An Evaluation of Different Cationic Lipids on Uptake of Liposomes by Endothelial Cells and Influence of the Lipid Type on Binding to Albumin Plasma Protein: A mechanistic approach in vitro

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ABSTRACT

In order to meet the increasing demands of a developing tumor mass, tumor endothelial cells actively participate in the formation of new blood vessels through a process known as neovascularization. Since tumor cells rely on the function of endothelial cells for a steady supply of oxygen and essential nutrients, any significant damage caused to tumor endothelial cells would ultimately result in tumor death. Unlike tumor cells which are located some distance away from the vascular wall, tumor endothelial cells are easily accessible to systemically applied therapeutics. Apart from that, tumor endothelial cells exhibit characteristic differences in membrane surface charge characteristics compared to endothelial cells lining vessels in quiescent tissues. Tumor endothelial cells have abundant anionic groups (i.e., glycosaminoglycans and phosphatidyl-serine) exposed on the outer leaflet of the plasma membrane which contribute to the negatively charged surface potential. This characteristic of the tumor vasculature can be exploited for therapy by using positively charged therapeutic drug carrier molecules. The positive surface charge potential of the drug carrier, combined with the net negatively charged tumor vascular surface, will facilitate the uptake of therapies by tumor endothelial cells resulting in the destruction of the vascular supply.

In this area, cationic liposomes have shown an ability to preferentially target tumor endothelial cells. We have used pegylated cationic liposomes (PCLs) in the present study. We have evaluated the ability of PCL preparations to interact with various endothelial cells; each PCL variety consisted of a different cationic lipid type, used at a fixed molar concentration and ratio to other liposome components, for the sake of comparison. Four endothelial cell lines representing different organ systems were used along with one mouse fibroblast cell line. Three endothelial cell lines HMEC-I, MS-1 VEGF and bEnd.3 are transformed cell lines, whereas
HUVEC is a primary (non-transformed) endothelial cell line. All of the cell lines were carefully selected on the basis of organ specificity; the endothelial cell lines thus represent an *in vitro* model of the respective tissue environment. In order to assess cell-PCL interactions, cell association studies and FACS analyses were performed under appropriate experimental conditions. We also characterized PCL formulations based on their physicochemical properties such as net surface charge potential and their size. To compare the relative toxicity level of the PCLs as a function of the specific cationic lipid employed, cell viability studies were performed. Liposomes are known to destabilize and rapidly clear from the circulation when administered intravenously.

Several studies suggest that plasma protein-liposome interaction is responsible for liposome’s untimely and undesirable fate. In order to mimic the *in vivo* environment, we evaluated plasma protein binding to PCLs; an ELISA kit was used to determine plasma protein’s relative affinity for binding PCLs. Albumin was the model protein used for this study owing to its abundant levels in blood, and since similar studies involving the use of PCLs have not been published to date. Moreover, the experimental conditions including concentration of plasma proteins used resemble the concentrations in blood for extrapolation purposes.

Studies have revealed the presence of anionic phospholipids on the external leaflet of plasma membrane of tumor endothelial cells. Phosphatidylserine (PS) is a highly abundant anionic phospholipid present in mammalian cells. The positively charged cationic liposomes undergo electrostatic interactions with negatively charged anionic phospholipids on tumor vasculature, thereby increasing the uptake of PCLs by tumor endothelial cells. Hence, we decided to measure the extent of interaction of our PCL preparations with PS. For this study, we used 96-well plate pre-coated with PS.
These studies revealed that the *in vivo* fate of liposomes may not only depend on the overall physicochemical properties (i.e., particle size and surface charge potential) of the PCLs, but on the specific chemistry of the cationic lipid used to achieve the desired physicochemical properties. Moreover, the specific lipid head group and acyl chain composition may influence the overall extent to which the different PCLs bind to plasma proteins.

The use of seven different PCL formulations on the basis of size, cationic charge potential and chemical composition offered a unique opportunity to investigate advantages and disadvantages of using the different PCL varieties.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>TOPIC</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRADUATE SCHOOL APPROVAL RECORD</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>x</td>
</tr>
</tbody>
</table>

1. BACKGROUND AND SIGNIFICANCE

1.1 Physiological barriers in conventional therapies .................. 2
1.2 Distribution through vascular compartments ........................ 4
1.3 Anti-vascular therapy as potential cancer treatment ............. 5
1.4 Characteristics of tumor blood vessels and their endothelial cells .............................. 6
1.5 Organ-specific endothelium ........................................... 8
1.6 Liposomes for targeting tumor vasculature ......................... 10
1.7 Cationic liposomes ................................................................ 13
1.8 Plasma protein-liposome interactions .................................. 16

2. STATEMENT OF HYPOTHESIS .............................................. 20

3. SPECIFIC AIMS ..................................................................... 20

4. MATERIAL AND METHODS

4.1 Materials ........................................................................... 22
4.2 Cell culture ...................................................................... 23
4.3 Formulations of Pegylated Cationic Liposomes (PCLs) ..................................... 24
4.4 Cell-PCLs toxicity assay.................................................................25
4.5 Cell-PCLs interaction assay ..............................................................26
4.6 Liposomal FACS analysis ...............................................................26
4.7 Enzyme Linked ImmunoSorbent Assay ............................................ 27
  4.7.1 PCL-Albumin binding study .......................................................27
  4.7.2 Effect of PEG on Liposome-Albumin binding......................... 29
  4.7.3 Effect of time on PCL-Albumin binding ....................................30
4.8 PCL-PS (Phosphatidylserine) interaction study.....................................30

5. RESULTS
  5.1 Physicochemical characteristics of different PCLs .......................... 32
  5.2 Cell-liposome toxicity study..........................................................33
  5.3 Cell-liposome interaction study.....................................................34
  5.4 FACS analysis for uptake of PCL formulations by different cell lines ....35
  5.5 Enzyme Linked ImmunoSorbent Assay .......................................... 36
    5.5.1 PCL-Albumin binding study ..................................................36
    5.5.2 Effect of PEG on Liposome-Albumin binding............................36
    5.5.3 Effect of time on PCL-Albumin binding ..................................37
  5.6 PCL-PS (Phosphatidylserine) interaction study ................................ 37

6. DISCUSSION .......................................................................................49

7. SUMMARY ..........................................................................................52

8. CONCLUDING REMARKS .................................................................53

9. REFERENCES .......................................................................................54
LIST OF FIGURES

Figure 1. Nanocarriers for vascular drug delivery ................................................... 2

Figure 2. Few tumor vascular endothelial cells supporting large number of tumor cells …6

Figure 3. Normal blood vessels Vs Tumor vasculature ..............................................7

Figure 4. Liposomes – flexibility to customize .............................................................10

Figure 5. Liposomes in multivesicular body ...............................................................14

Figure 6. Liposomes on luminal surface of fenestrae ...............................................14

Figure 7. Increase in amount of DOTAP in formulation enhances cell association ........16

Figure 8. Cationic lipid structures .............................................................................23

Figure 9. Vivaspin 2-centrifuge concentrators ..........................................................27

Figure 10. Cell-liposome toxicity data for PCLs..........................................................40

Figure 11. MS-I VEGF – PCLs interaction data ..........................................................41

Figure 12. HMEC-I – PCLs interaction data ...............................................................41

Figure 13. bEnd.3 – PCLs interaction data .................................................................42

Figure 14. HUVEC – PCLs interaction data ...............................................................42

Figure 15. L929 – PCLs interaction data ................................................................. 43

Figure 16. FACS data for MS-I VEGF cells ...............................................................44
Figure 17. FACS data for HMEC-I cells ................................................................. 44

Figure 18. FACS data for bEnd.3 cells ................................................................. 45

Figure 19. FACS data for HUVEC cells ............................................................... 46

Figure 20. FACS data for L929 ............................................................ 46

Figure 21a & 21b. PCL-Albumin Binding study ..................................................... 47

Figure 22. Effect of PEG on Liposome-Albumin study ........................................... 47

Figure 23. Effect of time on PCL-Albumin study .................................................. 48

Figure 24. PCL-PS interaction study ............................................................... 48

LIST OF TABLES

Table 1. Optimized centrifuge speed for PCLs ...................................................... 28

Table 2. Physicochemical parameters of PCLs ..................................................... 39

Table 3. Summary ............................................................................................. 52
ABBREVIATIONS:

DOPC - 1, 2-Dioleoyl-sn-Glycero-3-phosphocholine

DOTAP – 1, 2-Dioleoyl-3-Trimethylammonium-Propane

DDAB – 1, 2-dimethyl-dioctadecylammonium bromide

DODAP – 1, 2-Dioleoyl-3-dimethylammonium-propane

DMTAP – 1, 2-dimyristoyl-3-trimethylammonium propane

DOEPC – 1, 2-Dioleoyl-sn-glycero-3-ethylphosphocholine

DSTAP – 1, 2-distearoyl-3-trimethylammonium propane

DOPE – 1, 2-dioleoyl-3-phosphoethanolamine

PEG5000 – Polyethylene glycol 5000

DOPE-FITC – 1, 2-dioleoyl-3-phosphoethanolamine-fluorescein isothiocyanate

PBS – Physiological Buffer Solution

TCA – Trichloroacetic acid

SRB – Sulforhodamine B dye

BSA – Bovine Serum Albumin

FBS – Fetal Bovine Serum

MS-I VEGF – Murine Pancreatic Endothelial Cell line

HMEC-I – Human dermal Microvascular Endothelial Cell line

bEnd.3 – Mouse Brain Endothelial Cell line

HUVEC – Human Umbilical Vein Endothelial Cell line

L929 – Mouse Fibroblast Cell line

DMEM – Dulbecco’s Modified Eagle’s Medium

MEME – Minimum Essential Medium Eagle

EGM – Endothelial Cell Growth Medium

EBM – Endothelial Cell Basal Growth Medium
ELISA – Enzyme Linked ImmunoSorbent Assay
TMB – Tetramethylbenzidine
PCLs – Pegylated Cationic Liposomes
FACS – Flow Cytometry/ Fluorescence Activated Cell Sorting
PDGF – Platelet Derived Growth Factor
PS – Phosphatidylserine
1. BACKGROUND AND SIGNIFICANCE:

Cancer is a disease in which abnormal cells proliferate uncontrollably, invading other organs by spreading through blood and lymph systems. Cancer, a term credited to Greek physician Hippocrates (460-370 B.C.), is one of the oldest diseases known to man but still it is the one of the deadliest. Cancer is responsible for one in every four deaths in United States ranking second to the deaths caused by heart diseases (59). The 2008 statistics from American Cancer Society indicates that among the males, one in every two men has a risk of developing some kind of cancer and one in every four has a risk of dying from the disease. For females, statistics are not any better showing that one in every three females has a risk of developing cancer and one in five dying from this disease (60).

