Development and Characterization of LDV Peptide Targeted Nanocarriers for Paclitaxel Delivery: A Comparative Study of Micelles, Liposomes and Solid Lipid Nanoparticles

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DEVELOPMENT AND CHARACTERIZATION OF LDV PEPTIDE TARGETED NANOCARRIERS FOR PACLITAXEL DELIVERY: A COMPARATIVE STUDY OF MICELLES, LIPOSOMES AND SOLID LIPID NANOPARTICLES

by

Poonam Dattani

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By

Poonam Dattani
DEDICATION

This dissertation is dedicated to Yahowah, Yahoshua and Ruach HaKodesh.
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Development and Characterization of LDV Peptide Targeted Nanocarriers for Paclitaxel Delivery: A Comparative Study of Micelles, Liposomes and Solid Lipid Nanoparticles

Abstract

By Poonam Dattani

University of the Pacific
2019

Nanocarriers have been established as delivery vehicles to target cancer tumors. However, premature drug leakage is one of the major reasons for inefficient drug delivery of nanocarriers to the tumor. Drug diffusion out of the nanocarriers or destabilization of drug loaded nanocarriers by physiological interactions with blood cells, serum proteins, and cell membranes upon systemic administration contribute to premature drug release. In this study, targeted micelles, liposomes and solid lipid nanoparticles (SLNs) of similar composition were prepared and characterized to compare physicochemical characteristics, in vitro stability, in vitro release rates in release media and in vivo performance. Peptide Amphiphiles (PAs) formed micelles with critical micelle concentration (CMC) values ranging between 23.68 ± 0.72 µM to 38.76 ± 2.27 µM. Transmission Electron Microscopy (TEM) images confirmed the self-assembly of PAs into spherical structures where the largest sizes were seen for C16-(PEG2)_n-LDV micelles. Dynamic Light Scattering (DLS) results confirmed the presence of targeted liposomes and SLNs with sizes smaller than 100 nm. Forster Resonance Energy Transfer (FRET) studies revealed that targeted micelles, liposomes and SLNs were all stable upon dilution in aqueous medium, however the stability was significantly reduced in human serum, with micelles being the least stable and SLNs being the most stable. The same trend was observed for the in vitro release profiles, where targeted paclitaxel-loaded micelles (PTX-micelles) had the fastest release rate and paclitaxel-loaded SLNs (PTX-SLN) exhibited the slowest release rate. DLS results showed that sizes of PTX-
SLNs were smaller than PTX-liposomes (80.53 ± 5.37 nm vs 123.31 ± 5.87 nm). Cryogenic TEM observation showed increasing size in the order of PTX-micelles (6 to 12 nm) < PTX-SLN (10-120 nm) < PTX-liposomes (48-145 nm). Drug Loading Content (DLC) of PTX-SLN was greater than PTX-micelles and PTX-liposomes (7.45 ± 0.41 % vs 1.70 ± 0.42 % and 0.92 ± 0.09 %). Compared to initial aqueous dispersions, reconstituted spray dried formulations maintained their nanosize and paclitaxel content over 7 days at 4°C. In A375 melanoma xenograft mouse model, the tumor volumes were significantly smaller for mice treated with PTX-SLN compared to the control group. Furthermore, tumor volumes were significantly smaller for mice treated with PTX-SLN compared to those treated with PTX-micelles and PTX-liposomes. These studies demonstrate the potential of stable PTX-SLN for targeted delivery in cancer.
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Chapter 1: Introduction

1.1 Cancer: Chemotherapy and Limitations

Cancer is a disease characterized by the uncontrolled growth and spread of abnormal cells and it is the leading cause of death worldwide, accounting for an estimated 9.6 million deaths in 2018 [1]. The American Cancer Society (ACS) reported that in 2019, there will be an estimated 1,762,450 new cancer cases diagnosed and 606,880 cancer deaths in the United States [2]. It is also estimated that by 2030, 23.6 million new cases of cancer per year will occur, compared to 17 million cases that occurred in 2016 [3]. Treatment modalities of localized and metastasized cancer include immunological, photothermal, photodynamic, gene and hormone therapy. Surgery, radiation and chemotherapy are the first line treatment options for most types of cancer. Chemotherapeutic drugs have several limitations, for example they lack specificity towards cancer cells which results in toxic effects to healthy tissue, including bone marrow suppression, infertility and mucositis. Furthermore, the high distribution volume results in high concentrations of these drugs in healthy tissue. The development of anti-cancer drug formulations is challenging due to their poor aqueous solubility. For example, paclitaxel is hydrophobic and has a very low water solubility (<0.3 mg/mL), which can result in aggregation upon intravenous administration causing embolization of blood vessels. To overcome this issue, paclitaxel is currently formulated with Cremophor EL and dehydrated ethanol which is diluted with saline prior to intravenous administration. However, Cremophor EL is a toxic excipient and has been known to cause severe side effects such as hypersensitivity reactions, nephrotoxicity, cardiotoxicity and cardiotoxicity [4]. The efficacy of chemotherapeutic agents is also limited due to resistance mechanisms. For example, increased hydrostatic pressure of the tumor interstitium of tumor tissue can result in the outward convective interstitial flow resulting in drug transport away from the tumor. Furthermore, cancer cells can acquire resistance to a wide variety of drugs
through activation of anti-apoptotic pathways and over-expression of efflux pumps and this is known as multidrug resistance (MDR). The main protein involved in MDR is P-glycoprotein (P-gp), which is an ATP-dependent transporter located on the cell and nuclear membrane that non-selectively binds to neutral or positively charged molecules, of which many antineoplastic drugs fall under this category. The normal physiological role of P-gp is to protect the cellular entry of undesirable molecules through efflux mechanisms and to transport substances across the cells including drugs, steroids, polypeptides and bile acids. However, P-gp is over-expressed in intestinal, liver, pancreas, kidney, ovary and testicular cancers, which results in the pumping out of drug molecules from cells, resulting in sub-optimal therapeutic drug concentrations at the tumor site [5-9]. Overall, these factors highlight the necessity for new and more efficacious treatments for cancer.

1.2 Targeted Drug Delivery of Nanocarriers

Nanotechnology is an emerging therapeutic platform that is being used for the treatment of cancer and has shown great promise in the past few decades. Nanocarriers have gained increased interest over the years due to their ability to overcome obstacles faced with conventional chemotherapy, such as non-specificity and severe side effects. Specifically, nanocarriers are colloidal drug carrier systems less than 500 nm in size and can be used for encapsulation and delivery of hydrophobic and hydrophilic anti-cancer agents to improve bioavailability and therapeutic efficacy [10]. Several advantages of nanocarriers include, improved pharmacokinetics, pharmacodynamics and bioavailability, improved solubility and stability, ability to protect payload from degradation, facilitation of cellular uptake and cellular internalization, decreased non-specific toxicity, controlled release and site-specific delivery through active and passive targeting mechanisms. Furthermore, nanocarriers can help to overcome MDR through bypassing of P-gp efflux pumps on the cellular membrane [11].
1.2.1 Passive targeting. Targeting of nanocarriers to the tumor tissue is highly dependent on the structural differences of normal healthy tissue compared to tumor tissue. The pathophysiological abnormalities in the tumor microenvironment allow for the selective accumulation of nanocarriers into the tumor tissue. In healthy tissues, a counterbalance is attained between proangiogenic molecules (VEGF) and antiangiogenic molecules (VEGFR1). However, in tumor tissue, new vessels are formed and pro-angiogenic signaling is abnormally upregulated to ensure that growing tumor cells receive an adequate supply of oxygen and nutrients. This results in the formation of an abnormal vasculature network, characterized by irregular, chaotic, tortuous and leaky blood vessels. The endothelial surface is fenestrated with gaps (10 to 1000 nm) between the endothelial cells, enabling the extravasation and selective accumulation of nanocarriers into the interstitial space. However, tight junctions between normal endothelial cells do not allow nanocarriers to extravasate and this difference can be exploited to achieve tumor targeted drug delivery. Furthermore, the leaky tumor vasculature enables the extravasation of excess fluid into the tumor interstitium and the inability of non-functional lymphatic vessels to drain the excess fluid leads to an imbalance in the interstitial fluid pressure (IFP) [12, 13]. The increased vascular permeability and poor lymphatic drainage results in the selective accumulation and retention of nanocarriers in the tumor tissue and this is known as the Enhanced Permeability and Retention (EPR) effect. Despite these advantages, nanocarrier accumulation and retention may vary and have unpredictable outcomes due to the heterogeneity of tumor environment and variation in the degree of tumor vascularization and angiogenesis. Additionally, the elevated IFP of solid tumors may prevent the uptake and homogenous distribution of drugs in the tumor. Furthermore, nanocarriers can be recognized as foreign and undergo opsonization by the cells of the reticuloendothelial system (Mononuclear Phagocyte System, MPS). These factors combined may potentially reduce the bioavailability of drug at the tumor site and
reduce the efficacy of these drug delivery systems. Despite these limitations, there are several passive targeted nanocarriers commercially available, which demonstrate the potential of nanocarrier drug delivery systems in tumor therapy. Doxil and Myocet were the first liposome products to be approved by the FDA for the treatment of cancer, and were designed to improve the PK, PD and bioavailability of doxorubicin, which has a very short elimination half-life of 0.2 hours [14]. Myocet is an un-PEGylated liposome product, which demonstrated an improved elimination half-life of 2.5 hours. To further prolong the blood circulation time PEGylated liposomes were formulated (Doxil) which significantly increased the elimination half-life to 55 hours. Furthermore, Doxil has been shown to reduce cardiotoxic effects significantly compared to free doxorubicin [14].

1.2.2 Active targeting. Passive targeting strategies have several limitations as described above, therefore the development of nanocarriers with improved targeting abilities is of interest. Active targeting strategies are employed by grafting or incorporating ligands onto nanocarriers that specifically recognize and bind to receptors over-expressed in tumor cells. This results in enhanced cellular internalization rather than increased tumor accumulation. The most commonly studied targets and their ligands are listed in table 1.3 [15]. Integrins are transmembrane heterodimeric glycoprotein receptors comprising of 18 α and 8 β polypeptide subunits which non-covalently assemble into 24 different combinations [16]. Specifically, integrins mediate cell adhesion by interacting and binding to extracellular matrix glycoproteins or connective tissue such as fibronectin. Integrin mediated adhesion influences signaling cascades which in turn modulates standard cellular functions such as cell growth, motility, differentiation and proliferation. Collectively, these functions are fundamental to tissue integrity and the maintenance of normal cellular functions [17]. Many integrin receptors are over-expressed in cancer cells, which has been shown to correlate with tumor progression and growth [18]. For examples integrin α4β1 is over-expressed in
melanoma cells making it an ideal target for selective delivery of chemotherapeutic agents to cancer cells. This integrin recognizes a short peptide motif Leucine-Aspartic acid-Valine (LDV) within ligands fibronectin and VCAM1, which is key for regulating biological activity [19-22]. Therefore, employing synthetic peptides such as LDV could potentially be a useful strategy to target anti-cancer agents to cancer cells [23]. Peptides provide various advantages including therapeutic effects, small size to enhance delivery to the tumor, low risk of immune reactivity, ease of synthesis and low cost. Furthermore, nanocarriers provide a good platform for ligand conjugation through grafting or incorporation at controlled densities and orientations.
<table>
<thead>
<tr>
<th>Nanocarrier</th>
<th>Product</th>
<th>Drug</th>
<th>Composition</th>
<th>Approved indication</th>
<th>Approval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposomes</td>
<td>Doxil/Caelyx</td>
<td>Doxorubicin</td>
<td>HSPC:CH:mPEG-DSPE</td>
<td>Ovarian cancer Kaposi’s sarcoma Multiple myeloma Breast cancer</td>
<td>FDA (1995)</td>
</tr>
<tr>
<td>DaunoXome</td>
<td>Daunorubicin</td>
<td>Daunorubicin</td>
<td>DSCP:CH</td>
<td>HIV-associated Kaposi’s sarcoma</td>
<td>FDA (1996)</td>
</tr>
<tr>
<td>Mepact</td>
<td>Mifamurtide</td>
<td>DOPS:POPC</td>
<td>osteosarcoma</td>
<td>FDA (1999)</td>
<td></td>
</tr>
<tr>
<td>Myocet</td>
<td>Doxorubicin</td>
<td>EPC:CH</td>
<td>Metastatic breast cancer</td>
<td>EMA (2000)</td>
<td></td>
</tr>
</tbody>
</table>
Table 1.2: List of FDA/EMA approved nanocarriers for anti-cancer therapy

<table>
<thead>
<tr>
<th>Nanocarrier</th>
<th>Product</th>
<th>Drug</th>
<th>Composition</th>
<th>Approved indication</th>
<th>Approval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymeric micelle</td>
<td>Genexol-PM</td>
<td>Paclitaxel</td>
<td>mPEG-PDLLA</td>
<td>Breast/lung/pancreatic</td>
<td>Europe and Korea</td>
</tr>
<tr>
<td>Nanoparticle</td>
<td>Abraxane</td>
<td>Paclitaxel</td>
<td>Albumin</td>
<td>Advanced non-small cell lung cancer</td>
<td>FDA (2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Metastatic breast cancer</td>
<td>EMA (2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Metastatic pancreatic cancer</td>
<td></td>
</tr>
</tbody>
</table>
Table 1.3: List of cancer targets and their targeting ligands

<table>
<thead>
<tr>
<th>Target</th>
<th>Extracellular matrix protein/ligand</th>
<th>Targeting Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>αVβ3 integrin</td>
<td>Fibronectin/basement membrane protein</td>
<td>RGD, RYD</td>
</tr>
<tr>
<td>α4β1 integrin</td>
<td>Fibronectin/VCAM-1, CSF</td>
<td>LDV, IDAPS</td>
</tr>
<tr>
<td>α5β1 integrin</td>
<td>Fibronectin/Invasin/Platelet</td>
<td>LDV, RGD, PHSRN</td>
</tr>
<tr>
<td>Folate receptor</td>
<td>Folate</td>
<td>Folic acid</td>
</tr>
<tr>
<td>Transferrin receptor</td>
<td>Transferrin</td>
<td>Transferrin</td>
</tr>
<tr>
<td>HER2 receptor</td>
<td>Not known</td>
<td>Trastuzumab</td>
</tr>
</tbody>
</table>
1.2.3 Factors influencing drug targeting and delivery of nanocarriers. The EPR effect is influenced by the physicochemical properties of nanocarriers, including size, shape and surface charge and this in turn affects cellular uptake, internalization and circulation half-life. To increase the efficacy of drug targeting and delivery, the physicochemical properties of nanocarriers can be optimized to an ideal range (table 1.4).

a) Size. Nanocarriers with sizes above 400 nm will not be able to extravasate into the leaky fenestrations of the endothelial cells due to entrapment in the liver and spleen and phagocytosis by macrophages and Kupffer cells of the MPS. Conversely, nanocarrier sizes that are too small (<6 nm) are likely to be excreted by the kidneys. Larger particles of around 100 nm may not be able to penetrate deep into the finer vasculature and tissues of the tumor, compared to smaller sized nanocarriers of around 20 nm [24]. Cellular uptake is significantly influenced by the size of nanocarriers and this was demonstrated by preparing chitosan nanoparticles of various sizes and characterizing cellular uptake. The results showed reduced non-phagocytic cellular internalization at sizes above 150 nm and increased phagocytic cellular uptake with sizes above 150 nm [25]. Taking these factors into consideration, ideal nanocarrier sizes range between 10 nm to 100 nm for optimal drug targeting and delivery.

b) Surface charge. The surface charge of nanocarriers also influences phagocytic and non-phagocytic cellular uptake, agglomeration, blood circulation time, opsonization and renal elimination. In general, nanocarriers with a zeta potential ≥ + 30 mV or ≤ - 30 mV are preferred to maintain good colloidal stability and prevent aggregation in a physiological environment. Positively charged nanocarriers are taken up by cells more effectively compared to neutral or negatively charged nanocarriers due to the strong interactions with the negatively charged cell membrane. For example, it was reported that positively charged chitosan nanoparticles were more readily internalized in non-phagocytic cells compared to negatively charged nanoparticles [25]. However, positively charged nanocarriers are known
to have increased blood clearance and cause hemolysis and aggregation of platelets, due to opsonization and recognition by the MPS [24]. Also, various positively charged lipids used for formulating nanocarriers are cytotoxic through destabilization of the cell membrane, therefore careful consideration must be given when selecting materials for formulating nanocarriers. Overall, neutral or negatively charged nanocarriers are preferred due to prolonged circulation time, reduced cytotoxicity and evasion of renal elimination.

