Liposomes with selective and controllable surface reactivity: Applications for tumor specific thrombosis

by

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ABSTRACT

The possibility to selectively induce thrombosis in tumor blood vessels offers an interesting approach to attack solid tumors. With the well known propensity of phosphatidylserine (PS) containing membranes to bind blood coagulation proteins with high affinity and catalyze coagulation reactions, it is hypothesized that a tumor vasculature-targeted liposome containing PS may provide a means to achieve a therapeutic response based on this principle. This thesis presents a stepwise approach to the design of such liposomes.

In order to develop thrombogenic liposomes that are able to induce tumor specific blood coagulation while avoiding unwanted side effects, several features must be engineered into the formulation. First, the PS liposome surface should be protected in circulation to prevent unwanted blood coagulation reactions and rapid plasma removal of such liposomes. Results from Chapters 2 and 3 have demonstrated that an elevated level (15 mol%) of poly(ethylene glycol)-conjugated lipids (PEG-lipids) is required to prevent the PS-mediated binding of blood coagulation proteins and the rapid removal of PS liposomes. While adequate protection of the PS liposome surface is essential in the circulation, exposure of the thrombogenic PS liposome surface is necessary to trigger the blood coagulation reactions once the liposomes have reached tumor blood vessels. Results from Chapter 4 demonstrate the potential of combining PEG-lipids which have different rates of desorption from PS liposomes to balance PS surface exposure and protection. In addition to controlling the surface thrombogenicity of the PS liposomes, localizing the blood coagulation response within the tumor blood vessels is desirable. A monoclonal antibody (Ab) that recognizes a

surface marker selectively expressed on the tumor vascular endothelium was conjugated to the PS liposomes in an attempt to demonstrate localization of the coagulation response. The in vitro results from Chapter 5 demonstrate the potential of PS liposomes targeted to activated endothelial cells to selectively trigger thrombogenesis. Although specific delivery of the Ab-conjugated PS liposomes to tumor vasculature was not demonstrated in LS180 tumor bearing mice, attributes important to the in vivo targeting of such liposomes are discussed. The results from these studies provide the basis for developing thrombogenic liposomes that can be selectively activated in tumor vasculature to achieve therapeutic response.

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ABBREVIATIONS

Ab	antibody
AUC	area under the plasma concentration-time curve
BCA	bicinchoninic acid
BSA	bovine serum albumin
CA4P	Combretastatin A4 phosphate
CHE	cholesterylhexadecyl ether
CHOL	cholesterol
CL	cardiolipin
CMC	critical micelle concentration
DMPE-PEG 2000	1,2-dimyristyl-*sn*-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol) 2000]
DODAC	*N,N*-dioleoyl-*N,N*-dimethylammonium chloride
DOPC	1,2-dioleoyl-*sn*-glycero-3-phosphocholine
DOPE	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
DOPS	1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine]
DOTAP	1,2-dioleoyl-3-trimethylammonium propane
DOTMA	1,2-dioleoyl-3-*N,N,N*-trimethylaminopropane chloride
DPPE-PEG 2000	1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol) 2000]
DSPC	1,2-distearoyl-*sn*-glycero-3-phosphocholine
DSPE-PEG 350	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol) 350]
DSPE-PEG 750	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol) 750]
DSPE-PEG 2000	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol) 2000]
DSPE-PEG 2000-MAL	poly(ethylene glycol)-α-distearoylphosphatidylethanolamine,-ϖ-maleinmide
DSPE-PEG 5000	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol) 5000]
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EPC	egg phosphatidylcholine
FITC	fluorescein isothiocyanate
Fc	portion of the antibody molecule so called fragment crystallizable
Gla residues	γ-carboxyglutamic acid residues
G_{M1}	monosialoganglioside
H & E	hematoxylin and eosin
HBS	HEPES buffered saline
HER2	human epidermal growth receptor-2
HUVEC	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule-1
IL-1α	interleukin-1α
IL-4	interleukin-4

IL-13	interleukin-13
LUV	large unilamellar vesicles
MLV	multilamellar vesicles
MMP	matrix metalloproteinase
MPS	mononuclear phagocytic system
MRT	mean residence time
MW	molecular weight
PA	phosphatidic acid
P_B values	grams of associated protein per mole of lipid
PBS	phosphate buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PE-PEG	poly(ethylene glycol)-derivatized phosphatidylethanolamine
PEG	poly(ethylene glycol)
PEG-lipids	poly(ethylene glycol)-conjugated lipids
PG	phosphatidylglycerol
PI	phosphatidylinositol
POPE	1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphatidylethanolamine
POPE-PEG 2000	1-palmitoyl-2-oleoyl-*sn*-glycerol-3- phosphoethanolamine-N-[poly(ethylene glycol) 2000]
PS	phosphatidylserine
SM	sphingomyelin
SPDP	N-succinimidyl-3-(2-pyridyldithio) propionate
SUV	small unilamellar vesicles
T_c	chain melting temperature
TF	tissue factor
TFPI	tissue factor pathway inhibitor
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen-4

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To my parents,

Chapter 1 Introduction

1.1. Project Overview

Phosphatidylserine (PS) is well recognized for its role in blood coagulation, where membranes containing this lipid provide a catalytic surface for the proteolytic reactions of the blood coagulation cascade. Recently, solid tumor regression through selective thrombosis of tumor blood vessels has been achieved using a "coaguligand" which is a truncated tissue factor linked to a monoclonal antibody (Ab) targeted against tumor neo-vasculature [1, 2]. With the well known propensity of PS containing membranes to bind the high affinity blood coagulation factors and subsequently activate coagulation reactions, it is hypothesized here that a liposome-based, tumor vasculature-targeted formulation containing PS may have anti-vascular based therapeutic applications in cancer therapy. In this regard, such PS containing liposomes will have to exhibit unique attributes in order to limit liposome recognition and thrombogenesis in the central blood compartment, while permitting binding and PS-mediated thrombogenesis once at the tumor site. To achieve this goal, several formulation challenges must be overcome, based on manipulating the physicochemical attributes of liposomes to achieve the functional properties necessary for the intended application described in this thesis.

This introductory chapter will first present a brief overview of the physiological properties of the tumor vasculature and the various approaches taken to attack the tumor vasculature for tumor therapy. Since this thesis focuses on a strategy to induce blood clot formation within the tumor vasculature, the sections that follow will describe the blood coagulation process with particular emphasis on the role of phospholipid membranes in

blood coagulation. Lastly, aspects of liposome technology that are relevant to the design of novel liposome systems intended for triggering tumor specific thrombogenesis will be reviewed in light of how manipulations of the physicochemical properties of liposomes dictate the functional properties of liposomes.

1.2. Tumor vasculature – a potential target for the treatment of solid tumors

Conventional cancer treatments including surgery, chemotherapy and radiation therapy have been focused on the direct eradication of tumor cells. However, these therapeutic approaches are associated with several limitations. Treatment-related toxicity, inadequate delivery of therapeutic agents to tumor cells, and acquisition of resistance to these therapeutic modalities account for the limited clinical success of these conventional treatments. With the recognition by Folkman in the early 1970s that a blood supply to the tumor mass is essential for its growth, invasiveness and metastasis [3, 4], treatment strategies based on attacking the tumor vasculature or interfering with the tumor blood supply have been proposed [5]. These tumor vasculature-based therapeutic approaches offer the following advantages, which have been reviewed by Burrows et al [6]: 1) an amplified tumor cell killing effect could be produced by destroying the tumor blood supply, since each tumor blood vessel feeds many tumor cells; 2) tumor vascular endothelial cells are genetically more stable than tumor cells, and are thus more predictable in terms of response to therapy and less likely to develop resistance mechanisms when compared to heterogeneous tumor cell populations; 3) tumor vascular endothelial cells are freely accessible to agents that are injected intravenously; 4) the strategy can be applied to different solid tumor types since tumor blood vessels have similar morphological and biochemical properties.

Over the past few decades, intensive research has been devoted to understand the physiology and molecular biology of tumor vasculature in order to identify potential therapeutic targets. A variety of structural and physiological differences between normal and tumor vasculature have been discovered, which may be exploited for the design of novel anti-cancer therapeutics. The following sections will begin with a brief description of the properties of normal and tumor vasculature, followed by a review of the various tumor-vasculature based therapeutic strategies.

1.2.1. Properties of the normal vasculature

The normal vasculature is made up of endothelial cells and mural cells such as pericytes and smooth muscle cells that create a network of blood vessels and capillaries. The endothelial cells of this capillary network are generally mature, quiescent cells with minimal proliferative activity except during wound healing, bone repair and formation of the corpus luteum. These mature endothelial cells are characterized by the presence of a dense peripheral band of actin microfilaments that are important for maintaining endothelial cell structural integrity and for providing the capillaries and vessels the necessary mechanical stability [7, 8].

There are three major classes of blood capillaries: continuous, fenestrated and discontinuous (or sinusoidal), as defined by the endothelium and basement membrane structure, which in turn, dictates the permeability characteristics of the capillaries [9-11]. The continuous capillaries are characterized by the presence of tight junctions (~ 2 nm) and a continuous basement membrane. This type of vascular endothelium is found in muscles,

connective tissues, skin and heart, and is demonstrated to have the lowest permeability. Fenestrated capillaries are characterized by the presence of interendothelial cell junctions (40 – 60 nm) and typically a continuous basement membrane. This type of vascular endothelium is found in the kidney glomerulus, pancreas and other glands. Lastly, the sinusoidal type of endothelium is composed of fenestrations or pores up to 150 nm and a discontinuous basement membrane, and is the most permeable among the three types of normal vascular endothelium. The sinusoidal endothelium can be found in liver (with no basement membrane), spleen (with fragmented basement membrane) and bone marrow. The blood capillary network acts as the primary site of nutrient exchange between blood and tissues. Macromolecules extravasate in normal endothelium by transcapillary pinocytosis as well as via interendothelial cell junctions. The well organized structure and tightly regulated permeability observed in normal vascular endothelium provide normal tissues with ordered, regulated blood flow, where hypoxic and/or necrotic regions are not present. However, in diseased tissues such as solid tumors, drastic differences can be observed in the structure and physiology of the vasculature. The following sections will describe the properties of tumor vasculature, and how therapeutic approaches have been centered on the structural and physiological differences observed in tumor vasculature.

1.2.2. Properties of the tumor vasculature

Tumor blood vessels are structurally and physiologically different from their normal counterparts (see Figure 1.1 on P. 5). Tumor vasculature is composed of blood vessels that are derived from the pre-existing network of the host vasculature through a complex process termed angiogenesis in which new vessels are developed in response to increased

A. Normal vasculature
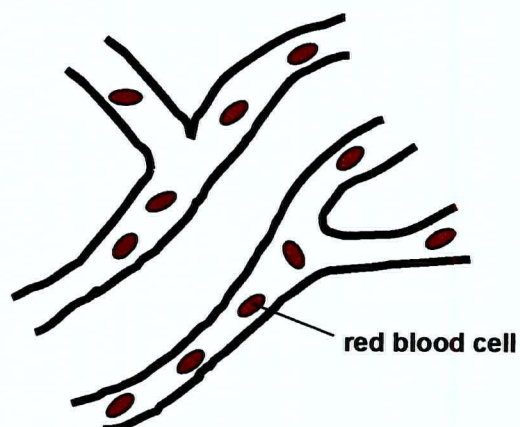
B. Tumor vasculature
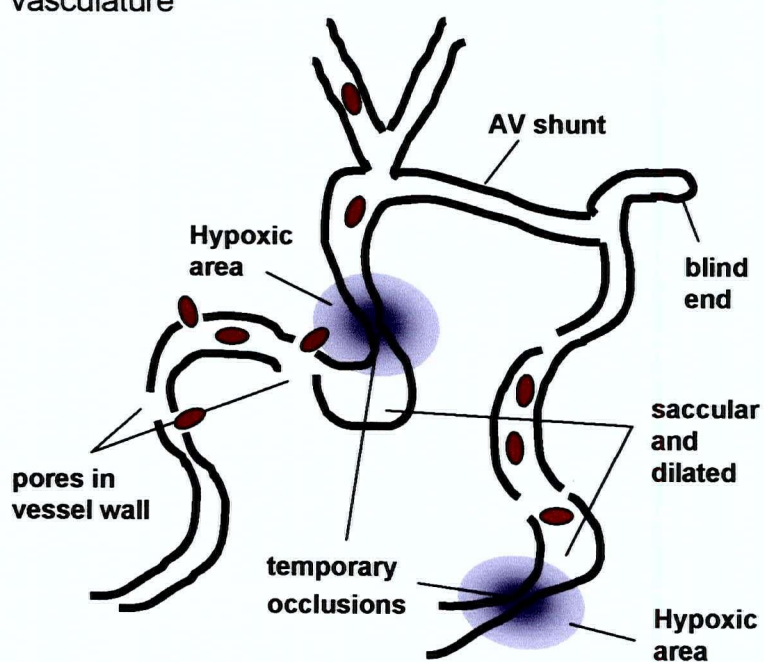
Figure 1.1. A schematic illustration of the normal and tumor vasculature. The normal vasculature is well developed with ordered, regulated blood flow (Panel A), while the tumor vasculature that is aberrantly branched and leaky, often with regions of hypoxia (Panel B).

stimuli from the tumor cells. As a result of these increased stimuli, tumor endothelial cells are highly proliferative, and the tumor vascular endothelium is often immature, in contrast to their normal counterparts [5]. Tumor vasculature also lacks smooth muscle cells or innervation, and therefore has an impaired capacity for autoregulation [12]. A collagen-rich matrix (i.e., the interstitium) separates the tumor cells from the feeding vessels, which can take up as much as half the volume of a solid tumor [13].

Studies utilizing various optical techniques revealed that tumor blood vessels are often unevenly distributed, aberrantly branched and twisted into tortuous shapes, dilated and saccular [14]. The walls of tumor blood vessels have large pores whose typical cut-off size vary from 380 – 780 nm, with a maximum up to 2 μm [15]. The pore dimension, confirmed by electron microscopy, indicated that the widened interendothelial junction is the major mode of macromolecular transport in tumors [16]. This significantly larger pore cut-off size of tumor blood vessels leads to a lack of selectivity in the extravasation of molecules into the tumor interstitium. The distribution of pores, open endothelial gaps and vesiculo-vacuolar organelles in tumor blood vessels has been shown by electron microscopy to be heterogeneous at both the core and the periphery of tumors [17].

Spatial and temporal heterogeneity in tumor blood flow and perfusion has been demonstrated [14]. The heterogeneous and chaotic microcirculation of tumors is attributed, in part, to the abnormal architecture of the tumor vasculature. As a result of heterogeneous perfusion, the interstitial pO_2 and pH are also spatially and temporally heterogeneous throughout the tumor [18]. The vascular permeability of tumor blood vessels is an order of

magnitude higher than that of their normal counterparts [19]; however, spatial and temporal heterogeneity in vascular permeability in tumor vessels has been observed, which restricts access to some regions of the tumor [20]. Furthermore, the lack of functional lymphatic system in tumors results in poor drainage and subsequent elevation in interstitial fluid pressure [9, 21, 22]. Considering that the tumor vasculature is strikingly different in its architecture and physiology, it is conceivable that delivery of therapeutic agents to all regions of a tumor in optimal quantities would remain a challenge, and that the substantially altered metabolic microenvironment in tumors would compromise the effectiveness of many conventional therapies [23].

1.2.3. Tumor vasculature-based therapeutic strategies

After a brief discussion of the structural and physiological differences between the tumor vasculature and its normal counterpart, the following sections will review the various tumor vasculature-based therapeutic strategies, which can be grouped into two general categories: 1) anti-angiogenesis treatments that prevent the formation/growth of new blood vessels in tumors, and 2) anti-vascular treatments which directly act on endothelial cells of the existing tumor vasculature. The former involves the development of agents that inhibit the complex process of angiogenesis which allows the formation of new blood vessels that nurture the tumor cells. These anti-angiogenic agents inhibit tumor growth in regions of neovascularization, but may not prevent growth of tumor cells along existing blood vessels or surviving tumor cells in regions fed by mature, non-proliferating vessels. The anti-vascular strategy involves the development of agents that act selectively on the tumor vasculature to block off the blood supply feeding the tumor cells or to produce massive

destruction of the tumor vascular endothelium, thus resulting in massive killing of the tumor cells by ischemic starvation.

1.2.3.1. Anti-angiogenesis approach

The elucidation of key events in tumor angiogenic process over the past decade has produced many rational targets for tumor therapeutics. Angiogenesis, which is the process of forming new capillary blood vessels from existent microvessels by sprouting, is an important determinant in the development of the primary tumor and the metastatic foci [3, 4], because tumors > 1 – 2 mm in diameter cannot receive nutrients through diffusion, and the growth of such tumors must rely on inducing a new blood supply. The process of tumor angiogenesis is a complex, multi-step cascade that involves sequential events including endothelial cell proliferation, migration and tubule formation, as well as proteolysis and remodeling of the extracellular matrix, which allow subsequent new vessel growth toward the tumor. Each of these steps in angiogenesis may be targeted for anti-angiogenic therapy. Further, a key feature of tumor angiogenesis is that this process occurs relatively selectively in tumors, since under normal physiological conditions, the process of angiogenesis occurs only during wound healing, bone repair and formation of the corpus luteum. With this relative selectivity, the search for tumor angiogenesis inhibitors has become an exciting research area. To date, more than 300 potential angiogenesis inhibitors have been identified, and the pace of discovering new agents is rapid. The sections that follow will review some key angiogenesis inhibitors developed over the decade that are under clinical trials, based on the mechanisms of action of the various agents.

The first group of agents are those that target the matrix metalloproteinases (MMPs). MMPs are essential for tumor growth, migration, angiogenesis, invasion and metastasis, because these are enzymes that degrade the tumor extracellular matrix. One key MMPs inhibitor is marimastat which has undergone randomized phase III clinical trials; however, the results have failed to demonstrate any benefit [24]. Other MMPs inhibitors that have undergone phase I trials include COL-3 where disease stabilization was observed in patients with non-epithelial cancers [25], and halofuginone which is a potent inhibitor of collagen $\alpha 1$ and MMP-2 gene expression [26].

The second group of agents are those that target molecules involved in the angiogenic process. Of these, the angiogenic inducers represent a key target for the development of novel tumor therapeutics. Normally, the balance of angiogenic inducers and inhibitors is tipped such as inhibition is favored; however, under pathological conditions such as cancer, the activity of the angiogenic inducers is increased and is no longer restrained by the inhibitors, leading to continuous new capillary growth [27]. One well studied example of angiogenic inducers is vascular endothelial growth factor (VEGF) which possesses potent mitogenic activity on endothelial cells [28]. VEGF is a key player in tumor angiogenesis, and has been a focus of intense research. Recombinant human monoclonal Ab directed against VEGF (Avastin™) has been developed [29], and has undergone a phase I trial which showed the Ab was well tolerated in patients [30]. In addition to VEGF, the receptors for this growth factor has been identified, which include VEGFR-1 (Flt-1), VEGFR-2 (Flk-1, KDR) and VEGFR-3 (Flt-4). Among these three high affinity VEGF receptors, VEGFR-2 is considered to be the most important for angiogenesis [31], and an

anti-VEGFR-2 Ab has been shown to have anti-angiogenic effects in pre-clinical studies, and has now entered the early phase of clinical trials [32]. Small molecules have also been developed to inhibit the VEGF receptors. One example is semaxanib which inhibits autophosphorylation of VEGFR-2, and has been shown to have potent anti-angiogenic effects in pre-clinical studies [33]. Although semaxanib is the first VEGF receptor inhibitor that entered a phase III trial, its short half-life and high incidence of thrombo-embolic events when used in combination with cisplatin has led to discontinuation in its development [34].

Another group of target molecules in the angiogenic process that have potential for developing therapeutics is the integrin family of cell adhesion molecules. In particular, the intergrin $\alpha v \beta 3$ is a marker for angiogenic blood vessels, with selective expression in tumor vasculature but not in normal quiescent tissues [35]. This intergrin molecule has been shown to bind directly to MMP-2 on the surface of vascular endothelial cells during angiogenesis, suggesting its role in regulating the interaction of angiogenic endothelial cells and the extracellular matrix [36]. A humanized anti-$\alpha v \beta 3$ Ab (Vitaxin) has been developed [37], and has undergone a phase I trial with results demonstrating disease stabilization [38].

The third group of angiogenic inhibitors are those that are derived from the tumors including angiostatin and endostatin. The discovery of these tumor-derived angiogenic inhibitors has provided the molecular basis for the well-known clinical observation that removal of certain primary tumors can be followed by rapid growth of distant metastases. The group of investigators, led by O'Reilly, were able to show that a primary tumor produces angiogenic inducers in excess of inhibitors for its own neovascularization. But the

longer half-life of the inhibitor allows the inhibitor to reach distant metastases and to become in excess of the inducer secreted by the metastases, resulting in an inhibition of growth of the metastases due to suppression of angiogenesis [39]. This endogenous angiogenic inhibitor is given the name angiostatin. Later, the group led by O'Reilly has discovered another tumor-derived inhibitor named endostatin [40]. This angiogenic inhibitor is reported to be more potent than angiostatin, and is endothelial cell specific [41], and this inhibitor has become a focus of research. Studies have demonstrated that endostatin inhibits endothelial cell proliferation and migration [40, 42, 43], and that it induces endothelial cell apoptosis [44]. In vivo, repeated dosing of endostatin can induce tumor regression to dormant microscopic lesions without acquisition of resistance [41]. Currently, both angiostatin and endostatin are undergoing early stages of clinical trials, with disease stabilization observed in patients treated with the recombinant human form of angiostatin [33].

Other pharmacological agents that possess anti-angiogenic activity have also been evaluated. Of interest, the chronic administration of conventional chemotherapeutic agents at one-tenth to one-third of the maximum tolerated dose significantly increases the anti-angiogenic effect and anti-tumor activity in animal models [45, 46]. Clinical trials have been initiated to test the anti-tumor activity of these agents used alone or in combinations.

1.2.3.2. Anti-vascular approach

While the target of anti-angiogenic agents is the region of neovascularization in tumor, the target of the anti-vascular approach is mainly the existing vascular network of the

tumor. There have been two strategies taken to attack existing tumor vasculature: 1) identifying small molecules that damage the tumor vascular network by directly and specifically killing tumor vascular endothelial cells, and 2) developing monoclonal antibodies to deliver an effector molecule to induce occlusion within the tumor blood vessels. Both of these strategies aim to cause a rapid and catastrophic shutdown in the vascular function of the tumor to produce tumor cell death as a result of oxygen and nutrient deprivation. The following sections will present a brief description of these two strategies.

Small molecules as vascular targeting agents

The small-molecule vascular targeting agents currently of greatest interest include a group of agents that destabilize the tubulin cytoskeleton of proliferating endothelial cells. The original interest in the use of these compounds as vascular damaging agents was stimulated by studies with colchicine reported in the 1930s and 1940s [47]. However, colchicine had to be administered at doses approximating its maximum tolerated dose (MTD) in order to achieve significant vascular damaging effects in tumors, where lethal toxicity was observed in patients. Over the years, research has been centered on the identification of tubulin polymerization inhibitors that can be administered at much lower doses to achieve similar vascular damaging effects as colchicine. Currently, the leading compounds in this group include Combretastatin A4 phosphate (CA4P) and ZD6126 (AstraZeneca), where clinical trials have been initiated to determine their efficacy when used alone or in combinations with conventional cancer therapy. Dramatic reduction in tumor vascular volume was observed at approximately one-thirtieth and one-tenth of the MTD for ZD6126 and CA4P, respectively [48, 49]. The water soluble pro-drug CA4P,

when cleaved by endogenous phosphatases, gives the active form that is able to induce rapid, selective and extensive blood flow reduction, resulting in hemorrhagic necrosis in a human breast cancer model [48]. The rapidity in vascular-mediated necrosis observed in vivo is thought to be a result of the contraction of endothelial cells leading to the loss of the cobblestone morphology within minutes of CA4P exposure [50]. These rapid changes in endothelial cell morphology would dramatically compromise vessel integrity, resulting in occluded blood flow in tumor vasculature and tumor necrosis. The other lead compound, ZD6126, is also thought to act through a similar mechanism in vivo [49]. This hypothesis is supported by in vitro studies where both CA4P and ZD6126 have been shown to act directly on the three-dimensional shape of proliferating endothelial cells but much less on that of quiescent endothelial cells [49, 50]. Given that the tumor vasculature contains regions of active angiogenesis with highly proliferative endothelial cells, this is likely to account for the observed selectivity in the vascular damaging effects of these compounds on tumor vasculature.

Ligand-targeted effector molecules as vascular targeting agents

The strategy of targeting effector molecules to tumor vasculature was first exemplified by Huang et al in which they used a truncated form of tissue factor that was conjugated to a bi-specific Ab against an artificially induced marker for triggering tumor specific infarction [51]. Two other studies following the same approach but targeting to markers on the tumor vasculature have demonstrated tumor specific infarction and tumor regression [1, 2]. Unlike attacking the molecular targets (such as signal transduction proteins, growth factor receptors and transcription factors) of tumor endotheial cells to

induce cell death and vascular damage, targeting the effector molecules to extraneous markers of tumor vascular endothelium does not require these tumor markers to be functionally active in inducing a therapeutic effect. This may help reduce the variability in responses and the likelihood of developing resistance mechanisms.

The thrombogenic liposomes being characterized in this thesis will be targeted to an extraneous marker of the tumor vascular endothelium, vascular cell adhesion molecule-1 (VCAM-1), with the intention of targeting such liposomes to tumor vasculature to trigger tumor specific thrombogenesis. Since this approach relies on the selective formation of occlusive thrombi within the tumor vessels, the target marker should be preferentially expressed on the tumor vascular endothelium. Table 1.1 (P. 15) lists the markers which have been reported to be preferentially expressed on tumor vascular endothelial cells in humans, and VCAM-1 is one such example (P. 15). Ideally, the tumor vasculature marker for targeting would have the following properties: 1) it should be abundant on the tumor vascular endothelium through all regions of the tumor; 2) it should be present on the luminal surface of the tumor endothelium; 3) it should be absent in normal vascular endothelium or other cells in contact with blood; and 4) it should be absent from endothelial cells in non-malignant pathological lesions (e.g. areas of inflammation) that may be present in cancer patients [52].

Table 1.1.

Examples of selective markers of the tumor vasculature [a]

Tumor vascular markers	Location on tumor blood vessels	Expression in other tissues
Endoglin	Luminal surface of endothelium	Low expression on normal vascular endothelium; strong on inflamed endothelium, macrophages, and immature erythroid cells
E selectin	Luminal surface of endothelium	Vascular endothelium in acute inflammatory lesions
VCAM-1	Luminal surface of endothelium	Vascular endothelium in inflammatory lesions, activated macrophages, dendritic cells
Fibronectin ED-B domain	Abluminal surface of endothelium and extracellular matrix	Neovasculature in ovary and myometrium
$\alpha v \beta 3$	Vascular endothelium	Low expression on normal vascular endothelium; strong on wound granulation tissue
VEGF-receptor complex	Abluminal surface of endothelium	Renal glomerulus, corpus luteum, arthritic lesions, psoriatic lesions
VEGFR1	Upregulated in luminal and abluminal surface of endothelium	Low expression on normal vascular endothelium
TIE-2	Abluminal surface of vascular endothelium	Low expression on normal vascular endothelium; upregulated on remodeling vasculature
PSMA	Vascular endothelium	Brain, renal tubules, prostatic epithelium, colonic crypts

[a] Adapted from Reference [52].

1.3. Membrane involvement in blood coagulation

Since this thesis focuses on the strategy to induce selective blood clot formation within the tumor vasculature in order to block off the blood supply to tumor cells, an understanding of the blood coagulation process is necessary to explore the potential of utilizing the different components as agents to trigger the blood clotting reaction. In particular, there is considerable potential for amplification of the blood clotting response because one converting enzyme is able to activate more than one zymogen. Many of these enzymatic activation reactions require the presence of negatively charged phospholipid membranes. The following sections will provide an overview of the blood coagulation process, with particular emphasis on the role of the negatively charged PS and PS containing membranes.

1.3.1. Overview of the blood coagulation process

The blood coagulation process is a complex physiological process for homeostasis, and this introduction will focus mainly on the discussion of the role of phospholipids and membranes in blood coagulation. Following vascular injury, two mechanisms are set into action to stop blood loss: 1) the activation and aggregation of platelets at the site of injury to form the primary hemostatic plug, and 2) the initiation of the blood coagulation cascade that comprises a series of stepwise transformations of proteolytic zymogens into active coagulation factors, leading to the formation of an insoluble fibrin clot which strengthens the primary platelet plug. In addition to the ability of negatively charged liposomes to induce reversible platelet aggregates [53], many of these proteolytic reactions in the coagulation cascade are also greatly accelerated in the presence of phospholipid membranes [54]. Figure

1.2 (P. 18) provides a simplified scheme of the blood coagulation events. From a historical perspective, the events involved in blood coagulation are organized into two pathways, namely, the extrinsic and intrinsic pathways, which converge to a common reaction that produces thrombin and ultimately fibrin [55-57]. A brief description of these pathways is given below.

1.3.1.1. Extrinsic pathway

It is generally accepted that the extrinsic pathway is the principal initiating pathway of in vivo blood coagulation [57-59]. This pathway is so named because it relies on the action of the subendothelial cell membrane protein, tissue factor (TF). Tissue factor, which is a transmembrane glycoprotein [60], is not normally in contact with the bloodstream. The extracellular domain of TF acts as the receptor for factor VII/factor VIIa, and the one-to-one complex of TF/factor VIIa forms a catalytic unit that activates factors IX and X [61-65]. It has been suggested that initiation of the blood coagulation events may begin with the binding of trace amounts of circulating factor VIIa to TF, forming the TF/factor VIIa complex for the production of factor Xa [66]. The factor Xa initially formed is used to form the first traces of thrombin which provides several positive feedback reactions including the activation of factors V and VIII [67-69]. Factor Xa also cleaves and activates TF-bound factor VII [64, 70].

The massive formation of thrombin, however, is not from the action of factor Xa formed from the TF/factor VIIa complex. This initially formed factor Xa is rapidly
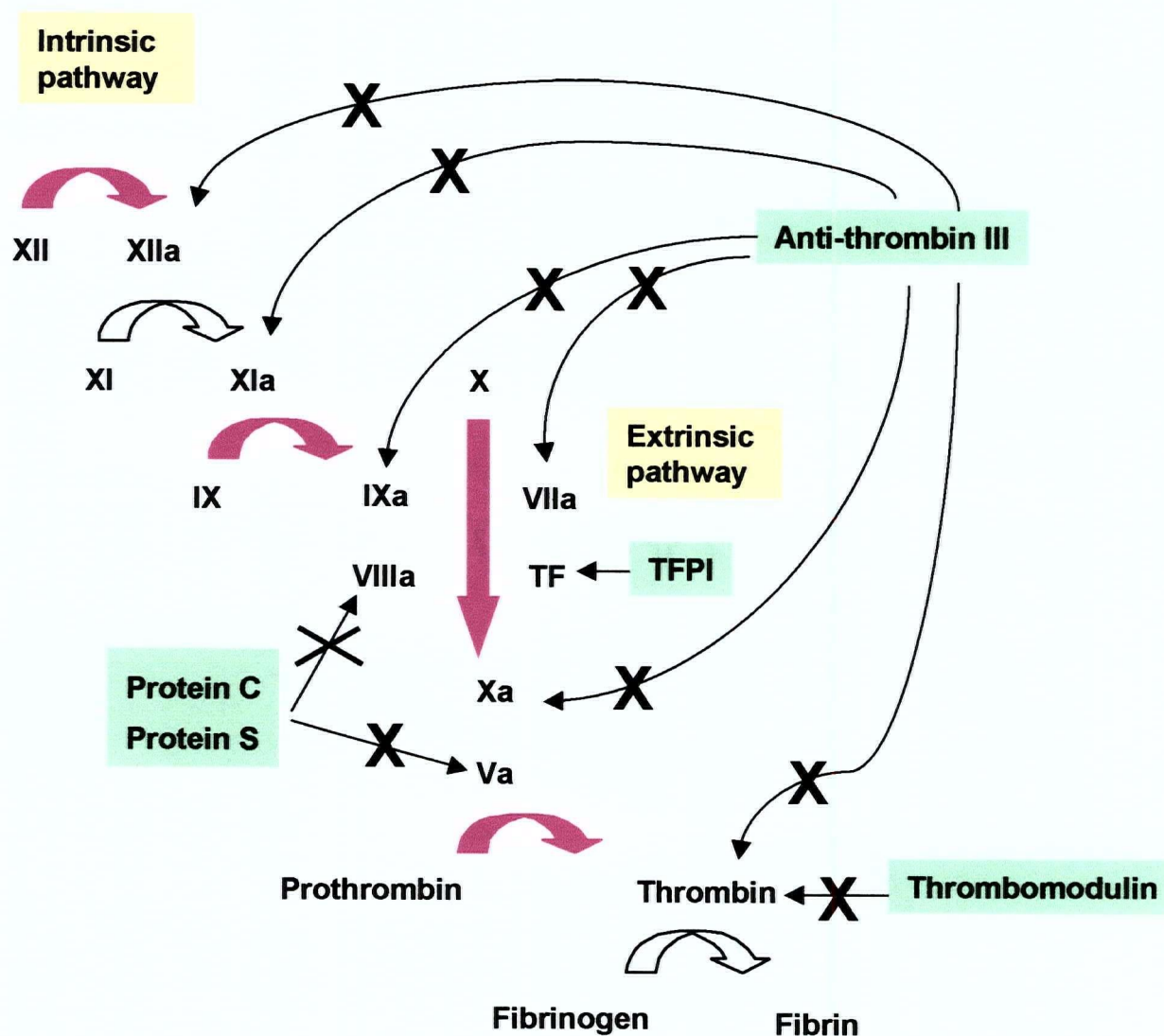
Figure 1.2. A simplified illustration of the extrinsic and intrinsic pathways of the blood coagulation cascade. Reactions which require the presence of a negatively charged membrane are indicated with a pink arrow, and the major regulators of the coagulation cascade are highlighted in green. Blood coagulation proteins are abbreviated with their respective roman numerals. TF, tissue factor; TFPI, tissue factor pathway inhibitor.

inactivated by the serine protease inhibitor, tissue factor pathway inhibitor (TFPI), which provides feedback inhibition to the TF/factor VIIa complex [58, 71]. In order to sustain the blood coagulation process, additional factor Xa production must occur through the action of factor IXa and its activated cofactor VIII, which are factors from the intrinsic pathway described below.

1.3.1.2. Intrinsic pathway

Once thought to be responsible for the initiation of the blood coagulation events, the intrinsic pathway is now generally accepted as a mechanism whereby blood coagulation reactions are maintained and propagated. This pathway begins upon exposure of the "contact" factors (factor XII, high MW kininogen and prekallikrein) to a negatively charged surface, which leads to the autoactivation of factor XII [72]. Factor XIIa then activates factor XI which subsequently activates factor IX. Recent studies have reported that thrombin is capable of activating factor XI in vitro, and have implied that thrombin, rather than factor XIIa, is the physiological activator of factor XI [73, 74]. Clinically, factor XII deficiency is not associated with abnormal bleeding, suggesting that factor XII is essentially bypassed during in vivo blood coagulation [75].

The more important reaction in the intrinsic pathway is the activation of factor X by the catalytic unit "tenase" formed from factor IXa (activated either by factor XIa or TF/factor VIIa complex) assembled with its cofactor VIIIa (activated by thrombin). This reaction provides the additional factor Xa that is needed for the assembly of the prothrombinase complex which is the catalytic unit that converts prothrombin to thrombin,

and overcomes the inhibitory effect of TFPI on the TF/factor VIIa complex. The final common pathway then begins with the prothrombinase complex which generates thrombin. This thrombin then catalyzes the conversion of fibrinogen to the insoluble fibrin for the stabilization of the primary platelet plug.

1.3.1.3. Major regulators of the blood coagulation reactions

In addition to TFPI, the major regulators of the blood coagulation reactions include anti-thrombin III, thrombomodulin, Protein C and Protein S, which act to inhibit critical blood coagulation reactions. Anti-thrombin III is a circulating serine protease inhibitor that inhibits the enzyme activity of thrombin, factors Xa, IXa, XIa, XIIa and VIIa [76-78]. This inhibitory effect can be potentiated by heparins and heparan sulfate. The activity of thrombin can also be inhibited by thrombomodulin which is an endothelial cell surface glycoprotein. Upon binding to thrombomodulin with high affinity, thrombin loses it ability to activate platelets or to convert fibrinogen, while changing its substrate specificity to become an efficient activator of protein C [79]. Activated protein C (APC) cleaves and inactivates factors VIIIa and Va, leading to the inactivation of the tenase and prothrombinase complexes, respectively [80, 81]. The proteolytic action of APC on factor Va is facilitated by Protein S which is a vitamin K-dependent glycoprotein [82, 83]. Protein S is also capable of inhibiting tenase and prothrombinase complexes directly [84-86].

1.3.2. Role of phospholipid membranes in blood coagulation

An understanding of the role of phospholipids and membranes in blood coagulation is necessary to explore the potential of utilizing liposome formulations that are being

characterized in this thesis for the application of triggering tumor specific thrombosis. Most of the blood coagulation reactions, including those which are anti-coagulant, are catalyzed by phospholipid membranes. Therefore, the use of phospholipid membranes for triggering tumor specific thrombosis can provide considerable potential for amplifying the blood coagulation response. Coagulation reactions that require phospholipid membranes are highlighted in Figure 1.2 (P. 18).

The requirement for blood coagulation protein assembly on phospholipid membranes to fully activate thrombosis has several advantageous features. First, binding to the lipid membranes not only increases the local concentration of the coagulation proteins by confining them in a two-dimensional space, but also facilitates the two-dimensional transfer of the activated coagulation proteins between the different complexes [87, 88]. For instance, the assembly of the prothrombinase complex is 500 times slower when one set of vesicle bound factor Xa is mixed with another set of vesicle bound factor Va than when both factors Xa and Va are added to the same set of vesicles [89]. Biologically, this has the effect of confining the cascade of blood coagulation events to the same membrane surface which helps to localize the blood clot formation at the site of injury. Secondly, the interactions of the coagulation proteins with the lipid membrane result in conformational changes that favor the juxtaposition and subsequent cleavage of the substrate. As an example, the interaction of factor VIIa to lipid bound TF results in a structural reorientation of factor VIIa which is thought to align its active site with the cleavage sites of its membrane bound substrates [90]. Furthermore, the lipid membrane is also effective in promoting the assembly of the anti-

coagulant protein complexes to provide feedback inhibition in close proximity to the membrane bound procoagulant enzyme complexes.

1.3.2.1. Role of PS in the coagulation protein-membrane interactions

Activity of the blood coagulation enzyme complexes depends critically on the lipid composition of the membrane, and a mixture of negatively charged phospholipids and neutral PC is essential for the important enzyme complexes such as tenase, prothrombinase and TF/factor VIIa [91-93]. Among the negatively charged phospholipids that are capable of enhancing the blood coagulation reactions when incorporated into PC membranes, PS is the most effective negatively charged phospholipid [94]. The catalytic efficiency of the prothrombinase complex has been shown to reach a maximum with 10 to 15 mol% PS in PC membranes, with a gradual decrease as the PS content increases further [95]. Other negatively charged phospholipids usually display a plateau of maximum activity at 25 to 50 mol% [94]. Interestingly, the interaction of factors IXa with VIIIa and factors Xa with Va is stereoselective, with much higher affinity toward phosphatidyl-L-serine than phosphatidyl-D-serine [93, 96]. The amino group and the serine moiety of the PS headgroup have been shown to have a distinctive role in the binding and activity of the tenase and prothrombinase complexes. Changing the pH or surface charge by incorporating cationic stearylamine has little effect on the interaction of the tenase and prothrombinase complexes with the phosphatidyl-L-serine containing membranes [93, 97].

Most of the membrane binding blood coagulation proteins are vitamin K dependent where the N terminal portion of these proteins are modified post-translationally with γ-

carboxyglutamic acid (Gla) residues, including prothrombin and factors VII, IX and X and the anticoagulant Protein C. The lipid-binding properties of these proteins are conferred by these Gla residues, and the mechanism of binding is once thought that the ionic calcium formed a bridge between the carboxyl group of these Gla residues with the phosphate headgroups of the phospholipids [98, 99]. However, with evidence showing the stereoselectivity of the binding of tenase and prothrombinase, the distinctive role of the amino group on the PS headgroup, and the insensitivity of the coagulation enzyme-membrane interaction to changes in pH and surface charge, a revised mechanism of interaction is proposed. It is thought that the protein-membrane association is mediated through the formation of a chelate complex between calcium and electron donating groups which are supplied by the Gla residues of the proteins, phosphate, amino and carboxyl groups of PS. This proposed mechanism is supported by the observation that excess calcium cannot disrupt the interactions of these Gla-modified coagulation proteins with the PS containing membrane but EDTA can [100, 101]. Recently hydrophobic interactions have also been suggested to contribute to the energy of these Gla-containing coagulation proteins binding to the PS containing membranes [102]. Furthermore, studies have revealed that a surface-exposed hydrophilic cleft, lined by lysine, serine and Gla residues, is created in the Gla domain as these Gla-containing coagulation proteins bind to PS containing membranes [103]. This cleft may be complementary in shape and charge to that of polar headgroup of PS, suggesting why PS is the optimal lipid for binding of the Gla-containing blood coagulation proteins.

Although PS can be found in eukaryotic cell membranes, this lipid is normally confined to the inner leaflet of the cell membrane such that blood coagulation reactions are not normally initiated. Three major mechanisms are involved in the maintenance of this asymmetric distribution of lipids between the two membrane leaflets. Two of these mechanisms require ATP as an energy source. They are the aminophospholipid translocase which transports PS and PE from the outer to the inner leaflet against a concentration gradient, and the ATP-dependent floppase which transport PS, PE and PC from the inner to the outer leaflet of the cell membrane at a rate much slower than the translocase [104, 105]. These two translocating activities work in concert to maintain a nonrandom phospholipid distribution across the cell membrane. The third mechanism is the lipid scramblase which can rapidly transport all major lipid classes back and forth at comparable rates [106, 107]. Activity of the lipid scramblase requires a high concentration of cytoplasmic calcium at which the synchronous activities of the translocase and the floppase are blocked [107, 108]. Conceivably, perturbations of these mechanisms would result in the loss of membrane asymmetry and the exposure of PS in the outer membrane leaflet. Increased procoagulant activity associated with PS exposure in the red blood cells has been observed in blood disorders such as sickle cell anemia and β-thalassemia [109, 110]; however, erythroid PS exposure is not predictive of thrombotic risk in mice with hemolytic anemia [111].

1.4. Liposome formulations: physicochemical and functional properties

The previous section highlighted the role of phospholipid membranes containing the negatively charged PS to propagate and amplify the blood coagulation process. Since liposomes, which are artificial vesicle membranes made up of phospholipids and cholesterol,

have been utilized as delivery systems for drugs and macromolecules, they appear to be a well-suited carrier system for the PS containing lipid membrane. An understanding of liposome physicochemical properties is thus central to applying these lipid-based delivery systems for the intended application described in this thesis. In the sections to follow, advances in modifying the physicochemical properties of liposomes to achieve various functional properties are reviewed in light of aspects of liposome technology that are relevant to the design of thrombogenic liposomes for selective, controllable tumor thrombogenesis.

