Development of Liposome Drug Delivery Systems for Anti-Glioma Therapy

by

Mohit L Jain

A thesis submitted in partial fulfilment for the requirements for the degree of MSc (by Research) at the University of Central Lancashire

January 2012
**Declaration**

I declare that while registered as a candidate for the research degree, I have not been a registered candidate or enrolled student for another award of the University or other academic or professional institution. No material contained in the thesis has been used in any other institution for an academic award and is solely my own work.

Signed
Acknowledgements

I express my whole-hearted thanks and gratitude to my supervisor Dr. Abdelbary Elhissi for his enormous help and motivation throughout my research. His kind and friendly nature made my MSc project memorable. I also thank my second supervisor Dr. Ka-Wai Wan for her valuable and kind support.

I would also like to thank Prof. Waqar Ahmed for his help and useful discussions. My sincere thanks go to Dr. Ka-Wai Wan, Dr. Gail Welsby, Dr. Philip Welsby and Tony Dickson for their help with tissue culture studies. I also thank Dave McCarthy for TEM pictures. I also thank Prof. Jaipaul Singh for his generous advice. I forward my appreciation towards my colleagues: Basel Arafat, Iftikhar Khan, Nozad Hussein, Huner Omer, Seema Jaiswal, Sneha Subramanian, Urwashi Naik, Samriddhi Lal and Oshadie Korale for their support and inspiration. I am also thankful to my friends: Alisha, Jane, Ritesh, Preet, Chinmay, Suresh and Sahil for their love, mental support and useful comments during stressful times.

I would also like to acknowledge Lipoid, Germany for their generous provision of phospholipids without which this project would not have been possible.

I also express my deep gratitude to my parents, sister (Divya) and relatives for their understanding and endless love. It was under their watchful eye that I gained so much experience and ability to face challenges head on.

My special thanks to my pet Hamster who kept me entertained all year. Lastly, I would like to thank Christian Bale and Christopher Nolan for releasing “The Dark Knight Rises” at the correct time giving me another reason to cherish this year.
Abstract

This study aims to investigate the potential of ethanol-based proliposomes in generating paclitaxel-loaded liposome delivery *in vitro*, by employing various phospholipid compositions.

Liposomes prepared using ethanol-based proliposome method successfully generated multilamellar vesicles. Three different lipid phases: SPC:Chol, HSPC:Chol or DPPC:Chol in 1:1 mole ratio were used in each liposomal formulation to compare their size, size distribution, zeta potential, pH and morphology. The size of the liposomes was then reduced into nanometre size range.

DPPC-liposomes entrapped 70-85% of the available paclitaxel compared to only 46-75% and 26-67% entrapped by liposomes made from SPC and HSPC respectively, using a range of paclitaxel concentration. The entrapment efficiency of liposomes was dependent on the lipid bilayer properties and ability of paclitaxel to modify surface charge.

*In vitro* studies revealed that paclitaxel alone was more toxic to U87-MG as well as SVG-P12 cell lines than liposome formulations. The cytotoxicity of liposomes was dependent on their entrapment efficiency and sustained drug release. Thus, DPPC-liposomes had a more cytotoxic effect on the cells than SPC and HSPC liposomes. However, Drug-free liposomes proved to be non-toxic to the cells, indicating that liposomes might enhance the efficacy of the entrapped drug. The properties of different liposome formulations were essential in understanding their drug delivery mechanism.
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APGSP</td>
<td>Autocrine and paracrine growth stimulatory pathways</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BCB</td>
<td>Blood cerebrospinal fluid barrier</td>
</tr>
<tr>
<td>BTB</td>
<td>Blood tumour barrier</td>
</tr>
<tr>
<td>BV</td>
<td>Bevacizumab</td>
</tr>
<tr>
<td>Chol</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DMPC</td>
<td>Dimyristoylphosphatidylcholine</td>
</tr>
<tr>
<td>DMPG</td>
<td>Dimyristoylphosphatidylglycerine</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPPC</td>
<td>Dipalmitoylphosphatidylcholine</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EE</td>
<td>Entrapment Efficiency</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle’s minimum essential medium</td>
</tr>
<tr>
<td>EPC</td>
<td>Egg phosphatidylcholine</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FTase</td>
<td>Farnesytransferase</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma multiforme</td>
</tr>
<tr>
<td>HSPC</td>
<td>Hydrogenated soya phosphatidylcholine</td>
</tr>
<tr>
<td>JCV</td>
<td>John Cunningham virus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>LUV</td>
<td>Large unilamellar vesicle</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MLV</td>
<td>Multilamellar vesicle</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum tolerated dose</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MVL</td>
<td>Multivesicular liposome</td>
</tr>
<tr>
<td>OH</td>
<td>Hydroxyl group</td>
</tr>
<tr>
<td>OLV</td>
<td>Oligolamellar vesicle</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer solution</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PCS</td>
<td>Photon correlation spectroscopy</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PFS6</td>
<td>Progression-free survival rate by 6 months</td>
</tr>
<tr>
<td>PI</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-L-Lysine</td>
</tr>
<tr>
<td>PTA</td>
<td>Phosphotungstic acid</td>
</tr>
<tr>
<td>RES</td>
<td>Reticuloendothelial system</td>
</tr>
<tr>
<td>REV</td>
<td>Reverse evaporation vesicle</td>
</tr>
<tr>
<td>SPC</td>
<td>Soya phosphatidylcholine</td>
</tr>
<tr>
<td>SUV</td>
<td>Small unilamellar vesicle</td>
</tr>
<tr>
<td>SVG-P12</td>
<td>Human glial cell line</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>T_m</td>
<td>Main phase transition temperature</td>
</tr>
<tr>
<td>U87-MG</td>
<td>Human glioblastome cell line</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>VMD</td>
<td>Volume median diameter (50% undersize)</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>ZP</td>
<td>Zeta potential</td>
</tr>
</tbody>
</table>
## Contents

Title Page................................................................................................................. 1
Declaration.............................................................................................................. 2
Acknowledgements............................................................................................... 3
Abstract.................................................................................................................. 4
List of abbreviations............................................................................................... 5
Contents................................................................................................................... 8
List of Figures......................................................................................................... 13

### CHAPTER 1:

**INTRODUCTION**

1.1 **Phospholipids**.............................................................................................. 17
1.2 **Liposomes**................................................................................................... 19
1.3 **Classification of liposomes**.......................................................................... 21
   1.3.1 Multilamellar vesicles (MLVs)................................................................... 22
   1.3.2 Small unilamellar vesicles (SUVs).............................................................. 23
   1.3.3 Large unilamellar vesicles (LUVs)............................................................ 24
   1.3.4 Oligolamellar vesicles (OLVs).................................................................. 24
   1.3.5 Multivesicular liposomes (MVLs).............................................................. 25
1.4 **Storage stability of liposomes**..................................................................... 25
1.5 **Proliposome technology**.............................................................................. 26
   1.5.1 Particulate-based proliposomes................................................................. 27
   1.5.2 Ethanol-based proliposomes..................................................................... 28
1.6 **Glioma**........................................................................................................ 29
CHAPTER 2:

METHODOLOGY

2.1 Materials.................................................................................47
2.2 Methods....................................................................................49
   2.2.1 Preparation of liposomes using the ethanol-based proliposome method…… 49
   2.2.2 Size reduction of liposomes..................................................49
   2.2.3 Laser diffraction size analysis for liposomes.................................50
   2.2.4 Photon correlation spectroscopy for liposomes...............................51
   2.2.5 Zeta potential analysis of liposomes........................................51
2.2.6 Transmission Electron Microscopy (TEM) ......................................................... 51
2.2.7 Determination of drug entrapment efficiency ............................................. 52
2.2.8 Cell culture technique ............................................................................. 53
2.2.9 Sub-culturing of the cells .......................................................................... 53
2.2.10 Calculation of cell viability ..................................................................... 54
2.2.11 Seeding of 96-well plates ......................................................................... 55
2.2.12 Evaluation of cytotoxicity using colourimetric tetrazolium-based MTT Assay ........................................................................................................ 56
2.2.13 Statistical analysis ..................................................................................... 57

CHAPTER 3:
CHARACTERISATION OF LIPOSOMES GENERATED FROM ETHANOL-BASED PROLIPOSOMES

3.1 Introduction ........................................................................................................ 59
3.2 Results and Discussion ...................................................................................... 59
3.2.1 Size analysis of liposomes before sonication ............................................. 59
3.2.2 Size distribution (Span) of liposomes before sonication ............................ 62
3.2.3 Zeta potential analysis of liposomes before sonication ............................... 63
3.2.4 Size analysis of the liposomes after probe sonication ............................... 64
3.2.5 Polydispersity Index (PI) of the liposomes after probe sonication ........... 66
3.2.6 Zeta potential analysis of liposomes after probe sonication ..................... 67
3.2.7 Morphology of the liposomes ..................................................................... 68
3.2.8 pH measurement of liposome formulations ............................................. 72
CHAPTER 4:

CYTOTOXICITY STUDIES OF LIPOSOMAL FORMULATIONS ON GLIOMA AND GLIAL CELLS IN VITRO

4.1 Introduction ........................................................................................................84
4.2 U87-MG cells ....................................................................................................85
4.3 SVG-P12 cells .................................................................................................86
4.4 Results and Discussion ..................................................................................87
   4.4.1 Introduction ...............................................................................................87
   4.4.2 Growth curve of U87-MG cells .................................................................88
   4.4.3 Growth curve of SVG-P12 cells .................................................................90
   4.4.4 Effects of liposomes, paclitaxel and controls on U87-MG cells ...............92
   4.4.5 Effects of liposomes, paclitaxel and controls on SVG-P12 cells ...............98
4.5 Conclusion ......................................................................................................104

CHAPTER 5:

GENERAL CONCLUSIONS AND FUTURE STUDIES

5.1 Introduction ......................................................................................................108
5.2 Characterisation of liposomes before sonication ............................................109
5.3 Characterisation of liposomes after sonication ..............................109
5.4 pH of liposome formulations......................................................110
5.5 Entrapment efficiency of paclitaxel in liposomes........................110
5.6 Tissue culture findings...........................................................112
5.7 Future studies...........................................................................115

CHAPTER 6:

REFERENCES 116
## List of Figures

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Title</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Chemical structure of phosphatidylcholine</td>
<td>17-18</td>
</tr>
<tr>
<td>1.2</td>
<td>Mechanism of liposome formation</td>
<td>19</td>
</tr>
<tr>
<td>1.3</td>
<td>Chemical structure of cholesterol</td>
<td>21</td>
</tr>
<tr>
<td>1.4</td>
<td>Classification of liposomes</td>
<td>22</td>
</tr>
<tr>
<td>1.5</td>
<td>A schematic representation of the blood-brain barrier</td>
<td>38</td>
</tr>
<tr>
<td>1.6</td>
<td>Chemical structure of paclitaxel</td>
<td>39</td>
</tr>
<tr>
<td>3.1</td>
<td>Size of liposomes generated from ethanol-based proliposome formulations with a range of paclitaxel concentrations</td>
<td>61</td>
</tr>
<tr>
<td>3.2</td>
<td>Size distribution of liposomes generated from ethanol-based proliposome formulations with a range of paclitaxel concentrations</td>
<td>62</td>
</tr>
<tr>
<td>3.3</td>
<td>Zeta potential of liposomes generated from ethanol-based proliposome formulations with a range of paclitaxel concentrations</td>
<td>64</td>
</tr>
<tr>
<td>3.4</td>
<td>Size of liposomes after probe sonication</td>
<td>65</td>
</tr>
<tr>
<td>3.5</td>
<td>PI of liposomes after probe sonication</td>
<td>66</td>
</tr>
<tr>
<td>3.6</td>
<td>Zeta potential of liposomes after probe sonication</td>
<td>67</td>
</tr>
<tr>
<td>3.7</td>
<td>TEM of SPC: Chol (1:1) liposomes containing 1 mg/ml paclitaxel concentration after size reduction</td>
<td>69</td>
</tr>
<tr>
<td>3.8</td>
<td>TEM of HSPC: Chol (1:1) liposomes containing 1 mg/ml paclitaxel concentration after size reduction</td>
<td>70</td>
</tr>
<tr>
<td>Section</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>3.9</td>
<td>TEM of DPPC: Chol (1:1) liposomes containing 1 mg/ml paclitaxel concentration after size reduction</td>
<td></td>
</tr>
<tr>
<td>3.10</td>
<td>pH of liposomes after probe sonication</td>
<td></td>
</tr>
<tr>
<td>3.11</td>
<td>Calibration curve of paclitaxel</td>
<td></td>
</tr>
<tr>
<td>3.12</td>
<td>Entrapment efficiency of liposomes by UV method</td>
<td></td>
</tr>
<tr>
<td>3.13</td>
<td>The amount of paclitaxel entrapped per 10 ml of formulation</td>
<td></td>
</tr>
<tr>
<td>4.1</td>
<td>Electron Micrograph of U87-MG cells showing epithelial morphology in low and high confluency</td>
<td></td>
</tr>
<tr>
<td>4.2</td>
<td>Growth curve of U87-MG cell line for 7 day period</td>
<td></td>
</tr>
<tr>
<td>4.3</td>
<td>Inverted light microscope photographs of U87-MG cells, on day 7 of growth curve, with different seeding densities</td>
<td></td>
</tr>
<tr>
<td>4.4</td>
<td>Growth curve of SVG-P12 cell line for 7 day period</td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>Inverted light microscope photographs of SVG-P12 cells, on day 7 of growth curve, with different seeding densities</td>
<td></td>
</tr>
<tr>
<td>4.6</td>
<td>Viability of U87-MG cell line tested with increasing concentrations of different drug compounds in 96-well plates</td>
<td></td>
</tr>
<tr>
<td>4.7</td>
<td>Inverted microscope photographs of the viability of U87-MG cells treated with paclitaxel-liposomal formulations and paclitaxel</td>
<td></td>
</tr>
<tr>
<td>4.8</td>
<td>IC\textsubscript{50} of paclitaxel-loaded liposomes, paclitaxel and PLL against U87-MG cells.</td>
<td></td>
</tr>
<tr>
<td>4.9</td>
<td>Viability of SVG-P12 cell line tested with increasing concentrations of different drug compounds in 96-well plates</td>
<td></td>
</tr>
<tr>
<td>4.10</td>
<td>Inverted microscope photographs of the viability of SVG-P12 cells treated with paclitaxel-liposomal formulations and paclitaxel</td>
<td>101</td>
</tr>
</tbody>
</table>
CHAPTER 1:

INTRODUCTION
1.1 Phospholipids

Phospholipids are essential components in the formulation of liposomes. They are amphipathic molecules which consist of hydrophilic (polar) headgroups and hydrophobic (non-polar) hydrocarbon chains (New, 1990a). The polar headgroups are made up of diverse molecules and non-polar hydrocarbon chains can differ in length and degree of saturation, resulting in different types of phospholipids, which may affect the bilayer permeability and surface charge of the resulting liposomes (Perrie and Rades, 2010). Phospholipids can be divided into synthetic phospholipids and natural phospholipids. Dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) are examples of synthetic phospholipids. Natural phospholipids include egg phosphatidylcholine (EPC) and soya phosphatidylcholine (SPC). Figure 1.1 shows the chemical structure of SPC, hydrogenated soya phosphatidylcholine (HSPC) and DPPC.

