RGD-targeted Solid Lipid Nanoparticles Containing Asiatic Acid for the Treatment of Cancer

By

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A thesis submitted in partial fulfilment for the requirements for the degree of Doctor of Philosophy (PhD) at the University of Central Lancashire

August, 2016
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.

I declare that no material contained in the thesis has been used in any other submission for an academic award and is solely my own work.

Signature of Candidate

Type of Award                  PhD (Doctor of Philosophy)

School                        Pharmacy and Biomedical Sciences
Abstract

Selective anti-cancer treatment and targeting is needed to help minimise unwanted side-effects from conventional chemotherapy. Advanced drug delivery systems (DDSs) that have the potential for passive and active targeting have been of significant interests in the past decades, particularly with the use of nano-based drug carriers. The concept of targeting to the tumour vasculature or angiogenic blood vessels has continued to be of interests in cancer research since 1970s, as it offers promising therapeutic applications for many solid tumours. Active targeting of DDSs relies on specific interactions between the ligand (generally conjugated onto the surface of DDSs) and its receptor, which is overexpressed on target tumour tissues. In this study, arginine-glycine-aspartic acid (RGD) tripeptide was used to target the αvβ3 integrin receptor that is overexpressed by both tumour endothelial cells and various tumour tissues, potentially providing a double-killing (by targeting tumour and tumour endothelial cells) effect.

Asiatic acid (AA) is an extract from a medical plant, *Centella Asiatica* and has demonstrated potential anti-inflammatory, as well as anti-angiogenic and anti-cancer properties. However, its poor water solubility (0.03 mg/mL) is one of the major limitations for its progression for clinical applications. To help overcome the solubility issue of AA and also to improve its therapeutic efficacy, AA was incorporated into RGD-containing solid lipid nanoparticles (SLNs) after optimisation of the SLN preparation comprised of glyceryl monostearate, glyceryl distearate and glyceryl tristearate lipids and subsequent characterisation on their physicochemical properties. The targeting ability of these AA-containing RGD-conjugated SLNs and their apoptotic / necrotic induction on cancer cells were assessed using U87 MG glioma cells and ECV-304 bladder cancer endothelial-like cells, which are known to express αvβ3 integrin. Moreover, the efficacy of both AA and AA-loaded SLNs were tested for the first time on *in vitro* 3D U87 MG tumour spheroids. Besides the anti-tumour efficacy, AA-containing RGD-SLNs were also investigated for their potential anti-angiogenic effect using various cellular assays of ECV-304 endothelial-like cells.

Results obtained from this study showed that RGD-targeted SLN formulations improved the cellular uptake of nanoparticles compared to non-RGD containing SLNs and thus enhanced drug accumulation and cytotoxic effect seen on U87 MG and ECV-304 cells. Furthermore, AA-containing SLNs showed prevention of spheroid formation, inhibition of spheroid growth and cytotoxic effect on U87 MG spheroids, where RGD-SLNs also showed improved spheroid penetration compared to non-RGD containing SLNs. In addition, AA-loaded RGD-SLNs showed potential anti-angiogenic effect by
demonstrating concentration-dependent inhibition of cell adhesion, migration and invasion, as well as disruptions in tube network of endothelial cells in in vitro angiogenesis assay.

To conclude, AA-loaded RGD-targeted SLNs showed promising anti-cancer and anti-angiogenic effects in vitro, which supports its development for future clinical applications and warrants further investigation on an appropriate in vivo angiogenesis model to maximise its clinical potential.
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<td>Two dimensional</td>
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<td>3D</td>
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<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAM</td>
<td>Chick Chorioallantoic Membrane</td>
</tr>
<tr>
<td>CAMs</td>
<td>Cell adhesion molecules</td>
</tr>
<tr>
<td>C6</td>
<td>Coumarin-6</td>
</tr>
<tr>
<td>C6-SLN or C6-MS-SLN</td>
<td>Coumarin-6 loaded solid lipid nanoparticles prepared with glyceryl monostearate</td>
</tr>
<tr>
<td>CBQCA</td>
<td>3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde</td>
</tr>
<tr>
<td>Cis</td>
<td>Cisplatin</td>
</tr>
<tr>
<td>c(RGDfK)</td>
<td>Cyclo-(Arg-Gly-Asp-D-Phe-Lys)</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>dd(H₂O)</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>DDS</td>
<td>Drug Delivery Systems</td>
</tr>
<tr>
<td>DL</td>
<td>Drug loading</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>D₂O</td>
<td>Deuterated water</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin hydrochloride</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>DS</td>
<td>Glyceryl distearate</td>
</tr>
<tr>
<td>DS-SLN</td>
<td>Solid lipid nanoparticles prepared with glyceryl distearate</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential Scanning Calorimeter</td>
</tr>
<tr>
<td>ECACC</td>
<td>European Collection of Cell Culture</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ECV-304</td>
<td>Human bladder carcinoma</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>EE</td>
<td>Entrapment efficiency</td>
</tr>
</tbody>
</table>
EGF Epidermal growth factor
EILDV Glu-Ile-Leu-Asp-Val
EMEM Eagle’s minimum essential medium
EGFR Epidermal growth factor receptor
EPR Enhanced Permeation and Retention
FACS Fluorescence-activated cell sorting
FBS Fetal bovine serum
FD Free drug concentration
FDA Food and Drug Administration
FGF-2 Fibroblast growth factor-2
FSC Forward scatter
GI Gastrointestinal
GFS Growth factors
GFR Growth factor reduced
GFRs Growth factor receptors
H₂O₂ Hydrogen peroxide
HBV Hepatitis B virus
HIF Hypoxia inducible factor
HPH High Pressure Homogenisation
HPLC High Performance Liquid Chromatography
HSA Human serum albumin
HUVEC Human Umbilical Vein Endothelial Cells
ICH International Conference on Harmonisation
IL-8 Interleukin-8
LDH Lactate Dehydrogenase
LUV Large unilamellar vesicles
M199 Medium 199
MLV Multilamellar vesicles
MMPs Metalloproteinases
MPS Mononuclear phagocyte system
MS Glyceryl monostearate
MS-SLN Solid lipid nanoparticles prepared with glyceryl monostearate
MTBE Methyl-tert-butyl ether
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
MW Molecular weight
MWCO Molecular weight cut-off
NAD(P)H Nicotinamide adenine dinucleotide (phosphate)
NEAA Non-essential amino acids
NLCs Nanostructured lipid carriers
NMR Nuclear Magnetic Resonance spectroscopy
OVA Ovalbumin
P40SLN Non-drug-loaded PEGylated solid lipid nanoparticles containing PEG(40)stearate
P100SLN Non-drug loaded PEGylated solid lipid nanoparticles containing PEG(100)stearate
P188 Poloxamer 188
PAMAM Poly(amidoamine)
PBS Phosphate buffered saline
PCL Polycaprolactone
PDGF Platelet derived growth factor
PDI Polydispersity index
PDLLA Poly(D,L-lactic acid)
PEG Polyethylene glycol also known as
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-SLN</td>
<td>Polyethylene oxide or polyoxyethylene Pegylated solid lipid nanoparticles</td>
</tr>
<tr>
<td>PEG(40)stearate</td>
<td>Polyoxyethylene(40)stearate</td>
</tr>
<tr>
<td>PEG(100)stearate</td>
<td>Polyoxyethylene(100)stearate</td>
</tr>
<tr>
<td>PET</td>
<td>Polyethylene terephthalate</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGA</td>
<td>Poly(glutamic acid)</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PLA</td>
<td>Polylactic acid</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PLN</td>
<td>Polymer-lipid hybrid nanoparticles</td>
</tr>
<tr>
<td>Prx II</td>
<td>Peroxiredoxin II</td>
</tr>
<tr>
<td>PPI</td>
<td>Poly(propylene imine)</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyvinyl pyrrolidone</td>
</tr>
<tr>
<td>R²</td>
<td>Correlation coefficient</td>
</tr>
<tr>
<td>REDV</td>
<td>Arg-Glu-Asp-Val</td>
</tr>
<tr>
<td>RES</td>
<td>Reticuloendothelial system</td>
</tr>
<tr>
<td>RGD</td>
<td>Arg-Gly-Asp</td>
</tr>
<tr>
<td>RGD-SLN</td>
<td>RGD peptide-conjugated solid lipid nanoparticles</td>
</tr>
<tr>
<td>RP40SLN</td>
<td>Non-drug loaded RGD-targeted solid lipid nanoparticles containing PEG(40)stearate</td>
</tr>
<tr>
<td>RP100SLN</td>
<td>Non-drug loaded RGD-targeted solid lipid nanoparticles containing PEG(100)stearate</td>
</tr>
<tr>
<td>RSD %</td>
<td>Percent relative standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SLN</td>
<td>Solid lipid nanoparticle</td>
</tr>
<tr>
<td>SMANCS</td>
<td>Styrene maleic anhydride neocarzinostatin</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>SUV</td>
<td>Small unilamellar vesicles</td>
</tr>
<tr>
<td>SVG P12</td>
<td>Human foetal glial cell line</td>
</tr>
<tr>
<td>TD</td>
<td>Total drug concentration</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TS</td>
<td>Tristearin, Glyceryl tristearate</td>
</tr>
<tr>
<td>TS-SLN</td>
<td>Solid lipid nanoparticles prepared with glyceryl tristearate</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>U87 MG</td>
<td>Human glioblastoma-astrocytoma</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray diffraction</td>
</tr>
</tbody>
</table>
List of abstracts and publication

Papers


Abstracts


Chapter 1

Introduction
1.1 Uncontrolled cellular growth in cancer development

Cancer involves abnormal cellular growth that may be due to mutational and epigenetic changes resulting in uncontrolled cell division and differentiation (Deng et al., 2014). Cancer cells have the ability to invade nearby tissues and metastasise to other parts of the body via the bloodstream and the lymphatic system making them more difficult to eradicate. There are special characteristics of cancer cells that can be distinguished from normal cells (Figure 1.1) and are also suggested as the hallmarks of cancer (Hanahan and Weinberg, 2011, 2000).

Cancer cells have the ability to generate their own growth signals to divide and grow rapidly using processes like mitogenic autocrine stimulation (Witsch et al., 2010) and overexpression of cell surface receptors like integrins to aid in transmission of pro-growth signals (Giancotti and Ruoslahti, 1999).

To maintain homeostasis within normal tissues, anti-proliferative signals (i.e. cytostatic anti-growth signals) are produced to keep cells at the quiescence state. However cancer cells do not perceive external “not to grow” signals and continue growing. For example, signalling molecule transforming growth factor beta (TGFβ) delivered from cytokines inhibits the growth of normal tissues, whereas cancer cells can disrupt this signal either by downregulating the TGFβ receptors or showing dysfunctional mutant receptors (Fynan and Reiss, 1993; Hanahan and Weinberg, 2000).

Principally, in normal cells, the rate of cell growth is balanced with growth factors and other cellular proteins that control cell death. When cells are not needed or damaged, they are destroyed with programmed cell death which is also known as cell suicide or apoptosis and is mediated by proteolytic enzymes, caspases (Elmore, 2007). However in the case of cancer cells, they have lost the capacity of cell suicide by apoptosis, which disrupts the cell life cycle balance and cause uncontrolled cell growth. They manage to inhibit apoptosis by overexpression of anti-apoptotic proteins like B-cell lymphoma 2 (Bcl-2) (Korsmeyer, 1992; McDonnell and Korsmeyer, 1991) or inactivation of pro-apoptotic regulator genes via mutations like p53 tumour suppressor genes (Harris, 1996), where inactivation of p53 protein is encountered in more than 50 % of all human cancers (Hanahan and Weinberg, 2000; Harris, 1996).

Normal cells have an intrinsic, cell-autonomous program which limits their multiplication, whereas cancer cells become immortalise and can show indefinite replicative capacity (Hanahan and Weinberg, 2000) (Figure 1.2). During the doubling of normal cells, telomere, which prevent end-to-end fusion of chromosomes is getting shorter and eventually after certain amount of cell division they cannot protect the ends
of chromosomal DNA anymore and result in cell death (Blasco, 2005; Shay and Wright, 2000). However, cancer cells can protect the length of telomere by producing telomerase enzyme and hence enabling cancer cells go through many cell divisions (Hayflick, 1997; Wright et al., 1989).

With relevance to the proposed investigation, cancer cells can induce angiogenesis in order to have their own blood vessels for further nutrient and oxygen supply to be able to grow and metastasise (Judah Folkman, 1971). Details of tumour angiogenesis in relation to the understanding of this investigation are given in greater depth in Section 1.2.

Metastasis of the cancer is the reason of 90% of human cancer deaths (Sporn, 1996). Metastasis is an extremely complex multi-step process, which includes infiltration of cancer cells into adjacent tissues, intravasation (the transendothelial migration of cells into vasculature that manage to survive through the circulatory system) and subsequent extravasation and colonisation in the new location of the body (van Zijl et al., 2011). Alterations in cell adhesion molecules (CAMs) play an important role during the tumour metastasis, including mutational inactivation of E-cadherin (Christofori and Semb, 1999), reduction the expression of CAMs in the immunoglobulin superfamily (Johnson, 1991) and overexpression of favourable integrins (i.e. \( \alpha_3\beta_1 \) and \( \alpha_v\beta_3 \)) (Varner and Cheresh, 1996), as well as upregulating proteases genes and downregulating protease inhibitor genes (Chambers and Matrisian, 1997).

These common characteristics of tumourigenesis apply to all solid tumours which are the main interest of this investigation. In addition, the \( \alpha_v\beta_3 \) integrin is also the main target of this project as it plays an extremely critical role in these complex tumourigenesis processes and is discussed in greater detail in Section 1.3.
Figure 1.1 The hallmarks of cancer. Most of the cancers acquire similar functional capabilities during their development. (Copied without permission from Hanahan and Weinberg (2011))

Figure 1.2 Normal cell division and cancer cell division (adapted from National Cancer Institute, 2014).
1.2 Tumour angiogenesis and vasculature

Angiogenesis is known as formation of new blood vessel from pre-existing vessels and besides occurring in cancer, it is also seen in normal physiological processes like embryonic development and wound healing as well as seen in other pathophysiological processes like inflammation and rheumatoid arthritis (Carmeliet, 2005; Folkman, 1995). However, angiogenesis in normal processes shows differences from pathophysiological angiogenesis by having better control over the balance of anti- and pro-angiogenic factors. Angiogenesis is usually quiescent except transient regulated angiogenesis occur in female reproductive cycle and also in injured tissues (Bergers and Benjamin, 2003; Fidler and Ellis, 1994). On the other hand, in tumour angiogenesis the balance of positive and negative controls are lost and constant growth of new vessels are observed (Bergers and Benjamin, 2003; Dvorak, 1986).

Although transportation of oxygen and nutrients to cells is provided to tumour cells by diffusion, oxygen deficiency occurs after tumour tissues reach to 1 – 2 mm in size, which leads to a cellular hypoxia. Hypoxia then triggers angiogenic switch for the formation of new blood vessels, which is a fundamental feature of the progression of malignancy. Recent studies have demonstrated that tumour hypoxia activates cellular hypoxia inducible factor (HIF) transcription and this induces the expression of various pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), tumour necrosis factor-α (TNF-α) or platelet derived growth factor (PDGF) (Carmeliet, 2000; Carmeliet and Jain, 2000; Semenza, 1998). Angiogenesis is a multi-stage process (Figure 1.3), which involves the activation of endothelial cell proliferation, degradation of basement membrane of the vasculature using proteases (i.e. matrix metalloproteinases (MMPs)), migration of endothelial cells, formation of blood vessels and vascular remodelling (Danhier et al., 2010; Heissig et al., 2003). Angiogenic switch can be triggered by signals including metabolic stress like low oxygen and low pH as well as mechanical stress, genetic mutations and inflammatory response. These triggering signals can result in enhanced expression of pro-angiogenic factors by cancer cells or stromal cells (such as, VEGF) and reduced expression of anti-angiogenic factors, such as thrombospondin-1, by stromal cells and cancer cells (Danhier et al., 2012a; Francavilla et al., 2009).

Tumour vasculature is architecturally and functionally different from normal blood vessels. In contrast to normal physiological vasculature, tumour vessels are reported to be tortuous, irregularly shaped, excessively branched, dilated, blind ended and highly disorganised (Bergers and Benjamin, 2003; Carmeliet and Jain, 2000). In addition, the walls of tumour vasculature have various openings (endothelial fenestrations), widened
junctions between endothelial cells and a discontinuous or absent basement membrane which results in a permeable and leaky vasculature (Bergers and Benjamin, 2003; Carmeliet and Jain, 2000; Hashizume et al., 2000). These could be due to the overexpression of VEGF and other pro-angiogenic factors that promote the tumour angiogenesis (Bergers and Benjamin, 2003; Carmeliet and Jain, 2000). Furthermore, highly branched tortuous tumour vasculature results in functional disruption of the vessels and blood flow (Brown and Giaccia, 1998; Carmeliet and Jain, 2000) and lead to poor tumour microcirculation and lymphatic drainage.

Figure 1.3 Multi-step progression of tumour angiogenesis and roles of the αvβ3 integrin in angiogenesis. (Copied without permission from Danhier et al. (2012a))
Introduction

Tumour angiogenesis does not only support tumour growth but is also crucial for tumour metastasis by providing an adequate route of exit for cancer cells to escape from the primary tumours, enter into the blood stream and localised in another part of the body where metastases then develop into secondary tumours and may initiate secondary tumour vascularisation (Bruce R. Zetter, 1998; Folkman, 2002). The enhanced density of immature, highly permeable vasculature of tumour blood vessels with disordered basement membrane and poor endothelial cell-cell junctions provide an increase in the entry of cancer cells into the bloodstream (Bruce R. Zetter, 1998; Dvorak et al., 1995). The relationship between tumour metastasis and angiogenesis have been shown with in vivo experiments where animals with primary tumour were treated with anti-angiogenic therapeutics and consequently, reduction in vascularisation resulted in a decrease of tumour metastasis (Mori et al., 1995; O'Reilly et al., 1994; Weinstat-Saslow et al., 1994).

1.3 Integrins in cancer development

Integrins are divalent cation-dependent heterodimeric cell adhesion molecules formed by two subunits of α and β. There are 18 different types of α and 8 different types of β subunits that can non-covalently assemble and form at least 24 different heterodimer integrin proteins (van der Flier and Sonnenberg, 2001). Integrins consist of three domains which these are a longer extracellular domain (consist of > 1600 amino acids), a transmembrane region and a short cytoplasmic domain (20 – 50 amino acids, except β4 subunit) (Hynes, 2002). As well as providing cell-cell and cell-matrix adhesions, integrins are also involved in the regulation of cellular signalling pathways (Hood and Cheresh, 2002). They can bind to extracellular matrix (ECM) proteins, growth factors (GFs), immunoglobulin, MMPs and cytokines. Signalling properties and ligand binding features of an integrin are determined by the combination of α and β subunits. While some integrins (e.g. αvβ3) are able to bind several ligands, others (e.g. α5β1) recognise a unique ligand. Most of the integrins recognise their corresponding ECM molecule with specific peptide sequence. It is known that Arg-Gly-Asp (RGD) tripeptide is recognised by various integrins, such as αvβ3, α5β1, αibβ3, α6β4 and αvβ1 (Plow et al., 2000); moreover, Glu-Ile-Leu-Asp-Val (EILDV) and Arg-Glu-Asp-Val (REDV) peptides are recognised by α4β1 (Komoriya et al., 1991; Massia and Hubbell, 1992). For example, ECM molecules of fibronectin and vitronectin bind to αvβ3 integrin receptors through their RGD peptide (Wu et al., 2001).
Integrin-mediated ligation induces integrin clustering and following intracellular signal transductions which modulates various cellular aspects such as changes in cellular shape, proliferation, cell motility, differentiation, gene expression and cell survival/apoptosis (Hood and Cheresh, 2002; Hynes, 2002). Besides inducing signals on their own, integrins can also collaborate with growth factor receptors (GFRs) to regulate many cellular processes (Danhier et al., 2012a).

1.3.1 αvβ3 Integrin Expression in Cancer

Integrin receptors are also involved in adhesive events of tumour progression including tumour growth, cancer cell migration, invasion and metastasis. β3 integrin expression is mostly correlated with metastatic potential of malignant tumours (Switala-Jelen et al., 2004; Timar et al., 1996). For example, pancreatic tumour growth and metastasis have been shown to be accelerated by exogenous expression of β3 integrin in pancreatic carcinoma cell lines lacking β3 integrin using in vivo experimental mouse model (Desgroisellier et al., 2009; Weis and Cheresh, 2011). Tumour metastasis requires the detachment of cancer cells from primary tumour to migrate, enter the circulation and colonise. During the metastasis process cell migrates effectively on ECM substrates where cell adhesion via receptor clustering is required so that cells can pull themselves along a migration path (Danhier et al., 2012a; Mizejewski, 1999; Switala-Jelen et al., 2004).

A wide variety of endothelial cell integrins are reported to take parts in angiogenesis. However, αvβ3 is one of the most important and extensively studied integrins for angiogenesis (Brooks et al., 1994; Kumar, 2003; Z. Liu et al., 2008). The expression of αvβ3 integrin on endothelial cells is induced by several pro-angiogenic growth factors including TNF-α, interleukin-8 (IL-8) and fibroblast growth factor-2 (FGF-2) (Avraamides et al., 2008; Brooks et al., 1994). It has been shown that integrin αvβ3 is associated with proteolytically active MMP-2 to regulate basement membrane degradation and to rearrange the ECM (Brooks et al., 1996). Moreover, it has been reported that αvβ3 integrin helps endothelial cells to evade apoptosis by binding to vitronectin that provides protection to endothelial cells from apoptosis through the activation of different signalling cascades (Francavilla et al., 2009).

According to the clinical data reported, αvβ3 integrin receptors are overexpressed on angiogenic endothelial cells and blood vessels of human tumour biopsy samples, whereas expression of αvβ3 is not observed in normal blood vessels (Avraamides et al., 2008; Danhier et al., 2012a). Moreover, overexpression of αvβ3 integrin is seen in
malignant breast cancer which is associated with enhanced bone metastasis (Liapis et al., 1996; Sloan et al., 2006; Takayama et al., 2005). Additionally, glioblastoma tissues also widely express α₃β₃ integrin on peripheral surface and plays a role in glioma cell invasion (Bello et al., 2001). Furthermore, α₃β₃ integrins are extensively expressed in prostate and pancreatic tumours which result in enhanced bone and lymph node metastasis, respectively (Hosotani et al., 2002; McCabe et al., 2007). Melanoma, ovarian and cervical cancers also overexpress α₃β₃ integrins which is associated with promotion of tumour growth, proliferation and metastasis (Albelda et al., 1990; Gruber et al., 2005; Landen et al., 2008; Nip et al., 1992).

### 1.3.2 RGD peptide for targeting α₃β₃ integrins

The RGD tripeptide sequence (Arg-Gly-Asp) was first discovered as a cell attachment site in fibronectin (D'Souza et al., 1991; Pierschbacher and Ruoslahti, 1984). Later, it was reported that RGD-recognition site is the minimum essential peptide sequence found in many ECM molecules (i.e. fibronectin, vitronectin, fibrinogen, laminin, etc.) and binds to α₃β₃ integrin receptors (Temming et al., 2005; F. Wang et al., 2013). Steric conformation of the RGD peptide may affect its peptide-ligand affinity (Liu, 2006). It has been shown that cyclisation of RGD peptide improves the binding characteristics compared to linear RGD peptides. It is because cyclisation gives rigidity to the peptide structure and hence enhances the selectivity of the promiscuous RGD-sequence for a specific integrin subtype (Temming et al., 2005; F. Wang et al., 2013). On the other hand, linear peptides show lower affinity which could be due to their flexibility in solutions. It is also reported by (Bogdanowich-Knipp et al., 1999) that, linear RGD peptides are susceptible to chemical degradation due to the reaction of the aspartic acid residue (D) with the peptide backbone, whereas cyclisation provides a protection against the chemical degradation of peptides due to the rigidity and cyclic RGDS have been shown to be more stable. In the light of these data, due to its higher binding affinity to α₃β₃ integrins and more stability towards chemical degradations, cyclic-Arg-Gly-Asp-D-Phe-Lys, [c(RGDfK)] was chosen to be exploited for this project for conjugation to the solid lipid nanoparticles (SLNs).
1.3.3 Conformational changes in $\alpha_v\beta_3$ integrin

It has been reported that, most of the integrins, including endothelial integrins, have “on” and “off” mechanisms. With the aid of analysing crystal structures of integrins (Liddington and Ginsberg, 2002; Xiong et al., 2001), it was suggested that the extracellular domain of inactive $\alpha_v\beta_3$ integrin is folded or bent, hiding its RGD-recognition site and hence preventing ligand binding. Additionally, shorter cytoplasmic domain shows close proximity of the two subunits with each other. In contrast, activated $\alpha_v\beta_3$ integrin has a straighter or unbent extracellular domain, exposing its RGD-binding site as well as showing a separation between two subunits in cytoplasmic domain (Figure 1.4) (Hodivala-Dilke et al., 2003).

Figure 1.4 Conformational changes in $\alpha_v\beta_3$ Integrin. During activation of the integrin, extracellular part extends and straightens, at the same time in the cytoplasmic part the tails of the subunits show separation (copied without permission from Danhier et al. (2012a)).
1.4 Challenges in cancer treatment

Extensive research has been carried out on various cancer therapies to help reduce the burden of cancer to patients and with the hope to cure the disease. Conventional strategies for the treatment of cancer include; surgery, radiotherapy and chemotherapy. The choice of treatment used depends on the type and stage of tumour progression.

The initial step for the treatment of solid tumours is generally the removal of the tumour and the surrounding affected tissue by surgery. It is a relatively effective kind of treatment, however surgery cannot be applied to treat the spread of cancer cells in the body (metastasis) and it cannot be guaranteed for the complete removal of the affected tissues, as there may be sites of cancer cells that could not be reached or identified in surgery. In addition, surgical treatment may sometimes trigger a faster progression of metastasis of the remaining tumour cells (e.g. dormant cancer cells) in the body (Feng and Chien, 2003). In most cases, it is not enough to cure cancer with a single treatment and therefore after surgery, treatment is supported with radiotherapy and/or chemotherapy as well, particularly when the tumours have metastasised.

1.4.1 Limitations of chemotherapy

Chemotherapy is a medical term which is used for cytotoxic and cytostatic drugs and these drugs are respectively used to kill tumour cells and to prohibit cell proliferation. Efficacy of chemotherapy depends on the concentration of the drug reaching to the tumour area and the duration of the exposure of the drug on tumour cells. Advantageously, this therapy not only kills the malignant cells at primary tumour area but also can destroy the metastases as well (King and Robins, 2006). However, delivering sufficient amounts of chemotherapeutic agents to the desirable sites is a challenge due to the pathophysiology of tumour cells and the hypoxic environment that tumour cells grow in.

Chemotherapy drugs kill the rapidly dividing cells generally by disturbing their cell cycle, such as inhibiting the functioning of metabolites, DNA binding, ribosome-inactivation etc., as shown in Table 1.1.
Table 1.1 Examples of anti-cancer drugs used in chemotherapy (Katzung et al., 2009).

<table>
<thead>
<tr>
<th>Classification</th>
<th>Mechanism of Action</th>
<th>Anti-cancer Agents</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-metabolites</strong></td>
<td>Inhibit the use of the metabolites within the cell division and disturb the cell cycle of proliferating cells at S-phase and cause cell death</td>
<td>• Fluorouracil</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Methotrexate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Capecitabine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Mercaptopurine</td>
</tr>
<tr>
<td><strong>Alkylating Agents</strong></td>
<td>Damage DNA by alkylation of the guanine base of DNA and interfere with cell proliferation</td>
<td>• Busulfan</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Carmustine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Chlorambucil</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Melphalan</td>
</tr>
<tr>
<td><strong>Vinca Alkaloids</strong></td>
<td>Disturb the cell cycle of proliferating cells at metaphase by inhibiting tubulin polymerization and cause cell death.</td>
<td>• Vincristine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Vinblastine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Vindesine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Vinorelbine</td>
</tr>
<tr>
<td><strong>Anthracyclines</strong></td>
<td>Intercalate the base pairs of the DNA and RNA strands and results in inhibiting the DNA/RNA synthesis.</td>
<td>• Daunorubicin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Doxorubicin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Epirubicin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Idarubicin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Valrubicin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Mitoxantrone</td>
</tr>
<tr>
<td><strong>Platinum Analogues</strong></td>
<td>Cause cell death by forming crosslinks between DNA strands</td>
<td>• Cisplatin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Oxaliplatin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Carboplatin</td>
</tr>
</tbody>
</table>
In recent years, various anticancer agents have been discovered either by extracting from plants (Balunas and Kinghorn, 2005) or designed and synthesised using molecular docking and combinatorial chemistry (Ferreira et al., 2015; Geromichalos, 2007; Hoelder et al., 2012; Sharma and Sharma, 1997). Although these drugs are quite successful in cancer treatment, especially in in vitro studies, they have several common in vivo problems. One of the major challenges of anti-cancer agents is their toxicity towards normal tissues. Since anti-neoplastic drugs are effective on rapidly dividing cells, they display non-specific toxicity and affect not only the tumour cells, but also healthy cells that have a relatively high cell turnover rate. This includes cells associated with the renal system, bone marrow, cardiac tissues and the gastrointestinal (GI) tract (Feng and Chien, 2003; Sharma and Sharma, 1997). Due to the effects on these normal rapidly dividing cells, thrombocytopenia, alopecia and adverse effects on the GI system are common problems associated with chemotherapy (Feng and Chien, 2003).

Unfortunately, the drugs that are more effective in treatment often also show more toxicity in the body (Feng and Chien, 2003). Such non-specificity in cytotoxicity leads to a reduction of the necessary administered dose and thereby reducing the drug concentration available at the required target tumour areas, which may lessen the effectiveness of the treatment. In addition, during the period of therapy, cancer cells can develop resistance against the drugs as a defence mechanism, which further hampers the efficacy of the drugs. Thus, high doses of drug(s) at the initial stages of the therapy are often used to help destroy as many malignant cells as possible before they have a chance to develop resistance (King and Robins, 2006), which may mean more side-effects for the patients.

Apart from the unwanted toxic side-effects, anti-cancer agents are often poorly water-soluble, which makes them difficult to be administered and leading to the use of organic co-solvents or surfactants which have their own undesirable side effects (Feng and Chien, 2003; Sharma and Sharma, 1997). Therefore, better formulation design is required to ensure an effective delivery and bioavailability is achieved.
1.5 Novel strategies in cancer treatment

Conventional cancer therapies have their own limitations and side-effects arising mainly from the lack of specificity for tumours and using these treatments is not sufficient to stop the progression of advanced tumours and metastasis of cancer. To improve the efficacy of anti-cancer therapy, in recent years various treatment methods have been developed by researchers. Some have focused on developing and investigating new cancer treatment strategies as summarised in Table 1.2, and others have investigated on developing better drug carrier systems to deliver the drugs to the tumour areas more efficiently (Table 1.3).

Table 1.2 Novel strategies used as anti-cancer treatments

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Application/Mechanism of Action</th>
<th>Examples</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hormone therapy</td>
<td>It is used in the treatment of hormone sensitive cancers like breast cancer and prostate cancer</td>
<td>Tamoxifen, Toremifene (Fareston®), Fulvestrant (Faslodex®), Aromatase inhibitors (AIs), Ovarian ablation for breast cancer, androgen suppression therapy</td>
<td>(Lim et al., 2013; Shaw and Oliver, 2009; Stokes and Chan, 2003)</td>
</tr>
<tr>
<td>Immunotherapy</td>
<td>It is used in the treatment of cancer by inducing the immune response of the body to kill the tumour cells</td>
<td>Melacine (Approved in Canada), OncoVAX (phase III), Sipuleucel-T (FDA approved), H6 gene modified cellular vaccine (AGI-101H) (completed phase II)</td>
<td>(Kozłowska et al., 2013; Levy et al., 2013)</td>
</tr>
<tr>
<td>Photodynamic</td>
<td>It uses light-sensitive</td>
<td>Photofrin, Foscan</td>
<td>(Allison and)</td>
</tr>
<tr>
<td>Therapy</td>
<td>Drug, photosensitiser, and light to damage the target tissue selectively</td>
<td>Moghissi, 2013; Brown et al., 2004</td>
<td></td>
</tr>
<tr>
<td>------------------------------</td>
<td>-------------------------------------------------------------------</td>
<td>-------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Tyrosine kinase inhibitors</td>
<td>They compete to bind the tyrosine kinase receptors which have important role in tumour cell proliferation and interfere with cell proliferation</td>
<td>Imatinib, Gefitinib (Iressa), Trastuzumab, Centuximab (Ranieri et al., 2013)</td>
<td></td>
</tr>
<tr>
<td>Angiogenesis/metastasis inhibitors</td>
<td>They are used to inhibit the formation of new tumour blood vessels and aims to prevent metastasis</td>
<td>Bevacizumab (Avastin), Neovastat, Vitaxin (Mackey et al., 2012; Ranieri et al., 2014; Ribatti, 2009)</td>
<td></td>
</tr>
<tr>
<td>Gene silencing</td>
<td>It is used to silence genes related with tumour growth, angiogenesis, metastasis and resistance to chemotherapy</td>
<td>Oblimersen (Bora et al., 2012; Deng et al., 2014; Miele et al., 2012)</td>
<td></td>
</tr>
<tr>
<td>Prophylactic vaccination</td>
<td>Vaccines generate immune response either using tumour related antigens or tumour specific antigens and provide tumour specific prophylactic or therapeutic treatment</td>
<td>Vaccine for hepatitis B virus (HBV) and human papilloma virus types 16 and 18 (HPV16 and 18) (Aly, 2012)</td>
<td></td>
</tr>
<tr>
<td>Gene therapy</td>
<td>It is used either to Germ line gene (Fujita et al.,</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
replace the damaged gene with the healthy gene or to complete the missing gene to express the required protein which is deficient or missing in the body

<table>
<thead>
<tr>
<th>Drug Delivery Systems (DDS)</th>
<th>therapy (approved in United States of America)</th>
<th>2014; Ibraheem et al., 2014; Jia et al., 2012)</th>
</tr>
</thead>
<tbody>
<tr>
<td>They are used to carry the anti-cancer agents to the target tumour area and they are aimed to minimise the side-effects of anti-cancer agents and to maximize the drug efficiency on the target.</td>
<td>Abraxane®</td>
<td>(Gao et al., 2013; Haley and Frenkel, 2008; Kreuter, 2012)</td>
</tr>
<tr>
<td>Doxil™</td>
<td>DaunoXome™</td>
<td></td>
</tr>
</tbody>
</table>

1.6 Drug delivery systems for applications in cancer

It has been more than one century since Paul Ehrlich first suggested the idea of the “Magic Bullet” in the early 1900s for selectively targeting a drug to specific disease-causing cells or tissues (Ehrlich, 1907; Ehrlich and Sachs, 1905; Strebhardt and Ullrich, 2008). Such a concept has since been realised in antibodies and targeted drug delivery systems.

The aim of an effective drug delivery systems for the treatment of cancer can be listed as follows (Danhier et al., 2010):

- To reduce side effects and toxicity caused by chemotherapy
- To decrease the interaction of the anti-cancer agents with healthy tissues
- To protect drugs against possible premature degradation in the body
- To prolong the circulation time of the drug in the bloodstream
- To improve the therapeutic index of the drug by increasing the drug concentration at the tumour area over healthy tissues.
To overcome the solubility issues of hydrophobic drugs for parenteral drug delivery

To improve drug uptake and intracellular delivery

Nanoparticulate drug delivery systems offer an alternative to conventional chemotherapy providing a better life quality to patients with reduced side effects and enhanced anti-cancer efficacy (Pisano et al., 2013). Since 1970s, various types of drug delivery systems have been designed and developed for delivering the therapeutic agents to the desired area in the body; including lipid-based, polymer-based and protein-based nanosized drug carrier systems and are summarized in Table 1.3.

Table 1.3 Examples to drug delivery systems widely studied in cancer research

<table>
<thead>
<tr>
<th>Drug Delivery Systems</th>
<th>Main Excipient</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin-based Nanoparticles</td>
<td>Albumin; Ovalbumin (OVA), Bovine serum albumin (BSA), Human serum albumin (HSA)</td>
<td>(Elzoghby et al., 2012)</td>
</tr>
<tr>
<td>Liposomes</td>
<td>Phospholipids</td>
<td>(Maurer et al., 2001; Sharma and Sharma, 1997)</td>
</tr>
<tr>
<td>Polymeric Micelles</td>
<td>Amphiphilic polymers e.g. polyethylene glycol (PEG) and polyvinyl pyrrolidone (PVP)</td>
<td>(Lu and Park, 2013)</td>
</tr>
<tr>
<td>Polymer Conjugates</td>
<td>Water soluble synthetic polymers; e.g. PEG, poly(glutamic acid) (PGA) and N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers</td>
<td>(Vicent and Duncan, 2006)</td>
</tr>
<tr>
<td>Dendrimers</td>
<td>Polymers; e.g. Poly(amidoamine) (PAMAM), poly(propylene imine) (PPI) and poly-L-lysine</td>
<td>(Kesharwani et al., 2014)</td>
</tr>
<tr>
<td>Solid Lipid Nanoparticles</td>
<td>Solid lipids; e.g. triglycerides, partial glycerides, fatty acids and</td>
<td>(Mehnert and Mäder, 2012)</td>
</tr>
</tbody>
</table>
### Polymeric Nanoparticles

<table>
<thead>
<tr>
<th>Saturated phospholipids</th>
</tr>
</thead>
</table>

- Polymers; e.g. Poly(lactic-co-glycolic acid) (PLGA), Polylactic acid (PLA) and Polycaprolactone (PCL) (T. Patel et al., 2012)

Albumin-based particles are among the first published drug delivery systems in history in 1974 (Kramer, 1974), which led to the first clinically approved nanoparticles, paclitaxel-loaded albumin formulation (Abraxane®), for breast cancer treatment in 2005 and 2008 by the FDA and MHRA, respectively. Albumin-based nanoparticles are prepared using albumin protein; OVA, BSA or HAS (Table 1.3) and the therapeutic agent is incorporated into the particle matrix using electrostatic interactions between charged therapeutic agent and charged amino acids found in the structure of albumin (Elzoghby et al., 2012). Albumin is able to electrostatically adsorb either positively (e.g. ganciclovir) or negatively charged (e.g. oligonucleotide) molecules (Irache et al., 2005; Weber et al., 2000). The size range of these nanoparticles are generally found around 50 to 300 nm (Elzoghby et al., 2012).

Liposomes are bilayered spherical vesicles made up of natural or synthetic phospholipids, and were first reported by Bangham and his co-workers in 1964 for the study of biomembrane dynamics (Bangham and Horne, 1964). However, as a drug delivery system, liposomes were first introduced in 1970s by Gregoriadis and colleagues (Gregoriadis et al., 1974) and up to now, it is believed that they are one of the most studied drug carrier systems. Phospholipids are also important component of cell membrane structure. They are amphiphilic structures containing a hydrophobic tail and a hydrophilic head. Liposomes are formed by self-assembling of these amphiphilic phospholipids into bilayers. During the liposome formation, phospholipids arrange their hydrophobic tails toward the centre of the bilayer; whilst their hydrophilic heads lie on the outside the bilayer interacting with aqueous surroundings (Malam et al., 2009).

Liposomes are colloidal vesicles and due to the number of the layers and the size of the liposomes, they can be classified as;
Multilamellar vesicles (MLV) [100 nm – 10 µm]
Large unilamellar vesicles (LUV) [> 100 nm]
Small unilamellar vesicles (SUV) [25-100 nm] (Sharma and Sharma, 1997)

Five different liposomal formulations of the anti-cancer agents are available in the market (Table 1.4) such as doxorubicin (Doxil®) (Caelyx® in Europe) and (Myocet®), daunorubicin (DaunoXomeTM) and Cytarabine (DepoCyt®) have been approved for clinical use since 1990s and very recently approved (2012) vincristine (Onco-TCS, Marqibo®) which are all unilamellar formed liposomes and carrying water soluble anti-cancer drugs.

<table>
<thead>
<tr>
<th>Drug delivery system</th>
<th>Marketed Name</th>
<th>Formulation</th>
<th>Therapeutic agent</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposome</td>
<td>Doxil® (Caelyx® in EU)</td>
<td>PEGylated unilamellar liposome</td>
<td>Doxorubicin</td>
<td>Ovarian and breast cancer, Kaposi's sarcoma, multiple myeloma</td>
</tr>
<tr>
<td></td>
<td>DaunoXome</td>
<td>Unilamellar liposome</td>
<td>Daunorubicin</td>
<td>Kaposi's sarcoma</td>
</tr>
<tr>
<td></td>
<td>Myocet®</td>
<td>Unilamellar liposome</td>
<td>Doxorubicin</td>
<td>Breast cancer</td>
</tr>
<tr>
<td></td>
<td>DepoCyt®</td>
<td>Unilamellar liposome</td>
<td>Cytarabine</td>
<td>Leukaemia, glioblastoma, lymphomatous meningitis</td>
</tr>
<tr>
<td></td>
<td>Onco-TCS</td>
<td>Unilamellar liposome</td>
<td>Vincristine</td>
<td>Non-hodgkin lymphoma</td>
</tr>
<tr>
<td>Polymeric micelles</td>
<td>Genexol-PM®</td>
<td>PEG-PLA micelle</td>
<td>Paclitaxel</td>
<td>Lung, breast, ovarian cancer</td>
</tr>
</tbody>
</table>
In parallel to the studies of albumin-based and liposomal nanoparticles for drug delivery, polymer-based therapeutics was also developed as drug carriers. By the mid-1970s, first polymer-drug conjugates were proposed by Ringsdorf (1975) and his concept of covalently bound polymer therapeutic conjugates still forms the basis for much of the work carried out today in this area (Larson and Ghandehari, 2012). Polymer drug conjugates are nanoparticulate systems and consist of three parts; a water-soluble polymer backbone, a linker and the therapeutic agent. They can be classified into two main groups; polymer-drug and polymer-protein conjugates. Moreover, polymers can carry anti-cancer drugs to the target tumour tissues passively or actively through EPR effect by covalently conjugating the drug molecules to the polymer backbone using a suitable linker of interest (Vicent and Duncan, 2006).

SMANCS is the polymer-protein conjugate, designed and synthesised by Maeda and co-workers, (styrene co-maleic anhydrate polymer conjugated to neocarzinostatin anti-cancer protein) in 1990s which was the first polymer-protein conjugate approved for clinical use and released on market as anti-cancer therapeutic (Maeda, 2001a) to be used in the treatment of hepatocellular carcinoma (Duncan, 2006). Following this, another polymer-protein conjugate entered the anti-cancer therapeutics market, PEG-L-asparaginase (Oncaspar) which is used in the treatment of acute lymphoblastic leukaemia (Duncan, 2006).

<table>
<thead>
<tr>
<th>Polymer conjugate</th>
<th>Zinostatin</th>
<th>Styrene maleic anhydride (SMA)-neocarzinostatin (NCS) (SMANCS)</th>
<th>Oncaspar®</th>
<th>PEG-drug</th>
<th>Lasparaginase</th>
<th>Leukaemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin-based nanoparticles</td>
<td>Abraxane®</td>
<td>Albumin-drug</td>
<td>Paclitaxel</td>
<td>Breast cancer</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

One of the main drawbacks of polymer-drug conjugates is their wide polydispersity. Therefore, dendrimers offer a greater advantage of providing a relatively monodisperse systems. As first dendrimers; the repetitive growth of branching using low molecular
weight amines was first reported by Buhleier in 1978 (Buhleier et al., 1978). However the term “dendrimers” was first used by Tomalia’s group in 1984 publishing the synthesis of PAMAM dendrimers which then has become one of the most studied dendrimers (Tomalia et al., 1985, 1984).

Dendrimers are highly branched, three dimensional globular, mono-dispersed macromolecules which are prepared using either natural or synthetic polymers. They can be found in 1-100 nm size range (Kesharwani et al., 2014). Branched dendrimers are prepared by polymerisation of monomer units and the dendrimer size can be controlled by controlling the amount of monomers that undergo polymerisation. They contain three architectural components; the core, the branches and the terminal functional groups (Kesharwani et al., 2014) and are made up of layers which each layers forms a new generation and each generation doubles the end groups (active sites) of the dendrimers (Nanjwade et al., 2009). Similar to polymer-drug conjugates, their versatility allow them to include various drug molecules and targeting groups on the carrier system. Drugs/therapeutic agents can be conjugated either to the end groups or entrapped within the branches (Alexander-Bryant et al., 2013).

Following the development of polymer-based nanocarriers, polymeric micelles were also introduced as drug delivery systems which are formed by the self-assembly of amphiphilic polymers in aqueous solutions. The hydrophobic tails of the polymers assembles inside the micelles and form the core; whilst the hydrophilic heads of the polymers lie on the outside of the micelles and form the shell. This enables hydrophobic drugs to be entrapped inside the micelles, while hydrophilic drugs are adsorbed on the surface of the particles where they can interact with the hydrophilic components of the polymers. Generally, the average size of polymeric micelles is within the 20–80 nm range depending on the structure of the polymers employed (Lu and Park, 2013). Genexol-PM is a FDA (Food and Drug Administration) approved micellar paclitaxel formulation made up of PEG and poly(D,L-lactic acid) (PDLLA) and is currently under investigation at phase III/IV clinical studies in patients with recurrent breast cancer (Oerlemans et al., 2010).

Finally, in the early 1990s the concept of solid lipid microparticle was first introduced and the formulation aimed for oral drug delivery (Eldem et al., 1991). Later in mid-1990s Muller’s and Gasco’s research groups developed first solid lipid nanoparticles (SLNs) with applications to both cancer treatment and topical drug delivery (Morel et
al., 1998; Müller et al., 2002; Zara et al., 1999). The concept will be explained in detail in following section (Section 1.6.2). To improve the drug loading and release kinetics of SLNs, a second generation lipid nanoparticles which is known as nanostructured lipid carriers (NLCs) were proposed at the beginning of 2000s. While SLNs were made up of solid lipids, NLC formulations contain a mixture of both solid and liquid lipids in their structure providing less order lipid matrix (Das et al., 2012; Müller et al., 2002; Severino et al., 2012a, 2012b). The limitation of encapsulation water-soluble therapeutic agents by SLN and NLC formulations resulted in the emergence of third generation of SLNs where drug loading polymers were incorporated into the lipid matrix which is called polymer-lipid hybrid nanoparticles (PLN) (Hadinoto et al., 2013).