1.4.1. Components of liposomes

Liposomes are spherical lipid envelopes constructed from bilayer membranes, which form upon hydration of amphipathic lipids such as glycerophospholipids and sphingolipids in an aqueous medium. The amphipathic nature of these lipids is characterized by the presence of a polar or hydrophilic head group region and non-polar or hydrophobic region, where the polar regions tend to orient toward the aqueous environment, whereas the hydrophobic regions are sequestered away from water. The lipid bilayer organization creates an effective permeability barrier between the interior and the exterior aqueous compartments of liposomes, which makes liposomes suitable for delivering hydrophilic agents in the interior aqueous core and hydrophobic agents solubilized within the hydrophobic regions. The diversity in the chemical nature of lipids, the building blocks of liposomes, is the key feature that imparts tremendous versatility in the selection of the right lipid component to achieve the necessary physicochemical properties of liposomes for particular functional objectives. Figure 1.3 (P. 27) presents a summary of the structures of

major lipids used in liposome formulations including glycerophospholipids, sphingolipids and cholesterol. In the following sections, the chemical nature of these lipids will be briefly described, and how the physicochemical properties of liposomes are modulated by varying the lipid composition will be reviewed.

1.4.1.1. Glycerophospholipids and sphingolipids

One of the primary components of most biological membranes is the glycerophospholipid or phospholipid in short. Figure 1.3 (P. 27) illustrates the typical phospholipid structure and examples of some species that are commonly used in formulating liposomes. This group of lipid molecules is characterized by two fatty acyl chains linked via ester bonds to a glycerol backbone at the C-1 and C-2 positions, with a polar or charged moiety attached to the C-3 position forming the head group region. The hydrophobicity of the acyl chains is determined mainly by the acyl chain length and the degree of saturation. Usually, the length of acyl chains found in liposomes can range from 12 to 18 carbons, and up to three double bonds can be found in the acyl chains. The hydrophobicity of the acyl chain composition is one major determinant in the rate of desorption of individual lipid species from the liposome membrane [112, 113], which in turn, affects the overall lipid composition that leads to altered functional properties of liposomes.

The polar head group region of these phospholipids is chemically diverse, with different charges and degree of bulkiness (see Figure 1.3 on P. 27). At physiological pH, all phospholipids have a negative charge on the phosphate group. The head groups of phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylglycerol (PG) and

General structure of phospholipid

head group

glycerol
backbone

acyl
chains

R

O

$O=P-O^-$

O

$H_2C-\overset{\overset{H}{|}}{C}-CH_2$

O O

$O=$ $=O$

Common head groups

$CH_2-CH_2-\overset{\overset{CH_3}{|}}{\underset{\underset{CH_3}{|}}{N}}-CH_3$ choline (PC)

$CH_2-CH_2-NH_3$ ethanolamine (PE)

H   phosphate (PA)

$CH_2-\overset{\overset{H}{|}}{\underset{\underset{COO^-}{|}}{C}}-NH_3$ serine (PS)

$CH_2-\overset{\overset{OH}{|}}{CH}-CH_2-OH$ glycerol (PG)

inositol (PI)
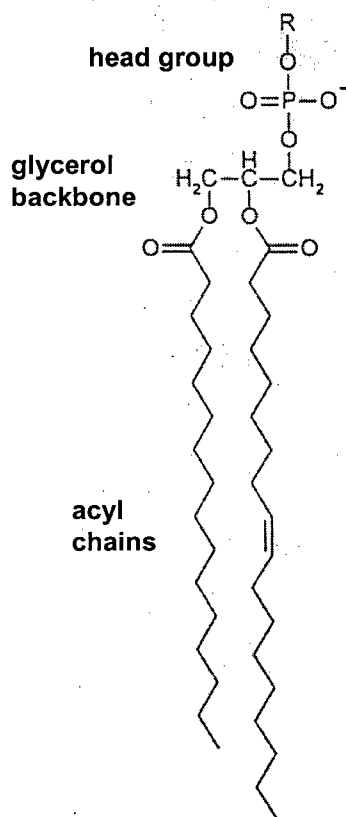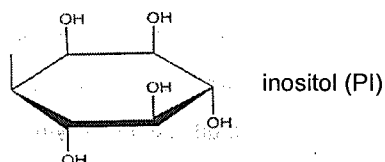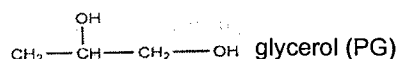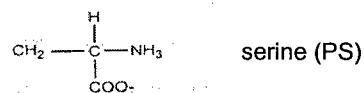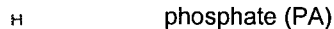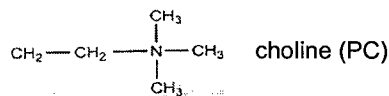
Common acyl chains

Saturated fatty acid chains

Lauric (12:0) $CH_3(CH_2)_{10}COOH$
Myristic (14:0) $CH_3(CH_2)_{12}COOH$
Palmitic (16:0) $CH_3(CH_2)_{14}COOH$
Stearic (18:0) $CH_3(CH_2)_{16}COOH$

Unsaturated fatty acid chains

Palmitoleic (16:1$^{\Delta 9}$) $CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
Oleic (18:1$^{\Delta 9}$) $CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
Linoleic (18:1$^{\Delta 9,12}$) $CH_3(CH_2)_4CH=CHCH_2CH=$
    $CH(CH_2)_7COOH$
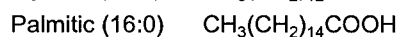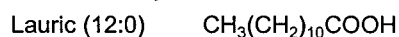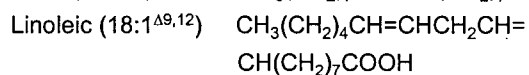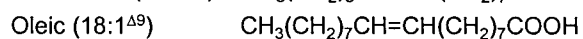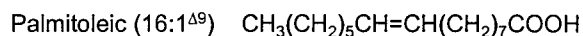
General structure of sphingolipid

HO

R-NH

OR'
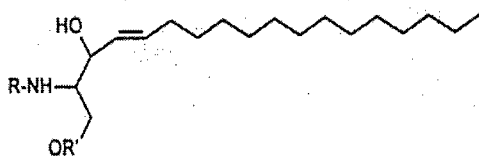
R = H; R' = H   sphingosine
R = COR"; R' = H   ceramide
R = COR"; R' = phoshocholine   sphingomyelin
(where R" = hydrocarbon chain)

cholesterol

HO
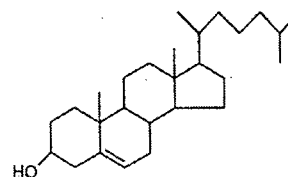
Figure 1.3. Structures of lipids commonly used in liposomes

phosphatidylinositol (PI) are negatively charged since the charge on the phosphate is not being neutralized, whereas the head groups of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are zwitterionic. Liposomes incorporated with PS, PA, PG or PI thus have a negatively charged surface at physiological pH. As described in previous sections, the negatively charged PS incorporated into PC-based membranes will constitute substantial thrombogenic activity; therefore, this lipid will be one of the major components used to formulate the thrombogenic liposome to be applied in tumor specific thrombosis. However, the inclusion of negative charges on the membrane surface will substantially influence the biological fate of systemically administered liposomes, which will be discussed further in Section 1.4.3.

Sphingolipids are another major class of lipids, which contain a special long chain amino alcohol sphingosine as the central unit with a fatty acid chain conjugated through an amide link to the nitrogen of sphingosine (see Figure 1.3 on P. 27). Similar to the glycerophospholipids, the head groups of sphingolipids are diverse, and the fundamental structural unit common to all sphingolipids is ceramide which bears a simple –OH on C-1 of the sphingosine molecule. Derivatives of ceramide include 1) sphingomyelins which contain a phosphocholine headgroup, 2) glycosphingolipids which contain sugar moieties linked directly to the –OH at C-1 of the ceramide, and 3) gangliosides which contain a bulky and polar headgroup made of several sugar units.

1.4.1.2. Cholesterol (CHOL)

Another lipid commonly found in membranes is CHOL which belongs to the family of sterols. Although the structure of CHOL is drastically different from those observed in phospholipids and sphingolipids (see Figure 1.3 on P. 27), it can also be incorporated into the bilayer membrane because of its amphipathic nature. The polar 3-β-hydroxy group constitutes the hydrophilic region of CHOL, whereas the relatively rigid and planar steroid nucleus, consisting of a 4-ring system, constitutes the hydrophobic region of CHOL. Within the bilayer, CHOL orients itself in such a manner that the hydrophobic steroid nucleus aligns itself with the first ten carbons of phospholipid acyl chains and the polar 3-β-hydroxy group is positioned along with the carbonyl ester bond of the phospholipid. CHOL by itself does not form a bilayer, but requires the presence of other bilayer forming lipids. The theoretical maximum level of CHOL that can be incorporated into a PC containing liposome bilayer is 77 mol% based on the packing of lipids [114]; however, only up to 50 mol% CHOL is typically used in phospholipid based liposome formulations due to the appearance of CHOL microcrystals at higher concentrations. Although CHOL is a small neutral lipid, it serves to modulate various properties of liposomes, both physical and biological, which will be further described in Sections 1.4.2 and 1.4.3.

1.4.1.3. Phase behavior of lipids in aqueous environment

The three major classes of lipids described above are virtually water insoluble. In addition to an ability to adopt the bilayer organization, lipids can also adopt other highly organized macromolecular aggregates in a phase distinct from the aqueous environment when mixed with water or buffers, as illustrated in Figure 1.4 (P. 31). The thermodynamic

driving force for the formation of these aggregates is the hydrophobic interactions among lipid molecules, which result from the system's achieving the greatest thermodynamic stability by minimizing the entropy decrease that is caused by the ordering of water molecules around the hydrophobic acyl chains [115]. The nature of this self association depends on 1) the nature of the polar head groups and hydrophobic acyl chains, and 2) the aqueous environmental conditions such as pH and temperature [116-118]. This phenomenon in which lipids are able to adopt a number of structurally distinct phases upon hydration is termed lipid polymorphism, and has been extensively studied and reviewed in the literature [116-118].

The molecular basis of lipid polymorphism can be explained through a generalized, dimensionless "shape" parameter of lipids, which is defined by S where $S = v/a_o l_c$ (see Figure 1.4 on P. 31) [114, 119]. Here, a_o is the optimal area per molecule at the lipid-water interface, v is the volume per molecule and l_c is the length of the fully extended acyl chain (in the case of phospholipids and sphingolipids). The shape parameter is a simplistic term to reflect the effects of a large variety of complex molecular forces and of the aqueous environment in which lipids are dispersed. In general, lipids which prefer a shape corresponding to $S < 1$ are referred to as "cone" shaped and adopt the inverted micellar or hexagonal phase, whereas lipids with $S \approx 1$ are cylindrical and bilayer-forming, and lipids with $S > 1$ are "inverted cone" shaped thus preferring the micellar phase. Lipids that are non bilayer forming can be incorporated into the bilayer, provided that the amount of bilayer forming lipids is sufficient. For example, 20 – 25 mol% bilayer forming lipids such as PC is
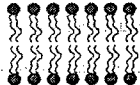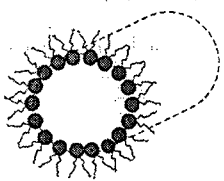
Phase	**Molecular Shape**	**Examples of lipids**

Micellar   **Inverted cone**

- Lysophospholipids
- Detergents
- PEG-lipids

Bilayer   **Cylinder**

- Phosphatidylcholine
- Phosphatidylserine
- Phosphatidylglycerol
- Phosphatidylinositol
- Phosphatidic acid
- Cardiolipin
- Sphingomyelin

Hexagonal   **Cone**

- Unsaturated phosphatidylethanolamine
- Cardiolipin / Ca^{2+}
- Phosphatidic acid / Ca^{2+} (pH < 6)
- Phosphatidylserine (pH < 4)

Figure 1.4. Phase behavior of lipids based on the molecular shape that the individual lipid adopts.

required to stabilize hexagonal phase preferring lipids such as DOPE into a bilayer structure [120]. Other factors which modulate lipid polymorphism include 1) length and degree of saturation of the acyl chains, 2) size, charge and hydration of the headgroup region which is sensitive to the pH and the presence of other ions in the aqueous medium, and 3) temperature [114, 116].

1.4.2. Physicochemical properties of liposomes

The previous sections are focused on the various types of lipids used in liposomes and the different macromolecular aggregate structures that can exist in aqueous environment. This section will begin with a description of the gel-liquid crystalline phase transition of the bilayer, in which different physicochemical properties (such as membrane fluidity and permeability) are exhibited by these two different phases. How these physicochemical properties can be modulated by varying lipid composition such as acyl chain length and CHOL content will be discussed in view of the functional performance of liposomes as delivery systems.

The key physical phenomenon of bilayers involves the melting of the acyl chains in the L_β to L_α bilayer transition as temperature increases (also known as gel-liquid crystalline phase transition). This calorimetric behavior of the bilayer is extensively studied using differential scanning calorimetry where the chain melting temperature (T_c) and the enthalpy (ΔH) of the transition are recorded. Below T_c, the two acyl chains are not lying perpendicularly to the plane of membrane but are tilted at an angle to maximize the Van der Waals interactions with each other, in such a manner that the bilayer is relatively rigid and

highly ordered with virtually no molecular motion of the acyl chains (which can be quantified by the order parameter, s, where s $\approx$ 1 in this case). This is referred as the gel phase of the bilayer. As temperature increases, the acyl chains adopt conformations (e.g. gauche) other than the all-trans configuration as observed in the gel phase. This increases the volume occupied by the acyl chains and thus reduces the overall length of the chain, resulting in a decrease in the bilayer thickness during the chain melting transition. Above T_c, the bilayer is fluid and disordered, exhibiting rapid rotations of lipids along the long axis (with the order parameter s $\approx$ 0 in this case). This is referred as the liquid crystalline phase of the bilayer. This gel to liquid crystalline phase transition has been studied and reviewed extensively in the literature [121, 122].

Increasing the acyl chain length and the degree of saturation increases T_c and enthalpy of the gel-liquid crystalline phase transition. For instance, with each two-carbon unit is added to the acyl chain of the saturated PC species, an increase in T_c by ~ 20 °C with a concomitant increase in enthalpy is observed. The head groups of phospholipids can also influence T_c, as well as ions such as calcium which can increase T_c of acidic phospholipids (reviewed by [122]). Increasing saturation in the acyl chains can increase the order which has been correlated to reduced membrane permeability [123]. It has also been observed that membranes are more permeable to water and solutes at or above T_c. If lipids exhibiting drastically different T_c are mixed together to form liposomes, separation of the lipid with the highest T_c into gel-phase domains with the others in the liquid crystalline phase can occur at temperatures below the T_c of this lipid. Formation of these domains in the bilayer results in

unstable liposome preparations, where content leakage can occur through the packing defects in the boundary between the gel and liquid crystalline phases.

The effects of CHOL on the physicochemical properties of liposomes are profound. One noteworthy observation is the ability of CHOL to decrease the enthalpy of the gel-liquid crystalline phase transition as its content in the bilayer is increased. With > 32 mol% CHOL in a PC containing bilayer, the enthalpy of the gel-liquid crystalline transition is virtually zero [121]. Because CHOL inserts into the bilayer and reduces the freedom of motion of the first ten carbons of the acyl chains in the bilayer, CHOL is able to increase the order of the acyl chains. Above T_c, CHOL increases the order and the molecular packing of the liquid crystalline bilayer compared to one without CHOL, and decreases the bilayer permeability. Below T_c, CHOL decreases the order of the rigid gel bilayer compared to one without CHOL, and increases the bilayer permeability. The effect of CHOL on the biological fate of liposomes will be discussed in the next section.

1.4.3. Biological fate of systemically administered liposomes

Since the thrombogenic liposomes being developed in this thesis will be targeted to the tumor vascular endothelial cells which are exposed to the bloodstream, intravenous injection represents the preferred route of administration of such liposomes. The following discussion of the biological fate of liposomes will be centered on the intravenous route of administration, although liposomes have also been administered through a number of different routes such as intraperitoneal, subcutaneous, intramuscular, and transdermal routes. Once intravenously injected into the bloodstream, liposomes are exposed to numerous blood

components such as serum proteins and cellular interactions. These interactions may cause liposome destabilization leading to leakage of the encapsulated agents and liposome removal by the mononuclear phagocytic system (MPS). The following sections will begin with the description of MPS which is the biological system responsible for the removal of liposomes in circulation, followed by the various formulation factors that affects the circulation longevity of liposomes.

1.4.3.1. The mononuclear phagocytic system (MPS)

The removal of liposomes and other exogenously derived particulates from bloodstream is inextricably linked with cellular uptake. The group of cells responsible for the removal of such particles from circulation include cells primarily from the liver, spleen and bone marrow, which together comprise the MPS and constitute the host defense system [124, 125]. The main features of the cells of MPS are that they are highly phagocytic, mononuclear, and present in many organs of the body where cells can be settled in tissues (as macrophages) or migratory in circulation (as monocytes). The mechanisms which govern the recognition of injected liposomes as foreign particles and the subsequent removal from circulation are complicated.

It is generally presumed that the important factor in this detection process is the binding of serum components to the liposome surface (reviewed in [124, 126-128]. Serum components which promote the phagocytosis of foreign particles are termed opsonins, and they often act as "bridges" between the phagocytic cell and the substrate such as liposomes. There are two types of opsonins: non-specific and specific [125]. Nonspecific opsonins

function by increasing the adhesiveness of the particle surface or of the phagocyte or both (e.g. fibronectin [129]). Specific opsonins work by interacting specifically with their corresponding receptors to mediate phagocytosis. In addition, the examples given below belong to the complement system which is composed of multiple cascade pathways that mediate liposome removal and lysis via pore formation. These specific opsonins include 1) immunoglobulins such as IgG which bind to Fc receptors found on phagocytic cells [129, 130], 2) complement proteins such as C3b and iC3b which bind to CR1 and CR3 receptors, respectively [131, 132], 3) β2-glycoprotein I that recognizes negatively charged lipids such as PS and PA and binds to the β2-glycoprotein I receptor [133], and 4) C-reactive protein that binds to Fc receptors [134, 135].

In addition to the opsonins, another group of serum components termed dysopsonins retard or inhibit phagocytosis of pathogens or particles by altering the surface properties of either the phagocytes or the particles or both, leading to less adherence of particles to the phagocyte [136]. Some well known dysopsonins for microorganisms include IgA and α1-acid glycoprotein [137, 138]. However, the dysopsonic agent for liposomes has not been identified, and is only inferred in some studies as serum factors of high molecular weights (> 100 kDa) [139-141]. The role of these dysopsonic agents awaits further exploration.

To further illustrate the complexity of the removal of liposomes from circulation by MPS, some investigators using the liver perfusion model have demonstrated that different animal species have different mechanisms in the liver extraction of liposomes from the circulation [142-145]. These investigators have explored the uptake of liposomes in rat and

mouse livers, and found that liver uptake of liposomes is a serum independent mechanism in mice, whereas this process is strongly dependent on serum in rats.

1.4.3.2. Factors affecting liposome removal from circulation

After an introduction to the MPS which is the biological system responsible for the removal of liposomes from circulation, the following sections will discuss a number of liposome formulation characteristics including liposome dose, size and composition that influence liposome removal from circulation. It is generally believed that increasing the circulation longevity of liposomes in blood will enhance the therapeutic efficacy and benefits of liposomal drug delivery [146, 147]. Thus, significant effort has been contributed to understand and exploit various liposome physicochemical properties that extend the survival of liposomes in the plasma compartment.

Dose

There is a relationship between liposome dose and circulation longevity where increased liposome circulation lifetimes have been observed with an increase in liposome dose. It is thought that large doses of liposomal lipid may deplete certain plasma opsonins which are responsible for the subsequent phagocytosis by MPS cells, resulting in increased liposome circulation lifetimes [148]. It is also possible that the phagocytic cells involved in liposome uptake and removal become saturated, since injection of an excess lipid dose results in an increase in liposome blood levels and a concomitant suppression of liposome uptake in liver and spleen [149]. However, under these conditions, lipid accumulation in

other MPS sites such as bone marrow and lung was observed [150]. Chronic administration of liposomes at high doses also impairs the ability of MPS cells to remove liposomes [151].

Size

As described in Section 1.2, the capillary network serves to selectively determine the size of macromolecules which can penetrate the blood vessels and access the tissues, depending on the type of fenestrations or junctions the capillary network has. It is conceivable that the sites to which liposomes are accessible after intravenous administration are thus dependent upon the liposome size. Three types of liposomes can be generated and classified according to size and lamellarity: 1) multilamellar vesicles (MLV, 1 – 100 μm), 2) large unilamellar vesicles (LUV, 50 – 500 nm), and 3) small unilamellar vesicles (SUV, 25 – 40 nm). For large liposomes (> 500 nm diameter), phagocytosis by fixed macrophages and blood monocytes appear to be involved in the uptake [149]. Liposomes > 1 μm are removed rapidly from circulation, typically within 15 min, where they are mechanically trapped within the spleen [152]. For small liposomes (≤ 80 nm diameter) which are thought to be able to pass through the fenestrated liver vascular endothelium, the uptake of these liposomes may involve liver parenchymal cells as well as macrophages, and it has been suggested that these two elimination pathways are parallel [153, 154]. Whereas the uptake process by macrophages is saturable, the uptake process by the liver parenchymal cells has been hypothesized to be linear and nonsaturable [154]. For LUVs that exhibit a diameter of 100 – 200 nm, they are eliminated more slowly from the bloodstream, and are the preferred liposome size for the delivery of anti-cancer agents to solid tumors.

Lipid composition

The diversity in structures and chemical nature of lipids, as presented in Section 1.4.1, can modify a number of physicochemical properties of liposomes including 1) the surface characteristics such as charge and hydrophilicity/hydrophobicity, and 2) the bilayer packing and fluidity of liposomes. Modifications to these liposome properties, in turn, can drastically alter the elimination kinetics of liposomes from circulation. For instance, inclusion of positively charged lipids such as DODAC and DOTMA which are used for DNA delivery results in rapid blood removal, exhibiting circulation times in the order of minutes and in vivo protein binding values of 500 – 800 g proteins per mole of lipid recovered [127, 155]. However, the removal of liposomes incorporated with negatively charged lipids is not solely dependent on the negative charge on the liposome surface. Liposomes containing PI circulate for extended period of time, although it is negatively charged at physiological pH [153, 156]. Inclusion of the negatively charged PS and CL into the neutral PC based liposomes results in circulation times of these liposomes in the order of minutes, with in vivo protein binding values being 2-fold higher than those obtained for PC based liposomes [157]. It has also been suggested that the removal of liposomes may be mediated by MPS recognition of specific head groups such as PS [158, 159]. Therefore, when formulating the PS-based thrombogenic liposomes described in this thesis, it is important to realize the rapid removal and protein interactions of these liposomes, and to undertake strategies to overcome these interactions. In addition to surface charge, other modifications to the liposome surface include conjugated ligands such as whole antibodies (Ab) and polymers (e.g. poly(ethylene glycol)), and the effects of such modifications will be explored in detail in later sections.

The effects of altering bilayer lipid packing on the in vivo stability of liposomes is best exemplified by the inclusion of CHOL in liposome formulations. In the absence of CHOL, pure gel-phased DSPC liposomes (with T_c > physiological temperature) are eliminated very rapidly with 50% of injected dose eliminated in less than 2 min. A relatively stable formulation can be achieved when CHOL is incorporated at 30 – 50 mol% into pure DSPC liposomes, where 50% of the injected lipid were eliminated in 5 – 6 h [160]. It is thought that the rapid removal of pure DSPC bilayer is due to the formation of membrane surface defects, where portions of hydrophobic acyl chains are exposed on the liposome surface to which plasma proteins can adsorb and subsequently mediate liposome uptake by MPS [160]. CHOL which is able to modulate the packing of lipids in the bilayer may prevent the formation of such defects in liposome membranes. Furthermore, CHOL can prevent liposome destabilization and content leakage mediated by plasma lipoproteins and other proteins such as albumin [157, 161-164]. Many apolipoproteins, namely apo A-I, A-II, A-IV, B, C and E, have shown to interact with liposomes and disrupt the liposome membrane (reviewed by [164]). Of these, apo A-I is the major apolipoprotein responsible for liposome destabilization, where it inserts itself into the liposome membrane [165]. CHOL can also stabilize liposomes in the circulation by preventing lipid transfer from liposomes to lipoproteins [166].

1.5. Advances in liposome technology

After a brief presentation of the physicochemical properties and the biological fate of liposomes, the following sections are focused on the development of liposome formulations for therapeutic applications, with the goal of improving the delivery of the therapeutic agents to treat diseases such as cancer. With each advancement in the technology over the

2nd generation:
Sterically stabilized liposomes
• **Drug carriers with extended circulation longevity**

Surface targeting

Thermosensitive

Programmable fusogenic

pH-sensitive

3rd generation:
Multi-functional liposomes
• **Sophisticated carriers with multiple components for specific target site delivery**

1st generation:
Conventional liposomes
• **simple carriers of therapeutic agents**

Figure 1.5. Schematic illustration of the characteristics of the various generations of liposomes.

past three decades, the complexity of the liposome formulation has increased. Figure 1.5 (P. 41) summarizes the characteristics of each generation of liposomes. One key advancement was the development of poly(ethylene glycol)-conjugated lipids (PEG-lipids) to stabilize liposomes in vivo, which has revitalized the role of liposomes as a delivery system. Physicochemical properties of these polymer-lipid derivatives will be discussed in the context of achieving unique liposome attributes including targeting capabilities and controllable surface properties which are the key elements in the thrombogenic liposomes to be developed in this thesis.

1.5.1. Simple carrier of therapeutic agents

Soon after the ability of phospholipids to self associate into enclosed spherical liposomes was discovered by Alec Bangham [167], it was recognized that liposomes could serve as drug carriers. Indeed, liposomes have been developed as effective carriers for the delivery of a wide range of therapeutic agents such as anti-cancer drugs, anti-microbial agents, genes and anti-sense molecules (reviewed in [168]). The following discussion will focus on the role and design of liposomes in the delivery of anti-cancer drugs for the treatment of solid tumors, which is more relevant to the context of this thesis.

Liposome encapsulation of anti-cancer drugs has been shown to provide a number of potential benefits including 1) protection of the encapsulated drug molecule from chemical or metabolic degradation after injection, 2) reduced toxicity due to the ability of liposomes to alter the tissue distribution of the drug that results in reduced drug exposure to healthy tissues, and 3) increased anti-tumor activity resulted from extended exposure and selective

accumulation of liposomal drug in tumor tissues [169-172]. As an example, delivery of doxorubicin in liposomes (100 nm diameter) to solid tumors was increased five-fold over free drug with reduced toxicity to the heart and gastrointestinal tract [173, 174]. The success of using liposomes to deliver anti-cancer drugs has been relied on the strategy of passive tumor targeting. As described in Section 1.2.2, the increased permeability of tumor vasculature together with the poor lymphatic drainage within the tumor enables selective accumulation of liposomes within tumors [175]. Thus, with this strategy of passive targeting, it is beneficial to develop liposome formulations which are capable of circulating in bloodstream for long period of time in an attempt to increase localization in tumors.

Based on the physicochemical properties of bilayers described previously, the choice of lipids in designing liposomes for drug delivery to solid tumors typically involved a combination of long-chained PC or SM with CHOL (30 – 50 mol%) to produce neutral LUVs that exhibit low membrane permeability and high stability toward plasma protein interactions. These liposomes, relatively simple in their design and composition, are referred as first generation or conventional liposomes. The drug molecules are often encapsulated by means of a transmembrane proton gradient, where the encapsulation efficiency may approach 100% for compounds such as doxorubicin [169, 176, 177]. Although the conventional liposomes have half lives (t $_{\frac{1}{2}\beta}$) of about 5 h, these liposomes are still recognized by the MPS, with subsequent accumulation in liver and spleen [178]. If the disease site is within the MPS, it is useful to exploit these conventional liposomes to target to these areas. However, if the disease site is beyond the MPS, localization of these liposomes in the MPS may limit the versatility of such liposomes as drug carriers in vivo.

1.5.2. Liposome surface modifications by PEG-lipid technology

Several strategies have been undertaken to reduce opsonization of liposomes and subsequent uptake by the MPS. Based on the observation that red blood cells can avoid opsonization and MPS recognition, the incorporation of monosialoganglioside (G_{M1}), a special lipid found in the outer membrane of red blood cells, into conventional liposomes resulted in prolonged circulation and reduced MPS uptake in mice [179]. However, one drawback of using G_{M1} in liposomes is that this lipid is difficult to purify or synthesize, and is available only from animal sources in small quantities [180].

A major advancement was made when the synthetic hydrophilic polymer, poly(ethylene glycol) (PEG), was attached to the liposome surface, providing extended circulation longevity and reduced MPS recognition. Three key observations leading to the application of PEG technology to liposomes include: 1) the ability of PEG to exclude proteins when it is used for purification and crystal growth [181], 2) its ability to protect therapeutic proteins and extend their circulation lifetimes [182-184], and 3) its ability to reduce protein adsorption onto surfaces covalently coated with this polymer [185]. Because of the reduced recognition by the MPS, the term "stealth" is often used to describe such property of these liposomes [186]. Investigators from early studies proposed that the liposomes are sterically stabilized by the presence of the head groups on the liposome surface, and hence these "stealth" liposomes are also termed as sterically stabilized liposomes [187, 188].

There are a number of methods to modify the liposome surface with PEG. PEG polymer can be first derivatized to a lipid anchor (usually PE) to form the PEG conjugated-lipid (PEG-lipid) which is then added to the lipid mixture for the production of liposomes [189, 190]. Or, the PEG polymer can be reacted with pre-formed liposomes containing activated groups on the liposome surface [191]. More recently, PEG-lipids can be incorporated into liposomes by incubating free PEG-lipids with liposomes where the PEG-lipids can insert into the bilayer spontaneously [192, 193]. Among these methods, incorporating the conjugated PEG-lipids before liposome formation is preferred because of the availability of PEG-lipids, the ease and the reproducibility of such production methods. Since PEG-lipids are extensively used in liposomes to provide better in vivo liposome stability, a description of the physicochemical properties of PEG-lipids is given below, followed by a review of their mechanisms in protecting liposomes from protein and cellular interactions.

1.5.2.1. Physicochemical properties of PEG-lipids

As the name has suggested, PEG-lipids consist of two components: the hydrophilic PEG polymer with commonly used molecular weights (MW) of 350 to 5000, and the hydrophobic lipid anchor including PE, ceramide and CHOL [180, 194-196]. These PEG-lipids having a hydrophilic polymer and a hydrophobic lipid anchor fit into the category of amphipathic molecules. The structure of the most commonly used PEG-lipid, DSPE-PEG 2000 is shown in Figure 1.6 (P. 46). The PEG polymer can be conjugated to the lipid anchor by different chemistries that result in different linkages (see Figure 1.7 on P. 47), which in turn, dictate the in vivo stability of these polymer-lipid conjugates [180, 197]. For instance,

A.
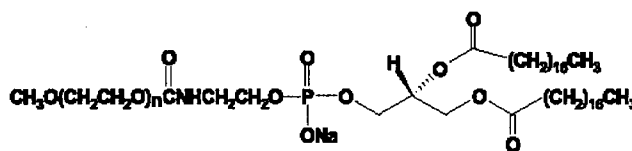
B.
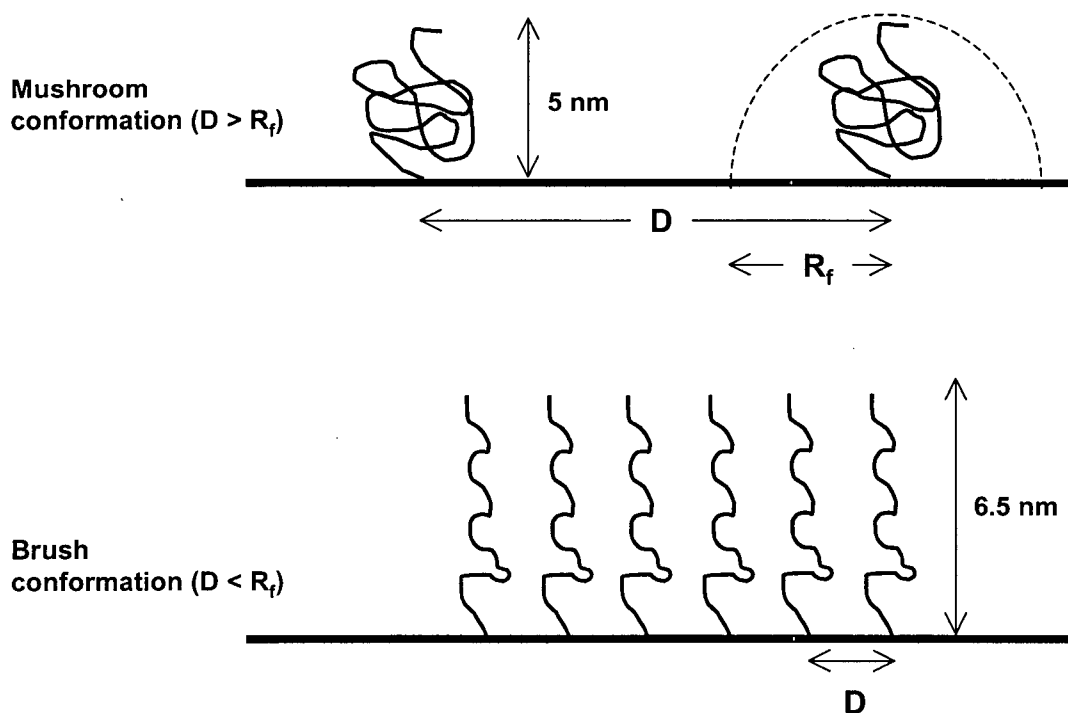
Figure 1.6. Structure of DSPE-PEG 2000 and the mushroom versus brush conformation of PEG 2000. Panel A shows the most commonly used PEG-lipid, DSPE-PEG 2000 (number of repeating units per polymer chain, n = 45). Panel B shows the "mushroom" versus "brush" conformation of the PEG 2000 polymer, where the adoption of conformation depends on the grafting distance between the polymers (D) and the Flory radius (R_f) of the polymer. The extensions of PEG 2000 from the liposome surface for the mushroom and brush conformations are also illustrated.

A.
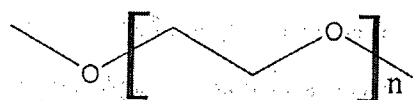
	Number of repeating units per polymer chains (n)	
Methoxy-PEG	PEG 350	n = 8
	PEG 550	n = 13
	PEG 750	n = 17
	PEG 2000	n = 45
	PEG 5000	n = 110

B.

succinate
linkage
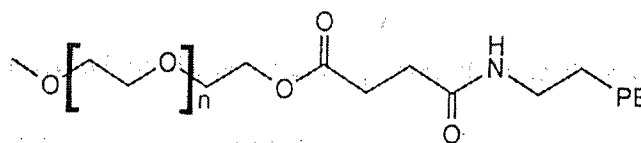
carbamate
linkage
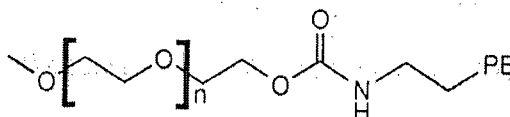
amide
linkage
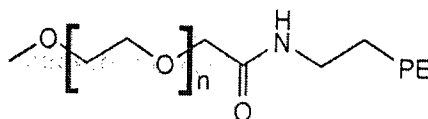
direct
linkage
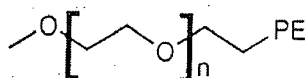
Figure 1.7. PEG polymers and various linkages commonly used in PEG-lipids. Panel A shows the chemical structure of methoxy-PEG, with the number of repeating units per polymer chain (n) listed for commonly used PEG polymers. Panel B shows the various linkages to phosphatidylethanolamine (PE).

the succinate linkage in the PEG-lipid is more susceptible to cleavage by esterases in blood than the carbamate linkage, resulting in a loss of the PEG polymer from the liposome surface [197]. The wide variety in the PEG polymer sizes, lipid anchors and linkage chemistries available for synthesizing PEG-lipids gives rise to different physicochemical properties of these lipid derivatives, including different phase preferences and different retention properties of PEG-lipids in the liposomes, as described below.

For PEG-lipids which have short polymer chains such as DSPE-PEG 350, they form bilayers in aqueous medium, while PEG-lipids such as DSPE-PEG 2000 or DSPE-PEG 5000 which have longer polymer chains form micelles [198, 199]. This observation can be explained through the relative shape adopted by these molecules, where an increase in the MW of the PEG polymer leads to a more "cone-shaped" PEG-lipid that prefers the micellar phase in aqueous medium [198]. The PEG-lipids which have longer polymer chains (e.g. PEG 2000 or PEG 5000) can be incorporated into liposomes; however, there is a maximum amount to which these PEG-lipids can be incorporated without disrupting the bilayer structure. For instance, up to 15 – 20 mol% DSPE-PEG 2000 can be incorporated into liposome bilayer of different compositions, and solubilization of the bilayer into micelles will occur above this level of PEG-lipid [200-202]. It appears that the transition from bilayer to micelles with PEG-lipid incorporation is not heavily dependent on the composition of the bilayer, but rather on the polymorphic properties of these lipid derivatives [200]. When using PEG polymer of the same MW, the hydrophobicity of the lipid anchor determines the rate of desorption of the PEG-lipid from the liposomes [112, 197, 203-205]. Lipid anchors that have short and/or unsaturated acyl chains have the

propensity to desorb from the liposome bilayer, while lipid anchors that have long, saturated acyl chains are retained in the liposome bilayer.

When PEG-lipids are incorporated into liposomes at levels where no bilayer solubilization occurs, the PEG polymer can adopt two different structural states, the "mushroom" or the "brush", depending on the grafting density of PEG on the liposome surface (see Figure 1.6 on P. 46) [206]. Mushrooms form at low grafting densities where adjacent PEG chains do not interact laterally, while brushes form at higher grafting densities where adjacent PEG chains overlap laterally [207]. The extension of the PEG polymer from the liposome surface has been reported, and it is generally accepted that the extension lengths are approximately 5 and 6.5 nm for mushroom and brush regimes of PEG 2000, respectively [207, 208].

1.5.2.2. Mechanisms of liposome protection by PEG-lipids

Several studies have reported that liposomes containing PEG-lipids, unlike conventional liposomes, have long circulation times that are independent of the administered lipid dose in the range of $4 - 400$ µmol/kg [178, 187]. The plamsa concentration versus time curve of these pegylated liposomes follows a single exponential process over nearly two log units, in contrast to that observed for conventional liposomes which exhibit at least two exponential processes (both types of liposomes are of $100 - 120$ nm in diameter).

One proposed mechanism of prolonged circulation longevity of pegylated liposomes involves the high degree of hydrophilicity of the PEG polymer [191]. Being soluble in all proportions in water, PEG is one of the most hydrophilic polymers [209]. An early study

has reported an increase in the circulation longevity of conventional liposomes by grafting the PEG polymer onto the liposome surface, and the investigators have attributed this effect to the ability of the PEG polymer to increase the liposome surface hydrophilicity, which presumably reduces MPS uptake [191]. Another group of investigators has also believed that the increased hydration provided by the PEG polymer is responsible for improving liposome stability in vivo [210]. Other groups have suggested that the high flexibility of the attached PEG polymer, in addition to its hydrophilicity, gives rise to an impermeable polymeric "cloud" over the liposome surface that is able to reduce protein adsorption even at relatively low polymer concentrations [211, 212]. But the most widely accepted mechanism of the increased circulation longevity of pegylated liposomes involves the ability of PEG to provide a steric repulsive barrier that reduces protein binding, membrane-membrane contact and subsequent liposome aggregation and cellular uptake [207, 208, 213].

The fact that pegylated liposomes are able to avoid the MPS, thus exhibiting "stealth" properties does not imply that they are not recognized by the MPS. Similar to the conventional liposomes, the pegylated liposomes are also eventually taken up by MPS organs, though at levels lower than that of conventional liposomes [173]. Recent studies have also shown surprising results where pegylated liposomes, given at doses lower than 1 μmol/kg, do not exhibit long circulating properties but instead are rapidly eliminated from bloodstream to the liver and spleen [214, 215]. It now appears that the PEG polymer on the liposome surface serves to slow down the rate of plasma protein adsorption and the rate of phagocytosis [191, 216].

In this thesis, PEG-lipids will be incorporated into the PS-based thrombogenic liposomes to serve several functional properties described as follows : 1) to protect the PS-based liposomes from the rapid removal by MPS such that these liposomes can be adequately accumulated within the tumor vasculature, 2) to provide controllable shielding of the PS liposome surface from the coagulation proteins such that unwanted coagulation reactions in bloodstream are avoided while coagulation reactions are initiated once these liposomes have reached the tumor vasculature, based on the ability of PEG-lipids to desorb from liposomes at different rates, and 3) to attach a targeting ligand onto the thrombogenic liposomes through a reactive group on the PEG polymer terminus to improve liposome localization at the target site. The controllable steric stabilization properties and the targeting capabilities exhibited by PEG-modified liposomes will be further discussed in the next section.

1.5.3. Liposomes with multiple functional components

Advances are being made in several areas of liposome design in an attempt to improve tumor specific drug release from the liposome carrier and intracellular delivery. In general, these liposomes are sophisticated in their design, with multiple functional components serving for specific purposes such as tumor site-specific delivery of therapeutic agents, controllable content release and drug release due to environmental changes.

1.5.3.1. Liposomes with controllable steric stabilization

Controllable steric stabilization has been applied to the design of "programmable" fusogenic liposomes which are formulated with the cationic lipid DOTAP and the non-

bilayer forming lipid DOPE [217, 218]. The inclusion of DOTAP serves to increase cellular association of the liposome formulation through charge interactions, and the inclusion of the hexagonal phase adopting lipid DOPE serves to induce destabilization of the liposome bilayer with subsequent release of content. However, in the absence of PEG-lipids, DOTAP/DOPE based liposomes are not stable and are removed rapidly from the bloodstream. The strategy of controllable steric stabilization with PEG-lipids is thus applied to provide these liposome formulations with transient stability, such that destabilization of such liposomes at target sites is not prevented. This strategy involves the use of PEG-lipids with lipid anchors of different acyl compositions such that the rates of PEG-lipid desorption from liposomes are different [218]. These programmable fusogenic liposomes have been shown to enhance the anti-tumor activity of the encapsulated anti-cancer drug mitoxantrone [217], and to control the intracellular delivery of antisense oligonucleotides [219]. It is believed that the strategy of controllable steric stabilization can be applied to the design of PS-based thrombogenic liposomes to control the interactions of the PS liposome surface with blood coagulation proteins.