**Fig.1.1 Chemical structure of phosphatidylcholines (Source: Zhao et al., 2004)**

Soya phosphatidylcholine (SPC)
Phosphatidylcholines (PCs) are primarily used phospholipids in liposome preparation. PCs are neutral or zwitterionic with pH ranging from strongly acidic to strongly alkaline. PCs are water insoluble lipids so they self-assemble in aqueous media, with hydrocarbon chains being oriented away from the aqueous phase. Mechanical agitation will quickly cause lipid bilayers to form liposomes (New, 1990a) (Fig. 1.2).
1.2 Liposomes

Liposomes are microscopic phospholipid bilayer vesicles having a size range between 25 nm and 20 μm. Liposomes were discovered in 1960s by Dr Alec Bangham (Bangham et al., 1965; Torchilin, 2005). Liposomes have the ability to entrap hydrophilic therapeutic agents in their aqueous central compartment and hydrophobic therapeutic agents (ligands, polymers or macromolecules) can be entrapped within their phospholipid bilayers or can be attached to the liposome surfaces (Torchilin, 2005). The advantage of liposomes is that,
they are biodegradable and non-toxic because they are made of naturally occurring materials that are present in the biological membranes (Naderkhani, 2011). Drugs loaded in liposomes may exhibit a continuous release or targeted delivery in manners that are dependent on liposome size, bilayer composition and liposome surface properties.

The mechanism suggested by Lasic (1988) described the formation of liposomes. In this experiment, the aqueous phase was added to the dry phospholipid film that led to the hydration of outer monolayer to exceed that of the inner layers. The increase in hydration resulted in increasing the surface area of the polar heads and caused the formation of “blisters” (Lasic, 1988, Saupe, 1977). These blisters were converted into phospholipid bilayers which further developed into tubular fibrils. This process increased the contact area of the lipid with the aqueous phase. The bilayer sheets then consisted of hydrophobic moieties exposed to the thermodynamically unstable aqueous phase. The bilayers may be compelled to seal off and form multilamellar vesicles (Lasic, 1988). Liposome bilayers exhibit a gel phase (well-ordered) below the lipid phase transition temperature \( T_m \) and a disorderly fluid phase above the \( T_m \) of the phospholipid employed. Therefore, the hydration procedure for liposome formation should be carried out at a temperature above the phase transition temperature \( T_m \) of the selected phospholipid (Lian and Rodney, 2001; Elhissi et al., 2006).

The incorporation of cholesterol into the lipid bilayer has demonstrated a significant effect on the properties of liposomes. Cholesterol enhances the stability of the lipid bilayers by forming highly ordered and rigid phase with fluid-like characteristics depending on the type of phospholipid involved in the liposome (Lee et al., 2005). The four hydrocarbon rings makes the molecular structure of cholesterol strongly hydrophobic while at the same
time the hydroxyl group (OH) makes the end of cholesterol weakly hydrophilic (Fig. 1.3).

The molar ratio of phospholipid to cholesterol in lipid bilayers commonly used in the liposome formation is 1:1 respectively (Cooper and Hausman, 2009).

**Fig.1.3. Chemical structure of cholesterol (Source: Sigma Aldrich, UK)**

![Chemical structure of cholesterol](image)

### 1.3 Classification of liposomes

Liposomes are classified depending on their morphology into multilamellar liposome vesicles (MLVs), small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs) oligolamellar vesicles (OLVs) and multivesicular liposomes (MVLs) (Fig. 1.4).
1.3.1 Multilamellar vesicles (MLVs)

Multilamellar liposome vesicles (MLVs) are commonly referred to as “conventional liposomes” and consist of several concentric phospholipid bilayers, and have a typical size range between 0.1 µm and 20 µm (Fig. 1.4). They are prepared by the thin film hydration method. A thin film of lipid is prepared by dissolving the phospholipid with or without cholesterol in an organic solvent (e.g. chloroform) within a round bottom flask. Evaporation of the organic solvent under vacuum using rotary evaporator causes the formation of a thin film of lipid on the inner walls of the flask. Addition of water with
shaking causes the formation of MLVs (Bangham et al., 1965; Elhissi et al., 2006). This procedure is carried out above the phase transition temperature ($T_m$) of the phospholipid. The solvent evaporated by rotary evaporator is collected via a condenser for disposal or reuse.

1.3.2 Small unilamellar vesicles (SUVs)

A small unilamellar vesicle (SUV) consists of a single phospholipid bilayer which makes a liposome having a size that ranges between 25 and 100 nm (Fig. 1.4). Batzsri and Korn (1973) prepared SUVs by injection of an ethanolic solution of phospholipid into the aqueous phase above the $T_m$ of the phospholipid, with appropriate dilution and mixing. Generally, SUVs are manufactured by probe sonication of MLVs dispersions. As an alternative to the method introduced by Batzsri and Korn (1973), probe sonication of large liposomes can generate SUVs (New, 1990b). In this method, the probe of the sonicator is immersed in the liposome dispersion and operated at the highest frequency to disrupt the MLVs to form SUVs. The probe is composed of an inert or biologically friendly metal like titanium. The probe is tuned to the oscillating electric current frequency such that the probe can oscillate in harmony with the liposome vesicles. Probe sonication can generate extensive heat rapidly due to the high power input into preparation by the tip of the probe. Due to increased gas exchange and high temperature, there is high risk of lipid bilayer degradation. Thus, while processing heat labile samples such as liposomes, the samples must be kept cold and the sonication must be performed in short burst intermitted with cooling periods (Santos et al., 2009).
1.3.3 Large unilamellar vesicles (LUVs)

Large unilamellar vesicles (LUVs) consist of a single phospholipid bilayer, similar to SUVs but having a larger size that falls in the range of 0.1 µm to 1 µm (Fig. 1.4). These liposomes are known to provide high hydrophilic drug entrapment compared to that of MLVs. LUVs are prepared by injection of an ethereal phospholipid solution into an aqueous phase previously heated above the T\textsubscript{m} of the injected phospholipid (Deamer and Bangham, 1976). The drawbacks associated with this method are that the population is not homogenous (70-190 nm) and the disclosure of encapsulated drug to high temperature or organic solvents (Chauhan \textit{et al}., 2012; Scieren \textit{et al}., 1978). In another method, Kirby and Gregoriadis (1984) prepared dehydrated-rehydrated vesicles by mixing aqueous drug solution and suspension of drug-free SUVs, followed by freeze-drying. The SUVs convert into LUVs which may typically have a vesicle size of 1 µm or less after rehydration.

1.3.4 Oligolamellar vesicles (OLVs)

MLVs possessing only two or three phospholipid bilayers are known as oligolamellar vesicles (OLVs) (Fig. 1.4). Szoka and Papahadjopoulos (1978) introduced a method known as Reverse phase evaporation method. This method produces a mixture of OLVs and LUVs and they are termed as reverse evaporation vesicles (REVs). REVs provide up to 62% of entrapment of the aqueous phase. Alternatively, ethanol-based proliposomes may generate oligolamellar liposomes (Perrett \textit{et al}., 1991). Ethanol-based proliposomes are concentrated ethanolic mixtures of phospholipids that generate liposomes upon addition of aqueous phase and shaking (Perrett \textit{et al}., 1991).
1.3.5 Multivesicular liposomes (MVLs)

When a large liposome vesicle similar in size to an MLV, enclose a group of liposomes, then the subsequent vesicle is known as multivesicular liposome (MVL) (Kim et al., 1983) (Fig. 1.4). According to the experiments conducted by Kim et al., (1983) high encapsulation (about 89%) of hydrophilic drugs was achieved. The drug was dissolved in the aqueous phase of water-in-oil emulsion, where oil phase consisted of phospholipid, neutral oil such as triolein and organic solvents. MVLs were formed by the addition of aqueous sucrose solution and aliquots of the emulsion, followed by the evaporation of the organic solvent at warm temperature. MVLs prepared by this method have advantages over MLVs prepared by the thin-film method as MVLs may have high storage stability and easy production scale up (Kim et al., 1987).

1.4 Storage stability of liposomes

Liposome formulations using synthetic or natural phospholipids (e.g. phosphatidylcholine) aims to reduce drug toxicity and increase the accumulation of drug at the target site (Lian and Rodney, 2001). However, liposomes are unstable as liquid dispersions and the liability of the phospholipids to degrade by oxidation or hydrolysis can cause liposome aggregation followed by leakage of entrapped material. Lipid hydrolysis may be increased at certain pH values of the dispersion (Grit et al., 1993). Oxidation may be reduced by incorporation of antioxidants (Hunt and Tsang, 1981) or by reduction of storage temperature to 4°C (Hernandez-Caselles et al., 1990).
For the stability of liposomes, freeze-drying of the liposome aqueous dispersions may be performed. This may however lead to a destabilising effect on the bilayers, which can be minimised by the addition of cryoprotectants such as trehalose or sucrose (Crowe et al., 1987) before freezing. Van Winden and Crommelin (1997) suggested a method to maintain the residual water content in the liposomes at a minimum level to prevent the increase of vesicular size on rehydration and increase the shelf-life and stability of the lyophilised liposomes and formulations.

Spray drying is another method employed to increase the storage life of liposomes. Skolka-Basnet et al. (2000) applied one-step spray-drying method on liposomes entrapping verapamil or metronidazole with or without cyclodextrin. They observed that the entrapment efficiency and size distribution of liposomes measured before drying were still maintained after one year of storage of the liposome powder at 4°C.

1.5 Proliposome technology

Proliposome technologies such as particulate-based proliposomes (Payne et al., 1986a, b) and ethanol (solvent)-based proliposomes (Perrett et al., 1991) have been suggested to deliver convenient and economic options when compared to spray-drying or freeze-drying of liposomes. Proliposomes can also overcome the difficulty of manufacturing of liposomes on a large scale due to the instability problems (liability of phospholipid to hydrolysis, oxidation and subsequent loss of entrapped drug) high costs and unsuitability
of scaling up of liposomes prepared from conventional methods e.g. thin film method (Kensil and Dennis, 1981; Grit et al., 1989; Hunt and Tsang, 1981).

1.5.1 Particulate-based proliposomes

Particulate-based proliposomes involve carbohydrates as soluble carrier materials layered with phospholipids to form MLVs upon addition of water above T<sub>m</sub> (Payne et al., 1986). This type of proliposome is prepared by attaching a flask comprising the carrier particles to a rotary evaporator. The organic phase of lipid is added through a feed-line in a portion-wise manner to coat the carrier, under low pressure. Evaporation of the organic solvent under vacuum using rotary evaporator causes the formation of particulate-based proliposomes. The carrier particles involved in the formation of these proliposomes may be sodium chloride, lactose, fructose, glucose (Payne et al., 1986a), mannitol (Zhang and Zhu, 1999), or sorbitol (Payne et al., 1986a, b; Payne et al., 1987; Lee et al., 1995, 1996; Ahn et al., 1995a, b; Chung, 1999; Hwang et al., 1997; Song et al., 2002; Jung et al., 2000; Elhissi et al., 2006). Another type of particulate-based proliposome, named bead-based proliposomes, was prepared in order to scale up proliposomes using fluidised-bed coating (Kumar et al., 2001; Chen and Alli, 1987; Katare et al., 1990).

Liposomes prepared from this method have high entrapment efficiency for lipophilic compounds such as nicotine (around 45 to 58%) (Chung, 1999), Amphotericin B (100%) (Payne et al., 1987), salmon calcitonin (20%) (Song et al., 2002), ciprofloxacin (96%) and CM3 peptide (100%) (Desai et al., 2002). However, the entrapment efficiency of hydrophilic materials was generally low (in the range of 4 to 10%) but the entrapment can be maximised by increasing the phospholipid to drug ratios (Ahn et al., 1995 a).
1.5.2 Ethanol-based proliposomes

Ethanol-based proliposomes are ethanolic lipid solutions which, depending on the hydration procedure, generate oligolamellar liposomes (Perrett et al., 1991) or multilamellar vesicles (Turánek et al., 1997), upon addition of aqueous phase above the T_m of the employed lipid. Agitation of the liposome formulation may produce MLVs and SUVs while non-agitated samples may form LUVs with intermittent MLVs (Deo et al., 1997).

Liposomes prepared from this method have been shown to be responsible for high entrapment efficiency for hydrophilic drugs. The entrapment efficiency ranged from 65 to 80% depending on the composition of phospholipid (Perrett et al., 1991) and 30 to 85% depending on the hydration method (Turánek et al., 1997). There was a small effect of the hydration rate on the entrapment efficiency of carboxyfluorescein (CF), however hydration temperatures employed was important in influencing the entrapment efficiency as high temperature (60°C) provided an effective entrapment (approx. 80%) rather than at low temperature (20°C) where the entrapment was around 50% (Turánek et al., 1997).

These liposomes may provide different entrapment efficiency for different compounds. For instance, they have showed entrapment of 69% and 65% for antibiotics gentamycin and neomycin respectively, 81% for CF, 85%, 62% and 87% for β-D-GlcNAc-norMurNac-L-Abu-D-isoGln, muramyl dipeptide and adamantylamide dipeptide immunomodulators respectively (Turánek et al., 1997). Hydrophobic drugs may also have high entrapment efficiencies with these liposomes. Entrapment efficiency of 93 to 98% was observed for
levonorgestrel depending on type of alcohol employed in the formulation (Deo et al., 1997).

1.6 Glioma

Cancer, clinically known as malignant neoplasm, includes a wide range of diseases involved in uncontrollable and abnormal cell growth. Glioma represents the most common cancer of the central nervous system (CNS), accounting for about 46% of intracranial tumours. Glioma is a type of tumour that is derived from glial cells and it includes tumours of oligodendrial, astrocytic, ependymal or a mixed source of cells (Danyu et al., 2011; Riemenschneider et al., 2010). The average survival probability in patients with glioma is 14.6 months (Danyu et al., 2011). The underlying causes of glioma have not yet been identified, but amongst the reported environmental risk factors is the exposure to high-intensity ionising radiation. However other factor that is relevant to the victims of glioma have been reported, for instance 5-10% of glioma cases possess genetic predisposition (Riemenschneider et al., 2010).

According to the World Health Organisation (WHO), there are 100 different types of brain tumours depending on the pathological diagnosis. Gliomas may originate in the brain, central nervous system or from elsewhere hence, they can be classified into either primary or secondary tumours (Lesniak and Brem, 2004).
1.6.1 Classification according to cell type

Gliomas are termed according to the histological features of the cell. These features are: Ependymomas, astrocytomas, oligodendrogliomas and mixed gliomas. **Ependymomas** ascend from ependymal cells lining the brain ventricles and center of the spinal cord. They are greyish, soft red tumours which contain mineral calcification or cysts. They account for only 2-3% of all primary tumours but are most common in children under the age of three. Treatment options include surgical removal of these tumours and radiation therapy (Hayashi *et al.*, 2012).

**Astrocytoma** tumours arise from astroglia, star-shaped glial cells, which form the supportive tissue in the brain. These tumours have been graded by the World Health Organisation (WHO) (Section 1.6.2) to identify the normal and abnormal characteristics of tumour cells.

**Oligodendrogliomas** are tumours that arise from oligodendrocytes, making up the supportive tissue of the brain. The location of the tumour is in the cerebral hemisphere. This tumour frequently occurs in young, middle-aged adults and may be found in children. Standard treatments include surgery, radiation therapy and chemotherapy. A combination of radiation and chemotherapy may also be included using temozolomide or PCV [procarbazine, CCNU (Lomustine) and Vincristine] (Levin *et al.*, 1980).
Oligoastrocytomas are a type of brain tumour which arises from mixed glial source. They are commonly found in the temporal or frontal lobes and anywhere in the cerebral hemispheres of the brain (Viswanathan et al., 2012).