The numbers of different methods for the treatment of cancer have evolved till now but the most common and conventional treatments employed upon diagnosis of cancer are surgery, radiation and chemotherapy. In general, surgery is the first-line treatment approach if the malignant tumor is benign. If a malignant tumor has begun to spread surgery, radiation or chemotherapy is used alone or in combination. However, some common solid tumors like those of breast, lung, pancreas, colorectum and brain remain poorly sensitive to most of the tumor treatments. One of the major problems with chemotherapy is the poor drug penetration in the tumor interstitial environment. This is because of the heterogeneous character of the vasculature and the existence of high flow resistance in the neovasculature as well as high pressure gradients within the tumor interstitial space (1, 2, 3). Additional problems faced with conventional chemotherapeutic agents in the treatment of cancer are the development of resistance for the drug and toxicity issues that distresses the quality of life of the patient. The solution to these problems as well as a major challenge is to develop a targeting system to concentrate drugs closely to the tumor site so as to
avoid their over-distribution all over the body. For the development of such a drug delivery system a variety of drug carriers have been evaluated. Some of the particle-like drug carriers are liposomes, microspheres and nanoparticles, and polymeric micelles (Fig. 1). Soluble carriers normally used are monoclonal antibodies and their fragments, peptides, modified plasma proteins and polysaccharides (21). Polymers made of various chemical compositions as well as ongoing research on erythrocytes have shown their potential as future biodegradable and cellular carriers.

**Figure 1. Nanocarriers for vascular drug delivery (Ref. 61)**

### 1.1 Physiological barriers in conventional therapies

The tumor microenvironment presents unique barriers to specific cancer treatment modalities. Hypoxia in a solid tumor is a pathophysiologic consequence of structurally and functionally disrupted blood microcirculation, and the relapse of diffusion conditions (4). Such hypoxic conditions are strongly associated to tumor progression leading to malignancy as well as resistance to the various cancer treatments (4, 5). Hypoxia has long been known to cause
inhibition of radiation treatment and resistance to several types of chemotherapy (6). Oxygen being a potent radiosensitizer, well oxygenated cells require one-third of the radiotherapy dose of anoxic cells to achieve an adequate cell killing effect (7). A significant number of clinical trials have demonstrated the relationship between tumor hypoxia and the therapeutic outcome for patients treated with radiotherapy for cervical carcinoma (8), soft tissue sarcoma (9), and head and neck cancer (10). Other than radiation treatment, hypoxic conditions have a direct effect on effectiveness of chemotherapeutic drugs like mephalan, bleomycin and etoposide (11). These drugs require adequate amount of molecular oxygen for maximal efficiency. High level of hypoxia decreases the rate of cell division and eventually results in cell cycle arrest, which can decrease the efficacy of drugs that are more active against dividing tumor cells.

Other than hypoxia, distinct characteristics of the tumor vasculature also results in insufficient delivery and reduced effectiveness of drugs. Heterogeneous tumor perfusion and long diffusion paths can result in inadequate concentrations of systemically delivered drug in tumor tissue (12, 13). Inadequate amount of drug delivered can have negative consequences like development of resistance for the drug regimen or re-growth of tumor after indication of relapse for some period. Also, high interstitial fluid pressure (IFP) in tumors acts as a hurdle for effective drug delivery by decreasing transcapillary fluid flow and convective transport of compounds from the bloodstream into the tumor interstitium (2). This is particularly important for large molecules like antibodies and other protein therapeutics which depend primarily on convective force rather than the simple diffusion for transport (14).

The resulting pH gradient between the acidic extracellular tumor microenvironment and basic intracellular pH also impart a barrier to delivery of the chemotherapeutic agents. Most of the commonly used chemotherapeutic agents are either weak bases (pKa 5.5-6.8) or weak acids (7.8-
8.8). In acidic extracellular regions, the weak bases, such as doxorubicin or vinblastine, usually exist in a charged state (15, 16). This charged state reduces their transport across the plasma membrane which causes considerable reduction in their cellular uptake in vitro. Surprisingly, the effectiveness of drugs like paclitaxel and topotecan is impaired due to low pH even though their chemical structures do not predict pH-dependent ionizations.

1.2 **Distribution through the vascular compartment**

The blood and oxygen to tumor for its growth are supplied through existing blood vessels and new blood vessels that emerge from the already existing blood vessels. These new blood vessels are called angiogenic blood vessels (17). The movement of molecules passing through the blood vessels is governed by the vascular morphology which includes number, length, diameter and geometric arrangement of various blood vessels as well as the blood flow rate. Although the tumor vasculature grow from pre-existing vessel networks, and the mechanism of angiogenesis are similar, its pattern can be completely different based on tumor type, tumor growth rate and its location (18). The organization and rate of blood flow are different not only in different kinds of tumors but also between a primary tumor and its transplants. On the basis of rate of perfusion the tumor can be divided in four different regions: a) an avascular and necrotic region; b) a semi-necrotic region; c) a stabilized region with microcirculation and d) an advancing front (19). The intra-tumor blood flow analysis in transplanted tumors in animals was carried out using nuclear magnetic resonance (NMR), positive emission tomography (PET) and functional computed tomography (19). It was found that blood flow rate in necrotic and semi-necrotic tumor regions are low. On the other hand, the blood flow in non-necrotic regions was observed to be uneven with possibility of having higher perfusion rates than the surrounding normal host normal tissues. Taking in account the sequential and spatial diversity in blood perfusion along with disparity in
vascular morphology, it is expected that the overall distribution of therapeutic agents in tumors is heterogeneous and that the overall uptake of drugs decreases with increase in tumor size (19).

1.3 Vascular targeting therapy as a potential cancer treatment

As mentioned above, the flow of blood to the tumor is supplied through the mature and neovasculature; neovasculature (or angiogenic blood vessels) emerge from the already existing blood vessels. Hence, the development of novel drug delivery systems for targeting tumor vessels may result in significant destruction of tumor vasculature structure and function. Anti-vascular treatment can be divided in two categories: anti-angiogenic and vascular targeting therapy. Anti-angiogenic treatment intends to inhibit the new blood vessel formation whereas vascular targeting therapy aims to selectively destruct mature vasculatures leading to secondary tumor cell death (18).

Some of the additional reasons that manifest vascular targeting therapy as potential anti-cancer treatment are (a) therapeutic molecules or treatment system has greater accessibility to endothelial cells of tumor vasculature compared to most neoplastic cells; (b) probability of occurrence of mutations in endothelial cells is less thus increasing overall effectiveness in long-term therapy; (c) anti-vascular therapy checks-off the functional blood supply to tumor, thus preventing formation of malignant neoplasma; (d) since large number of tumor cells are dependent on fewer endothelial cells for blood and nutrition, destruction of tumor vasculature will result in considerable neoplastic cell kill (Fig. 2) (20).
1.4 Characteristics of tumor blood vessels and their endothelial cells

There are characteristic differences in biology of tumor blood vessels and normal blood vessels. The blood vessels nourishing tumors are abnormal (13). Tumor capillaries directly supplying blood and nutrients to cancer cells are irregularly shaped, being excessively thin in some areas and forming thick, wavy clumps in others. These malformations give rise to turbulent and uneven blood flow resulting in variety in distribution of blood to different areas within the tumor (22, 23). The endothelial cell lining in the inner surface of tumor capillaries is fenestrated having gaps of between 100 and 150 nm in diameter with size of fenestrae often changing in response to local mediators. In contrast, this lining in normal blood vessels is a smooth, tightly-packed sheet. These loose interconnections and focal intercellular openings in tumor capillaries are likely to be responsible for much of the vessel leakiness (24, 25). Here, the extravasation of proteins from tumor vessels resulting from these openings or pores is slower than calculated. An abnormally small hydrostatic gradient from inside of tumor vessels to interstitial areas which results from high interstitial pressure within the tumor reduces the convective motion of macromolecules (Fig. 3).
Although both, anionic and cationic sites are present on the endothelial cell surface, tumor endothelia have an overabundance of anionic sites (35). Most of the vascular lining is covered by a dynamic and highly complex polymeric meshwork called glycocalyx. The components of this layer are proteoglycans, glycosaminoglycans (GAGs), glycoproteins as well as some bound plasma proteins (26). It is involved in the regulation of some critical functions like vessel permeability, transport of red blood cells and coordination of interaction of leukocytes with inflamed vascular region (26). The proteoglycans functions as the ‘backbone’ molecule of the glycocalyx. These proteoglycan molecules consist of a core protein to which one or more negatively charged glycosaminoglycan (GAG) chains are attached. There are five types of glycosaminoglycan (GAG) chains namely heparan sulfate, chondroitin sulfate, dermatan sulfate, keratin sulfate, and hyaluronan (hyaluronic acid) and heavily contribute to the overall net negative charge (26, 27). Heparan sulfate proteoglycans represent approximately 50-90% of the total proteoglycan content present in the glycocalyx (26, 27).