**c) Shape.** Less extensively studied is the role of nanoparticle geometry on drug targeting and delivery. Most therapeutic carriers under investigation or in clinical practice are spherical in shape and this is due to the presumption that size is the principle parameter of interest and there is a difficulty in developing methods for non-spherical nanoparticles. The shape of nanoparticles can affect velocity, diffusion and adhesion to blood vessel walls. The targeting ability is also affected due to the available surface area for targeting ligands and the degree to which particles fit contours of target cell membranes. The role of geometry on phagocytosis has been studied, where polystyrene nanoparticles of various shapes were prepared and tested in alveolar rat macrophages. Results showed that internalization of ellipse-shaped nanoparticles was dependent on the point of attachment, where internalization occurred within a few minutes from the pointed end and no internalization when in contact with the flat side. For spherical polystyrene nanoparticles, internalization into macrophages occurred at all points of attachment due to symmetry [26]. These results showed that development of non-spherical nanoparticles may prove to be a useful strategy for evading phagocytosis. The role of geometry on non-phagocytic cellular internalization in human cervical carcinoma epithelial HELA cells has also been studied through formulation of cubed and cylindrical nanoparticles of various sizes and shapes. Results showed that rod-shaped, high aspect ratio (AR=3, d=150 nm, h=450 nm) nanoparticles internalized much faster compared to rod-shaped particles with a lower aspect
ratio (AR=1, d=200 nm, h=200 nm) [27]. A possible explanation for this is that higher aspect ratio particles have larger surface areas for contact with the cell membrane, resulting in faster cellular uptake. This is highlighted by another study that reported that antibody coated rod-shaped nanoparticles compared to spherical were more effective at targeting endothelial cells due to the large surface area and thus increased interactions of antibody binding sites to cell membrane receptors [28].

\[d\) Surface chemistry.\] It is well known that less than 10% of nanocarriers administered intravenously can reach their target site and this partially due to removal by the MPS in the liver and spleen. Opsonization occurs when antibodies or other substances bind to foreign particles, making them more susceptible to phagocytosis and these opsonins interact with nanocarriers through electrostatic, ionic, van der waals, hydrophobic and hydrophilic forces [29]. In general, nanocarriers that are highly charged and hydrophobic are more susceptible to opsonization, and it is therefore essential to modify the surface of nanocarriers to help avoid elimination by the immune system and prolong the blood circulation time. One of the most common strategies is to coat the surface of nanocarriers with a hydrophilic polymer known as polyethylene glycol (PEG), to produce stealth characteristics. In 1997, Abuchowski reported that PEGylation to bovine liver catalase significantly reduced immunogenicity and prolonged blood circulation time and since then PEG has been used frequently in nanocarriers such as micelles, liposomes and lipid nanoparticles [30]. PEG forms a hydrophilic flexible layer on the surface of nanocarriers which prevents adsorption of opsonins by steric hinderance and subsequent disruption of nanocarriers. Depending on the molecular weight, PEG can form mushroom or brush-like structures surrounding the nanocarriers. For example, low PEG (5KDa) densities between 0.5 to 0.7 mol% form mushroom-like structures to maximize surface coverage. At higher densities, PEG chains extend to avoid overlap with existing PEG chains forming a brush-like
model. There is an optimal range of PEG required to form stable nanocarriers, for example it has been reported that thermodynamically stable liposomes are formed when formulated with 5 to 7 mol% PEG and further increases to above 10 mol% destabilize liposomes due to repulsion between PEG chains [31]. The advantageous effects of PEG are clearly demonstrated by the two commercially available liposome products Doxil (PEGylated) and Myocet (non-PEGylated), where Doxil has a significantly longer blood circulation time compared to Myocet [14]. It has also been reported that PEGylated polymeric nanoparticles demonstrated higher plasma concentrations and reduced accumulation in the liver compared to non-pegylated polymeric nanoparticles. Furthermore, 10% of nanoparticles were remaining 6 hours post injection compared to only 0.4% for non-pegylated NP’s [32]. These results clearly demonstrated the importance of modifying the surface of nanocarriers to prolong circulation time and reduce uptake by the MPS.
Table 1.4: Physicochemical properties of nanocarriers and their impact on drug targeting and delivery

<table>
<thead>
<tr>
<th>Physicochemical property</th>
<th>Strategy</th>
<th>Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>20nm to 100nm</td>
<td>EPR effect</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased tumor penetration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduced renal elimination</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduced uptake by MPS</td>
</tr>
<tr>
<td>Charge</td>
<td>neutral/anionic</td>
<td>Increased cellular uptake</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduced opsonization</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prolonged circulation time</td>
</tr>
<tr>
<td>Shape</td>
<td>rod</td>
<td>Increased surface area for cellular interactions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased cellular uptake</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Evasion of macrophages</td>
</tr>
<tr>
<td>Surface chemistry</td>
<td>PEGylation</td>
<td>Reduced opsonization</td>
</tr>
<tr>
<td></td>
<td></td>
<td>prolonged circulation time</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased physical stability</td>
</tr>
</tbody>
</table>
1.3 Nanocarriers

Nanocarriers are spherical aggregates, ranging between 20 nm to 400 nm in size and are commonly used to encapsulate hydrophobic and hydrophilic drugs. Nanocarriers can be classified into two categories: organic and inorganic nanoparticles. Examples of the latter include carbon nanotubes and mesoporous silica nanoparticles, however these are less common due to non-biocompatibility and toxicity. Organic nanocarriers include liposomes, micelles, dendrimers, lipid and polymeric nanoparticles and these are generally preferred due to low toxicity and biocompatibility of lipid and polymeric materials. Phospholipids are the major component of the biological cell membrane, therefore their biocompatible and biodegradable nature makes them key components in drug delivery systems. The self-assembly of lipids is dependent on the nature of the hydrophilic and hydrophobic regions and the type of nanocarrier formed is largely dependent on the packing constraints of lipids to minimize exposure of the hydrophobic regions to the aqueous environment. The geometry of a nanocarrier is defined by the packing parameter, where $V$ is the surfactant tail volume, $a$ is the effective area per molecule at the interface and $l$ is the surfactant tail length \[33\]. Therefore, if these factors are known, the geometry of a nanocarrier can be predicted.

Equation \[ P = V/l \]

When $P=1$, a lamellar liquid crystalline structure is formed giving rise to vesicle or liposome formation. For example, the biological cell membrane is composed of phospholipids arranged in a bilayer or lamellar structure. When $P>1$, amphiphilic lipids self-assemble to form reversed structures such as reversed micelles, reversed hexagonal and reverse cubic structures. Packing parameters $<1$ form normal micelle, hexagonal or cubic phases.
1.3.1 Micelles. Micelles are nanosized (10 nm to 100 nm) colloidal aggregates that result from the self-assembly of lipid or polymeric amphiphilic molecules in aqueous solution. At low amphiphilic concentrations, amphiphiles exist as monomers and at higher concentrations amphiphiles aggregate to form micelle structures and the concentration at which this occurs is known as the Critical Micelle Concentration (CMC). Micelles are dynamic structures that are in continuous equilibrium with monomers, where monomers are continually being exchanged between micelles. During micelle formation, the hydrophobic segments of amphiphiles aggregate to form a hydrophobic core that encapsulates and controls the release of hydrophobic drugs. The hydrophilic segment forms a hydrophilic shell, ensuring aqueous solubility, stabilization of the micelles and prevention of opsonization and uptake by the MPS. Micelles have several advantages including increased solubility of hydrophobic drugs and formulation using a variety of polymeric or lipid amphiphilic molecules to fine-tune the CMC and stability of micelles. Furthermore, the small sizes of micelles enable rapid accumulation into the tumor. Targeting ligands can be grafted onto amphiphilic molecules to impart active targeting properties to improve drug targeting and delivery to the tumor tissue. Despite these advantages, micelles have poor drug loading efficiency and poor in vivo stability. For example, surfactant-based micelles exhibit high CMC values leading to reduced stability when administered systemically [34]. For this reason, various strategies have been employed to improve the stability of micelles including, cross-linking and covalent drug entrapment [34, 35]. Compared to surfactant-based micelles, polymeric micelles demonstrate significantly lower CMC values and self-assembly occurs at concentrations exceeding the CMC. These systems demonstrate better stability and prolonged circulation times in vivo [36, 37]. Some commonly used materials used to form micelles include poloxamers, PEG-PLA polymers, PEGylated polyglutamic acid, PEG-PAA and lipid-PEG conjugates.
1.3.2 **Liposomes.** Liposomes are the most widely used nanocarrier systems and represent the majority of FDA approved nanomedicine products (table 1.1, table 1.2). Liposomes are formed from amphiphilic molecules, composed of a phospholipid bilayer that entraps hydrophobic drugs and surrounds a hydrophilic core for entrapping hydrophilic drugs. Liposomes can be formed from natural or synthetic lipids, where multiple bilayers result in the formation of large multilamellar vesicles (MLV). These can be downsized to single bilayer liposomes known as small unilamellar vesicles (SUV). Like micelles, liposomes are versatile drug delivery systems as their lipid composition, size, charge and surface chemistry can be fine-tuned to achieve optimal drug delivery characteristics.

Compared to micelles, the size range of liposomes tends to be larger, ranging between 50 nm to 500 nm and this range depends on the method of production and the choice of lipids. The surface charge of liposomes depends on the choice of lipids used, for example cationic and anionic lipids will produce positively or negatively charged liposomes respectively. The surface chemistry of liposomes can also be modified by coating with PEG polymers to help evade the immune system and prolong blood circulation. Liposomes can also be grafted with targeting ligands to impart specific targeting properties. Despite these advantages, drug loading in liposomes remains a challenge, where encapsulation of hydrophilic drugs is low and requires active loading techniques to increase the drug loading efficiency. Furthermore, encapsulation of hydrophobic drugs inside the bilayer was shown to destabilize the lipid bilayer membrane resulting in aggregation and instability of vesicles [38]. Like micelles, liposomes also have the potential to become unstable upon in vivo administration. For example, lipids with low transition temperatures result in a more fluid-like flexible membrane structure which increases the permeability and drug leakage from the membrane at physiological body temperature.
1.3.3 Solid lipid nanoparticles. SLNs are nanosized colloidal structures with sizes ranging from 50 to 1000 nm. Typically, SLNs are composed of a solid lipid phase stabilized by a surfactant. Examples of lipids used are triglycerides, free fatty acids, fatty acid alcohols, waxes and steroids, which are all used for dispersion and entrapment of hydrophobic drugs. The lipid molecules are solid at room and body temperatures, which enables them to have better stability and prolonged drug retention compared to micelles and liposomes. SLNs have several advantages, that include excellent physically stability, good release profiles, high drug loading capacity, biodegradability and non-toxicity of lipid carriers. However, despite these advantages SLNs can become unstable through gelation, lipid particle growth and polymorphic transitioning [39].

1.4 Significance and Statement of the Problem

Despite the development of numerous anti-cancer nanocarriers in the pre-clinical phase, very few have been successfully developed and approved for clinical use. This is due to the low delivery efficiencies of anti-cancer nanomedicines to the tumor which is highlighted by a recent meta-analysis of pre-clinical data suggesting that a median of 0.7% of the injected dose of nanocarriers reaches the target tumors [40, 41]. One of the major reasons for low delivery to the tumor is the poor stability of anti-cancer drug-loaded nanocarriers. Upon systemic administration, drug diffusion out of the nanocarriers or interactions with blood cells, cellular membranes, serum proteins and other biomacromolecules can result in premature drug release before reaching the target tumor site. Therefore, developing a stable anti-cancer nanocarrier system is key for the successful clinical translation of these nanomedicines.
1.5 Purpose

The purpose of this dissertation is to develop targeted micelles, liposomes and SLNs of similar composition and to evaluate their potential by comparing physicochemical characteristics, in vitro stability, in vitro release rates and in vivo efficacy. These studies will provide a more comprehensive evaluation of the stability of different types of nanocarrier systems, providing further insights into the selection of suitable nanocarrier systems for clinical development.

1.6 Specific Aims

The specific aims of this dissertation are as follows:

Aim 1: To design, prepare and characterize LDV-targeted micelles, liposomes and solid lipid nanoparticles of similar composition.

Aim 2: To compare the in vitro stability and in vitro drug release of LDV-targeted micelles, liposomes and solid lipid nanoparticles. The stability of micelles, liposomes and solid lipid nanoparticles can be assessed in aqueous medium and biological media using Forster Resonance Energy Transfer (FRET). Anti-cancer drug paclitaxel can be loaded into micelles, liposomes and solid lipid nanoparticles and in vitro release characteristics can be determined. Nanocarriers can also be spray dried to maintain long-term stability.

Aim 3: To compare the in vivo anti-tumor efficacy of selected LDV-targeted paclitaxel-loaded micelles, liposomes and solid lipid nanoparticles using mouse tumor models.
Chapter 2: Design and Synthesis of LDV Peptide Amphiphiles

2.1 Introduction

Amphiphiles are composed of hydrophobic and hydrophilic regions which self-assemble in aqueous solutions to form a variety of structures including micelles and vesicles. Amphiphiles can be characterized by the hydrophilic-lipophilic balance (HLB), which is an empirical parameter indicating the degree of hydrophilicity and lipophilicity. HLB is related to the number, weight of hydrophobic and lipophilic groups as well as the structure of the amphiphile and there are several methods that can be used to determine this value [42]. Typically, for non-ionic surfactants, the HLB scale ranges between 1 and 20. For ionic surfactants, the HLB scale ranges between 0 to 40, where 0 indicates complete hydrophobicity and 40 indicates a stronger hydrophilic character of the amphiphile. The HLB of amphiphiles must be optimum to enable formation of micelles and this can be achieved by altering the hydrophobic and hydrophilic segments. Therefore, determination of HLB values can be used to predict surfactant properties and those surfactants with HLB values within the scale can be used to assess the potential self-assembly into micelles. In this study, novel PAs were designed, where the hydrophobic region is composed of a C16 fatty acid chain and the hydrophilic region is comprised of tripeptide LDV targeting region and ethylene oxide linker units (Figure 2.1). PAs were selected for synthesis based on optimum HLB values and assessed for their ability to form micelles. The general process of Solid Phase Peptide Synthesis (SPPS) is illustrated in figure 2.2 and involves the building of a peptide chain on a solid resin. The first amino acid already attached to the resin is coupled with subsequent amino acids using a series of deprotection and coupling steps. Specifically, this involves the deprotection of temporary protecting groups on the N-terminal of the resin-attached amino acid and subsequent coupling of the next amino acid. This process is
repeated until the desired peptide sequence is obtained, followed by detachment of the peptide from the resin.

2.2 Materials

Peptide materials: Fmoc-L-Val-Wang resin, Fmoc-L-Asp-OH, Fmoc-L-leu-OH, Fmoc-8-amino-3,6-dioxaoctanoic acid (Fmoc-ADA-OH), 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU), Hydroxybenzotriazole (HOBt), (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), N,N-Diisopropylethylamine (DIPEA), N,N′-Diisopropylcarbodiimide (DIC) were purchased from Chem-Impex International, Inc (IL, USA). Solvents used were of HPLC grade, dichloromethane (DCM), Dimethylformamide (DMF) and acetonitrile were purchased from fisherscientific (PA, USA). Piperidine, trifluoroacetic acid (TFA), Triisopropyl silane (TIS) and stearic acid were obtained from Acros organics (NJ, USA) and used as supplied.

2.3 Method

2.3.1 Design of PAs and calculation of HLB values. Three PAs were designed with varying lengths of hydrophilic linker units: C16-(PEG2)_2-LDV, C16-(PEG2)_4-LDV, C16-(PEG2)_6-LDV (Table 2.1). To predict surfactant properties of PAs, the HLB values for each PA was calculated using the Davies method (Table 2.2) [42]. This method was chosen since it is useful for calculating HLB values of ionic surfactants. The carboxylic acid groups of PAs are ionized at physiological pH 7.4 and thus the overall net charge of -1 makes the PA an anionic structure. This method takes into account the hydrophilicity and hydrophobicity of chemical groups using a parameter group number. Each chemical group is assigned a group number, which can be used to calculate the HLB value using equation 2. For all PAs, carboxylic acid groups present in the peptide and oxygen present in the ethylene oxide linker
are hydrophilic groups and carbon-hydrogen groups present in the C16 fatty acid chains are hydrophobic groups.

Equation 2:

\[
HLB = 7 + \sum \text{hydrophilic group numbers} + \sum \text{hydrophobic group numbers}
\]

2.3.2 Solid Phase Peptide Synthesis of peptide amphiphiles. PAs were synthesized using standard Fmoc-chemistry in a polypropylene vessel on a peptide synthesis shaker. Briefly, wang resin preloaded with valine (Fmoc-L-Val-WR) was soaked in DMF for 30 minutes to swell the resin. Next, the protecting group Fmoc was removed from the amino group of L-valine by incubating with 20% piperidine in DMF for 30 minutes. The resin was then washed with DMF and DCM three times each. Following deprotection, Fmoc-L-Asp-OH was coupled to valine at a molar ratio of 3 by treating with DIC and HOBT in DMF for 3 hours. The same deprotection-coupling steps were used for the coupling of Fmoc-L-leu-OH. Next, two, four or six Fmoc-ADA-OH (PEG2) linker units were coupled to L-leucine by treating with HATU, HOBT and DIPEA in DMF for 3 hours. For conjugation of palmitic acid, the Fmoc group was removed from the final PEG2 linker unit using 20% piperidine in DMF and the resin was washed using the same procedure as described above. The resin was then treated with a mixture of PyBOP and DIPEA in DMF:DCM (50:50) for 3 hours. The resin was then washed and the PA was cleaved from the resin by adding a mixture of TFA:TIS:H₂O (95:2.5:2.5) for 3 hours. The mixture was removed from the peptide synthesis vessel and TFA was evaporated using nitrogen gas until <0.2 ml of solution was remaining. Cold isopropyl ether was added to precipitate out the crude PA, which was separated from isopropyl ether using centrifugation at 3000 rpm. Crude PAs were then suspended in DI water, lyophilized and subsequently purified by reverse phase High Performance Liquid
Chromatography (RP-HPLC) using an Agilent 1200 HPLC system equipped with a diode array detector (DAD). Samples were dissolved in methanol and loaded onto a zorbax agilent C-18 column (4.6 x 150mm, 5µm) equilibrated with 90% eluent A (0.1% TFA in water) and 10% eluent B (0.1% TFA in acetonitrile) for 30 minutes. PAs were eluted using a linear gradient method of 10 to 95% eluent B in 20 minutes at 1.0 mL/min. The eluate was monitored at 280 nm. The PAs were then re-lyophilized for 48 hours and the lyophilized powders were stored at -80°C. The identity of PAs was confirmed by ESI-MS (Electro Spray Ionization Mass Spectrometry).