1.6.2 Classification according to grade

Gliomas could be further classified according to their grades based on a system that has been introduced by the WHO. Grade - I glioma is known to be non-invasive, least advanced, least aggressive and have good prognosis. Low grade gliomas (WHO grade II) are not benign but can be differentiated (non-anaplastic). This type of glioma represents moderately increased cellular density, infrequent nuclear atypia, and absence of mitotic activity, necrosis and endothelial proliferation. They tend to be growing slow and infiltrating tumours (ability to grow in the surrounding tissue). The most common type of tumour in childhood is the low grade astrocytoma with 10-15% diagnosis of high grade gliomas (Pollack, 1994) which makes a total of approximately 50-70 cases of glioma per year in the U.K.

Grade-II gliomas can be removed by surgery, however some microscopic cells may remain behind causing the tumour to grow again in some years. Radiation therapy and chemotherapy may also be suggested for the treatment. This type of glioma generally has good prognosis (survival rate of up to 5 years) and regular attention on the recurrence of symptoms is necessary (Marquet et al., 2007).

Grade III astrocytomas are undifferentiated (anaplastic) group of abnormal cells having tentacle-like projections. The tumour grows into the surrounding tissue completely which
renders them difficult to be removed by surgery. The histological features of this type of glioma represent increased cellular density, distinct nuclear atypia, marked mitotic activity, and absence of necrosis and endothelial proliferation (Marquet et al., 2007). The treatment options are based on the location and size of the tumour. Surgery and biopsy may be carried for diagnosis and reduction of symptoms.

**Grade IV** astrocytoma is also known as glioblastoma multiforme (GBM) or high-grade glioma, which is the most aggressive and frequent primary brain tumour. These tumours represent up to 50% of all primary brain tumours and 20% of all intracranial tumours with poor prognosis (Danyu et al., 2011; Riemenschneider et al., 2010). GBM is generally found in the cerebral brain hemispheres, but they can also be found in spinal cord. This type of glioma is capable of rapid growth causing symptoms such as increased brain pressure, seizures, headaches, loss of memory and behavioural changes. It is the most malignant type of brain tumour with 60-75% of astrocytic tumours and 12-15% of all brains tumours. GBM has tendency to migrate into normal brain cells and extremely infiltrative property making them very difficult to be treated by standard therapies except for increasing the survival time of the patients (Zhang, et al., 2012). Chemotherapeutic options are same as those used for treatment of grade-III gliomas.

1.6.3 Classification according to location

Gliomas can also be classified according to their location, being above or below the tentorium that separates the cerebrum from the cerebellum. Supratentorial is located in the cerebrum. This affects mostly 70% of infected adults. By contrast, infratentorial is located
in the cerebellum. This affects mostly 70% of infected children. Pontine is located in the pons of the brain stem which is also another area for tumour growth.

1.7 Standard treatment options for glioma

Current standard remedy for newly diagnosed patients is surgery followed by radiation therapy and using the adjuvant chemotherapeutic agent temozolomide which is an oral alkylating agent (Rahman et al., 2010). Several forms of radiation therapies such as conventional external beam radiation, stereotactic radiation and conformal may be carried out depending on the progression of the tumour. Chemotherapeutic options include Carmustine (BCNU), Lomustine (CCNU), procarbazine, temozolomide and cisplatin. In some cases, biodegradable wafers of BCNU (Gliadel®) are also implanted in the tumour cavity for targeted delivery in case of high-grade gliomas (Bota et al., 2007).

These treatment options have side-effects which may lead to neurological disabilities. Pathways that control the angiogenesis (formation of new blood vessels) are usually observed in both paediatric and adult tumours (Liang et al., 2005). Clinical studies have reported that GBM is highly vascularized and dependent on angiogenesis. The structure of vasculature in GBM is tortuous, disorganised and functional abnormality leading to acidosis, disruption of the BBB, hypoxia, increased interstitial pressure and tissue necrosis. Vascular endothelial growth factor-A (VEGF-A) is the principle growth factor expressed by GBM cells. Its receptor, vascular endothelial growth factor receptor-2 (VEGFR-2) is known to mediate signal transduction, which is expressed in the endothelial cells associated to glioma. Anti-angiogenic therapies may restore normal function of blood vessels, increase oxygen delivery and enhance the production of cytotoxic agents to inhibit
tumour progression and therapeutic resistance (Jain et al., 2009). Strategies for targeting tumour angiogenesis and tumour endothelial cells that aim at tumour vasculature regression have been employed (Rahman et al., 2010; Palanichamy et al., 2006; Chi et al., 2009).

1.8 Anti-angiogenic therapies

1.8.1 Antibody therapies

Bevacizumab (BV) is an IgG1 recombinant humanised monoclonal antibody (MAb) that acts against free vascular endothelial growth factor-A (VEGF-A) in the circulation, preventing activation of pro-angiogenic pathway upon attachment to VEGF receptor. BV was the first anti-angiogenic therapy approved by the Food and Drug Administration (FDA) for the treatment of recurrent glioblastoma multiforme (rGBM) in 2009 (Chi et al., 2009). Originally, bevacizumab was developed for the treatment of non-small cell lung and metastatic cancers. This antibody has also been approved by the European Medical Agency for its use in the treatment of kidney and breast cancer. BV has been shown to produce approx. 20 to 40% response rate and increase progression-free survival rate by 6 months (PFS6) to approx. 30 to 50% (Perry et al., 2010), which is greater than temozolomide producing only 21% PFS6 (Yung et al., 2000). BV therapy causes significant reductions in peritumoral oedema often decreasing the need of high dose intake and corticosteroid use. BV can be very effective in treating brain tumours. However, the adverse effects of BV include hypertension, bowel perforation and renal thrombotic microangiopathy (Eremina et al., 2011).
1.8.2 Small molecule anti-cancer drugs

Small molecule anticancer agents are comparatively low molecular weight compounds with improved penetration through the blood brain barrier. These compounds may act on multiple molecularly related receptors tyrosine kinases (Rahman et al., 2010).

1.8.3 Other Treatments

Cediranib is an indole-ether quinazoline that inhibits all subtypes of the VEGF receptors; some platelet derived growth factors (PDGF) and c-Kit receptors. Sorafenib is another compound that inhibits a broad range of kinases. Other tyrosine kinase inhibitors include imatinib, erlotinib and gefitinib (Rahman et al., 2010). Other therapies such as molecular targeted drugs may interfere with intracellular signalling pathways and various drug carriers may target specific cancer cell surface molecules. Examples include various types of epidermal growth factor receptor (EGFR) inhibitors like cetuximab. Drugs that target intracellular molecules include farnesyltransferase (FTase) inhibitor tipifarnib and rapamycin inhibitor temsirolimus (Van Meir, et al., 2010; Ohgaki and Kleihues, 2007; Furnari et al., 2007).

Antisense therapies like protease inhibitors (e.g. marimastat and tamoxifen) are also included to block the signalling of malignant cells to produce proteins for tumour cell reproduction and alter the ability of malignant cells to interfere with the normal cells. Immunotherapeutic options such as immunotoxins (e.g. diptheria) are also available to
inhibit tumour growth. Interferons also inhibit tumour growth by stimulating the immune system.

Gene therapy is another method for inhibiting tumour growth by making tumour cells liable to drug therapy and restoring the normal function of tumour suppressors (Iwami et al., 2010). microRNAs are conserved sequences of 20-23 base pair long. They may be effective in controlling the angiogenic process by binding to messenger RNA via complementary sequences (Rahman et al., 2010). DNA can also be delivered to the patients by altering the tumour cells in vitro and transferring them back into the patient. It can also be delivered by injecting the tumour mass along with a vector that carries a gene for encoding cytokines or toxins. Another approach is when the vector is systematically administered while the gene is delivered locally to the target cells. However, the major drawbacks in gene therapy are low-efficiency of available gene-vectors and lack of selectivity of the vectors for targeted delivery (Lesniak and Brem, 2004).

Despite of these advances, the types of drugs used for different targets have been mostly disappointing in patients, with non-demonstrated survival benefits. Poor intratumoral accumulation due to the blood brain barrier and high interstitial pressure restricting the amount of drug to exert its effects on the cancer cells which is considered a major problem that limits the therapeutic efficacy (Van Meir, et al., 2010).
1.9 Difficulties in CNS drug delivery

The treatment options for gliomas have been inadequate due to the lack of efficient drug delivery methods. The effective therapies for glioma are restricted due to the presence of the blood brain barrier (BBB), the blood-tumour barrier (BTB) and the blood-cerebrospinal fluid barrier (BCB). The tight junctions in the BBB do not allow the exchange or influx of molecules or ions from the systemic circulation to the CNS (Fig. 1.5). The impermeability of the cerebral capillary endothelium to the ions, peptides and macromolecules imposes a challenge to researchers at improving drug delivery by focusing on augmenting the permeability of drug through the BBB (Lesniak and Brem, 2004).
Fig. 1.5. A schematic representation of the blood-brain barrier. (Source: Drappatz et al., 2007)
1.10 Paclitaxel

Paclitaxel, a diterpinoid is a promising anti-tumor drug having poor water solubility but can be dissolved in organic solvents. It was first isolated from Western yew (Taxus brevifolia; Family Taxaceae) in 1967, having molecular formula C_{47}H_{51}NO_{14} and molecular weight of 853 Da (Wani et al., 1971; Singla et al., 2001; Panchagnula, 1998) (Fig. 1.6).

*Fig. 1.6. Chemical Structure of Paclitaxel (Source: Singla et al., 2002)*

Paclitaxel has been shown to have a significant anti-cancer activity against ovarian carcinoma, head and neck cancers, breast cancer, lung cancer and AIDS related Kaposi’s sarcoma (Rowinsky and Donehower, 1995). Paclitaxel is considered as significant in chemotherapy advancement for the past 20 years and is the first of a new class of microtubule stabilizing agents. It has been known to cause apoptosis by disruption of
normal tubule function necessary for cell division (Sharma and Straubinger, 1994; Hennenfent and Govindan, 2006; Slavin and Chhabra, 2007). Paclitaxel also causes induced apoptosis of cancer cells by binding to Bcl-2 (B-cell leukemia 2) apoptosis stopping protein and arresting their function. (Henley and Isbill, 2007). The potential efficacy of paclitaxel against brain tumours have also been reported (Tseng and Bobola, 1999).

Tissue culture studies have reported the cell kinetic effects of paclitaxel resulting in proliferation of cells during G₂ or M phase of cell cycle (Schiff and Horwitz, 1980). It also showed significant cytotoxic effects against various human malignant gliomas *in vitro* as well as *in vivo* (Hruban *et al*., 1989; Rowinsky *et al*., 1990). The clinical dosage of paclitaxel can be dissolved in Cremophor® EL (Poly-oxyethylated castor oil) and ethanol (50:50 v/v) and diluted before parenteral injection. However, there are serious side-effects caused by Cremophor® EL such as nephrotoxicity, hypersensitivity reactions, neurotoxicity, laboured breathing, hypotension and lethargy in the patients (Singla *et al*., 2002). Although premedication with antihistamine and corticosteroids reduces hypersensitivity, minor side-effects have been reported in 5 to 30% of patients (Weiss *et al*., 1990). Therefore, improvements have been made in order to increase the aqueous solubility of paclitaxel without using Cremophor® EL, in order to reduce the side effects caused by the drug vehicles and improve the therapeutic efficacy. The alternatives include the use of liposomal-based formulations (Crosasso *et al*., 2000; Singla *et al*., 2002).
1.11 Liposomal drug delivery system

Liposome formulations have been extensively studied at the molecular level both in vivo and in vitro for drug delivery owing to their ability to increase the accumulation of chemotherapeutics in the tumours (Paolo et al., 2008). The amphipathic (hydrophobic and hydrophilic) properties of liposomes permit a wide range of drugs to be loaded into liposomes and hence the encapsulated drug can be protected from metabolic degradation (Paolo et al., 2008). Liposomes may have favourable pharmacokinetic properties in vivo depending on their surface properties and composition of the lipid bilayers, possibly providing a prolonged half-life in the blood circulation. Several studies have demonstrated the effects of doxorubicin liposomes (Caelyx®), for targeting brain tumours (Danyu et al., 2011; Verreault et al., 2011). Danyu et al., (2011) showed the anti-glioma effects of these liposomes modified with angiopep-2 using another liposomal drug formulation namely irinotecan (Iriniphore C™) and vincristine (anti-tumour drugs); and their results suggested tumour blood vessel normalisation of structure and function (Verreault et al., 2011).

Incorporation of paclitaxel in the liposomes can reduce the drug toxicity to normal tissues and eliminate the hypersensitivity reactions caused by Cremophor EL vehicle. The drug release from liposome vesicle is comparatively rapid but not instantaneous depending on the alterations in therapeutic index and drug biodistribution mediated by liposomes. Liposomes also reduce the dose-limiting toxicity of paclitaxel by significant elevation of maximum tolerated dose (MTD) (Sharma et al., 1993; Cabanes et al., 1998; Fetterly and Straubinger, 2003). Fine et al., (2006) in a randomised study using paclitaxel and tamoxifen alone on brain tumours concluded that paclitaxel has higher deposition in the metastatic brain tumours leading to decreased expression of the P-glycoproteins, as
compared to tamoxifen. Sampedro et al., (1994) employed different phospholipid compositions like L-Dimyristoylphosphatidylcholine (DMPC) and L-Dimyristoylphosphatidylglycerol (DMPG) with cholesterol. Multilamellar vesicles were prepared using the standard thin film hydration method with a drug to lipid weight ratio of 1:15. The paclitaxel was entrapped in liposomes and used against L1210 cells (Mouse lymphocytic leukaemia cell line), causing higher in vitro cytotoxicity than that of paclitaxel alone. An in vitro study was carried out to silence VEGF expression in U251 (Human glioblastoma astrocytoma) cell lines, by VEGF shRNA (short hairpin RNA) as an adjuvant therapy and treatment with various concentrations of paclitaxel-loaded liposomes. The results showed a significant decrease in VEGF expression of the cells making them sensitized to liposomal formulations in terms of apoptosis, changes in morphology, cell viability and formation of colonies (Yu et al., 2012).

1.12 Factors affecting liposome drug delivery

Liposomes provide several opportunities to improve cancer therapy via different mechanisms. Liposomes contribute in the formulation of hydrophilic and lipophilic drug agents and provide a sustained release of drugs to enhance or alter the pharmacokinetic profiles and increase the therapeutic index. Entrapment of the drug in liposomes can result in increased drug exposure duration of the tumour cells. Liposomal drug formulations can also provide specific pharmacokinetic alterations and enhance tumour deposition. However, liposomes may possess different properties depending on their size, surface charge and entrapment efficiency of the drug (Straubinger et al., 2004).
It was reported that the liposome size of 100-200 nm was optimum for their increased accumulation in tumours (Gabizon and Papahadjopoulos, 1988; Liu et al., 1992). These studies have emphasized that the accumulation of liposomes was dependent on their blood circulation time. However, the results did not prove the actual liposomal accumulation from blood space into the tumour cells, since their accumulation is dependent on their concentration present in the blood, their transfer from blood to the tumour and their contact with the tumour cells. Liposomal circulation time and their concentration in the blood vary in terms of their uptake by macrophages in the reticuloendothelial system (RES). It should be noted that tumour accumulation of liposomes is independent of their circulation time in the blood. During the initial stages of glioma, the BBB is functional around the tumour, but as the disease progresses, it produces a large amount of tumour angiogenesis and the gap of vascular endothelium goes to 50-300 nm with increased permeability (Danyu et al., 2011). Drugs entrapped in unilamellar liposomes, which have diameters ranging from 50 to 200 nm are small enough to escape the RES and possibly pass the BBB for targeting the tumour site (Uchiyama et al., 1995; Di Paolo et al., 2008). Vesicle distribution and clearance after systemic administration is affected by liposomes size. If the size of the liposome is large (>200 nm) then it can be easily cleared by the cells of RES (Lian and Rodney, 2000).