Under normal physiological conditions anionic phospholipids are mostly missing from the external leaflet of plasma membrane of mammalian cells (28). PS (phosphatidylserine) is the most profuse anionic phospholipid of the plasma membrane and is present on the internal leaflet.
of the plasma membrane in most cell types (28, 29, 35). PI (phosphatidylinositol), the second most abundant anionic phospholipid holds the same position as PS in most of the normal cells (30). Phospholipids PA (phosphatidic acid) and PG (phosphatidylglycerol) are present in minor amounts and are situated in the internal leaflet of plasma membrane like other anionic phospholipids (31). In general, the neutral phospholipids are asymmetrically distributed in the plasma membrane. The tumor vasculature as well as pathological and physiological conditions like apoptosis, cell activation, inflammation, injury and malignant transformation leads to the loss of the asymmetric distribution of anionic phospholipids PS and PE. Normally, this asymmetric distribution is maintained by ATP-dependent aminophospholipid translocase that catalyze the transport of aminophospholipids from the external leaflet to the internal leaflet of the plasma membrane (32). Inhibition of enzyme aminophospholipid translocase or the activation of a Ca\(^{2+}\)-dependent enzyme scramblase that transports phospholipids bidirectionally results in loss of PS and PE asymmetry in the plasma membrane (33, 34). These anionic phospholipids exposed on the outer leaflet of the plasma membrane furnish tumor vasculatures with a substantial negative charge density compared to blood vessels in normal healthy tissues.

1.5 Organ-specific endothelium

Endothelial cells of blood vessels nourishing different organs and having different area of circulations display diversity in terms of expression of antigens, production of vasoactive factors, metabolic properties, response to growth factors, and vulnerability of pathological insults (36, 37). Studies carried out so far have suggested that both, genetic (38) and environmental influences (39) are responsible for diverse character of the vascular system in different organs. These unique genetic and environmental conditions influence the expression of specific molecules on endothelial cell surface, which in turn contributes to the formation of vascular
structure of specific organ. Studies have been carried out to identify differentially expressed endothelial cell determinants that could possibly be utilized for selective delivery of therapeutic agents to a given tissue. McIntosh et al., figured out presence of distinct structural and biochemical characteristics in the vasculatures of different organs. Study suggested that this difference could be exploited to transport therapeutic molecules exclusively to the lung (40). Separate studies have established that the efficacy of VEGF in promoting angiogenesis differs significantly in among the different organs (40). Studies performed using cDNA expression profiles created on the endothelial cells revealed considerable organ specific differences in manifestation of different proteins like tyrosine kinase receptors, chemokine receptors, and proteins concerned with controlling efflux of toxic substances (41). Human dermal microvascular endothelial cells (HMEC-I) expresses cell adhesion molecule ICAM-I, CD44, CD31 and CD36 along with other cell surface molecule typically associated with endothelial cells. It was observed that mouse brain endothelial cells bEnd.3 manifested detectable levels of tyrosine kinase receptor called PDGF-Rβ (PDGF=Platelet Derived Growth Factor), the chemokine receptor CXCR-2 and P-glycoprotein (41). On the other hand, same results were not obtained for endothelial cells involved in pulmonary circulation (41). In terms of growth factors, the brain and liver endothelial cells exhibited the highest increase in cell proliferation in response to bFGF (basic Fibroblast Growth Factor), whereas EGF (Endothelial Growth Factor) turned out to be most potent mitogen for lung and uterus-derived endothelial cells (41). Such a diverse nature of endothelial cells in various organs may help to target specific organ by exploiting that particular feature or may even create opportunities to address complex issues involving the treatment.
1.6 Liposomes for targeting tumor vasculature

Liposomes are well-recognized drug delivery vehicles. Liposomes are microscopic, closed vesicles which enclose an internal aqueous space separated from the external medium by a bilayer membrane composed of phospholipids identical to phospholipids segment that make up the cell membranes. The structure of a liposome highly resembles the basic structure of a cell. They are also relatively biocompatible. Liposome presents the potential to deliver drugs to desired target within the body and to reduce the systemic toxicity. Thus, there is growing interest that improved formulation and better targeting strategies will lead the way to better treatment options.

Figure 4. Liposomes - flexibility to customize (Ref. 62)

Liposomes can be widely classified on basis of size, morphology, composition, method of preparation and functions. Briefly, liposomes exist in two common varieties from size standpoint of view: Small Unilamellar Vesicles (SUVs) and Large Unilamellar Vesicles (LUVs). SUVs have typically size range from 200nm to 800nm. Intermediate Unilamellar Vesicles
(IUVs) having size range from 100nm to 200nm are considered among SUVs. SUVs are single-shelled vesicles produced as a result of high-intensity ultrasonication (55). Because of size reduction techniques like ultrasonication and extrusion SUVs approach a limited maximum diameter leading to the formation of mostly homogeneous liposome populations. LUVs have size range from 200nm to 800nm. The hydrophilic drugs are entrapped in internal aqueous core whereas hydrophobic drugs get entrapped in the bilayer membrane. Two major obstacles in drug therapy in relation to drug biodistribution and specificity can be overcome by using liposomal formulations. Encapsulated or entrapped drug molecules in liposomes are protected from external degrading conditions, and from the passive targeting to the tissues or organs such as the liver, spleen, and bone marrow that bear discontinuous endothelium (43). The physicochemical properties of liposomes are net surface charge (zeta potential), particle size, hydrophobicity and fluidity, transition temperature of liposomal formulation, drug loading capacity and packing of lipid bilayer. These physicochemical properties are dependent on type and ratio of lipids used, surface coating and method of formulation and these properties influence their stability and the type of plasma protein that bind to them.

One of the major disadvantages faced by conventional liposomes is the rapid elimination from the blood circulation by body defense systems. These systems limit the application of many liposomal formulations. This problem is encountered usually when the target organ or tissue is beyond the mononuclear phagocyte system (MPS) which leads to efficient uptake by the macrophages resulting in untimely removal from the circulation (46). Macrophages are unable to recognize liposomes as such but the selected serum protein bound to the liposomes aids in recognition by macrophages and ultimately clearance. These serum proteins are called opsonins.
Some of them known to date are immunoglobulins, fibronectin, beta 2-glycoprotein, C-reactive protein (CRP), and beta 2-macroglobulin (55).

Several attempts have been made to manipulate the factors affecting physicochemical properties of liposomal formulation in order to minimize the liposomal uptake by MPS (45, 46). In this context, an attempt to manipulate lipid type used in the formulation was made which in turn would alter lipid bilayer fluidity. Reduction in the transfer of phospholipids from the lipid bilayer to high density lipoproteins (HDL) can be achieved by incorporating cholesterol (CHOL) in the liposomal formulation (45). Also, liposomes prepared by using the saturated phosphatidylcholine (PC) or sphingomyelin (SM) demonstrated greater stability in blood compared to those having a relatively high unsaturated fatty acyl chain content (46). The faster elimination of larger liposomes from circulation compared to the smaller ones suggests the ability of macrophages to distinguish between the sizes of foreign particles (48). Liposome surface characteristics have been shown to influence rate of liposome clearance (48). For example, negatively charged liposomes are found to have a shorter half-life in blood than do neutral liposomes. Positively charged liposomes circulate for a significantly shorter period of time, probably owing to the relatively abundant presence of anionic proteins in blood (46).

Among the diverse polymers investigated for liposome surface coating in order to prolong the blood circulation time of liposomes, poly-(ethylene glycol) (PEG) is widely used as polymeric steric stabilizer (55). Although liposome surface modification by PEG can be achieved in many ways, the most commonly used method is to anchor PEG in the liposomal membrane via a cross-linked lipid (for example: DOPE-PEG, DSPE-PEG). Increased circulation time achieved with PEG coating is due to decreased binding or interaction of liposomes with plasma proteins and cell-surface proteins (49, 50). This leads to the reduction in uptake by MPS. The direct evidence
or mechanism of decreased binding of liposomes with plasma components is not completely understood but one possible explanation for the decrease in interaction is due to steric hindrance effect generated by surface coated methoxy-PEG molecules (51). The hydrophilic shell of PEG prevents the aggregation among the liposomal particles and decreases the interaction between liposomes and plasma proteins. This property of PEG is not only due to the molecular mass and uniformity of bound polymer but also due to its significant conformational flexibility (52).

1.7 Cationic Liposomes:

Cationic liposomes have proven to be a suitable carrier for targeting tumor microvasculature with ability to accumulate selectively in angiogenic tumor endothelial cells (54). Cationic lipids are mixed in specific ratio with other helper lipids in formulation of liposomes to achieve desired degree of positive charge. It is believed that this positive charge enables cationic liposomes to interact with negatively charged surface of endothelial cells of tumor vasculature.

In biodistribution study, 4 hrs after the intravenous injection of cationic liposomes, blood vessels of lung, lymph node, ovary and anterior pituitary showed greatest cationic liposome uptake (53). Liver and spleen demonstrated macrophages as major site for cationic liposome uptake. Irrespective to its charge, liposomes showed significant accumulation in liver. In liver, liposomes are found to interact more with Kupffer cells followed by interaction with endothelial cells (53). Increase in cationic lipid content also improved heterogeneous accumulation of cationic liposomes in tumor vasculatures. With the increase in cationic lipid content from 10 to 50% the association of cationic liposomes in tumor vasculatures increased significantly from 14 to 27%, respectively (52). Notably, there was no significant change in localization of liposomes in tumor interstitial area with increase in cationic charge. Studies performed to understand the effect of
tumor type and microenvironment showed no significant differences in accumulation of cationic liposomes in vascular area of the two different tumors, MCAIV and LS174t (53). There was significantly lower accumulation of cationic liposomes in normal vasculature (~4%) compared to the tumor vasculature (~25-28%). The speculated reason is that sluggish and irregular tumor blood flow in tumor vasculature promotes the interaction of cationic liposomes with its anionic sites compared to the normal vasculature. Although specific mechanism is not known but it is hypothesized that cells internalize cationic liposomes through process of endocytosis and their interaction with vasculatures is governed by negatively charged proteoglycans (20).