1.6.1 Solid lipid nanoparticles (SLNs) as drug delivery systems used for cancer

SLNs are spherical shaped colloidal particles and have a size distribution between 100 – 400 nm (Joshi and Müller, 2009). It has been suggested that SLNs combine the advantages of traditional drug delivery systems such as polymeric nanoparticles, liposomes and emulsions, meanwhile avoid or minimise the drawbacks associated with them. Proposed advantages include enhanced drug stability, improved drug loading, controlled release, increased physical stability, avoidance of using organic solvents and fast and effective production process (Mehnert and Mäder, 2012; Müller et al., 2000; Wissing et al., 2004; zur Mühlen et al., 1998)

SLN carriers are composed of solid lipids which are in solid state at both room and body temperatures (Mehnert and Mäder, 2012). During the formation of SLNs solid lipids are stabilised with emulsifiers in an aqueous dispersion and they show resemblance to nano-emulsions by replacing inner liquid lipid with solid lipids. Using of solid lipids instead of liquid oils reduces the mobility of associated drugs and provides controlled drug release (Mehnert and Mäder, 2012). While drug was dissolved or dispersed in the solid lipid matrix, surfactants are adsorbed on the surface of the SLNs to stabilise the lipid core and prevent the agglomeration of the particles (Mehnert and Mäder, 2001).

SLNs can be prepared using various lipids including triglycerides (Venkateswarlu and Manjunath, 2004), partial glycerides (K. Patel et al., 2012), fatty acids (Mohanty et al., 2014), steroids (Vighi et al., 2013) or waxes (Finke et al., 2013) and stabilised with lipid-based and polymer-based emulsifiers and water. SLNs are compatible with many surfactants (both ionic and non-ionic) including poloxamer 188, sodium glycocholate,
lecithin, polysorbate 80 etc. which are approved by drug regulatory agencies (Mehnert and Mäder, 2012).

Widely employed SLN preparation techniques consists of using high pressure homogenisation (HPH), ultrasonication and microemulsion methods (Mehnert and Mäder, 2012; Müller et al., 2011). Preparation of SLNs using HPH technique was first developed by Müller and co-workers at the beginning of 1990s (Müller et al., 2011, 1995). The technique relies on pushing the liquid formulation through a narrow gap (few microns) with high pressure (100 – 2000 bar) (Mehnert and Mäder, 2012; Wissing et al., 2004) and very short distance causes acceleration of the liquid to very high velocity. As a result, very high shear stress and cavitation forces breaks down particles to the submicron range (Mehnert and Mäder, 2012). HPH offers two different approaches to prepare SLNs, which are hot and cold homogenisation techniques (Mehnert and Mäder, 2012; Müller et al., 2011; Wissing et al., 2004). While hot homogenisation technique provides lower particle sizes and narrower size distribution when compared with cold homogenisation, cold homogenisation allows temperature sensitive-drugs to be formulated in SLNs more efficiently. Ultrasonication, on the other hand, relies on the cavitation in aqueous dispersions caused by powerful ultrasound where the cavitation results disintegration of the particles to submicron sizes (Mehnert and Mäder, 2012; Müller et al., 2011). The preparation steps of crude pre-emulsion for particle size reduction with hot homogenisation of HPH and ultrasonication techniques are the same where they include melting of the lipid and dissolving/dispersing the drug in the lipid with an appropriate surfactant mixture, pre-mix the dispersion using high speed homogeniser to form a crude pre-emulsion and finally reduction of particle size using either HPH or ultrasonication (Mehnert and Mäder, 2012). Preparation of SLNs using microemulsion technique was first developed by Gasco’s research group (Gasco, 1997) where hot microemulsion at 65 – 70 °C containing lipid, surfactant, co-surfactant and water, is dispersed in cold water (2 – 3 °C) under stirring (Mehnert and Mäder, 2012; Wissing et al., 2004). Typical volume ratios of hot microemulsion to cold water are around 1:25 to 1:50, v/v (Mehnert and Mäder, 2012).

Each SLN preparation technique has its own advantages and disadvantages. Whilst HPH provides a better opportunity for scaling up the formulations, there are various different parameters that need to be optimised for a successful SLN preparation, which includes size of nozzle gap, pressure of the system and the number of cycles (Mehnert and Mäder, 2012). These ultimately result in longer SLN optimisation time and the common problem of nozzle blockage during production is a challenge. Preparation of SLNs with microemulsion technique may not require advanced particle size reduction.
equipment. However, extensive dilution of the formulations results in lower lipid and drug content compared to HPH technique (Mehnert and Mäder, 2012). Additionally, high concentration of surfactants and co-surfactants are necessary to achieve small particle sizes and this may cause undesirable toxicities (Mehnert and Mäder, 2012; Wissing et al., 2004). Furthermore, ultrasonication is an easier, relatively quicker and a cheaper technique to apply in comparison to HPH; however ultrasonication may cause small amounts of undesired microparticles and metal contamination (Mehnert and Mäder, 2012; Wissing et al., 2004). Nevertheless, these can be purified using centrifugation and filtration techniques which was also suggested in the literature previously (Garanti et al., 2016; Hellstern et al., 2001; Luo et al., 2006). It is important to note that both HPH and ultrasoniation methods also result in a rise in temperature of the formulation, which may damage temperature-sensitive drugs or excipients. In this project, SLNs were prepared using ultrasonication method due to the quicker application time, easier handling of the instrument, avoidance of blockage risk during production and a good control and reproducible batch production by controlling the temperature using ice-baths.

SLN formulations have been widely studied for various administration routes, including pulmonary (Liu et al., 2008), oral (Harde et al., 2011), topical (Souto et al., 2007), ocular (Shen et al., 2010) and parenteral administration (Joshi and Müller, 2009; Wissing et al., 2004). Indeed, a broad range of drugs has been successfully encapsulated into SLNs and studied for the treatment of different diseases, such as fungal (Patel and Patravale, 2011) and HIV infections (Aji Alex et al., 2011), Parkinson’s Disease (Tsai et al., 2011), schizophrenia (Kumar and Randhawa, 2013), and cancer (Wong et al., 2007) etc. Besides small molecular drugs, various macrotherapeutic agents have also been successfully incorporated into SLNs like peptides and proteins (Almeida and Souto, 2007; Fan et al., 2014), and nucleic acids (Bondi et al., 2007; Montana et al., 2007; Torrecilla et al., 2015).

Currently, commercial use of SLNs is limited in cosmetic industry (Pardeike et al., 2009) and to our knowledge, no available pharmaceutical SLN formulations have reached the clinical phase. This could be because SLN technology is relatively a new area when compared with other lipid- and polymer-based nanoparticles and hence more studies may be required before entering the clinical trial stage. However, the success of lipid-based drug delivery systems such as liposomes in clinical applications gives hopes for SLN systems since both systems are made up of physiological lipids and there is already a wealth of knowledge regarding the metabolism and potential effects of these lipids. Liposomes, as previously mentioned in Section 1.6, have various
successful examples in the market like Doxil® and DaunoXome, along with other products that are currently in clinical trials (Allen and Cullis, 2013; Chang and Yeh, 2012). For example, DiCERNA Encore DCR-MYC liposomal-based lipid nanoparticle which recently entered into Phase Ib/2 trial aims to treat patients with hepatocellular carcinoma using small inhibitory RNA (siRNA) as therapeutic agent (Esmond and Chung, 2014). The potential of delivering macromolecules using these lipid-based drug delivery systems opens up another avenue for improved therapeutic applications.

The pre-clinical investigations of SLN formulations have shown potential for applications for cancer chemotherapy (Kim et al., 2015; Williams et al., 2003; Zara et al., 2002). Table 1.5 and 1.6 show the recent examples of pre-clinical studies of SLNs to target the tumour areas via intravenous administration. Both passive (Table 1.5) and active (Table 1.6) targeting methods have been employed for SLN drug delivery of anti-cancer agents. Current results show that the incorporation of anti-cancer agents into SLNs have significantly improved its efficacy when compared to free drug solutions (Jose et al., 2014; Singh et al., 2016). SLNs have also shown to prolong circulation time and improve drug accumulation to tumour areas as well as demonstrating better tumour regression than free drug solutions (Kim et al., 2015; Mosallaei et al., 2013). Conjugation of an active targeting ligand onto the surface of SLNs has displayed even more accumulation of the drug in the tumour sites and enhanced efficiency compared to non-active targeted counterparts (Han et al., 2014; Jain et al., 2015). Furthermore, it was reported that PEGylation of the SLNs helped to increase circulation time of particles and escape from macrophage uptake (Jang et al., 2016). It is worth to emphasise that anti-cancer agents incorporated into SLN formulations showed better therapeutic efficacy compared to their marketed products (Kim et al., 2015; Mosallaei et al., 2016, 2013; Singh et al., 2016) as well as displaying lower side effects (Yuan et al., 2014), which reinforce the possibility of developing SLNs in pharmaceutical markets for anti-cancer therapy in the near future.
Table 1.5 Examples of drugs encapsulated in SLNs and their potential benefits to target tumours via passive targeting following intravenous administration

<table>
<thead>
<tr>
<th>Active SLN System</th>
<th>Target Pre-clinical stage</th>
<th>Relevant effects</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Docetaxel SLN</td>
<td>Colorectal and Melanoma</td>
<td>In vitro and in vivo ● Higher cell uptake ● Improved tumour regression compared with marketed product (Taxotere)</td>
<td>(Mosa Illaei et al., 2013)</td>
</tr>
<tr>
<td>Camptothecin PEG-SLN</td>
<td>Colon Cancer</td>
<td>In vitro and in vivo ● Prolonged circulation and increased tissue distribution ● Reduced macrophage uptake ● Enhanced tumour accumulation</td>
<td>(Jang et al., 2016)</td>
</tr>
<tr>
<td>Resveratrol SLN</td>
<td>Brain Cancer</td>
<td>In vitro and in vivo ● Increased concentration of resveratrol in brain compared to free resveratrol solution</td>
<td>(Jose et al., 2014)</td>
</tr>
<tr>
<td>Docetaxel SLN</td>
<td>Breast Cancer</td>
<td>In vitro and in vivo ● Similar extend of therapeutic efficacy observed with marketed formulation (Taxotere) ● Reduced myelosuppression toxicity to bone marrow cells, a side effect seen with Taxotere</td>
<td>(Yuan et al., 2014)</td>
</tr>
<tr>
<td>7-Ethyl-10-hydroxycamptothecin (SN38) PEG-SLN</td>
<td>Colorectal Cancer</td>
<td>In vitro and in vivo ● Enhanced in vitro efficiency compared to free drug solution ● Higher in vivo therapeutic efficacy compared to marketed product, irinotecan</td>
<td>(Mosa Illaei et al., 2016)</td>
</tr>
<tr>
<td>Temozolomide SLN</td>
<td>Brain Cancer</td>
<td>In vitro and in vivo ● Increased Temozolomide concentration in brain compared to free Temozolomide solution ● Reduced toxicity seen compared to free Temozolomide</td>
<td>(Huang et al., 2008)</td>
</tr>
</tbody>
</table>
Table 1.6 Examples of drugs encapsulated in SLNs and their potential benefits to target tumour area via active targeting following intravenous administration

<table>
<thead>
<tr>
<th>Active</th>
<th>SLN</th>
<th>Ligand</th>
<th>Target</th>
<th>Pre-clinical stage</th>
<th>Relevant effects</th>
<th>Ref.</th>
</tr>
</thead>
</table>
| Docetaxel       | SLN   | Lactoferrin | Brain Cancer            | *In vitro* and *in vivo* | ● Increased DTX concentration in brain compared with those of SLN and marketed formulation (Taxotere)  
● Improved particle accumulation in brain                                                                                                               | (Singh et al., 2016)          |
| Paclitaxel      | PEG-SLN | Cetuximab | Lung and Breast Cancer | *In vitro* and *in vivo* | ● Enhanced *in vivo* stability  
● Improved tumour accumulation of targeted nanoparticles compared to non-targeted SLNs  
● Better tumour regression compared to marketed products (Taxol and Genexol-PM)                                                                   | (Kim et al., 2015)            |
| Doxorubicin     | SLN   | Galactose   | Lung Cancer             | *In vitro* and *in vivo* | ● Higher cellular uptake, higher efficiency and cytotoxicity was observed compared to non-targeted SLNs and Dox solution                                                                                           | (Jain et al., 2015)           |
| Docetaxel and Curcumin | PEG-SLN | Folic acid | Breast Cancer           | *In vitro* and *in vivo* | ● An improved therapeutic effect observed after folic acid conjugation  
● Co-encapsulation of actives improved docetaxel efficacy                                                                                              | (Pawar et al., 2016)          |
| Plasmid DNA and Doxorubicin | PEG-SLN | Transferri n | Lung Cancer             | *In vitro* and *in vivo* | ● Using active targeting ligand improved tumour accumulation and therapeutic efficacy  
● Combination of chemotherapy and gene therapy improved the efficacy of treatment                                                                   | (Han et al., 2014)            |
Since SLNs are made up of lipids that are in the solid state at room and body temperature, SLNs provide better particle stability in terms of drug leakage and particle aggregation in comparison to other lipid-based drug delivery systems such as nanoemulsions and liposomes that exploit lipids in the liquid state (Mehnert and Mäder, 2012; Wissing et al., 2004). Furthermore, in contrast to polymer-based nanoparticles, SLNs do not contain organic solvents that can potentially cause toxic clinical effects and SLNs also have the feasibility of to scaling up (Mehnert and Mäder, 2012). Additionally, SLNs provide excellent compatibility with hydrophobic drugs like asiatic acid, provide controlled drug release, prolong drug circulation time, protect drugs from pre-mature degradation and the possibility of active targeting (Mehnert and Mäder, 2012; Müller et al., 2011; Üner and Yener, 2007; Wissing et al., 2004; Wong et al., 2007). SLNs are made up of physiological lipids and excipients that are known as GRAS (generally recognised as safe) which minimise the potential of unexpected toxicities (Mehnert and Mäder, 2012; Müller et al., 2011). More importantly, the feasibility of producing nano-sized SLNs allow intravenous delivery to circulate in the microvascular system and exploit the tumour microenvironment for targeted drug delivery via EPR effect which will be explained in detail in Section 1.9.1 (Mehnert and Mäder, 2012; Torchilin, 2006; Wong et al., 2007). Besides, SLNs have also been shown to prevent macrophage uptake by coating the surface with hydrophilic PEG coating (Jang et al., 2016; Üner and Yener, 2007). These advantages provide the bases of the proposed research study.

1.7 Barriers for drug delivery systems with parenteral administration

Administration of drug-targeting systems generally relies on parenteral drug delivery. The difficulty of drug administration in in vivo studies is the body’s defence mechanism which protects itself against incoming foreign materials (i.e. drugs and drug carriers) using various physical, chemical and immunogenic barriers (Perrie and Rades, 2012).

Clearance of foreign materials via the kidney is limited because kidney is able to excrete particles with size under 10 nm (Venturoli and Rippe, 2005). On the other hand, due to the relatively much larger sizes of drug carriers, the body uses mononuclear phagocyte system (MPS) and opsonisation to remove these large particles from the body which makes the MPS as the biggest barrier in the systemic circulation for a drug carrier (Jones et al., 2013). MPS (also known as the reticuloendothelial system, RES) is a defence mechanism of the body which eliminates and removes foreign substances; such as proteins and bacteria. The MPS includes
Kupffer cells of the liver, alveolar macrophages of the lung, the spleen cells, lymph nodes and bone marrow as fixed cells; and blood monocytes and tissue macrophages as mobile cells (Hillery et al., 2002).

For example, when a drug carrier enters into the polar aqueous medium of systemic circulation, it makes direct contact with plasma proteins including immunoglobulins and complement proteins (Chonn et al., 1992). The surface of the foreign particles is tagged by these plasma proteins (opsonins) which is also known as opsonisation, to provide easier identification for MPS clearance (Senior, 1987). The phagocytes have the specific recognition sites to bind these opsonins. As a result, phagocytes easily recognise the undesired particles in the circulation which then engulf and remove them from the bloodstream (Hillery et al., 2002; Perrie and Rades, 2012).

Therefore to avoid opsonisation, MPS recognition and receptor-mediated phagocytosis, drug carrier systems should have minimised interactions with plasma proteins inside the systemic circulation.

1.8 Significant factors to avoid the clearance of drug delivery systems

There are significant factors that play important roles on opsonisation and clearance of drug delivery systems; like particle size (Carrstensen et al., 1992), surface charge (Malik et al., 2000) and hydrophilicity/hydrophobicity of the surface (Carrstensen et al., 1992; Gabizon and Papahadjopoulos, 1992). The effects of these parameters on clearance are explained as follows:

- **Particle size**: The particles having sizes above 100 nm are tend to be cleared by macrophages of the MPS (Alexis et al., 2008; Perrie and Rades, 2012). As it is studied by Carrstensen et al. (1992), MPS uptake is size-dependent and clearance increases with increasing the particle size. Therefore, to avoid the uptake by macrophages, it is necessary to prepare particles less than 100 nm in size.

- **Surface charge of the particles**: Charged particles are removed faster than uncharged or slightly-charged particles (Carrstensen et al., 1992). When positively charged particles enter into the systemic circulation, they have higher chance to interact with the biological components in the blood stream and this may cause quick uptake by the MPS (Malik et al., 2000). The study had performed by Malik et al. (2000) showed that positively charged dendrimers are
more haemolytic than negatively charged dendrimers which makes cationic particles not suitable for parenteral administration.

- **Hydrophilicity/hydrophobicity of the particles:** The particles with hydrophobic surface are rapidly recognised as foreign and thus opsonised by plasma proteins. Therefore to decrease the opsonisation, hydrophilic coating provided by the surface grafting of polymers is generally used to mask the system. The commonly used polymer is the poly(ethyleneglycol) (PEG). PEGylation or sometimes known as “stealth” coating provides steric stabilisation. Coating the nanoparticles with PEG polymer increases the hydrophilicity of the surface of drug delivery systems and provides protection of carriers against the varied blood opsonins by creating steric hindrance with highly hydrated groups of PEG. PEGylation minimises the electrostatic and hydrophobic interactions with plasma proteins (Jokerst et al., 2011). As a result PEGylation provides a longer circulation time for drug carriers to escape from the circulation and to reach the target area. Studies conducted by Allen et al. (1991) and Blume and Cevc (1990) are successful examples for the significant reduction of macrophage uptake *in vitro* and improvement of circulation time *in vivo* of PEGylated liposomes. Since PEG provides many advantages like high solubility in aqueous systems; low toxicity, immunogenicity and antigenicity inside the body; and avoidance MPS clearance, it is employed in many particulate drug delivery systems as coating agent (Torchilin, 2012).

It is worth mentioning that a therapeutic agent or its carrier system needs to stay in the blood stream for a sufficient time to be able to reach the target tissues and to mediate its action. Therefore it is extremely important to design a drug delivery system that is shielded from the MPS clearance to have sufficient circulation time in the body.

### 1.9 Targeted drug delivery

Drug targeting can be achieved either by passive targeting, where the drug carrier is designed to exploit the natural conditions of the target tissue/organ to provide the preferred accumulation of the drug at the desired area, or by active targeting, where a targeting group (ligand) is associated with the drug delivery system and/or the drug to enable specific interaction with the desirable target site or receptors on target tissues.
1.9.1 Passive Targeting

The tumour vasculature is different to normal vasculature. As mentioned before, it has incomplete leaky vessels, lack of pericytes lining which surrounds endothelial cells and has abnormalities in the basement membrane due to the rapidly dividing endothelial cells during tumour angiogenesis (Bergers and Benjamin, 2003; Carmeliet and Jain, 2000; Hashizume et al., 2000). The fenestrations between these endothelial cells are larger than the fenestrations in normal endothelial lining (Bergers and Benjamin, 2003; Carmeliet and Jain, 2000; Hashizume et al., 2000). These gaps can be found of sizes between 100 – 780 nm, depending on the type of cancer (Byrne et al., 2008; Hobbs et al., 1998). Therefore it is easier for nano-carriers with sizes less than 200 nm to pass through the tumour endothelium. Moreover, drug delivery systems tend to have longer retention times in tumour area due to the poor lymphatic drainage (Byrne et al., 2008; Haley and Frenkel, 2008b), as a result this provides longer interaction time for drugs/nano-carriers with tumour tissues. This is also known as Enhanced Permeation and Retention (EPR) effect, which was first introduced by Maeda using SMANCS (Maeda, 2001b; Maeda et al., 2000). Figure 1.5 illustrates this phenomenon.

![Diagram of normal tissue vs tumour tissue](image)

**Figure 1.5 Difference in tumour and normal vasculature.** Passive targeting of nanocarrier systems using the EPR effect (adapted from Prakash et al. (2011)).
1.9.2 Active Targeting

Active drug targeting relies on the specific interactions between the ligand and its receptor. A typical actively targeted drug delivery system includes three components; a therapeutic agent, a drug delivery system and the targeting ligand as illustrated in Figure 1.6.

![Figure 1.6 Three components of a typical active targeted drug delivery system; containing the drug, carrier and the targeting group.](image)

The targeting group is chosen to interact with a receptor / recognition site that is overexpressed by tumour cells or the tumour vasculature and not expressed or slightly expressed by normal tissue which this allows selective active targeting to the tumour cells or tumour vasculature. The aim of active targeting is to improve cellular internalisation and accumulation of the therapeutic agent by the tumour cells and minimises the interaction with normal cells. The ligand targeted drug delivery systems accumulate inside the tumour vasculature via the EPR effect and then they are internalised by tumour cells/endothelium via ligand-receptor interactions (Inuma et al., 2002).

While designing the actively targeted carriers, the target can be either tumour cells or tumour vascular endothelial cells (Byrne et al., 2008). For example, transferrin plays an important role in the regulation of tumour growth and overexpression of transferrin receptors are seen on cancers cells (Gatter et al., 1983; Yang et al., 2009) which makes them as a potential target. Up to now three different transferrin-targeted drug delivery systems achieved to enter in clinical studies which are CALAA-01 (Phase I), a transferrin receptor-targeting cyclodextrin-based nanoparticle containing siRNA directed against the M2 subunit of ribonucleotide reductase (Safety Study of CALAA-01 to Treat Solid Tumor Cancers, 2016); MBP-426 (Phase Ib/II), a transferrin receptor-targeting liposome containing oxaliplatin (Study of MBP-426 in Patients With Second Line Gastric, Gastroesophageal, or Esophageal Adenocarcinoma, 2016) and SGT-53 (Phase I), a transferrin receptor-targeting liposome containing plasmid DNA encoding p53 (Safety Study of Infusion of SGT-53 to Treat Solid Tumors, 2016). The success of
transferrin-targeted nanocarriers indicates the potential of actively targeted nanoparticles for application in cancer chemotherapy. Besides transferrin, folate receptors, epidermal growth factor receptors and glycoproteins (type of glycoprotein differs due to the type of cancer) are also highly expressed on the tumour cell surface (Table 1.7) which are widely exploited as targets for actively targeted drug delivery systems (Danhier et al., 2010).

On the other hand, targeting the tumour vasculature using nanoparticles aims to destroy tumour endothelium and/or prevent the formation of new blood vessels which indirectly results in the death of cancer cells with abolishing the oxygen and nutrient supply. It was first suggested by Judah Folkman in 1971 that tumours need blood vessel to survive and grow, however cutting off that blood supply, tumours could be starved into remission (J Folkman, 1971; Ribatti, 2008). Actively targeted drug delivery systems that can specifically bind to receptors on tumour blood vessels could improve the uptake and accumulation of the drug inside the endothelial cells resulting in enhanced efficacy. The examples of different target receptors and their ligands which are widely used in active targeting strategy are given in Table 1.7.

Table 1.7 Examples for target receptors and their ligands

<table>
<thead>
<tr>
<th>Target</th>
<th>Ligand</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transferrin receptor</td>
<td>Transferrin</td>
<td>(Yang et al., 2009; Yhee et al., 2013)</td>
</tr>
<tr>
<td>Folate receptor</td>
<td>Folate</td>
<td>(Low and Kularatne, 2009)</td>
</tr>
<tr>
<td>Glycoproteins</td>
<td>Lectin</td>
<td>(Bies et al., 2004; Clark and Mao, 2012)</td>
</tr>
<tr>
<td>The epidermal growth factor receptor (EGFR)</td>
<td>Epidermal growth factor (EGF), transforming growth factor-α (TGF-α), amphiregulin, betacellulin, heparin-binding EGF (HB-EGF), and epiregulin</td>
<td>(Laskin and Sandler, 2004; Mendelsohn and Baselga, 2006)</td>
</tr>
</tbody>
</table>
**HER-2**
Trastuzumab; a monoclonal antibody
(Harries and Smith, 2002; Martin-Castillo *et al.*, 2013)

**$\alpha_\text{v}\beta_3$ integrin**
RGD peptide
(Shan *et al.*, 2015; Shuhendler *et al.*, 2012)

### Targeting of tumour vasculature

<table>
<thead>
<tr>
<th>Target</th>
<th>Ligand</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular endothelial growth factors (VEGF)</td>
<td>rhuMAb VEGF (avastin); recombinant humanized monoclonal antibody to VEGF</td>
<td>(Bakbak <em>et al.</em>, 2016; Gordon <em>et al.</em>, 2001; Margolin <em>et al.</em>, 2001)</td>
</tr>
<tr>
<td>VEGF receptors (VEGFR-1) (VEGFR-2)</td>
<td>Monoclonal antibodies or tyrosine kinase inhibitors</td>
<td>(Tugues <em>et al.</em>, 2011)</td>
</tr>
<tr>
<td>$\alpha_\text{v}\beta_3$ integrin</td>
<td>RGD peptide</td>
<td>(Dubey <em>et al.</em>, 2011, 2004)</td>
</tr>
<tr>
<td>Vascular cell adhesion molecule-1 (VCAM-1)</td>
<td>Sulphoraphane</td>
<td>(Kim <em>et al.</em>, 2012; Shan <em>et al.</em>, 2012)</td>
</tr>
<tr>
<td>The matrix metalloproteinases (MMPs)</td>
<td>Inhibitors of MMPs (MMPI); e.g. Marimastat (BB2516), Prinomastat (AG3340), BMS 275291, BAY 12-9566, Neovastat (AE-941)</td>
<td>(Sridhar and Shepherd, 2003)</td>
</tr>
</tbody>
</table>

### 1.9.3 RGD-targeted strategies

Targeting $\alpha_\text{v}\beta_3$ integrin with RGD-based strategies including conjugation to small molecule drugs (Arap *et al.*, 1998; Burkhart *et al.*, 2004; Kim and Lee, 2004), therapeutic peptides and proteins (Dickerson *et al.*, 2004; Yokoyama and Ramakrishnan, 2004), to various drug delivery systems (i.e. polymers, liposomes, SLNs etc.) (Chang *et al.*, 2015; Miura *et al.*, 2013; Shuhendler *et al.*, 2012) and also as RGD-equipped imaging agents (Cai and Conti, 2013; Wang and Liu, 2013) have been widely studied (Temming *et al.*, 2005).
Introduction

Due to the expression of integrins both on tumour endothelial cells and various cancer cell types as well as their role in tumour progression and angiogenesis, provides double targeting and makes them important therapeutic targets. Furthermore, integrin $\alpha_\beta_3$ receptors are known to be poorly expressed on non-angiogenesis activated endothelial cells which provides selective targeting for the RGD-containing therapeutics. Moreover, therapeutics designed to target the $\alpha_\beta_3$ integrin may also behave as antagonist (like Vitaxin, a humanised monoclonal antibody against the $\alpha_\beta_3$ integrin) by interfering the binding of receptors to its natural ligands (Gutheil et al., 2000). As a result, a RGD-containing peptide-targeted chemotherapy might achieve a dual effect of drug targeting and receptor antagonism as well.

Recently published RGD-conjugated SLNs (Shan et al., 2015; Shuhendler et al., 2012) demonstrated a significant improvement in tumour accumulation in vivo and showed promising ability to prevent metastasis compared to control. Moreover, conjugation RGD peptide to liposomes provided enhanced delivery of the anti-cancer p53 gene into both the endothelial and tumour cells and led to a significant tumour growth inhibition in a syngeneic mouse tumour model (Mondal et al., 2013). Additionally, Qin et al. (2014) showed the enhanced and precise targeting effect of dual targeted liposomes with transferrin and RGD peptide compared with the transferrin-targeted liposomes or RGD-targeted liposomes which demonstrates promising anti-cancer efficacy of RGD-targeted systems.

1.10 Asiatic acid (AA)

AA, a pentacyclic triterpenoid (Figure 1.7), is one of the active components of Centella Asiatica which is a medicinal plant generally found in Asia including India and China. Centella Asiatica is widely used in Chinese and Indian traditional medicine and has four different active components which are; asiaticoside, madecassoside, madasiatic acid and asiatic acid and these components were first isolated by Bontems back in 1941 (Bontems, 1941).
Introduction

Currently, AA is used as the active constituent in the cosmetic industry for skin and hair treatments and it is also employed in the wound healing (Abdul et al., 2002). Both asiatic acid and asiaticoside are the components of Madecassol which is a titrated extract of Centella Asiatica. Madecassol is an approved commercial medicine and is used for the treatment of keloids, the proliferation of connective tissue, and hypertrophic scars via oral administration (Abdul et al., 2002).

Recent studies have demonstrated the various possible therapeutic applications of AA. For example, AA showed anti-oxidant feature which significantly suppressed the ultraviolet-A-modulated reactive oxygen species production and lipid peroxidation which cause pre-mature aging (Soo Lee et al., 2003). Furthermore, AA contributed to the regulation of the carbohydrate metabolism by modulating the key regulatory enzymes in diabetic rats (Ramachandran and Saravanan, 2013). Moreover, AA demonstrated anti-inflammatory (Huang et al., 2011; Punturee et al., 2004), hepatoprotective (Ma et al., 2009) and also protective effect against neurotoxicity (Jew et al., 2000; Krishnamurthy et al., 2009).

In addition, as shown in Table 1.8, AA also showed anti-cancer activities against various cancer cell lines such as SK-MEL-2 human melanoma cells (Park et al., 2005), HepG2 human hepatoma cells (Lee et al., 2002) and U87 MG human glioblastoma cells (Cho et al., 2006). AA displayed apoptosis-induced cancer cell death with SW480 human colon cancer cell (Tang et al., 2009), U87 MG glioma cells (Cho et al., 2006), MCF-7 and MDA-MB-231 breast cancer cells (Hsu et al., 2005) through mitochondrial apoptotic pathway by the activation of both caspase 9 and caspase 3. It was also reported by Park et al. (2005) that AA induces time- and concentration- dependent apoptosis in human melanoma SK-MEL-2 cells through generation of reactive oxygen species and activation of cascade-3. Although AA caused cell death via apoptosis for all the cancer cell lines reported, cell cycle arrest was shown at different phases for

Figure 1.7 Diagram showing the molecular structure of asiatic acid.
Introduction

different cell lines (Table 1.8) (Hsu et al., 2005; Ren et al., 2016; Zhang et al., 2013). Most importantly, recent in vivo animal studies of anti-cancer effect of AA have displayed significant inhibition of in vivo tumour volume and weight for glioma and lung cancer (Kavitha et al., 2014; Wu et al., In Press). It was also reported by Kavitha et al. (2014) that AA reduced the viability of glioma cells with better efficacy than temozolomide at equimolar concentrations, which is a marketed medicine for glioma treatment. Besides anti-tumour activity, AA was also reported to show anti-angiogenic properties as well (Kavitha et al., 2011) via inhibiting the pro-angiogenic effect of VEGF. As it was previously explained in Section 1.2, malignant tumours induce angiogenesis because they need blood vessels to be able to grow and metastasise. Therefore, this anti-angiogenic property of AA is also a very important factor for the consideration of this project, where a potential of achieving a double therapeutic effect (anti-tumour and anti-angiogenic effects) against tumours and a stronger anti-cancer efficacy can be realised for this investigation.

Table 1.8 Pre-clinical anti-cancer effect of AA on various different cancer cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Pre-clinical stage</th>
<th>Relevant effects and mechanism of action</th>
<th>Ref.</th>
</tr>
</thead>
</table>
| HL-60 human leukemia cells | In vitro | ● Time and concentration cytotoxicity  
● Inhibition of cell proliferation through dose-dependent apoptosis  
● Induction of apoptosis by downregulating anti-apoptotic proteins Bcl-2, Mcl-1 and surviving  
● Inhibition of ERK and p38 phosphorylation, intracellular signalling proteins | (Wu et al., 2015) |
| LN18, U87 MG, and U118 MG human glioblastoma cells | In vitro and in vivo | ● Reduction of glioma cell viability with better efficacy than temozolomide at equimolar concentrations  
● Reduction of tumour volume in vivo  
● Reduction U87 MG xenografts growth in vivo  
● Capable to cross blood brain barrier  
● Inhibition of cell proliferation by inducing apoptosis via activating caspases, downregulating Bcl2 family members and surviving, inducing endoplasmic reticulum stress and increase free intracellular calcium release | (Kavitha et al., 2014) |
| SW620 human colon adenocarcinoma, HepG2 human hepatoblastoma | In vitro | ● Concentration-dependent cytotoxicity  
● Cell death mainly caused by apoptosis | (Jirasripongpun et al., 2012) |
<p>| MCF-7 and | In vitro | ● Inhibition of cell growth by inducing apoptosis | (Hsu et |</p>
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Experiment</th>
<th>Effects</th>
</tr>
</thead>
</table>
| MDA-MB-231 | In vitro | ● Activation of ERK1/2 and p38, pro-apoptotic function  
● Activation of caspase 9 pathway  
● Inhibition of cell cycle arrest at the S-G2/M phase via enhancing p21/Cdc2 interactions and reducing the expression of cyclinA, cyclinB1, Cdc25C, and Cdc2 |
| U87 MG | In vitro | ● Induction of time- and concentration-dependent cell death  
● Cell death mainly due to apoptosis  
● Induction of apoptosis through activation of caspase 3 and 9 pathways and due to increase in free calcium levels |
| SW480 | In vitro | ● Inhibition of cell growth via apoptosis through increase in mitochondrial membrane permeability and release of mitochondrial cytochrome c  
● Activation of caspase 9 and caspase 3 pathways |
| A549 and H1299 | In vitro and in vivo | ● Induction of concentration- and time-dependent apoptosis via mitochondrial damage and generating reactive oxygen species  
● Significant inhibition of in vivo tumour volume and weight  
● Induction of apoptosis in vivo  
● Reduction in the production of proliferating cell nuclear antigen |
| RPMI 8226 | In vitro | ● Concentration- and time-dependent inhibition of cell growth  
● Cell cycle arrest at the G2/M phase  
● Reduction of FAK levels and inhibition of signal transduction mediated by FAK |
| SKOV3 and OVCAR-3 | In vitro | ● Cell cycle arrest at the G0/G1 phase  
● Inhibition the expression of proteins CDK4, CDK6, CDK2, cyclin E and cyclin D1 and enhanced the expression of the CDK inhibitors p27 and p21 which takes role in cell cycle arrest  
● Cell death mainly by apoptosis through caspase 9 and 3 activation and alteration of the Bax-to-Bcl-2 ratio  
● Inhibition of PI3K/Akt/mTOR signalling which is important for cell survival and proliferation |
| HepG2 | In vitro | ● Concentration-dependent cell cytotoxicity and apoptosis  
● Induction of apoptosis through increased expression of p53 and elevated release of free intracellular calcium |
| SK-MEL-2 | In vitro | ● Concentration- and time-dependent cell cytotoxicity and apoptosis  
● Increase in intracellular reactive oxygen species  
● Increase in Bax levels, pro-apoptotic factor  
● Induction of apoptosis through caspase 3 activation |
Promising successful *in vitro* and *in vivo* anti-cancer efficacy of AA against various different cancer cell lines and xenograft proves that AA has potential chemotherapeutic activity and this supports further pre-clinical and clinical evaluations of AA against cancer. However, the main problem with AA is its poor water solubility. It is practically insoluble in water and has an aqueous solubility of 0.03 mg/mL (Liu *et al*., 2009) which makes it a challenging task to formulate AA for parenteral administration. In this project, AA was encapsulated in solid lipid nanoparticles (SLNs) to overcome its solubility problems and enable AA to be delivered via intravenous administration using a suitable formulation with targeting ability to the tumour sites to enhance the therapeutic efficacy of AA.

1.11 Spheroids as an *in vitro* 3D tumour spheroid model

Multicellular tumour spheroids are 3-dimensional (3D), spherical cell clusters which are formed by self-assembly (Mehta *et al*., 2012) and was first adapted for cancer research by Sutherland and co-workers in the early 1970s (Inch *et al*., 1970; Sutherland *et al*., 1971). Studies have indicated that 3D tumour spheroids show better resemblance to *in vivo* tumour tissues than that of 2-dimensional (2D) monolayer cell cultures which are widely used for *in vitro* cancer studies (Goodman *et al*., 2008; Mehta *et al*., 2012; Sarisozen *et al*., 2014). Tumour spheroids with 3D architecture mimic *in vivo* tumours displaying similar microenvironment; pathophysiology and complexity as well as having similar cell morphology, gene expression, growth kinetics and drug response (Barrera-Rodríguez and Fuentes, 2015; De Witt Hamer *et al*., 2007; Goodman *et al*., 2007; Hirschhaeuser *et al*., 2010; Mehta *et al*., 2012; Sarisozen *et al*., 2014).

It was reported as spheroids having larger diameters than 400 µm develop zones (Figure 1.8) (Friedrich *et al*., 2009; Mehta *et al*., 2012). The outer layer of the spheroids is called proliferation zone where cells of the spheroid actively proliferates, whereas inner layer is called quiescent zone and in this zone cells are in non-proliferative state due to limited oxygen and nutrient supply. Spheroids with diameters larger than 500 µm develop secondary necrosis in the centre which this can also be seen in some *in vivo* tumours as well (Friedrich *et al*., 2009; Mehta *et al*., 2012).
Figure 1.8 The structure of 3D tumour spheroids. Spheroids show a gradient of oxygen, metabolites and nutrients within themselves. This results in central necrotic core which is surrounded by quiescent viable cells and actively proliferating peripheral cells. (Figure was copied without permission from Mehta et al. (2012))

Despite to 2D monolayer cells, 3D tumour spheroids provide suitable platform to study the penetration of drugs and drug delivery systems into spheroids (Hirschhaeuser et al., 2010; Mehta et al., 2012). Cells of spheroids are closely packed presenting tight junctions and cell-cell interactions similar to in vivo studies (Kunz-Schughart et al., 2004; Mehta et al., 2012). Additionally, they show mass transport limitations of oxygen, nutrients and metabolites where gradients of oxygen and nutrient distribution is seen which this causes gradient proliferation profile and accumulation of metabolic waste inside the spheroids as well as formation of hypoxic regions (Friedrich et al., 2009; Goodman et al., 2007; Kunz-Schughart et al., 2004; Mehta et al., 2012). Therefore these physiological barriers serve resemblance to the in vivo drug delivery which provides relevant 3D model for studying penetration of drugs and drug delivery systems.

Besides providing better model for penetration studies, spheroids serve suitable 3D tumour model for studying multicellular resistant caused by upregulation of pathways and overexpression of efflux pump (Jang et al., 2003; Sarisozen et al., 2014). It has been reported that growing cells in monolayer cultures affect the phenotype of the cells and as a results affect their response to the anti-cancer treatments (Goodman et al., 2008). However, growing cells as 3D spheroids can preserve cellular genomic properties due to cell-cell communications and cell-matrix interactions (Barrera-Rodriguez and Fuentes, 2015; De Witt Hamer et al., 2007; Friedrich et al., 2009; Goodman et al., 2008; Oloumi et al., 2002). Due to the physiological barriers served by
tumour spheroids, the efficacy of some drug candidates and nanoparticle systems have been reported as reduced compared to the results had obtained with 2D monolayer cells (Friedrich et al., 2009; Hirschhaeuser et al., 2010; Mehta et al., 2012).

Tumour spheroids are reported as to be in intermediate complexity (Kunz-Schughart, 1999; Mikhail et al., 2013) compared to monolayer cell cultures and complex *in vivo* tumours (Figure 1.9). 3D tumour spheroids were described as they mimic *in vivo* avascular tumour nodules, micrometastases or intervascular regions of large solid tumours (Friedrich et al., 2009; Ivascu and Kubbies, 2007). Due to the resemblance to *in vivo* tumours and providing high throughput screening convenience, it is believed that tumour spheroid models will contribute to economical savings in cancer research by reducing animal tests (Carver et al., 2014; Friedrich et al., 2009).

![Figure 1.9 3D tumour spheroid models as intermediary between 2D monolayer cell cultures and animal models. (Copied without permission from Mikhail et al. (2013))](image-url)
1.12 Aims and objectives of the project

1.12.1 Aim of the project

The main aim of this project was to investigate the possibility of improving the therapeutic efficacy of a poorly water-soluble drug, asiatic acid (AA) by incorporating it into a lipidic nano-based drug delivery system (solid lipid nanoparticles) that contain the RGD peptide for targeting to the $\alpha_\text{v}\beta_3$ integrin, which is overexpressed in cancer cells and the tumour vasculature and potentially mediate a double-killing effect. Considering the lack of effective drug delivery systems for AA and the extensive evidence in the literature for its potential in treating various conditions, including cancer and anti-inflammatory diseases, this drug warrants a systematic development of an effective drug delivery system for AA using a novel type of SLNs that have the ability to target cancer cells selectively. Therefore, the main objectives of this project were:

- To develop an effective SLN formulation to maximise the delivery capacity of AA.
- To study the compatibility and efficiency of AA entrapped in SLNs that are made from different glyceryl stearates and determine their in vitro efficacy of the AA-containing SLN formulations.
- To examine the effects of SLN surface modifications (PEGylation and RGD conjugation) on their physicochemical properties and in vitro anti-cancer efficacy of SLNs.
- To evaluate the potential mechanism of cell-killing induced by the AA-SLNs.
- To investigate the targeting ability of RGD-conjugated SLNs to cancer cells (U87 MG and ECV-304) containing $\alpha_\text{v}\beta_3$ integrin receptor.
- To explore the anti-cancer efficiency of RGD-targeted AA-containing SLN formulations with a 3D multicellular U87 MG tumour spheroid model.
- To analyse the possible anti-angiogenic potential of AA-containing RGD-SLNs towards endothelial-like ECV-304 cells.
1.12.2 Summary of the Chapters

**Chapter 1** – Illustrates the literature search related to the work undertaken in the project. This includes background information about cancer progression, metastasis, angiogenesis, the role of integrins in cancer, major challenges in the treatment of cancer, drug delivery systems as a novel treatment approach for cancer therapy, SLNs and rational and benefits of using RGD-based strategies.

**Chapter 2** – Presents all chemical materials and experimental methods used for this project. Additionally, development and validation of a novel HPLC method for the analysis of AA in AA-containing SLN formulations is described in this Chapter.

**Chapter 3** – Shows the development of suitable SLN formulations using three different glyceryl stearates and investigates the compatibility and efficiency of three different types of AA-loaded SLNs. The physicochemical characterisation of the particles (± AA) in terms of particle size, PDI, surface charge, entrapment efficiency/drug loading, and drug crystallinity were analysed using instruments of zetasizer, HPLC, DSC and XRD. Additionally, the most promising AA-loaded SLN formulations were chosen based on the *in vitro* anti-cancer effect towards U87 MG glioma cells for the subsequent investigations (cellular uptake studies and apoptosis assay) and surface modifications which were carried out in the next Chapter.

**Chapter 4** – Describes the surface modifications applied to SLN formulations and examines the targeting ability of RGD-conjugated SLNs. PEGylation of the particles was achieved by incorporating PEG stearates into the formulations. For the production of RGD-SLNs, RGD peptide was first covalently conjugated to PEG stearates by a two-step chemical synthesis where RGD-PEG stearate conjugates were obtained and followed with the addition of this RGD-PEG stearate conjugates into SLNs during the SLN manufacturing phase (Section 2.3.1). Both PEGylated and RGD-targeted SLNs were studied with two different PEG stearate molecules differing in chain length (PEG(40)stearate and PEG(100)stearate) and the effect of PEG chain length on their physicochemical properties and *in vitro* efficacy of SLNs was investigated.

**Chapter 5** – Analyses the effect of RGD-targeted AA-containing SLN formulations on 3D multicellular tumour spheroids. U87 MG cell spheroids were prepared and optimised using liquid-overlay method. The efficacy of RGD-targeted AA-loaded SLN formulations at preventing spheroid formation, inhibiting spheroid growth, spheroid penetration/uptake and cytotoxicity were investigated by comparing its effect observed with free AA and other AA-SLNs (AA-MS-SLNs and AA-PEG-SLNs).
Chapter 6 – Examines the anti-angiogenic potential of RGD-targeted AA-loaded SLNs using endothelial-like ECV-304 cells. In this chapter potential anti-angiogenic property of RGD-SLNs (± AA) was studied by testing its cytotoxicity (to stop proliferation of endothelial cells), cellular uptake (to show RGD targeting ability), inhibition of cell adhesion, migration and invasion (to test the inhibition ability of RGD-SLNs on endothelial cell-like model) and inhibition of tube formation (to examine the inhibition effect on angiogenesis) and the results were compared to the effect observed with free AA and other AA-SLNs (AA-MS-SLNs and AA-PEG-SLNs).

Chapter 7 – Presents a general conclusion for the project and gives possible future investigations that could be performed to provide further insights on the challenges encountered in the project.