Different types of cytotoxic drugs have been entrapped in the neutral or sterically stabilized liposomes for cancer therapy. However, studies have indicated that cationic liposomes selectively target the chronic inflammation sites and angiogenic vessels in tumours (Thurston et al., 1998). The reports also suggest that angiogenic endothelial cells bind and internalise cationic liposomes but not other liposome types. Cationic liposomes
can be essential in inhibiting new vessel formation or destructing the pre-existing tumour vessel. These liposomes can also enhance the therapeutic properties of the entrapped drug by anti-vascular targeting and increasing the accumulation of drug at the tumour site (Denekamp et al., 1984; Los et al., 2001). In another study, the influence of surface charge on the kinetics and uptake of the liposomes into tumour vasculature was investigated in vivo. The histological distribution of cationic liposomes revealed a rapid uptake in angiogenic tumour sites whereas anionic and neutral liposomes exhibited comparatively slow extravasation after intravenous injection (Krasnici et al., 2003).

Drug entrapment during liposome preparation and subsequent release after administration are two essential properties that define the efficacy of drug delivery systems. The process of incorporation of the drug into the liposomes is known as drug loading. The liberation of the drug is the reverse phenomenon in which the drug is released from the solid state and become absorbed for pharmacological action. The in vitro release of the drug can be a quality control for investigating the internal structure of the liposome, interaction between the liposome and drug, and predict its in vivo behaviour (Chorny et al. 2002). Drug loading and drug release are dependent on the physicochemical properties of the liposomes and drug, and their interaction with the surrounding environments. The amount of drug loaded in the vesicles determines the rate and duration of drug release from the system (de Villiers et al., 2009). If the maximum loading capacity of the vesicle is reached, then further increase in the drug loading can decrease the entrapment efficiency. Changes in the preparation method employed and modification of the pH can also affect entrapment efficiency of the drug (Gaber et al., 2006; Lecaroz et al., 2006).
Thus, liposomes can be potential drug delivery vehicles by limiting the drug systemic distribution volume while avoiding any toxic effects on normal tissues, active targeting via tumour selective ligands on the particle surface and passive accumulation of permeable tumour vasculature.

1.13 Aim of the thesis

The aim of this study was to design liposomes (entrapping paclitaxel) from ethanol-based proliposomes and investigate their cytotoxic effects on grade IV glioma and normal glial cell lines.
CHAPTER 2:

METHODOLOGY
2.1 MATERIALS

List of all the chemicals and consumables obtained from different suppliers are detailed in Table 2.1.

**Table 2.1. Materials used in the preparation of liposomes and performing tissue culture technique**

<table>
<thead>
<tr>
<th>Supplier/Country</th>
<th>Materials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipoid, Switzerland</td>
<td>• SPC</td>
</tr>
<tr>
<td></td>
<td>• HSPC</td>
</tr>
<tr>
<td></td>
<td>• DPPC</td>
</tr>
<tr>
<td>Lonza, Switzerland</td>
<td>• EMEM (Eagle’s minimum essential medium)</td>
</tr>
<tr>
<td></td>
<td>• Non-essential amino acid solution</td>
</tr>
<tr>
<td></td>
<td>• L-glutamine (cell culture tested, 99.0 – 101.0 %)</td>
</tr>
<tr>
<td>Fisher Scientific, UK</td>
<td>• Trypsin-EDTA solution</td>
</tr>
<tr>
<td></td>
<td>• Ethanol (Absolute and 70%)</td>
</tr>
<tr>
<td></td>
<td>• 96-well plates (sterile with lids)</td>
</tr>
<tr>
<td></td>
<td>• 50 ml centrifuge tubes (sterile)</td>
</tr>
<tr>
<td></td>
<td>• Tissue culture flask 75 cm² (sterile)</td>
</tr>
<tr>
<td></td>
<td>• Serological pipettes (sterile)</td>
</tr>
</tbody>
</table>
| Sigma, UK                                                                 | - Cholesterol ≥ 99% grade  
|                                                                          | - 15 ml glass vials        
|                                                                          | - PLL (poly-L-lysine) hydrobromide (molecular weight 30,000-70,000) 
|                                                                          | - Dextran (molecular weight 5,000 approx.) 
|                                                                          | - DMSO (Dimethyl sulfoxide; suitable for culture) 
|                                                                          | - Thiazolyl blue tetrazolium bromide 
|                                                                          | - FBS (Fetal bovine serum) 
|                                                                          | - PBS (Phosphate buffer saline) tablets 
|                                                                          | - Trypan blue solution (0.4% liquid, sterile filtered) 
|                                                                          | - Syringe filters (0.2 and 0.45 µm) 
|                                                                          | - Syringe needles 
|                                                                          | - Sterile pipette tip boxes |
| European collection of cell cultures, UK                                | - U87-MG cell line         
|                                                                          | - SVG-P12 cell line        |
2.2 METHODS

2.2.1 Preparation of liposomes using the ethanol-based proliposome method

In this method, the lipid phase (Phospholipid: Cholesterol, 1:1 mole ratio) (50 mg) was dissolved in an absolute ethanol (60 mg) at 70°C for 1 min in a 15ml glass vial. This produced a clear ethanolic solution comprising lipid to ethanol ratio of 5:6 w/w. Paclitaxel was then dissolved in a range of concentrations (0.5, 1, 1.5 and 2 mg per ml i.e. 0.06, 0.12, 0.18 and 0.24 mM per ml of liposomal formulation) within the lipid phase and ethanol to dissolve both lipids and drug. Aqueous (water) phase (10 ml), above the T\text{m} of the lipid (T\text{m} of SPC, HSPC and DPPC are -20°C, 50°C and 41°C respectively), was then added immediately to avoid lipid phase solidification. Liposomes were generated upon vigorous hand shaking and vortexing (Fisons Whirlimixer, UK) for 4 min. Liposomal formulations were then kept for annealing above the T\text{m} of the lipids for 2 h followed by their size and zeta potential characterisation. This procedure of preparation and characterisation remained the same for all the three phospholipids (i.e. SPC, HSPC and DPPC) used separately in each formulation.

2.2.2 Size reduction of liposomes

Size reduction of liposomes was conducted using probe sonication. In this method, liposome dispersion (10 ml) was placed in a small beaker (50 ml) and the probe of the sonicator (Sonics Vibra-cell-CV33, USA) was immersed in the dispersion and operated at the highest frequency for a maximum of 10 min, while cooling the beaker in a water bath at regular intervals. The size of liposomes was ascertained following centrifugation (Jouan
Robotics A-14, France) at 10,000 rpm to remove the titanium particles leached from the probe. The size and polydispersity of the sonicated liposomes (in the supernatant) were analysed using photon correlation spectroscopy (PCS) by recording the $Z_{\text{average}}$ and polydispersity index (PI) respectively. Size measurements below 200 nm indicated the formation of SUVs and LUVs. Ideally this was accompanied by a PI of 0.3 or less. While performing sonication procedure, care against overheating of the sample was taken. PCS is explained in more details in section 2.2.4

2.2.3 Laser diffraction size analysis for liposomes

Laser diffraction technique was used for size analysis of liposomes. A laser beam is emitted from laser-producing helium lamp so that it is incident on particles in the sample. The beam is then diffracted at an angle, measured by a photodetector to calculate the size distribution of particles based on their volume. The measurements were performed using the Malvern Mastersizer 2000 (Malvern instruments Ltd., UK). This was carried out by the addition of 70 ml of deionised water to the cone dispersion unit (Hydro2000 SM, UK) of the instrument. Adequate amount of sample was added to the dispersion unit in order to reach the green area of the obscuration range. Size and size distribution were presented as the volume median diameter (VMD) (50% undersize) and span respectively. Span = (90% undersize – 10% undersize) / VMD.
2.2.4 Photon correlation spectroscopy analysis for liposomes

A drawback of laser diffraction technique is that it measures size of particles at the micrometres size range more accurately than particles in the submicron range. Therefore, photon correlation spectroscopy was used to measure the size of the liposomes in the nanometre range after probe sonication. This technique relies on the Brownian motion of the particles using the Zetasizer instrument (Zetasizer nano, Malvern Instruments Ltd., UK). Size and size distribution were presented by the $Z_{\text{average}}$ and polydispersity index (PI) respectively.

2.2.5 Zeta potential analysis of liposomes

Zeta potential (ZP) of the liposomes was carried out using laser Doppler velocimetry (LDV) principle again with the help of Zetasizer instrument (Zetasizer nano, Malvern Instruments Ltd., UK). The principle relies on the Doppler shift in a laser beam used to measure the velocity in semi-transparent or transparent fluid flows. The ZP was measured by adding the sample (700 µl) in a disposable zeta cell and setting the temperature at 25°C.

2.2.6 Transmission Electron Microscopy (TEM)

In this setup, a drop of liposome dispersion was placed on carbon-coated copper grids (400 mesh) (TAAB Laboratories Equipment Ltd., UK), which was negatively stained with 1% phosphotungstic acid (PTA), and then viewed and photographed using a TEM (Philips CM 120 Bio-Twin TEM, Philips Electron Optics BV, the Netherlands).
2.2.7 Determination of drug entrapment efficiency

The entrapment efficiency (EE) of paclitaxel in liposomes was analysed using UV spectrophotometer (Jenway 7315 Spectrophotometer, UK). A calibration curve of paclitaxel was obtained by dissolving 10 mg of paclitaxel in 100 ml of absolute ethanol and serial dilution was carried out to achieve concentrations from 10 mg/100 ml to 1 mg/100 ml. The absorbance values of the diluted samples were recorded at a wavelength of 270 nm. Using these absorbance values, a calibration curve of paclitaxel in mg/100ml against absorbance at 270 nm was plotted. R-squared value and a linear equation were also obtained.

For analysis of EE of paclitaxel in liposomes, syringe filters (0.450 µm) were used to pass the liposomes through it at least three times, using a 5ml syringe. The filter was then washed using HPLC water until the solution runs clear. Then, the filter was placed in absolute ethanol half way and paclitaxel that did not pass with the liposomes was extracted using a syringe. This fraction of paclitaxel was regarded as un-entrapped. The absorbance of the un-entrapped paclitaxel in ethanol was measured at 270 nm using a UV spectrophotometer. The absorbance values were then substituted in the linear equation of the calibration curve to obtain the un-entrapped amount of paclitaxel in the liposomes. This amount was then subtracted from the total amount of paclitaxel in the liposomes to calculate the amount of entrapped paclitaxel. This procedure was repeated for all liposomal formulations. The EE of paclitaxel was calculated from the following equation:
Amount of paclitaxel entrapped (mg)

\[
EE (\%) = \frac{\text{Amount of paclitaxel in liposome dispersion (mg)}}{\text{Amount of paclitaxel in liposome dispersion (mg)}} \times 100
\]

2.2.8 Cell Culture Technique

The cell culture procedures were performed aseptically in a cell culture hood (Gelaire Flow Laboratories BSB 4A, Italy). All the cell culture materials were sterilised by autoclaving before use. The media was warmed to 37°C (Grant Instruments Sub28 water bath, UK) before sub-culturing the cells. The working surfaces and hands were always sprayed with 70% ethanol to maintain the sterile conditions and avoid the risk of contamination. U87-MG (grade 4 glioma, passage number 13) and SVG-P12 (glial cells, passage number 7) cell lines were used for the cell culture experiments. EMEM (Eagle’s minimum essential medium) was used as a media for growing the cells. EMEM was supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine and 0.1 mM non-essential amino acids. Dextran and poly-L-lysine were prepared by dissolving in media. MTT was prepared by dissolving 5 mg in 1 ml of PBS solution. Paclitaxel was dissolved in ethanol (76 µl) followed by addition of the media.

2.2.9 Sub-culturing of the cells

The cells (U87-MG and SVG-P12) were obtained from European Collection of cell cultures (ECACC, UK) and grown to 80-95% confluence, as confirmed by the inverted microscope (Leica Microsystems DMIL, Germany). The cells were grown in 75 cm² tissue
culture flasks and incubated in a CO₂ incubator (New Brunswick, an Eppendorf Company Galaxy 170S, UK) at 37°C. The U87-MG cells were passaged every 2 days and SVG-P12 cells were passaged every 4-5 days due to the difference in growth rates. The subculture procedure was carried out aseptically in the cell culture hood. In this process, the medium was first removed using the 10ml pipette without disturbing the cells. The cells were then rinsed three times with PBS solution (10ml). Adherent cells were detached by adding 0.25% trypsin-EDTA solution (2 ml) to the cells and incubated for 2 min at 37°C. Gentle agitation of the culture flask was carried out to help detachment of the cells. This was confirmed using the inverted microscope. Fresh media (4-5 ml) was added to the detached cells. The cell suspension was then centrifuged (Sigma 3-16PK centrifuge, Germany) at 1000x g for 5 min. The supernatant was discarded and the cell pellet was obtained. The cell pellet was re-suspended three times in the appropriate media (10 ml) using a syringe and needle (23 G; 0.6 mm X 25 mm) to ensure disaggregation of the cells.

2.2.10 Calculation of cell viability

The total number of viable cells was assessed by trypan blue exclusion and the cells were then added to new culture flasks at the appropriate seeding density. To evaluate the viable cell count the homogenous cell suspension (100 µl) obtained from the sub-culture technique was mixed with trypan blue (100 µl). The suspension was placed on the Improved Neubauer Haemocytometer slide with a cover slip properly placed on top of the chamber. The cells were observed under a compound microscope (Nikon Eclipse e200, Japan) at 10x magnification. The cells that had taken up the dye (i.e. stained blue) were non-viable. Each large square of the haemocytometer is 1 mm² in area with a depth of 0.1 mm. Therefore, each large square provides 1 mm² x 0.1 mm = 10⁻⁴ cm³ or 10⁻⁴ ml of cells.
The number of cells per large square is the number of cells \( \times 10^4 \) per ml. Five large squares were counted followed by the addition of the number of viable cells and their average. The average number of cells was then multiplied by the dilution factor from stock (x2) and \( 10^4 \). The following equation was used to calculate the volume of cell suspension required for addition to the media for the preferred cell density to seed the cells in the 96-well microtitre plate:

\[ C_1 \times V_1 = C_2 \times V_2 \]

Where,

\( C_1 = \text{Concentration of cells per ml} \)
\( V_1 = \text{Volume of cell suspension required} \)
\( C_2 = \text{Density of cells per well} \)
\( V_2 = \text{Total volume required to seed the 96-well microtitre plates} \)

### 2.2.11 Seeding of 96-well plates

The cells were sub-cultured and counted as previously detailed. A seeding density of \( 1 \times 10^5 \) cells per well was used to seed the 96-well plates for testing the compounds (i.e. liposomal-paclitaxel formulations, paclitaxel alone, drug-free liposomes, poly-L-lysine as a positive control and Dextran as a negative control). Paclitaxel was dissolved in 76 \( \mu \)l of ethanol and added to the media warmed at 37°C. The cells were seeded into the inner rows and columns of the 96-well plates while the outer rows and columns were seeded with PBS solution to avoid evaporation around the perimeter. The cells in the 96-well plates were grown in 5% CO\(_2\) incubator at 37°C for a period of 24 hours. The positive and negative controls were added (i.e. concentration of 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 3, 5
mg/ml) in the 10 columns of the 96-well plates respectively. Zero represents media (200 µl) without the compounds. Similarly, paclitaxel-loaded Liposomes, paclitaxel and paclitaxel-free liposomes were added (i.e. concentration of 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 1.5, 2 mg/ml) in the 96-well plates. The cells were further incubated in 5% CO₂ incubator at 37°C for 72 h to analyse the cytotoxicity of the compounds.

