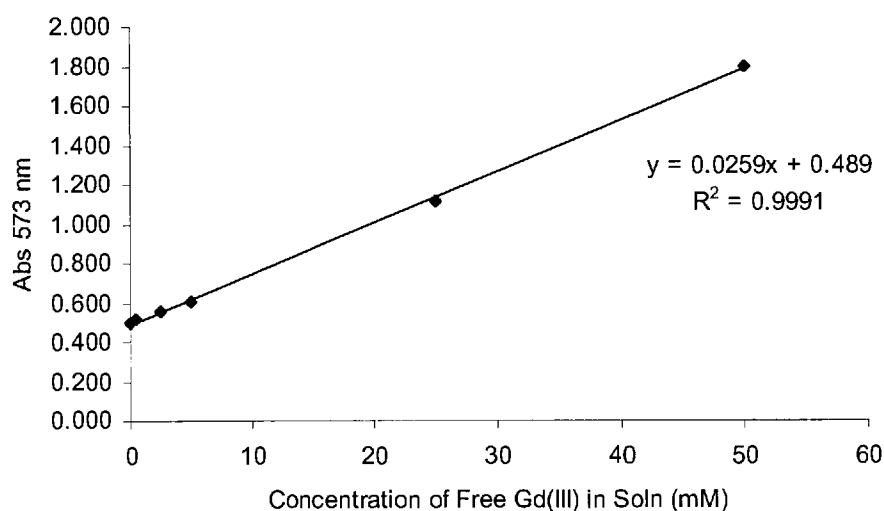


Figure 9.7: Gd(III) Xylenol Orange Titration Curve

The presence of free gadolinium in a sample was assessed by measuring the absorbance of a test solution (990 μL of Xylenol orange solution, 10 μL of sample solution) at 573 nm and using the titration curve to determine the amount of free Gd(III) ions present.

Alternatively the amount of free Gd(III) ions present could be calculated directly from the Beer Lambert equation (Extinction coefficient (ϵ) = 20,700 $\text{L mol}^{-1} \text{cm}^{-1}$)

$$[\text{Free Gd(III)}] = A_{573} / \epsilon$$

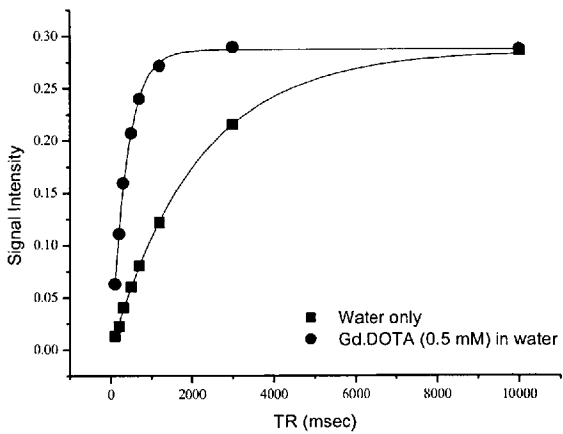
9.3 Calculating T₁ Relaxation Times

To calculate T₁ relaxation values for a given compound, a sample of the compound dissolved in an appropriate solvent, would be evaluated using serial spin echo sequences with variable TR times (time to repeat), but constant TE times (Time of Echo). Signal intensity readings from the sample are acquired at each individual TR value. The T₁ is extrapolated from a plot (Signal Intensity Vs TR) of this data using an exponential equation (see Equation 9.1).

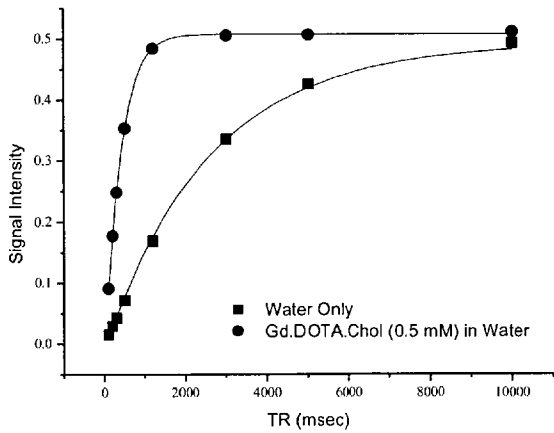
$$S = S_0(1 - e^{-TR/T_1})$$

Equation 9.1: T₁ Equation

Example plots of T₁ data and the calculated T₁ values from that data are shown in. Figure 9.8