Chapter 8 – Published references used for the thesis
Chapter 2

Materials and Methods
2.1 Apparatus

2.1.1 General laboratory equipment

Centrifuge used for the purification of the formulations was purchased from Sigma Centrifuge, Germany; while benchtop Spectrafuge 24D was from Jencons-Pls, UK. pH meter was obtained from Hanna Instruments, UK. Whatman filter papers were provided from Fisher Scientific, UK. Sonication of compounds was carried out with Ultrasonic cleaner from VWR International, UK. Hot plates used during the studies were from Fisher Scientific, UK. Vacuum oven was obtained from Townson & Mercer, UK. Freeze-dryer was obtained from ScanVac CoolSafe Freeze Drying, Labocene, Denmark. Incubator used for in vitro drug release studies was purchased from Sanyo Incubator, Japan. Filter device Amicon 0.5 mL centrifugal tubes (3K Da molecular weight cut-off, (MWCO), mini dialysis device Slide-A-Lyzer (3.5K Da MWCO 2mL) and HPLC vials were purchased from Fisher Scientific, UK. Spectra/Por® dialysis membrane (3.5K Da MWCO) was obtained from Spectrum Labs, USA.

2.1.2 Instruments used for the preparation of the SLNs

Organic solvents were evaporated using a Buchi rotary evaporator, Switzerland. Crude formulation was homogenised with ultra-turrax T8 homogeniser from IKA, Germany and probe sonicator was obtained from Vibra Cell Sonics, USA.

2.1.3 Cell culture equipment

All cell culture studies were performed in class II microbiological safety cabinets from Labcaire Systems Limited, UK. The incubation of the cells was carried out in Sanyo CO₂ incubator, Japan. Haemocytometer slide used for cell counting was obtained from Marienfeld, Germany. All sterile culture flasks (25 and 75 cm³), sterile plates (6-well, 12-well, 24-well and 96-well) (Nunc™ MicroWell™), sterile centrifuge tubes (15 and 50 mL), serological pipettes and Mr. Frosty™ freezing containers were purchased from Fisher Scientific, UK. Disposable syringes were obtained from Becton Dickinson, UK while Millex® sterile syringe filters (0.45 µm and 0.22 µm pore size) were from Millipore, UK. Inserts for 24-well plates, transparent PET (Polyethylene terephthalate) membrane, 8 µm pore size were obtained from VWR International, UK.
2.1.4 Microscopy equipment

Cells were visualised with Leica DMIL light microscope from Leica Microsystems GmbH, Germany whereas light microscope images were taken with MShot camera, China and processed with MShot Digital Imaging System software, China. Fluorescence imaging for monolayer cells was carried out with Carl Zeiss fluorescence microscope from Carl Zeiss Microscopy GmbH, Germany. Fluorescence images were taken with AxioCam MRm Zeiss camera, Germany and processed with Zen lite 2012 software, Germany. Fluorescence imaging for 3D tumour spheroids was conducted with Zeiss LSM510 confocal microscope from Zeiss LSM, Germany. Confocal images were studied using LSM/FCS software, Germany. Scanning electron microscopy (SEM) samples were gold coated under vacuum using JFC-1200 Fine Coater from JEOL, Japan and then studied using FEI Quanta 200 SEM, USA.

2.1.5 Analytical Instruments

Photon correlation spectroscopy, Malvern Zetasizer used for size/zeta potential/PDI measurements was purchased from Malvern Instrument, UK. Zeta potential cells which a specific for zeta potential measurements were also obtained from Malvern Instruments, UK.

High Performance Liquid Chromatography (HPLC) analysis was performed using Agilent HPLC 1260 Infinity from Agilent Technologies, USA and data was studied with Agilent’s software OpenLAB CDS CS Workstation, Agilent Technologies, USA. HPLC studies were carried out with a C18 reverse phase column, Synergi 4u Max-RP 80A (250 x 4.60 mm, particle size 4 µ) from Phenomenex Ltd, Germany.

DSC823e Mettler Differential Scanning Calorimeter (DSC) was obtained from Mettler Toledo, Switzerland. Flat base 40 µL standard DSC pans with non-pin-holed lid were used for the DSC analysis and purchased from Mettler Toledo, UK.

X-ray diffraction (XRD) studies were performed using the Bruker powder X-ray diffractometer, D2 PHASER with LYNXEYE™ detector from Bruker, Germany. The data was studied using Diffrac.Suite Eva software version 3.0, Germany.

Nuclear Magnetic Resonance (NMR) spectroscopy was carried out with ¹H NMR Bruker Fourier 300 (300 MHz) with broadband decoupling from Bruker, Germany. NMR tubes were obtained from GOSS Scientific, UK. NMR spectra were processed using MestReNova 10.0.2 software, Spain.
UV-Vis spectrophotometer was obtained from Jenway, UK; while Genios Pro microtiter plate reader was purchased from Tecan, Austria.

Flow cytometry analysis was performed using Becton Dickinson FACS Aria, USA; equipped with an argon laser (488 nm) and emission filter for 519 nm. Results were studied with FACSDiva software, USA and Flowing Software 2.5.1, Finland.

2.2 Materials

2.2.1 General Chemicals

Phosphate buffered saline (PBS) tablets, acetic acid, agarose were obtained from Sigma Aldrich, UK. Chloroform, tetrahydrofuran (THF), methanol, acetonitrile, ethanol and acetone at HPLC grade and dimethyl sulfoxide (DMSO, analytical grade) were obtained from Fisher Scientific, UK. CBQCA Protein Quantitation Kit was purchased from Invitrogen/Life Technologies, UK.

2.2.2 Materials for SLN preparation and RGD conjugate synthesis

Asiatic acid (AA), tristearin (TS), polyoxyethylene(40)stearate (PEG(40)stearate), 4-nitrophenyl chloroformate, tripentylamine, sodium bicarbonate, deuterated water (D$_2$O), coumarin-6 (C6) were purchased from Sigma Aldrich, UK. Glyceryl monostearate (Imwitor 900K, MS) was donated by Cremer Oleo, Germany; phospholipon 90H was obtained from Lipoid, Germany; poloxamer 188 was purchased from BASF, Germany and glyceryl distearate (Precirol ATO 5, DS) was a kind gift from Gatetfosse, France. Polyoxyethylene(100)stearate (PEG(100)stearate) was obtained from VWR International, UK. Toluene, Methyl-tert-butyl ether (MTBE) and isopropanol at HPLC grade were purchased from Fisher Scientific, UK. Deuterated chloroform was provided from GOSS Scientific, UK. Cyclo-(Arg-Gly-Asp-D-Phe-Lys) (cRGDfK) peptide was purchased from Eurogentec, Belgium.

2.2.3 Chemicals for cell culture and in vitro studies

SVG P12, the human foetal glial cell line, and the U87 MG human glioblastoma-astrocytoma, epithelial-like cell line were obtained from American Tissue Culture Collection (ATCC), while ECV-304, human bladder carcinoma, endothelial like cells
were from European Collection of Cell Culture (ECACC). Eagle’s minimum essential medium (EMEM) with Earle’s Balanced Salt Solution without L-glutamine, Medium 199 with Earle’s Balanced Salt Solution with L-glutamine (M199), non-essential amino acids, sodium pyruvate, L-glutamine, 0.25 % trypsin-EDTA were obtained from Lonza, Belgium. Bovine Serum Albumin (BSA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), doxorubicin hydrochloride (DOX), DMSO (sterile, cell culture grade), paraformaldehyde were obtained from Sigma Aldrich, UK; Foetal bovine serum (FBS) was obtained from Biosera, UK. Collagen I rat tail, BD biosciences basement membrane matrix, BD biosciences growth factor reduced (GFR) basement membrane matrix, crystal violet were purchased from VWR International, UK. ProLong™ Gold Antifade Mountant was obtained from Fisher Scientific, UK whereas the mounting media Vectashield® with 4’,6-diamidino-2-phenylindole (DAPI) nuclear stain was provided from Vector Laboratories, USA. Accumax™ Cell Counting Solution in Dulbecco's Phosphate-Buffered Saline (DPBS) was provided from Merck Millipore, UK. Trypan Blue solution (0.4 % w/v) was also purchased from Sigma Aldrich, UK. Alexa® Fluor 488-annexin V PI double staining apoptosis assay kit was obtained from Invitrogen/Life Technologies, UK.

2.3 Methods

2.3.1 Preparation of AA-loaded solid lipid nanoparticles (SLNs)

SLNs were prepared by solvent evaporation and hot homogenisation technique (Figure 2.1) which was adapted from Venkateswarlu and Manjunath (2004). At the first stage of the project (Chapter 3), SLNs were prepared using three different glyceride lipids which were glyceryl monostearate (MS), glyceryl distearate (DS) and glyceryl tristearate (TS) as illustrated in Figure 2.2. The lipids were intentionally picked as glyceryl stearates with different number of stearate groups in order to study the effect of number of stearate chains on SLN matrix (physicochemical characterisation) and AA loading (drug entrapment efficiency, loading and yield). AA was used as the model drug throughout the project. Briefly, AA (1.5 mg/mL), lipid (TS, MS or DS, 0.45 mmol) and phospholipon 90 H (200 mg) were dissolved in a mixture of methanol and chloroform (1:1, v/v, 10 mL). The organic solvents were then evaporated and the lipid-drug mixture was melted at the same temperature (65 °C). Aqueous poloxamer 188 solution (10 mg/mL), pre-heated to the same temperature as the melted lipid-drug mixture, was mixed with the lipid phase. The mixture was homogenised (at 25000 rpm) for 10 min using homogeniser to prepare a pre-emulsion. The subsequent crude pre-emulsion
underwent ultrasonication for 25 min to obtain nanoemulsions. The nanoemulsions were left to cool down to room temperature to form AA-loaded SLNs (AA-SLNs). In order to have a valid comparison between each SLN, the mole numbers of glyceryl stearates were used same (0.45 mmol) for all three formulations and also the concentrations of other excipients (phospholipon 90 H and poloxamer 188) were kept same as well. To prepare drug-free SLNs, the same procedure was followed without using AA. Prepared formulations were then stored in the fridge (2-8 °C) until further use. All samples were tested within one day of preparation.

Titanium particles, which came from the titanium probe of ultrasonicator during the particle size reduction process, were removed by centrifugation for 30 min at 4500 rpm (3893 g) following SLNs preparation (Hellstern et al., 2001). Then formulations were filtered using Millex syringe filters (0.45 μm) to ensure the removal of all titanium particles remained after centrifugation (Luo et al., 2006). All physical characterisations and biomedical applications of SLNs were conducted after purifying the particles. The yield was calculated using the remained total drug after purification and the initial starting amount of drug as described in Equation 2.1.

\[
\text{Yield (\%) = \frac{\text{Remained total drug after purification}}{\text{initially added drug}} \times 100}
\]

(Equation. 2.1)

At the second stage of the project (chapter 4), surface modifications were introduced to the SLNs prepared in Chapter 3. PEGylated SLNs were obtained with addition of PEG(40)stearate or PEG(100)stearate (5 %, mol of PEGstearate/mol of lipid) in the lipid phase (Üstündağ-Okur et al., 2015; Wan et al., 2008); whilst RGD-targeted SLNs were prepared with addition of RGD-conjugates (synthesised in Chapter 4, RGD-PEG(40)stearate or RGD-PEG(100)stearate) in the lipid phase along with PEGstearates (10 %, mol of RGD-conjugate/mol of PEGstearate) (Shan et al., 2015; Shuhendler et al., 2012). SLN preparation process was carried out without any changes from the protocol above. Likewise, fluorescent nanoparticles were prepared by adding coumarin-6 (C6) fluorescent dye (~25 μg/mL) in the lipid phase prior to SLN preparation. The same purification technique was applied to remove the titanium particles from PEGylated, RGD-targeted and C6-loaded SLNs as well. The quantification of fluorescent dye content of nanoparticles was carried out using an UV spectrophotometer.
Figure 2.1 Preparation of SLNs: A) melting lipid and drug (Buchi Rotavapor, 2016); B) mixing aqueous phase with melted lipid phase; C) homogenisation of the mixture; D) size reduction using probe sonicator; E) SLN formulation.
Figure 2.2 Molecular structures of asiatic acid (AA), glyceryl monostearate (MS), glyceryl distearate (DS) and glyceryl tristearate (TS).
2.3.2 HPLC method development and validation

Drug content and release studies are two of the most important pharmaceutical parameters to evaluate the success of SLNs as drug delivery systems. Therefore, a rapid and efficient analytical method is essential for the evaluation of these parameters. Despite the fact that literature suggests various HPLC methods for quantification of AA (Pan et al., 2010; Rafamantanana et al., 2009; Rafat et al., 2008; Thongnopnua, 2008; Zheng and Wang, 2009), none of the methods provides a successful technique to analyse AA content within SLN systems. Therefore, it was necessary to develop a completely new HPLC method for the analysis of AA within SLNs. It is important to note that, the new analytical quantification technique must serve reproducible and reliable data for long term application; hence the validation of the developed method is a crucial aspect (Ermer, 2001; Ermer and Ploss, 2005). In this study, the validation of the developed HPLC method was assessed in terms of linearity, specificity, precision and accuracy.

2.3.2.1 HPLC method

Quantitative HPLC analysis was carried out with Agilent’s liquid chromatography system (Agilent HPLC 1260 Infinity from Agilent Technologies, USA) and results were analysed with Agilent’s software OpenLAB CDS CS Workstation. A C18 reverse phase column was used throughout the study. An isocratic mobile phase system was used which consisted of acetic acid solution (1 %, v/v), tetrahydrofuran, and acetonitrile (33:5:62, v/v/v). Flow rate of the mobile phase was 1.8 mL/min, sample injection volume was 20 µL and column temperature was kept at 45 °C throughout the HPLC analysis to prevent any possible precipitation of the lipid based SLN nanoparticles inside the column. The wavelength for UV detection was 206 nm. Total analysis run time was 6 min. System was equilibrated using mobile phase at least 1 h prior to sample injection.

2.3.2.2 Linearity

The linearity of the method was examined to show the direct proportional relationship of the results versus drug concentration in the sample and was achieved by plotting the area under the curve (AUC) against AA concentrations for ten different standard solutions (0.0, 10.0, 25.0, 50.0, 75.0, 100.0, 150.0, 200.0, 250.0, 300.0 µg/mL). Three
independent series of standard solutions were used and each standard solution was injected in triplicate. The linearity of the method was examined with linear regression analysis using correlation coefficient ($R^2$) of the linear regression line for the response versus concentration plot (Shabir, 2003). According to literature (Shabir, 2003), $R^2 > 0.999$ is generally considered as acceptable fit of the data; therefore the $R^2$ obtained in this study, 0.9993, was acceptable for AA analysis within the concentration range studied (Figure 2.3).

**2.3.2.3 Specificity**

Specificity of a HPLC method is the ability to assess unequivocally the analyte in the presence of other components of the sample such as surfactants, lipids and solvents used (Shabir, 2003; Tache and Albu, 2007). Method specificity was confirmed comparing the response of the solution which contains only AA with the response of the solutions of non-drug-loaded-SLNs, AA-loaded SLNs (AA-SLNs) and solvent used to dissolve the samples (Figure 2.4 – 2.5).

As it is shown in Figure 2.4, the retention time for AA peak was at 2.5 min. The chromatograms of non-AA-loaded-SLNs and solvent system used do not show any overlap or interference with AA peak at 2.5 min (Figure 2.4 – 2.5). Since there were no interferences coming from excipients of non-AA-loaded-SLNs, it is safe to say that the method is specific for AA analysis.

**2.3.2.4 Precision**

Precision of an analytical method is related with the repeatability and reproducibility of the technique and can be represented as the percent relative standard deviation (RSD %). The degree of repeatability of a HPLC method can be evaluated according to the International Conference on Harmonisation (ICH) guidelines by analysing three samples of three different concentrations with three repeats (9 determinations) under same experimental conditions. Moreover for the determination of injection repeatability, The US Food and Drug Administration (FDA) guidelines suggests to inject same sample ten times under same chromatographic conditions (Shabir, 2003; A.C. Silva et al., 2012).

Table 2.1 shows the results for degree of repeatability where three different concentrations of AA (300.0, 100.0, 25.0 µg/mL) were injected in triplicates and less
than 2 % RSD values were obtained. At the same time Table 2.2 displays the results for injection repeatability where same concentration of AA (300.3 µg/mL) was injected 10 times and less than 1 % RSD was observed. According to literature suggestions (Shabir, 2003), the HPLC method developed is precise for AA analysis.

2.3.2.5 Accuracy

Accuracy of an analytical method is the closeness of the measured results to the actual data. It can be calculated using recovery method which is basically the (calculated data/ actual data) x 100. Each standard solution was injected in triplicate.

Table 2.3 shows the data obtained for method accuracy. Acceptable method accuracy is required to be 100 ± 2 % (Shabir, 2003). Therefore the method accuracy for newly developed HPLC method for Asiatic acid is acceptable.

Figure 2.3 Standard calibration curve of AUC against AA concentration of 0 – 300 µg/mL (N=3).
Figure 2.4 A) HPLC chromatogram of solvent system alone (THF:methanol, 65:35, v/v); B) HPLC chromatogram of AA alone dissolved in THF:methanol (65:35, v/v).
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Figure 2.5 Specificity assessment of the HPLC method in the presence of SLN formulations. Non-drug loaded SLNs A) MS; B) DS and C) TS. AA-loaded SLNs D) AA-MS; E) AA-DS; F) AA-TS.
Table 2.1 HPLC data obtained for method precision using three different concentrations of AA.

<table>
<thead>
<tr>
<th>AA&lt;sup&gt;a&lt;/sup&gt; (µg/mL)</th>
<th>AA&lt;sup&gt;b&lt;/sup&gt; (µg/mL)</th>
<th>SD</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>299.97</td>
<td>2.31</td>
<td>0.770</td>
</tr>
<tr>
<td></td>
<td>297.88</td>
<td>3.66</td>
<td>1.227</td>
</tr>
<tr>
<td></td>
<td>299.04</td>
<td>3.20</td>
<td>1.071</td>
</tr>
<tr>
<td>100</td>
<td>101.11</td>
<td>1.98</td>
<td>1.95</td>
</tr>
<tr>
<td></td>
<td>97.17</td>
<td>1.88</td>
<td>1.93</td>
</tr>
<tr>
<td></td>
<td>98.99</td>
<td>2.02</td>
<td>2.04</td>
</tr>
<tr>
<td>25</td>
<td>21.13</td>
<td>0.35</td>
<td>1.65</td>
</tr>
<tr>
<td></td>
<td>25.53</td>
<td>0.51</td>
<td>1.99</td>
</tr>
<tr>
<td></td>
<td>22.95</td>
<td>0.46</td>
<td>2.00</td>
</tr>
</tbody>
</table>

<sup>a</sup>AA standard concentration; <sup>b</sup>Average measured AA concentration
Table 2.2 HPLC data obtained for the injection repeatability (instrumental precision) using same concentration of AA.

<table>
<thead>
<tr>
<th>AA(^a) (µg/mL)</th>
<th>AA(^b) (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>299.8521</td>
</tr>
<tr>
<td></td>
<td>302.3474</td>
</tr>
<tr>
<td></td>
<td>297.7284</td>
</tr>
<tr>
<td></td>
<td>293.9412</td>
</tr>
<tr>
<td>300</td>
<td>298.5425</td>
</tr>
<tr>
<td></td>
<td>301.1616</td>
</tr>
<tr>
<td></td>
<td>299.5335</td>
</tr>
<tr>
<td></td>
<td>295.6224</td>
</tr>
<tr>
<td></td>
<td>301.9757</td>
</tr>
<tr>
<td></td>
<td>296.7373</td>
</tr>
<tr>
<td>Average</td>
<td>298.7442</td>
</tr>
<tr>
<td>SD</td>
<td>2.769568</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>0.92707</td>
</tr>
</tbody>
</table>

\(^a\)AA standard concentration; \(^b\)Average measured AA concentration

Table 2.3 Results obtained for method accuracy

<table>
<thead>
<tr>
<th>Asiatic acid (µg/mL)</th>
<th>Accuracy (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>99.654</td>
<td>0.349</td>
</tr>
<tr>
<td>100</td>
<td>98.935</td>
<td>1.350</td>
</tr>
<tr>
<td>25</td>
<td>98.088</td>
<td>1.975</td>
</tr>
</tbody>
</table>
2.3.3 Evaluation of Coumarin-6 Concentration

The concentration of the Coumarin-6 was determined using UV-Vis spectrophotometer (Jenway, UK). Samples were dissolved in methanol:chloroform (1:1, v/v) and the calibration curve was obtained within the concentration range of 0 - 25 µg/mL (Figure 2.6). Samples were analysed at 455 nm.

![Calibration curve for Coumarin-6 fluorescent dye.](image)

2.3.4 Size Analysis and Zeta Potential

Measurement of size, polydispersity index (PDI) and zeta potential of SLNs was performed by photon correlation spectroscopy using Malvern Zetasizer Instrument (Malvern Instrument, UK) at 25 °C. Size measurements were carried out on diluted samples (dilution factor (DF):5) with double distilled water. Zeta potential measurements were determined in special zeta potential cells by mixing SLN formulation with NaCl solution (0.9% w/v), 1:1 (v/v). All of the measurements were carried out in triplicate.
2.3.5 Entrapment efficiency of SLNs

Total (TD) and free drug (un-entrapped drug; FD) concentrations of SLN formulations was analysed using HPLC instrument for the assessment of entrapment efficiency. Total drug concentration of SLNs is the drug content remained in the formulation after purification and was determined by taking known volumes from each formulation and dissolving them in HPLC solvent; tetrahydrofuran, methanol mixture (65:35, v/v). Samples were subsequently injected into HPLC using the HPLC method as described in Section 2.3.2.1. Free drug concentration is the un-entrapped drug and was separated from SLNs employing Amicon centrifugal filters. SLN formulation (0.5 mL) was placed into the upper chamber of the centrifugal filter and centrifuged at 15,500 rpm for 30 min. After centrifugation, supernatant which contains water and free compounds molecular weight less than 3K Da, passed through the filter to the bottom chamber where free AA was found as well. Free drug concentration was again analysed by diluting supernatant with HPLC solvent and analysing with HPLC instrument. Entrapment efficiency (EE) was calculated using Equation 2.2; whilst drug loading (DL) was calculated with Equation 2.3.

\[
EE\ (\%) = \frac{(TD-FD)}{TD} \times 100
\]  
(Equation 2.2)

\[
DL\ (\%) = \frac{\text{Weight of entrapped drug}}{\text{(Total weight of all excipients + drug)}} \times 100
\]  
(Equation 2.3)

2.3.6 In vitro drug release

*In vitro* drug release studies were performed for AA-loaded MS-, DS- and TS-SLN at 37 °C using the dialysis bag method by adopting the solvent system of phosphate buffer saline (PBS, pH 7.4) and absolute ethyl alcohol (40:60, v/v), (Li et al., 2009). First of all, dialysis membranes (3.5 kDa) which were cut into appropriate sizes were kept in double distilled water 1 h before the experiment in accordance to manufacturer’s instructions. SLN formulations (10 mL) were added into the dialysis membranes and both ends of the membrane were securely tied with nodes and thread to prevent any possible leakage of the formulation. Membranes were then placed into dissolution media (150 mL) containing glass vessels and a rotational movement was provided with a magnetic stirrer at 300 rpm. Sample aliquots (3 mL) were removed from the dissolution vessel at certain time intervals. To maintain sink conditions, the same volume of fresh dissolution media was added back to the system. The amount of drug released out from the formulations was determined over 72 h and samples were analysed using HPLC method as described in section 2.3.2.1.
2.3.7 X-ray diffraction studies (XRD)

Bruker XRD was used to study the crystallinity of the AA in the lipid matrix of SLNs. Powder form samples were placed into the sample holders and scanned from 5° to 50° with a scan type coupled two theta/theta using Scintillation counter and 1-dimensional LYNXEYE detector exposing to Cu Kα radiation (30 kV, 10 mA). Prior to the experiment samples were frozen overnight at -20 °C freezer and freeze dried for 24 h using a freeze dryer to obtain powder form of formulation. To determine the crystallinity of AA incorporated with SLNs, four different samples were necessary to study for each specific formulation. These are AA alone (pure drug), blank SLN without any AA, SLN with AA (AA-SLN) and physical mixture which was prepared by physically mixing of freeze dried non-AA-loaded-SLN and AA. Crystallinity of the drug within the SLNs was decided by comparing four samples all together.

2.3.8 Differential Scanning Calorimeter (DSC)

Crystallinity of the drug with the SLNs was also studied with Mettler DSC. Samples used for DSC performance were the same as the samples used for XRD analysis. Briefly, freeze dried SLN samples were weighed in standard aluminium pans (40 µL) approximately 5 mg and sealed with a lid. The lid was punctured with a fine needle to allow water/moisture evaporate from sample if there is any. An empty pan including a lid was used as a reference for the study. Analysis was carried out under a nitrogen environment (50 mL/min). To perform DSC analysis two different methods were used. One of these methods was using a heating rate of 10 °C/min and the second was applying a faster heating rate of 100 °C/min in the range of 5 – 400 °C.

2.3.9 Scanning electron microscope (SEM)

Studying the surface morphology of SLNs could provide an idea about the shape and size of the nanoparticles. Here the surface morphology of the SLNs was monitored with SEM. Briefly; SEM samples were prepared by placing a drop of the SLN dispersion on a metallic stub covered with a glass lamella. Samples were left at room temperature to completely dry before imaging with SEM. JFC-1200 Fine Coater was used to coat the samples with gold for 2 min under vacuum. Samples were then studied with SEM microscope at 20 kV.
2.3.10 Cell Culture Studies

All cell culture studies were performed under sterile conditions in a class II microbiological tissue culture hoods. Equipment used for culturing were sterilised with either autoclave machine or 70 % ethanol solution whereas un-sterile reagents were sterilised with filtering using sterile syringes and syringe filters of pore sizes 0.22 µm (for reagents) and 0.45 µm (for SLNs) in the sterile tissue culture hoods. Culture media and materials were warmed up to 37 °C in a water bath prior to studying with the cell lines. Moreover, all the working surfaces and items (i.e media/reagent bottles) were sterilised with 70 % ethanol before cell culture manipulations. Experiments were carried out with SVG P12, U87 MG and ECV-304 cell lines (Figure 2.7) with passage numbers 6-12, 15-25 and 140-160, respectively. SVG P12 and U87 MG cells were maintained in complete EMEM cell culture media supplemented with 10 % fetal bovine serum (FBS), 1 % L-glutamine (2 mM), 1 % sodium pyruvate (1 mM) and 1 % non-essential amino acids (NEAA) while ECV-304 cells were cultured in complete M199 media supplemented with 10 % FBS and 1 % L-glutamine at 37 °C in a humidified incubator under 5 % CO₂ atmosphere. Cell culture conditions were given in Table 2.4 with details.

2.3.10.1 Subculture of cells

Cells were grown to confluency (~ 80-90 %) as observed with a light microscope prior to subculture. While passaging the cells, the media in the flasks were aspirated, cells were washed with PBS (5 mL if 75 cm³ flask, 2 mL if 25 cm³ flask) and the PBS subsequently was removed from the flasks. While passaging cells, 0.25 % trypsin-EDTA solution was added (2 mL if 75 cm³ flask, 1 mL if 25 cm³ flask) into the flasks to detach adherent cells and flasks were returned to the incubator for 3-5 min incubation. The flask was tapped gently to aid detachment of the cells, which was observed under the light microscope. Fresh media (5 mL) was added into the flask to neutralise the activity of the trypsin. Cell suspension was then placed in a centrifuge tube and centrifuged for 5 min at 1000 rpm. The supernatant was aspirated and cell pellet was re-suspended into fresh media. To ensure cells are evenly distributed in the media, cell suspension was pipetted up and down to help break up any clumps. The total number of viable cells were counted using trypan blue (as described in section 2.3.9.2) and then cells were placed into new flasks at desired split ratios. After addition of appropriate amount of culture media (5 or 10 mL culture media per 25 cm³ and 75 cm³ flasks, respectively), flasks were incubated at 37 °C under 5 % CO₂ atmosphere.
Figure 2.7 Typical cellular morphology of confluent cells: A) SVG P12 human foetal glial cells; B) U87 MG human glioblastoma astrocytoma cells; C) ECV-304 human bladder carcinoma cells.
Table 2.4 Cell lines and culture conditions used.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Name</th>
<th>Supplier</th>
<th>Culture Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>SVG P12</td>
<td>Human foetal glial cells</td>
<td>ATCC</td>
<td>10 % FBS, 1 % L-glutamine (2 mM), 1 % sodium pyruvate (1 mM) and 1 % non-essential amino acids</td>
</tr>
<tr>
<td>U87 MG</td>
<td>Human glioblastoma-astrocytoma</td>
<td>ATCC</td>
<td>10 % FBS, 1 % L-glutamine (2 mM), 1 % sodium pyruvate (1 mM) and 1 % non-essential amino acids</td>
</tr>
<tr>
<td>ECV-304</td>
<td>Human bladder carcinoma</td>
<td>ECACC</td>
<td>10 % FBS and 1 % L-glutamine</td>
</tr>
</tbody>
</table>

2.3.10.2 Viability assessment with trypan blue

Cell count was carried out using trypan blue exclusion where homogeneous cell suspension (100 µL) was mixed with trypan blue solution (1:1, v/v) and a drop of this mixture was placed in between cover slip and haemocytometer. Trypan blue is a negatively charged chromophore which is commonly used for cell counting to distinguish between live and death cells. When mixed with cells, live cells with intact cell membrane can exclude the material so they look bright and colourless under the microscope; however it is able to pass through the ruptured membrane of dead cells where they look blue under the microscope (Louis and Siegel, 2011). A haemocytometer has two separate counting chambers which each chamber has 9 large squares with 1 mm length as shown in Figure 2.8. The depth between the slide and the coverslip is measured 0.1 mm if the correct coverslip is placed on top of the haemocytometer. As a result, each large square provides a defined volume of 0.1 mm$^3$ (1 mm x 1 mm x 0.1 mm = 10$^{-6}$ cm$^3$ or 10$^{-4}$ mL). The average number of viable cells per mL in a single cell suspension can be obtained by counting the number of cells per large square (a mean of 4 squares) and then multiply it by the dilution factor (x 2) and x 10$^4$ (Figure 2.8).
Figure 2.8 Schematic representation of haemocytometer used for counting cells. The shaded regions show areas used for cell-counting. Each large square provides a defined volume of 0.1 mm$^3$ (1 mm x 1 mm x 0.1 mm = 10$^{-4}$ cm$^3$ or 10$^{-4}$ mL).

Figure 2.9 Reduction of MTT (water-soluble) by viable cells to a water-insoluble formazan salt.
2.3.10.3 Freezing down of cells for preparation of cell bank

In order to prepare cell banks and have sufficient cell stocks, cells were routinely frozen down every two or three months. During the freezing down process, cells were sub-cultured as described in section 2.3.9.1 and after counting the cells, the cell suspension was centrifuged for 5 min at 1000 rpm. The supernatant was aspirated and cells were re-suspended in freezing media which is culture media with 10 % DMSO at a cell density of 1 x 10^6 cells/mL. Cell suspension was then transferred into sterile cryogenic vials (1 mL) and vials were placed in Mr. Frosty™ freezing container which is designed to achieve cooling rate 1 °C/min (Freshney, 2010). The container was immediately placed in -80 °C freezer and left there for 24 h. After that vials were transferred to liquid nitrogen vapour (- 192 °C) for long term storage.

2.3.10.4 Cell recovery from cell bank

Frozen cells were thawed by placing cryogenic vials directly to 37 °C. This thawing process took approximately 2 min. Cells were then placed into flasks with excessive media (20 mL) to minimise the concentration of DMSO used as cryoprotectant in freezing media. After that flasks were put inside an incubator and incubated for overnight at 37 °C under 5 % CO₂ conditions to let cells attach to the flask. Following day, media was aspirated and replaced with fresh culture media.

2.3.10.5 Cell viability with MTT assay

The colorimetric MTT assay was first developed by (Mosmann, 1983) for evaluation of cytotoxicity of anti-cancer agents and nowadays it is one of the most commonly used cell viability assays in anti-cancer research to assess the cell survival after various treatments. The assay is based on reduction of MTT which is a water-soluble substance to a water-insoluble purple coloured formazan salt Figure 2.9. It has been considered that viable cells reduce MTT to formazan salt with their mitochondrial enzyme, succinate dehydrogenase by transferring a proton from NAD(P)H coenzyme to MTT and oxidised to NAD(P)⁺ (Riss et al., 2004). Here the MTT assay was used to assess the growth of each cell line studied and also to evaluate the cytotoxicity of SLNs (± AA) against monolayer cells and three dimensional (3D) tumour spheroids.
2.3.10.5.1 Growth curves of cell lines

Cells were detached with 0.25 % trypsin-EDTA solution and cell count was determined as previously described in section 2.3.9.1 and 2.3.9.2. Cell suspension was diluted to prepare $1 \times 10^4$, $1 \times 10^5$, $1 \times 10^6$ cells/mL and seeded in 96-well plates (100 µL) to give $1 \times 10^3$, $1 \times 10^4$ and $1 \times 10^5$ cells/well. To prevent evaporation, cells were seeded only into the inner wells of the 96-well plates and perimeter was filled with PBS. Plates were kept in the incubator at 37 °C, 5 % CO$_2$ environment and the growth of the cells were observed over 10 days. Five mg/mL MTT solution was prepared in PBS and sterilised by filtering with 0.22 µm pore size filters. Twenty µL of the solution was added to each well and plates were incubated for 5 h. Media with MTT (120 µL) was removed carefully to prevent disturbing the formazan crystals. Purple crystals were dissolved by addition of DMSO (100 µL) into the wells and subsequently incubation for 30 min at 37 °C. Then plates were analysed with Tecan microtitre plate reader at 595 nm. Further dilution of the wells was necessary when absorbance obtained was greater than 1; due to the limitation of the spectrophotometric analysis. In this case, 20 µL dissolved salt was placed into a clean well and diluted with fresh 80 µL of DMSO. Another reading of the plate was taken and obtained result was multiplied by 5 (dilution factor).

MTT assay was performed every day using one row of the plate (n=6) for 10 days and the experiment was triplicated to obtain a growth curve for each cell line. Therefore, the growth curves for SVG P12, U87 MG and ECV-304 cells were obtained with the average absorbance studied from a total of 18 replicates (n=18), as seen in Figure 2.10. Cell doubling times were calculated as 2 days for SVG P12 and U87 MG and 1 day for ECV-304, respectively. For all further experiments, it was decided to use a seeding density of $2 \times 10^3$, $5 \times 10^3$ and $1 \times 10^3$ cells/well for SVG P12, U87 MG and ECV-304 cells, respectively to be able to obtain confluent cells (~80- 90 %) by day 3 and 4, unless otherwise stated.

2.3.10.5.2 Evaluation of SLN (± AA) cytotoxicity

MTT assay was also used for evaluation of the cytotoxicity of AA and SLN formulations (± AA). SLNs were sterilised by syringe filtering (0.45 µm pore size) in the sterile tissue culture hood and all dilutions were prepared using sterile culture media. Briefly, 100 µL of cell suspension of $2 \times 10^3$ cells/well for SVG P12, $5 \times 10^3$ cells/well for U87 MG and $1 \times 10^3$ cells/well for ECV-304 were seeded into the inner rows of 96-well plates and plates were incubated for 24 h. The media was replaced with 100 µL fresh media and then cells were treated with different concentrations of SLNs for 24, 48 and 72 h. MTT
solution (20 μl of 5 mg/mL in PBS) were added to each well and plates were incubated for 5 h followed by spectrophotometric analysis at 595 nm using the Tecan microtiter plate reader as described in previous section. Cell viability was expressed in percentage by comparing to control (untreated cells) (n=18).

2.3.10.6 Qualitative cellular uptake studies with fluorescence imaging

Zeiss fluorescence microscopy was used to determine the cellular uptake of the SLNs by SVG P12, U87 MG and ECV-304 cells. Tracking the SLNs inside the cells were achieved by employing coumerin-6 (C6), fluorescent dye, loaded SLNs (± AA) (Rivolta et al., 2011; Zhang et al., 2013) which were prepared as described in section 2.3.1. Cells (2.5x10^5) were seeded on sterile cover slips placed in 6-well plates in a volume of 1 mL supplemented culture media and incubated at 37 °C with 5 % CO₂ for 24 h. The media was removed by aspiration, replaced with fresh media containing SLN (± AA) (46 μg/mL AA-equivalent concentration) and incubated at 4 or 37 °C (for temperature-dependent study) for 2 h. Meanwhile, 0.1 mg/mL of C6 was dissolved in ethanol/media mixture (1:1, v/v), diluted to an appropriate concentration (≈ 4 μg/mL) with culture media and given to cells as a control group for better comparison between C6 and C6-loaded SLNs treatments. At the end of the incubation time, media containing the SLNs/C6 was removed from the cells by aspiration and the cells on the cover slips were washed three times with PBS to remove any SLNs that have not been taken up by the cells. Cells were then fixed with 4 % w/v paraformaldehyde (PFA) in PBS for 15 min and later washed three times with PBS. Quenching any free aldehyde presented on the cover slips was achieved by incubating cells with 0.3 M glycine at room temperature for 15 min. After that cells were again washed with PBS for three times to remove glycine. Finally, DAPI containing mounting medium was used to mount cells on glass microscope slides. One drop of mounting medium was added on the microscope slide and the cover slips with the cells were placed facing down on the slide. Mounting medium left overnight (12 – 15 h) in a dark place to dry out and cover slips were then secured by painting with colourless nail polish around the perimeter of the slips. The slides were dried before imaging with Zeiss fluorescence microscope. DAPI imaging for nucleus staining was performed using Zeiss filter set 49 (DAPI; excitation 365 nm and emission 445/50 nm) and C6 imaging was carried out with Zeiss filter set 38 (Endow GFP; excitation 470/40 nm and emission 525/50 nm). Random fields of adherent cells were recorded. Slides were stored in the dark at 4-8 °C and studied within one week of preparation. Zen lite 2012 software was used to process the images.
Figure 2.10 Growth curves for the cell lines studied. A) SVG P12 cells seeded at $1 \times 10^2$, $1 \times 10^3$ and $1 \times 10^4$ cells/well; B) U87 MG cells seeded at $1 \times 10^2$, $1 \times 10^3$ and $1 \times 10^4$ cells/well; C) ECV-304 cells seeded at $1 \times 10^3$ and $1 \times 10^4$ cells/well. Data represent mean ± SD. (n=18)
2.3.10.7 Quantitative cellular uptake studies of SLNs using flow cell cytometry

Quantitative cellular uptake studies of the SLNs by SVG P12, U87 MG and ECV-304 cells were carried out with a BD FACSARia flow cell cytometry. Prior to the experiment, cells (1x10^6 cells/mL, 1 mL) were seeded in 6-well plates and incubated for 24 h. Plates were then treated with SLNs (± AA) (46 µg/mL AA-equivalent concentration) containing C6 or without C6 or C6 alone and incubated for 1, 3 and 5 h at 4 or 37 °C for time- and temperature-dependent cell uptake analysis. To study uptake at 4 °C, cells were incubated for 1 h at 4 °C prior to the experiment and the treatment media containing SLNs were chilled at 4 °C before given to the cells as well. After appropriate incubation time, cells were washed with PBS three times and detached from the surface with 0.25 % trypsin-EDTA solution (500 µL) by incubating at 37 °C. Cell suspension was then centrifuged at 1000 rpm for 5 min, supernatant was discarded and cells were re-suspended in cold PBS (300 µL). Propidium iodide (PI) (1 %, v/v) was added into the cell suspension at a final concentration 5 µg/mL and immediately analysed using BD FACSARia flow cytometer equipped with 488 nm filter. PI staining of cells corresponds to naturally occurring dead cells and PI-stained cells were excluded from the data analysis (Martins et al., 2012).

Data were collected with 10,000 events per sample and processed using with FACSDiva software and Flowing Software 2.5.1. Relative differences in cells were gated using forward scatter (FSC) and side scatter (SSC). Untreated cells and non-C6 containing nanoparticles were used as negative controls. Cell-associated fluorescence intensity of C6 was measured with FITC channel 530/30-nm whereas PI was detected with PE-Texas Red channel 616/23-nm.

In the competitive binding experiment which was adapted from (Chen et al., 2012; Guo et al., 2014), excess c(RGDfK) peptide (0.1 mg/mL) in cell culture media was incubated with both U87 MG and ECV-304 cells 1 h prior to the addition of C6 containing RGD targeted SLNs (C6-RGD-SLNs) and then continued incubation with C6-RGD-SLNs for additional 3 h at 37 °C. The cells then washed and prepared for the analysis with flow cell cytometry as described above.
2.3.10.8 Apoptosis Assay

Annexin V is a phospholipid binding protein where it has high affinity for phosphatidylserine lipid in the presence of Ca$^{2+}$ (Vermes et al., 1995). Phosphatidylserine is found at the inner leaflet of the plasma membrane where it translocates at the earlier stages of apoptosis and expose itself onto the outer surface of the cell and provides a sensitive apoptosis detection probe for the Annexin V protein (Fadok et al., 1992; Vermes et al., 1995). Propidium iodide (PI), a DNA stain, is able to permeate easily inside the membrane to stain the DNA in cells that have lost their membrane integrity. Therefore, it was used as a marker to distinguish dead cells.

The induction of apoptosis was measured by BD FACSaria flow cytometry for all SLN formulations (± AA), AA and cisplatin on SVG P12, U87 MG and ECV-304 cells. Apoptosis assay was performed with Alexa® Fluor 488-annexin V PI double staining apoptosis assay kit and analysis protocol was adapted from manufacturer’s instructions. SVG P12, U87 MG and ECV-304 cells (1 x 10$^5$ cells/mL, 1 mL) were seeded in each well of sterile 6-well plates. The cells were incubated for 24 h at 37 °C with 5 % CO$_2$ before the experiment and subsequently treated with SLN (± AA), AA (concentrations of 40, 60 and 80 µM) and cisplatin (50 and 100 µM) for 24, 48 and 72 h. Cisplatin was used as a positive control for apoptosis at all time-points tested (Kondo et al., 1995; Li et al., 2012). Following treatments, media containing SLNs, AA or cisplatin was aspirated and kept for further analysis as well as the PBS obtained from the wash. Cells were subsequently detached by addition of trypsin (500 µL) at 37 °C. Detached cells were combined with previously kept media and PBS obtained from the washed and centrifuged all together at 1000 rpm for 5 min to obtain a cell pellet. The cell pellet was then washed with PBS once to remove any debris and resuspended in the binding buffer, as provided by the apoptosis assay kit, which contained PI (diluted by 1:200 v/v) and annexin V conjugated to Alexa® Fluor 488 (diluted by 1:100 v/v). The samples were incubated at room temperature for 15 min and then kept at 4 °C. In order to avoid any further cell death, samples were immediately analysed by flow cytometry.

Annexin V staining was detected with FITC channel 530/30-nm whereas PI was detected with PE-Texas Red channel 616/23-nm and performed at 488 nm. Data were collected with 10,000 events per sample and processed using with FACSDiva software. Relative differences in cells were gated using forward scatter (FSC) and side scatter (SSC). In this study, viable cells were reported as annexin V negative/PI negative; early apoptotic cells as annexin V positive/PI negative; late apoptotic as annexin V positive/PI positive and necrotic cells as annexin V negative/PI positive.
(Figure 2.11) as also previously reported by J. Wang et al. (2014), L. Wang et al. (2014) and S. Wang et al. (2012).

**Figure 2.11** Schematic representation of the analysis of the apoptosis assay with Annexin V and PI staining.

### 2.3.11 Statistical Analysis

Results were expressed as mean ± standard deviation (SD) from three independent experiments and were analysed using Microsoft Excel 2010 software to calculate the significance between groups using the student’s t-test (two-tail). A difference was considered to be significant if the P-value was less than 0.05 and very significant if less than 0.001.
Chapter 3

Preparation of AA-loaded Solid Lipid Nanoparticles for Anti-Cancer Therapy
3.1 Introduction

Poor water-solubility is a major obstacle in pharmaceutical formulations, resulting in formulation instability and low drug bioavailability (Bunjes, 2010; Kipp, 2004). Approaches for achieving complete dissolution include usage of organic solvents (e.g. DMSO) or surface active solubilisation agents which may cause toxicities (Kipp, 2004). In addition, preparation of salt forms of the drugs can successfully work for the improvement of solubility, however the technique is limited with acidic/basic drugs, which could be costly and may alter pharmacological activity due to change in the molecular structure (Kalepu and Nekkanti, 2015; Kipp, 2004). Incorporation of drugs with drug delivery systems is another approach to overcome the poor water-solubility problem which are extensively studied in the literature (Kalepu and Nekkanti, 2015; Kipp, 2004; van Hoogevest et al., 2011).

Likewise, beside the successful potential therapeutic activities mentioned in Chapter 1, the major limitation of AA comes from its poor water-solubility (0.03 mg/mL) (Liu et al., 2009), where it limits its effective oral and parenteral drug delivery, as well as its potential clinical applications. In this study, solid lipid nanoparticles (SLNs) were used to improve the poor water-solubility of AA as well as providing tumour specific targeted delivery.

Novel AA-loaded SLNs (AA-SLNs) were prepared with three different glyceryl stearates including glyceryl mono- (MS), di- (DS) and tri-stearate (TS), and their physical characteristics have been systematically investigated. The in vitro drug release from the prepared AA-SLNs was determined and cytotoxicity studies were evaluated using both SVG P12 (human foetal glial) and U87 MG (human glioblastoma-astrocytoma) cells. In addition, systematic studies of the effectiveness of cellular uptake for the SLNs and their apoptotic effect were assessed using flow cell cytometry and fluorescence imaging.

U87 MG and SVG P12 cells are the model cells used in this study. Since U87 MG cells contain the receptor of interest, αvβ3 receptor, the cell line was intentionally chosen to assess the targeting ability of RGD (Arginine-glycine-aspartic acid) conjugated SLNs prepared in chapter 4 (Miura et al., 2013). SVG P12 cells are immortalised normal brain cells and were used as control cell line (Khoury et al., 2015). Additionally, both cells are well established, extensively studied cell lines for cancer research.

There have been no reports on AA-loaded SLNs for parenteral delivery in the literature. Therefore this study has the importance of being a stepping stone as a preliminary study for AA-SLNs in anti-cancer research via parenteral administration.
3.2 Methods

The methods used for SLN preparation, characterisation and in vitro studies were summarised in Section 2.3.1 – Section 2.3.10.