2.4 Results and Discussion

The results show that all PAs have HLB values ranging between 36.55 and 37.45, which is within the HLB scale for ionic surfactants (table 2.3). The results also show that as the number of PEG2 units increases, the HLB value increases. PAs with two, four and six PEG2 linkers were chosen for synthesis and characterization as they are within the HLB scale and have high HLB values that may improve the aqueous solubility of PAs without PEG2 linkers. Mass Spectrometry confirmed the formation of all PAs. The spectrum displayed the protonated molecular ion peak \([\text{M}+\text{H}]^+\) at m/z 874.57 for C16-(PEG2)_2-LDV (Figure 2.3). For C16-(PEG2)_4-LDV, the major peak is shown at 1186.6 m/z corresponding to the sodiated molecule \([\text{M}+\text{Na}]^+\). Similarly, the peak at 605 Da is representative of the doubly charged sodiated PA \([\text{M}+2\text{Na}]^{2+}\) (figure 2.4). The presence of C16-(PEG2)_6-LDV was confirmed by doubly charged protonated molecular ion peak \([\text{M}+\text{H}]^{2+}\) at 728.1 m/z and a doubly charged protonated sodiated ion \([\text{M}+\text{H}+\text{Na}]^{2+}\) at 739 m/z (figure 2.5). HPLC purity of all PAs were >95%. PAs were further characterized for self-assembly into micelles and incorporation into liposomes and SLNs.
Table 2.1: Design of peptide amphiphiles

<table>
<thead>
<tr>
<th>Peptide Amphiphile</th>
<th>Fatty acid chain length</th>
<th>Number of hydrophilic PEG2 linkers</th>
<th>Exact mass (Da)</th>
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<tr>
<td>C16-(PEG2)$_2$-LDV</td>
<td>C16</td>
<td>2</td>
<td>873.57</td>
</tr>
<tr>
<td>C16-(PEG2)$_4$-LDV</td>
<td>C16</td>
<td>4</td>
<td>1163.2</td>
</tr>
<tr>
<td>C16-(PEG2)$_6$-LDV</td>
<td>C16</td>
<td>6</td>
<td>1453.86</td>
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Figure 2.1: Chemical structure of LDV-peptide amphiphile
Table 2.2: HLB numbers for chemical groups using the Davies and Rideal method [42]

<table>
<thead>
<tr>
<th>Hydrophilic groups</th>
<th>Lipophilic groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical group</td>
<td>Group number</td>
</tr>
<tr>
<td>-SO4Na+</td>
<td>38.7</td>
</tr>
<tr>
<td>-COO-H+</td>
<td>21.2</td>
</tr>
<tr>
<td>-COO-Na+</td>
<td>19.1</td>
</tr>
<tr>
<td>-N (tertiary amine)</td>
<td>9.4</td>
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<tr>
<td>-O-</td>
<td>1.3</td>
</tr>
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</table>

Table 2.3: HLB values for peptide amphiphiles

<table>
<thead>
<tr>
<th>Peptide Amphiphile</th>
<th>HLB value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16-(PEG2)\textsubscript{2} -LDV</td>
<td>37.5</td>
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<td>C16-(PEG2)\textsubscript{4} -LDV</td>
<td>37.95</td>
</tr>
<tr>
<td>C16-(PEG2)\textsubscript{6} -LDV</td>
<td>38.45</td>
</tr>
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</table>
Figure 2.2: Flow chart for solid phase peptide synthesis steps and LDV peptide amphiphile characterization
Figure 2.3: ESI-MS spectrum of C16-(PEG2)₂-LDV peptide amphiphile
Figure 2.4: ESI-MS spectrum of C16-(PEG2)₄-LDV peptide amphiphile
Figure 2.5: ESI-MS spectrum of C16-(PEG2)_6-LDV peptide amphiphile
Chapter 3: Design and Preparation of LDV-targeted Nanocarriers

3.1 Introduction

A major challenge associated with nanotechnology for cancer therapy is the retention of drug inside nanocarriers following intravenous administration, as drug should remain inside or complexed to the nanocarrier until it reaches the target cancer cells. The most extensively studied nanocarriers are micelles and liposomes and the stability of these systems is a critical factor for effective drug delivery. Micelles are attractive drug delivery vehicles and have several advantages such as small size for exploiting the EPR effect, hydrophobic core for loading of poorly soluble anti-cancer agents and hydrophilic shell for stability resulting in long circulation kinetics. The chemical composition and molecular weight of the amphiphiles can also be adjusted to achieve effective drug release behavior, physical stability and drug loading [43]. However, poor stability is a significant challenge that hampers the clinical translation of micelles. Specifically, micelles are dynamic systems and dilution upon intravenous administration can reduce surfactant concentrations below the CMC, resulting in premature drug release before reaching the target tumor site. Micelles are also prone to dissociation through interactions with blood components such as albumin and apolipoproteins. Compared to micelles, liposomes have several advantages: they are able to encapsulate both hydrophilic and hydrophobic drugs and they are mechanically stronger making them less prone to breakage and dissociation upon dilution [44]. However, the use of liposomes is also limited by disadvantages such as drug leakage from the bilayer membrane, poor physical stability, low encapsulation efficiency, short shelf-life and destabilization through interactions with serum proteins. Compared to liposomes and micelles, SLNs have the potential to provide better control over drug release and drug delivery since the lipid components are solid at both room and body temperature. This enables improved retention of drug molecules inside the nanocarrier, reduced leakage of drug molecules and prolonged
release of drug molecules in vivo. SLNs also have good physical stability and good in vitro release characteristics. However, SLNs also have several disadvantages including unpredictable gelation tendency, polymorphic transitioning and low drug loading efficiency [45]. Therefore, there is a necessity to develop nanocarriers that are better able to retain the drug before reaching the target site, are resistant to disruption from interactions with serum proteins and therefore offer better control over drug release and drug delivery. To develop nanocarriers with optimum physicochemical characteristics, the size, charge and morphology can be determined to help understand or predict the in vivo behavior of nanocarriers. For determination of size, Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS) are commonly used. DLS measures the hydrodynamic diameter of nanocarriers dispersed in aqueous solution and this method is widely preferred due to the non-invasive fast measurement times and size determination of nanocarriers in its native colloidal state. This method typically relies on Rayleigh Scattering from the nanoparticles in solution that are undergoing Brownian motion [46]. This is the random movement of particles in a liquid that occurs as a result of the continuous bombardment from solvent molecules of the surrounding medium. In general, larger particles move more slowly in solution compared to smaller particles and the velocity of brownian motion is defined the translational diffusion coefficient. Therefore, the size of the nanocarrier refers to how the particle diffuses within the solution and can be calculated from the translation diffusion coefficient using the Stokes-Einstein equation [46, 47]:

Equation 3.1:

\[ d(H) = \frac{kT}{3\pi\eta D} \]

Where \(d(H)\) is the hydrodynamic diameter, \(D\) is the translational diffusion coefficient, \(k\) is the Boltzmann’s constant, \(T\) is the absolute temperature and \(\eta\) is the viscosity. Despite frequent
use of this method, DLS has several limitations such as poor resolution of particle sizes and the inability to determine nanocarrier morphology, porosity and all particle populations present in a polydisperse sample. In contrast, TEM helps to overcome these disadvantages by direct observation of samples, which allows for morphology characterization as well as size determination. The morphology of nanocarriers is a characteristic that has been shown to dictate the efficacy of targeting and cellular uptake. It was shown that antibody coated rod-shaped nanoparticles compared to spherical were more effective at targeting endothelial cells due to the large surface area and thus increased interactions of antibody binding sites to cell membrane receptors [28]. For size and morphology characterization, nanocarrier samples are adsorbed onto a support film, followed by negative staining to view dehydrated structures under an electron beam. Negative staining can be achieved by applying heavy metals salts to the sample, where the hydrated regions in and around the particle become occupied. As the stain is dried, an amorphous electron-dense replica of the particle is formed. Upon imaging, the electron beam primarily interacts with the stain, enhancing the contrast of the image, where the image is light in areas occupied by the nanocarrier and dark in areas surrounding the nanocarrier. Despite the usefulness of this method, sample preparation is expensive, time consuming and requires drying of nanocarriers. This can cause the sample to shrink, break down or agglomerate, producing a sample image that is no longer representative of the native colloidal state [48]. In addition to size and morphology, the charge or zeta potential is a fundamental parameter that dictates nanocarrier behavior such as aggregation, interaction with cell membranes and other surfaces such as serum proteins [49]. The zeta potential is defined as the difference in the electrical potential on the shear surface of the nanocarrier and the electrical potential of the solution and is determined by an electrophoresis experiment that measures the velocity of the nanocarriers using Laser Doppler Velocimetry (LDV). This is followed by application of the Henry equation to
calculate the zeta potential (Equation 3.2), where $z$ is the zeta potential, $U$ is the electrophoretic mobility, $\varepsilon$ is the dielectric constant, $\eta$ is the viscosity and $f(ka)$ is Henry’s function [47]. Positively charged or neutral nanocarriers will be preferentially taken up by the negatively charged membrane, although cationic lipids and polymers are known to be toxic to cells.

Equation 3.2: $U = \frac{2\varepsilon z f(ka)}{3\eta}$

PAs were assessed for their ability to self-assemble into micelles. At low surfactant concentrations, surfactant molecules exist as monomers and adsorb at the liquid surface. As the concentration of surfactant increases, the surface tension of the solution decreases and the surface becomes saturated with surfactant molecules. Upon further addition of surfactant, surfactant monomers start to self-assemble into micelles and the concentration at which this occurs is known as the Critical Micelle Concentration (CMC). PAs were predicted to have amphiphilic properties from calculated HLB values and so it was hypothesized that these PAs would self-assemble into micelles, where the fatty acid chains aggregate away from the aqueous solution to form a hydrophobic core and the LDV-PEG2 region faces the aqueous solution forming a hydrophilic corona (figure 3.1). The CMC can be determined by determining the concentration at which an inflection point occurs when physicochemical properties such as surface tension are plotted as a function of concentration. Light scattering methods can also be used, where a sharp increase in turbidity is observed. A commonly used method for CMC determination is known as the fluorescence probe technique. Here, a hydrophobic fluorescent dye known as pyrene, demonstrates sensitivity to the polarity of the surrounding medium and consequent changes in spectral properties [50]. Below the CMC, pyrene is solubilized in water and above the CMC, when micelles are forming, pyrene
partitions into the hydrophobic core. As a result, the pyrene emission spectrum exhibits changes in fluorescent intensity and vibrational structure of emission spectrum. Specifically, there are 5 vibrionic bands. The intensity of the third (383 nm) vibrionic band is significantly enhanced in a hydrophobic environment and the intensity of the first peak is increased in a polar environment (figure 3.7). Therefore, the ratio of the 3<sup>rd</sup> and 1<sup>st</sup> peak intensities in the emission spectrum provides a measure of the apparent polarity of the environment. An increase in this ratio, indicates a decrease in polar environment of the pyrene. The CMC can be determined from the inflection points of pyrene intensity ratio and surfactant concentration plots.

Figure 3.1: Schematic representation of the structure and formation of micelles from LDV-peptide amphiphiles in aqueous solution.
To compare the stability of liposomes with micelles, the lipid compositions must be as similar as possible. Micelles are formed from single chain amphiphiles, due to steric effects of the hydrocarbon chain and steric conditions determined by the charged head group. In contrast, double-chained amphiphiles favor the formation of bilayers, due to the steric restriction associated with the second hydrocarbon chain [51]. For micelle formation, single chain palmitic acid was conjugated with peptide-linker segments to confer amphiphilic properties. To form liposomes of similar composition to these micelles, palmitic acid derived double-chain amphiphilic phospholipids can be used, ensuring that the fatty acid chain length of micelles and liposomes are kept the same. Based on this rationale, a synthetic phospholipid named dipalmitoylphosphatidylcholine (DPPC) was selected for liposome formation since it is composed of two palmitic acid fatty acid chains attached to a phosphatidylcholine head-group, enabling the formation of a phospholipid bilayer. To impart similar head group properties, PAs were incorporated into DPPC liposomes. It was hypothesized that the palmitic fatty acid chain of PAs would insert into the DPPC bilayer, facilitating the anchoring of the hydrophilic (PEG2)_n-LDV head group to the liposome surface (figure 3.2). The two most common methods to produce liposomes are sonication and extrusion, where each method produces different mean sizes and size distributions. Thin lipid films or lipid cakes are hydrated in aqueous medium and stacks of crystalline bilayers become fluid and swell. The hydrated lipid sheets detach during agitation and self-close to form MLVs, which are heterogeneous in size and number of lamellae and range between a few hundred nanometers to several microns. MLVs are concentric bilayers separated by narrow aqueous spaces and can be downsized to SUVs ranging between 20 nm to 100 nm by energy input in the form of sonic energy or mechanical energy. For sonication, acoustic energy is applied to lipid suspensions from a bath or probe tip sonicator. Smaller sized liposomes can be produced by increasing the sonication time and this process is relatively fast. For application of
mechanical energy to produce liposomes, a lipid suspension is passed through a polycarbonate membrane with a specific pore size, and this results in vesicle sizes close to the diameter of the pore size. Compared to sonication, this process is slower but produces reproducible sizes and size distributions [52].
Figure 3.2: Schematic representation of the structure and formation of liposomes from LDV-peptide amphiphiles and DPPC lipids in aqueous solution using the thin-film hydration method.
To design SLNs of similar composition to micelles and liposomes, a palmitic acid derived solid lipid was used to form the hydrophobic solid core component. Tripalmitin is a triglyceride obtained by acylation of three hydroxy groups of glycerol by palmitic acid (C16:0/C16:0/C16:0). The high melting point of tripalmitin (66°C to 68°C) means that at room temperature and physiological body temperature, tripalmitin is solid, which may significantly reduce the mobility of drug molecules within the lipid core, preventing premature drug release. To stabilize the solid lipid core and impart targeting properties, PAs were incorporated (figure 3.3). SLNs can be prepared via a variety of methods including high pressure homogenization (HPH), solvent emulsification/evaporation, supercritical fluid technology, ultrasonication or high-speed homogenization. Many of these methods are often characterized by poorly controlled conditions, which lead to poly-disperse sizes and size distributions. Microfluidic technology has been shown to overcome these disadvantages by providing better control of particle sizes and stability of lipid nanoparticles through optimization of parameters such as the Flow Rate Ratio (FRR), the total flow rate (TFR), choice of solvents, temperature and microfluidic chip design [53-60]. A typical microfluidic micro-mixer contains a microfluidic cartridge comprising of moulded channels with staggered herringbone structures. The microfluidic chips are designed to have two inlet channels that merge into a microchannel, where one channel is used to pump lipid mixtures in ethanol and the second channel is used to pump aqueous solutions. SLNs are formed when these two phases mix through staggered herringbone microchannels, in which chaotic flow occurs subjecting the fluid to a repetitive series of a rotational flow profile. This design increases the rate of mixing because the grooves cause the streams of fluid to twist over each other, allowing rapid and controlled mixing of both phases. During mixing, when a specific ethanol concentration is reached, lipids precipitate and start to grow and form nanoparticles (figure 3.3).
Figure 3.3: Schematic representation of the structure and formation of solid lipid nanoparticles from LDV peptide amphiphiles and tripalmitin lipid in aqueous solution, using microfluidic technology.
3.2 Materials

PAs were synthesized as described in section 2. DPPC lipids and liposome extruder was purchased from Avanti polar lipids (AL, USA). Tripalmitin and pyrene was purchased from Sigma-Aldrich (MO, USA). Solvents including DCM, methanol, ethanol and chloroform were purchased from fisherscientific (Pittsburg, PA). Copper formvar 200 mesh grids were purchased from Ted Pella Inc (Redding, CA).

3.3 Methods

3.3.1 Liposome preparation. Liposomes were formed by thin-film hydration. A mixture of DPPC and PA were dissolved in chloroform: methanol = 2:1 (v/v) at a molar ratio of 1:0.05. Organic solvents were evaporated to dryness under a stream of nitrogen gas and solvent traces were removed by leaving the glass tubes under vacuum overnight. The dried lipid film was then hydrated by the addition of DI water at a temperature between 50°C to 55°C, followed by vortexing periodically for 30 minutes to detach any lipid from the glass surface. Hydration was carried out in a water bath and the temperature was maintained above the main phase transition temperature (T_m) of lipids (41°C for DPPC). This process resulted in the formation of MLVs, which were subjected to six freeze-thaw cycles with liquid nitrogen and heated DI water. MLVs were downsized by extruding 21 times through 0.1μm-pore polycarbonate filters to produce SUVs. Liposomes were prepared for each PA (C16-(PEG2)2-LDV, C16-(PEG2)4-LDV, C16-(PEG2)6-LDV) and each liposome composition was prepared in triplicates.
3.3.2 Solid lipid nanoparticle preparation. The NanoAssmblr (Benchtop, Precision NanoSystems Inc., Vancouver, Canada) was used to prepare SLNs by microfluidic mixing. Specifically, a microfluidic cartridge comprising of moulded channels with staggered herringbone structures was used to formulate SLNs by mixing of the organic (tripalmitin in ethanol) and aqueous (PA in water) phases at a molar ratio of 1:0.5. The organic and aqueous phases were pumped into two inlets of the microfluidic chip using disposable syringes and mixing was controlled by setting the FRR to 3:1 and the TFR to 15 mL/min. At all steps of preparation, the organic and aqueous phases were kept at 75°C by keeping solutions in the oven and installing a heat block into the NanoAssemblr instrument. Next, the mixture was collected from the NanoAssemblr and transferred into a G2 slide-A-Lyzer dialysis cassette (MWCO 10,000) and dialyzed against DI water to remove traces of ethanol. Specifically, the dialysis membrane was hydrated in DI water for 2 minutes prior to insertion of SLN sample. The dialysis cassette was stirred in DI water, which was exchanged every hour for 3 hours. Post-dialysis, the sample was removed from the dialysis cassette and used for further characterization. SLNs were prepared for each PA (C16-(PEG2)2-LDV, C16-(PEG2)4-LDV, C16-(PEG2)6-LDV) and all SLN compositions were prepared in triplicate.