Studies carried out using cationic liposomes-DNA complexes have shown that of the liposomes associated with angiogenic endothelial cells, 85% of them were on the luminal surface of endothelial cells or multivesicular bodies or vesicles inside the cells (Fig. 5) (54). At 20 min after the injection, 51% of liposomes on endothelial cell surface were found associated with fenestrae (Fig. 6). Although fenestrae constitute only 4% of luminal endothelial cell surface in tumor vasculature, presence of negative charge in fenestrae diaphragm may explain this behavior of cationic liposomes. Since liposomes bound to the fenestrated vessels do not internalize, it
suggests that this interaction is only charge dependent. Non-fenestrated regions on blood vessels may internalize liposomes by receptor mediated uptake (53, 54). No extravasation was observed during four hour time period when liposomes underwent change in form of diffuse coating on luminal surface to discreet dots. Such kind of liposome uptake by angiogenic tumor blood vessels was limited to cationic liposomes whereas electroneutral, anionic and sterically stabilized liposomes did not show such pattern of uptake. These observations suggest that accumulation of cationic liposomes at angiogenic tumor vasculature is a result of uptake by the endothelial cells and not due to pertaining extravasation in tumor blood vessels.

In order to understand the overall behavior of cationic liposomes, it is worth specifying properties dependent on type of cationic lipids used in the formulation. Alteration in physicochemical properties like size and surface charge of liposomes can influence its involvement in electrostatic interactions and uptake by target cells. From the studies carried out using cationic lipids DOTAP, DSTAP, DMTAP, DODAP and DDAB, liposome size varied among them but fell within range of 111 to 161 nm which is typical for targeting tumor vasculature (20). Significant decreases in the liposome size can be achieved by increasing the cationic lipid content from 0 to 10% and this, in turn, improves also the stability of sonicated paclitaxel-loaded liposomal formulations (20). DOTAP containing formulations were found to have the highest zeta potential (27.4 ± 5.9 mV) as well as showed significant increase in endothelial cell association in vitro on increasing DOTAP content from 10 to 50%. DDAB containing formulation showed the lowest zeta potential values of 13.5 ± 2.7 mV (20). The contribution of the distinct physicochemical properties from the cationic lipid used determines the overall therapeutic profile of the formulation. For this purpose, characterization and optimization steps of cationic lipids are required to prepare the most effective formulation.
1.8 Plasma protein-Liposome interactions

The *in vivo* studies of promising lipid carriers and liposomes revealed major hurdles, thus limiting their ability to be selective to their targets. When administered *in vivo* liposomes were found to be rapidly removed from the circulation by the cells, and to organs that encompass the reticuloendothelial system (RES). Such early clearance of liposomes from the circulation is influenced by several physical and chemical properties like size, lipid composition, surface charge as well as surface coatings (48). Early efforts were made to explain this behavior of liposomes in circulation based on its interaction with plasma proteins. The studies demonstrated that plasma proteins rapidly interact with the phospholipid bilayer of liposome membranes upon incubation with plasma or serum. Several studies suggested that the liposome clearance was associated with the biophysical property of the liposome bilayer and its interaction with certain plasma proteins (48). These studies support the connection between plasma protein-liposome interactions and clearance (46, 47, 48, 49). The studies support destabilization process which ultimately cause liposome breakdown and opsonization. These studies support efforts to alter the
unique physicochemical properties of liposomal formulations to improve liposome stability in blood and binding to cellular targets on cancer and/or endothelial cells.

Studies have revealed a number of factors that influence plasma protein-liposome interactions and clearance rates but the major parameters playing a role are surface charge, surface coating and lipid dose (43). Cationic liposomes have shown considerably more complement activation and protein binding compared to neutral liposomes in an *in vitro* experiment. Studies of cationic liposomes composed of 50% cationic lipid DOTMA (1, 2-dioleyl-3-N,N,N-trimethylaminopropane chloride) revealed extensive binding to plasma proteins leading to extremely rapid clearance from the circulation of mice (43). Even negatively charged liposomes have shown rapid clearance from the circulation owing to protein-bilayer membrane interactions. Anionic liposomes composed of phosphatidylserine (PS), phosphatidic acid (PA) or cardiolipin (CL) were cleared rapidly from the circulation whereas those composed of phosphatidylglycerol (PG) or phosphatidylinositol (PI) demonstrated an extended circulation period. From these experiments it can be derived that the presence of surface charge alone does not necessarily explain the nature of liposome membrane-plasma protein interactions (43). The chemical makeup of the lipids used in development of liposome formulation must also be involved in this interaction. Factors like specific lipid head group and acyl chain composition may contribute significantly in driving interaction with different plasma proteins. In support of this assumption, it is observed that liposomal formulations prepared from different PI sources (plant and bovine liver as sources) demonstrated different rates of clearance. The major difference between these two PI sources is in the saturation and length of acyl chains, with bovine liver PI being 18:0 and plant PI being 18:2. Liposomal formulation of bovine liver PI showed twice the binding to the plasma proteins than the other formulation (43). Addition of cholesterol in preparation of
liposomes have resulted in stabilizing as well as fluidizing membrane effects and enhanced circulation of liposomes in the body. Cholesterol potentially acts by preventing the formation of membrane surface defects, thereby eliminating potential sites for protein adsorption (45).

Previously noted, surface coating with PEG have resulted in the reduction of protein binding by approximately eight-folds.

The interaction of liposomes with plasma proteins alters the targeting property of the formulation. Cationic liposomes, when administered into the bloodstream vascular targeting purposes, rapidly binds with negatively charged plasma proteins and lipoprotein that cover the liposomal surface. Protein adsorption to the surface of cationic liposomes may also alter the surface charge potential. This might reduce the affinity of cationic liposomes for tumor endothelial cells. The interaction of liposomes with plasma proteins may also decrease the therapeutic effectiveness and promote rapid clearance.

To date we have identified some of the key blood proteins interacting with liposomes in the circulation that ultimately leads to their uptake by the reticuloendothelial system. Not enough research is performed yet to understand the impact of albumin on the in vivo fate of the liposomes. Albumin is the most abundant plasma protein. It binds to both neutral as well as charged liposomes in circulation. Albumin has been associated with liposome destabilization (42). Complements and related proteins are also found to bind liposome which results in rapid uptake by macrophages (49). As far as the interaction of immunoglobulins and liposomes is concerned, immunoglobulins may be specific for certain lipids or may be involved in non-specific interactions. Anti-phospholipid antibodies are shown to be present in diseased as well as healthy individuals and these antibodies might be able to recognize phospholipids on the basis of the liposome membrane properties (44). Antibodies against hexagonal phase phospholipids show
anticoagulant effects that have been characterized (47). Fibronectin along with other extracellular proteins boost the phagocytic activity of peripheral blood leukocytes. Purified fibronectin bound to liposomes of varied composition increase liposome uptake by 10-fold (50). A major protein isolated from rapidly cleared liposomes is 𝛽2-glycoprotein I. The amount of association of 𝛽2-glycoprotein I with anionic liposomes correlates with their clearance rates. This indicates the role of 𝛽2-glycoprotein I in mediating the uptake of anionic liposomes by macrophages (51). The levels of 𝛽2-glycoprotein I in plasma are about 200-fold lower than the other major proteins bound to liposomes and its biological function is suggested to act as a cofactor for the binding of antiphospholipid antibodies. Thus, liposome-blood protein interactions alter the physicochemical properties of the carrier system as well its circulation times upon administration.

In order to overcome liposome-plasma protein problems described above, stealth liposome technology has shown promising results in preclinical studies (56). When high molecular weight polymers like polyethylene-glycol (PEG) are coupled to the liposomal surface, polymer provides sufficient protection to the liposomes from plasma protein in blood. This prolongs the plasma clearance profile of liposomal formulation, thereby, improving overall therapeutic effect (57). Since each PEG molecule is assessed to bind about 136 water molecules, PEG-coating on liposomal surface can provide stearic hindrance to the binding of plasma protein on liposome surface (58).
2. STATEMENT OF HYPOTHESIS

**Hypothesis # 1:** The extent of pegylated cationic liposomes (PCLs) uptake by different human endothelial cell lines will vary as a function of the cationic lipid used to prepare the liposome.

**Hypothesis # 2:** The binding of albumin to the different PCLs will vary according to the cationic lipid variety used. The extent of binding will be different, even though the molar concentration for the different cationic lipids used is the same.

**Hypothesis # 3:** Pegylated cationic liposome (PCL) will show relatively reduced binding to albumin plasma protein compared to non-pegylated liposome variety using the same cationic lipid type.

**Hypothesis # 4:** Different PCLs composed of either unsaturated or saturated cationic lipid type will vary in their ability to bind to albumin plasma protein.

**Hypothesis # 5:** The extent of interaction of different PCLs with phosphatidylserine (PS) will vary as a function of cationic lipid used to prepare the liposome.

3. SPECIFIC AIMS

- To evaluate the relative toxic effects of the different pegylated cationic liposomes (PCLs) on different endothelial cell lines *in vitro*.

- To evaluate the overall extent to which various PCLs are taken up by the various endothelial cell lines *in vitro*.

- To evaluate the extent of binding of plasma protein albumin to the different PCLs *in vitro*. 
• To evaluate the extent of plasma protein albumin binding to PCLs as a function of time in vitro.

• To evaluate the role of PEG on the binding of albumin to the different types of cationic liposomes in vitro.