	Water	Gd(DOTA) ⁻
T ₁	2156	390
Std. Error	27.8	8.2
Data Fit (R ²)	0.9998	0.9988



	Water	Gd.DOTA. Chol
T ₁	2937	446
Std. Error	115.7	13.2
Data Fit (R ²)	0.9990	0.9981

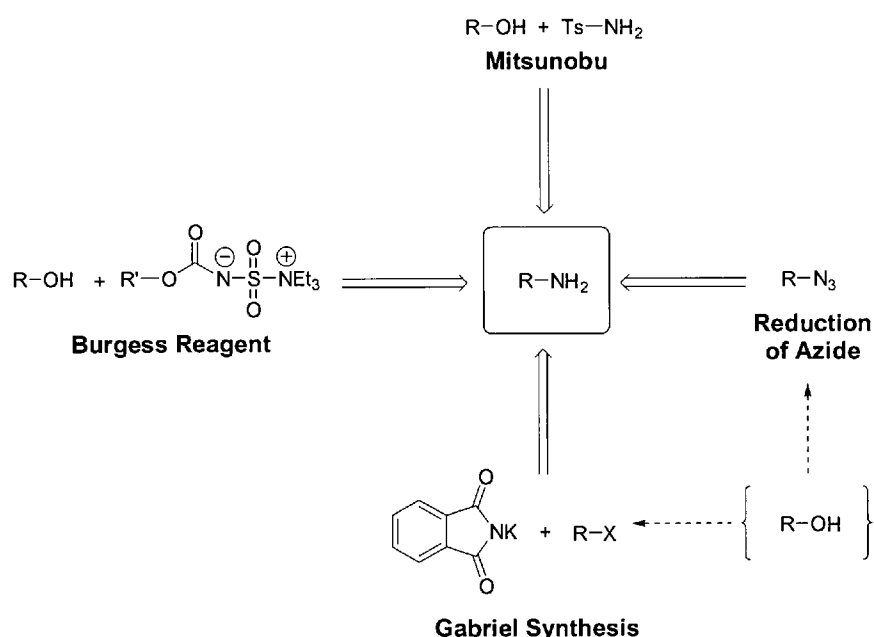
Figure 9.8: T₁ Data for Gd(DOTA)⁻ (25) and Gd.DOTA.Chol (6)

9.4 Synthesis of Polyamines

The efficiency of polyamine synthesis relies heavily upon the effectiveness of the synthesis of amines. Generally this is accomplished by the conversion of primary alcohols to primary amines and subsequently primary amines to secondary amines. Quaternary amines can be easily made from the corresponding primary amine.

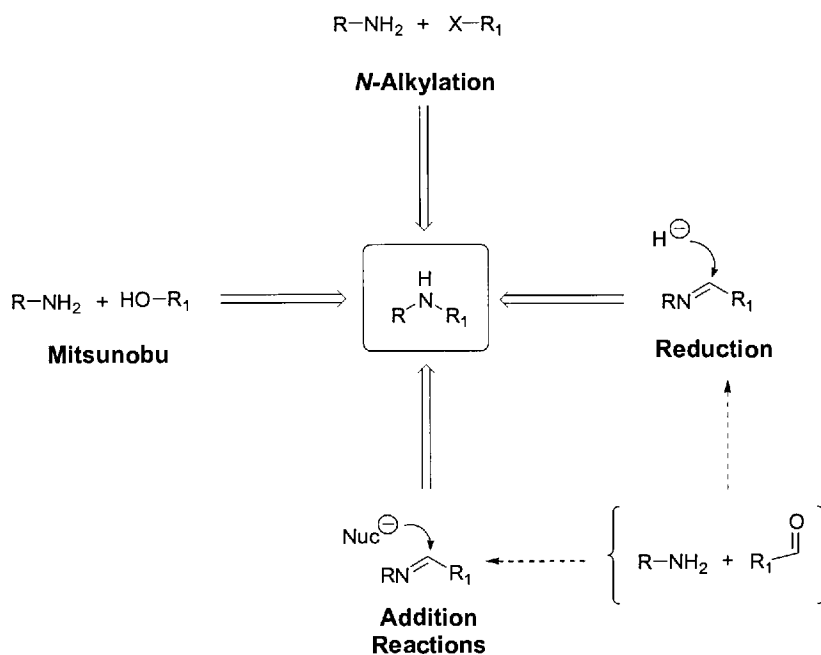
Scheme 9.1 and

Scheme 9.2 gives an overview of methods used for the synthesis primary and secondary amines respectively.