1.5.3.2. Liposomes with targeting capabilities

The use of ligand directed targeting of liposomes have been investigated over the past two decades attempting to increase the accumulation and localization of liposomes in diseased sites such as tumors. This approach relies on the differential over-expression of target molecules on the target cells. The types of ligand commonly used include whole antibodies (Ab), Ab fragments and small peptides or molecules (see Table 1.2, P. 53). There are two key factors to be considered in the conjugation of ligand (especially whole Ab) to

Table 1.2.

Examples of ligands commonly used for liposomes with surface targeting information

Ligands	Target	References
• *Ab and Ab fragments*		
Mab N12A5	HER2/*neu*	[220]
RhuMabHER2 F(ab)'	HER2/*neu*	[221]
Mab 34A	Lung endothelium (gp 112)	[222]
Anti-myosin Ab	Infarcted myocardium	[223]
Anti-laminin Ab	Injured vascular bed	[223]
Mab F10.2	ICAM-1	[224]
Mab F(ab)' against E-selection	E-selectin	[225]
Mab against CD19	B cell lymphoma	[226]
• *Peptide*		
Laminin pentapeptide	Laminin receptor	[227]
• *Natural receptor ligands*		
Folate	Folate receptor	[228]
• *Other ligands*		
Avidin/Streptavidin	Biotin	[229]
Plasminogen	Fibrin	[230]

liposomes. First, different coupling chemistries can result in different orientations of the whole Ab on liposome surface. For instance, the use of maleimide chemistry gives rise to random orientations of the Ab due to modifications of primary amine groups on the Ab, and the use of hydrazide chemistry gives rise to Ab oriented with the antigen binding site exposed for interactions (see Figure 1.8 on P. 55) [231]. However, higher coupling efficiency is associated with the maleimide coupling chemistry [231]. Secondly, the ligand can be conjugated to the reactive group either on a lipid such as PE (i.e., ligand is on the liposome surface) [232-234] or on the PEG terminus of the PEG-lipid (i.e., ligand is extended away from the liposome surface) [222, 230, 231]. Figure 1.9 (P. 56) illustrates the different locations on liposomes to which the ligand, a whole Ab in this case, can be attached. The strategy of attaching the whole Ab to the surface of conventional liposomes has been shown to increase the removal of the liposomes from bloodstream which is mediated by the Fc region of the whole antibody, resulting in increased uptake by the MPS and short circulation longevity [235, 236]. Such rapid removal from blood will prevent the Ab-coupled liposomes from reaching the target diseased sites located outside the MPS. To overcome such problem, PEG-lipids have been included in the Ab-coupled conventional liposomes to extend the circulation longevity; however, the PEG polymer interferes with the binding of the Ab to its target molecule [237, 238]. Strategies of attaching the Ab to the PEG terminus were therefore developed to provide the liposomes with long circulation longevity and effective binding to target molecules [222, 230, 231]. Use of Ab fragments and small peptides as ligands have also been suggested to help reduce the Fc region-mediated removal of the Ab-coupled liposomes [227, 239, 240].

A. Conjugation with biotin-avidin interaction

Ab—BIOTIN　　+　　AVIDIN　　+　　BIOTIN—◯

Ab—BIOTIN— AVIDIN— BIOTIN—◯
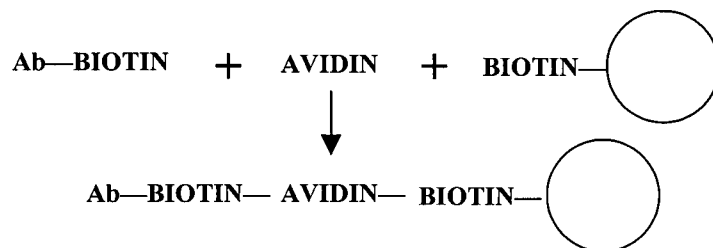
B. Conjugation through MAL/PDP chemistry: Ab in random orientation

Ab—SH

+

MAL

PE

Ab—N

+

PDP

PE

PE

PE
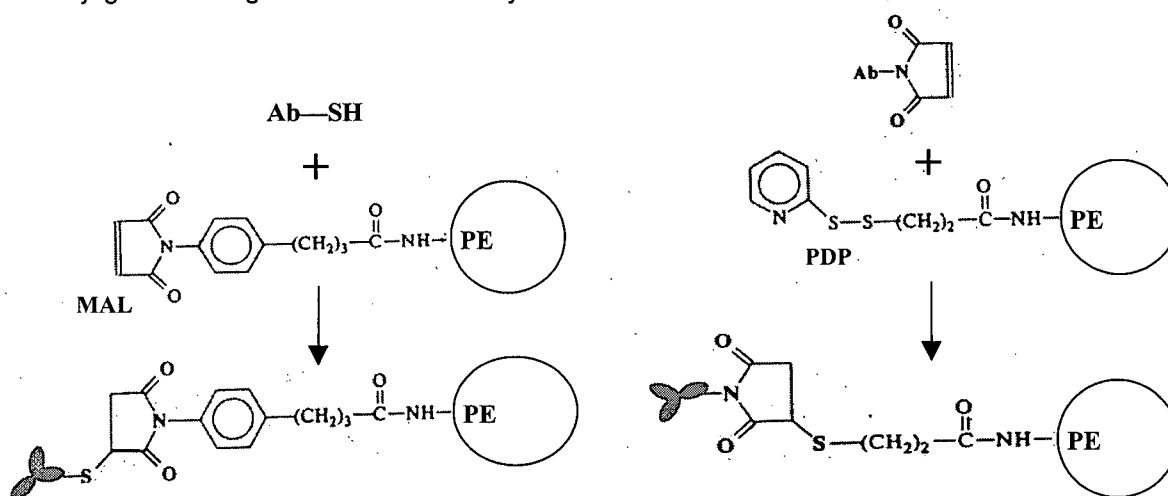
C. Conjugation through hydrazide chemistry: Ab with antigen binding site exposed for binding

Ab—CHO

+

$NH_2NH-C-CH_2-NH-C-O-[(CH_2)_2-O]_n-C-NH-$ PE

HYDRAZIDE　　PEG

$-CH=NNH-C-CH_2-NH-C-O-[(CH_2)_2-O]_n-C-NH-$ PE
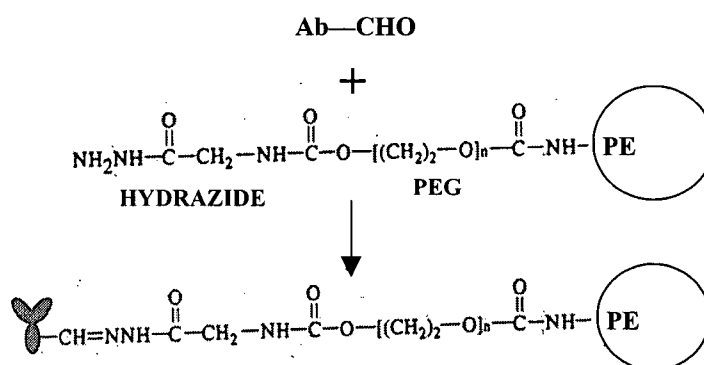
Figure 1.8. Schematic illustration of some commonly used conjugation chemistries. An antibody (Ab) is used as an example of a targeting ligand in this diagram. Panel A shows the conjugation of Ab to liposomes through biotin-avidin interaction. Panel B illustrates the conjugation reaction through MAL/PDP chemistry: the reaction on the left shows an Ab with a free thiol group coupling to a liposome with MAL group, and the reaction on the right shows an Ab with a MAL group coupling to a liposome with PDP group. The Ab conjugated through this reaction chemistry would be in random orientation. Panel C illustrates the conjugation reaction through hydrazide chemistry. This reaction would yield conjugated Ab with the antigen binding sites exposed for binding, and shown here is a liposome with the hydrazide group at PEG terminus. MAL, maleimide; PDP, pyridyldithioproprionate.
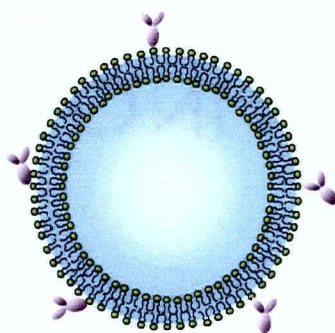
A. Conventional ligand-conjugated liposome
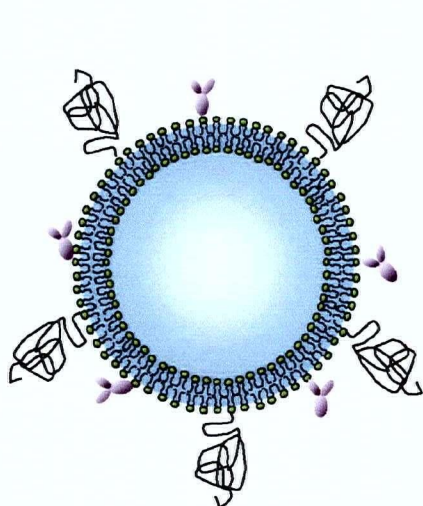
B. Pegylated ligand-conjugated liposome
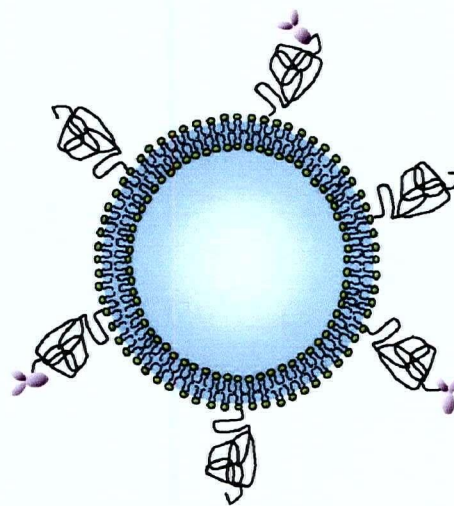
C. "Pedant-typed" ligand-conjugated liposome

Figure 1.9. Types of ligand-conjugated liposomes. A whole antibody is used to exemplify the different positions (either on the surface or on the distal end of PEG) where the ligand can be chemically conjugated to the liposome. Depending on the coupling chemistry, the orientation of the antibody can be random (as illustrated here) or specifically exposing the antigen binding site of the antibody.

Several studies have shown improved therapeutic activity of the encapsulated agent using liposomes with the targeting ligand attached at PEG terminus over those without targeting ligand. These successful in vivo models include improved efficacy of encapsulated agents such as doxorubicin which is targeted to CD-19+ human B-cell lymphoma [226] and to HER2-overexpressing human breast cancer xenografts [241, 242], and amphotericin B which is targeted to the vascular endothelial surface in the lung against invasive pulmonary aspergillosis in mice [243]. Of the successful examples described above, target sites such as lymphoma B cells and lung vascular endothelium represent areas from the intravascular compartment which are readily accessible to the drug-loaded Ab-conjugated liposomes. Thus, accessibility of the target molecule/cell plays an important role in the targeting of the ligand-coupled liposomes [244-246]. Targeting to the HER2-overexpressing human breast cancer xenografts illustrates the importance of having a target molecule that is selectively and highly (10^6 HER2/cell) expressed and is capable of internalization of the liposomes after binding to facilitate targeted intracellular drug delivery [221, 247].

1.5.3.3. Liposomes with novel drug release mechanisms

In addition to improving the accumulation of liposomes in diseased sites such as tumors, strategies that improve the rate and extent of release of the encapsulated agent from liposomes have also been developed. Indeed, PEG-containing liposome formulations have been shown to have slow release of encapsulated anti-cancer drugs and the drug may not be freely available within the tumor, although these liposome formulations of chemotherapeutics did achieve a significant reduction in tumor growth rate [248]. Thus,

development of techniques that allow increased rates of drug release from these liposome carriers combined with techniques that allow substantial accumulation in tumors is highly desirable. The two most widely investigated novel drug release mechanisms in the literature include drug release through local hyperthermia or changes in environmental pH, and are described in the following sections. Similar to the thrombogenic liposomes to be developed here, these liposomes are designed to have multiple components to achieve specific functional properties such as transient liposome stabilization and transformable capabilities to allow triggered release of the therapeutic agents in an effort to improve the availability of the therapeutic agent within its biological site of action.

Drug release through local hyperthermia

Local hyperthermia has been used clinically in the treatment of solid tumors. It appears to be beneficial to design liposomes with T_c which is close to the temperature used in local hyperthermia (2 – 3 °C above physiological temperature), such that a greater rate of release of the liposomal contents is achieved as the liposomes pass through the locally heated solid tumor, providing a higher local concentration of the therapeutic agent. This type of liposomes was first described by Yatvin et al in 1978 [249, 250]. Over the years, the design of these liposomes has become much more sophisticated, where PEG-lipids are incorporated for steric stabilization and a specific lipid (lysolipid) is used for enhancing the release of encapsulated agents in the range of clinically attainable hyperthermia (39° to 40 °C) [251]. This sophisticated thermosensitive liposome formulation has been used to encapsulate doxorubicin and tested in a human squamous cell carcinoma xenograft, with a

rate of complete tumor regression at 60 days post treatment of 17/20 [252, 253]. A recent review has summarized the development of thermosensitive liposomes [254].

Drug release through changes in environmental pH

pH-Sensitive liposomes were first described by Yatvin et al in 1980 [255, 256]. These liposome carriers were designed to contain specific agents that are capable of triggering liposome destabilization as a result of pH changes in the environment, usually making use of the acidic lysosome after liposomes are taken up into cells. These agents include pH-titratable lipids which are used in combination with fusogenic DOPE, acid-labile lipids which are hydrolyzed upon low pH releasing a membrane destabilizing agent, and pH-sensitive peptides which can mediate membrane fusion at low endosomal pH [257]. Although these liposomes have increased in vitro cytoplasmic delivery, the incorporation of the pH-sensitive agents often increases the rate of removal of the liposomes from circulation [257], which has been shown to require 10 mol% DSPE-PEG 2000 for in vivo stabilization [258].

1.6. Rationale and hypothesis of thesis project

Solid tumor regression through selective thrombosis of tumor blood vessels has been recently achieved using the "coaguligand" which is a truncated tissue factor linked to a monoclonal antibody targeted against the tumor vasculature [1, 2]. With the well-recognized ability of PS containing membranes to initiate the blood coagulation reactions, it is proposed that appropriately designed liposomal carriers for PS may have therapeutic applications in anti-cancer therapy that may be used alone or combined with other drugs inside the liposomes. In this regard, it is hypothesized that selective exposure of the

thrombogenic PS liposome surface within the tumor vasculature will induce tumor specific blood coagulation, resulting in occlusion in blood flow and tumor infarction. Central to testing the hypothesis is the ability to transiently protect the thrombogenic PS membrane surface from the blood clotting factors such that re-exposure of the thrombogenic PS membrane surface is allowed within the tumor vasculature to trigger the blood coagulation reactions. Further, conjugation of a monoclonal antibody against a marker that is selectively expressed on tumor vasculature will improve the accumulation and localization of the thrombogenic PS liposomes.

1.6.1. Principles of designing thrombogenic liposomes

The concept of how the ideal thrombogenic liposome should behave is illustrated in Figure 1.10 (P. 61). Upon intravenous injection, these PS-based liposomes should be well protected by the PEG-lipids such that the potential of triggering unwanted blood coagulation reactions in systemic circulation is minimized, and the removal by MPS is avoided. Once these liposomes reach the tumor vasculature, they will bind to the selectively over-expressed marker on the tumor vascular endothelial cells through ligand mediated interactions. The exposure of the PS liposome surface will then occur after target binding. Ideally, the loss of the protective PEG coating should occur only within the tumor vasculature. Once PS liposome surface exposure is achieved, blood coagulation reactions should proceed and produce multiple occlusions to tumor blood flow, resulting in massive tumor cell death due to deprivation of oxygen and nutrients.

The ideal thrombogenic liposome in vivo

Stage 1: thrombogenic liposome surface protected in blood stream
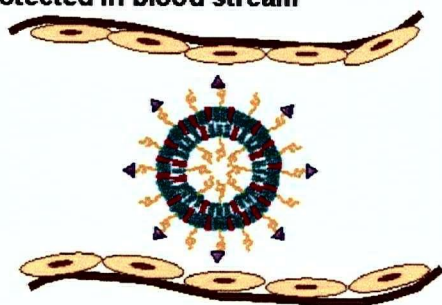
Stage 2: binding to marker in tumor vascular endothelium

tumor vascular endothelium

loss of PEG coating

binding of clotting factors

Stage 4: site-specific blood clotting in tumor vasculature
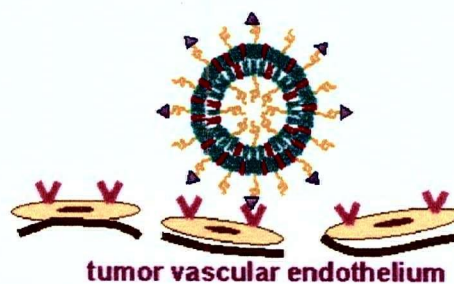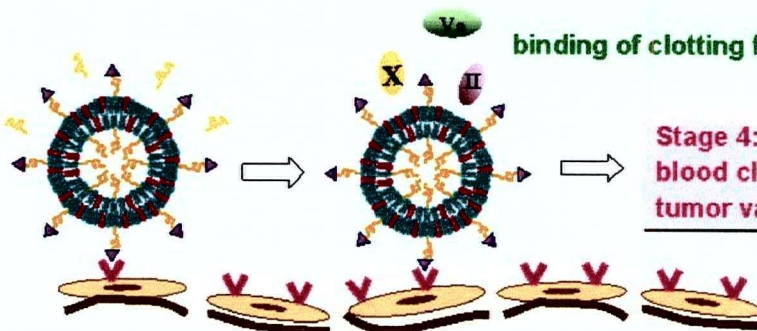
Stage 3: re-exposure of thrombogenic surface & trigger of blood clotting

Figure 1.10. Schematic illustration of the ideal thrombogenic liposome in vivo.

1.7. Summary of research objectives

The overall objective of this thesis is to develop novel, tumor vasculature-targeted thrombogenic liposomes containing PS for triggering tumor specific thrombosis. With the present liposome technology, sophisticated liposomal formulations with controllable surface properties and improved targeting capabilities can be designed. The focus of this thesis is to engineer the components in the thrombogenic liposomes to achieve the functional properties necessary for inducing tumor specific thrombosis, with the specific objectives for the thesis listed as follows:

1. **To develop a PS-based liposomal formulation with optimal surface thrombogenicity which is coupled with effective shielding of the thrombogenic liposome surface by PEG-lipids.** This is described in Chapters 2 and 3, where these properties of the thrombogenic liposomes were evaluated in terms of their ability to interact with blood clotting factors and plasma proteins.

2. **To control the surface reactivity of the PS-based liposomes by optimizing the rate of PEG-lipid exchange out of the liposomes.** This is described in Chapter 4, where a series of in vitro and in vivo studies were conducted to determine the effectiveness of PEG-lipids containing various acyl chain compositions in controlling the interactions of PS-based liposomes with blood coagulation proteins and the circulation longevity of these liposomes.

3. **To demonstrate the initiation of a localized, controllable blood coagulation response via targeting of an Ab-conjugated PS containing liposome to a tumor vasculature marker.** This is described in Chapter 5, where a series of in vitro and in vivo studies were performed to determine the binding of such liposomes to the target marker and to evaluate the clotting activities of these liposomes subsequent to target binding.

In the final chapter of this thesis (Chapter 6), approaches to improve the physicochemical attributes of the thrombogenic liposomes for achieving the functional objective of the liposomes will be discussed, and formulation issues remaining to be resolved will also be addressed.

Chapter 2   In vitro characterization of thrombogenic liposomes [*]

2.1. Introduction

Phosphatidylserine has long been recognized for its involvement in the blood coagulation cascade reactions [87]. Since DOPS containing membranes are the most effective in binding the high affinity blood coagulation proteins with subsequent activation of the blood coagulation reactions [94], this lipid was selected as one of the major components in the thrombogenic liposomes that are being developed in this thesis. The contents of DOPS in the liposomes can be varied to optimize thrombogenicity. However, for the applications in this thesis, the thrombogenic PS liposome surface must also be temporarily protected to prevent unwanted blood coagulation reactions from occurring in the general circulation. In this chapter, the effectiveness of PEG-lipids in controlling surface exposure and PS-mediated blood coagulation reactions is investigated.

The physical, chemical and biological properties of liposomes containing PEG-lipids have been extensively studied over the past decade. Much of this interest arose from attempts to utilize PEG polymer as a "cloaking" agent in order to extend blood residence times of various macromolecule-based pharmaceutical formulations. For liposomal delivery systems, PEG-lipids provided a synthetic alternative to ganglioside G_{M1}, a naturally occurring glycosylated lipid that had been shown to significantly increase the circulation longevity of 100 nm diameter liposomes into which it had been incorporated [187]. It is widely known that PEG-lipids can reduce plasma protein binding and cellular surface interactions at a PEG grafting density of 5 mol%. As described in Chapter 1, the

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[*] **Data except Figure 2.1 have been published in Biochim Biophys Acta 1510: 56-69, 2001.**

characteristics of reduced protein binding and MPS clearance associated with PEG

containing liposomes are due to the steric repulsive barrier imparted by the PEG polymer

extended away from the liposome surface [207, 208, 213]. These characteristics can also be

attributed to the high degree of water binding by the hydrophilic PEG polymer on the

surface, and the ability of the random coil conformation of PEG to provide a "mushroom-

shaped" steric barrier to many proteins and larger biological structures [191, 210, 212].

The degree of surface binding inhibition provided by PEG on the membrane surface

depends on the molecular weight of the PEG polymer, the concentration of PEG-lipid in the

bilayer and the molecular weight (size) of the membrane binding ligand. For example,

liposome-cell interactions can be reduced with as little as 0.5 mol% PE-PEG 2000

incorporation, whereas inhibition of liposome binding for small macromolecules (i.e. $M_r <$

20,000) requires between 5 – 7 mol% PE- PEG 2000 or 10 – 20 mol% PE-PEG 750 [259,

260]. It may not be surprising, then, that 5 mol% DSPE-PEG 2000 is typically employed in

liposomes for intravenous drug delivery in order to limit liposome interactions with cells and

reduce binding of small molecular weight components present in plasma.

The vast majority of studies with PEG-containing membranes have been performed

with liposomes composed of lipids exhibiting no net charge such as PC, SM and CHOL.

Indeed, when more reactive lipids such as the negatively charged phospholipid PS were

incorporated into liposomes containing 5 mol% PE-PEG 2000, these previously sterically

stabilized liposomes became readily recognized by cells of the MPS and were removed very

rapidly from the circulation [190, 238]. Consequently, there is a need to better characterize

the steric stabilization effects of PEG-lipids in liposomes that display reactive lipid

membrane surfaces in order to control their exposure for the intended application described

in this thesis.

In this chapter as well as the next, the steric stabilization properties of PEG-lipids

incorporated into liposomes containing DOPS were investigated. In particular, this chapter

is focused on the effect of PEG-lipids on the well-characterized binding interactions of

blood coagulation proteins with PS containing liposomes. With this system, the influence of

PEG composition and content on PS containing lipid surfaces are evaluated at different

levels: 1) quantitative evaluation of the binding of specific blood coagulation proteins, and

2) determination of functional activity of membrane bound proteins and protein complexes.

It is demonstrated here that the properties of the steric repulsive PEG barrier in liposomes

containing DOPS are substantially modified from those observed with neutral liposomes.

2.2. Materials and Methods

2.2.1. Materials

All lipids were obtained from Avanti Polar Lipids (Alabaster, AL) except for

[^{3}H]DSPE-PEG 2000 which was obtained from Northern Lipids (Vancouver, BC). The

[^{3}H]- and [^{14}C]CHE were from NEN/Dupont (Mississauga, ON). Cholesterol, ellagic acid,

Sepharaose CL-4B were purchased from Sigma (St. Louis, MO). All blood coagulation

proteins were purchased from ICN (Aurora, OH), and the thrombin chromogenic substrate

S-2238 was purchased from Chromogenix (Molndal, Sweden). Bio-Gel A-15m and A-5m

size exclusion gel and gel filtration standards were obtained from Bio-Rad (Mississauga,

ON). The micro bicinchoninic acid (micro BCA) protein assay kit was purchased from

Pierce (Rockford, IL). The Oregon Green 514 protein labeling kit was purchased from

Molecular Probes (Eugene, OR).

2.2.2. Preparation of large unilamellar liposomes

Lipids were prepared in chloroform solution and subsequently dried under a stream

of nitrogen gas. The resulting lipid film was placed under high vacuum for a minimum of 2

h. The lipid film was hydrated in HBS (Hepes 20 mM/ NaCl 150 mM buffer, pH 7.5) at 65

°C to form multilamellar vesicles. The resulting preparation was frozen and thawed five

times prior to extrusion 10 times through stacked polycarbonate filters (Poretics Co.,

Canada) with an extrusion apparatus (Lipex Biomembranes, Vancouver, BC) at 65 °C [261,

262]. The resulting mean liposome diameter obtained following extrusion was 100 – 120

nm as determined by quasi-elastic light scattering using the Nicomp submicron particle sizer model 370/270 operating at a wavelength of 632.8 nm.

2.2.3. Incorporation of DSPE-PEG 2000 into liposomes

Incorporation and retention of DSPE-PEG 2000 in liposomes after preparation were determined by size exclusion chromatography. Briefly, liposomes with traces of $[^{14}C]CHE$ (as a general lipid marker) and $[^{3}H]DSPE-PEG$ 2000 (as the PEG-lipid marker) were applied to a 42 cm × 1.3 cm Bio-Gel A-15m column (50 – 100 mesh), and were eluted with HBS at a flow rate of 0.5 mL/min regulated by a peristaltic pump. Aliquots from the 1-mL column fractions were counted directly in 5.0 mL Pico-fluor 40 scintillation fluid (Packard Biosciences, The Netherlands).

2.2.4. In vitro clotting time assay

This assay was based on the activated partial thromboplastin time. Human plasma was collected into citrated tubes (9 mL blood to 1 mL citrate) from four normal, healthy volunteers, and pooled to make one batch of plasma for studies in this chapter. An ellagic acid solution was used freshly prepared as follows: 1 mg ellagic acid was dissolved in 33.3 mL HBS and filtered through a 0.8 μm syringe filter. Human citrated plasma (50 μL) was pre-incubated with 10^{-5} M ellagic acid (50 μL) and liposomes (50 μL) for two minutes at 37 °C. Calcium (8.75 mM as final concentration) was then added to initiate the clotting reaction. The time at which the mixture changed from a liquid to a viscous gel was recorded, and was noted as the time for the clotting reaction to be completed.

2.2.5. In vitro chromogenic assay for factor Xa activity

Formation of catalytically active prothrombinase protein complexes (factor Xa and factor Va) on liposome surfaces was determined employing a chromogenic substrate that is cleaved by enzymatically active thrombin [263]. Briefly, the "prothrombinase complex cocktail" contained the components for the prothrombinase conversion under the following conditions: 8.0 nM (0.2 unit) factor Xa, 0.2 nM factor Va, 6 mM $CaCl_2$, and liposomes at various concentrations. These mixtures were incubated in Tris 50 mM/NaCl 120 mM buffer (pH 7.8) for five minutes at 37 °C. Prothrombin (1 mM) was added to the cocktail, and the final mixture (150 μL) was incubated for three minutes. The conversion of prothrombin to thrombin was stopped by the addition of EDTA (15 mM final concentration). S-2238, which is a specific chromogenic substrate of thrombin, was added at 0.4 mM, and the rate of chromogen formation was monitored at 405 nm with a plate reader equipped with kinetic analysis software (Dynex Technologies Inc., Chantilly, VA). A calibration curve was obtained under the same conditions in the presence of lipids with known amounts of thrombin, and the amount of thrombin formed in the assay were determined from the calibration curve.

2.2.6. Prothrombin binding to liposomes

Bovine prothrombin was labeled with the fluorescent dye Oregon Green 514, containing a reactive succinimidyl ester moiety that reacts with primary amines of the protein to form dye-protein conjugates. The fluorescently labeled prothrombin was incubated with various liposome composition at lipid concentrations of 0.2 and 0.4 mg/mL

in the presence of 2.0 mM Ca^{2+} at 37 °C for 15 min, and the above reaction conditions were based on a previous study on prothrombin binding to PS membranes [264]. The mixture was then separated using Microcon 100 ultrafiltration devices (Millipore, Bedford, MA) by centrifugation at 3000 × g for 15 min. The filtrate, containing free protein, was measured for fluorescence with excitation and emission wavelengths set at 506 and 526 nm, respectively. The amount of prothrombin bound to liposomes was determined using a calibration curve constructed with known amounts of fluorescent labeled prothrombin and correcting for protein recovery using liposome-free prothrombin solutions.

2.3. Results

2.3.1. Clotting activities of two different neutral phospholipids as the bulk lipid component in thrombogenic liposomes

The effect of different neutral phospholipids used as the bulk lipid component in the thrombogenic liposome formulation was first investigated. Here, the different combinations of neutral lipids used were EPC/CHOL and DSPC/CHOL with or without incorporation of 20 mol% DOPS (Figure 2.1, P. 72). The CHOL content in the liposomes was maintained 45 mol%. The comprehensive procoagulant activity of the various liposomes was determined using an assay which measured the activated partial thromboplastin time, where exogenously added liposomes provided the catalytic membrane surface for blood coagulation. In the presence of 20 mol% DOPS, EPC/CHOL and DSPC/CHOL liposomes were substantially more procoagulant than those without DOPS. For instance, the clotting times for EPC/CHOL or DSPC/CHOL liposomes without DOPS were approximately 300 s for liposome concentrations between 0.01 – 0.1 mM, whereas the incorporation of 20 mol% DOPS into either liposomes led to a decrease in the clotting times to about 120 – 150 s. Increasing the liposome concentration to 1 mM imparted slight thrombogenic activities to the neutral EPC/CHOL and DSPC/CHOL liposomes, with clotting times decreased to about 240 s. The increase in clotting time observed with 20 mol% PS liposomes as the liposome concentration increased is consistent with an excess of the catalytic surface. This excess catalytic surface results in surface-dilution effects of the blood coagulation proteins, leading to decreased thrombogenic activity [54]. In general, 20 mol% DOPS/DSPC/CHOL liposomes were somewhat more procoagulant than 20 mol% DOPS/EPC/CHOL, and consequently DSPC/CHOL was chosen as the bulk lipid component in thrombogenic PS liposome formulations.
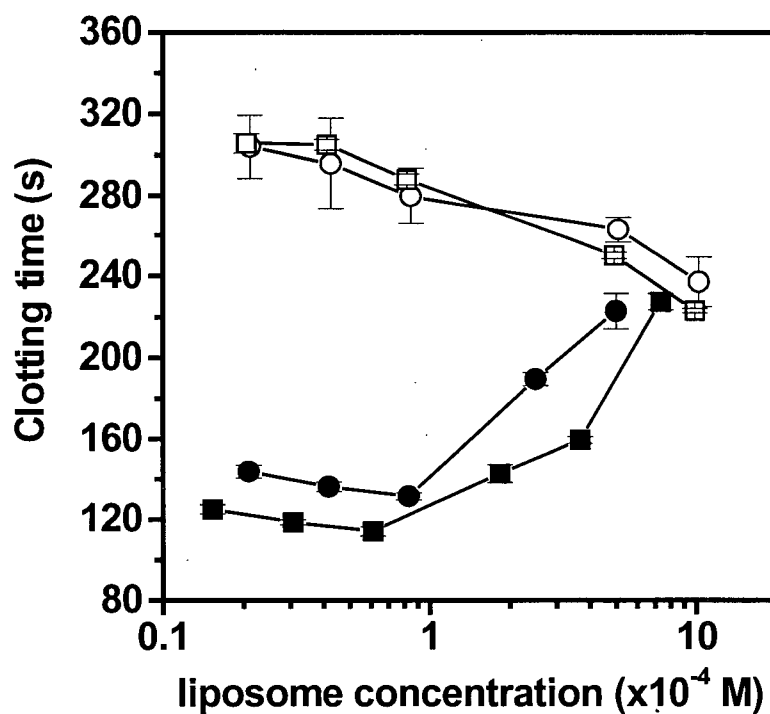
Figure 2.1. Comparison of clotting activity of EPC (circle) and DSPC (square) as the bulk neutral lipid component in liposomes with (solid symbols) or without (open symbols) 20 mol% DOPS. All the liposomes contained 45 mol% CHOL. The clotting times were determined in triplicate with the in vitro clotting time assay, where liposomes were pre-incubated with human citrated plasma, ellagic acid (10^{-5} M) for 2 min and calcium was subsequently added to initiate the clotting reactions, as described under Section 2.2.4. The error bars represent S.D.

2.3.2. Clotting activities of liposomes containing various mole percentages of DOPS

Next, the clotting times of DSPC/CHOL liposomes with various mol% DOPS were compared in order to establish baseline procoagulant activity of the liposomes as well as conditions for comparative experiments (Figure 2.2, P. 74). In the absence of liposomes in the assay mixture, the time to form the fibrin clot was approximately 350 s, while the addition of neutral DSPC/CHOL liposomes at 0.02 mM and 0.5 mM decreased the clotting time to 310 s and 255 s, respectively. The inclusion of 10 – 20 mol% DOPS in DSPC/CHOL liposomes decreased the clotting times to approximately 120 s when the liposome concentration was below 0.1 mM. This reduction in clotting times reflected that the presence of a negatively charged phospholipid membrane surface enhanced the blood coagulation reactions. The clotting time of 20 mol% DOPS liposomes increased to 152 s as the liposome concentration was increased to 0.36 mM; however, the clotting times of 10 mol% DOPS liposomes were maintained at approximately 120 s throughout the range of liposome concentrations used in the assay. These results indicate that 10 mol% DOPS liposomes exhibited optimal thrombogenic activity, and were utilized to evaluate the effectiveness of DSPE-PEG 750 and DSPE-PEG 2000 in impeding the propagation of the blood coagulation cascade.

2.3.3. Effect of PEG-lipids on the functional activity of membrane bound blood coagulation proteins

The effect of PEG-lipids on the catalytic activity of the well studied prothrombinase complex was first investigated. The prothrombinase complex consists of factors Xa and Va
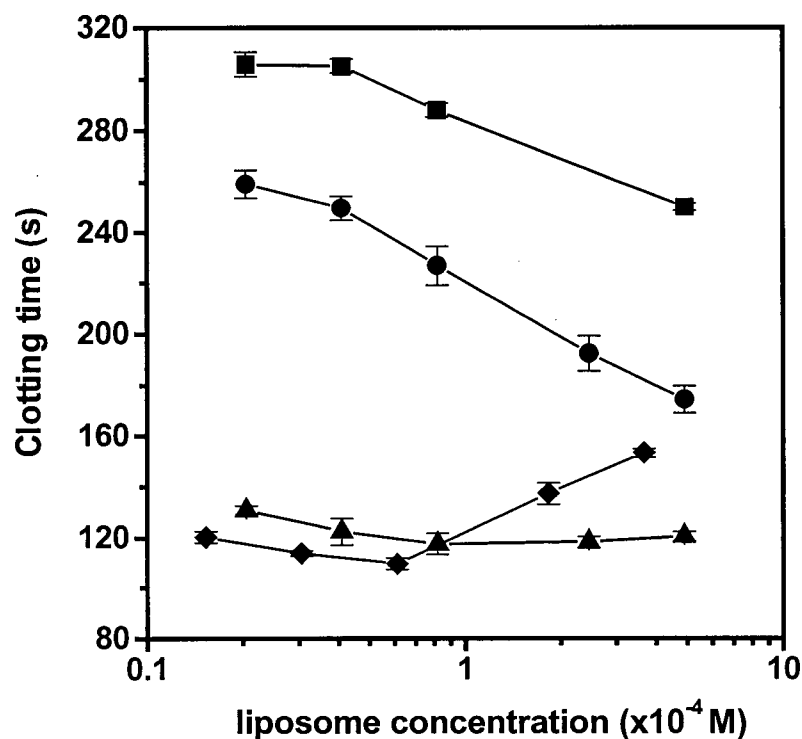
Figure 2.2. Clotting activity of DSPC/CHOL liposomes with different mole percentages of DOPS. The clotting times were measured for 0 mol% (■), 5 mol% (●), 10 mol% (▲) and 20 mol% (◆) of DOPS incorporated in DSPC/CHOL with the in vitro clotting time assay, where liposomes were pre-incubated with human citrated plasma, ellagic acid (10^{-5} M) for 2 min and calcium was subsequently added to initiate the clotting reactions, as described under Section 2.2.4. Data points were determined in triplicate, and the error bars represent S.D.

assembled on negatively charged membrane surfaces, and is responsible for the proteolytic activation of prothrombin to thrombin. The rate of thrombin formation by the prothrombinase complex in the presence of liposomes was monitored using a chromogenic substrate that is activated by thrombin, and the results are summarized in Table 2.1 (P. 76). No substrate activation was observed in the absence of liposomes. The rate of thrombin formation was negligible for the neutral DSPC/CHOL liposomes (0 mol% DOPS). Incorporating 10 mol% DOPS into DSPC/CHOL liposomes resulted in a rate of thrombin formation of 1.94 mol thrombin/min/mol factor Xa. The effectiveness of DSPE-PEG 750 and DSPE-PEG 2000 in inhibiting the assembly and the catalytic activity of the prothrombinase complex on the DOPS membrane surface were evaluated. With 10 mol% DSPE-PEG 750 or 5 mol% DSPE-PEG 2000 in 10 mol% PS liposomes, the rates of thrombin formation were 2.48 and 1.96 mol thrombin/min/mol factor Xa, respectively, which were not statistically different from that for the non-pegylated DOPS liposomes ($p > 0.05$). Only by elevating the PEG-lipid content to 20 mol% for DSPE-PEG 750 or $10 - 15$ mol% for DSPE-PEG 2000 could the rate of thrombin formation be substantially reduced to < 0.465 mol thrombin/min/mol factor Xa (minimum detectable rate).

In addition to the prothrombinase complex, the PS membrane surface is required for the proteolytic activation of several additional blood coagulation proteins and propagation of the blood coagulation cascade [54, 265]. Also, full clot formation requires the release of thrombin from the prothrombinase complex which can then enzymatically convert fibrinogen to fibrin. Thus, the impact of PEG-lipids on the comprehensive

Table 2.1

Rate of thrombin formation in the presence of various liposomes assayed by the in vitro chromogenic assay

Liposomes	mol Thrombin/min/mol factor Xa ± S.D. [a]
DSPC/CHOL 55:45	N. D. [b]
DOPS/DSPC/CHOL 10:45:45	1.94 ± 0.24 [c]
DSPE-PEG 2000/DOPS/DSPC/CHOL 5:10:40:45	1.96 ± 0.34 [c]
DSPE-PEG 2000/DOPS/DSPC/CHOL 10:10:35:45	N. D.
DSPE-PEG 2000/DOPS/DSPC/CHOL 15:10:30:45	N. D.
DSPE-PEG 750/DOPS/DSPC/CHOL 10:10:35:45	2.48 ± 0.18
DSPE-PEG 750/DOPS/DSPC/CHOL 20:10:25:45	N. D.

[a] Data represent the average ± S.D. of three experiments. Liposome concentrations used were 75 µM, and the rate of thrombin formation was determined as described in Section 2.2.5.

[b] N. D. represents non-detectable levels which were characterized by the assay system to be below 0.46 mol thrombin/min/mol factor Xa.

procoagulant activity of DOPS liposomes was determined using the in vitro clotting time assay which measured the activated partial thromboplastin time where exogenously added liposomes provided the catalytic membrane surface. The percent inhibition of clotting activity of 10 mol% DOPS liposomes by DSPE-PEG 750 or DSPE-PEG 2000 is presented in Figure 2.3 (P. 77). The inhibitory effect of DSPE-PEG 750 was sigmoidal, as reflected by the increase in the percent inhibition of the clotting activity for 10 mol% DOPS liposomes. When incorporated at ≤ 10 mol%, DSPE-PEG 750 inhibited approximately 15% of the clotting activity for 10 mol% DOPS liposomes. When the level of DSPE-PEG 750 in DOPS liposomes was increased to 20 mol%, the inhibition of clotting activity of 10 mol% PS liposomes was increased to 85%. A similar trend was observed with DSPE-PEG 2000 where low levels of the PEG-lipid in the DOPS liposomes provided only modest inhibition to the clotting activity. Specifically, when DSPE-PEG 2000 incorporated at 5 mol%, 40% of the clotting activity was inhibited for 10 mol% DOPS liposomes. This inhibitory effect was increased to approximately 80% when the level of DSPE-PEG 2000 was increased to 15 mol% in the DOPS liposomes. These results clearly indicate that DSPE-PEG 2000 incorporated at low levels (e.g. 5 mol%) is not effective in preventing the PS-mediated clotting reactions, although this level of DSPE-PEG 2000 is effective in preventing protein interactions with neutral liposomes.

2.3.4. Inhibitory effect of DSPE-PEG 2000 on prothrombin binding to DOPS liposomes

The results thus far demonstrate the ability of DSPE-PEG 2000 to effectively inhibit the clotting activity of DOPS liposomes. However, its ability to prevent proteins
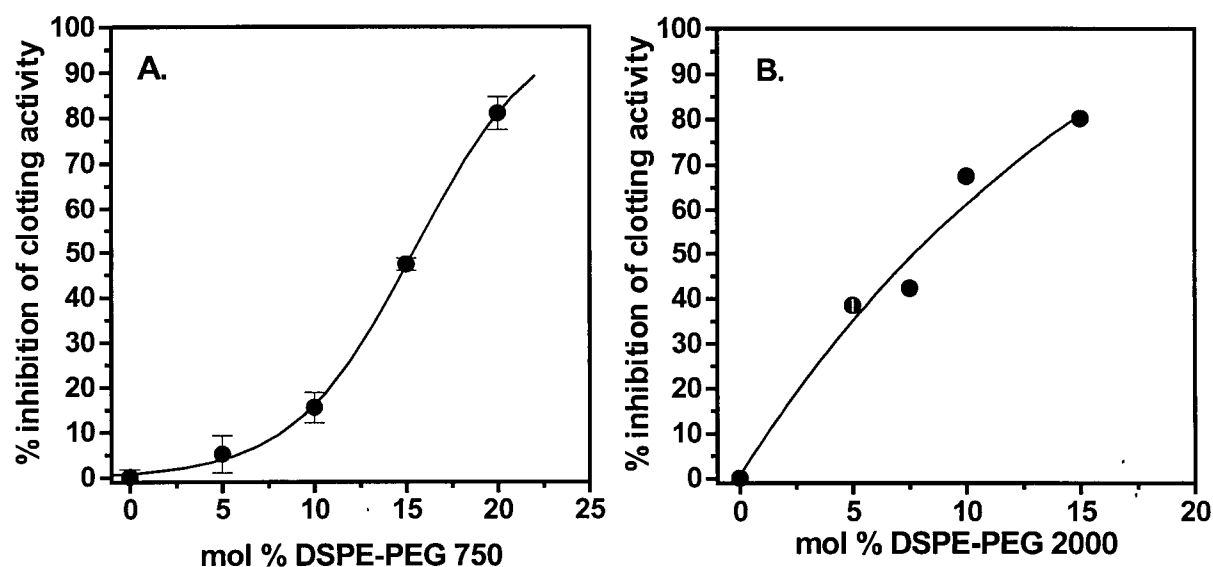
Figure 2.3. The effect of PEG-lipids on the clotting activity of DOPS liposomes. The PEG-lipids used here were (A) DSPE-PEG 750 and (B) DSPE-PEG 2000 in 10 mol% DOPS liposomes. The % inhibition was calculated as follows: % inhibition = $(t_{PEG} - t_{PS})/(t_{blank} - t_{PS}) \times 100$ where t represented the clotting time of each type of liposome as determined by the in vitro clotting time assay described in Section 2.2.4. The liposome concentrations used in (A) and (B) were 0.4 mM and 0.2 mM, respectively, and were pre-incubated with human citrated plasma, ellagic acid (10^{-5} M) for 2 min, and calcium was subsequently added to initiate the clotting reactions. Data points were determined in triplicate, and the error bars represent S.D. In some cases, the size of the symbol is larger than the size of the error bar.

from accessing the PS membrane surface is yet to be determined. Here, the binding of fluorescently labeled prothrombin to the 10 mol% DOPS liposomes containing various percentages of DSPE-PEG 2000 was investigated. Fluorescent derivatization of prothrombin did not significantly alter the binding properties of prothrombin to 10 mol% DOPS liposomes, as free vs. bound protein fractions were similar to those for prothrombin binding to PS containing liposomes reported previously using light scattering techniques [264]. Negligible prothrombin association with liposomes was observed in DSPC/CHOL liposomes (with no DOPS incorporated). Incorporation of DSPE-PEG 2000 at 5 mol% into 10 mol% DOPS liposomes resulted in 28% and 37% inhibition of prothrombin binding at protein/lipid wt. ratios of 0.25:1 and 0.1:1, respectively (Figure 2.4, P. 80). Increasing the amount of DSPE-PEG 2000 to 10 mol% or 15 mol% substantially enhanced the inhibition of prothrombin binding to the 10 mol% DOPS liposomes, where prothrombin binding was inhibited by 75 – 85% using 15 mol% DSPE-PEG 2000 at protein to lipid wt. ratios of 0.1:1 and 0.25:1.

