THE STABILITY PROFILES OF NOVEL PEPTIDE MODIFIED LIPOSOMES DESIGNED TO TREAT METASTATIC BREAST CANCER

BY

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ABSTRACT

Metastatic breast cancer is the second most commonly diagnosed cancer worldwide. Therefore, improved chemotherapeutics are desperately needed. One promising potential drug construct involves the use of drug delivery vesicles. A major advantage associated with the use of drug delivery vesicles such as liposomes to transport otherwise unencapsulated cytotoxic agents is their ability to improve the therapeutic and pharmacological properties of these chemotherapeutic drugs. While the encapsulation of cytotoxic agents in liposomes have proven to be quite effective clinically, these drug constructs do in fact lack targeting capabilities to selectively bind cancerous versus non-cancerous cells. Another advantage associated with use of liposomes is that they can easily be surface-modified to incorporate targeting ligands designed to preferentially bind specific upregulated cell surface receptors, such as integrins, present on cancer cells. In fact, the α3β1 integrin is known to be overexpressed in metastatic breast cancer rendering it as a potentially promising target for this type of drug delivery. Therefore, in this study a unique single stranded peptide construct known to selectively bind the α3β1 integrin has been incorporated directly into the liposomal bilayer. Furthermore, in order to verify that the presence of the peptide within the bilayer did not negatively influence the overall structural integrity of the liposome, stability studies were conducted. These results suggest that we have designed a stable, targeted liposomal construct intended for the treatment of metastatic breast cancer.

ACKNOWLEDGEMENTS

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APPROVED:

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DDV	Drug Delivery Vesicle
EPR	Enhanced Permeability and Retention
HER-2	Human Epidermal Growth Factor Receptor 2
CMC	Critical Micelle Concentration
MLV	Multilamellar Vesicle
ULV	Unilamellar Vesicle
LUV	Large Unilamellar Vesicle
SUV	Small Unilamellar Vesicle
EREM	Extended-Release Epidural Morphine
MDR	Multidrug Resistance
Tm	Phase Transition Temperature
DPPC	Dipalmitoylphsophatidylcholine
DSPC	Distearoylphosphatidylcholine
HSPC	Hydrogenated Soy Phosphatidylcholine
PEG	Polyethyleneglycol
RES	Reticuloendothelial System
CUR	Curcumin
CXB	Celecoxib
HA	Hyaluronic Acid
NBD	NF-κB Essential Modulator-Binding Domain Peptide
TAT	Cell-Penetrating Peptide

TNF-α	Tumor Necrosis Factor α
ECM	Extracellular Matrix
MMP	Matrix Metalloproteinase
PEG_{2000}	DSPE-polyethylene-glycol-2000
PEG_{750}	DSPE-polyethylene-glycol-750
PBS	Phosphate Buffered Saline

CHAPTER I

BREAST CANCER INTRODUCTION

1.1 BREAST CANCER

Breast cancer is the most commonly diagnosed, and the second leading cause of cancer death among women world-wide, and also accounts for 30% of all new cancer diagnoses amongst women annually in the United States (Torre et al. 2017, Siegel, Miller and Jemal 2019). Therefore, recent research has become increasingly focused on developing new, more efficacious chemotherapeutics for treatment of this disease. Currently, the "gold standard" therapy for metastatic breast cancer often involves Taxane drugs such as paclitaxel (Figure 1.1) (Chowdhury et al. 2019), or other commonly used drugs such as doxorubicin (Figure 1.2).

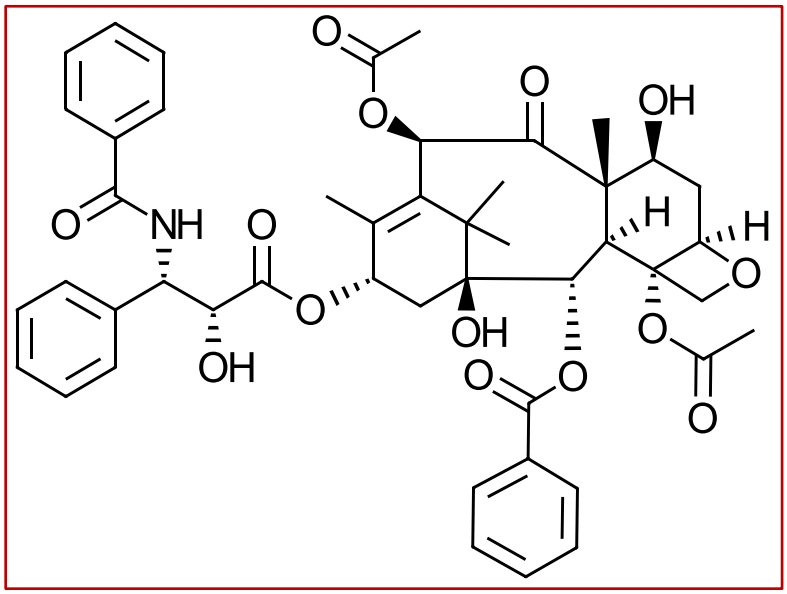

Figure 1.1 : The molecular structure of Paclitaxel.

The Taxanes are derived from plants of the genus *Taxus*, with paclitaxel found in the bark of *Taxus brevifolia* or the Pacific Yew tree (Gelderblom et al. 2001). Paclitaxel is a lipophilic diterpenoid pseudoalkaloid which promotes the stabilization of microtubules by interfering with the depolymerization of these structures during mitosis, which is essential to successful cellular replication (Singla, Garg and Aggarwal 2002, Horwitz 1992).

Doxorubicin is another common chemotherapeutic agent utilized to treat breast cancer. This cytotoxic agent is an aromatic polyketide anthracycline which originated from a strain of *Streptomyces Peucetius Var. Caesius* in the 1970s (Figure 1.2) (Arcamone 1981). Mechanistically, this drug exerts its cytotoxic capabilities through intercalation into DNA and disruption of topoisomerase-II-mediated DNA religation, which is particularly potent to rapidly replicating cancer cells (Thorn et al. 2011). Although both paclitaxel and doxorubicin have proven to be somewhat efficacious in the treatment of breast cancer, there are many negative side-effects associated with unencapsulated, non-targeted drugs. For

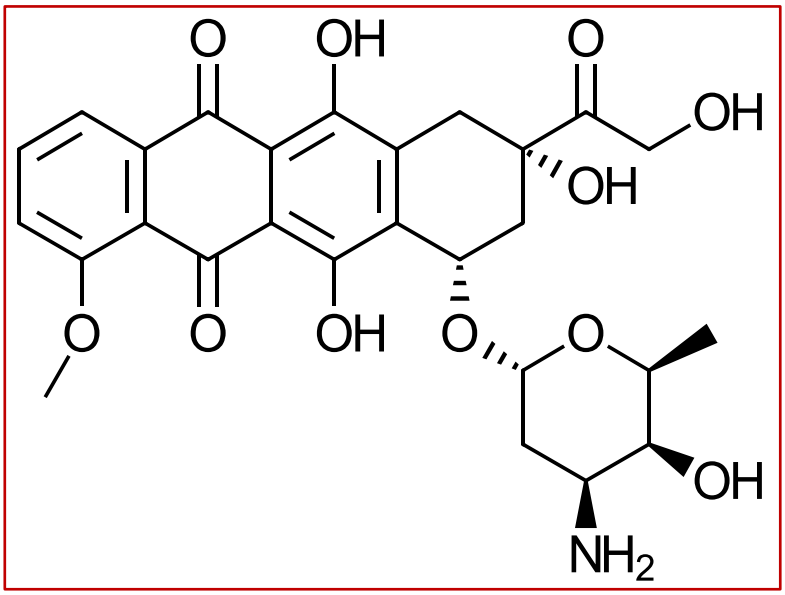

Figure 1.2 : The molecular structure of Doxorubicin.

example, the emulsifier Cremophor EL which is utilized to solubilize paclitaxel is quite toxic resulting in significant side effects to include nephrotoxicity, neurotoxicity, and fatal hypersensitivity reactions to name a few (Singla et al. 2002). As for doxorubicin, cardiomyopathy, severe inflammation, and alopecia are all known side effects attributed to this drug (Singal and Iliskovic 1998, Gabizon et al. 1994). Therefore, the utilization of nanocarriers as drug delivery systems to encapsulate cytotoxic agents such as these have become of particular interest as they can serve as a barrier between the drug and healthy tissue to potentially reduce these unintended, deleterious side-effects while the drug is in transit to solid tumors (Khan et al. 2019).

1.2 NANOCARRIER-BASED DRUG DELIVERY

The utilization of nanocarriers as drug delivery vesicles (DDV) possess the potential to improve the pharmacological properties of free drugs largely in part by altering the drug pharmacokinetics and biodistribution (Mosgoeller, Prassl and Zimmer 2012). For

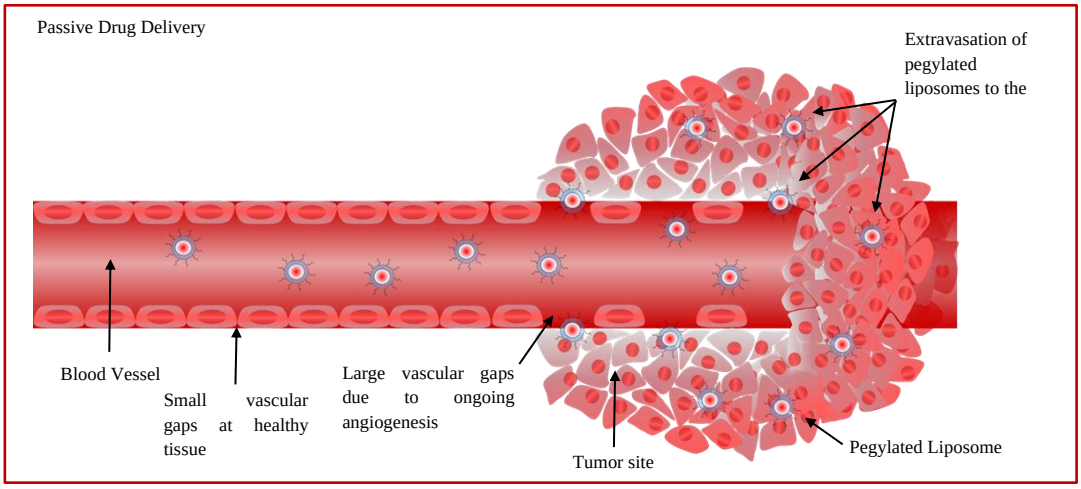

Figure 1.3 : Diagram representing passive drug delivery via the EPR effect involving extravasation of cytotoxic agent to the tumor site through large vascular gaps present in the tumor microenvironment (adapted from (Khan et al. 2018)).

example, DDVs allow for the cytotoxic agent to preferentially accumulate at the tumor-site relative to non-cancerous tissue such that there is an effective cytotoxic dose localized to the site of malignancy (Din et al. 2017). This is due in part to a phenomenon known as the enhanced permeability and retention (EPR) effect first described by Matsumura and Maeda in 1986 (Figure 1.3) (Kalyane et al. 2019, Matsumura and Maeda 1986). In tumorous tissues, deregulated angiogenesis initiates the formation of large vascular gaps up to 400 nm, thus contributing to the increased permeability of the tumor (Yuan et al. 1995). Furthermore, the tumor microenvironment is also characterized by a poor lymphatic system resulting in elevated interstitial pressure and enhanced retention of fluid in tumor tissues (Padera, Meijer and Munn 2016). Therefore, these characteristics regarding the tumor microenvironment have the potential to contribute to the selective accumulation of nanocarriers 10-50 fold higher at the tumor site relative to non-cancerous tissue following passive drug delivery (Lyer et al. 2006). Another potential benefit of DDVs is through encapsulating the cytotoxic agent, the DDV provides a barrier between the cytotoxic agent and the healthy tissue which minimizes direct contact between the two while the drug is in transit. This property of DDVs further reduces the unintended negative side-effects of chemotherapeutics relative to the utilization of unencapsulated free drug (Bozzuto and Molinari 2015). In fact, the maximum tolerated dose in mice of encapsulated doxorubicin is 55 mg/kg, while the dose of unencapsulated doxorubicin is limited to 18 mg/kg (Parr et al. 1997). An additional distinctive advantage associated with the use of nanocarriers is their ability to increase the solubilization of hydrophobic drugs, which many chemotherapeutics tend to be, thereby decreasing the possibility of embolism formation upon being administered intravenously as well as the need for toxic solubilizing agents

such as Cremophor EL (Kydd et al. 2017). In order to alleviate the many limitations unencapsulated cytotoxic agents possess via DDVs, there are many different types of nanocarriers that may be utilized such as dendrimers, micelles, and liposomes to name a few, some of which have been successfully utilized in recent research.