• To evaluate the extent of interaction of different PCLs with phosphatidylserine (PS) in vitro.
4. MATERIALS AND METHODS

4.1 Materials

For the preparation of pegylated cationic liposomes (PCLs) DOPC (1,2-Dioleoyl-sn-Glycero-3-phosphocholine), DOTAP (1,2-Dioleoyl-3-Trimethylammonium-Propane), DDAB (1,2-dimethyl-dioctadecylammonium bromide), DODAP (1,2-Dioleoyl-3-dimethylammonium-propane), DMTAP (1,2-dimyristoyl-3-trimethylammonium propane), DOEPC (1,2-Dioleoyl-sn-glycero-3-ethylphosphocholine), DSTAP (1,2-distearoyl-3-trimethylammonium propane), Stearylamine, Cholesterol, DOPE-PEG\textsubscript{5000} (1,2-dioleoyl-3-phosphoethanolamine polyethyleneglycol 5000), and DOPE-FITC were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Sulforhodamine B (SRB) for cell viability assay was purchased from Sigma Aldrich (St.Louis, MO). PBS (phosphate-buffered saline) without calcium or magnesium was purchased from Biowhittaker (Walkersville, MD).
4.2 Cell culture

Immortalized murine endothelial cell line MS1 VEGF (ATCC, CRL-2460) and mouse brain endothelial cell line bEnd.3 (ATCC, CRL-2299) were purchased from American Type Culture Collection (ATCC, Manassas, Virginia). Both cell lines were maintained in a humidified 5% CO₂ atmosphere at 37°C using Dulbecco's Modified Eagle Medium (DMEM) purchased from ATCC (Cat. No. 30-2002) supplemented with 10% FBS (fetal bovine serum)-Fetalclone I
(optimized for hybridomas) obtained from Hyclone (Logan, UT). Mouse fibroblast cells of the permanent cell line L929 (ATCC, CCL-1) were grown in Minimum Essential Medium Eagle (MEME) (ATCC, Cat. No. 30–2003) supplemented with 10% FBS. Human umbilical vein endothelial cells HUVEC were derived from individual donors and purchased from Cambrex Bioscience (Clonetics, CC-2519, Walkersville, MD). Human primary endothelial cells were acquired at passage 5 and used until passage 9, when the population doubling was stable at approximately 15 hours. HUVEC were maintained in microvascular endothelial cell growth medium EGM-2MV consisting of 2% FBS and required growth supplements, purchased as a single bullet kit (Cambrex Bioscience/Clonetics, CC-3202, Walkersville, MD). Human dermal endothelial cells HMEC-1 were obtained from the Centers for Disease Control and Prevention (Atlanta, GA) and cultured in EGM-2MV consisting of 5% FBS and required growth factors. HMEC-1 cells demonstrate stable growth kinetics regardless of passage numbers (Ades et al. 1992). All cell lines were grown in a Revco ELITE III cell culture incubator (Kendro Laboratory, Asheville, NC) with 5% CO$_2$ at 37°C.

### 4.3 Formulation of Pegylated Cationic Liposomes (PCLs)

DOPC (35%), cholesterol (10%), DOPE-PEG$_{5000}$ (5 mol%) and the appropriate cationic lipid type (as shown in figure 6) were mixed to prepare liposomes, typically 50 mol% was used. The ratios of all the lipids were kept constant but different types of cationic lipids were employed to prepare different PCLs. Typically, 5% of FITC was included as part of liposome preparation for studies involving fluorescence detection. The concentration of lipids used to prepare liposomes was typically 10 µmol/mL. The chloroform used to prepare lipid stock was evaporated after lipids were mixed in mentioned ratios using a Buchi Rotovapor R-200 (Buchi Labortechnik AG, Flawil, Switzerland) for about 25 minutes, or until a dried thin lipid film was formed. Additional
trace amount of organic solvent were removed from the film by drying for 2 hours in a vacuum environment using a Labconco freeze dryer (Labconco, Kansas City, MO). The film was then hydrated with warm PBS in an inert atmosphere containing N₂ gas, incubated at 37°C, and vortexed sporadically. For size reduction, liposomes were sonicated in a bath type sonicator (Laboratory Supplies, Hicksville, NY) for 10 minutes. This produced a uniformed population of small unilamellar vesicles. Different PCLs were characterized by evaluating through their liposome size and zeta potential at 25°C in distilled water using a 90PLUS particle size and zeta potential analyzer (Brookhaven Instruments, NY) and each value denote the mean ± SD for ten different determinations.

4.4 Cell-PCLs Toxicity Assay

Cells were seeded at 1 × 10⁴ cells/well in a 48-well plate and incubated for 24 hours in a 37°C cell culture incubator set at 5% CO₂. Then, 100 nmol/mL of different PCLs were added to each well. After 24 hours of incubation, sulforhodamine B (SRB) assay was performed (Dandamudi et al. 2006; Kalra et al. 2006; Skehan et al. 1990). In this assay, after the incubation the plate was washed twice with 1 ml/well of 1X PBS. Then cells were fixed by adding 100 µL of 50% TCA (100%w/v trichloroacetic acid solution) to each well and the plates were stored at 4°C for 1 hour. After that, in order to remove unfixed cells and TCA, the plates were thoroughly washed with distilled water at least five times. Once the unfixed cells along with cellular debris were washed off from each well, fixed cells were stained by adding 200 µL of 0.4% SRB per well and plates were stored in dark for 30 minutes. To get rid of excess SRB plates were washed by 1% acetic acid and then air dried. The protein-bound SRB dye was dissolved by adding 1 mL of PBS and, then, fluorescence intensity was measured at excitation wavelength of 540/20 nm and emission
wavelength of 590/20 nm using FLX800 Fluorescence Microplate Reader (Bio-tech Instruments, Winooski, VT).

4.5 Cell-PCLs Interaction Assay

Cells were seeded at $1 \times 10^4$ cells/well in a 48-well plate and incubated for 24 hours in a 37°C cell culture incubator set at 5% CO$_2$. Then, 100 nmol/mL of different FITC-labeled PCLs were added to each well. The plate was incubated for 24 hours. After incubation, contents were aspirated and cells were washed with PBS at least twice. The fluorescence intensity was measured after washing the plate with a FLX800 Microplate Fluorescence Reader (Biotek Instruments, Winooski, VT). The fluorescence intensity of labeled PCLs taken up by cells was determined at a fixed excitation wavelength of 540/20 nm and emission wavelength of 590/20 nm. In this assay cell association was measured using fluorescence intensity (arbitrary units) measured after washing the plate.

4.6 Liposomal FACS Analysis

To measure the extent of uptake of the different PCLs FACS analysis was performed for each cell line. Cells were seeded in a 6-well plate with $5 \times 10^5$ cells/well in their respective media. Plate was incubated for 24 hours in a 37°C cell culture incubator set at 5% CO$_2$. The amounts of 100 nmol/mL different FITC-labeled PCLs were then added to the wells. The plate was incubated for 24 hours in a 37°C cell culture incubator set at 5% CO$_2$. Then the contents from wells were aspirated, washed three times with PBS, trypsinized, and washed once by centrifuging with PBS. The cells were then analyzed with a FACScaliber (BD Biosciences).
4.7 Enzyme Linked ImmunoSorbent Assay (ELISA)

ELISA was performed on polystyrene microtitre 96-well plate pre-coated with anti-albumin antibody. For this, Human Albumin ELISA Kit was purchased from Immunology Consultants Laboratory, Inc. (E-80AL, Lot # 2). For the ELISA study protease free human albumin (Fraction V) was purchased from Fisher Bioreagents (cat. no. BP264210).

FIGURE 9. Vivaspin 2 centrifuge concentrators

4.7.1) PCL-Albumin Binding Study

Samples and Controls: To generate samples and controls for the PCL-albumin binding study, first 500 nmole/ml of different PCL formulations were added to a 48-well plate containing 0.5ml of FBS-free DMEM media (randomly selected media). In sample wells, 150 µL of 40mg/mL of human albumin solution in buffer was added. Control wells were devoid of any albumin. After 24 hours, the contents from the well were taken out and transferred to Vivaspin 2 centrifuge tubes with membrane MWCO of 1,000,000. Vivaspin 2 centrifuge concentrators were bought from Sartorius Biolab, Germany (Pdt. No. : VS0262) and used for recovery of molecules above molecular weight of 1,000,000 from dilute solutions. We employed Vivaspin 2 for separation of
free albumin in the media from the albumin bound to the PCL formulations. Liposomal formulations have molecular sizes higher than the molecular weight cut-off for the membrane in Vivaspin 2 tube whereas free human albumin (MW of 66,000 Da) as well as media components easily passes through the membrane. Thus, PCLs with bound albumin will be retained on the membrane of the Vivaspin 2 tube upon centrifugation. For this purpose, centrifugation speeds for each PCL formulation was optimized in order to achieve consistent and reasonable amount of recovery. The optimized speed for obtaining 100 µL of each sample as well as control of PCL formulation is shown in table 1:

<table>
<thead>
<tr>
<th>Pegylated Cationic Liposomes (PCLs) 500 nmol</th>
<th>Centrifugation speed (g Force)</th>
<th>Time in minutes</th>
<th>Volume of Retention</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC/DOTAP/CHOL./PEG5000</td>
<td>1500 g Force</td>
<td>3 minutes</td>
<td>100 µl</td>
</tr>
<tr>
<td>DOPC/DDAB/CHOL./PEG5000</td>
<td>1500 g Force</td>
<td>3 minutes</td>
<td>100 µl</td>
</tr>
<tr>
<td>DOPC/DODAP/CHOL./PEG5000</td>
<td>2500 g Force</td>
<td>3 minutes</td>
<td>100 µl</td>
</tr>
<tr>
<td>DOPC/DMTAP/CHOL./PEG5000</td>
<td>1500 g Force</td>
<td>3 minutes</td>
<td>100 µl</td>
</tr>
<tr>
<td>DOPC/DOEPC/CHOL./PEG5000</td>
<td>1500 g Force</td>
<td>3 minutes</td>
<td>100 µl</td>
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<tr>
<td>DOPC/DSTAP/CHOL./PEG5000</td>
<td>3000 g Force</td>
<td>3 minutes</td>
<td>100 µl</td>
</tr>
<tr>
<td>DOPC/Stearylamine/CHOL./PEG5000</td>
<td>1500 g Force</td>
<td>3 minutes</td>
<td>100 µl</td>
</tr>
</tbody>
</table>

Table 1. Optimized centrifugation speed for sample PCLs

The centrifuge used was accuSpin 3R from Fisher Scientific. The samples and controls achieved after centrifugation of the Vivaspin 2 tube were recovered using pipette in recovery cap. These samples were diluted to 200 µL using serum-free DMEM media. Before centrifuging samples in Vivaspin 2, the tube was washed by centrifuging dilute albumin solution in order to prevent non-specific binding on the membrane surface.
ELISA: After the samples and controls were obtained, an ELISA plate was used as per the company protocol. Only serum-free DMEM media was used as a blank to account for values resulting from any non-specific binding. For blank, control and experimental samples, each sample was attributed duplicate wells in order to ensure consistency during analysis. For the blank sample, 100 µL of only serum-free media was added to the wells. 100 µL of control 1 containing DOTAP-PCL only in serum-free media was added in the wells. Similarly, 100 µL control 2 containing DDAB-PCL only in serum-free media was added in other two wells. Same procedure was followed for all the controls. Then 100 µL of sample 1 containing albumin bound DOTAP-PCL was added in the wells. Similarly, other samples were seeded and so forth. Microtiter plate was incubated at room temperature for one hour. Following incubation, the contents of the wells were aspirated and each well was efficiently washed with wash solution four times and, finally, the plate was inverted on absorbent paper to blot the excess fluids from the wells. Then 100 µL of enzyme-antibody conjugate was added to each well and incubated for thirty minutes at room temperature. Again, after incubation the wells were washed as mentioned earlier. Then, 100 µL of TMB substrate solution was added in each well and the plate was incubated for precisely ten minutes at room temperature. After ten minutes, 100 µL of stop solution was administered in each well. Absorbance was determined at 450 nm in µQuant microplate reader (Biotek instruments, Inc.).