2.3.5. Incorporation of DSPE-PEG 2000 into PS liposomes

From the results of the *in vitro* studies, 15 mol% DSPE-PEG 2000 was the most effective to reduce the PS-mediated blood coagulation protein interactions with liposomes. At such high levels, there are potential concerns regarding the incorporation efficiency of this PEG-lipid. In particular, formation of micelles has been demonstrated when the level of DSPE-PEG 2000 was more than 15 mol% in liposomes [198, 200, 201], which is close to the level used in the present study. Therefore, the incorporation of 15 mol% DSPE-PEG 2000 into 10 mol% DOPS liposomes was examined with size exclusion chromatography
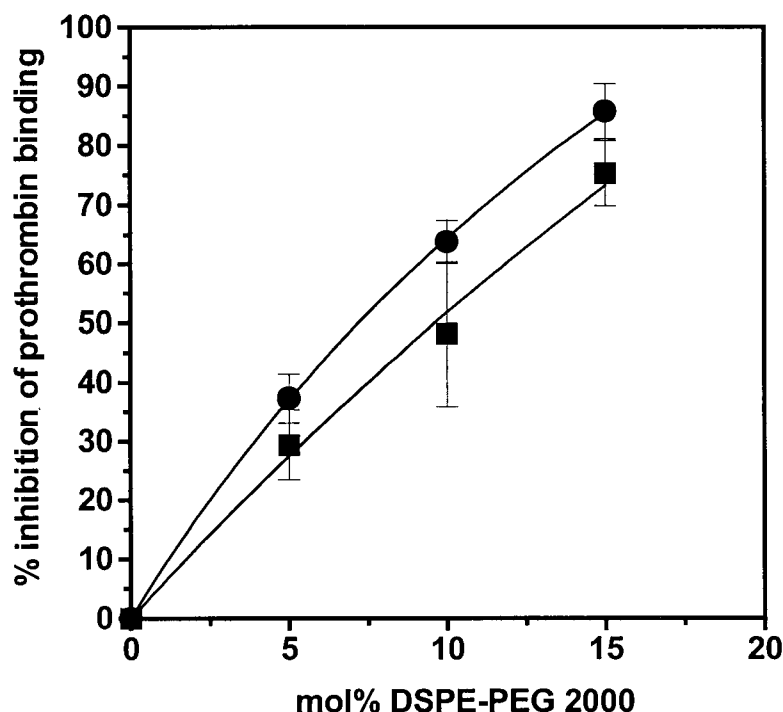
Figure 2.4. The effect of DSPE-PEG 2000 on the binding of prothrombin to 10 mol% DOPS liposomes. Two different prothrombin to liposome ratios, 0.1 w/w (●) and 0.25 w/w (■), were used. The fluorescently labeled prothrombin was incubated with various liposomes at concentrations of 0.2 and 0.4 mg/mL in the presence of 2.0 mM Ca^{2+} at 37 °C for 15 min. The percentage of inhibition of prothrombin binding was calculated as follows: % inhibition = $(F_{PEG} - F_{PS})/(F_{control} - F_{PS}) \times 100$ where F represented the fluorescence of the unbound protein in the filtrate after centrifugation with the Microcon 100 ultrafiltration device which was determined as described in Section 2.2.6, and the subscripts represented the different types of liposomes tested. Data are reported as the average of three experiments, with the error bars representing S.D.

where liposomes would be separated from free micellar DSPE-PEG 2000. The ability of the Bio-Gel A-15m gel filtration column to resolve liposomes from free micellar DSPE-PEG 2000 was first confirmed by applying liposomes and free DSPE-PEG 2000 to the column immediately after mixing. As shown in the inset of Figure 2.5 (P. 82), liposomes eluted at the exclusion volume of the column, while free DSPE-PEG 2000 eluted near the inclusion volume. This demonstrates that free micellar DSPE-PEG 2000 could be separated from liposomes in mixed solutions, and this could be used to determine the retention of DSPE-PEG 2000 in liposomes when prepared at high incorporation levels.

Figure 2.5 (Panel A) shows that no free DSPE-PEG 2000 peak was observed in the elution profile of the stock preparation (54 mM) of 10 mol% DOPS liposomes containing 15 mol% DSPE-PEG 2000. Identical elution profiles were obtained when the stock liposomes were diluted to the concentrations used in the in vitro experiments. Based on the ratio of the radiolabeled DSPE-PEG 2000 and liposome marker ([^{14}C]CHE), the amount of DSPE-PEG 2000 present in the DOPS liposome containing fractions reflected a DSPE-PEG 2000 composition of over 14 mol% for all of the liposome concentrations. For comparison, DSPC/CHOL liposomes containing 15 mol% DSPE-PEG 2000 were assayed with the same technique, and a similar elution profile was observed, with over 14 mol% DSPE-PEG 2000 being incorporated into the liposomes (Figure 2.5, Panel B). These data indicate that DSPE-PEG 2000 content up to 15 mol% can be incorporated into liposomes, and that free PEG-lipid could not be detected during the preparation or the dilution of liposomes containing up to 15 mol% DSPE-PEG 2000.
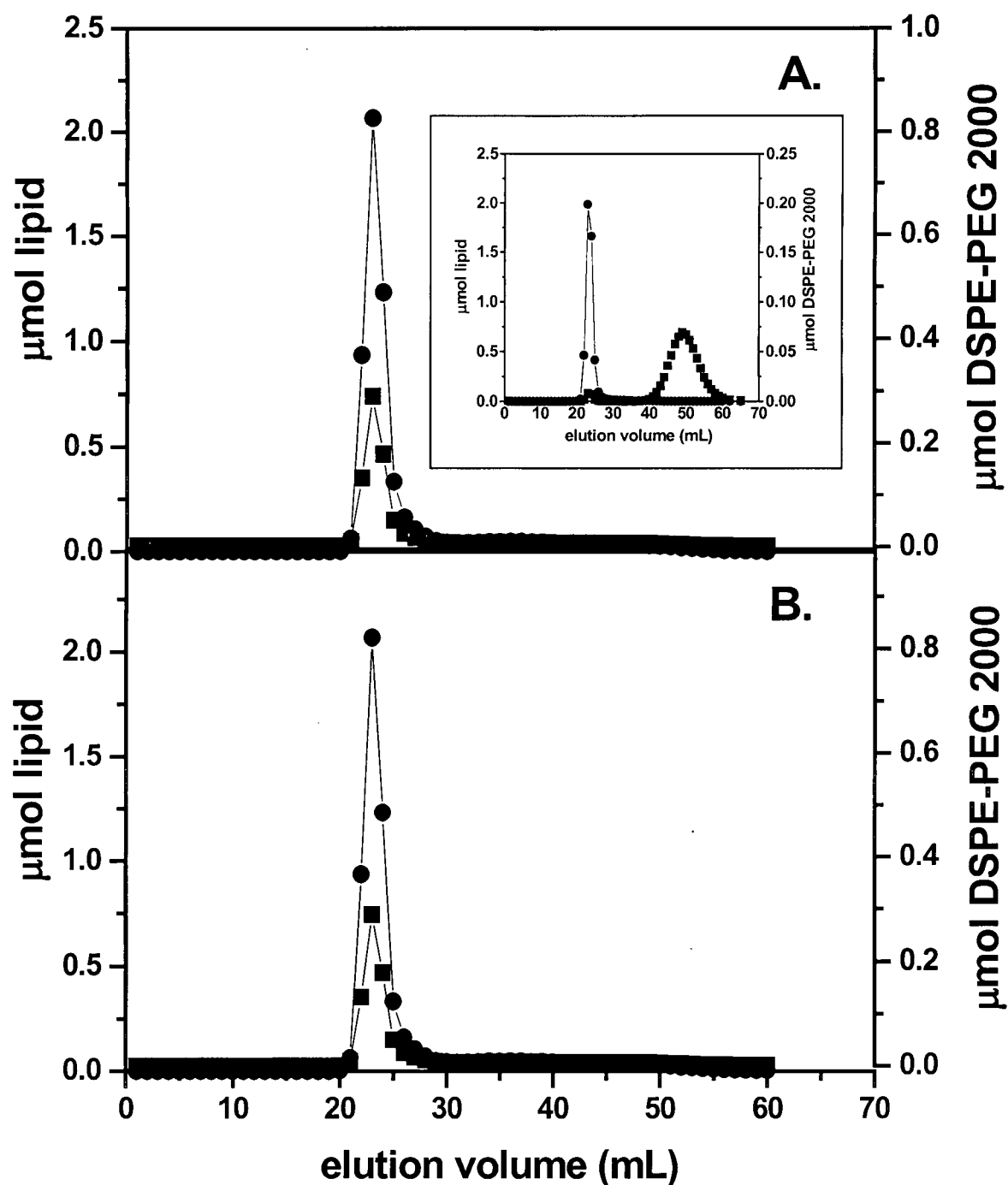
Figure 2.5. Size exclusion chromatography with Bio-Gel A-15m column of (A) 10 mol%
PS liposomes and (B) DSPC/CHOL liposomes, each containing 15 mol% DSPE-PEG 2000.
Both types of liposomes were labeled with traces of [^{14}C]CHE (●) as a lipid marker and
[^{3}H]DSPE-PEG 2000 (■), and were assayed at stock concentrations (54 mM). The
calibration of the Bio-Gel A-15m column (42 cm × 1.3 cm) with DOPS/DSPC/CHOL
10:45:45 liposomes (●) and DSPE-PEG 2000 micelles (■) was shown in the inset.

2.4. Discussion

Surface modification of liposomes with the hydrophilic polymer PEG has provided a major advance in drug delivery applications due to the ability of this polymer to reduce protein binding and plasma removal of liposomes. As described in Chapter 1, the mechanism by which the PEG polymer protects the liposome surface has been attributed to 1) the hydrophilicity of the polymer that effectively provides a "water shell" to the liposome surface, 2) the flexibility of the polymer that adopts a large number of conformations, resulting in a "probability cloud" which sterically protects the liposome surface, and 3) the ability of PEG to act as a steric repulsive barrier [208, 210, 211]. Most of the studies on PEG-lipids have focused on the protection of neutral liposome surfaces from plasma protein binding and subsequent MPS uptake. In this context, several studies have demonstrated that 5 to 7 mol% DSPE-PEG 2000 is optimal for protection of such liposomes [190, 210]. However, the use of PEG-lipids to protect a biologically active membrane surface has not been well documented. In this chapter, the ability of PEG-lipids to protect the biologically active PS containing liposome surface was tested under in vitro conditions.

One of the first questions that arose in this study was the feasibility of incorporating PEG-lipids at elevated levels into liposomes. This is due to reports that DSPE-PEG 2000 incorporation beyond 7 – 8 mol% into phospholipid liposomes can lead to alternate lipid phases primarily arising from DSPE-PEG micelle formation [190, 210]. However, the results demonstrated that DSPE-PEG 2000 can be readily incorporated into DSPC/CHOL-based liposomes up to 15 mol% without the generation of micelles. Further, the in vivo retention and stability of the PEG-lipids in PS containing liposomes will be evaluated in the next chapter.

In contrast to neutral liposomes such as DSPC/CHOL where 5 to 7 mol% DSPE-PEG 2000 provides protection for protein binding, biologically active liposomes containing PS with these levels of PEG-lipids are still capable of interacting with relatively large proteins. Only when the PEG-lipids were present at higher mole percentages was prothrombin binding substantially inhibited. This observation is somewhat surprising since studies by Needham et al. [259] have demonstrated that the k_{on} for similarly sized proteins or lysolipid micelles (compared to 1800 $Å^2$ per molecule surface coverage for prothrombin, see ref. [98]) interacting with a membrane surface is decreased approximately 100-fold when DSPE-PEG 750 or DSPE-PEG 2000 are incorporated into PC bilayers at 12% and 6%, respectively. The fact that 20 mol% DSPE-PEG 750 or 15 mol% DSPE-PEG 2000 are required to substantially inhibit prothrombinase protein interactions with PS containing liposomes indicates that these protein molecules can penetrate through the PEG layer at lower polymer levels (e.g. 5 mol% DSPE-PEG 2000). Similar PEG content dependencies observed for inhibition of prothrombin binding, chromogenic prothrombinase activity and complete clotting activity indicate that even the large ternary protein prothrombinase complex (in excess of 7000 $Å^2$ surface coverage) can associate on PS membranes containing < 10 mol% PEG 2000.

Three key features may account for the altered steric stabilization effects of DSPE-PEG observed for the protein membrane interactions studied here. First, the electrostatic attraction that exists between negatively charged PS molecules and binding ligands on the coagulation proteins could provide some of the energy necessary for the additional work required to move proteins through the PEG layer. This could decrease the effect of PEG on

k_{on} for PS-coagulation protein binding compared to non-specific protein-neutral membrane interactions. Second, the high binding affinities of coagulation proteins for PS-containing liposomes (K_D values ranging from 7×10^{-8} M to 2.5×10^{-10} M, see ref. [266, 267]) arise primarily from significant decreases in k_{off} where collisional binding efficiencies > 30% have been documented for proteins of the prothrombinase complex [266]. This is in contrast to the very inefficient contact phase of protein adsorption to neutral membrane surfaces [266, 267]. Consequently, the ability of prothrombin and other blood coagulation proteins to bind to PS-containing liposomes in the presence of 5 mol% PEG 2000 may be kinetically driven due to the very slow release of protein once initial contact with the membrane has been made through transient openings in the steric PEG barrier that have been shown to occur at PEG 2000 concentrations as high as 10 mol% [268].

The third membrane binding feature for prothrombin, Factor X and Factor V that may impact on the steric stabilization properties of PEG-lipids is their multivalent interaction with PS at the membrane surface. Eight PS molecules are required to bind one prothrombin molecule and the total PS requirement for binding all three proteins of the prothrombinase complex is between 40 – 50 molecules [269]. Assuming an average area per PS molecule of approximately 60 Å^2, 27% of the area covered by one prothrombin molecule is occupied by PS, reflecting a 2.7-fold enrichment of PS in this region. Similarly, the approximately 7000 Å^2 covered by the complete prothrombinase complex (prothrombin, Factor X and Factor V) would be predicted to have a lipid composition consisting of between 40 – 50 mol% PS. Previous studies have demonstrated that these proteins are capable of inducing lateral separation of PS in PS/PC liposomes [264]. Such membrane

microdomains enriched in PS would be largely devoid of DSPE-PEG, thereby providing potential nucleation sites for protein binding where the steric barrier associated with PEG-lipids would be absent.

We propose that compression of the packing area per PEG molecule at the liposome surface is necessary as DSPE-PEG 2000 is being excluded from the protein binding sites under this PS-enriched protein binding microdomain model. This prediction can be made due to the observation that prothrombinase protein binding is mediated mainly through the PS head group at the lipid/water interface, and protein insertion into the hydrophobic portion of the bilayer is minimal [270]. Previous studies have documented that the packing area per PEG molecule was 960 Å^2 and 480 Å^2 for levels of 5 and 10 mol% incorporation respectively, based on a PEG volume of 31,500 Å^3 [213]. We propose that DSPE-PEG 2000 can be further compressed to a PEG packing area of 330 Å^2 per molecule and an extension of 100 Å from surface at 15 mol% incorporation (see Figure 2.6, P. 87). For PS liposomes containing 5 and 10 mol% DSPE-PEG 2000, this means that 67% and 33% of liposome surface can be made available for protein binding if the PEG molecule is compressed to 330 Å^2. The level of prothrombin binding to PS liposomes with 5 and 10 mol% DSPE-PEG 2000 was approximately 70% and 30%, respectively, of that obtained in the absence of PEG which is consistent with the PEG compression model described above. The combined effects of densely packed PEG and extension of the polymer barrier from the membrane surface under these conditions appear to provide steric inhibition of even high affinity protein-membrane interactions. It remains to be determined whether much smaller
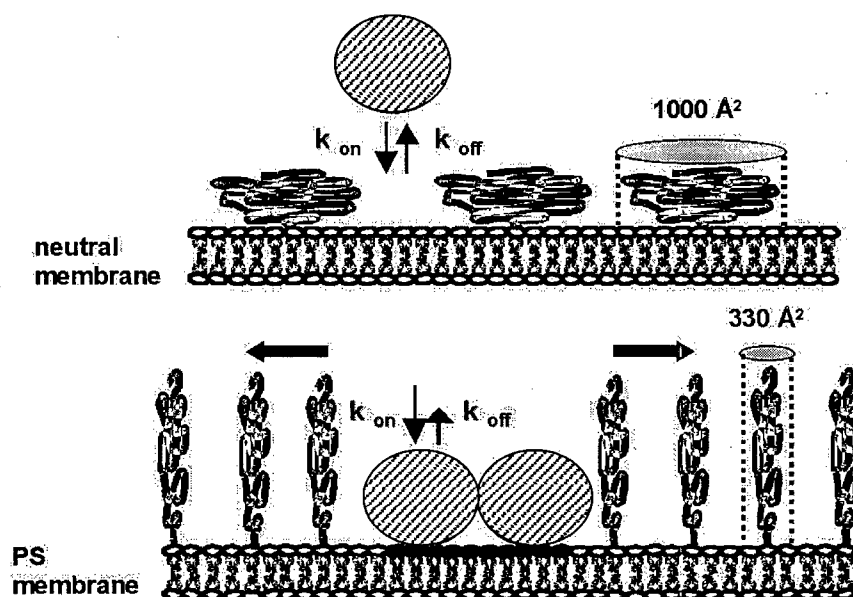
Figure 2.6. An illustration of the PS-enriched protein binding microdomain model. DSPE-PEG 2000 is laterally excluded from the protein binding site (indicated by black arrows) when proteins bind to the PS membrane surface mediated by the PS head group (black circles). Compression of the packing area per PEG molecule at the liposome surface from 1000 Å^2 (at 5 mol% DSPE-PEG 2000) to 330 Å^2 is proposed to be necessary for accommodating the proteins bound.

molecules are able to penetrate to the liposome surface under these densely packed conditions.

In this chapter, the effectiveness of PEG-lipids in preventing the PS-mediated blood coagulation protein binding and the subsequent clotting reactions has been evaluated, and it is found that an elevated level (> 10 mol%) of DSPE-PEG 2000 was necessary to inhibit the clotting activity of the PS membranes. These results suggest that the altered steric stabilization effects of PEG in membranes containing significant concentrations of reactive lipid species may important implications in liposome design where appropriate concentrations of surface grafted PEG and reactive lipids may be used to select for specific interactions with plasma components based on molecular size and membrane binding affinities. In the following chapter, the ability of PEG-lipids to protect the PS containing liposomes against the biological milieu will be evaluated.

Chapter 3   In vivo protection of the thrombogenic liposomes by PEG-lipids[*]

3.1. Introduction

This chapter addresses the approach to the in vivo protection of the thrombogenic PS liposome surface.   As described in Chapter 1, when liposomes are injected into the bloodstream, they are recognized and taken up by cells of the MPS which reside mainly in the liver and the spleen. The presence of PEG polymer, usually at 5 – 8 mol% DSPE-PEG 2000 on the neutral liposome surface, can reduce MPS recognition, where the PEG polymer acts as a steric barrier that reduces the rate of plasma protein binding and cellular interactions. This protection from MPS removal by the PEG polymer has been well characterized using neutral liposomes composed of PC, SM and CHOL; however, inclusion of the negatively charged PS in these neutral liposomes has led to rapid removal of the liposomes from bloodstream, with high lipid levels in the MPS and low lipid accumulation in target site [190, 238]. This would be an obstacle to be overcome in the development of a PS liposome formulation capable of triggering tumor specific thrombogenesis. Furthermore, the thrombogenic PS liposome surface should be protected in the bloodstream to prevent unwanted blood clot formation and subsequent occlusion from occurring in the organs leading to organ necrosis.

Results from the in vitro studies of Chapter 2 have shown that an elevated PEG-lipid content was effective in reducing the binding of the blood coagulation proteins such as prothrombin to DOPS liposomes, and in inhibiting the clotting activity of the DOPS liposomes. In light of these results, it was postulated that PEG-lipids, incorporated at an

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[*] Figure 3.1, Table 3.3 and 3.4 have been published in Biochim Biophys Acta 1510: 56 – 69, 2001.

elevated level, may be effective in protecting the PS liposome surface from the high affinity, PS-mediated binding of plasma proteins, and from recognition by the MPS. Again, PEG 750 and PEG 2000 incorporated at different mole percentages in the liposomes have been used here, and their ability in the protection of the PS liposome surface was evaluated based on 1) circulation longevity of the liposome formulations, and 2) level of plasma protein binding and 3) accumulation in MPS organs including liver and spleen. It is demonstrated here that DOPS liposomes with 15 mol% DSPE-PEG 2000 exhibit extended circulation longevity comparable to that of "stealth" liposomes (5 mol% DSPE-PEG 2000/DSPC/CHOL), with reduced plasma protein binding and accumulation in liver and spleen.

3.2. Materials and Methods

3.2.1. Materials

All lipids were obtained from Avanti Polar Lipids (Alabaster, AL) except for [^{3}H]DSPE-PEG 2000 which was obtained from Northern Lipids (Vancouver, BC). The [^{3}H]- and [^{14}C]CHE were from NEN Life Science Products (Boston, MA). Cholesterol and Sepharaose CL-4B were purchased from Sigma (St. Louis, MO). Bio-Gel A-15m size exclusion gel and gel filtration standards were obtained from Bio-Rad (Mississauga, ON). The micro bicinchoninic acid (micro BCA) protein assay kit was purchased from Pierce (Rockford, IL).

3.2.2. Preparation of liposomes

The preparation of LUV followed the extrusion procedure as described in Section 2.2.2 [261, 262]. The resulting mean liposome diameter obtained was 100 – 120 nm as determined by quasi-elastic light scattering using the Nicomp submicron particle sizer model 370/270, as described in Section 2.2.2.

3.2.3. Plasma pharmacokinetics and tissue distribution of liposomes

All of the in vivo studies were completed using protocols approved by the University of British Columbia's Animal Care Committee. These studies met or exceeded the current guidelines of the Canadian Council of Animal Care. Female CD-1 mice (7 – 8 weeks old) were purchased from Charles River Laboratories (St. Constant, PC). Liposomes, labeled with [^{3}H]CHE as a non-exchangeable, non-metabolizeable lipid marker [271], were injected

as a single IV bolus via lateral tail vein over 10 s with a lipid dose of 50 mg/kg and an injection volume of 200 μL into the CD-1 mice (~ 22 g). At various times, three mice from each group were terminated by CO_2 asphyxiation. Blood was collected by cardiac puncture, and was placed into EDTA-coated or heparin-coated microtainer collection tubes (Becton-Dickinson). After centrifuging the blood samples at 4 °C for 15 min at 1000 × g, plasma was isolated and visually showed no hemolysis. Aliquots of the plasma obtained were counted directly in 5.0 mL scintillation fluid. The limit of lipid quantitation in this case was 1 μg per mL plasma. Tissues including liver, spleen and lungs were also harvested from each group of mice 24 h post liposome injection to determine the biodistribution of liposomes. Briefly, 0.5 mL Solvable (Packard BioScience Co.) was added to whole organs (spleen and lungs) or liver homogenate (50% w/v), and the mixture was incubated at 50 °C overnight. After cooling to room temperature, 50 μL EDTA 200 mM, 200 μL hydrogen peroxide 30%, and 25 μL HCl 10 N were added, and the mixture was incubated for one hour at room temperature. The mixture was added with 5.0 mL scintillation fluid and counted 24 h later. The limit of lipid quantitation in tissues was 1 μg. Plasma removal studies with more sampling time points were conducted for two liposome formulations (10 mol% DOPS liposomes with and without 15 mol% DSPE-PEG 2000). Plasma was isolated at 0.25, 0.5, 1, 2, 8, 24 and 48 h for 10 mol% DOPS liposomes without 15 mol% DSPE-PEG 2000 and at 0.5, 1, 2, 4, 8, 16, 24 and 48 h. Plasma pharmacokinetic parameters (total body clearance, CL; mean residence time, MRT; volume of distribution at steady-state, Vss) for these two formulations were determined by non-compartmental analysis using WinNonlin software (version 1.5; Pharsight Corp., Mountain View, CA). First order elimination was assumed, and three or four time points were used in the estimation of the terminal elimination phase.

AUC_{0-48h} and $AUMC_{0-48h}$ were calculated using the linear trapezoidal rule. MRT was estimated as the ratio of $AUMC_{0-48h}/AUC_{0-48h}$, CL as $Dose_{IV}/AUC_{0-48h}$ and Vss as $Dose_{IV}/[(AUMC_{0-48h})/(AUC_{0-48h})^2]$. For DOPS liposomes containing 15 mol% DSPE-PEG 2000, organs including brain, liver, kidney, heart, lung and spleen were harvested from mice 24 h post injection of the liposomes. Organs were then fixed in 10% buffered formalin, embedded in paraffin, and 5-μm sections were prepared on glass slides, followed by H & E staining (Wax-It, Aldergrove, BC).

Retention of DSPE-PEG 2000 in liposomes recovered from mouse followed the procedure using size exclusion chromatography as described in Section 2.2.3. The liposomes used were labeled with traces of $[^{14}C]CHE$ (as a general lipid marker) and $[^{3}H]DSPE$-PEG 2000 (as the PEG-lipid marker). Plasma was obtained as described above, and was applied to a 42 cm × 1.3 cm Bio-Gel A-15m column (50 – 100 mesh). Sample was then eluted with HBS at a flow rate of 0.5 mL/min regulated by a peristaltic pump. Aliquots from the 1-mL column fractions were counted directly in 5.0 mL Pico-fluor 40 scintillation fluid (Packard Biosciences, The Netherlands).

3.2.4. In vivo recovery and analysis of liposome-associated proteins

Blood from mice injected with radiolabeled liposomes (150 mg/kg) was collected into heparin-coated microtainers, as described before. Plasma was isolated, and was applied to a 1.0 cm × 18 cm Sepharose CL-4B column, and was eluted with HBS containing 2.5 mM $CaCl_2$ (pH 7.5) at a flow rate of 5.87 mL/h regulated by a peristaltic pump. Column fractions were analyzed for radioactivity to determine the fractions containing the

liposomes, and the two fractions with the highest radioactivity were pooled for the quantification of total protein associated with the recovered liposomes. The proteins associated with the recovered liposomes were extracted and delipidated according to the procedure described by Wessel and Flugge, since lipids interfere with protein assays [272]. The extracted proteins were re-suspended in 20 μL distilled water. Quantification of the proteins was performed using the micro BCA (bicinchoninic acid) protein assay procedure. The extracted proteins was diluted to 0.15 mL with Milli-Q water, and 0.15 mL protein assay reagent was added. The mixture was incubated for 2 h at 37 °C. After the mixture was cooled to room temperature, the absorbance at 570 nm was measured with a plate reader (Dynex Technologies, Chantilly, VA). The extracted proteins were quantified by a standard curve consisting of known amounts of bovine serum albumin, which was linear in the range of $0 - 17.5$ μg/mL ($r^2 = 0.9967$).

3.2.5. Assessment of clotting activity of liposomes recovered from mouse plasma

Liposomes, labeled with [3H]CHE, were injected intravenously as a single bolus dose into mice at 150 mg/kg, and blood was collected by cardiac puncture from the mice 10 min post liposome injection and processed as described in Section 3.2.4. The plasma was then isolated by column chromatography as described above, and fractions containing the liposomes were pooled for the testing of clotting activity by the in vitro clotting time assay using mouse plasma as described in Section 2.2.4.

3.2.6. Statistical analysis

One-way ANOVA was performed to detect differences among different groups, with Newman-Keuls test as post-hoc analysis. A p-value of < 0.05 was considered significant.

3.3. Results

3.3.1. Effect of PEG-lipids on the plasma removal and the tissue distribution of PS containing liposomes

3.3.1.1. Plasma removal of liposomes

Liposomes containing PS are rapidly eliminated from the circulation, a phenomenon believed to be due to extensive binding of plasma proteins and uptake by the MPS [273]. This can be seen in Figure 3.1 (P. 96), which presents the plasma removal of 10 mol% DOPS liposomes containing various amounts of DSPE-PEG 750 (Figure 3.1, Panel A) or DSPE-PEG 2000 (Figure 3.1, Panel B). As early as 1 h after liposome injection, the effect of DOPS inclusion on the plasma removal of DSPC/CHOL liposomes can be seen. The circulation longevity of DSPC/CHOL liposomes was dramatically decreased with the inclusion of 10 mol% DOPS, as reflected by the plasma lipid concentration which was reduced from 0.712 mg/mL to 0.003 mg/mL 1 h post-injection. This represents a decrease in the injected dose remaining in plasma at 1 h from 60% to < 1%.

As described earlier, it is well known that 5 – 8 mol% DSPE-PEG 2000 commonly used in DSPC/CHOL liposomes can extend the circulation longevity of the neutral liposomes, and prevent plasma proteins from interacting with this neutral liposome surface. However, from the in vivo data presented in Figure 3.1 (Panel B, P. 96), the use of 5 mol% DSPE-PEG 2000 in the DOPS liposomes was not effective in protecting the liposomes from removal. This is reflected by the lower plasma lipid concentration of 0.088 mg/mL for this liposome at 1 h, compared to 1.0 mg/mL observed for the DSPC/CHOL containing
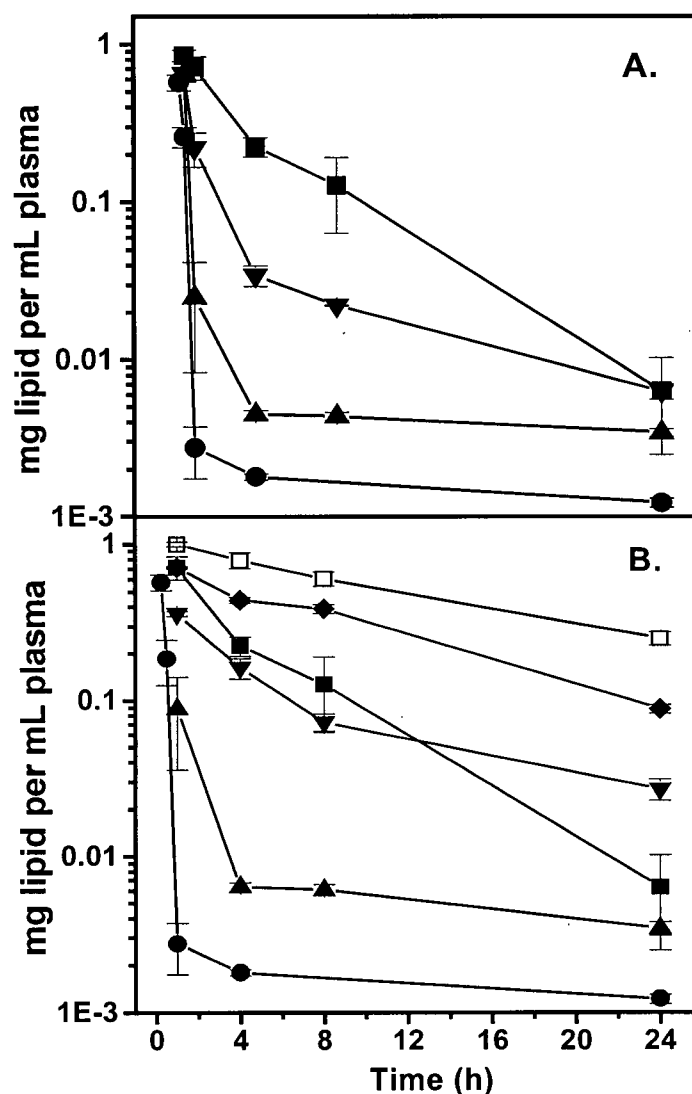
Figure 3.1. The effect of incorporating DSPE-PEG 750 or DSPE-PEG 2000 on the plasma removal of DOPS liposomes. Three CD-1 mice (~ 22 g) were used for each data point, and the error bars represent S.D. The liposomes were injected as a single IV bolus dose of 50 mg/kg. The plasma removal curves for the following liposomes were determined and plotted in (A): DSPC/CHOL 55:45 (■), DOPS/DSPC/ CHOL 10:45:45 (●), DSPE-PEG 750 incorporated at 10 mol% (▲) and 20 mol% (▼) in DOPS 10%/DSPC/CHOL 45%. The plasma removal curves for the following liposomes were determined and plotted in (B): DSPC/CHOL 55:45 (■), DOPS/DSPC/CHOL 10:45:45 (●), DSPE-PEG 2000 incorporated at 5 mol% (▲), 10 mol% (▼) and 15 mol% (◆) in DOPS 10%/DSPC/CHOL 45%, and DSPE-PEG 2000/DSPC/CHOL 5:50:45 (□).

5 mol% DSPE-PEG 2000 which is devoid of DOPS. It is necessary to increase the DSPE-PEG 2000 content to 15 mol%, which is the maximum incorporation level without inducing micelle formation [198], in order to prolong the circulation longevity of the 10 mol% DOPS liposomes, with a lipid concentration of 0.71 mg/mL at 1 h. The plasma lipid concentrations at 24 h were 0.251, 0.112 and 0.003 mg/mL for DSPC/CHOL containing 5 mol% DSPE-PEG 2000 and DOPS liposomes containing 15 and 5 mol% DSPE-PEG 2000, respectively. These plasma concentrations corresponded to 18%, 8% and 0.2% of the injected lipid dose remaining in plasma for the three liposome formulations mentioned above, respectively. These results demonstrated that only in the presence of an elevated level of DSPE-PEG 2000 would the plasma removal of DOPS containing liposomes approach those observed for PS free sterically stabilized neutral liposomes (5 mol% DSPE-PEG 2000 in DSPC/CHOL).

Although DSPE-PEG 750 or DSPE-PEG 2000 generally provided protection for the DOPS liposomes in the bloodstream, as reflected by the trend that increasing PEG-lipid content in the 10 mol% DOPS liposomes increased the plasma lipid concentrations of such liposomes (Figure 3.1, P. 96), the protective effect of DSPE-PEG 2000 on the plasma removal of DOPS liposomes was more profound than that of DSPE-PEG 750. When DSPE-PEG 750 was incorporated at its maximum level of 20 mol% in 10 mol% DOPS liposomes, the plasma lipid concentration at 1 h for this liposome was only 0.21 mg/mL, which was 3.4-fold lower than 0.71 mg/mL observed for DOPS liposomes containing 15 mol% DSPE-PEG 2000 at 1 h. The plasma lipid levels at 24 h for DOPS liposomes containing 20 mol% DSPE-PEG 750 or 15 mol% DSPE-PEG 2000 were 0.006 and 0.112 mg/mL, respectively, which corresponded to 0.4% and 8% of injected lipid dose remaining in plasma.

3.3.1.2. Lipid accumulation in tissues

The trends observed in liposome plasma removal correlated closely with liposomal lipid levels observed in the major MPS organs, namely, the liver and the spleen. Specifically, the plasma liposome levels were inversely proportional to the liver and spleen liposome recovery 24 h after intravenous injection, where liposome formulations with very low plasma lipid concentrations at 24 h had very high lipid recovery in the liver and the spleen. Table 3.1 (P. 99) summarizes the lipid levels for various liposomes in the liver, spleen and lung 24 h post injection. The effect of DOPS on MPS uptake of DSPC/CHOL liposomes can be seen from the significant increases in lipid recovery in liver from 0.44 to 0.63 mg/g tissue and in spleen from 0.98 to 2.47 mg/g tissue ($p < 0.05$). These increases in lipid levels in liver and spleen corresponded to an increase from a total of 62% to > 90% of the injected lipid dose accumulated in these two MPS organs. These percentages are consistent with values reported in previous studies [180, 190].

Incorporating 10 mol% DSPE-PEG 750 or 5 mol% DSPE-PEG 2000 did not decrease the amounts of lipid accumulated in the MPS organs, with a total of > 90% of the injected lipid dose accumulated in liver and spleen. This is not statistically different from the levels observed for 10 mol% DOPS liposomes without any PEG-lipid protection ($p > 0.05$). Only when incorporating DSPE-PEG 750 at its maximum level of 20 mol%

Table 3.1.

Effect of DSPE-PEG 750 and DSPE-PEG 2000 on the tissue distribution of DOPS liposomes 24 hours post injection

Liposomes	mg lipid per g tissue +/- S.D. [a]		
	liver	spleen	lungs
DSPC/CHOL 55:45	0.44 ± 0.07 (52%) [b]	0.98 ± 0.47 (10%) [b]	0.030 ± 0.003 (0.4%)
DSPC/CHOL/DSPE-PEG 2000 50:45:5	0.17 ± 0.03 (21%) [d]	0.31 ± 0.11 (3%)	0.056 ± 0.002 (1%)
DOPS/DSPC/CHOL 10:45:45	0.63 ± 0.09 (71%) [b,c]	2.47 ± 0.29 (20%) [b,c]	0.011 ± 0.001 (0.2%)
DOPS/DSPC/CHOL/DSPE-PEG 750 10:35:45:10	0.70 ± 0.03 (72%) [c]	2.35 ± 0.61 (21%) [c]	0.012 ± 0.002 (0.2%)
DOPS/DSPC/CHOL/DSPE-PEG 750 10:25:45:20	0.47 ± 0.03 (57%)	1.09 ± 0.09 (9%)	0.016 ± 0.004 (0.2%)
DOPS/DSPC/CHOL/DSPE-PEG 2000 10:40:45:5	0.56 ± 0.12 (72%) [c]	2.11 ± 0.41 (22%) [c]	0.010 ± 0.002 (0.2%)
DOPS/DSPC/CHOL/DSPE-PEG 2000 10:35:45:10	0.42 ± 0.02 (49%)	1.24 ± 0.27 (12%)	0.018 ± 0.003 (0.3%)
DOPS/DSPC/CHOL/DSPE-PEG 2000 10:30:45:15	0.19 ± 0.03 (20%) [d]	0.79 ± 0.13 (6%)	0.063 ± 0.009 (1%)

[a] Data represent the averages ± S.D. of three mice. The injected lipid dose was 50 mg/kg as a single IV bolus, and the liposomes were labeled with [^{3}H]CHE (as a general lipid marker) and prepared as described in Section 2.2.2. The amount of lipid per gram of tissue was determined as described in Section 3.2.3. Shown in parentheses are the percent injected doses recovered in each organ. Data were analyzed by one-way ANOVA and Newman-Keuls test with $p < 0.05$. The limit for quantitating the amount of liposomes (labelled with [^{3}H]CHE) by liquid scintillation counting was 0.001 mg.

[b] Lipid levels in liver and spleen for 10 mol% DOPS liposomes were statistically different from those observed with DSPC/CHOL liposomes.

[c] Lipid levels in liver and spleen for DOPS liposomes with 10 mol% DSPE-PEG 750 or 5 mol% DSPE-PEG 2000 were not statistically different from those observed with DOPS liposomes without PEG protection.

[d] Lipid levels in liver for DOPS liposomes with 15 mol% DSPE-PEG 2000 were not statistically different from those observed with DSPC/CHOL liposomes with 5 mol% DSPE-PEG 2000.

or 10 mol% DSPE-PEG 2000 into 10 mol% DOPS liposomes did the amounts of lipid accumulated in the MPS organs decrease to those observed for DSPC/CHOL liposomes, which corresponded to a total of ~ 60% of injected dose in liver and spleen. When 15 mol% DSPE-PEG 2000 was incorporated into the DOPS liposomes, the amount of lipid accumulated in the two MPS organs was reduced to a total of 26% of injected lipid dose, which was not statistically different from those observed for DSPC/CHOL with 5 mol% DSPE-PEG 2000, a liposome formulation known for its ability to evade the MPS organs (p > 0.05). The lipid levels observed in the MPS organs here are consistent with those reported previously for DSPC/CHOL with 5 mol% DSPE-PEG 2000 [178]. Although the skin and carcass were not collected here for radioactivity determination, it has been shown that these tissues exhibited enhanced lipid accumulation in case of sterically stabilized liposomes [180]. The amount of lipid distributed to the lung constituted a very low percentage of the injected dose for all of the liposome formulations studied here, with a maximum of 1% of injected dose observed for the 5 mol% DSPE-PEG 2000 in DSPC/CHOL and the 10 mol% DOPS liposomes protected by 15 mol% DSPE-PEG 2000.