3.3.3 Characterization of micelles, liposomes and solid lipid nanoparticles.

a) Determination of the critical micelle concentration (CMC). The CMC was determined using the pyrene fluorescence method. Stock solutions of PA were prepared in methanol at 2 mg/mL and calculated volumes of the stock solutions were added to glass scintillation vials. Next, a stock solution of pyrene in dichloromethane was prepared at 0.6µM and 50µl of this solution was added to each vial. The mixture was vortexed to ensure a homogenous solution of PAs and pyrene. Organic solvents were evaporated under a stream of nitrogen gas and left under a fume hood overnight to form a thin film of PA and pyrene. The dry films were then hydrated with DI water to achieve PA concentrations ranging
between 0.01µM to 100µM. Samples were equilibrated in a water bath at 37°C and shaken at 85 rpm. Following equilibration, the fluorescence spectra was recorded using a spectrofluorometer (Shimadzu Spectrofluorometer RF-5301 PC Shimadzu corporation, Pleasanton, USA). Samples were measured at an excitation wavelength of 337 nm and an emission wavelength range of 350 nm and 500 nm. The first (I1) 373 nm and third (I3) 383 nm vibrionic emission peak fluorescence intensities were recorded and the ratio of the third and first vibrionic emission peak fluorescence intensities was plotted against log concentration of PAs. This method was carried out for all three PAs (C16-(PEG2)2-LDV, C16-(PEG2)4-LDV, C16-(PEG2)6-LDV) and all experiments were performed in triplicate.

b) Size, PDI and charge characterization of micelles, liposomes and SLNs. The size, PDI and zeta potential of blank liposomes and blank SLNs were determined using DLS on the Zetasizer ZS 90 (Malvern Instruments, Ltd., Malvern, UK). For nanocarrier size and PDI, measurements were taken in a disposable microcuvette at 25°C. For zeta potential, samples were diluted in DI water and inserted into a disposable folded capillary zeta cell using a syringe. For each sample, measurements were taken three times using the auto run setting. The morphology and size of blank micelles was determined using TEM on a Philips CM120 BioTwin. Micelles were prepared from PAs as described above. A drop of each micelle solution was placed onto a formvar 200-mesh copper grid coated with carbon and left to dry in open air. Next, the sample was stained using a drop of 2% w/v phosphotungstic acid (pH 3) to enhance the contrast of the sample and excess solution was removed using filter paper. After air-drying, the copper grid was transferred onto the microscope sample holder for imaging.
3.4 Results and Discussion

The formation of micelles is a thermodynamic process and is driven by the increase in entropy when hydrophobic regions of the amphiphile are removed from water and aggregate to form a hydrophobic core. This hydrophobic effect decreases the ordered structure of the water molecules that surround the micelle structure, to attain a minimum free energy state. Micelles are formed at the CMC, which is characterized by an inflection point when physicochemical properties such as surface tension undergo sharp changes at a specific surfactant concentration. Factors that influence micellization include, the structure and length of the hydrophobic and hydrophilic regions, electrolyte addition and temperature. In general, an increase in length of the hydrocarbon chain results in an increase in micelle size and a decrease in the CMC. An increase in hydrophilic head group length (ethylene oxide chain length) renders micelles more hydrophilic with higher CMC values. In this study, the CMC was determined at 37°C in water using the pyrene fluorescence probe method and all three PAs formed micelles with a CMC ranging between 23.7µM to 38.8µM (table 3.1 and figures 3.4 to 3.6) which are typical values for low molecular weight surfactants. Micelles with the largest number of PEG2 linker units (C16-(PEG2)_6-LDV) had the largest CMC values. This can be explained by the fact that micelle formation is a result of a balance of intermolecular forces, where attractive forces occur through hydrophobic interactions between hydrophobic fatty acid chains and repulsive forces arise from steric or electrostatic interactions between the polar PEG2-LDV head groups [61, 62]. As the number of PEG2 units increases, the repulsive interactions are larger compared to the hydrophobic interactions and thus larger concentrations of PAs are needed to form micelles [63, 64]. In contrast, micelles with lower CMC values (C16-(PEG2)_2-LDV, C16-(PEG2)_4-LDV) are more resistant to dissociation upon dilution. The size of micelles was not determined by DLS and this is likely due to the dynamic nature of micelles and difficulty in determining micelle size in the
presence of two populations (micelle aggregates and monomers). Therefore, direct observation of micelles by TEM showed that all blank micelles were spherical in morphology and sizes ranged between 55 nm to 295 nm, where C16-(PEG2)$_6$-LDV micelles had the largest sizes (figure 3.8). This is likely due to the longer number of ethylene oxide units and subsequent increased repulsive forces resulting in a looser packing density, forming larger micelle structures [65]. Conversely, hydrophobic interactions of micelles with shorter hydrophilic head groups predominated, resulting in tighter packing of hydrophobic fatty acid chains, forming smaller micelles. The size of blank liposomes was in the range of 92.51 ± 0.29 nm to 102.73 ± 0.12 nm and the size of SLNs were smaller in the range of 78.46 ± 4.31 nm to 86.93 ± 3.49 nm (table 3.2). These results indicate that the sizes of blank micelles, liposomes and SLNs are in the ideal range for tumor targeting via the EPR effect. The PDI values for liposomes and SLNs were ≤2, which indicates a narrow size distribution. The magnitude of the zeta potential provides an indication of the colloidal stability. Specifically, zeta potential values more positive than +30 mV or more negative than -30 mV will repel each other decreasing the tendency to flocculate, indicating good colloidal stability. For liposomes, the zeta potential values were negative ranging between -34.47 ± 0.25 mV and -39.47 ± 1.33 mV. The zeta potential values for SLNs ranged between -45.46 ± 19.90 mV and -65.55 ± 8.36 mV. The negative zeta potential values can be attributed to the charged carboxylate group of aspartic acid in the LDV peptide region, indicating good colloidal stability.
Figure 3.4: Critical micelle concentration plot of I_3/I_1 ratio of pyrene v/s concentration of C16-(PEG2)_2-LDV peptide amphiphiles (n=3)
Figure 3.5: Critical micelle concentration plot of \( \frac{I_3}{I_1} \) ratio of pyrene v/s concentration of C16-(PEG2)_4-LDV peptide amphiphiles (n=3)
Figure 3.6: Critical micelle concentration plot of $I_3/I_1$ ratio of pyrene v/s concentration of C16-(PEG2)$_6$-LDV peptide amphiphiles (n=3)
Figure 3.7: Fluorescence spectra of pyrene for LDV-targeted micelles loaded with pyrene
Table 3.1: Critical micelle concentration values for LDV-targeted micelles (n=3)

<table>
<thead>
<tr>
<th>Peptide Amphiphile</th>
<th>CMC (μM)</th>
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<tbody>
<tr>
<td>C16-(PEG2)$_2$-LDV</td>
<td>28.31 ± 3.85</td>
</tr>
<tr>
<td>C16-(PEG2)$_4$-LDV</td>
<td>23.68 ± 0.72</td>
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<tr>
<td>C16-(PEG2)$_6$-LDV</td>
<td>38.76 ± 2.27</td>
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</table>
Figure 3.8: Transmission Electron Microscope images of LDV-targeted blank micelles
Table 3.2: Comparison of size, zeta potential and PDI of LDV-targeted blank micelles, blank liposomes and blank solid lipid nanoparticles (n=3)

<table>
<thead>
<tr>
<th>Nanocarrier</th>
<th>Size (nm)</th>
<th>Zeta potential (mV)</th>
<th>PDI</th>
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<tr>
<td>C16-(PEG2)$_2$-LDV micelle</td>
<td>54 ± 4.90</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>C16-(PEG2)$_4$-LDV micelle</td>
<td>55 ± 10.0</td>
<td>ND</td>
<td>ND</td>
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<td>C16-(PEG2)$_6$-LDV micelle</td>
<td>295 ± 65.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C16-(PEG2)$_2$-LDV liposome</td>
<td>97.32 ± 0.45</td>
<td>-39.47 ± 1.33</td>
<td>0.200</td>
</tr>
<tr>
<td>C16-(PEG2)$_4$-LDV liposome</td>
<td>92.51 ± 0.29</td>
<td>-34.47 ± 0.25</td>
<td>0.174</td>
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<tr>
<td>C16-(PEG2)$_6$-LDV liposome</td>
<td>102.73 ± 0.12</td>
<td>-35.33 ± 0.10</td>
<td>0.137</td>
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<tr>
<td>C16-(PEG2)$_2$-LDV SLN</td>
<td>78.46 ± 4.31</td>
<td>-45.46 ± 19.90</td>
<td>0.209 ± 0.004</td>
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<tr>
<td>C16-(PEG2)$_4$-LDV SLN</td>
<td>87.07 ± 1.79</td>
<td>-65.55 ± 8.363</td>
<td>0.207 ± 0.002</td>
</tr>
<tr>
<td>C16-(PEG2)$_6$-LDV SLN</td>
<td>86.93 ± 3.49</td>
<td>-61.92 ± 3.078</td>
<td>0.186 ± 0.010</td>
</tr>
</tbody>
</table>
Chapter 4: In Vitro Stability and Drug Release Characterization

4.1 Stability of LDV-targeted Micelles, Liposomes and SLNs by FRET

4.1.1 Introduction. One of the major challenges with the use of nanocarriers, is their instability when administered systemically. For example, micelles are known to disassemble when diluted below the CMC upon systemic administration. Additionally, nanocarriers can destabilize and disrupt due to interactions with blood components, causing premature drug release and reduced anti-cancer efficacy. Nanocarriers can be loaded with fluorescent dyes that are used to probe the stability upon dilution and under physiological conditions. For example, Forster Resonance Energy Transfer (FRET) is a distance-dependent process where energy is transferred from an excited fluorophore (donor dye) to another fluorophore (acceptor dye) and has been used to probe the stability of various nanocarriers including polymeric micelles and liposomes [66, 67]. Specifically, donor and acceptor FRET dyes (DiO and Dil) can be loaded into the hydrophobic core of micelles, the bilayer of liposomes and the solid lipid core of SLNs. Upon excitation of the donor dye, energy is transferred to the acceptor dye and energy is released at the emission wavelength of the acceptor molecules, due to the close proximity of the dyes within the hydrophobic regions of the nanocarriers. Typically, energy transfer can only occur if the dyes are within 10 angstroms of each other. When nanocarriers are disrupted, the dyes are released and the larger distance between them results in a loss of energy transfer between donor and acceptor dyes. High FRET efficiencies indicate the presence of physically stable nanocarriers and low FRET efficiencies indicate poor physical stability of nanocarriers.
Figure 4.1: Schematic representation of intact and disrupted FRET dye-loaded micelles

Figure 4.2: Schematic representation of intact and disrupted FRET dye-loaded liposomes
Figure 4.3: Schematic representation of intact and disrupted FRET dye-loaded solid lipid nanoparticles
4.1.2 Materials. PAs were synthesized as described in the section 2. (CA, USA). DPPC lipids and liposome extruder was purchased from Avanti polar lipids (AL, USA). Tripalmitin was purchased from Sigma-Aldrich (MO, USA). FRET dyes 3,3’-dioctadecyloxacarbocyanine perchlorate (DiO) and 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (Dil) were purchased from Invitrogen (Carlsbad CA, USA). Dialysis membranes MWCO 1000 Da were purchased from Spectrum Laboratories (Rancho Dominguez, CA, USA). Solvents including DCM, methanol, ethanol, N,N’-dimethyl formamide (DMF) and chloroform were purchased from fisherscientific (Pittsburg, PA).

4.1.3 Methods. FRET dyes DiO and Dil were loaded into micelles, liposomes and SLNs using the same methods for preparation of blank nanocarriers. Table 4.1 summarizes the methods, compositions, lipid concentrations and drug loading of FRET dye-loaded nanocarriers.

a) Preparation of FRET dye-loaded micelles. FRET pair dyes DiO and Dil were loaded into micelles through the dialysis membrane technique. PAs were mixed together with FRET dyes in DMF and left shaking in a water-bath at 50⁰C for approximately 1 hour. The mixture was then transferred into dialysis membrane tubing (MWCO 1000) and dialyzed against 1 liter of water, which was exchanged three times over 24 hours. After dialysis, micelle samples were removed by transferring into glass vials and covering with aluminum foil prior to characterization. Micelles were prepared from all three PAs and each micelle composition was prepared in triplicates.

b) Preparation of FRET dye-loaded liposomes. Liposomes were prepared using the thin-film hydration and extrusion method. A mixture of DPPC, PA and FRET dyes were dissolved in chloroform: methanol = 2:1 (v/v) at a molar ratio of 1:0.5:0.02. Organic solvents were evaporated to dryness under a stream of nitrogen gas and solvent traces were removed
by leaving the glass tubes under vacuum overnight. The dried lipid film was then hydrated by the addition of aqueous solution at a temperature between 50°C to 55°C, followed by vortexing periodically for 30 minutes to detach any lipid from the glass surface. Hydration was carried out in a water bath and the temperature was maintained above the main phase transition temperature ($T_m$) of lipids (41°C for DPPC). This process resulted in the formation of MLVs, which were subjected to six freeze-thaw cycles with liquid nitrogen and heated DI water. MLVs were downsized by extruding 21 times through 0.1µm-pore polycarbonate membrane filters. Liposomes were prepared using all three PAs and each liposome composition was prepared in triplicates. Following preparation, liposome suspensions were protected with aluminum foil prior to characterization.

**c) Preparation of FRET dye-loaded solid lipid nanoparticles.** The NanoAssembler (Benchtop, Precision NanoSystems Inc., Vancouver, Canada) was used to prepare SLNs by microfluidic mixing. Specifically, a microfluidic cartridge comprising of moulded channels with staggered herringbone structures was used to formulate SLNs by mixing of the organic (tripalmitin and FRET dyes in ethanol) and aqueous (PA in water) phases at a molar ratio of 1:0.5:0.02. The organic and aqueous phases were pumped into two inlets of the microfluidic chip using disposable syringes and mixing was controlled by setting the FRR to 3:1 and the TFR to 15mL/min. At all steps of preparation, the organic and aqueous phases were kept at 75°C by keeping solutions in the oven and installing a heat block into the NanoAssembler instrument. Next, the mixture was collected from the NanoAssembler and transferred into G2 slide-A-Lyzer dialysis cassettes (MWCO 10,000) and dialyzed against DI water to remove traces of ethanol. Specifically, the dialysis membrane was hydrated in DI water for 2 minutes prior to dialysis. Then the dialysis cassette was stirred in DI water, which was exchanged every hour for 3 hours. Post-dialysis, the sample was removed from the dialysis cassette and
used for further characterization. SLNs were prepared using all three PAs and all SLN compositions were prepared in triplicate.

