Formulation and preliminary evaluation of delivery vehicles for the boron neutron capture therapy of cancer

By

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BSc (Hons)

A thesis submitted in partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

of the

University of Portsmouth

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February 2015
ABSTRACT

Boron neutron capture therapy (BNCT) is a method for selectively destroying malignant (normally glioma) cells whilst sparing normal tissue. Irradiation of $^{10}\text{B}$ (large neutron capture cross-section) with thermal neutrons effects the nuclear fission reaction: $^{10}\text{B} + ^{1}\text{n} \rightarrow ^{7}\text{Li}^+ + \alpha + \gamma$; where the penetration of $\alpha$-particles and $7\text{Li}^+$ is only 8 and 5 µm, respectively, i.e., within a single cell thickness, assuming $^{10}\text{B}$ can be preferentially located within glioma cells. Poor selectivity is the main reason why BNCT has not become a mainstream cancer therapy. Carboranes, a third generation of high boron-containing, low-toxicity, BNCT compounds, are currently being investigated.

Towards the aim of increasing malignant cell targeting specificity, this thesis investigates monodispersed dipalmitoylphosphatidylcholine (DPPC) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) SUV liposome formulations containing carboranes derivatised with delocalised lipophilic cations (DLCs), specifically the dequalinium bis nido carborane salt. AFM studies showed the loaded liposomes appeared stable (63 days, 4°C, if re-probing was employed). The integrity of the liposome membrane in serum, as reflected by %latency and %retention experiments using a fluorescent marker (calcein), was found to be high for both types of liposomes prepared using cholesterol. Successful entrapment of carboranes was demonstrated by the Nile Red method and by ICP-MS measurements. The liposomes were of sufficient size (80-100 nm) to pass through the blood brain barrier (BBB). The cationic moiety of the carborane salt allowed selective targeting of glioma mitochondria, thought to be due to differences in mitochondrial membrane potentials between malignant and non-neoplastic cells. Specific targeting of IN699 (glioma, WHO grade IV) and SC1800 (non-neoplastic astrocyte) cells with the carborane salt was evidenced by live cell (fluorescence) imaging.

Spray drying was used as an alternative method of formulating agents for BNCT treatments for liver and lung cancers, where the larger (micrometre-diameter) particles do not need to pass across the BBB. Polyvinylpyrrolidone / o-carborane co-spray-dried microparticles were produced. $^1\text{H}$ NMR studies revealed the high temperatures (180 °C) of the spray drying process did not degrade the PVP. Mean particle diameters ($x_{90}$) were in the 2 – 10 µm range, with finer fractions being present ($x_{10} \approx 1 – 2$ µm), and were therefore considered suitable for delivery to the lungs. SEM imaging showed the particles to be spherical, with dimples and cavities caused by the spray drier nozzle characteristics, as typical with the spray drying process. Some small irregularly-shaped crystalline particles, thought to be o-carborane, were observed by SEM, although the proportion accounted for less that than in the formulation (10 %w/w). An attempt was made to map the boron content in spray-dried powders on a surface using EDS, although the low atomic weight of boron made detection not possible. Cytotoxicity studies, using human glioblastoma U-87 MG (cancerous) and human fetal lung fibroblast MRC-5 (non-neoplastic) cells, revealed the PVP / o-carborane co-spray-dried particles to be non-toxic.
DEDICATION

This PhD is dedicated solely to “The Ancients of Days”,

The Lord that raiseth the poor from the dung hill- THE ALMIGHTY GOD!
ACKNOWLEDGEMENTS

Firstly, I would like to give thanks to ALMIGHTY JEHOVAH NESSY for HIS mercies that endureth forever.

I appreciate my First Supervisor, Dr John Tsibouklis – Unmh! Dr John – Thanks so much. You are really one in a million! You are a father indeed. Thanks so much for all your help. Ha! I cannot forget your immersed contribution you have played in this project and in my life as a whole. You have made all nook and cranny of the School of Pharmacy and Biomedical Sciences enjoyable for me.

Thanks also goes to my second supervisor, Dr James R. Smith for the knowledge, endurance and effort GOD gave you to pilot this project to the shore. You picked me up when no one was there to support me. You invested all your time and effort for the success of the project. I will never forget your unquantifiable contributions. You are highly intelligent! God bless you and your family. You will live long to reap all the fruits of your labour. God will bless you and prosper all your handiwork. Many thanks to you once again.

I have learnt a lot from you both morally and academically – check in my small booklet I titled “James and John”. May the Lord help you both, give you and your families’ long life, prosperity and ALL your desires in life, in Jesus Name. Amen.

Thanks also goes to Dr Eugen Barbu – always ready to listen to me when things seemed to go wrong and to give advice. Thanks also to Dr Marta Roldo, the late Dr Tom Nevell, Val, Theresia Stich and to all the members of the Biomaterials and Drug Delivery (BADD) group. Thanks to Drs Sam Higgins and Rhiannon Lloyd for cell culture work. Thanks to Dr Dimitris Fatouros at Aristotle University of Thessaloniki, Greece; Dr Gianpiero Calabrese, University of Kingston, London; and Prof Sophia Antimisiaris, University of Patras, Greece. Many thanks also go to all those who contributed in one way or another to the success of the project.

Fuazi Mohammed, as I always tell you, is it possible to forget someone always being there? Come rain, come sunshine, you are unforgettable. Always ready to render help – This is Fauzi! Thank you.

I appreciate my parents for their support spiritually with their prayers every second, financially, morally and in all things. They really stood by me. I thank you my mother for taking care of Jomiloju (My only child) from day one when I had to go and complete my studies. Thanks to my father and mother in the times of extreme Hot and Cold!

The bible says, ”Seest thou a man diligent in his business? He shall stand before Kings; he shall not stand before mean men. (Proverbs Chapter 22 verse 29): http://saharareporters.com/2014/11/19/boko-haram-shoots-nigerian-army-brigade-commander#.VGy2m7_mtRM.email. ---- This is Col. A. B. A. Popoola!

I am very grateful to my elder sister, the husband (Col. A. B. A. Popoola) for their financial support and prayers. Also, to the three Hebrew soldiers of the Lord (Ayobami, Opeyemi (Baba jeje) and Tomiwa (My General!) thank you.
"E se Baba ati Iya fun wa kale o, Daddy and MamaJJ.” Ori ni eyin mejeji je fun wa o, e ko ni di iru lailai loruko Jesu.Amin. Iku ko ni pa yin,arun ko ni se yin lo jo o. E pe, pe pe fun wa o. Unmh! E se mo du pe!

Thanks goes to the family of Ronke Oni (Prayer warrior), Kehinde Ojo, Peju Adeuti, Taiwo Olatunde Oggunosun “The distinguish Artist” Ogbon ati oye ti Olorun fun e ko ni pare lailai and “The Law”(Lawyer Anuoluwapo Oggunosun). Many thanks to you all.

Thanks to Oluwajomilolu, Jesutofunmi Olusanya for disturbing grandma with your powerful English language “Grandma get up from the bed, I am busy, I am eating, don’t disturb me, that’s grandma’s shoe, I am charging grandma’s phone (at age one)……Lol! I am grateful to Eniola and Olugbenga Olatunde Oggunosun, Shola and Testimony Oni, Peculiar and Favour Adeuti.

To my darling husband, Major Olugbenga Olubukola Olusanya of Nigerian Army, Armour Division. O se oko mi. I ponder why you are being loved by old and young; little did I know the secrets are in the scripture (Proverbs chapter 22 verse 29, Proverbs chapter 13 verse 3, Psalm 16 verse 8 and Psalm 27) and that is the reason your work and quietness speak for you before the Generals, the Senior officers, the young ones and your colleagues. Thanks for being there at all times-“Baba jeje”

Omo Olowu Oduru,
Omo aji fepe sere
Omo ata gba ni yanrin
Omo eduku mede mede
Omo imale afi ede ja
Omo ake ni gbo keru ba ara ona,
Omo ape na ola,
Omo tun wo aporo,
Tun wo na da?
Eru mo du ni
Omo iran ku odo, oni irun ere
Omo eduku mede mede
Omo oloko ti e rere
Omo ai fi a ni adire rago,
Awon to ma Kun, lo ma Kun!

My Sweetheart thanks so much for your support spiritually with your prayers, financially, materially and materially.
Iru oko bi ti re o se owon. Thanks so much my Love. E se gan ni!
AUTHOR’S DECLARATION

Whilst registered as a candidate for the PhD degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of myself and not submitted for any other academic award.

Temidayo Olajumoke Bolanle Olusanya
Ise Ni Ogun Ise
(Yoruba and English Translations)

Ise ni ogun `se
Mura si ise ore mi
Ise ni a fi n deni giga
Bi a ko ba re ni fehin ti
Bi ole la a ri
Bi a ko ba reni gbekele
A te ra mo se eni
Iya re le lowo lowo
Baba re si le leshin le kan
Bi o ba gboju le won
O te tan ni mo so fun o
Ohun ti a ko ba jiya fun
Se kii le tojo
Ohun ti a ba fara sise fun
Ni npe lowo eni
Apa lara, Igunpa ni iye kan
Bi aiye ba nfe o loni
Ti o balowo lowo
Won a ma fe o lola
Jeki o wa ni ipo atata
Akiye a ma ye o si terin-terin
Jeki o deni ti n rago
Ko o ri bi aiye ti nyinmu si o
Eko si tun so ni doga
Mura ki o ko dara-dara
Iya nbo fomo ti ko ghon
Ekun mbe fomo ti o nsa kiri
Ma fowuro sere ore mi
Mura si ise ojo n lo!

The antidote to poverty is hard work
So work hard, dear friend
Success only comes through hard work
When there is no one to rely on
It's like we are lazy
When there is no one to trust
We focus on our work
Your mother may be rich
Your father may own a thousand and one horses
If you rely on their wealth
You are sinking fast to your shame.
Whatever we do not work for
Will not last
It is what we strive for
That last
Your arms and elbows are your instruments of work
If you are feted by society today
If you are still rich,
They will fete you tomorrow as well.
When you hold an esteemed position
You will be regarded with great joy
Just wait and become a pauper and
And see how you will be treated with disdain
Education can take you to the top
So study hard
The unwise will surely regret their folly
The result for lack of planning is tears
Don’t waste your early years
Make hay while the sun shines.
Standing in the mist of Unknown,
I look around to see the unseen,
Oh! What a waste of time untold,
Searching for the enemy within.

Standing in the midst of unknown,
Science told me the enemy is within,
Growing gradually unknown,
In those whose temple unscreened!

Standing in the mist of unknown,
The battle begins unfold,
The enemy within untold,
That the battle against it is on course,
Soon to be won in all
Whose temple are screened and scanned!

Standing in the midst I know,
That the battles against it are told,
By the comrades of science we know,
That cancer, the enemy within,
Will soon be cancelled assured!

T.O.B Olusanya
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iv</td>
</tr>
<tr>
<td>Author’s declaration</td>
<td>vi</td>
</tr>
<tr>
<td>Words of admonitions and encouragements</td>
<td>vii</td>
</tr>
<tr>
<td>Cancer poem</td>
<td>viii</td>
</tr>
<tr>
<td>Table of contents</td>
<td>ix</td>
</tr>
<tr>
<td>List of abbreviations</td>
<td>xiv</td>
</tr>
<tr>
<td>List of symbols</td>
<td>xvii</td>
</tr>
<tr>
<td>List of figures</td>
<td>xix</td>
</tr>
<tr>
<td>List of tables</td>
<td>xxiv</td>
</tr>
</tbody>
</table>

## CHAPTER ONE:

### Introduction

1.1 Cancer

1.1.1 Characterisation of tumours

1.1.2 Naming cancers

1.2 Brain tumours

1.2.1 Brain anatomy

1.2.2 Brain tumours

1.3 Liver cancer

1.3.1 Structure of the liver

1.3.2 Liver cancer

1.4 Lung cancer

1.4.1 Structure of the lungs

1.4.2 Lung cancer

1.5 Cancer therapies

1.5.1 Surgery

1.5.2 Radiotherapy

1.5.3 Chemotherapy

1.5.3.1 Brain tumour chemotherapy using temozolomide

ix
2.2.1.1 Preparation of phosphate buffered saline (PBS, pH 7.4) 67
2.2.1.2 Preparation of calcein stock solution 68
2.2.1.3 Preparation of serum stock solution from rat blood 68
2.2.1.4 Preparation of Nile Red stock solution 68
2.2.1.5 Preparation of o-carborane stock solution 69
2.2.1.6 Preparation of DPPC/DSPC liposomes using thin film method 70
2.2.1.7 Preparation of DPPC/DSPC liposomes for fluorescence displacement, particle size measurement and ICP-MS 71
2.2.2 Calcein stability measurements 72
2.2.3 Nile Red entrapment measurements 74
2.2.4 Particle sizing and ζ-potential 74
2.2.5 ICP-MS Boron analysis 75
2.2.6 Scanning electron microscopy 75
2.2.7 Atomic force microscopy 76
2.3 Results and discussion 77
2.3.1 Particle sizing, PDI and ζ-potential 77
2.3.2 Effect of re-probing on liposome properties 78
2.3.3 Stability of liposome properties after one week 80
2.3.4 AFM 82
2.3.5 SEM 90
2.3.6 Stabilisation of liposomes with cholesterol and serum 91
2.3.7 Nile red experiments 97
2.3.8 ICP-MS measurements of o-carborane incorporation 101
2.4 Summary 102
2.5 References 104

CHAPTER THREE: 108
Efficacy of specific formulations of o-carborane 108
3.1 Introduction 108
3.1.1 Targeting cancer cell mitochondria 108
3.1.2 Mito dyes 110
3.1.3 Use of carborane DLC derivatives for BNCT 112
3.1.4 Aims 113
3.2 Materials and methods 115
  3.2.1 Chemical and instrumentation 115
  3.2.2 Synthesis of dequalinium \textit{bis nido o-carborane} salt(DC) 115
  3.2.3 Cell culture 116
  3.2.4 MTS assay 117
  3.2.5 Microscopy 122
  3.3 Results and discussion 123
  3.3.1 Fluorescence imaging of DC 123
  3.3.2 MTS assay 124
  3.3.3 Targeting glioma cells with DC 132
  3.4 Summary 136
  3.5 References 138

\textbf{CHAPTER FOUR:} 141
\textit{Spray-dried particles containing \textit{o-carborane}} 141
  4.1 Introduction 141
    4.1.1 Spray drying 141
    4.1.2 Polyvinylpyrolidone 143
    4.1.3 Aims 144
  4.2 Materials and methods 145
    4.2.1 Materials 145
    4.2.2 Methods 145
      4.2.2.1 Spray drying 145
      4.2.2.2 Particle sizing 146
      4.2.2.3 NMR 147
      4.2.2.4 SEM and EDS 147
      4.2.2.5 Tissue culture and cytotoxicity studies 147
  4.3 Results and discussion 148
    4.3.1 Spray drying 148
    4.3.2 $^1$H NMR of PVP/o-carborane 149
    4.3.3 Particle sizing 152
    4.3.4 Morphology 156
    4.3.5 Cytotoxicity studies 163
4.4 Summary 168
4.5 References 169

CHAPTER FIVE:
Conclusion and further work

5.1 Summary 172
5.2 Further work 174
5.3 References 176
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABM</td>
<td>Astrocytes Basal Medium</td>
</tr>
<tr>
<td>$A_c$</td>
<td>Mean Absorbance for DC concentration $c$</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
</tr>
<tr>
<td>AGM2</td>
<td>Trade mark for a recommended medium</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>$A_o$</td>
<td>Mean Absorbance for number of DC</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
</tr>
<tr>
<td>BC</td>
<td>Before Christ</td>
</tr>
<tr>
<td>BNCT</td>
<td>Boron Neutron Capture Therapy</td>
</tr>
<tr>
<td>BPA</td>
<td>1,4-dihydroxyborylphenylalanine</td>
</tr>
<tr>
<td>BSH</td>
<td>Disodium Mercaptoundecahydro-closo dodecaborate</td>
</tr>
<tr>
<td>CFS</td>
<td>Cerebrospinal Fluids</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
</tr>
<tr>
<td>CUP</td>
<td>Carcinoma of Unknown Primary Origin</td>
</tr>
<tr>
<td>DC</td>
<td>Dequalinium <em>bis nido</em> Carborane salt</td>
</tr>
<tr>
<td>DF</td>
<td>Dilution Factor</td>
</tr>
<tr>
<td>Dia</td>
<td>Diameter</td>
</tr>
<tr>
<td>DLC</td>
<td>Delocalised Lipophilic Cations</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>DMPC</td>
<td>1,2-dimyrystoyl-sn-glycerol-3-phosphocholine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide (CH$_3$)$_2$SO.</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPPC</td>
<td>1,2-dipalmitoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DSPC</td>
<td>1,2-distearol-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>ECs</td>
<td>Endothelial Cells</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EGCG</td>
<td>Epigallocatechin gallate</td>
</tr>
<tr>
<td>EM</td>
<td>Emission</td>
</tr>
<tr>
<td>$F_{AT}$</td>
<td>Fluorescence Value after adding Triton</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Term</td>
</tr>
<tr>
<td>--------------</td>
<td>------</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>F&lt;sub&gt;BT&lt;/sub&gt;</td>
<td>Fluorescence Value before adding Triton</td>
</tr>
<tr>
<td>FR</td>
<td>Folate Receptor</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma Multiforme</td>
</tr>
<tr>
<td>Gd/x</td>
<td>A type of Whatman syringe filter</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's Balanced Salt Solution</td>
</tr>
<tr>
<td>HLET</td>
<td>Higher Linear Energy Transfer</td>
</tr>
<tr>
<td>ICPMS</td>
<td>Inductive Coupled Plasma Mass Spectrophotometry</td>
</tr>
<tr>
<td>IN699</td>
<td>Glioma cell line IN699</td>
</tr>
<tr>
<td>LC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Lethal concentration at 50%</td>
</tr>
<tr>
<td>LUVs</td>
<td>Large Unilamella Vesicles</td>
</tr>
<tr>
<td>Max</td>
<td>Maximum</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug Resistance</td>
</tr>
<tr>
<td>MIM</td>
<td>Mitochondria Inner Membrane</td>
</tr>
<tr>
<td>Min</td>
<td>Minimum</td>
</tr>
<tr>
<td>MLVs</td>
<td>Multilamella Vesicles</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinase enzymes</td>
</tr>
<tr>
<td>MPS</td>
<td>Mononuclear Phagocytes</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MtDNA</td>
<td>Matrix of extra chromosomal mitochondria DNA</td>
</tr>
<tr>
<td>MTG</td>
<td>Mito Tracker Green</td>
</tr>
<tr>
<td>MTIC</td>
<td>5-(3-methyltriazen-1-yl)imidazole-4-carboximide</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium</td>
</tr>
<tr>
<td>MTT</td>
<td>(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>NADH</td>
<td>Oxidised Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve Growth Factor</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NY</td>
<td>New York</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered-Saline</td>
</tr>
<tr>
<td>PC</td>
<td>Prostate Cancer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PCPB</td>
<td>Para-carboxyphenylboronic acid</td>
</tr>
<tr>
<td>PDI</td>
<td>Polydispersity Index</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PES</td>
<td>A type of syringe filter</td>
</tr>
<tr>
<td>PMS</td>
<td>Phenazine Methosulfate</td>
</tr>
<tr>
<td>RES</td>
<td>Reticuloendothelial System</td>
</tr>
<tr>
<td>SC1800</td>
<td>Astrocytes</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscope</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short ribonucleic acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering Ribonucleic Acid</td>
</tr>
<tr>
<td>SUVs</td>
<td>Small Unilamella Vesicles</td>
</tr>
<tr>
<td>Tf</td>
<td>Transferin</td>
</tr>
<tr>
<td>TJs</td>
<td>Tight Junctions</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer Chromatography</td>
</tr>
<tr>
<td>TMS</td>
<td>Tetramethylsilane</td>
</tr>
<tr>
<td>TMZ</td>
<td>Temozolomide</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Vc</td>
<td>Viability expressed as a fraction</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelia Growth Factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
</tbody>
</table>
LIST OF SYMBOLS

°C  Degrees Celsius
µg  Microgram
µm  Micrometre
1,12-C₂H₁₂B₁₀  Para carborane
1,2-C₂H₁₂B₁₀  Ortho carborane
1,7-C₂H₁₂B₁₀  Meta carborane
C  Concentration
CHCl₃  Chloroform
D  Diffusion coefficient
Da  Dalton
D_H  Hydrodynamic diameter
f  Particle frictional coefficient
g  Gram
h  Hour
K  Boltzman Constant
k  Kilo or Spring constant
kHz  KiloHertz
kV  Kilovolts
L  Length
M  Molar
mA  Milliamps
mg  Milligram
mL  Millilitre
min  Minute
mM  Millimolar
mmol  Millimoles
mol⁻¹  Per mole
mV  Millivolts
N  Newton
n  Number of occurrence
nm  Nanometre
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Meaning</th>
</tr>
</thead>
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<td>Pump rate</td>
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<tr>
<td>$\lambda_{ex}$</td>
<td>Excitation wavelength</td>
</tr>
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</table>
LIST OF FIGURES

CHAPTER ONE: Introduction

Figure 1.1. The origin of cancer: uncontrolled cell growth. 1
Figure 1.2. Structure of the brain: (a) lateral view, and (b) mid-sagittal sectional view [57] 2
Figure 1.3. Anterior (left) and posterior view (right) of the liver [76]. 9
Figure 1.4. Causes of liver cancer [84]. 13
Figure 1.5. Structure of the lung [94]. 14
Figure 1.6. Structure of Temozolomide. 15
Figure 1.7. The differences in radiation path between proton therapy and standard x-ray radiotherapy for the treatment of brain tumours. 19
Figure 1.8. The BNCT reaction. 21
Figure 1.9. The application of BNCT in a hospital setting [164]. 23
Figure 1.10. Chemical structures of (a) BSH and (b) BPA. 26
Figure 1.11. The three isomers of carborane: (a) ortho-carborane (1,2-C_2H_12B_10), (b) meta-carborane (1,7-C_2H_12B_10), and (c) para-carborane (1,12-C_2H_12B_10); ○ = BH, ● = CH. 27
Figure 1.12. Schematic showing the cells and tight junctions that comprise the BBB [209]. 29
Figure 1.13. Schematic diagram of the transport pathways across the BBB [215]. 30
Figure 1.14. Idealised structures of (a) liposomes and (b) phospholipids [237] 33
Figure 1.15. Different types of liposomes. Components: phospholipids (blue), PEG lipid (orange), antibody or targeting ligand (red), cationic lipid/helper lipid (black cross). Adapted from [237]. 34
Figure 1.16. Chemical structure of cholesterol 35

CHAPTER TWO: Liposomes loaded with o-carborane for BBB delivery

Figure 2.1. A charged particle surrounded by oppositely charged ions. The $\zeta$-potential is the potential at the slipping plane. Adapted from [22]. 61
Figure 2.2. Chemical structure of calcein. 63
Figure 2.3. Chemical structures of (a) DPPC, (b) DSPC, and (c) Nile Red. 65
Figure 2.4. (a) Sephadex G-50 columns used in gel permeation chromatography \((n = 3)\), and (b) schematic showing separation of encapsulated (liposomes) and non-encapsulated calcein.

Figure 2.5. Origin of AFM tip broadening artefact. The red line shows a 2D view of the artefact with corresponding oversized diameter measurement \((d)\); the true diameter (height, \(h\)) is shown. The yellow arrow shows the scan direction.

Figure 2.6. Representative AFM images of unloaded DPPC liposomes obtained after 1, 19, 35, 49 and 63 days \((a – e, \text{ respectively; } n=4)\).

Figure 2.7. Representative AFM images of \(o\)-carborane loaded DPPC liposomes obtained after 1, 19, 35, 49 and 63 days \((a – e, \text{ respectively; } n=4)\).

Figure 2.8. Representative AFM images of unloaded DSPC liposomes obtained after 1, 19, 35, 49 and 63 days \((a – e, \text{ respectively; } n=4)\).

Figure 2.9. Representative AFM images of \(o\)-carborane loaded DSPC liposomes obtained after 1, 19, 35, 49 and 63 days \((a – e, \text{ respectively; } n=4)\).

Figure 2.10. Breakdown of liposomes to form lipid bilayer islands/rafts on mica.

Figure 2.11. Liposomes (a) unloaded and (b) loaded with \(o\)-carborane. The latter structure may be more stable due to increased non-polar interactions and so might be less susceptible to collapse to form lipid bilayer islands/rafts.

Figure 2.12. SEM images of unloaded \((a,b,c)\) and \(o\)-carborane \((1000 \mu\text{L})\) loaded \((d,e,f)\) DSPC liposomes on mica.

Figure 2.13 Mean particle sizes of unloaded and \(o\)-carborane loaded DSPC liposomes obtained from SEM images \((3 \text{ images, } n = 10)\); error bars = SD.

Figure 2.14. Particle size variation for different formulations of DPPC and DSPC liposomes incorporating calcein, with and without cholesterol in PBS and serum \((\text{error bar = SD; } n = 3)\).

Figure 2.15a. Latency of DPPC liposomal dispersions formulated with and without cholesterol in PBS and serum. Formulations were incubated at 37 °C. Data are presented as mean ± SD \((n=3)\).

Figure 2.15b. Retention of DPPC liposomal dispersions formulated with and without cholesterol in PBS and serum. Formulations were incubated at 37 °C.
Data are presented as mean ± SD (n=3).

Figure 2.16a. Latency of DSPC liposomal dispersions formulated with and without cholesterol in PBS and serum. Formulations were incubated at 37 °C. Data are presented as mean ± SD (n=3).

Figure 2.16b. Retention of DSPC liposomal dispersions formulated with and without cholesterol in PBS and serum. Formulations were incubated at 37 °C. Data are presented as mean ± SD (n=3).

Figure 2.17a. Nile Red incorporation into DPPC liposomes loaded with different volumes of o-carborane.

Figure 2.17b. Nile Red incorporation into DSPC liposomes loaded with different volumes of o-carborane.

Figure 2.18 Nile Red incorporation into DSPC liposomes loaded with different volumes of o-carborane.

CHAPTER THREE: Efficacy of specific formulations of o-carborane

Figure 3.1. Schematic showing the extrinsic and intrinsic pathways of apoptosis. Adapted from [2].

Figure 3.2. Cross-sectional image of a single mitochondrion [5].

Figure 3.3. Chemical structures of some common DLCs: (a) dequalinium chloride, (b) rhodamine-123 chloride, (c) Nile Blue chloride, (d) Mitotracker Red CMXRos, (e) Mitoracker Green, and (f) JC-1.

Figure 3.4. Chemical structure of dequalinium bis nido o-carborane salt (DC).

Figure 3.5. MTS assay: formation of coloured formazan derivative by viable cells.

Figure 3.6. Arrangement of a 96 well plate for an MTS assay.

Figure 3.7 (a). Bright field and (b-f) fluorescence images of DC crystals. Filter sets: (b) 20 ‘Rhodamine’, (c) 00 ‘Texas Red’, (d) 37 ‘GFP’, (e,f) 10 ‘FITC’. Bar = 60 µm (120 in f).