1.2.1 DENDRIMERS AS DRUG DELIVERY SYSTEMS

Dendrimers are highly branched polymers composed of a central core from which generations of dendrons grow with terminal surface groups at the end of the branches (Figure 1.4) (Lee and Larson 2009). The advantages associated with the use of dendrimers as drug delivery systems include biocompatibility, biopermeability across biological membranes, and their ability to encapsulate or conjugate a large variety of many different drugs (Madaan et al. 2014). In fact, there have been many successful studies using dendrimers to treat metastatic breast cancer. For example, She et al. has utilized a

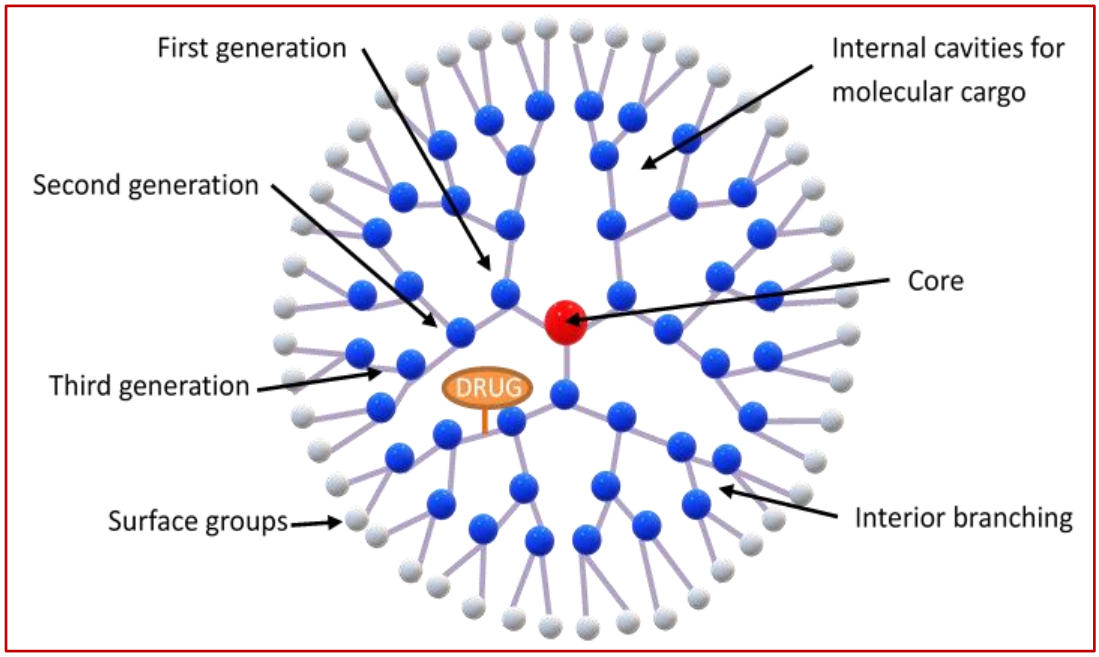

Figure 1.4 : Structure of a dendritic macromolecule.

dendronized heparin-doxorubicin conjugate as a pH-sensitive DDV for the treatment of 4T1 breast cancer cells both *in vitro* as well as modeled in female BALB/c mice *in vivo* (She et al. 2013). In this study, the dendrimer drug formulation was observed to have rapid drug release in acidic conditions characteristic of the tumor microenvironment contributing to its high effectiveness in 4T1 breast cancer cell killing and antiangiogenic properties with no significant toxicity to the healthy organs of both tumor-bearing and healthy mice. Another successful study utilizing dendrimers to treat breast cancer involves the conjugation of Trastuzumab, a common monoclonal antibody used to treat human epidermal growth factor receptor 2 (HER-2) positive breast cancer, directly to the dendrimer (Miyano et al. 2010). In this study, Miyano et al. demonstrates significant internalization and cytotoxicity of the dendrimer-drug conjugate in HER-2-positive SKBR3 breast cancer cells compared to a significantly less cytotoxic effect observed in the non-malignant breast cell line MCF-7. Although dendrimers have demonstrated some success in the treatment of breast cancer, there are major disadvantages associated with these systems. For example, it is well known that all classes of dendrimers present cytotoxic and hemolytic properties primarily due to the strong cationic charcteristics of these nanoparticles resulting in cell destabilization, leakage of cytoplasmic proteins, and subsequent lysis (Mendes, Pan and Torchilin 2017).

1.2.2 MICELLES AS DRUG DELIVERY SYSTEMS

The use of micelles has also been of particular interest as drug delivery systems. Micelles are composed of amphiphilic molecules that are oriented with their hydrophilic polar head groups facing the outside of the DDV while the hydrophobic tails are located internally (Figure 1.5). Synthetically, micelles are formed spontaneously by aggregation of amphiphiles as the critical micelle concentration (CMC) is reached (Oerlemans et al. 2010). Polymeric micelles are small yet versatile in size ranging from 5 to 100 nm in diameter, characterized by their block-copolymers, and are sterically stable due to their hydrophilic shell (Oerlemans et al. 2010). In fact, there have been many successful studies utilizing micelles for the delivery of cytotoxic agents. For example, Li et al. has developed ultra-small polymeric micelles for the delivery of the drug docetaxel intended to treat metastatic breast cancer (Li et al. 2014). Li et al. reports that due to the small size of the nanoparticles, they were quite effective at lymphatic delivery displaying high chemotherapeutic efficacy

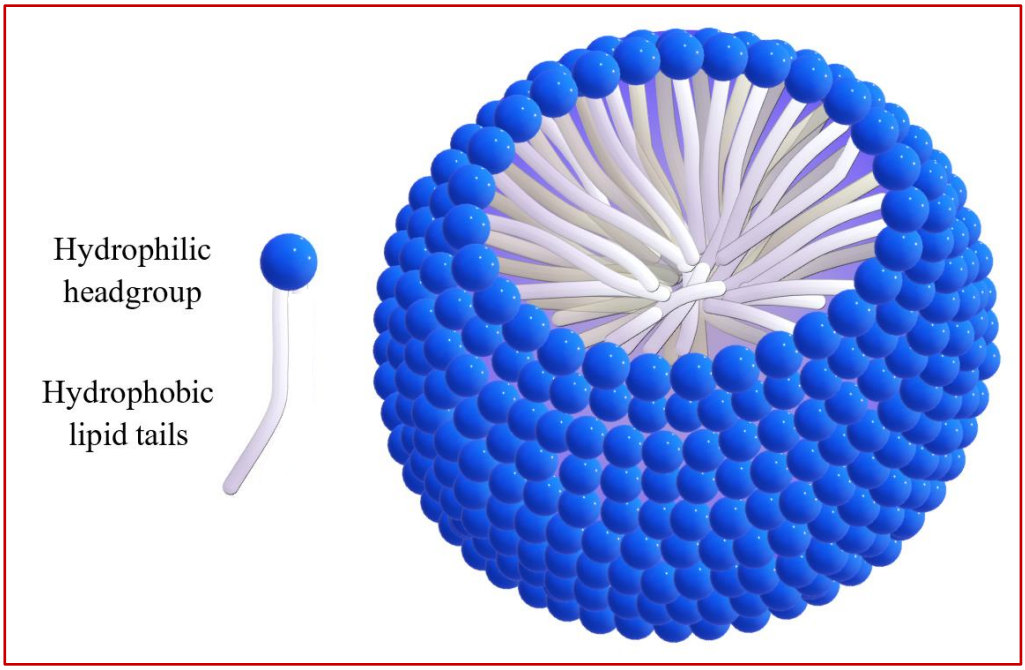

Figure 1.5 : Figure depicting the structure of a micelle.

against breast cancer metastases of 4T1 tumors in female BALB/c mice. Although micelles have proven to be somewhat efficacious DDVs, there are some inherent disadvantages associated with the use of these particular DDVs. For example, significant dilution occurs when micelles are introduced into physiological conditions such as the blood stream or the lymphatic fluid. This results in destabilization of the DDV and potential subsequent leakage of encapsulated cytotoxic agent prior to its arrival at the tumor site (Hanafy, El-Kemary and Leporatti 2018). Another considerable disadvantage associated with the use of micelles as DDVs is the fact that they are somewhat limited with respect to encapsulation of hydrophilic chemotherapeutic agents such as doxorubicin (Xu, Ling and Zhang 2013). Liposomes however, have the potential to retain stability while in transit through the blood stream as well as being able to accommodate both hydrophilic and hydrophobic drugs (Khan et al. 2018).

1.2.3 LIPOSOMES AS DRUG DELIVERY SYSTEMS

1.2.3.1 HISTORY OF LIPOSOMES

Liposomes were first described in 1961 by Dr. Alec D. Bangham after observing images of phospholipids spontaneously forming closed bilayer membrane systems (Bangham and Horne 1964). This was accomplished utilizing electron microscopy to observe the dispersions of dry phospholipids when added to a solution of negative stains. During the late 1970s to early 1980s the liposome's versatility and utility began to be realized after further investigation of its stability, behavior in the human body, cellular interactions, as well as their performance as DDVs (Figure 1.6) (Sharma et al. 2010). Today, liposomes are successfully used in a variety of scientific fields such as biophysics

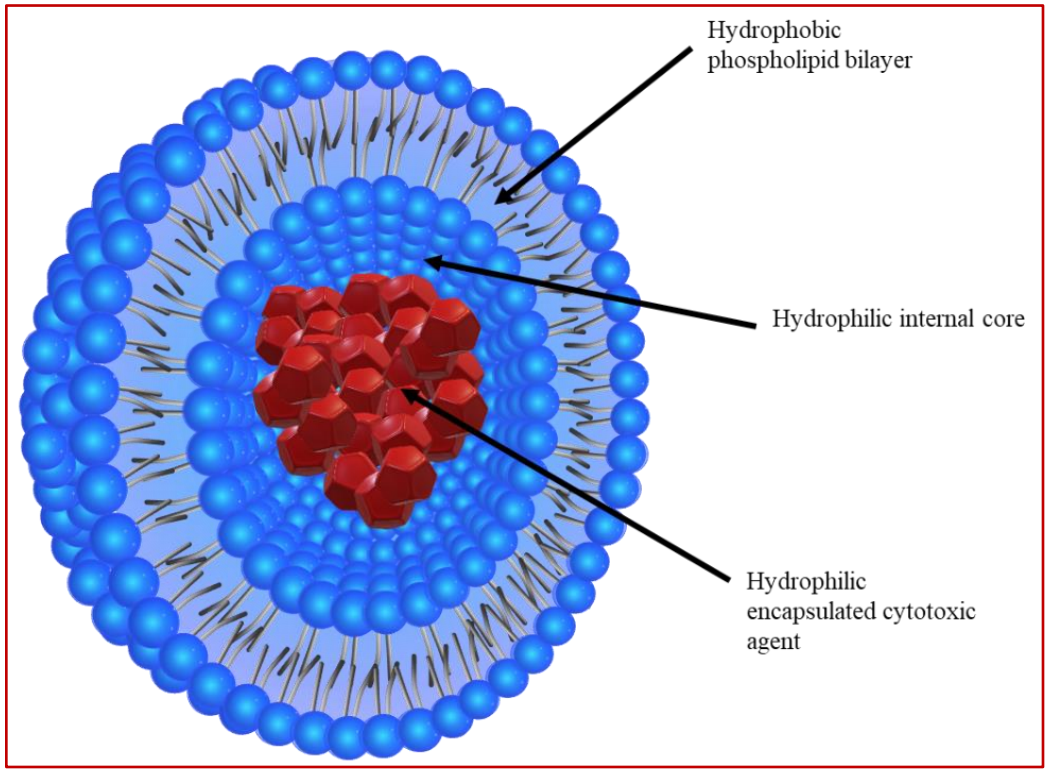

Figure 1.6 : Figure depicting the structure of a liposome encapsulating cytotoxic agent.

to model properties of cell membranes, chemistry to investigate catalysis and energy conversion, biology to study cell function, and pharmacology to develop effective DDVs (Sharma et al. 2010). In fact, liposomes have been of particular interest in recent research as they are quite versatile in their application in the medical field such as tumor imaging, vaccine and gene delivery, as well as the treatment of infections and cancer (Allen and Cullis 2013). Although liposomal applications are wide in scope due to their biocompatibility, the main focus here is liposomes utilized as DDVs for the treatment of breast cancer.