3.3.1.3. Analysis of DOPS liposome removal from plasma with or without 15 mol% DSPE-PEG 2000

Since 15 mol% DSPE-PEG 2000 in DOPS liposomes provided effective protection of the liposome surface that resulted in extended circulation longevity, a more detailed pharmacokinetic (PK) analysis was conducted on this formulation, and compared to 10 mol% DOPS liposomes with no PEG-lipid. Table 3.2 (P. 102) summarizes the pharmacokinetic parameters for 10 mol% DOPS liposomes with and without 15 mol%
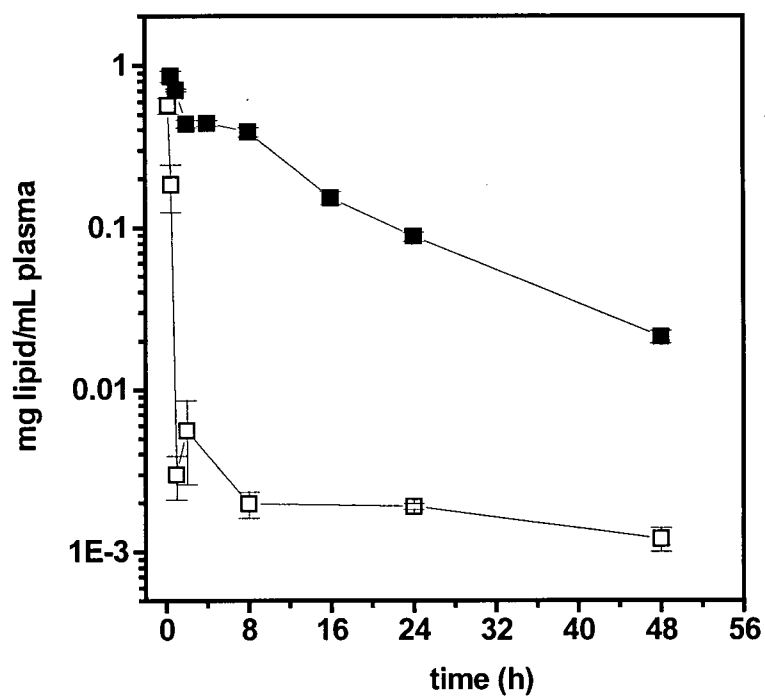
Figure 3.2. Plasma removal profiles for 10 mol% DOPS liposomes (□) and 10 mol% DOPS liposomes containing 15 mol% DSPE-PEG 2000 (■) injected as a single 50 mg/kg IV bolus dose into CD-1 mice via lateral tail vein. Three mice were used for each time point with the error bars representing S.D. In some cases, the size of the symbol is larger than the size of the error bar.

Table 3.2.

Pharmacokinetic parameters for two liposome formulations in CD-1 mice [a]

	DOPS/DSPC/CHOL 10:45:45	DOPS/DSPE-PEG 2000/DSPC/CHOL 10:15:30:45
$AUC_{0-48\ h}$ [b,c]	$0.529\ mg \bullet h \bullet mL^{-1}$	$8.429\ mg \bullet h \bullet mL^{-1}$
$AUMC_{0-48\ h}$	$1.911\ mg \bullet h^2 \bullet mL^{-1}$	$91.838\ mg \bullet h^2 \bullet mL^{-1}$
MRT_{last}	3.6 h	11 h
CL	$1.8\ mL \bullet h^{-1}$	$0.12\ mL \bullet h^{-1}$
V_{ss}	40 mL	1.6 mL
R^2	0.8818	0.9764
Corr (x:y)	-0.9390	-0.9881

[a] CD-1 mice (~ 22 g) were injected with 50 mg/kg lipid as a single IV bolus dose via the lateral tail vein, and blood was collected and processed as described in Section 3.2.3. For 10 mol% DOPS liposomes, blood samples were collected at 0.25, 0.5, 1, 2, 8, 24 and 48 h, and for 10 mol% DOPS liposomes containing 15 mol% DSPE-PEG 2000 at 0.5, 1, 2, 4, 8, 16, 24 and 48 h. Three mice were used for each time point.

[b] Noncompartmental analysis based on the linear trapezoidal rule was performed using the software WinNonlin version 1.5 to estimate the values of various pharmacokinetic parameters. Three to four time points were used for the estimation of the terminal elimination phase. In the calculations, the dose was entered as an IV bolus with no lag time and first order elimination was assumed. Uniform weighting was used.

[c] $AUC_{0-48\ h}$, area under curve from time of dosing (t = 0 h) up to the last measured concentration (t = 48 h); $AUMC_{0-48\ h}$, area under moment curve from time of dosing (t = 0 h) up to the last measured concentration (t = 48 h); MRT_{last}, mean residence time which is calculated based on values up to the last measured concentration (t = 48 h) and is not extrapolated to infinity; CL, total body clearance (estimated); V_{ss}, volume of distribution at steady state (estimated); R^2, coefficient of determination for the points used in the estimation of the first order rate constant associated with the terminal portion of the plasma concentration-time curve via linear regression; Corr (x:y), correlation coefficient for the points used in the estimation of the first order rate constant associated with the terminal portion of the plasma concentration-time curve via linear regression.

DSPE-PEG 2000 after an IV bolus dose of 50 mg/kg into CD-1 mice. Plasma liposome concentrations at various times (see Figure 3.2, P. 101) and pharmacokinetic parameters (see Table 3.2, P. 102) were determined as described in Section 3.2.3. As early as 30 min after injection, the plasma levels for 10 mol% DOPS protected with 15 mol% DSPE-PEG 2000 (0.86 mg/mL) were significantly higher than those without 15 mol% DSPE-PEG 2000 (0.18 mg /mL; t-test, $p < 0.05$).

With the inclusion of 15 mol% DSPE-PEG 2000, the $AUC_{0-48\,h}$ and the MRT $_{last}$ for 10 mol% DOPS liposomes were increased by 16-fold and 3-fold, respectively. This showed that the inclusion of this PEG-lipid can dramatically improve the circulation longevity of 10 mol% DOPS liposomes. Total body clearance (CL) for 10 mol% DOPS liposomes was decreased from 1.8 to 0.12 mL $\bullet$ h^{-1} after including 15 mol% DSPE-PEG 2000, and the Vss was also decreased from 40 to 1.6 mL. The results for 10 mol% DOPS liposomes corroborated the observation that these liposomes rapidly distributed to tissues such as the liver and the spleen which are the major organs of MPS responsible for the uptake of particulate matters.

3.3.1.4. In vivo retention of DSPE-PEG 2000 in liposomes

From the plasma removal data of various PEG-lipid containing DOPS liposomes, 15 mol% DSPE-PEG 2000 is the most effective in extending the circulation longevity of DOPS liposomes. The retention of DSPE-PEG 2000 in liposomes after intravenous administration was thus investigated to ensure that the extended circulation longevity of DOPS liposomes was due to the protective effect of DSPE-PEG 2000. PS-free DSPC/CHOL liposomes and

10 mol% DOPS liposomes, both containing 15 mol% DSPE-PEG 2000, were injected into mice to determine the amount of DSPE-PEG 2000 retained by the liposomes in plasma at various times post injection (see Table 3.3, P. 105). The level of DSPE-PEG 2000 in 10 mol% DOPS liposomes decreased slightly from 14.3 mol% at 1 h to 13.4 mol% at 24 h, and in DSPC/CHOL liposomes from 14.5 mol% at 1 h to 12.6 mol%. These decreases in the amounts of DSPE-PEG 2000 in the two liposomes, though small, were significant ($p <$ 0.05). However, an elevated level (> 12 mol%) of DSPE-PEG 2000 remained associated with the 10 mol% DOPS liposomes and DSPC/CHOL liposomes over a period of 24 h to protect these liposomes in the circulation. These results also demonstrated that the retention of elevated levels of DSPE-PEG 2000 was not specific to PS containing liposomes.

3.3.2. Evaluation of liposome-protein interactions after in vivo injection

The extended circulation time of sterically stabilized DSPC/CHOL liposomes has been attributed to the ability of PEG-lipids to reduce protein binding and cellular interactions [188, 274]. In this study, the amount of protein associated with liposomes recovered from mice 30 min post injection was determined with the micro BCA protein assay (Table 3.4, P. 106). The parameter, P_B value, was used to indicate the amount of protein (in grams) associated with each mole of lipid recovered from mouse plasma in a single determination because of low lipid recovery from mouse plasma. Liposomes with 10 mol% DOPS in the absence of DSPE-PEG 2000 had the highest amount of associated protein per mole of recovered liposomal lipid (20.0 g protein/mol lipid). In contrast, the amount of protein associated with the sterically stabilized 5 mol% DSPE-PEG 2000/DSPC/CHOL

Table 3.3.

Mole percentages of DSPE-PEG 2000 remaining in liposomes recovered from murine plasma at various hours post injection

Liposomes	Time (h)	mol% DSPE-PEG 2000 remaining in liposomes ($\pm$ S.D.) [a]
DOPS/DSPC/CHOL/DSPE-PEG 2000 10:30:45:15	1	14.3 ± 0.1
	4	13.9 ± 0.2
	24	13.4 ± 0.1 [c]
DSPC/CHOL/DSPE-PEG 2000 40:45:15	1	14.5 ± 0.4
	4	14.1 ± 0.1
	24	12.6 ± 0.2 [c]

[a] Data represent the averages $\pm$ S.D. of three mice. The injected lipid dose was 50 mg/kg as a single IV bolus.

[b] The liposomes were dual-labeled with [^{14}C]CHE (as a general lipid marker) and [^{3}H]DSPE-PEG 2000 and prepared as described in Section 2.2.2.

[c] The levels of DSPE-PEG 2000 in the two liposomes at 24 h were significantly different from those observed at 1 and 4 h, analyzed using one-way ANOVA and Newman-Keuls test with $p < 0.05$.

Table 3.4.

P_B values [a] for liposomes recovered from murine plasma 30 min post injection

Liposomes	in vivo P_B value (g protein/mol lipid)
DSPE-PEG 2000/DSPC/CHOL 5:50:45	5.86
DOPS/DSPC/CHOL 10:45:45	20.0
DOPS/DSPC/CHOL/DSPE-PEG 2000 10:40:45:10	7.49
DOPS/DSPC/CHOL/DSPE-PEG 2000 10:30:45:15	4.03

[a] P_B value was defined as the amount of protein associated with each mole of lipid recovered from murine plasma 30 min post injection in a single determination. CD-1 mice were injected with liposomes in a single IV bolus dose of 50 mg/kg . Due to low recovery of lipids from animals, plasma from four mice was pooled and chromatographed on a Sepharose CL-4B column under similar conditions. Column fractions containing the liposomes were pooled, and the amount of protein was determined by the micro BCA assay as described in Section 3.2.

liposomes was 5.86 g protein/mol lipid. The incorporation of 10 mol% DSPE-PEG 2000 into the 10 mol% PS liposomes provided moderate reduction in the amount of liposome associated protein (7.49 g protein/mol lipid). When 15 mol% DSPE-PEG 2000 was incorporated into the 10 mol% DOPS liposomes, the amount of associated protein was reduced to 4.03 g protein/mol lipid, which reflected a five-fold reduction when compared to that of the non-pegylated PS containing liposomes. Taken together, 15 mol% DSPE-PEG 2000 provided protection for 10 mol% DOPS liposomes, as reflected by extended circulation longevity, reduced plasma protein binding and decreased accumulation in the MPS organs of such liposome formulation.

3.3.3. Clotting activity of PS liposomes with various mole percentages of DSPE-PEG 2000 recovered from mice post injection

Once the liposomes are injected into the bloodstream, plasma components begin to interact with the liposome surface; thus, it is important to address whether the DOPS liposome surface retains functional thrombogenic activity in vivo. DOPS liposomes containing various mole percentages of DSPE-PEG 2000 injected into mice at 150 mg/kg were recovered 10 min post injection. Here, a higher lipid dose was used to overcome the problem of low lipid recovery as described earlier. The clotting activity of 10 mol% DOPS liposomes with various mole percentages of DSPE-PEG 2000 recovered post injection is shown in Figure 3.3 (P. 108). The results demonstrate that the clotting activity of 10 mol% DOPS liposomes injected into mice was not affected by plasma components. Importantly, protection of the 10 mol% DOPS liposome surface in the circulation depended on an elevated level (15 mol%) of DSPE-PEG 2000 in the liposomes. DOPS liposomes
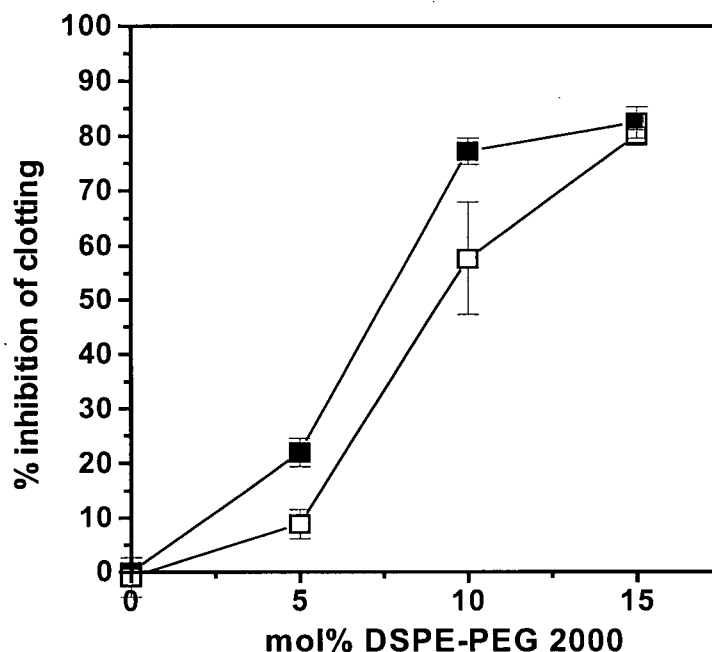
Figure 3.3. The clotting activity of 10 mol% DOPS liposomes with various mole percentages of DSPE-PEG 2000 with (□) and without (■) injection into mice. Liposomes were injected at 150 mg/kg IV as a single bolus dose into female CD-1 mice (~ 22 g), and were recovered from murine plasma by column chromatography 10 min post injection. Pooled column fractions containing the liposomes were tested for clotting activity by the in vitro clotting time assay as described in Section 3.2.5., and the % inhibition of clotting was calculated as follows: % inhibition = $(t_{PEG} - t_{PS})/(t_{blank} - t_{PS}) \times 100$ where t represented the clotting time of each type of liposome. Data points represent triplicates, and the error bars S.D.

containing 5 and 10 mol% DSPE-PEG 2000 injected into mice were slightly more procoagulant than those that had not been injected. This is presumably due to the binding of the blood coagulation proteins to these liposomes, leading to slight increase in clotting activity; however, these proteins were not identified.

To demonstrate that 15 mol% DSPE-PEG 2000 can protect the thrombogenic surface of the DOPS liposomes and prevent unwanted clot formation in organs leading to potential blood flow occlusion, major organs were harvested and processed from mice 24 h post injection of DOPS liposomes containing 15 mol% DSPE-PEG 2000, as described in Section 3.2.3. In particular, signs of blood coagulation (such as fibrin clot formation, packed erythrocytes or occlusive platelet aggregates) and signs of tissue damage (such as pyknosis and cytolysis), which were observed previously in tumors harvested from mice injected with the "coaguligand", were examined in tissue sections stained with hematoxylin and eosin (H & E). As shown in Figure 3.4 (P. 109), the harvested tissues including brain, liver, kidney, heart, lung and spleen were healthy, and exhibited a uniform morphology without any sign of blood coagulation including fibrin clots, packed erythrocytes or occlusive platelet aggregates.
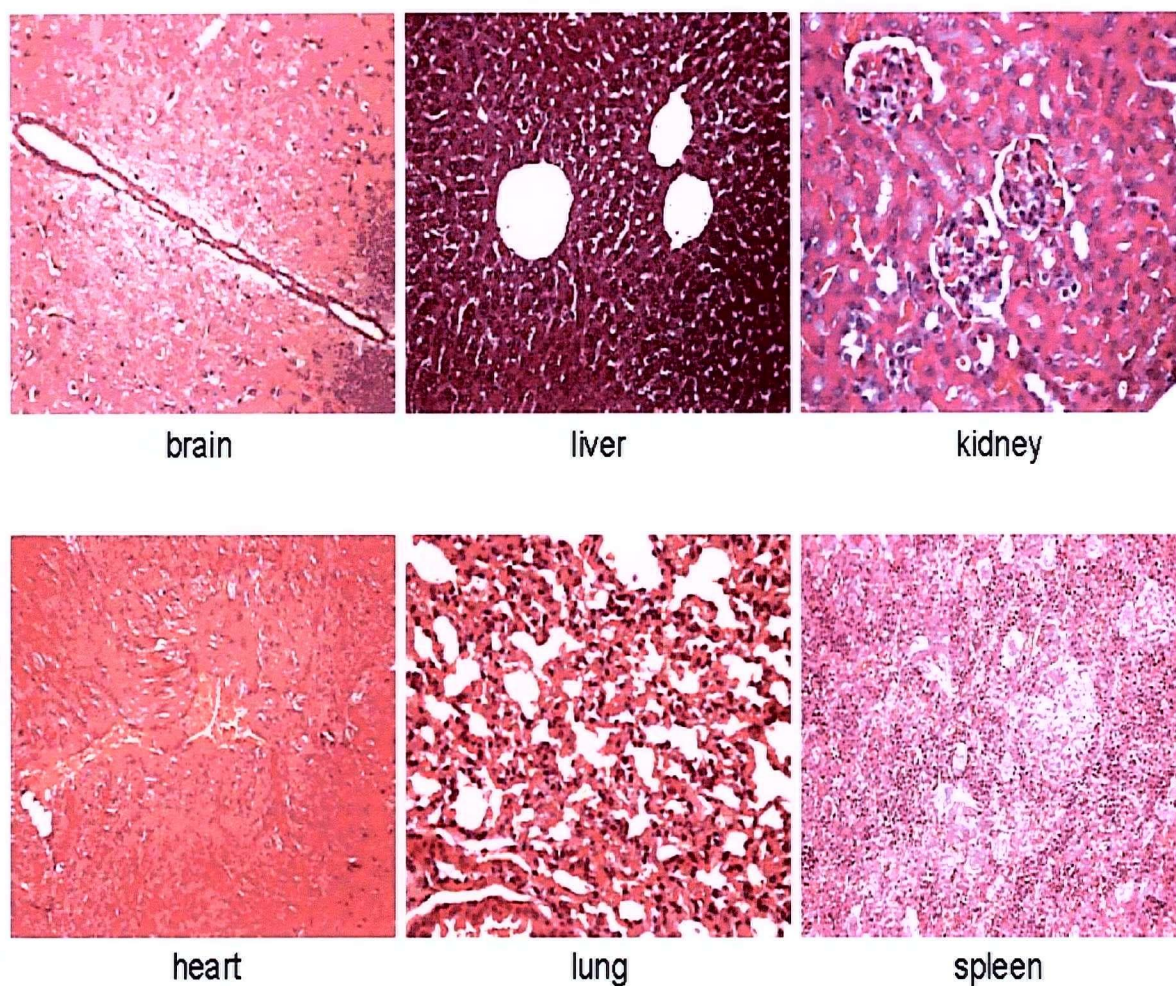
Figure 3.4. H & E staining of major organs harvested from CD-1 mice 24 hours post liposome injection. Mice were injected with 10 mol% DOPS liposomes containing 15 mol% DSPE-PEG 2000 as a single IV bolus dose of 50 mg/kg. Organs were harvested and processed as described in Section 3.2.3 (magnification: × 200).

3.4. Discussion

As described in Chapter 1, the removal of liposomes is generally thought to be due to plasma protein binding or opsonization, where the coating of serum opsonins on the liposome surface leads to MPS recognition and subsequent uptake mainly into liver and spleen. However, studies have shown that hepatic uptake in mouse liver is independent of serum components, in contrast to rat hepatic uptake which showed a strong dependence on serum components [142-145]. Since the plasma removal profiles of the various liposome formulations in this thesis were established using mouse models, a closer look at the mechanism of how PS liposomes are eliminated from bloodstream in the mouse would be necessary before considering the role of PEG-lipids in the protection of these liposomes.

Using the liver perfusion model, investigators have demonstrated that the uptake of PS containing liposomes can be decreased only by a pre-perfusion of liposomes of the same composition (i.e., PS containing), and the levels of PS liposomes accumulated in the mouse liver remained high when liposomes of different composition (i.e., PC/CHOL) were used in pre-perfusion [143]. They have also demonstrated that mouse hepatic uptake of PS containing liposomes is sensitive to the PS levels in the liposomes, and that 3 mol% PS was adequate for liver uptake to reach its maximum. It is well known that apoptotic cells expose PS on the membrane surface because of a failure to maintain membrane asymmetry, and these cells are quickly recognized and phagocytosed by macrophages [275, 276]. Phagocytosis of the apoptotic cells can be inhibited stereospecifically by PS (the L-isoform) but not other negatively charged phospholipids [275]. Taken together, these studies have suggested that PS containing liposomes or cell membranes are taken up by a specific mechanism which is different from that for neutral (PC/CHOL) liposomes. These findings

have led to the recent identification of a potential receptor which is specific for recognizing PS on apoptotic cells [277]. Interestingly, studies have also identified a 50-kDa serum glycoprotein, β_2-glycoprotein I, that binds to PS liposomes through electrostatic and hydrophobic interactions [278], and reduces PS-dependent blood clotting activity [279]. In particular, the uptake of β_2-glycoprotein I-treated PS containing liposomes by mouse macrophages was shown to be unaffected by the "shielding effect" of β_2-glycoprotein I, thus providing evidence for the existence of mechanisms other than those dependent on direct PS recognition by macrophages [279]. Given that β_2-glycoprotein I is able to precipitate negatively charged liposomes [279], in vivo aggregation of these liposomes (including PS containing liposomes) is likely. This, in turn, can be a potential mechanism for the rapid removal of such liposomes, which is supported by a study that demonstrated the role of liposome aggregation in liposome removal from circulation [280].

The pharmacokinetic studies reported here are the first to document effective protection of PS liposomes from plasma protein binding and from MPS removal by using PEG-lipids. The data demonstrate that increasing levels of DSPE-PEG in the DOPS liposomes increase the circulation longevity of DOPS containing liposomes and decrease the amounts of protein associated with the DOPS liposomes. Specifically, 15 mol% DSPE-PEG 2000 was required in the DOPS liposomes to display plasma removal curves, protein binding levels as well as liver and spleen uptake that were comparable to those for traditional "stealth" 5 mol% DSPE-PEG 2000/DSPC/CHOL liposomes. From the results obtained in Chapter 2, where an elevated level of DSPE-PEG 2000 was needed to inhibit the PS-mediated, high affinity binding of the blood coagulation proteins, it is conceivable that

an elevated level of DSPE-PEG 2000 (> 10 mol%) is thus necessary to prevent the multiple

PS-mediated removal processes of such liposomes. DSPE-PEG may play several roles in

prolonging the circulation longevity of DOPS liposomes. As addressed in Chapter 1, the

hydrophilicity of PEG polymer on the DOPS liposome surface would create a "water shell"

that helps reduce recognition by MPS [191, 210]. The steric repulsive effect exerted by the

PEG polymer may prevent or slow down proteins such as β_2-glycoprotein I from

approaching the PS liposome surface, and membrane-membrane contact with the

macrophages. The PEG polymer may also be able to shield the PS head group and prevent

direct PS macrophage interactions, which has been suggested to be involved in the removal

of PS liposomes.

The in vitro data from Chapter 2 have shown that both DSPE-PEG 2000 and DSPE-

PEG 750 at maximum incorporation levels were effective in inhibiting 80% of the clotting

activity of DOPS liposomes; however, the in vivo data presented here have shown that

DSPE-PEG 2000 was more effective than DSPE-PEG 750 in the protection of DOPS

liposomes from removal. While 20 mol% PEG 750 and 15 mol% PEG 2000 are likely to

prevent membrane-membrane contact, their ability to prevent plasma proteins from

approaching may be different. Considering the molecular weights and incorporation levels

of these two PEG polymers, it is conceivable that 15 mol% PEG 2000 would impart a more

dense polymer barrier on the liposome surface than 20 mol% PEG 750. Thus, the work

anticipated for the penetration of plasma proteins such as β_2-glycoprotein I to PS liposome

surface would be more for PEG 2000. Although 20 mol% PEG 750 could inhibit the

assembly of the enzyme complexes for blood coagulation reactions on the PS liposome

surface, it is likely that individual proteins could still access the PS membrane surface, which is supported by the observation that prothrombin binding was not completely abolished even in the presence of 15 mol% PEG 2000. If this is the case, proteins that are bound to the PS liposome surface may not be completely shielded by the PEG 750 barrier, given that PEG 750 extends approximately 3.5 nm away from membrane surface at maximum incorporation level [207] whereas β_2-glycoprotein I would extend approximately 6 nm from the surface (based on a hydrodynamic radius of 3 nm from proteins of similar molecular weights [281]).

The clotting activity of PS liposomes has been shown to be reduced by pre-treating such liposomes with β_2-glycoprotein I [279]; however, the clotting activity of 10 mol% DOPS liposomes in this study recovered from mouse plasma after injection was not affected (see Figure 3.3, P. 108). This is likely to be due to the lower affinity of β_2-glycoprotein I for the PS liposomes than blood coagulation factors such as prothrombin (based on the dissociation constants for β_2-glycoprotein I and prothrombin are 14 μM and 70 nM, respectively [267, 282]). Although the 10 mol% DOPS liposomes and those incorporated with 5 mol% DSPE-PEG 2000 have been shown to retain their surface reactivity toward the blood coagulation proteins after injected into the bloodstream, the potential of these liposomes to induce disseminated intravascular coagulation in bloodstream is anticipated to be low. This is based on the observation that these liposomes were being eliminated very rapidly from circulation, where the injected dose remaining in plasma for these liposomes was < 5% after 1 h in circulation and < 0.2% after 24 h. Thus, in an attempt to further demonstrate the ability of PEG-lipids to shield the PS liposome surface and to address the

potential toxicity of these thrombogenic liposomes, DOPS liposomes containing 15 mol%

DSPE-PEG 2000 were used. Indeed, the presence of an elevated level of DSPE-PEG 2000

in the DOPS liposomes prevented unwanted clot formation in organs that could lead to

potential blood flow occlusion (see Figure 3.4, P. 110).

In this chapter, the ability to protect the 10 mol% DOPS liposomes from plasma

proteins and from MPS uptake has been demonstrated using DSPE-PEG 2000 at an elevated

level of incorporation (> 10 mol%). These results closely matched those of specific in vitro

assays for inhibition of PS-mediated blood coagulation protein binding. Although

protection of the thrombogenic PS liposome surface is important for designing liposome

formulations that are intended for tumor specific thrombogenesis, re-exposure of the

thrombogenic liposome surface within the tumor blood vessels is also necessary for

triggering the blood coagulation responses. In the next chapter, the ability to control the

surface reactivity of these PS containing liposomes will be explored, using PEG-lipids that

can desorb from the PS liposomes with different rates.

Chapter 4   Control of the thrombogenicity of phosphatidylserine containing liposomes by exchangeable poly(ethylene glycol)-conjugated lipids in vitro and in vivo *

4.1. Introduction

The protection of PS containing membrane surface from the clotting factors and plasma proteins using PEG-lipids has been demonstrated in the previous chapters. By incorporating > 10 mol% DSPE-PEG 2000 in the 10 mol% DOPS liposomes, circulation longevity was extended, and the high affinity, PS-mediated interactions with the clotting factors were inhibited. While this is necessary to facilitate liposome access to tumor site and avoid unwanted thrombogenic activity in the central blood compartment, once the liposomes have reached the target site, i.e., tumor blood vessels, exposure of the PS liposome surface is necessary to trigger the blood coagulation reactions. Thus, the thrombogenic PS liposomes need to be designed such that they are only temporarily protected by PEG-lipids to prevent blood coagulation reactions in the general circulation. The transformation of the well-protected PS liposome surface to one that is reactive toward various clotting factors is dependent on the retention of the steric PEG barrier on the PS liposome surface. As described in Chapter 1, it has been shown that 5 mol% POPE-PEG 2000 is less effective in extending the circulation time of DSPC/CHOL liposomes than the same mole percentage of DSPE-PEG 2000 present in the liposomes [197]. This is mainly due to the increased exchange of the entire PE-PEG conjugate out of the DSPC/CHOL liposome when POPE was used as the lipid anchor [197]. Other reports have demonstrated that the rate of PEG-lipid exchange is dependent on the acyl chain composition [112, 203-205]. The exchangeability of PEG-lipids into and out of membrane bilayers has also been

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* **Data except Figure 4.6 and Table 4.2 have been published in Biochim Biophys Acta 1560: 37-50, 2002.**

demonstrated in studies where the acquisition of the PEG barrier on the liposome surface was achieved by the spontaneous insertion of free micellar PEG-lipids into pre-formed liposomes [192, 193, 283]. This PEG-lipid exchange phenomenon has been utilized to provide controlled drug release for improved therapeutic activity [217], and controlled intracellular delivery for anitsense oligonucleotides [219].

The aim of this chapter is to use PEG 2000-conjugated PE with acyl chain lengths ranging from 14 to 18 carbons to investigate the kinetics of PE-PEG transfer into and out of 10 mol% DOPS liposomes, as well as how this affects PS membrane surface reactivity. Since protection of the lipid membrane surface from protein and cellular binding is dependent on the retention of the PEG polymer barrier on the membrane surface, the transfer of PEG-lipids into or out of liposomes should also modulate the reactivity of this biologically active PS membrane surface which is capable of eliciting protein binding reactions. These properties were evaluated by determining: 1) the clotting activity of 10 mol% DOPS liposomes as PE-PEG is exchanged in and out of the membrane, and 2) the plasma levels of 10 mol% DOPS liposomes stabilized by various PE-PEG following intravenous administration. It is projected that the degree of protection of the PS containing liposomes will correlate with the PE-PEG levels in the liposomes. This chapter demonstrates that PE-PEG 2000 with a lipid anchor of appropriate chain length can be used to transiently regulate membrane surface reactivity of liposomes containing reactive lipid species such as PS. This may have important implications in the delivery of biologically active lipids, as well as the design of liposomes containing reactive surfaces for therapeutic

applications and, in particular, the application pursued here towards the development of

liposomes that can induce tumor specific thrombosis triggered by controlled exposure of PS.

4.2. Materials and methods

4.2.1. Materials

All lipids were obtained from Avanti Polar Lipids except for [^{3}H]DSPE-PEG 2000, [^{3}H]DPPE-PEG 2000 and [^{3}H]DMPE-PEG 2000 which were obtained from Northern Lipids (Vancouver, BC). The [^{3}H]- and [^{14}C]CHE were from NEN Life Science Products (Boston, MA). Cholesterol and ellagic acid were purchased from Sigma-Aldrich Canada (Oakville, ON). Bio-Gel A-15m size exclusion gel was obtained from Bio-Rad (Mississauga, ON).

4.2.2. Preparation of various liposomes

The preparation of LUVs followed the extrusion procedure as described in Section 2.2.2. The resulting mean liposome diameter obtained was 100 – 120 nm as determined by quasi-elastic light scattering using the Nicomp submicron particle sizer model 370/270, as described in Section 2.2.2 [261, 262]. For making "heavy" MLVs, the lipid film (EPC/CHOL 55:45) was hydrated in 300 mM sucrose to a concentration of 100 mg/mL at 65 °C with vortex mixing. The resulting preparation was centrifuged at 1600 × g for 10 min in eppendorf tubes, and the bottom sucrose layer was removed with a 1-cc syringe and 20-gauge needle. The MLVs were then washed three times with 0.5 mL HBS at 1600 × g for 10 min, and the pellet was resuspended in HBS. The recovery of MLVs in the pellet was always greater than 95% [284].

4.2.3. Incorporation and retention of PEG-lipids in liposomes

Incorporation of PE-PEG in liposomes after hydration and extrusion (thin film method) was determined by size exclusion chromatography. Briefly, liposomes labeled with trace levels of $[^{14}C]CHE$ and tritiated PE-PEG were applied to a 42 cm × 1.3 cm Bio-Gel A-15m column (50 – 100 mesh), and were eluted with HBS at a flow rate of 0.5 mL/min regulated by a peristaltic pump. To elute under acidic conditions, citrate 300 mM solution (pH 4) was chosen as the elution buffer. Aliquots (0.5 mL) from the 1-mL column fractions were mixed with 5.0 mL Pico-fluor 15 scintillation fluid, and the level of radioactivity was determined using a Packard scintillation counter model 1900 TR. To determine the amount of PE-PEG transferred from pure PE-PEG micelles to liposomes, 5 μmol 10 mol% DOPS/DSPC/CHOL liposomes were incubated with micellar PE-PEG (0.882 μmol) with a final volume of 0.4 mL for various times, and the resulting mixture was fractionated on a 27 cm × 0.7 cm Bio-Gel A-15m column by gravity. The amount of PE-PEG incorporated into liposomes by the micelle transfer method and by the thin film method was expressed in μmol PE-PEG/μmol total lipid. The external PE-PEG grafting density for the pegylated liposomes prepared by micelle transfer method was estimated as follows: mol% PE-PEG = 2 × (mol $_{PE-PEG}$/ mol $_{total\ lipid}$) × 100%, based on the assumptions that PEG-lipids are inserted only into the outer monolayer of the LUVs during the transfer process and phospholipids are distributed equally between the two monolayers.

4.2.4. In vitro PEG-lipid transfer assay

Donor liposomes (LUVs) containing various types of tritiated PEG-lipids were incubated with acceptor MLVs (EPC/CHOL) at 37 °C for various times at a donor to

acceptor mole ratio of 1:100. Donor LUVs were separated from the acceptor MLVs by centrifugation at 1600 × g for 10 min. The sample was washed twice with 0.5 mL HBS at 1600 × g for 10 min. Both the supernatant (donor LUVs) and the pellet (acceptor MLVs) were collected for scintillation counting. Recovery, as determined by radioactivity, was over 95% for donor and acceptor liposomes, respectively.

4.2.5. In vitro clotting time assay

This assay was based on the activated partial thromboplastin time, and has been described in Section 2.2.4. Human plasma was collected into citrated tubes (9 mL blood to 1 mL citrate) from four normal, healthy volunteers, and pooled to make one batch of plasma for studies in this chapter.

4.2.6. Correlation of PEG surface density and inhibition of clotting activity

Correlation of PEG surface density and the percent inhibition of clotting activity was derived from data in Figure 4.3 (P. 132). The calculations were carried out as described below for DMPE-PEG. The DMPE-PEG levels at 0 and 24 h were 0.105 and 0.065 μmmol/μmol lipid, respectively. Therefore, the total DMPE-PEG loss was 0.040 μmmol/μmol lipid. Assume there was 1 μmol lipid and the phospholipids were distributed equally between the two monolayers [285], the amount of DMPE-PEG on the outer monolayer was equal to 0.052 μmmol. Assume that only DMPE-PEG on the outer monolayer were available for transfer, the amount of DMPE-PEG remaining on the outer monolayer at 24 h was 0.012 μmmol. The surface density of DMPE-PEG on the outer

monolayer at 24 h was calculated as follows: surface density (in mol%) = 0.012 μmmol PEG/0.5 μmol lipid × 100% = 2.4.

The same calculations were conducted for DPPE-PEG. The percent inhibition of clotting activity was then correlated with the surface density of each of the PE-PEG. Since the levels of DSPE-PEG in the 10 mol% DOPS LUVs were relatively constant, indicating a very small amount of PE-PEG has desorbed from the LUVs, the DSPE-PEG levels at t = 0 h and 24 h were correlated with the corresponding percent inhibition of clotting activity directly.

4.2.7. Plasma removal of liposomes

All of the in vivo studies were completed using protocols approved by the University of British Columbia's Animal Care Committee. These studies met or exceeded the current guidelines of the Canadian Council of Animal Care. Liposomes, labeled with traces of [^{14}C]CHE and various [^{3}H]PEG-lipids, were injected as a bolus dose via the lateral tail vein into 20 – 22 g female Balb/c mice. The total lipid dose was 50 mg/kg with an injection volume of 200 μL. At various times after liposome injection (t = 0.25, 0.5, 1 and 2), 25 μL blood was collected into microcapillary tubes pre-rinsed with 200 mM EDTA solution by nicking the tail vein. The blood was then added to 200 mM EDTA solution (200 μL) and centrifuged at 1000 × g for 10 min. Aliquots of the supernatant were counting directly in 5 mL Pico-fluor 40 scintillation fluid. At 4 and 24 h, three mice were terminated by CO_2 asphyxiation. Blood was collected by cardiac puncture, and was placed into EDTA-coated microtainer collection tubes. After centrifuging blood samples for 15 min at 1000 × g,

plasma was isolated and visually showed no hemolysis. Aliquots (100 μL) of the plasma obtained were counted directly in 5.0 mL scintillation fluid. The limit of lipid quantitation was 1 μg per mL plasma. The plasma data collected were analyzed with the software WinNonlin (version 1.5) to calculate the values of $AUC_{0-24\,h}$ for the various liposomes.

4.2.8. Statistical analysis

One-way ANOVA was performed to detect differences among different groups, with Newman-Keuls test as post-hoc analysis. A p-value of < 0.05 was considered significant.

4.3. Results

4.3.1. Incorporation of PE-PEG into DOPS and neutral liposomes

It has been demonstrated in the previous chapters that an elevated level (> 10 mol%) of DSPE-PEG 2000 is needed to reduce plasma protein interactions with 10 mol% DOPS liposomes, where this PEG-lipid was retained in the 10 mol% DOPS liposomes under in vitro and in vivo experimental conditions. For the studies in this chapter, it was first necessary to examine the incorporation efficiency of the other two PEG-lipids, DMPE-PEG 2000 and DPPE-PEG 2000. The incorporation of various PE-PEG species into neutral and PS containing liposomes was achieved using two different methods. In one procedure, PE-PEG was added to the lipid mixture before making the dried lipid film for hydration (referred to as the thin film method here). The pegylated liposomes produced by the thin film method would have an approximately equal distribution of the PE-PEG in the inner and outer monolayer [286], whereas PE-PEG incorporation from addition of micelles (referred to as micelle transfer method) results in asymmetric PE-PEG distribution, where the PE-PEG is found exclusively in the outer monolayer [180, 203]. Table 4.1 (P. 125) summarizes the results of PEG-lipid incorporation into liposomes by the two different methods. The incorporation of DPPE- and DSPE-PEG 2000 into 10 mol% DOPS/DSPC/CHOL and DSPC/CHOL liposomes was approximately 14 mol% using the thin film method with an input level of 15 mol%. These results are consistent with previous studies by Kenworthy et al and Hristova et al [198, 200, 201]. The incorporation of DMPE-PEG 2000 into 10 mol% DOPS/DSPC/CHOL liposomes was significantly less efficient compared to that of DPPE- and DSPE-PEG 2000, where the liposomes after hydration and extrusion contained 10.5 mol% DMPE-PEG 2000 (Table 4.1, P. 125). This lower level of DMPE-PEG 2000

Table 4.1
Incorporation of various PEG-lipids into DOPS and neutral liposomes [a]

	10 mol% DOPS/DSPC/CHOL		DSPC/CHOL	
	μmol PE-PEG/μmol total lipid	mol%	μmol PE-PEG/μmol total lipid	mol%
Thin film method				
DMPE-PEG 2000 [b]	0.105 ± 0.006	10.5 [d, e, f]	0.120 ± 0.009	12.0 [d, f]
DMPE-PEG 2000 (pH 4)	0.123 ± 0.003	12.3 [d, f]		
DPPE-PEG 2000	0.132 ± 0.001	13.2 [e, f]	0.139 ± 0.009	13.9 [f]
DSPE-PEG 2000	0.140 ± 0.003	14.0 [f]	0.144 ± 0.004	14.4 [f]
Micelle transfer method [c]				
DMPE-PEG 2000 [b]	0.045 ± 0.002	9.08 [e]		
DPPE-PEG 2000	0.056 ± 0.003	11.2 [e]		
DSPE-PEG 2000	0.058 (72 h)	11.7		

[a] Reported values represent means of three independent determinations and the standard deviations. Statistical analysis was performed using ANOVA and Newman-Keuls test with $p < 0.05$.

[b] DMPE-PEG 2000 was incorporated into 10 mol% DOPS/DSPC/Chol liposomes hydrated at pH 7.5 and 4.0. Other PEG-lipids were incorporated into liposomes hydrated at pH 7.5. The input level of PEG-lipids for the thin film method was 15 mol% for the two types of liposomes.

[c] The 10 mol% DOPS/DSPC/Chol liposomes were incubated with PE-PEG micelles for 24 hours (DMPE-PEG 2000 and DPPE-PEG 2000) or 72 hours (DSPE-PEG 2000). The mixture was separated on Bio-Gel A-15m column as described in Section 4.2 to determine the amount of PEG-lipids incorporated into the liposomes by the micelle transfer method, which was calculated as follows: mol% PE-PEG = 2 × (mol $_{PE-PEG}$/mol $_{total\ lipid}$) × 100% to reflect the PE-PEG density on the outer monolayer, based on the assumptions that PE-PEG are inserted only into the outer monolayer of LUV during the transfer process and phospholipids are distributed equally between the two monolayers.

[d] Incorporation of DMPE-PEG 2000 in 10 mol% DOPS liposomes was statistically different from that in DSPC/CHOL liposomes and from that incorporated at pH 4.

[e] Incorporation of DMPE-PEG 2000 in 10 mol% DOPS liposomes by thin film method was not statistically different from that incorporated by micellar transfer method, while incorporation of DPPE-PEG 2000 in 10 mol% DOPS liposomes by thin film method was statistically different from that incorporated by micellar transfer method.

[f] Levels of DMPE-PEG 2000 incorporated into 10 mol% DOPS liposomes and into DSPC/CHOL liposomes were statistically different from those observed for DPPE-PEG 2000 and DSPE-PEG 2000, respectively.

incorporation was reflected by a peak of free micellar DMPE-PEG 2000 observed in the gel filtration column elution profile. Similarly, incorporation of DMPE-PEG 2000 into DSPC/CHOL liposomes was significantly lower than that observed with the other two types of PEG-lipids. The DSPC/CHOL liposomes after hydration and extrusion contained 12 mol% DMPE-PEG 2000, compared to 13.9 mol% and 14.4 mol% observed for DPPE-PEG 2000 and DSPE-PEG 2000, respectively.

The PE-PEGs used in the present studies were conjugated through a carbamate linkage that imparts a net negative charge on the phosphate moiety at physiological pH [194, 287]; thus, the negative surface charge on PS could potentially impede the incorporation of DMPE-PEG 2000 into the PS containing liposomes due to charge repulsion. The effect of protonating the PS carboxyl moiety on DMPE-PEG 2000 incorporation was investigated. At pH 4.0, approximately 97% of the carboxylate PS headgroups are protonated (based on pK = 5.5 [288]), and this led to a modest yet statistically significant increase in the incorporation of DMPE-PEG 2000 into 10 mol% DOPS/DSPC/CHOL liposomes from 10.5 mol% to 12.3 mol% ($p < 0.05$), which was similar to that obtained with neutral DSPC/CHOL liposomes (12.0 mol%, see Table 4.1, P. 125).