Figure 3.8. Combined bright field and fluorescence images of (a,b,c) glioma (IN699) cells and (d,e,f) astrocytes (SC1800) cells treated with DC (0.1 mM). Images acquired 3.5 h after incubation. Bar = 60 µm.

Figure 3.9. Combined bright field and fluorescence images of (a,b,c) glioma...
(IN699) cells and (d,e,f) astrocytes (SC1800) cells treated with DC (0.01 mM). Images acquired 3.5 h after incubation. Bar = 60 µm.

Figure 3.10. Combined bright field and fluorescence images of (a,b,c) glioma (IN699) cells and (d,e,f) astrocytes (SC1800) cells treated with DC (0.001 mM). Images acquired 3.5 h after incubation. Bar = 60 µm.

Figure 3.11. Combined bright field and fluorescence images of (a,b,c) glioma (IN699) cells and (d,e,f) astrocytes (SC1800) cells treated with no DC (control). Images acquired 3.5 h after incubation. Bar = 60 µm.

CHAPTER FOUR: Spray-dried particles containing o-carborane

Figure 4.1. Structure of PVP.

Figure 4.2. Literature 1H NMR spectrum of PVP [26].

Figure 4.3. 1H NMR spectrum of ‘as received’ PVP

Figure 4.4. 1H NMR spectrum of spray-dried PVP (2 %w/v).

Figure 4.5. 1H NMR spectrum of PVP (2 %w/v) co-spray-dried with o-carborane (0.2 %w/v).

Figure 4.6. 1H NMR spectrum of ‘as received’ o-carborane.

Figure 4.7. An example Sympatec particle size distribution output plot from sample #1 run 1 of 3 (full results in Appendix 1); x10 = 1.31 µm; x50 = 3.47 µm; x90 = 8.48 µm; SMD = 2.50 µm; VMD = ; x16 = 1.65 µm; x84 = 7.15 µm; x90 = 13.43 µm; S/V = 2.40 m²/cm³; S/m = 23973.67 cm²/g.

Figure 4.8. Particles diameters of spray-dried particles determined using spray drying (mean ± SD).

Figure 4.9. SEM images of (a,b) ‘as received’ PVP and (c,d) ‘as received’ o-carborane: Magnification (a) 100×, (b) 2200×, (c) 100×, (d) 800×.

Figure 4.10. SEM images of spray-dried PVP (inlet temperature 180 °C):
Sample (a) #1, (b) #2, (c) #3, (d) #4; magnification 2200×.

Figure 4.11. SEM images of spray-dried PVP (inlet temperature 130 °C):
Sample (a) #5, (b) #6; magnification 2200×.

Figure 4.12. SEM images of co-spray-dried PVP / o-carborane (inlet temperature 180 °C): Sample (a) #11, (b,c,d) #12 (different areas), (e,f) #13 (different areas); magnification 2200×.

Figure 4.13. SEM images of co-spray-dried PVP/o-carborane (inlet
temperature 180 °C), sample #13; magnification 400×.

Figure 4.14. SEM and EDS images of co-spray-dried PVP / o-carborane sample #11: (a) SEM; (b) B, (c) C, (d) O, (e) Na, (f) Au.

Figure 4.15. SEM and EDS images of ‘as received’ PVP / borax mixture (1:1 wt%): (a) SEM; (b) B, (c) C, (d) O, (e) Na, (f) Al.

Figure 4.16 SEM and EDS images of borax: (a) SEM; (b) B, (c) C, (d) N, (e) O, (f) Na.

Figure 4.17a. Effect of spray-dried PVP / o-carborane (samples #2, #8 and #11; combined results) on MRC-5 cell proliferation.

Figure 4.17b. MRC-5 cell death caused by spray-dried PVP / o-carborane (samples #2, #8 and #11; combined results).

Figure 4.18a. Effect of spray-dried PVP / o-carborane (samples #2, #8 and #11; combined results) on U87-MG cell proliferation.

Figure 4.18b. U87-MG cell death caused by spray-dried PVP / o-carborane (samples #2, #8 and #11; combined results).
LIST OF TABLES

CHAPTER ONE: Introduction
Table 1.1. Main categories of cancer. 1
Table 1.2. Cancer prefixes and meanings. 7
Table 1.3. Brain physiological structures and function [57]. 8

CHAPTER TWO: Liposomes loaded with o-carborane for BBB delivery
Table 2.1. Particle size, PDI and ζ-potential values for DPPC and DSPC liposomes with different volumes of incorporated o-carboranes. 61
Table 2.2a. Effect of re-probing DPPC liposomes containing various loadings of o-carborane on particle size, PDI and ζ-potential (n = 3). 77
Table 2.2b. Effect of re-probing DSPC liposomes containing various loadings of o-carborane on particle size, PDI and ζ-potential (n = 3). 79
Table 2.3a. Mean particle sizes and PDI values of DPPC liposomes before and after storage at 3-5 °C for 1 week; n = 3; SD = standard deviation. 80
Table 2.3b. Mean particle sizes and PDI values of DSPC liposomes before and after storage at 3-5 °C for 1 week; n = 3; SD = standard deviation. 81
Table 2.4. Time variation of particle sizes (diameters) of DPPC and DSPC liposomes unloaded and loaded with o-carborane (1000 µL) determined using AFM. 88
Table 2.5. Time variation of particle sizes (heights) of DPPC and DSPC liposomes unloaded and loaded with o-carborane (1000 µL) determined using AFM. 88
Table 2.6. Particle size, PDI and ζ-potential values for DPPC and DSPC liposomes incorporating calcein, with and without cholesterol in PBS and serum (n=3). 92
Table 2.7. Statistical differences (p values) between particle sizes for DPPC and DSPC liposomes incorporating calcein, with and without cholesterol in PBS and serum (one-way ANOVA, post-hoc analysis). 94
Table 2.8. Particle size, PDI and ζ-potential values for DPPC and DSPC liposomes incorporating Nile Red and differing volumes of o-carborane (n=2). 98
Table 2.9. ICP-MS detected volumes of o-carborane with different DPPC and 101
DSPC liposome o-carborane inclusions (n=3).

CHAPTER THREE: Efficacy of specific formulations of o-carborane

Table 3.1. Dilutions used for MTS assay.
Table 3.2a. MTS data obtained for IN699 cell line after 24 h.
Table 3.2b. MTS data obtained for SC1800 cell line after 24 h.
Table 3.3a. MTS data obtained for IN699 cell line after 48 h.
Table 3.3b. MTS data obtained for SC1800 cell line after 48 h.
Table 3.4a. MTS data obtained for IN699 cell line after 72 h.
Table 3.4b. MTS data obtained for SC1800 cell line after 72 h.
Table 3.5. LC\textsubscript{50} values of DC exposed to IN699 and SC1800 cells.

CHAPTER FOUR: Spray-dried particles containing o-carborane

Table 4.1. Samples for spray drying and instrument inlet temperature settings.
Table 4.2. %Yields obtained for various spray-dried formulations.
Table 4.3a. Statistical differences between x\textsubscript{90} data obtained from various spray-dried formulations; * \( p < 0.05 \), ** \( p < 0.01 \); *** \( p < 0.001 \); NS = no significant difference.
Table 4.3b. Statistical differences between x\textsubscript{50} data obtained from various spray-dried formulations; * \( p < 0.05 \), ** \( p < 0.01 \); *** \( p < 0.001 \); NS = no significant difference.
Table 4.3c. Statistical differences between x\textsubscript{10} data obtained from various spray-dried formulations; * \( p < 0.05 \), ** \( p < 0.01 \); *** \( p < 0.001 \); NS = no significant difference.
Chapter 1

Introduction

1.1 Cancer

Cancer is the name given to abnormal growth of cells in the body system initiated by genetic mutations in proto-oncogenes, tumour suppressor genes or DNA repair genes [1]. The first investigations of cancer were reported in 1600 BC in Egypt, where Papyrus discovered various cancers and the use of natural products for their treatment [2].

In the UK, one third of people will develop one form of cancer in their lifetime [3]. The diagnosis rate in males between 25 and 35 years of age is over a 10 000 cases in 100 000 people; the rate for females is over 2 000 cases for the same population. The survival rate of a certain cancer, which depends on the type of neoplastic tissue, is described as the patient survival after 5 years since diagnosis [4]. The occurrence of malignant tumours is higher than benign tumours [5].

The origin of cancer starts from a cell that has undergone genetic mutation [6,7]. The elucidation of cancer depends on knowing when a normal cell becomes cancerous [8]. The body is made up of many types of cells, which are the basic units of life [9]. For a healthy body living, cells grow and multiply to produce more daughter cells. However, when critical cell damage occurs, cell take another route to obliterate the damage through a pathway that results in apoptosis (cell suicide) for the replacement of old cells (cell
senescence) (Figure 1.1) [10,11]. This programmed cell death (apoptosis) is guarded by signalling pathways.

Figure 1.1. The origin of cancer: uncontrolled cell growth.

There are many factors that contribute to the onset of cancer, which may be grouped under the terms physical (environmental hazards, smoking, diet, UV exposure), chemical (occupational hazards, nitrosamines, asbestos) and biological (viruses, bacteria) carcinogenesis. These activate the method that leads to the deregulation of genes for proliferation of neoplastic tissues [12]. Different types of cancer are dominant in different parts of the world [13]. For example, in Japan, stomach cancer is well known; in the US, colon is prominent; skin cancer is a major type in Australia, caused by excessive exposure
to sunlight, which can lead to melanoma [14]. The high incidences of one type of cancer over another are related to different hereditary factors (genetic) and geographical/environment location [15]. Tobacco smoking causes more danger to health because it contains more than 24 chemicals that are liable to cause cancer, especially to the lung, mouth, larynx, oesophagus, stomach, pancreas, kidney and bladder.

Tumour protein P53, described as ‘the guardian of the genome’ (named after its function), is coded by TP53 in humans and triggers cell suicide. It is found on the short arm of chromosome 17 and codes for a protein that prompts apoptosis (cell death) that is imperative in tumourigenesis [16]. In half of all cancers, mutation in p53 can be observed [17].

1.1.1 Characterisation of tumours

Tumours can be categorised as either liquid or solid. Leukaemia (cancer of bone marrow and blood) and lymphomas are examples of liquid tumours, where the neoplastic cells precursors are usually mobile. Solid tumours present the key cancer burden with cancer starting in tissues including breast, lung, colon, prostate and ovary constituting ca. 80% of all malignant neoplasms [18]. Solid tumours consist of epithelial or mesenchymal cells that are usually stationary (immobile). Only one or two mutation are needed to cause a malignant liquid tumour [19] and they do not require as much of the pathway to be stimulated because their precursors cells are in the mobile phase and are invasive. Solid tumours require a minimum of 3 mutations to cause a malignancy in adults and each of the mutation causes one alteration or another to their pathways. Chromosome translocations are not common in solid tumours [1].
Necrosis occurs among collections of cells, visualised under microscope as a zone of impaired cells where the release of the inflammatory mediators cause infection or lysis within the system. Apoptosis takes place during tissue remodelling [20-22] and necroptosis, paroaptosis and autophagy are various cell-type dependent methods routes for apoptosis [23].

Not all tumours are cancerous and some cancers, such as leukaemia, do not form tumours [24]. Tumour categorisation depends on morphological and histopathological forms of the tumour [25]. Tumour cells can be categorised as either benign or malignant [26]. Benign tumours are non-cancerous (suffix –oma), are generally not life-threatening and do not have the ability to spread (invade or metastatise) to non-affected cellular areas and grow locally very slowly. They can be removed surgically by a procedure known as debulking [27], which is a very good solution due to the well-defined boundary of limitation. Malignant (cancer) tumours cells are morbidly debilitating and invade neighbouring tissues, enter blood vessels and metastasize to different sites in the body that make them spread sporadically and become cancerous [28]. Malignant melanoma is an aggressive neoplasm that is connected with great risk of death [29].

The malignancy sequence is based on the capacity of a cell to affect other neighbouring cells due to activation of matrix metalloproteinase enzymes (MMPs) and angiogenesis [30-32]. The advancement of neoplasm leads to metastasis which in turns causes the porosity deformation of neoplastic cells enhancing tissue leakages (intravasation) [33]. This causes a great change in proliferation, morphological assessment of the cell and tissues structural design. In a normal cell, the tissue does not need any supply of oxygen from vascularised tissues.
Tumours can be grouped into Grades 1 to 4, the higher end being the more aggressive and unsafe [34-37]. The grades are used to understand patient’s health status and prognosis. Staging is another method used in tumour classification; it provides a measure of the extent of metastasis. For central nervous system (CNS) tumours, staging is assessed by the results from CT or MRI scans, or by cerebrospinal fluid examination [38].

Cancers spread over the body in two ways: invasion and metastasis [39,40]. The former is a straight migration of cancer cells into neighbouring tissues while metastasis is the enablement of cancer cells to spread into blood vessels and lymphatic tissues, move around the blood stream and invade normal cells elsewhere [41,42].

Angiogenesis is the growth of new blood vessels [43]. Directly or indirectly, this process involves the vascular endothelia growth factor (VEGF), vascular permeability factor (VPF) or vasculotropin [44,45]. Migration, genetic signalling and multiplication of endothelial cells (ECs) are activated by VEGF and also ECs hyper-permeability in order for plasma protein to get into extravascular regions.

A normal cell can become cancerous through a series of morphological alterations and synthesis of cell type-specific proteins, but mostly through mutational modification. The capability of a cell to have unlimited division is known as immortalisation and is one of the main prerequisites of tumourigenesis, carcinogenesis or oncogenesis [46]. A single gene is not solely responsible for oncogenesis [47]: a transformation in 3 types of genes - oncogenes, tumour-suppressor genes and stability genes - leads to the development of cancer [24]. Therefore, cancer could be described as a genetic disease that has undergone
abnormal cell division without control, developed from different unfavourable conditions, e.g., heredity, menstrual irregularities or physical state [48].

Cancer is not just one disease but a group of heterogeneous diseases of different malignant neoplastic growths. There are more than 100 cancers that have a common hallmark of unchecked growth that advances without limitation [7,49,50]. Hallmarks of cancer involve six biological abilities: proliferative signalling, evading growth suppression, resistance to cell death, enablement of replicative immortality, inducement of angiogenesis and activation of invasion and metastasis [50].

Cancer can form in any part of the body, although the name of any particular cancer indicates where it originated. A primary cancer indicates the site of the cancer is where it originated, whereas a secondary cancer denotes one that has metastasised or infiltrated to form a tumour elsewhere in the body [51]. A typical example is colon cancer (primary) that spreads to the liver (secondary cancer): this is then called metastatic colon cancer and cancer that begins in melanocytes of the skin is melanoma (primary cancer). A brain tumour might be primary or secondary: if it starts from the brain, it is primary (benign or malignant), but if the cancer metastasises to the brain from other organs, such as from skin, eye, colon, breast etc, then the cancer is denoted a secondary brain cancer [52].

In only a very low percentage of cases are patients having a secondary tumour in which the origin (primary site) is not known, i.e., cancer of unknown primary origin (CUP) [53-55]. CUP is a set of heterogeneous neoplasms that share unique clinical features, but without a specific site of origin at the time of diagnoses [56]. This may be because the primary
tumour is too small to be detected by imaging tests and/or is cleared away before the secondary tumour begins; there may also be many primary sites. CUP can appear anywhere, but are mostly found in the lymph nodes, liver, lungs, bones or skin.

1.1.2 Naming cancers

Various methods have developed for naming cancer to differentiate them from one another, e.g., carcinomas, sarcomas, lymphomas and leukaemia (Table 1.1). Latin prefixes are used to indicate the location of the primary (Table 1.2). For instance, the prefix “osteo” means bone, i.e., cancer that originates in bone is osteosarcoma, while “adneo” means gland: adenocarcinoma.

Table 1.1. Main categories of cancer.

<table>
<thead>
<tr>
<th>Category</th>
<th>Origin</th>
</tr>
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<tbody>
<tr>
<td>Carcinoma</td>
<td>Skin or in tissues that cover internal and external organs. Different types: adenocarcinoma, basal cell carcinoma, squamous cell carcinoma and transitional cell carcinoma.</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>Bone, cartilage, fat, muscle, blood vessels or other connective or supportive tissue.</td>
</tr>
<tr>
<td>Leukaemia</td>
<td>Blood-forming tissue such as the bone marrow. Causes large numbers of abnormal blood cells to be produced and enter the blood.</td>
</tr>
<tr>
<td>Lymphoma and myeloma</td>
<td>Cells of the immune system.</td>
</tr>
<tr>
<td>Central nervous system cancers</td>
<td>Brain tissues and spinal cord.</td>
</tr>
</tbody>
</table>
Table 1.2. Cancer prefixes and meanings.

<table>
<thead>
<tr>
<th>Prefix</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adeno</td>
<td>Gland</td>
</tr>
<tr>
<td>Chondro</td>
<td>Cartilage</td>
</tr>
<tr>
<td>Erythro</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>Hemangio</td>
<td>Blood vessels</td>
</tr>
<tr>
<td>Hepato</td>
<td>Liver</td>
</tr>
<tr>
<td>Lipo</td>
<td>Fat</td>
</tr>
<tr>
<td>Lympho</td>
<td>Lymphocyte</td>
</tr>
<tr>
<td>Melano</td>
<td>Pigment cell</td>
</tr>
<tr>
<td>Myelo</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>Myo</td>
<td>Muscle</td>
</tr>
<tr>
<td>Osteo</td>
<td>Bone</td>
</tr>
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</table>

1.2 Brain tumours

1.2.1 Brain anatomy

The brain and the spinal cord form the two parts of the central nervous system (CNS). The brain can be divided into three categories: fore-brain (prosencephalon), the mid-brain (mesencephalon) and the hind-brain (rhombencephalon) (Figure 1.2; Table 1.3) [57,58]. The prosencephalon consists of the diencephalon (cerebral cortex) and telencephalon [57,59,60] and it is used for the collection and sensory information processing, thinking, perceiving and understanding language and controlling of motor neurons. The diencephalon is situated at the posterior region of the forebrain, which also consists of the thalamus and hypothalamus [58] that are for controlling motor neurons, relaying sensory information and for autonomic purpose. The telencephalon is the largest part of the brain and consists of two main parts: the cerebral hemisphere and the median region (the lamina terminalis) [58].
The midmain (mesencephalon) [58] is the integral part of the brainstem that joins with the hindbrain and the forebrain, *i.e.*, with the pons underneath and the diencephalon on top [57]. The midbrain is for auditory, motor functioning and visual responses. The mid- and hindbrain are jointly known as the brainstem.
Table 1.3. Brain physiological structures and function [57].

<table>
<thead>
<tr>
<th>Brain structure</th>
<th>Function</th>
</tr>
</thead>
</table>
| Basal Ganglia                        | • Involved in cognition and voluntary movement  
• Diseases related to damages of this area are Parkinson’s and Huntington’s |
| Brainstem                            | • Relays information between the peripheral nerves and spinal cord to the upper parts of the brain  
• Consists of the midbrain, medulla oblongata, and the pons |
| Broca’s Area                         | • Speech production  
• Understanding language |
| Central Sulcus (Fissure of Rolando)   | • Deep groove that separates the parietal and frontal lobes |
| Cerebellum                           | • Controls movement coordination  
• Maintains balance and equilibrium |
| Cerebral Cortex                      | • Outer portion (1.5 mm to 5 mm) of the cerebrum  
• Receives and processes sensory information  
• Divided into cerebral cortex lobes |
| Cerebral Cortex Lobes                | • Frontal Lobes: involved with decision-making, problem solving and planning  
• Occipital Lobes: involved with vision and colour recognition  
• Parietal Lobes: receives and processes sensory information  
• Temporal Lobes: involved with emotional responses, memory and speech |
| Cerebrum                             | • Largest portion of the brain  
• Consists of folded bulges called ‘gyri’ that create deep furrows |
| Corpus Callosum                      | • Thick band of fibres that connects the left and right brain hemispheres |
| Cranial Nerves                       | • Twelve pairs of nerves that originate in the brain, exit the skull, and lead to the head, neck and torso |
| Substantia Nigra                     | • Helps to control voluntary movement and regulates mood |
| Wernicke’s Area                      | • Region of the brain where spoken language is understood |
| Medulla Oblongata                    | • Lower part of the brainstem that helps to control autonomic functions |
| Meninges                             | • Membranes that cover and protect the brain and spinal cord |
| Olfactory Bulb                       | • Bulb-shaped end of the olfactory lobe  
• Involved in the sense of smell |
| Pineal Gland                         | • Endocrine gland involved in biological rhythms  
• Secrets the hormone melatonin |
| Pituitary Gland                      | • Endocrine gland involved in homeostasis  
• Regulates other endocrine glands |
| Pons                                 | • Relays sensory information between the cerebrum and the cerebellum |
The hindbrain (rhombencephalon) runs from the spinal cord and consists of metencephalon (pons and cerebellum) and myelencephalon (medulla oblongata) [58]. The pons and cerebellum are used to maintain balance equilibrium, for coordination and sensory information. The myelencephalon comprises the medulla oblongata that is important for controlling breathing, heart rate and digestion.

1.2.2 Brain tumours

Brain tumours (gliomas) are extremely heterogeneous both morphologically and genetically [61], and include various types such as astrocytomas, oligodendrogliomas and ependymomas [62]. High grade gliomas, especially glioblastoma multiforme (GBM, a grade IV glioma) are highly terminal and resistant to many different modalities of therapy, including chemotherapy [63,64].

The brain consists of neurones (or nerve cells) and glial cells (supporting tissues). The former represent the part of the brain that allows it to convey electrical messages to different part of the body, whereas the glial tissues allow the passage of blood vessels in and out of the brain, which is protected by membranes/lining known as meninges. Primary brain tumours may originate from any of these types of cells. Secondary tumours, which are more common, originate from other parts of the body, such as lung, skin, prostate and breast.

Clinical diagnosis of glioma is usually obtained from histology [65]. Around 5000 cases of brain tumours are diagnosed monthly [66]. They are mostly found in the elderly, although
do occur across a range of ages [67,68]. Malignant brain tumour proliferation is rapid and even when debulking surgery has been performed, micro-invasive infiltration into normal cell regions can occur [28]. Brain tumours do not metastasise to other organs and thus are primary tumours. Patients with gliomas generally have a very poor prognosis [69]: The radio-chemotherapeutic (temozolomide, Section 1.5.3.1) which is the current standard of treatment has a 5-year survival of 65% and a 7.5 year median survival [70,71]. Leaving behind cancerous tissue due to uncertainties in the surgical margin and multiple cancer sites is a major problem with surgery [72-74].

1.3 Liver cancer

1.3.1 Structure of the liver

The liver is a cone-shaped, dark reddish-brown organ located beneath the lower rib cage under the diaphragm in the upper right-hand portion of the abdominal cavity. In a healthy adult, the liver weighs 1200-1500 kg and the length is about 8 inches long, being larger in men than in women. The liver is described as a “workhorse” since the majority of metabolic activities are undertaken in this organ [75]. It consists of two main lobes, each comprising of many lobules which are connected to small ducts. The left and right lobes are anteriorly parted by a peritoneum known as the Falciform ligament (Figure 1.3), with these lobes being parted posteriorly by Fissure for ligamentum venosum [76]. There are two primary sources of blood supply to the liver: the hepatic artery (oxygenated blood) and the hepatic portal vein (deoxygenated blood, venous blood), containing nutrients.
The functions of the liver can be separated into three main areas: metabolism, filtration and secretion. In the former, the liver breaks down nutrients, and some drugs, in the blood supplied from the spleen, stomach and intestinal tract. After this, the metabolites are either transported to other tissues or retained in the liver for future usage, e.g., storage of glycogen. As a vascular filter, unwanted materials are removed from the blood to maintain homeostasis. The blood vessels in the liver lobes hold ca. 10% of the blood in the body. The liver also secretes a product known as bile to help sequester liver waste products and also to aid fat digestion. A numbers of diseases can arise from malfunctioning of the liver, such as hereditary disorder, drug-induced damage and cancer.

1.3.2 Liver cancer

Liver cancer is the sixth most common cancer and the third most common of all deaths, universally having a poor prognosis [77]. Hepatocellular carcinoma (HCC) is most popular malignant neoplasm of the liver [78]. Others include hepatoblastoma in children, Cholangio carcinoma in adults and angiosarcoma from blood vessels [77].
Cancer of the liver is a malignant neoplasm syndrome. The diagnosis of liver cancer at late stage has a poor prognosis that has become a bottle neck for health practitioners [79,80]. Cancer stem cells (CSCs) are the basis of the majority of solid tumours [81]. The liver is more prone to cancer due to the large number of metabolisms that take place, such as xenobiotic, lipid, carbohydrate, amino acid and hormone metabolism, in addition to degradation of plasma protein, storage of vitamins and metals [82]. There are many causes of liver cancer, e.g., hepatocellular carcinoma (Figure 1.4); one of the major causes is the change of homeostasis balance of S-adenosylmethionine (SAMe) synthesis and catabolisation in the liver [83].

Figure 1.4. Causes of liver cancer [84].

1.4 Lung cancer

1.4.1 Structure of the lungs

The lungs are used for gas exchange and are both located in a single compartment [85]; they are composed of a bronchial tree [86,87] that occupies the majority of the thoracic cavity (Figure 1.5).
The lungs are a soft, spongy mesh of tissues, comprising of alveolar cells and elastic connective tissues [88]. The lungs are separated from each other by the mediastinum [89,90]. Further down the trachea, there is a separation into the left and right primary bronchi. These structures are asymmetrically branched [86] into continuously decreasing airways (bronchioles) until they terminate in tiny sacs called alveoli [91]. Blood vessels enter the lungs at a structure called the hilum. The right lung is made up of three lobes and is shorter, wider and of greater volume than the left lung since it grows faster [92] while the left lung comprises two lobes, being longer and narrower than the right and having a hollow called cardiac notch. Each of the lungs is surrounded by double layered membrane,
called the pleural [91]. The visceral pleural is strongly attached to the periphery layer of
the lung. Through the hilum, the visceral pleural is extended with the parietal pleura that
covers the wall of the thoracic cavity. The small space between the visceral and the parietal
pleurae is known as the pleural cavity [91], which has a film of fluid that is produced by
the pleura. The fluid serves as an emollient to decrease friction and also helps to hold the
lung layers together as the alveoli inflate and deflate during gas exchange [93].