Similarly, seeding densities of 1 x 10⁴, 1 x 10⁵ and 1 x 10⁶ cells per well were used for performing the growth curves of the cell lines for a period of 7 days. Five 96-well plates containing 1 x 10³, 1 x 10⁴ and 1 x 10⁵ cells per well were grown in 5% CO₂ incubator at 37°C for a period of 24 h to 168 h (i.e. 24 h for day 1, 48 h for day 2, 72 h for day 3, 96 h for day 4 and 168 h for day 7 growth curves).

**2.2.12 Evaluation of cytotoxicity using colourimetric tetrazolium-based MTT assay**

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was developed by Mosmann (1983) for analysing the cytotoxicity of anticancer agents and determination of *in vitro* cytotoxicity of polymers (Sgouras and Duncan, 1990). In this assay, a mitochondrial enzyme, succinate dehydrogenase, reduces the MTT in viable cells to a water-insoluble blue-coloured salt called formazan (Slater *et al*, 1963). MTT (20 µl) was added to the cells 5 h before the end of the incubation period. After the completion of MTT incubation, MTT-containing media (220 µl) was carefully removed to avoid removal of the blue crystals precipitated at the bottom of the plate. DMSO (100 µl) was then added to the MTT-treated cells. The plates were incubated for a further 30 min at 37°C before spectrophotometric analysis at 612 nm using a microtitre plate reader (Tecan GENios Plus,
Switzerland). The viability of the cells exposed to the compounds was expressed as the percentage of untreated control cells (n = 18 ± S.D.).

The IC$_{50}$ values (i.e. concentration resulting in 50% inhibition of cell growth) of the liposomes and paclitaxel were calculated graphically from the cell viability curves obtained by considering the absorbance of the media containing cells as 100% (Yang et al., 2007 and Sharma et al., 1996).

2.2.13 Statistical analysis

Statistical significance was measured using the analysis of variance (ANOVA) and student’s $t$-test as appropriate. All values were expressed as the mean ± standard deviation of the mean. Values of P < 0.05 were regarded as significantly different.
CHAPTER 3:

CHARACTERISATION OF LIPOSOMES GENERATED FROM ETHANOL-BASED PROLIPOSOMES
3.1 Introduction

Proliposome technologies such as particulate-based proliposomes (Payne et al., 1986a, b) and ethanol (solvent)-based proliposomes (Perrett et al., 1991) have been advocated to overcome the instability of liposomes and provide convenient and economic options when compared to spray-drying or freeze-drying of liposomes. Ethanol-based proliposomes are ethanolic lipid solutions which, depending on the hydration procedure, generate oligolamellar liposomes (Perrett et al., 1991) or multilamellar vesicles (Turánek et al., 1997), upon addition of aqueous phase above the $T_m$ of the employed lipid.

3.2 Results and Discussion

3.2.1 Size analysis of liposomes before sonication

The effect of paclitaxel concentrations (ranging from 0.5 mg/ml to 2 mg/ml) and the phospholipid compositions on the size and size distribution of the liposomes generated from ethanol-based proliposomes was studied. Figure 3.1 shows the effect of formulation on the VMD of liposomes produced from SPC, HSPC and DPPC in 1:1 mole ratio with cholesterol. The size differences in the paclitaxel-loaded liposomes were recorded and compared with paclitaxel-free liposomes.

As shown in Figure 3.1, the VMD of the SPC-liposomes containing 1.5 mg/ml (3.78 μm ± 0.08) and 2 mg/ml (3.83 μm ± 0.09) paclitaxel concentrations were slightly larger than that of paclitaxel-free SPC-liposomes (3.12 μm ± 0.07) (P<0.05). However, the VMD of SPC-liposomes containing 0.5 mg/ml (3.28 μm ± 0.08) and 1 mg/ml (3.45 μm ± 0.08) paclitaxel concentration showed no significant difference when compared to that of paclitaxel-free
SPC-liposomes (P>0.05). This suggests that the VMD of SPC-liposomes increased with the increase in paclitaxel concentrations (1.5 and 2 mg/ml) while at low drug concentrations (0.5 mg/ml and 1 mg/ml) no effect on VMD of liposomes has occurred. The average difference in the VMD of SPC-liposomes containing maximum paclitaxel concentration (2 mg/ml) and to that of paclitaxel-free SPC-liposomes was 0.71 µm (less than 1 µm).

The VMD of all HSPC-liposomes containing paclitaxel were higher when compared to that of paclitaxel-free HSPC-liposomes (P<0.05). The VMD of paclitaxel-free HSPC-liposomes was 3.33 µm ± 0.25, while the average VMD of HSPC-liposomes containing 2mg/ml paclitaxel concentration was 8.78 µm ± 0.28. The VMD of HSPC-liposomes containing maximum paclitaxel concentration was 5.45 µm higher than paclitaxel-free HSPC-liposomes (P<0.05). The VMD of HSPC-liposomes containing 0.5 mg/ml paclitaxel concentration was 4.49 µm ± 0.26, showing an increase of 1.16 µm from the paclitaxel-free HSPC-liposomes (P<0.05). This demonstrates the continuous increase in the size of HSPC-liposomes as the concentration of paclitaxel was increased.

A similar trend was observed for the DPPC liposomes. The VMD of all the DPPC-liposomes containing paclitaxel were significantly larger than the VMD of paclitaxel-free DPPC-liposomes (P<0.05). The average VMD of paclitaxel-free DPPC-liposomes was 2.61 µm ± 0.16, while the average VMD of DPPC-liposomes with maximum paclitaxel concentration was 4.88 µm ± 0.15, showing that liposome size has almost doubled as a result of drug inclusion within formulation (Fig.3.1). The VMD of all the DPPC-liposomes increased with the increase in paclitaxel concentration but not as high as HSPC-liposomes did.
The VMD of HSPC-based liposomes were higher than those of the corresponding DPPC-liposome formulations (P<0.05) especially when paclitaxel was included in the formulations. The VMD of all the paclitaxel containing DPPC-liposomes were higher than that of the corresponding SPC-liposome formulations (P<0.05) except for the VMD of DPPC-liposomes containing 0.5 mg/ml paclitaxel (3.42 µm ± 0.14), where there was no significant difference (P>0.05). Amongst the phospholipids used, paclitaxel concentration was most influential to the size of vesicles made from HSPC:Chol (1:1). This is possibly attributed to the higher hydrophobicity of the longer acyl chains in HSPC phospholipid and repulsive interactions between water molecules at the interface; causing them to aggregate (Zhao et al., 2005; Zhao et al., 2004; Zhao et al., 2004).

**Fig 3.1. Size of liposomes generated from ethanol-based proliposome formulations with a range of paclitaxel concentrations (n=5 ± sd)**
3.2.2 Size distribution (Span) of liposomes before sonication

Size distribution of liposomes was represented by measurement of Span which is a term introduced by Malvern Instruments Ltd to express the polydispersity of particles. In general, no effect was seen on the Span when paclitaxel was included within the proliposome formulations and the Span values of all formulations were around 2 (Fig. 3.2). However, the span of liposomes made from SPC:Chol (1:1) was increased (P<0.05) by inclusion of 0.5 mg/ml paclitaxel. No further increase of SPC-made liposomes was observed by inclusion of higher drug concentrations (Fig. 3.2). Paclitaxel concentration did not affect the span of HSPC-liposomes or DPPC liposomes and no significant difference (P>0.05) was detected between formulation upon inclusion of a range of paclitaxel concentrations (Fig. 3.2).

Fig. 3.2. Size distribution of liposomes generated from ethanol-based proliposome formulations with a range of paclitaxel concentrations (n=5 ± sd)
3.2.3 Zeta potential analysis of liposomes before sonication

The zeta potential (ZP) of all the liposomes before sonication were in the negative range (Fig. 3.3). A slight effect of formulation on the ZP of liposomes was observed, so that the ZP of formulations was in the range between approximately -1.5 and -6.5 mV. The average ZP of the paclitaxel-free SPC-liposomes was -1.82 mV ± 0.09 and increased to -3.57 mV ± 0.28 upon inclusion of 0.5 mg/ml paclitaxel (P<0.05). Inclusion of higher concentrations of the drug tended to reduce the charge intensity and this was also observed for liposomes made from HSPC or DPPC (Fig. 3.3). Thus, overall, only a slight or no difference in ZP was seen when drug-free liposomes were compared with liposomes having the highest paclitaxel concentration (i.e. 2mg/ml). It is possible that changes in ZP are related to changes in the VMD of liposomes. Further research is needed to understand why the ZP tended to increase at low paclitaxel concentration and revert to the original ZP value upon inclusion of higher drug concentrations.

The ZP of HSPC-liposomes did not show any significant difference with the increase in paclitaxel concentration (P>0.05). By contrast, the ZP of DPPC-liposomes displayed a similar effect as that of SPC-liposomes. In this case, the ZP of all the DPPC-liposomes containing paclitaxel increased (becoming more negative) upon inclusion of paclitaxel concentration.
**Fig. 3.3**. Zeta potential of liposomes generated from ethanol-based proliposome formulations with a range of paclitaxel concentrations (n=5 ± sd)

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPC:Chol (1:1)</td>
<td>-3.5 ± 0.2</td>
</tr>
<tr>
<td>HSPC:Chol (1:1)</td>
<td>-4.7 ± 0.3</td>
</tr>
<tr>
<td>DPPC:Chol (1:1)</td>
<td>-6.0 ± 0.4</td>
</tr>
</tbody>
</table>

3.2.4 Size analysis of the liposomes after probe sonication

Size of liposomes was reduced in order to convert MLVs into SUVs having a size range of 100-200 nm. Size reduction was carried out using a probe sonicator for a maximum period of 10 min followed by centrifugation at 10,000 rpm to remove the titanium particles which were released by the probe sonicator. The sonication time was limited to 10 min in order to avoid the leakage of paclitaxel from liposomes and decomposition of phospholipids as a result of sonication induced heating. Therefore, intermittent cooling of liposomes was performed during sonication.
The effect of probe sonication on the size of liposomes was investigated. When compared to the size of MLVs, the size of liposomes was decreased by approximately 95% or more, regardless of formulation (Fig. 3.1 and Fig. 3.4). This indicates that drug inclusion and lipid composition did not retard size reduction and hence MLVs were successfully fragmented into nano-sized liposomes (100-200 nm) (Fig. 3.4). Also, liposomes made from HSPC:Chol (1:1) had larger size than liposomes made from DPPC:Chol (1:1) or SPC:Chol (1:1). This indicates that size of sonicated vesicles was affected by lipid phase composition. Furthermore, the effect of paclitaxel concentration on liposome size after sonication was minimal (Fig. 3.4).

**Fig. 3.4. Size of liposomes after probe sonication (n=5 ± sd)**
3.2.5 Polydispersity Index (PI) of the liposomes after probe sonication

The PI for all the liposomes was found to be below 0.3 (Fig. 3.5), indicating that sonication has generated liposomes with relatively narrow size distribution, regardless of lipid type and drug concentration. The PI of paclitaxel-free SPC-liposomes was 0.09 ± 0.05 and it increased to 0.18 for SPC-liposomes containing the maximum paclitaxel concentration (Fig 3.5). The PI of HSPC-liposomes increased with 1.5 mg/ml paclitaxel concentration (from 0.17 ± 0.03 to 0.21 ± 0.03), whereas it decreased to 0.08 for HSPC vesicles having the maximum paclitaxel concentration (P<0.05) (Fig. 3.5), indicating very slight effect of paclitaxel on vesicle polydispersity. As shown in Fig. 3.5, it is difficult to correlate paclitaxel concentration with the PI value, regardless of phospholipid type. The low PI for all formulations indicates that the sonication time selected was appropriate and no further processing of the vesicles is needed.

Fig. 3.5. PI of liposomes after probe sonication (n=5 ± sd)
3.2.6 Zeta potential analysis of liposomes after probe sonication

The ZP of probe sonicated liposomes was studied. For SPC and HSPC made liposomes, very slight but significant effect (P<0.05) of drug concentration was observed on the ZP (Fig. 3.6). For the HSPC or SPC made liposomes, especially at low drug concentrations the ZP was around 0 mV, indicating a neutral surface charge. This was not the case for the DPPC-made vesicles since higher drug concentration made the surface charge more intense (i.e. more negative ZP values). The increased negativity of the ZP for the SPC and HSPC liposomes as a result of increasing the drug concentration demonstrated a very slight trend, but with statistically significant differences between the formulations (Fig. 3.6). Compared with liposomes prior to sonication (Fig. 3.3), the ZP of paclitaxel-free SPC-liposomes after sonication was almost neutral whereas the ZP of SPC-liposomes containing the maximum paclitaxel concentration decreased in intensity by approximately 54% as compared to the pre-sonicated liposomes (Fig. 3.3 and Fig. 3.6). This suggests that vesicle size has an effect on ZP values (Howard and Levin, 2010).