**d) Stability characterization.** The dilution stability of micelles, liposomes and SLNs was determined by diluting 10-fold in aqueous solution or methanol (control) and recording the fluorescence spectra (excitation 484 nm, emission 495 nm to 600 nm) using a fluorescence spectrophotometer (Shimadzu Spectrofluorometer for micelles and liposomes and a SpectraMax M2 microplate reader for SLNs). FRET ratios were calculated using fluorescence intensity values of dyes: \( \frac{I_{DIL}}{I_{DIL}+I_{DIO}} \), where lower ratios represent increased micelle, liposome or SLN disassembly. For stability in serum, micelles, liposomes and SLNs were diluted 10-fold in human serum or in 0.1% triton X-100 (control) and the FRET ratios were calculated using the same method. The results were plotted using GraphPad Prism software and a non-linear fit of FRET ratio’s was generated by least squares and the rate constants (K) were determined. The rate constants were statistically compared using one-way ANOVA followed by Tukey post-test. A p value <0.05 was considered statistically significant.
Table 4.1: Preparation micelles, liposomes and solid lipid nanoparticles loaded with FRET dyes

<table>
<thead>
<tr>
<th></th>
<th>Liposomes</th>
<th>Micelles</th>
<th>Solid lipid nanoparticles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Composition (Molar ratio)</td>
<td>DPPC: C16-(PEG2)₆-LDV 1:0.05</td>
<td>C16-(PEG2)₆-LDV</td>
<td>Tripalmitin:C16-(PEG2)₆-LDV 1:0.05</td>
</tr>
<tr>
<td>Hydrophobic fluorescent dyes</td>
<td>FRET dyes DiO + Dil</td>
<td>FRET dyes DiO + Dil</td>
<td>FRET dyes DiO + Dil</td>
</tr>
<tr>
<td>Method of preparation</td>
<td>Thin-film hydration Freeze-thaw Extrusion</td>
<td>Dialysis Bag</td>
<td>Microfluidics</td>
</tr>
<tr>
<td>Lipid Concentration (mg/mL)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Loading of each dye (wt %)</td>
<td>2.2</td>
<td>2.2</td>
<td>2.2</td>
</tr>
</tbody>
</table>
4.1.4 Results and discussion. The effectiveness of nanocarriers for tumor targeting is dependent on the ability to remain intact before reaching the target tumor site. Therefore, nanocarriers must remain intact and circulate in the blood for a sufficient amount of time to allow accumulation at the tumor site. Specifically, premature drug release can occur when micelles are administered intravenously and diluted below the CMC. Furthermore, nanocarriers are prone to protein adsorption, leading to opsonization and phagocytosis by macrophages of the RES in the blood circulation or in the liver and spleen. Serum proteins can also partition into the nanocarriers, causing disruption and premature drug release. Therefore, for effective drug targeting and delivery to occur, nanocarriers must be able to withstand the effects of dilution and remain stable in the presence of serum. FRET dyes were loaded into micelles, liposomes and SLNs and these nanocarriers were characterized for their ability to remain intact upon dilution and in the presence of serum. When micelles, liposomes and SLNs were diluted in excess aqueous solution, the fluorescence spectrum remained unchanged, showing a major emission peak at 570 nm upon excitation at the appropriate wavelength (figures 4.4 to figure 4.24). This indicates energy transfer due to the close proximity of dyes in the bilayer of liposomes and in the core of micelles and SLNs. For all nanocarriers except C16-(PEG2)_2-LDV micelles, FRET efficiencies remained high (≥0.85), indicating good stability upon dilution (table 4.2 and table 4.3). When micelles, liposomes and SLNs were disrupted in methanol, FRET dyes were released and diffused apart, resulting in loss of energy transfer. As a result, a shift in the emission peak from 570 nm to 505 nm was observed. When micelles, liposomes and SLNs were diluted in human serum, an increase in the fluorescence intensity at 505 nm and a decrease at 570 nm was observed over 8 hours, indicating nanocarrier disassembly or FRET dye molecule release over time (figure 4.25 to figure 4.45). The rate of nanocarrier disassembly was the fastest for
micelles, followed by liposomes and SLNs, with rate constant values of $2.77 \pm 0.39$ hr$^{-1}$, $0.61 \pm 0.09$ hr$^{-1}$ and $0.20 \pm 0.05$ hr$^{-1}$ respectively (table 4.5). Upon statistical analysis of rate constants, there were significant differences between nanocarriers ($p<0.0001$). The Tukey test revealed a significant difference between the rate constants of micelles vs liposomes ($p<0.0001$) and micelles vs SLNs ($p<0.0001$). However, there was no significant difference between the rate constants of liposomes and SLNs ($p=0.16$). For micelles in serum, the FRET efficiency decreased from 1 to 0.51 over 1 hour. At 3 hours, the FRET ratio ($\approx 0.42$) reached a plateau, indicating complete micelle disassembly or FRET molecule release at this time. For liposomes in serum, the decrease in FRET efficiency over 1 hour was less compared to micelles (1 to 0.74) and the FRET ratio plateaued at 5 hours ($\approx 0.45$). Compared to micelles and liposomes, SLNs were the most stable in serum with only a small decrease in the FRET ratio from 1 to 0.88 over 1 hour and a steady decrease to $\approx 0.63$ over 8 hours. When micelles, liposomes and SLNs were disrupted in triton X-100, a shift in the emission peak from 570 nm to 505 nm was observed, indicating complete disassembly or FRET molecule release. Overall, the results demonstrate that micelles were more prone to destabilization in the presence of serum compared to liposomes and SLNs. Micelles formed from low molecular weight amphiphiles are dynamic structures and the continuous exchange of amphiphiles between micelles contributes to poor stability. However, compared to micelles, the phospholipid bilayer of liposomes imparts increased rigidity and improved mechanical properties, leading to increased retention of FRET dye molecules and increased resistance to disruption by serum proteins. SLNs demonstrated higher stability over micelles and liposomes, which is likely due to rigid solid nature of the lipid core, which affords increased FRET dye retention and resistance to disruption by serum proteins.
Figure 4.4: Normalized FRET efficiency of LDV-targeted micelles when diluted 10-fold in aqueous solution (n=3)
Figure 4.5: Time resolved emission spectra of Dil + DiO loaded C16-(PEG2)_2-LDV micelles diluted 10-fold in aqueous solution.

Figure 4.6: Emission spectra of Dil + DiO C16-(PEG2)_2-LDV micelles diluted 10-fold in methanol.
Figure 4.7: Time resolved emission spectra of Dil + DiO loaded C16-(PEG2)$_4$-LDV micelles diluted 10-fold in aqueous solution.

Figure 4.8: Emission spectra of Dil + DiO C16-(PEG2)$_4$-LDV micelles diluted 10-fold in methanol.
Figure 4.9: Time resolved emission spectra of Dil + DiO loaded C16-(PEG2)_6-LDV micelles diluted 10-fold in aqueous solution

Figure 4.10: Emission spectra of Dil + DiO C16-(PEG2)_6-LDV micelles diluted 10-fold in methanol
Figure 4.11: Normalized FRET efficiency of LDV-targeted liposomes when diluted 10-fold in aqueous solution (n=3)
Figure 4.12: Time resolved emission spectra of Dil + DiO loaded C16-(PEG2)$_2$-LDV liposomes diluted 10-fold in aqueous solution

Figure 4.13: Emission spectra of Dil + DiO C16-(PEG2)$_2$-LDV liposomes diluted 10-fold in methanol
Figure 4.14: Time resolved emission spectra of Dil + DiO loaded C16-(PEG2)$_4$-LDV liposomes diluted 10-fold in aqueous solution

Figure 4.15: Emission spectra of Dil + DiO C16-(PEG2)$_4$-LDV liposomes diluted 10-fold in methanol
Figure 4.16: Time resolved emission spectra of Dil + DiO loaded C16-(PEG2)_6-LDV liposomes diluted 10-fold in aqueous solution.

Figure 4.17: Emission spectra of Dil + DiO C16-(PEG2)_6-LDV liposomes diluted 10-fold in methanol.
Figure 4.18: Normalized FRET efficiency of LDV-targeted solid lipid nanoparticles when diluted 10-fold in aqueous solution (n=3)
Figure 4.19: Time resolved emission spectra of Dil + DiO loaded C16-(PEG2)2-LDV solid lipid nanoparticles diluted 10-fold in aqueous solution

Figure 4.20: Emission spectra of Dil + DiO C16-(PEG2)2-LDV solid lipid nanoparticles diluted 10-fold in methanol
Figure 4.21: Time resolved emission spectra of Dil + DiO loaded C16-(PEG2)$_{4}$-LDV solid lipid nanoparticles diluted 10-fold in aqueous solution

Figure 4.22: Emission spectra of Dil + DiO C16-(PEG2)$_{4}$-LDV solid lipid nanoparticles diluted 10-fold in methanol
Figure 4.23: Time resolved emission spectra of Dil + DiO loaded C16-(PEG2)$_6$-LDV solid lipid nanoparticles diluted 10-fold in aqueous solution

Figure 4.24: Emission spectra of Dil + DiO C16-(PEG2)$_6$-LDV solid lipid nanoparticles diluted 10-fold in methanol
Table 4.2: Normalized FRET efficiencies of DiO + Dil dye-loaded micelles, liposomes and solid lipid nanoparticles when diluted 10-fold in aqueous solution (n=3)

<table>
<thead>
<tr>
<th>Nanocarrier</th>
<th>Average Normalized FRET efficiency at 1 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16-(PEG2)₂-LDV micelles</td>
<td>0.68 ± 0.01</td>
</tr>
<tr>
<td>C16-(PEG2)₄-LDV micelles</td>
<td>0.88 ± 0.02</td>
</tr>
<tr>
<td>C16-(PEG2)₆-LDV micelles</td>
<td>0.87 ± 0.02</td>
</tr>
<tr>
<td>C16-(PEG2)₂-LDV liposomes</td>
<td>0.85 ± 0.01</td>
</tr>
<tr>
<td>C16-(PEG2)₄-LDV liposomes</td>
<td>0.86 ± 0.00</td>
</tr>
<tr>
<td>C16-(PEG2)₆-LDV liposomes</td>
<td>0.85 ± 0.01</td>
</tr>
<tr>
<td>C16-(PEG2)₂-LDV SLNs</td>
<td>0.95 ± 0.01</td>
</tr>
<tr>
<td>C16-(PEG2)₄-LDV SLNs</td>
<td>0.99 ± 0.00</td>
</tr>
<tr>
<td>C16-(PEG2)₆-LDV SLNs</td>
<td>0.97 ± 0.00</td>
</tr>
</tbody>
</table>
Table 4.3: Average normalized FRET efficiencies of DiO + Dil FRET dye-loaded micelles, liposomes and solid lipid nanoparticles when diluted 10-fold in aqueous solution (n=3)

<table>
<thead>
<tr>
<th>Nanocarrier</th>
<th>Average Normalized FRET efficiency at 1 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16-(PEG2)n-LDV micelles</td>
<td>0.81 ± 0.11</td>
</tr>
<tr>
<td>C16-(PEG2)n-LDV liposomes</td>
<td>0.85 ± 0.01</td>
</tr>
<tr>
<td>C16-(PEG2)n-LDV SLNs</td>
<td>0.97 ± 0.02</td>
</tr>
</tbody>
</table>
Figure 4.25: Normalized FRET efficiency of LDV-targeted micelles when diluted 10-fold in human serum (n=3)
Figure 4.26: Time resolved emission spectra of Dil + DiO loaded C16-(PEG2)$_2$-LDV micelles diluted 10-fold in human serum

Figure 4.27. Emission spectra of Dil + DiO C16-(PEG2)$_2$-LDV micelles diluted 10-fold in triton X-100
Figure 4.28: Time resolved emission spectra of Dil + DiO loaded C16-(PEG2)$_4$-LDV micelles diluted 10-fold in human serum

Figure 4.29: Emission spectra of Dil + DiO C16-(PEG2)$_4$-LDV micelles diluted 10-fold in triton X-100
Figure 4.30: Time resolved emission spectra of Dil + DiO loaded C16-(PEG2)$_6$-LDV micelles diluted 10-fold in human serum

Figure 4.31: Emission spectra of Dil + DiO C16-(PEG2)$_6$-LDV micelles diluted 10-fold in triton X-100
Figure 4.32: Normalized FRET efficiency of LDV-targeted liposomes when diluted 10-fold in human serum (n=3)
Figure 4.33: Time resolved emission spectra of Dil + DiO loaded C16-(PEG2)$_2$-LDV liposomes diluted 10-fold in human serum

Figure 4.34: Emission spectra of Dil + DiO C16-(PEG2)$_2$-LDV liposomes diluted 10-fold in triton X-100
Figure 4.35. Time resolved emission spectra of Dil + DiO loaded C16-(PEG2)$_4$-LDV liposomes diluted 10-fold in human serum

Figure 4.36: Emission spectra of Dil + DiO C16-(PEG2)$_4$-LDV liposomes diluted 10-fold in triton X-100
Figure 4.37: Time resolved emission spectra of Dil + DiO loaded C16-(PEG2)$_6$-LDV liposomes diluted 10-fold in human serum

Figure 4.38: Emission spectra of Dil + DiO C16-(PEG2)$_6$-LDV liposomes diluted 10-fold in triton X-100
Figure 4.39: Normalized FRET efficiency of LDV-targeted solid lipid nanoparticles when diluted 10-fold in human serum (n=3)
Figure 4.40: Time resolved emission spectra of Dil + DiO loaded C16-(PEG2)₂-LDV solid lipid nanoparticles diluted 10-fold in human serum

Figure 4.41: Emission spectra of Dil + DiO C16-(PEG2)₂-LDV solid lipid nanoparticles diluted 10-fold in triton X-100
Figure 4.42: Time resolved emission spectra of Dil + DiO loaded C16-(PEG2)$_4$-LDV solid lipid nanoparticles diluted 10-fold in human serum

Figure 4.43: Emission spectra of Dil + DiO C16-(PEG2)$_4$-LDV solid lipid nanoparticles diluted 10-fold in triton X-100
Figure 4.44: Time resolved emission spectra of Dil + DiO loaded C16-(PEG2)_6-LDV solid lipid nanoparticles diluted 10-fold in human serum

Figure 4.45: Emission spectra of Dil + DiO C16-(PEG2)_6-LDV solid lipid nanoparticles diluted 10-fold in triton X-100
Table 4.4: Normalized FRET efficiencies of all DiO + Dil FRET dye-loaded micelles, liposomes and solid lipid nanoparticles in human serum (n=3)

<table>
<thead>
<tr>
<th>Nanocarrier</th>
<th>Normalized FRET efficiency n serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16-(PEG2)$_2$-LDV micelles</td>
<td>0.51 ± 0.01</td>
</tr>
<tr>
<td>C16-(PEG2)$_4$-LDV micelles</td>
<td>0.47 ± 0.01</td>
</tr>
<tr>
<td>C16-(PEG2)$_6$-LDV micelles</td>
<td>0.54 ± 0.02</td>
</tr>
<tr>
<td>C16-(PEG2)$_2$-LDV liposomes</td>
<td>0.79 ± 0.01</td>
</tr>
<tr>
<td>C16-(PEG2)$_4$-LDV liposomes</td>
<td>0.69 ± 0.02</td>
</tr>
<tr>
<td>C16-(PEG2)$_6$-LDV liposomes</td>
<td>0.75 ± 0.01</td>
</tr>
<tr>
<td>C16-(PEG2)$_2$-LDV SLNs</td>
<td>0.89 ± 0.03</td>
</tr>
<tr>
<td>C16-(PEG2)$_4$-LDV SLNs</td>
<td>0.90 ± 0.01</td>
</tr>
<tr>
<td>C16-(PEG2)$_6$-LDV SLNs</td>
<td>0.86 ± 0.03</td>
</tr>
</tbody>
</table>
Table 4.5: Average normalized FRET efficiencies and rate constants of DiO + Dil FRET dye-loaded micelles, liposomes and solid lipid nanoparticles (n=3)

<table>
<thead>
<tr>
<th>Nanocarrier</th>
<th>Normalized FRET efficiency in serum at 1 hour</th>
<th>Rate constant K (1/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16-(PEG2)$_n$-LDV micelles</td>
<td>0.51 ± 0.04</td>
<td>2.77 ± 0.39</td>
</tr>
<tr>
<td>C16-(PEG2)$_n$-LDV liposomes</td>
<td>0.74 ± 0.05</td>
<td>0.61 ± 0.09</td>
</tr>
<tr>
<td>C16-(PEG2)$_n$-LDV SLNs</td>
<td>0.88 ± 0.02</td>
<td>0.20 ± 0.05</td>
</tr>
</tbody>
</table>
4.2 In Vitro Release of Paclitaxel-Loaded Targeted Micelles, Liposomes and SLNs

4.2.1 Introduction. Paclitaxel was loaded into micelles, liposomes and SLNs and characterized for size, morphology, charge, drug loading efficiency and drug release. Paclitaxel is a natural anti-cancer agent extracted from taxus with broad spectrum anti-tumor effects. Paclitaxel is a widely used therapeutic agent that works through cell tubulin polymerization and inhibition of depolymerization, preventing the formation of normal mitotic apparatus. However, there are significant challenges associated with the formulation and administration of paclitaxel due to poor aqueous solubility ($\leq 0.3\mu g/mL$), requiring the use of additional solvents and excipients for effective administration. For example, Taxol is a commercialized FDA approved product comprising of paclitaxel dissolved in polyethoxylated castor oil (Cremophor EL) and dehydrated ethanol, which is then diluted in 0.9% normal saline prior to intravenous administration. Unfortunately, Taxol has several limitations, including precipitation upon dilution and severe side effects due to cremophor EL, resulting in reduced therapeutic efficacy. To eliminate such side effects and improve drug solubility, paclitaxel can be loaded into micelles, liposomes and SLNs.

4.2.2 Materials. PAs were synthesized as described in section 2. (CA, USA) and GenScript (Piscataway, NJ). DPPC lipids and liposome extruder was purchased from Avanti polar lipids (AL, USA). Solvents including DCM, methanol, ethanol and chloroform were purchased from fisherscientific (Pittsburg, PA). Paclitaxel was purchased from LC laboratories. Tripalmitin was purchased from Sigma-Aldrich (MO, USA). Float-A-Lyzer G2 dialysis tubes (MWCO 10,000) were purchased from Spectrum Laboratories (Rancho Dominguez, CA). Pierce Slide-A-Lyzer G2 dialysis cassettes (MWCO 10,000) were purchased from VWR (CA, USA).
4.2.3 Methods.