1.4.2 Lung cancer
Data collected by WHO suggest that lung diseases are responsible for 71% of the total
disease burden worldwide [95]. Lung cancer (small cell carcinoma) causes the largest
number of cancer deaths in men and women in developed countries and worldwide
[96,97], accounting about 1/6th of all deaths [98-102]. Lung cancer accounts for 1.35
million of the overall cases globally and 1.18 million deaths recorded in 2002, while in
2008, this had increased to 1.6 million reported cases and 1.4 million deaths [102]. Lung
cancer incident rates in men are more prominent in Southern Europe, North America and
Eastern Asia. In women, the highest incidence rate are mainly in Northern Europe,
Australia, North America and New Zealand [102]. The majority of the lung cancers burden
would be eradicated if only people could do away with cancer-associated life-styles, e.g.,
tobacco products in-take and westernised nutrition [102]. Increasing the price of tobacco,
having counter-advertisements, prohibiting smoking in public could lead to a reduction in
the number of incidences of this disease [103]. Annual data collected from WHO predicts
tobacco consumption is leading to a lung cancer epidemic that will last for several years
[96,102]; however, recent advances in early detection technology [104,105] and targeted
therapies show future promise [105].
Lung cancers used to be treated solely using radiotherapy, whereas now, surgery, combined surgery-radiotherapy, chemotherapy and intra bronchial arterial infusion have led to slight increased survival rates [106,107]. Patients with early stage lung cancer can be treated with thoracic surgery (cure rate \( \approx 80\% \)). Advanced lung cancers are usually treated with combined therapies.

1.5 Cancer therapies

The best strategy for the treatment of cancer cells is early discovery of cancer which is important in the treatment and eradication of cancerous cells; using targeted therapeutic agents that stop the advancement of early neoplastic tissue development [108]. In this way the problem of malignancy can be overcome before getting to the late-stage because the sooner a cancer is detected and treatment begins, the better the chances that the treatment will be effective [29,48,108-113]. Cancer therapy focused on inducing cell death of tumour cells whilst causing low toxicity or no harm to the normal cells [114]. The best cancer therapy effectively attacks the main parameters that cause tumours, the heterogeneity and growth capability [111]. Therapeutic interventions include surgery, radiotherapy and chemotherapy and combinations thereof. The sections below outline these methods with a particular emphasis on brain tumour therapies.

1.5.1 Surgery

Surgical therapy is invasive and it is the main technique for treatment of the brain tumour because of problem of detecting brain tumour at early stage [115]. The only problem in surgery is the removal of the tumour without damaging other vital areas (cognitive, motor neurons, reflexes \( etc \)) in the brain. To confirm the historical type, classification, grade and
potential aggressiveness of cancer, an invasive biopsy is often carried out. Surgery is often recommended initially for the removal of brain tumours.

1.5.2 Radiotherapy

Radiation is a non-invasive treatment and is often used immediately after surgery. It is also useful for killing tumours at an early stage of their development. Radiation is administered in very low dosage (1.8–2.0 Gray (GY)) and given to patients on a weekly basis to allow normal tissues to recover from sub-lethal destruction [116]. Radiation treatment is often a slow process and carries the risk of reducing mental reasoning and initiating secondary tumours in the area of irradiation [117]. In children, radiation can affect brain development [118]. The performance of radiation therapy is mostly prevented by invasive diffusion characteristics of the brain tumour coupled with radiation resistance in populations.

1.5.3 Chemotherapy

Chemotherapeutic agents have a low therapeutic index. Chemotherapy is toxic and can affect not only cancer cells but also healthy cells which lead to severe systemic effects and all too often to patient deaths [119-121]. Chemotherapy treatment of brain cancer is not common due to the BBB that prevents passage of a wide range of potential anticancer drugs.

Using more than one drug (combinational chemotherapy) is a widely accepted practice in oncology, although multidrug resistance (MDR) by neoplastic cells is common [122]. This is due to the drug efflux character of p-glycoprotein or multidrug resistance protein [123]. The former is an ATP-dependent transporter in charge of cellular extrusion of many drugs and is found in many tissues (e.g., luminal brain of the cerebral endothelium).
Administrations of drugs are typically spaced up to three weeks between treatments to allow repopulation of injured proliferating cells [116]. During this time, however, neoplastic cells can also recover and thus the efficiency of chemotherapy can become limited.

1.5.3.1 Brain tumour chemotherapy using temozolomide

Temozolomide (TMZ; Temodar, Temodal, Temcad; 5-(3-methyltriazen-1-yl) imidazole-4-carboximide, MTIC; Figure 1.6) is an orally administered drug for the treatment of Grade IV gliomas; it has a high oral bioavailability [124-128]. It is also used for malignant melanoma (anaplastic astrocytoma) [124]. TMZ works by methylating DNA, usually at N-7 or O-6 positions of guanine residues [125,127,128]. This initiates the apoptosis process of the tumour cells. The drug is not as popular as it once was in the 1970s and 80s [127], and research is now being carried out in combining the therapy with chloroquine [129], epigallocatechin gallate (EGCG, green tea) and other agents [126-130].

Figure 1.6. Structure of Temozolomide.

1.5.4 Radiochemotherapy

Radiochemotherapy is combination of radiation and chemical therapy that has been used with mixed success in the treatment of adult brain tumours [131]. Both radiotherapy and
chemotherapy are administered in multiple doses allowing time for the recovery of normal cells between the treatments [116]. A study has shown that temozolomide is the best and a generally well-tolerated drug for the treatment of gliomas and its efficacy can be enhanced when used in combination with radiotherapy [27]. Median survival times are improved from 12.1 to 14.6 months for the addition of temozolomide to radiotherapy treatment [27].

1.5.5 Proton therapy

Much recent media interest has been paid to proton therapy. In conventional x-ray radiation, the energy from primary photons and secondary electrons are projected as a beam that penetrates beyond the cancer site which can adversely affect normal cells [132]. Proton therapy is a form of radiotherapy, but a beam of protons is directed towards the cancer, causing ionisation and therefore DNA damage to the cancer, but does not penetrate beyond the cancer site; the penetration depth can be precisely controlled (Figure 1.7). This minimises the damage to normal cells. Clinical use of the technique came into place in the 1970s and is increasing used around the world [133], particularly for paediatric malignancies [134], which has helped in cure rate of most childhood neoplasms offering a 5 year survival rate of 56 – 75% [135,136].

1.5.6 Photodynamic therapy

Photodynamic therapy involves introducing a photosensitiser, such as pholofrin, to the tumour and exciting it at the correct wavelength to produce an oxygen radical/some other reactive oxygen species that can stimulate apoptosis whilst reducing systemic toxicity [137]. The treatment is mainly restricted to cutaneous malignancies and those of the
bladder that can be readily reached by light, although is being extended to treatment of malignant gliomas [138] with reported increases in median survival [139].

Figure 1.7. The differences in radiation path between proton therapy and standard x-ray radiotherapy for the treatment of brain tumours.

1.5.7 Gene therapy
Gene therapy is a medical technique that offers enormous potential for solving the challenges of cancer worldwide by correcting the disease at a biomolecular level. If a specific gene can be inserted or a mutant gene ‘switched off’ then the disorder can be treated ‘at source’ [140]. The therapeutic aim is to introduce genetic biomaterial into the cell that codes for the missing or deficient protein or siRNA/shRNA [141-146]. Gene therapy has the capability of withstanding a prolong administration of a single dose. Genes are hydrophilic, charged and are large molecules, however, so mechanisms of passing the BBB are required.
1.6 Boron neutron capture therapy

1.6.1 Principle of BNCT

In 1936, Locher proposed a treatment for cancer using neutrons [147] that was later extended to the technique known as Boron Neutron Capture Therapy (BNCT) [148-151]. This technique is a non-conventional radio-chemotherapy technique [152-156] that provides a way of selectively destroying malignant cells and sparing the normal cells [28,157]. Boron (\(^{10}\)B) has a large neutron capture cross-section and when the element is irradiated with low-energy (thermal) neutrons, a nuclear fission reaction occurs to yield high-linear-energy-transfer (HLET) \(\alpha\)-particles (1.5 MeV) and a recoiling \(^7\text{Li}^+\) (0.8 MeV; ca. 2.4 MeV for both fragments) [158-162] (Figure 1.8). The penetration of the \(\alpha\)-particles and \(^7\text{Li}^+\) into surrounding tissues is only 8 and 5 \(\mu\)m, respectively, and so the highly-ionising energy dose is confined to the original \(^{10}\)B containing cell or at least to the adjacent cells. Since the collateral damage is very low, this in principle, allows for a particularly targeted therapy (Figure 1.9). For this to occur, a sufficient quantity of \(^{10}\)B must be selectively localised into the cancer cells, and the correct number of thermal neutrons must be aimed at the \(^{10}\)B-containing cancer cells [163].
Chapter 1: Introduction

Figure 1.8. The BNCT reaction.

Figure 1.9. The application of BNCT in a hospital setting [164].
1.6.2 Type of cancer treated with BNCT

BNCT treatment has mainly been focused on gliomas, but other cancers have also been investigated, such as head and neck, and liver cancers [28]. Also, non-malignant diseases, such as rheumatoid arthritis, are useful in BNCT treatment [165]. Penetration of the low-energy thermal neutron beam, from a nuclear reactor, through tissues is the limiting factor [166]. GBM brain tumours have been regarded as the most appropriate for research study owing to its aggressiveness [167] and poor prognosis [165]. The high mortality seen in incidences of lung cancer [165] prompt a wide-spectrum use of BNCT for this cancer type [168,169]. A majority of patients with lung cancer would die from this disease if there was no other way of treatment other than surgical excision of the affected cancer part of the lung [165]. BNCT is therefore an attractive therapeutic approach [170].

1.6.3 Early history of BNCT

Despite the apparent simplicity of BNCT, the technique has had a chequered history, with progress encountering a number of barriers. The first BNCT treatment was performed on a glioma patient in 1951 [171] and was followed by the treatment of around 40 patients into the early 1960s [172]. Many of these patients, however, suffered from scalp radiodermatitis. In the US, BNCT was carried out on 18 patients using the less toxic and more boron-rich compound disodium decahydrodecaborate (Na₂B₁₀H₁₀), although many resulted in brain necroses and interest in the technique declined in the US for a while. Japan continued with BNCT in a clinical setting in 1968 using disodium mercaptoundecahydro-closo-dodecaborate (BSH; Na₂B₁₂H₁₁SH) [163]. Here, the tumour was treated with BNCT during an operative procedure and this increased the 5-year survival rate of the patent giving renewed hope for the technique [173]. Since then BNCT
has increased in popularity, although has never become mainstream owing to problems associated with poor tumour selectivity, accessibility of thermal neutron beams in a hospital environment (rather than a nuclear reactor), neutron beam penetration issues and lack of randomised clinical trials with equivocal demonstration of therapeutic efficacy [28,163].

1.6.4 Boron actives for BNCT

The quest for new boron-containing compounds for use in BNCT has continued since the conceptualisation of the technique. These molecules generally fall into first, second and third generation compounds.

1.6.4.1 First generation compounds

In the 1950s, sodium borate (Borax), boric acid and pentaborates were used in BNCT to meet the demand for low toxicity, high tumour uptake and tumour concentration of 20 $\mu$g $^{10}\text{B} \text{ g}^{-1}$ (per gram of tumour) [174]. Despite these conditions, however, patients still suffered from disease recurrence [175], mainly owing to the poor tumour retentions and attained and low tumour/brain ratios [175]. Consequently, in the 1960s, various arylboronic acids were used that had lower toxicities, increased tumour/brain concentration ratios and had good retention abilities [176]. Para-carboxyphenylboronic acid (PCPB) was marked as being particularly promising [177].
1.6.4.2 Second generation compounds

BSH and boro-phenylalanine (BPA; 1,4-dihydroxyborylphenylalanine) are second generation boron compounds and have both passed clinical trial stage (Figure 1.10) [158,178].

![Chemical structures of (a) BSH and (b) BPA.](image)

BSH and BPA are both effective in selective tumour cells uptake. The former compound was initiated by Hatanaka’s group and has been applied successfully for the treatment of human glioblastoma in BNCT [179,180]. BSH has a higher %B than first generation compounds and has a low toxicity.

BPA is an analogue of the amino acid phenylalanine. The large uptake of amino acids by rapidly dividing cancer cells provides the mechanism for the specific incorporation BPA into cancer cells [181]. BPA was used at any early stage in the BNCT treatment of melanoma [182].
1.6.4.3 Third generation compounds

Carboranes, caged molecules based on boron, exhibit high molar boron content, neutral charge and good stability [183]. They are therefore ideal candidate molecules, the third class of compounds, for use in BNCT [184]. These molecules, however, are highly hydrophobic, making their delivery through the physiological medium very difficult [184,185].

Carboranes may be synthesised by the reaction of acetylene, or its derivatives, with boron hydrides. The polyhedral structure of carboranes exhibits three different forms: ortho, meta or para (Figure 1.11) [178,186]. The carboranes are regarded as biocompatible materials [187] and their anions confirm little coordinating power [188], but for application in BNCT, structural modifications are often necessary to improve the aqueous solubility of these molecules [187,189]. The ionic carborane derivatives have the ability to target tumour cells for BNCT [190].

Figure 1.11. The three isomers of carborane: (a) ortho-carborane (1,2-C₂H₁₂B₁₀), (b) meta-carborane (1,7-C₂H₁₂B₁₀), and (c) para-carborane (1,12-C₂H₁₂B₁₀); ○ = BH, ● = CH.
1.7 The blood brain barrier

Over a century ago, Paul Ehrlich (1885) demonstrated that many water-soluble dyes injected intravenously into the blood circulation were unable to pass into the brain [191]. His successor, Goldman (1913) injected dyes into the CFS and found that the brain has been stained but did not include peripheral organs, suggesting only small molecules could pass through what became known as the blood brain barrier (BBB) [192]. This structure impedes the influx of materials from blood to the brain, with brain blood capillary endothelial cells causing the low permeability due to tight junctions (Figure 1.12) [193]. The barrier is important for the regulation of the homeostasis of the brain’s (neural) microenvironment of ions, nutrients and signalling and prevents entry of pathogens [194]. The BBB, however, has proven to be a bottle neck for the delivery of therapeutic agents to the brain to treat a variety of central nervous system disorders [195-197].

The BBB consists of three types of cell membranes: the endothelium (or basal-membrane; connected with tight-junctions, TJs), pericytes (necessary in angiogenesis regulation and endothelial proliferation) and astrocytes (which stimulate BBB properties in the endothelial membrane) [198]. The TJs are an important part of the integrity of the BBB [199]; they, along with biological mechanisms, inhibit the free diffusion of blood-borne solutes from the blood into the brain [200-204]. An efflux system further enhances the BBB by pumping drug molecules out of cells [197,200,205-207]. For these reasons, fewer than 5% of known drug molecules are able to reach the brain, regardless of their potential in vitro [208].
1.7.1 Transport pathways across the blood-brain barrier

The BBB acts as both a physical and biological barrier for regulating and maintaining important nutrients and essential materials in the brain (Figure 1.13) [201]. The TJs between neighbouring endothelial cells provide the physical barrier. This forces most molecular traffic to take a transcellular route across BBB instead of allowing paracellular movement. The biological barrier is essentially the layer of endothelial cells and their cell membranes that have various receptors (‘gatekeepers’) and mechanisms for allowing the influx and efflux of important endogenous biomaterials (substrates), such as sugars, amino acids, nucleotides and inorganic ions [203,210-214]. Small hydrophobic molecules, such as barbiturates and ethanol, are able to use the transcellular route. Small gaseous molecules
(O₂, CO₂) are also able to use this route. Hydrophilic molecules, large and small, such as histamine, peptides and proteins, are not able to pass via the transcellular route.

Figure 1.13. Schematic diagram of the transport pathways across the BBB [215].

The BBB prevents systemic chemotherapy to the brain and central nervous system. Strategies for brain drug delivery may be broadly classified as being either neurosurgical-based (invasive), pharmacologic-based, or physiologic-based.

For biomolecules to successfully cross the BBB through passive diffusion, high lipid solubility and a molecular mass range of 400 – 500 Da are required. Since small molecules do not normally penetrate the BBB [211,216,217], much recent research has been focused on developing biomaterial-based nano- and microcarrier systems for drug delivery [218].
These have included the use of liposomes [219-224], since they are small (ca. 100 nm), biodegradable and can protect the active drug from degradation [225-227].

1.8 Liposomes

For effective delivery of drugs to the brain, many approaches are under investigation, including modulation of BBB, inter-cerebral infusion/injection, nanoparticulate drug delivery, the use of pro-drugs, chemical delivery and delivery via liposomes [228]. Research efforts towards liposome-facilitated drug delivery are rationalised in terms of the biodegradability, non-toxicity, non-immunogenicity and also in terms of their capacity to accommodate a wide range of drug molecules, irrespective of hydrophilicity [229,230]. A good drug delivery system ensures that the active drug is present at the site of action at the right time and for right duration [228]. Drug carriers are necessary in pharmaceutical formulations for many reasons [231]: (a) to enhance drug targeting [228], (b) to overcome solubility issues, (c) to protect drugs (such as protein and DNA) from undesired interactions that could lead to hydrolysis, enzymatic degradation, loss of original structure or activity, and (d) reduce drug toxicity.

Drugs can be chemically modified using pro-drug techniques or by making use of a biological carrier system/drug delivery vehicle [232], which can be divided into two major groups: soluble carrier systems and particulate carriers. The former may comprise of natural or synthetic water soluble polymers, where drugs are conjugated to the delivery vehicle. Examples of the latter system include liposomes, microspheres and nanoparticles, where the drug is either surface-bound or entrapped within the carrier.
The physiological environment of the targeted site plays an important role in drug targeting as the drug may accumulate naturally at the targeted site (passive targeting). Alternatively, drugs can be targeted to the site using target-specific recognition components, e.g., the addition of a galactose group to a drug carrier system can enhance targeting of galactose receptors on the surface of liver cells (active targeting) [228].

1.8.1 Structure of liposomes

Liposomes are artificially prepared vesicles made of a lipid bilayer [233,234] and are composed of naturally-derived phospholipid bilayers with mixed lipid chains [235,236] (Figure 1.14). Their size may vary from 30 nm to many micrometres [237]. Phospholipids are a class of lipid which occur naturally as biomolecules that play an important role in human physiology, serving as components of biological membranes. The hydrophilic head is the phosphate group which is negatively charged and the hydrophobic tail is a fatty acid. Phospholipids are cholesterol-like compounds found in all layers of the skin, except the stratum-corneum [237,238]. Asymmetrical phospholipids are commonly used for liposomal dispersion (Figure 1.14b) [228,238]. The biphasic properties of liposomes make them attractive as a vehicle carrier for both lipophilic and hydrophilic drugs. This enables them to have different time latencies and retention capacities [239].
Liposomes are classified according to their size, lamellarity and numbers of bilayers or vesicles [240]. For example, liposomes may be unilamella (small unilamella, SUVs, < 100 nm; or large unilamella, LUVs) or multilamella, MLV. SUVs are circular in shape (typically 20 nm in diameter) [240] and are homogenously distributed which makes them unique among other vesicles [241]. The low encapsulation efficiency (ca. 0.1 – 1 %) reduces their capacity as drug vehicles. LUVs (> 100 nm) have high encapsulation
efficiencies [242]. MLVs are up to 5 or more bilayers (diameter > 100 nm) and may contain SUVs and/or LUVs; their encapsulation capacity is generally poor.

Further variation in liposome structure and properties can be achieved using various inclusions and graftings (Figure 1.15). Conventional liposomes, so far outlined, are comprised of phospholipids in an aqueous compartment that form lipid bilayers. Rigidity can be improved by the addition of cholesterol (Figure 1.16) [243].

![Figure 1.15. Different types of liposomes. Components: phospholipids (blue), PEG lipid (orange), antibody or targeting ligand (red), cationic lipid/helper lipid (black cross). Adapted from [237].](image)

The bilayers they form consist of hydrophilic and hydrophobic layers, which make it possible for the encapsulation of lipophilic drugs in the hydrophobic area and also for lipophilic (hydrophobic) drugs to be encapsulated in the hydrophilic region. PEGylated (or long circulating) liposomes have their surfaces coated with a polyethylene glycol (PEG) that allows them to resist being coated with opsonin, a plasma protein which makes liposomes more easily recognised by the reticuloendothelia system or mononuclear
phagocytic system (MPS) in the liver, causing longer circulation and release profiles [234,244]. With PEGylated liposomes, the circulation time is not dependent on the dose administered [245]. Other liposome types include targeted liposomes (ligand bearing or immunoliposomes – those involving targeted antibodies as ligands), cationic liposomes (for genetic material delivery) and deformable/elastic liposomes.

![Chemical structure of cholesterol](image)

**Figure 1.16. Chemical structure of cholesterol.**

### 1.8.2 Use of liposomes for chemotherapy

Liposomes are ideally suited for nano-drug delivery systems: actives may be entrapped within the lipophilic or hydrophilic regions within the liposome structures [149,246-248]. A promising application is based on the uptake of liposomes by the reticuloendothelial system (RES) for treatment of infection or for vaccination. Liposomes have been used for treating infections [249-251], developing vaccines [252-254] and gene delivery [244].

In addition, the ability of SUVs to persist in the circulatory system serves as an access into neoplastic tissue [235,255,256]. This is important for developing novel chemotherapy
techniques [246], where higher efficiencies for delivering toxic tumouricidal agents to the tumour are required [257]. Encapsulation of potent drugs in liposomes reduces their toxic adverse side-effects [258].

Almost all anti-cancer drugs have been encapsulated in liposomes using diverse technologies [259]. The suitability of liposomes to act as anticancer drug carriers depends on the potency of the drug they carry [260]. The successful encapsulation of doxorubicin and daunorubicin in liposome has resulted in two products – doxil and daunoxome [258], which have been clinically approved [259] for the treatment of Kapos’s sarcoma [261]. Over-expressing cells have also been targeted using immunoliposomes [262] and thermosensitive liposomes [263].

1.8.3 Drug delivery to the brain using liposomes

Therapeutic drug delivery to the brain through the intravenous route is problematic due to the blood-brain-barrier (BBB), a physiological natural barrier that restricts the number of potential drug molecules from entering the brain. Small (MW < 400 – 600 g mol\(^{-1}\)) lipophilic compounds are able to diffuse across the BBB, but most drugs in the circulatory system are not able to pass through [264]. Immunoliposomes (antibody-directed liposomes; Figure 1.15) provide a method of site-specific drug delivery providing steps are taken do reduce their rapid clearance by the retroendothelial system, such as PEGylation [265]. Huwyler \textit{et al.} encapsulated daunomycin, an anti-cancer agent, into PEGylated liposomes (85 nm), each coupled to 30 OX26 antibodies [266,267]. These were found to accumulate successfully in brain tissue, overcoming the BBB limitation. Similar transport
across the BBB was achieved with liposomes (< 100 nm) containing various nerve growth factors (NGFs; 34% encapsulation) [268].

1.8.4 Use of liposomes for BNCT

Liposomes are ideal carrier systems as they accommodate large numbers of boron containing species for selective delivery to neoplastic tissues [149,267]. Boronated compounds may be inserted within the vacant polar inner space of liposomes or, in the case of polyhedral boranes, encapsulated within the lipid double-layers [267,269]. The *in vitro* delivery of boron species by a liposomal carrier was introduced by Yanagie *et al.* [270]. A number of methods, systems and species have been explored.

Pan *et al.* passively entrapped BSH and an amino-boron compound Na₃(B₂₀H₁₇NH₃) into folate receptor (FR)-targeted SUV PEGylated liposomes for specific delivery, achieving encapsulation efficiencies of 6 and 15%, respectively [271]. Boronated polyamine derivatives were also investigated. The FR-targeted liposomes delivered large quantities of boron to tumour cells (1584 µg/10⁹ cells), exceeding the lethal dose (20-35 µg/g tumour). The same amino-boron compound entrapped in PEG-2000-distearoyl phosphatidylethanolamine liposomes was investigated by Feakes *et al.* [272]. As with most PEGylated liposomes, long circulation times were observed, with a peak concentration of 47 µg of B/g tumour being reached after 48 h. Awad *et al.* studied interactions between BSH and liposomes that offered suggestions for the strong binding and intracellular uptake (cancerous tissue is rich in choline) of this boron species in glioblastoma patients prior to BNCT [186].
Justus et al. used double-tailed lipids covalently bound to carboranes, producing a double negatively charged head group, to making liposomes [273]. These were found to be less toxic than \((\text{B}_{12}\text{H}_{11}\text{S})^2\), the only acceptable cluster compound, and used in clinical trials in Japan, at the time of the investigation. Miyajima et al. also produced double-tailed boron lipids and thus liposomes [274]. Transferrin (Tf) was loaded into the PEGylated liposomes to induce specificity, since the Tf receptor is upregulated in tumour cells. Nakamura et al. synthesised a nido-carborane lipid and incorporated this into 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC)/cholesterol/PEG based liposomes [275]. Li et al. produced a similar boronated, two-tailed lipid and highlighted that the absence of need for DSPC, high B loading rates could be achieved [276]; unfortunately, in this particular case, the lipid was found to be cytotoxic in vivo.

Another approach has been to covalently attach carboranes to cholesterol that is used to prepare the liposomes, as investigated by Feakes et al. [277]. The nido-form provided further negative charge on the liposomes exterior and was thought to contribute to the enhanced tumour specificity. Thirumamagal et al. also made three carboranyl cholesterol derivatives, with the boron cages replacing the B and C rings of cholesterol, one compound of which was stably incorporated into the liposome; low cytotoxicity was demonstrated [278].

Martini et al. incorporated BPA into mixed neutral (zwitterionic) and cationic liposomes and analysed the location of this boronated molecule [279]. The boron-substituted aromatic ring was maintained in the hydrophobic region, with the amino group oriented out towards
the polar head exterior. Altieri et al. found cationic liposomes, using two carborane derivatives, to be at least 30× more effective in terms of achieving cellular $^{10}$B concentrations as uncharged carriers [280]. Salvati et al. incorporated a carborane containing eight closed icosahedral cages into various liposomes made from positive, negative and neutral phospholipids [231]. The latter zwitterionic liposomes, in this study, were found to uptake the carborane species in higher quantities.

1.9 Project aims

The three main aims of this project are:

1. To formulate, characterise and evaluate the efficacy of a liposomal-based delivery system containing a model carborane ($o$-carborane) for BNCT that would be sufficiently small (< 100 nm) to potentially cross the BBB (Chapter 2);

2. To derivatise a carborane with a mitochondrial dye (a delocalised lipophilic cation) that shows specificity for cancer cells (based on mitochondrial membrane potentials) in order to make a targeted BNCT molecule and then to test its efficacy \textit{in vitro} using cancer and control cells (Chapter 3);

3. To investigate the use of a spray-dried $o$-carborane formulation for use in BNCT treatment of cancer. Since the particle size will be > 100 nm and therefore unable to transport across the BBB, the microparticles may have potential for BNCT treatment for liver and lung cancer; the latter being particularly attractive since the spray-dried formulation could be delivered as an inhaled powder to the lungs, providing further targeting (Chapter 4).
This project adds to and complements work carried out by the research supervisors and their collaborators, notably Dr Dimitris Fatouros (University of Thessaloniki, Greece), Prof Sophia Artimisiaris (University of Patra, Greece) and Dr Gianpiero Calabresse (University of Kingston, UK). The novelty/contributions to knowledge resulting from this PhD work are highlighted in Chapter 5.
1.10 References


Chapter 1: Introduction


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Chapter 2

Liposomes loaded with o-carborane for BBB delivery

2.1 Introduction

2.1.1 Preparation of liposomes using the thin film method

Liposome properties are determined by lipid composition, size, surface charge (zeta potential; \( \zeta \)-potential) and method of preparation [1]. For the latter, these include the: thin film method, sonication method [2], injection method [3,4], extrusion method [5], French press method [6], microfluidisation method, reverse-phase evaporation method [7,8], dehydrated – rehydrated vesicle method [9,10], giant vesicle method [10,11], detergent depletion method [12,13], large unilamella vesicle from Cochleate method [14], and one step method [15]. The thin film method, described below, is one of the most common and is used in this thesis.