4.7.2) Effect of PEG on Liposome-Albumin binding

Samples and controls: To generate samples and controls to study effect of PEG on liposome-albumin binding, first 500 nmole/ml of different pegylated and non-pegylated cationic liposome formulations were seeded in 48-well plate containing 0.5ml of FBS-free DMEM media (randomly selected media). In sample wells, 150 µL of 40mg/mL of human albumin solution in
buffer was added. Control wells were devoid of any albumin. After 24 hours contents from the wells were taken out and transferred to Vivaspin 2 centrifuge tubes with membrane MWCO of 1,000,000. Samples and controls in Vivaspin 2 tubes were centrifuged at centrifugal speed mentioned in table 1 and recovered in recovery cap using pipette. These samples were diluted to 200 µL using serum-free DMEM media. Then samples and controls were seeded in ELISA plate for one hour incubation and ELISA protocol as mentioned above was followed. Lastly, absorbance was determined at 450 nm in µQuant microplate reader.

4.7.3) Effect of time on PCL-Albumin Binding

To generate samples and controls to determine effects of time on PCL-albumin binding, first 500 nmole/ml of different PCL formulations were seeded in 48-well plate containing 0.5ml of FBS-free DMEM media (randomly selected media). In sample wells, 150 µL of 40mg/mL of human albumin solution in buffer was added. Control wells were devoid of any albumin. After different time points of 30 minutes, 12 hours and 24 hours, contents from the wells were taken out and transferred to Vivaspin 2 centrifuge tubes with membrane MWCO of 1,000,000. Samples and controls in Vivaspin 2 tubes were centrifuged at centrifugal speed mentioned in table 1 and recovered in recovery cap using pipette. These samples were diluted to 200 µL using serum-free DMEM media. Then samples and controls were seeded in ELISA plate for one hour incubation and ELISA protocol as mentioned above was followed. Lastly, absorbance was determined at 450 nm in µQuant microplate reader.

4.8 PCL-Phosphatidylserine (PS) Interaction Study

In order to determine interaction of different PCLs with anionic phospholipid phosphatidylserine, abundantly present in external leaflet of plasma membrane of tumor
endothelial cells, 96-well plate pre-coated with phosphatidyserine complexed with beta 2-glycoprotein-1 that came along with anti-phosphatidyserine ELISA kit (ALPCO diagnostics, Windham, NH; Cat. No. 35-7207) was used. Different FITC-labeled PCL formulations were prepared. Then, 100 nmoles/ml of different PCLs diluted in PBS buffer was added in 96-well plate. Each sample was replicated twice by adding in two wells across the plate. Plate was allowed to stand for one hour in dark at room temperature. After one hour of incubation, contents of the plates were aspirated. Plate was then washed with appropriate volume of PBS buffer at least three times. After washing the plate, it was blotted on paper to remove any buffer solution retained in wells. Finally, the fluorescence intensity was measured using a FLX800 Microplate Fluorescence Reader (Biotek Instruments, Winooski, VT). The fluorescence intensity of FITC-labeled PCLs bound to phosphatidyserine (PS) was determined at a fixed excitation wavelength of 540/20 nm and emission wavelength of 590/20 nm.
5. RESULTS

5.1 Physicochemical Characteristics of different PCLs

The physicochemical parameters such as liposomal size and cationic surface charge potential may play an important role in defining the targeting ability of the final formulation. Cationic liposomes with size range of 100 – 150 nm and zeta potential range of 20-25 mV are previously shown to target tumor vasculature (20). Here, seven formulations of pegylated cationic liposomes (PCLs) were prepared with different types of cationic lipids. Each formulation had a fixed amount of cationic lipid (i.e. 50% Cationic lipid). The influence of these cationic lipids (i.e. DOTAP, DDAB, DODAB, DMTAP, DOEPC, DSTAP and Stearylamine) on the final liposomal formulation was determined in terms of surface charge and particle size distribution.

To ensure a homogeneous liposomal formulation, liposomes were sonicated for 10 min. The particle size and surface charge potential of these sonicated samples are presented in Table 1. The particle sizes measured were found to be in range of 130 to 180 nm. DODAP and stearylamine containing liposomes formed relatively large size liposomes compared to DOTAP, DDAB, DOEPC and DMTAP. DMTAP-PCLs were the smallest in size (i.e. 119.0 ± 1.5 nm) whereas DSTAP formed PCLs with moderate size of 156.5 ± 1.4 nm.

After size analysis we determined surface charge potential for each preparation type. As shown in Table 2, DOTAP-PCLs were found to have highest values for zeta potential in relation to other cationic lipid PCLs analyzed. The zeta potential of DOEPC-PCL was close to that of DOTAP followed by DMTAP-PCL. First generation cationic lipid stearylamine containing PCLs showed the lowest values for zeta potential when compared to all of the second generation cationic lipid preparation types.
5.2 Cell-Liposome Toxicity Study

We determined the toxic effects of the different PCL formulations at a fixed concentration of 100 µM and at a percent of cationic lipid (50 mol%) relative to other fixed liposome components. We have employed the Sulforhodamine B (SRB) assay to determine the toxic effects of different PCL formulations. Sulforhodamine B (aka Kiton Red) is a fluorescent dye which binds electrostatically to basic amino acid residues of trichloroacetic acid-fixed cells (63). This dye requires mild acidic conditions to bind to basic amino acid residues of fixed cells whereas under mild basic conditions (which can be generated using Physiological Buffer Solution) it can be extracted from cells and solubilize for detection using a Fluorescence microplate reader.

The cell-liposome toxicity study was reported for four different cell lines (three endothelial cell lines – MS-1 VEGF, HMEC-I, bEnd.3 and one mouse fibroblast cell line L929) treated with seven different PCLs (containing 50% cationic lipids – DOTAP, DDAB, DODAP, DMTAP, DOEPC, DSTAP and Stearylamine). We observed that all of the endothelial cell lines were less susceptible to the toxic effects of the various cationic lipids used when compared to the first generation cationic lipid stearylamine. For example, at a fixed concentration of PCLs, stearylamine showed a percent viability in range of 10 to 20% on average. However, the second generation cationic lipids DSTAP, DOEPC, DDAB, DOTAP, DMTAP and DODAP generally reported viability values between 69.7 ± 9.3% and 96.7 ± 6.5%. Moreover, the fibroblast cell line L929 was observed to be more susceptible to the toxic effects of cationic lipids compared to endothelial cell lines. In general, DSTAP-PCL showed the highest percent of cell viability regardless of the cell line used. As noted, stearylamine was generally the most toxic. The overall toxicity profile of PCLs from least to most toxic was observed as follows: DSTAP = DODAP = DDAB = DOTAP = DOEPC = DMTAP < Stearylamine.
5.3 Cell-Liposome Interaction Study

The cell-liposome interaction studies for the five cell lines (four endothelial cell lines MS-I VEGF, HMEC-I, bEnd.3 and HUVEC; one mouse fibroblast cell line L929) were carried out using different FITC-labeled PCLs (100 nmole/ml). The cells were seeded in 48-well plate followed by seeding with different PCL formulations after 24 hours and incubating for another 24 hours. The fluorescence intensity for cell association studies was measured at sensitivity of 40,000 in Fluorescence microplate reader. Interestingly, DSTAP-PCL demonstrates significantly higher cell association for all cell lines (including fibroblast cell line L929) compared to any other cationic lipid formulations. For cell lines MS-I VEGF, HMEC-I, and L929, the cationic lipid formulations of DOTAP, DDAB and DOEPC show moderate uptake by cells. For mouse brain endothelial cell line bEnd.3, DOEPC-PCL shows interaction with cells at same level as DSTAP-PCL which is followed by DDAB-PCL. Without exception the, the first generation cationic lipid stearylamine-PCL preparation demonstrated a limited ability to associate with all the cell lines evaluated, compared to other preparation. In most cases, the reduced uptake of DODAP-PCL and DMTAP-PCL by endothelial cells was slightly better than stearylamine-PCL preparation. Remarkably, for the primary human umbilical endothelial cell line HUVEC, the uptake of stearylamine-PCLs was higher than that of DOTAP, DDAB and DMTAP-PCLs. In general, the cell association profile of PCLs for all the cell lines can be interpreted as follows: DSTAP > DOEPC = DOTAP = DDAB > DMTAP > Stearylamine > DODAP.
5.4 FACS Analysis for uptake of PCL formulations by different cell lines

To understand the cationic lipid dependent binding of PCLs to various endothelial cell lines, FACS analysis was performed and correlated with the cell association data obtained. For FACS analysis, FITC-labeled PCLs were used with incubation time of 24 hours in cells seeded in a 6-well plate. FACS is a powerful tool that has been used to characterize the cells based on their uptake of different FITC-labeled PCL formulations (20). The analysis revealed a strong shift to the left for stearylamine and DODAP-PCLs. On the contrary, DOTAP, DSTAP, DDAB, DMTAP and DOEPC shifted towards the right suggesting relatively high degree of interaction between these PCL types and the cells. The shift towards the right side indicates the favorable uptake of PCLs by the cells. This observation correlates with the cell association data where stearylamine and DODAP-PCLs showed the least degree of cell interaction. For primary human umbilical endothelial cell line (HUVEC), DSTAP and DOEPC-PCLs exhibited the greatest uptake when compared to any other cationic lipid preparation. For bEnd.3 and HMEC I cell lines, DMTAP-PCLs showed uptake similar to stearylamine-PCLs, favoring the lower association observed in the cell association study. Hence, we can divide PCL formulations in two different categories based on FACS and cell association data; one category which consists of DSTAP, DOEPC, DOTAP, DDAB and DMTAP-PCLs demonstrating superior uptake by most of the cell lines analyzed here, DSTAP being the best among them. However, it is difficult to categorize DMTAP-PCLs on superior side according to cell association data and FACS analysis for bEnd.3 and HMEC I, but based on FACS analysis of the other cell lines it may be categorized as preparation that interacts moderately with the cells. The other category includes stearylamine and DODAP-PCLs shows an inferior uptake for all the cell lines employed.
5.5 Enzyme Linked ImmunoSorbent Assay (ELISA)