1.2.3.2 LIPOSOME CLASSIFICATION

Liposomes are generally classified by size (ranging from 10 nm to 2.5 µm) and number of bilayers, or lamellarity (Laouini et al. 2012). Liposomes are most commonly organized into two major categories, multilamellar vesicles (MLVs) and unilamellar vesicles (ULVs). ULVs are further divided into two subcategories: large unilamellar

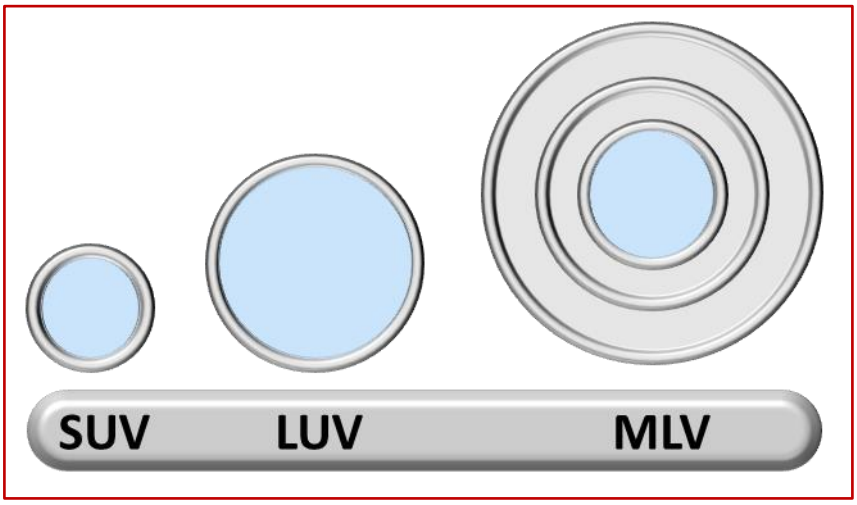

Figure 1.7 : Schematic diagram depicting structural differences between SUVs, LUVs, and MLVs which are classified based on size and number of bilayers.

vesicles (LUVs) and small unilamellar vesicles (SUVs) (Figure 1.7). MLVs consist of many phospholipid bilayers, which have an average diameter of 1 to 5 μm (Laouini et al. 2012). The structure of MLVs are composed of many phospholipid bilayers entrapped in one another decreasing in size, with each phospholipid bilayer separated by an aqueous polar solution. When MLVs encapsulate a drug, such as the Extended-Release Epidural Morphine (EREM) or DepoDur™ utilized for post-cesarean pain, the nanocarrier slowly releases the morphine cargo into the epidural space for approximately two days as compared to conventional, single-dose neuraxial morphine sulfate with is limited to the first postoperative day (Bulbake et al. 2017). This slow, sustained release of drug from DepoDur™ is attributed to the systemic degradation of each bilayer of the MLV. Although a slow releasing DDV may be particularly effective for long lasting pain relief, it is undesirable for cancer treatment which requires rapid chemotherapeutic release. This is due in part to the fact that tumor cells require exposure to a swift, lethal dose of the drug in order to circumvent challenges such as multidrug resistance (MDR) (Liang et al. 2010). In fact, MDR can be attributed to a variety of factors that occur during cancer treatment such as changes in tumor-site physiology as well as tumor cell acquired resistance where resistant tumor cells survive treatment from a previous population that was initially sensitive to chemotherapy (Liang et al. 2010). Therefore, unilamellar vesicles, which can be obtained after many rounds of MLV sonication and extrusion, are far more adequate for the effective treatment of cancer. LUVs have a single bilayer, possess a mean diameter of 120-200 nm, and have the potential to store a greater amount of chemotherapeutic cargo relative to vesicles smaller in size (Blanco, Shen and Ferrari 2015). However, the relatively large size of LUVs has the potential to result in significant splenic filtration of the particles

causing dramatic decrease in circulation times of the drug (Blanco et al. 2015). On the other hand, SUVs are the smallest of the liposomes which also have a single bilayer and a diameter ranging from 50 – 100 nm in diameter, subsequently allowing for greater circulation times in part by organ filtration circumvention (Laouini et al. 2012). Furthermore, SUVs can be surface-modified with polymers that provide the DDV with stealth like properties for immune system evasion. Collectively, these properties allow liposomes to circulate *in vivo* long enough to accumulate in great enough abundance such that a lethal cytotoxic dose of the drug can be delivered to the tumor site. Furthermore, SUVs have the increased propensity when compared to LUVs to successfully extravasate through large vascular fenestrations at the tumor site as previously described by the EPR effect (Blanco et al. 2015).

1.2.3.3 LIPOSOME COMPONENTS

Phospholipids are found naturally in cell membranes and are therefore ideal candidates for liposomes intended for *in vivo* use as they are biocompatible. A phospholipid is composed of a polar head group which contains a phosphate bound to a glycerol backbone esterified to two fatty acid chains (Figure 1.8). Different types of phospholipids are characterized by their length and saturation of their fatty acid tails as well as different head groups. These characteristics can alter the stability and physical properties of the membrane. Phospholipids are amphiphilic in nature due to the fact that they are composed of a hydrophilic polar head group and a hydrophobic non-polar tail group. The lipids form a spherical lipid bilayer as the diacyl tails interdigitate with one another forming a hydrophobic membrane, while the hydrophilic head groups are oriented both internally forming an aqueous core as well as externally toward the extra-liposomal space (Okamoto et al. 2018). The membrane can therefore carry hydrophobic drugs in the bilayer, and the internal aqueous core can accommodate hydrophilic drugs (Khan et al. 2007).

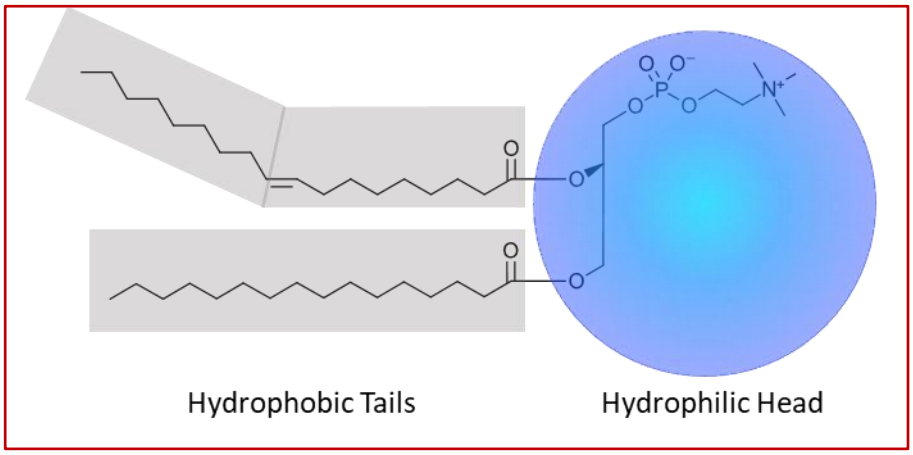

Figure 1.8 : Structural diagram of a phospholipid which is composed of a hydrophilic head group containing a phosphate as well as two hydrophobic, fatty acid tails.

There are many different types of phospholipids which are characterized by their different head groups, as well as the length and degree of saturation of the diacyl tails. This in part lends to phospholipids having different phase transition temperatures (Tm), which is the point at which phospholipids transition from a gel to a liquid crystalline state. For example, phospholipids with long, saturated diacyl tails have a relatively high Tm and form rigid bilayer membranes with minimal permeability via strong hydrophobic interactions, while their shorter/unsaturated counterparts have lower transition temperatures and therefore form less stable membranes. Thus, a fine balance must be achieved when selecting phospholipids used for these constructs such that the drug delivery vehicle is stable while in circulation, yet retains the ability to release its cargo upon arrival at the tumor site. Therefore, phospholipids with transition temperatures near physiological temperatures tend to be optimal for liposomal DDVs (Kitayama et al. 2014). As such, many researchers have focused on dipalmitoylphosphatidylcholine (DPPC), distearoylphosphatidylcholine (DSPC), and hydrogenated soy phosphatidylcholine (HSPC) (Figure 1.9) (Kitayama et al. 2014) with transition temperatures of 41 ºC, 55 ºC, and 52 ºC respectively (Table 1.1) (Li et al. 2015) for optimal, stable liposomal formulation with relatively ideal drug delivery capabilities.

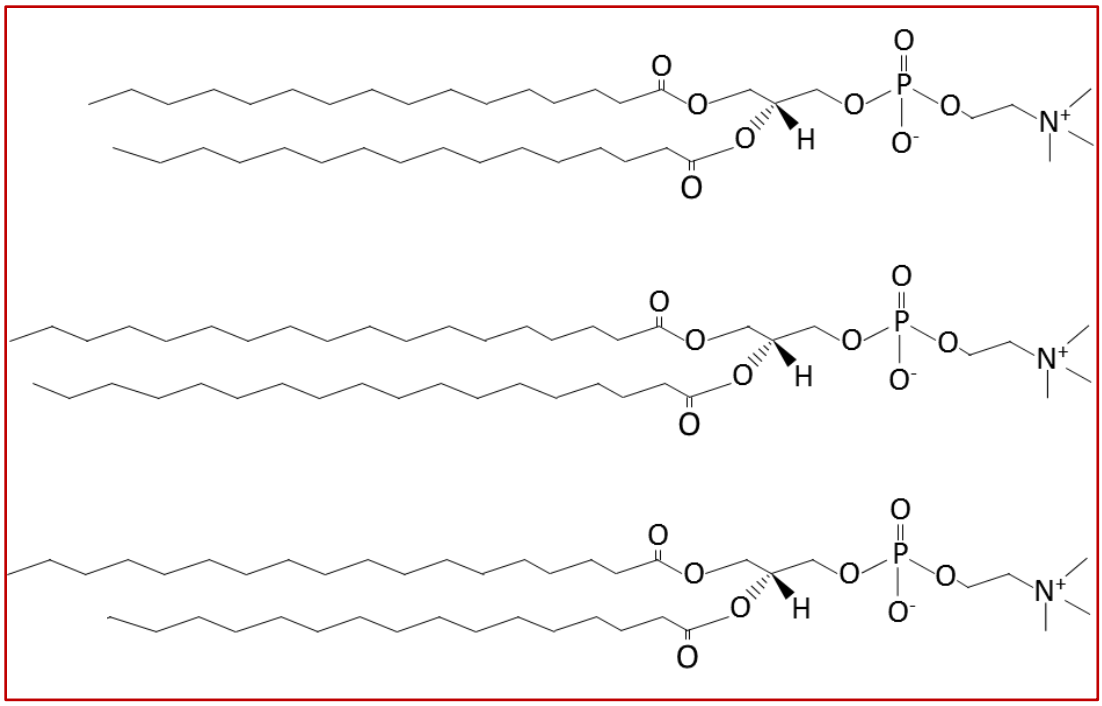

Figure 1.9 : Structures of Dipalmitoylphosphatidylcholine (DPPC) (top), Distearoylphosphatidylcholine (DSPC) (middle), and Hydrogenated Soy Phosphatidylcholine (HSPC) (bottom).

Phospholipids	Transition Temperatures ºC
Dipalmitoylphosphatidylcholine (DPPC)	41 ºC
Distearoylphosphatidylcholine (DSPC)	55 ºC
Hydrogenated Soy Phosphatidylcholine (HSPC)	52 ºC

Table 1.1 : Transition temperatures of the three common phospholipids utilized in liposomal DDV formulations (Li et al. 2015).

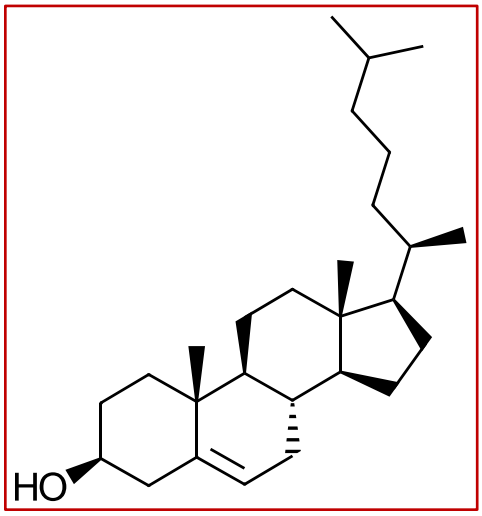

Figure 1.10 : Structure of cholesterol.

Another component essential to stable liposomal formulation is cholesterol (Figure 1.10). Cholesterol, which is also found in biological membranes, is composed of a tetracyclic fused ring skeleton with a single hydroxyl group located at carbon 3, a double bond between carbons 5 and 6, and an iso-octyl hydrocarbon side chain at carbon 17, and orients itself in the membrane according to polarity (Urich 1994). Thus, the hydroxyl group orients itself alongside the polar phosphate head groups of the phospholipids, leaving the hydrophobic steroid rings buried within the hydrophobic membrane (Figure 1.11) (Ohvo-Rekila et al. 2002). The addition of cholesterol provides further rigidity to the liposomal membrane which results in a decrease in liposomal permeability at temperatures slightly above the respective T_m that is proportional to the relative concentration of cholesterol added (Demel and Kruyff 1976).