Lipids are mixed with chloroform or methanol and a high-vacuum rotor evaporator pump is used to remove the organic solvent to form a thin film around the walls of the flask [16,17]. The aqueous phase for the thin film to be re-hydrated must be pre-warmed to just above the phase transition temperature for the lipid (related to carbon chain length and liposome rigidity). This solution is then agitated to allow removal of the lipid thin film from the wall of the flask. MLVs are then produced. Drugs to be encapsulated may be added to the solution after re-hydration.
2.1.2 Characterisation of liposomes

Liposomes are also characterised in terms of their size, size distribution, ζ-potential, stability and trapping efficiency of any incorporated moiety/drug, as detailed below [18].

2.1.2.1 Liposome size

Dynamic Light Scattering (DLS), using a Nanosizer or Zetasizer, is typically used to characterise liposome particle sizes. The Zetasizer determines the size of particles by measuring the speed of their movement caused by Brownian motion in a liquid. The speed is used to calculate the hydrodynamic particle diameter ($D_H$) using the Stoke-Einstein equation (Equation 2.1),

$$D_H = \frac{kT}{f} = \frac{kT}{3\pi \eta \phi} \quad \text{(Equation 2.1)}$$

where $k$ = Boltzmann constant, $T$ = absolute temperature, $f$ = particle frictional coefficient, $\eta$ = solvent viscosity and $D$ = diffusion coefficient.

Large particles move slowly whilst smaller ones move more rapidly. Size extrusion chromatography is another widely used method [19,20]. The polydispersity index (PDI) (Equation 2.2) provides a measure of the variation in particle size of a sample. The value is between 0 and 1, 0 being that of a perfectly monodispersed distribution,

$$PDI = \frac{\sigma^2}{D_H^2} \quad \text{(Equation 2.2)}$$

where $\sigma$ = standard deviation of the distribution (nm) and $D_H$ = hydrodynamic diameter.
2.1.2.2 Surface charge of liposomes

The surface charge on liposomes can be measured from the ζ-potential (mV). When a charged particle is suspended in an ionic liquid, oppositely charged ions are strongly attracted and surround the charged particle. Other ions far out in the solution are less attracted and form a diffuse layer (Figure 2.1). In this layer, there is an imaginary boundary and ions within it will travel along with the particle as moves in the liquid. However, ions outside the boundary will not move and this is known as the slipping plane. ζ-Potential is a potential that occurs between the particle surface and the dispersing liquid that depends on the distance from the particle surface. It is the potential at the slipping plane and is measured using the Helmholtz-Smoluchowski equation (Equation 2.3) [21],

\[
\zeta = \frac{4\pi \mu \eta}{D}
\]  

(Equation 2.3)

where \( \mu = \) electrophoretic mobility, \( \eta = \) viscosity and \( D = \) dielectric constant of the medium at the boundary.

The ζ-potential is the degree of repulsion between the particles; the higher the values, in either positive or negative direction, the more the stable the particle. Limits of -31 or +31 mV are often used, where agglomeration is expected between these values.

The outer surfaces of liposomes have the greatest impact on their physical stability, storage and in vivo studies.
Figure 2.1. A charged particle surrounded by oppositely charged ions. The $\zeta$-potential is the potential at the slipping plane. Adapted from [22].

### 2.1.3 Stability of liposomes

Pharmaceutical products need a shelf life of 24 months and therefore the chemical and physical stability of liposome formulations are very important [23]. Phospholipids can hydrolyse to form fatty acids 1- and 2-acyl-lyso-phospholipid upon storage [24]. The complexing agent ethylenediamine tetraacetic acid (EDTA) can be used to minimise this problem. The rancidity of lipids can be reduced by $\alpha$-tocophenol, which causes a reduction in auto-oxidation of lipids caused by the action of light, metal ions and temperature. Cholesterol can be used together with $\alpha$-tocophenol to increase liposome shelf-life [25,26]. As a physical method for stabilising liposomes, freeze drying (cryoprotection) [27] and dehydration (lyoprotection) [28,29] may also be used. The amount of drugs liposomes are able to retain (retention) and the duration of their retention (latency), measures of
membrane integrity, are important parameters for storage and *in vivo* studies. They can be measured by quantifying the release of calcein (Figure 2.2), a small-water soluble hydrophilic marker dye contained in the hydrophilic interior of the liposome, after the addition of Triton X-100 to disrupt the lipid bilayer (Equations 2.4 and 2.5) [30],

\[
\text{% Latency} = \frac{DF \times (F_{AT} - F_{BT})}{DF \times F_{AT}} \times 100\% \quad \text{(Equation 2.4)}
\]

where \( DF \) = dilution factor, \( F_{BT} \) = fluorescence value before adding Triton X-100, \( F_{AT} \) = fluorescence value after adding Triton X-100, and

\[
\text{% Retention} = \frac{\% \text{ Latency at } t_x}{\% \text{ Latency at } t_0} \times 100\% \quad \text{(Equation 2.5)}
\]

where \( t_x \) = time after a particular time period (*e.g.*, 24 h), and \( t_0 \) = initial time.

![Chemical structure of calcein](image)

Figu re 2.2. Chemical structure of calcein.

A high latency means lower amounts of calcein has been released (higher encapsulation) before the addition of Triton X-100. The property is dependent on the integrity of the liposomal membrane, as well as the physical and chemical properties of the drug delivery
system. A high retention signifies larger calcein release (higher encapsulation) after the Triton X-100 addition.

2.1.4 Aims

The aim of the work outlined in this Chapter was to form small (< 100 nm dia.), stable SUV liposomes of dipalmitoylphosphatidylcholine (DPPC) and DSPC liposomes (Figure 2.3), with and without cholesterol, incorporating \( \alpha \)-carborane for BCNT. These novel particles, produced \textit{via} the thin-film method, should have the potential to cross the BBB and avoid recognition by macrophages [31]. Stability was assessed in terms of \( \zeta \)-potential and retention of the hydrophilic marker calcein (Figure 2.2) in phosphate-buffered-saline (PBS, pH 7.4) and human serum. \( \alpha \)-Carborane incorporation was measured using spectrophotometry (displacement of Nile Red, Figure 2.3c) and directly using inductively coupled plasma (ICP) mass spectrometry for \(^{10}\text{B} \) content.

![Chemical structures of DPPC, DSPC, and Nile Red](image)

\( \text{Figure 2.3. Chemical structures of (a) DPPC, (b) DSPC, and (c) Nile Red.} \)
2.2 Materials and methods

The experiments were divided into three main sections: (a) preparation of DPPC and DSPC liposomes, using the thin-film method, incorporating different o-carborane volumes and assessing particle sizes, \( \zeta \)-potentials and stability; (b) increasing stability using cholesterol and assessing stability in near-physiological conditions (serum); and (c) measuring degree of loading (Nile Red entrapment and ICP-MS).

2.2.1 Preparation of liposomes

DPPC and DSPC were obtained from Lipoid, Germany. Potassium chloride, potassium phosphate \( \text{K}_2\text{HPO}_4 \), cholesterol, calcein, Nile Red and Sephadex G-50 were provided by Sigma-Aldrich (Dorset, UK). Syringe filters (0.2 μm, PES 25 mm; GD/X sterile) were obtained from Whatman. Ethanol, chloroform, sodium chloride, sodium phosphate \( \text{NaH}_2\text{PO}_4 \), sodium hydroxide and hydrochloric acid were obtained from Fisher Scientific (Loughborough, UK). NaOH (20%) and HCl (2M) were prepared in the laboratory. o-Carborane was obtained from Alfa Aesar, UK.

2.2.1.1 Preparation of phosphate buffered saline (PBS, pH 7.4)

PBS (1 L) was made containing NaCl (8.00 g), KCl (0.19 g), \( \text{KH}_2\text{PO}_4 \) (0.2 g), \( \text{Na}_2\text{HPO}_4 \) (1.7 g) and adjusted to pH 7.4 using NaOH (20%) and HCl (2M).
2.2.1.2 Preparation of calcein stock solution

Calcein stock solution (50 mM) was prepared by dissolving calcein (0.3113 g, 622.55 g mol\(^{-1}\)) in distilled water (ca. 8 mL) with dropwise addition of NaOH (10% w/v) until the powder was completely dissolved. The pH of the calcein solution was adjusted to pH 7.4 (with addition of 2M HCl, if necessary). The solution was then made up to 10 mL, filtered, placed in an amber bottle wrapped in aluminium foil and stored in the fridge (4 °C).

2.2.1.3 Preparation of serum stock solution from rat blood

Plasma stock solution was made using whole blood: rat blood (20 mL) was placed in a beaker and left for ca. 30 min to clot. This was facilitated by placing it in an incubator (37 °C for 30 min). A long Pasteur pipette was used to separate the clotted blood from the wall of the beaker. The clotted blood was then transferred into separate Eppendorf tubes, cooled (4 °C, 10 min) and centrifuged (3000 rpm, 10 min) to separate the plasma from the blood. The serum appeared as a clear yellowish colour liquid on top of the clotted blood. The serum was then transferred into new Eppendorf tubes using a plastic pipette. This was then further centrifuged (3000 rpm, 10 min) to allow further clearing of the plasma and the separated serum was placed in Eppendorf tubes and stored in the freezer for future use.

2.2.1.4 Preparation of Nile Red stock solution

For assessment of liposome \(\sigma\)-carborane loading, a Nile Red solution in CHCl\(_3\) was required to disperse the lipids before forming the thin-film. The final concentration of Nile Red in the lipid dispersion was required to be 2.5 \(\mu\)M, with a final dispersion volume of 3 mL (3 mL \(\times\) 3 (for triplicates) = \(7.5 \times 10^{-3}\) \(\mu\)moles = \(2.5 \times 10^{-3}\) \(\mu\)moles ml\(^{-1}\), required for
the final dispersion). To achieve this, a stock solution of Nile Red (2.39 μg mL\(^{-1}\) = 7.5 × 10\(^{-3}\) μmoles × 318.38 g mol\(^{-1}\)) in CHCl\(_3\) was prepared as follows: Nile Red (3.6 mg) was dissolved in CHCl\(_3\) (15 mL) to make Solution 1 (0.24 mg mL\(^{-1}\)); CHCl\(_3\) (9 mL) was added to an aliquot of Solution 1 (1 mL) to make Solution 2 (23.9 μg mL\(^{-1}\)); CHCl\(_3\) (9 mL) was added to an aliquot of Solution 2 (1 mL) to make the stock Nile Red Solution (2.39 μg mL\(^{-1}\)). This was then wrapped in foil and placed in the fridge (4°C).

### 2.2.1.5 Preparation of o-carborane stock solutions

In accordance with the literature method \(^1\), the lipid : o-carborane molar ratios were mixed to be 1:0.5, 1:1, 1:10, 1:20 and 1:40. These were prepared using DPPC and, separately, DSPC (15 mg) to achieve a final lipid concentration of 5 mg mL\(^{-1}\). The concentrations were thus, for DPPC = 5 mg mL\(^{-1}\) / 734 g mol\(^{-1}\) = 6.812 × 10\(^{-3}\) mmol mL\(^{-1}\) = 6.8 mM, and for DSPC = 5 mg mL\(^{-1}\) / 790.15 g mol\(^{-1}\) = 6.3 × 10\(^{-3}\) mmol mL\(^{-1}\) = 6.3 mM. To obtain the required lipid : o-carborane molar ratios of 1:0.5, 1:1, 1:10, 1:20 and 1:40, the concentrations of o-carborane needed were therefore 3.4, 6.8, 68, 136 and 272 mM, respectively, for the DPPC lipid, and 3.2, 6.3, 63, 126 and 252 mM for the DSPC lipid. In the case of the lowest o-carborane concentration (3.4 mM) for the DPPC lipid, the mass of o-carborane = 3.4 × 10\(^{-3}\) mmol mL\(^{-1}\) × 3 mL = 10.2 × 10\(^{-3}\) mmol in 3 mL = 10.2 × 10\(^{-3}\) × 144.23 g mol\(^{-1}\) = 1.5 mg o-carborane in 3 mL dispersion. Similarly, for all the o-carborane solutions for the required ratios (1:0.5, 1:1, 1:10, 1:20 and 1:40), the mass of DPPC and DSPC were 1.5, 3.0, 30, 60 and 120 mg, and 1.4, 2.8, 28, 56 and 112 mg, respectively.
Two stock o-carborane solutions to prepare the dilutions for the DPPC and DSPC lipids were produced, as follows. For the highest mass (120 mg), 1 mL of CHCl₃ was required, and therefore, for the total mass (1.5 + 3.0 + 30 + 60 + 120 mg = 214.5 mg), for the DPPC lipids, the required CHCl₃ volume = 214.5 mg / 120 mg × 1 mL = 1.788 mL; this volume was then added to o-carborane (214.5 mg). Similarly, 220.2 (1.4 + 2.8 + 28 + 56 + 112) mg / 112 mg × 1 mL = CHCl₃ (1.788 mL) was added to o-carborane (214.5 mg). These Eppendorf tubes were then wrapped in foil and placed in the fridge (4°C).

2.2.1.6 Preparation of DPPC/DSPC liposomes using the thin-film method

DPPC and DSPC liposomes were prepared using the standard thin-film hydration method: DPPC/DSPC (250 µL) from the stock solution (200 mg per 10 mL, 20 mg mL⁻¹)² were placed into three separate flasks. This gave a lipid mass of 5 mg (DPPC, 6.8 mM; DSPC, 6.3 mM³), required for the low systemic toxicity and normal tissue uptake, equivalent to ca. 20 µg / ¹⁰⁸B / g tumour). The organic solvent was evaporated under reduced pressure (Buchi 461 rotary evaporator, Switzerland) at a temperature 10 °C > lipid phase transition temperature (chain melting temperature, $T_c = 42 \, ^°C$ DPPC, 54 °C DSPC [35]) and then dried (N₂ stream) [33,36] to yield thin films at the air-water interface. These were rehydrated at 60 °C with PBS (3 mL; buffer solution, pH 7.4) and dispersed using a vortex [33] to produce empty liposomes [37].

Loaded liposomes were prepared using calcein (3 mL; 50 mM) that was pre-warmed > $T_c$ of DPPC (45 °C; 10 min) and added to the prepared thin film. This dispersion was vortexed (5 min) to assist uniform dispersion. To decrease the size of liposomal
dispersions, the mixture was placed in the bath sonicator (30 min) [33,36]. The round-bottomed flask was placed at the air-water interface in the sonicator to ensure optimal particle size reduction [38].

For the preparation of SUVs, the suspension was subjected to probe sonication (120 s at 2 s intervals; Gex 400 Probe Sonicator, Germany) [39]. To remove of Ti fragments, MLVs or liposomal aggregates, the liposome dispersion was centrifuged (5 min; 3000 rpm). The samples were then annealed [39] for at least 1 h at the liposome preparation temperature (50-60 °C; above their $T_m$ in the water bath) to remove any structural disabilities [39] before loading them onto the prepared gel-chromatography columns.

For the addition of cholesterol, the lipid : cholesterol ratio was maintained at 2:1, i.e., 500 µL : 250 µL, to make 750 µL for all 3 columns (250 µL $\cong$ 5 mg per column). For the absence of cholesterol, 750 µL of lipid was used. The lipid (+ cholesterol) was placed in a round-bottomed flask and the CHCl$_3$ removed.

**2.2.1.7 Preparation of DPPC/DSPC liposomes for fluorescence displacement, particle size measurements and ICP-MS**

DPPC and DSPC liposomes containing o-carborane, as with the unloaded liposomes, were prepared using the same thin-film hydration method as provided in Section 2.2.1.6. Different volumes of o-carborane stock solution (0, 12.5, 25, 250, 500, 1000 µL; Section 2.2.1.5), CHCl$_3$ (1 mL) and Nile Red (1000 µL; Section 2.2.1.4) were pipetted into a round-bottomed flask. The solvent was removed under reduced pressure (Buchi Rotavapor...
R) to yield a thin-film. The remainder of the procedure was carried out as outlined in Section 2.2.1.6.

2.2.2 Calcein stability measurements

Sephadex G-50 (10 g) was dispersed in PBS (200 mL) and air bubbles removed by sonication (30 min; FS400 Decon ultrasonic bath, Germany) before loading into empty Sephadex G-50 columns (1 cm × 35 cm; n = 3) [33, 39-41]. To render calcein-loaded vesicles osmotically stable, non-encapsulated calcein was separated from liposomes containing calcein (Section 2.2.1.6) on a Sephadex G-50 column (gel-permeation-chromatography, Anachem, UK) using PBS (pH 7.4) as the eluent (Figure 2.4); pre-saturation of the column with each lipid, allowed recoveries > 94% [42].

Figure 2.4. (a) Sephadex G-50 columns used in gel permeation chromatography (n = 3), and (b) schematic showing separation of encapsulated (liposomes) and non-encapsulated calcein.
Prior to any liposome sample measurements, the fluorescence intensity ($\lambda_{em} = 495$ nm, $\lambda_{ex} = 520$ nm; Varian Inc., USA) of PBS (blank, pH 7.4) was measured (cuvette). Samples from the 3 columns (S1, S2, S3) were placed in a water bath (37 °C). An aliquot of each solution (40 μL) was placed into separate Eppendorf tubes containing PBS (4 mL; pH 7.4) [39]. The fluorescence intensity was measured immediately ($t_0 = 0$ h). The solution was transferred into the original Eppendorf tube, Triton X-100 (10%; 400 μL) added and the tube agitated (electrical vortex, 5 s, 2000 rpm) to break down the liposomes and release the encapsulated calcein [32,39]. The fluorescence was then re-measured. This was repeated after time intervals of 1, 2, 3, 4, 5, 6, 7, 8, 9, 12 and 24 h.

For fluorescence measurements in serum, a similar method was used. A blank fluorescence reading was obtained from a solution of serum (125 μL) in PBS (500 μL; pH 7.4). The combined volume of samples from the columns were divided by 4 and used for each reading, i.e., 200 μL of sample was obtained from each column, and 50 μL of serum used for each reading. Fluorescence measurements (cuvette) and the procedure for adding Triton X-100 were performed as above, after adding the sample (40 μL) to PBS (4 mL, pH 7.4).

Sample dilution (1 mL in PBS, 1 mL; pH 7.4) was necessary when fluorescence measurements were > 700 for the initial and final measurement, i.e., before and after the addition of Triton X-100.
%Latency and %retention of liposomes, with and without cholesterol, in serum at 37 °C were calculated at time intervals of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 24 h (Equations 2.4 and 2.5, Section 2.1.3). In Equation 2.4, $DF = 1.1$.

A paired t-test was used to evaluate statistical differences between % latency and %retention results. A probability value < 0.05 was considered to be significant.

### 2.2.3 Nile Red entrapment measurements

The fluorescence intensities of liposomal solutions containing Nile Red were measured under identical conditions to those stated for the calcein content measurements ($\lambda_{em} = 495$ nm, $\lambda_{ex} = 520$ nm; Varian Inc., USA; Section 2.2.2).

### 2.2.4 Particle sizing and $\zeta$-potential

Dynamic light scattering (DLS; measurement angle, 173 °) data were obtained for liposomes with and without entrapped o-carborane and electrophoresis measurements performed using a Nano-ZS Zeta at 25 °C (4 mW He-Ne laser $\lambda = 633$ nm, photodiode detector; Malvern instrument, UK). The PDI (Section 2.1.2.1) and $\zeta$-potential (Section 2.1.2.2) were also recorded at 25 °C.
2.2.5 ICP-MS Boron analysis

An aliquot of conc. sulphuric acid (500 μL) was added to each of the liposome solutions (600 μL; each liposome formulation (3 mL) containing o-carborane, 0, 12.5, 25, 250, 500 and 1000 μL) in boron-free glass vials. The solutions became darkened and were allowed to cool to 24 °C (very important due to the exothermic reaction in the next step). Hydrogen peroxide (100 μL) was then added to each tube and when the solutions became decolourised, the volumes were adjusted to 2 mL by addition of distilled water (800 μL). The $^{10}$B content of the mineralised (‘digested’) samples and supernatant were then determined using an inductive coupled plasma-mass spectrometer (ICP-MS; Agilent Technologies, 700 Series) operating at 1550 W, with a carrier gas flow rate of 0.91 L min$^{-1}$, make up gas flow of 0.24 L min$^{-1}$ and a nebuliser pump rate of 0.1 rps. Beryllium and $^{10}$B standard solutions (1000 ppm) were used in the calibration.

2.2.6 Scanning electron microscopy

An aliquot of a solution containing DSPC liposomes (5 μL), unloaded and o-carborane (1000 μL) loaded, was placed on a surface of freshly-cleaved muscovite mica (Agar Scientific, Stansted, Essex, UK) and left to dry for 2 min. The surface was then rinsed with distilled water, dried in a N$_2$ stream and sputter-coated with Au/Pd in an Argon atmosphere (< 0.2 Torr, 18 mA for 5 – 10 min; Polaron E5000 SEM coating-unit, Quorum Technologies Ltd., East Grinstead, UK). SEM imaging was performed immediately using a JEOL JSM-6060LV SEM instrument (resolution = 4.5 nm, acceleration voltage = 15 kV). Particle sizes were obtained from the scale bar and means and SDs determined.
2.2.7 Atomic force microscopy

Liposome samples were prepared as for SEM (Section 2.2.6), although without Au/Pd coating. Atomic force microscopy (AFM) studies were carried out using a Multi-Mode/NanoScope IV scanning probe microscope, Bruker, Santa Barbara, CA, USA and were performed in air under ambient conditions ($T = 23 \, ^\circ\text{C}, \, \text{RH} = 21\%$) using the J-scanner (max. xy = 200 µm). Scanning was performed in Tapping mode using Si cantilevers with integrated tips ($t = 3.6–5.6$ µm, $l = 140–180$ µm, $w = 48–52$ µm, $\nu_p = 288–338$ kHz, $k = 12–103$ N m$^{-1}$, $R < 7$ nm; model: OTESPA, Bruker, France), and an RMS amplitude of 0.8 V. The images were subsequently processed and dimensions measured using NanoScope Analysis software (V 1.4, Bruker, Santa Barbara, CA, USA).
2.3 Results and discussion

2.3.1 Particle sizing, PDI and ζ-potential

The particle sizes, PDIs and ζ-potentials of the prepared DPPC/DSPC liposomes unloaded and loaded with o-carborane (0 to 1000 μL), and those that were probed once, were determined immediately after annealing using the zeta seizer are shown in Table 2.1.

Table 2.1. Particle size, PDI and ζ-potential values for DPPC and DSPC liposomes with different volumes of incorporated o-carboranes.

<table>
<thead>
<tr>
<th>Liposomes + o-carborane / μL</th>
<th>DPPC, Mean ± SD</th>
<th>DSPC, Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d / nm</td>
<td>PDI</td>
</tr>
<tr>
<td>0</td>
<td>67.9 ± 5.1</td>
<td>0.27 ± 0.13</td>
</tr>
<tr>
<td>12.5</td>
<td>74.7 ± 6.8</td>
<td>0.16 ± 0.07</td>
</tr>
<tr>
<td>25</td>
<td>66.5 ± 8.1</td>
<td>0.18 ± 0.08</td>
</tr>
<tr>
<td>250</td>
<td>73.7 ± 11.8</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>500</td>
<td>78.1 ± 9.0</td>
<td>0.26 ± 0.14</td>
</tr>
<tr>
<td>1000</td>
<td>86.7 ± 11.7</td>
<td>0.18 ± 0.12</td>
</tr>
</tbody>
</table>

The particle sizes are ca. 80 nm with those of DSPC being larger than those of DPPC. This was expected since the chain length of the former is larger than that in DPPC. Particle sizes were seen to increase linearly with increased o-carborane loading, suggesting successful incorporation. This has been observed elsewhere using different liposomes [34]. Since the particle sizes are < 100 nm, these liposomes show promise for delivery across the BBB.
All PDI values, irrespective of o-carborane loading, were < 0.5 confirming a very evenly monodispersed, homogeneous particle size distribution (Table 2.1).

The net particle charges on the prepared liposomes, as reflected by their ζ-potentials, are summarised in Table 2.1. All values were between -30 and +30 mV, indicating that the particles were not stable and therefore are likely to agglomerate. This is probably due to the high ionic strength of the PBS medium [34]. Awad et al. observed the ζ-potential to decrease sigmoidally, from -5 to -40 mV, with increasing BSH concentrations and attributed this to an interaction between the boron species and the lipid bilayer [32]. In the current study, the ζ-potential was found to decrease with increasing o-carborane loading, although this parameter is not concentration.

2.3.2 Effect of re-probing on liposome properties

To further decrease particle size, liposome formulations were re-probed. Table 2.2a and 2.2b show the effect of re-probing (before and after, 5 min) the DPPC and DSPC liposomes loaded with o-carborane on particle size, PDI and ζ-potential (n = 3).

The particle sizes prior to re-probing were greater than those observed in Section 2.3.1 probably due to instability of the liposomes upon storage. Re-probing significantly reduced the particle size of both DPPC and DSPC liposomes (Tables 2.2a and 2.2b) to a range suitable for drug delivery across the BBB (< 100 nm) [43]. Again, particle sizes increased with o-carborane loading and all sizes were larger for DSPC liposomes compared with those of DPPC, due to the larger lipid chain length of the former. PDI values for all
liposomes were again < 0.5 indicative of very evenly monodispersed and homogenous particle size distributions. Values of between -30 and +30 mV were again observed for the \( \zeta \) -potentials, suggesting agglomeration may result.

Table 2.2a. Effect of re-probing DPPC liposomes containing various loadings of \( o \)-carborane on particle size, PDI and \( \zeta \) -potential (n = 3).