3.2.1 Evaluation of cytotoxicity of doxorubicin, poloxamer 188, ethanol and water

Cytotoxicity studies were carried out using the MTT assay as described in section 2.3.10.5. Doxorubicin hydrochloride (DOX), a well-studied and marketed anti-cancer drug, is used as positive control in this chapter to verify system suitability for cell cytotoxicity studies using reagents like MTT, the spectrophotometer and cell lines (U87 MG and SVG P12). Toxicity of poloxamer 188, the surfactant used to prepare the SLNs, was also tested. Briefly, DOX and poloxamer 188 (P188) were dissolved in the culture media and diluted to appropriate concentrations (0.125-50 µg/mL and 0.05-10 mg/mL for DOX and P188, respectively). Ethanol (4 %, v/v) was used in the preparation of AA stock solution for the treatment of SVG P12 and U87 MG cells. The formulations were prepared as described in Section 2.3.1 where double distilled water was used as the dispersion medium. Ethanol and double distilled water were also tested on cells as negative controls and they were mixed with culture media at appropriate concentrations before given to the cells. Cytotoxicity of these four compounds was performed time-dependently for 24, 48 and 72 h.

3.3 Results

3.3.1 Physicochemical characterisation of the SLNs

3.3.1.1 Preparation and characterisation of SLNs

To study the effect of the stearate chain number on the SLN matrix in terms of physicochemical characteristics and drug loading; SLNs were prepared with three different glyceryl stearates which were glyceryl monostearate (MS), glyceryl distearate (DS), and glyceryl tristearate (TS). SLNs were first characterised by measuring the particle size, PDI and zeta potential on a Malvern Zetasizer by dynamic light scattering and electrophoretic light scattering, respectively, and the results are presented in Table 3.1. The particle size of the SLNs was measured as mean diameter, whereas PDI and zeta potential represents the size distribution (homogeneity) and surface charge of the particles, respectively.
Non-drug-loaded SLNs showed particle size between 94 – 137 nm, where sizes of all three SLN particles are significantly different than each other ($p<0.001$). AA-loaded SLNs displayed slightly higher particle size than non-drug-loaded SLNs in the range of 126–141 nm where there was no significant difference between the sizes of AA-MS- and AA-DS-SLNs. However, AA-TS-SLN size was significantly smaller ($p<0.001$). It has been observed that the increase in number of stearate chains of the lipid molecules resulted in a significant decrease in particle size of SLNs (± AA) ($p<0.001$). TS, which is the most hydrophobic in the series of lipids studied, resulted in relatively smaller particles (hydrophobicity of the lipids tested (TS > DS > MS)). The more hydroxyl group the glyceride contains (no of hydroxyl group MS > DS > TS), the more polar it becomes, which enhances its surface-active property as well as its ability to interact with the polymeric surfactants. This may have contributed to the changes of particle size (Mäder, 2006).

**Table 3.1 Average size, PDI, zeta potential, entrapment efficiency, yield and drug loading of SLNs of different glyceryl stearates.**

<table>
<thead>
<tr>
<th>SLN samples</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta (mV)</th>
<th>EE (%)</th>
<th>Yield (%)</th>
<th>Drug Loading (%) (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>137.1 ± 3.2</td>
<td>0.25 ± 0.003</td>
<td>-0.13 ± 0.71</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DS</td>
<td>122.6 ± 1.0</td>
<td>0.22 ± 0.003</td>
<td>-0.04 ± 0.04</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TS</td>
<td>94.5 ± 2.7</td>
<td>0.22 ± 0.016</td>
<td>-0.78 ± 1.05</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>AA-MS</td>
<td>141.7 ± 1.7</td>
<td>0.21 ± 0.010</td>
<td>-0.02 ± 0.03</td>
<td>98.1 ± 0.05</td>
<td>73.8 ± 0.3</td>
<td>5.0 ± 0.25</td>
</tr>
<tr>
<td>AA-DS</td>
<td>141.3 ± 2.5</td>
<td>0.23 ± 0.012</td>
<td>-0.33 ± 0.14</td>
<td>98.1 ± 0.60</td>
<td>36.5 ± 0.5</td>
<td>2.1 ± 0.30</td>
</tr>
<tr>
<td>AA-TS</td>
<td>126.9 ± 0.5</td>
<td>0.24 ± 0.008</td>
<td>-0.54 ± 1.26</td>
<td>99.9 ± 0.04</td>
<td>86.5 ± 2.3</td>
<td>3.1 ± 0.12</td>
</tr>
</tbody>
</table>
Tumours have leaky vasculature with pore size of 100 – 780 nm (Byrne et al., 2008) and it has been suggested that nanoparticles in the size range of 10 – 200 nm are able to pass through these pores and accumulate in tumours and tumour vasculature (Torchilin, 2000). Therefore, the size range of nanoparticles obtained in this study seems feasible for use in anti-cancer treatment.

All nanoparticles produced displayed a narrow size distribution with low polydispersity index (Figure 3.1) and they all possessed a small negative surface charge between −1 and 0 mV (Table 3.1), which is a desirable property to avoid macrophage opsonisation and unintended toxicity (Carrstensen et al., 1992). Even though higher surface charge is considered to provide stronger electrostatic repulsion and hence longer stability for carrier systems, polymeric surfactants like P188 can provide steric stabilisation for longer storage of nanoparticles (Müller et al., 2000).

Figure 3.1 Representation of particle size distribution of AA-loaded MS, DS, TS formulations by intensity

SLN particles were then characterised in terms of entrapment efficiency (Equation 2.2, Section 2.3.4), drug loading (Equation 2.3, Section 2.3.4) and formulation yield (Equation 2.1, Section 2.3.1) using HPLC (Table 3.1). Although different lipid preparations have displayed variations in particle size; drug entrapment efficiencies of these nanoparticles have shown to be similar, 98 – 99 % (Table 3.1) which is an indication that AA was compatible with all glyceryl stearates tested. However, the yield of the formulations showed significant differences (p<0.001). The main reason of the decrease in the yield was losing lipids during the purification process where centrifugation and filtration took place.

The MS-SLN formulation has shown to have a significantly higher drug loading capacity than TS-SLN (p<0.001). This could be due to the crystalline nature of the TS-
SLN where it is possible to see the sharper XRD peaks of AA-TS-SLN as illustrated in Figure 3.2. Previous reports suggested that drug loading capacity of lipids increases with a decrease in the lipid crystallinity (A. C. Silva et al., 2012). Therefore, partial glycerides may have more crystal imperfections in their lattice structure in which more drug can be incorporated (Müller et al., 2000; Westesen et al., 1997).

Table 3.2 shows the physicochemical characteristics of MS-SLNs with coumarin-6 (C6). C6 was incorporated into MS-SLNs as fluorescent dye with and without AA for fluorescence imaging. Analysis of the C6-loaded SLNs (C6-SLNs) showed just a slight increase in the particle size of the MS-SLNs ($p>0.05$), whilst similar PDI and zeta potentials were obtained for C6-MS-SLN compared to MS-SLNs; indicating that the incorporation of C6 did not cause a significant change to the physicochemical characteristics of the SLN systems.

**Table 3.2 Particle size, polydispersity index (PDI) and zeta potential of C6-SLNs**

<table>
<thead>
<tr>
<th>SLN Samples</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>145.9 ± 4.1</td>
<td>0.23 ± 0.011</td>
<td>-0.16 ± 0.68</td>
</tr>
<tr>
<td>AA-MS</td>
<td>145.1 ± 4.4</td>
<td>0.22 ± 0.003</td>
<td>-0.25 ± 0.59</td>
</tr>
</tbody>
</table>
Figure 3.2 XRD spectra for drug alone (AA) (blue), non-AA-loaded SLNs (green), AA-SLN (red) and the physical mixture of AA and non-AA-loaded SLNs (purple) of three formulations A) MS-SLN; B) DS-SLN; and C) TS-SLN.
3.3.1.2 In vitro comparison of drug release kinetics from SLN formulations

The main reason of performing this study was to compare the effect of three different solid lipids (MS, DS and TS) on the release of the drug, AA, from the resultant SLN formulations to help understand the possible release mechanisms for subsequent cellular activity testing. The in vitro drug release studies were performed using dialysis bag method where PBS (pH 7.4)/ethanol mixture (40:60 v/v) was used as the release media at 37 °C. Theoretically, in vitro release conditions were expected to mimic in vivo conditions as much as possible. However, since AA has very low drug solubility (0.03 mg/mL in water), ethanol was included into the release media in order to ensure drug dissolution at sink condition (Shen and Burgess, 2012) and provide measurable drug concentrations (above the limit of quantitation of HPLC) in samples aliquoted at appropriate time points using HPLC (Luan et al., 2013). Since this is a common problem for hydrophobic drugs, such a model exploiting ethanol as the solvent for drug release evaluation from SLNs is extensively seen in the literature (Akanda et al., 2015; Kakkar et al., 2011; Li et al., 2009). Alternatively, surfactants added to the dissolution media (Harivardhan Reddy and Murthy, 2005; Huang et al., 2013; H. Hu et al., 2013) or changing the pH of the media (Kumar et al., 2007) have also been observed.

A biphasic drug release pattern is often seen with SLN particles (Akanda et al., 2015; Huang et al., 2013; Nayak et al., 2010; Neves et al., 2013), where a burst release is seen initially and then followed by a sustained release profile. The drug in the outer shell and on the particle surface tend to be released relatively faster as a burst release, whereas drug in the core of the lipid matrix tend to be released slower as a sustained release due to the increased diffusional distance and hindering effects by the surrounding solid lipid shell (Huang et al., 2013; Müller et al., 2000; Wissing et al., 2004). Figure 3.3 shows the release profiles of the drug, AA, from SLNs over 48 h. A relatively faster (burst) release pattern was seen, about 30 – 40 % of the total drug, within 2 h followed by a slower, sustained release of the drug over 48 h. Even though the burst release suggested that relatively small amount of the drug was found in the outer shell of the SLNs, sustained release of AA from SLNs indicated that relatively higher amount of AA was found in the lipid core.

As shown in Figure 3.3, TS showed the slowest AA release rate when compared with MS- and DS- nanoparticles. This is likely to have been attributed to the hydrophobic nature of TS; where it could retain the hydrophobic drug for longer, delaying drug release. On the other hand, nanoparticles prepared with MS allowed more drugs to be released at the same time points tested. Similar trends were also observed by Vivek and co-workers, where the effect of the lipid matrix on the in vitro release of olanzapine...
was highlighted (Vivek et al., 2007). The degree of crystallinity of the lipid matrix may also be another reason for the slower release profile of TS observed. It was suggested by Nayak et al. (2010) that a less ordered lipid matrix helps drug to release out faster. As an example; in the study of Nayak et al. (2010), Curcuminoids-loaded MS- and TS-lipid nanoparticles were compared and a faster drug release profile was also observed from MS particles. Likewise; in the present study, it is also possible to see the sharper XRD peaks of AA-TS-SLNs, as illustrated in Figure 3.2, where it suggests that TS-SLNs likely has a higher degree of crystallinity than MS- and DS-SLNs; therefore this might also contributed to the slower drug release rate of TS-SLNs.

![Drug Release Graph](image)

**Figure 3.3** The *in vitro* drug release profiles of AA-loaded MS-, DS- and TS-SLNs formulations over 48 h in ethanol:PBS media (60:40 v/v) at 37 °C.

### 3.3.1.3 Thermal Analysis and X-Ray Diffraction to study the physical state of AA

The diffraction pattern of the drug, AA, non-drug-loaded SLNs and drug-loaded SLNs (AA-SLNs) for all formulations prepared using three different solid lipids (MS, DS and TS) was analysed using thermal analysis and x-ray diffraction techniques, as illustrated in Figure 3.2, 3.4 and 3.5. The DSC thermograms for AA, AA-SLNs, non-drug-loaded SLNs and the physical mixtures of non-drug-loaded SLNs with AA suggested that AA might not be in the crystalline state. The thermograms showed a single exothermic peak for AA crystals and an endothermic peak at 332.8 °C, where the endothermic peak represents the melting of the drug (Figure 3.4). However, freeze-dried AA-loaded
SLNs and physical mixtures that contained the AA concentration of interest did not show such a peak (Figure 3.4).

In fact, SLNs presents a major challenge for thermal analysis. This is particularly the case when there is a major difference between the melting points of the lipid constituents and the melting point of the drug. During a DSC heat scan, lipids within the SLNs reach their melting point at 40 – 70 °C (melting point for MS, DS, TS, phospholipon 90H and poloxamer 188 are 54 – 64, 50 – 60, 72, 55, and 52 °C respectively; melting point data was obtained from related MDSD forms of Cremer Oleo, Gattefosse, Sigma Aldrich, Lipoid and BASF, respectively), and the scan continues for further 250 °C until the melting point of AA is reached (325 °C). One possible reason for the absence of endotherm of drug melting in the DSC thermograph could be due to the dissolution of AA in the molten lipids before the drug melts. Such limitation has been previously reported when DSC was used to analyse drug’s physical form in SLNs (Damian et al., 2000; Ghanbarzadeh et al., 2015; Kelidari et al., 2015).

To prohibit the possible melting of AA in the physical mixture, a second method was employed where the heat flow was dramatically increased to 100 °C/min, not allowing a long period of time for the drug to be dissolved by the lipids; however it was still not possible to observe any drug peaks for the physical mixture (Figure 3.5).

The XRD spectrum of AA crystals (Figure 3.2) displayed sharp peaks at 2θ scattered angles 7.62, 12.55, 13.66 and 15.03 (Khunathum and Ritthidej, 2010; Liu et al., 2009; Zhao et al., 2010). These peaks were also detected in the physical mixture of all three types of formulations using MS, DS and TS. Nevertheless, neither of these peaks was detected in AA-loaded SLNs (Fig. 3.2). The absence of AA peaks found in AA-loaded SLN spectra suggested that the majority of AA was in the amorphous state within the nanoparticles. Common broader peaks with less intensity were also observed around 2θ scattered angles 19, 21 and 23 for all of the nanoparticle formulations which most likely they belong to the solid lipid matrix (Figure 3.2).

3.3.1.4 SEM Imaging for morphological confirmation of the SLNs

SEM images of the AA-loaded SLN particles for MS, DS, and TS lipids are shown in Figure 3.6. This illustrated the spherical geometry and the uniformity of SLN morphology of the SLNs tested. SEM images also confirmed the particle size of the SLNs as less than 200 nm, which was in agreement with the data obtained using the zetasizer shown in Table 3.1.
Figure 3.4 DSC thermograms obtained with 10 °C/min heating rate for drug alone (AA) (blue), AA-SLN (red), non-AA-loaded SLN (green) and the physical mixture of AA and non-AA-loaded SLN (purple) of three formulations A) MS; B) DS; and C) TS.
Figure 3.5 DSC thermograms obtained with 100 °C/min heating rate for drug alone (AA) (blue), AA-SLN (red) non-AA-loaded SLN (green) and the physical mixture of AA and non-AA-loaded SLN (purple) of three formulations A) MS-SLN; B) DS-SLN; and C) TS-SLN.
Figure 3.6 SEM images of AA-loaded A) MS-SLN; B) DS-SLN; and C) TS-SLN.
3.3.2 In vitro testing of SLNs (± AA)

3.3.2.1 In vitro cytotoxicity of AA-SLNs

Cytotoxicity of AA and all three types of lipid nanoparticles (± AA) has been studied towards U87 MG human glioblastoma and SVG P12 human foetal glial cell lines using the MTT colorimetric cell viability assay (Mosmann, 1983). DOX treatment was used as a positive control (0.125 – 50 µg/mL) on U87 MG and SVG P12 cells (Figure 3.7), which showed IC_{50} value at 34.5, 0.9 and 0.7 µM (Table 3.3) for 24, 48 and 72 h against U87 MG, where similar results to those published previously (Nair K et al., 2011; Xiang et al., 2011). The concentration and time-dependent cytotoxic effect of AA on SVG P12 and U87 MG cells was observed in the concentration range 0-0.05 mg/mL, as presented in Figure 3.8 A and B, respectively. It is clearly seen that AA is more toxic towards U87 MG glioma cells than the normal SVG P12 glial cells at all time points tested (24, 48 and 72 h). As shown in Table 3.3, the IC_{50} of AA on U87 MG cells was found to be between 0.019-0.03 mg/mL (i.e. 38.8 – 61.0 µM), whilst a slightly higher IC_{50} was required for SVG P12 cells (59.3 – 71.6 µM).

Similarly, time-dependent cytotoxicity of all three drug-loaded nanoparticles was also studied towards two different cell lines. TS and DS particles exhibited a lower cytotoxicity on U87 MG glioma cells than MS particles did following 72 h treatment, which correlated to the slower rates of drug release observed for AA-TS and AA-DS nanoparticles (Figure 3.3). If less amount of AA was being released from the SLNs, less cytotoxicity was expected at the same time point tested. As shown in Figure 3.9, toxicity of MS-AA increased as time of incubation increased. By 48 h almost all the tested cells were killed after treatment with 51 μg/mL AA-equivalent concentration of the AA-MS formulations.

Because of the SLNs acting as a carrier as well as a barrier for drug release, AA alone was more toxic towards glioma cells than AA-loaded SLNs when comparing the time points studied. Similar observation was seen by Zanotto-Filho et al. (2013) where they could only obtain similar cytotoxicity of drug alone with drug-loaded nanoparticles after 96 h, suggesting that not 100 % of the entrapped drug was released within 72 h of the treatment time. In one of the studies by Venkateswarlu and Manjunath (2004), where they studied the drug release from triglyceride-SLNs at different pH, TS-SLN showed only less than 20 % drug release in 24 h at pH 7.4, which also supports the data collected from this study why no significant differences were observed between TS-SLN and AA-TS-SLN treated cells (Figure 3.11 A and B).
Figure 3.7 Cell viability following treatment with DOX on A) SVG P12 cells and B) U87 MG cells for 24, 48 and 72 h. (N=3, n=18, ± SD)
Figure 3.8 Cell viability following treatment with AA on A) SVG P12 cells and B) U87 MG cells for 24, 48 and 72 h. (N=3, n=18, ± SD)
Table 3.3 Concentration of doxorubicin and asiatic acid against U87 MG and SVG P12 cells that result in 50 % viability (IC50)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Exposure time (h)</th>
<th>IC50 values for SVG P12*</th>
<th>IC50 values for U87 MG*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/mL</td>
<td>µM</td>
<td>µg/mL</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>24</td>
<td>20 ± 1</td>
<td>34.5 ± 1.83</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.5 ± 0.03</td>
<td>0.9 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.4 ± 0.05</td>
<td>0.7 ± 0.09</td>
</tr>
<tr>
<td>Asiatic Acid</td>
<td>24</td>
<td>35 ± 2</td>
<td>71 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>33 ± 2</td>
<td>67 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>29 ± 3</td>
<td>59 ± 6.1</td>
</tr>
</tbody>
</table>

Experiments were repeated at least three times (N=3, n=18 ± SD).

Noticeable significant differences were observed in the cytotoxicity of AA alone and AA-MS towards SVG P12. After treatment of SVG P12 cells with 50 µg/mL of AA, cell viability was determined as 17, 11 and 7 % following 24, 48 and 72 h incubation, respectively; whilst 100, 61 and 42 % cell viability was observed at the same AA-equivalent concentration of AA-MS following 24, 48 and 72 h treatment ($p<0.001$). On the other hand, at the same AA-equivalent concentration, AA-MS was significantly more toxic against U87 MG cells with 57, 9 and 7 % cell viability following 24, 48 and 72 h incubation, respectively ($p<0.001$). This indicates that MS-SLN reduced the cytotoxicity of AA towards normal cells whilst having a greater effect towards glioma cells ($p<0.001$).

The non-drug-loaded SLNs using both glyceryl mono- and di-stearate (MS- and DS-SLN) showed a higher toxicity towards U87 MG cells but had minimal cytotoxicity on normal SVG P12 glial cells between the concentration range 0.02 – 0.05 mg/mL (AA-equivalent concentration), as shown in Figure 3.9 – 3.10. To investigate the potential cytotoxic effect of the surfactant (poloxamer 188, P188) used in the formulations, P188 was dissolved in the cell culture media as described in section 3.2.1 and tested on the cells in the range of concentrations found in SLNs. Nevertheless, P188 did not show
any toxicity towards either of the cell lines (Figure 3.12) at the equivalent concentrations used in the SLN formulations. Similar observation was also obtained by Schöler et al. (2002) with macrophages and it was also noted that P188 did not exert any cytotoxic effect as well. Nanotoxicology applied to SLNs was systemically reviewed by Doktorovova et al. (2014) where they summarised the SLN cytotoxicity obtained so far by various studies and discussed the possible reasons behind these toxicities. It was revealed that cancer cells tend to be more susceptible to SLN cytotoxicity than non-cancerous cell lines. Cytotoxicity depends on the lipid matrix and the SLN concentration used (Doktorovova et al., 2014). These conclusions are also in agreement with the data obtained from our study. Schöler et al. (2002) suggested that the cytotoxicity of SLNs is most likely caused by products from the enzymatic degradation of SLNs including free fatty acids. Furthermore, it was reported by Fermor et al. (1992) that fatty acids like stearic and oleic acids have extremely high cytotoxicity on both cancer and normal cell lines tested. Therefore, the fatty acids as degradation products from the cellular uptake of the SLNs or from the fatty acids, as impurities of the glyceryl mono- and di-stearate lipids, could have also contributed to the cytotoxicity of non-drug-loaded SLNs. Nevertheless, the cytotoxicity results obtained against SVG P12 and U87 MG showed better SLN compatibility with normal cell lines than glioma cells, since blank SLNs did not show any toxicity against SVG P12 (Figure 3.9).

The effect of double-distilled water, used as the dispersion media in SLN formulations, and ethanol, used to help the dissolution of AA prior to AA treatment, towards the cytotoxicity of SVG P12 and U87 MG cells were also tested as control studies. Both of the treatments was prepared in the range of concentrations found in SLNs and AA stock solution and incubated for 72 h. Neither of them showed toxicity to the two cell lines studied (Figure 3.13).

As confirmed by flow cell cytometry (Figure 3.14 – 3.16), U87 MG cells tend to have higher uptake of nanoparticles than SVG P12 cells. Consequently, a significantly higher toxicity was seen with U87 MG cells when compared with SVG P12 cells (Figure 3.9). Based on the drug release profile (Figure 3.2) and preferential cytotoxicity of MS-SLNs towards U87 MG cells, formulations based on MS-SLNs were chosen to be further investigated using fluorescence imaging, fluorescence-activated cell sorting (FACS) analysis and apoptosis assay.
Figure 3.9 Cell viability following treatment with MS-SLN on A) SVG P12 cells and B) U87 MG cells for 24, 48 and 72 h. (N=3, n=18, ± SD)
Figure 3.10 Cell viability following treatment with DS-SLN on A) SVG P12 cells and B) U87 MG cells for 24, 48 and 72 h. (N=3, n=18, ± SD)
Figure 3.11 Cell viability following treatment with TS-SLN on A) SVG P12 cells and B) U87 MG cells for 24, 48 and 72 h. (N=3, n=18, ± SD)
### Table 3.4 Concentration of different formulations against U87 MG and SVG P12 cells that result in 50% viability (IC$_{50}$)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Exposure time (h)</th>
<th>IC$_{50}$ values for SVG P12*</th>
<th>IC$_{50}$ values for U87 MG*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg/mL µM</td>
<td>mg/mL µM</td>
</tr>
<tr>
<td>MS-SLN</td>
<td>24</td>
<td>N.D. N.D.</td>
<td>N.D. N.D.</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>N.D. N.D.</td>
<td>0.056 ± 0.005 114 ± 10.2</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>N.D. N.D.</td>
<td>0.042 ± 0.002 85 ± 4.1</td>
</tr>
<tr>
<td>AA-MS-SLN</td>
<td>24</td>
<td>0.088 ± 0.004 180 ± 8.1</td>
<td>0.056 ± 0.005 114 ± 10.2</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.064 ± 0.006 130 ± 12.2</td>
<td>0.038 ± 0.002 77 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.046 ± 0.003 94 ± 6.1</td>
<td>0.03 ± 0.002 61 ± 4.1</td>
</tr>
<tr>
<td>DS-SLN</td>
<td>24</td>
<td>0.096 ± 0.002 196 ± 4.1</td>
<td>0.09 ± 0.004 184 ± 8.1</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.07 ± 0.003 143 ± 6.1</td>
<td>0.038 ± 0.002 77 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.066 ± 0.002 135 ± 4.1</td>
<td>0.03 ± 0.001 61 ± 2.0</td>
</tr>
<tr>
<td>AA-DS-SLN</td>
<td>24</td>
<td>0.07 ± 0.002 143 ± 4.1</td>
<td>0.066 ± 0.001 135 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.066 ± 0.002 135 ± 4.1</td>
<td>0.036 ± 0.002 73 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.062 ± 0.001 126 ± 2.0</td>
<td>0.028 ± 0.003 57 ± 6.1</td>
</tr>
<tr>
<td>TS-SLN</td>
<td>24, 48, 72</td>
<td>N.D. N.D.</td>
<td>N.D. N.D.</td>
</tr>
<tr>
<td>AA-TS-SLN</td>
<td>24, 48, 72</td>
<td>N.D. N.D.</td>
<td>N.D. N.D.</td>
</tr>
</tbody>
</table>

*IC$_{50}$ values of AA-SLNs were given based on AA-equivalent concentrations and values of non-AA-loaded SLNs were given based on AA-SLN-equivalent concentrations (N=3, n=18 ± SD). N.D. means no IC$_{50}$ values were determined using the concentrations tested."
Figure 3.12 Cell viability following treatment with poloxamer 188 on A) SVG P12 cells and B) U87 MG cells for 24, 48 and 72 h. (N=3, n=18, ± SD)
Figure 3.13 Cell viability following treatment with A) water and B) ethanol on SVG P12 and U87 MG cells after 72 h incubation. (N=3, n=18, ± SD)
3.3.2.1.1 Summary of in vitro cell cytotoxicity studies

- AA showed time- and concentration-dependent toxicity towards both SVG P12 normal and U87 MG cancer cells. However AA was significantly more toxic to U87 MG cells compared to normal SVG P12 cells especially at AA concentration of 0.025 mg/mL ($p<0.001$) (Figure 3.8).
- Even though concentration-dependent cytotoxicity was seen by MS-SLN and DS-SLN (± AA) following treatment on U87 MG glioma cells, TS-SLN (± AA) did not display any toxicity towards neither U87 MG cells nor SVG P12 cells (Figure 3.14). This could be the reason of slower rates of drug release observed for AA-TS-SLN (Figure 3.3)
- AA-DS-SLN and non-AA-containing DS-SLN showed similar cytotoxicities on both U87 MG and SVG P12 cells at all concentrations (Figure 3.14). Slower rates of drug release for AA-DS-SLN could again be the reason for not observing any significantly different cytotoxicity ($p>0.05$) between AA-DS-SLN and DS-SLN (Figure 3.3 and 3.14).
- Non-drug loaded MS- and DS-SLN showed higher toxicity towards U87 MG cells but minimal cytotoxicity on normal SVG P12 cells (Figure 3.14) and TS-SLN did not display any toxicity. The toxicity of non-drug-containing SLNs might be caused by enzymatic degradation products of particles which results in the release of free fatty acids (Schöler et al., 2002) and free fatty acids like stearic and oleic acids were reported to show concentration- and cell-type-dependent toxicity (Fermor et al., 1992). Additionally, cancerous cells are reported to be more susceptible to cytotoxicity of SLNs (± drug) than normal cells (Doktorovova et al., 2014) which explains the reason why SLNs without AA is more toxic to U87 MG cells compare to SVG P12 cells at the same concentrations (Figure 3.14).
- Since AA-MS-SLN showed preferential cytotoxicity towards U87 MG glioma cells and MS-SLN (± AA) displayed minimal toxicity towards normal cells, MS-SLN were chosen to be further studied using fluorescence imaging, FACS analysis and apoptosis assay.
Figure 3.14 A summary graph of Figure 3.9, 3.10 and 3.11; showing cell viability following treatment with MS-SLN, DS-SLN and TS-SLN (± AA) on A) SVG P12 cells and B) U87 MG cells after 72 h incubation.
3.3.2.2 Intracellular uptake of AA-SLNs

Internalisation of a drug delivery system by the tumour cells is extremely important to be able to show its therapeutic efficacy successfully. If the nanoparticle cannot be taken up by the target cells, the drug can still enter the cells after being released from the particles (Chen, 2010). However in this condition, the drug cannot be delivered exclusively to the tumour cells but also possibly be dispersed to the normal cells as well; which this may result in lower therapeutic efficacy (Chen, 2010).

Endocytosis is known as the main entry route for various nanoparticles to transport across the cellular membrane and it is an energy-dependent mechanism. It can be halted by incubating cells at low temperature (4 °C) (Martins et al., 2012). In this study, intracellular uptake of AA-SLNs was studied using fluorescence microscope and flow cytometry at both 4 and 37 °C to evaluate the cellular uptake of AA-SLNs.

Since the fluorescent dye, coumarin-6 (C6), was not covalently conjugated to the surface of the SLNs, cells were treated with free C6 under the same conditions as C6-loaded MS-SLNs to help confirm the cellular uptake of nanoparticles. Cells treated with the free dye alone showed relatively weaker fluorescence intensities (Figure 3.15B and 3.16B) in comparison to C6-loaded MS-SLNs (C6-MS-SLNs) where they showed intense green fluorescence (Figure 3.15A and 3.16A). Similar study has been carried out using flow cytometry as well, in which non-AA-loaded SLNs and free C6 were used as negative controls (Figure 3.17A). The acquired data suggest that both free dye and non-dye-loaded SLNs showed negligible fluorescence with both cell lines studied (Figure 3.17A).

Based on the fluorescence images (Figure 3.15A and 3.16A), SLNs tend to diffuse inside the cytoplasm of SVG P12 cells. On the other hand, a tendency of particle accumulation around the nuclei was observed with U87 MG cells. Both endocytic and energy-independent non-endocytic mechanisms have been proposed for the cellular uptake of SLNs (Martins et al., 2012). To confirm an energy-dependent process was essential for cellular uptake of the SLNs prepared in this study, experiments were carried out at both 4 °C and 37 °C. Treatment of cells with the C6-SLNs at 4 °C (Figure 3.15C and 3.16C) resulted in substantially lower fluorescence intensities than cells incubated at 37 °C (Figure 3.15A and 3.16A), which suggested an energy-dependent endocytic pathway was operated by these nanoparticles. This is further supported by findings from flow cytometry, where intense fluorescence was seen from cells treated with C6-SLNs (± AA) formulations after incubation at 37 °C (Figure 3.17). On the contrary, cells treated with SLNs following incubation at 4 °C displayed only weak
fluorescence intensity (Figure 3.17). Figure 3.17 B and C illustrated the flow cytometry overlay histograms of control cells and cells treated with free C6, MS-SLN (− drug, + coumarin) and AA-MS-SLN (+ drug, + coumarin) for 3 h. It was clearly seen that MS-SLN (− drug, + coumarin) and AA-MS-SLN (+ drug, + coumarin) did not show significant differences in terms of shifting on the FITC-A scale, indicating that similar number of fluorescent cells detected and hence similar cellular internalisation observed. Therefore, it could be inferred that cellular internalisation of the nanoparticles was unaffected by drug encapsulation.

It is also worth mentioning that internalisation of MS-SLNs by cancer cells was significantly higher ($\rho<0.05$) than normal cells (Figure 3.17A). Furthermore, a significant increase ($\rho<0.05$) in cellular uptake of the particles by U87 MG cells was observed from 1 to 3 h incubation of the cells with particles; whereas from 3 to 5 h incubation did not result in a significant difference ($\rho>0.05$) in the cellular uptake of the particles (Figure 3.17A).
Figure 3.15 Fluorescence microscopy images of SVG P12 cells: A) MS-SLN at 37 °C, B) free coumarin-6 dye at 37 °C, C) MS-SLN at 4 °C. The blue colour represents DAPI staining, green colour represents coumarin-6 staining and last column represents the merged images of DAPI and coumarin-6 staining.
Figure 3.16 Fluorescence microscopy images of U87 MG cells: A) MS-SLN at 37 °C, B) free coumarin-6 dye at 37 °C, C) MS-SLN at 4 °C. The blue colour represents DAPI staining, green colour represents coumarin-6 staining and last column represents the merged images of DAPI and coumarin-6 staining.
Figure 3.17 A) Flow cytometry data for U87 MG and SVG P12 cell uptake (mean fluorescence ± SD) of coumarin-6-loaded solid lipid nanoparticles following 1 h, 3 h, or 5 h incubation at 37 and 4 °C. Control cells are untreated cells; C-6 represents free coumarin-6; MS represents non-drug-loaded but coumarin-6 containing MS-SLNs; and AA-MS represents AA and coumarin-6-containing MS-SLNs. Flow cytometry histogram overlays for B) SVG P12 cells and C) U87 MG cells (control cells, free coumarin-6, non-AA-loaded SLN uptake at 4 °C and A-SLN uptake at 37 °C).
3.3.2.3 Cell death mechanism induced by SLNs (± AA)

Apoptosis assay was performed with Alexa® Fluor 488-Annexin V PI double staining apoptosis assay kit and viable cells were reported as Annexin V −/PI −; early apoptotic cells as Annexin V +/PI −; late apoptotic cells as Annexin V +/PI + and necrotic cells as Annexin V −/PI +, as employed by J. Wang et al. (2014) and S. Wang et al. (2012).

Cisplatin treatment (50 and 100 µM) was used as a positive control of induction of apoptosis at all time points, as shown from studies by Kondo et al. (1995) and Li et al. (2012). In Figure 3.18 and 3.19, both U87 MG and SVG P12 cells died mainly by apoptosis and relatively smaller proportions of cells died by necrosis following 24, 48 and 72 h of treatment of cisplatin. In addition, both free AA and AA-loaded MS-SLNs induced U87 MG and SVG P12 cell death via apoptosis or necrosis in a concentration-dependent manner. At a lower concentration (40 µM) of AA alone, approximately 11 – 13 % of the cell population was apoptotic and cell death was mainly caused by necrosis following 24 – 72 h of treatment on U87 MG cells (Figure 3.19). On the other hand, increasing the concentration of AA from 40 to 80 µM shifted the killing mechanism of the drug to more apoptosis than necrosis. For instance, when 60 µM AA was tested, the percentage of apoptotic cells detected was ~58 %, ~68 % and ~66 % after 24, 48 and 72 h incubation, respectively. Whilst ~82 %, ~89 % and ~86 % of apoptotic cells were detected when a higher concentration (80 µM) of AA was tested following 24, 48 and 72 h, respectively (Figure 3.19). Apoptotic cell death with cisplatin treatment showed both time- and concentration-dependent pattern on both SVG P12 and U87 MG cells. However the increase in time from 24 to 72 h did not appear to result in as profound a significant increase in the percentage of U87 MG cells undergoing apoptosis as that observed from the increase in concentration of AA treatment. Such effects were also found for SVG P12 cells, although the extent of apoptosis measured was slightly higher for AA treatment on SVG P12 (~ 34 %) than on U87 MG cells (~ 13 %) for the first 24 h of treatment (p<0.001), suggesting the sensitivity of SVG P12 cells for AA-induced apoptotic cell death. The sustained release of AA from the formulations meant that comparable extent of apoptosis with AA alone was only observed from the AA-loaded MS-SLNs following 72 h treatment. For instance following 24 h of treatment of U87 MG cells, induction of apoptosis by AA-MS-SLN was only seen as ~20 % and ~28 % for 60 and 80 µM AA-equivalent concentrations, respectively; whereas these results were significantly increased to ~71 % and ~85 % (p<0.001) following 72 h treatment. More importantly, MS-SLNs reduced the cytotoxicity of AA towards normal cells but continued enabling AA-induced apoptotic cell death towards glioma cells.
Figure 3.1 Flow cytometry analysis of SVG P12 cells stained with Annexin V- Alexa® Fluor 488 and PI following 24, 48 and 72 h (A, B and C respectively) treated by cisplatin 50 and 100 µM; AA 40, 60 and 80 µM; AA-MS 40, 60 and 80 µM; MS 40, 60 and 80 µM AA-equivalent concentrations respectively. (Early apoptosis and late apoptosis; N=3 ± SD)
Figure 3.19 Flow cytometry analysis of U87 MG cells stained with Annexin V- Alexa® Fluor 488 and PI following 24, 48 and 72 h (A, B and C respectively) treated by cisplatin 50 and 100 µM; AA 40, 60 and 80 µM; AA-MS 40, 60 and 80 µM; MS 40, 60 and 80 µM AA-equivalent concentrations respectively. (Early apoptosis and late apoptosis; N=3 ± SD)
Non-AA-loaded MS-SLNs showed toxicity on U87 MG cells at concentrations higher than 50 µM AA-SLN-equivalents (after 72 h). The Annexin V data obtained help to explain the killing mechanism of MS-SLNs is via both apoptosis and necrosis when progressive increase in Annexin V stained cells from 15 to 50 % was noted after 72 h incubation with MS-SLNs at equivalent SLN concentration of AA-SLNs (40 to 80 µM). It is believed that the free fatty acids, such as stearic acid, found in the formulations can cause endoplasmic reticulum stress-mediated apoptosis (Zhang et al., 2011), therefore these findings are also in agreement with the possible apoptotic effect of MS-SLNs against U87 MG cells observed.

3.4 Conclusion

Asiatic acid was successfully encapsulated in SLNs using three different glycerides. Due to their small size, high drug entrapment efficiency and desirable drug release, the monostearate form of SLNs (AA-MS-SLNs) provided the most potential for anti-cancer effect towards glioma cells. DSC and XRD studies suggested that the majority of the drug appear to be in the amorphous form in the nanoparticles. Fluorescence microscopy and FACS analysis also confirmed the preferential uptake of SLNs by glioma cells in comparison to normal cells (p<0.05). The obtained data also indicate that both apoptotic and necrotic mechanisms of cell death were induced by the drug, AA, alone or with SLNs, depending on the concentration applied to the cells. With optimal drug encapsulation efficiency and minimal toxicity towards healthy cells, SLNs containing AA have the potential to effectively treat glioma. A targeted form of SLNs containing AA will subsequently be evaluated.
Chapter 4

Preparation and Characterisation of Surface Modified PEGylated and RGD-targeted AA-SLNs
4.1 Introduction

As also mentioned in Section 1.6, targeted drug delivery systems can be designed to overcome undesired issues of conventional chemotherapy including reduction of side effects and undesired toxicity caused by chemotherapy treatment, improvement of side specific delivery of drugs, providing protection to therapeutic agents from possible chemical degradations etc., which makes them more attractive for cancer research. However, passive targeted delivery via EPR effect has some limitations due to the variations in the degree of tumour angiogenesis and vascularisation. Additionally, because of the interstitial fluid pressure of solid tumours may not allow successful uptake and penetration of drug delivery systems in tumours (Danhier et al., 2010). On the other hand, active drug targeting relies on the specific interactions between the actively targeting ligand and its receptor which is overexpressed by tumour cells or the tumour vasculature and not expressed or slightly expressed by normal tissue to be able to achieve high specificity (Bamrungsap et al., 2012). Results obtained with active targeting ligands demonstrates an enhanced uptake of nanoparticles by tumour cells and hence improved therapeutic activity (Byrne et al., 2008; Guo et al., 2014). To improve the cytotoxic efficacy of AA- containing MS-SLN formulations (prepared in Chapter 3), arginine-glycine-aspartate (RGD) peptide was conjugated on the SLNs to target αvβ3 integrin receptors which are overexpressed on tumour endothelial cells and various tumour cells including U87 MG glioma cells (Zhang et al., 2006) which are used in this Chapter.

In this study, PEG-stearate with two different chain lengths (number of repeating unit 40 and 100; molecular weight 2000 and 5000, respectively) (Figure 4.1) was used for the preparation of PEGylated MS-SLN and the effect of this surface modification on the physicochemical and biological properties of MS-SLN was systemically investigated prior to subsequent RGD peptide conjugation. The reason of choosing stearate conjugated PEG among other PEG-lipids was the structural similarity of stearate to the glyceryl monostearate lipid used in MS-SLN which could provide better compatibility to the SLNs prepared (Howard et al., 2012; Yuan et al., 2013, 2008).

Although poloxamer and poloxamine polymeric surfactants can also provide steric hindrance on the surface of the particles (Müller et al., 1997, 1996), it was shown by Bazile et al. (1995) and Mosqueira et al. (1999) that PEG-coated particles provided longer circulation half-life than poloxamer (F68)-coated nanoparticles. Probably this is one of the reasons for the inclusion of PEG in SLNs prepared with poloxamer 188 from the literature, which further improves surface hydrophilicity and steric hindrance (Chen
Besides providing a stealth effect to the nanoparticles for enhanced circulation time, PEG also acts as a linker to conjugate to the targeting ligand on the surface of the particles. Although there is no agreement on the ideal PEG chain length for optimal shielding effect, in general a long chain length of PEG (MW ≥ 2000) has shown less macrophage uptake and therefore tends to demonstrate longer circulation half-life (Cruz et al., 2011; Duan and Li, 2013). The chain length efficiency of PEG as a linker has displayed variable results, depending on the targeting receptor, the conjugated ligand and the particle used (Stefanick et al., 2013). Since no specific data was available in the literature regarding the best PEG chain length for RGD peptide-targeted nanoparticles, two different chain lengths of PEG were employed in this study to systematically investigate the targeting ability of RGD-conjugated SLNs. These were PEG(40)stearate (MW: 2000) and PEG(100)stearate (MW: 5000), as shown in Figure 4.1.

![Molecular structures of PEG(40)stearate and PEG(100)stearate.](image)

PEG chain length and density are important parameters that can affect the PEG coating in various ways within different types of drug carriers. For example, it was suggested for liposomes to contain 5 – 7 mol % of PEG-lipid to be able to have the optimum efficiency of the PEGylation (Barenholz, 2001; Tirosh et al., 1998). For PEGylated SLNs the relationship between PEG density and in vivo circulation time was studied by Zhao et al. (2012) where they showed that up to 10 mol % of PEG-lipid could prolong the circulation time, however it did not exhibit any further improvement when more than 10 mol % PEG-lipid was used. Based on the available data from the literature, two concentrations of PEG-stearate (5 and 10 mol %, (n/n)) were employed for this investigation, to study the effects of PEG density on the physical characterisation and cell cytotoxicity towards U87 MG cancer cells and SVG P12 normal cells, as intermediates for the subsequent studies of RGD-SLNs. Mol % (n/n) represents the mole of PEG over mole of lipid (i.e. MS).
Therefore, this chapter investigates the effect of surface modifications applied to SLNs in terms of PEGylation and RGD conjugation, using two different chain lengths, on the physicochemical characteristics and biological functions of SLNs. A cyclo-(arginine-glycine-apartate-D phenylalanine-lysine) (c(RGDfK)) peptide was covalently conjugated to the PEG-stearate linker and formulated with SLNs to produce RGD-targeted nanoparticles. RGD targeting ability of SLNs were also studied in terms of cytotoxicity and cellular uptake with SVG P12 and U87 MG cells in comparison with PEGylated and unmodified SLNs using MTT assay, fluorescence microscope and flow cell cytometry. Similar techniques were also used by Chen et al. (2012) and Jain et al. (2015) for the determination of targeting ability of nanoparticles. Furthermore, the apoptotic effects of these particles were also studied with flow cell cytometry using Annexin V PI apoptosis assay.

SVG P12 normal cells were used as control cell line. The integrin receptor of αvβ3 was selected for active targeting of SLNs as U87 MG cells used in this study has previously shown overexpression of this cell surface receptor which was detected as 1.28 ± 0.46 × 10^5 (Zhang et al., 2006). In addition, c(RGDfK) is a well-studied peptide in terms of stability, biocompatibility, specificity and high affinity towards αvβ3 integrin receptor (Haubner et al., 1996; Pierschbacher and Ruoslahti, 1987) and was used here for specific targeting of αvβ3 receptor (Chen et al., 2012; Wei et al., 2014).

4.2 Methods

The methods used for SLN preparation with PEG-stearate and RGD-PEG stearate, characterisation of SLNs and in vitro studies were given in Section 2.3.1 – Section 2.3.10.

4.2.1 Synthesis of c(RGDfK)-PEG stearate targeting conjugate

The conjugate was synthesised by a two-step reaction. In the first step of the reaction, the hydroxyl group (-OH) of polyoxyethylene(100)stearate (PEG(100)stearate) was activated with p-nitrophenyl chloroformate (p-NPC) and then in the second step cyclo-(arginine-glycine-apartate-D phenylalanine-lysine) (c(RGDfK)) peptide was conjugated to the activated nitrophénylcarbonate end of the polymer with a substitution reaction as illustrated in Figure 4.2 (Shan et al., 2015; Shuhendler et al., 2012). To investigate the
Preparation and Characterisation of Surface Modified PEGylated and RGD-targeted AA-SLNs

effect of PEG linker length on the targeting ability of SLNs, c(RGDfK)-PEG stearate conjugate was synthesised with both PEG(40)stearate and PEG(100)stearate. Details of the methods used are described below in this Section.

PEG stearate (10 g for PEG(100)stearate and 4 g for PEG(40)stearate) was dissolved in toluene (75 mL) under nitrogen at 60 °C. After complete dissolution of PEG stearate, tripylyamine (3.2 mL) and p-NPC (0.430 g) was added into the solution and left stirring at 60 °C under nitrogen for 24 h. The solution was then cooled to 30 – 40 °C and methyl-tert-butyl ether (MTBE, 100 mL) was gradually added into the solution with constant stirring. The volume of the solution was halved by evaporation with a rotary evaporator (at 40 °C) and the p-nitrophenylcarbonate-PEG stearate (NPC-PEG stearate) was precipitated from solution by cooling to ~4 °C overnight. The precipitate was separated by vacuum filtration on a sintered Buchner glass funnel (porosity: #4).

For purification of the product, the solid material obtained from filtration was completely dissolved in 20 mL of methanol and re-precipitated by adding isopropanol drop by drop at 0 °C. The resultant solution was kept at ~4 °C overnight to promote precipitation and then the precipitant was separated by vacuum filtration. The final NPC-PEG stearate product was dried under vacuum overnight using a vacuum oven at room temperature. This process was repeated twice.