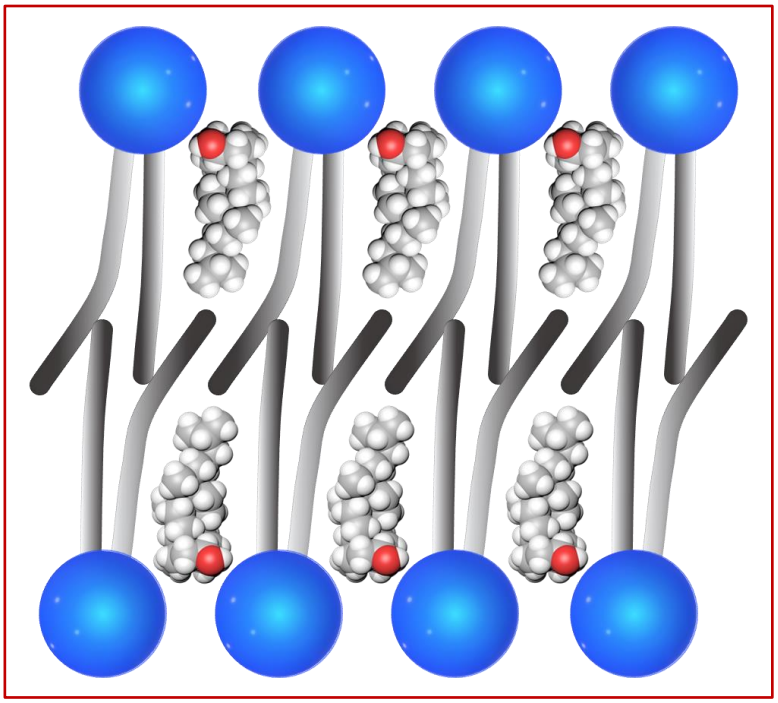

Figure 1.11 : Diagram depicting cholesterol in a phospholipid bilayer interacting with the fatty acid tails oriented parallel to and buried within the membrane.

Another component that has been revolutionary to the clinical success of this particular DDV is the surface modification involving the addition of polyethylene glycol (PEG) (Figure 1.12). PEG is a biocompatible polymer that was identified in the 1980s for its ability to provide “stealth like” properties to liposomes while in transit (Moghimi and Szebeni 2003). This is primarily due to the fact that PEG has the potential to protect the liposome from the patient’s immune system thus extending blood circulation time of the DDV (Veronese and Harris 2002) via reduced immunogenicity and antigenicity (Bulbake et al. 2017). This in part attributed to the fact that PEGylated liposomes have the potential to diminish opsonization by macrophages (Malam, Loizidou and Seifalian 2009). While in transit, liposomes are exposed to a variety of proteins that have the potential to adhere to the surface of the liposome most often due to size, with larger objects having more adhesion

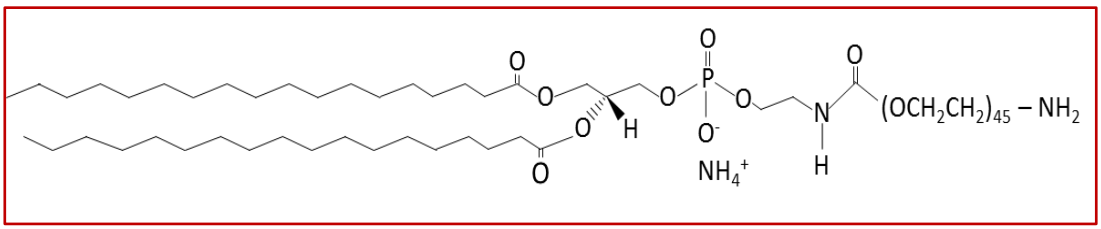

Figure 1.12 : The molecular structure of PEG_{2000}.

compared to smaller ones (Yan, Scherphof and Kamps 2005). Many of the liposome-associated blood proteins are classified as opsonins, meaning they target the liposome for destruction specifically by the adherence of these particles to be recognized by receptors on the surface of macrophages as well as hepatocytes for cellular uptake and renal clearance (Kamps, Morselt and Scherphof 1999). The increased circulation times of current liposomal therapies is widely attributed to the PEGylated property of these liposomes which suppresses protein adherence to the liposome surface due to the presence of the steric barrier characteristic of the polymer brushes (Yan et al. 2005). In fact, in a study done by Gabizon et. al, up to 70% of the administered dose of non-PEGylated liposomes were removed from circulation and found in reticuloendotheial system (RES) tissues (Gabizon and Martin 1997). On the other hand, the clinically approved liposome Doxil®, which is modified with PEG_{2000}, remains in circulation much longer with only 10-15% of the of the drug being accumulated in the RES.

Overall, the addition of PEG to the liposomal membrane has the capability to improve liposomal circulation times, which allows for increased accumulation in the tumor microenvironment and subsequent passive delivery of the drug to tumor cells (Barenholz 2012). However, while the use of PEGylated liposomes has exhibited promising clinical success in the treatment of metastatic breast cancer, future work involves further improving these constructs for more efficacious active drug delivery (Figure 1.13) (Large et al. 2018).

This form of targeted delivery generally involves the addition of a targeting ligand to drug formulations, which are specific to certain upregulated cancer cell surface receptors in order to selectively bind cancerous versus non-cancerous cells (Figure 1.14).

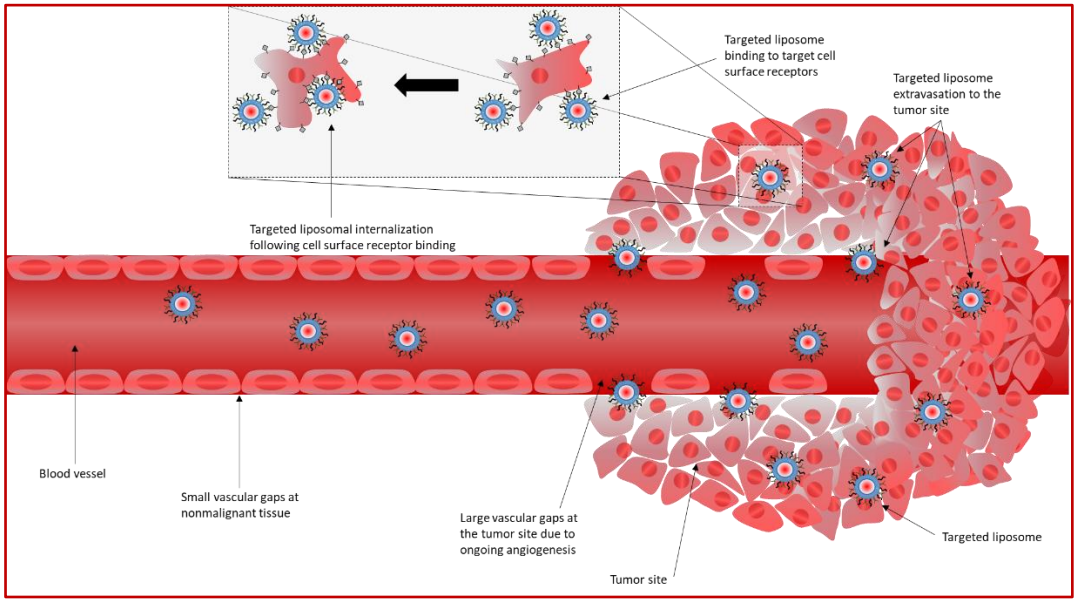

Figure 1.13 : Diagram representing active targeted drug delivery involving extravasation of the targeted liposomes to the tumor site through large vascular gaps present in the tumor microenvironment. Once in the tumor microenvironment, internalization of the targeted liposome follows the targeting ligand binding its specific receptor (adapted from (Khan et al. 2018)).

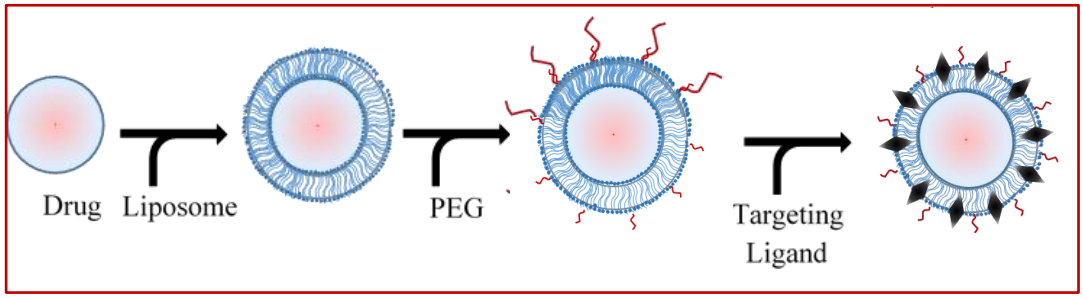

Figure 1.14 : Depiction of innovations in drug delivery for chemotherapeutic agents.

1.3 TARGETED LIPOSOMES

Liposomes can be surface-modified in order to accommodate the addition of targeting ligands specific to various known upregulated cancer cell surface receptors, thus allowing for improved drug/cancer cell colocalization. Furthermore, this can also dramatically enhance cellular internalization of the drug, particularly with receptors known to undergo receptor-mediated endocytosis (Jani and Krupa 2019). There are currently a variety of potential targeting ligands that may be utilized with liposomes that have recently been reported with varying levels of success such as vitamins, antibodies, and peptides (Noble et al. 2014).

1.3.1 VITAMINS AS TARGETING LIGANDS

There are several studies that utilize vitamins as targeting ligands on the surface of liposomes for the treatment of a variety of cancers (Fields 2007, Goren et al. 2000). For example, in a recent study by Nguyen et al., liposomes intended to treat metastatic breast cancer and loaded with doxorubicin are surface modified with folic acid or vitamin B9 conjugated to the lipids (Nguyen et al. 2019). In this study, it was determined that the presence of the vitamin B9 targeting ligand significantly enhanced cellular uptake of liposomes as well as tumor shrinkage in 4T1 tumor-bearing mice up to 50% (Nguyen et al. 2019). While there are numerous other similar studies that could be mentioned here, it should be noted that there are several disadvantages associated with liposomes surface-modified with vitamins intended for this purpose. For example, low tumor specificity has been observed due to the fact that many targeting receptors that bind various vitamins are also present at similar expression rates on normal cells relative to cancer cells (Jiang et al.

2019). Furthermore, the pre-saturation of vitamin targeting ligands is known to occur while in circulation as they are known to bind various other moieties non-specifically. Thus, this negatively affects receptor/ligand recognition between the cancer cell and the drug following arrival at the tumor site (Sapra 2003).

1.3.2 ANTIBODIES AS TARGETING LIGANDS

In order to overcome low target specificity associated with vitamins as targeting ligands, some researchers are investigating the potential of antibodies used as targeting ligands (Kirpotin, Park and Hong 1997, Fields 2007, Khan et al. 2019). More recently, Dumont et al. formulated liposomes encapsulating doxorubicin and decorated with the MM-302 antibody which is specific to HER-2 known to be overexpressed in metastatic breast cancer (Dumont et al. 2019). In this study, Dumont et al. found that unlike its non-targeted counterpart, MM-302 targeted liposomes controlled both primary and metastatic tumor burden in 4T1 BALB/c mice. Furthermore, the targeted drug formulation also prevented growth of new tumors in 90% of mice when treated with this DDV (Dumont et al. 2019). However, it is worth noting that while antibody targeting ligands do have the potential to offer high affinity and specificity towards their targets, there are some disadvantages associated with their use. For example, aggregation can occur between immunoliposomes due to their large size, which can contribute to potential cross-linking of antibody molecules (Sapra 2003). This then leads to quick clearance of immunoliposomes from circulation, resulting in minimal drug accumulation at the tumor site. Furthermore, antibodies used as targeting ligands also possess the potential to induce negative immunogenic response in the patient (Jiang et al. 2019, Sapra 2003).

1.3.3 PEPTIDES AS TARGETING LIGANDS

Peptides have also been utilized as targeting ligands and have many advantages when compared to either vitamins or antibodies. For example, peptides used as targeting ligands have high tumor specificity, are moderate in size and therefore have a low propensity for aggregation, and tend not to induce negative immunogenic responses in patients (Jiang et al. 2019). Furthermore, peptides can be easily synthesized, manipulated, and customized specific to the desired receptor of interest, and there have been many peptide-modified liposomal-based formulations recently reported (Fields 2007, Khan et al. 2007, Jaafari and Foldvari 2002, Medina et al. 2001). For example, Dai et al. has proposed a targeted liposomal DDV that is specific to the α3 receptor, which is upregulated in triple negative breast cancer (Dai et al. 2014). This formulation involved doxorubicin-loaded stealth liposomes that were surface-modified with a cyclic octapeptide specific to α3. Overall, the peptide-modified liposomes demonstrated significantly increased cellular uptake and decreased tumor weight by approximately 50 mg when compared to their non-targeted counterparts (Dai et al. 2014). In another recent study, Sun et al. has developed liposomes containing coencapsulated curcumin (CUR) and celecoxib (CXB), which have been previously shown to have an enhanced synergistic cytotoxic effect (Sun et al. 2019). These liposomes were also surface-modified with hyaluronic acid (HA) to bind the overexpressed CD44 receptor and a fusion peptide composed of NF-κB essential modulator-binding domain peptide (NBD) in order to bind and block the NF-κB pathway, along with the cell-penetrating peptide (TAT) in order facilitate cellular uptake. This pathway is known to induce persistent activation of tumor cells and tumor-associated stromal cells which leads to invasion and migration, metastasis, and inflammation through

the activation of the tumor necrosis factor α (TNF-α) pathway. Thus, NF-κB pathway suppression is highly desirable. In this study, *in vivo* results using the targeted liposomal based formulation in 4T1 breast tumor-bearing mice demonstrated an enhanced cytotoxic effect on cancer cells with increased cellular uptake, macrophage migration inhibition, an antimetastastic effect, and anti-inflammatory properties when compared to non-targeted liposomal treatment. Furthermore, TNF-α was suppressed by 50% when 4T1 cells were treated with the targeted liposome formulation. In any event, results from this and numerous other similar studies that have been recently reported suggest the very promising potential of using liposomes surface-modified to accommodate peptides used as targeting ligands in order to treat various types of cancer.