<table>
<thead>
<tr>
<th>Vol. of ( o )-carborane / ( \mu )L</th>
<th>1000</th>
<th>500</th>
<th>250</th>
<th>25</th>
<th>12.5</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>Probe</td>
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<td></td>
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</tr>
<tr>
<td>1</td>
<td>Before</td>
<td>d / nm</td>
<td>547.9</td>
<td>234.6</td>
<td>99.3</td>
<td>56.7</td>
</tr>
<tr>
<td></td>
<td>PDI</td>
<td></td>
<td>1.000</td>
<td>0.608</td>
<td>0.318</td>
<td>0.204</td>
</tr>
<tr>
<td>1</td>
<td>After</td>
<td>d / nm</td>
<td>69.0</td>
<td>65.9</td>
<td>60.5</td>
<td>58.8</td>
</tr>
<tr>
<td></td>
<td>PDI</td>
<td></td>
<td>0.105</td>
<td>0.116</td>
<td>0.48</td>
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<tr>
<td></td>
<td>( \zeta ) / mV</td>
<td>-0.365</td>
<td>-2.24</td>
<td>-1.04</td>
<td>-0.416</td>
<td>1.53</td>
</tr>
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<td>2</td>
<td>Before</td>
<td>d / nm</td>
<td>114.5</td>
<td>106.9</td>
<td>88.74</td>
<td>85.04</td>
</tr>
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<td></td>
<td>PDI</td>
<td></td>
<td>0.479</td>
<td>0.544</td>
<td>0.245</td>
<td>0.341</td>
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<td>2</td>
<td>After</td>
<td>d / nm</td>
<td>81.5</td>
<td>80.6</td>
<td>70.1</td>
<td>64.8</td>
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<td></td>
<td>PDI</td>
<td></td>
<td>0.357</td>
<td>0.413</td>
<td>0.168</td>
<td>0.199</td>
</tr>
<tr>
<td></td>
<td>( \zeta ) / mV</td>
<td>-4.31</td>
<td>-16.3</td>
<td>-16.5</td>
<td>-24.5</td>
<td>-35.2</td>
</tr>
<tr>
<td>3</td>
<td>Before</td>
<td>d / nm</td>
<td>1046.0</td>
<td>136.4</td>
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<td>89.2</td>
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<td>1.000</td>
<td>0.445</td>
<td>0.442</td>
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<tr>
<td>3</td>
<td>After</td>
<td>d / nm</td>
<td>75.5</td>
<td>50.2</td>
<td>44.1</td>
<td>43.5</td>
</tr>
<tr>
<td></td>
<td>PDI</td>
<td></td>
<td>0.171</td>
<td>0.243</td>
<td>0.3000</td>
<td>0.260</td>
</tr>
<tr>
<td></td>
<td>( \zeta ) / mV</td>
<td>-2.52</td>
<td>-13.5</td>
<td>-13.1</td>
<td>-22.9</td>
<td>-20.8</td>
</tr>
<tr>
<td>Mean \pm SD</td>
<td>After</td>
<td>d / nm</td>
<td>75.3±6.2</td>
<td>65.6±15.2</td>
<td>58.2±13.2</td>
<td>55.7±11.0</td>
</tr>
<tr>
<td></td>
<td>PDI</td>
<td></td>
<td>0.21±0.13</td>
<td>0.26±0.15</td>
<td>0.32±0.16</td>
<td>0.21±0.05</td>
</tr>
<tr>
<td></td>
<td>( \zeta ) / mV</td>
<td>-2.40±1.98</td>
<td>-10.68±7.44</td>
<td>-10.21±8.12</td>
<td>-15.94±13.47</td>
<td>-18.16±18.51</td>
</tr>
</tbody>
</table>
Table 2.2b. Effect of re-probing DSPC liposomes containing various loadings of o-carborane on particle size, PDI and ζ-potential (n = 3).

<table>
<thead>
<tr>
<th>Vol. of o-carborane / µL</th>
<th>1000</th>
<th>500</th>
<th>250</th>
<th>25</th>
<th>12.5</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>150.5</td>
<td>146.9</td>
<td>145.1</td>
<td>94.7</td>
<td>93.2</td>
<td>82.8</td>
</tr>
<tr>
<td>PDI</td>
<td>0.280</td>
<td>0.217</td>
<td>0.179</td>
<td>0.136</td>
<td>0.177</td>
<td>0.215</td>
</tr>
<tr>
<td>1</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>After</td>
<td>111.0</td>
<td>105.8</td>
<td>96.51</td>
<td>93.5</td>
<td>82.7</td>
<td>59.5</td>
</tr>
<tr>
<td>PDI</td>
<td>0.167</td>
<td>0.220</td>
<td>0.180</td>
<td>0.169</td>
<td>0.159</td>
<td>0.262</td>
</tr>
<tr>
<td>ζ / mV</td>
<td>-3.92</td>
<td>-5.46</td>
<td>-11.8</td>
<td>-23.7</td>
<td>-20.2</td>
<td>-7.88</td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>Before</td>
<td>150.8</td>
<td>136.9</td>
<td>132.9</td>
<td>91.0</td>
<td>81.7</td>
<td>79.0</td>
</tr>
<tr>
<td>PDI</td>
<td>0.220</td>
<td>0.131</td>
<td>0.186</td>
<td>0.092</td>
<td>0.135</td>
<td>0.176</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After</td>
<td>98.3</td>
<td>97.3</td>
<td>96.3</td>
<td>90.1</td>
<td>76.7</td>
<td>71.3</td>
</tr>
<tr>
<td>PDI</td>
<td>0.186</td>
<td>0.204</td>
<td>0.160</td>
<td>0.143</td>
<td>0.098</td>
<td>0.134</td>
</tr>
<tr>
<td>ζ / mV</td>
<td>-4.37</td>
<td>-12.4</td>
<td>-17.4</td>
<td>-3.21</td>
<td>-31.5</td>
<td>-22.3</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
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<tr>
<td>Before</td>
<td>588.4</td>
<td>130.3</td>
<td>79.7</td>
<td>73.0</td>
<td>86.9</td>
<td>80.5</td>
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<tr>
<td>PDI</td>
<td>0.956</td>
<td>0.487</td>
<td>0.215</td>
<td>0.163</td>
<td>0.212</td>
<td>0.186</td>
</tr>
<tr>
<td>3</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After</td>
<td>85.8</td>
<td>81.4</td>
<td>64.3</td>
<td>39.3</td>
<td>39.0</td>
<td>38.8</td>
</tr>
<tr>
<td>PDI</td>
<td>0.209</td>
<td>0.285</td>
<td>0.249</td>
<td>0.307</td>
<td>0.304</td>
<td>0.246</td>
</tr>
<tr>
<td>ζ / mV</td>
<td>-2.06</td>
<td>-16.0</td>
<td>-17.9</td>
<td>-2.99</td>
<td>-26.8</td>
<td>-21.0</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>After</td>
<td>98.4±12.6</td>
<td>94.9±12.4</td>
<td>85.7±18.5</td>
<td>74.3±30.4</td>
<td>66.1±23.7</td>
<td>56.6± 6.5</td>
</tr>
<tr>
<td>PDI</td>
<td>0.19±0.02</td>
<td>0.24±0.04</td>
<td>0.20±0.05</td>
<td>0.21±0.01</td>
<td>0.19±0.11</td>
<td>0.21±0.07</td>
</tr>
<tr>
<td>ζ / mV</td>
<td>-3.45±1.22</td>
<td>-11.30±5.36</td>
<td>-15.7±3.39</td>
<td>-9.97±11.89</td>
<td>-26.12±5.68</td>
<td>-17.06±7.98</td>
</tr>
</tbody>
</table>

2.3.3 Stability of liposome properties after one week

Since the ζ-potentials of the liposomes suggested that agglomeration may be an important factor, the particle sizes were re-measured after 1 week of storage in the fridge (3-5 °C). Liposomes were probed once before storage but not re-probed after the storage period. Particle sizes and PDI values of DPPC and DSPC liposomes are shown in Tables 2.3a and 2.3b, respectively.
Before particles were left in storage, particle size measurements indicated that increased \( o \)-carborane incorporation increased particle sizes for both DPPC and DSPC liposomes. At lower concentrations, the particles sizes were within the 100 nm acceptable range. For DSPC liposomes, particle sizes increased to very high values with increasing volumes of \( o \)-carborane. PDI values were generally < 0.5, reflecting high monodispersity and homogeneity, although some DSPC liposomes at higher \( o \)-carborane incorporations slightly exceeded this limit. After storage, liposome sizes generally increased although to no observable pattern and not obviously related to \( o \)-carborane content. The liposomes were not re-probed, although this would probably have reduced the particle sizes to values < the required 100 nm. This is investigated in the next section.

Table 2.3a. Mean particle sizes and PDI values of DPPC liposomes before and after storage at 3-5 °C for 1 week; n = 3; SD = standard deviation.

<table>
<thead>
<tr>
<th>Volume of ( o )-carborane / µL</th>
<th>Before ( d / \text{nm} )</th>
<th>PDI</th>
<th>After ( d / \text{nm} )</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>262.0 ± 191.8</td>
<td>0.44 ± 0.33</td>
<td>394.9 ± 385.2</td>
<td>0.71 ± 0.30</td>
</tr>
<tr>
<td>500</td>
<td>139.3 ± 37.6</td>
<td>0.41 ± 0.12</td>
<td>170.4 ± 62.5</td>
<td>0.31 ± 0.10</td>
</tr>
<tr>
<td>250</td>
<td>100.7 ± 18.4</td>
<td>0.21 ± 0.06</td>
<td>120.2 ± 26.1</td>
<td>0.35 ± 0.20</td>
</tr>
<tr>
<td>25</td>
<td>86.3 ± 4.6</td>
<td>0.26 ± 0.22</td>
<td>93.7 ± 4.8</td>
<td>0.19 ± 0.08</td>
</tr>
<tr>
<td>12.5</td>
<td>83.4 ± 5.5</td>
<td>0.24 ± 0.02</td>
<td>106.1 ± 15.1</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>0</td>
<td>61.0 ± 19.5</td>
<td>0.30 ± 0.18</td>
<td>113.3 ± 28.8</td>
<td>0.43 ± 0.32</td>
</tr>
</tbody>
</table>
Table 2.3b. Mean particle sizes and PDI values of DSPC liposomes before and after storage at 3-5 °C for 1 week; n = 3; SD = standard deviation.

<table>
<thead>
<tr>
<th>Volume of o-carborane / µL</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d / nm</td>
<td>PDI</td>
</tr>
<tr>
<td>---------------------------</td>
<td>--------</td>
<td>-------</td>
</tr>
<tr>
<td>1000</td>
<td>3917.2 ± 3614.7</td>
<td>0.46 ± 0.16</td>
</tr>
<tr>
<td>500</td>
<td>2335.6 ± 2707.4</td>
<td>0.57 ± 0.44</td>
</tr>
<tr>
<td>250</td>
<td>1802.1 ± 2347.4</td>
<td>0.61 ± 0.45</td>
</tr>
<tr>
<td>25</td>
<td>245.0 ± 165.6</td>
<td>0.52 ± 0.35</td>
</tr>
<tr>
<td>12.5</td>
<td>82.3 ± 5.5</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>0</td>
<td>76.0 ± 2.6</td>
<td>0.15 ± 0.13</td>
</tr>
</tbody>
</table>

2.3.4 AFM

AFM was used to investigate the stability of liposomes containing o-carborane over longer periods than one week. In these studies, the liposomes were re-probed prior to measuring sizes with the AFM. This technique also has the advantages of observing the morphology of the liposomes and checking for agglomeration. Line profiles were used to obtain the particle sizes from the AFM images. Diameter \((d)\) and height \((h)\) values were obtained to check which property was more appropriate for the particle size measurements. This is because diameter measurements are known to be influenced by broadening artefacts caused by the larger AFM tip moving across a smaller sample (Figure 2.5). Images of DPPC and DSPC liposomes without and with o-carborane (maximum volume, 1000 µL) obtained after 1, 19, 35, 49 and 63 days after preparation are shown in Figures 2.6 – 2.9.
Chapter 2: Liposomes loaded with $o$-carborane for BBB delivery

Figure 2.5. Origin of AFM tip broadening artefact. The red line shows a 2D view of the artefact with corresponding oversized diameter measurement ($d$); the true diameter (height, $h$) is shown. The yellow arrow shows the scan direction.

The unloaded liposomes, both DPPC and DSPC, often appeared as agglomerated islands/rafts, probably composed of lipid bilayers, with attached isolated spherical liposomes (Figure 2.10). These islands were largely unseen for the loaded liposomes. This may be due to poor sampling technique or some increased stability of the loaded liposomes resulting from more hydrophobic interactions between $o$-carborane and the hydrophobic lipid tails (Figure 2.11).

In terms of particle size, loaded liposomes, both DPPC and DSPC, were generally larger than their unloaded counterparts (Tables 2.4 and 2.5) as observed in previous measurements (Section 2.3.1).
Figure 2.6. Representative AFM images of unloaded DPPC liposomes obtained after 1, 19, 35, 49 and 63 days (a – e, respectively; n=4).

This pattern was reflected in both diameter and height AFM measurements, although the former were much larger due to the expected tip broadening effect (Figure 2.5). Interestingly, the AFM height measurements gave appreciably (an order of magnitude) lower values than diameters obtained from the zeta sizer. This suggests that the AFM determined diameters may not be as affected by the tip broadening artefact as first thought, possibly due to the tip being sharp. The low height values may be due to compression of the liposomes by the AFM tip.
Figure 2.7. Representative AFM images of o-carborane loaded DPPC liposomes obtained after 1, 19, 35, 49 and 63 days (a – e, respectively; n=4).

The loaded liposomes generally appeared intact after 63 days of storage suggesting a successful formulation had been achieved. The fact that the liposomes needed re-probing to produce particles in the correct size range is not a limitation, since the product would be stored in a laboratory fridge before administering it to the patient. In other words, it is not like a commercial drug where long term stability outside of a laboratory environment needs to be demonstrated.
Chapter 2: Liposomes loaded with $\sigma$-carborane for BBB delivery

Figure 2.8. Representative AFM images of unloaded DSPC liposomes obtained after 1, 19, 35, 49 and 63 days (a – e, respectively; n=4).
Figure 2.9. Representative AFM images of \(\sigma\)-carborane loaded DSPC liposomes obtained after 1, 19, 35, 49 and 63 days (a – e, respectively; \(n=4\)).
Table 2.4. Time variation of particle sizes (diameters) of DPPC and DSPC liposomes unloaded and loaded with $o$-carborane (1000 µL) determined using AFM.

<table>
<thead>
<tr>
<th>Time / days</th>
<th>DPPC Unloaded</th>
<th>DPPC Loaded</th>
<th>DSPC Unloaded</th>
<th>DSPC Loaded</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>86.4 ± 24.8</td>
<td>121.4 ± 26.7</td>
<td>83.3 ± 20.6</td>
<td>130.9 ± 26.2</td>
</tr>
<tr>
<td>19</td>
<td>89.0 ± 37.2</td>
<td>103.2 ± 20.4</td>
<td>87.0 ± 24.0</td>
<td>108.3 ± 21.5</td>
</tr>
<tr>
<td>35</td>
<td>83.6 ± 22.1</td>
<td>101.0 ± 28.0</td>
<td>66.5 ± 16.2</td>
<td>108.7 ± 22.6</td>
</tr>
<tr>
<td>49</td>
<td>85.7 ± 21.4</td>
<td>62.0 ± 22.7</td>
<td>58.5 ± 15.4</td>
<td>89.4 ± 20.4</td>
</tr>
<tr>
<td>63</td>
<td>77.4 ± 25.2</td>
<td>35.3 ± 11.2</td>
<td>100.0 ± 36.2</td>
<td>113.0 ± 22.2</td>
</tr>
</tbody>
</table>

Table 2.5. Time variation of particle sizes (heights) of DPPC and DSPC liposomes unloaded and loaded with $o$-carborane (1000 µL) determined using AFM.

<table>
<thead>
<tr>
<th>Time / days</th>
<th>DPPC Unloaded</th>
<th>DPPC Loaded</th>
<th>DSPC Unloaded</th>
<th>DSPC Loaded</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.3 ± 2.7</td>
<td>16.8 ± 4.91</td>
<td>6.8 ± 2.1</td>
<td>19.9 ± 4.3</td>
</tr>
<tr>
<td>19</td>
<td>11.4 ± 7.02</td>
<td>14.0 ± 3.4</td>
<td>8.1 ± 3.5</td>
<td>18.6 ± 5.3</td>
</tr>
<tr>
<td>35</td>
<td>13.1 ± 5.7</td>
<td>7.8 ± 3.7</td>
<td>8.6 ± 3.7</td>
<td>20.6 ± 4.0</td>
</tr>
<tr>
<td>49</td>
<td>11.6 ± 4.5</td>
<td>4.8 ± 0.31</td>
<td>9.2 ± 3.9</td>
<td>21.3 ± 6.4</td>
</tr>
<tr>
<td>63</td>
<td>8.2 ± 3.1</td>
<td>4.5 ± 1.1</td>
<td>7.4 ± 2.2</td>
<td>18.3 ± 5.1</td>
</tr>
</tbody>
</table>
Chapter 2: Liposomes loaded with \( o \)-carborane for BBB delivery

Figure 2.10. Breakdown of liposomes to form lipid bilayer islands/rafts on mica.

Figure 2.11. Liposomes (a) unloaded and (b) loaded with \( o \)-carborane. The latter structure may be more stable due to increased non-polar interactions and so might be less susceptible to collapse to form lipid bilayer islands/rafts.
2.3.5 SEM

To further investigate liposome morphology and to compare particle sizing data obtaining via the zeta sizer and AFM, SEM images were obtained (Figure 2.12) and particle diameters measured (Figure 2.13). The unloaded and o-carborane loaded (1000 µL) liposomes were mainly spherical and featureless, with the loaded structures again appearing to be larger than the unloaded liposomes. This was confirmed by measuring the diameters of these structures (Figure 2.13).

![SEM images of unloaded and o-carborane loaded liposomes](image)

**Figure 2.12** SEM images of unloaded (a,b,c) and o-carborane (1000 µL) loaded (d,e,f) DSPC liposomes on mica.
Other studies have shown that \( o \)-carborane loaded 1,2-dimyristoyl-\( sn \)-glycero-3-phosphocholine (DMPC) liposomes were also larger than their unloaded counterparts [43]. Sizes were in the range measured using laser diffraction methods (Malvern Nanosizer, Sections 2.3.1-2.3.3). This supports the theory that particle sizes obtained via AFM height measurements were subject to compression and the tip-broadening artefact was not a major limitation (Section 2.3.4); AFM diameter, rather than height, measurements were therefore concluded to be satisfactory for obtaining particle size data.

![Figure 2.13](image_url)  
**Figure 2.13.** Mean particle sizes of unloaded and \( o \)-carborane loaded DSPC liposomes obtained from SEM images (3 images, \( n = 10 \)); error bars = SD.

### 2.3.6 Stabilisation of liposomes with cholesterol and serum

To assess the stability of the liposome formulations in physiologically-relevant media, the leaching of the water-soluble, hydrophilic fluorescent marker calcein from these vehicles
was investigated. To improve the stability of DPPC and DSPC liposomes, the effect of cholesterol inclusions were investigated. Cholesterol is known to stabilise liposome formations by increasing rigidity and preventing leakage [44]. These studies were carried out by following the release of calcein from the liposomes after treatment with Triton X-100, which disrupts the lipid bilayers. DPPC and DSPC liposomes containing calcein were first prepared as described previously (Sections 2.2.1.6 and 2.2.2) and particle sizes, PDI values and ζ-potentials obtained in PBS medium and in rat serum (37 °C) (Table 2.6). Statistical differences in particle diameters (Figure 2.14) are summarised in Table 2.7.

Table 2.6. Particle size, PDI and ζ-potential values for DPPC and DSPC liposomes incorporating calcein, with and without cholesterol in PBS and serum (n=3).

<table>
<thead>
<tr>
<th>Liposomes + Calcein&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d / nm</td>
</tr>
<tr>
<td>DPPC</td>
<td>42.0 ± 5.2</td>
</tr>
<tr>
<td>DPPC+S</td>
<td>64.4 ± 3.1</td>
</tr>
<tr>
<td>DPPC+C</td>
<td>68.2 ± 5.7</td>
</tr>
<tr>
<td>DPPC+C+S</td>
<td>98.8 ± 1.0</td>
</tr>
<tr>
<td>DSPC</td>
<td>58.6 ± 5.8</td>
</tr>
<tr>
<td>DSPC+S</td>
<td>76.4 ± 2.6</td>
</tr>
<tr>
<td>DSPC+C</td>
<td>81.5 ± 8.6</td>
</tr>
<tr>
<td>DSPC+C+S</td>
<td>99.8 ± 1.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> S = serum (PBS when S not stated); C = cholesterol.
DSPC liposomes were larger than DPPC liposomes in all cases (p < 0.05), as observed previously, due to the longer chain-length of the former lipid. For both types of liposomes, in the absence of cholesterol, particle diameters were larger in serum than in PSB buffer; the liposomes in serum were larger still when the particles incorporated cholesterol. The latter observation was expected since cholesterol is known to interface between the lipid bilayers, increasing their stability and hence increasing their size.

Figure 2.14. Particle size variation for different formulations of DPPC and DSPC liposomes incorporating calcein, with and without cholesterol in PBS and serum (error bar = SD; n = 3).
Table 2.7. Statistical differences (p values) between particle sizes for DPPC and DSPC liposomes incorporating calcein, with and without cholesterol in PBS and serum (one-way ANOVA, post-hoc analysis).

<table>
<thead>
<tr>
<th></th>
<th>DPPC</th>
<th>DPPC+S</th>
<th>DPPC+C</th>
<th>DPPC+C+S</th>
<th>DSPC</th>
<th>DSPC+S</th>
<th>DSPC+C</th>
<th>DSPC+C+S</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC</td>
<td></td>
<td>0.001</td>
<td>0.000</td>
<td>0.001</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>DPPC+S</td>
<td></td>
<td>0.974</td>
<td>0.000</td>
<td>0.820</td>
<td>0.109</td>
<td>0.009</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>DPPC+C</td>
<td></td>
<td></td>
<td>0.000</td>
<td>0.298</td>
<td>0.469</td>
<td>0.058</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>DPPC+C+S</td>
<td></td>
<td></td>
<td></td>
<td>0.000</td>
<td>0.001</td>
<td>0.009</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>DSPC</td>
<td></td>
<td>0.007</td>
<td>0.001</td>
<td>0.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSPC+S</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.884</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSPC+C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSPC+C+S</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2.15a and b and Figure 2.16a and b show the stability of the DPPC and DSPC liposomes in terms of %latency and %retention over a 24 h period in PBS (pH 7.4), with and without the presence of cholesterol. Latency refers to the amount of encapsulated calcein (hydrophilic marker) within the DPPC/DSPC liposomes at a given time (Equation 2.4), whereas retention refers to the amount of calcein left after the liposomes have been dispersed in Triton X-100 to leach out the calcein (Equation 2.5). Both parameters provide a measure of the membrane integrity.
Figure 2.15a. Latency of DPPC liposomal dispersions formulated with and without cholesterol in PBS and serum. Formulations were incubated at 37 °C. Data are presented as mean ± SD (n=3).

Figure 2.15b. Retention of DPPC liposomal dispersions formulated with and without cholesterol in PBS and serum. Formulations were incubated at 37 °C. Data are presented as mean ± SD (n=3).
Chapter 2: Liposomes loaded with $\sigma$-carborane for BBB delivery

Figure 2.16a. Latency of DSPC liposomal dispersions formulated with and without cholesterol in PBS and serum. Formulations were incubated at 37 °C. Data are presented as mean $\pm$ SD (n=3).

Figure 2.16b. Retention of DSPC liposomal dispersions formulated with and without cholesterol in PBS and serum. Formulations were incubated at 37 °C. Data are presented as mean $\pm$ SD (n=3).
For DSPC liposomes in PBS media, the %latency was almost always higher when vesicles had been prepared with cholesterol rather than without (Figure 2.15a). This was expected owing to the known influence of cholesterol to increase DSPC liposome stability [39,45,46]. Cholesterol also increased the capacity of the vehicles to accommodate calcein (Figure 2.15b). Consistent with literature reports, the latency of DSPC/cholesterol liposomes was higher than their DPPC/cholesterol congeners [47]. For the DPPC liposomes, however, cholesterol slightly reduced liposome stability over most of the time points investigated (Figure 2.16a), although the calcein retention was greater with cholesterol (Figure 2.16b).

For both DPPC and DSPC liposomes, without cholesterol, %latency was significantly reduced in serum compared with those in PBS media (Figures 2.15b and 2.16b). The inclusion of cholesterol, however, increased the %latency to levels similar to those in PBS media for DSPC liposomes and for much above this in the case of DPPC liposomes (ca. 100%; Figure 2.15b). Retention values for both DPPC and DSPC liposomes with cholesterol in serum were generally the highest. Thus, over a 24 h per period, the integrity of the lipid membrane for both types of cholesterol-containing liposomes in serum media was high when cholesterol was used in their preparation.

2.3.7 Nile red experiments

Nile red, another fluorescent marker, was incorporated into o-carborane loaded liposomes to displace the boron compound and thus offering a quantitative method for determining o-
carborane entrapment. This would also provide information as to whether $o$-carborane was incorporated into the liposomes at a sufficient quantity to be useful for the BNCT process.

DPPC and DSPC liposomes containing a fixed volume of Nile Red (1000 $\mu$L; 2.5 $\mu$M) and a range of $o$-carborane volumes (0, 12.5, 25, 250, 500 and 1000 $\mu$L) for entrapment were prepared (Section 2.2.1.6 and 2.2.1.7). The liposomes were characterised in terms of particle size, PDI and $\zeta$-potential as previously performed (Section 2.3.1; Table 2.8).

Table 2.8. Particle size, PDI and $\zeta$-potential values for DPPC and DSPC liposomes incorporating Nile Red and differing volumes of $o$-carborane (n=2).

<table>
<thead>
<tr>
<th>Liposomes + $o$-carborane / $\mu$L</th>
<th>DPPC, Mean ± SD</th>
<th>DSPC, Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$d$ / nm</td>
<td>PDI</td>
</tr>
<tr>
<td>0</td>
<td>63.3 ± 28.9</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>12.5</td>
<td>65.8 ± 31.2</td>
<td>0.31 ± 0.09</td>
</tr>
<tr>
<td>25</td>
<td>67.5 ± 29.4</td>
<td>0.21 ± 0.04</td>
</tr>
<tr>
<td>250</td>
<td>73.8 ± 28.1</td>
<td>0.26 ± 0.09</td>
</tr>
<tr>
<td>500</td>
<td>82.6 ± 24.5</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>1000</td>
<td>87.6 ± 31.1</td>
<td>0.19 ± 0.01</td>
</tr>
</tbody>
</table>

These results confirm that liposomes were again successfully produced and with the desired particle sizes required (< 100 nm). DPPC particles were smaller than DSPC liposomes and PDI and $\zeta$-potential values were also in agreement with those obtained previously (Section 2.3.1). Since the mean particle size increased with increasing $o$-carborane volume, as observed previously, this suggests that increasingly higher volumes
of o-carborane used in the preparation were being encapsulated in the liposomes. The purpose of this experiment, of course, was to prove this was indeed the case by measuring the fluorescence intensity of the liposome formulations: entrapped Nile Red should occupy the remaining space in the liposomes not filled with o-carborane. In other words, higher fluorescence intensities should be recorded with samples than contained lower volumes of the entrapped boron compound. It was interesting to note that particle size increased with o-carborane volume though, since the void space should be filled with Nile Red solution and therefore similar particle sizes would have been expected independent of o-carborane volumes.