Fig. 3.6. Zeta potential of liposomes after probe sonication (n=5 ± sd)
3.2.7 Morphology of the liposomes

Figures 3.7, 3.8 and 3.9 are TEM images for the 1 mg/ml paclitaxel concentrations for SPC, HSPC and DPPC liposomes respectively. The TEM-images were taken after probe sonication of liposomes and clearly showed the vesicles were SUVs. The size of SPC, HSPC and DPPC liposomes were approx. 125 nm, 175 nm and 155 nm and these values correlate with the size analysis study. Thus probe sonication method applied in this study was successful at converting the MLVs into liposomes in the nanometre size range.
Fig 3.7. TEM of SPC: Chol (1:1) liposomes containing 1 mg/ml paclitaxel concentration after size reduction
Fig. 3.8. TEM of HSPC: Chol (1:1) liposomes containing 1 mg/ml paclitaxel concentration after size reduction
Fig. 3.9. TEM of DPPC: Chol (1:1) liposomes containing 1 mg/ml paclitaxel concentration after size reduction
3.2.8 pH measurement of liposome formulations

The effect of paclitaxel concentrations and lipid composition on the pH of liposomes was analysed. The pH of all SPC and HSPC liposomes was slightly acidic, while the pH of DPPC-liposomes was neutral to slightly basic (Fig. 3.10). It was observed that the pH of formulations increased by increasing the paclitaxel concentration. In case of SPC-liposomes, the pH of paclitaxel-free liposomes was 6.06 ± 0.01 whereas the pH of liposomes having the maximum paclitaxel concentration was 6.72 ± 0.02 (P<0.05). Similarly, the pH of HSPC paclitaxel-free liposomes was 6.08 ± 0.03 while the pH of liposomes with maximum paclitaxel concentration was 6.84 ± 0.2 (P<0.05). For each paclitaxel concentration, the pH was significantly but slightly different when using different phospholipids. The pH of formulations was the highest for DPPC liposomes followed by HSPC and then SPC formulations. For each type of phospholipid, when the highest drug concentration formulation was compared to the drug-free one, the pH of SPC and HSPC liposomes increased by 0.71 and 0.76 respectively while the pH of DPPC-liposomes increased by 0.6. The pH of SPC and HSPC-liposomes increased slightly with the increase in drug concentration (P<0.05). However, the pH of DPPC-liposomes was maintained until 1.5 mg/ml paclitaxel concentration with no significant difference. This study overall suggests minor differences in the pH of liposome formulations containing different lipids and different paclitaxel concentrations. The pH of DPPC liposomes seems to be the closest to pH values of the blood (pH 7.4) and from that particular perspective they seem to be highly appropriate excipients for the anticancer drug paclitaxel.
3.2.9 Entrapment efficiency of paclitaxel in liposomes

The entrapment efficiency of paclitaxel in liposomes was analysed using a UV spectrophotometer (as described in Section 2.2.7). A calibration curve of paclitaxel was obtained by dissolving 10 mg of paclitaxel in 100 ml of ethanol and serial dilution was carried out to achieve drug concentrations from 10 mg/100 ml to 1 mg/100 ml. Figure 3.11 shows the calibration curve of paclitaxel in mg/100 ml against absorbance at 270 nm. R-squared value and a linear equation were obtained to carry out the entrapment efficiency of paclitaxel in the liposomes.
According to Figure 3.12, the entrapment efficiency of paclitaxel decreased with the increase in paclitaxel concentration (P<0.05) and that was independent of phospholipid composition. The entrapment efficiency of paclitaxel in SPC-liposomes was 75% ± 5.3 for 0.5 mg/ml concentration whereas it decreased by almost 30% when 2 mg/ml paclitaxel was included (46% ± 6.7) (P<0.05). Similarly, paclitaxel entrapment efficiency in HSPC-liposomes for 0.5 mg/ml was 67.2% ± 4.7 and decreased by approximately 41% when using 2 mg/ml paclitaxel concentration (26.25% ± 3.8). The entrapment efficiency in DPPC liposomes was generally higher than that in SPC or HSPC vesicles. For instance, the entrapment efficiency of the drug in DPPC-liposomes was 85.6% ± 2.7 and decreased by only around 15% using 2 mg/ml paclitaxel concentration (70.1% ± 1.8) (P<0.05).
The difference in zeta potential with different paclitaxel concentrations may correlate with the different entrapment efficiencies using liposome formulations with different lipid compositions. Figure 3.6 clearly demonstrates that higher drug concentrations have conferred more negative surface charge on the DPPC liposomes. The ability of higher drug concentrations to exert that effect on SPC and HSPC vesicles was less, which correlates well with the entrapment efficiency findings. This gives a strong indication that the accommodation of paclitaxel within the liposome bilayers is responsible for the negative zeta potential values.

**Fig. 3.12. Entrapment efficiency of liposomes by UV method (n=5 ± s.d.)**
3.2.10 Amount of paclitaxel entrapped in liposomes

Figure 3.13 represents the amount of paclitaxel entrapped in 10 ml of liposome formulation using UV spectrophotometric analysis. The amount of paclitaxel entrapped in the formulations was dependent on the composition of phospholipid and paclitaxel concentration. In case of SPC and DPPC liposomes, the amount of entrapped paclitaxel increased as the concentration of paclitaxel increased in the liposomes. However, the amount of entrapped paclitaxel in HSPC-liposomes decreased with the highest concentration (20 mg/10 ml). SPC-liposomes containing 20 mg paclitaxel originally, could only entrap around 9.2 mg. In fact, there was no significant difference between the paclitaxel entrapped in SPC-liposomes containing 15 mg and 20 mg paclitaxel originally; which displayed a plateau phase. This indicates that SPC-liposomes might have reached a limit where its bilayers could not entrap more paclitaxel.

HSPC-liposomes had less entrapment efficiency than SPC and DPPC-liposomes (Fig. 3.12). The plateau phase was reached when HSPC-liposomes contained 15 mg paclitaxel but could only entrap around 7.3 mg. The entrapped amount for HSPC-liposomes containing 20 mg paclitaxel decreased to approx. 5.25 mg. The formulation started losing its drug entrapment property and could not entrap paclitaxel efficiently with the highest drug concentration as compared to the previous drug concentrations.

DPPC-liposomes displayed a linear increase in entrapping paclitaxel with its increasing concentration. With highest paclitaxel concentration, the amount of paclitaxel entrapped was around 14 mg, which was higher that SPC and HSPC formulations.
Fig. 3.13. The amount of paclitaxel entrapped per 10 ml of formulation (n=5 ± s.d.)

Studies suggest that cholesterol used along with DPPC can make the tertiary system of paclitaxel and DPPC mixed bilayer more stable and facilitate molecular interactions between them. Cholesterol containing lipids are more rigid and stable than lipids used alone. It has been reported that drug-free DPPC-liposomes possess two thermal transitions: a sharp acyl chain melting transition temperature of 42.3 °C and a pre-transition temperature of 35.4°C. Incorporation of paclitaxel can eliminate the pre-transition temperature without changing the main phase transition temperature, leading to a flexible bilayer. It has also been studied that increasing the concentration of paclitaxel beyond 0.5 mg/ml does not disturb the bilayer of DPPC vesicles suggesting the formation of stable drug delivery systems than HSPC. Paclitaxel incorporation into the lipid bilayers of DPPC liposomes was also found to be higher than that of HSPC-liposomes. According to
previous studies, the drug was found to be entrapped in C1-C8 carbon atoms of the acyl chain i.e. in the outer hydrophobic bilayer zone of DPPC liposomes. Paclitaxel binds to the carbon atoms of DPPC by its C13 side chain, which is hydrophobic due to the presence of two aromatic rings. However, the presence of double bonds at the middle of two acyl chains of HSPC-liposomes causes reduction in the phase transition (54°C) and pre-transition temperature upon inclusion of paclitaxel. This might explain the inability of paclitaxel to entrap efficiently into HSPC-liposomes (Campbell et al., 2001; Bernsdorff et al., 1999; Balasubramanian et al., 1994; Zhao et al., 2007).

It has also been found that the stability of paclitaxel in saturated phospholipids is dependent on the chain length with DPPC (16:0) liposomes being more capable of entrapping higher proportions of paclitaxel than HSPC (18:0) liposomes. The interaction between phospholipid and paclitaxel is known to be nonspecific and dependent on the hydrophobic force or van der Waals force. The lower van der Waals interactions between hydrocarbon chains of short acyl chains leads to compact and rapid binding of paclitaxel with phospholipids of shorter chain; and increased intermolecular spacing between the bilayers. Whereas, the stronger van der Waals interactions in HSPC-liposomes can hinder and act as a physical barrier for the movement of paclitaxel in the bilayers; and form unstable systems at air/water interface when compared to phospholipids with shorter acyl chain. DPPC possesses a first-order phase transition which is a transition between liquid-expanded and liquid-condensed states whereas HSPC possess only liquid-condensed phase. This may be due to the high hydrophobicity of longer acyl chain phospholipids and repulsive interactions between water molecules at the interface (Zhao et al., 2005; Zhao et al., 2004; Zhao et al., 2004).
Some studies have suggested that naturally occurring or unsaturated phospholipid such as SPC can entrap hydrophobic drugs more efficiently as compared to HSPC due to their low gel to liquid-crystalline phase transition temperature ($T_m=-20^\circ$C). The liposomes prepared from this type of phospholipid along with cholesterol, are flexible enough to entrap more hydrophobic molecules. The liposomes prepared from HSPC liposomes are highly rigid and can disallow the penetration of hydrophobic drugs. However, DPPC-liposomes have shown high molecular interactions with paclitaxel due to their first-order phase transition behaviour.

It has also been shown that, paclitaxel interaction with lipid bilayers causes an increase in the surface charge intensity of the liposomes. This means that, the charge intensity on the liposomes increases with the increase in the entrapment of paclitaxel in their vesicles. This property is dependent on high molecular interactions between paclitaxel and phospholipids. Negative charge increases the physical stability of liposomes by preventing their fusion and aggregation due to electrostatic repulsion (Kan et al., 2011; Zhang et al., 2005; Kirby et al., 1980; Senior and Gregoriadis, 1982; Zolnik et al., 2010).

Thus, the difference in ZP with different paclitaxel concentrations may correlate with the different EE using liposome formulations with different lipid compositions. Figure 3.6 clearly represents that higher paclitaxel concentrations have conferred more negative surface charge on the DPPC liposomes. The ability of higher drug concentrations to exert that effect on SPC and HSPC vesicles was comparatively less, which correlates well with the EE findings. This gives a strong indication that the accommodation of paclitaxel within the liposome bilayers is responsible for the negative ZP values.
3.3 Conclusion

Liposomes were prepared from Ethanol-based proliposomes by employing different phospholipid compositions: SPC, HSPC or DPPC with cholesterol in equimole ratio. Paclitaxel, an anti-cancer drug, was incorporated during liposome preparation. The size differences in the paclitaxel-loaded liposomes were recorded and compared with paclitaxel-free liposomes. The size of all the formulations increased with the increase in paclitaxel concentration. However, paclitaxel concentration was most influential to the size of vesicles made from HSPC:Chol (1:1), due to the aggregation caused by higher hydrophobicity of their acyl chains and repulsive interactions between water molecules at the interface. The size distribution of the formulations remained almost the same with increasing paclitaxel concentration. ZP values of the formulations were in the negative range and showed a slight increase in the charge intensity upon inclusion of paclitaxel.

Probe sonication of the liposomes was carried out in order to convert MLVs into SUVs, having a size range of 100 to 200 nm. The technique was successful in converting liposomes into nanometre range. The PI for all the liposomes was found to be below 0.3, indicating that sonication has generated liposomes with relatively narrow size distribution, regardless of lipid type and drug concentration. Phospholipid composition and drug concentration had minor influence on the pH of the formulations. The pH of DPPC liposomes seemed to be the closest to pH values of the blood (pH 7.4) and from that particular perspective they seem to be highly appropriate excipients for the anticancer drug paclitaxel.
The entrapment efficiency of paclitaxel in DPPC liposomes was generally higher than that in SPC or HSPC vesicles. DPPC-liposomes were also able to entrap high amount of paclitaxel with increasing concentration of paclitaxel in the formulation. However, SPC-liposomes reached a limit where they could only entrap less 50% of the original paclitaxel amount. HSPC-liposomes displayed a decrease in the amount of entrapped paclitaxel at highest drug concentration, after reaching a plateau phase. High entrapment efficiency of paclitaxel in DPPC-vesicles may be attributed to the fact that incorporation of paclitaxel makes DPPC bilayer more stable and flexible. Low van der Waals forces between the short acyl chains can increase the molecular interactions between paclitaxel and DPPC. The property of first-order phase transition possessed by DPPC is also an important factor in the binding of paclitaxel to the bilayers of the lipid. However, HSPC liposomes limit the incorporation of hydrophobic materials in their bilayers due to their long hydrophobic acyl chains causing repulsive reactions with water molecules at the interface. SPC is also known to entrap hydrophobic drugs more efficiently as compared to HSPC due to their low phase transition temperature. SPC-liposomes are flexible enough to entrap more hydrophobic molecules.

ZP of the liposomes after probe sonication indicated that higher paclitaxel concentrations conferred more negative surface charge on the DPPC liposomes. The ability of higher drug concentrations to exert that effect on SPC and HSPC vesicles was comparatively less. This gave a strong indication that the accommodation of paclitaxel within the liposome bilayers was responsible for the negative ZP values and it was clearly correlated with the EE findings.
Overall, DPPC-liposomes may be the best drug carrier systems for paclitaxel because of their unique properties to entrap high amounts of hydrophobic drugs.
CHAPTER 4:
CYTOTOXICITY STUDIES OF LIPOSOMAL FORMULATIONS ON GLIOMA
AND NORMAL GLIAL CELLS IN VITRO
4.1 Introduction

Tissue culture is defined as *in vitro* growth of cells obtained from a multicellular organism and is an essential tool for the understanding of cell biology of an organism at a simpler level. This is achieved by allowing researchers to selectively determine one variable at a time in a manipulated cellular environment and preventing the exposure of an organism to lethal and other pharmacological agents. Moreover, the continuous cell growth can be analysed for their development and differentiation and regulation of gene expression.

Tissue culture experiments can be divided into two categories: cultures that apply primary cell cultures and cultures that apply secondary cell lines. Primary cultures consist of cells which are obtained from an animal, segregated and grown in a culture plate. But most primary cultures do not survive for longer periods of time. By contrast, secondary cell lines can grow, proliferate and become adapted to the *in vitro* conditions for very long periods of time. These clonal cell lines, grown in unlimited quantities, advocate a more homogenous basis of cell materials rather than do primary cultures. Nevertheless the indefinite growth of a cell in a culture medium suggests that it lacks the differentiated properties of “normal” cells and transforms into a cancer cell. Therefore, to avoid this from happening, the properties of the differentiated cells of an original organism must be understood.
4.2 U87-MG cell line

U87-MG is a human glioblastoma-astrocytoma epithelial-like cell line (Fig. 4.1) first obtained from a grade IV cancer patient (Pomtén et al., 1968). Grade IV glioma is also known as glioblastoma multiforme (GBM) which is the most common malignant brain tumour. The cellular and growth behaviour of these cells both in situ and in vitro can be influenced by a wide range of expressions of growth factors and their receptors, representing the involvement of intricate autocrine and paracrine growth stimulatory pathways (APGSP) in the tumours. One such important growth factor is platelet-derived growth factor (PDGF). Malignant astrocytomas mainly produce PDGF A and B chain and their receptors, indicating that APGSP are essential in the pathogenesis of tumour in vitro (Maxwell et al., 1990; Hermanson et al., 1992; Nister et al., 1991).
4.3 SVG-P12 cell line

SVG-P12 is a human glial fibroblast-like cell line. The first established culture from human origin was obtained by transfecting primary human fetal glial cells from brain material dissected from 8 to 12 week old embryos with DNA from an origin-defective-mutant (ori) of SV40 virus. This cell line is able to support the multiplication of John Cunningham virus (JCV). The cells may also be useful in detection and cultivation of other
human neurotropic viruses. This cell line has an indefinite life span in culture medium established by its continuous rapid multiplication and propagation using repeated tissue culture techniques (Major EO, United States Patent, 1987).

4.4 Results and Discussion

4.4.1 Introduction

The media used to support the growth of U87-MG and SVG-P12 cell lines in this experiment was Eagle’s minimal essential medium (EMEM). The medium contain nutrients that are necessary for cell growth and is suitable for culturing mammalian cells in vitro. The media was supplemented with 10% Fetal Bovine serum (FBS), 1 mM Sodium pyruvate, 1 mM L-glutamine and 1% mM non-essential amino acids (Section 2.2.8). Many factors required for attachment and growth are provided by these additional nutrients. FBS is a serum supplement used for in vitro cell culture of eukaryotic cells. It contains very low levels of antibodies and some growth factors that are suitable for cell growth. Sodium pyruvate provides an additional energy source for cells and acts as a protective against the toxic effects of hydrogen peroxide. The amino acid L-glutamine is essential in adhesion, supporting the attachment of nitrogen-containing glycoproteins to cells. Non-essential amino acids reduce the burden of metabolism on the cells by increasing cell proliferation. The cells are derived from complete organisms and often cannot grow in vitro without the presence of hormones, amino acids and growth factors (Cooper, 2000). The procedure for carrying out the cell culture technique and growing conditions required for cell growth were described in sections 2.2.8 to 2.2.12.
In the present experiment, liposomal formulations of paclitaxel using different phospholipid compositions with equimole ratios of cholesterol (SPC:Chol, HSPC:Chol or DPPC:Chol) were developed. DPPC-liposomes showed a higher entrapment efficiency of paclitaxel than that of SPC and HSPC liposomes.