\textit{a)} Preparation of paclitaxel-loaded micelles, liposomes and SLNs. Paclitaxel-loaded nanocarriers (PTX-liposomes, PTX-micelles and PTX-SLN) were prepared using similar methods as for blank nanocarriers (table 4.6). For PTX-liposomes, the hydrophobic components, DPPC, PA and paclitaxel were dissolved in chloroform: methanol = 2:1 (v/v). Organic solvents were evaporated to dryness under a stream of nitrogen gas and solvent traces were removed by leaving the glass tubes under vacuum overnight. The dried lipid film was then hydrated by the addition of DI water at a temperature between 50°C to 55°C, followed by vortexing periodically for 30 minutes to detach any lipid from the glass surface. Hydration was carried out in a water bath and the temperature was maintained above the main phase transition temperature (T\textsubscript{m}) of lipids (41°C for DPPC). This process resulted in the formation of MLVs, which were subjected to six freeze-thaw cycles with liquid nitrogen and heated DI water. MLVs were downsized by extruding 21 times each through 0.4µm, 0.2µm, and 0.1µm-pore polycarbonate filters to produce SUVs. Paclitaxel was loaded into micelles using the solvent evaporation method. In a glass tube, PAs and paclitaxel were dissolved in methanol. Then methanol was evaporated under nitrogen gas and solvent traces were removed under vacuum overnight. Next, the dry lipid film was hydrated by the addition of DI water at a temperature between 50°C to 55°C, followed by vortexing and sonicating periodically for 30 minutes to detach any lipid from the glass surface. The resulting mixture was then centrifuged at 3000 rpm for 1 hour to remove excess paclitaxel and yield a clear micelle solution. The NanoAssmblr (Benchtop, Precision NanoSystems Inc., Vancouver, Canada) was used to prepare PTX-SLN by microfluidic mixing. Specifically, a microfluidic cartridge comprising of moulded channels with staggered herringbone structures was used to formulate PTX-SLN by mixing of the organic (tripalmitin and paclitaxel in ethanol) and aqueous (PA in water) phases. The organic and aqueous phases were pumped into two inlets.
of the microfluidic chip using disposable syringes and mixing was controlled by setting the 
FRR to 3:1 and the TFR to 15 mL/min. At all steps of preparation, the organic and aqueous 
phases were kept at 75°C by keeping solutions in the oven and installing a heat block into the 
NanoAssemblr instrument. Next, the mixture was collected from the NanoAssemblr and 
transferred into G2 slide-A-Lyzer dialysis cassettes (MWCO 10,000) and dialyzed against 
water to remove traces of ethanol. Specifically, the dialysis membrane was hydrated in DI water for 2 minutes prior to insertion of SLN sample. Then the dialysis cassette was stirred 
in DI water, which was exchanged every hour for 3 hours. PTX micelles, PTX-liposomes 
and PTX SLNs were prepared using all three PAs and each composition was prepared in 
triplicates.

b) Paclitaxel loading efficiency for micelles, liposomes and SLNs. Samples were 
prepared by diluting nanocarriers 10-fold in methanol and vortexing to ensure complete 
solubilization of lipids and paclitaxel. Paclitaxel concentration was determined using 
isocratic RP-HPLC (HPLC Agilent 1200 series, Agilent Technologies Inc.) using a C18 
column (Agilent 4.6 mm x 250, 5µm) at 25°C. The mobile phase consisted of 
acetonitrile/water (70/30, v/v) at a flow rate of 1.0 mL/min. Paclitaxel was detected with 
ultraviolet (UV) detection at 227 nm using 20µl injection volumes. A calibration curve was 
generated by plotting known concentrations of paclitaxel against peak area using a series of 
standards ranging between 1µg/ml to 100 µg/ml. The standard curve was used to determine 
the concentration of paclitaxel in PTX-micelles, PTX-liposomes and PTX-SLNs. Drug 
Loading Content (DLC) and Drug Loading Efficiency (DLE) was determined using equations 
4.1 and 4.2:

Equation 4.1  \[ DL(\%) = \frac{\text{Amount of PTX in nanocarrier}}{\text{Amount of PTX loaded nanocarriers}} \times 100 \]

Equation 4.2  \[ DLE(\%) = \frac{\text{Amount of PTX in nanocarrier}}{\text{Amount of PTX used for the preparation nanocarriers}} \times 100 \]
Table 4.6: Preparation of paclitaxel-loaded micelles, liposomes and solid lipid nanoparticles

<table>
<thead>
<tr>
<th></th>
<th>PTX-Liposomes</th>
<th>PTX-Micelles</th>
<th>PTX-Solid Lipid Nanoparticle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Composition (Molar ratio)</td>
<td>DPPC: C16-(PEG2)ₙ-LDV (1:0.05)</td>
<td>C16-(PEG2)ₙ-LDV (1:0.05)</td>
<td>Tripalmitin: C16-(PEG2)ₙ-LDV (1:0.05)</td>
</tr>
<tr>
<td>Paclitaxel loading (wt%)</td>
<td>1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Method of preparation</td>
<td>Thin-film hydration</td>
<td>Solvent evaporation</td>
<td>Microfluidics</td>
</tr>
<tr>
<td></td>
<td>Freeze-thaw</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Extrusion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydration medium</td>
<td>water</td>
<td>water</td>
<td>water</td>
</tr>
<tr>
<td>Final lipid Concentration (mg/mL)</td>
<td>15</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
c) *Determination of size, PDI, zeta potential and morphology.* The size, PDI and zeta potential of PTX-liposomes and PTX-SLNs were determined using DLS on the Zetasizer ZS 90 (Malvern Instruments, Ltd., Malvern, UK). For nanocarrier size, PDI and zeta potential, measurements were taken in a disposable microcuvette at 25°C. For each sample, measurements were taken three times. The morphology and size of PTX-micelles, PTX-liposomes and PTX-SLNs was determined by Nanoimaging Services Inc (San Diego, CA) using a FEI Tecnai T12 electron microscope (serial number D1100), operating at 120keV equipped with a FEI Eagle 4k x 4k CCD camera. For Electron Microscope (EM) grid preparation, 3µl of each nanocarrier sample was applied to a cleaned 400-mesh copper grid and blotted away with filter paper. Next, vitrification was carried out in liquid ethane and these vitreous ice grids were transferred into the EM using a cryostage that maintains the grids at a temperature below -170°C. Images of each grid were acquired at multiple scales to assess the overall distribution of the specimen. After identifying potentially suitable target areas for imaging at lower magnifications, high magnification images were acquired at nominal magnifications of 110,000x (0.10 nm/pixel), 52,000x (0.21 nm/pixel) and 21,000x (0.50 nm/pixel). The images were acquired at a nominal underfocus of -5.0µm to -1.0µm and electron doses of ~10-25 e/Å².

d) *Determination of in vitro drug release.* Dialysis tubes (Float-A-Lyzer G2, MWCO 10,000) were used to determine in vitro release profiles of PTX-micelles, PTX-liposomes and PTX-SLNs. Dialysis devices containing 1mL of nanocarrier sample was placed into 4 liters of DI water at 37°C and magnetically stirred. At predetermined time points up to 24 hours, aliquots (20 µl) of nanocarrier sample were removed from the dialysis tube and paclitaxel content was analyzed using HPLC as described above. The release profiles were analyzed using non-linear regression analysis performed by GraphPad Prism software. The release rate constants and time to release 50% of drug (t50%) from micelles,
liposomes and SLNs were determined and statistically compared using one-way ANOVA followed by the Tukey multiple comparison test. A P-value of <0.05 was considered statistically significant.

4.2.4 Results and discussion.

a) Physicochemical characterization of paclitaxel-loaded nanocarriers. The physicochemical characterization and drug loading of PTX-micelles, PTX-liposomes and PTX-SLNs such as hydrodynamic size, zeta potential and DLC are summarized in tables 4.7 and 4.8. DLS results showed that there was no significant difference between the hydrodynamic sizes of all PTX-liposomes and these ranged between 117.27 ± 8.08 nm and 129.00 ± 8.32 nm. The hydrodynamic sizes of all PTX-SLNs was also similar and ranged between 74.40 ± 4.39 nm and 84.4 ± 2.77 nm. The hydrodynamic sizes of micelles could not be determined by DLS, therefore cryoTEM was used to determine the size and morphology of PTX-micelles (figure 4.46). For these micelles, the images showed roughly round particles between 6 nm to 12 nm, which are significantly smaller than the sizes of blank micelles characterized by TEM. This effect of size reduction of drug-loaded micelles compared to blank micelles has previously been observed and may be explained by the intermolecular interactions between paclitaxel and the core of the micelle [68, 69]. Paclitaxel is physically incorporated in the core of micelles, enhancing the hydrophobic interactions resulting in the formation of smaller particles. CryoTEM imaging of PTX-liposomes revealed unilamellar particles with rounded to angular appearance, ranging between 48-145 nm in their longest dimension. PTX-liposomes had an interior density that was similar to the density of the surrounding aqueous medium, indicating the presence of a hydrophilic core that is commonly found in liposomes. The outer layer of the particles resembled a lipid bilayer which has a thickness of 6 to 7.5 nm in width. The sample also contained many short narrow particles ranging between 20 to 40 nm in length and 5 to 7 nm in width as well as
some round to oblong particles that were 20 to 40 nm in their longest dimension, which are likely different views of lipid-based, disk-shaped particles. PTX-SLN were also imaged and revealed particles ranging from 10 to 120 nm that were mostly round to oblong and occasionally angular in appearance. Particles had an interior density that was slightly denser than the surrounding aqueous solution, indicating the presence of a lipid core. The sample also contained a few short narrow particles with a length of 12 to 30 nm and a width of 6 to 14 nm. In general, DLS results showed that sizes of PTX-SLN were smaller than PTX-liposomes (80.53 ± 5.37 nm vs 123.31 ± 5.87 nm) and PDI values were ≤0.2. CryoTEM results confirmed the smaller size range of PTX-SLN (10-120 nm) compared to PTX-liposomes (48-145 nm) and PTX-micelles had the smallest sizes between 6 to 12 nm. Although the smaller sizes of PTX-micelles may increase uptake and tumor penetration, the size of these micelles fall outside the ideal nanocarrier size range (10-100 nm), where sizes smaller than 10 nm may be easily excreted by the kidneys. PTX-liposomes and PTX-SLN showed ideal size ranges. Zeta potential values ranged between -23.61 ± 10.23 mV and -51.65 ± 10.82 mV for PTX-micelles, PTX-liposomes and PTX-SLN, which is attributed to the carboxylate group present in the peptide. The highly negative charge of these nanocarriers provides good colloidal stability and may help to prevent interactions with opsonins [70, 71]. However, negatively charged particles may prevent efficient cellular uptake due to the negatively charged cellular membrane[72, 73].

b) Paclitaxel loading efficiency of micelles, liposomes and SLNs. Drug loading into micelles depend upon the partitioning of lipophilic drug molecules into the micelle core and the extent to which this occurs can be described by the partition coefficient. In particular an increase in the length of the hydrophobic region will improve the partitioning behavior of hydrophobic drugs into the micelle core. There are several factors that affect the drug loading efficiency of micelles, which include the compatibility of the drug with the core forming
component of the micelle, the hydrophobicity, nature and length of the core-forming segment, the length and nature of the corona-forming component, the interactions between the drug and core and the method of micelle preparation. In general, the larger the hydrophobic segment, the larger the core for increased drug entrapment. An increase in the hydrophilic segment results in an increase in the CMC and reduced drug loading [74-76]. For all PTX-micelles, the DLC and DLE was low: ranging between 1.28 ± 0.07 wt % to 2.11 ± 0.46 wt % and 12.88 ± 0.75 % to 21.13 ± 4.59 % (table 4.7) which is typically seen for micelles with DLC’s ranging between 1-20 wt% [77]. The highest DLC was achieved for C16-(PEG2)_2-LDV PTX-micelles, which is likely due to the increased hydrophobicity and lower CMC value. Like micelles, efficient drug loading is highly dependent on the compatibility of drug molecules with the hydrophobic segment of the nanocarrier. For efficient drug loading into liposomes, the drug must be significantly hydrophobic with high log P values (≥ 3) for partitioning into the hydrophobic region of the phospholipid bilayers. Paclitaxel has a log P value of 3.6 and can be loaded into liposomes, which has been demonstrated through the development of Lipusu currently on the market and LEP-ETU currently undergoing clinical trials [38, 78]. However, the bulky and asymmetric nature of paclitaxel makes liposomal drug loading challenging and often leads to paclitaxel leakage or precipitation immediately after preparation or during storage. Consequently, it was shown that only ≤3.3 mol% can be loaded into lipid bilayers, resulting in poor drug loading efficiency [79]. In the initial studies, paclitaxel was loaded into liposomes at similar lipid concentrations to micelles (1mg/mL), however these vesicles were unstable and aggregated, which was likely due to an insufficient amount of lipid available to effectively retain paclitaxel in the phospholipid bilayer. Furthermore, initial drug loading at 10 wt% resulted in destabilization of vesicles, indicating that an optimal balance between lipid concentration and paclitaxel concentration was required to produce stable vesicles. Subsequently, stable PTX-liposomes
were produced when prepared at higher lipid concentrations (15 mg/mL) and lower initial DLC at 1 wt%, which resulted in drug loading capacities ranging between 0.83 ± 0.41 wt% to 1.00 ± 0.01 wt% and DLE’s ranging between 63.93 ± 31.85 % and 106.51 ± 0.68 % (table 4.7). Compared to PTX-micelles and PTX-liposomes, PTX-SLNs demonstrated the highest DLC and DLE with values ranging between 7.14 ± 0.17 wt% to 7.91 ± 0.87 wt% and 71.49 ± 1.66 % to 79.15 ± 8.65 % respectively (table 4.7). This is likely due to the use of high melting point triglycerides that help to increase paclitaxel incorporation and retention inside the lipid matrix. These results showed that paclitaxel was efficiently loaded into PTX-micelles, PTX-liposomes and PTX-SLNs and the variation in DLE’s may be due to nanocarrier structure, lipid compatibility with drug and methods used for producing nanocarriers.

a) Determination of in vitro drug release. Upon systemic administration, nanocarriers must extravasate into the tumor and either undergo interstitial drug release or intracellular release. Therefore, nanocarriers must be designed to demonstrate drug release in a controlled sustained release manner for enabling drug accumulation in the tumor. For example, Doxil is a liposomal formulation encapsulating doxorubicin and delivers 10 to 15 times more drug to the tumor compared to free doxorubicin. However, the bioavailability of Doxil is only 40 to 50% due to slow release, indicating that the excessive stability of this formulation is problematic [80]. Conversely, a polymeric micelle formulation Genexol-PM encapsulating paclitaxel has been shown to be too unstable upon systemic administration, where paclitaxel partitions out of the micelle and interacts with serum proteins, resulting in premature drug release before reaching the target site [80]. Therefore, the key challenge is to develop nanocarriers that maintain stability before reaching the tumor whilst allowing for efficient release when reaching the tumor site. In this study, the in vitro release profiles of PTX-micelles, PTX-liposomes and PTX-SLNs were compared to provide an indication of the
ability of these nanocarriers to retain drug under sink conditions. Compared to PTX-liposomes and PTX-SLNs, the release rate of paclitaxel from micelles was the fastest with ≥95% of the drug being released over 4 hours (figure 4.49). This is likely due to the dynamic nature of micelles, disassembly of micelles or rapid diffusion of paclitaxel from the micelles under sink conditions. Paclitaxel was released from liposomes at a slower rate than PTX-micelles, with ≈20% of drug release by 4 hours and ≈60% released over 24 hours (figure 4.50). These results demonstrate that PTX-liposomes have improved drug retention properties over PTX-micelles, presumably due to the more rigid and mechanical structure of the phospholipid bilayer membrane. All PTX-SLNs exhibited the slowest release rate compared to PTX-micelles and PTX-liposomes, where only 3-13% of PTX was released over 4 hours and 16-40% released over 24 hours (figure 4.51). These results showed a significant increase in drug retention in PTX-SLNs compared to PTX-micelles and PTX-liposomes, which is likely due to the core-forming high melting point triglyceride, which may have significantly decreased the mobility of paclitaxel molecules within the lipid core, reducing drug leakage [81]. In contrast, PTX-liposomes are formed from DPPC lipids which have a gel-to-liquid phase transition temperature (41°C) that is close to the body temperature, increasing the membrane fluidity and probability of paclitaxel molecules leaking out of the phospholipid bilayer membrane [82, 83]. The differences in release rates between PTX-micelles, PTX-liposomes and PTX-SLNs are described by release rate constants and t50% values presented in table 4.8. The release rate constants were the highest for PTX-micelles compared with PTX-liposomes and PTX-SLNs (0.80 ± 0.09/hr vs 0.08 ± 0.03/hr and 0.02±0.01/hr). One-way ANOVA results showed that there was a significant difference between the t50% values of PTX-micelles, PTX-liposomes and PTX-SLNs (p=0.03). Specifically, the t50% values for PTX-micelles were significantly lower compared to PTX-liposomes and PTX-SLNs. The Tukey test revealed a significant difference with PTX-
micelles vs PTX-SLNs (p=0.03) and PTX-liposomes vs PTX-SLNs (p=0.06). However, there was no significant difference in t_{50\%} values of PTX-micelles vs PTX-liposomes (p=0.84).
Figure 4.46: CryoTEM image of paclitaxel-loaded LDV targeted-micelles at a magnification of 52,000x. Observed in the sample are small particles that vary in size (yellow arrows), and larger densities that possibly represent clusters of small particles (blue arrow). Scale Bar: 200 nm
Figure 4.47: CryoTEM image of paclitaxel-loaded LDV-targeted liposomes at a magnification of 52,000x. Observed in the sample are unilamellar particles with a round angular appearance and with an interior density similar to that of the surrounding aqueous solution (red arrow); elongated particles (cyan arrow); round to elongated faint particles (green arrow); small particles (yellow arrow). Scale Bar: 200 nm
Figure 4.48: CryoTEM image of paclitaxel-loaded LDV-targeted solid lipid nanoparticles at a magnification of 52,000x. Observed in the sample are particles of various sizes that have an interior density that is slightly more dense than that of the surrounding buffer and are roughly round (orange arrow); angular or oblong in shape (purple in shape); small and elongated particles (cyan arrow); small particles (yellow arrow). Scale Bar: 200 nm
Figure 4.49: In vitro release profiles of paclitaxel-loaded LDV-targeted micelles in aqueous solution (n=3)

Figure 4.50: In vitro release profiles of paclitaxel-loaded LDV-targeted liposomes in aqueous solution (n=3)
Figure 4.51: In vitro release profiles of paclitaxel-loaded LDV-targeted solid lipid nanoparticles in aqueous solution (n=3)

Figure 4.52: Summary of in vitro release profiles of paclitaxel-loaded LDV targeted micelles, liposomes and SLNs in aqueous solution
Table 4.7: Size, PDI, Zeta Potential, DLC and DLE of paclitaxel-loaded micelles, liposomes and solid lipid nanoparticles (n=3)