1.4 CELL SURFACE RECEPTORS (INTEGRINS)

Integrins are non-covalently associated heterodimeric subunits designated as α and β respectively (Figure 1.15). Integrins are receptors that are known to bind most extracellular matrix (ECM) proteins such as collagen, fibronectin, and laminin (Hamidi and Ivaska 2018). Integrins are also involved in cell-cell adhesion as well as cell signaling pathways (Humphries, Byron and Humphries 2006). In cancer cells, specific integrins are known to be overexpressed, which may provide a potential target for targeted DDVs to not only improve drug-cell colocalization, but also improve drug internalization in cancer cells via receptor-mediated endocytosis (Alday-Parejo, Stupp and Ruegg 2019).

The α3β1 integrin is one particular cell surface receptor that is known to be overexpressed in metastatic breast cancer (Mitchell et al. 2010). This cell surface receptor has a number of different functions such as cell-cell adhesion, cell signaling, as well as

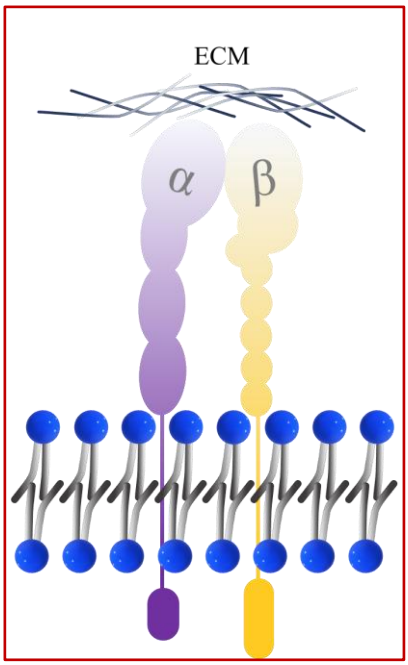

Figure 1.15 : Depiction of α and β subunits interacting with the extracellular matrix.

interactions with ECM proteins such as laminin, collagen, and fibronectin (Mitchell et al. 2010). It is also believed that the α3β1integrin is directly involved in metastasis through interaction with vascular laminins and matrix metalloproteinases (MMP) such as MMP-9 (Pouliot and Kusuma 2013). While somewhat unclear, it is believed that a defect in this integrin's cell signaling pathway in cancer cells plays a role in interfering with adhesion to the ECM as well as inducing ECM degradation, which has the potential to contribute to metastatic migration (Kusuma, Anderson and Pouliot 2011). Furthermore, when the α3β1 integrin is inhibited, cell migration and invasion are further decreased, emphasizing the receptors role in metastasis (Wang et al. 2004). Another factor that makes the α3β1 integrin an attractive target for peptide modified liposomes is its ability to undergo phagocytosis, which is a type of receptor-mediated endocytosis that engulfs large particles (Coopman et al. 2017). For example, in a study performed by Coopman et al., monoclonal antibodies specific to α3 were found to be cointernalized with gelatin and colocalized internally in acid vesicles (Coopman et al. 2017). In any event, the upregulation and receptor-mediated endocytosis characteristic of the α3β1 integrin distinguishes this receptor as a viable target for DDVs. Importantly, the peptide sequence to which this integrin binds in type IV collagen is known. This peptide sequence (GEFYFDLRLKGDK) can be found within the non-triple helical region of collagen IV (Parkin et al. 2010). Thus, this peptide sequence is known to bind both single stranded peptides just as avidly as triple helical peptides (Lauer, Gendron and Fields 1998). Furthermore, a C_{16} palmitic acid tail can be conjugated to the peptide at the C-terminus, which allows for the peptide sequence to remain at the liposomal surface while being anchored to the bilayer via hydrophobic interactions. Therefore, in this study, a unique single stranded α3β1 integrin-specific targeting peptide C_{16}-

GEFYFDLRLKGDK-NH_2 synthesized by ChinaPeptides was incorporated into the liposomal bilayer in order to develop novel peptide-modified targeted liposomes intended to treat metastatic breast cancer (Figure 1.16).

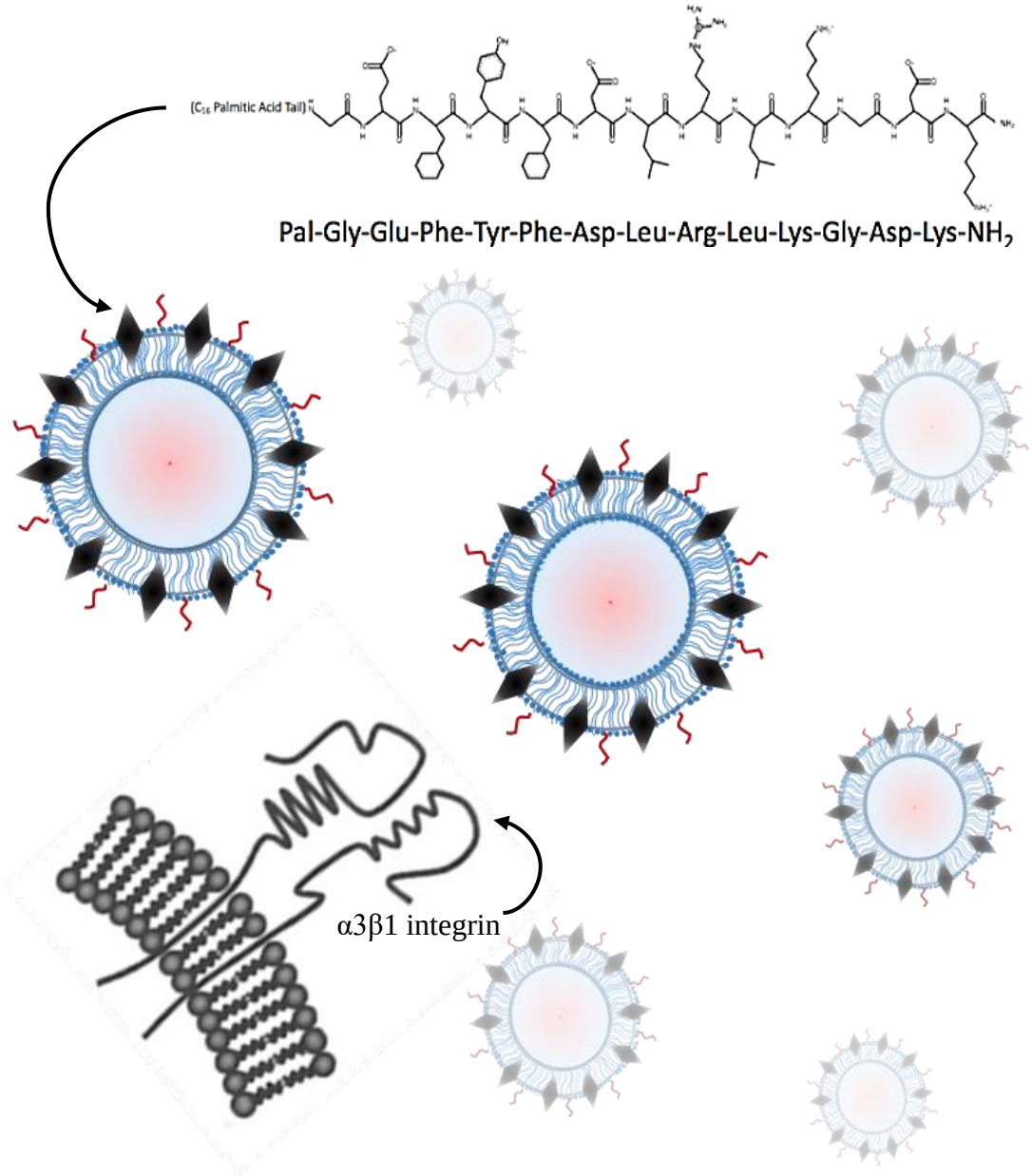

Figure 1.16 : Structure and sequence of the peptide generated using software from Tulane University "PepDraw" (top) as well as depiction of targeted liposomes specific to the $\alpha_3\beta_1$ integrin (bottom) (adapted from Fields, 2007).

CHAPTER II

EXPERIMENTAL PROCEDURE

2.1 MATERIALS AND METHODS

2.1.1 CHEMICALS FOR LIPOSOME PREPARATION

The phospholipids HSPC, DSPE-polyethylene-glycol-2000 (PEG_{2000}), DSPE-polyethylene-glycol-750 (PEG_{750}), and cholesterol utilized to synthesize liposomes were all purchased from Avanti Polar Lipids (Birmingham, AL). The peptide sequence, C_{16}-GEFYFDLRLKGDK-NH_2, was purchased from ChinaPeptides (Pudong New Area, Shanghai, China). All other chemicals were purchased from VWR (West Chester, Pennsylvania) unless stated otherwise.

2.1.2 LIPOSOME PREPERATION

HSPC and cholesterol were combined in a 1:1 molar ratio and if PEG was added, then the molar ratio of HSPC, cholesterol and PEG was 1:1:0.1 respectively. In the case of targeted liposomes, the peptide sequence C_{16}-GEFYFDLRLKGDK-NH_2 was added to the previously mentioned lipid mixture at a 1:1:0.02 molar ratio without PEG and 1:1:0.1:0.02 with PEG. The powdered liposome components were then dissolved in an organic phase mixture of methanol, tert-butyl ether, and chloroform (1:2:2.4) by stirring in a round bottom flask with a magnetic stir bar for thirty minutes in order to

achieve a homogenous solution. The organic phase was then separated under reduced pressure by rotary evaporation (Buchi, New Castle, DE), leaving a thin lipid film at the bottom of the flask which is then dried overnight in a desiccator containing phosphorous pentoxide. The aqueous phase, consisting of rhodamine 6G (400 μM) in phosphate buffered saline (PBS) was heated for ten minutes at 62°C before hydrating the lipid film in the round bottom flask spontaneously producing MLVs. Once the lipid film was hydrated by the aqueous phase, the solution was then allowed to sonicate for five to ten minutes. The solution then was then heated in a water bath set ten degrees above HSPC T_m in order to ensure adequate rhodamine encapsulation followed by one minute of freezing in an ice bath. This cycle is repeated five times per sample with vortexing in-between rounds to ensure a high encapsulation of rhodamine 6G in the vesicles. The MLVs formed from lipid film hydration are then subjected to ten cycles of extrusion through 100 nm double-stacked polycarbonate membrane filters (VWR, West Chester, Pennsylvania) utilizing a Lipex Extruder (Northern Lipids, Inc., Vancouver, British Columbia) at pressures typically between 250-700 psi. The newly formed ULVs of 100 nm in diameter are then added to a column to separate unencapsulated fluorophore from fluorophore-loaded liposomes by size exclusion chromatography using a Sephadex-G50 medium grade resin gel column (Amersham Biosciences, Piscataway, NJ) pre-conditioned with PBS (pH 7.4). In the figure below, the process of liposome preparation is summarized (Figure 2.1).

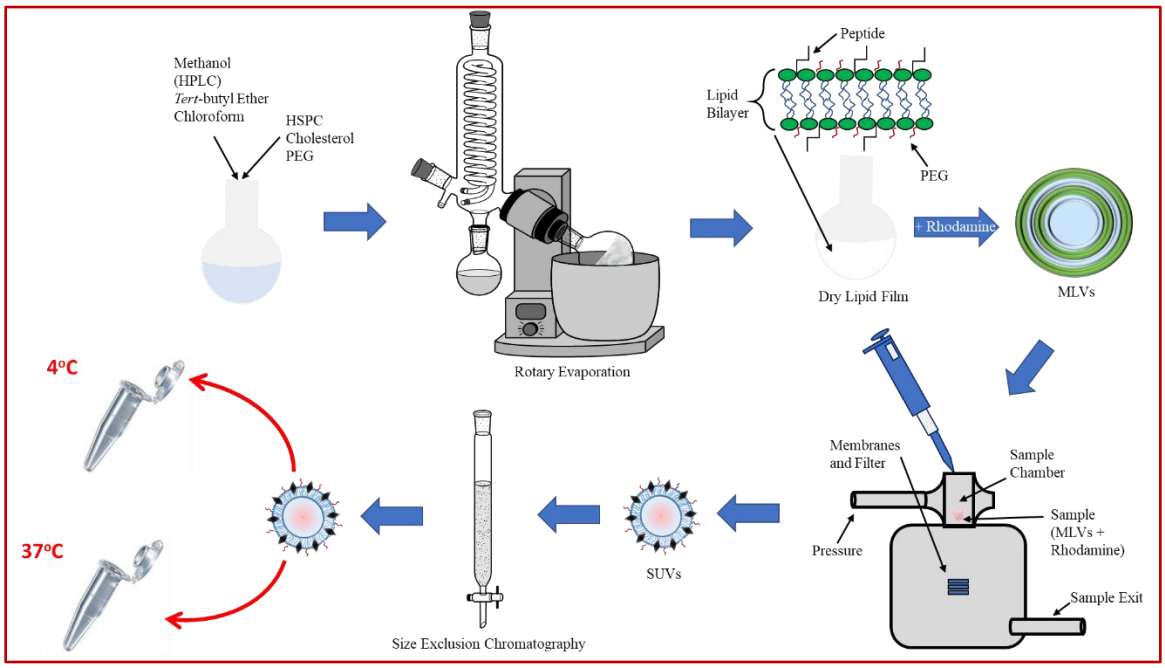

Figure 2.1 : Outline of liposome preparation and storage.