In the current chapter, the cytotoxicity of different paclitaxel concentrations, loaded in the liposomes, against U87-MG grade IV glioma and SVG-P12 glial cell lines was determined using the MTT assay (Section 2.2.11). The cytotoxicity using liposomes were then compared to the cytotoxicity of paclitaxel alone (i.e. without liposomes). The controls used in the cell culture experiments were dextran (negative control, to ensure that there is no cytotoxic effect on the cells) and PLL (positive control, to ensure that there is cytotoxic effect on the cells). The difference of cytotoxicity in U87-MG and SVG-P12 cells following the use of liposome formulations, paclitaxel and controls were also compared. Growth curves of both the cell lines were also carried out for a period of 7 days to determine the seeding density for the cells. Therefore, seeding densities of $1 \times 10^3$, $1 \times 10^4$ and $1 \times 10^5$ cells per well were used to obtain the appropriate seeding density.

### 4.4.2 Growth curve of U87-MG cells

The growth curve of the cells was plotted by considering the day 1 absorbance (at 612 nm) values as 100% for each seeding density (Fig. 4.2). The cell growth was observed with each seeding density but at different rate. The absorbance of cells, having $1 \times 10^3$ seeding density, was 0.0541 for day 1 and 0.1455 for day 7. On the other hand, $10^4$ seeding cells displayed absorbance of 0.1021 on day 1 and 0.3150 on day 7. However, the absorbance of cells, having $10^5$ seeding density, was 0.3308 for day 1 and 0.9936 for day 7. The cell
growth with these seeding densities almost tripled on the 7\textsuperscript{th} day but the absorbance values of $10^5$ cells were higher than the other cells.

The growth of the cells having $10^4$ seeding density is more than that of $10^5$ seeding density cells by day 7 in terms of percent growth. The growth rate of $10^5$ cells decreased due to the fact that the cell colonies occupied the available substrate as a result of the mechanism known as contact inhibition (Galle \textit{et al.}, 2009). However, the absorbance values for $10^4$ and $10^3$ cells are lower than that of $10^5$ cells. Higher absorbance helps in a wider comparison of percent cell viability while assessing the cytotoxicity of the compounds which are in minute to high concentrations (0.001 mg/ml through 2 or 5 mg/ml). Therefore, the seeding density of $10^5$ considered as appropriate for carrying out cytotoxicity testing. Figure 4.3 shows that $10^5$ seeding density cells are more confluent than $10^3$ or $10^4$ cells on day 7 of growth curve.

\textbf{Fig.4.2. Growth curve of U87-MG cell line for 7 day period}
**Fig. 4.3. Inverted light microscope photographs of U87-MG cells, on day 7 of growth curve, with different seeding densities**

![Images of inverted light microscope photographs of U87-MG cells with different seeding densities](image)

**1 x 10^3 seeding density**  **1 x 10^4 seeding density**  **1 x 10^5 seeding density**

### 4.4.3 Growth curve of SVG-P12 cells

The growth of the cells having seeding densities of $10^3$, $10^4$ and $10^5$ per well increased by approximately 80%, 109% and 178% respectively and displayed absorbance values of 0.0818, 0.2142 and 0.7142 respectively on day 7 (Fig. 4.4). Higher absorbance was shown by the cells having cell seeding density of $10^5$. This confirms that $10^5$ would be an appropriate seeding density for SVG-P12 cell line to significantly compare the effects of formulations and controls on them. Figure 4.5 shows that $10^5$ seeding density cells are more confluent than $10^3$ or $10^4$ cells on day 7 of growth curve.
Fig. 4.4. Growth curve of SVG-P12 cell line for 7 day period

![Growth curve graph](image)

Fig. 4.5. Inverted light microscope photographs of SVG-P12 cells, on day 7 of growth curve, with different seeding densities

![Microscope images](image)

4.4.4 Effects of liposomes, paclitaxel and controls on U87-MG cells
After selecting the $10^5$ seeding density for the U87 cells, they were tested using a range of formulations to assess their cytotoxicity on the cell line. Figure 4.6 shows the various formulations on the viability of U87 cells by plotting the concentrations in log-scale. Dextran was used as a negative control. It was observed that with the increase in dextran concentration, the cell viability increased by approximately 9% (109% ± 0.28) for 5mg/ml dextran concentration (P<0.05), considering the absorbance value of the media as 100%. Likewise, cell growth was also observed in case of drug-free liposomes. Cell viability with drug-free SPC-liposomes, HSPC-liposomes and DPPC-liposomes increased by approximately 5%, 2% and 3% (P<0.05) respectively with 2mg/ml concentration, but not as high as dextran (P<0.05). The cell viability increased more in case of drug-free SPC-liposomes as compared to the drug-free HSPC and DPPC-liposomes (P<0.05).

PLL, used as a positive control, was toxic to the cells. The cell viability with PLL decreased by approximately 94% (viability= 6.03% ± 1.35) with 5 mg/ml concentration (P<0.05). Similarly, paclitaxel was also responsible for the reduction in cell growth by approximately 97% (viability= 3.24% ± 0.45) with the highest concentration (2 mg/ml) (P<0.05). Decrease in cell viability was also observed in case of paclitaxel loaded liposomes with increasing paclitaxel concentration in the liposomes. The viability of U87 cells with the treatment of SPC-liposomes, HSPC-liposomes and DPPC-liposomes decreased by approximately 78% (21.65% ± 2.17 cell viability), 65% (45% ±0.28 cell viability) and 94% (5.73% ± 0.55 cell viability), respectively when 2 mg/ml paclitaxel concentration was used. However, paclitaxel proved to be more lethal to the U87 cells with 2 mg/ml concentration than PLL with 5 mg/ml concentration, by approximately 3% (P<0.05).
Cytotoxicity of paclitaxel was also higher than paclitaxel in liposome formulations by approximately 17%, 42% and 3% when using SPC, HSPC and DPPC respectively (P<0.05) (Figure 4.6). Nevertheless, DPPC-liposomes showed higher cytotoxicity than SPC-liposomes and HSPC-liposomes by approximately 16% and 39% respectively when using 2 mg/ml paclitaxel (P<0.05). The reduced cytotoxicity of paclitaxel in liposome formulations as compared to non-liposomal paclitaxel might be attributed to sustained drug release upon using liposomes, or because of the nutritional values of phospholipid and cholesterol of liposomes.

Fig. 4.6. Viability of U87-MG cell line tested with increasing concentrations of different drug compounds in 96-well plates. (n=18, N=3 ± sd)
Figure 4.7 shows the light microscope images of the viability of U87-MG cells after 72 hours of incubation with paclitaxel-liposome formulations and paclitaxel alone. The toxicity of liposome formulations was lower than paclitaxel. However, the cell viability with DPPC-liposomes was less than SPC and HSPC liposomes. High cytotoxicity of DPPC-liposomes might be explained by high amount of paclitaxel entrapped in their vesicles whereas, SPC and HSPC formulations entrapping comparatively lower amount of paclitaxel; displayed low cytotoxicity.

Figure 4.8 represents the IC$_{50}$ (half maximal inhibitory concentration) of the paclitaxel-loaded liposomes, paclitaxel and PLL against U87-MG cells. In other words, IC$_{50}$ represents the drug concentration required for 50% inhibition of cell viability in vitro. Significantly higher IC$_{50}$ values compared to that of paclitaxel (0.2 mg/ml ± 0.09) and PLL (0.3 mg/ml ± 0.05) were observed for all liposomal-paclitaxel formulations (P<0.05). HSPC formulation (IC$_{50}$ = 1.81 mg/ml ± 0.13) was even less toxic than the SPC formulation (IC$_{50}$ =1.15 mg/ml ± 0.25) and in turn they both were less toxic than DPPC paclitaxel formulation having IC$_{50}$ value of 0.52 mg/ml ± 0.08 (P<0.05).
Fig. 4.7. Inverted microscope photographs of the viability of U87-MG cells treated with paclitaxel-liposomal formulations and paclitaxel.

Paclitaxel-SPC-Liposomes

Paclitaxel-HSPC-Liposomes

Paclitaxel-DPPC-Liposomes

Paclitaxel
The less toxic nature of liposomes may be attributed to the sustained release of paclitaxel when the liposomes comes in contact with medium and cells (Crosasso et al., 2000). Studies indicate that it takes time for the encapsulated paclitaxel to be released from the liposomes due to the stability of lipid bilayers as a result of inclusion of cholesterol (Yang et al., 2007). Increased IC$_{50}$ of the liposomes entrapping paclitaxel indicate that they were less toxic than paclitaxel. This has been attributed to fact that the paclitaxel was retained within the phospholipid bilayers or attached to the surface of the liposomes. This suggest that ethanol-based proliposome technology has successfully generated liposomes having sustained release properties by demonstrating a depot effect (part of paclitaxel is stored in the lipid bilayer as a depot, so the longer the action of paclitaxel, the larger the depot) (Song et al., 2006; Horowitz et al., 1992). This indicates that, paclitaxel-loaded liposomes having a size of approximately 100 to 200 nm would remain stable in biological...
environments; however, further studies should be conducted *in vivo* to find if a correlation with *in vitro* findings can be established. Also, all the liposomal formulations (paclitaxel-loaded as well as paclitaxel-free) contained ethanol in minute quantities (76 µl) required during preparation. Ethanol was also used to dissolve paclitaxel along with the media of the cell culture. Ethanol is toxic to cells, however, the fact that U87-MG cell viability was increased with the treatment of paclitaxel-free liposomes proves that ethanol, present in such trace amount, was not responsible for cell death. Also, paclitaxel-loaded liposomes were toxic to the cells, indicating that it is paclitaxel which was responsible for cytotoxicity (Figure 4.7).

The cytotoxicity exhibited by the liposome formulations had correlation with the entrapment efficiency studies. DPPC formulations displayed higher cytotoxicity compared to SPC and HSPC formulations, and these results were consistent with the repetition of experiments. Studies indicate that increased local concentration of the drug at the cellular site is dependent on the amount of drug present in the liposomes. The efflux of drug from HSPC formulations was comparatively lower than SPC and DPPC formulations. High entrapment efficiency of paclitaxel in the liposomes would lead to high intracellular uptake of paclitaxel *in vitro*. This might explain the difference in the cytotoxic effects of the paclitaxel-loaded liposomal formulations (Gregoriadis, 1988).
4.4.5 Effects of liposomes, paclitaxel and controls on SVG-P12 cells

Using $10^5$ seeding density for the SVG-P12 cells, they were tested with different compounds to assess their cytotoxicity. Figure 4.9 shows the effect of formulation on the viability of SVG-P12 cells by plotting concentrations in log-scale. As for U87-MG cells, dextran was used as a negative control. It was observed that with the increase in the dextran concentration, the cell viability increased by approximately 13% (113% ± 0.75) using 5mg/ml dextran (P<0.05), considering the absorbance value of the media as 100%. Likewise, cell growth was observed in case of drug-free liposomes. Cell viability increased by 2% using drug-free SPC-liposomes drug-free HSPC-liposomes and by 3% using drug-free DPPC-liposomes respectively when the liposome concentration was 2mg/ml, but not as high as dextran (P<0.05). There was no significant difference between the effects of drug-free SPC-liposomes, HSPC-liposomes and DPPC-liposomes (P>0.05).

PLL, as a positive control, was toxic to SVG-P12 cells. The viability of the cells when treated with PLL, decreased by approximately 96% (cell viability of 3.98% ± 0.66) using 5 mg/ml concentration (P<0.05). Similarly, paclitaxel was responsible for the reduction in cell growth by approximately 78% (cell viability of 21.77% ± 1.05) with the highest concentration (2 mg/ml) (P<0.05). The decrease in cell viability was also observed in case of paclitaxel loaded liposomes with increasing liposome concentrations. The viability of SVG-P12 cells, when treated with paclitaxel-loaded SPC-liposomes, HSPC-liposomes and DPPC-liposomes, decreased by approximately 44% (cell viability of 56.17% ± 1.2), 38% (62.39% ± 1.95 cell viability) and 45% (55.12% ± 2.06 cell viability), respectively using 2 mg/ml paclitaxel concentration. Paclitaxel proved to be less lethal to the SVG-P12 cells than PLL (P<0.05). However, cytotoxicity of paclitaxel and PLL was higher than all
paclitaxel loaded liposomes (P<0.05). The results suggest that liposomal formulations of paclitaxel are less toxic to glial cells than to U87 cells by approximately 30%, 20% and 45% when treated with paclitaxel-loaded SPC-liposomes, HSPC-liposomes and DPPC-liposomes respectively.

Fig.4.9. Viability of SVG-P12 cell line tested with increasing concentrations of different formulations in 96-well plates. (n=18, N=3 ± sd)
Figure 4.10 shows the light microscope images of the viability of SVG-P12 cells after 72 hours of incubation with paclitaxel-liposome formulations and paclitaxel alone. The toxicity of liposome formulations was lower than paclitaxel. However, paclitaxel-liposomes and paclitaxel-alone were less toxic to SVG-P12 cells than U87-MG cells.

The IC₅₀ of paclitaxel and PLL was 0.87 mg/ml ± 0.11 and 0.3 mg/ml ± 0.06 respectively. Paclitaxel was less toxic to the SVG-P12 cells as compared to the U87 cells by 18%. Liposomal formulations were also toxic to the SVG-P12 cells but their toxicity was less than 50%. This implies that the liposomal formulations would need more than 2 mg/ml paclitaxel to kill 50% of SVG-P12 cells. Thus, IC₅₀ of liposome formulations could not be determined.
Fig. 4.10. Inverted microscope photographs of the viability of SVG-P12 cells treated with paclitaxel-liposomal formulations and paclitaxel.
Paclitaxel have been known to be cytotoxic on a wide variety of human cell lines such as malignant brain tumour cells (U87-MG, U373, H80 and D324), breast adenocarcinoma (MCF-7), lung carcinoma (A549), cervical carcinoma (HeLa), colon adenocarcinoma (HT-29), ovarian adenocarcinoma (OVG-1) and pancreatic adenocarcinomas (PC-Sh and PC-Zd); and rat brain tumour cell lines (9L and F98) (Cahan et al., 1994; Liebmann et al., 1993). The reason for increased cytotoxicity by paclitaxel in vitro have been credited to the fact that paclitaxel acts as an inhibitor of cell proliferation in vitro by interfering with the cell cycle development (Straubinger et al., 2004; De Brabander et al., 1981). Paclitaxel works by blocking the cell cycle at G2/M phase and altering the arrangement of spindle microtubules thereby causing cell death (Jordan et al., 1993). Similar anti-mitotic mechanism, upon treatment with paclitaxel, may have taken place suggesting the decrease in viability of U87-MG and SVG-P12 cell lines.