Figures 2.17a and b show the variation of fluorescence intensity for DPPC and DSPC liposomes, respectively, containing Nile Red and differing volumes of o-carborane. As expected, the highest fluorescence intensities, for both DPPC and DSPC liposomes, were obtained with formulations containing no o-carborane and intensities decreased progressively with increasing volumes of this compound. These results semi-quantitatively suggest that the volume of o-carborane used to prepare the liposomes was successfully entrapped within these vesicles. To formally quantify o-carborane incorporation, the boron content was next determined from digested formulations using ICP-MS.
Chapter 2: Liposomes loaded with \( o \)-carborane for BBB delivery

Figure 2.17a. Nile Red incorporation into DPPC liposomes loaded with different volumes of \( o \)-carborane.

Figure 2.17b. Nile Red incorporation into DSPC liposomes loaded with different volumes of \( o \)-carborane.
2.3.8 ICP-MS measurements of o-carborane incorporation

To quantitatively assess the volume of o-carborane entrapped within the DPPC and DSPC liposomes, formulations were digested with conc. H$_2$SO$_4$ / H$_2$O$_2$ (Section 2.2.5) and analysed using ICP-MS for boron content (Table 2.9; Figure 2.18).

Table 2.9. ICP-MS detected volumes of o-carborane with different DPPC and DSPC liposome o-carborane inclusions (n=3).

<table>
<thead>
<tr>
<th>Formulated vol. o-carborane / µL</th>
<th>Mean ICP-MS detected vol. o-carborane / µL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPPC</td>
</tr>
<tr>
<td>0</td>
<td>3.05 ± 1.05</td>
</tr>
<tr>
<td>12.5</td>
<td>12.34 ± 7.52</td>
</tr>
<tr>
<td>25</td>
<td>23.57 ± 9.56</td>
</tr>
<tr>
<td>250</td>
<td>207.83 ± 155.50</td>
</tr>
<tr>
<td>500</td>
<td>398.18 ± 165.81</td>
</tr>
<tr>
<td>1000</td>
<td>1021.57 ± 370.05</td>
</tr>
</tbody>
</table>

The results show a clear match between the actually incorporated volumes of o-carborane with those actually determined. The gradients of the plots (Figure 2.18) were very close to 1 (DPPC: 0.995; DSPC: 1.249).
Figure 2.18. Comparison of detected versus actually incorporated volumes of $o$-carborane into DPPC and DSPC liposomes using ICP-MS.

2.4 Summary

DPPC and DSPC SUV liposomes with particle diameters of ca. 80 – 100 nm containing $o$-carborane were successfully produced using the thin film method. Those of DSPC had slightly larger particle sizes as reflected by their longer carbon chain. For both DPPC and DSPC liposomes, particle diameter increased with increasing entrapped $o$-carborane volumes. The liposomes had a monodispersed, homogeneous particle size distribution (PDI < 0.5), although possessed $\zeta$-potentials in a range indicative of instability (between -30 and
+30 mV). However, this was probably due to high ionic strength of the PBS medium and re-probing was found to reduce agglomerated particle to the required size range over storage at 3 – 5 °C for 1 week. AFM studies showed that the loaded liposomes generally appeared intact after 63 days of storage if re-probing was employed. The integrity of the liposome membrane in serum, as reflected by %latency and %retention experiments using a fluorescent marker (calcein), was found to be high for both DPPC and DPPC liposomes prepared using cholesterol. Co-entrapment of o-carborane with Nile Red into the liposomes semi-quantitatively confirmed the required volume of the boron compound successfully became entrapped with the liposomes. This was proven quantitatively using ICP-MS measurements.
2.5 References


44. M. J. Ostro and P. R. Cullis: 'Use of liposomes as injectable-drug delivery systems', Amer. J. Hospital Pharm., 1989, 46(8), 1576-1587.


Chapter 3

Efficacy of specific formulations of $o$-carborane

3.1 Introduction

In Chapter 2, stable DPPC and DSPC SUV liposomes that incorporated $o$-carborane were successfully formulated and characterised. Since the focus was on the integrity and stability of the liposomes themselves, to keep costs to a minimum, a model, non-specific $o$-carborane was used. In other words, this carborane is unable to target cancer cells that would be key for the BNCT approach. In this Chapter, however, since knowledge of the liposome formulation has been obtained, attention is now moved to targeting cancer cells with carborane derivatives.

3.1.1 Targeting cancer cell mitochondria

Mitochondria are organelles that are present in the majority of cells and are often described as the ‘power houses’ since they are the main source of adenosine triphosphate (ATP) needed for respiration (energy generation) [1,2]. They also play a key role in programmed cell death (apoptosis) (Figure 3.1) [3,4]. Each organelle consists of a matrix of extra-chromosomal mitochondrial DNA (mtDNA) and enzymes surrounded by a double membrane (Figure 3.2) [5,6]. The outer membrane is permeable to small molecules (nucleotides, sugars and salts), whilst the inner, folded (into cristae) layer is selectively permeable, allowing passage of pyruvic acid and $O_2$ into the organelle and removal of ATP and $CO_2$ [6].
Chapter 3: Efficacy of specific formulations of $o$-carborane

Figure 3.1. Schematic showing the extrinsic and intrinsic pathways of apoptosis. Adapted from [2].

Figure 3.2. Cross-sectional image of a single mitochondrion [5].
At the mitochondria inner membrane (MIM; Figure 3.2), oxygen is reduced to water by the passage of electrons from NADH and succinate via an enzymatic series of electron donors and acceptors [7]. This produces a proton gradient across the MIM known as the mitochondria cell membrane potential ($\Delta \Psi_m$) [7,8]. This property conveniently provides a measure of the energetic condition of the mitochondria and the level of dysfunction [9-11]. For example, the $\Delta \Psi_m$ of neoplastic cells are quite different from non-neoplastic cells [12]: $-60$ mV for neoplastic cells [13], and lower values being obtained from non-neoplastic cells, reflective of the damaged mitochondrial metabolic activities in cancer [10]. In addition, in-vitro $\Delta \Psi_m$ values are also estimated to be 180-200 mV [14] compared to 130-150 mV [15] for those measured in vivo. Brain mitochondria are regulated by $\Delta \Psi_m$ [16] which plays an important role in cancer metabolism (aerobic glycolysis), as proposed by Warburg in 1930 [3,10], which causes resistance to cell death. It is therefore an ideal select target for cancer therapy [3].

3.1.2 Mito dyes

There are quite a number of mitochondrial dyes, some of which make use of the differing in vitro $\Delta \Psi_m$ values between neoplastic and non-neoplastic cells to providing visual distinctions between these two cell populations (Figure 3.3) [17]. Many of these dyes are delocalised lipophilic cations (DLCs), i.e., they have an extended conjugated $\pi$-electron system, are hydrophobic and bear a positive charge (via one or more nitrogen atoms) [18]; a few key examples are shown in Figure 3.3. Dequalinium chloride, a dication consisting of two quaternary quinolinium rings separated by a dodecylene chain (Figure 3.3a), exhibits some anticarcinoma activity, prolonging survival in mice [19]; it has also been used extensively as an antiseptic [20].
Figure 3.3. Chemical structures of some common DLCs: (a) dequalinium chloride, (b) rhodamine-123 chloride, (c) Nile Blue chloride, (d) Mitotracker Red CMXRos, (e) Mitoracker Green, and (f) JC-1.
Rhodamine-123 chloride is a green fluorescent dye that is used to visualise mitochondria and to estimate mitochondrial activity (Figure 3.3b) [18]. MitoTracker Green (MTG) also selectively accumulates in the mitochondria and is used in confocal microscopy and flow cytometry; the dye covalently binds to mitochondrial proteins via cysteine SH groups (Figure 3.3e) [21]. JC-1 is a novel cationic carbocyanine dye (Figure 3.3f), which exists as a monomer at low concentration and at higher concentrations, forms aggregates with a different emission maximum and hence can be a sensitive $\Delta \Psi_m$ marker: mitochondria with high values of $\Delta \Psi_m$ appear orange and low values appear green [22].

The selective accumulation of DLCs in the mitochondria is a direct consequence of the Nernst Equation (Equation 3.1):

$$\Delta \psi (mV) = 61.5 \log_{10} \left( \frac{[\text{DLC}]_{\text{in}}}{[\text{DLC}]_{\text{out}}} \right) \quad \text{(Equation 3.1)}$$

Hence, there will be a ten-fold increase in DLC species for every 61.5 mV increment in $\Delta \psi$, and so there will be an increased concentration of DLC in cancer cells compared with normal cells [13,23].

### 3.1.3 Use of carborane DLC derivatises for BNCT

DLCs can be derivatised with boron-containing compounds to produce mitrochondrial-targetted BNCT agents [24-26]. They can either be through convolent attachment or using the ionic salt structure, replacing the chloride ion with a carborane anion. The latter approach has had some recent attention: dequalinium bis nido $o$-carborane salt (DC; Figure
3.4) was synthesised by Calabrese et al. [24], although technical difficulties in finding appropriate filter-sets to investigate the uptake of this compound by cells proved difficult. Specific targeting of cancer cells using an analogous rhodamine-123 bis nido o-carborane salt was successful however, with fluorescence being observed in prostate cancer (PC3) cell lines, but not in an equivalent non-neoplastic control (PNT2) [24].

![Chemical structure of dequalinium bis nido o-carborane salt (DC).](image_url)

**Figure 3.4.** Chemical structure of dequalinium bis nido o-carborane salt (DC).

### 3.1.4 Aims

The aim of the work in this chapter was to synthesise DC and to assess its ability to selectively target the mitochondria of glioma (GMB) cells, whilst leaving non-neoplastic cells (astrocytes) intact. The cell lines investigated were IN699 (GMB, grade IV) and SC1800 (non-neoplastic astrocytes, a control), and were considered more appropriate for BNCT studies than the prostate cell lines used previously. DC has been synthesised before for use as a BNCT agent, although suitable fluorescence filter sets were not available for use with this compound [24]. Prior to treating cells, the cytotoxicity of DC was examined.
using an MTS assay, since the agent itself should not be cytotoxic: cells should only be damaged once the compound has reached its target and the BNCT nuclear reaction initiated.

An MTS assay is known as a one-step MTT assay, which is a colourimetric method of assessing cell viability: enzymes in viable cells reduce colourless 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) to a purple-coloured formazan product (490 – 500 nm), in the presence of phenazine methosulfate (PMS) in PBS buffer (Figure 3.5) [27]. Therefore dead cells will produce a less intensely coloured solution.

Figure 3.5. MTS assay: formation of coloured formazan derivative by viable cells.
3.2 Materials and methods

3.2.1 Chemicals and instrumentation

Dequalinium chloride was purchased from Sigma (Dorset, UK). α-Carborane was purchased from BOC Sciences (NY, USA). Potassium hydroxide pellets were purchased from Acros Organics (NJ, USA). Methanol, toluene and petroleum ether were obtained from Fisher Scientific (Loughborough, UK). Deuterated chloroform, deuterated methanol and tetramethylsilane (TMS) for NMR were purchased from Cambridge Isotope Laboratories, Inc. (USA). Silica gel filter plates for thin layer chromatography (TLC) (5 cm × 10 cm; silica gel 60 F254) were sourced from Merck (Darmstadt, Germany).

1H, 13C and 11B NMR spectra were obtained using a JEOL Eclipse+ 400 MHz NMR instrument (Peabody, USA), operating at a field strength of 9.389766 T.

3.2.2 Synthesis of dequalinium bis nido α-carborane salt (DC)

Synthesis of DC in the same quantities was carried out as described elsewhere [24]. TLC confirmed the reaction had gone to completion.

TLC: \( R_f = 0.57 \) (DC), 0.20 (dequalinium chloride), 0 (α-carborane) (10:9:1 methanol : petroleum ether : toluene).

1H NMR (400 MHz, CD3OD): \( \delta 8.31 (dd, J_1 = 8.42 Hz, J_2 = 1.10 Hz, 2H), 8.08 (d, J = 8.42 Hz, 2H), 8.02 (dd, J_1 = 8.06 Hz, J_2 = 1.46 Hz, 2H), 7.68 (ddd, J_1 = 7.51 Hz, J_2 = 7.51 Hz, J_3 = 1.10 Hz, 2H), 6.75 (s, 2H), 4.49 (t, J = 8.42 Hz, 4H), 2.76 (m, 6H), 1.89–1.81 (m, 4H), 1.67 (m, 4H), 1.53 (m, 4H), 1.41 (m, 8H-B); Lit. 1H NMR (400 MHz, CD3OD): \( \delta 8.32 (dd, J_1 = 8.42 Hz, J_2 = 1.28 Hz, 2H), 8.08 (d, J = 8.79 Hz, 2H), 8.02 (dd, J_1 = 6.87 Hz, J_2 = 1.36 Hz, 2H)) \)

115
Hz, 2H), 7.69 (dd, J₁ = 8.42 Hz, J₂ = 6.96 Hz, J₃ = 1.28 Hz, 2H), 6.75 (s, 2H), 4.50 (t, J = 
8.28 Hz, 4H), 2.76 (m, 6H), 1.86–1.79 (m, 4H), 1.68 (m, 4H), 1.53 (m, 4H), 1.41 (m, 8H-
B) [24].

$^{13}$C NMR (100.5 MHz, CD₃OD): $\delta$ 157.63 (2C), 155.16 (2C), 139.58 (2C), 134.48 (2C),
126.04 (2C), 123.92 (2C), 118.02 (2C), 117.06 (2C), 104.23 (2C), 55.83 (2C), 29.14 (2C),
28.93 (2C), 28.36 (2C), 26.20 (2C), 20.92 (2C); Lit. $^{13}$C NMR (100.5 MHz, CD₃OD): $\delta$
158.40 (2C), 155.87 (2C), 140.31 (2C), 135.12 (2C), 126.73 (2C), 124.62 (2C), 118.68
(2C), 117.77 (2C), 104.83 (2C), 44.56 (2C), 29.93 (2C), 29.68 (2C), 29.66 (2C), 29.03
(2C), 26.91 (2C), 21.47 (2C) [24].

$^{11}$B NMR (128.2 MHz, CDCl₃): $\delta$ -4.06 (s, 1B), -9.32 (s, 1B), -10.60 (s,1B), 14.63 (s, 2B),
-15.49 (s, 1B), -18.33 (s, 2B), -23.22 (s, 2B), -34.49 (s, 1B), -39.23 (s, 1B); Lit. $^{11}$B NMR
(128.2 MHz, CDCl₃): $\delta$ -14.63 (s, 2B), -15.65 (s, 1B), -18.30 (s, 2B), -23.22 (s, 2B), -34.52
(s, 1B), -39.23 (s, 1B) [24].

### 3.2.3 Cell culture

GBM IN699 cells were obtained from the Institute of Neurology, London, where they were
derived from the supratentorial Glioblastoma cells from a 15-year-old treatment-naïve
male [28]. They were grown within P75 flasks (Cellstar filter cap cell culture flask, 250
mL, 75 cm²; Greiner) in DMEM & glutamax (Dulbecco’s Modified Eagle’s Medium;
Gibco Life Technologies) supplemented with 10% foetal bovine serum (FBS; Clonetics
AGM SingleQuots kits). SC1800 astrocyte cells were obtained from ScienCell Research
Laboratories (#1800), where they were isolated from the human cerebral cortex and frozen.
at passage 1, prior to being packaged and distributed in vials (1 × 10^6 cells mL\(^{-1}\)) [29].

Cells were grown in P25 flasks (Cellstar filter cap cell culture flask, 50 mL, 25 cm\(^2\); Greiner) in AGM2 media, consisting of Clonetics ABM astrocyte basal medium (Lonza), 10% human serum (Sigma), 1% L-glutamate (Lonza), 0.1% ascorbic acid (Lonza), 0.1% gentamycin & antimycin (Lonza), 0.1% external growth factor (Lonza) and 0.25% insulin (Lonza). Flasks of cells were incubated (37 °C, 5% CO\(_2\)), unless otherwise stated.

Cells were removed from flasks by typanation (removing existing media and washing with HBSS (Hanks’ Balanced Salt Solution; Gibco Life Technologies)). They were then incubated in TrypLE Express (Gibco Life Technologies) for 5 – 10 min until they became detached. Cells were then quenched with growth media to form a suspension, which was then centrifuged (1000 rpm, 5 min; Boeco centrifuge C-28A; Boeckel & Co) to form a pellet. This was then resuspended in growth media to form a homogeneous cell suspension and transferred to flasks containing growth media. The incubated cells were allowed to grow and split once ca. 80% confluency was obtained.

### 3.2.4 MTS assay

IN699 and SC1800 cell lines were used for the MTS assay [30]. Cells were cultured and seeded in three 96 well plates, one for each of the three time points: 24, 48 and 72 h. Each well plate had 24 wells seeded with IN699 cells and 24 wells seeded with SC1800s. Wells were set out for different concentrations of DC and appropriate controls (no DC and no cells; Figure 3.6); experiments were made in triplicate. The concentration range chosen was from 0.01 to 1 mM.
Figure 3.6. Arrangement of a 96 well plate for an MTS assay.

The dilutions were made from a stock solution which had a concentration of 1 mM. The quantities of stock and media solutions prepared were calculated as follows: since one well takes 100 μL, calculation was made for the amount of 1 mM stock solution and media for 100 μL required. For a concentration of 0 mM (the control) for example, 100 μL of media was needed and 0 μL of the stock solution. For a concentration of 0.01 mM, 100 μL \times 0.01 = 1 μL of the stock solution has to be mixed with 100 μL – 1 μL = 99 μL of media etc. The ratios of stock solution/media are shown in Table 3.1.
Table 3.1. Dilutions used for MTS assay.

<table>
<thead>
<tr>
<th>[DC 5] / mM</th>
<th>Volume of stock solution [1 mM] / μL</th>
<th>Volume of DMSO Media / μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.01</td>
<td>1</td>
<td>99</td>
</tr>
<tr>
<td>0.1</td>
<td>10</td>
<td>90</td>
</tr>
<tr>
<td>0.25</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>0.5</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>0.75</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

\[= 261 \mu L \]
\[= 439 \mu L \]

The total amount of needed stock solution for the 6 dilutions was 266 μL (Table 3.1), but since the work was carried out in triplicates and as there were three plates, the amount of stock solution needed was \(261 \mu L \times 3 \times 3 = 2349 \mu L\) for each cell line. The stock solutions and also the dilutions were prepared for each cell line separately since the cells required different media. The same calculation procedure was used for the media: \(439 \mu L \times 3 \times 3 = 3951 \mu L\) for each cell line.

Since the media and the stock solution were on an open bench and the drug treatment took place in the hood where it is worked under sterile conditions, it was decided to have more stock solution and media prepared than was required to allow for filtering of the solution (and hence loss of material). Stock solution (5 mL) and media (10 mL) were prepared for each cell line.
For 5 mL of stock solution (1 mM), the quantity of DC required was:

\[ m = \frac{Conc \times Vol \times MW}{1000} = 3.617 \text{ mg} \]  
(Equation 3.2)

This mass of DC (723.49436 g mol\(^{-1}\)) was dissolved in media (5 mL), which consisted of media (4.95 mL, 1% DMSO) and DMSO (0.05 mL, to achieve improved DC solubility). An additional aliquot of DMSO (150 µL) was added to improve solubility, and so the total stock volume was 5.15 mL containing 3.88% DMSO. To check the concentration value of the stock solution (\(C\)), which might have changed from 1 mM, a re-calculation was made:

\[ C = \frac{n}{V} \]  
(Equation 3.3)

where \(n\) = number of moles of DC and \(V\) = volume of solution (0.00515 L), and

\[ n = \frac{m}{MW} = 5.0 \times 10^{-6} \text{ mol} \]  
(Equation 3.4)

and therefore

\[ C = \frac{5.0 \times 10^{-6}}{0.00515} = 9.7 \times 10^{-4} \text{ mol L}^{-1} = 0.97 \text{ mM} \approx 1 \text{ mM} \]  
(Equation 3.5)

Therefore, the concentration after adding additional DMSO was still \(ca.\) 1 mM.

For the 10 mL of media + DMSO solutions, DMSO (0.1 mL) were mixed with media (9.9 mL) to achieve a DMSO content of 1%. To achieve the same DMSO% as in the stock solutions, DMSO (300 µL) were added. The media + DMSO solutions then had 3.88% of DMSO.
After all stock solutions and media + DMSO solutions had been prepared, they were placed in a water bath (37 °C, 15 min). The hood was then prepared and all solutions were filtered with a syringe filter to obtain sterility. The six dilutions were then prepared: 1 mL per concentration, for each cell line in the Eppendorf tubes. The 96 well plates were then retrieved from the incubator, all media was removed from the wells and then the dilution / stock solution/ media + DMSO (100 μL) were pipetted into the respective wells. The well plate was labelled from the outside (so as not to forget which well had been pipetted with which concentration) and put back into the incubator.

For the first time point (24 h), after 21.5 h had elapsed, one Eppendorf tube containing MTS solution was removed from the freezer and allowed to defrost for 30 min in the open lab (MTS solution was transferred previously from the original bottle into Eppendorf tubes and wrapped in tin foil, since MTS solution is light sensitive!). After 30 min, the MTS solution (yellow) was vortexed. Then the first 96 well plate was removed from the incubator, MTS solution (10 μL) was pipetted into each well which contained media (ratio 1 : 10 → 10 μL MTS solution : 100 μL media), the plate was closed and wrapped with tinfoil and put in the incubator at 37 °C for a further 2 h. During this time, the MTS (a yellow tetrazole) was reduced to purple formazan, a natural process in living cells [31]. After the 2 h, the well plate was put into the plate reader and absorbance values obtained (490 nm). The received data is displayed as an Excel data file that was saved for subsequent data analysis. The same procedure was repeated with the remaining two plates for the second (48 h) and the third (72 h) time points.
3.2.5 Microscopy

IN699 and SC1800 cell lines were grown in a 24 well plate, as described previously (Section 3.2.3). Aliquots containing various concentrations of DC (0, 0.1, 0.001 and 0.0001 mM) in cell-specific media (for IN699 and for SC1800) were added to the cells (in triplicate), i.e., to obtain IN699 + DC (0, 0.01, 0.001, 0.0001 mM; \( n = 3 \)) and SC1800 + DC (same concentrations; \( n = 3 \)).

Live cell imaging was performed using a Zeiss Axiovert 200M (inverted) microscope (Carl Zeiss, Welwyn Garden City, Herts, UK) contained in an incubator (37 °C, 5% CO\(_2\), humid atmosphere). A 10× objective was used and bright field images and fluorescence (filter set no. 10 ‘FITC’; Zeiss) were consecutively acquired every 15 min for 6 h (V5.4, Perkin Elmer). Maximum dye penetration before the onset of photobleaching occurred at 3.5 h. Images were obtained and overlayed using Volocity software (V6.1.1, Perkin Elmer). In addition, fluorescence imaging (20× and 40×) of the DC crystals were also carried out using a variety of filter sets (no. 10 ‘FITC’, exc. 450 – 490 nm, em. 515 – 565 nm; 37 ‘GFP’, exc. 450/50 nm, em. 510/50 nm; 20 ‘Rhodamine’, exc. 546/12 nm, em. 575 – 640 nm; and 00 ‘Texas Red’, exc. 530 – 585 nm, em. 615 nm; Zeiss).
3.3 Results and discussion

3.3.1 Fluorescence imaging of DC

Bright field and fluorescence images of DC crystals are shown in Figure 3.7.

Figure 3.7 (a). Bright field and (b-f) fluorescence images of DC crystals. Filter sets: (b) 20 ‘Rhodamine’, (c) 00 ‘Texas Red’, (d) 37 ‘GFP’, (e,f) 10 ‘FITC’. Bar = 60 µm (120 in f).
An intense fluorescence signal was displayed over a range of wavelengths for DC (Figure 3.7), despite this not being observed in previous studies [24]. The reason why this was not observed in previous studies is unclear. The very intense fluorescent signals for the solid material showed promise for subsequent in vitro studies where diluted DC solution would be employed.

3.3.2 MTS assay

Absorbance values from the MTS assay after 24, 48 and 72 h are shown in Tables 3.2, 3.3 and 3.4, respectively, for both IN699 and SC1800 cell lines. The %Viability is calculated as (Equation 3.6):

\[
\% Viability = \frac{A_c}{A_0} \times 100\% \quad \text{(Equation 3.6)}
\]

where \( A_c \) = absorbance at DC concentration \( c \) (3 runs in triplicate \( \therefore n = 9 \)), and \( A_0 \) = absorbance at of media in the absence of DC.

%Viability plots vs. DC concentrations were then obtained (Figures 3.8 – 3.10) in order to calculate the \( LC_{50} \) values (Table 3.5), which is the concentration at which 50\% of the cells are viable (acquired by interpolation).
SDs were calculated using Equations 3.7 – 3.8:

\[
V_c \pm v_c = \frac{A_c \pm a_c}{A_0 \pm a_0}
\]  
\text{(Equation 3.7)}

where \(V_c\) = viability expressed as a fraction (not %) for DC concentration \(c\), \(v_c\) = SD in viability for DC concentration \(c\), \(A_c\) = mean absorbance for DC concentration \(c\), \(a_c\) = SD of absorbance for DC concentration \(c\), \(A_0\) = mean absorbance for no DC, and \(a_0\) = SD of absorbance for no DC, and therefore \(v_c\) can be calculated as:

\[
\frac{v_c}{V_c} = \sqrt{\left(\frac{a_c}{A_c}\right)^2 + \left(\frac{a_0}{A_0}\right)^2}
\]  
\text{(Equation 3.8)}

The \(LC_{50}\) values (Table 3.5) were greater for the IN699 cell line compared with the SC1800s for all the 3 time points tested (24, 48 and 72 h). This is probably due to the inherent resistance of cancer cells to cytotoxic agents (DC, as with many chemicals, will become cytotoxic at some concentration). The reason for recovery in viability for both cell lines grown at 48 and 72 h is uncertain but may be due to layered cell growth in a media in which DC is poorly soluble. Importantly, however, the \(LC_{50}\) values are much higher, in the mM range, than the concentrations required for BNCT (and tumour concentration of 20 \(\mu\)g \(^{10}\)B g\(^{-1}\) (per gram of tumour) [32]) and therefore DC will not be toxic to these types of cells. It is the BNCT nuclear reaction that is required to generate species (\(\alpha\) particles) that destroy the cells (preferentially the cancer cells).
Table 3.2a. MTS data obtained for IN699 cell line after 24 h.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>0.01</th>
<th>0.1</th>
<th>0.25</th>
<th>0.5</th>
<th>0.75</th>
<th>1.0</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>[DC]/mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RUN 1</td>
<td>n = 1</td>
<td>0.373</td>
<td>0.547</td>
<td>0.314</td>
<td>0.180</td>
<td>0.199</td>
<td>0.186</td>
<td>0.499</td>
</tr>
<tr>
<td>RUN 1</td>
<td>n = 2</td>
<td>0.370</td>
<td>0.493</td>
<td>0.300</td>
<td>0.215</td>
<td>0.236</td>
<td>0.200</td>
<td>0.561</td>
</tr>
<tr>
<td>RUN 1</td>
<td>n = 3</td>
<td>0.375</td>
<td>0.257</td>
<td>0.328</td>
<td>0.212</td>
<td>0.273</td>
<td>0.094</td>
<td>0.506</td>
</tr>
<tr>
<td>RUN 2</td>
<td>n = 1</td>
<td>0.406</td>
<td>0.571</td>
<td>0.315</td>
<td>0.185</td>
<td>0.207</td>
<td>0.210</td>
<td>0.519</td>
</tr>
<tr>
<td>RUN 2</td>
<td>n = 2</td>
<td>0.374</td>
<td>0.513</td>
<td>0.319</td>
<td>0.226</td>
<td>0.234</td>
<td>0.181</td>
<td>0.590</td>
</tr>
<tr>
<td>RUN 2</td>
<td>n = 3</td>
<td>0.373</td>
<td>0.285</td>
<td>0.336</td>
<td>0.213</td>
<td>0.260</td>
<td>0.095</td>
<td>0.545</td>
</tr>
<tr>
<td>RUN 3</td>
<td>n = 1</td>
<td>0.433</td>
<td>0.585</td>
<td>0.333</td>
<td>0.198</td>
<td>0.219</td>
<td>0.225</td>
<td>0.548</td>
</tr>
<tr>
<td>RUN 3</td>
<td>n = 2</td>
<td>0.395</td>
<td>0.541</td>
<td>0.330</td>
<td>0.233</td>
<td>0.239</td>
<td>0.190</td>
<td>0.598</td>
</tr>
<tr>
<td>RUN 3</td>
<td>n = 3</td>
<td>0.391</td>
<td>0.297</td>
<td>0.355</td>
<td>0.191</td>
<td>0.272</td>
<td>0.101</td>
<td>0.569</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>0.388</td>
<td>0.454</td>
<td>0.326</td>
<td>0.206</td>
<td>0.238</td>
<td>0.164</td>
<td>0.548</td>
</tr>
<tr>
<td>SD</td>
<td></td>
<td>0.021</td>
<td>0.134</td>
<td>0.016</td>
<td>0.018</td>
<td>0.027</td>
<td>0.053</td>
<td>0.035</td>
</tr>
<tr>
<td>%Viability</td>
<td></td>
<td>70.73</td>
<td>82.87</td>
<td>59.39</td>
<td>37.55</td>
<td>43.33</td>
<td>30.00</td>
<td>100.00</td>
</tr>
<tr>
<td>%SD</td>
<td></td>
<td>5.93</td>
<td>25.03</td>
<td>4.78</td>
<td>4.12</td>
<td>5.63</td>
<td>9.80</td>
<td>9.07</td>
</tr>
</tbody>
</table>
Table 3.2b. MTS data obtained for SC1800 cell line after 24 h.