5.5.1) PCL-Albumin Binding Study

ELISA studies were performed to determine the ability of human albumin to bind different PCL preparations. PCLs were incubated with human albumin containing growth media, whereas the controls were exposed to the growth medium only. We hypothesized that the different PCL preparations will vary in their ability to interact with human albumin owing to their unique physicochemical properties (i.e., membrane surface charge potential, membrane fluidity and particle sizes). In general, the PCL preparations did not show any significant differences in their ability to bind plasma protein albumin. But when DOTAP, DODAP and DSTAP-PCLs are taken into account, DOTAP-PCL binds to albumin plasma protein significantly more than DODAP and DSTAP-PCLs. Here, DOTAP and DODAP are unsaturated cationic lipid whereas DSTAP is saturated cationic lipid. Among all three, DOTAP-PCL has the highest surface charge potential. Thus, for the binding of plasma protein albumin, surface charge potential may play more important role than other factors like particle size and degree of saturation in cationic lipid.

5.5.2) Effect of PEG on Liposome-Albumin Binding

In order to determine the effect of PEG on the binding of cationic liposomes to the plasma protein albumin, pegylated as well as non-pegylated cationic liposomes were incubated with albumin. Pegylated variety of DOTAP, DMTAP and DSTAP cationic liposomes showed significantly less binding to plasma protein albumin compared to their respective non-pegylated variety. On the other hand, the pegylated variety of DDAB, DODAP, DOEPC and stearylamine did not show a significant reduction in albumin binding compared to the non-pegylated cationic liposome variety. Both, DMTAP and DSTAP are saturated cationic lipids and their liposomal
preparations have relatively higher surface charge potential. Thus, due to relatively rigid lipid bilayer and higher surface charge, the incorporation of PEG layer in PCL confers a greater steric hindrance to albumin binding than their non-pegylated counterparts. But on the other hand, DOTAP is unsaturated cationic lipid. Hence, degree of saturation may not play that important role as surface charge potential for PEG layer to contribute steric hindrance to albumin binding to liposome surface. Stearylamine has a significantly lower surface charge potential compared to the majority of other cationic liposomes used in the study. Thus, the use of PEG_{5000} in stearylamine-PCL may not result in considerable reduction in surface charge potential contributed by the cationic lipid.

**5.5.3) Effect of time on PCL-Albumin Binding Study:**

To study the effect of time of incubation of PCLs with albumin on extent of albumin binding, we treated PCLs DOTAP and DSTAP with albumin at different time points of 30 min, 12 hours and 24 hours. This study was carried out for DOTAP and DSTAP since both PCLs demonstrate relatively high PCL uptake. Also, DOTAP-PCL and DSTAP-PCL represent unsaturated and saturated cationic liposomes, respectively. Hence, binding of albumin to these PCLs at different time points will reveal the influence of membrane fluidity on rate of protein adsorption. This study showed no significant difference in binding of albumin plasma protein at different time points.

**5.6 PCL-PS (Phosphatidylserine) Interaction Study**

Phosphatidylserine (PS) is the most prominent anionic phospholipid present on the external leaflet of the plasma membrane of tumor endothelial cells. In order to determine the extent of interaction of positively charged PCLs with negatively charged phospholipid PS, we performed
PCL-PS interaction study using 96-well plate pre-coated with PS. Here, DOTAP, DDAB and DMTAP-PCLs showed relatively more interaction with PS compared to DODAP, DOEPC, DSTAP and stearylamine-PCLs. DOTAP and DMTAP-PCLs have relatively high surface charge potentials compared to other PCLs, possibly contributing to the superior level of their binding to PS in this study.
RESULTS

Physicochemical Parameters of different PCLs

<table>
<thead>
<tr>
<th>PEGYLATED CATIONIC LIPOSOMAL (PCLs) FORMULATIONS</th>
<th>PARTICLE SIZE (nm)</th>
<th>ZETA POTENTIAL (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC/DOTAP/CHOL./PEG 5000</td>
<td>135 ± 2.3</td>
<td>25.4 ± 2.4</td>
</tr>
<tr>
<td>DOPC/DDAB/CHOL./PEG 5000</td>
<td>131.3 ± 1.2</td>
<td>15.8 ± 1.5</td>
</tr>
<tr>
<td>DOPC/DODAP/CHOL./PEG 5000</td>
<td>176.3 ± 1.5</td>
<td>16.0 ± 2.3</td>
</tr>
<tr>
<td>DOPC/DMTAP/CHOL./PEG 5000</td>
<td>119.0 ± 1.5</td>
<td>20.2 ± 1.8</td>
</tr>
<tr>
<td>DOPC/DOEPC/CHOL./PEG 5000</td>
<td>124.6 ± 0.6</td>
<td>22.0 ± 3.2</td>
</tr>
<tr>
<td>DOPC/DSTAP/CHOL./PEG 5000</td>
<td>156.5 ± 1.4</td>
<td>18.58 ± 1.10</td>
</tr>
<tr>
<td>DOPC/STEARYLAMINE/CHOL./PEG 5000</td>
<td>178.8 ± 1.1</td>
<td>9.8 ± 2.3</td>
</tr>
</tbody>
</table>

Table 2. Physicochemical parameters of PCLs

Particle size and surface charge potential for different PCL formulations. The particle sizes were measured using Brookhaven Instruments Corporation (BIC), 90Plus Particle Sizing Software Ver. 3.57. Surface charge potential was measured using Brookhaven Instruments Corporation (BIC), PALS Zeta Potential Analyzer Ver. 3.28.
Figure 10. Cell-liposome toxicity study data for PCLs formulation

Figure 10 shows the percent of cell-liposome toxicity as an effect of various PCLs on different cell lines. Here, SRB assay was used to determine percent of cell viability. At a given concentration of 100 nmol/ml, stearylamine-PCLs demonstrate significantly high cytotoxicity for all the four cell lines. DSTAP and DODAP containing PCLs exhibit relatively high cell viability for all cell lines compared to the other PCLs evaluated.
Cell-PCLs Interaction Study

Figure 11. MS-1 VEGF - PCLs Interaction data

Figure 11 shows the cell interaction study of MS-1 VEGF cells incubated in 48-well plate for 24 hours and treated with 100 nmol/ml of different PCLs for 24 hours. Stearylamine and DODAP-PCLs showed the least amount of cell associations whereas DSTAP-PCLs showed the highest degree of uptake. DOTAP, DDAB and DOEPC-PCLs show relatively moderate amount of binding.

Figure 12. HMEC-1 - PCLs Interaction data
Figure 12 shows cell interaction study for the HMEC I cell line upon treatment with different PCLs for 24 hours. Stearylamine, DODAP and DMTAP-PCLs exhibited the lowest degree of cell interaction whereas DSTAP-PCL showed the greater degree of uptake. DOTAP, DDAB and DOEPC-PCLs show moderate interaction with HMEC-I cells.

**Figure 13. bEnd.3 - PCLs interaction data**

Figure 13 shows interactions of various PCLs with bEnd.3 cells over a 24 hr period. DODAP, stearylamine and DMTAP-PCLs show the least cell interaction whereas DSTAP and DOEPC-PCLs demonstrate highest interaction followed by DDAB and DOTAP-PCLs.

**Figure 14. HUVEC-PCLs interaction data**
Figure 14 shows interaction of primary HUVEC cells with different PCLs upon incubation for 24 hours and treated with 100 nmol/ml of different PCLs for 24 hours. Here, DSTAP-PCLs shows highest interaction followed by DOEPC, stearylamine and DOTAP-PCLs. DMTAP and DDAB-PCLs shows less interaction followed by DODAP-PCL which exhibit least association.

![L929-PCLs Interaction](image)

**Figure 15. L929-PCLs interaction data**

Figure 15 shows the interaction of different PCLs with mouse fibroblast cell line L929 after 24 hour incubation and treated with 100 nmol/ml of different PCLs for 24 hours. Same as for other cell lines, DSTAP-PCLs shows highest cell interaction whereas DOEPC, DDAB and DOTAP-PCLs exhibit moderate interaction. DMTAP-PCLs followed by stearylamine and DODAP containing PCLs show least amount of interaction.
FACS analysis to study uptake of different PCLs by cell lines

MS-I VEGF cells:

Figure 16 shows FACS analysis for MS-1 VEGF cells upon treatment with different PCLs for 24 hours. Key: BLANK, DODAP-PCL, Stearylamine-PCL, DMTAP-PCL, DOEPC-PCL, DOTAP-PCL, DSTAP-PCL and DDAB-PCL. After 24 hours of incubation, data shows lesser uptake of DODAP-PCL and Stearylamine-PCL compared to other PCL formulation which shows relatively same pattern of uptake.

HMEC-I cells:

Figure 17. FACS data for HMEC-I cells
Figure 17 shows FACS analysis for HMEC-I cells upon treatment with different PCLs for 24 hours. Key: BLANK, DODAP-PCL, Stearylamine-PCL, DMTAP-PCL, DOEPC-PCL, DOTAP-PCL, DSTAP-PCL and DDAB-PCL. DODAP-PCL, stearylamine-PCL and DMTAP-PCL demonstrate relatively lower uptake by HMEC-I cells since lines appear more leaning towards the blank line. DOEPC-PCL, DOTAP-PCL and DDAB-PCL exhibit same pattern of cell uptake followed by DSTAP-PCL.