Scheme 9.1: Synthesis of Primary Amines

Primary alcohols can be converted to primary amines using a number of methods (see Scheme 9.1). A direct transformation can be achieved using either the Burgess Reagent [193] to produce a carbamate protected amine which can then be subsequently deprotected to give the free primary amine, or alkylation of *p*-toluenesulfonamine (or *N*-Boc-*p*-toluenesulfonamide) under Mitsunobu conditions [194], [195]. Primary amines can also be made from reduction of the corresponding azide [196] or from the corresponding halide derivative plus potassium phthalimide (Gabriel Synthesis) [197]. Azide and halide derivatives can easily be made from the corresponding primary alcohol using simple functional group inter-conversion reactions [198].



Scheme 9.2: Synthesis of Secondary Amines from Primary Amines

Secondary amines are easily made from addition reactions to primary amines (see Scheme 9.2). The most obvious disconnection of a secondary amine would lead to a primary amine and corresponding halide. Although *N*-alkylation (Hoffman Alkylation) [199] appears simple, there is an inherent lack of reaction control which leads to the production of over-alkylated products. Reaction of a primary amine with an aldehyde results in production of imine, which can then either be subsequently reduced (reductive amination) or reacted with a nucleophile to give a secondary amine [199]. Alternatively the secondary amine can be produced from condensation of an alcohol with an amine under Mitsunobu reaction conditions [194], [195].

9.5 Miscellaneous Data

DOTA.Chol (27)

Figure 9.9 shows the HPLC and MS of DOTA.Chol (27).