The incorporation of PE-PEG into 10 mol% DOPS/DSPC/CHOL liposomes by the micelle transfer method is also summarized in Table 4.1 (P. 125). The values reported for PE-PEG incorporation using the micelle transfer method were PE-PEG levels observed in the 10 mol% DOPS liposomes after 24 h for DMPE-PEG 2000 and DPPE-PEG 2000 and after 72 h for DSPE-PEG 2000, with time points where the transfer process achieved > 90%

of the estimated maximum. The kinetics of PE-PEG insertion from micelles to DOPS liposomes is shown in Figure 4.1 (Panel A, P. 128). Assuming that the PEG-lipids were inserted only into the outer monolayer of the LUVs during the transfer process and phospholipids were distributed equally between the two monolayers [180, 203], the amount of PEG-lipids transferred to the liposomes was first calculated as mole percentage of total lipid, and this value was then multiplied by two to approximate the PEG-lipid density in the outer monolayer which was equivalent to that achieved when preparing the liposomes by thin film method (see Table 4.1, P. 125). The external grafting density of DMPE-PEG 2000 in 10 mol% DOPS liposomes was comparable using the two different methods. DPPE-PEG 2000 had slightly but significantly lower external grafting densities in the outer monolayer of 10 mol% DOPS liposomes using the micelle transfer method as compared to the thin film method ($p < 0.05$). The grafting densities achieved in the outer monolayer for DPPE-PEG 2000 and DSPE-PEG 2000 were 11 and 12 mol% respectively using the micelle transfer method, as compared to 13 and 14 mol% respectively using the thin film method.

4.3.2. Insertion of PE-PEG into DOPS liposomes from micelle addition and subsequent inhibition of clotting activity of DOPS liposomes

The transfer of various PE-PEG from micelles into 10 mol% DOPS liposomes as a function of time is shown in Figure 4.1 (P. 128). Pure PE-PEG micelles were added to pre-formed 10 mol% DOPS containing liposomes to achieve a final concentration of 2.205 mM, which was above the CMC reported for DSPE-PEG 2000 (CMC of 2.2 – 10.8 μM [192]). At a PE-PEG to liposome mole ratio of 0.176:1, not all of the PE-PEG were transferred into the DOPS liposomes, and any unincorporated PE-PEG was separated from the liposomes
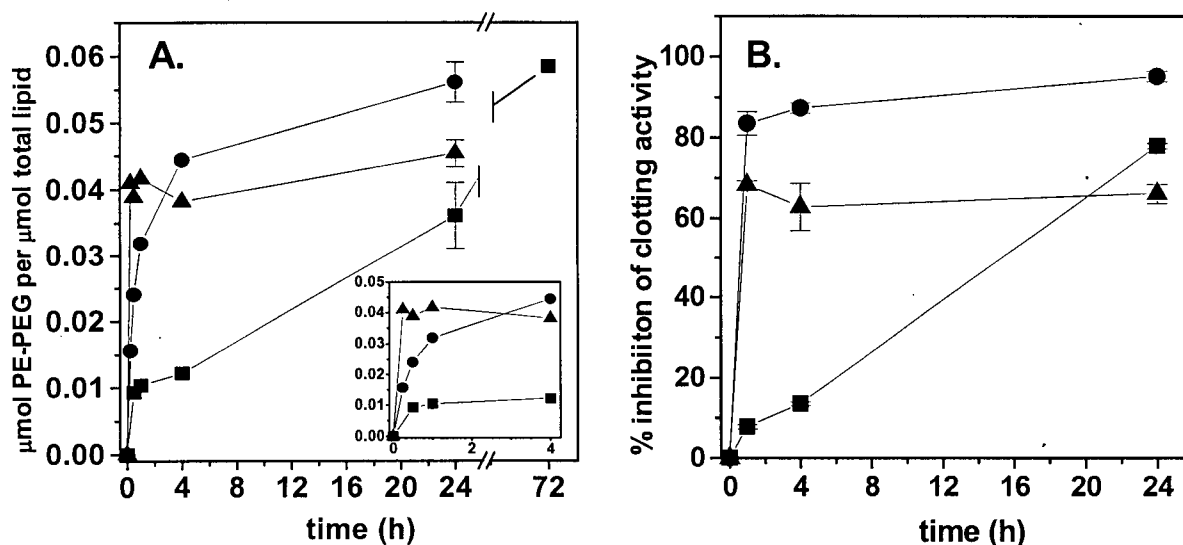
Fig. 4.1. Kinetics of PEG-lipid insertion into DOPS containing liposomes from micelle addition and the subsequent inhibition of clotting activity of DOPS liposomes. Panel A shows the time course for DMPE-PEG 2000 (▲), DPPE-PEG 2000 (●) and DSPE-PEG 2000 (■) transfer from micelles into 10 mol% DOPS/DSPC/CHOL liposomes at 37 °C. The inset highlights differences in the kinetics of PEG-lipid transfer over the first 4 hours. The amount of PEG-lipids transferred into liposomes is expressed as μmol PE-PEG per μmol total lipid with each data point representing a single determination except at 24 h where the amount of PEG-lipid transferred was determined in triplicate as described in Section 4.2.3. Panel B shows the % inhibition of clotting activity as DMPE-PEG 2000 (▲), DPPE-PEG 2000 (●) and DSPE-PEG 2000 (■) transferred into 10 mol% DOPS/DSPC/CHOL liposomes from micelles over 24 h. The % inhibition of clotting activity was calculated as follows: % inhibition = $(t_{PEG} - t_{PS})/(t_{Blank} - t_{PS}) \times 100$, where t represents the clotting time of each type of liposome as determined by the in vitro clotting time assay. Data points were determined in triplicates, and the error bars represent S.D.

with a Bio-Gel A-15m column according to established procedures [289]. Mixing PE-PEG micelles with liposomes did not have any significant effect on liposome size, which remained in the range of 100 – 120 nm during the 24 h incubation period with the PE-PEG micelles. After 4 h, only 0.012 μmol DSPE-PEG 2000 has been inserted into 1 μmol 10 mol% DOPS/DSPC/CHOL liposomes, as compared to 0.038 μmol DMPE-PEG 2000 and 0.045 μmol DPPE-PEG 2000. After 24 h, the transfer of DMPE-PEG 2000 and DPPE-PEG 2000 into the 10 mol% DOPS liposomes was nearly complete. The amounts of DMPE-PEG 2000 and DPPE-PEG 2000 transferred into 10 mol% DOPS liposomes after 24 h were 0.045 and 0.056 μmol PE-PEG/μmol total lipid, respectively. The transfer of DSPE-PEG 2000 into 10 mol% DOPS liposomes required 72 h to reach a similar level of 0.058 μmol PE-PEG/μmol total lipid.

The inhibition of surface reactivity of 10 mol% DOPS liposomes by PE-PEG was evaluated by monitoring the clotting activity of the DOPS liposomes at various times following PE-PEG insertion. The transfer of micellar DMPE-PEG 2000 and DPPE-PEG 2000 into 10 mol% DOPS liposomes resulted in rapid inhibition of clotting activity (Figure 4.1, Panel B, P. 127). The percent inhibition of clotting achieved following the incubation of PS liposomes with micellar DMPE-PEG 2000 and DPPE-PEG 2000 reached levels of 65% and 82% within one hour, respectively. In contrast, the transfer of micellar DSPE-PEG 2000 into 10 mol% DOPS liposomes was slow, and this resulted in significantly much slower generation of clotting activity inhibition, with only 8% inhibition achieved after one hour (ANOVA; $p < 0.01$).

4.3.3. Transfer of PE-PEG from neutral and anionic LUVs into MLVs and subsequent restoration of clotting activity of DOPS liposomes

The loss of various PE-PEG from neutral DSPC/CHOL and PS containing LUVs was examined by incubating the LUVs with a 100-fold molar excess of EPC/CHOL MLVs. The MLVs were used to model the biological milieu where membranes from the vascular endothelium and other cellular components in the bloodstream constitute a large lipid pool that may interact with intravenously injected liposomes, leading to possible interbilayer exchange of lipids. Here, the MLVs provided a lipid pool to accept the PEG-lipids that transfer out of the LUVs (see Figure 4.2 on P. 131 and Figure 4.3 on P. 132). From a previous study, approximately 10% of the total lipid is contained in the outermost bilayer of an MLV; thus, a 10-fold excess in surface area of MLV acceptors is available for PE-PEG transfer [285]. A rapid decrease in DMPE-PEG 2000 membrane content was observed in both 10 mol% DOPS liposomes and neutral DSPC/CHOL liposomes, where approximately 40% of DMPE-PEG 2000 transferred out of the two types of liposomes within 15 min following the addition to the MLVs.

As the acyl chain length of the lipid anchor increased from 14 carbons (DMPE) to 18 carbons (DSPE), the rate of transfer of the PEG-lipids from LUVs to MLVs was reduced such that the level of DSPE-PEG 2000 in 10 mol% DOPS liposomes and in DSPC/CHOL liposomes remained relatively constant over 24 h. This observation is consistent with results from previous studies where the rate of PEG-lipid transfer was decreased with increasing acyl chain length [112, 203-205]. Desorption of various PE-PEG from LUVs in the absence of MLVs under similar conditions was negligible, with no micellar PE-PEG detected in fractions isolated following column chromatography.
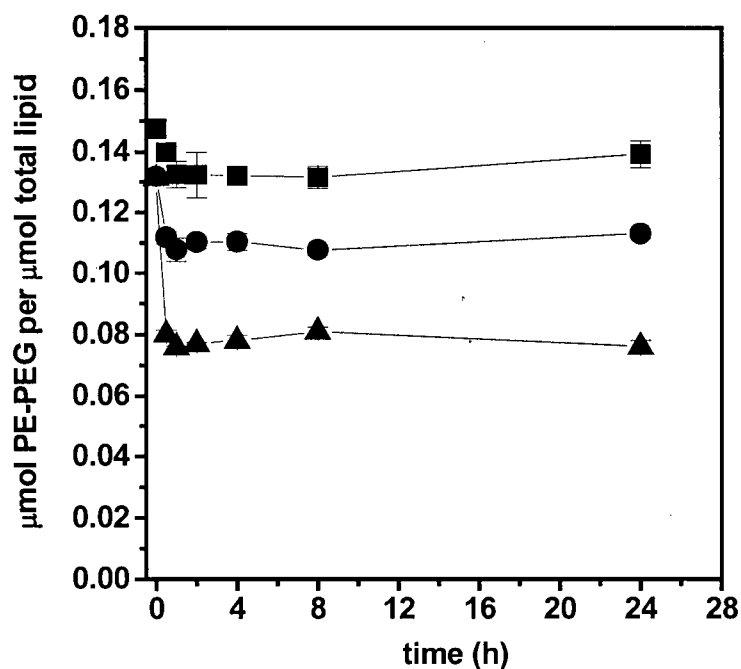
Figure 4.2. Kinetics of PEG-lipid transfer from neutral LUV into MLV. Time courses are shown for DMPE-PEG 2000 (▲), DPPE-PEG 2000 (●) and DSPE-PEG 2000 (■) transferring from DSPC/CHOL LUV to EPC/CHOL MLV (in 100-fold molar excess) at 37 °C. Data points were determined in triplicate using the in vitro PEG-lipid transfer assay as described in Section 4.2.4, and the error bars represent S.D. In some cases, the size of the symbol is larger than the size of the error bar.
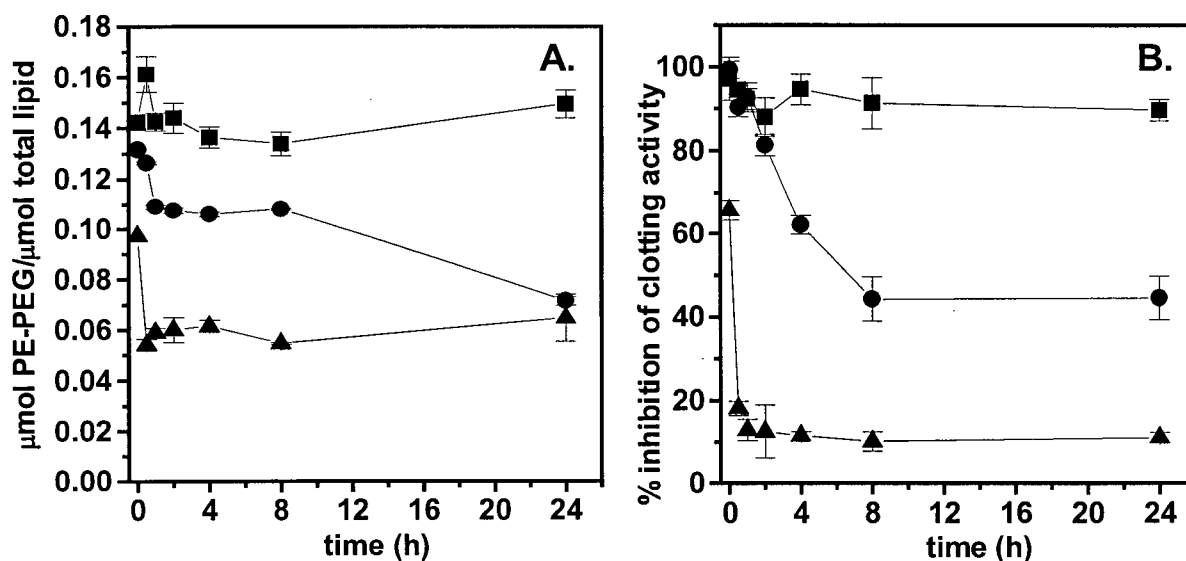
Figure 4.3. Kinetics of PEG-lipid transfer from DOPS LUV into MLV and the subsequent restoration of clotting activity. Panel A shows the time course for DMPE-PEG 2000 (▲), DPPE-PEG 2000 (●) and DSPE-PEG 2000 (■) transferred from 10 mol% DOPS/DSPC/CHOL LUV to EPC/CHOL MLV (in 100-fold molar excess) at 37 °C. Data points were determined in triplicate using the in vitro PEG-lipid transfer assay as described in Section 4.2.4, and the error bars represent S.D. Panel B shows the % inhibition of clotting activity as DMPE-PEG 2000 (▲), DPPE-PEG 2000 (●) and DSPE-PEG 2000 (■) transferred out from the 10 mol% DOPS liposomes into EPC/CHOL MLV over 24 hours. The % inhibition of clotting activity was calculated as follows: % inhibition = (t_{PEG} − t_{PS})/(t_{Blank} − t_{PS}) x 100, where t represents the clotting time of each type of liposome as determined by the in vitro clotting time assay. Data points were determined in triplicate, and the error bars represent S.D.
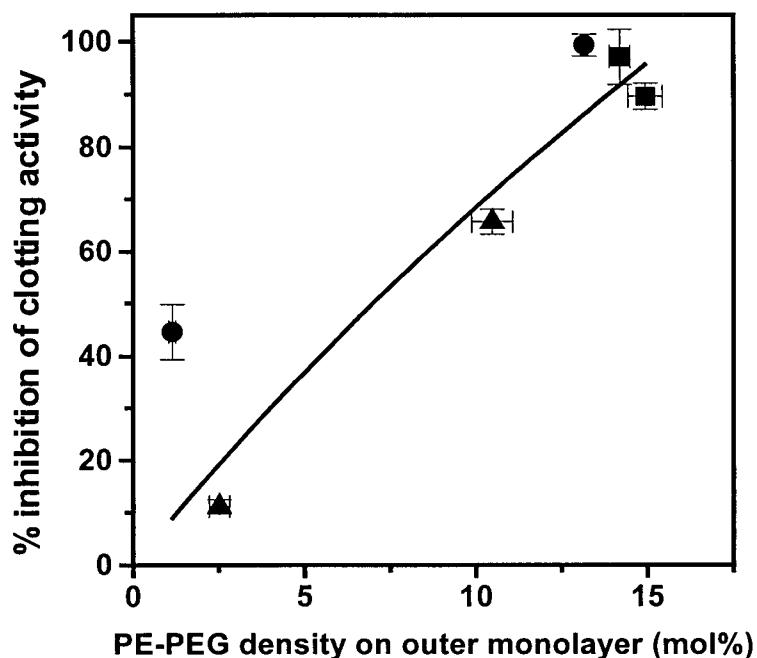
Figure 4.4. The % inhibition of clotting activity as a function of PEG-lipid level in 10%
DOPS/DSPC/CHOL liposomes. Data points represent the % inhibition of clotting activity
and the PE-PEG level in the 10 mol% DOPS/DSPC/CHOL liposomes before or after 24-
hour incubation with the EPC/CHOL MLV (derived from Figure 4.3). The 10 mol% DOPS
liposomes were made with DMPE-PEG 2000 (▲), DPPE-PEG 2000 (●) or DSPE-PEG
2000 (■) incorporated by the thin film method. Error bars represent S.D.

The clotting activity of 10 mol% DOPS liposomes containing various types of PEG-lipids was examined as the PEG-lipids were transferring out of the liposomes (Figure 4.3, Panel B, P. 132). The transfer of approximately 40% DMPE-PEG 2000 out of 10 mol% DOPS liposomes within 15 min resulted in a dramatic decrease in the inhibition of clotting activity from 67% to 18%. In contrast, the percent inhibition of clotting activity for DSPE-PEG 2000 remained constant for 24 h. This is consistent with data previously observed which indicate that DSPE-PEG 2000 is not readily lost from the PS liposomes. For DPPE-PEG 2000 which has a rate of transfer that is intermediate between those of DMPE-PEG 2000 and DSPE-PEG 2000, the inhibition of clotting activity gradually decreased from 99% to 45% within 8 h.

Figure 4.4 (P. 133) presents a composite of blood clotting activity obtained as a function of the PEG-lipid content in 10 mol% DOPS liposomes derived from Figure 4.3 (P. 132). Results from this study reveal a trend which reflects a dependence of the inhibition of clotting on the PE-PEG surface density in the DOPS membrane. As the PEG surface density increased in the DOPS membrane, the percent inhibition of clotting activity increased, similar to results observed in previous chapters.

4.3.4. Effect of exchangeable PE-PEG on the plasma levels of DOPS and neutral liposomes after intravenous injection

To examine the effect of PE-PEG transfer on the plasma removal profiles of PS containing LUVs and DSPC/CHOL LUVs, these two types of liposomes containing various PE-PEGs incorporated at > 10 mol% were injected into mice, and plasma liposome concentrations were determined by monitoring a non-exchangeable lipid label, [^{14}C]CHE

[271] (see Figure 4.5, P. 136). The plasma data collected were used to calculate the AUC_{0-24} $_h$ values for the various liposomes using the software WinNonlin (version 1.5), based on the linear trapezoidal rule as described in Chapter 3 (see Table 4.2, P. 138). The rapid transfer of DMPE-PEG 2000 out of both types of liposomes resulted in rapid removal of liposomes from the circulation. Approximately 10% and 35% of the injected liposomal lipid remained in plasma after 1 h for 10 mol% DOPS liposomes and DSPC/CHOL liposomes, respectively, and the $AUC_{0-24\,h}$ values for the above two types of liposomes were 0.399 and 0.935 mg • h • mL^{-1}, respectively. In contrast, the plasma levels of 10 mol% DOPS liposomes and DSPC/CHOL liposomes containing the slowly exchanged DSPE-PEG 2000 were much higher, with 66% and 82% of the liposomal lipid remaining in the plasma after 1 h, respectively. The AUC_{0-24h} values for these two liposome systems were 5.018 and 9.150 mg • h • mL^{-1}, producing 13-fold and 10-fold increases in the $AUC_{0-24\,h}$ values, respectively, when compared to those determined for liposomes containing DMPE-PEG 2000.

The plasma removal profile of 10 mol% DOPS liposomes containing PE-PEG was similar for systems incorporating DPPE-PEG 2000 and DMPE-PEG 2000. This is in contrast to the results obtained with neutral DSPC/CHOL liposomes where DPPE-PEG 2000 containing systems were removed from the bloodstream much slower than DMPE-PEG 2000 liposomes. This PS-mediated removal of liposomes was most prominent in liposomes prepared with the same amount of DPPE-PEG 2000, where the $AUC_{0-24\,h}$ value for DSPC/CHOL-based liposomes was 4.112 mg • h • mL^{-1} and the $AUC_{0-24\,h}$ value for DOPS-based liposomes was 0.465 mg • h • mL^{-1}, resulting in 9-fold change in $AUC_{0-24\,h}$ values.

Panel A

Panel B

(i)

mg liposomal lipid per mL plasma

(9.27%)

(0.29%)

(0.25%)

time (h)

(i)

mg liposomal lipid per mL plasma

(28.5%)

(5.76%)

(0.53%)

time (h)

(ii)

mg PE-PEG 2000 per mL plasma

(8.87%)

(2.94%)

(2.00%)

time (h)

(ii)

mg PE-PEG 2000 per mL plasma

(23.3%)

(3.73%)

(2.10%)

time (h)

(iii)

mg PEG-lipid to mg liposomal lipid

time (h)

(iii)

mg PEG-lipid to mg liposomal lipid

time (h)
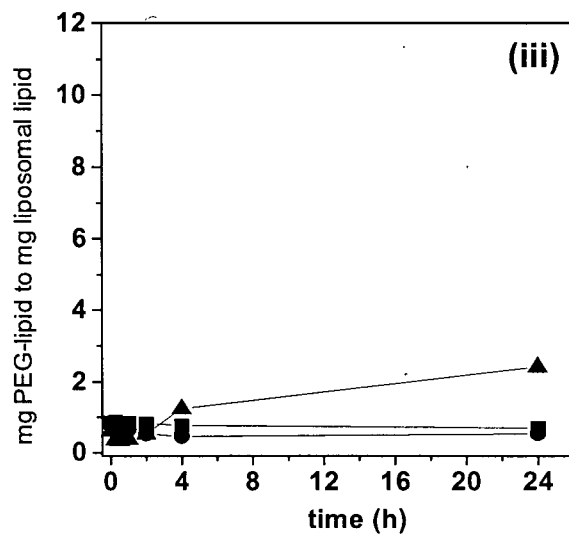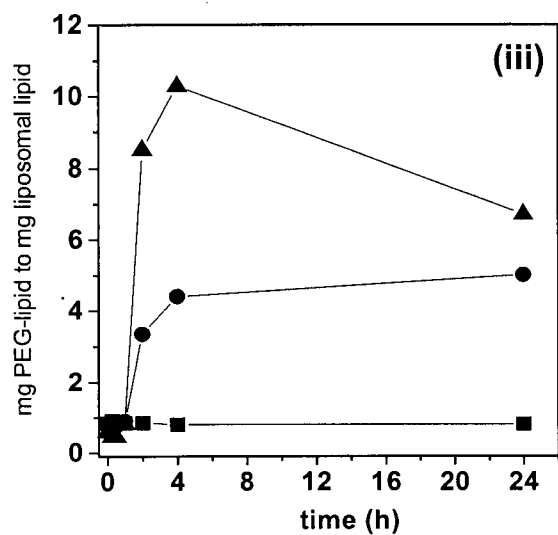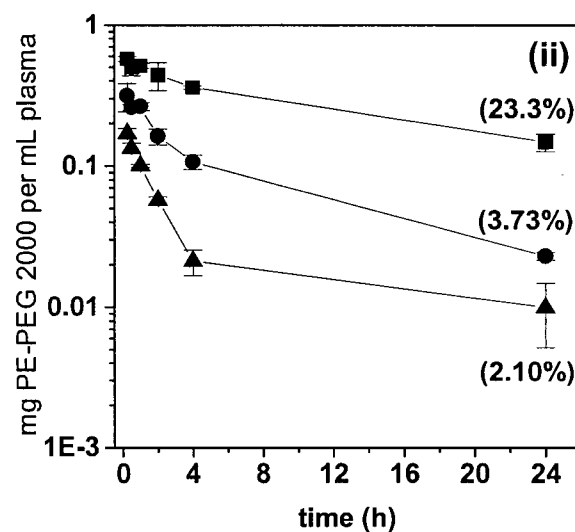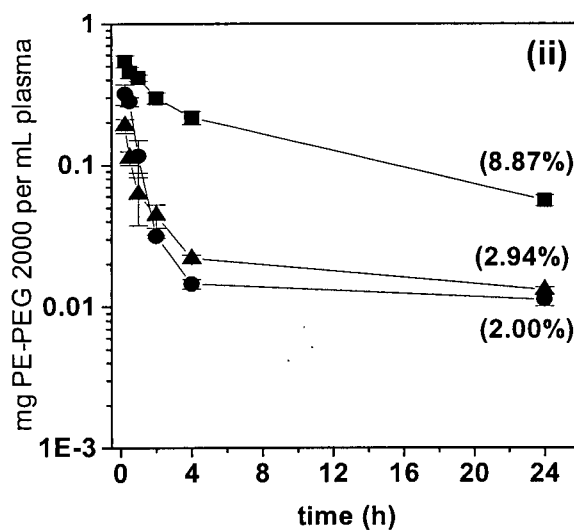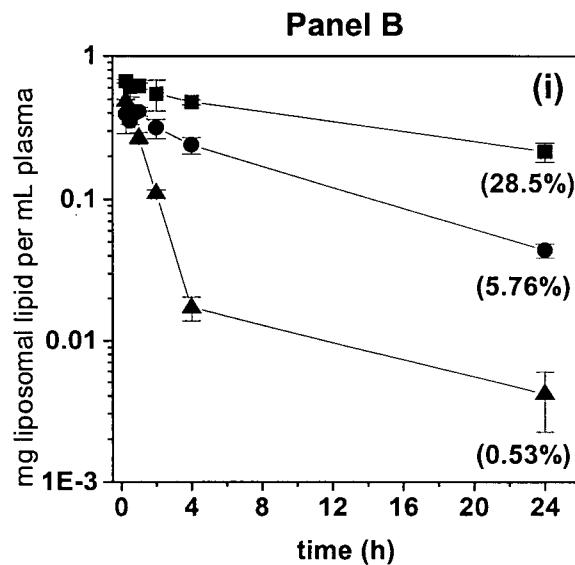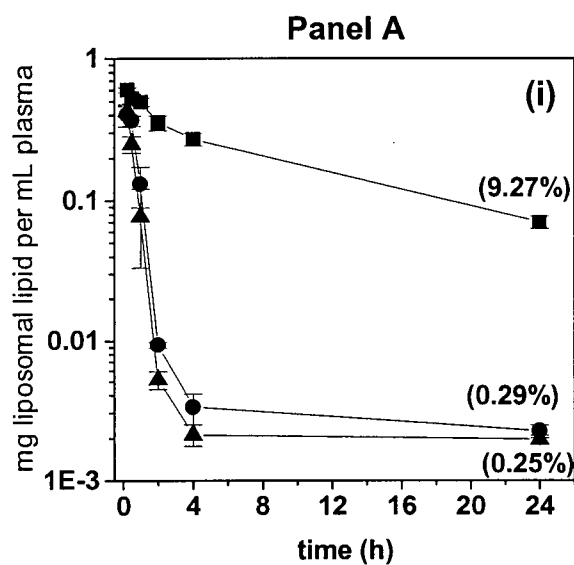
Figure 4.5. The effect of various PEG-lipids on the plasma removal of DOPS and neutral liposomes. Three Balb/c mice (20 – 22 g) were used for each data point, and the error bars represent S.D. The lipid dose was injected as a single IV bolus of 50 mg/kg. The levels of various PE-PEG species present in 10 mol% DOPS liposomes and in DSPC/CHOL liposomes were those reported in Table 4.1. Panel A shows (i) the plasma levels of liposomal lipids, (ii) the plasma levels of PEG-lipids, and (iii) the PEG-lipid to liposomal lipid ratio in plasma for DMPE-PEG 2000 (▲), DPPE-PEG 2000 (●) and DSPE-PEG 2000 (■) incorporated in 10 mol% DOPS liposomes. Panel B shows (i) the plasma levels of liposomal lipids, (ii) the plasma levels of PEG-lipids, and (iii) the PEG-lipid to liposomal lipid ratio in plasma for DMPE-PEG 2000 (▲), DPPE-PEG 2000 (●) and DSPE-PEG 2000 (■) incorporated in DSPC/CHOL liposomes. The percentages of injected dose remaining in plasma for liposomal lipids and PEG-lipids 24 h post injection are shown in parentheses.

Table 4.2.

AUC_{0-24h} values for 10 mol% DOPS liposomes and DSPC/CHOL liposomes containing various PE-PEG [a,b]

	10 mol% DOPS liposomes (mg • h • mL^{-1})	DSPC/CHOL liposomes (mg • h • mL^{-1})
DMPE-PEG 2000	0.399	0.935
DPPE-PEG 2000	0.465	4.112
DSPE-PEG 2000	5.018	9.150

[a] Balb/c mice (20 – 22 g) were injected with 50 mg/kg lipid as a single IV bolus dose via the lateral tail vein, and blood was collected and processed as described in Section 4.2.7. Three mice were used to determine the plasma lipid levels for each time point.

[b] Non-compartmental analysis based on the linear trapezoidal rule was performed using the software WinNonlin version 1.5 to estimate the values of various pharmacokinetic parameters, as described in Chapter 3. AUC_{0-24h} is the area under curve from time of dosing (t = 0 h) up to the last measured plasma concentration (t = 24 h).

The amount of PE-PEG remaining in plasma was also monitored in order to correlate liposome removal properties with retention of the PEG-lipids in the liposomes. Figure 4.5 (P. 136) demonstrates that the plasma removal of the three PEG-lipids studied followed the same trend as that of the liposomal lipids where DMPE-PEG 2000 itself and the liposomes containing this PEG-lipid had the fastest removal from the bloodstream. DSPE-PEG 2000 and the liposomes containing this PEG-lipid were removed most slowly from circulation. After 24 h, the plasma levels of DSPE-PEG 2000 were 4-fold and 10-fold higher than those of DMPE-PEG 2000 in 10 mol% DOPS liposomes and DSPC/CHOL liposomes respectively. It is interesting to note that the relative amounts of PE-PEG in plasma were higher than those of liposomal lipid for the liposome systems which were eliminated rapidly from the bloodstream. For instance, the percentage of DMPE-PEG 2000 remaining in plasma at 24 h for both 10 mol% DOPS liposomes and DSPC/CHOL liposomes was approximately 2 – 3% compared to 0.3 – 0.5% of liposomal lipid remaining for the two types of liposomes, as determined from [^{14}C]CHE. This divergence of liposome and DMPE-PEG 2000 plasma removal is further evidenced in the PEG to liposomal lipid ratio in plasma (Figure 4.5, P. 136), where this ratio did not remain constant but increased over time for DMPE-PEG 2000 incorporated in both types of liposomes and for DPPE-PEG 2000 incorporated in the PS containing liposomes. Since DMPE-PEG 2000 is able to transfer out of liposomes rapidly, it may possibly interact with plasma components that act as an acceptor for DMPE-PEG 2000, leading to longer circulation times of the PEG-lipid compared to the intact liposomes. This is supported by a previous finding that DMPE-PEG 2000 can exchange into the plasma lipoprotein pool [290].

4.3.5. Effect of combinations of exchangeable and non-exchangeable PE-PEG on the plasma removal of DOPS liposomes

Since the rapid desorption of DMPE-PEG 2000 and DPPE-PEG 2000 resulted in rapid removal of the 10 mol% DOPS liposomes formulated with these two PEG-lipids, combinations of exchangeable and non-exchangeable PEG-lipids were used in an attempt to balance PS surface exposure with circulation longevity of such liposomes. Here, combinations of DPPE-PEG 2000 (as the exchangeable PE-PEG) and DSPE-PEG 2000 (as the non-exchangeable PE-PEG) were used, and the effects on the plasma removal profiles of 10 mol% DOPS liposomes were studied. The results are shown in Figure 4.6 (P. 141). In general, the circulation longevity of the 10 mol% DOPS liposomes was dependent on the amount of the exchangeable DPPE-PEG 2000 used in the DOPS liposomes. This result has important implications in the development of the thrombogenic PS liposomes described in this thesis. It demonstrates the possibility of controlling the exposure of the PS liposome surface and the circulation longevity of the PS containing liposomes through an appropriate combination of exchangeable and non-exchangeable PEG-lipids.
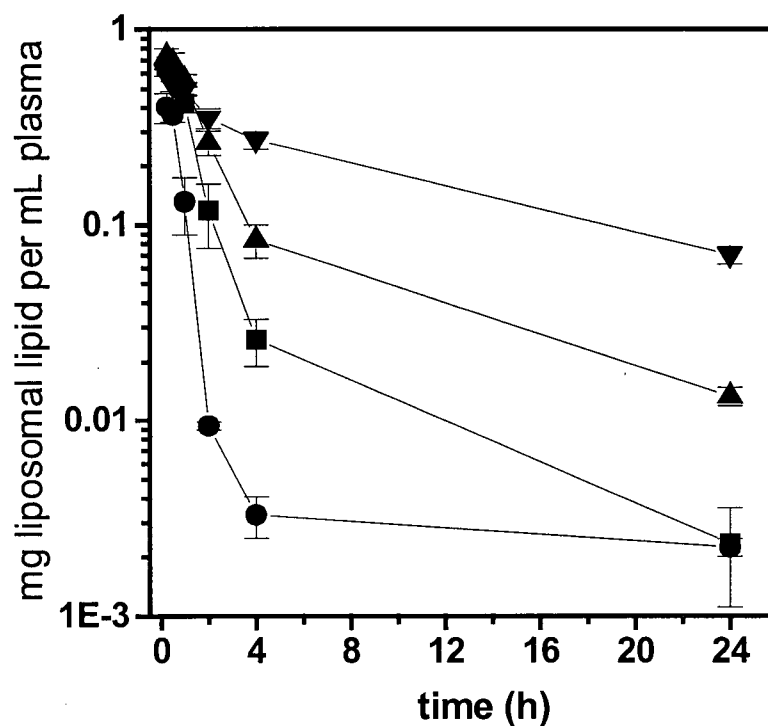
Figure 4.6. The effect of combinations of exchangeable and non-exchangeable PE-PEG 2000 on the plasma removal profiles of PS containing liposomes. In this study, 10 mol% DOPS/DSPC/CHOL with the following PEG content was injected into Balb/c mice at 50 mg/kg as a single IV bolus: 15 mol% DSPE-PEG 2000 (▼), 10 mol% DSPE-PEG 2000/5 mol% DPPE-PEG 2000 (▲), 5 mol% DSPE-PEG 2000/10 mol% DPPE-PEG 2000 (■), and 15 mol% DPPE-PEG 2000 (●). Three mice were used for each data point, and the error bars represent S.D.

4.4. Discussion

In the development of thrombogenic PS liposomes for selective thrombosis in tumor blood vessels, control of the thrombogenic surface exposure is crucial and requires transient shielding of the liposomal PS surface. Retention of a surface grafted PEG polymer coating is necessary for the protection of the thrombogenic PS liposome surface in the general circulation. However, the PEG polymer coating on the PS liposome surface may prevent interactions with the clotting factors and the initiation of blood coagulation once they have localized in the tumor vasculature. Controlled loss of the PEG coating is therefore necessary to trigger the blood coagulation reactions mediated by the PS membrane surface within the tumor blood vessels. Indeed, current trends in designing liposomes with surface grafted PEG are to achieve: 1) temporary stabilization of liposomes in circulation to allow adequate accumulation at the target site, and 2) subsequent transformation of liposomes to a reactive or unstable form to facilitate target cell interaction/fusion and content release through the loss of PEG polymer [203, 217, 219, 291-293]. Furthermore, in designing pegylated liposomes, it is possible to control membrane-dependent enzymatic activity by controlling the access of proteins/enzymes to the liposome surface via inclusion of appropriate levels of the surface grafted PEG polymer coating [294, 295]. In this chapter, the ability to control the surface reactivity of PS containing membranes toward high affinity clotting factors in a time dependent fashion is demonstrated through the use of exchangeable PE-PEG. This enhances the understanding of how to design liposomes for inducing tumor selective thrombosis.

Several features have been identified in governing the transfer rate of PEG-lipids, including the headgroup and the acyl chain of PEG-lipids, the liposomal lipid composition

and the concentration of the acceptor membrane [112, 113, 204, 296, 297]. The hydrophilic

PEG polymer (MW 2000) on the various lipid anchors used in this study was conjugated to

the amino group on PE, resulting in the generation of a negatively charged PE-PEG at

physioloigical pH [194, 287]. The negative charge on PE-PEG has a modest effect on

reducing DMPE-PEG 2000 membrane incorporation into the negatively charged 10 mol%

DOPS membrane. This is likely due to the charge repulsion between the DMPE-PEG 2000

and DOPS head groups. Although the effect of the molecular weight of the PEG polymer

was not investigated in this study, it has been documented that an increase in the molecular

weight of the PEG polymer would lead to an increase in the desorption rate of PEG-lipids

from vesicles [204].

Among the three types of PE-PEG used, DMPE-PEG 2000 was observed to have the

lowest incorporation into both neutral and PS containing liposomes and the fastest rate of

transfer into and out of the liposomes. This result is consistent with previous studies [112,

203-205]. Considering the acyl chain lengths of DMPE-PEG 2000 and of the remaining

lipid components (DSPC/CHOL ± 10 mol% DOPS), formation of packing defects in the

lipid bilayer is probable because of the four-carbon mismatch in the acyl chain lengths [298,

299]. These packing defects may make the incorporation of DMPE-PEG 2000 into the

liposomes less favorable, thus limiting the amount of DMPE-PEG 2000 that can be

incorporated into liposomes. Alternatively, the smaller length/width axial ratio of this PEG-

lipid may result in a more preferred partitioning into highly curved micellar structures [180,

218]. Desorption of lipids or lipid conjugates involves the formation of a transition state

which entails a significant displacement of the acyl chain into the aqueous phase [300]. This

requires energy to disrupt the lipid-lipid van der Waals interactions and the hydrogen-

bonded water to accommodate the lipid acyl chain in the aqueous phase. Thus, increasing

the hydrophobicity by extending the acyl chain of the lipid anchor would make the

desorption of PEG-lipids energetically more unfavorable.

Results from the in vitro clotting assay presented in Figure 4.4 (P. 133) demonstrate

a trend which reflects a dependence of the inhibition of clotting on the PE-PEG surface

density in the DOPS containing liposomes. However, there is one outlying point where the

% inhibition of clotting for DPPE-PEG protected DOPS liposomes at 24 h was higher than

that for DMPE-PEG protected DOPS liposomes, although the levels of these two PEG-lipids

in the DOPS liposomes were similar. This higher % inhibition of clotting by DPPE-PEG

reflects that the PS-mediated blood coagulation reactions are somehow prevented, and the

reasons underlying this observation are yet to be determined. One possible explanation is

that the binding of blood coagulation proteins such as those of the prothrombinase complex

to PS containing membranes requires the formation of PS enriched microdomains in the

membrane. It is possible that membrane re-organization may occur during the desorption of

DPPE-PEG from the PS liposomes, which in turn could prevent the formation of such PS

enriched microdomains. The temperature at which the in vitro clotting assay was conducted

was 37 °C, which DPPE-PEG at this temperature may undergo gel to liquid crystalline phase

transition that could generate a non-homogeneous distribution of these two phases in the

DOPS membrane. This, in turn, may possibly prevent the formation of the PS enriched

microdomains that allow interactions with the blood coagulation proteins.

Under in vitro conditions in the absence of an acceptor membrane, PE-PEG did not desorb from 10 mol% DOPS LUVs or from DSPC/CHOL LUVs. The presence of an acceptor membrane is thus necessary for the desorption of PEG-lipids from LUVs. In this study, 20 mM MLVs were used in the in vitro transfer assay, and under high acceptor vesicle concentrations, it is possible to enhance the rate of PE-PEG desorption. This may be due to an increase in the collisional frequency of the pegylated LUVs and the acceptor MLVs that may facilitate transfer of the lipid [296, 297]. Although fusion-mediated lipid transfer has been suggested previously as an alternative means of PE-PEG transfer [301], the data presented here do not support this mechanism because recovery of the LUVs in the supernatant, as determined by the [^{14}C]CHE lipid marker, was always > 95%. Given that membrane-membrane contact can be inhibited by surface grafted PEG polymer at 1 – 2 mol% [203], fusion-mediated lipid transfer may be sterically hindered in this case where PEG surface density was elevated (> 10 mol%). Other possible mechanisms whereby PE-PEG can be transferred into MLVs include direct transfer of the PE-PEG monomer or transfer through the formation of intermediate PE-PEG micelles or oligomers after desorption of the PE-PEG monomers from the LUVs bilayer. The MLVs in the in vitro PEG-lipid transfer assay act as a lipid pool, which provide a large surface area to accept the PEG-lipids as they are desorbing from liposomes, similar to the large membrane surface from the vascular endothelium and other cellular components in the bloodstream. Another potential PEG-lipid acceptor in vivo is the lipoprotein family which is known to be involved in lipid transfer [290, 302]. The data presented here showed that the relative amount of DMPE-PEG 2000 in plasma was higher than that for the liposomal lipids which were

eliminated rapidly from the bloodstream. It is possible that DMPE-PEG 2000 was transferred to the lipoproteins, resulting in longer circulation lifetimes.

The results from the in vivo studies clearly demonstrated that the negatively charged PS liposomes containing DMPE-PEG or DPPE-PEG were eliminated from the bloodstream more rapidly than the neutral DSPC/CHOL liposomes containing either of these PE-PEG. It is well documented that the plasma removal of PS containing liposomes is very rapid (> 90% of injected dose removed within minutes) due in part to PS-mediated plasma protein interactions with the liposome surface and subsequent MPS recognition. As DMPE-PEG and DPPE-PEG exchanged out of the PS liposomes, PS would be exposed to the high binding affinity plasma proteins. It is likely that the interaction between the high affinity plasma proteins and the PS containing liposomes (with a free energy of association of about –9 kcal/mol for Factor X [303]) may provide additional energy to overcome the energy barrier for PE-PEG desorption from PS membranes. This may result in further PE-PEG loss from PS liposomes and more PS being exposed to plasma proteins for interactions.

In designing "transiently stabilized" liposomes, it is important to balance the necessity of achieving circulation longevity for target accumulation, and the importance of transforming the liposomes to a form that is capable of initiating site-specific reactions such as drug release, cell fusion or selective access of proteins. For pegylated liposomes, this balance is dependent on the rate at which the grafted PEG desorb from the liposomes. In this chapter, the ability to control the membrane surface reactivity of PS containing liposomes in a time-dependent fashion by using exchangeable PEG-lipids of various acyl

chain lengths has been demonstrated. For the intended application pursued in this thesis, PS containing liposomes must also achieve tumor site specific accumulation and localization of to minimize the potential for disseminated intravascular coagulation. In the next chapter, studies were conducted to address the targeting of these liposomes to a marker of the tumor vascular endothelium through the use of monoclonal antibodies.

Chapter 5   Targeting of antibody conjugated, phosphatidylserine-containing liposomes to vascular cell adhesion molecule 1 for controlled thrombogenesis*

5.1. Introduction

In the development of a thrombogenic PS containing liposome for tumor specific thrombogenesis, the PS containing liposomes must selectively localize within the tumor vasculature in order to induce blood coagulation in a manner that will be of therapeutic utility. One way to achieve this selectivity is to exploit altered features of the vascular endothelium within tumors. In solid tumors, the vasculature is often marked by antigens that are over-expressed and/or selectively expressed in the endothelial lining [52, 304]. One such antigen is the vascular cell adhesion molecule 1 or VCAM-1.