To synthesise c(RGDfK)-PEG stearate conjugate (RGD-PEG), c(RGDfK) was dissolved in 0.1 M sodium bicarbonate at room temperature and NPC-PEG stearate was slowly added into the solution under stirring. c(RGDfK) and NPC-PEG stearate were mixed in 2:1 (n/n) ratio and the solution was stirred overnight at room temperature. Unreacted c(RGDfK) and p-nitrophenol were removed by dialysis against 3 L of double distilled water dd(H2O) using Slide-A-Layzer® 3500 MWCO mini dialysis unit for 72 h, changing the dialysis medium every 12 h. The c(RGDfK)-PEG stearate conjugate was obtained by freeze-drying. The molecular structure of NPC-PEG stearate product and c(RGDfK)-PEG stearate conjugate was analysed by 1H NMR in CDCl3 solvent or in D2O, respectively (Figure 4.3). The acquired NMR spectra were also confirmed with those obtained from Shan et al. (2015) and Shuhendler et al. (2012).
Figure 4.2 Synthesis of c(RGDfK)-PEG stearate targeting conjugate.

The conjugate was synthesised by a two-step reaction where in the first step hydroxyl group of PEG-stearate was activated with p-NPC to form NPC-PEG stearate and then in the second step conjugated with c(RGDfK) peptide to produce covalently bonded c(RGDfK)-PEG stearate. In this experiment both PEG(40)stearate and PEG(100)stearate were used to synthesise two different products: c(RGDfK)-PEG(40)stearate and c(RGDfK)-PEG(100)stearate for the preparation of RGD-SLNs.
Preparation and Characterisation of Surface Modified PEGylated and RGD-targeted AA-SLNs

(See legend on next page)
Figure 4.3 The $^1$H NMR spectra of PEG stearate (top), NPC-PEG stearate (middle) and c(RGDfK)-PEG stearate (bottom). A) Belongs to the synthesis which starting material is PEG(40)stearate and B) belongs to the synthesis which starting material is PEG(100)stearate. Red boxes illustrate chemical shifts attributed by NPC molecule; blue boxes indicate the chemical shifts attributed by c(RGDfK) and black boxes represent no peak attributed by NPC molecule.

The yield of the reaction was calculated using Equation 4.1 below and the reaction yield is presented in Table 4.1.

Yield (%) = experimental weight of the product / theoretical weight of the product x 100

(Equation 4.1)

<table>
<thead>
<tr>
<th>Yield of the reaction</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RGD-PEG(40) stearate</td>
<td>77.93 %</td>
</tr>
<tr>
<td>RGD-PEG(100) stearate</td>
<td>85.42 %</td>
</tr>
</tbody>
</table>

Table 4.1 Yield of the RGD-PEG stearate reaction

4.2.2 Evaluation of peptide quantity with CBQCA assay

The c(RGDfK) peptide content within RGD-PEG and RGD targeted SLNs (RGD-SLN) was determined using the 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) protein quantitation kit (Balasundaram et al., 2006; Shan et al., 2015). The assay was first designed by Liu et al. (1991) for ultrasensitive determination of primary amines by capillary electrophoresis. CBQCA reagent reacts specifically with primary amines where the non-fluorescent molecule CBQCA transforms into a fluorescent conjugate in the presence of cyanide catalyst (Figure 4.4). The assay was followed in accordance to manufacturer’s instructions. To assess the peptide quantity, known amount of RGD-PEG was dissolved in dd(H$_2$O) and 135 µL of this solution was placed in a 96-well plate. KCN solution (5 µL of a 20 mM solution) was added to the same well of the RGD-PEG solution and the reaction was initiated by the addition of CBQCA reagent (10 µL of a 2 mM solution). The plate was covered with an aluminium foil to protect from light and incubated at room temperature for 1 h with continuous shaking at 150 rpm. After the incubation period, fluorescence emission of the sample was measured at 550 nm with excitation wavelength at 485 nm. Each and every experiment had a negative control using dd(H$_2$O) (n=3), whilst PEG-SLN formulation without any peptide
was used for peptide quantitation of RGD-SLNs. The fluorescence measurement of the controls was subtracted from the fluorescence obtained for each RGD-containing sample.

Figure 4.4 In the presence of a cyanide catalyst, the transformation of the non-fluorescent molecule CBQCA into a fluorescent molecule when reacting with primary amine groups.

RGD peptide loading efficiency on surface of SLNs was calculated using Equation 4.2:

\[
\text{RGD loading efficiency (\%) = } \frac{\text{RGD quantity presented in the formulation}}{\text{theoretical RGD quantity}} \times 100
\]  

(Equation 4.2)

4.2.3 Assessment of the linearity of CBQCA assay for RGD peptide quantitation

In order to confirm the linearity of the technique, a calibration curve was prepared with a well-known standard protein, bovine serum albumin (BSA), provided by the company within the kit. BSA standard solutions were prepared in the concentration range of 0 – 1000 ng in dd(H₂O) and the assay was carried out as described in Section 4.2.2. The R² obtained was larger than 0.999 (Figure 4.5A) and therefore linearity of the technique was confirmed as acceptable (Shabir, 2003). After confirmation of the working ability of the assay with BSA protein, another calibration curve was prepared with c(RGDfK) peptide to provide a direct comparison with RGD targeted SLNs, between the concentration range of 0 – 0.666 µg/mL in dd(H₂O). The R² of the RGD peptide calibration curve was also found larger than 0.999 which confirms the linearity of the assay (Shabir, 2003) with RGD peptide as well (Figure 4.5B).
Figure 4.5 Assessment of the linearity of the CBQCA protein quantitation assay. Calibration curves were prepared using standard solutions with A) BSA protein in the range of 0 – 1000 ng; and B) RGD peptide in the range of 0 – 0.666 µg/mL.
4.2.4 Evaluation of the cytotoxicity of the PEG-stearates

*In vitro* toxicity of the compounds used in the preparation of the formulations is important. PEG (40) and (100) stearates were dissolved in cell culture media at the equivalent concentrations used in the SLN formulations and tested on SVG P12 and U87 MG cells. Assessment of cell cytotoxicity of PEG-stearates was determined with MTT assay as explained in Section 2.3.9.5.

4.2.5 Stability studies of the SLNs

Stability studies of the SLNs were carried out at ~4 °C over a period of two months. Nanoparticles were routinely analysed for their particle size, PDI and zeta potential.
4.3 Results and Discussion

4.3.1 Evaluation of the physicochemical characteristics of SLNs

4.3.1.1 Preparation and characterisation of PEGylated and RGD-targeted SLNs (± AA)

Due to the small particle size, narrow PDI, faster drug release rate and the preferential cytotoxicity towards U87 MG cells, MS-SLNs were chosen for further studies. In this Chapter, the effects of surface modifications with PEG and RGD peptide conjugation to MS-SLNs were investigated. Both PEGylated and RGD-targeted SLNs (RGD-SLNs) were prepared with solvent evaporation and hot homogenisation technique as described in Section 2.3.1. The influence of PEG chain length on the physicochemical properties of SLNs and linker efficiency for targeting ability of RGD-SLNs was systemically studied. Two different PEG-stearate concentrations were employed for the optimisation of PEG-SLNs. Optimum PEG density on the SLN surface was determined based on the physicochemical and cytotoxicity data of SLNs produced.

Data relating to particle size, PDI, surface charge, entrapment efficiency, drug loading and formulation yield for PEGylated SLNs (PEG-SLNs) and RGD-SLNs can be seen in Table 4.2 and Table 4.3, respectively. Whilst non-drug containing PEG-SLNs showed particle size in the range of 85 – 95 nm, AA-containing PEG-SLNs (AA-PEG-SLNs) displayed slightly bigger particle size between 97 – 120 nm. However, a noticeable significant difference ($p<0.001$) was observed between the particle sizes of SLNs with or without PEG; where the addition of PEG significantly reduced the particle size of SLNs. This was also observed in previous studies conducted by Fang et al. (2012), Luo et al. (2015), Üstündağ-Okur et al. (2015) and Yuan et al. (2008). PEG-stearate is an amphiphilic molecule containing both hydrophilic and hydrophobic parts. It is believed that the amphiphilic property of the PEG-stearate can reduce the surface tension of the particles and hence decrease the particle size (Fang et al., 2012).
Table 4.2: Average size, PDI, zeta potential, entrapment efficiency, yield and drug loading of PEGylated SLNs prepared with PEG-(40)/(100)-stearates.

<table>
<thead>
<tr>
<th>SLN samples</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta (mV)</th>
<th>EE (%)</th>
<th>Yield (%)</th>
<th>Drug Loading (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS-SLN</td>
<td>137.1 ± 3.2**</td>
<td>0.25 ± 0.003</td>
<td>-0.13 ± 0.71</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AA-MS</td>
<td>141.7 ± 1.7**</td>
<td>0.21 ± 0.010</td>
<td>-0.02 ± 0.03</td>
<td>98.1 ± 0.05</td>
<td>73.8 ± 0.3</td>
<td>5.0 ± 0.25</td>
</tr>
<tr>
<td>P40SLN</td>
<td>85.0 ± 5.9</td>
<td>0.24 ± 0.010</td>
<td>-0.14 ± 1.52</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(10 % n/n)</td>
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<tr>
<td>P40SLN</td>
<td>89.8 ± 9.4</td>
<td>0.23 ± 0.022</td>
<td>-0.23 ± 1.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(5 % n/n)</td>
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<tr>
<td>AA-P40SLN</td>
<td>97.4 ± 1.7</td>
<td>0.20 ± 0.009</td>
<td>-0.63 ± 2.25</td>
<td>99.3 ± 0.20</td>
<td>95.3 ± 2.1</td>
<td>5.5 ± 0.1</td>
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<tr>
<td>(10 % n/n)</td>
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<tr>
<td>AA-P40SLN</td>
<td>120.0 ± 4.1</td>
<td>0.21 ± 0.009</td>
<td>-0.09 ± 1.47</td>
<td>99.5 ± 0.01</td>
<td>96.6 ± 1.9</td>
<td>5.9 ± 0.1</td>
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<tr>
<td>(5 % n/n)</td>
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<tr>
<td>P100SLN</td>
<td>83.8 ± 2.2</td>
<td>0.22 ± 0.012</td>
<td>-1.60 ± 2.96</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>(10 % n/n)</td>
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<tr>
<td>P100SLN</td>
<td>91.3 ± 2.8</td>
<td>0.22 ± 0.021</td>
<td>-0.04 ± 0.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>(5 % n/n)</td>
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<tr>
<td>AA-P100SLN</td>
<td>101.8 ± 2.2</td>
<td>0.22 ± 0.007</td>
<td>0.60 ± 2.01</td>
<td>99.1 ± 0.40</td>
<td>82.3 ± 4.4</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td>(10 % n/n)</td>
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</tr>
<tr>
<td>AA-MS</td>
<td>119.3 ± 0.20</td>
<td>0.15 ± 1.1</td>
<td>98.9 ± 83.7</td>
<td>4.7 ± 0.1</td>
<td></td>
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</tr>
</tbody>
</table>
Preparation and Characterisation of Surface Modified PEGylated and RGD-targeted AA-SLNs

For ease of comparison, selected data obtained from MS-SLNs in Chapter 3 was also included in the first two rows of the table.

Even though a previous study performed by Fang et al. (2005) showed that the increase in molecular weight of PEG used in the formulation resulted in a decrease in the size of polymeric nanoparticles, the increase in molecular weight of PEG-stearate (from 2000 to 5000) did not show any significant change within the particle size of PEG-SLNs prepared in this study (Table 4.2). On the other hand, increasing the PEG concentration (from 5 mol % to 10 mol %) resulted in a reduction of particle size (Table 4.2) which had also been seen by Üstündağ-Okur et al. (2015) and Yuan et al. (2013). Whilst RGD-SLNs showed particle size range of 92 – 93 nm, AA-RGD-SLNs displayed slightly larger particle size range of 110 – 115 nm, similar to that observed for PEG-SLNs (Table 4.3). This suggests that RGD conjugation to the surface of SLNs did not cause any significant particle size change in comparison to PEG-SLNs.

Table 4.3 Average size, PDI, zeta potential, entrapment efficiency, yield and drug loading of RGD-targeted SLNs prepared with RGD-PEG-(40)/(100)-stearates.

<table>
<thead>
<tr>
<th>SLN samples</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta (mV)</th>
<th>EE (%)</th>
<th>Yield (%)</th>
<th>Drug Loading (%) (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P100SLN</td>
<td>1.9</td>
<td>0.011</td>
<td>0.23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5 % n/n)</td>
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</tbody>
</table>

Table 4.3 Average size, PDI, zeta potential, entrapment efficiency, yield and drug loading of RGD-targeted SLNs prepared with RGD-PEG-(40)/(100)-stearates.

<table>
<thead>
<tr>
<th>SLN samples</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta (mV)</th>
<th>EE (%)</th>
<th>Yield (%)</th>
<th>Drug Loading (%) (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP40SLN</td>
<td>92.0 ± 3.4</td>
<td>0.24 ± 0.008</td>
<td>-0.11 ± 0.15</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RP100SLN</td>
<td>93.1 ± 4.1</td>
<td>0.22 ± 0.009</td>
<td>-0.24 ± 0.20</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AA-RP40SLN</td>
<td>110.2 ± 1.7</td>
<td>0.19 ± 0.012</td>
<td>-0.29 ± 0.27</td>
<td>99.0 ± 0.3</td>
<td>85.9 ± 2.7</td>
<td>5.2 ± 0.2</td>
</tr>
<tr>
<td>AA-RP100SLN</td>
<td>115.7 ± 2.2</td>
<td>0.19 ± 0.008</td>
<td>-0.5 ± 0.51</td>
<td>99.1 ± 0.2</td>
<td>89.0 ± 1.6</td>
<td>4.96 ± 0.1</td>
</tr>
</tbody>
</table>
Both PEG-SLNs and RGD-SLNs gave narrow size distribution showing PDI < 0.3 similar to MS-SLNs. Furthermore, introducing the surface modifications to MS-SLNs did not cause a significant difference in zeta potential and drug entrapment efficiency of the particles (Table 4.2 – 4.3) when compared with MS-SLNs. However, SLNs with PEG resulted in significantly higher formulation yield than SLNs without PEG ($p<0.001$). For example, the formulation yield was $\sim 73.8\%$ for MS-SLNs, whilst SLNs prepared with PEG(40)stearate (P40-SLN) showed $\sim 95 – 96\%$ and SLNs prepared with PEG(100)stearate (P100-SLN) also displayed $\sim 82 – 83\%$ formulation yield. The reason for this could be attributed to the reduction in loss of SLNs during centrifugation and filtration in the SLN purification step (Chapter 2, Section 2.3.1) when smaller particle sizes of PEG-SLNs were obtained following PEG conjugation to SLNs.

The physicochemical characteristics of SLNs such as particle size, PDI and surface charge play a very important role in their successful applications. It is acknowledged that particles with sizes less than 200 nm tend to show longer circulation whilst particles less than 20 – 30 nm in size are likely to be eliminated by renal excretion (Gaumet et al., 2008; Moghimi et al., 2001). With a relatively narrow PDI distribution (< 0.3) and applicable surface charge (0 – 1), as well as a feasible particle size range (85 – 120 nm), the SLNs prepared in this study enable intravenous drug delivery and tumour accumulation (Torchilin, 2000; Carrstensen et al., 1992).

For fluorescence imaging, fluorescent dye, coumarin-6 (C6), was entrapped into SLNs with and without AA. No significant increase ($p>0.05$) of particle size was observed following C6 entrainment in SLN ± AA (Table 4.4). Similar PDI and surface charges were also obtained. Therefore, the inclusion of C6 into SLNs did not affect the physicochemical characteristics of C6-SLNs prepared in this study, in terms of their size, PDI and surface charge.
Table 4.4 Average particle size, PDI and zeta potential of Coumarin-6-loaded SLNs

<table>
<thead>
<tr>
<th>SLN Samples</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P40SLN</td>
<td>94.1 ± 4.1</td>
<td>0.24 ± 0.012</td>
<td>−0.16 ± 1.15</td>
</tr>
<tr>
<td>P100SLN</td>
<td>95.1 ± 1.3</td>
<td>0.23 ± 0.010</td>
<td>−0.29 ± 1.48</td>
</tr>
<tr>
<td>AA-P40SLN</td>
<td>123.8 ± 3.1</td>
<td>0.19 ± 0.003</td>
<td>−0.35 ± 1.61</td>
</tr>
<tr>
<td>AA-P100SLN</td>
<td>126.3 ± 2.7</td>
<td>0.19 ± 0.008</td>
<td>−0.29 ± 1.18</td>
</tr>
<tr>
<td>AA-RP40SLN</td>
<td>118.7 ± 1.0</td>
<td>0.24 ± 0.009</td>
<td>−0.16 ± 0.16</td>
</tr>
<tr>
<td>AA-RP100SLN</td>
<td>120.7 ± 2.1</td>
<td>0.24 ± 0.002</td>
<td>−0.32 ± 0.48</td>
</tr>
</tbody>
</table>

4.3.1.2 Evaluation of peptide quantity both in RGD-PEG conjugates and RGD-SLN formulations

Coupling targeting ligands directly to the surface of particles is commonly seen in the literature, especially for solid lipid nanoparticles and liposomes (T.-Y. Lee et al., 2007; Shilpi et al., 2015; Torchilin et al., 2001; Zheng et al., 2014). However, this technique often results in significant variations of coupling yield and may also lead to conformational and chemical changes to the ligand and thus reduces the binding affinity (Hansen et al., 1995; Stefanick et al., 2013; Veronese, 2001). Since this type of ligand coupling results in batch-to-batch variations and inconsistency of ligand activity and cellular uptake, RGD-PEG conjugate was synthesised separately and subsequently purified before incorporating it into the SLN preparation in the current study.

Peptide quantity in the RGD-PEG conjugates and RGD-SLN formulations were assessed using the CBQCA assay and the RGD loading efficiency was calculated using Equation 4.2. The results are presented in Table 4.5. RGD-PEG40-SLN (RP40SLN) formulations were found to contain 34 – 39 µM whereas RGD-PEG100-SLN (RP100SLN) formulations consisted of 27 – 30 µM RGD peptides. The integrin αvβ3 binding affinity of c(RGDfK) peptide is 46.83 – 49.9 nM (IC50), as found by Shi et al. (2011) and Debordeaux et al. (2015) with a competitive displacement assay against
Preparation and Characterisation of Surface Modified PEGylated and RGD-targeted AA-SLNs

radio labelled-c(RGDfK) bound to U87 MG cells. This suggests that the available RGD content found in all of the formulations prepared should have been adequate to target U87 MG cells.

Table 4.5 The quantity of RGD peptide found in the prepared formulations and their loading efficiencies.

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Peptide Content (µg/mL)</th>
<th>µM</th>
<th>RGD Loading Efficiency on SLN surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGD-PEG40</td>
<td>94.62 ± 10.06</td>
<td>157.69 ± 16.76</td>
<td>N.D.</td>
</tr>
<tr>
<td>RGD-PEG100</td>
<td>45.04 ± 5.16</td>
<td>75.03 ± 8.65</td>
<td>N.D.</td>
</tr>
<tr>
<td>RP40SLN</td>
<td>23.88 ± 3.45</td>
<td>39.79 ± 5.76</td>
<td>94.61 %</td>
</tr>
<tr>
<td>A-RP40SLN</td>
<td>20.83 ± 4.26</td>
<td>34.71 ± 7.10</td>
<td>82.41 %</td>
</tr>
<tr>
<td>RP100SLN</td>
<td>18.35 ± 5.51</td>
<td>30.57 ± 9.18</td>
<td>96.78 %</td>
</tr>
<tr>
<td>A-RP100SLN</td>
<td>16.44 ± 3.77</td>
<td>27.1 ± 6.76</td>
<td>86.68 %</td>
</tr>
</tbody>
</table>

N.D. means no loading efficiency was determined for RGD-PEG40 stearate and RGD-PEG100 stearate conjugates.

RP40SLN, AA-RP40SLN, RP100SLN and AA-RP100SLN stand for non-drug loaded RGD-PEG40-SLN, AA-loaded RGD-PEG40-SLN, non-drug loaded RGD-PEG100-SLN and AA-loaded RGD-PEG100-SLN, respectively.

4.3.1.3 Stability studies of the SLNs

Changes to the physicochemical properties can be used as indicators to the long-term stability of the prepared formulations. Storage stability of a formulation is one of the most important parameters and plays a major role while deciding whether the formulation is successful or not. From an industrial point of view, formulations with short shelf-life are not desired since production would be costly. Additionally, unstable formulations with very short shelf-lives could aggregate before in vivo/clinical applications and may therefore not demonstrate its desired efficacy and reduces its therapeutic outcomes. Therefore, the mean particle size, PDI and surface charge of the SLNs following storage for 60 days were monitored and the results are shown in Table 4.5, 4.6 and 4.7. Long time storage of aqueous formulations like SLNs can be achieved by freeze-drying process (Mehnert and Mäder, 2012). However, preliminary studies of freeze-dried SLNs (± cryoprotectant) prepared in this study showed increase in particle size after re-dispersion of freeze-dried formulation into water. On the other
Preparation and Characterisation of Surface Modified PEGylated and RGD-targeted AA-SLNs

hand, storing of SLNs in buffers may change the electrolyte (ion) concentration in the formulation. It was previously reported by Freitas (1999) that increasing electrolyte concentration in the aqueous SLN formulations results in increase in particle size. Because of these reasons, storage stability testing of SLNs was evaluated in purified water.

Only MS-SLNs showed a slight increase in size (~ 10 nm) after 15 days (p<0.05) and remained unchanged afterwards (Table 4.5). However, no significant differences were observed in terms of PDI and zeta potential of all the SLNs. More importantly, PEG-SLNs and RGD-SLNs remained stable over 2 months without showing any significant change in particle size, PDI and zeta potential (Table 4.6 and 4.7). It is believed that the steric hindrance provided by PEG coating prevents and reduces the aggregation of SLNs in vivo as well as during storage (Knop et al., 2010). This is the most likely reason for the improved stability of the PEG-SLNs and RGD-SLNs observed in this study.

Table 4.6 Effect of time of storage (at 4 °C) on particle size, PDI and surface charge of MS-SLNs

<table>
<thead>
<tr>
<th>SLN Samples</th>
<th>Days</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS-SLN</td>
<td>0</td>
<td>132.9 ± 3.27</td>
<td>0.21 ± 0.021</td>
<td>–0.54 ± 0.56</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>145.4 ± 5.99</td>
<td>0.22 ± 0.015</td>
<td>–0.86 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>145.6 ± 3.30</td>
<td>0.21 ± 0.024</td>
<td>–0.90 ± 0.84</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>141.2 ± 2.91</td>
<td>0.24 ± 0.011</td>
<td>–0.87 ± 0.55</td>
</tr>
<tr>
<td>AA-MS-SLN</td>
<td>0</td>
<td>135.7 ± 3.03</td>
<td>0.21 ± 0.013</td>
<td>–0.49 ± 0.85</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>143.4 ± 6.27</td>
<td>0.22 ± 0.012</td>
<td>–1.27 ± 1.86</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>137.2 ± 3.73</td>
<td>0.22 ± 0.003</td>
<td>–0.09 ± 1.52</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>136.0 ± 1.31</td>
<td>0.246 ± 0.006</td>
<td>–0.82 ± 0.77</td>
</tr>
</tbody>
</table>
### Table 4.7 Effect of time of storage (at 4 °C) on particle size, PDI and surface charge of P40SLNs and P100SLN

<table>
<thead>
<tr>
<th>SLN Samples</th>
<th>Days</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P40SLN</td>
<td>0</td>
<td>94.7 ± 4.07</td>
<td>0.25 ± 0.006</td>
<td>−0.46 ± 0.95</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>99.2 ± 2.38</td>
<td>0.21 ± 0.007</td>
<td>−0.83 ± 0.64</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>99.1 ± 0.93</td>
<td>0.22 ± 0.001</td>
<td>−0.45 ± 1.22</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>102.3 ± 1.65</td>
<td>0.25 ± 0.006</td>
<td>−0.78 ± 0.33</td>
</tr>
<tr>
<td>AA-P40SLN</td>
<td>0</td>
<td>116.7 ± 3.45</td>
<td>0.20 ± 0.003</td>
<td>−0.57 ± 1.05</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>118.2 ± 3.55</td>
<td>0.20 ± 0.007</td>
<td>−0.64 ± 0.86</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>115.8 ± 2.06</td>
<td>0.19 ± 0.008</td>
<td>−0.26 ± 0.94</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>115.3 ± 0.55</td>
<td>0.20 ± 0.008</td>
<td>−0.33 ± 0.77</td>
</tr>
<tr>
<td>P100SLN</td>
<td>0</td>
<td>90.1 ± 0.96</td>
<td>0.22 ± 0.009</td>
<td>−0.64 ± 0.65</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>86.0 ± 0.83</td>
<td>0.22 ± 0.005</td>
<td>−0.03 ± 1.06</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>88.3 ± 0.31</td>
<td>0.23 ± 0.003</td>
<td>−0.20 ± 0.93</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>88.7 ± 1.03</td>
<td>0.23 ± 0.005</td>
<td>−0.29 ± 1.14</td>
</tr>
<tr>
<td>AA-P100SLN</td>
<td>0</td>
<td>118.7 ± 4.03</td>
<td>0.21 ± 0.011</td>
<td>−0.88 ± 1.25</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>115.2 ± 0.93</td>
<td>0.18 ± 0.019</td>
<td>−0.14 ± 1.07</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>112.7 ± 0.87</td>
<td>0.19 ± 0.010</td>
<td>−1.49 ± 0.91</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>114.0 ± 1.45</td>
<td>0.19 ± 0.003</td>
<td>−0.16 ± 1.48</td>
</tr>
</tbody>
</table>
**Table 4.8 Effect of time of storage (at 4 °C) on particle size, PDI and surface charge of RP40SLNs and RP100SLN**

<table>
<thead>
<tr>
<th>SLN Samples</th>
<th>Days</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RP40SLN</strong></td>
<td>0</td>
<td>99.1 ± 3.35</td>
<td>0.26 ± 0.011</td>
<td>−0.62 ± 0.93</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>92.6 ± 2.09</td>
<td>0.23 ± 0.007</td>
<td>−0.60 ± 1.36</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>93.1 ± 1.09</td>
<td>0.23 ± 0.004</td>
<td>−0.29 ± 1.06</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>92.4 ± 1.66</td>
<td>0.23 ± 0.002</td>
<td>−0.51 ± 1.55</td>
</tr>
<tr>
<td><strong>AA-RP40SLN</strong></td>
<td>0</td>
<td>113.5 ± 2.35</td>
<td>0.19 ± 0.005</td>
<td>−1.50 ± 0.69</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>112.6 ± 1.88</td>
<td>0.17 ± 0.003</td>
<td>−1.68 ± 0.88</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>111.9 ± 1.10</td>
<td>0.17 ± 0.014</td>
<td>−0.71 ± 1.01</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>113.8 ± 0.68</td>
<td>0.18 ± 0.003</td>
<td>−1.09 ± 0.79</td>
</tr>
<tr>
<td><strong>RP100SLN</strong></td>
<td>0</td>
<td>96.1 ± 2.73</td>
<td>0.23 ± 0.007</td>
<td>−0.37 ± 1.15</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>95.4 ± 1.79</td>
<td>0.21 ± 0.003</td>
<td>−1.52 ± 1.11</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>93.8 ± 1.45</td>
<td>0.21 ± 0.008</td>
<td>−0.79 ± 0.81</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>94.2 ± 1.89</td>
<td>0.22 ± 0.005</td>
<td>−0.74 ± 0.69</td>
</tr>
<tr>
<td><strong>AA-RP100SLN</strong></td>
<td>0</td>
<td>120.8 ± 3.49</td>
<td>0.18 ± 0.011</td>
<td>−0.91 ± 0.56</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>118.3 ± 1.61</td>
<td>0.19 ± 0.007</td>
<td>−0.86 ± 0.99</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>119.6 ± 0.42</td>
<td>0.18 ± 0.015</td>
<td>−0.76 ± 1.04</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>119.5 ± 1.01</td>
<td>0.20 ± 0.017</td>
<td>−0.74 ± 0.84</td>
</tr>
</tbody>
</table>
4.3.2 In vitro studies of PEGylated and RGD-targeted SLNs (± AA)

4.3.2.1 Evaluation of in vitro cytotoxicity

The cytotoxicity of PEG(40)stearate and PEG(100)stearate was studied prior to SLN preparation towards SVG P12 and U87 MG cells within the concentration range of 0 – 0.5 mg/mL. It has been observed that increase in the concentration of PEG-stearates increases the toxicity towards both SVG P12 and U87 MG cells (Table 4.9 – 4.10, Figure 4.6 – 4.7).

A variety of PEG-stearate density was used to prepare PEGylated SLNs in the literature (Kakkar et al., 2015; Wan et al., 2008; Yuan et al., 2013; Zhao et al., 2012). However, since PEG-stearates showed concentration-dependent toxicity (Table 4.9 – 4.10, Figure 4.6 – 4.7), based on the minimal toxicity towards both SVG P12 and U87 MG cells recorded for PEG(40)stearate and PEG(100)stearate, PEG 5 and 10 mol % densities were selected as the optimal PEG densities for future SLN preparation. The highest PEG(40)stearate concentrations applied to the cells were 0.064 and 0.128 mg/mL and PEG(100)stearate were 0.161 and 0.321 mg/mL for 5 and 10 mol % densities, respectively. These concentrations correspond to > 90 % cell viability for both SVG P12 and U87 MG cells.

Time-dependent cytotoxicity was observed for all PEG-SLNs tested against SVG P12 and U87 MG cells. Furthermore, an increase in PEG concentration (from 5 mol % to 10 mol %) incorporated to the SLN formulations enhanced the cytotoxicity of AA-loaded PEG-SLNs towards both SVG P12 and U87 MG cells (Figure 4.8 and 4.9). For example, U87 MG cells treated with 0.031 mg/mL AA-equivalent concentration of AA-P40SLN demonstrated significantly different (p<0.05) cell viability following 24 h incubation; showing ~ 67 % viable cells with 5 mol % PEG and ~ 33 % viable cells with 10 mol % PEG containing P40SLNs (Figure 4.9). Likewise, a significant difference was also observed in U87 MG cells following treatment with 0.024 mg/mL AA-equivalent concentration of 5 and 10 mol % PEG containing AA-P40SLNs. For example, while 5 mol % P40SLNs resulted in ~ 100 %, ~ 54 % and ~ 43 % cell viabilities, 10 mol % P40SLNs ~ 85 %, ~ 48 % and ~ 35 % cell viabilities following 24, 48 and 72 h incubation, respectively.
Figure 4.6 Cell viability of SVG P12 cells following treatment with A) PEG40-stearate; B) PEG100-stearate after 24, 48, and 72 h.
Figure 4.7 Cell viability of U87 MG cells following treatment with A) PEG40-stearate; B) PEG100-stearate after 24, 48, and 72 h.
Table 4.9 Concentration of different formulations against U87 MG and SVG P12 cells that result in 50 % viability (IC\textsubscript{50}).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Exposure time (h)</th>
<th>IC\textsubscript{50} values for SVG P12*</th>
<th>IC\textsubscript{50} values for U87 MG*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/mL µM</td>
<td>mg/mL µM</td>
<td></td>
</tr>
<tr>
<td>PEG40Stearate</td>
<td>24</td>
<td>0.58 ± 0.03 290 ± 1.5 0.68 ± 0.04 340 ± 2.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.39 ± 0.02 195 ± 1.0 0.36 ± 0.02 180 ± 1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.37 ± 0.02 185 ± 1.0 0.36 ± 0.02 180 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>P40SLN (5 %)</td>
<td>24</td>
<td>N.D. N.D. N.D. N.D.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.078 ± 0.002 159.6 ± 4.1 0.062 ± 0.001 126.8 ± 2.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.070 ± 0.002 143.2 ± 4.1 0.056 ± 0.002 114.5 ± 4.1</td>
<td></td>
</tr>
<tr>
<td>P40SLN (10 %)</td>
<td>24</td>
<td>N.D. N.D. 0.11 ± 0.005 225 ± 10.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.074 ± 0.004 151.4 ± 8.1 0.048 ± 0.004 98.2 ± 8.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.064 ± 0.004 130.9 ± 8.1 0.040 ± 0.002 81.4 ± 4.1</td>
<td></td>
</tr>
<tr>
<td>AA-P40SLN (5 %)</td>
<td>24</td>
<td>0.040 ± 0.002 81.8 ± 4.1 0.035 ± 0.003 71.6 ± 6.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.036 ± 0.002 73.6 ± 4.1 0.025 ± 0.005 51.1 ± 10.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.030 ± 0.001 61.3 ± 2.0 0.023 ± 0.002 47.0 ± 4.1</td>
<td></td>
</tr>
<tr>
<td>AA-P40SLN (10 %)</td>
<td>24</td>
<td>0.038 ± 0.006 77.5 ± 12.2 0.028 ± 0.004 57.2 ± 8.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.030 ± 0.002 61.3 ± 4.1 0.024 ± 0.002 49.1 ± 4.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.028 ± 0.002 57.2 ± 4.1 0.021 ± 0.004 42.9 ± 8.1</td>
<td></td>
</tr>
</tbody>
</table>

*IC\textsubscript{50} values of AA-SLNs calculated based on AA-equivalent concentrations and values of non-AA-loaded SLNs were given based on AA-SLN-equivalent concentrations (N=3, n=18 ± SD). N.D. means no IC\textsubscript{50} values were determined using the concentrations tested.
Table 4.10 Concentration of different formulations against U87 MG and SVG P12 cells that result in 50 % viability (IC<sub>50</sub>).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Exposure time (h)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; values for SVG P12*</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; values for U87 MG*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg/mL µM</td>
<td>mg/mL µM</td>
</tr>
<tr>
<td>PEG100Stearate</td>
<td>24</td>
<td>N.D. N.D.</td>
<td>N.D. N.D.</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.78 ± 0.02 156 ± 4.0</td>
<td>0.74 ± 0.04 148 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.70 ± 0.02 140 ± 4.0</td>
<td>0.70 ± 0.02 140 ± 4.0</td>
</tr>
<tr>
<td>P100SLN (5 %)</td>
<td>24</td>
<td>N.D. N.D.</td>
<td>N.D. N.D.</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.080 ± 0.005 163.6 ± 10.2</td>
<td>0.060 ± 0.002 122.7 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.062 ± 0.004 126.8 ± 8.1</td>
<td>0.054 ± 0.002 110.4 ± 4.1</td>
</tr>
<tr>
<td>P100SLN (10 %)</td>
<td>24</td>
<td>N.D. N.D.</td>
<td>N.D. N.D.</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.064 ± 0.004 130.9 ± 8.1</td>
<td>0.048 ± 0.004 98.2 ± 8.1</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.046 ± 0.004 94.1 ± 8.1</td>
<td>0.036 ± 0.004 73.6 ± 8.1</td>
</tr>
<tr>
<td>AA-P100SLN (5 %)</td>
<td>24</td>
<td>0.060 ± 0.002 122.7 ± 4.1</td>
<td>0.050 ± 0.003 102.3 ± 6.1</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.043 ± 0.002 87.9 ± 4.1</td>
<td>0.035 ± 0.005 71.6 ± 10.2</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.032 ± 0.004 65.4 ± 8.1</td>
<td>0.026 ± 0.002 53.2 ± 4.1</td>
</tr>
<tr>
<td>AA-P100SLN (10 %)</td>
<td>24</td>
<td>0.042 ± 0.004 85.9 ± 8.1</td>
<td>0.039 ± 0.001 79.8 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.037 ± 0.002 75.7 ± 4.1</td>
<td>0.027 ± 0.002 55.2 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.030 ± 0.002 61.3 ± 4.1</td>
<td>0.023 ± 0.002 47.0 ± 4.1</td>
</tr>
</tbody>
</table>

*IC<sub>50</sub> values of AA-SLNs were given based on AA-equivalent concentrations and values of non-AA-loaded SLNs were given based on AA-SLN-equivalent concentrations (N=3, n=18 ± SD). N.D. means no IC<sub>50</sub> values were determined using the concentrations tested.
Similarly; AA-P100SLN treatment applied on U87 MG cells showed cell viability differences at 0.024, 0.031 and 0.051 mg/mL AA-equivalent concentrations between 5 and 10 mol % PEG density following 24, 48 and 72 h incubation (Figure 4.11).

However, 5 and 10 mol % PEG100 densities of AA-P100SLN formulation did not result in a significant change ($p>0.05$) in cell viability of SVG P12 cells at concentrations lower than 0.051 mg/mL, as shown in Figure 4.10. The only cytotoxic difference of 5 and 10 mol % PEG100 density could be seen was at 0.051 mg/mL AA-equivalent concentration towards SVG P12 cells; where ~ 45 % and ~ 18 % cell viability was recorded for 5 mol % PEG containing P100SLNs, and ~ 15 % and ~ 8 % cell viability was observed with 10 mol % PEG following 48 and 72 h incubation, respectively.

On the other hand, AA-P40SLNs showed higher cytotoxic differences towards SVG P12 cells compared to AA-P100SLNs when PEG40 density was changed from 5 mol % to 10 mol %. For instance, treatment of 0.031 mg/mL AA-equivalent concentration of 5 and 10 mol % PEG containing AA-P40SLNs showed ~ 62 % and ~ 47 % cell viability with 5 mol % PEG, whereas ~ 46 % and ~ 37 % cell viability were observed for 10 mol % PEG following 48 and 72 h incubation, respectively (Figure 4.8).

Moreover, PEGylation of the SLNs significantly reduced the concentration required to kill 50 % of the cells (IC$_{50}$), of the AA-loaded SLNs as well. Whilst AA-MS-SLN requires 0.056, 0.038 and 0.03 mg/mL AA-equivalent concentration to result in 50 % cell viability of U87 MG cells following 24, 48 and 72 h incubation (Chapter 3), AA-P40SLN showed 0.035, 0.025, 0.023 mg/mL (with 5 mol % PEG content) and 0.028, 0.024, 0.021 mg/mL (with 10 % PEG content), respectively; and AA-P100SLN required 0.050, 0.035, 0.026 (with 5 mol % PEG content) and 0.039, 0.027, 0.023 mg/mL (with 10 % PEG content) AA-equivalent concentration to kill 50 % of U87 MG cells following 24, 48 and 72 h treatment, respectively (Table 4.9 – 4.10). The decrease in the IC$_{50}$ values could be due to the higher uptake and accumulation of the PEG-SLNs by SVG P12 and U87 MG cells, as confirmed using flow cell cytometry (Figure 4.20). Similar observation was also observed by Fang et al. (2012) where it was reported that chemotherapeutic agent 3-Chloro-4-[(4-methoxyphenyl)amino]furo[2,3-b]quinolone decreased IC$_{50}$ values of the treated cells after incorporation within PEGylated monostearate SLNs.

In addition to the AA-loaded PEG-SLNs, the cytotoxicity of non-drug-loaded blank PEG-SLNs was also assessed towards SVG P12 and U87 MG cells. P100SLNs (both 5 and 10 mol %) did not show any toxicity towards SVG P12 cells at concentrations lower than 0.051 mg/mL (Figure 4.10), whilst P40SLN with 10 mol % PEG density
showed toxicity starting from 0.031 mg/mL AA-equivalent concentration with ~ 80 % cell viability following 72 h incubation (Figure 4.8). At 0.051 mg/mL AA-equivalent concentration, P40SLN with 10 mol % PEG resulted in SVG P12 viability as 80 %, 68 % and 60 % following 24, 48 and 72 h incubation, respectively; whereas 5 mol % PEG containing P40-SLN gave ~ 80 % cell viability only after 72 h treatment at the same concentration (Figure 4.8). Consequently all PEG-SLNs (both PEG40 and PEG100) with 10 mol % PEG density showed higher toxicity towards both SVG P12 and U87 MG cells than all PEG-SLNs (both PEG40 and PEG100) with 5 mol % PEG density did. Therefore, due to the lower cytotoxicity of PEG-SLNs towards SVG P12 normal cells, 5 mol % PEG containing the SLNs was chosen for further conjugation to RGD peptide and for fluorescence microscopy, flow cell cytometry and apoptosis assay. Therefore, from this point onwards, the terminology used for PEG-SLN or RGD-PEG-SLN refers to 5 mol % PEG content, unless otherwise stated.
Figure 4.8 Cell viability of SVG P12 cells following treatment with A) P40SLN (5 %). B) P40SLN (10 %) after 24, 48, 72 h.
Figure 4.9 Cell viability of U87 MG cells following treatment with A) P40SLN (5 %). B) P40SLN (10 %) after 24, 48, 72 h.
Figure 4.10 Cell viability of SVG P12 cells following treatment with A) P100SLN (5 %). B) P100SLN (10 %) after 24, 48, 72 h.
Figure 4.11 Cell viability of U87 MG cells following treatment with A) P100SLN (5 %). B) P100SLN (10 %) after 24, 48, 72 h.
4.3.2.1.1 Summary of cytotoxicity findings so far

- The cytotoxicity of PEG(40)stearate and PEG(100)stearate was studied prior to SLN preparations and concentrations based on minimal toxicity towards cells tested were employed in PEGylation of SLNs (5 mol % and 10 mol %) (Figure 4.6 and 4.7).

- A time-dependent cytotoxic effect was observed with PEG-SLNs and increasing PEG density from 5 mol % to 10 mol %, increased the cytotoxicity towards cells tested (Figure 4.12).

- AA-containing SLNs prepared with PEG(100)stearate showed significantly lower toxicity (p<0.05) towards U87 MG cells when compared to SLNs with PEG(40)stearate (Figure 4.12). This might have been due to steric hindrance applied by the longer PEG chain length (PEG(100)stearate).

- Since non-drug loaded 10 mol % PEG-SLNs showed higher toxicity on SVG P12 normal cells, 5 mol % PEG-SLNs were chosen to use in further studies including RGD-conjugation. Therefore, as also described in Chapter 2, Section 2.3.1, RGD-containing SLNs were prepared conjugating RGD peptide to 5 mol % PEG-SLNs. **Most importantly, from this point onwards, the terminology used for PEG-SLN or RGD-PEG-SLN refers to 5 mol % PEG content, unless otherwise stated.**
Figure 4.12 A summary graph of Figure 4.8, 4.9, 4.10 and 4.11; showing cell viability following treatment with P40SLN and P100SLN (± AA) on A) SVG P12 cells and B) U87 MG cells after 72 h incubation. “-5” and “-10” represents 5 and 10 mol % PEG-stearate content in the SLN formulations, respectively.
RGD peptide was conjugated to SLN particles to actively target the αvβ3 integrin receptor, which is known to be overexpressed by U87 MG cells (Zhang et al., 2006). Conjugation of the RGD peptide improved the cytotoxicity of AA-loaded nanoparticles with both RP40SLN and RP100SLN. AA-RP40SLN showed improvement in the cytotoxicity towards U87 MG by reducing cell viability at lower concentrations when compared with non-RGD-containing AA-PEG40-SLN. For example, AA-RP40SLN showed significantly different extent of cytotoxicity towards U87 MG cells (p<0.05) at 0.024 mg/mL AA-equivalent concentration for both 48 and 72 h treatments when it resulted in ~ 42 % and ~ 34 % cell viability following 48 and 72 h incubation, respectively (Figure 4.13B), whilst ~ 54 % and ~ 43 % U87 MG viability were observed following 48 and 72 h incubation (Figure 4.9A), respectively, for AA-P40SLN. Likewise, AA-RP100SLN had shown significantly different cytotoxicity results when compared with AA-P100SLN counterpart (p<0.001) in the treatment of U87 MG cells. For example, following 24, 48 and 72 h incubation with AA-RP100SLN resulted in ~ 62 %, ~ 15 % and ~ 12 % cell viability (Figure 4.14B) whilst treatment with AA-P100SLN showed ~ 100 %, ~ 67 % and ~ 42 % alive cells (Figure 4.11A), respectively at 0.031 mg/mL AA-equivalent concentration.

The non-drug loaded PEG- and RGD- SLNs (with either PEG40 or PEG100 chain) appeared to be slightly less cytotoxic (p>0.05) towards U87 MG cells where MS-SLN gave IC50 of 0.056 and 0.042 mg/mL AA-equivalent concentration following 48 and 72 h, respectively (Chapter 3, Table 3.4) but IC50 values of 0.062 and 0.056 mg/mL for P40SLN (Table 4.9); 0.060 and 0.054 mg/mL for P100SLN (Table 4.10); 0.066 and 0.048 mg/mL for RP40SLN (Table 4.11) and 0.048 and 0.046 mg/mL for RP100SLN (Table 4.11) were found following 48 and 72 h incubation, respectively. Since greater amount of AA can be accommodated in PEG-SLN formulations (Table 4.2), less SLNs are therefore require to induce the same cytotoxic effect. Similarly, the reason for the lower IC50 (i.e. greater toxicity) observed for non-AA-loaded RGD-SLN s in comparison to the PEG-SLNs counterparts may have been due to the greater cellular uptake of the RGD-containing SLNs, as later confirmed using flow cell cytometry (Figure 4.20) and fluorescence imaging (Section 4.3.2.2).

The RGD-targeted SLNs using PEG100 chain length showed greater cytotoxic effect towards U87 MG cells when compared with those prepared with PEG40 (Figure 4.13 and 4.14). There is no significant difference in cytotoxicity of AA-P40SLN and AA-RP40SLN after 24 h incubation, however, a significant difference (p<0.05) was obtained only following 48 and 72 h incubation (Table 4.9 and 4.11, respectively). On the other hand, AA-P100SLN and AA-RP100SLN showed profound differences
(p<0.001) in terms of their cytotoxicity towards U87 MG cells at all time points tested (Table 4.10 and 4.11, respectively). This is in agreement with the flow cytometry data obtained to confirm higher cellular uptake of RP100SLN by U87 MG cells when compared with RP40SLN (Figure 4.20). The observations related to the cellular uptake of PEG- and RGD- SLNs will be discussed in Section 4.3.2.2.