<table>
<thead>
<tr>
<th>Nanocarrier</th>
<th>DLC (wt%)</th>
<th>DLE (%)</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16-(PEG2)₂-LDV micelle</td>
<td>2.11 ± 0.46</td>
<td>21.13 ± 4.59</td>
<td>-</td>
<td>-</td>
<td>-30.86 ± 5.16</td>
</tr>
<tr>
<td>C16-(PEG2)₄-LDV micelle</td>
<td>1.28 ± 0.07</td>
<td>12.88 ± 0.75</td>
<td>(6-12)*</td>
<td>-</td>
<td>-28.06 ± 6.04</td>
</tr>
<tr>
<td>C16-(PEG2)₆-LDV micelle</td>
<td>1.72 ± 0.44</td>
<td>17.17 ± 4.44</td>
<td>-</td>
<td>-</td>
<td>-11.9 ± 0.4</td>
</tr>
<tr>
<td>C16-(PEG2)₂-LDV liposome</td>
<td>0.83 ± 0.41</td>
<td>63.93 ± 31.85</td>
<td>123.67 ± 3.86</td>
<td>0.117</td>
<td>-40.13 ± 1.29</td>
</tr>
<tr>
<td>C16-(PEG2)₄-LDV liposome</td>
<td>0.93 ± 0.32</td>
<td>72.03 ± 24.65</td>
<td>117.27 ± 8.08 (48-145)*</td>
<td>0.264</td>
<td>-39.82 ± 3.34</td>
</tr>
<tr>
<td>C16-(PEG2)₆-LDV liposome</td>
<td>1.00 ± 0.01</td>
<td>106.51 ± 0.68</td>
<td>129.00 ± 8.32</td>
<td>0.235</td>
<td>-40.47 ± 1.05</td>
</tr>
<tr>
<td>C16-(PEG2)₂-LDV SLN</td>
<td>7.91 ± 0.87</td>
<td>79.15 ± 8.65</td>
<td>74.4 ± 4.39</td>
<td>0.172</td>
<td>-59.87 ± 6.15</td>
</tr>
<tr>
<td>C16-(PEG2)₄-LDV SLN</td>
<td>7.14 ± 0.17</td>
<td>71.49 ± 1.66</td>
<td>82.8 ± 5.56 (10-120)*</td>
<td>0.210</td>
<td>-55.69 ± 1.81</td>
</tr>
<tr>
<td>C16-(PEG2)₆-LDV SLN</td>
<td>7.26 ± 1.19</td>
<td>72.55 ± 11.91</td>
<td>84.4 ± 2.77</td>
<td>0.187</td>
<td>-39.39 ± 9.36</td>
</tr>
</tbody>
</table>

*Sizes of paclitaxel nanocarrier determined by cryoTEM.
Table 4.8: Average size, DLC, DLE, rate constants and $t_{50\%}$ values for paclitaxel-loaded micelles, liposomes and solid lipid nanoparticles ($n=3$)

<table>
<thead>
<tr>
<th>Nanocarrier</th>
<th>DLC (%)</th>
<th>DLE (%)</th>
<th>Size (nm)</th>
<th>Rate constant K (1/hr)</th>
<th>$t_{50%}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTX-micelles</td>
<td>1.70 ± 0.41</td>
<td>17.06 ± 4.12</td>
<td>-</td>
<td>0.80 ± 0.09</td>
<td>0.87 ± 0.10</td>
</tr>
<tr>
<td>PTX-liposomes</td>
<td>0.92 ± 0.08</td>
<td>80.82 ± 22.61</td>
<td>123.31 ± 5.98</td>
<td>0.08 ± 0.03</td>
<td>9.15 ± 3.12</td>
</tr>
<tr>
<td>PTX-SLN</td>
<td>7.44 ± 0.41</td>
<td>74.39 ± 4.15</td>
<td>80.53 ± 5.37</td>
<td>0.02 ± 0.01</td>
<td>51.41 ± 30.99</td>
</tr>
</tbody>
</table>
4.3 Spray Drying of Paclitaxel-loaded LDV Targeted Micelles, Liposomes and SLNs

4.3.1 Introduction. One of the challenges faced with lipid-based nanocarriers is their relative instability in aqueous dispersions. Lipid nanocarriers can undergo physical and chemical degradation, resulting in decreased safety and loss of therapeutic efficacy. Chemical degradation can occur through the oxidation of fatty acid chains and the hydrolysis of ester bonds, resulting in the generation of free fatty acids, lysophospholipids and phosphoglycerol compounds. Physical instability occurs through vesicle fusion, aggregation and drug leakage [84]. These factors combined affect the quality of the final product, resulting in poor therapeutic efficacy and safety concerns. For example, commercially available liposome products such as DOXIL are known to become unstable in solution, where drug leakage and liposome aggregation occurs [85]. To overcome these obstacles, aqueous dispersions of nanocarriers can be converted into a dry powder which can be stored over a long period of time but can be reconstituted at the time of administration. As a result, stabilization is achieved by reducing the water content and the shelf-life is increased. Currently, lyophilization or freeze-drying are the most common methods for drying pharmaceutical products. Freeze-drying involves the freezing of nanocarriers in aqueous solution followed by the removal of water from frozen samples by sublimation under vacuum. However, during this process, the nanoparticles are subjected to freezing and drying stresses, which may result in nanocarrier destabilization. For example, freezing can cause the disruption of liposome bilayer membrane structure due to the liquid-ice interface resulting in aggregation and vesicle fusion [86]. Furthermore, freeze-drying requires expensive equipment and may take several days to complete. To overcome the limitations faced with freeze-drying, spray drying can be used to convert lipid-based nanocarriers into dry powders using a single step. Spray drying is carried out through three stages, namely atomization, dehydration and powder collection. Initially, the feedstock solution which is the nanocarrier
in aqueous solution is atomized into a hot drying gas such as nitrogen. Atomization involves the application of an energy source that acts on a bulk liquid, resulting in the liquid break up into individual spray droplets. Following atomization, microparticle formation occurs through the conversion of atomized spray droplets into solid particles. The solid particles are then separated from the process gas stream using a cyclone and this principle is based on the density difference between the particle and gas. During the spray drying process, heat and high shearing forces can result in the degradation of lipid components, therefore optimization of formulation and process parameters is critical to maintain the integrity of nanocarriers during spray drying. For example, carbohydrate excipients such as mannitol, sucrose or trehalose are added into the feed to serve as bulking agents or protectants that result in the formation of microparticles embedded with nanocarriers [78, 87]. Other excipients such as amino acids and peptide sequences have also proved useful in spray drying by protecting against thermal stresses and denaturation, stabilization against aggregation and oxidation and reducing the hygroscopicity of various formulations [88]. For example, L-leucine has been used as an excipient for spray drying with lipid-based nanocarriers, due to hydrophobic and surfactant-like properties that enable migration to the surface of particles during the drying process, resulting in deaggregation and stabilization of particles [89-91]. In this study, selected PTX-micelles, PTX-liposomes and PTX-SLNs were spray dried with trehalose or a combination of trehalose and L-leucine.
4.3.2 Materials. PAs were synthesized at GenScript (Piscataway, NJ). DPPC lipids and liposome extruder were purchased from Avanti polar lipids (AL, USA). Solvents including DCM, methanol, ethanol and chloroform were purchased from fisherscientific (Pittsburg, PA). Paclitaxel was purchased from LC laboratories. Tripalmitin and D-(-)-Trehalose Dihydrate was purchased from Sigma-Aldrich (MO, USA). Pierce Slide-A-Lyzer G2 dialysis cassettes (MWCO 10,000) were purchased from VWR (CA, USA). L-leucine, aluminum stubs and carbon adhesive tapes were provided by AstraZeneca (SSF, USA).

4.3.3 Methods.

a) Spray drying of paclitaxel-loaded micelles, liposomes and SLNs. Initially, one of each type of nanocarrier was selected to determine the feasibility for spray drying. Upon evaluation, PTX-micelles formed from C16-(PEG2)_4-LDV PAs were chosen since this nanocarrier has the lowest CMC value, small size and suitable water solubility for formulation preparation. PTX-liposomes and PTX-SLNs formed from C16-(PEG2)_4-LDV PAs were then selected for spray drying for direct comparison. PTX-micelles, PTX-liposomes and PTX-SLNs were prepared using the cosolvent evaporation method, thin-film hydration/extrusion method and microfluidic technology as previously described. To determine which excipients are required to form stable nanocarriers upon spray drying, nanocarriers were spray dried in trehalose only or in a mixture of trehalose and l-leucine. For feedstock preparation, 50mg of PTX-micelles, PTX-liposomes and PTX-SLNs were mixed with excipients (trehalose, L-leucine) dissolved in water at 2.5 wt% (table 4.9) and the total feedstock concentration was 20 mg/ml. The feedstock solution was then spray dried using a custom designed small-scale pharmaceutical spray dryer. The dryer inlet temperature was maintained at 60°C; the dryer outlet temperature was maintained at 50°C. The drying gas flow rate was maintained at 850 slpm (standard liters per minute) with an atomization gas flow rate of 15 slpm. The collector jacket temperature and the cyclone jacket temperatures
were maintained at 50⁰C. The feedstock solution was fed into the spray dryer at rate of 3 mL/min, which resulted in the deposition of spray dried material in a collector. Next, the collector containing the spray dried powders was transferred into a glove box and the powders were then subsequently transferred into glass sample jars using a spatula. The tare and gross weights of the collector and sample jars were then recorded and the production yield was calculated from the mass ratio of the collected powders to the total solid in the feed. Sample handling was performed in a glove box with constant flushing of nitrogen. Prior to characterization, spray dried powders were kept in a dry box at low relative humidity (≤5%).

**b) Moisture content of spray dried powders.** The residual moisture content of all spray-dried powders were determined by the oven vaporizer Karl Fisher coulometric titration method using a Metrohm 874 oven sample processor (Switzerland). The oven temperature was set to 150⁰C and the gas flow to 75mL/min. Samples were prepared by weighing out approximately 15mg of spray-dried powder into vials and sealed with caps. Blank samples were also prepared by sealing an empty vial. All samples were prepared in triplicate and analyzed using Tiamo 2.5 software. The moisture content was determined using the equation 4.3.

\[
\text{Equation 4.3: } \quad \text{Moisture Content (\%)} = \frac{\text{Water Detected (ug)}}{\text{Sample Mass (ug)}}
\]
c) **Differential Scanning Calorimetry (DSC).** DSC measurements for all spray-dried powders were performed using a differential scanning calorimeter (DSC Q2000 TA, USA). Approximately 15mg of spray dried powder was loaded into an aluminum pan and hermetically sealed. The sample was heated at the scanning rate of 2°C/min to 150°C in a nitrogen atmosphere. All samples were prepared in triplicate and analyzed using the TA instrument explorer Qseries software.

d) **Morphology of spray dried powders.** The morphology of spray-dried powders were evaluated using Scanning Electron Microscopy (SEM). Sample preparation was carried out in a glove box under constant flushing with nitrogen and the relative humidity was kept below 5%. Spray-dried powders were deposited onto an aluminum stub coated with conductive carbon tape. The aluminum stubs coated with sample were then sputter coated with gold-palladium for 60 seconds under high vacuum. SEM imaging was performed using a JSM-IT100 microscope (JOEL Ltd., USA), operating at an acceleration voltage of 20 kV.

e) **Reconstitution and characterization of paclitaxel-loaded nanocarriers.** Spray dried powders were reconstituted with purified water and agitated for approximately 5 minutes by hand until all the powder had dissolved. The final lipid concentration upon reconstitution was 0.5 mg/mL. Following reconstitution, the size, PDI and charge were characterized using the mobius zeta Potential and DLS detector (Wyatt, CA, USA). The paclitaxel content was determined using the HPLC method previously described.

f) **Determination of stability.** The stability of paclitaxel-loaded nanocarriers before and after spray drying was carried out by determination of size, PDI and paclitaxel content over 7 days at room temperature and at 4°C. The size and PDI was determined using the mobius zeta Potential and DLS detector (Wyatt, CA, USA). Paclitaxel content was determined using the HPLC method previously described.
Table 4.9: Composition of spray dried formulations

<table>
<thead>
<tr>
<th>Name of formulation</th>
<th>Component (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTX-micelles/trehalose</td>
<td>nanocarrier (2.5 wt%)</td>
</tr>
<tr>
<td></td>
<td>trehalose (97.5 wt%)</td>
</tr>
<tr>
<td>PTX-liposomes/trehalose</td>
<td>nanocarrier (2.5 wt%)</td>
</tr>
<tr>
<td></td>
<td>trehalose (97.5 wt%)</td>
</tr>
<tr>
<td>PTX-SLN/trehalose</td>
<td>nanocarrier (2.5 wt%)</td>
</tr>
<tr>
<td></td>
<td>trehalose (97.5 wt %)</td>
</tr>
<tr>
<td>PTX-micelles/trehalose/leucine</td>
<td>nanocarrier (2.5 wt%)</td>
</tr>
<tr>
<td></td>
<td>trehalose (77.5 wt%)</td>
</tr>
<tr>
<td></td>
<td>leucine (20 wt%)</td>
</tr>
<tr>
<td>PTX-liposomes/trehalose/leucine</td>
<td>nanocarrier (2.5 wt%)</td>
</tr>
<tr>
<td></td>
<td>trehalose (77.5 wt%)</td>
</tr>
<tr>
<td></td>
<td>Leucine (20 wt%)</td>
</tr>
<tr>
<td>PTX-SLN/trehalose/leucine</td>
<td>nanocarrier (2.5 wt%)</td>
</tr>
<tr>
<td></td>
<td>trehalose (77.5 wt %)</td>
</tr>
<tr>
<td></td>
<td>leucine (20 wt%)</td>
</tr>
</tbody>
</table>
4.3.4 Results and discussion. Nanocarriers prepared and stored as aqueous dispersion are known to undergo physical and chemical changes over time. Therefore, conversion of nanocarriers dispersions to a powder form is highly desired to improve the long-term stability. The drying method, process parameters and formulation excipients need to be carefully selected to ensure that nanocarriers can be re-dispersed without any significant size change, aggregation or loss of drug content. Furthermore, the spray dried powders should remain stable with low residual moisture content, to prevent chemical degradation of the formulation components. In this study, selected PTX-micelles, PTX-liposomes and PTX-SLN were successfully spray dried with either trehalose or a mixture of trehalose and L-leucine to stabilize the nanocarriers. The choice of spray drying excipients did not significantly affect the yield, which ranged between 71.5% to 83.5% across all spray dried powder formulations (table 4.10 and figure 4.53). Many spray dried powders contain carbohydrates in their amorphous state and since amorphous components are thermodynamically unstable and there is a driving force for crystallization to occur, a high transition temperature of powders is highly desirable. Trehalose comprises the bulk of the spray dried formulations and was selected due to the high transition temperature. Increased moisture content can significantly decrease the transition temperature of spray dried powders, since water acts as a plasticizer, causing the mobilization of amorphous content [92]. Consequently, powders with increased moisture content can have reduced transition temperatures, increasing the chances of forming physically unstable powders. The oven KF results showed that the residual moisture content for all spray dried powders was very low at values <2% (table 4.11) and did not affect the transition temperatures of all spray dried formulations. This was demonstrated through the DSC results showing mid-point transition temperature values ranging between 95.07 ± 0.86 °C and 100.77 ± 0.00 °C (table 4.12) (figures 4.54 to 4.59) which are representative of trehalose. SEM images showed smooth
surface and spherical morphology of powders spray dried with trehalose. In contrast, powders spray dried with both trehalose and L-leucine showed corrugated particles with wrinkled morphology (figure 4.60). Formulations with wrinkled morphology are attributed to the presence of L-leucine which precipitates out earlier in the drying process due to its lower solubility. This results in a solid shell that collapses later in the drying process. In contrast, formulations with highly water-soluble excipients such as trehalose continually shrink as liquid droplet dries, eventually forming particles with smooth morphology [88, 93, 94]. To determine whether the nanocarriers had maintained their integrity after spray drying, the spray dried powders were reconstituted in purified water and the size/PDI was measured using DLS. The results showed that the size of PTX-liposomes decreased from 107 nm to 93.33 nm and the PDI decreased from 0.236 to 0.149 after spray drying with trehalose (table 4.13 and 4.14, figure 4.61 and 4.62). The same trend was observed for PTX-SLNss which showed a decrease in size from 90.80 nm to 74.20 nm (table 4.13 and table 4.14, figure 4.63). Particle size reduction in the presence of cryoprotectants has been recently observed and may be due to the interactions of trehalose with the nanocarrier [95]. Conversely, the sizes of liposomes increased from 107.60 nm to 138.53 nm when spray drying with trehalose and L-leucine (figure 4.61, table 4.13). This effect has previously been observed where concentrations of L-leucine at 1% (w/w) increased the size of liposomes significantly after spray drying [89]. This is likely due to the partitioning of hydrophobic amino acid L-leucine into the lipid membrane during drying, causing aggregation of the vesicles [89]. Furthermore, it has also been demonstrated that amino acids with hydrocarbon side chains can cause damage to the lipid membranes of vesicles during freeze/thaw processes [96]. L-leucine also influenced the size and PDI of PTX-SLNss since these powders did not dissolve completely upon reconstitution and required syringe filtration to remove undissolved particles. Despite these changes, all spray dried PTX-liposomes and PTX-SLNss maintained
nanosizes close to 100 nm upon reconstitution. The stability of reconstituted PTX-SLN and PTX-liposomes was determined by measuring the size, PDI and paclitaxel content over 7 days at room temperature or at 4°C (figures 4.67 to 4.72). The results showed no significant change in size, PDI and paclitaxel content over 7 days at 4°C. There was also no significant change in the size and PDI of PTX-SLN and PTX-liposomes at room temperature over 7 days. However, a decrease in the paclitaxel content of nanocarriers was observed over 7 days at room temperature except for PTX-SLN and PTX-micelles spray dried in trehalose.