VCAM-1 is present on the vascular endothelia of many human malignant tumors including neuroblastoma [305], renal carcinoma [306], non small cell lung carcinoma [307], Hogkin's disease [305], angiosarcoma [308], and colon carcinoma [309]. In normal human tissues, weak and patchy VCAM-1 expression is confined to a few vessels in the thyroid, thymus and kidney [308], and in the mouse to vessels in the heart and lung [1, 310]. Other investigators have demonstrated the use of VCAM-1 as a target molecule to evaluate the in vivo targeting and anti-tumor activity of the truncated tissue factor-based "coaguligand" [1]. Thus, VCAM-1 serves as an appropriate target molecule for the initial characterization of the targeting capabilities of the thrombogenic PS liposomes, which would be conjugated with an anti-VCAM-1 Ab to the distal end of the PEG polymer on the liposome surface. Human VCAM-1 is a 100 – 110 kDa transmembrane glycoprotein with seven C2-type

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* **Data including Figures 5.2 to 5.6 have been submitted as a manuscript to Biochim. Biophys. Acta.**

immunoglobulin domains, with VLA-4 as the principal ligand [311]. Its expression can be induced by inflammatory cytokines such as IL-1α, IL-4, IL-13 and tumor necrosis factor alpha [312, 313]. Its role in vivo is thought to regulate leukocyte migration across blood vessel walls and provide attachment points for developing endothelium during angiogenesis [314].

This chapter addresses the approach to target the thrombogenic PS liposomes against VCAM-1, with both in vitro and in vivo characterization of such liposome formulations. The aim of the in vitro studies presented here is to demonstrate the ability of the target bound anti-VCAM-1 Ab conjugated thrombogenic PS liposomes to catalyze the blood coagulation reactions. First, binding of such liposomes to target was evaluated using two in vitro model systems, an ELISA plate based assay and a cell based assay using IL-1α treated HUVECs. Next, the clotting activity of the target bound thrombogenic PS liposomes was evaluated by the in vitro clotting assay. The results from these in vitro studies provided proof-of-concept evidence to support further characterization of the formulation in animal models. Since selective accumulation and localization of the thrombogenic PS liposomes within the tumor vasculature is crucial to the therapeutic value of such liposomes in anti-tumor therapy, the aim of the in vivo studies was to evaluate delivery of the anti-VCAM-1 Ab conjugated thrombogenic PS liposomes to the tumor vasculature in tumor bearing mice. Attributes important to assuring in vivo anti-tumor activity are discussed.

5.2. Materials and methods

5.2.1. Materials

All lipids were purchased from Avanti Polar Lipids (Alabaster, AL) except for

DSPE-PEG 2000-MAL which was from Shearwater Polymers Inc. (Huntsville, AL). The

[^{3}H]CHE was obtained from NEN Life Sciences Products (Oakville, ON, Canada).

Cholesterol, DTT, ellagic acid and Sephadex G-50 size exclusion gel were purchased from

Sigma. Recombinant human IL-1α was purchased from R&D Systems. Mouse anti-human

VCAM-1 Ab, labeled with FITC, was obtained from Calbiochem. Cross reactivity of Ab

has been tested with direct ELISA. Humanized mouse monoclonal anti-HER2 Ab

(Herceptin) and normal rat IgG (Caltag Laboratories, Burlingame, CA) were used as

irrelevant Ab. Bio-Gel A-15m size exclusion gel was obtained from Bio-Rad (Mississauga,

ON, Canada). N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) was purchased from

Pierce.

5.2.2. Tumor and hybridoma cell lines

The human colon carcinoma cell line LS180 was purchased from the American

Tissue Culture Collection (ATCC, Manassas, VA) and maintained in Minimum Essential

Medium with Earle's salt and non-essential amino acids (Stem Cell Technologies,

Vancouver, BC) plus 10% fetal bovine serum.

The hybridoma cell line VIII-6G10 (produces mouse IgG$_1$ against human VCAM-1)

was purchased from the American Tissue Culture Collection (Manassas, VA), and was sent

to Antibody Solutions (Palo Alto, CA) for production in bioreactors. The mouse anti-human

VCAM-1 Ab was purified by affinity chromatography, and was supplied in PBS with a concentration of 2.2 mg/mL. This Ab was kept in aliquots at 4 °C until use.

The hybridoma cell line M/K-2.7 (produces rat IgG_1 against murine VCAM-1) was a generous gift from Dr. Sophia Ran (Southwestern Medical Center, University of Texas). This cell line was maintained in RPMI 1640 medium (Stem Cell Technologies, Vancouver, BC) supplemented with 1% L-glutamine, 0.05 mM β-mercaptoethanol, and 10% fetal bovine serum. The rat anti-mouse VCAM-1 Ab was produced by the method of ascites formation in mice [315, 316]. All of the in vivo studies were completed using protocols approved by the University of British Columbia's Animal Care Committee. These studies met or exceeded the current guidelines of the Canadian Council of Animal Care. Briefly, female SCID/Rag 2 mice (BC Cancer Agency, Vancouver, BC) were primed with 0.5 mL pristane (Sigma, St Louis, MO) injected into the peritoneal cavity. After 10 days, 2×10^6 M/K-2.7 hybridoma cells were injected into the peritoneal cavity. Ascites fluid was collected from each mouse with a 3-mL syringe, and was incubated at room temperature for 30 min followed by an incubation at 4 °C overnight. The fluid was subsequently centrifuged at $3000 \times g$ for 10 min, and the supernatant was isolated and pooled. The rat anti-mouse VCAM-1 Ab was then isolated from the supernatant according to the procedure in the ImmunoPure (Protein G) IgG Purification Kit from Pierce (Rockford, IL). This Ab was kept in aliquots at 4 °C until use, and the concentrations of the aliquots were in the range of 6 to 10 mg/mL (in PBS).

5.2.3. Preparation of various liposomes

The preparation of LUVs followed the procedure described in Section 2.2.2. The resulting mean LUVs diameter was 100 – 120 nm as determined by quasi-elastic light scattering using the Nicomp submicron particle sizer model 370/270. The MLVs were prepared with Egg PC/Chol (55:45 mole ratio) as described in Section 4.2.2., and they were used as a membrane sink to promote PEG-lipid exchange.

5.2.4. Conjugation of thiolated anti-VCAM-1 Ab to liposomes

The anti-VCAM-1 Ab and the irrelevant Ab were conjugated to the liposomes according to established procedures described as follows [233, 317]. SPDP was dissolved in ethanol, and 80 μL of this solution (12.5 mM) was diluted with 920 μL HBS to give a final concentration of 1 nmol/μL. The Ab (3 – 5 mg) was reacted with SPDP in a 1:5 Ab to SPDP mole ratio for 25 min at room temperature, and was subsequently passed down a Sephadex G-50 column equilibrated with sodium acetate buffer (100 mM sodium acetate/50 mM NaCl, pH 4.5). Fractions containing the Ab with absorbance at 280 nm greater than 1 were pooled, and were added to DTT powder to give a concentration of 25 mM DTT. This mixture was incubated at room temperature for 25 min. The thiolated Ab was then isolated by size exclusion chromatography with a Sephadex G-50 column equilibrated with HBS, and was immediately added to liposomes containing 1 mol% DSPE-PEG 2000-MAL (10 mM final concentration) at various initial Ab to lipid ratios. The extent of Ab modification was determined by estimating the Ab concentration from its absorbance at 280 nm (molar extinction coefficient at 280 nm = 9.52×10^4) and the 2-thiopyridone concentration from its

absorbance at 343 nm (molar extinction coefficient at 343 nm = 8080) with an aliquot of the reaction mixture prior to size exclusion chromatography.

The conjugation reaction was carried out at room temperature for 18 h. The mixture was passed down a Bio-Gel A-15m column equilibrated with HBS at the end of reaction to separate the unreacted Ab from the Ab-conjugated liposomes. The concentration of the Ab-conjugated liposomes post column was determined by liquid scintillation counting from aliquots mixed with 5.0 mL Pico-fluor 15 scintillation fluid (Packard Biosciences, The Netherlands) and counted with a Packard scintillation counter model 1900 TR. The amount of Ab conjugated to the liposomes was determined using the Pierce Micro BCA protein assay kit in the presence of 0.5% Triton X-100 to disrupt the liposomes (0.3 μmol lipid used for assay). The mean resulting diameter of the Ab conjugated liposomes was determined to be 120 – 130 nm as described previously in Section 2.2.2.

5.2.5. Liposome binding to surface bound VCAM-1

This assay was based on a previously described procedure [318]. Briefly, VCAM-1 (1 μg/well) was coated onto Maxisorp 96-well plate (Nalge Nunc International, Rochester, NY) overnight at 4 °C. The plate was then blocked with 10% fetal bovine serum for 1 h at 37 °C. Liposomes were then added to the wells, and were incubated for 2 h at 37 °C. The wells were washed three times with PBS, followed by the addition of 5% Triton X-100. After a 15-min incubation at 37 °C, the content from each well was transferred to a scintillation vial with three rinses of PBS, and the radioactivity was determined by liquid

scintillation counting. The study was repeated once due to limited availability of anti-VCAM-1 Ab.

5.2.6. Induction and assessment of VCAM-1 expression on HUVECs by IL-1α

HUVECs were purchased from Clonetics (Walkersville, MD), and were cultured in EGM-2 medium supplemented with BulletKit (Clonetics, Walkersville, MD). Cells in the 3rd to 5th passages were used, and all studies were completed with cells at ~ 70% confluence. HUVECs were stimulated with 6.25 ng/mL IL-1α for 4 h at 37 °C. At the end of incubation, HUVECs were washed with PBS/0.1% BSA twice, and were incubated with PBS/2.5 mM EDTA for 5 min to dislodge cells. Cells (2×10^5/sample) were pelleted and resuspended in 0.1 mL PBS/10% human serum containing the FITC-labeled mouse anti-VCAM-1 Ab and incubated on ice for 30 min. Cells were then washed twice with ice cold PBS/0.1% BSA, and resuspended in 0.4 mL ice cold PBS/0.1% BSA/propidium iodide for flow cytometry analysis using an EPICS Elite ESP flow cytometer (Beckman-Coulter, Miami, FL) equipped with an Enterprise 621 laser (Coherrent, Santa Clara, CA) set to measure fluorescence of FITC. The flow cytometer scale was calibrated with Quantum 24 or 25 beads (Flow Cytometry Standards, San Juan, Puerto Rico), and 10^4 events were collected for each sample. Data were analyzed with the ExpoMFA software. A previously described protocol was followed to quantify the number of Ab binding sites, using Simply Cellular beads (Flow Cytometry Standards, San Juan, Puerto Rico) as a standard to determine the fluorescence intensity per Ab [319].

5.2.7. Liposome binding to IL-1α stimulated HUVECs

HUVECs were stimulated with IL-1α as described above. Subsequently, 1×10^6 cells were placed in polypropylene tubes, and [^{3}H]CHE labeled liposomes were added to achieve a final liposomal lipid concentration of 2 mM. Following an incubation at 4 °C for 4 h, the cells were centrifuged at 300 × g for 5 min, and washed three times with PBS. Cells were solubilized with 0.9% Triton X-100 in PBS, and the contents were transferred to a scintillation vial for liquid scintillation counting. The experiment was repeated once due to limited availability of the anti-VCAM-1 Ab.

5.2.8. In vitro clotting time assay

This assay was based on a procedure previously utilized for assessing procoagulant activity of PS-containing liposomes [289, 320]. Human plasma was collected into citrated tubes (9 mL blood to 1 mL citrate) from four normal, healthy volunteers, and pooled to make one batch of plasma for studies in this chapter. Briefly, Maxisorp Nunc-Immuno tubes were coated with VCAM-1 and pre-blocked with 10% serum. Liposomes (250 nmol in 0.5 mL HBS) were added and incubated for 2 h at 37 °C. The tubes were then washed three times with PBS. Human citrated plasma (125 µL), 10^{-5} M ellagic acid (125 µL) and HBS (125 µL) were then added to the tubes, which were then incubated for 2 min at 37 °C. Calcium (8.75 mM, final concentration) was added to initiate the clotting reaction, and the mixture (0.5 mL total) was gently shaken. The time at which the mixture turned into a viscous gel was recorded, and noted as the time required for the clotting reaction to be completed. As indicated, MLVs were added to the tubes and incubated for 15 min, prior to

the addition of the plasma mixture. The amount of lipid remained associated with the tube was assayed as described previously.

5.2.9. Immunohistological analysis of VCAM-1 expression in LS180 tumor bearing mice

All of the in vivo studies described in the following sections were completed using protocols approved by the University of British Columbia's Animal Care Committee. These studies met or exceeded the current guidelines of the Canadian Council of Animal Care. The mice used for establishing subcutaneous LS180 tumor models were female severe combined immune deficient mice (SCID) with a mutation in the Rag 2 gene that resulted in a complete block in lymphocyte development and minimal production of immunoglobulins or T-cell receptors. Female SCID/Rag 2 mice were inoculated unilaterally with 1×10^6 LS180 cells subcutaneously. Tumor size was measured with a caliper, and calculated with the following equation: tumor volume $(cm^3) = (L \times W^2)/2$ where L is the length and W is the width of the tumor. When the size of LS180 tumors reached $0.11 - 0.17 \ cm^3$, the tumors were removed from the mice, fixed in 10% buffered formalin for a maximum of 5 days, embedded in paraffin, and 5 µm sections on glass slides prepared (Wax-It, Aldergrove, BC). Slides were then de-paraffinized in two changes of xylene, then hydrated stepwise in 100%, 95% and 70% ethanol. Endogenous peroxidase activity was blocked by a 0.3% v/v solution of H_2O_2 in methanol. Slides were then washed with distilled water, followed by three changes of PBS. The next step was the incubation of the primary Ab (diluted with PBS) with the sections for 1 h. The primary Ab used was goat anti-mouse VCAM-1 (R&D systems, Minneapolis, MN). Tissues without the addition of the primary Ab were used as

negative controls. Blocking agents and secondary antibodies were from the Vectastain Elite

goat ABC kit (Vector Laboratories, Burlingame, CA), and the slides were processed

according to the procedure described in the kit. The slides were visualized with

ImmunoPure Metal Enhanced DAB Substrate kit (Pierce, Rockford, IL). Slides were

counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene and mounted with

Permount (Fisher Scientific).

5.2.10. Plasma removal and tissue distribution of Ab-conjugated PS containing liposomes in LS180 tumor bearing mice

Female SCID/Rag 2 mice were inoculated unilaterally with 1×10^6 LS180 cells

subcutaneously. When the LS180 tumor size reached $0.11 - 0.17$ cm^3, liposomes labeled

with traces of [^{3}H]CHE were injected via the lateral tail vein into $20 - 22$ g tumor bearing

SCID/Rag 2 mice. The total lipid dose was 50 mg/kg with an injection volume of 200 µL.

At various times after liposome injection (t = 0.25, 0.5 and 8 h), 25 µL blood was collected

into microcapillary tubes pre-rinsed with 200 mM EDTA solution by nicking the tail vein.

The blood was then added to 200 mM EDTA solution (200 µL) and centrifuged at $1000 \times g$

for 10 min. Aliquots of the supernatant were counting directly in 5 mL Pico-fluor 40

scintillation fluid. At 1, 4 and 24 h, three mice were terminated by CO_2 asphyxiation. Blood

was collected by cardiac puncture, and was placed into EDTA-coated microtainer collection

tubes. After centrifuging blood samples for 15 min at $1000 \times g$, plasma was isolated and

visually showed no hemolysis. Aliquots (100 µL) of the plasma obtained were counted

directly in 5.0 mL scintillation fluid. The limit of lipid quantitation was 1 µg per mL

plasma. The plasma data collected were used to calculate the $AUC_{0-24 h}$ values for the

various liposomes using the software WinNonlin (version 1.5) as described before. Tumor, liver, spleen, lung, heart and kidney were also harvested from each group of mice to determine liposome accumulation in these tissues at 1, 4 and 24 h post liposome injection. The amounts of lipid in these tissues were determined according to the procedure described in Section 3.2.3. Tumor samples harvested from the mice were also fixed in 10% buffered formalin for immunohistological studies to detect the localization of the Ab-conjugated DOPS liposomes in the LS180 tumor. Tumor samples were processed and slides prepared as described in Section 5.2.8 using a donkey anti-rat IgG Ab (Jackson Immunoresearch Laboratories Inc., West Grove, PA) to detect the rat Ab conjugated to the liposomes.

5.2.11. Statistical analysis

One-way ANOVA was performed to detect differences among different groups, with Newman-Keuls test as post-hoc analysis. A p-value of < 0.05 was considered significant.

5.3. Results

5.3.1. Conjugation of anti-VCAM-1 Ab to PS containing liposomes

The anti-VCAM-1 Ab was conjugated to PS containing liposomes based on a well established procedure [233, 317], and Figure 5.1 (P. 160) shows the schematic illustration of the coupling chemistry. The coupling efficiencies (Figure 5.2, in parentheses, P. 161) for PS liposomes containing 2 mol% DSPE-PEG 2000 were between 55-61%, which is comparable to other procedures that employed a thioether linkage under similar reaction conditions [233, 234, 317, 321]. However, an increase in DSPE-PEG 2000 level to 14 mol% substantially decreased the coupling efficiencies. When the initial Ab to lipid ratio was 45 µg Ab/µmol lipid, the coupling efficiencies was 51%, but when the initial ratio increased to 60 µg Ab/µmol lipid, the measured levels of coupled Ab did not increase and therefore the efficiency of the reaction was less than 40%. An initial ratio of 45 µg Ab/µmol lipid produced similar final Ab to lipid ratios for PS liposomes containing 2 or 14 mol% DSPE-PEG 2000 (23 − 27 µg Ab/µmol lipid). Thus, this ratio was used for subsequent conjugation reactions to produce liposomes that had similar Ab to lipid ratios for the evaluation of target binding. At these final Ab to lipid ratios of 23 − 27 µg Ab/µmol lipid, it is estimated to have 15 − 17 copies of Ab per liposome, assuming that one µmol of 100-nm liposomes contains 6.23×10^{12} vesicles [285] .

5.3.2. Binding of anti-VCAM-1 Ab-conjugated PS liposomes to target VCAM-1

Binding of the Ab-conjugated PS liposomes containing 2 or 14 mol% DSPE-PEG 2000 to VCAM-1, as evaluated by the ELISA plate-based assay, was comparable when
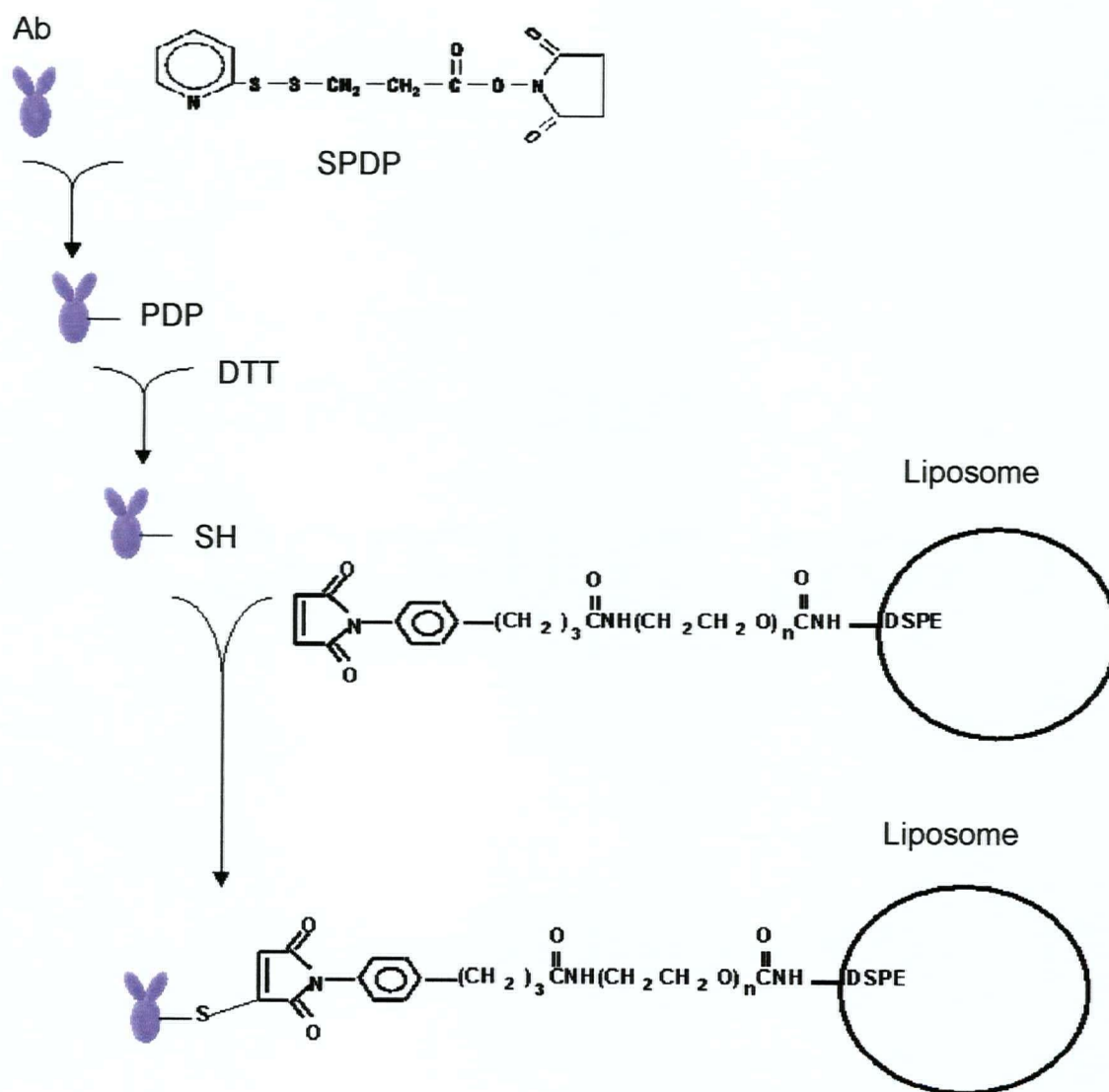
Figure 5.1. An illustration of the coupling chemistry involved in the conjugation of the Ab to the PS containing liposomes. The Ab was first modified with SPDP which reacts with the primary amines on the Ab. The free thiol group on the Ab was generated after DTT reduction, and would react with the maleimide group on the distal end of the PEG linked to DSPE. The reactive lipid for the conjugation was DSPE-PEG 2000-MAL, and the amount was 1 mol% in the liposomes.
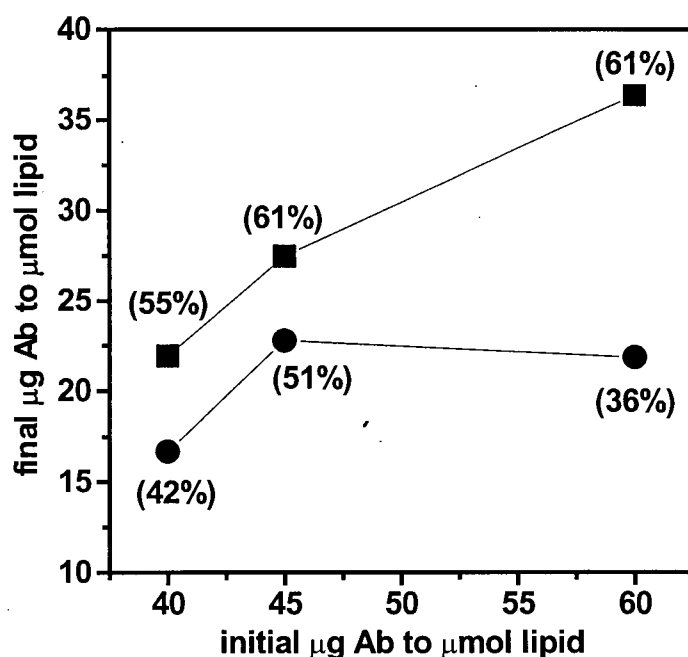
Figure. 5.2. Coupling efficiency of anti-VCAM-1 Ab to PS containing liposomes. The anti-VCAM-1 Ab was conjugated to the following liposome compositions using maleimide chemistry as described in Section 5.2.4: (■) DOPS/DSPC/CHOL/DSPE-PEG2000/DSPE-PEG 2000-MAL 10:42:45:2:1 (mole ratio) or (●) DOPS/DSPC/CHOL/DSPE-PEG2000/DSPE-PEG 2000-MAL 10:30:45:14:1 (mole ratio). The percent efficiencies for various initial Ab to lipid ratios are shown in parentheses, and were calculated as follows: % = final Ab to lipid ratio / initial Ab to lipid ratio × 100%.

using lipid concentrations up to 0.5 mM lipid added (Figure 5.3, P. 163). The amount of lipid recovered for the Ab-conjugated PS liposomes with 2 mol% DSPE-PEG 2000 reached maximum binding levels of 3.2 nmol per well, representing a binding efficiency of ~ 1.6% at 2 mM lipid added. However, at lower lipid concentrations (e.g. 0.2 mM), the binding efficiency was increased to ~ 11%. The amount of binding for liposomes prepared with 14 mol% DSPE-PEG 2000 added at concentrations of > 1 mM was less than that observed for the formulation prepared with 2 mol% DSPE-PEG 2000. The amount of lipid recovered for Ab-conjugated PS liposomes with 2 mol% DSPE-PEG 2000 at 2.0 mM lipid was 6- and 11-fold higher than those recovered for PS liposomes conjugated with an irrelevant Ab or with no Ab, respectively. For Ab-conjugated PS liposomes with 14 mol% DSPE-PEG 2000, the amount of lipid recovered was 9- and 20-fold higher, respectively. The in vitro binding assay described above demonstrated that in a cell-free system, specificity could be achieved for an anti-VCAM-1 Ab conjugated liposomes, and that non-specific binding due to the presence of PS was not substantially higher than that observed for liposomes that lack PS.

The binding of liposomes to VCAM-1 was also evaluated with HUVECs stimulated with 6.25 ng/mL IL-1α for 4 h, which has been optimized based on IL-1α concentration and duration of stimulation (Figure 5.4, P. 165). Flow cytometric analysis indicated that approximately 32% of the HUVECs were stained positive with an FITC-labeled anti-VCAM-1 Ab, and it was estimated that these cells have 1×10^6 antigen binding sites per cell. It has been suggested that target molecules on cells should have substantial levels of over-expression, in the range of $10^5 - 10^6$ copies per cell, to achieve effective targeting [221]. Although not all
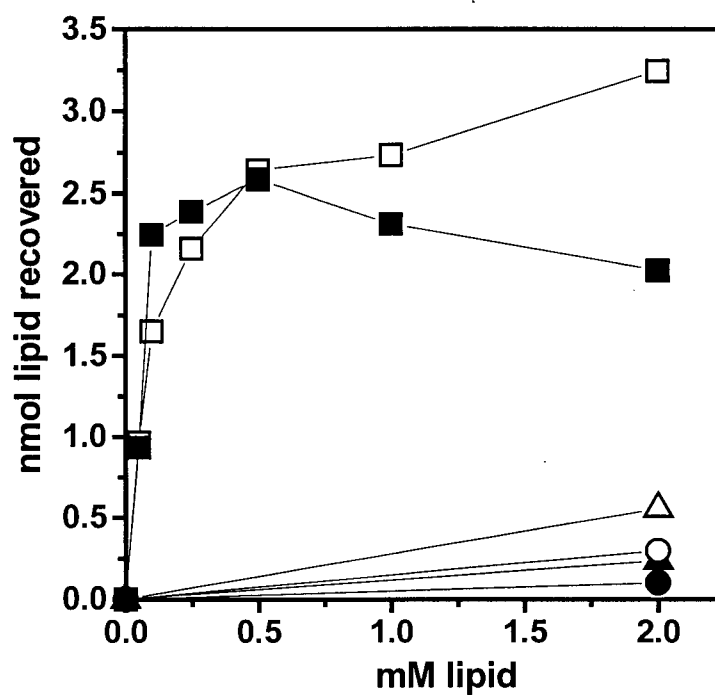
Figure 5.3. Binding of anti-VCAM-1 Ab-conjugated PS liposomes (squares), irrelevant Ab conjugated PS liposomes (triangles) and PS liposomes without Ab (circles) containing 2 mol% (open symbols) or 14 mol% (solid symbols) DSPE-PEG 2000 to human VCAM-1 coated ELISA plates. Liposomes (100 µL) at various concentrations were added to the ELISA plates pre-blocked with 10% fetal bovine serum, and were incubated at 37 °C for 2 h. Each data point represents averages of two experiments.

of the cells exhibited this level of expression in this study, it can be argued that VCAM-1 is a good target candidate since targeting of the Ab-conjugated thrombogenic PS liposomes to the endothelial cells within the tumor vasculature will not require binding to all endothelial cells.

The stimulated HUVECs were incubated with the pegylated PS liposomes with or without conjugated anti-VCAM-1 Ab for 4 h. Based on other studies, maximum binding of Ab-conjugated liposomes was observed after this incubation period [247, 322]; therefore, the binding experiments here were carried out for 4 h. In the presence of the conjugated Ab, 4.8 and 4.2 nmol lipid were associated with 1×10^6 stimulated HUVECs for PS liposomes containing 2 and 14 mol% DSPE-PEG 2000, respectively. By comparing binding to HUVECs that were stimulated with IL-1α to that obtained with HUVECs that were not exposed to IL-1α and which did not express VCAM-1, the relative increases in cell associated lipid for PS liposomes containing 2 and 14 mol% DSPE-PEG 2000 were calculated to be 180% and 380%, respectively (Figure 5.4, P. 165). The binding of the anti-VCAM-1 Ab-conjugated PS liposome to IL-1α treated HUVECs was comparable to other studies which targeted liposomes to cell adhesion molecules such as ICAM-1 and E-selectin, where liposomes conjugated with Ab provided 5- to 12-fold increases in cell associated lipids over those without Ab [224, 322, 323]. Although the presence of 14 mol% DSPE-PEG 2000 compromised the coupling efficiency of the Ab to the reactive group of PS liposomes (Figure 5.2, P. 161), this level of PEG-lipid did not substantially interfere with the binding of the Ab-conjugated PS liposomes to VCAM-1. In fact, the data shown in Figure
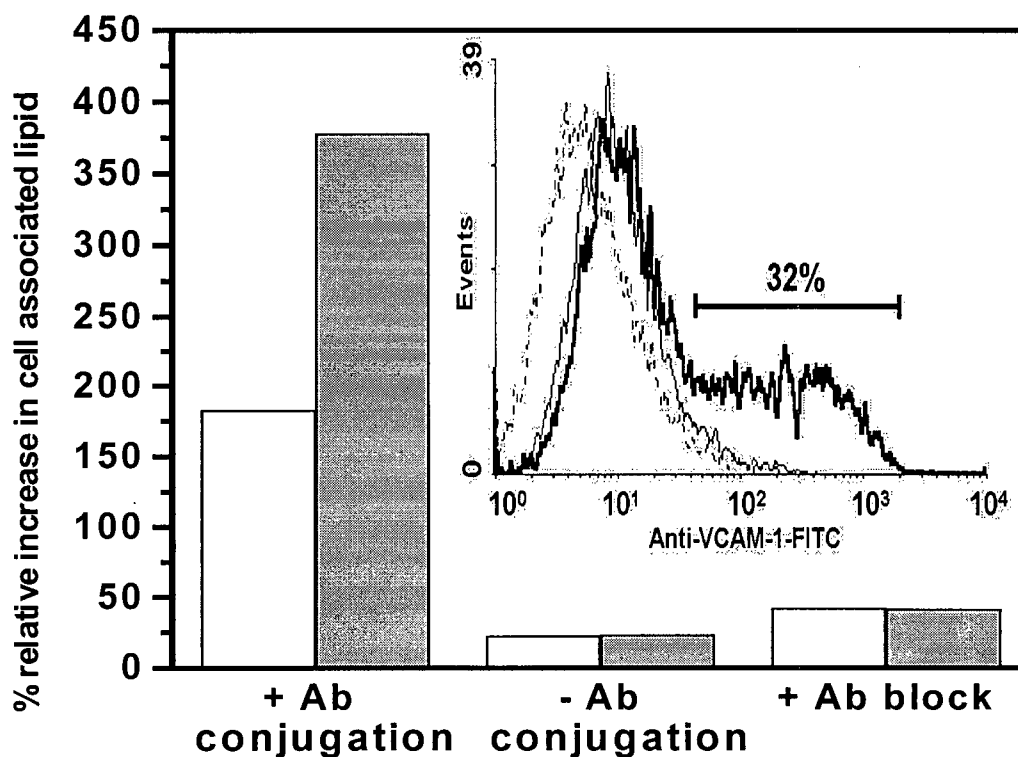
Figure 5.4. Binding of PS liposomes with or without Ab conjugation containing 2 mol% (white bar) or 14 mol% (gray bar) DSPE-PEG 2000 to HUVEC at 4 °C for 4 h. HUVEC after stimulation with IL-1α were also blocked with free anti-VCAM-1 Ab for 1 h before incubation with Ab-conjugated PS liposomes containing 2 or 14 mol% DSPE-PEG 2000. The percent relative increase in cell associated lipid was calculated as follows: % relative increase = $(L_{stim} - L_{unstim}) / L_{unstim} \times 100\%$, where L_{stim} represents the amount of lipid per 10^6 cells for IL-1α stimulated HUVEC and L_{unstim} represents the amount of lipid per 10^6 cells for unstimulated HUVEC. Two independent experiments were done for each group. Representative FACS profile showing VCAM-1 expression on HUVEC post IL-1α stimulation at 37 °C for 4 h is presented in inset. Dotted line, unstimulated HUVEC stained with IgG_1-FITC; thin solid line, unstimulated HUVEC stained with anti-VCAM-1-FITC; thick solid line, stimulated HUVEC stained with anti-VCAM-1-FITC.

5.4 (P. 165) suggest that the Ab-conjugated PS liposomes with 14 mol% DSPE-PEG 2000 bound more efficiently than the Ab-conjugated PS liposomes with 2 mol% DSPE-PEG 2000. To demonstrate the specificity of the Ab-conjugated pegylated PS liposome for the target VCAM-1, IL-1α treated HUVECs were pre-incubated with free anti-VCAM-1 Ab prior to the addition of liposomes. Under these conditions, binding values were reduced to those observed with liposomes which did not have a conjugated Ab, demonstrating that the binding reaction was specific when using the anti-VCAM-1 Ab conjugated liposomes.

5.3.3. Assessment of the clotting activity of Ab-conjugated PS liposomes post binding

In designing a thrombogenic PS liposome formulation that is targeted to the tumor vasculature for triggering tumor specific thrombosis, it is important for the PS liposome carrier to maintain its thrombogenicity after binding to its target. Therefore, after demonstrating target specific binding, we evaluated the clotting activity of the bound Ab-conjugated PS liposomes in ELISA tubes after removal of unbound liposomes, where the lipid concentration used was one that reached maximum binding level. As shown in Figure 5.5 (P. 167), all PS liposomes without Ab conjugation, which exhibited negligible binding to the surface-bound VCAM-1, showed very low clotting activities. The amounts of lipid recovered for Ab-conjugated PS liposomes containing 2 and 14 mol% DSPE-PEG 2000 were comparable, which were 6.6 nmol and 5.0 nmol per tube, respectively. When these amounts of the specified liposomes were added directly to the clotting assay (i.e., liposomes were not bound to a surface) the clotting activities measured were comparable to those measured with the bound liposomes (compare Figure 5.5, bars 1 and 2 to bars 6 and 7, P. 167). Importantly, only the Ab-conjugated PS liposomes containing 2 mol% DSPE-PEG
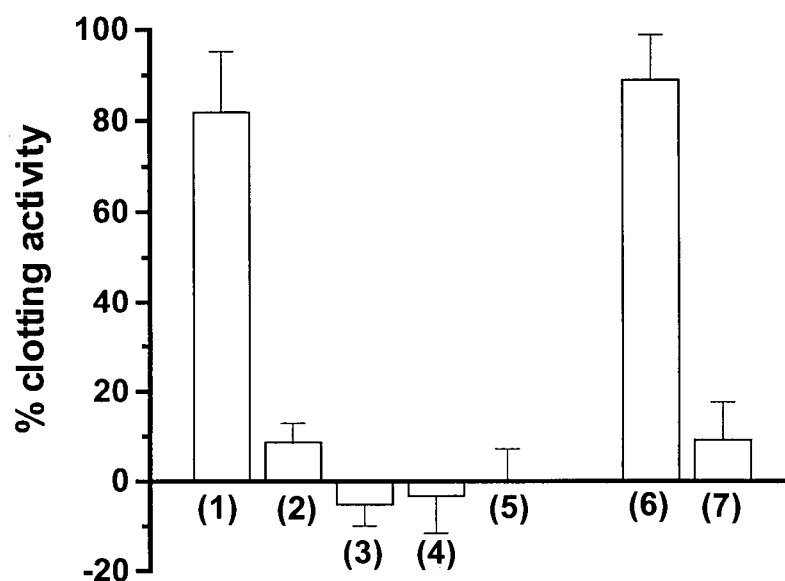
Figure 5.5. Clotting activity of anti-VCAM-1 Ab-conjugated PS liposomes post binding. Various liposomes were allowed to bind to VCAM-1 coated ELISA tubes for 2 h, and the clotting activities were then assayed. The % clotting activity was calculated as follows: % = ($t_{blank} - t_{test}$) / ($t_{blank} - t_{PS}$) × 100%, where t_{blank}, t_{PS} and t_{test} represent the clotting times for HBS, 10% PS liposomes in solution and test liposomes, respectively. The various liposomes tested following addition to the VCAM-1 coated ELISA tubes include: (1) PS 10%/PEG 2000 2%/VCAM-1 Ab, (2) PS 10%/PEG 2000 14%/VCAM-1 Ab, (3) PS 10%/PEG 2000 2%, (4) PS 10%/PEG 2000 14%, (5) PS 10%, all with DSPC/CHOL as bulk lipids. Importantly, all tubes were washed three times prior to addition of plasma. As controls, PS 10%/PEG 2000 2%/VCAM-1 Ab (6) and PS 10%/PEG 2000 14%/VCAM-1 Ab (7) were added to the plasma in same amounts as those that were bound to the ELISA tubes to demonstrate the clotting activity of these liposomes free in solution. Triplicates were done for each group, and the error bars represent S.D.

2000 exhibited substantial clotting activity (~ 80%) when bound to the ELISA tubes. Tubes with bound Ab-conjugated PS liposomes with 14 mol% DSPE-PEG 2000 gave < 10% clotting activity. These results are consistent with those observed in previous chapters, which demonstrated that elevated levels of a non-exchangeable PEG-lipid inhibits PS clotting activity [289].

5.3.4. Controllable surface thrombogenicity of VCAM-1 bound PS liposomes using exchangeable PEG-lipids

The exchangeable DMPE-PEG 2000 was used to demonstrate that loss of PEG from the PS liposomes is crucial to the promotion of thrombi formation for those Ab-targeted liposomes prepared with elevated levels of PEG-lipids [320]. The clotting activities of the Ab-conjugated PS liposomes prepared with maximum levels of PEG-lipid incorporation (9 mol% for DMPE-PEG 2000 or 14 mol% for DSPE-PEG 2000) were measured after binding and following an incubation with MLVs to serve as a "sink" for the PEG-lipids (Figure 5.6, P.169). Both anti-VCAM-1 Ab-conjugated PS liposomes containing 9 mol% DMPE-PEG 2000 or 14 mol% DSPE-PEG 2000 exhibited similar levels of bound lipid following an incubation with the VCAM-1 coated ELISA tubes, with 4.8 and 5.0 nmol lipid per tube, respectively. The levels of bound lipid recovered were comparable under conditions where MLVs were added. This indicated that MLVs did not displace the Ab-conjugated PS liposomes from the binding sites. The Ab-conjugated PS liposomes protected by DMPE-PEG 2000 recovered approximately 97% clotting activity upon incubation with MLVs (sample 1), which was substantially higher than that observed from those same liposomes but in the absence of an MLVs incubation (sample 2). The Ab-conjugated PS liposomes protected by the non-exchangeable DSPE-PEG 2000 exhibited < 10% clotting activity under
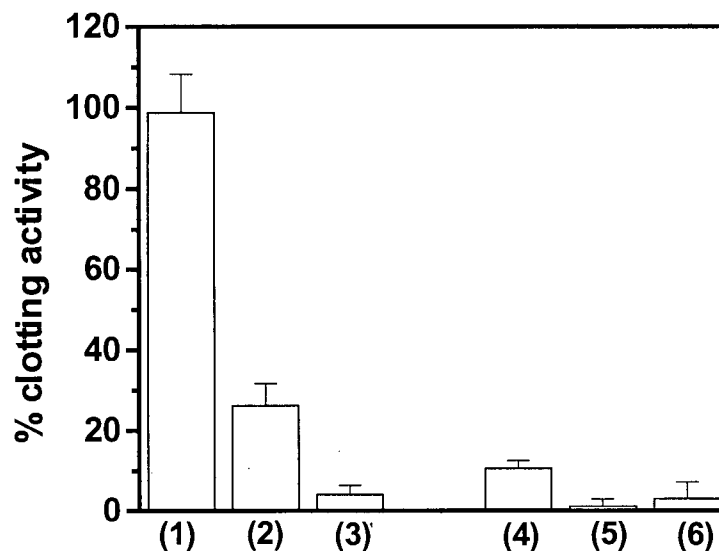
Figure 5.6. Restoration of clotting activity upon the exchange of DMPE-PEG 2000 out from the bound Ab-conjugated PS liposomes post binding. The non-exchangeable DSPE-PEG 2000 was included as a control. The test conditions were as described in Fig. 5.5 and the liposomes tested include: (1) PS 10%/DMPE-PEG 9%/VCAM-1 Ab + MLV incubation, (2) PS 10%/DMPE-PEG 9%/VCAM-1 Ab - MLV incubation, (3) PS 10%/DMPE-PEG 10% + MLV incubation, (4) PS 10%/DSPE-PEG 14%/VCAM-1 Ab + MLV incubation, (5) PS 10%/DSPE-PEG 14%/VCAM-1 Ab - MLV incubation, (6) PS 10%/DSPE-PEG 15% + MLV incubation. All liposomes had DSPC/CHOL as bulk lipids. The MLV were used as a "sink" to capture PEG-lipids which exchanged out of the various PS liposomes, and were added in 100-fold molar excess (0.6 μmol in 0.5 mL HBS). Triplicates were done for each liposome, with error bars representing S.D.

the same conditions regardless of whether the ELISA tubes were incubated with or without MLVs (sample 4 and 5). This clearly demonstrates that surface bound anti-VCAM-1 Ab-conjugated PS liposomes can promote thrombus formation and that this activity can be regulated through the use of exchangeable PEG-lipids.

5.3.5. In vivo targeting of the anti-VCAM-1 Ab conjugated thrombogenic PS liposomes

The studies described above demonstrated that the anti-VCAM-1 Ab conjugated PS liposomes can be selectively targeted to VCAM-1 using in vitro model systems such as VCAM-1 coated ELISA plates and IL-1α stimulated HUVECs. Importantly, these surface associated VCAM-1 targeted PS liposomes maintained their surface reactivity toward the blood coagulation factors. In moving toward the in vivo application of these VCAM-1 targeted thrombogenic PS liposomes, the first step is to evaluate the delivery of these liposomes to the in vivo target site, i.e., VCAM-1 expressing tumor blood vessels.