<table>
<thead>
<tr>
<th>[DC] / mM</th>
<th>0.01</th>
<th>0.1</th>
<th>0.25</th>
<th>0.5</th>
<th>0.75</th>
<th>1.0</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUN 1 n = 1</td>
<td>0.395</td>
<td>0.368</td>
<td>0.190</td>
<td>0.115</td>
<td>0.102</td>
<td>0.114</td>
<td>0.434</td>
</tr>
<tr>
<td>RUN 1 n = 2</td>
<td>0.286</td>
<td>0.198</td>
<td>0.165</td>
<td>0.080</td>
<td>0.082</td>
<td>0.091</td>
<td>0.479</td>
</tr>
<tr>
<td>RUN 1 n = 3</td>
<td>0.501</td>
<td>0.169</td>
<td>0.173</td>
<td>0.086</td>
<td>0.081</td>
<td>0.163</td>
<td>0.316</td>
</tr>
<tr>
<td>RUN 2 n = 1</td>
<td>0.379</td>
<td>0.340</td>
<td>0.236</td>
<td>0.112</td>
<td>0.099</td>
<td>0.124</td>
<td>0.473</td>
</tr>
<tr>
<td>RUN 2 n = 2</td>
<td>0.302</td>
<td>0.195</td>
<td>0.128</td>
<td>0.074</td>
<td>0.088</td>
<td>0.095</td>
<td>0.413</td>
</tr>
<tr>
<td>RUN 2 n = 3</td>
<td>0.421</td>
<td>0.152</td>
<td>0.176</td>
<td>0.090</td>
<td>0.083</td>
<td>0.168</td>
<td>0.324</td>
</tr>
<tr>
<td>RUN 3 n = 1</td>
<td>0.379</td>
<td>0.338</td>
<td>0.235</td>
<td>0.106</td>
<td>0.099</td>
<td>0.118</td>
<td>0.489</td>
</tr>
<tr>
<td>RUN 3 n = 2</td>
<td>0.304</td>
<td>0.207</td>
<td>0.122</td>
<td>0.072</td>
<td>0.087</td>
<td>0.090</td>
<td>0.437</td>
</tr>
<tr>
<td>RUN 3 n = 3</td>
<td>0.434</td>
<td>0.180</td>
<td>0.191</td>
<td>0.094</td>
<td>0.082</td>
<td>0.175</td>
<td>0.360</td>
</tr>
<tr>
<td>Mean</td>
<td>0.378</td>
<td>0.238</td>
<td>0.179</td>
<td>0.092</td>
<td>0.089</td>
<td>0.126</td>
<td>0.414</td>
</tr>
<tr>
<td>SD</td>
<td>0.071</td>
<td>0.085</td>
<td>0.040</td>
<td>0.016</td>
<td>0.008</td>
<td>0.034</td>
<td>0.066</td>
</tr>
<tr>
<td>%Viability</td>
<td>91.29</td>
<td>57.61</td>
<td>43.34</td>
<td>22.30</td>
<td>21.53</td>
<td>30.52</td>
<td>100.00</td>
</tr>
<tr>
<td>%SD</td>
<td>22.50</td>
<td>22.44</td>
<td>11.88</td>
<td>5.25</td>
<td>3.98</td>
<td>9.53</td>
<td>22.59</td>
</tr>
</tbody>
</table>
Table 3.3a. MTS data obtained for IN699 cell line after 48 h.

<table>
<thead>
<tr>
<th>[DC] / mM</th>
<th>0.01</th>
<th>0.1</th>
<th>0.25</th>
<th>0.5</th>
<th>0.75</th>
<th>1.0</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUN 1 n = 1</td>
<td>0.295</td>
<td>0.308</td>
<td>0.067</td>
<td>-0.045</td>
<td>0.042</td>
<td>0.516</td>
<td>0.281</td>
</tr>
<tr>
<td>RUN 1 n = 2</td>
<td>0.294</td>
<td>0.213</td>
<td>0.080</td>
<td>-0.093</td>
<td>-0.048</td>
<td>-0.056</td>
<td>0.185</td>
</tr>
<tr>
<td>RUN 1 n = 3</td>
<td>0.323</td>
<td>0.381</td>
<td>0.079</td>
<td>0.020</td>
<td>0.043</td>
<td>0.046</td>
<td>0.230</td>
</tr>
<tr>
<td>RUN 2 n = 1</td>
<td>0.300</td>
<td>0.332</td>
<td>0.051</td>
<td>-0.033</td>
<td>0.056</td>
<td>0.515</td>
<td>0.286</td>
</tr>
<tr>
<td>RUN 2 n = 2</td>
<td>0.294</td>
<td>0.205</td>
<td>0.107</td>
<td>-0.089</td>
<td>-0.025</td>
<td>-0.042</td>
<td>0.184</td>
</tr>
<tr>
<td>RUN 2 n = 3</td>
<td>0.317</td>
<td>0.389</td>
<td>0.087</td>
<td>0.043</td>
<td>0.049</td>
<td>0.026</td>
<td>0.239</td>
</tr>
<tr>
<td>RUN 3 n = 1</td>
<td>0.312</td>
<td>0.106</td>
<td>-0.029</td>
<td>0.053</td>
<td>0.088</td>
<td>0.117</td>
<td>0.335</td>
</tr>
<tr>
<td>RUN 3 n = 2</td>
<td>0.223</td>
<td>0.198</td>
<td>-0.006</td>
<td>0.086</td>
<td>0.104</td>
<td>0.128</td>
<td>0.362</td>
</tr>
<tr>
<td>RUN 3 n = 3</td>
<td>0.247</td>
<td>0.166</td>
<td>0.039</td>
<td>0.061</td>
<td>0.087</td>
<td>0.089</td>
<td>0.329</td>
</tr>
<tr>
<td>Mean</td>
<td>0.289</td>
<td>0.255</td>
<td>0.053</td>
<td>0.000</td>
<td>0.044</td>
<td>0.149</td>
<td>0.270</td>
</tr>
<tr>
<td>SD</td>
<td>0.033</td>
<td>0.100</td>
<td>0.045</td>
<td>0.067</td>
<td>0.051</td>
<td>0.217</td>
<td>0.065</td>
</tr>
<tr>
<td>%Viability</td>
<td>107.19</td>
<td>94.55</td>
<td>19.53</td>
<td>0.15</td>
<td>16.27</td>
<td>55.12</td>
<td>100.00</td>
</tr>
<tr>
<td>%SD</td>
<td>28.57</td>
<td>43.57</td>
<td>17.26</td>
<td>24.73</td>
<td>19.25</td>
<td>81.51</td>
<td>34.03</td>
</tr>
</tbody>
</table>
Table 3.3b. MTS data obtained for SC1800 cell line after 48 h.

<table>
<thead>
<tr>
<th>[DC] / mM</th>
<th>0.01</th>
<th>0.1</th>
<th>0.25</th>
<th>0.5</th>
<th>0.75</th>
<th>1.0</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUN 1 n = 1</td>
<td>0.311</td>
<td>0.125</td>
<td>-0.031</td>
<td>0.037</td>
<td>0.078</td>
<td>0.115</td>
<td>0.403</td>
</tr>
<tr>
<td>RUN 1 n = 2</td>
<td>0.346</td>
<td>0.210</td>
<td>-0.013</td>
<td>0.078</td>
<td>0.089</td>
<td>0.112</td>
<td>0.424</td>
</tr>
<tr>
<td>RUN 1 n = 3</td>
<td>0.324</td>
<td>0.220</td>
<td>0.036</td>
<td>0.053</td>
<td>0.080</td>
<td>0.097</td>
<td>0.375</td>
</tr>
<tr>
<td>RUN 2 n = 1</td>
<td>0.321</td>
<td>0.113</td>
<td>-0.024</td>
<td>0.057</td>
<td>0.090</td>
<td>0.127</td>
<td>0.356</td>
</tr>
<tr>
<td>RUN 2 n = 2</td>
<td>0.248</td>
<td>0.195</td>
<td>0.001</td>
<td>0.083</td>
<td>0.101</td>
<td>0.125</td>
<td>0.359</td>
</tr>
<tr>
<td>RUN 2 n = 3</td>
<td>0.255</td>
<td>0.174</td>
<td>0.042</td>
<td>0.066</td>
<td>0.086</td>
<td>0.099</td>
<td>0.323</td>
</tr>
<tr>
<td>RUN 3 n = 1</td>
<td>0.312</td>
<td>0.106</td>
<td>-0.029</td>
<td>0.053</td>
<td>0.088</td>
<td>0.117</td>
<td>0.335</td>
</tr>
<tr>
<td>RUN 3 n = 2</td>
<td>0.223</td>
<td>0.198</td>
<td>-0.006</td>
<td>0.086</td>
<td>0.104</td>
<td>0.128</td>
<td>0.362</td>
</tr>
<tr>
<td>RUN 3 n = 3</td>
<td>0.247</td>
<td>0.166</td>
<td>0.039</td>
<td>0.061</td>
<td>0.087</td>
<td>0.089</td>
<td>0.329</td>
</tr>
<tr>
<td>Mean</td>
<td>0.287</td>
<td>0.167</td>
<td>0.002</td>
<td>0.064</td>
<td>0.089</td>
<td>0.112</td>
<td>0.363</td>
</tr>
<tr>
<td>SD</td>
<td>0.044</td>
<td>0.043</td>
<td>0.030</td>
<td>0.016</td>
<td>0.009</td>
<td>0.014</td>
<td>0.034</td>
</tr>
<tr>
<td>% Viability</td>
<td>79.25</td>
<td>46.10</td>
<td>0.47</td>
<td>17.54</td>
<td>24.58</td>
<td>30.90</td>
<td>100.00</td>
</tr>
<tr>
<td>% SD</td>
<td>14.15</td>
<td>12.63</td>
<td>8.26</td>
<td>4.72</td>
<td>3.28</td>
<td>4.84</td>
<td>13.09</td>
</tr>
</tbody>
</table>
Table 3.4a. MTS data obtained for IN699 cell line after 72 h.

<table>
<thead>
<tr>
<th>[DC] / mM</th>
<th>0.01</th>
<th>0.1</th>
<th>0.25</th>
<th>0.5</th>
<th>0.75</th>
<th>1.0</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUN 1 n = 1</td>
<td>0.404</td>
<td>0.303</td>
<td>0.032</td>
<td>0.035</td>
<td>0.050</td>
<td>-0.020</td>
<td>0.388</td>
</tr>
<tr>
<td>RUN 1 n = 2</td>
<td>0.401</td>
<td>0.239</td>
<td>0.016</td>
<td>0.027</td>
<td>0.039</td>
<td>0.046</td>
<td>0.362</td>
</tr>
<tr>
<td>RUN 1 n = 3</td>
<td>0.371</td>
<td>0.247</td>
<td>0.055</td>
<td>0.008</td>
<td>0.025</td>
<td>0.057</td>
<td>0.255</td>
</tr>
<tr>
<td>RUN 2 n = 1</td>
<td>0.372</td>
<td>0.278</td>
<td>0.042</td>
<td>0.045</td>
<td>0.045</td>
<td>-0.060</td>
<td>0.369</td>
</tr>
<tr>
<td>RUN 2 n = 2</td>
<td>0.363</td>
<td>0.252</td>
<td>0.024</td>
<td>-0.008</td>
<td>0.021</td>
<td>0.039</td>
<td>0.341</td>
</tr>
<tr>
<td>RUN 2 n = 3</td>
<td>0.395</td>
<td>0.226</td>
<td>0.029</td>
<td>0.016</td>
<td>0.018</td>
<td>0.035</td>
<td>0.235</td>
</tr>
<tr>
<td>RUN 3 n = 1</td>
<td>0.374</td>
<td>0.297</td>
<td>0.075</td>
<td>0.046</td>
<td>0.067</td>
<td>-0.027</td>
<td>0.370</td>
</tr>
<tr>
<td>RUN 3 n = 2</td>
<td>0.368</td>
<td>0.287</td>
<td>0.048</td>
<td>0.011</td>
<td>0.034</td>
<td>0.078</td>
<td>0.351</td>
</tr>
<tr>
<td>RUN 3 n = 3</td>
<td>0.388</td>
<td>0.256</td>
<td>0.058</td>
<td>0.009</td>
<td>0.025</td>
<td>0.067</td>
<td>0.237</td>
</tr>
<tr>
<td>Mean</td>
<td>0.382</td>
<td>0.265</td>
<td>0.042</td>
<td>0.021</td>
<td>0.036</td>
<td>0.024</td>
<td>0.323</td>
</tr>
<tr>
<td>SD</td>
<td>0.015</td>
<td>0.027</td>
<td>0.019</td>
<td>0.018</td>
<td>0.016</td>
<td>0.048</td>
<td>0.062</td>
</tr>
<tr>
<td>%Viability</td>
<td>118.16</td>
<td>82.03</td>
<td>13.05</td>
<td>6.51</td>
<td>11.12</td>
<td>7.41</td>
<td>100.00</td>
</tr>
<tr>
<td>%SD</td>
<td>23.26</td>
<td>17.90</td>
<td>6.38</td>
<td>5.86</td>
<td>5.40</td>
<td>14.87</td>
<td>27.26</td>
</tr>
</tbody>
</table>
Table 3.4b. MTS data obtained for SC1800 cell line after 72 h.

<table>
<thead>
<tr>
<th>[DC] / mM</th>
<th>0.01</th>
<th>0.1</th>
<th>0.25</th>
<th>0.5</th>
<th>0.75</th>
<th>1.0</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>RUN 1 n = 1</td>
<td>0.163</td>
<td>0.043</td>
<td>-0.115</td>
<td>0.009</td>
<td>0.016</td>
<td>0.034</td>
<td>0.065</td>
</tr>
<tr>
<td>RUN 1 n = 2</td>
<td>0.244</td>
<td>0.167</td>
<td>-0.076</td>
<td>0.006</td>
<td>0.026</td>
<td>0.025</td>
<td>0.187</td>
</tr>
<tr>
<td>RUN 1 n = 3</td>
<td>0.267</td>
<td>0.223</td>
<td>-0.015</td>
<td>0.024</td>
<td>0.068</td>
<td>0.032</td>
<td>0.243</td>
</tr>
<tr>
<td>RUN 2 n = 1</td>
<td>0.173</td>
<td>0.067</td>
<td>-0.101</td>
<td>0.014</td>
<td>0.025</td>
<td>0.051</td>
<td>0.086</td>
</tr>
<tr>
<td>RUN 2 n = 2</td>
<td>0.249</td>
<td>0.119</td>
<td>-0.057</td>
<td>0.022</td>
<td>0.044</td>
<td>0.036</td>
<td>0.155</td>
</tr>
<tr>
<td>RUN 2 n = 3</td>
<td>0.134</td>
<td>0.195</td>
<td>-0.014</td>
<td>0.019</td>
<td>0.066</td>
<td>0.033</td>
<td>0.193</td>
</tr>
<tr>
<td>RUN 3 n = 1</td>
<td>0.175</td>
<td>0.077</td>
<td>-0.106</td>
<td>0.012</td>
<td>0.032</td>
<td>0.058</td>
<td>0.100</td>
</tr>
<tr>
<td>RUN 3 n = 2</td>
<td>0.258</td>
<td>0.121</td>
<td>-0.054</td>
<td>0.021</td>
<td>0.045</td>
<td>0.044</td>
<td>0.160</td>
</tr>
<tr>
<td>RUN 3 n = 3</td>
<td>0.142</td>
<td>0.209</td>
<td>-0.010</td>
<td>0.030</td>
<td>0.071</td>
<td>0.036</td>
<td>0.203</td>
</tr>
<tr>
<td>Mean</td>
<td>0.201</td>
<td>0.136</td>
<td>-0.061</td>
<td>0.017</td>
<td>0.044</td>
<td>0.039</td>
<td>0.154</td>
</tr>
<tr>
<td>SD</td>
<td>0.053</td>
<td>0.066</td>
<td>0.041</td>
<td>0.008</td>
<td>0.021</td>
<td>0.010</td>
<td>0.060</td>
</tr>
<tr>
<td>%Viability</td>
<td>129.86</td>
<td>87.72</td>
<td>-39.46</td>
<td>11.24</td>
<td>28.20</td>
<td>24.99</td>
<td>100.00</td>
</tr>
<tr>
<td>%SD</td>
<td>60.82</td>
<td>54.53</td>
<td>-30.81</td>
<td>6.64</td>
<td>17.33</td>
<td>11.70</td>
<td>54.62</td>
</tr>
</tbody>
</table>
Table 3.5. \( LC_{50} \) values of DC exposed to IN699 and SC1800 cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>( t / h )</th>
<th>( LC_{50} / \text{mM} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>IN699</td>
<td>24</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.17</td>
</tr>
<tr>
<td>SC1800</td>
<td>24</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.14</td>
</tr>
</tbody>
</table>

3.3.3 Targeting glioma cells with DC

IN699 and SC1800 cells in a 24 well plate were each treated with an aliquot of DC (either 0.1, 0.01 or 0.001 mM) and combined bright field and fluorescence images were acquired using a time-lapse live cell imaging microscope (Figures 3.8 – 3.11). At all DC concentrations investigated, the fluorescent DC compound was observed in the IN699 cells, but importantly, not in the SC1800 cells. These results show that DC has been very successfully targeted to the glioma cells, but not the non-neoplastic astrocytes and therefore would be a good candidate for BNCT treatments.
Figure 3.8. Combined bright field and fluorescence images of (a,b,c) glioma (IN699) cells and (d,e,f) astrocytes (SC1800) cells treated with DC (0.1 mM). Images acquired 3.5 h after incubation. Bar $= 60 \mu$m.

The highest concentration (0.1 mM $\cong LC_{50}$, Table 3.4) used was to ensure adequate fluorescence intensity could be observed, bearing in mind that the solution would be diluted once placed in the well containing the cells. Proof of principle was demonstrated using this high concentration, although image acquisition was only continued for around 6 h: the images shown in Figures 3.8 – 3.11 were all acquired after 3.5 h; IN699 were less intensely stained before this time, suggesting this was the approx. time period for DC crossing into these cells. That IN699 cells were stained at the lowest DC concentration (0.001 mM), leaving the SC1800 cells unstained means successful targeting was achieved ca. 2 orders of magnitude below the $LC_{50}$ value (ca. 0.1 mM, Table 3.4). Therefore, selective targeting was achieved using non-cytotoxic concentrations of DC.
Figure 3.9. Combined bright field and fluorescence images of (a,b,c) glioma (IN699) cells and (d,e,f) astrocytes (SC1800) cells treated with DC (0.01 mM). Images acquired 3.5 h after incubation. Bar = 60 µm.
Figure 3.10. Combined bright field and fluorescence images of (a,b,c) glioma (IN699) cells and (d,e,f) astrocytes (SC1800) cells treated with DC (0.001 mM). Images acquired 3.5 h after incubation. Bar = 60 µm.

A control experiment was performed where IN699 and SC1800 cells were acquired using bright field and fluorescence imaging in the absence of DC to check that the fluorescence from the IN699 cells was not being produced from some auto-fluorescence phenomenon (Figure 3.11). As expected, no fluorescence was observed, confirming the successful targeting of IN699 cells with DC.
Figure 3.11. Combined bright field and fluorescence images of (a,b,c) glioma (IN699) cells and (d,e,f) astrocytes (SC1800) cells treated with no DC (control). Images acquired 3.5 h after incubation. Bar = 60 µm.

3.4 Summary

Having established that DPPC and DSPC SUV liposomes of the correct size range for transport across the BBB (ca. 80 – 100 nm) containing o-carborane could be successfully produced, the work in this chapter set about to investigate targeted delivery. The unselective o-carborane was successfully converted into a dequalinium salt (DC) that should be able to selectively target the mitochondria of cancer cells. A high concentration of boron, necessary for BNCT, would thus be accumulated preferentially in cancer cells. In contrast to a previous study [24], DC was found to be fluorescent. Cytotoxicity of the compound was tested against a glioma cell line (IN699) and control non-neoplastic
(SC1800) cells, although $LC_{50}$ values (0.36 mM and 0.18 mM for IN699 and SC1800 cells, respectively, for 24 h) were much greater than the concentrations of DC necessary for BNCT treatments. $LC_{50}$ values obtained after 48 and 72 h, were of the same magnitude. Importantly, DC was exclusively accumulated in IN699 cells but not in SC1800 cells, demonstrating successful targeting. This was found over a range of DC concentrations, with the lower end (0.001 mM) being $ca.$ 2 orders of magnitude below the $LC_{50}$. Thus, DC would be an ideal non-cytotoxic boron-containing agent for use in BNCT treatment of cancer.
3.5 References


Chapter 3: Efficacy of specific formulations of o-carborane


Chapter 4

Spray-dried particles containing o-carborane

4.1 Introduction

In the previous two chapters, the aims have been to produce a stable liposome formulation with particles of the correct size to carry across the BBB (Chapter 2), which could contain a derivatised carborane to achieve cancer cell selectivity (Chapter 3). These goals have been met and attention is now turned to producing an alternative method of formulating agents for BNCT. Spray drying is a technique that produces particles from a liquid feed, although the particle size is usually in the 1 – 10 µm range. Such particles would be too large to cross the BBB, but might be useful carriers for BNCT agents to cancers other than brain tumours, such as those of the liver or lung. In the case of liver cancer, such formulations might be delivered orally or via injection since they are likely to end up in this organ. For lung cancer, delivery could be administered via a metred-dose inhaler system. These two cancer types are described before detailing the principles of spray drying.

4.1.1 Spray drying

Spray drying involves converting a solution or suspension into solid particles using a drying technique and is extensively used in the pharmaceutical and food/agricultural industries [1-3]. It is used as a method for microencapsulation [4,5], where fine particles/droplet are enveloped into efficacious larger particles [5]. Spray drying is one of a number of microencapsulation techniques, such as spray cooling, spray chilling, air
spray-dried particles containing o-carborane suspension coating, extrusion, centrifugal extrusion, freeze drying, rotational suspension separation, co-crystallisation, liposome entrapment, interfacial polymerisation and molecular inclusion [4,6]. Spray drying is particularly attractive as it is a one-step method [7] that changes a liquid feed to a dried particulate form with the ability to control particle size, density, morphology and solvent residue [8]. The products are usually micrometre-sized, amorphous particles [8,9], which are often hygroscopic [7]. The physical and chemical properties of the feed and the instrumental conditions, such as air flow rate, inlet temperature, pump speed and feed concentration [10].

The spray drier typically comprises of a feed solution, an atomiser, a gas inlet, a drying chamber, a cyclone and a collector [3,10]. The first process involves converting the liquid into a spray form via a nozzle and a compressed gas (usually air) when in contact with the hot chamber. The larger the drying chamber, the faster the rate of evaporation to form dried solid particles; these are then removed from the gas flow by means of a cyclone and collected. The technique involves centrifugal, kinetic and electrostatic forces [11].

The start of the drying process takes place during atomisation since the air inlet temperature normally is around 150 – 220 °C [12]. The air flow rate controls the residency time of the product in the hot drying compartment; when the residency time is increased, the amount of moisture removed is higher and the reduced velocity in air flow increases the recovery of the product (less going to exhaust) [13,14].

The drying process is also made more effective by using elevated inlet temperatures. The outlet temperature is usually monitored, although is not controlled operationally. Inlet temperature, together with pump speed (liquid flow rate), aspirator rate (rate of drying air
in the chamber) and concentration of the feed solution, all influence the morphology and size of the spray-dried particles produced. For small particles, the aspirator should be set high, the inlet temperature should also be high and dilute feed solutions should be used.

Spray drying allows formulations to be produced with a particle size that is ideal for drug delivery to the lungs. It is particularly useful for making dry powder inhaler (DPI) particulates [15,16]. The particle size broadly determines the locations within the airway the dispersed powders will be deposited [15,17]. For example, the smallest particles will end up in the lungs, slightly larger ones will reach the bronchioles and the largest particles will only travel as far as the trachea. Spray drying is useful in that it produces fairly narrow regions of particle size distributions, thus increasing the effectiveness of this delivery method.

### 4.1.2 Polyvinylpyrrolidone

Polyvinylpyrrolidone (PVP, povidone; Figure 4.1) is a water soluble polymer that is effective as a drug delivery vehicle for dispersions of micro-particles [18,19]. Historically, PVP was used for treatment of trauma victims as a blood plasma substitute and was later integrated to various applications in pharmaceutics, medicine and industry [20]. PVP is used as an emulsifier, disintegrant and binding agent in pharmaceutics and agroculture [21-23]. It is also a food additive: a stabiliser, E1201, as is PVPP (crosopovidone), E1202. The polymer also enhances solubility of drugs and can help prevent recrystallisation. PVP can be useful in helping to disperse drugs or reagents that are poorly water soluble. PVP has been approved by the Food and Drug Administration (FDA) in the US [23,24], although
there has been a report of skin allergic reactions to PVP / povidone. An oral formulations of PVP was also noted to cause damage to the pulmonary vascular region when injected [25].