Overall, the short-term stability studies showed that the reconstituted nanocarriers were stable for up to 7 days at 4°C. Further studies are required to characterize the long-term stability of these formulations.
Table 4.1: Yields of spray dried formulations

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTX-micelles/trehalose</td>
<td>71.5</td>
</tr>
<tr>
<td>PTX-liposomes/trehalose</td>
<td>79.5</td>
</tr>
<tr>
<td>PTX-SLN/trehalose</td>
<td>80.5</td>
</tr>
<tr>
<td>PTX-micelles/trehalose/leucine</td>
<td>74.5</td>
</tr>
<tr>
<td>PTX-liposomes/trehalose/leucine</td>
<td>83.5</td>
</tr>
<tr>
<td>PTX-SLN/trehalose/leucine</td>
<td>79.5</td>
</tr>
</tbody>
</table>

Figure 4.53: Yields of spray dried formulations
Table 4.11: Residual moisture content of spray dried formulations (n=3)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Water (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTX-micelles/trehalose</td>
<td>1.29 ± 0.05</td>
</tr>
<tr>
<td>PTX-liposomes/trehalose</td>
<td>1.85 ± 0.01</td>
</tr>
<tr>
<td>PTX-SLN/trehalose</td>
<td>1.42 ± 0.01</td>
</tr>
<tr>
<td>PTX-micelles/trehalose/leucine</td>
<td>1.08 ± 0.03</td>
</tr>
<tr>
<td>PTX-liposomes/trehalose/leucine</td>
<td>1.44 ± 0.04</td>
</tr>
<tr>
<td>PTX-SLN/trehalose/leucine</td>
<td>1.01 ± 0.03</td>
</tr>
</tbody>
</table>

Table 4.12: Transition temperatures of spray dried formulations (n=3)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Tg Onset (°C)</th>
<th>Mid-Point Tg (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTX-micelles/trehalose</td>
<td>97.65 ± 0.83</td>
<td>100.77 ± 0.00</td>
</tr>
<tr>
<td>PTX-liposomes/trehalose</td>
<td>94.26 ± 2.97</td>
<td>98.91 ± 0.00</td>
</tr>
<tr>
<td>PTX-SLN/trehalose</td>
<td>96.33 ± 0.02</td>
<td>98.10 ± 0.10</td>
</tr>
<tr>
<td>Liposomes/Trehalose/Leucine</td>
<td>90.98 ± 0.80</td>
<td>95.17 ± 1.43</td>
</tr>
<tr>
<td>Micelles/Trehalose/Leucine</td>
<td>92.11 ± 0.09</td>
<td>95.07 ± 0.86</td>
</tr>
<tr>
<td>SLN/Trehalose/Leucine</td>
<td>95.39 ± 0.77</td>
<td>98.23 ± 0.77</td>
</tr>
</tbody>
</table>
Figure 4.54: DSC-thermogram of spray dried PTX-micelles/trehalose

Figure 4.55: DSC-thermogram of spray dried PTX-liposomes/trehalose
Figure 4.56: DSC-thermogram of spray dried PTX-SLN/trehalose

Figure 4.57: DSC-thermogram of spray dried PTX-micelles/trehalose/leucine
Figure 4.58: DSC-thermogram of spray dried PTX-liposomes/trehalose/leucine

Figure 4.59: DSC-thermogram of spray dried PTX-SLN/trehalose/leucine
Figure 4.60: Scanning Electron Microscope images of spray dried formulations (PTX-micelles/trehalose, PTX-liposomes/trehalose, PTX-SLN/trehalose, PTX-SLN/micelles/trehalose/leucine, PTX-liposomes/trehalose/leucine, PTX-SLN/trehalose/leucine).
Table 4.13: Sizes of paclitaxel-loaded liposomes and solid lipid nanoparticles before and after spray drying (SD) (n=3)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Size before SD (nm)</th>
<th>Size after SD in trehalose (nm)</th>
<th>Size after SD in trehalose/leucine (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTX-liposomes</td>
<td>107.60 ± 3.10</td>
<td>93.33 ± 2.95</td>
<td>138.53 ± 17.31</td>
</tr>
<tr>
<td>PTX-SLN</td>
<td>90.80 ± 1.11</td>
<td>74.20 ± 2.95</td>
<td>121.20 ± 8.98</td>
</tr>
<tr>
<td>PTX-micelles</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 4.14: PDI of paclitaxel-loaded liposomes and solid lipid nanoparticles before and after spray drying (SD) (n=3)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>PDI before SD</th>
<th>PDI after SD in trehalose</th>
<th>PDI after SD in trehalose/leucine</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTX-liposomes</td>
<td>0.236 ± 0.001</td>
<td>0.149 ± 0.007</td>
<td>0.237 ± 0.003</td>
</tr>
<tr>
<td>PTX-SLN</td>
<td>0.236 ± 0.002</td>
<td>0.224 ± 0.005</td>
<td>0.131 ± 0.025</td>
</tr>
<tr>
<td>PTX-micelles</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
Figure 4.61: Size of paclitaxel-loaded liposomes before and after spray drying
Figure 4.62: PDI of paclitaxel-loaded liposomes before and after spray drying
Figure 4.63: Size of paclitaxel-loaded solid lipid nanoparticles before and after spray drying
Figure 4.64: PDI of paclitaxel-loaded solid lipid nanoparticles before and after spray drying
Figure 4.65: Size of reconstituted spray dried paclitaxel-loaded liposomes and solid lipid nanoparticles measured over 7 days at 4°C (n=3)

Figure 4.66: PDI of reconstituted spray dried paclitaxel-loaded liposomes and solid lipid nanoparticles measured over 7 days at 4°C (n=3)
Figure 4.67: Size of reconstituted spray dried paclitaxel-loaded liposomes and solid lipid nanoparticles measured over 7 days at room temperature (n=3)

Figure 4.68: PDI of reconstituted spray dried paclitaxel-loaded liposomes and solid lipid nanoparticles measured over 7 days at room temperature (n=3)
Figure 4.69: Graph showing the paclitaxel content of spray dried micelles, liposomes and solid lipid nanoparticles measured over 7 days at 4°C

Figure 4.70: Graph showing the paclitaxel content of spray dried micelles, liposomes and solid lipid nanoparticles measured over 7 days at room temperature
Chapter 5: In Vivo Efficacy of Paclitaxel-loaded Micelles, Liposomes and SLNs

5.1. Introduction

To evaluate the in vivo efficacy of drug-loaded nanocarriers, xenograft models of human tumors grown in immunodeficient mice are developed by injection or implantation of human tumor cells in mice. Once tumor growth has been established, mice are injected with drug-loaded nanocarriers and the anti-cancer efficacy can be determined by evaluating tumor growth progression over time and determination of the tumor mass at the end of the study. Other parameters such as body weight can be monitored throughout the course of the study to determine toxic effects of nanocarriers. To maximize therapeutic efficacy, nanocarriers must demonstrate sufficient stability for extended in vivo circulation half-life, reduced drug leakage during circulation, maintenance of nanosize for tumor penetration and degradation for reduced systemic toxicity [97]. In this study, nanocarriers spray dried with trehalose were selected to compare the in vivo anti-cancer efficacy of PTX-micelles, PTX-SLNs and PTX-liposomes.

5.2 Materials

Spray dried PTX-micelles, PTX-liposomes and PTX-SLNs were prepared at AstraZeneca (SSF, CA). Cremophor EL was purchased from Spectrum Chemicals. The following materials were purchased by LoveLace Biomedical: A375 adherent malignant human melanoma cell line was obtained from ATTC (VA, USA). NCI Athymic NCr-nu/nu mice were purchased from Charles River Laboratories (San Diego CA, USA).

5.3 Methods

The in vivo animal study was conducted by Lovelace Biomedical (Albuquerque, NM) according to protocol (No. FY17-078) reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). The tumor regression effect was evaluated in 3 to 5-week-old (15 to 25g) female NCI Athymic NCr-nu/nu mice obtained from Charles River
Laboratories. All animals were injected with A375 human melanoma cells at a target of $10^6$ cells per animal by subcutaneous injection into the right or left posterior flank or on the back. Following tumor growth, animals were randomized into five treatment groups with targeted minimum group sizes of six mice per treatment group: (Trehalose, Taxol, PTX-micelles, PTX-liposomes and PTX-SLNs). PTX-micelles, PTX-liposomes and PTX-SLNs were provided to LoveLace Biomedical in spray dried powder form and were reconstituted in sterile water for injection prior to in vivo administration. Each group was treated by tail vein injection twice weekly and treatments were given at a paclitaxel dose of 50µg/kg for up to 4 weeks. The tumor volume was measured three times weekly using equation 5.1[98]:

Equation 5.1 \[ V = \text{length} \ast (\text{width})^2/2. \]

The body weight of mice in each group was measured three times weekly. Tumor volume and weight and body weight were determined at necropsy. Mice were euthanized based on the moribund/euthanasia criteria according to LoveLace biomedical protocol, which includes, difficulty or inability to reach food or water, reluctance to move when stimulated to do so, 25% or greater pre-challenge body weight loss from the highest body weight measurement and tumor volume sizes reaching over 4000 mm$^3$. The data was analyzed by GraphPad Prism and the software was used to plot tumor volume v/s treatment day and the differences in tumor volume were statistically evaluated. The differences in tumor volume between groups were compared using repeated-measures one-way ANOVA (mixed-effects model) followed by Tukey post hoc comparisons. A p value <0.05 was considered statistically significant. The tumor growth inhibition (TGI) was calculated using equation 5.2, where T indicates the mean tumor volume of the treatment group and C indicates the mean tumor volume of the control group [99]. In vitro-in vivo correlations were analyzed by simple linear regression.
Equation 5.2 \[ \text{TGI (\%)} = (1 - \frac{T}{C}) \times 100 \]

5.4 Results and Discussion

The anti-tumor efficacy of PTX-micelles, PTX-liposomes and PTX-SLNs was evaluated in tumor-bearing mice. For the control group (trehalose), PTX-micelle group and PTX-liposomes group, a number of mice were either moribund or had died on day 26 and 29 (figure 5.1). The statistical analysis results showed significant differences in tumor volumes between groups \((p<0.0001)\). Tukey’s multiple comparison test revealed statistically significant differences in tumor volumes between control and taxol groups \((p<0.01)\) and between control and PTX-SLN \((p<0.01)\) groups, where tumor volumes in taxol and PTX-SLN groups were smaller than those in the control group. There was no statistically significant difference in tumor volumes between the control group vs PTX-micelles \((p=0.97)\) or PTX-liposomes \((p=0.99)\). When comparing the treatment groups, PTX-SLNs demonstrated statistically significantly smaller tumor sizes compared to PTX-liposomes \((p<0.02)\) and PTX-micelles \((p<0.001)\). After 24 days of treatment, the percent tumor growth inhibition was only 9.7% and 20.2% for PTX-micelles and PTX-liposomes compared to the control (table 5.1). Compared to PTX-micelles and PTX-liposomes, PTX-SLNs inhibited the tumor growth as much as 50.3% (table 5.1). At the end of the study, the reduction in tumor mass was the lowest for mice treated with PTX-SLNs and the largest for mice in the control, PTX-micelles and PTX-liposome groups (figure 5.2, table 5.2). These tumor mass results correlated well the measured tumor volumes at the end of the study \((R^2=0.93)\) (figure 5.3). For all groups, there was no significant body weight loss observed (figure 5.4). The results shown in figure 5.5 and 5.6 show that the tumor growth inhibition effect of PTX-micelles, PTX-liposomes and PTX-SLNs correlate well with the in vitro \(t_{50}\%\) \((R^2=0.94)\) and the in vitro rate constants in human serum \((R^2=0.65)\). Overall, the results showed that PTX-SLNs demonstrated higher therapeutic efficacy compared to PTX-micelles and PTX-liposomes. A
plausible conclusion from the in vitro-in vivo correlation results is that the $t_{50\%}$ and rate constants determined in human serum is indicative of the ability of the nanocarrier to stay in the blood circulation, leading to higher drug concentrations in the tumor and thus increased efficacy.
Figure 5.1: Graph showing the average tumor volume over 29 days for mice treated with trehalose, Taxol, PTX-micelles, PTX-liposomes and PTX-solid lipid nanoparticles (n=8)

Table 5.1: Percent tumor growth inhibition values with respect to control at 24 days

<table>
<thead>
<tr>
<th>Nanocarrier</th>
<th>% tumor growth inhibition with respect to control at 24 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTX-micelles</td>
<td>9.7</td>
</tr>
<tr>
<td>PTX-liposomes</td>
<td>20.2</td>
</tr>
<tr>
<td>PTX-SLN</td>
<td>50.3</td>
</tr>
</tbody>
</table>
Figure 5.2: Graph showing the terminal tumor mass at the end of the study for the various treatments
Table 5.2: Average tumor mass at the end of the study for mice treated with trehalose, Taxol, PTX-micelles, PTX-liposomes and PTX-solid lipid nanoparticles

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Average tumor mass at the end of the study (grams)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trehalose</td>
<td>2.38 ± 1.95</td>
</tr>
<tr>
<td>PTX-micelles</td>
<td>2.31 ± 1.81</td>
</tr>
<tr>
<td>PTX-liposomes</td>
<td>2.23 ± 1.57</td>
</tr>
<tr>
<td>Taxol</td>
<td>1.76 ± 1.21</td>
</tr>
<tr>
<td>PTX-SLN</td>
<td>1.55 ± 0.85</td>
</tr>
</tbody>
</table>
Figure 5.3: Graph showing the correlation between tumor mass and tumor volume at the end of the study.

Figure 5.4: Average body weights of mice treated with trehalose, Taxol, PTX-micelles, PTX-liposomes and PTX-solid lipid nanoparticles.
Figure 5.5: Graph demonstrating in vitro-in vivo correlation between time to release 50% of drug (\(t_{50\%}\)) and percent tumor growth inhibition for PTX-micelles, PTX-liposomes and PTX-solid lipid nanoparticles.
Figure 5.6: Graph demonstrating in vitro-in vivo correlation between rate constants and percent tumor growth inhibition for PTX-micelles, PTX-liposomes and PTX-solid lipid nanoparticles
Chapter 6: Summary and Conclusions

Cancer is one of the major causes of death worldwide and chemotherapy is the mainstay of treatment. However, conventional chemotherapy has limitations such as the development of multidrug resistance, low selectivity for target tissue and toxic side effects that have negative consequences for the health and well-being of cancer patients. Many chemotherapeutic agents such as paclitaxel have poor aqueous solubility, requiring the use of other excipients to aid solubilization for effective intravenous administration. For example, paclitaxel (Taxol) is currently formulated with cremophor EL, which is a toxic excipient and has been known to cause severe side effects [38]. To overcome these challenges, nanocarriers can be used to encapsulate and deliver hydrophobic anti-cancer agents to tumor tissue by passive and active targeting mechanisms. Despite these advantages, premature drug leakage is one of the major reasons for inefficient drug delivery to the tumor. Drug diffusion out of the nanocarriers or destabilization of drug loaded nanocarriers by physiological interactions with blood cells, serum proteins, and cell membranes upon systemic administration contribute to premature drug release. Therefore, developing stable anti-cancer nanocarriers is key for the successful clinical translation of these nanomedicines.

In this study, targeted micelles, liposomes and SLNs of similar composition were prepared and their potential was evaluated by comparing physicochemical characteristics, in vitro stability, in vitro release rates and in vivo efficacy. Micelles were formed through the self-assembly of PAs comprising of an LDV peptide region conjugated to single chain palmitic acid via PEG2 hydrophilic linkers. PAs self-assembled in aqueous solution at CMC values ranging between $23.68 \pm 0.72\mu M$ to $38.76 \pm 2.27\mu M$. Liposomes and SLNs of similar composition to micelles were prepared using palmitic acid derived lipids and PAs to impart targeting properties. In the vitro stability studies using FRET showed that targeted micelles, liposomes and SLNs were stable when diluted in aqueous medium over 24 hours, however
the stability decreased in the presence of human serum, where micelles were the least stable and SLNs the most stable. The same trend was observed for the in vitro paclitaxel release profiles, where targeted PTX-micelles had the fastest release rate and PTX-SLNs exhibited the slowest release rate. PTX-micelles, PTX-liposomes and PTX-SLNs were spray dried to arrest drug passive diffusion and enhance long term stability. The results showed that compared to initial aqueous dispersions, spray dried formulations maintained their nanosize and paclitaxel content over 7 days at 4°C. At room temperature, the paclitaxel content of PTX-micelle/trehalose and PTX-SLN/trehalose formulations were maintained over 7 days and all formulations maintained their nanosize. PTX-micelles, PTX-liposomes and PTX-SLNs were further evaluated to determine the anti-cancer in vivo efficacy in an A375 melanoma xenograft mouse model. The results showed a significant delay in tumor growth for mice treated with PTX-SLNs compared with control, PTX-micelle and PTX-liposome groups. Overall, the in vitro results showed that targeted PTX-SLNs were the least prone to disruption in human serum and were able to encapsulate paclitaxel over longer periods of time under sink conditions compared to PTX-micelles and PTX-liposomes. Furthermore, PTX-SLNs demonstrated superior anti-cancer efficacy in vivo compared to PTX-micelles and PTX-liposomes and could be promising vehicles for drug targeting and delivery. Future work could involve exploring other nanocarriers for stability characterization. The use of other materials for nanocarrier preparation such as polymers could be investigated. Additionally, escalation of dose can be implemented to achieve better therapeutic efficacy with tumor regression.
REFERENCES