However, SVG-P12 cells showed less sensitivity to paclitaxel and paclitaxel-loaded liposomes than U87-MG cells. This may be due to the reason that the rate at which tumour cells are killed is dependent on their growth curve. Growth curve analysis of the cell lines plays a crucial role in understanding the cell proliferation and effect of anti-tumour agents on them. Tumour cells that display a rapid and unregulated growth are more sensitive to the cytotoxic effects of anti-mitotic and anti-tumour drug such as paclitaxel and exposed to mitotic cell death by rapid intracellular uptake of the drug. Paclitaxel interfere with DNA replication in the cell cycle and inhibits the mitotic division of the cells attempting to divide. While performing tissue culture experiments, U87-MG cells reached high confluency (80-90%) in 2 days while SVG-P12 cells achieved the similar confluency in 4-5 days, for further sub-culturing of the cells. The growth and division of normal cells such
as SVG-P12, in tissue culture conditions, are similar to that of U87-MG cells. However, the growth rate of normal cells decreases once they cover the bottom of the culture flask and remain as a monolayer. Growth inhibition may be caused by the exhaustion of growth factors in the medium. On the other hand, glioma cells continue to grow until they overlap with surrounding cells and form clumps. This may be due to the fact that they are unresponsive to the signals that cause the ceasing of growth and division of their normal counterparts. This might explain the rapid decrease in the viability of U87-MG cells as compared to that of SVG-P12 cells when treated with paclitaxel and liposome formulations (Karp, 2002). It can be observed that SVG-P12 cells treated with paclitaxel-loaded liposomes were less sensitive to the effect of paclitaxel at 2 mg/ml as compared to the increased toxicity in U87-MG cells by paclitaxel (Figures 4.6, 4.7, 4.8, 4.9 and 4.10).

It was also observed that the drug-free formulations did not show any toxic effect on the glioma as well as normal glial cell lines. Studies have shown that drug-free liposomes displayed non-toxic effect or effect equal to that of negative control on AsPC1 cells (Human pancreatic tumour cell line) \textit{in vitro} (Graeser et al., 2009). This may be due to the fact that liposomes are prepared from naturally occurring substances such as phospholipids and cholesterol which are major components of biological membranes essential for cellular functions. Phosphatidylcholine forms a major component of the cell membranes and are found in the exoplasmic membrane leaflets. In fact, liposome vesicles might enhance the efficacy of the drugs by binding to the cells and releasing them in a sustained manner (al-Suwayeh et al., 1996; Lasic et al., 1995).
4.5 Conclusion

This study was aimed to investigate the in vitro cytotoxicity of paclitaxel-loaded liposome formulations and paclitaxel on a grade IV glioma (U87-MG) cell line and a normal glial (SVG-P12) cell line. The cytotoxicity study was carried out using MTT reduction assay. Liposome formulations and paclitaxel-alone had toxic effects on both the cell lines. However, liposomes were less toxic to the cells compared to paclitaxel. This is attributed to the sustained release of paclitaxel from the liposomes due to the stability of the lipid bilayers. The drug was retained for a longer time in the liposome vesicles. This indicated that ethanol-based proliposome technology was successful in generating liposomes having sustained release properties by demonstrating a depot effect. Paclitaxel-loaded liposomes having a size of approximately 100 to 200 nm would remain stable in biological environments.

The cytotoxicity exhibited by the liposome formulations had correlation with the entrapment efficiency studies. DPPC-liposomes displayed higher cytotoxicity compared to SPC and HSPC formulations, and these results were consistent with the repetition of experiments. Studies indicate that increased local concentration of the drug at the cellular site is dependent on the amount of drug present in the liposomes. The efflux of drug from HSPC formulations was comparatively lower than SPC and DPPC formulations. High entrapment efficiency of paclitaxel in the liposomes would lead to high intracellular uptake of paclitaxel in vitro, with sustained release.
Paclitaxel is an anti-mitotic and anticancer drug. It inhibits the cell proliferation *in vitro* by interfering with the cell cycle development. This might explain the toxic nature of paclitaxel against U87-MG and SVG-P12 cells. However, paclitaxel was relatively less toxic to SVG-P12 cells than U87-MG cells. This may be due to the fact that the rate at which tumour cells are killed is dependent on their growth curve. Growth curve analysis of the cell lines played a crucial role in understanding the cell proliferation and effect of anti-tumour agents on them. Tumour cells that display a rapid and unregulated growth are more sensitive to the cytotoxic effects of anti-mitotic and anti-tumour drug such as paclitaxel and exposed to mitotic cell death by rapid intracellular uptake of the drug. Paclitaxel interfere with DNA replication in the cell cycle and inhibits the mitotic division of the cells attempting to divide. Since, the growth of glioma cells *in vitro* is uncontrolled compared to normal glial cells, paclitaxel may have interfere with DNA replication in the cell cycle and inhibited the mitotic division of the glioma cells rapidly.

Drug-free liposomes proved to be non-toxic to both the cell lines by showing effect similar to that of negative control (Dextran). Liposomes are prepared from naturally occurring substances such as phospholipids and cholesterol, which are major components of biological membranes essential for cellular functions. Phosphatidylcholine forms a major component of the cell membranes and are found in the exoplasmic membrane leaflets. Liposome vesicles might in turn enhance the efficacy of the entrapped drugs by releasing them in a sustained manner.
Finally, ethanol-based proliposomes employed using different phospholipids composition and drug concentration may provide a potential delivery for both hydrophobic and hydrophilic drugs. The properties of different liposome formulations were essential in understanding their drug delivery mechanism *in vitro*. However, to ascertain whether the correlation with *in vitro* findings can be established, further studies should be conducted *in vivo*. 
CHAPTER 5:

GENERAL CONCLUSIONS AND FUTURE STUDIES
5.1 Introduction

It is well established that liposomes can deliver paclitaxel and provide beneficial effects on the pharmacology and toxicology of the drug. Paclitaxel-loaded liposome formulations not only avoid the acute toxicity of Cremophor EL vehicle but also change the drug efficacy (Sharma et al., 1993; Sharma et al., 1995; Sharma et al., 1996; Sharma et al., 1997; Cabanes et al., 1998). Furthermore, liposomes prepared from ethanol-based proliposomes can overcome the difficulty of manufacturing liposomes on a large scale. Moreover, proliposomes offer an approach to avoiding the instability problems of liposomes in aqueous media (Kensil and Dennis, 1981; Grit et al., 1989; Hunt and Tsang, 1981).

In this project, paclitaxel-loaded liposomes were prepared from ethanol-based proliposomes. Ethanol-based proliposomes are ethanolic solutions of phospholipid which produce liposomes on addition of aqueous phase above the Tm of the lipid phase (Perrett et al., 1991). It was observed that the physicochemical properties of formulations, entrapment efficiency of paclitaxel in the liposomes and the cytotoxicity of the formulations were influenced by lipid composition (SPC:Chol, HSPC:Chol and DPPC:Chol in 1:1 mole ratio) and the concentration of paclitaxel in liposome dispersions.
5.2 Characterisation of liposomes before sonication

Liposomes prepared from ethanol-based proliposomes were MLVs as shown by TEM study. The VMD of SPC, HSPC and DPPC liposomes increased with increasing paclitaxel concentration. Compared with paclitaxel-free liposomes, vesicles containing paclitaxel (2mg/ml) had VMD measurements that were larger by approximately 0.7, 5.5 and 2.2 µm respectively (Section 3.2.1). Amongst the phospholipids used, paclitaxel concentration was most influential to the size of vesicles made from HSPC:Chol (1:1). This is possibly attributed to the higher hydrophobicity of the longer acyl chains in HSPC phospholipid and repulsive interactions between water molecules at the interface; causing them to aggregate. The ZP values of all formulations had negative values. Inclusion of higher concentrations of paclitaxel tended to reduce the charge intensity of SPC and DPPC liposomes while the ZP of HSPC-liposomes did not show any significant difference with the increase in paclitaxel concentration (Section 3.2.3). The size distribution of HSPC and DPPC liposomes did not show any significant increase with the increase in paclitaxel concentration.

5.3 Characterisation of liposomes after sonication

MLVs were successfully fragmented into nano-sized liposomes (100-200 nm) using probe sonication for 10 min. It was observed that the effect of paclitaxel concentration on liposome size after sonication was minimal. HSPC liposomes had larger size than liposomes prepared from DPPC and SPC suggesting the influence of lipid composition (Section 3.2.4). The PI of all the formulations was below 0.3, indicating that sonication
time selected was appropriate to form homogenous liposomes (Section 3.2.5). The ZP of SPC and HSPC liposomes was slightly influenced by paclitaxel concentration. At low drug concentrations, the ZP of these formulations was around 0 mV, indicating a neutral surface charge. However, DPPC-liposomes had more intense surface charge upon using higher paclitaxel concentrations (Section 3.2.6).

5.4 pH of liposome formulations

For each paclitaxel concentration, the pH of liposomes was slightly different when using different phospholipid compositions (Section 3.2.8). The pH of all SPC and HSPC liposomes was acidic, while it was neutral to slightly basic for DPPC-liposomes. For each phospholipid type, when the highest paclitaxel concentration formulation was compared with the paclitaxel-free ones, the pH increased. The results suggest that the pH of formulations were influenced by paclitaxel concentration and lipid composition. The pH of DPPC liposomes was found to be the closest to the pH of blood (pH 7.4) making DPPC liposomes highly appropriate vehicles for the anticancer hydrophobic drug paclitaxel.

5.5 Entrapment efficiency of paclitaxel in liposomes

The entrapment efficiency of paclitaxel in liposomes, analysed using a UV spectrophotometer, decreased with the increase in paclitaxel concentration. It was observed that the entrapment of paclitaxel in DPPC liposomes (85.6% ± 2.7) was generally higher than that in SPC (75% ± 5.3) or HSPC (67.2% ± 4.7) vesicles when low drug concentration was used. The entrapment efficiency of the drug in DPPC liposomes, using 2
mg/ml paclitaxel concentration, decreased by only around 15% compared to a decreased by 30% and 41% for SPC and HSPC liposomes respectively (Section 3.2.9 and 3.2.10).

The difference in ZP with different paclitaxel concentration was in correlation with the difference in entrapment efficiency using formulations with different lipid phases. The ZP values in Fig 3.6 notified that higher paclitaxel concentrations was able to confer more negative surface charge on the DPPC liposomes while the capability of paclitaxel to exert that effect on SPC and HSPC liposomes was less. This associated well with the entrapment efficiency findings. This also provides a strong indication that incorporation of paclitaxel in the bilayers of liposomes accounts for the negative ZP values. Previous studies indicate that incorporation of paclitaxel in DPPC-liposomes makes DPPC bilayer more stable and flexible. Low van der Waals forces between the short acyl chains can increase the molecular interactions between paclitaxel and DPPC. The property of first-order phase transition possessed by DPPC is also an important factor in the binding of paclitaxel to the bilayers of the lipid. However, HSPC liposomes limit the incorporation of hydrophobic materials in their bilayers due to their long hydrophobic acyl chains causing repulsive reactions with water molecules at the interface. SPC is also known to entrap hydrophobic drugs more efficiently as compared to HSPC due to their low phase transition temperature. SPC-liposomes are flexible enough to entrap more hydrophobic molecules.
5.6 Tissue culture findings

The cytotoxicity of paclitaxel-loaded liposomes against U87-MG grade IV glioma and SVG-P12 glial cell lines was determined using MTT assay. The cell seeding density of $10^5$ cells/well was chosen for both the cell lines to carry out the cytotoxicity studies (Section 4.4.2 and 4.4.3). It was observed that paclitaxel alone was more toxic to U87-MG and SVG-P12 cells than liposomes formulations. However, it was toxic by 17% and 42% containing SPC and HSPC liposomes. DPPC-liposomes showed higher cytotoxicity than SPC and HSPC formulations. The results were in correlation with the entrapment efficiency findings. However, paclitaxel-free liposomes increased the viability of U87 cells indicating the non-toxic nature of drug-free liposomes.

In case of SVG-P12 cells, the cytotoxicity of liposomal formulations was well below 50% while paclitaxel was significantly toxic to the glial cells (around 91%). Liposomes proved to be less toxic to SVG-P12 cells than U87 cells indicating the specific targeting of liposomes in terms of toxicity. Again, paclitaxel-free liposomes contributed to the cell viability of SVG-P12 cells.

Liposome formulations and paclitaxel-alone had toxic effects on both the cell lines. However, liposomes were less toxic to the cells compared to paclitaxel. This is attributed to the sustained release of paclitaxel from the liposomes due to the stability of the lipid bilayers. The drug was retained for a longer time in the liposome vesicles. This indicated that ethanol-based proliposome technology was successful in generating liposomes having sustained release properties by demonstrating a depot effect. Paclitaxel-loaded liposomes
having a size of approximately 100 to 200 nm would remain stable in biological environments.

The cytotoxicity exhibited by the liposome formulations had correlation with the entrapment efficiency studies. DPPC-liposomes displayed higher cytotoxicity compared to SPC and HSPC formulations, and these results were consistent with the repetition of experiments. Studies indicate that increased local concentration of the drug at the cellular site is dependent on the amount of drug present in the liposomes. The efflux of drug from HSPC formulations was comparatively lower than SPC and DPPC formulations. High entrapment efficiency of paclitaxel in the liposomes would lead to high intracellular uptake of paclitaxel in vitro, with sustained release.

Paclitaxel is an anti-mitotic and anticancer drug. It inhibits the cell proliferation in vitro by interfering with the cell cycle development. This might explain the toxic nature of paclitaxel against U87-MG and SVG-P12 cells. However, paclitaxel was relatively less toxic to SVG-P12 cells than U87-MG cells. This may be due to the fact that the rate at which tumour cells are killed is dependent on their growth curve. Growth curve analysis of the cell lines played a crucial role in understanding the cell proliferation and effect of anti-tumour agents on them. Tumour cells that display a rapid and unregulated growth are more sensitive to the cytotoxic effects of anti-mitotic and anti-tumour drug such as paclitaxel and exposed to mitotic cell death by rapid intracellular uptake of the drug. Paclitaxel interfere with DNA replication in the cell cycle and inhibits the mitotic division of the cells attempting to divide. Since, the growth of glioma cells in vitro is uncontrolled compared to normal glial cells, paclitaxel may have interfered with DNA replication in the cell cycle and inhibited the mitotic division of the glioma cells rapidly.
Drug-free liposomes proved to be non-toxic to both the cell lines by showing effect similar to that of negative control (Dextran). Liposomes are prepared from naturally occurring substances such as phospholipids and cholesterol, which were useful for growing the cells. They are major components of biological membranes essential for cellular functions and regulating cell cycle. Phosphatidylcholine forms a major constituent of the cell membranes, and are found in the exoplasmic membrane leaflets. Liposome vesicles might in turn enhance the efficacy of the entrapped drugs by releasing them in a sustained manner and prove to be non-toxic.

These studies have demonstrated that ethanol-based proliposomes offer an appropriate means of generating drug delivery systems. DPPC-liposomes may be the best drug carrier systems for paclitaxel because of their unique properties to entrap high amounts of hydrophobic drugs.
5.7 Future studies

Due to time restrictions, several studies have not been performed. For instance, it would be essential to understand why the ZP of liposomes before sonication tended to increase at low paclitaxel concentration and revert to the original ZP value upon inclusion of higher paclitaxel concentrations which were consistent with repetition of the experiment 5 times.

Ethanol-based proliposomes containing different lipid compositions and varying paclitaxel concentrations should be investigated for their in vivo behaviour in terms of drug release, clearance and biodistribution to ascertain whether the correlation with in vitro findings can be established.

Applying different liposomal-based systems may also be helpful to understand the differences in the behaviour of liposomes in blood circulation. Also, applying gene therapy via liposomes for glioma treatment may produce desired results.
CHAPTER 6:
REFERENCES


Schieren, H., Rudolph, S., Findelstein, M.m Coleman, P. and Weissmann, G., Comparison of large unilamellar vesicles prepared by a petroleum ether vaporization method with


following intravenous treatment with lipid-based nanoparticulate formulations of irinotecan (Irinophore C™), doxorubicin (Caelyx®) or vincristine. *BMC cancer.*, 124 (2011) 1-18.