2.1.3 PRESENCE OF THE PEPTIDE

To validate the peptide was effectively incorporated in the liposomal membrane, absorbance scans were conducted from 240-320 nm to probe for the aromatic amino acid residues phenylalanine and tyrosine present in the peptide's sequence utilizing a Tecan infinite M200 fluorescence plate reader.

2.1.4 FLUOROPHORE RELEASE READINGS

To determine the fluorophore leakage rate of the liposomes, liposomes were stored at 37°C for four days to mimic physiological conditions as well as 4°C for thirty days to represent storage conditions. Fluorophore release measurements were taken utilizing a Tecan infinite M200 fluoresence plate reader (Tecan Research Triangle Park, NC) to quantify rhodamine 6G at $\lambda_{excitation}$ = 525 nm and $\lambda_{emission}$ = 555 nm using a quartz plate (Molecular Devices, Sunyvale, CA). Fluorescent measurements were determined at various time points throughout the trials at both temperatures of liposomes in either PBS (intact liposomes) or ethanol (broken liposomes). Percent fluorophore leakage was calculated by comparing readings of intact liposomes to broken liposomes which represents 100% dequenching.

CHAPTER III

EXPERIMENTAL RESULTS

3.1 PRESENCE OF THE PEPTIDE

Absorbance scans ranging from 240-320 of liposomes demonstrated that the targeting ligand was in fact effectively incorporated in the targeted liposomal formulations relative to the non-targeted systems (Figure 3.1-3.3). This was observed with liposomes containing no PEG, PEG_{750}, and PEG_{2000}.

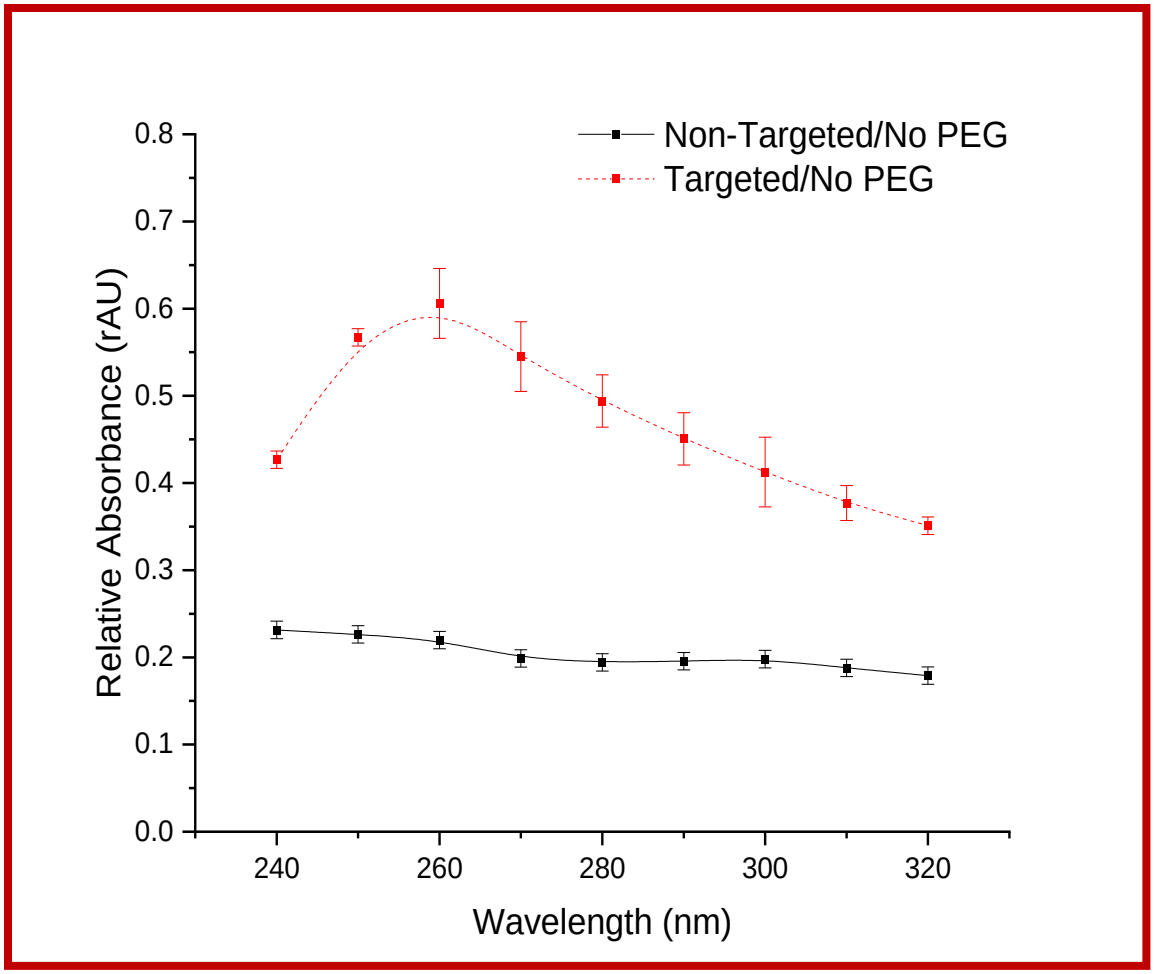

Figure 3.1 : Graph depicting the relative absorbance values of both targeted (red) and non-targeted (black) liposomes containing no PEG.

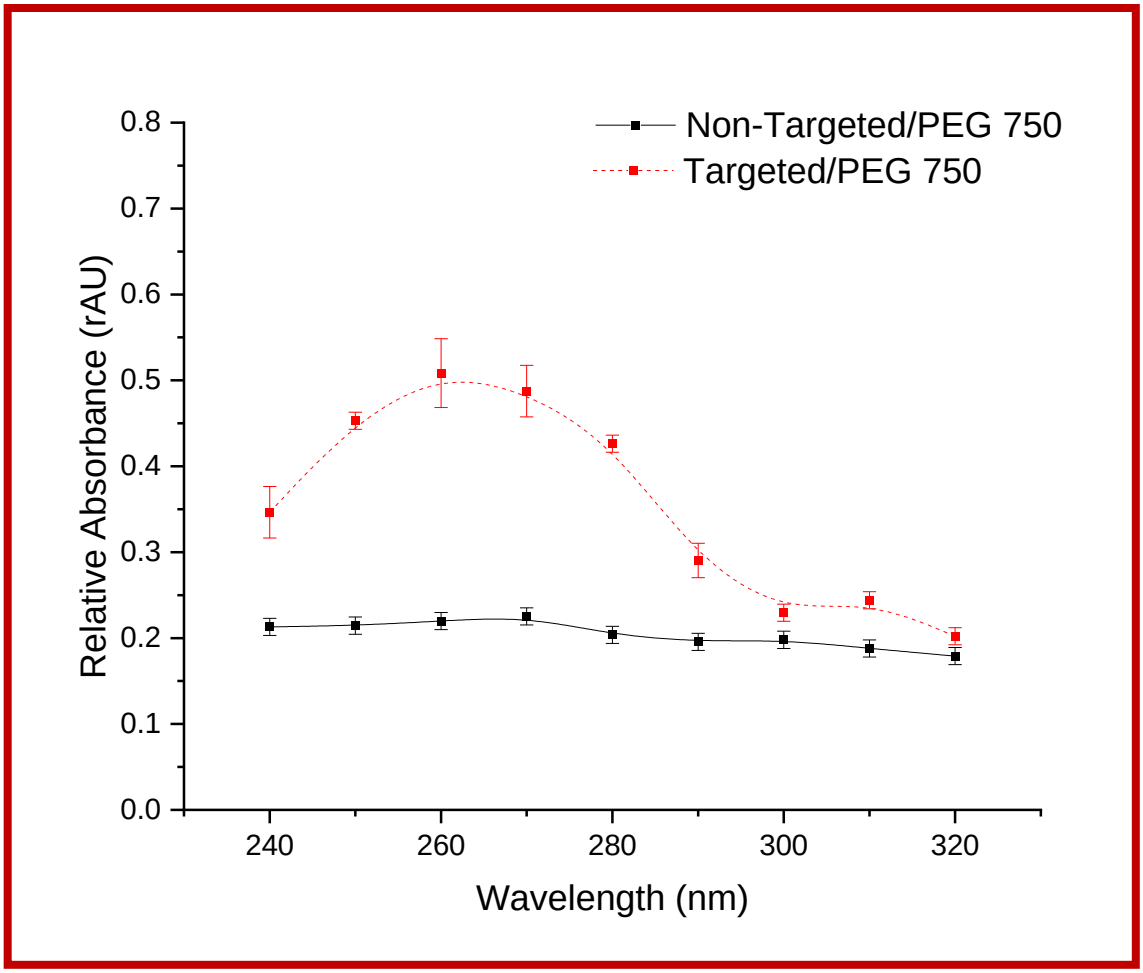

Figure 3.2 : Graph depicting the relative absorbance values of both targeted (red) and non-targeted (black) liposomes containing PEG_{750}.

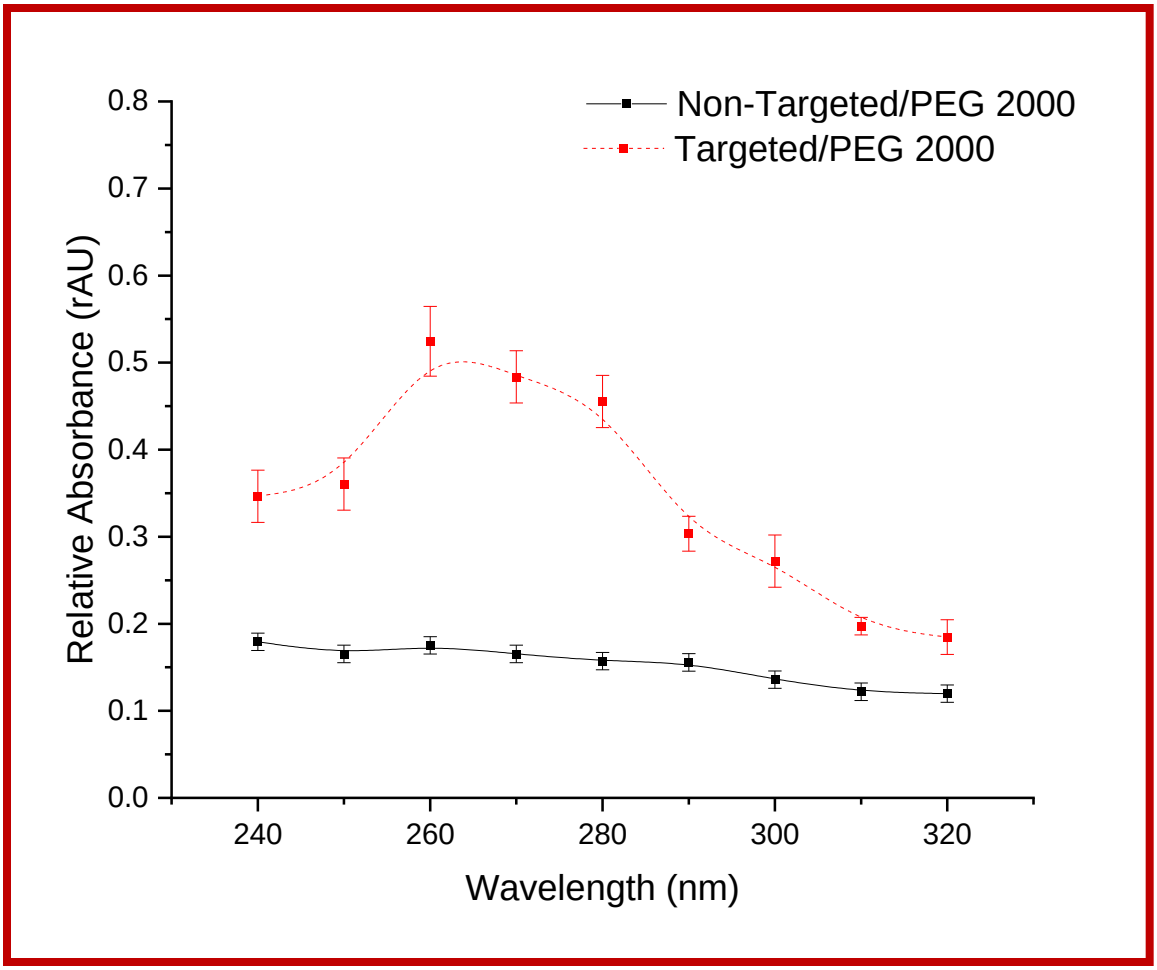

Figure 3.3 : Graph depicting the relative absorbance values of both targeted (red) and non-targeted (black) liposomes containing PEG_{2000}.