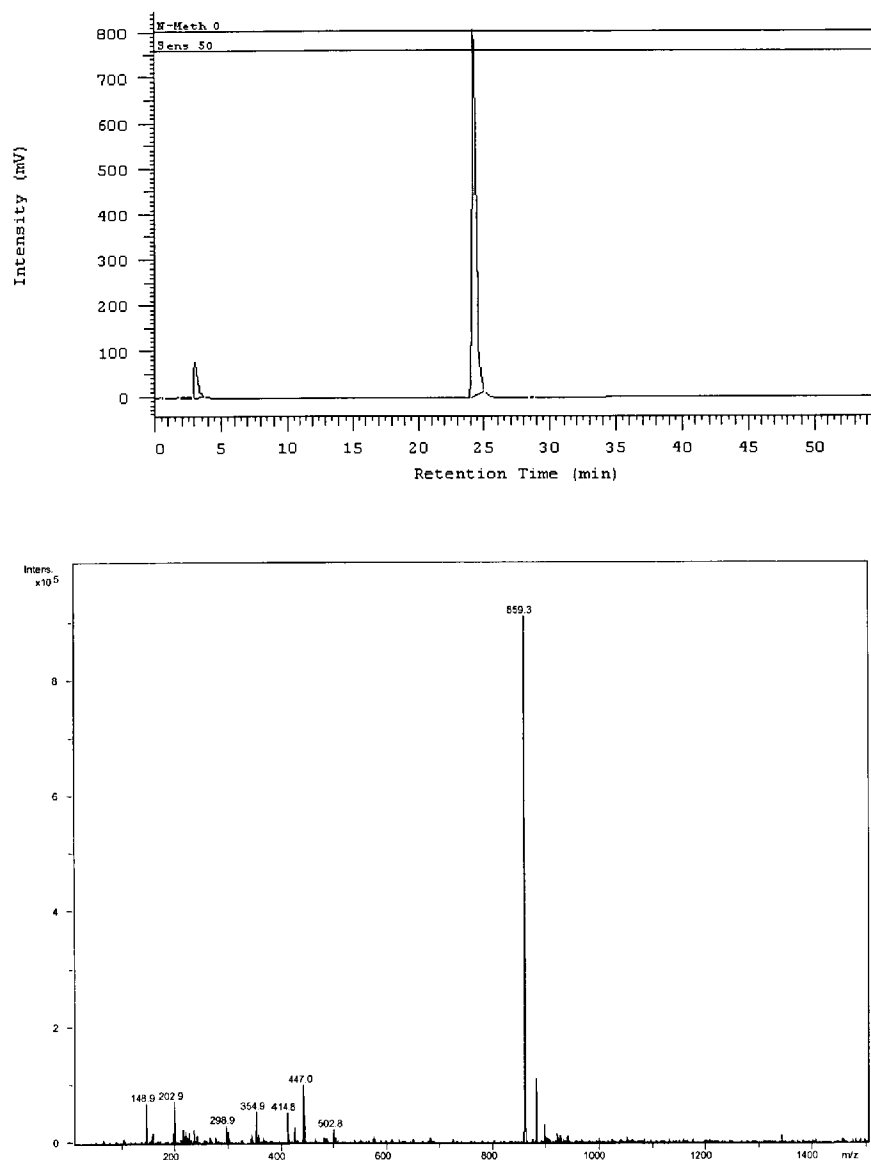


Figure 9.9: HPLC and MS of DOTA.Chol (27)

(HPLC conditions: See page 173. Mass Spectrum: Positive ionisation ESI)

DOTA.DSPE (28)

Figure 9.10 shows the HPLC and MS of DOTA.DSPE (28).

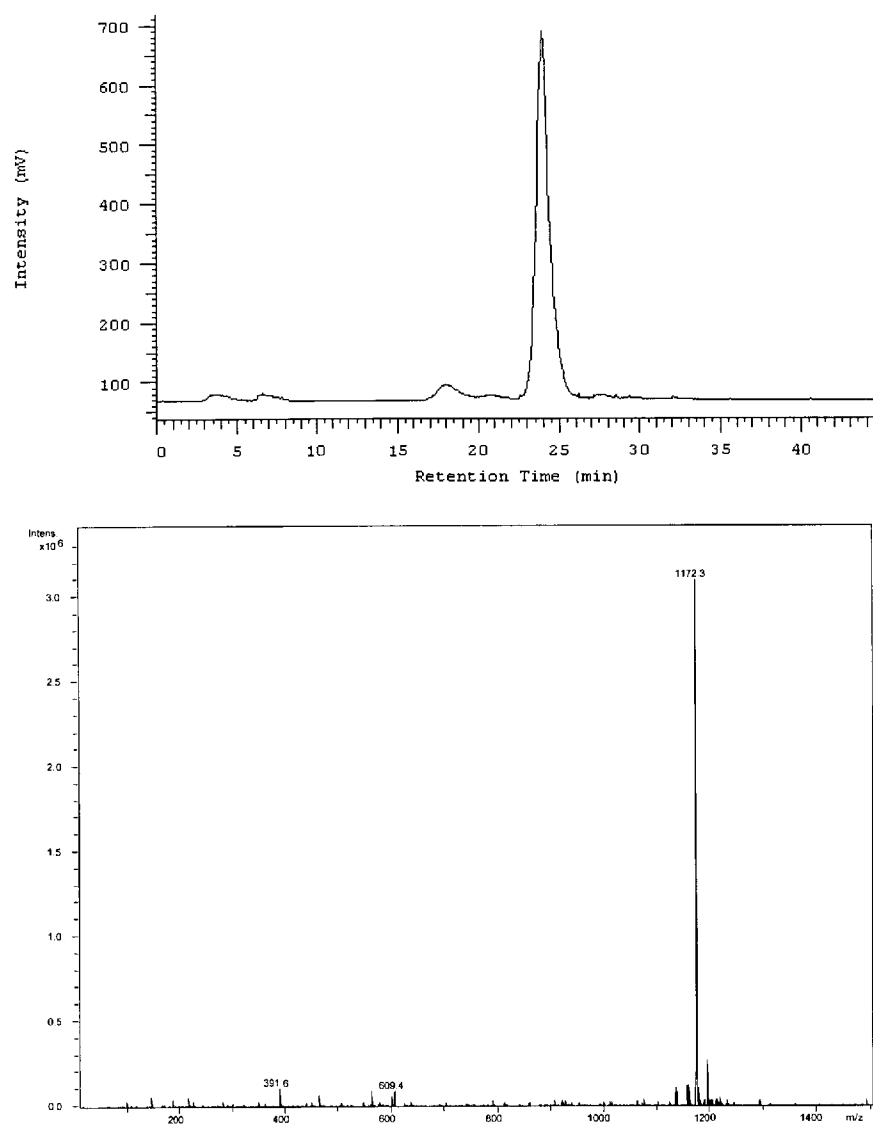


Figure 9.10: HPLC and MS of DOTA.DSPE (28)