![Structure of PVP](image)

Figure 4.1. Structure of PVP.

4.1.3 Aims

The aim of the work in this chapter was to produce PVP / \( o \)-carborane co-spray-dried powders that could be used as boron-delivery vehicles for BNCT treatments via a PDI or similar mechanism. In this initial work, focus was resorted back to the cheaper model compound \( o \)-carborane, rather than using the more expensive DC compound (Chapter 3). PVP was used as an excipient, solubilising the \( o \)-carborane and also controlling the loading rate and boron content. Since spray drying produces particles in the micrometre range, the formulated particles would not be expected to cross the BBB. Particle diameters can be optimised for delivery to the lungs by controlling the spray drying parameters; this will be important for BNCT treatments of lung cancer. For delivery to the liver, particle size is less critical and the delivery mechanism would be anticipated to be the normal clearance mechanism. Cytotoxicity tests were also carried out.
4.2 Materials and methods

4.2.1 Materials

PVP (MW 360,000 g mol$^{-1}$) was purchased from Sigma-Aldrich (Dorset, UK); $o$-carborane and di-sodium tetraborate (Na$_2$B$_4$O$_7$.10H$_2$O) were obtained from Alfa Aesar, UK and Fisher Scientific (Loughborough, UK), respectively. Distilled water (MilliQ) was used to prepare PVP / $o$-carborane solutions to be spray-dried. The cell culture reagents trypsin, Dulbecco’s Modified Eagle’s Medium (DMEM), Fetal Bovine Serum (FBS) and Penicillin-Streptomycin (PS) were obtained from Gibco.

4.2.2 Methods

4.2.2.1 Spray drying

Aqueous solutions of PVP (1 and 2 %w/v) with and without $o$-carborane (0.2 %w/v) were prepared (100 mL, 50 mL for $o$-carborane containing solutions) for spray drying (Table 4.1). Solutions were agitated (3000 rpm, electronic mini shaker) until all solids had dissolved (ca. 1 h) and left overnight (16 h). Spray drying was performed using a Buchi B-290 spray drier (Buchi, Manchester, UK) using high inlet temperature (180 °C) and low inlet temperature (130 °C) conditions; other spray drying conditions were kept constant (air flow rate 52 mm, aspirator 100 %, pump speed 10 %, nozzle cleaner setting 3; Table 4.1). After spray drying, %recoveries were calculated and the powders stored in vials in a desiccator.
Table 4.1. Samples for spray drying and instrument inlet temperature settings.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Product</th>
<th>w/v% PVP</th>
<th>w/v% o-carborane</th>
<th>Inlet temperature / °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>PVP</td>
<td>2</td>
<td>0</td>
<td>180</td>
</tr>
<tr>
<td>#2</td>
<td>PVP</td>
<td>2</td>
<td>0</td>
<td>180</td>
</tr>
<tr>
<td>#3</td>
<td>PVP</td>
<td>2</td>
<td>0</td>
<td>180</td>
</tr>
<tr>
<td>#4</td>
<td>PVP</td>
<td>2</td>
<td>0</td>
<td>180</td>
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<td>PVP</td>
<td>1</td>
<td>0</td>
<td>180</td>
</tr>
<tr>
<td>#11</td>
<td>PVP + o-carborane</td>
<td>2</td>
<td>0.2</td>
<td>180</td>
</tr>
<tr>
<td>#12</td>
<td>PVP + o-carborane</td>
<td>2</td>
<td>0.2</td>
<td>180</td>
</tr>
<tr>
<td>#13</td>
<td>PVP + o-carborane</td>
<td>2</td>
<td>0.2</td>
<td>180</td>
</tr>
</tbody>
</table>

4.2.2.2 Particle sizing

Particle sizing was performed on the dry powder PVP / o-carborane samples. The powders were dispersed using compressed air (2 bar) via the RODDOS dry powder dispenser (RODOS, Sympatec GmbH Germany) prior to sizing analysis with a Sympatec HELOS laser diffractometer (HELOS Sympatec). The particle size distribution was recorded as $x_{10}$, $x_{50}$ and $x_{90}$ values using WINDOW 4.0 software (Sympatec). The values presented were the mean of triplicate runs. Statistical analysis was performed using IBM Statistics SPSS (Version 21) using a one-way ANOVA and post-hoc analysis (Tukey test).
4.2.2.3 NMR

$^1$H, $^{13}$C and $^{11}$B NMR spectra were obtained using a JEOL Eclipse+ 400 MHz NMR instrument (Oxford Instruments, UK), operating at a field strength of 9.389766 T. PVP and o-carborane samples were dissolved in either CDCl$_3$ or d-MeOD.

4.2.2.4 SEM and EDS

Dry powders were sprinkled onto double-sided, carbon-loaded disks attached to nickel SEM stubs. Excess powder was removed using a N$_2$ stream and the samples were then sputter-coated with Au/Pd in an Argon atmosphere (< 0.2 Torr, 18 mA for 5 – 10 min; Polaron E5000 SEM coating-unit, Quorum Technologies Ltd., East Grinstead, UK). SEM imaging was performed immediately using a JEOL JSM-6060LV SEM instrument (resolution = 4.5 nm, acceleration voltage = 15 kV). EDS (Inca Oentral FET×3 with high-angle, ultra-thin window Si(Li) detector; Oxford Instruments, Oxford, UK) analysis was also undertaken on some of the samples investigated by SEM. A spot size of 60 and a working distance of 11 was used for EDS.

4.2.2.5 Tissue culture and cytotoxicity studies

Human glioblastoma U-87 MG (cancer cell line) and human fetal lung fibroblast MRC-5 (normal cell line) cells were grown in culture at 37 °C in DMEM, supplemented with FBS (10 %v/v), penicillin (100 μg/mL), streptomycin (100 μg/mL) in a humidified atmosphere containing CO$_2$ (5 %v/v). The medium was changed every 2-3 days and cells were sub-cultured by trypsinisation before reaching ~80 % confluence in tissue culture flasks.
The effect of PVP / o-carborane materials (#2, #8 and #11; Table 4.1) on U-87 MG and MRC-5 cell proliferation capacity and viability was assessed in vitro. These samples were diluted in DMEM and homogenised prior to their addition in cell cultures. Briefly, cells at an initial density of $10^5$ cells/mL were seeded in a 24-well tissue culture plate. After 2 h following cell attachment to the substrate of the plate, material suspensions were added at various concentrations (0.1 mg/mL, 0.5 mg/mL and/or 1.0 mg/mL) before cells being allowed to further growth for 48 h. At this point, cells were detached by trypsinisation and the cell density (number of cells/mL) was determined using a Neubauer plate (cell counting plate). Cell proliferation was expressed as percentage (%) of cell growth compared to control-untreated cell cultures. Cellular death was also assessed using the trypan-blue dye exclusion method and reported as cell viability (% of trypan blue negative cells in culture). The data presented were calculated based on at least four separate measurements from two biological replicate experiments.

4.3 Results and discussion

4.3.1 Spray Drying

Spray drying successfully produced white, fine powdered products of PVP / o-carborane (Table 4.2). Lower %yields (%recoveries) were obtained using 1 w/v% PVP than when using 2 w/v% PVP and consequently, co-spray-dried formulations using o-carborane were carried using the higher PVP concentration; similar %yields were obtained. No obvious changes to %yield were found when the inlet temperature from 130 to 180 °C or when co-spray drying was employed (180 °C).
Table 4.2. %Yields obtained for various spray-dried formulations.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>%Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1</td>
<td>31.0</td>
</tr>
<tr>
<td>#2</td>
<td>28.0</td>
</tr>
<tr>
<td>#3</td>
<td>30.5</td>
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<td>#4</td>
<td>31.0</td>
</tr>
<tr>
<td>#5</td>
<td>20.5</td>
</tr>
<tr>
<td>#6</td>
<td>35.0</td>
</tr>
<tr>
<td>#7</td>
<td>13.5</td>
</tr>
<tr>
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4.3.2 $^1$H NMR of PVP/o-carborane

To check that the integrity of PVP had not been compromised during the spray drying process, $^1$H NMR experiments were performed. The literature spectrum was also obtained and used to help assign peaks (Figure 4.2) [26]. The spectra before and after spray drying (Figures 4.3 and 4.4), both in the absence of o-carborane, looked very similar. The signal corresponding to H3 appeared slightly differently in that the sharp, single peak within the doublet shifted slightly up field. This may be associated with hydrogen bonded water, as spray drying is known to produce hygroscopic, very fine amorphous powders. Peaks H1, H2, H4 and H5 were very similar to those in the literature [26], suggesting the PVP was unaffected by the spray drying process, as might be expected since there have been numerous reports on the spray drying of this material [27-29].
Chapter 4: Spray-dried particles containing o-carborane

Figure 4.2. Literature $^1$H NMR spectrum of PVP [26].

Figure 4.3. $^1$H NMR spectrum of ‘as received’ PVP.
Figure 4.4. $^1$H NMR spectrum of spray-dried PVP (2 %w/v).

A $^1$H NMR spectrum of spray-dried PVP / o-carborane (Figure 4.5) was also obtained to see whether any peaks contained in o-carborane (Figure 4.6) were also present. Such peaks were not apparent in the co-spray-dried product, although this was probably due to the high MW of the PVP: the spectrum was simply swamped with the backbone CH$_2$ and pendant pyrrolidone signals relative to the o-carborane BH and CH signals; in any case, the latter reagent was only present at 10 %w/v. Thus the $^1$H NMR data was inconclusive as to whether the o-carborane had been incorporated into the microparticles. The technique did establish, however, that PVP was unchanged by the spray-drying process, and particularly the high temperatures (180 °C) involved.
Chapter 4: Spray-dried particles containing o-carborane

4.3.3 Particle sizing

Particle diameters of spray-dried PVP and PVP/ o-carborane were measured using laser defraction (Sympatec particle sizer). Figure 4.7 shows a typical output profile of the sizing results. Two plots are displayed: a particle diameter frequency plot and a cumulative frequency graph of particle size from which median and interquartile range data is
automatically generated. In the example, the mean particle diameter is ca. 3 µm, although finer particles (‘fines’) and larger particles, the latter of which may be agglomerates, can be observed. To take into account the spread in size data, it is conventional to report particle sizes as $x_n$ values. For example, an $x_{90}$ value refers to the particle diameter exhibited by 90% of the particles; in other words, 90% of the particles will have a diameter $\leq x$. The $x_{50}$ and $x_{10}$ are also often stated, which have similar definitions, and have progressively smaller values since less of the particles will have these particular size thresholds. The ‘bell-shaped’ curve (‘envelope’), although noting the log-scale axis, can have shoulders on either side of the peak maximum; shoulders on the right of the maximum almost always are an indication of agglomerated particles. Repeating the sizing measurement at higher pressures can reduce this effect, although the final delivery device operational pressure, e.g., in a PDI, needs to be considered. The $x_{10}$, $x_{20}$ and $x_{50}$ particle size data are presented and discussed in the remainder of the this Chapter (Table 4.3a,b,c and Figure 4.8), although the particle size envelope data are not shown. Statistical differences between particle size data are shown in Tables 4.4a,b,c.

Figure 4.7. An example Sympatec particle size distribution output plot from sample #1 run 1 of 3 (full results in Appendix 1); $x_{10} = 1.31$ µm; $x_{50} = 3.47$ µm; $x_{90} = 8.48$ µm; SMD = 2.50 µm; VMD = $x_{16} = 1.65$ µm; $x_{84} = 7.15$ µm; $x_{99} = 13.43$ µm; $S_V = 2.40$ m²/cm³; $S_m = 23973.67$ cm²/g.
The $x_{90}$ data shows that mean particle diameters were ca. 2 – 10 µm, although #11 was 17.27 ± 3.77 µm. No statistical differences ($p > 0.05$) were observed between different batches of spray-dried PVP (#1 to #4), in the absence of o-carborane, using an inlet temperature of 180 °C. When this temperature was decreased to 130 °C, again, no significant differences ($p > 0.05$) in $x_{90}$ values were observed compared to the higher temperature products, although batch differences were seen between #1 and #7 ($p < 0.05$), and #2 and #7 ($p < 0.05$) only. Statistical differences between $x_{90}$ values were also generally not observed ($p > 0.05$) when the PVP concentration was lowered to 1 %w/v. This temperature reduction, however, did lead to a lowering of the %yield (Section 4.3.1). When PVP was co-spray-dried with o-carborane, $x_{90}$ values were not affected ($p > 0.05$), although for #11, $x_{90}$ values were all much higher ($p < 0.01$ or $p < 0.001$) than all the other samples. The reason for this batch variation is uncertain, but may be due to variations in humidity perhaps causing agglomeration. This is examined further is Section 4.3.4.

Particle sizes characterised by $x_{50}$ and $x_{10}$ values followed a very similar trend to the $x_{90}$ values; $x_{10}$ sizes were much less than $x_{50}$ and, in turn, less than $x_{90}$ sizes, due to the definition of the $x_n$ term (Section 4.3.3).
Chapter 4: Spray-dried particles containing \( o \)-carborane

Figure 4.8. Particles diameters of spray-dried particles determined using spray drying (mean ± SD).

Table 4.3a. Statistical differences between \( x_{90} \) data obtained from various spray-dried formulations; * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \); NS = no significant difference.

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4.3.4 Morphology

The morphology of the spray-dried powders of PVP with and without o-carborane was investigated using SEM. Prior to this, imaging of the ‘as received’ PVP and o-carborane was performed (Figure 4.9). The former consisted of large, smooth-sided crystals with
dimensions typically in the 100 – 500 µm range (Figure 4.9a,b). The particles of o-carborane appeared smaller, 5 – 100 µm (Figure 4.9c,d).

![SEM images of (a,b) ‘as received’ PVP and (c,d) ‘as received’ o-carborane: Magnification (a) 100×, (b) 2200×, (c) 100×, (d) 800×.](image1)

SEM images of spray-dried PVP, produced in the absence of o-carborane and using an inlet temperature of 180 °C, all showed small, rounded particles of relatively uniform size (< 5 µm; Figure 4.10). Dimples and hollows in the particles were observed and have been encountered in numerous other spray-dried products [30]: they are thought to result from the nozzle characteristics as the emerging droplet is formed [31]. The particle surfaces appeared smooth, although in some cases, smaller particles were attached, possibly being
due to inter-particulate forces or capillary forces causing agglomeration. No obvious differences in morphology were seen between different batches of spray-dried PVP (samples #1 to #4; Figure 4.10). No obvious changes in morphology or particle size were observed when the inlet temperature was reduced to 130 °C (Figure 4.11), in agreement with the particle size data (Section 4.3.3).

![SEM images of spray-dried PVP](image)

(c) (d)

Figure 4.10. SEM images of spray-dried PVP (inlet temperature 180 °C): Sample (a) #1, (b) #2, (c) #3, (d) #4; magnification 2200×.
SEM imaging of PVP co-spray-dried with o-carborane (Figure 4.12), in agreement with the particle sizing data (Section 4.3.3), generally showed similarly sized particles, although some particles were larger than 10 µm. In some of the SEM images of the co-spray-dried product, small irregularly-shaped crystalline particles were seen, which were not encountered in the pure spray-dried PVP products. These crystals appeared to be similar to those seen in the ‘as received’ o-carborane material (Figure 4.9d). It may be that the o-carborane, which is not water-soluble, although assisted to form suspensions by the viscosity/swelling of the PVP, was largely unchanged by the spray drying process. A less-magnified image of the PVP/o-carborane spray dried product revealed there to be a significant number of such small crystals, thought to be o-carborane (Figure 4.13); these were not apparent in similar images of pure spray-dried PVP (not shown). The extent of this phase separation is unknown. To address this situation, EDS mapping was carried out.
Figure 4.12. SEM images of co-spray-dried PVP / o-carborane (inlet temperature 180 °C): Sample (a) #11, (b,c,d) #12 (different areas), (e,f) #13 (different areas); magnification 2200x.
Chapter 4: Spray-dried particles containing o-carborane

An SEM image of co-spray-dried PVP/o-carborane (sample #11) with corresponding EDS element maps for boron, carbon, oxygen, sodium and gold (elements giving rise to the highest signals) are shown in Figure 4.14. The SEM image shows the characteristic morphology of the spray-dried product observed previously. The assumption was that if the PVP was intimately mixed with o-carborane, the boron map would appear very similar to the SEM image. However, the boron signal (Figure 4.14b) was quite low and darker depressions marked regions where the particles resided, suggesting that the boron content was less than on the double-sided, adhesive, carbon tape. It is known that elements with atomic masses below carbon often not shown in EDS studies and therefore this might explain the absence of boron in the EDS map of the co-spray-dried particles. The carbon map shows an image expected from particles adhered to the carbon-loaded adhesive tape. Carbon atoms are also present in PVP and so the minimum brightness in the carbon map still remains quite high (Figure 4.14c). The oxygen map showed brighter areas where the particles were located, perhaps indicative of the higher oxygen atom concentration from

Figure 4.13. SEM images of co-spray-dried PVP/o-carborane (inlet temperature 180 °C), sample #13; magnification 400×.
the C=O bond on the pyrrolidine ring compared with oxygen containing compounds associated with the carbon-loaded resin (Figure 4.14d). The sodium and gold maps did not reveal any significant contrast (Figure 4.14e,f).

Figure 4.14. SEM and EDS images of co-spray-dried PVP / o-carborane sample #11: (a) SEM; (b) B, (c) C, (d) O, (e) Na, (f) Au.
Chapter 4: Spray-dried particles containing o-carborane

To investigate whether boron-mapping was possible using this technique, a mixture of PVP and Na$_2$B$_4$O$_7$.10H$_2$O (1:1 %w/w) was ground using a mortar and pestle and transferred to a double-sided adhesive carbon disc on to an Al SEM stub (as in Section 4.2.2.4) and analysed using SEM and EDS mapping (Figure 4.15). The boron map (Figure 4.15b) again showed an inversion of the expected contrast, as observed in the boron map for sample #11 (Figure 4.14b). The contrast in the maps of all the other elements were as expected, i.e., the presence of O and Na and an absence of C on areas covered with particles (Na$_2$B$_4$O$_7$.10H$_2$O), and a strong Al signal for the SEM stub. To further confirm these observations, pure, ground Na$_2$B$_4$O$_7$.10H$_2$O crystals were similarly investigated (Figure 4.16). Again, similar image contrast was observed suggesting that boron-mapping is not effective using this technique: the atomic mass is too low.

4.3.5 Cytotoxicity studies

To check for cytotoxicity, the spray-dried powders (PVP #2 and #8, and PVP / o-carborane #11) were exposed to human glioblastoma U-87 MG (cancerous) and human fetal lung fibroblast MRC-5 (non-neoplastic) cell lines. Cell proliferation and cell death were determined over a range of PVP / o-carborane concentrations (0-1.0 mg/mL; Figures 4.17-4.18). In all cases, cell proliferation did not fall below 87% and values were otherwise greater than 90%. Similarly, the highest cell death value was only 2.5%. No obvious change in cell proliferation or cell death was noted over the duration of the experiment (48 °C Therefore, the PVP / o-carborane co-spray-dried product was described as being non-cytotoxic at the concentrations investigated. This is in agreement with the known low toxicity data of the constituent PVP [32,33] and o-carborane [34] compounds.
Figure 4.15. SEM and EDS images of ‘as received’ PVP / borax mixture (1:1 wt%): (a) SEM; (b) B, (c) C, (d) O, (e) Na, (f) Al.
Figure 4.16. SEM and EDS images of borax: (a) SEM; (b) B, (c) C, (d) N, (e) O, (f) Na.
Chapter 4: Spray-dried particles containing o-carborane

Figure 4.17a. Effect of spray-dried PVP / o-carborane (samples #2, #8 and #11; combined results) on MRC-5 cell proliferation.

Figure 4.17b. MRC-5 cell death caused by spray-dried PVP / o-carborane (samples #2, #8 and #11; combined results).
Figure 4.18a. Effect of spray-dried PVP / o-carborane (samples #2, #8 and #11; combined results) on U87-MG cell proliferation.

Figure 4.18b. U87-MG cell death caused by spray-dried PVP / o-carborane (samples #2, #8 and #11; combined results).
4.4 Summary

In this chapter, PVP (2 %w/v) / o-carborane (0.2 %w/v) spray-dried microparticles were produced with the aim providing a boron-delivery system for use in BNCT treatment of liver and lung cancers. Since particle sizes resulting from spray drying procedures are in the micrometre range, the ability of these to cross the BBB and thus BNCT treatments for brain tumours was not considered. \(^1\)H NMR studies revealed the high temperatures (180 °C) of the spray drying process did not degrade the PVP. Mean particle diameters \((x_{90})\) were in the 2 – 10 µm range, with finer fractions being present \((x_{10} \equiv 1 – 2 \mu m)\), and were therefore considered suitable for delivery to the lungs. Further optimisation of particle sizes could be carried out to decrease \(x_{90}\), for example, by changing air and liquid flow conditions. SEM imaging showed the particles to be spherical, with dimples and cavities caused by the spray drier nozzle characteristics, as typical with the spray drying process. Some small irregularly-shaped crystalline particles, thought to be o-carborane, were observed by SEM, although the proportion accounted for less than that in the formulation (10 %w/w). An attempt was made to map the boron content in spray-dried powders on a surface using EDS, although the low atomic weight of boron made detection not possible. Cytotoxicity studies, using human glioblastoma U-87 MG (cancerous) and human fetal lung fibroblast MRC-5 (non-neoplastic) cells, revealed the PVP / o-carborane co-spray-dried particles to be non-toxic, as might have been expected.
4.5 References


Chapter 5

Conclusions and further work

5.1 Summary

DPPC and DSPC SUV liposomes with particle diameters of *ca.* 80 – 100 nm containing *o*-carborane were successfully produced using the thin film method. Those of DSPC had slightly larger particle sizes as reflected by their longer carbon chain. For both DPPC and DSPC liposomes, particle diameter increased with increasing entrapped *o*-carborane volumes. The liposomes had a monodispersed, homogeneous particle size distribution (PDI < 0.5), although possessed ζ-potentials in a range indicative of instability (between -30 and +30 mV). However, this was probably due to high ionic strength of the PBS medium and re-probing was found to reduce agglomerated particle to the required size range over storage at 3 – 5 °C for 1 week. AFM studies showed that the loaded liposomes generally appeared intact after 63 days of storage if re-probing was employed. The integrity of the liposome membrane in serum, as reflected by %latency and %retention experiments using a fluorescent marker (calcein), was found to be high for both DPPC and DPPC liposomes prepared using cholesterol. Co-entrapment of *o*-carborane with Nile Red into the liposomes semi-quantitatively confirmed the required volume of the boron compound successfully became entrapped with the liposomes. This was proven quantitatively using ICP-MS measurements.

Having established that DPPC and DSPC SUV liposomes of the correct size range for transport across the BBB (*ca.* 80 – 100 nm) containing *o*-carborane could be successfully
produced, the work in this chapter set about to investigate targeted delivery. The unselective \( o \)-carborane was successfully converted into a dequalinium salt (\( \text{DC} \)) that should be able to selectively target the mitochondria of cancer cells. A high concentration of boron, necessary for BNCT, would thus be accumulated preferentially in cancer cells. In contrast to a previous study [1], \( \text{DC} \) was found to be fluorescent. Cytotoxicity of the compound was tested against a glioma cell line (IN699) and control non-neoplastic (SC1800) cells, although \( LC_{50} \) values (0.36 mM and 0.18 mM for IN699 and SC1800 cells, respectively, for 24 h) were much greater than the concentrations of \( \text{DC} \) necessary for BNCT treatments. \( LC_{50} \) values obtained after 48 and 72 h, were of the same magnitude. Importantly, \( \text{DC} \) was exclusively accumulated in IN699 cells but not in SC1800 cells, demonstrating successful targeting. This was found over a range of \( \text{DC} \) concentrations, with the lower end (0.001 mM) being \( ca. \) 2 orders of magnitude below the \( LC_{50} \). Thus, \( \text{DC} \) would be an ideal non-cytotoxic boron-containing agent for use in BNCT treatment of cancer.

In the final chapter, PVP (2 %w/v) / \( o \)-carborane (0.2 %w/v) spray-dried microparticles were produced with the aim providing a boron-delivery system for use in BNCT treatment of liver and lung cancers. Since particle sizes resulting from spray drying procedures are in the micrometre range, the ability of these to cross the BBB and thus BNCT treatments for brain tumours was not considered. \( ^1 \)H NMR studies revealed the high temperatures (180 °C) of the spray drying process did not degrade the PVP. Mean particle diameters (\( x_{90} \)) were in the 2 – 10 \( \mu \)m range, with finer fractions being present (\( x_{10} \approx 1 – 2 \mu \)m), and were therefore considered suitable for delivery to the lungs. Further optimisation of particle sizes could be carried out to decrease \( x_{90} \), for example, by changing air and liquid flow
conditions. SEM imaging showed the particles to be spherical, with dimples and cavities caused by the spray drier nozzle characteristics, as typical with the spray drying process. Some small irregularly-shaped crystalline particles, thought to be o-carborane, were observed by SEM, although the proportion accounted for less that than in the formulation (10 %w/w). An attempt was made to map the boron content in spray-dried powders on a surface using EDS, although the low atomic weight of boron made detection not possible. Cytotoxicity studies, using human glioblastoma U-87 MG (cancerous) and human fetal lung fibroblast MRC-5 (non-neoplastic) cells, revealed the PVP / o-carborane co-spray-dried particles to be non-toxic, as might have been expected.

5.2 Further work

This project could be continued in various directions, which are outlined below.

The encapsulation of DC and the rhodamine 123 carborane salt into the DPPC and DSPC liposomes would be an obvious next stage since the current studies have focused on one of these carborane derivatised mitochondrial dyes, but not in its liposomal carrier form. It would also be useful to ascertain the mitochondrial membrane potentials of the cells of interest to check suitability or fine-tune selection of such carborane derivatised compounds.

Spray drying of PVP containing DC and the rhodamine 123 carborane salt could also be carried out, since the current work used o-carborane as a model carborane material. Further
optimisation of the spray drying parameters might enable smaller particles to be formed, which would improve the ability to target the lungs. Currently, the particle size ($x_{90}$) is ca. 2 – 10 µm, although finer particles are present ($x_{10} = 1 – 2$ µm; $x_{50} = 2 – 8$ µm). ICP MS should be performed on the spray dried particles to determine the boron content.

Another direction would be to carry out the BNCT reaction on cells that have been treated with the derivatised carboranes. The correct dosage would need to be optimised and cells could be imaged before and after the BNCT reaction to check for efficacy. Flow cytometry could also be used in conjunction with fluorescence imaging. Some of these studies could also be performed in the presence of mixed cultures of cells: cancer and non-neoplastic cells, or even with tissues. In vivo experiments could eventually be performed.
5.3 References