Interestingly, AA alone (Chapter 3, Table 3.3) and RGD-SLNs showed similar IC₅₀ values for U87 MG cells (Table 4.11). For instance, treatment with AA alone on U87 MG cells resulted in IC₅₀ values of 0.03, 0.02 and 0.019 mg/mL (Chapter 3, Table 3.3), whilst AA-RP40SLN displayed 0.034, 0.022 and 0.020 mg/mL (Table 4.11), and AA-RP100SLN gave IC₅₀ values of 0.033, 0.024 and 0.020 mg/mL (Table 4.11) following 24, 48 and 72 h incubation, respectively. This illustrates one of the advantages of using RGD-targeted SLNs for the delivery of AA.
Figure 4.13 Cell viability following the treatment with RP40SLN against A) SVG P12 and B) U87 MG.
Figure 4.14 Cell viability following the treatment with RP100SLN against A) SVG P12 and B) U87 MG.
**Table 4.11** Concentration of different formulations against U87 MG and SVG P12 cells that result in 50 % viability (IC$_{50}$).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Exposure time (h)</th>
<th>IC$_{50}$ values for SVG P12*</th>
<th>IC$_{50}$ values for U87 MG*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg/mL</td>
<td>µM</td>
</tr>
<tr>
<td>RP40SLN</td>
<td>24</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.074 ± 0.002</td>
<td>151.4 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.064 ± 0.002</td>
<td>130.9 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.074 ± 0.004</td>
<td>151.4 ± 8.1</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.068 ± 0.004</td>
<td>98.2 ± 8.1</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.046 ± 0.004</td>
<td>94.1 ± 8.1</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.034 ± 0.001</td>
<td>69.5 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.030 ± 0.004</td>
<td>61.3 ± 8.1</td>
</tr>
<tr>
<td>AA-RP40SLN</td>
<td>24</td>
<td>0.054 ± 0.005</td>
<td>110.4 ± 10.2</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.042 ± 0.003</td>
<td>85.9 ± 6.1</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.031 ± 0.002</td>
<td>63.4 ± 4.1</td>
</tr>
<tr>
<td>AA-RP100SLN</td>
<td>24</td>
<td>0.064 ± 0.005</td>
<td>110.4 ± 10.2</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.042 ± 0.003</td>
<td>85.9 ± 6.1</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.031 ± 0.002</td>
<td>63.4 ± 4.1</td>
</tr>
</tbody>
</table>

*IC$_{50}$ values of AA-SLNs were given based on AA-equivalent concentrations and values of non-AA-loaded SLNs were given based on AA-SLN-equivalent concentrations (N=3, n=18 ± SD). N.D. means no IC$_{50}$ values were determined using the concentrations tested.
4.3.2.1.2 Summary of RGD-SLN cytotoxicity findings

- It is known that U87 MG cells express ~ 1.28 x 10⁵ αvβ₃ receptors per cell (Zhang et al., 2006). Therefore conjugation of RGD peptide to the SLNs helped to increase the cytotoxicity of AA-containing particles with both RP40SLN and RP100SLN as a result of possible RGD peptide – αvβ₃ receptor binding.
- AA-RP40SLN and AA-RP100SLN showed significantly higher toxicity (p<0.05 and p<0.001, respectively) compared to AA-P40SLNs and AA-P100SLNs counterparts and AA-MS-SLN (p<0.001).
- Non-drug-containing RGD-SLNs showed slightly higher toxicity than PEG-SLNs towards U87 MG glioma cells which could be due to the increased uptake of RGD-SLNs as a result of ligand-receptor binding (Figure 4.12, 4.15 and 4.22).
- Even though AA-P40SLN showed higher toxicity than AA-P100SLN towards U87 MG cells (Figure 4.12), it was observed that AA-RP40SLN and AARP100SLN displayed similar toxicity on U87 MG cells (Figure 4.15) which could also be attributed to ligand-receptor binding.
Figure 4.15 A summary graph of Figure 4.13 and 4.14; showing cell viability following treatment with RP40SLN and RP100SLN (± AA) on A) SVG P12 cells and B) U87 MG cells after 72 h incubation.
4.3.2.2 Intracellular uptake of PEGylated and RGD-targeted SLNs

The internalisation of the SLNs by SVG P12 normal cells and U87 MG glioma cells were confirmed using fluorescence imaging and flow cell cytometry both at 4 and 37 °C. SLNs were fluorescent labelled by incorporating the C6 fluorescent dye. Since control cells (Figure 4.17) and C6 alone did not show significant fluorescence intensity, as confirmed by both fluorescence microscopy and flow cell cytometry (C6 intensity was confirmed in Chapter 3, Section 3.3.2.2), the observed green-coloured fluorescence within cells after SLN treatments was attributed to C6-loaded SLNs. For fluorescence imaging, nuclei staining were achieved using DAPI stain (Figure 4.17).

Cellular uptake of SLNs and RGD-targeted nanoparticles have been reported as receptor-mediated endocytosis via clathrin- and/or caveolae-mediated pathway, which is an energy-dependent process (Chai et al., 2014; Martins et al., 2012; Yao et al., 2015). Therefore, examination at 4 and 37 °C help confirm the involvement of endocytosis of the nanoparticles prepared in this study, similar to other studies from the literature (Martins et al., 2012; Zhang et al., 2013). As demonstrated from the fluorescence images (Figure 4.18 – 4.21) and flow cell cytometry data (Figure 4.22), incubating cells with PEG- or RGD- SLNs at 4 °C highly reduced the internalisation of the nanoparticles (Figure 4.18 – 4.21). In contrast, incubation at 37 °C drastically showed higher fluorescence intensities (Figure 4.18 – 4.21), once again, indicating that SLN uptake is an energy-dependent process, similar to the observations discussed in Chapter 3 for MS-SLNs. Furthermore, surface modifications applied in this study did not cause a change in the apparent intracellular localisation of the tested SLNs because as reported in Chapter 3, Section 3.3.2.2 with MS-SLNs as well, that SLNs tend to accumulate around the nuclei of U87 MG cells (Figure 4.20 and 4.21), whereas a tendency of particle diffusion was observed inside the cytoplasm of SVG P12 cells (Figure 4.18 and 4.19).

Flow cell cytometry analysis (Figure 4.22) also shows that drug loaded and non-drug loaded P40SLN and P100SLN particles did not display significantly different cellular uptake (p>0.05) with neither U87 MG cells nor SVG P12 cells. This indicates that drug encapsulation did not have an influence on cellular uptake of these nanoparticles.

Significantly greater cellular uptake (p<0.05) of PEG- and RGD- SLNs by U87 MG cancer cells was evident when compared with SVG P12 cells (Figure 4.22). This was similar to the data obtained for MS-SLNs in these two cell lines (Chapter 3; Figure 3.17). Likewise, Costa et al. (2013) also demonstrated that nanoparticles were
internalised by breast cancer cells more effectively than by normal cells, which is a favourable property for cancer treatment.

Internalisation of nanoparticles by cells can be influenced by various factors. These factors relate to particle size (Wan et al., 2008; Zauner et al., 2001), cell type and seeding density (Zauner et al., 2001), surface properties such as charge and hydrophobicity (Carrstensen et al., 1992; Kobayashi et al., 2014), and also temperature (Martins et al., 2012; Zhang et al., 2013).

In comparison to MS-SLNs (Chapter 3, Section 3.3.2.2), it was found that the cellular uptake of PEG-SLNs (PEG(40)stearate or PEG(100)stearate) by both SVG P12 and U87 MG cells was much higher than that seen with MS-SLNs, similar to observations made obtained by Wan et al. (2008) and Yuan et al. (2008) where PEGylation of the SLNs improved the cellular uptake of nanoparticles. This could have been due to the smaller particle size induced by PEG-SLNs when the particle size of C6-loaded MS-SLNs (~145 nm) and C6-PEG-SLNs (94–125 nm) was compared. It was demonstrated by Carrstensen et al. (1992); Wan et al. (2008) and Zauner et al. (2001) that reduction of particle size favours cell uptake of nanoparticles. Moreover, Xu et al. (2012) explained that internalisation of large nanoparticles could be difficult because larger particles lead to higher stretching energies of the cell membrane. Therefore, cell membrane dynamics may also play an important role when determining the rate of nanoparticle uptake.

PEGylation of the nanoparticle also alters the hydrophobicity and hydrophilicity balance of the nanoparticle surface, which may lead to higher cellular uptake by cancer cells (Wan et al., 2008). Wan et al. (2008) also added that incorporation of PEG-stearates (melting point ~ 50 °C) to nanoparticles may reduce the melting point of the solid lipids that were used to prepare these nanoparticles, and hence resulting in lower viscosity of nanoparticles, enabling easier entering to cells.

More importantly, RGD-targeted SLNs showed greater uptake by U87 MG cells than that of PEG-SLNs. The increase in the internalisation of the RP40SLN compared to P40SLN was significantly (p<0.05) higher (Figure 4.22); whereas the difference of the cellular uptake of RP100SLN and P100SLN was even higher (p<0.001) (Figure 4.22 and Figure 4.23), which confirms the intended aim of mediating receptor (αvβ3)–ligand (RGD) specific binding of SLNs. When comparing cellular uptake results obtained in this study, it is apparent that RP100SLNs resulted in ~50% greater cellular uptake than MS-SLNs and ~20% greater uptake than P100SLNs, whereas RP40SLNs were
uptaken ~ 40 % more than MS-SLNs and ~ 15 % more than P40SLNs following 3 h of incubation. These are comparable with the data reported by Zeng et al. (2014) where C6-loaded RGD-containing liposomes showed 50 % increase in uptake compared to non-RGD containing liposomes by HUVEC cells.

The effects of PEG chain length as a linker on the targeting ability of the carrier systems were extensively studied in the literature (Cruz et al., 2011; Hattori and Maitani, 2004; Kawano et al., 2010; Saw et al., 2015; Shiokawa et al., 2005; Stefanick et al., 2013). However, there is no consensus on one particular PEG chain length for targeting. Stefanick et al. (2013) found that using shorter PEG linker length than PEG2000 enhanced the targeting to HER2 receptors with HER2- or VLA-4-antagonistic peptides, whilst Kawano et al. (2010) and Shiokawa et al. (2005) found that increasing PEG chain length from PEG2000 to PEG5000 improved the efficacy of the liposomes and microemulsions, respectively, via folate receptor targeting. There has not been a systematic analysis on the influence of PEG chain length on RGD targeting ability of SLNs as a drug delivery system.

In the current study, it was observed that RP100SLN with a longer chain length aids higher cellular uptake by U87 MG cells than RP40SLN that possesses a shorter chain length (Figure 4.22). This data was also in agreement with the cytotoxicity data obtained for RGD-SLNs against U87 MG cells where a larger cytotoxic increase was seen with RP100SLN than RP40SLN when comparing their corresponding PEG-SLN counterparts. RGD-SLNs with a longer PEG chain showing a higher cellular uptake suggests that ligand carrying longer chain probably enables a better interaction with the integrin receptors compared to the shorter chain (Gabizon et al., 1999). PEG arm length should be chosen carefully for an optimal particle surface structure to ensure receptor-ligand recognition. Too long a chain length may cause conformational “folding in” of the targeting moiety (RGD) inside the PEG globule, hence inhibiting effective receptor-ligand binding (Sawant et al., 2008); whereas a short chain length has the risk to be shielded by the surrounding polymers and result in reduced receptor-ligand binding (Sawant et al., 2008). PEG-stearate is an amphiphilic polymer and possesses both hydrophilic and hydrophobic parts. The hydrophobic part, stearate group, of PEG-stearate associates with the lipid core of SLNs and the hydrophilic part orientates on the surface where the targeting ligand also presents (Fang et al., 2012; Wan et al., 2008). Fang et al. (2012) predicted the structure of PEGylated SLNs using transmission electron microscopy (TEM) and reported it as shown in (Figure 4.16). Therefore, from the available literature, it is believed that the shorter PEG chain used in this study (PEG40stearate) may not have been long enough to reach out away from
the shield of the poloxamer 188 used for the preparation of these SLNs, hence a reduced cellular interaction with RP40SLNs was observed in comparison with that seen using RP100SLNs where a longer PEG chain (RGD100stearate) was employed.

Figure 4.16 Schematic representation of drug-loaded PEGylated SLNs adapted from (Fang et al., 2012).

For an effective integrin-mediated cell adhesion, (Kantlehner et al., 1999) found the minimal distance required between the ligand and the surface as ~ 35 Å. The length of a PEG monomer is 3.5 Å (Jokerst et al., 2011); therefore this makes the length of polymers PEG40 and PEG100 used in this study 140 and 350 Å, respectively, which means that PEG-stearates used in this study are convenient for targeting to the integrin receptors on the surface of U87 MG cells. However, no X-Ray crystallography testing of the drug delivery system was carried out in this investigation, as it was deemed to be beyond the scope of the project. Nevertheless, the targeting ability of the RGD-SLNs to U87 MG cells was demonstrated with a competitive binding experiment where free RGD peptide was pre-incubated with the cells before treatment with RGD-SLNs. As shown in Figure 4.22, pre-treatment with free RGD peptide reduced the cellular uptake of RGD-SLNs by 8 – 10 %. Therefore, this suggests that it is highly probable that an integrin receptor-mediated endocytosis was involved during the internalisation of the RGD-SLNs.
Figure 4.17 Fluorescence images of control cells without any treatment A) SVG P12 cells and B) U87 MG cells. Blue colour represents DAPI nucleus staining, green colour represents Coumerin-6 (C6) containing nanoparticles and the last column illustrates the merged images of DAPI and C6. This confirms that cells do not show any green-coloured fluorescence emission without treatment with C6-containing SLNs.
Preparation and Characterisation of Surface Modified PEGylated and RGD-targeted AA-SLNs

Figure 4.18 Fluorescence images of SVG P12 cells treated with P40SLN and P100SLN; A) P40SLN at 37 °C; B) P40SLN at 4 °C; C) P100SLN at 37 °C and D) P100SLN at 4 °C. Blue colour represents DAPI nucleus staining, green colour represents Coumarin-6 (C6)-containing nanoparticles and the last column illustrates the merged images of DAPI and C6.
Figure 4.19 Fluorescence images of SVG P12 cells treated with RP40SLN and RP100SLN; A) RP40SLN at 37 °C; B) RP40SLN at 4 °C; C) RP100SLN at 37 °C and D) RP100SLN at 4 °C. Blue colour represents DAPI nucleus staining, green colour represents Coumarin-6 (C6)-containing nanoparticles and the last column illustrates the merged images of DAPI and C6.
Figure 4.20 Fluorescence images of U87 MG cells treated with P40SLN and P100SLN: A) P40SLN at 37 °C; B) P40SLN at 4 °C; C) P100SLN at 37 °C and D) P100SLN at 4 °C. Blue colour represents DAPI nucleus staining, green colour represents Coumerin-6 (C6)-containing nanoparticles and the last column illustrates the merged images of DAPI and C6.
Figure 4.21 Fluorescence images of U87 MG cells treated with RP40SLN and RP100SLN; A) RP40SLN at 37 °C; B) RP40SLN at 4 °C; C) RP100SLN at 37 °C and D) RP100SLN at 4 °C. Blue colour represents DAPI nucleus staining, green colour represents Coumarin-6 (C6)-containing nanoparticles and the last column illustrates the merged images of DAPI and C6.
Preparation and Characterisation of Surface Modified PEGylated and RGD-targeted AA-SLNs

(See legend on next page)
Figure 4.22 Cell uptake studies of coumarin-6-loaded solid lipid nanoparticles in U87 MG and SVG P12 cells following 1, 3, or 5 h incubation at 37 or 4 °C using flow cell cytometry (mean fluorescence ± SD). Control cells are untreated cells; SLN without “A-” represents non-drug-loaded but coumarin-6 (C6)-containing SLNs; and “A-” represents AA and C6-containing MS-SLNs.

![Flow cytometry histograms](image)

Figure 4.23 Flow cytometry histogram overlays for A) SVG P12 cells and B) U87 MG cells; (control cells; RP100SLN uptake at 4 °C; P100SLN uptake at 37 °C; and RP100SLN uptake at 37 °C).

**4.3.2.3 Cell death mechanism induced by PEG-/RGD- SLNs (± AA)**

It is important to understand the mechanism of cell death induced by the SLNs ± drugs to help maximise potential anti-cancer therapeutic response. Cell death mechanism was determined using Alexa® Fluor 488-annexin V PI double staining apoptosis assay kit as described in Section 2.3.13. The aim of repeating the experiment with PEG-/RGD- SLNs was to determine the possible effects of surface modifications on the cell death mechanism of the drug delivery system towards SVG P12 and U87 MG cells. For this purpose, two different AA-equivalent concentrations (40 and 60 µM) of the PEG-/RGD- SLN formulations were used for the treatment of cells following 24, 48 and 72 h incubation to compare with the data previously obtained for AA alone and AA-MS-SLN treatment at equivalent concentrations.

Both PEG-SLNs and RGD-SLNs induced cell death of U87 MG and SVG P12 cells via both apoptosis and necrosis (Figure 4.24 – 4.25). As discussed in Chapter 3 Section 3.3.2.3, apoptotic cell death following AA-MS-SLN treatment was time- and concentration-dependent on both SVG P12 and U87 MG cells. Owing to the sustained release of AA from MS-SLNs, comparable extent of U87 MG cell apoptosis with AA
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alone was only seen with AA-MS-SLNs following 72 h. SLNs modified with PEG and RGD also displayed time- and concentration-dependent apoptotic cell death (Figure 4.24 – 4.25). However, comparable extent of apoptosis of U87 MG cells with AA alone was observed at an earlier time point (48 h) following RGD-SLN treatment. Additionally, a significant increase (p<0.001) in the total apoptosis of U87 MG cells was observed after PEG-/RGD-SLNs treatment of 40 µM AA-equivalent concentration compared to both AA alone and AA-MS-SLN treatment at all time points tested. The maximum difference of total apoptosis using 40 µM AA-equivalent concentrations between AA alone/AA-MS-SLN and PEG-/RGD-SLN formulations was observed following 72 h incubation. According to the data obtained after 72 h incubation, ~ 12 % and ~ 13 % total apoptotic cells were seen with cells treated with AA alone or AA-MS-SLN, respectively; whereas ~ 33 %, ~ 40 %, ~ 73 %, and ~ 69 % apoptotic cells were observed following AA-P40SLN, AA-P100SLN, AARP40SLN or AA-RP100SLN treatments (p<0.001), respectively (Figure 4.24). Therefore, the enhanced apoptotic effect of RGD-SLNs was evident, whilst the PEG chain length had less significant effect in this case. This is also true when a higher concentration (60 µM AA-equivalent concentration) was used. For example, whilst ~ 67 % and ~ 71 % apoptotic cell death was seen with AA alone and AA-MS-SLN, respectively, ARP40SLN and ARP100SLN showed ~ 75 % and ~ 81 % apoptotic cell death following 72 h (Figure 4.24). Consequently, these results not only indicate an improved apoptotic effect of RGD-SLNs by providing a greater and quicker progression from early to late phase apoptosis compared to AA-MS-SLN and PEG-SLN formulations, but they also demonstrate that RGD-SLNs increased apoptotic death of U87 MG cells more than that seen with AA alone. The increase in the induction of apoptosis of U87 MG cells by RGD-SLNs is most probably due to the increase in the cellular uptake of the nanoparticles by U87 MG cells via receptor-ligand binding (active targeting) and hence enhanced intracellular drug accumulation, as illustrated in the flow cytometry and fluorescence imaging data obtained in this study. Similar improved apoptotic results were also found by Liu et al. (2010) and Z. Wang et al. (2012) after RGD conjugation on the surface of liposomes and dextran-oleate conjugates, respectively.

A higher total apoptotic effect with PEG-SLNs was also seen in SVG P12 cells when comparing the data obtained for AA-MS-SLNs at both 40 and 60 µM AA-equivalent concentrations for all time points tested Figure 4.25. This could be due to the increased uptake of PEG-SLNs compared to MS-SLNs as confirmed with flow cytometry in Section 4.3.2.2, Figure 4.22.
The cell death mechanism induced by non-drug loaded PEG-/RGD- SLNs was also investigated and the killing mechanism of PEG-SLNs and RGD-SLNs was found to be via both apoptosis and necrosis. The increase in the apoptotic cell death by these SLNs was mainly seen following 72 h. As also mentioned before in Chapter 3, Section 3.3.2.3 the reasons for the cytotoxicity and the apoptotic effect of the non-drug loaded SLNs possibly due to the stearic acids present in SLN formulations as an impurity of the raw supplied lipid or the digestion product of glyceryl stearate lipids following cellular treatment (Mu et al., 2001; Zhang et al., 2011).
Figure 4.24 Flow cytometry analysis of U87 MG cells stained with annexin V-Alexa® Fluor 488 and PI following 24, 48 and 72 h incubation (A, B and C, respectively) treated with P40SLN, P100SLN, AA-P40SLN, AA-P100SLN, RP40SLN, RP100SLN, AA-RP40SLN and AA-RP100SLN at 40 and 60 µM AA-equivalent concentrations respectively. (N=3 ± SD)
Figure 4.25 Flow cytometry analysis of SVG P12 cells stained with annexin V- Alexa® Fluor 488 and PI following 24, 48 and 72 h (A, B and C respectively) treated by P40SLN, P100SLN, AA-P40SLN, AA-P100SLN, RP40SLN, RP100SLN, AA-RP40SLN and AA-RP100SLN 40 and 60 µM AA-equivalent concentrations respectively. (N=3 ± SD)
4.4 Conclusions

- Surface-modified SLNs were successfully prepared by incorporating PEG and RGD peptide into MS-SLN formulation obtained from Chapter 3.

- PEGylated SLN formulations were produced using two different PEG stearate molecules differing in chain length (PEG(40)stearate and PEG(100)stearate). Each PEG stearate was used with two different concentrations in the formulations (5 and 10 mol %) and the ideal PEG density was decided according to the cytotoxicity results obtained with SVG P12 and U87 MG cells. As a result, SLNs containing 5 mol % PEG were chosen for further investigation of cellular uptake and cell death mechanism as well as RGD peptide conjugation.

- Surface modifications to MS-SLNs prepared in Chapter 3 improved the yield of the PEG-/RGD-SLNs from 73.8 % to 82 – 96 %, as well as significantly improving cellular uptake and cytotoxicity of the formulations towards U87 MG glioma cells (IC_{50} of MS-SLN and RGD-SLN, respectively 0.03 and 0.02 mg/mL.)

- Improvement in efficacy of SLNs after RGD conjugation is most likely to be attributed to the receptor-ligand binding of RGD peptide with integrin receptors (\(\alpha_v\beta_3\)) found on the surface of the U87 MG cells, particularly when binding was reduced if competitively inhibited by RGD peptide alone.

The improved cell death and uptake of the RGD-targeted SLNs towards U87 MG cells demonstrated in this study confirms the potential of using the formulation for the treatment of glioma. To investigate further clinical relevance and potential of targeting to tumour tissues that overexpress integrin receptors (especially, \(\alpha_v\beta_3\)) on their surface, the effects of RGD-SLN formulations on multicellular tumour spheroids, which are accepted as models with intermediate complexity between monolayer cells and in vivo tumours (Santini and Rainaldi, 1999), were tested in Chapter 5.
Chapter 5

Anti-cancer effects of RGD-targeted SLNs on multicellular tumour spheroids
5.1 Introduction

Successful *in vitro* anti-cancer effects do not always translate to effective *in vivo* activity (Johnson *et al.*, 2001; Mikhail *et al.*, 2013; Phillips *et al.*, 1990). The main reason for this could relate to the common use of 2-dimensional (2D) monolayer cellular models for candidate drug screening, where there is often a lack of cell-cell interactions, adequate extracellular matrix support, and the 3-dimensional (3D) architecture inherently developed by tumour cells at different stages of tumour progression.

On the other hand, 3D tumour spheroids are of intermediate complexity between the *in vitro* monolayer cell cultures and the highly complex *in vivo* tumour models such as xenografts, which reflect particular aspects of 3D cellular interactions and provide resemblance to the *in vivo* tumour tissues (Hirschhaeuser *et al.*, 2010; Kunz-Schughart, 1999; Mikhail *et al.*, 2013). A recent study conducted by De Witt Hamer *et al.* (2007) revealed that genomic profile of human glioma is preserved in tumour spheroids but not in primary monolayer cultures. Therefore, it is believed that tumour spheroids are more suitable and relevant 3D *in vitro* cellular model to study the clinical potential of nanomedicine (Liang *et al.*, 2015; Mehta *et al.*, 2012; Mikhail *et al.*, 2013).

In this study, U87 MG tumour spheroids were employed as 3D *in vitro* tumour model by using the liquid overlay method (Metzger *et al.*, 2011; Mikhail *et al.*, 2013). The efficacy of the previously prepared AA-loaded MS-SLN, P40SLN, P100SLN, RP40SLN, RP100SLN formulations and free drug was tested on these spheroids by 1) monitoring the inhibition of spheroid formation and growth using phase-contrast microscope; 2) evaluating spheroid cell viability using MTT assay; and 3) determining spheroid penetration and cellular uptake using confocal microscopy and flow cell cytometry.
5.2 Methods

5.2.1 Spheroid cultivation

Spheroid cultivation was carried out in the same way as culturing monolayer cells, as described in Section 2.3.9. The spheroids were incubated in a sterile environment, under humidified atmosphere of 5 % CO₂ at 37 °C. The same cell culture media and conditions used for U87 MG cells were also employed for tumour spheroids (Table 2.4).

5.2.1.1 Preparation of agarose-coated plates for spheroid culturing

Agarose coating of sterile 96-well plates were prepared by adapting the method proposed by Friedrich et al. (2009). To dissolve agarose in PBS solution (1 % w/v), the agarose-PBS mixture was continuously stirred at 90 °C until the solution became clear. Dissolved agarose was autoclaved for sterilisation. In order to avoid solidifying the agarose solution prematurely at low temperatures, all the plates and pipette tips were warmed up at least 1 h at 37 °C inside an incubator prior to the addition of agarose solution (50 µL) to each well. Cooling down the agarose solution slowly is important to ensure smooth and concave surfaces are obtained. The agarose-coated plates were left to cool down and solidify for at least 3 h inside the sterile laminar flow hood. Then they were sealed using parafilm and covered with aluminium foil to protect from light and kept in the refrigerator (4 °C) for future use. Plates were kept in the refrigerator for a maximum of one week for any testing.

5.2.1.2 Spheroid formation

Multicellular U87 MG tumour cell spheroids were prepared by liquid overlay method (Friedrich et al., 2009; Ho et al., 2012). U87 MG cells were detached using 0.25 % trypsin-EDTA solution as described in Section 2.3.9.1 and a single cell suspension was prepared in EMEM media without changing the supplement content (10 % FBS, 1 % L-glutamine, 1 % sodium pyruvate and 1 % NEAA). Cells were counted accurately using trypan blue exclusion (Section 2.3.9.2) and appropriate amount of cells (100 µL) (e.g. for spheroid growth curve 1 x 10⁴, 3 x 10⁴ and 5 x10⁴ cells/mL) were seeded into previously prepared agarose-coated 96-well plates. An extra volume of EMEM media (100 µL, containing all the supplements) was added into the wells to ensure tumour spheroids have enough culture media for surviving following 4 days till spheroid formation completes. Plates were then maintained in a humidified incubator at 37 °C under 5 % CO₂. The spheroid formation was confirmed using an inverted light
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microscope. After spheroid formation (4 days), which gave spheroids diameter of less than 400 µm, were incubated with either AA alone or SLN formulations (± AA) for various purposes.

5.2.2 Spheroid growth curves

The growth of U87 MG spheroids at three seeding densities was monitored using a phase-contrast microscope (Leica DMIL microscope) by measuring the dimensions of the spheroids every day over a 10-day period. U87 MG cells were detached and counted as previously described in Section 2.3.9.1 and 2.3.9.2. Single cell suspension was diluted to prepare 1 x 10^4, 3 x 10^4 and 5 x10^4 cells/mL, and seeded in 96-well plates (100 µL) pre-coated with agarose to give 1 x 10^3, 3 x 10^3 and 5 x10^3 cells/well. An extra 100 µL of culture media was added into the wells (makes total volume 200 µL) to provide enough nutrients for cells until spheroid formation completes (day 4). Cultures were maintained by replacing 100 µL of the media on day 4 and 7 (Vinci et al., 2012). Spheroid growth was determined using Leica DMIL inverted phase-contrast microscope by taking images with MShot camera every 24 h for 10 days (n=9). Spheroid diameter was determined by measuring and taking the average of two perpendicular diameters as shown in Equation 5.1. Images were processed with MShot Digital Imaging System software. The growth, in terms of volume of the tumour spheroids, was calculated using Equation 5.2 (Filippovich et al., 1997; Maftouh et al., 2014; Ni et al., 2015; Qu et al., 2005)

\[
\text{Diameter} = \frac{(D1 + D2)}{2} \quad \text{Equation 5.1}
\]

\[
\text{Volume of a spheroid} = \frac{4}{3} \pi \left(\frac{D}{2}\right)^3 \quad \text{Equation 5.2}
\]

5.2.3 Inhibition of tumour spheroid formation and growth assay

Evaluation on the inhibition of tumour spheroids growth by the prepared SLN formulations was performed by either incubating the cells with the SLN formulations (± AA) simultaneously or by adding the formulations after the tumour spheroids have been established on Day 4 post-seeding. In both cases, the effects on spheroid formation and growth of the spheroids were compared with cells received no treatment (i.e. control wells contained only cell culture media).

Briefly, U87 MG cells (3 x 10^3 cells/well) were seeded in pre-coated agarose 96-well plates together with either AA alone (as control) or SLN formulations (± AA) (0 – 0.051
mg/mL AA-equivalent concentrations) and incubated at 37 °C and 5 % CO₂. The changes in the formation or morphological structure of spheroids were followed using Leica DMIL light microscope after 72 h incubation and the images of the spheroids were recorded using MShot camera.

Alternatively, following spheroid formation by Day 4, the spheroids were treated with either AA alone or SLN formulations (± AA) in the range of 0 – 0.051 mg/mL AA-equivalent concentrations and continued routine cell culture incubation at 37 °C and 5 % CO₂. The changes in the spheroid growth were again monitored every 24 h for the subsequent 5 days and recorded using Leica DMIL light microscope and MShot camera. The dimensions of the spheroids were measured using MShot Digital Imaging System software and the spheroid volumes were calculated using Equation 5.2. The percent growth inhibition of the spheroids was calculated using Equation 5.3, comparing treated spheroids with the control spheroids (spheroids that was not applied any treatment).

\[
\text{% Growth inhibition} = \frac{V_i}{V_0} \times 100 \% \tag{5.3}
\]

\(V_i\) = Volume of the spheroids at \(i^{th}\) day after treatment

\(V_0\) = Volume of the spheroids at day 0 (before treatment given)

### 5.2.4 Evaluation of tumour spheroid viability

The viability of the tumour spheroids were assessed using the MTT assay (Mosmann, 1983). Ho et al. (2012) have modified the MTT assay for the evaluation of cell viability of spheroids and this method was further adapted for the study in Chapter 5. U87 MG cells (3 x 10³ cells/well) were seeded in the agarose pre-coated 96-well plates and incubated for 4 days to form spheroids at 37 °C under 5 % CO₂ atmosphere. Following 4 days of spheroid growth, plates were treated with either AA alone or SLN formulations (± AA) in the range of 0 – 0.051 mg/mL AA-equivalent concentrations by replacing 50 % of the culture media with drug/formulation-containing media and plates were incubated for a further 72 h at 37 °C under 5 % CO₂ environment. At the end of the treatment period (72 h), spheroids were transferred into new 96-well plates without agarose coating and 40 µL of MTT solution (5 mg/mL) was added into each well. The spheroids were incubated with MTT solution for 5 h at 37 °C and then MTT + media mixture was carefully removed completely (240 µL) from the plates before addition DMSO (100 µL) into the wells to dissolve the formazan crystals. After incubation of DMSO for 30 min, absorbance was recorded at 595 nm using Tecan microtitre plate.
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reader. Cell viability of the spheroids was presented in percentage by comparing with untreated (control) cells (N=3, n=9).

5.2.5 SLN uptake by tumour spheroids using confocal fluorescence microscopy

Nanoparticle uptake by tumour spheroids was visualised using Zeiss LSM510 confocal microscope. Briefly, spheroids after 4 days of growth were incubated with C6-loaded MS-SLN, PEG-SLN, RGD-SLN formulations or C6 alone for 3 or 6 h at 37 °C in 5 % CO₂ conditions. Then, the spheroids were harvested, centrifuged at 1000 rpm 5 min and the supernatant was discarded. The spheroids were washed with PBS (3 times) and fixed with paraformaldehyde (4 %, w/v) for 20 min. The spheroids were then washed again with PBS (2 times) and placed on cover slips for imaging. Z-stack images of spheroids were recorded with ~ 5.7 µm intervals starting from the top of each spheroid using argon laser at 488 nm. The images were recorded using 10 x objective and processed using Zeiss LSM Image Browser version 4.2 software, USA. Z-projection images were also recorded to show the general distribution of C6 in the spheroids.

5.2.6 SLN uptake by tumour spheroids using flow cell cytometry

For a better comparison of SLN uptake between monolayers of cells and 3D cultures, the quantitative analysis of nanoparticle uptake by spheroids was determined using flow cell cytometry. Briefly, spheroids were grown for 4 days and treated with either C6-loaded SLNs or C6 alone for 3 and 5 h at 37 °C under 5 % CO₂ atmosphere. Spheroids were then harvested and washed with PBS (3 times). Subsequently, AccuMax® cell dissociation solution was added to the tubes and incubated at 37 °C for 20 min with occasional pipetting to dissociate the spheroids. In order to prevent damage to the spheroids, AccuMax® cell dissociation solution was used rather than using 0.25 % EDTA-trypsin. Following cell dissociation, the cells were centrifuged at 1000 rpm for 5 min and the supernatant was discarded. Cells were re-suspended in PBS (300 µL) and immediately analysed by flow cell cytometry under the same settings described for SLNs uptake by monolayer U87 MG cells (Section 2.3.11).
5.3 Results and Discussion

5.3.1 Spheroid formation and growth curves

Various spheroid preparation techniques have been explored in the literature, including “hanging drops” (Kelm et al., 2003; Timmins and Nielsen, 2007), liquid overlay (Friedrich et al., 2009; Sarisozen et al., 2014), spinner flask (Lin et al., 2008; Nyberg et al., 2005), low attachment plates (Takaishi et al., 2009), polymeric scaffolds (Fischbach et al., 2007), microfluidic devices and spheroids on a chip (Ong et al., 2008; Torisawa et al., 2007). Each of these techniques has deficiencies in uniformity of spheroid size, low throughput, requirement of special equipment and high shear forces.

The liquid overlay method was chosen in this study to produce spheroids as it provides unified and reproducible spheroids with a single tumour spheroid per well, which is relatively easier to handle without the need of specialised equipment and it can produce a large number of spheroids for high-throughput screening. To demonstrate the reproducibility of spheroid size studied in this investigation, the coefficient of variation (CV) of spheroid diameters on day 4 was calculated using three different seeding cell densities (1 x 10^3, 3 x 10^3 and 5 x 10^3 cells/well) of U87 MG cells and the subsequent spheroid production yields were calculated by monitoring the efficiency of formation of one spheroid per well with regular shapes (Sarisozen et al., 2014; Vinci et al., 2012). The production yield of the tumour spheroids were found to be 80.0 ± 3.3 %, 94.4 ± 3.8 % and 90.0 ± 6.6 % with intraplate CV of 2.41 %, 2.52 % and 3.66 % for 1 x 10^3, 3 x 10^3 and 5 x 10^3 cells/well of U87 MG cells, respectively.

Ivascu and Kubbies (2007) studied the diversity of different breast cancer cell spheroids and classified them according to their morphology as compact spheroids, tight aggregates and loose aggregates. Compact spheroids are defined as having tight cell-cell adhesion, compactness, regular shapes and hardly distinguishable individual cell margins where relatively long trypsinisation time and mechanical force are required for the dissociation of these spheroids into individual cells. Tight aggregates, on the other hand, do not show full compaction like compact spheroids and hence shorter trypsinisation time with low mechanical shear is often required. Loose aggregates are also defined as loose association of cells, which can be easily dissociated with gentle agitation (Ivascu and Kubbies, 2007). According to this classification, the tumour spheroids obtained in this study can be classified as tight spheroids. Similar observation with U87 MG tumour spheroids was also noted by Vinci et al. (2012) where tight spheroids showing spherical, dense and regular structure were obtained.
After 24 h incubation of U87 MG cells (at all seeding densities tested), they showed tight aggregates with irregular shapes. Spheroids with more compact and regular shapes were seen after 48 h, as illustrated in Figure 5.1.

**Figure 5.1 Formation of U87 MG spheroids.** The figure shows the spheroid formation following incubation for: A) 1 day; B) 2 days and C) 3 days after seeding at $1 \times 10^3$, $3 \times 10^3$ and $5 \times 10^3$ cells/well. Scale bars represent 200 µm.
Due to the irregular shapes, dimensions of the spheroids obtained following 24 h incubation of the cells was not included into the spheroid growth curves (Figure 5.2). As shown in Figure 5.2 and 5.3, the spheroids grew in a linear fashion at all of the cell seeding densities tested (1 x 10^3, 3 x 10^3 and 5 x 10^3 cells/well). The increase in growth of the spheroids can be seen in Figure 5.3 where images of the spheroids on day 4, 6, 8 and 10 of incubation are presented. Spheroids have shown a faster growth rate with smaller initial cell numbers (1 x 10^3 and 3 x 10^3 cells/well) whilst a slower growth rate was observed with spheroids whose seeding density was 5 x 10^3 cells/well, especially after day 5. Mean growth rates of spheroids were ~40 – 50 µm per day for seeding densities at 1 x 10^3 or 3 x 10^3 cells/well, and ~30 – 40 µm for seeding density at 5 x 10^3 cells/well.

Three-dimensional tumour spheroids are of increasing interest for cancer research since they resemble in vivo tumour tissues in terms of proliferation gradients, enhanced drug resistant and limited mass transport (Iwascu and Kubbies, 2007; Kunz-Schughart, 1999). However studies have shown that the physiological state of spheroids depends on the spheroid size (Friedrich et al., 2009). It has been reported that spheroids with smaller sizes (< 200 µm) can display cell-cell and cell-matrix interactions; however they may not show pathophysiologica properties like having hypoxic areas in the centre of the spheroids or providing proliferation gradients (Friedrich et al., 2009; Hirschhaeuser et al., 2010; Iwascu and Kubbies, 2007), therefore they may not mimic in vivo tumours completely. On the other hand, large spheroids with the diameter > 500 µm develop central secondary necrosis (Friedrich et al., 2009; Hirschhaeuser et al., 2010; Iwascu and Kubbies, 2007) and cause cell death in the centre of the spheroid. Therefore, choosing spheroids with appropriate dimensions to test the SLN formulations prepared in this study is important. It has been suggested that spheroids that are ≤ 400 µm employed for spheroid-based drug screening studies (Friedrich et al., 2009) are big enough to show cell-cell, cell-matrix interactions and pathophysiological properties that mimic in vivo tumours, yet small enough for not developing central secondary necrosis. Additionally, findings suggest that incubating spheroids for 96 h provides enough time for a spheroid to form (Friedrich et al., 2009). In our study, 3 x 10^3 cells/well reached spheroid diameter of 325 – 375 µm after 4 days (96 h) of incubation (Figure 5.2). Therefore, spheroids with an initial cell number of 3 x 10^3 cells/ well were chosen for subsequent experiments on spheroid growth inhibition, cytotoxicity and nanoparticle uptake.
Figure 5.2 Growth of U87 MG spheroids over 10 days. Cells were initially seeded at three different seeding densities of $1 \times 10^3$, $3 \times 10^3$ and $5 \times 10^3$ cells/well and growth was monitored using a phase-contrast microscope every 24 h over 10 days. (N=3; ± SD)
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1 x 10^3 cells/well 3 x 10^3 cells/well 5 x 10^3 cells/well

Figure 5.3 Images of growth of U87 MG spheroids. Cells were initially seeded at three different seeding densities of 1 x 10^3, 3 x 10^3 and 5 x 10^3 cells/well, and growth was monitored using a phase-contrast microscope every 24 h over 10 days. Figure shows images of spheroids taken following incubation for A) 4 days; B) 6 days; C) 8 days, and D) 10 days. Scale bars represent 200 µm.
5.3.2 Inhibition of spheroid formation

The cells (3 x 10^3 cells/well) seeded simultaneously with AA showed complete spheroid inhibition with AA concentrations ≥ 31 µg/mL and spheroid damage with AA concentration at 24 µg/mL (Figure 5.4). Since IC_{50} value of AA with U87 MG cells after 24 h incubation is 30 µg/mL (Chapter 3, Table 3.3), the spheroid formation is most likely inhibited due to the cell death induced by AA for concentrations higher than 30 µg/mL. Although cell aggregation was seen at 24 µg/mL AA, damage to the surface of the spheroids and prevention of the compact spheroid formation with regular shape and also inhibition of spheroid growth were evident after 72 h incubation. At a concentration of 11 µg/mL, AA did not show any apparent effect on spheroid formation, as the spheroids look like control spheroids without any treatment (Figure 5.4).

Both AA-RP40SLNs and AA-RP100SLNs inhibited spheroid formation completely at AA-equivalent concentrations ≥ 40 µg/mL following 24 h incubation (Figure 5.5 and 5.6). This data is indeed comparable with the IC_{50} values of the formulations (34 and 33 µg/mL, respectively following 24 h incubation). At 31 µg/mL AA-equivalent concentration, both formulations resulted in loose aggregates which can be dissociated with simple pipetting (Figure 5.5 and 5.6). Spheroids were evidently damaged when 24 µg/mL AA-equivalent concentration was applied to cells, where growth was inhibited and compactness was lost (Figure 5.5 and 5.6). However, when non-drug loaded RP40SLNs and RP100SLNs were tested, there was no visible damage or negative effect on spheroid formation (Figure 5.7 and 5.8), which demonstrate that the effect seen by AA-RP40SLN and AA-RP100SLN formulations was attributed to the drug, AA.

When the effect of PEG-SLNs were studied on spheroid formation, it was seen that P40SLNs showed similar effects on spheroids as RGD-SLN formulations; providing complete inhibition of spheroid formation with AA-equivalent concentrations ≥ 40 µg/mL and loose aggregates at 31 µg/mL, as well as spheroid growth inhibition and surface damage at 24 µg/mL AA-equivalent concentrations (Figure 5.9A). A higher concentration was required to facilitate the same effects for AA-P100SLNs and AA-MS-SLNs as complete spheroid inhibition was only observed at 51 µg/mL AA-equivalent (Figure 5.9C and 5.10A), whilst loose aggregates were seen at 40 µg/mL and non-compact, tight aggregates were detected at 31 µg/mL AA-equivalent concentrations. Like RGD-SLNs, the non-drug containing PEG-SLNs (Figure 5.9B and 5.9D) and MS-SLNs (Figure 5.10B) did not also show any effect on spheroid formation.
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Figure 5.4 Inhibition of U87 MG spheroid formation with AA. Images were taken following simultaneous incubation with AA concentration of 0 – 51 µg/mL for: A) 1 day; B) 2 days and C) 3 days using initial seeding density of $3 \times 10^3$ cells/well. Scale bars represent 200 µm.
Figure 5.5 Inhibition of U87 MG spheroid formation with AA-RGD100SLNs. Images were taken following simultaneous incubation with AA-equivalent concentration of 0 – 51 µg/mL for: A) 1 day; B) 2 days and C) 3 days using initial seeding density of 3 x 10^3 cells/well. Scale bars represent 200 µm.
Figure 5.6 Inhibition of U87 MG spheroid formation with AA-RGD40SLNs. Images were taken following simultaneous incubation with AA-equivalent concentration of 0 – 51 µg/mL for: A) 1 day; B) 2 days and C) 3 days using initial seeding density of 3 x 10^3 cells/well. Scale bars represent 200 µm.
Figure 5.7 Inhibition of U87 MG spheroid formation with RGD100SLNs. Images were taken following simultaneous incubation with AA-equivalent concentration of 0 – 51 µg/mL for: A) 1 day; B) 2 days and C) 3 days using initial seeding density of 3 x 10^3 cells/well. Scale bars represent 200 µm.
Figure 5.8 Inhibition of U87 MG spheroid formation with RGD40SLN. Images were taken following simultaneous incubation with AA-equivalent concentration of 0 – 51 µg/mL for: A) 1 day; B) 2 day and C) 3 days using initial seeding density of 3 x 10^3 cells/well. Scale bars represent 200 µm.
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Figure 5.9 Inhibition of U87 MG spheroid formation. Seeded cells were simultaneously incubated with A) AA-P100SLN; B) P100SLN; C) AA-P40SLN and D) P40SLN using initial seeding density of $3 \times 10^3$ cells/well. Images were taken following 3 day incubation with AA-equivalent concentration of 0 – 51 µg/mL. Scale bars represent 200 µm.
Figure 5.10 Inhibition of U87 MG spheroid formation. Seeded cells were simultaneously incubated with A) AA-MS-SLN and B) MS-SLN using initial seeding density of $3 \times 10^5$ cells/well. Images were taken following 3 day incubation with AA-equivalent concentration of 0 – 51 µg/mL. Scale bars represent 200 µm.
5.3.3 Inhibition of spheroid growth

Inhibition of spheroid growth was assessed by monitoring the change in the spheroid volume every 24 h and to compare the effect of the formulations at 51 µg/mL AA-equivalent concentrations, the ratio of change of U87 MG spheroid volume (calculated using Equation 5.3) are presented graphically in Figure 5.12 and 5.13.

As shown in Figure 5.11 – 5.20, the control spheroids, continued to increase in size and volume, reaching 404 % of the initial volume after five days (Figure 5.12). In contrast, treatment on established spheroids (after day 4) with 51 µg/mL of AA showed immediate decrease in spheroid volume following 24 h incubation resulting in a percentage change in the ratio of tumour spheroid volume on first day of treatment as ~ 83 % (Figure 5.11B and 5.12). However, rather than seeing a continual reduction of spheroid sizes, an increase in spheroid volume was observed after day 2 following treatment with AA, resulting in the percentage of change ratio as ~ 86 %, ~ 103 %, ~ 126 % and ~ 130 %, corresponding to 2, 3, 4, 5 days, respectively (Figure 5.11 C and D and Figure 5.12). Even though AA treatment reduced the growth rate of spheroids significantly when compared to the growth rate of the control spheroids (p<0.001), the continued growth of the spheroids examined implies that a single dose of AA may not be enough to inhibit the spheroid growth and destroy all the cells completely. This is not absolutely unexpected, as it is known that the standard dose administration of temozolomide, which is an effective chemotherapy drug used in the treatment of malignant glioma, is given through 5 sequential days for an interval of 4 weeks (Khan et al., 2002). Günther et al. (2003) also showed that single administration of temozolomide reduced the growth rate of U87 MG tumour spheroids, however the spheroids also continued to grow at a slower rate, and only after the second round of treatment with temazolomide that the U87 MG tumour spheroids were killed.