(HPLC conditions: See page 175. Mass Spectrum: Positive ionisation ESI)

DTPA.Chol (29)

Figure 9.11 shows the HPLC and MS of DTPA.Chol (29).

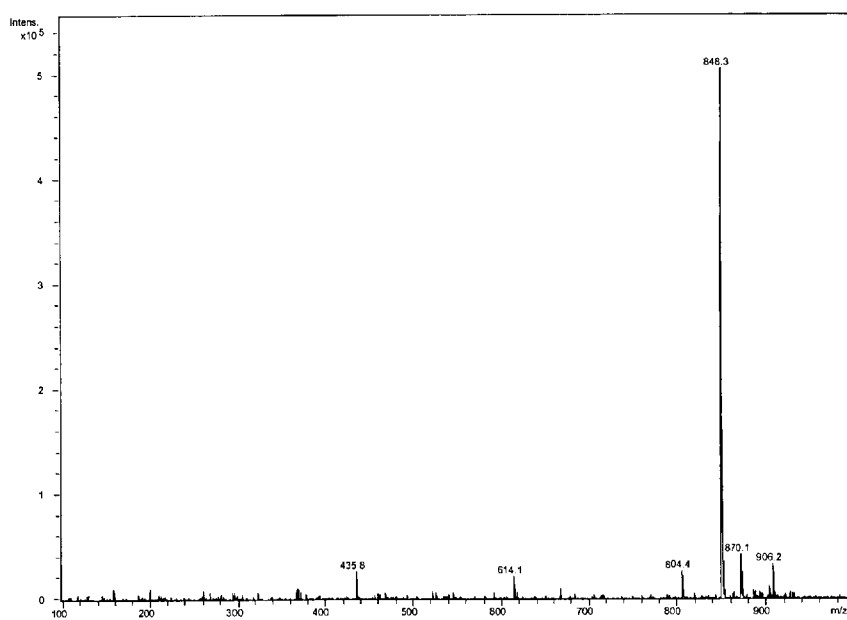
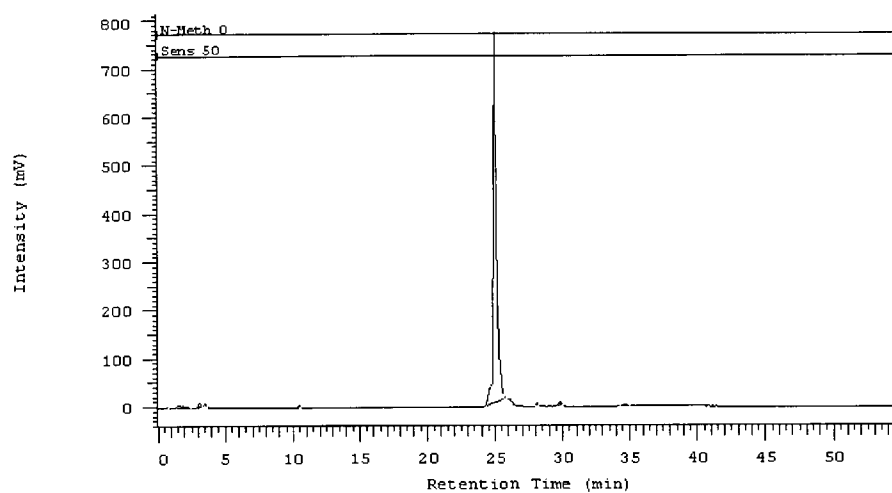


Figure 9.11: HPLC and MS of DTPA.Chol (29)

(HPLC conditions: See page 179. Mass Spectrum: Positive ionisation ESI)