3.2 LIPSOME STABILITY: LEAKAGE RATES

In order to evaluate the effect the α3β1 integrin-specific targeting peptide has on overall liposomal stability, rhodamine loaded liposomes both with and without the peptide, designated as targeted and non-targeted respectively, were prepared. The stability profiles of liposomes encapsulating rhodamine 6G was determined by quantifying fluorophore release from the liposomal core at 4 and 37° at select time points over a four-week period. The fluorescence intensity was measured at $\lambda_{excitation}$=525 nm and $\lambda_{emission}$=555 nm. Complete release of fluorophore from the vesicles following ethanol treatment at each time point yields 100% dequenching and a saturated signal. Fluorophore that remains encapsulated in the liposomal core results in a quenched fluorescence reading and over time as rhodamine slowly leaks from the vesicles, the fluorescence reading will increase. The percent leakage of fluorophore released from the vesicles is quantified by evaluating the fluorescence intensity of rhodamine from intact liposomes (measured in PBS) relative to a control with 100% fluorophore leakage (ethanol-treated liposomes). Overall, the presence of the targeting ligand contributed no significant destabilization effect in vesicles prepared in this study. In fact, liposomes stored at 4°C had minimal fluorophore leakage over the thirty days which is expected as they were stored at low temperature preventing much of the phospholipid fluidity and liposome destabilization resulting in only about 25% leakage over the course of the study (Figure 3.4-3.6). In contrast, liposomes stored at 37°C did have increasing leakage rates over time reaching approximately 40 to 50% leakage in four days for vesicles both with and without the peptide amphiphile (Figure 3.7-3.9). This observation was also expected based on the T_m of 52°C for HSPC. Furthermore, it is important to note that there was no significant difference in leakage rates between the

targeted and non-targeted liposomes containing no PEG, PEG_{750}, and PEG_{2000} stored at both 37°C and 4°C further indicating that the presence of the peptide has no significant effect on liposomal stability (Figure 3.4-3.9).

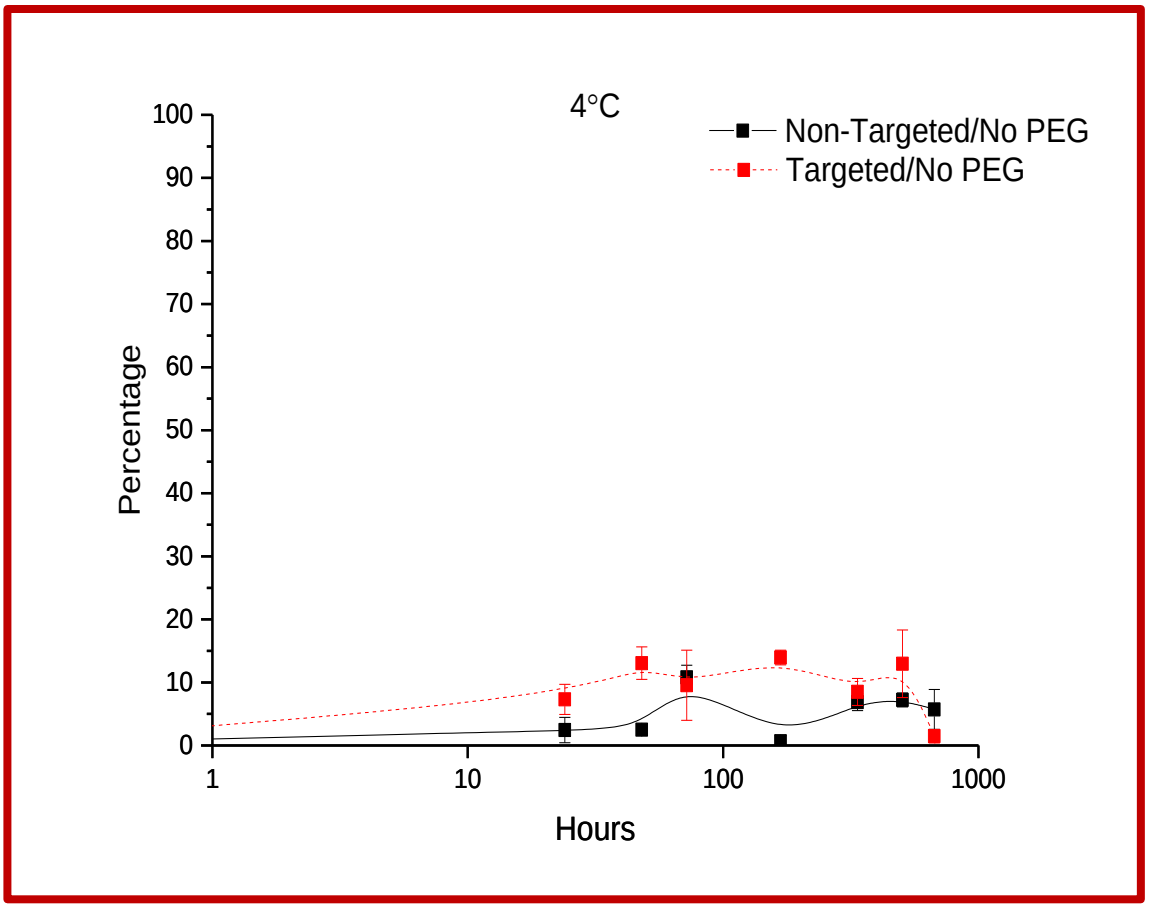

Figure 3.4 : Leakage rates of non-targeted (black) and targeted (red) HSPC liposomes at 4ºC over 4 weeks.

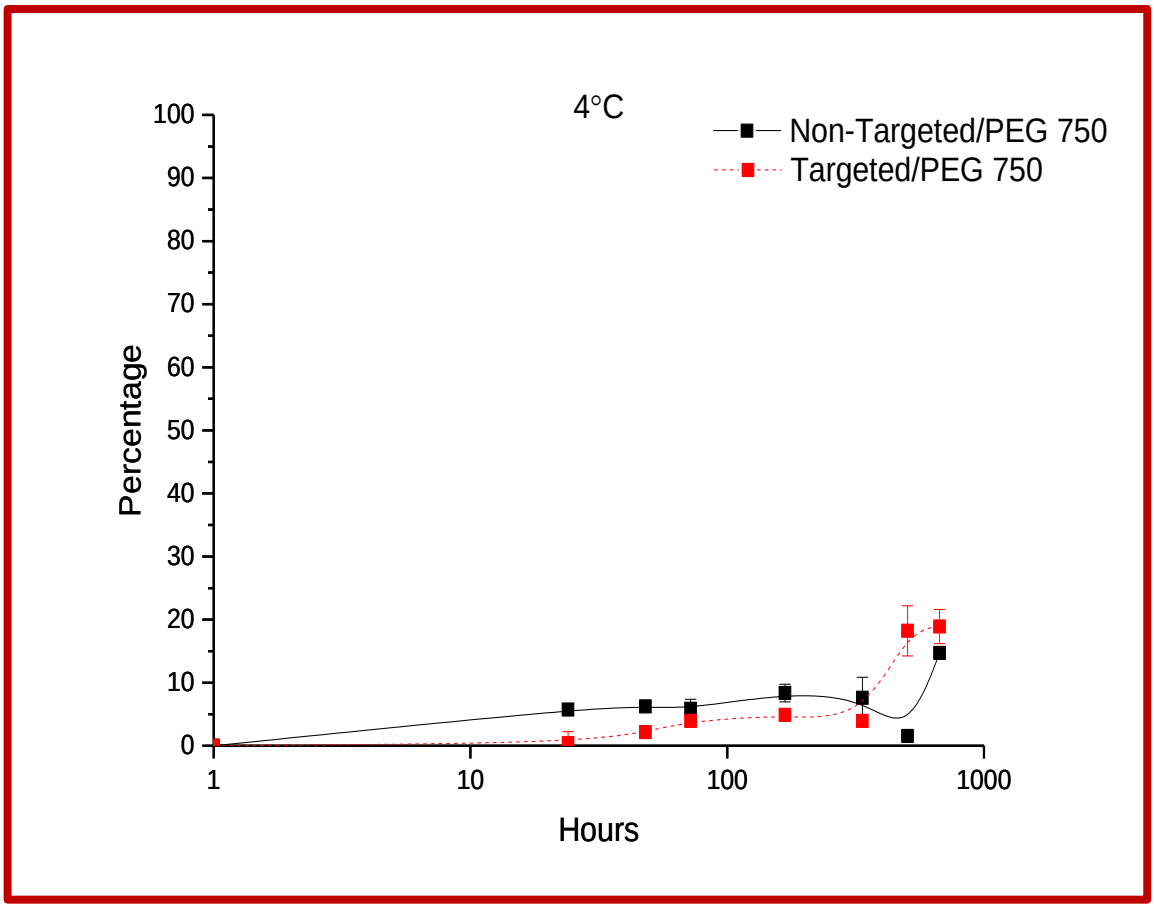

Figure 3.5 : Leakage rates of non-targeted (black) and targeted (red) HSPC and PEG 750 liposomes at 4ºC over 4 weeks.

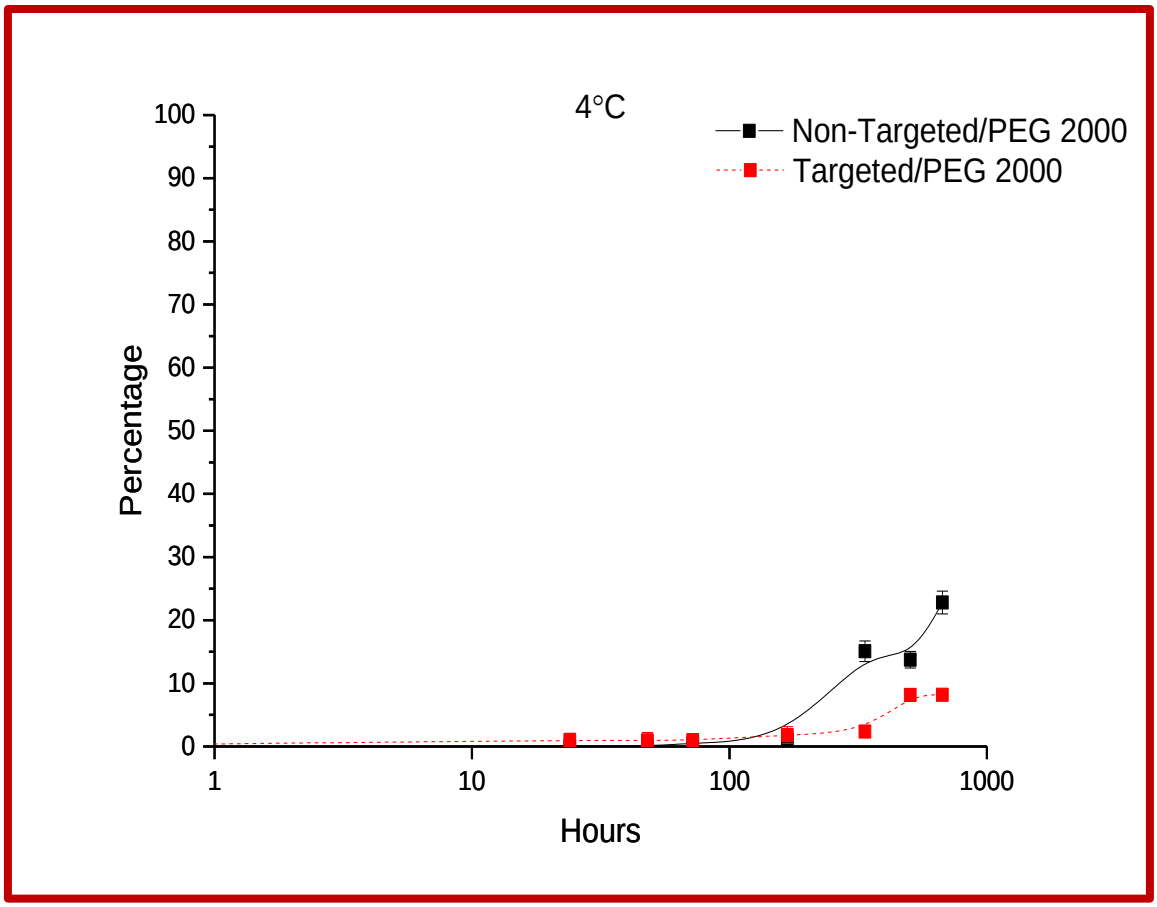

Figure 3.6 : Leakage rates of non-targeted (black) and targeted (red) HSPC and PEG 2000 liposomes at 4ºC over 4 weeks.