Likewise, Vinci et al. (2012) studied the concentration-dependent effects of different drugs on U87 MG tumour spheroids. While all the treatments given showed concentration-dependent responses, results varied on the reduction of tumour spheroid volume; some stopped the spheroid from growing and some only reduced the growth rate. Therefore, future studies on varying the dosing regimen for AA-containing nanoparticulate drug delivery systems would be invaluable.
Anti-cancer effects of RGD-targeted SLNs on multicellular tumour spheroids

<table>
<thead>
<tr>
<th>Control</th>
<th>11 µg/mL</th>
<th>24 µg/mL</th>
<th>31 µg/mL</th>
<th>40 µg/mL</th>
<th>51 µg/mL</th>
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**Figure 5.11** Inhibition of U87 MG spheroid growth following incubation with free AA on established U87 MG spheroids (after Day 4). Images were taken after drug treatment (AA concentration 0 – 51 µg/mL) for: A) 0 day; B) 1 day; C) 3 days and D) 5 days incubation. Scale bars represent 200 µm.
In contrast, an increase in the incubation time helped to promote a reduction of tumour spheroid volume following treatments with AA-RP100SLNs and AA-RP40SLNs (Figure 5.12, Figure 5.14B and Figure 5.15B). Spheroids treated with AA-RP100SLNs and AA-RP40SLNs (Figure 5.12, Figure 5.14B and Figure 5.15B), showed a delay in reducing the spheroid volume, as the percentage change in the ratio of spheroid volume was recorded as ∼ 104 % and ∼ 102 %, respectively, following 24 h incubation with the formulations. Nevertheless, this was followed by ∼ 94 %, ∼ 92 %, ∼ 90 % and ∼ 87 % change ratio with AA-RP100SLN formulation and ∼ 97 %, ∼ 93 %, ∼ 92 % and ∼ 90 % change ratio with AA-RP40SLN formulations after 2, 3, 4 and 5 days incubation, respectively (Figure 5.12, Figure 5.14 and 5.15 C – D). Therefore, there was a sustained response regarding the ratio of spheroid volume change after 24 h. Contrary to the treatment with AA alone, RGD-targeted SLNs (AA-RP100SLNs and AA-RP40SLNs) not only stopped tumour growth, but also helped to reduce the volume of the spheroids when compared to the initial spheroid volumes (Figure 5.12). The sustained release of the drug from SLNs may have resulted in this continued growth inhibition observed from the data, which supports the potential development of such drug delivery system for the delivery of AA.

It was reported by Mikhail et al. (2013) that high intracellular binding and consumption of the drug by peripheral cells of the spheroids reduce the effectiveness of docetaxel and therefore limits the toxicity of docetaxel to cells distant from the surface. To overcome this, drug was suggested to be incorporated into nanoparticles which would minimise the interaction with the peripheral cells and thus may improve penetration and efficacy (Mikhail et al., 2013). In light of this, this also helps explain the immediate reduction in tumour volume observed in this study following 24 h incubation with AA but its inability to sustain this effect over 5 days. On the other hand, since AA is carried by SLNs with greater cellular uptake and penetration, as well as being released over a longer period of time, greater effects on the reduction of spheroid volumes were obtained in this study. Such effect is further enhanced by the targeting ligand, RGD.

Complementary to the data obtained for Chapter 3 and 4 on the higher uptake of RGD-targeted SLNs by U87 MG cells, the synergistic effect of AA and RGD-mediated reduction in spheroid volume observed in this study. In comparison with the RGD-SLN preparations, there was not a significant decrease in the spheroid volume following treatment with AA-P100SLNs and AA-P40SLNs, but these PEGylated SLN formulations also stopped tumour spheroid growth during the course of treatment for over 5 days (Figure 5.13, 5.16 and 5.17). The effect was still more desirable than that achieved with AA alone, where AA was not effective at reducing spheroid volume
Anti-cancer effects of RGD-targeted SLNs on multicellular tumour spheroids

(Figure 5.12 and 5.13). This could have been due to the slow release of AA from the SLN preparations and nanoparticle accumulation inside the spheroids. However, as shown previously in Chapter 4, Section 4.3.2.2, since PEG-SLNs are not taken up by U87 MG cells as much as RGD-SLNs, this may have been the reason why no significant reduction of the spheroid volume was observed with PEG-SLNs when compared with RGD-targeted SLNs. Similarly, tumour spheroid growth was stopped by AA-MS treatment over 4 days of incubation, where no significant change in spheroid volume was observed. Only a slight, but insignificant increase in volume was calculated on day 5 of treatment (Figure 5.13 and 5.18 A and B).

The non-drug loaded SLNs were also tested on tumour spheroids as controls. As seen in Figure 5.12 and 5.13; and Figure 5.18 – 5.20, spheroids continued growing in the presence of SLNs, indicating no beneficial effects on the spheroids. However, spheroids treated with RGD-SLNs and PEG-SLNs seems like reduced the growth rate slightly when compared with control spheroids. MS-SLN, on the other hand showed almost the same growth rate like control spheroids which contains no treatment. This could be due to the nanoparticle uptake capability of the U87 MG cells where it is shown in Chapter 4, Section 4.3.2.2 that PEG-SLNs and RGD-SLNs were taken up more by U87 MG cells than MS-SLNs.
Figure 5.12 Percentage change in volume ratio of U87 MG spheroids over 5 days following treatments with RGD-SLNs. Spheroids were treated with 51 µg/mL AA-equivalent concentration of AA, AA-RP100SLN, RP100SLN, AA-RP40SLN and RP40SLN. The percentage of volume change was based on the comparison of spheroid volume at i\textsuperscript{th} day with volume of the spheroids at day 0. (Equation 5.3)
Figure 5.13 Percentage change in volume ratio of U87 MG spheroids over 5 days following treatments with non-RGD SLNs. Spheroids were treated with 51 µg/mL AA-equivalent concentration of AA-P100SLN, P100SLN, AA-P40SLN, P40SLN, AA-MS-SLN and MS-SLN. The percentage of volume change was based on the comparison of spheroid volume at i<sup>th</sup> day with volume of the spheroids at day 0. (Equation 5.3)
Anti-cancer effects of RGD-targeted SLNs on multicellular tumour spheroids

<table>
<thead>
<tr>
<th>Control</th>
<th>11 µg/mL</th>
<th>24 µg/mL</th>
<th>31 µg/mL</th>
<th>40 µg/mL</th>
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**Figure 5.14** Images illustrating inhibition of U87 MG spheroid growth following treatment with AA-RP100SLNs. Images were taken following incubation with formulations containing 0 – 51 µg/mL of AA-equivalent concentrations for A) 0 day; B) 1 day; C) 3 days and D) 5 days. Scale bars represent 200 µm.
Anti-cancer effects of RGD-targeted SLNs on multicellular tumour spheroids

Figure 5.15 Images illustrating inhibition of U87 MG spheroid growth following treatment with AA-RP40SLNs. Images were taken following incubation with formulations containing 0 – 51 µg/mL of AA-equivalent concentrations for A) 0 day; B) 1 day; C) 3 days and D) 5 days. Scale bars represent 200 µm.
Anti-cancer effects of RGD-targeted SLNs on multicellular tumour spheroids

Figure 5.16 Images illustrating inhibition of U87 MG spheroid growth following treatment with AA-P100SLNs. Images were taken following incubation with formulations containing 0 – 51 µg/mL of AA-equivalent concentrations for A) 0 day; B) 1 day; C) 3 days and D) 5 days. Scale bars represent 200 µm.
Figure 5.17 Images illustrating Inhibition of U87 MG spheroid growth following treatment with AA-P40SLNs. Images were taken after incubation with formulations containing 0 – 51 µg/mL of AA-equivalent concentrations for A) 0 day; B) 1 day; C) 3 days and D) 5 days. Scale bars represent 200 µm.
Anti-cancer effects of RGD-targeted SLNs on multicellular tumour spheroids

Figure 5.18 Images illustrating inhibition of U87 MG spheroid growth following treatment with MS-SLNs. Images were taken following A) 0 day prior to AA-MS-SLN treatment; B) 5-day incubation after AA-MS-SLN treatment; C) 0 day prior to MS-SLN treatment and D) 5-day incubation after MS-SLN treatment. All concentrations are expressed as AA-equivalent concentration (0 – 51 µg/mL). Scale bars represent 200 µm.
Anti-cancer effects of RGD-targeted SLNs on multicellular tumour spheroids

Figure 5.19 Images illustrating inhibition of U87 MG spheroid growth following treatment with RP40SLNs and RP100SLNs. Images were taken following A) 0 day prior to RP40SLN treatment; B) 5 day incubation after RP40SLN treatment; C) 0 day prior to RP100SLN treatment and D) 5 day incubation after RP100SLN treatment. All concentrations are expressed as AA-equivalent concentration (0 – 51 µg/mL). Scale bars represent 200 µm.
Anti-cancer effects of RGD-targeted SLNs on multicellular tumour spheroids

Figure 5.20 Images illustrating inhibition of U87 MG spheroid growth following treatment with P40SLNs and P100SLNs. Images were taken following A) 0 day prior to P40SLN treatment; B) 5 day incubation after P40SLN treatment; C) 0 day prior to P100SLN treatment and D) 5 day incubation after P100SLN treatment. All concentrations were expressed as AA-equivalent concentration (0 – 51 µg/mL). Scale bars represent 200 µm.
5.3.4 Evaluation of spheroid cytotoxicity of SLN formulations

In addition, spheroid size determination assay is one of the most popular techniques used to evaluate the effects of anti-cancer agents on the sizes of spheroids and it has advantages of being a rapid and easily handled technique for the visualisation of spheroids using phase-contrast imaging and for determining the dimensions of the testing spheroids (Dhanikula et al., 2008; Q. Hu et al., 2013; Mikhail et al., 2013; Vinci et al., 2012). However, the loss of spheroid integrity may not be indicative for total cell death, whilst spheroid integrity may not reflect the presence of viable cells either (Hirschhaeuser et al., 2010; Sarisozen et al., 2014). Therefore, besides size determination of the spheroids examined, it was important to use a technique that would provide a measure of spheroid cell survival.

Various analytical assays have been exploited to assess the cytotoxicity effect of anti-cancer agents on 3D tumour spheroids. For example, assessment of clonogenic survival using colony formation assay (Wartenberg et al., 1998; Watanabe et al., 2007), LDH (lactate dehydrogenase) release assay (Ho et al., 2012; Perche and Torchilin, 2012), live/dead cell identification assays (Shin et al., 2013), and APH (acid phosphatase) assay (Mikhail et al., 2013).

In one of their studies, Ho et al. (2012) had shown that MTT assay is a suitable method for high throughput screening of cytotoxicity with spheroids and the assay was validated by comparing the results obtained with LDH release assay using spheroids prepared with several different cell lines. In this study, MTT assay was used to determine the cell viability of tumour spheroids (Barrera-Rodríguez and Fuentes, 2015; Dilnawaz and Sahoo, 2013; Ho et al., 2012). Since MTT assay was used for the cytotoxicity testing of monolayers of cells in previous chapters (Chapter 3 and 4), it was also used to determine the cell viability of tumour spheroids to help make a direct comparison.
Anti-cancer effects of RGD-targeted SLNs on multicellular tumour spheroids

**Figure 5.21 Cell viability of U87 MG spheroids.** Spheroids were treated with A) AA-containing SLN formulations and B) non-AA containing RGD− / PEG− / MS−SLNs following 72 h incubation.
Figure 5.21 illustrates the cell viability data obtained with U87 MG tumour spheroids treated with AA or different AA-containing SLN formulations within the range of 0 – 51 µg/mL AA-equivalent concentration, which is the same concentration range applied to U87 MG monolayer cells in Chapter 3 and 4. The aim of using the same range of AA-equivalent concentration on both monolayers and spheroids is to help identify any differences in behaviour of the formulations towards the two cell culture models. As shown in Figure 5.21, the cytotoxic effect of AA and AA-SLN formulations towards U87 MG tumour spheroids was reduced when compared with the levels obtained for U87 MG monolayer cells. For example, whilst the percentage of viability measured for U87 MG monolayer cells was ~ 7 % following 72 h incubation with 50 µg/mL of AA, U87 MG tumour spheroids showed ~ 60 % viability at the same AA concentration. Likewise, although a concentration of 51 µg/mL AA-equivalent with all the tested AA-SLN formulations resulted in ~ 85 – 90 % reduction of cell viability (relative to the control) towards U87 MG monolayer cells, only ~ 40 – 50 % reduction of cell viability was observed against the tumour spheroids despite incubation at the same concentration and time (72 h). Generally, both AA and AA-loaded SLN formulations showed concentration-dependent toxicities towards U87 MG tumour spheroids, in a similar manner as that observed on U87 MG monolayer cells. However, the cytotoxic effect of both AA and AA-SLNs was significantly reduced ($p<0.001$) towards U87 MG tumour spheroids when compared with the monolayer model. A higher dose of drug is required to achieve the same cytotoxic effect on spheroids as that needed for the monolayer of cells. E.g. 0.051 mg/mL of AA-RP40SLN formulation is required for 50% reduction of cell viability on U87 MG spheroids, when compared with only 0.02 mg/mL towards U87 MG monolayer of cells. This could be due to the poor penetration of the AA/AA-SLNs into tumour spheroids and/or resistance of tumour spheroids occurring from cell-cell and cell-matrix interactions. Anti-cancer treatment given to tumour spheroids is only in contact with the cells present on the surface of the spheroids and drugs need to diffuse through the spheroid to be able to interact with cells present inside the spheroid. Whilst cells grown as monolayers in culture plates lose various important signals, tissue phenotypes and key regulators (Horning et al., 2008; G. Y. Lee et al., 2007). Tumour spheroids mimic in vivo tumours showing diffusional limits to mass transport of drugs, nutrients and other factors, which limits drug diffusion though the spheroids and result in lower therapeutic effects compared to treatments of monolayer cells (Dilnawaz and Sahoo, 2013; Mikhail et al., 2013). Therefore, this supports the lower penetration (Section 5.3.5), uptake (Section 5.3.5) and cytotoxicity observed with U87 MG spheroids in comparison to U87 MG monolayers of cells. Kim et al. (2010) and Wartenberg et al. (1998) also demonstrated that penetration of one of the widely used
anti-cancer agent, doxorubicin, was limited to the periphery of the spheroids and could not diffuse through the spheroid more than the outer few cell layers.

More importantly, RGD-targeted SLNs showed a greater extent of cytotoxicity towards U87 MG spheroids than that achieved by AA alone when 51 µg/mL AA-equivalent concentration following 72 h incubation was used. AA-RGD-SLN also showed the highest cytotoxicity towards U87 MG spheroids in comparison to non-RGD containing SLNs (e.g. AA-PEG- and AA-MS- SLNs) at all concentrations tested following 72 h incubation (Figure 5.21). This supports the potential beneficial effects seen with RGD-targeted SLNs for tumour spheroid growth inhibition (Figure 5.12 and 5.13) and MTT cytotoxicity assays (Figure 5.21) when compared with drug or other formulations tested. The results are also in agreement with Jiang et al. (2013) and X. Wang et al. (2013) where they also demonstrated that RGD modification on the surface of nanoparticles improved the delivery and efficacy of paclitaxel and doxorubicin towards tumour spheroids by improving the penetration of the particles. These positive effects observed are likely to be due to the higher uptake and accumulation of RGD-SLN in the spheroids.

Even though AA-MS-SLN showed significantly least toxicity (p<0.05) among all SLN formulations at 0.024 mg/mL AA-equivalent concentration, at higher AA-SLN concentrations the toxicity difference became insignificant (Figure 5.21A). Furthermore, non-drug-containing RGD-, PEG- and MS- SLNs did not cause significant toxicity on U87 MG spheroids (p>0.05) as also clearly seen in Figure 5.21B, which indicates that reduction of cell viability of spheroids was due to the effects of AA.

5.3.5 Penetration of the C6-containing SLNs into tumour spheroids

In order to visualise the penetration of SLNs into spheroids, C6-loaded MS-SLN, P40SLNs, P100SLNs, R40SLNs and R100SLNs were incubated with the spheroids for 3 or 6 h prior to confocal imaging. Such a visualisation technique has also been exploited to evaluate the penetration of nanoparticles into spheroids (Jiang et al., 2013; Ruan et al., 2015; Sarisozen et al., 2014; X. Wang et al., 2013).

Z-stack imaging was started from the top of the spheroids, recorded with ~ 5.7 µm intervals and carried on till the green colour coming from coumarin-6 disappears. Generally, the nanoparticles showed a successful penetration into the spheroids up to ~ 40 – 50 µm in depth (Figure 5.22 – 5.25). However, from ~ 40 – 50 µm onwards, the intensity of C6-containing nanoparticles displayed a gradual decrease and rather than
showing in the core of the z-stack image slices, SLNs appeared by the edges of the spheroids. Figure 5.22A and 5.23A show the z-stack images of the penetration of C6-RP40SLN and C6-RP100SLN formulations into the spheroids, respectively, following 3 h incubation, and Figure 5.22B and 5.23B show the images of the same SLN formulations after 6 h incubation. A slight increase in the fluorescence intensity could be seen in the core of the image slice when comparing the images obtained after 3 or 6 h at ~ 51 – 63 µm for RGD-SLNs (Figure 5.22 and 5.23). It is clearly seen that images obtained after 6 h incubation have higher fluorescence intensity than that observed following 3 h incubation. On the other hand, images obtained for P40SLN, P100SLN and MS-SLN formulations following 6 h incubation displayed relatively weaker fluorescence intensities compared to that shown by RGD-SLNs (Figure 5.24 and 5.25), which correlates to the higher uptake of RGD-SLNs than other SLN preparations shown in Chapter 3 and 4 and later in this section. In fact, MS-SLNs showed the least fluorescence emission amongst all formulations tested when fluorescence could only be recorded up to 112 µm in depth and was lost after that (Figure 5.25A). As demonstrated by Goodman et al. (2007) and Ng and Pun (2008), penetration of the nanoparticles into spheroids is highly dependent on their particle sizes. It was suggested that particles with sizes less than 100 nm have more chances to penetrate deeper into spheroids than particles with sizes more than 100 nm. This could be the reason for the poorer penetration of MS-SLNs into the spheroids when compared with PEG–/RGD– SLNs whose particle size range is smaller.

C6 alone was also showed much weaker fluorescence intensity and lesser extent of penetration into the spheroids as fluorescence could only be recorded up to 77 µm in depth (Figure 5.25B). This data further supports the trend that RGD-SLNs showed better anti-tumour activity, deeper penetration and higher accumulation towards the tumour spheroids than other formulations did (RGD-SLNs > PEG-SLNs > MS-SLNs > C6).
Figure 5.22 Penetration of C6-RP40SLN formulation into U87 MG spheroids. Images were obtained following A) 3 h or B) 6 h incubation. Scale bars represent 200 µm.
Figure 5.23 Penetration of C6-RP100SLN formulation into U87 MG spheroids. Images were obtained following A) 3 h or B) 6 h incubation. Scale bars represents 200 μm.
Anti-cancer effects of RGD-targeted SLNs on multicellular tumour spheroids

Figure 5.24 Penetration of C6-P40SLN and C6-P100SLN formulations into U87 MG spheroids. Spheroids were treated with A) C6-P40SLN or B) C6-P100SLN formulations following 6 h incubation. Scale bars represent 200 µm.
Figure 5.25 Penetration of C6-MS-SLN and C6 alone into U87 MG spheroids. Spheroids were treated with A) C6-MS-SLN formulations or B) C6 alone following 6 h incubation. Scale bars represent 200 µm.
Projections are the brightest structures in the 3D z-stack images compressed onto a single plane which provides a simple way of visualising all the objects at once in the different planes of a stack. Figure 5.26 shows the z-projection of the stacks belonging to the spheroids treated with RP40SLN and RP100SLN formulations following 6 h incubation. Z-projections of the spheroids confirm the 3D shape and closely packed compact cell structure of the U87 MG tumour spheroids obtained in this study.

Figure 5.27 shows the results from the quantitative nanoparticle uptake study using flow cell cytometry. Significant reduction ($p<0.001$) of fluorescence intensities was observed with all of the formulations tested towards U87 MG spheroids when compared to the results obtained on U87 MG monolayer cells (Chapter 3 and 4). Another noticeable difference observed in the flow cytometry study on the tumour spheroids (Figure 5.27) is the difference in the extent of cellular uptake of between RP40SLN and RP100SLN formulations. Whilst RP100SLNs showed higher uptake of than RP40SLNs towards U87 MG monolayer cells, the opposite behaviour was observed in the case of uptake by U87 MG spheroids. Even though there is not a significant difference ($p>0.05$) between the uptake of these two formulations by U87 MG spheroids, there was a larger decrease in the recorded fluorescence intensities for RP100SLN than RP40SLN preparations when compared to the results obtained from U87 MG monolayer cells. It is acknowledged that physicochemical properties of the drugs including charge, shape, size, molecular weight and water solubility all affect the rate of diffusion through a tissue (Minchinton and Tannock, 2006). It is possible that RP100SLNs with a longer PEG chain, which has a higher molecular weight and lower water solubility (compared to PEG 40 that RP40SLN contains) has a less favourable diffusion rate than RP40SLNs. The apparent differences observed in this study warrant a more thorough systematic assessment on the effects of linkers towards uptake by spheroids and monolayers of cells.
Figure 5.26 Projection of the z-stacks images of U87 MG spheroids. Spheroids were treated with A) RP40SLN or B) RP100SLN formulations following 6 h incubation.
Anti-cancer effects of RGD-targeted SLNs on multicellular tumour spheroids

Figure 5.27 Flow cytometry data for cell uptake by U87 MG spheroids following treatment with SLN formulations and free C6. A) Mean fluorescence (± SD) of coumarin-6-loaded solid lipid nanoparticles following 3 h, or 5 h incubation at 37 °C. Control cells are untreated cells; “C6” represents C6 dye alone treatment. B) Flow cytometry histogram overlays (control cells; C6 uptake; MS-SLNs uptake; PEG-SLNs uptake and RGD-SLNs uptake at 37 °C).
5.4 Conclusion

- Multicellular U87 MG spheroids were successfully prepared using the liquid overlay method. The effect of MS-SLNs, P40SLNs, P100SLNs, RP40SLNs, RP100SLNs and free drug, AA, on in vitro 3D tumour spheroid model was investigated.

- Treatments with these formulations between 0 – 51 µg/mL AA-equivalent concentrations on tumour spheroids were not as effective as those found on U87 MG monolayer cells. This could be attributed to the physical and physiological barrier posed by the spheroids, which resulted in the lower penetration and uptake of SLN formulations into spheroids, as demonstrated quantitatively in the flow cytometry analysis and qualitatively from confocal imaging in this study.

- Delivery of AA using SLNs has shown more favourable effects towards the tested spheroids than treatment of the drug alone. In the spheroid growth inhibition assay, it was evident that the growth inhibition effect of AA was not effective after 24 h, whilst a more sustained effect (over 5 days) on preventing spheroid growth was demonstrated through treatment with AA-containing SLN formulations.

- The RGD-targeted SLN formulations (e.g. RP40SLNs and RP100SLNs) showed the best results in terms of inhibition of tumour spheroid formation, cytotoxicity, spheroid penetration and cellular uptake, which is in agreement with the previous data acquired on U87 MG monolayer of cells (Chapter 3 and 4).

Improved efficacy of RGD-SLNs could be due to the ligand–receptor interactions provided by integrin receptors (αvβ3) and RGD peptide. This will be further investigated on endothelial-like cells to understand their anti-angiogenic potential in Chapter 6.
Chapter 6

In vitro anti-angiogenesis studies with AA-containing SLN formulations
6.1 Introduction

Tumour angiogenesis is one of the necessary processes for the progression of a cancerous tissue to metastasise and spread around the body, which often result in high mortality from patients (Folkman, 1995; Judah Folkman, 1971; Skobe et al., 1997). For instance, sustained excessive angiogenesis seen in malignant gliomas is one of the main causes for uncontrolled glioma growth, development of drug resistance and metastasis, which makes malignant gliomas considered as incurable tumours (Kavitha et al., 2011). Therefore, anti-angiogenic therapy is seen as a feasible strategy in the treatment of cancer where it can kill the tumour vasculature and starve the tumour tissues to death. Unlike conventional anti-cancer treatment, anti-angiogenic therapy also has a lower probability of causing drug resistance due to their vascular targeting ability (Hayes et al., 1999).

Interactions of cells with the extracellular matrix (ECM) play an important role in various biological processes including tumour angiogenesis and metastasis (Campbell et al., 2010; Felding-Habermann et al., 2001; Guan, 1997) and integrins found on the surface of the cells are one of the major cell adhesion molecules required for ECM attachment (Guan, 1997; Parise et al., 2000). Overexpression of αvβ3 integrin receptors is also found on tumour angiogenic endothelial cells, as well as seen on various malignant tumour tissues (Danhier et al., 2012b; Zitzmann et al., 2002). The αvβ3 integrin is reported to be strongly involved in the regulation of angiogenesis (Danhier et al., 2012a). RGD-related peptides are commonly used to help bind to αvβ3 integrin (Danhier et al., 2012a). It was previously reported that disintegrins of snake venoms can bind to the integrin surface receptors using their RGD motif and inhibit cancer cell adhesion between cells and various ECM components (Li and Sakaguchi, 2004; Saviola et al., 2015; Wang et al., 2010). It was also demonstrated that soluble RGD peptides and their derivatives can inhibit cancer cell migration and invasion in vitro (Gehlsen et al., 1988; Georgoulis et al., 2012; Li et al., 2011). Therefore, using RGD peptide as a targeting ligand not only targets tumour tissues but also targets tumour endothelium as well, providing double targets for treating cancer. Moreover, αvβ3 integrin is known to be poorly expressed on normal non-proliferative cells or non-angiogenesis activated endothelial cells, which makes it a more selective target for treatment.

Although cell motility is also a necessary characteristic implicated in various normal biological processes such as that involved in immune response, tissue repair and embryonic development (Valster et al., 2005), but migration and invasion of cancer cells also play a crucial role in angiogenesis and tumour metastasis (Hood and
In vitro anti-angiogenesis studies with AA-containing SLN formulations

Cheresh, 2002) that are of interest in this Chapter. In order to migrate and invade effectively, integrins are required to bind to ECM molecules and regulate the intracellular signalling pathways (Hood and Cheresh, 2002).

ECV-304 cells were chosen as they provide an endothelial cell-like culture model to study anti-cancer and anti-angiogenic efficacy of in vitro cancer cell treatments. ECV-304 cells were first described as immortalised endothelial cell line from human umbilical vein (Takahashi et al., 1990). However later genetic analyses performed by Brown et al. (2000) showed that ECV-304 cells are genetically identical to a human bladder cancer cell line, T24/83. Nevertheless, ECV-304 cells express many endothelial cell properties which are not observed in T24/83 cell lines (Suda et al., 2001). ECV-304 cells have extensively been used in cancer research as an endothelial cell culture model for the study of tube formation (Jeon et al., 2005; Zheng and Liu, 2007), endothelial cell migration and invasion (Wang et al., 2010), cell adhesion (Li et al., 2010) and angiogenesis (Hughes, 1996). Most importantly, Genersch et al. (2003) reported the expression of αvβ3 integrin receptors on the surface of ECV-304 cells which is the target of the RGD peptide conjugated SLNs prepared for this study. Therefore, it was chosen as the cellular model to evaluate the anti-angiogenic potential of the SLNs.

Besides the effective anti-cancer properties of AA shown in previous chapters on U87 MG cells and various cancer cell lines reported in the literature, it was shown by Kavitha et al. (2011) that AA can also act as anti-angiogenic agent by inhibiting the pro-angiogenic effects of VEGF and human gliomas in endothelial cell culture models, as well as reducing endothelial cell migration, invasion and tube formation. RGD-SLNs have also been shown to inhibit the invasion of breast cancer cells similar to disintegrins or soluble RGD peptides by binding to αvβ3 integrin receptors due to the RGD peptide conjugated to SLN surface (Shan et al., 2015). Therefore, following on from the previous chapters where AA-MS-SLNs is chosen as the optimised SLN system, the combination of AA and RGD with the potential of enhancing the anti-angiogenic and cytotoxic effects of AA have been explored in this Chapter using ECV-304 cells as the cellular model.

Same as the other experiments performed in Chapter 2-4, the cytotoxic effect on ECV-304 cells was assessed using the MTT assay (Mosmann, 1983), whilst the targeting ability of RGD-SLNs was investigated using flow cell cytometry. As shown in Chapter 5 that the uptake of cells in monolayer of cells and tumour spheroids is different, it was

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therefore necessary to study the cellular uptake of these targeted SLNs by ECV-304 cells, which was qualitatively analysed by fluorescence microscopy. Any changes of the cell death mechanism were further studied using the Annexin V PI staining apoptosis assay. More importantly, the anti-angiogenic potential of AA-RGD-SLNs was evaluated based on their ability to inhibit cell adhesion, migration and invasion. The proficiency of RGD-targeted SLNs in the inhibition of cell adhesion was assessed using fibronectin binding assay, whereas inhibition of endothelial cell migration and invasion following treatment with RGD-SLNs (± AA) was investigated using transwell chambers where the cells need to migrate from the top to the bottom chamber. Similarly, the ability of AA and AA-containing SLNs in the inhibition of tube formation formed by ECV-304 cells was examined on Matrigel-coated 96-well plates.

6.2 Methods

Methods used for cytotoxicity, cellular uptake and mechanisms of toxicity of SLNs (± AA) towards ECV-304 cells were given in Chapter 2, Section 2.3.10.5 – 2.3.10.8.

6.2.1 Adhesion assay

To evaluate the effect of RGD-targeted SLNs binding to αvβ3 integrin receptor on ECV-304 and U87 MG cancer cell adhesion, an in vitro adhesion assay was carried out as previously reported Chen et al. (2009) and Shan et al. (2015), with minor modifications. Briefly, a flat bottom 96-well plate was first coated with 5 µg/mL of fibronectin (50 µL) in PBS and incubated at 4 °C overnight. The coated wells were then washed three times with PBS containing 0.2 % bovine serum albumin (BSA) and blocked with 2 % BSA in PBS (150 µL) for 1 h at room temperature.

Cells were harvested as previously described in Chapter 2, Section 2.3.9.1, re-suspended (5 x 10⁴ cells/mL) in serum-free cell culture media (M199 for ECV-304 and EMEM for U87 MG cells) containing various concentrations of non-AA-containing RP40SLN and RP100SLN (RGD concentration range 0 – 10 µM) and pre-incubated at 37 °C for 30 min prior to seeding into fibronectin-coated 96-well plates. After removing the blocking solution (BSA, 2 %) from 96-well plates, each well was washed three times with PBS containing 0.2 % BSA and then cells were seeded (100 µL) in the coated 96-well plate and incubated for 1 h at 37 °C, 5 % CO₂. The reason of using non-drug-loaded RGD-SLNs was to prevent any possible cell death due to AA toxicity.
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towards the cells tested. After the incubation period, unbound cells (100 µL) were removed, followed by washing gently (three times) with PBS containing 0.2 % BSA. Serum-free media (100 µL) and 20 µL of MTT solution (5 mg/mL) were then added to the wells containing the bound cells. Following a further 3 h incubation at 37 °C, MTT-containing media (120 µL) was gently removed and the formazan crystals were dissolved with DMSO (100 µL) for 30 min at 37 °C. The plates were analysed by UV spectrometer (Tecan microtitre plate reader) at 595 nm. The extent of binding was compared to control cells that did not receive any treatment.

6.2.2 Migration assay

To further determine the effect of RGD-targeted SLN binding to ανβ3 integrin receptor on ECV-304 and U87 MG cancer cell migration, a modified in vitro transwell migration assay was performed as previously reported (Kavitha et al., 2011; Shan et al., 2015; Wang et al., 2010). The migration assay was carried out using a transwell (Boyden chamber) made up of an 8.0 µm pore size polycarbonate filter. Cells were harvested at concentration of 5 x 10⁵ cells/mL as previously described in Chapter 2, Section 2.3.9.1 and re-suspended in serum-free cell culture media (M199 for ECV-304 and EMEM for U87 MG cells). A cell suspension (100 µL) together with 100 µL of SLN (± AA) (total volume 200 µL) suspended in serum-free media at appropriate concentrations (20 to 40 µg/mL, AA-equivalent concentration) were placed in the top chamber, whilst the complete cell culture media (containing 10 % FBS) was placed in the bottom chamber. The transwells containing the cells was incubated for 16 h to allow cell migration. Then the transwells were washed with PBS twice and fixed with formaldehyde solution (4 % w/v). Cells were then stained with crystal violet dye (0.1 % w/v in double distilled water) for 1 h. The filters of the transwell were washed with PBS extensively, to ensure removal of the excess crystal violet stain. The non-migrated cells on the top part of the filters were removed with cotton swabs. The transwells were allowed to dry for 2 h, and then microscopic images of migrated cells were taken using Leica DMIL light microscope from Leica Microsystems GmbH, Germany. The crystal violet dye was dissolved in 300 µL of 10 % (v/v) acetic acid and incubated on an orbital shaker for 1 h at room temperature. The plates were analysed at 595 nm with the Tecan microtitre plate reader, expressed as a fraction of control.
6.2.3 Invasion assay

An *in vitro* invasion assay using ECV-304 cells was carried out to help examine the effect of RGD-targeted SLN binding on inhibiting invasion of ECV-304. The method was adapted from previously reported assay by Friedli *et al.* (2009) and Wang *et al.* (2010). Transwells (8.0 µm pore size polycarbonate filters) were pre-coated with Matrigel matrix, which acts as the basement membrane matrix with reduced growth factor. Matrigel was first defrosted overnight on ice and prior to coating, the transwells and pipette tips were pre-cooled at -20 °C for 1 h. During the coating process, Matrigel matrix was kept on ice at all times to prevent pre-mature polymerisation of Matrigel at room temperature. Pre-cooled transwells were then placed into 24-well plates on ice in the sterile culture hood and 50 µL of 1 mg/mL Matrigel matrix (diluted with ice-cold serum-free sterile cell culture media) was placed on the filters in the top part of the chamber. For completion of polymerisation, plates were incubated at 37 °C for 3 h.

ECV-304 cells were harvested as previously described in Chapter 2, Section 2.3.9.1 and re-suspended in serum-free M199 culture media at concentration of 5 x 10^5 cells/mL. Excess liquid in transwells were removed carefully with sterile pipette tips and cell suspension (100 µL) was placed in the upper part of the chamber together with 100 µL of SLNs (± AA) (total volume 200 µL) containing serum-free media at appropriate concentrations (20 to 40 µg/mL, AA-equivalent concentration) and 700 µL complete cell culture media (containing 10 % FBS) was placed in the bottom chamber. Cells were allowed to migrate for 16 h and subsequently analysed as described in Section 6.2.2. for the migration assay.

6.2.4 Tube formation assay

Matrigel tube formation assay was employed for the assessment of SLN (± AA) formulations on *in vitro* angiogenesis (Jeon *et al.*, 2005; Kavitha *et al.*, 2011). The Matrigel matrix, acting as the basement membrane matrix was thawed as described in Section 6.2.3 overnight on ice. Similarly, all pipette tips and plates were pre-cooled at -20 °C for 1 h prior to use. Un-diluted Matrigel (50 µL) was placed in 96-well plates and kept at 37 °C overnight to allow completion of the Matrigel polymerisation. ECV-304 cells were trypsinised as previously described in Chapter 2, Section 2.3.9.1 and suspended in M199 media. Cells (100 µL of 1 x 10^5 cells/mL) were then seeded into the pre-coated 96-well plates simultaneously with 100 µL of SLNs (± AA) suspended in M199 media using appropriate concentrations of treatment (20 µg/mL for AA treatment;
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30 µg/mL for SLNs (± AA) treatment) at 37 °C under 5 % CO₂ for 8 h. The resultant tubular structures of the cells were photographed using Leica DMIL light microscope from Leica Microsystems GmbH, Germany.

6.3 Results and discussion

6.3.1 Evaluation of in vitro cytotoxicity of AA-SLN towards ECV-304 cells

Similar to the results on U87 MG and SVG cells, time- and concentration- dependent activity were observed for all formulations tested (DOX, AA, AA-containing SLNs) towards ECV-304 cells (Figure 6.1 to 6.4). The IC₅₀ values for DOX and AA (Table 6.1) are significantly higher (p<0.001) than that observed previously with those seen on U87 MG (0.2 ± 0.05 µg/mL for DOX; 19 ± 1 µg/mL for AA) following 72 h incubation under the same conditions (Section 3.3.2.1; Table 3.3).

It is clearly seen in Figure 6.2 – 6.4 that surface modifications of the SLN particles improved the cytotoxic efficacy of the formulations towards ECV-304 cells, similar to that reported previously on U87 MG cells in Chapter 4. Whilst the IC₅₀ values for AA-MS were found to be 0.074, 0.064 and 0.042 mg/mL following 24, 48 and 72 h incubation, respectively (Table 6.2, Figure 6.2), the cytotoxicity effect of surface-modified SLNs was greater as lower IC₅₀ values were obtained with PEG-SLN (0.050 – 0.034 mg/mL) (Table 6.2, Figure 6.3) and between 0.048 – 0.028 mg/mL for RGD-SLN (Table 6.3, Figure 6.4) after 24 – 72 h incubation. AA-MS-SLN showed cytotoxic effect only at 0.051 mg/mL AA-equivalent concentration displaying ~ 82 %, ~ 65 % and ~ 33 % viable cells (Figure 6.2) following 24, 48 and 72 h incubation, respectively, cytotoxic effect of AA-P40SLN and AA-P100SLN (Figure 6.3) were observed even at lower AA-equivalent concentrations (0.024 – 0.051 mg/mL). This could have been attributed to the higher cellular uptake of PEG-SLN by ECV-304 cells compared to MS-SLN as shown later in Section 6.2. However, SLN formulations with two different PEG chain lengths seem not to have any significant difference (p>0.05) in terms of cytotoxicity towards ECV-304 cells, although AA-P40SLN formulations showed slightly higher toxicity on ECV-304 cells than AA-P100SLN formulations.
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Figure 6.1 Cell viability of ECV-304 cells following treatment with A) DOX; B) AA after 24, 48, and 72 h at 37°C and 5 % CO₂.
Table 6.1 Concentration of doxorubicin (DOX) and AA against ECV-304 cells that result in 50 % viability (IC\textsubscript{50}).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Exposure time (h)</th>
<th>IC\textsubscript{50} values for ECV-304*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>µg/mL</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>24</td>
<td>5 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.4 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.3 ± 0.03</td>
</tr>
<tr>
<td>Asiatic Acid</td>
<td>24</td>
<td>36 ± 2</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>35 ± 4</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>30 ± 2</td>
</tr>
</tbody>
</table>

Experiments were repeated at least three times (N=3, n=18 ± SD).

Figure 6.2 Cell viability of ECV-304 cells following treatment with A) MS-SLN formulation after 24, 48, and 72 h at 37°C and 5 % CO\textsubscript{2}.
However, compared to U87 MG glioma cells, higher IC_{50} values were observed with ECV-304 bladder cancer cells, which could have been due to the sensitivity of drugs and resistance towards ECV-304 cells. In one of their studies, Kim et al. (1997) reported that ECV-304 cells showed overexpression of peroxiredoxin II (Prx II), which provides protection for cells from oxidative damage caused by hydrogen peroxide (H_{2}O_{2}). Yo et al. (2002) suggested that overexpression of Prx II in cells, like that found in gastric cancer and in ECV-304 cells, bring more resistance to anti-cancer drugs. Therefore, this might result in a higher IC_{50} with AA and SLN formulations (± AA) towards ECV-304 cells in comparison to U87 MG cells. This observation is also in agreement with the previously reported cytotoxicity results by Xiong et al. (2015) where nanographene nanoparticles were used as drug delivery system and resulted in lowest cytotoxicity towards ECV-304 cells compared to other cancer cells tested, including ovarian cancer A2780 and A2780/T cells and cervical cancer HeLa cells.

AA-containing RGD-SLNs, enhanced cytotoxicity towards ECV-304 cells after 72 h treatment (IC_{50} between 0.028 – 0.030 mg/mL incubation, Table 6.3) compared to both PEG-SLNs and MS-SLNs with IC_{50} between 0.034 – 0.036 mg/mL and 0.042 mg/mL for PEG-SLNs and MS-SLNs, respectively (Table 6.2). Similar to the effects seen on U87 MG and SVG cells, which could be attributed to the specific binding of RGD ligand to α_{v}β_{3} integrin receptors on the surface of ECV-304 cells (Genersch et al., 2003), thereby providing a better internalisation of AA-containing RGD-SLNs into ECV-304 cells via integrin-mediated endocytosis (Cai et al., 2011). Similar observations were also obtained by Cai et al. (2011) and Zhan et al. (2010) with RGD-targeted micelles and by Chang et al. (2015) and Zeng et al. (2014) for RGD-targeted liposomes where after particles conjugated to the RGD peptide showed greater cytotoxic effect when compared to the non-RGD-targeted particles. When comparing the cytotoxic results obtained in this study, it is apparent that RGD-SLNs resulted in ~ 1.5 fold greater cytotoxicity than AA-MS-SLNs and ~ 1.3 fold more cytotoxic than AA-PEG-SLNs after 72 h incubation, which are comparable with the data reported by Chang et al. (2015), where RGD-targeted liposomes showed 1.78, 1.89 and 1.19 fold higher cytotoxicity effect on MCF-7, HepG2 and A549 cells, respectively, compared to the non-RGD containing counterpart. Similar to the results obtained with PEG-SLNs, RGD-SLNs with two different PEG chain lengths seem also not to have any significant difference (p>0.05) in terms of cytotoxicity towards ECV-304 cells, even though AA-RP100SLN formulations appeared to show slightly higher toxicity on ECV-304 cells than AA-RP40SLN formulations.
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Figure 6.3 Cell viability of ECV-304 cells following treatment with A) P40SLN; B) P100SLN formulations after 24, 48, and 72 h at 37°C and 5 % CO₂.
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Figure 6.4 Cell viability of ECV-304 cells following treatment with A) RP40SLN; B) RP100SLN formulations after 24, 48, and 72 h at 37°C and 5% CO₂.
Table 6.2 Concentration of MS-SLN and PEG-SLN formulations against ECV-304 cells that result in 50 % viability (IC$_{50}$)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Exposure time (h)</th>
<th>IC$_{50}$ values for ECV-304*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg/mL</td>
</tr>
<tr>
<td>MS-SLNs</td>
<td>24</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.08 ± 0.003</td>
</tr>
<tr>
<td>AA-MS-SLNs</td>
<td>24</td>
<td>0.074 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.064 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.042 ± 0.004</td>
</tr>
<tr>
<td>P40SLNs</td>
<td>24</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.062 ± 0.005</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.056 ± 0.001</td>
</tr>
<tr>
<td>AA-P40SLNs</td>
<td>24</td>
<td>0.050 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.036 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.034 ± 0.003</td>
</tr>
<tr>
<td>P100SLNs</td>
<td>24</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.062 ± 0.005</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.057 ± 0.004</td>
</tr>
<tr>
<td>AA-P100SLNs</td>
<td>24</td>
<td>0.050 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.037 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.036 ± 0.003</td>
</tr>
</tbody>
</table>

*IC$_{50}$ values of AA-SLN were given based on AA-equivalent concentrations and values of non-AA-loaded SLNs were given based on AA-SLN-equivalent concentrations (N=3, n=18 ± SD). N.D. means no IC$_{50}$ values were determined using the concentrations tested.
Table 6.3 Concentration of RGD-SLN formulations against ECV-304 cells that result in 50 % viability (IC\textsubscript{50})

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Exposure time (h)</th>
<th>IC\textsubscript{50} values for ECV-304*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg/mL</td>
</tr>
<tr>
<td>RP40SLN</td>
<td>24</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.056 ± 0.005</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.044 ± 0.003</td>
</tr>
<tr>
<td>AA-RP40SLN</td>
<td>24</td>
<td>0.048 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.034 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.03 ± 0.003</td>
</tr>
<tr>
<td>RP100SLN</td>
<td>24</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.066 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.049 ± 0.006</td>
</tr>
<tr>
<td>AA-RP100SLN</td>
<td>24</td>
<td>0.048 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.034 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>0.028 ± 0.002</td>
</tr>
</tbody>
</table>

*IC\textsubscript{50} values of AA-SLNs were given based on AA-equivalent concentrations and values of non-AA-loaded SLNs were given based on AA-SLN-equivalent concentrations (N=3, n=18 ± SD). N.D. means no IC\textsubscript{50} values were determined using the concentrations tested.

6.3.2 Cellular uptake of C6-SLNs by ECV-304 cells

It is essential to determine the cellular uptake efficiency of the RGD-SLNs by ECV-304 cells to enrich understanding on the data for subsequent cell migration and invasion assays to investigate their potential as anti-angiogenic drug delivery systems for AA. Similar to the results for U87 MG and SVG cells, fluorescence imaging and flow cell cytometry were used here to provide qualitative and quantitative analysis of SLN internalisation. Although differences between the extent of uptake were present, comparative data was observed on 1) uptake of controls; 2) time-dependent uptake; 3) AA-containing SLNs; and 4) effectiveness of uptake between non-targeted and RGD-targeted SLNs by ECV-304 cells in comparison to the results obtained on U87 MG and
SVG P12 cells shown previously in Chapter 3 and 4. Similar to the data discussed in Chapter 3 and 4, cellular internalisation of free C6 fluorescent dye showed weak fluorescence intensity with both flow cell cytometry quantitatively (Figure 6.5) and fluorescence imaging qualitatively (Figure 6.6) indicating that the level of fluorescence seen following nanoparticle treatment was attributed to the SLN accumulation inside the cells. Same as that reported in SVG P12 and U87 MG cells (Chapter 3 and 4), encapsulation of AA into SLNs did not show any significant impact on SLN cellular uptake by ECV-304 cells. E.g. no differences were shown between MS-SLNs and AA-MS-SLNs (Figure 6.5). Therefore, only AA-containing C6-PEG-SLNs and C6-RGD-SLNs were investigated with ECV-304 cells (Figure 6.5).