5.3.5.1. Immunohistological analysis of VCAM-1 expression in SCID/Rag 2 mice

The first step in assessing the tumor binding to VCAM-1 targeted liposomes was to identify a tumor model which exhibits strong expression of VCAM-1 for the evaluation of the targeting of the anti-VCAM-1 Ab conjugated PS liposomes in vivo. A number of tumor models which are well established within our lab have been screened for the expression of VCAM-1. Among the various models, xenograft of human colon carcinoma LS180 showed the strongest VCAM-1 immunoreactivity, which is consistent with a study that demonstrated strong expression of VCAM-1 in human colon carcinoma [309]. Thus, the LS180 tumor

model was used to evaluate the plasma removal and tumor accumulation of the anti-VCAM-1 Ab-conjugated PS liposomes. Figure 5.7 (P. 172) shows the results for VCAM-1 immunoreactivity in various organs of SCID/Rag 2 mice bearing LS180 tumors. Strong immunoreactivity was observed on the blood vessels throughout the LS180 tumors. In addition, major organs including brain, heart, kidney, liver, lung, spleen and muscles were harvested and analyzed immunohistochemically for VCAM-1 expression. Brain, spleen and muscle collected from SCID/Rag 2 mice showed negligible VCAM-1 staining, while heart and lung exhibited weak and patchy VCAM-1 immunoreactivity. These observations are consistent with previous studies that reported weak level of VCAM-1 expression in mouse heart and lung [1, 310]. However, in this study, liver and kidney of the SCID/Rag 2 mice also showed weak VCAM-1 immunoreactivity that was confined to a few vessels in these tissues.

5.3.5.2. Plasma removal and tissue distribution of anti-VCAM-1 Ab conjugated PS liposomes in SCID/Rag 2 mice bearing LS180 tumors

For the in vivo targeting of the thrombogenic PS liposomes, a rat anti-mouse VCAM-1 Ab was used, and the conjugation of this Ab followed the same procedure as described in Section 5.2.3. Given its in vivo stability and its extended circulation longevity, the DOPS liposomes containing 14 mol% DSPE-PEG 2000 are most likely to target and accumulate in tumor blood vessels. Thus, the 10 mol% DOPS liposomes containing 14 mol% DSPE-PEG 2000 and 1 mol% DSPE-PEG 2000-MAL was used in the conjugation of the anti-VCAM-1 Ab, and in the evaluation of the plasma removal and tumor accumulation
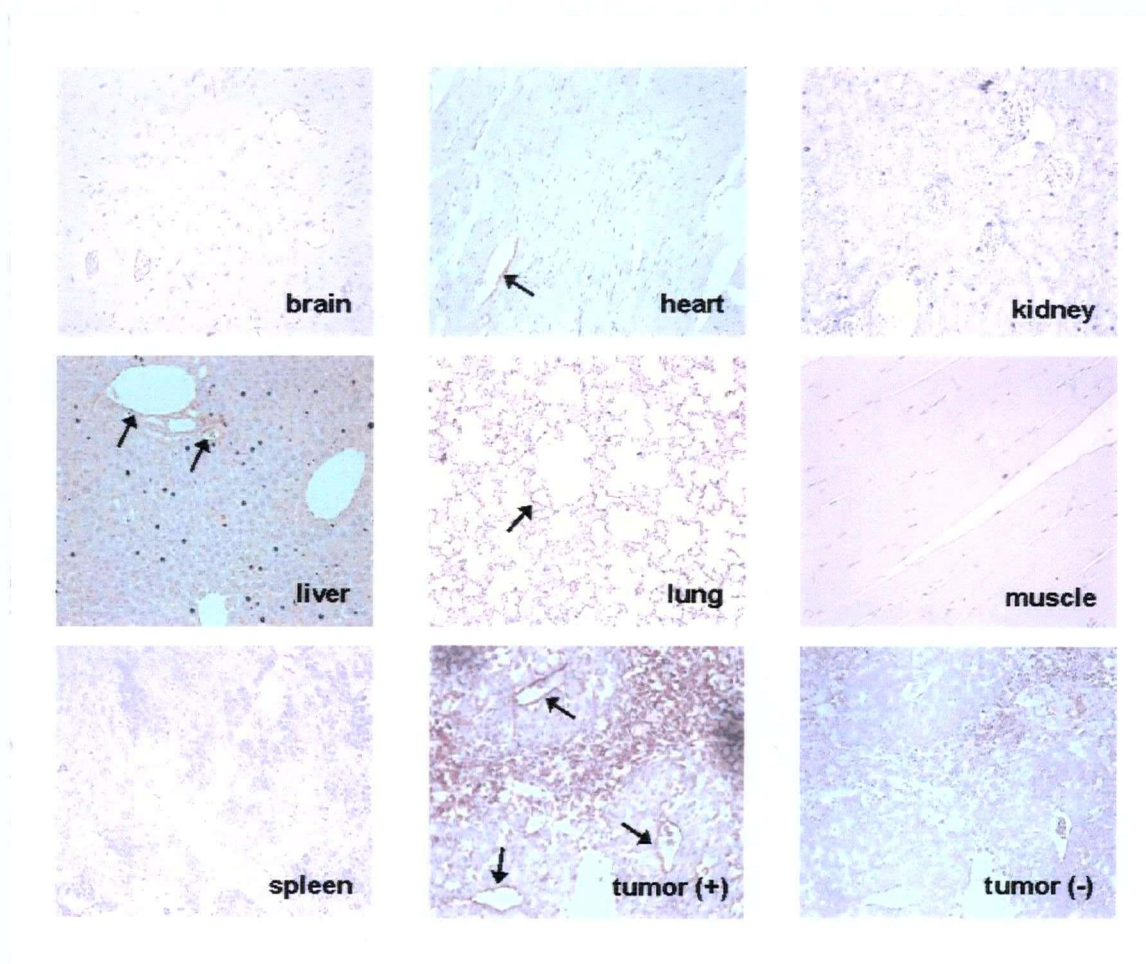
Figure 5.7. VCAM-1 like immunoreactivity in various organs of SCID/Rag 2 mice bearing LS180 tumors. Arrows in the diagram indicates area of immunoreactivity (magnification: × 200). Tumor (+) represents sections from the LS180 tumors stained with the primary anti-mouse VCAM-1 Ab, while tumor (-) represents tumor sections stained without the primary Ab as control. Other organ sections were also stained with or without the primary Ab. Four sections were obtained from each tissue sample, and were processed as described in Section 5.2.8. The size of LS180 tumors ranged from 0.11 to 0.17 cm^3. Three mice were used in the experiment.

of such liposomes. The amount of rat anti-mouse VCAM-1 Ab conjugated to the 10 mol%

DOPS liposomes containing 14 mol% DSPE-PEG 2000 was 22 μg Ab/μmol lipid, where 45

μg Ab/μmol lipid was used initially for the conjugation reaction. This is consistent with the

amount obtained from the conjugation of the mouse anti-human VCAM-1 Ab described

earlier (Figure 5.2, 161).

Plasma removal of the Ab-conjugated PS liposomes was studied in SCID/Rag 2 mice

with and without LS180 tumors (see Figure 5.8, P. 174). The removal of PS liposomes

without Ab conjugation was the slowest, with 20% and 14% of injected dose remaining in

plasma 24 h post injection and $AUC_{0-24\,h}$ values of 9.533 and 7.984 mg • h • mL^{-1} in mice

with and without tumors, respectively. Conjugation of the control rat Ab to PS liposomes

led to more rapid liposome removal. The percent injected dose remaining in plasma 24 h

post injection for these liposomes in mice with and without tumors were 4% and 3.8%,

respectively, and the $AUC_{0-24\,h}$ values for these liposomes in mice with or without tumors

were 3.760 and 3.654 mg • h • mL^{-1}, respectively. The anti-VCAM-1 Ab-conjugated PS

liposomes were eliminated more rapidly. Specifically, the plasma levels of the anti-VCAM-

1 Ab-conjugated PS liposomes at 4 and 8 h were an order of magnitude lower than those

obtained for PS liposomes with no Ab or with control conjugated rat Ab in mice with or

without tumors. Only 1% of injected dose remained in plasma 24 h post injection for anti-

VCAM-1 Ab-conjugated PS liposomes in mice with or without tumors. The $AUC_{0-24\,h}$

values for anti-VCAM-1 Ab-conjugated PS liposomes injected into mice with or without

tumors were 1.542 and 2.067 mg • h • mL^{-1}, respectively. In general, the presence of tumor

did not alter the in vivo behavior of the DOPS liposomes with no Ab, with control rat Ab or
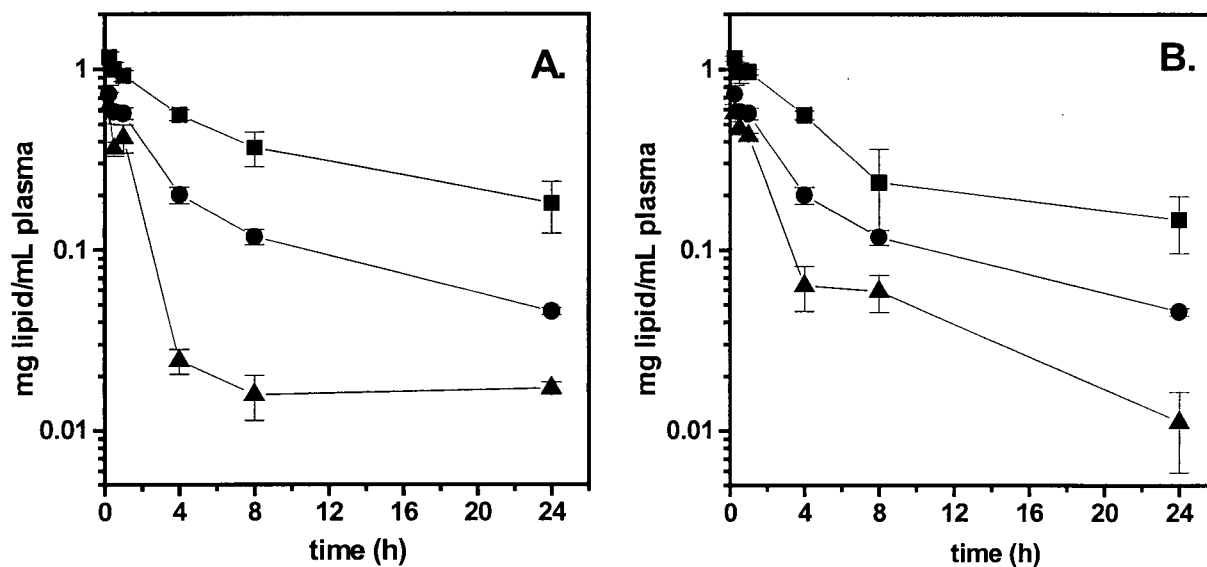
Figure 5.8. Plasma removal of 10 mol% DOPS/15 mol% DSPE-PEG 2000/DSPC/CHOL liposomes with no Ab (■), rat Ab control (●) or rat anti-mouse VCAM-1 Ab (▲) conjugated. Liposomes were injected as a single IV bolus dose of 50 mg/kg, and the mice weighed ~ 20 g. Panel A shows the plasma removal of the above liposomes in SCID/Rag 2 mice bearing LS180 tumors (tumor size 0.11 − 0.17 cm^3), while Panel B shows those in SCID/Rag 2 mice without LS180 tumors. Three mice were used for each data point, with the error bars representing S.D.

with anti-VCAM-1 Ab conjugated, as reflected by the observed $AUC_{0-24\,h}$ values for the various types of liposomes. However, the plasma lipid levels of DOPS liposomes conjugated with the anti-VCAM-1 Ab in mice with tumors were slightly yet significantly lower than those observed in mice without tumors at 4 and 8 h post injection (t-test, p < 0.05).

5.3.5.3. Liposome accumulation in tumors and various organs in SCID/Rag 2 mice

Liposome accumulation in LS180 tumors was studied over a 24-h time period, as shown in Figure 5.9 (P. 176). The amount of PS liposomes without Ab conjugation accumulated in tumor 24 h post injection was 0.105 ± 0.045 mg per gram of tumor, compared to 0.023 ± 0.015 mg accumulated per gram of tumor for PS liposomes conjugated with the anti-VCAM-1 Ab, and 0.051 ± 0.036 mg per gram of tumor for PS liposomes conjugated with the rat Ab control. However, the tumor lipid level for PS liposomes without Ab conjugation was not statistically significantly higher than that observed for the other two formulations (p = 0.125 for liposomes with anti-VCAM-1 Ab and p = 0.447 for liposomes with control rat Ab).

To determine if the conjugation of the anti-VCAM-1 Ab to the PS containing liposomes would offer any improvement in the accumulation of liposomes in LS180 tumors, the tumor targeting efficiency (T_E), which is defined as the AUC_{0-24h} in the tumor divided by the AUC_{0-24h} in plasma, was calculated for the various PS liposomes with no Ab, control rat Ab, or rat anti-mouse VCAM-1 Ab. Since this parameter has been used previously as a measure to determine the selectivity of tumor uptake properties for conventional liposomes
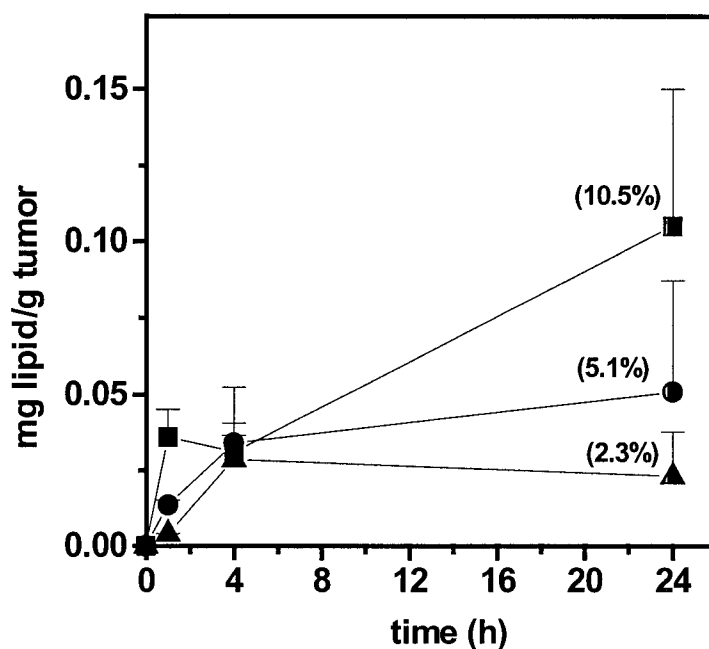
Figure 5.9. Accumulation of 10 mol% DOPS/15 mol% DSPE-PEG 2000/DSPC/CHOL liposomes with no Ab (■), rat Ab control (●) or rat anti-mouse VCAM-1 Ab (▲) conjugated in LS180 tumor bearing SCID/Rag 2 mice over a 24-hour period. The lipid dose was injected as a single IV bolus of 50 mg/kg, and the mice weighed ~ 20 g. The size of the tumors ranged from 0.11 to 0.17 cm^3. Shown in parentheses are the percent injected dose per gram of tumor for the three types of liposomes at 24 h post injection. Each data point represents the mean from three mice, with the error bars representing S.D shown in one direction for clarity. Data at 24 h were analyzed with one-way ANOVA and Newman-Keuls test with $p < 0.05$.

and sterically stabilized liposomes [171], it could be used in this case to evaluate the targeting capability of the Ab-conjugated PS liposomes to the VCAM-1 expressing LS180 tumors normalized to circulating plasma concentrations. The T_E value for the pegylated PS liposomes with no Ab conjugated was 0.15 which was the lowest among the three types of liposomes being studied here, although this liposome exhibited the highest tumor AUC_{0-24h} and plasma AUC_{0-24h} with values of 1.476 mg • h • g^{-1} and 9.533 mg • h • mL^{-1}, respectively (see Table 5.1, P. 178). In contrast, the pegylated PS liposomes conjugated with anti-VCAM-1 Ab, though having the lowest tumor AUC_{0-24h} (0.566 mg • h • g^{-1}) and plasma AUC_{0-24h} (1.542 mg • h • mL^{-1}), exhibited the highest T_E value of 0.37. However, the T_E value for PS liposomes conjugated with anti-VCAM-1 Ab is only 2.5- and 1.5-fold higher than those observed for PS liposomes with no Ab and with control rat Ab, respectively. These results demonstrate that the conjugation of anti-VCAM-1 Ab provided modest selectivity in liposome accumulation in LS180 tumors, and had lower absolute tumor accumulation values due to the more rapid removal from the circulation.

To investigate further the specificity in the localization of the anti-VCAM-1 Ab-conjugated PS liposomes in the LS180 tumors, LS180 tumors from SCID/Rag 2 mice injected with DOPS liposomes conjugated with control rat Ab or rat anti-mouse VCAM-1 Ab were collected 24 h post injection and sectioned. Tumor sections were then analyzed with an anti-rat IgG Ab. The immunohistological results from LS180 tumor sections are presented in Figure 5.10 (P. 179). As shown in the figure, negligible rat IgG immunoreactivity on the blood vessels throughout the LS180 tumor sections was observed for the PS liposomes conjugated with the control rat Ab. As for tumor sections collected

Table 5.1.

Plasma and tumor AUC_{0-24h} values of various liposomes after IV injection to SCID/Rag 2 mice bearing LS180 tumors

Liposomes	Tumor AUC_{0-24h} [a] $(mg \bullet h \bullet g^{-1})$	Plasma AUC_{0-24h} [a] $(mg \bullet h \bullet mL^{-1})$
DOPS 10%/DSPE-PEG 2000 15%/ DSPC/CHOL	1.476	9.533
DOPS 10%/DSPE-PEG 2000 15%/ DSPC/CHOL/rat Ab control	0.922	3.760
DOPS 10%/DSPE-PEG 2000 15%/ DSPC/CHOL/rat anti-mouse VCAM-1 Ab	0.566	1.542

[a] Tumor AUC_{0-24h} was calculated based on the values presented in Figure 5.9 using the trapezoidal rule. Plasma AUC_{0-24h} for each liposome formulation was estimated based on the plasma lipid levels presented in Figure 5.8, using non-compartmental analysis and linear trapezoidal rule from the software WinNonlin version 1.5 as described in Chapter 3. The lipid dose in this study was 50 mg/kg given as a single IV bolus via the lateral tail vein, and the mice weighed ~ 20 g. The size of the tumors ranged from 0.11 to 0.17 cm^3.
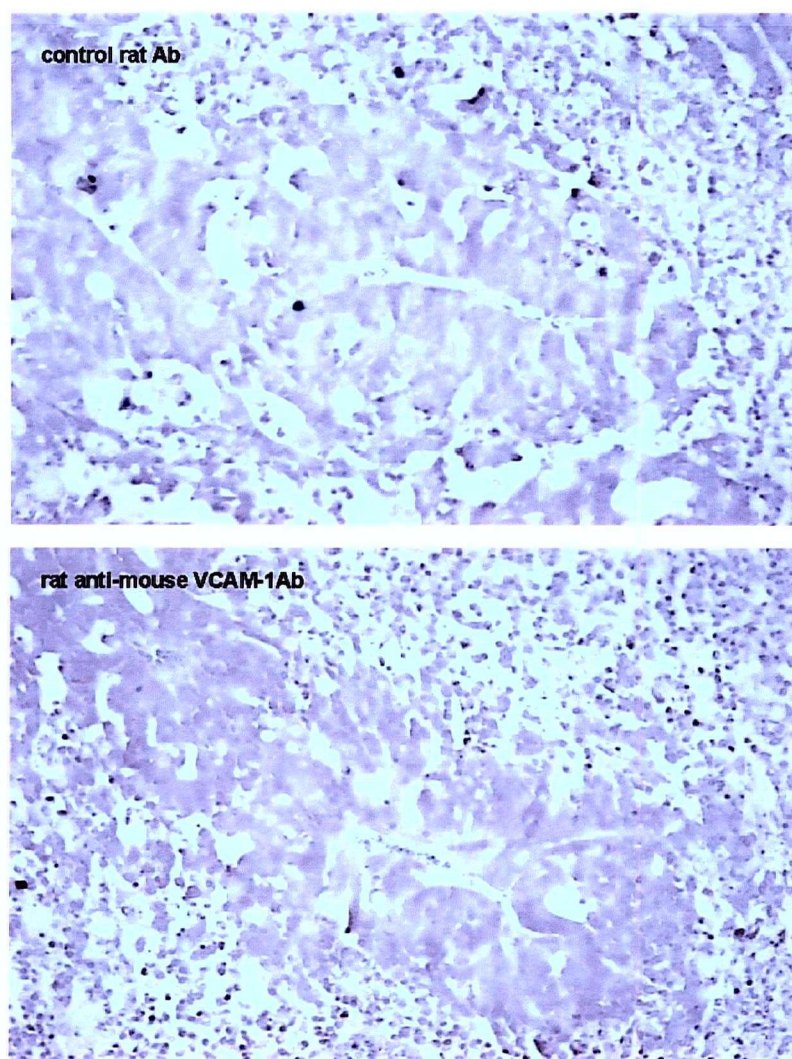
Figure 5.10. Rat IgG like immunoreactivity in LS180 tumor sections harvested from SCID/Rag 2 mice 24 h after the injection of DOPS 10%/DSPE-PEG 2000 15%/DSPC/CHOL liposomes conjugated with rat anti-mouse VCAM-1 Ab or rat Ab control (magnification: × 200). Six sections were obtained from each tumor sample, and were processed as described in Section 5.2.8 using a donkey anti-rat IgG Ab. Three mice were used in the experiment (tumor size 0.11 − 0.17 cm^3).

from mice injected with rat anti-mouse VCAM-1 Ab conjugated PS liposomes, negligible rat IgG immunoreactivity was also observed on the blood vessels throughout LS180 tumors. Since lipid accumulation in LS180 tumors was observed for the anti-VCAM-1 conjugated liposomes and the control rat Ab conjugated liposomes, it is possible that the two Ab's did not remain conjugated to the liposomes after injection into the bloodstream. Although the linkage used to attach the Ab to the PS liposomes has been shown in vitro to be stable in plasma [324], the linkage between the Ab and the PS liposomes may become more susceptible to cleavage when the liposomes are injected into bloodstream. As suggested previously, the attachment of macromolecules such as whole Ab may increase the propensity of PEG-lipids to desorb from the lipid bilayer [204]. Thus, it is possible that the entire Ab-PEG-lipid desorbed from the liposome membrane, resulting in a loss of the Ab from the liposomes. Based on the tumor lipid accumulation profiles (Figure 5.9, P. 176), the amounts of Ab present in the LS 180 tumors can be estimated (assuming that the Ab remained conjugated to the PS liposomes), with approximately 20 and 50 ng for the anti-VCAM-1 Ab and the control rat Ab, respectively. These levels of Ab approach the limit of detection for the immunohistological method used here (15 ng); thus, it is possible that these levels of Ab were not sufficient to be detected by the immunohistological method.

Table 5.2 (P. 181) shows the lipid levels in various tissues harvested 24 h after injection of various liposome formulations. In addition to organs of the MPS (liver and spleen), tissues that showed weak expression of VCAM-1 were also collected for analysis to determine if high levels of anti-VCAM-1 Ab-conjugated PS liposomes would be

Table 5.2.

Tissue lipid levels in SCID/Rag 2 mice bearing LS180 tumors 24 h post injection [a]

Liposomes	liver	spleen	lung	heart	kidney
		mg lipid / g tissue +/- S.D.			
DOPS 10%/DSPE-PEG 2000 15%/DSPC/CHOL/rat anti-mouse VCAM-1 Ab	0.438 ± 0.203 (37%)	0.214 ± 0.125 (0.5%)	0.051 ± 0.015 (0.9%)	0.033 ± 0.005 (0.4%)	0.042 ± 0.011 (0.5%)
DOPS 10%/DSPE-PEG 2000 15%/DSPC/CHOL	0.146 ± 0.115 * (13%)	0.533 ± 0.087 ** (2%)	0.045 ± 0.013 (0.9%)	0.030 ± 0.053 (0.3%)	0.050 ± 0.066 (0.6%)
DOPS 10%/DSPE-PEG 2000 15%/DSPC/CHOL/rat Ab control	0.572 ± 0.043 (48%)	0.332 ± 0.071 (1%)	0.034 ± 0.061 (0.7%)	0.043 ± 0.016 (0.5%)	0.055 ± 0.008 (0.7%)

[a] Data represent the averages ± S.D. of three mice. The injected lipid dose was 50 mg/kg as a single IV bolus, and the liposomes were labeled with [^{3}H]CHE (as a general lipid marker) and prepared as described in Section 5.2.2 and 5.2.3. The amount of lipid per gram of tissue was determined as described in Section 3.2.3. Shown in parentheses are the percent injected dose recovered from each tissue. Data were analyzed by one-way ANOVA and Newman-Keuls test with $p < 0.05$. * denotes the lipid level for DOPS 10%/DSPE-PEG 2000 15%/DSPC/CHOL in liver is statistically different from those values of the other two liposome formulations in liver, and ** denotes the lipid level for DOPS 10%/DSPE-PEG 2000 15%/DSPC/CHOL in spleen is statistically different from those values of the other two liposome formulations in spleen.

accumulated in these tissues. The lipid levels recovered in liver and in spleen for PS liposomes with no Ab conjugation were 0.146 and 0.533 mg lipid/g tissue, respectively. Compared to the observed liver lipid levels for PS liposomes conjugated with control rat Ab (0.572 mg lipid/g tissue) and with anti-VCAM-1 Ab (0.438 mg lipid/g tissue), the liver lipid levels for the PS liposome with no Ab were statistically significantly lower ($p < 0.05$). This is consistent with the results reported in previous chapters where 15 mol% DSPE-PEG 2000 can prevent MPS recognition of the PS liposomes. Approximately 50% of the injected lipid dose were accumulated in the liver for DOPS liposomes conjugated with control rat Ab or with anti-VCAM-1 Ab, and the mechanism for this lipid accumulation will be explored in the discussion section. The spleen lipid levels for PS liposomes with no Ab were statistically significantly higher than those observed for PS liposomes conjugated with control rat Ab (0.332 mg lipid/g tissue) and with anti-VCAM-1 Ab (0.214 mg lipid/g tissue), with $p < 0.05$.

For the three types of pegylated PS liposomes studied here, the lipid levels per gram of tissue in VCAM-1 expressing organs such as lung, heart and kidney were an order of magnitude lower than those observed in liver and spleen, and these lipid levels observed in lung, heart and kidney were not statistically different for the three types of PS liposomes, respectively ($p > 0.05$). The total percent of injected dose accumulated in lung, heart and kidney was < 2% for each of the three types of pegylated PS liposomes. These results reflect that the potential of unwanted blood coagulation in these tissues would be low, although these tissues exhibited low levels of VCAM-1 expression.

5.4. Discussion

Results from the in vitro assays shown in this chapter have clearly demonstrated that the VCAM-1 bound Ab conjugated PS liposomes were capable of triggering the blood coagulation responses upon exposure of the thrombogenic PS liposome surface. This surface exposure and thrombogenic activity would be triggered by exchanging out PEG-lipids after target cell binding. These results successfully provided proof-of-concept evidence that supports further development of this novel PS-containing liposome formulation into one that is applicable in vivo for the induction of tumor specific thrombogenesis. However, the results from the in vivo studies demonstrated that the lipid levels accumulated in LS180 tumors for the anti-VCAM-1 Ab conjugated DOPS liposomes were not different from those for DOPS liposomes with the control rat Ab or with no Ab conjugated. Some problems or limitations relating to the in vivo targeting of these thrombogenic PS liposomes are discussed below.

Although the coupling efficiency was slightly compromised in the presence of 14 mol% DSPE-PEG 2000 in the DOPS liposomes, this is the first study to document successful Ab conjugation and binding to target in the presence of a dense PEG barrier (> 10 mol%) on the liposome surface. With the ever increasing interest in developing liposomes with multiple components and lipid species, conjugation of ligands to liposomes grafted with a dense PEG barrier is likely, and the results obtained here would provide useful information to future design of ligand conjugated liposomes for targeted delivery. The elevated level (14 mol%) of DSPE-PEG 2000 in the DOPS liposomes most likely decreases the flexibility of the PEG spacer in the reactive PEG-lipid for the conjugation reaction. This dense PEG barrier is also likely to shield, at least partially, the reactive maleimide group at

the distal end of the PEG polymer in the reactive PEG-lipid. Taken together, these features appear to decrease the coupling efficiency of the Ab to DOPS liposomes containing 14 mol% DSPE-PEG 2000. Although coupling efficiency was reduced in the presence of 14 mol% DSPE-PEG 2000, binding to the VCAM-1 expressing HUVECs under such conditions was enhanced. One explanation for this phenomenon is that once the Ab was conjugated to the PEG terminus, the dense PEG barrier may not be able to completely shield the Ab, given that the whole Ab is much bulkier than the reactive maleimide group. In this case, the Ab would still be available for interacting with the target antigen under such conditions.

With the in vitro demonstration that the VCAM-1 bound DOPS liposomes maintained their thrombogenicity upon exposure of the PS liposome surface, the next step was to evaluate the delivery of these thrombogenic liposomes in vivo. Compared to the plasma removal of DOPS liposomes containing 14 mol% DSPE-PEG 2000, conjugation of the control rat Ab to this liposome formulation resulted in more rapid removal of such liposomes from bloodstream and concomitant increased accumulation in the liver. This observation is consistent with those previously reported where Ab conjugated liposomes were eliminated slightly faster than Ab free liposomes at Ab densities of < 50 μg/μmol lipid [231]. This enhanced plasma removal of Ab-conjugated PS liposomes, at first sight, may be explained by protein binding and subsequent cross-linking of the Ab-conjugated liposomes leading to aggregation mediated liposome removal. Considering that these Ab conjugated liposomes were protected by an elevated level of the non-exchangeable DSPE-PEG 2000 which has been shown to reduce protein binding and liposome aggregation, such explanation

is not very likely. Alternatively, such enhanced removal of the Ab conjugated liposomes may be related to Fc receptor mediated recognition and subsequent uptake by liver macrophages in mice and rats [235, 271]. The Fc receptor family is known to bind to the constant region (Fc) of Ab, and can be divided into three subfamilies of related molecules, designated FcγRI, II, and III. Of these, FcγRI (CD64) has a high affinity for the Fc portion of the IgG molecules (K_d of 10^{-8} to 10^{-9} M) [325]. Since the coupling chemistry used here would result in random orientation of Ab on the liposomes, it is likely to have a portion of the Fc region of the conjugated Ab exposed to the recognition and uptake by liver macrophages through the Fc receptor mediated mechanism. The conjugation of the rat anti-mouse VCAM-1 Ab to the DOPS liposomes, however, led to even more rapid removal of such liposomes from the bloodstream. At first sight, one may attribute this rapid removal of anti-VCAM-1 Ab conjugated DOPS liposomes to the accumulation of liposomes in VCAM-1 expressing tissues including the LS180 tumors. Such explanation is not supported by the low lipid levels (< 1% of injected dose) observed in the VCAM-1 expressing tissues such as lung, heart, kidney and tumor. The accumulation of the anti-VCAM-1 Ab conjugated DOPS liposomes was mainly in the liver, with > 40% of the injected dose found in this organ 24 h post liposome injection; however, accumulation of such liposomes in liver is not likely to be mediated through the binding of the anti-VCAM-1 Ab conjugated liposomes to the target VCAM-1, given that VCAM-1 expression in liver was only weak and patchy. It is believed that accumulation of the anti-VCAM-1 Ab conjugated DOPS liposomes in the liver is likely to be mediated by the Fc receptor uptake mechanism.

In mice without LS180 tumors, the plasma removal of the anti-VCAM-1 Ab conjugated DOPS liposomes was more rapid than that of the control rat Ab conjugated DOPS liposomes. This difference in plasma removal may possibly be due to the accumulation of the anti-VCAM-1 Ab conjugated DOPS liposomes in some other VCAM-1 expressing tissues which were not collected for immunohistological analysis in this study. One such example is the peripheral lymphoid tissue in which the dendritic cells are shown to exhibit VCAM-1 expression [326, 327]. Furthermore, VCAM-1 expression can be induced by cytokines whose levels are often increased in diseases such as cancer. If this is the case, then VCAM-1 may not be a good target for the tumor vasculature homing of the thrombogenic PS liposomes developed here due to a lack of tissue selectivity in its expression. In the future, specific delivery of anti-VCAM-1 Ab-conjugated PS liposomes has to be demonstrated. Although selective localization in tumor vasculature of the VCAM-1 targeted coaguligand was observed by Ran et al [1], this discrepancy in observations may be explained by the difference in mouse strain and in tumor model used to determine the targeting properties of the coaguligand and the thrombogenic PS liposomes studied here. Over-expression of adhesion molecules such as VCAM-1 on endothelial cells can be induced by inflammatory cytokines which are produced during disease states such as cancer [312, 313]. Thus, vascular beds other than that within the tumor may express VCAM-1, and this should be taken into consideration in future studies involved in the in vivo delivery of the thrombogenic liposomes using adhesion molecules as targets. Specific binding of the thrombogenic liposomes to tumor vascular bed has to be demonstrated in future. VCAM-1 expression in other mouse tissues studied here was not further evaluated because the major challenge to be overcome in the targeting of the thrombogenic liposomes to tumor

vasculature is the rapid removal of the anti-VCAM-1 Ab conjugated PS liposomes mediated by Fc receptor uptake mechanism that results in low tumor lipid accumulation. Due to a lack of tissue selectivity and low tumor lipid accumulation which remain to be the major challenges in the in vivo delivery of the thrombogenic liposomes, the ability of such liposomes to induce thrombogenic activity within tumor vasculature was not evaluated.

The results from the tumor accumulation study showed that the conjugation of the anti-VCAM-1 Ab provided only marginal improvement in liposome accumulation in LS180 tumors. Several factors may account for this observation. It is very likely that the anti-VCAM-1 Ab conjugated DOPS liposomes which are not bound to the tumor vasculature during the first few passages through the tumor vasculature are eliminated very quickly from bloodstream with subsequent accumulation in the liver, and would not have a chance to bind to the target site anymore. In vivo binding of liposomes has been suggested to be strongly influenced by the blood flow rate, the rate of liposomes passing through the target site, and the accessibility of the target [244]. Efficient targeting of liposomes has been demonstrated in the well-perfused, mouse lung endothelium where the target molecule (gp112, a glycoprotein) is highly expressed on the luminal membrane, with close to 60% of injected Ab conjugated liposomes accumulated in the lung after 30 min [222]. In contrast, the abnormal architecture of tumor vasculature and heterogeneous tumor blood flow may restrict liposomes from reaching all parts of the tumor vasculature, resulting in low tumor lipid levels in general. Another factor to be considered is the in vivo stability of the conjugated Ab to the reactive PEG-lipid. Although the linkage between the Ab and the liposome used here has been demonstrated to be stable in plasma [324], the possibilities of

the conjugated anti-VCAM-1 Ab being cleaved from the liposomes in vivo and/or the entire construct of Ab-PEG-lipid being exchanged out of the liposomes cannot be ruled out.

In summary, the in vivo results presented in this chapter did not demonstrate specific delivery of the thrombogenic PS liposomes to the tumor vasculature, and the limitations of in vivo targeting of such liposomes were discussed. In the final chapter of this thesis, approaches to improve the attributes of the thrombogenic PS liposome will be addressed.

Chapter 6   Summarizing Discussion

This thesis has followed the approach of attacking the tumor vasculature where thrombogenic PS liposome systems were targeted to a specific surface marker of the tumor vascular endothelium in an attempt to elicit tumor specific vascular occlusion, and the data provided proof-of-concept evidence which supports further development of such liposomes into one that is applicable to tumor specific thrombogenesis. Given that thrombosis is one common complication in cancer patients [328], future studies should demonstrate that these thrombogenic liposomes would interact with the tumor vasculature specifically to minimize undesirable thrombotic events in healthy tissues. Both efficacy (in terms of thrombogenic activity in tumors) and safety profiles of the thrombogenic liposomes should be established in the future. Studies which utilized anti-tumor vasculature approaches (such as the use of coaguligand or tubulin polymerization inhibitors) have demonstrated massive tumor cell death within the tumor core [1, 48]. However, complete tumor cell kill was not achieved with this approach, where a rim of viable tumor cells were still observed in the tumor periphery. The strategy of combining the vascular damaging agents with conventional anti-cancer treatments has been proposed, and it has demonstrated enhanced anti-tumor activity of the conventional therapies [329, 330]. Given that liposome systems are well known for delivering chemotherapeutics agents, the thrombogenic PS liposome systems developed here as vascular damaging agents could be optimized by encapsulating conventional cytotoxic agents, thus offering the potential of combining the two therapeutic approaches in a single delivery vehicle.

The work presented in this thesis has provided a stepwise approach to the design of the thrombogenic PS-based liposome formulations for tumor specific thrombogenesis, from the protection of the thrombogenic PS liposome surface (Chapters 2 and 3), to the control of the PS surface thrombogenicity (Chapter 4), and finally to the targeting of such PS liposome formulations to VCAM-1, a marker of the tumor vasculature (Chapter 5). Within each step, some formulation issues for designing the thrombogenic PS liposomes have been addressed. After obtaining a PS containing liposome formulation with optimal surface thrombogenicity, shielding of such liposome surface in vivo is necessary to prevent 1) unwanted blood coagulation reactions from occurring in the vasculature of normal tissues, and 2) recognition by cells of the MPS which are known to rapidly eliminate PS containing liposomes. While 5 mol% DSPE-PEG 2000 has been widely known for its ability to extend the circulation longevity of neutral liposomes such as DSPC/CHOL, this level of DSPE-PEG 2000 has been shown in previous reports and in Chapter 3 of this thesis to be ineffective in protecting the PS containing liposomes from rapid removal [190, 238]. Results from Chapters 2 and 3 are the first to document that an elevated level (> 10 mol%) of DSPE-PEG 2000 was necessary to prevent the binding of blood coagulation proteins and the rapid removal of the negatively charged DOPS liposomes by MPS. These results would have important implications in the design of pegylated liposome systems, as current trends in developing liposomes are moving toward the use of reactive lipid species to aid in the encapsulation of agents or to facilitate content release at target sites. Recently, some studies have demonstrated pegylated liposomes were eliminated rapidly from bloodstream upon repeated injections, which is apparently mediated by heat-labile molecules in the serum [214]. Repeated dosing of the pegylated PS liposomes is one area that has not been explored in this

thesis. It would be interesting to examine if elevated levels of DSPE-PEG 2000 would prevent such rapid removal from occurring upon repeated dosing.

While adequately protecting the thrombogenic PS liposome surface is crucial in the vasculature of normal tissues, exposure of the PS liposome surface is also essential for triggering the blood coagulation responses once the PS liposomes have reached the tumor vasculature. Work described in Chapter 4 presented an approach to control the surface thrombogenicity of the PS containing liposomes where PEG-lipids with different rates of desorption from liposomes were used to demonstrate time-dependent exposure of the thrombogenic PS liposome surface. The in vivo data presented in Chapter 4 demonstrated that both DMPE-PEG 2000 and DPPE-PEG 2000 desorbed rapidly from the PS containing liposomes leading to rapid removal of the PS liposomes from circulation. From a delivery point of view, such attributes of the PS liposomes that were formulated with these exchangeable PEG-lipids are undesirable, since rapid removal of the PS liposomes is likely to decrease the chance of these liposomes to accumulate in the tumor vasculature in vivo. Although combinations of exchangeable and non-exchangeable PEG-lipids were used to balance surface exposure with circulation longevity of PS liposomes (Figure 4.6, P. 141), this approach may not be able to provide satisfactory fine tuning of this balance. Ideally, exposure of the PS liposome surface should occur selectively within the tumor vasculature such that the potential of inducing unwanted blood coagulation in circulation and in the vasculature of normal tissues would be minimized. Recently, it has been shown that the linkage between the PEG polymer and the lipid anchor can be cleaved chemically [291, 292]. This offers an interesting, alternative approach to improve the selectivity of PS

liposome surface exposure for the intended application of tumor specific thrombogenesis. Development of new linkages between the PEG polymer and the lipid anchor that are sensitive to enzymatic degradation by enzymes selectively over-expressed in tumors (such as the matrix metalloproteinases) could be useful in improving the selectivity in the loss of PEG coating [331]. Subsequent evaluation of the in vivo efficacy and toxicity of the thrombogenic liposomes could then be initiated after the establishment of a strategy that allows selective loss of PEG coating within the tumor vasculature.

In addition to selective exposure of PS liposome surface, localization of blood coagulation responses within the tumor vasculature is desirable. A monoclonal, whole Ab that is directed against VCAM-1, a marker of the tumor vasculature, was conjugated to the thrombogenic PS liposomes in an attempt to address this issue. The in vitro data presented in Chapter 5 clearly demonstrated that the anti-VCAM-1 Ab conjugated PS liposomes maintained the clotting activity after binding to surface bound VCAM-1, provided that the PS liposome surface was exposed. These results thus provided proof-of-concept evidence for the potential of developing a PS containing liposome formulation to trigger tumor specific thrombogenesis. The in vivo data did not demonstrate the specific delivery of the anti-VCAM-1 Ab conjugated PS liposomes to the tumor vasculature, which is likely to be due, at least in part, to the Fc receptor mediated uptake of the Ab conjugated PS liposomes into liver, resulting in rapid removal of these liposomes [235, 271]. This can also be explained by the possible loss of the targeting ligand (i.e., the anti-VCAM-1 Ab) from the PS liposomes. Although the linkage between the Ab and the liposomes employed in this thesis has been previously shown to be stable in plasma [324], it is possible that such

linkages are more susceptible to cleavage in vivo. It is also possible that the entire construct of Ab-PEG-lipid desorbs from the PS liposomes [204]. Thus, further evaluation of the stability of the targeting ligand in vivo is required in future studies.

One important point to be addressed in future studies is the immunogenicity of whole Ab conjugated liposomes [332, 333]. While the use of Ab fragments such as F(ab') or scFv has been suggested to reduce the immunogenicity of ligand conjugated liposomes, the use of humanized whole Ab for coupling to liposomes may represent another way to circumvent the problem of immunogenicity [246]. In addition, the use of Ab fragments such as F(ab') or F(ab')$_2$ may offer a possible solution to the rapid removal of the Ab conjugated PS liposomes which is mediated in part by recognition of the Fc portion of the whole Ab. However, the use of Ab fragments is not without problems. Fragmentation of the whole Ab molecule into F(ab') or F(ab')2 can lead to 2-fold and 14-fold decrease in binding affinity, respectively [334]. Use of F(ab') fragments may result in reduction in binding avidity due to loss of bivalent binding ability [335-337], and may be inefficient due to a lack of sulfhydryl groups for reacting with the maleimide group on the liposomes [338]. Careful evaluation of the binding and of the immunogenicity of ligand conjugated liposomes should be carried out in the future.

In summary, the work presented in this thesis described a stepwise approach to the development of thrombogenic liposome formulations that may potentially be applied in triggering tumor specific thrombogenesis. The data from this thesis contribute not only to the design of sophisticated, multi-functional liposomes for targeted delivery, but also to the development of lipid-based therapeutics that attack the tumor vasculature.

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