**bEnd.3 cells:**

![FACS data for bEnd.3 cells](image)

Figure 18 shows FACS analysis of mouse brain endothelial cell line bEnd.3 upon 24 hour of incubation with different PCL preparations. Key: DODAP-PCL, BLANK, Stearylamine-PCL, DMTAP-PCL, DOTAP-PCL, DOEPC-PCL, DDAB-PCL and DSTAP-PCL. From the data, DODAP-PCL, Stearylamine-PCL and DMTAP-PCL lines are in left hand side around the blank sample whereas the other PCLs demonstrate same pattern of uptake by cells and appear on right hand side.
**HUVEC cell:**

![Graph showing FACS analysis of HUVEC cells]

**Figure 19. FACS data for HUVEC cells**

Figure 19 shows FACS analysis of primary human umbilical cell line HUVEC upon incubation with different PCL formulations for 24 hours. Key: BLANK, DODAP-PCL, Stearylamine-PCL, DDAB-PCL, DMTAP-PCL, DOTAP-PCL, DOEPC-PCL, and DSTAP-PCL. Blank, DODAP-PCL and Stearylamine-PCL are in vicinity of each other whereas other formulations are more on right hand side. DOEPC-PCL and DSTAP-PCL shows better uptake followed by DOTAP, DMTAP and DDAB containing PCLs.

**L929 cells:**

![Graph showing FACS analysis of L929 cells]

**Figure 20. FACS data for L929 cells**

Figure 20 shows FACS analysis data for mouse fibroblast cell line L929 upon incubating with different PCL formulations. Key: BLANK, DODAP-PCL, Stearylamine-PCL, DMTAP-PCL,
**DOEPC-PCL, DOTAP-PCL, DDAB-PCL** and **DSTAP-PCL**. Same as in endothelial cell lines, DODAP and Stearylamine PCLs appear more on the left hand side of the FACS histogram where as other PCLs are more on right hand side with DSTAP, DOTAP and DDAB showing better uptake followed by DOEPC and DMTAP containing PCLs.

**Enzyme Linked ImmunoSorbent Assay (ELISA)**

* a) **PCL-Albumin Binding Study**

Graph shows ELISA data determining the level of human albumin binding to different PCL formulations.

* b) **Effect of PEG on Liposome-Albumin Binding**

* P < 0.05

**Figure 21a. PCL-Albumin Binding Study for all PCLs**

**Figure 21b. PCL-Albumin Binding Study for DOTAP, DODAP and DSTAP-PCLs**

**Figure 22. ELISA to determine effect of PEG on Liposome-Albumin Binding**
c) Effect of time on Liposome-Albumin Binding

Figure 23. ELISA to determine effect of time on PCL-Albumin interaction

PCL-PS (Phosphatidylserine) Interaction Study

Figure 24. PCL-PS interaction study using FITC-labeled PCLs
6. DISCUSSION

We carried out the study to evaluate the ability of the different pegylated cationic liposomes (PCLs) to interact with four different endothelial cell lines and one fibroblast cell line. For this study, we prepared seven different PCLs composed of different types of cationic lipids used in a fixed ratio. For all the cell lines, stearylamine-PCL showed the least uptake followed by DODAP-PCL. On the other hand, DSTAP-PCL showed the highest binding efficiency for all cell lines where as DMTAP-PCL showed a binding capacity slightly above the range of DODAP-PCL. Other PCLs composed of DOTAP, DDAB and DOEPC, showed moderate binding to the cells. These data which indicate the relative level of interaction and uptake of PCLs by cells were supported by the cell-PCL interaction study as well as FACS analysis. These in vitro studies suggest that the behavior of PCLs is generally guided by its own physicochemical properties irrespective of the characteristics of the different organ-specific endothelial cells. The behavior and fate of the particular preparations is not necessarily guided by only their surface charge and particle size, but also by their structural properties such as the level of saturation, head groups and acyl chain length. Here, the first generation cationic lipid stearylamine, unlike second generation cationic lipids, showed a very high level of toxicity to the cells.

DOTAP, DMTAP and DSTAP have common chemical head groups but different acyl chain characteristics. DSTAP and DMTAP are also saturated lipids where as DOTAP belongs to the unsaturated lipid class. All three cationic lipid preparations have relatively high surface charge potential, but they display major differences in terms of their ability to interact with cells. Based on these studies, it is more probable that their behavior be driven by their acyl chains which influence the position of the polar head group within the bilayer, and therefore regulate the functional access of the liposomes to the cellular targets (64).
Plasma proteins are known to cause rapid clearance of liposomes from the circulation (46,48). We thus carried out studies to measure the extent of binding of different PCLs to albumin plasma protein given its relevance to systemic chemotherapy, and that such studies have not been carried out to date. Albumin is the most abundant plasma protein present in the blood. Our studies have revealed no significant difference among the different PCLs in terms of the extent of albumin bound to them. But taking in account DOTAP, DODAP and DSTAP we did find that DOTAP-PCL binds to albumin plasma protein significantly more than the DODAP and DSTAP-PCLs. Since DOTAP-PCL has highest zeta potential among all the formulations as well as significantly more binding to albumin than DODAP and DSTAP-PCLs, we can say that surface charge potential is contributing factor in binding of albumin plasma protein to PCLs.

High molecular weight polymer such as PEG_{5000} is used in preparation of liposomes in order to prolong their circulation period in blood (55). Since plasma proteins are known to cause destabilization and break down of liposomes, the inclusion of PEG provides the steric barrier to the binding of plasma proteins on the surface of liposomes (55, 57). Thus, PEG confers stability to liposomes against the destabilizing effect of plasma proteins in blood (55, 56, 57). To measure the role of PEG coating in our preparations we decided to perform albumin binding study for pegylated as well non-pegylated variety of cationic liposomes. DMTAP and DSTAP-PCLs show relatively less binding to albumin than their respective non-pegylated varieties. In contrast, DOTAP, DODAP, DOEPC, DDAB and stearylamine-PCLs show more binding to albumin than their respective non-pegylated varieties. Here, DMTAP and DSTAP are saturated whereas DOTAP, DODAP and DOEPC are unsaturated cationic lipids. Thus, PEG appears to confer steric hindrance to albumin binding depending on the degree of saturation of the cationic lipid used. PEG provides better protection against albumin protein binding to rigid bilayers (formed
by saturated cationic lipids) compared to when the unsaturated cationic lipids were used. We note that DDAB and stearylamine-PCLs have relatively low surface charge potentials compared to other PCL varieties. Hence, the nature of the interaction between (negatively charged) albumin and (positively charged) PCL may be determined by more than charge-mediated mechanisms. With the purpose of understanding the effect of time on the ability of PCLs to bind albumin, ELISA studies were carried out at different time-points (30 minutes, 12 hours and 24 hours). At all the time-points the amount of albumin bound to DOTAP and DSTAP-PCLs did not vary significantly. Hence, irrespective to the time, the amount of albumin bound to the PCLs is the same.

On the basis of the experimental results, the interaction of PCLs with endothelial cells is highly complex. Such factors as surface charge, membrane fluidity, size and ability to bind and avoid protein binding all appear to influence the process. Nonetheless, the outcome of our studies reveals which particular cationic lipid type should be use for targeting studies involving use of endothelial cells derived from various organs (i.e., brain, skins, pancreas and umbilical cord). Finally, our studies demonstrate how the different PCLs compare in terms of toxicity where the second generation cationic lipids held a significant advantage over the first generation type.
7. SUMMARY

From the overall profiles of studies we have carried out, cationic lipids can be categorized in the following rankings of preference:

<table>
<thead>
<tr>
<th>STUDY</th>
<th>OVERALL RANKING PROFILE FOR PREFERENCE OF CATIONIC LIPIDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeta potential analysis</td>
<td>DOTAP &gt; DOEPC &gt; DMTAP &gt; DSTAP &gt; DODAP = DDAB &gt; Stearylamine</td>
</tr>
<tr>
<td>Cell-Liposome toxicity study</td>
<td>DSTAP = DODAP = DDAB = DOTAP = DOEPC = DMTAP &lt; Stearylamine</td>
</tr>
<tr>
<td>Cell-Liposome interaction study</td>
<td>DSTAP &gt; DOEPC = DOTAP = DDAB &gt; DMTAP &gt; Stearylamine &gt; DODAP</td>
</tr>
<tr>
<td>FACS analysis</td>
<td>DSTAP = DOTAP = DDAB = DOEPC &gt; DMTAP &gt; DODAP = Stearylamine</td>
</tr>
<tr>
<td>Albumin binding to PCLs (ELISA)</td>
<td>DOTAP = DOEPC = DDAB = DODAP = DSTAP = DMTAP = Stearylamine ( DOTAP = DSTAP &gt; DODAP )</td>
</tr>
<tr>
<td>Effect of PEG on Liposome-Albumin binding</td>
<td>DSTAP = DMTAP = DOTAP &gt; DDAB = DODAP = DOEPC = Stearylamine</td>
</tr>
<tr>
<td>Effect of time on PCL-Albumin binding</td>
<td>DOTAP = DSTAP</td>
</tr>
<tr>
<td>PCL-PS Interaction Study</td>
<td>DDAB = DOTAP = DMTAP &gt; DOEPC = DSTAP &gt; DODAP = Stearylamine</td>
</tr>
</tbody>
</table>

Table 3. Summary
8. CONCLUDING REMARKS

- From the studies carried out so far, we can make concluding remarks that DSTAP-PCLs demonstrated the highest binding efficiency for all the cell lines. Also, DSTAP and DMTAP pegylated cationic liposome variety binds albumin significantly less compared to their non-pegylated variety.

- DOTAP-PCL demonstrated the highest surface charge potential as well as significantly more interaction with PS than DSTAP-PCL.

- The overall effect of DOTAP-PCL in present study is consistent with previous reports suggesting that DOTAP-PCL might represent the most efficient vascular targeting system. Also, the overall behavior of DSTAP-PCL in present study suggests a great deal of potential as an efficient vascular targeting system. Thus, both, DOTAP and DSTAP-PCLs might represent the most efficient preparation type for tumor vascular studies.


10. REFERENCES


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