Not unexpectedly, reducing the incubation temperature from 37 °C to 4 °C also drastically reduces the fluorescence intensity obtained from the cellular uptake of C6-SLNs by ECV-304 cells (Figure 6.5 – 6.8) which confirms that AA-SLN uptake in ECV-304 cells is also reliant on the endocytotic pathways (Martins et al., 2012). Similar experiment was performed by Takeuchi et al. (2003) where ECV-304 cells were treated with liposomes at 37, 20 and 4 °C. Reducing incubation temperature even to 20 °C significantly reduced the cellular uptake of liposomes indicating that uptake mechanism of nanoparticles relies on mainly endocytotic pathway as well (Takeuchi et al., 2003). As expected, increasing incubation time from, for example, 1 h to 3 h increased cellular internalisation of C6-SLNs. However, no apparent increase in terms of fluorescence intensity was observed following incubation between 3 h to 5 h, which might indicate saturation of particles.

Furthermore, MS-SLNs showed the lowest uptake by ECV-304 cells compared to PEG-SLNs and RGD-SLNs, in a similar manner as reported previously in Chapter 4 with U87 MG cells (Figure 6.5). This could have been related to the size of the nanoparticles where C6-MS-SLNs have the largest particle size (~ 146 nm) among all SLN formulations tested. It is acknowledged that uptake of smaller-sized particles are more favourable than larger ones (Carrstensen et al., 1992; Wan et al., 2008; Zauner et al., 2001).

Same as the uptake in the other two cell lines tested, RGD-targeted SLNs showed significantly higher cellular uptake by ECV-304 cells compared to PEG-SLNs and MS-SLNs at all time points tested (p<0.001) which is most probably due to the RGD-integrin specific binding. Competitive binding experiment using free c(RGDfK) peptide resulted in a reduction of cellular uptake of RGD-SLNs (p<0.05), which illustrates the involvement of integrin-mediated endocytosis (Figure 6.5).
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In contrast to the fluorescence images obtained with U87 MG cells (Figure 3.16, 4.18 and 4.19; Chapter 3 and 4) where C6-SLN showed accumulation around the nuclei of U87 MG cells, the C6-SLNs were found to be more diffused into the cytoplasm of ECV-304 cells. RGD-containing SLNs did not affect the intracellular localisation of SLNs and both RGD- and non-RGD- containing SLNs diffused into the cytoplasm of the cells (Figure 6.6 – 6.8). Cellular dynamics often result in differences in the intracellular trafficking behaviour of the nanoparticles. This is considered as an important aspect of understanding the biochemical and molecular mechanism of delivery of these nanoparticles, which warrants further examination in the future. However, as this falls outside the scope of the current study, no further evaluation was conducted at this stage of the investigation.
Figure 6.5 Flow cytometry data for ECV-304 cell uptake treated with SLN formulations and free C6. A) Mean fluorescence (± SD) of coumarin-6-loaded solid lipid nanoparticles following 1h, 3 h, and 5 h incubation at 37 °C. Control cells are untreated cells; *C6* represents C6 dye alone treatment. B) Flow cytometry histogram overlays (control cells; C6 uptake; MS-SLN uptake; PEG-SLN uptake (P100SLN) and RGD-SLN uptake (RP100SLN) at 37 °C)

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Figure 6.6 Fluorescence images of ECV-304 cells treated with MS-SLNs or free C6; A) Control cells at 37 °C; B) MS-SLNs at 37 °C; C) C6 at 37 °C and D) MS-SLNs at 4 °C. Blue colour represents DAPI nucleus staining, green colour represents Coumerin-6 (C6)-containing nanoparticles and the last column illustrates the merged images of DAPI and C6.
Figure 6.7 Fluorescence images of ECV-304 cells treated with P40SLNs or P100SLNs; A) P40SLNs at 37 °C; B) P40SLNs at 4 °C; C) P100SLNs at 37 °C and D) P100SLNs at 4 °C. Blue colour represents DAPI nucleus staining, green colour represents Coumerin-6 (C6)-containing nanoparticles and the last column illustrates the merged images of DAPI and C6.
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Figure 6.8 Fluorescence images of ECV-304 cells treated with RP40SLNs or RP100SLNs; A) RP40SLNs at 37 °C; B) RP40SLNs at 4 °C; C) RP100SLNs at 37 °C and D) RP100SLNs at 4 °C. Blue colour represents DAPI nucleus staining, green colour represents Coumerin-6 (C6)-containing nanoparticles and the last column illustrates the merged images of DAPI and C6.
6.3.3 Cell death mechanism of ECV-304 cells

Similar to U87 MG and SVG cells, the cell death mechanism of ECV-304 cells was further studied following the treatment with AA and AA-containing SLN formulations was investigated using Annexin V PI apoptosis assay (Koopman et al., 1994) and cisplatin (50 and 100 µM) also induced time- and concentration-dependent apoptosis of ECV-304 cells at all time points (Figure 6.9).

In addition, both free AA and AA-containing SLNs induced ECV-304 cell death via both apoptosis and necrosis pathways in a time- and concentration dependent manner. Same as that observed for U87 MG and SVG P12 cells, free AA showed immediate induction of apoptosis at an earlier time point (24 h), whilst AA-loaded SLNs displayed a slower action and reached to their peak following 72 h incubation which could have been due to the sustained release of AA from SLN formulations, as discussed in previous chapters (Chapter 3 and 4). A significantly greater extent of apoptosis was also observed from the treatments with AA alone or AA-containing formulations in comparison to non-AA preparations (Figure 6.9).

When it is compared to the results obtained for U87 MG cells (Chapter 3 and 4), it was found that AA-loaded SLN formulations act at a slower rate of inducing apoptosis towards ECV-304 cells. For example, whilst ~ 70 – 80 % apoptotic U87 MG cells were observed with AA-containing RGD-SLN formulations (30 µg/mL AA-equivalent concentration) following 48 h incubation, the same extent of apoptosis of ECV-304 cells were only observed following 72 h incubation with AA-loaded RGD-SLN. Similar to the lower extent of cytotoxicity shown earlier (Figure 6.1 to Figure 6.4), this could have been related to the sensitivity and resistance of ECV-304 cells towards AA-SLN formulations in comparison to U87 MG cells (Yo et al., 2002). The greater cellular uptake by ECV-304 cells seen from the flow cell cytometry results (Figure 6.5) also helps explain and complementary to the higher apoptotic induction of ECV-304 cells observed with AA-loaded RGD-SLN formulations when compared with AA-containing PEG-SLN and MS-SLN formulations at all time points tested (Figure 6.9).

It is important to note that non-drug loaded SLNs did not show the same extent of apoptosis as that seen with U87 MG cells following all 24, 48, and 72 h treatment (Figure 3.19 and Figure 4.24; Chapter 3 and 4). As discussed in Chapter 3, the possible reason for apoptosis induction with the non-drug containing SLNs was, possibly due to the stearic acids present in SLN formulations as an impurity of the raw supplied lipid or the digestion product of glyceryl stearate lipids following cellular treatment (Mu et al., 2001; Zhang et al., 2011). The reason for the significantly
reduced amount of apoptotic cells ($p<0.001$) observed here for ECV-304 cells could be due to the reduced sensitivity of ECV-304 cells towards fatty acid toxicity. For example, studies related to the toxicity of fatty acids on both ECV-304 and U87 MG cells revealed that treatment with 400 µM oleic acid did not result in ECV-304 cell death (Masi et al., 2011), whereas the same amount of oleic acid killed almost all of the U87 MG cells tested (Antal et al., 2014).

Additionally, the effect of using PEG linker with different chain lengths did not result in any significant differences to the extent of apoptosis induced by PEG-SLN or RGD-SLN. Moreover, higher apoptotic induction was observed with AA-RGD-SLN when compared with AA-PEG-SLN and MS-SLN at all time points tested, especially at 80 µM AA-equivalent concentration. This observation is in agreement with both cytotoxicity and cellular uptake data recorded for RGD-, PEG-, and MS- SLN, which could have been attributed to the ligand-receptor interactions of RGD-SLN.
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Figure 6.9 Flow cytometry analysis of ECV-304 cells stained with Annexin V-Alexa® Fluor 488 and PI. Cells were treated with Cisplatin (50 and 100 µM; represented as Cis-50 and Cis-100, respectively) as the positive control; AA, non-AA loaded and AA-loaded SLNs ± RGD ligand at 40 and 60 µM AA-equivalent concentrations following A) 24 h; B) 48 h; and C) 72 h incubation. AA-loaded SLNs are represented as “A”. MS, P40, P100, RP40 and RP100 represent MS-SLNs, P40SLNs, P100SLNs, RP40SLNs, RP100SLNs, respectively. (N=3 ± SD).
6.3.4 Inhibition of adhesion of ECV-304 and U87 MG cells

To explore the anti-angiogenic potential of the prepared RGD-SLNs, the inhibition of ECV-304 and U87 MG cell adhesion following treatment with RGD-SLNs was studied by binding to fibronectin-coated plates, where fibronectin is an abundant ECM protein, which plays an essential role in cell adhesion as well as binding to the integrin receptors via its RGD motif (Takahashi et al., 2007). It was observed that increasing the concentration of RGD-SLN formulations decreased cell adhesion of ECV-304 and U87 MG cells (at 10 µg/mL concentration ~ 20 – 25 % inhibition was observed). Concentration-dependent inhibition was seen with both RP40SLN and RP100SLN formulations and similar results of inhibition were obtained, so no significant impact of the PEG linker space was observed (Figure 6.10). As a negative control, MS-SLNs did not result in any adhesion inhibition. Complementary to the lower cellular uptake by ECV-304 cells seen from the flow cell cytometry data for RGD-SLNs following free RGD peptide treatment (Figure 6.5), the inhibition of ECV-304 cell and U87 MG cell adhesion to fibronectin-coated plates observed in this study after treatment with RGD-SLNs was most probably due to the specific binding of RGD-containing SLNs to integrin receptors (Shan et al., 2015). Shan et al. (2015) showed that RGD-targeted SLNs can also display inhibitory effect on cell adhesion like disintegrins and it was also demonstrated by showing the inhibition of breast cancer cell adhesion to fibronectin. Since the tested RGD concentrations were not specified by Shan et al. (2015), it was not possible to give a direct comparison between the RGD-SLN systems used in this study with Shan et al. (2015). However, concentration-dependent inhibition was also observed with their RGD-SLN particles, where the highest tested concentration of their RGD-SLNs (containing 10 % RGD-SLN) claimed to cause ~ 30 % inhibition of cell adhesion.
6.3.5 Inhibition of migration and invasion of cancer cells

To study migration and invasion of cells, transwell chambers were used where ECV-304 cells were allowed to migrate or invade towards a FBS gradient in the presence of AA or SLNs (± AA) (Figure 6.11A). For invasion assay, cells were expected to travel through a matrigel-coated transwell filters, where the matrigel was used to mimic ECM (Figure 6.11A). It was shown by Kavitha et al. (2011) that AA prevents cell migration and invasion of human umbilical vein endothelial cells (HUVEC) by inhibiting VEGF expression of cells; whereas Shan et al. (2015) demonstrated that RGD-SLNs are able to reduce invasion of aggressive breast cancer cells via RGD-integrin binding (Figure 6.11B). In this study the effect of AA and RGD-, PEG- and MS- SLNs (± AA) on ECV-304 cell migration and invasion was investigated on ECV-304 cells. The results obtained from the experiments can be seen in Figure 6.12 and 6.13. Concentrations of AA and SLN formulations (± AA) tested were chosen with the least toxicity towards ECV-304.
In vitro anti-angiogenesis studies with AA-containing SLN formulations

Even though it was reported by Shan et al. (2015) that non-drug containing RGD-SLNs showed concentration-dependent inhibition of breast cancer cell invasion, in this current study presented (Figure 6.12 and 6.13), there was not a significant inhibition effect observed with non-AA-containing RGD-SLNs on ECV-304 cell migration and invasion at 20 and 30 µg/mL AA-equivalent concentrations. Therefore, it was decided to test a higher concentration of the nanoparticles (40 µg/mL AA-equivalent) to investigate the possible inhibition effect of RGD-SLNs. As a result, at 40 µg/mL AA-equivalent concentration, RGD-SLNs showed ~ 10 % inhibition in migration and ~ 10 – 15 % inhibition in invasion of ECV-304 cells when compared with control cells (Figure 6.12 and 6.13), which confirm the concentration-dependent inhibition activity of RGD-SLNs. Additionally, non-AA or non-RGD-containing SLNs (e.g. MS-SLN and PEG-SLN formulations) did not show any effect on the migration and invasion of ECV-304 cells. Therefore, inhibition activity seen by RGD-SLNs could be attributed to the RGD ligand association with the SLNs.

Since AA shows toxicity towards ECV-304 cells above 20 µg/mL following 24 h incubation, only 20 µg/mL AA concentration was tested on ECV-304 cells, whereas both 20 and 30 µg/mL AA-equivalent concentration for SLN formulations was studied in the experiment. AA alone (20 µg/mL) resulted in ~ 20 % inhibition in cell migration and ~ 25 % inhibition in cell invasion (Figure 6.12 and 6.13) which correlate to the findings recorded by Kavitha et al. (2011) on HUVEC cells. Concentration-dependent inhibition of both migration and invasion of ECV-304 cells was observed after treatment with AA-loaded SLNs. For example, whilst AA-RGD-SLNs showed ~ 5 % inhibition in both migration and invasion of ECV-304 cells at 20 µg/mL AA-equivalent concentration, at a higher concentration (30 µg/mL AA-equivalent), ~ 15 – 20 % inhibition in migration and ~ 25 – 30 % inhibition in invasion of ECV-304 cells was noted. Therefore, no profound differences were shown between AA and AA-RGD-SLNs in terms of their anti-migrative and anti-invasive activity. AA alone (20 µg/mL) and AA-RP100SLN (30 µg/mL AA-equivalent) showed comparable degree of inhibition of migration and invasion of ECV-304 cells (Figure 6.12A). This may not perceive as ideal, but with the sustained cytotoxic effect observed with the SLN preparations and the continued apoptotic property, such comparable anti-migrative and anti-invasive effect may still be relevant for future studies.

It is worth mentioning that AA-RP100SLNs with longer PEG chain length showed higher degree of inhibition in both migration and invasion of ECV-304 cells at 30 µg/mL AA-equivalent concentration (~ 20 % inhibition in migration and ~ 30 % inhibition in invasion) compared to AA-RP40SLN formulations (~ 15 % inhibition in migration and ~
23 % inhibition in invasion). These results are in the agreement with the results obtained from the cellular uptake studies (Section 6.3.2) where flow cytometric data revealed greater uptake of C6-RP100SLNs by ECV-304 cells than C6-RP40SLNs did.

In addition, a higher degree of inhibition of ECV-304 cell invasion was observed with AA-SLNs than its effect on ECV-304 cell migration inhibition. For example, whilst AA-PEG-SLNs did not have any effect on the inhibition of cell migration at 30 µg/mL AA-equivalent concentration, they showed ~15 – 20 % inhibition of cell invasion. Likewise, AA-RGD-SLNs showed ~15 – 20 % inhibition of cell migration, whereas ~23 – 30 % inhibition of cell invasion was observed. The longer the resident time for the cells to be in contact with the SLN treatment, the greater the effects that the formulations may have had on the cells. Since there is not an obstacle for cells between the top and the bottom part of the chamber in migration assay, cells could have travelled relatively faster compared to that in the invasion assay, where the top chamber was coated with Matrigel matrix and movement of cells from the top to the bottom chamber would have been retarded, which would have allowed them to interact with the SLN formulation at the top of the transwell for longer. However, to fully confirm this assumption, it is acknowledged that further time-dependent migration and invasion studies would need to be conducted.
In vitro anti-angiogenesis studies with AA-containing SLN formulations

Figure 6.11 Schematic diagram of cell migration and invasion assay. A) Working principle of in vitro cell migration and invasion assay and B) Mechanism of action of AA and RGD-SLNs affecting cell migration and invasion.
In vitro anti-angiogenesis studies with AA-containing SLN formulations
In vitro anti-angiogenesis studies with AA-containing SLN formulations

Figure 6.12 Inhibition of ECV-304 cell migration. Cells were treated with AA (20 µg/mL); AA-MS-SLN, AA-P40SLN, AA-P100SLN, AA-RP40SLN, AA-RP100SLN (20 and 30 µg/mL, AA-equivalent concentration) and non-drug-containing MS-SLN, P40SLN, P100SLN, RP40SLN, RP100SLN (20, 30 and 40 µg/mL, AA-equivalent concentration). Concentrations were chosen at the least toxicity towards ECV-304 cells based on the cytotoxicity data obtained after 24 h. A) Percentage of cell migration. Bars represent mean ± SD of N=3 studies; B) Microscopy images of ECV-304 cell migration through transwell chambers after treatment with the aforementioned formulations at 30 µg/mL (AA-equivalent) and AA at 20 µg/mL. Cells were stained with crystal violet (0.1 % w/v).
In vitro anti-angiogenesis studies with AA-containing SLN formulations

A

% Invasion

AA-Equivalent Concentration (µg/mL)

20 µg/mL

30 µg/mL

40 µg/mL

Control MS P40 P100 RP40 AA-MS AA-P40 AA-RP40 AA alone Control MS P40 P100 RP40 AA-MS AA-P40 AA-RP40 AA-RP100

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In vitro anti-angiogenesis studies with AA-containing SLN formulations

**Figure 6.13 Inhibition of ECV-304 cell invasion.** Cells were treated with AA (20 µg/mL); AA-MS-SLN, AA-P40SLN, AA-P100SLN, AA-RP40SLN, AA-RP100SLN (20 and 30 µg/mL, AA-equivalent concentration) and non-drug-containing MS-SLN, P40SLN, P100SLN, RP40SLN, RP100SLN (20, 30 and 40 µg/mL, AA-equivalent concentration). Concentrations were chosen at the least toxicity towards ECV-304 cells based on the cytotoxicity data obtained after 24 h. A) Percentage of cell invasion. Bars represent mean ± SD of N=3 studies; B) Microscopy images of ECV-304 cell invasion through transwell chambers containing Matrigel after treatment with the aforementioned formulations at 30 µg/mL (AA-equivalent) and AA at 20 µg/mL. Cells were stained with crystal violet (0.1 % w/v).
6.3.6 Inhibition of tube formation

The main step of tumour angiogenesis is the formation and merging of tubes produced by endothelial cells forming a complex network of vessels which is necessary for tumour growth and metastasis (Figure 6.14). Kavitha et al. (2011) showed inhibition of tube formation of HUVEC cells following treatment with free AA by inhibiting VEGF expression (Figure 6.14). To study the possible anti-angiogenic effect of AA and AA-containing SLN formulations, in vitro tube formation assay was used where ECV-304 cells were allowed to form tubes on Matrigel matrix for 8 h in the presence of AA and SLN formulations (± AA). For this study 20 µg/mL of AA and 30 µg/mL AA-equivalent concentrations of SLN formulations were chosen to avoid the high cytotoxic effects of the treatments towards ECV-304 cells, as observed from the cytotoxicity results following 24 h incubation.

![Figure 6.14 Schematic representation of inhibition of tube formation of ECV-304 cells.](image)
In vitro anti-angiogenesis studies with AA-containing SLN formulations

The images obtained from the tube formation assay show extensive network and tube formation of ECV-304 cells in the control wells without any nanoparticle or drug treatment. It was previously reported that free RGD peptide can inhibit tube formation (Dobler et al., 2006). However, disruption of the tubular network or discontinued tubes was not observed in the wells treated with non-AA-containing RGD-SLN formulation (Figure 6.16 B and C). Nevertheless, notably thinner tubes were seen after RGD-SLN treatment when compared with the cells treated without either AA or RGD ligand (Figure 6.15 A, B, C and Figure 6.16 A). This disparity in the observations could have been due to the significantly lower RGD concentration used in this RGD-SLN preparations studied in this investigation (~1 µM) than the concentration of RGD employed (58 µM) in the study conducted by Dobler et al. (2006).

Disruptions of the tubular network, as well as discontinued and thin tubes were evident following incubation with AA and AA-containing SLN formulations. As previously reported by Kavitha et al. (2011), AA has the ability to inhibit tube formation in a concentration-dependent manner when tested in human umbilical vein endothelial cells (HUVEC) cells. The anti-angiogenic potential of AA and AA-containing SLNs is also confirmed with ECV-304 cells using the in vitro tube formation assay. Comparatively, more disruptions were found with AA-loaded RGD-SLN formulations (Figure 6.16 E and F) than other AA-SLNs (Figure 6.15 E, F and Figure 6.16 D), which complements the added advantage of RGD ligand conjugation to mediate RGD-targeted delivery of AA-containing SLNs.
Figure 6.15 Effect of AA and AA-SLN on tube forming ability of ECV-304 cells. A) Control; B) MS-SLN; C) P40SLN; D) AA; E) AA-MS-SLN and F) AA-P40SLN following 8 h incubation at 37 °C under 5 % CO\textsubscript{2} atmosphere. The arrows indicate the disruption of the tube network. Scale bars represent 100 µm.
In vitro anti-angiogenesis studies with AA-containing SLN formulations

Figure 6.16 Effect of AA and AA-SLNs on tube forming ability of ECV-304 cells. A) P100SLN; B) RP40SLN; C) RP100SLN; D) AA-P100SLN; E) AA-RP40SLN and F) AA-RP100SLN following 8 h incubation at 37 °C under 5 % CO₂ atmosphere. The arrows indicate the disruption of the tube network. Scale bars represent 100 µm.
6.4 Conclusion

Tumour angiogenesis plays an important role in tumour growth and metastasis. Anti-angiogenic therapy aims to inhibit the formation of tumour vasculatures, thereby disrupting the oxygen and nutrient supply to tumour tissues to prevent tumour growth and also metastasis (Benny and Pakneshan, 2009). Anti-angiogenetic therapy is considered as a feasible strategy, since tumour vasculature feeds tumour cells, destroying one blood vessel can result in the killing of many tumour cells (Jia et al., 2011). In addition, unlike tumour cells, endothelial cells are less likely to develop drug resistance due to their lower mutation rate (compared to tumour cells), it is also easier for an anti-cancer agent to access to endothelial cells than tumour tissues and so the same anti-angiogenic treatment can be effective in various solid tumours (Hallak et al., 2005; Hayes et al., 1999; Jia et al., 2011).

In previous chapters (Chapter 3, 4 and 5), the anti-tumour potential of AA and AA-containing SLN formulations was tested towards U87 MG and SVG P12 cells in vitro as well as testing on in vitro 3D U87 MG tumour spheroids, where positive outcomes of the AA-RGD-SLNs were shown and their potential mechanisms were revealed. In this chapter, the anti-angiogenic effect of AA-RGD-targeted SLNs was further demonstrated through the inhibition of tube formation, cell adhesion, cell migration and invasion assays. AA alone has been shown to have anti-angiogenic properties in the literature (Kavitha et al., 2011) and supported in this study too. There are also many RGD-targeted systems reported for as potential anti-angiogenic therapy that exploit the targeting ability of RGD peptide to αvβ3 integrins that are overexpressed by endothelial cells (Schraa et al., 2004; Sundaram et al., 2009). However, for the first time, the combined anti-angiogenic effects of AA and RGD-SLNs have been examined, as presented in this Chapter. Both AA and AA-SLNs showed cytotoxic effect towards ECV-304 cells in a time- and concentration- dependent manner. It was evident that the cytotoxic effect of SLNs was greater with AA-RGD-SLNs compared with AA-PEG-SLNs and AA-MS-SLN formulations. This further complements the greater cellular uptake and increased accumulation of RGD-SLNs by ECV-304 cells, as measured by flow cell cytometry (Figure 6.5) in this present study. It was found that both AA and AA-loaded SLN formulations induce ECV-304 cell apoptosis. Although the effect was more profound with AA alone, the AA-RGD-SLNs showed greater apoptotic effect than that without AA or RGD counterparts (Figure 6.9). Overall, anti-angiogenic potential of RGD-SLNs was demonstrated by showing the inhibitory effect towards cell adhesion, migration and invasion of ECV-304 cells. The RGD-SLN formulations were all more
In vitro anti-angiogenesis studies with AA-containing SLN formulations

active than that observed with other SLN formulations (PEG-SLNs and MS-SLNs). The combined effect of AA and RGD-SLNs was better than RGD-SLNs alone on the tube formation assay, which supports the anti-angiogenic potential of AA and its advantage of having a sustained release of the drug by the use of SLNs. From the data obtained, it is believed that AA-loaded RGD-SLNs do not only provide potential cytotoxic effect towards tumour cells, but these targeted nanoparticles also have the ability to mediate endothelial cells targeting and improve delivery of the drug to tumours and the tumour vasculature where an over-expression of αvβ3 integrin is found. To further explore this for clinical application, an in vivo angiogenic model will need to be pursued to help elucidate the benefits and challenges for in vivo administration.
Chapter 7

Overall Conclusion & Future Work
7.1 Overall objectives

The aim of this project was to investigate the possibility of developing RGD-targeted SLN formulations to improve the therapeutic anti-cancer activity and clinical applications of AA as well as promoting targeting to cancer cells more selectively by exploiting the tumour microenvironment and ligand-receptor interactions (RGD peptide – αvβ3 integrin receptor) using a nano- and lipid-based drug delivery system that has not been studied in the past.

7.2 General discussion

Cancer is one of the most widespread diseases of 21st century and it is one of the most serious causes of human mortality. According to the study published in Lancet Oncology by Bray et al. (2012), it was predicted that the incidence of the total cancer cases will increase from 12.7 million new cases in 2008 to 22.2 million by 2030 and cancer is defined as a life-threatening disease for future generations (Adams et al., 2010).

Although chemotherapy is quite successful in inhibition of cancer cell proliferation, progression as well as providing cancer cell death, anti-cancer drugs generally caused serious side-effects which some of them can be life-threatening (e.g. doxorubicin causes serious cardiotoxicity problems) and also reduces the quality of life of cancer patients. This is the result of non-specific actions of anti-cancer agents towards both cancerous and normal cells. Moreover, it was reported that only a small fraction of chemotherapeutic drugs reaches the tumour area which results in both enhanced drug toxicity and decreased therapeutic efficacy (Wong et al., 2007).

Drug delivery systems were suggested as a novel approach for cancer treatments to carry cytotoxic agents specifically to the tumour area and hence improve the therapeutic efficacy of the treatment by accumulation of the drug at tumour site and also reduce the side effects seen with conventional chemotherapy treatment. To achieve this, nano-based drug delivery systems have the passive targeting effect by exploiting the tumour microenvironment through the leaky vasculature and poor lymphatic drainage to escape from the circulation, to reach and accumulate at the tumour site(s) (Section 1.9.1). This type of passive targeting effect is widely applied in nano-based drug delivery systems employed for anti-cancer research and is known as the EPR effect (Maeda et al., 2000). This partly solves the non-specificity problem of
the chemotherapy. Furthermore, sophisticatedally designed surface modifications can further manipulate the biodistribution of nanoparticles with the aim of furthering drug uptake and penetration to tumour tissues (Mehnert and Mäder, 2012; Wong et al., 2007).

SLNs used in this project provide many benefits as drug delivery systems for cancer therapy. SLNs provide better stability profile compared to other lipid nanoparticles (e.g. emulsions and liposomes) and at the same time protect the therapeutic agent from potential degradations, provide controlled release, avoid the usage of organic solvents during manufacturing and enable simpler reproducible scale up processes. More importantly, they can effectively encapsulate anti-cancer agents that tend to be hydrophobic (Mehnert and Mäder, 2012; Wissing et al., 2004; Wong et al., 2007).

Amongst the different targeting ligands, RGD-targeted drug delivery systems were chosen for targeting the αvβ3 integrin which is widely expressed on both tumour endothelial cells and various cancer cell types (i.e. glioma, breast cancer, prostate cancer etc.). The double targeting characteristic of the RGD-based drug carriers are favourably used within various delivery systems as demonstrated in liposomes, micelles and SLNs (Cai et al., 2011; Chen et al., 2012; Shan et al., 2015; Zitzmann et al., 2002). The reported in vitro and in vivo studies all showed an achievement in enhanced therapeutic efficacy and drug accumulation (Danhier et al., 2012b; Mondal et al., 2013), including the study conducted in this investigation.

To date, various anti-cancer agents have been discovered from medicinal plants (Balunas and Kinghorn, 2005) and some of them are currently available for routine clinical use as cancer treatments (e.g. paclitaxel). Asiatic acid is an extract from medical plant, Centella Asiatica, which has shown anti-cancer activities towards many cancer cell lines including U87 MG glioma cells (Cho et al., 2006), which were also used in this study. However, one of the major problems of AA is its poor water-solubility (0.03 mg/mL) (Liu et al., 2009), which limits its clinical applications.

In this project, AA was successfully incorporated within SLN formulations to overcome its solubility and delivery problem. It also helped to mediate controlled release of the drug in the cancer cell line tested (Chapter 3). For this purpose, SLNs with three different glyceryl stearate lipids (MS, DS and TS) were employed and systematically characterised using various techniques as described in this thesis to help optimise and select the most desirable system for further conjugation of the targeting ligand, RGD peptide. Nanoparticles with small particle size (< 150 nm), narrow particle size
distribution (< 0.3), slight negative charged (< –1), high entrapment efficiency (95 – 99 %) were obtained. DSC and XRD studies suggested that the majority of the drug appear to be in the amorphous form in SLNs. Although varying the number of stearate chains within the tested lipids did not show a significantly different effect on the physicochemical properties of SLNs, it did, however, influence the amount of the drug released at particular time points and it also affected the cytotoxic effect of SLNs towards U87 MG cancer and SVG P12 control cells.

Data obtained from flow cell cytometry further confirmed the higher cellular uptake (~1.3 fold higher) of nanoparticles by U87 MG cancer cells than SVG P12 normal cells (Figure 3.17). Based on the drug release profile (a relatively faster release; Figure 3.3) and preferential cytotoxicity of MS-SLNs towards U87 MG cancer cells (IC_{50} for SVG P12 is 0.046 mg/mL and for U87 MG 0.03 mg/mL, following 72 h incubation) formulations based on MS-SLNs were chosen for further investigation including RGD conjugation.

After developing and optimising SLN carriers for the incorporation of AA, it was also important to improve the therapeutic targeting of AA-containing SLNs to tumour site (Chapter 4). For this purpose, MS-SLNs which provided the most potential for anti-cancer effect towards glioma cells were chosen and conjugation studies were carried out. Studying the effect of PEGylation was important to optimise the required PEG density prior to RGD peptide conjugation. Therefore two different PEG densities (5 mol % and 10 mol %) were employed and investigated in terms of the resultant physicochemical properties of the PEG-SLNs and their in vitro uptake and anti-cancer efficiency towards both U87 MG glioma cells and SVG P12 normal cells. Because of the minimal toxicity observed with 5 mol % PEG containing non-drug loaded PEG-SLNs towards normal cells, PEG 5 mol % as linker were chosen for RGD conjugation. Following chemical synthesis of covalently conjugated RGD peptide to PEG stearates, RGD-PEG stearate conjugates were incorporated into the SLN preparations to obtain RGD-targeted SLN formulations.

Since U87 MG cells are known to express the α_vβ_3 integrin receptor (Zhang et al., 2006) and the potential of using AA for brain tumour, this cell line was chosen to study the targeting ability of RGD-SLNs. Comparative results obtained with AA-RGD-SLN, AA-PEG-SLN and AA-MS-SLN formulations showed that of the incorporated RGD peptide improved the anti-cancer efficiency of all formulations (detected anti-cancer efficiency; AA-RGD-SLNs > AA-PEG-SLNs > AA-MS-SLNs) most probably due to the enhanced uptake of RGD-SLNs by U87 MG cells and increased accumulation of AA
inside the cells, as shown by the flow cell cytometry and fluorescence imaging. This is most likely attributed to the receptor-ligand binding of RGD peptide with integrin receptor (αvβ3) found on the surface of the U87 MG cells, particularly when binding was reduced when competitively inhibited by RGD peptide alone (Chapter 4).

After promising targeting efficiency was obtained with RGD-containing SLNs on monolayer cancer cells, the effect of these particles was tested on in vitro 3D tumour spheroids (Chapter 5), as tumour spheroids with their 3D architecture are believed to mimic in vivo tumours better when they display a similar microenvironment, pathophysiology and complexity, as well as having similar cell morphology, gene expression, growth kinetics and drug response (Barrera-Rodríguez and Fuentes, 2015; De Witt Hamer et al., 2007; Goodman et al., 2007; Hirschhaeuser et al., 2010; Mehta et al., 2012; Sarisozen et al., 2014). Therefore, tumour spheroids are considered to be in intermediate complexity when compared with monolayer cell cultures and the complex in vivo tumour models (Mikhail et al., 2013). Because of the resemblance of this in vitro spheroid model to the in vivo tumour systems, it was important to investigate the efficacy of the nanoparticles obtained in this study on spheroids to illustrate its potential in vivo application. It was found that treatments with AA-loaded SLN formulations on tumour spheroids were not as effective as those found on U87 MG monolayer cells, which could have been attributed to the physical and physiological barrier posed by the spheroids in comparison to the monolayers of cells, which resulted in lower penetration and uptake of SLN formulations into spheroids. Therefore, this was not entirely unexpected. Nevertheless, treatment with AA-loaded SLNs has shown more favourable effects towards the tested spheroids than treatment of the drug, AA, alone. When compared to other SLN formulations tested, RGD-SLNs demonstrated the best results in all the assays studied, including prevention of tumour formation, inhibition of tumour growth and penetration studies, which further confirmed the preferential interaction of the RGD-SLNs than non-targeted ones.

Because of the overexpression of αvβ3 integrin on tumour endothelial cells, RGD-based strategies are widely used in anti-angiogenic research to target the integrin receptors (Danhier et al., 2012a; Dubey et al., 2011, 2004). In addition, previously it was reported that AA not only shows anti-tumour effects but also displays anti-angiogenic properties (Kavitha et al., 2011). Therefore, the potential anti-angiogenic effect of AA-containing RGD-targeted SLNs was important and was investigated using endothelial-like ECV-304 cell lines (Chapter 6). It is known that angiogenesis is a multi-step process involves endothelial cell proliferation, migration, and adhesion and eventually vessel
formation (Danher et al., 2010; Heissig et al., 2003). Therefore, the effect of RGD-SLNs on the inhibition of cell adhesion, migration and invasion and prevention of tumour formation was evaluated to help further optimise the targeted AA drug delivery system. Overall, a higher inhibition efficacy was obtained with RGD-targeted SLNs compared to free AA, PEG-SLNs and MS-SLNs, which indicate the potential usage of AA-loaded RGD-SLNs as anti-angiogenic agent.

Cell suicide, apoptosis, occurs after a cellular damage and plays an important role in the development and health of multicellular organisms (Baek et al., 2015). Resisting cell death by evasion of apoptosis is known as one of the hallmarks of cancer (Hanahan and Weinberg, 2011, 2000), which may cause disruption of the balance between cellular growth and death due to dysregulation of apoptosis. It has been widely shown in the literature that AA can induce apoptosis in various cancerous cell lines (Kavitha et al., 2014; Lee et al., 2002; Park et al., 2005; Tang et al., 2009b). This was also supported in this study as well and additionally, it was still effective after incorporation to SLN formulations.

It was demonstrated for the first time that, not only AA can continue showing its functional therapeutic effects after incorporation into the nanoparticles, but also appropriate surface modifications can provide enhanced cellular uptake and intracellular drug accumulation, leading to improved cell cytotoxicity and apoptotic induction.
7.3 General conclusion

- AA was successfully encapsulated in SLNs using three different glycerides (MS, DS and TS) and the effect of the number of stearate chain on SLN preparation, characteristics and cell cytotoxicity were systemically investigated. Due to their small size, high drug entrapment efficiency and desirable drug release, the monostearate form of SLNs (AA-MS-SLNs) provided the most potential for anti-cancer effect towards glioma cells. Therefore, MS-SLNs were chosen for further investigations and surface modifications.

- Surface-modified SLNs were prepared by incorporating PEG and RGD peptide into MS-SLNs obtained from Chapter 3, and the effect of PEG chain length on in vitro cell culture studies (e.g. cell cytotoxicity, cellular uptake, apoptosis) was investigated using both PEG(40)stearate and PEG(100)stearate.

- It has been observed that SLNs prepared with PEG(40)stearate showed significantly higher cytotoxicity in comparison to SLNs prepared with PEG(100)stearate towards U87 MG cells ($p<0.05$). However, whilst RGD-SLNs prepared with either shorter (RP40SLN) and longer (RP100SLN) PEG chains both showed similar cytotoxicity against U87 MG cells; FACS analysis revealed significantly greater cellular uptake of RP100SLNs than RP40SLNs ($p<0.05$).

- Surface modifications improved the yield of the PEG-/RGD-SLNs from 73.8 % to 82 – 96 %, as well as significantly improving cellular uptake and cytotoxicity of the formulations towards U87 MG glioma cells ($IC_{50}$ of MS-SLN and RGD-SLN were 0.03 and 0.02 mg/mL, respectively)

- AA caused cell death of U87 MG, SVG P12 and ECV-304 cells via both apoptosis and necrosis. It was also demonstrated that AA continues to show its functional therapeutic effect after encapsulation within SLNs and is still able to induce apoptosis to a comparable extent. The incorporation of RGD-peptide onto the surface of SLNs also result in similar apoptotic effects as observed with non-RGD-targeted SLNs but at an earlier time points (e.g. from 72 h to 48 h).

- Reproducible 3D tumour spheroids of U87 MG cells were successfully prepared to test the anti-cancer effect of RGD-SLNs. Treatments with SLNs ($\pm$ AA) between 0 – 51 μg/mL AA-equivalent concentrations on tumour spheroids were not as effective as those found on U87 MG monolayer cells. The physical and physiological barriers posed by spheroids might have led to the lower
penetration and uptake of SLN formulations into spheroids, as demonstrated from the data obtained on FACS analysis and confocal imaging in this study.

- Treatment with AA-loaded SLNs has shown more favourable effects towards the tested spheroids than treatment of the drug, AA, alone. RGD-SLNs demonstrated the best results among all SLN formulations, in all the assays studied, including prevention of tumour formation, inhibition of tumour growth and penetration studies.

- Investigations of the combined anti-angiogenic effects of AA and RGD-SLNs was demonstrated for the first time in Chapter 6.

- Anti-angiogenic potential of RGD-SLNs was demonstrated by showing the inhibitory effect towards cell adhesion, migration and invasion of ECV-304 cells and the RGD-SLNs showed better effects than that seen with other SLN formulations.

- Although PEG chain length did not cause any significant difference in anti-angiogenic properties of PEG- and RGD- SLNs, the combined effect of AA and RGD-SLNs was better than RGD-SLNs alone on the tube formation assay, which supports the anti-angiogenic potential of AA and its advantage of having a sustained release of the drug by the use of SLNs.

- From the data obtained, it is believed that AA-loaded RGD-SLNs do not only provide potential cytotoxic effect towards tumour cells, but these targeted nanoparticles also have the ability to mediate endothelial cells targeting and improve delivery of the drug to tumours and the tumour vasculature where an over-expression of $\alpha_v\beta_3$ integrin is found.
7.4 Contributions to the literature

Although incorporation of AA within solid lipid nanoparticles were previously reported by Zhang et al. (2014) to investigate the effect of particles on intestinal absorption kinetics, there is very limited information available regarding interactions of these carriers with cells and their effects and mechanisms of actions in cancer cells. Therefore, this is the first systematic investigation on AA-loaded SLNs for parenteral delivery and testing on normal human brain cells as a control to cancer cells. In this project, the encapsulation of AA into SLNs, their physical and chemical characterisations, anti-cancer efficacy towards U87 MG, SVG P12 and ECV-304 cells, cellular uptake and cell death mechanisms were systemically investigated.

Likewise, non-drug-loaded RGD-targeted SLNs were previously reported (Shan et al., 2015; Shuhendler et al., 2012) to investigate their targeting ability towards breast cancer cells. However, the effect of RGD-targeted SLNs on delivering an anti-cancer drug effectively towards cancer cells and whether it improves the anti-cancer activity on these cells were missing. In this project, enhanced cell cytotoxicity was observed with RGD-SLN as a result of improved uptake and drug accumulation.

Furthermore, the anti-angiogenic effect of AA-containing RGD-targeted SLNs was investigated. To fully evaluate the effectiveness of the RGD-SLN, further studies on an in vivo angiogenic model such as the chicken embryo chorioallantoic membrane assay was planned and conducted. Preliminary data acquired also showed differences in the RGD-based SLNs when compared to the control, but these require further testing to prove consistency and reliability of the data to be statistically significant. For this reason, they have not been included for the publication of this thesis but will be used for on-going and future experimentations.

Moreover, there was limited information available regarding with the effect of PEG chain length on the targeting efficiency of RGD-conjugated nanoparticles. In this study, two PEG stearate molecules with different chain lengths were used (PEG(40)stearate and PEG(100)stearate) and RGD-SLN with longer PEG chain showed higher uptake and cell cytotoxicity than RGD-SLN with shorter PEG chain.

Investigation on the therapeutic efficacy of AA-containing SLNs and their targeting and penetration abilities towards U87 MG spheroid model was also successfully performed first time in the literature. Although cytotoxic effect of AA-SLN were not as effective as that observed on U87 MG monolayer cells, prevention of spheroid formation, inhibition
Overall Conclusion & Future Work

of tumour growth and cell cytotoxicity all supported a feasible approach towards a clinically acceptable formulation.

7.5 Future work

RGD-targeted SLN formulations prepared in this study showed enhanced uptake and hence improved therapeutic efficacy towards both U87 MG and ECV-304 cells which further supports the potential of RGD-based strategies for targeting the αvβ3 integrin as a treatment approach of cancer. However, is it possible to improve the enhanced therapeutic activity of AA-loaded RGD-targeted SLNs with an increase of the RGD ligand? In this study, 10 mol % RGD peptide was used for the preparation of RGD-SLNs. Future studies can focus on the optimisation of the ideal peptide concentration needed for obtaining better targeting effect with the AA-containing MS-SLN formulation. To do that, a range of RGD peptide concentrations (both less and more than 10 mol % RGD) can be used for the conjugation and then the targeting ability of RGD-SLNs can be examined using cell cytotoxicity studies and FACS analysis. Besides, the influence of RGD peptide concentration on anti-angiogenic activity can be evaluated using cell adhesion/migration/invasion and tube formation assays.

Although inhibition of spheroid growth was observed with AA-loaded SLN formulations and a slight reduction in spheroid size was achieved with RGD-SLNs, it was not possible to completely destroy or reduce the size of spheroid significantly. However, treatment of spheroids was carried out with only a single administration between 0 – 51 µg/mL (AA-equivalent concentrations). As a first step, the administered dose of both AA and AA-SLN can be increased and tested again on spheroids as a single administration. Nevertheless, it is known that chemotherapy drugs may also be given to cancer patients with a multi-dose administration (Khan et al., 2002). Therefore, as a second step a multi-dose administration regimen can be applied for treatment of tumour spheroids. For example, Günther et al., (2003) showed that a single administration of temozolomide to U87 MG spheroids only reduced the growth rate of spheroids; whilst the drug killed the tumour spheroids completely following another treatment. In this study, AA/AA-SLN can be administered for 2/3 consecutive days or every 3 days of incubation which is the suggested media changing time for spheroids (Vinci et al., 2012) to evaluate and determine the optimal dosing regimen. Since in vitro anti-angiogenic studies performed in Chapter 6 have shown promising results following treatment with AA and AA-containing SLNs, the anti-angiogenic effect of these SLN
Overall Conclusion & Future Work

formulations will also be tested in vivo using a chick chorioallantoic membrane (CAM) model. CAM is an extraembryonic membrane and has a dense capillary network which widely employed to study both angiogenesis and anti-angiogenesis (Richardson and Singh, 2003; Vargas et al., 2007). The relatively low cost in experimentation set-up, ease of preparation and absence of a developed immune systems are some of the main advantages of using the CAM model in comparison to mammalian systems (Richardson and Singh, 2003). The CAM blood vessels grow rapidly up to day 11 of incubation where rapid proliferation of endothelial cells and angiogenesis can be seen (Ribatti et al., 2006). The overexpression of receptors/proteins that are essential for angiogenesis such as α,β3 integrins and VEGF allows examination of the prepared targeted drug delivery system on the developing blood vessels in CAM, so that the anti-angiogenic effect of the formulations can be studied (Tartis et al., 2006). For example, in previous studies of Tartis et al. (2006), the targeting ability of RGD-containing lipid nanoparticles was examined using the CAM model and it revealed that, with the help of ultrasound, RGD-containing particles significantly increased fluorescent dye accumulation in the blood vessels. The in vivo CAM anti-angiogenesis model has already been designed for this project. Preliminary studies on the anti-angiogenic activity of AA-containing RGD-targeted SLNs on the in vivo CAM model showed promising results, which warrant systematic investigation to help develop a clinically feasible product for further in vivo tumour testing.
Chapter 8

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Ai) 24 h for U87 MG cells

Control  
Cis 50 µM  
Cis 100 µM  
AA 40 µM  
AA 60 µM  
AA 80 µM  
AA-MS 40 µM  
AA-MS 60 µM  
AA-MS 80 µM  
MS 40 µM  
MS 60 µM  
MS 80 µM
Aii) 48 h for U87 MG

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Aiii) 72 h for U87 MG

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Bi) 24 h for SVG P12

Control

Cis 50 µM

Cis 100 µM

AA 40 µM

AA 60 µM

AA 80 µM

AA-MS 40 µM

AA-MS 60 µM

AA-MS 80 µM

MS 40 µM

MS 60 µM

MS 80 µM
Bii) 48 h for SVG P12

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Biii) 72 h for SVG P12

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Figure: Quantitative apoptotic measurement in A) U87 MG and B) SVG P12 cells using Annexin V PI double staining apoptosis assay after treatment at different concentrations with cisplatin (Cis 50 and 100 µM); free asiatic acid (AA 40, 60 and 80 µM); AA-loaded MS-SLN (AA-MS 40, 60 and 80 µM); and non-drug-loaded MS-SLN (MS-SLN 40, 60 and 80 µM) following i) 24; ii) 48 and iii) 72 h.