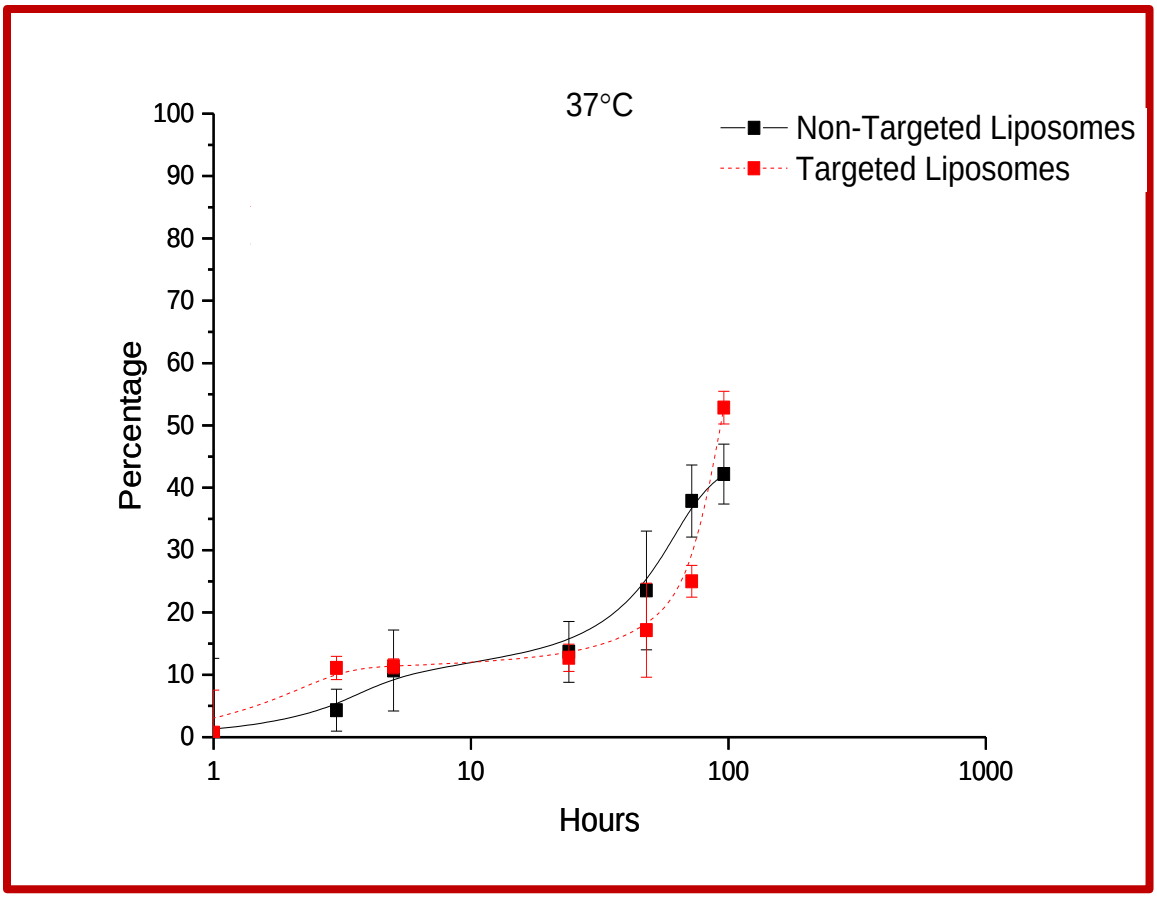

Figure 3.7 : Leakage rates of non-targeted (black) and targeted (red) HSPC liposomes at 37ºC over 4 days.

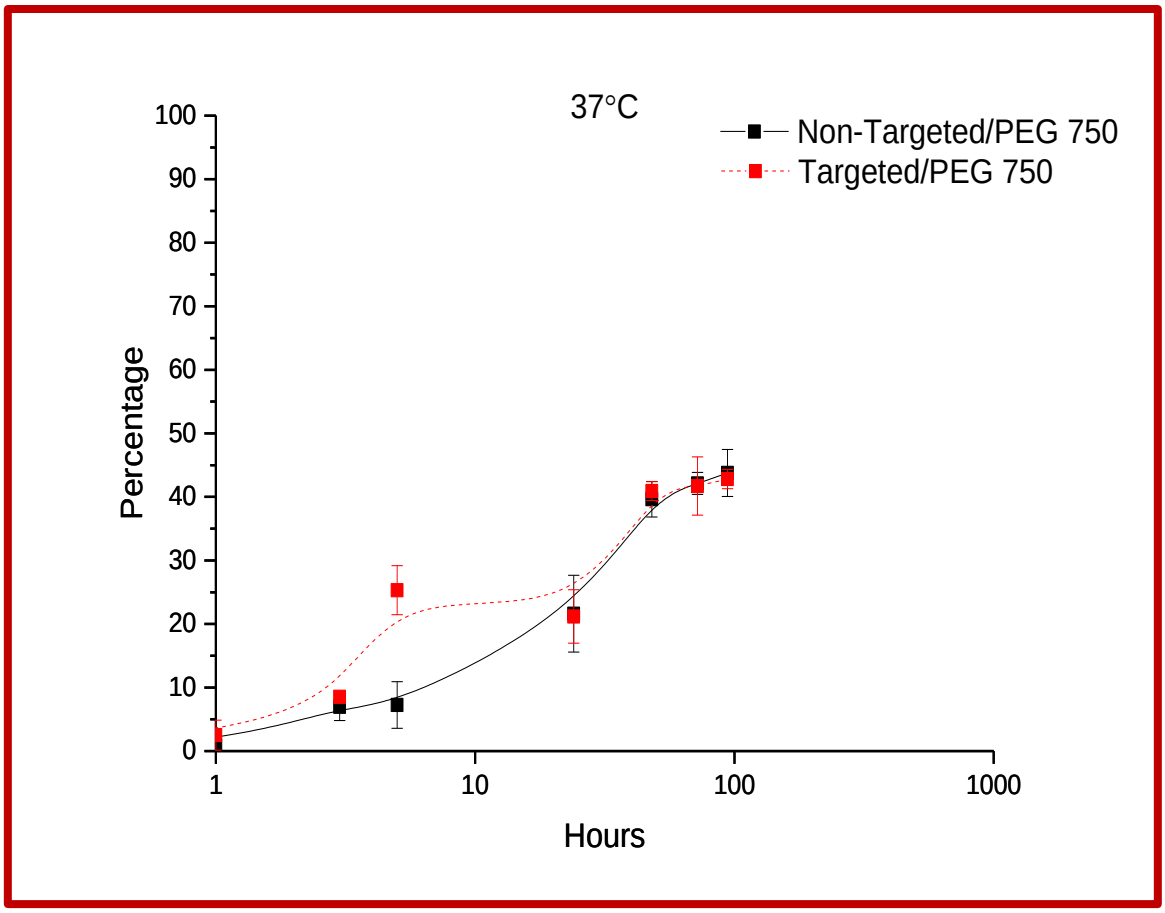

Figure 3.8 : Leakage rates of non-targeted (black) and targeted (red) HSPC and PEG 750 liposomes at 37 ºC over 4 days.

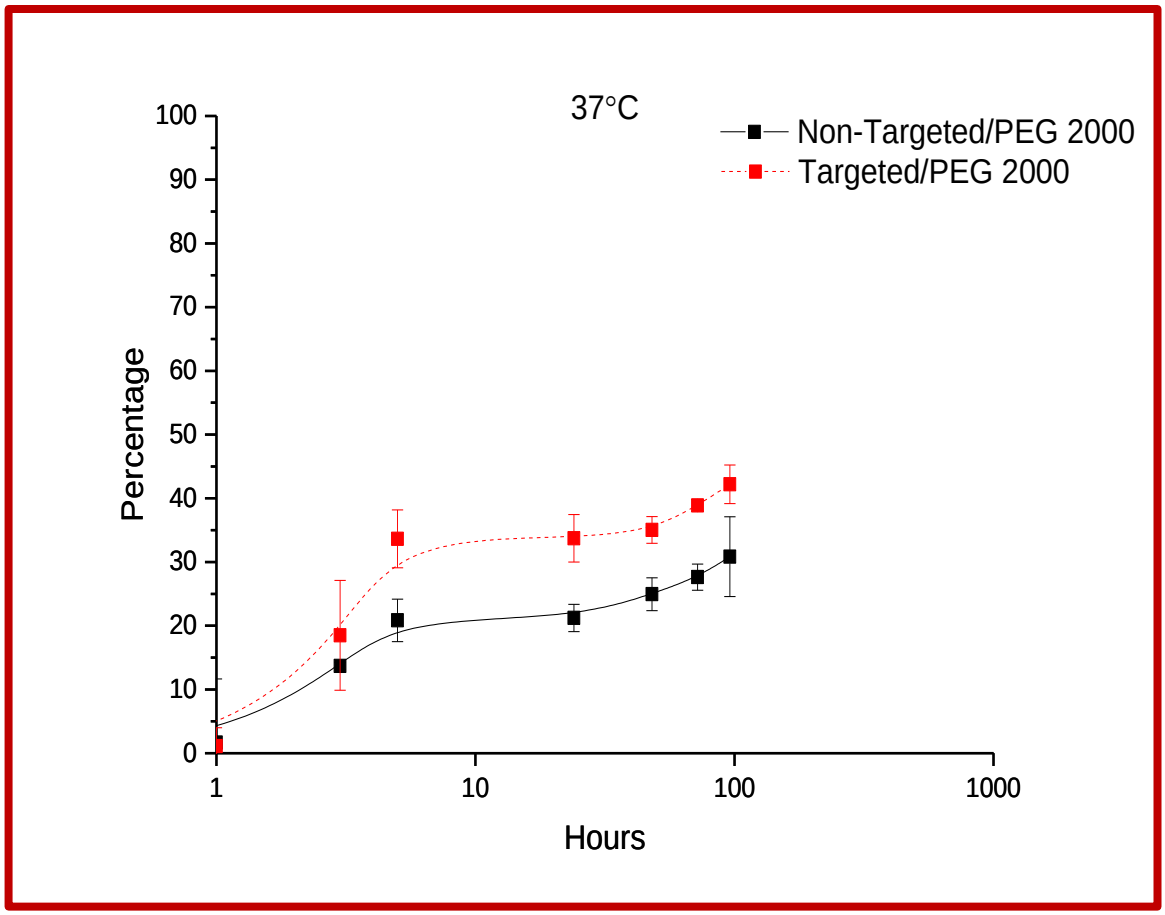

Figure 3.9 : Leakage rates of non-targeted (black) and targeted (red) HSPC and PEG 2000 liposomes at 37 ºC over 4 days.

CHAPTER IV

CONCLUSION

As stated in the introduction, breast cancer contributes to 30% of cancer diagnoses in women located in the United States annually and is therefore a particularly predominant disease that has acquired the attention of researchers interested in developing new, innovative chemotherapeutics. Due to a multitude of innovations involving drug delivery and design, liposomes have recently gained some attention regarding their potential as drug delivery vesicles due to their ability to improve therapeutic and pharmacological properties of otherwise unencapsulated chemotherapeutics. In fact, PEG modifications have significantly improved liposomal circulation times primarily due to the steric barrier this molecule provides which prevents interaction with many components of the RES. Although PEGylated liposomes such as the clinically approved drug Doxil® have experienced some therapeutic success, these systems are limited primarily due to the fact that they are not targeted. Current research has made significant efforts in developing stable, effective targeted drug delivery systems that are specific to cancer cell surface receptors that are upregulated. In fact, the α3β1 integrin is one particular cell surface receptor that has been shown in multiple studies to be significantly upregulated in metastatic breast cancer and is therefore a viable target for drug delivery. Another

advantage to targeting this cell surface receptor is its ability to undergo receptor-mediated endocytosis, which provides the potential for the drug delivery system to be internalized by the cancer cell. In this study, a unique α3β1 integrin specific targeting peptide has been incorporated directly into the liposomal bilayer, which is known to selectively bind this particular receptor. Experiments were conducted to verify the presence of the peptide within the liposomal bilayer utilizing absorbance spectroscopy. Subsequent stability studies were then conducted on liposomes both with and without the targeting ligand to determine if the peptide influenced the overall structural integrity of the liposome. No significant difference in stability was observed in either formulation, suggesting this targeted liposomal construct may be a stable system with potential targeting capabilities.

There is significant potential for future work with this project as this study indicates a stable targeted liposomal formulation has been synthesized with added potential targeting capabilities. Further work regarding this project will involve evaluating the relative selectivity of targeted versus non-targeted liposomes on metastatic breast cancer cells utilizing fluorescence microscopy. Following fluorescence microscopy studies, competitive displacement assays will be utilized in order to verify that the targeted liposomes are accumulating and binding the metastatic breast cancer cells in a receptor-mediated fashion. Ultimately cytotoxicity assays will be performed with both targeted and non-targeted liposomes in order to quantify the relative antineoplastic capabilities of each drug formulation to determine if in fact the targeted liposomal formulation improves overall cancer cell cytotoxicity.

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