STUDIES ON FACTORS INFLUENCING THE ELIMINATION OF LIPOSOMES FROM THE CIRCULATION

by

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ABSTRACT

This thesis concerns the interactions experienced by liposomal drug delivery systems as they circulate in the blood and their influence on elimination behaviour. These studies are divided into three areas, which investigate three factors influencing liposome elimination behaviour - lipid dose, poly(ethylene glycol) (PEG)-polymer coating and the generation of an immune response to liposome systems.

First, the influence of lipid dose on elimination has previously been attributed to 'saturation' of the free and fixed macrophages of the reticuloendothelial system (RES) at high lipid doses. Here the potential role of blood protein binding by liposomes is examined. Protein binding and elimination properties of two representative compositions of large unilamellar vesicles were examined in mice over a dose range of 10-1000 mg lipid / kg body weight. Although longer half-lives were observed for higher doses even the highest lipid doses of the most rapidly cleared liposome compositions failed to completely saturate RES uptake. However, these higher dose liposomes did bind significantly less protein as measured on a protein-to-lipid basis. These results suggest the existence of a specific pool of blood proteins that interacts with liposomes of a given composition, and is diluted over larger surface areas at higher lipid doses, resulting in less efficient protein-mediated RES uptake.

A second method of influencing liposome elimination behaviour is through "steric stabilization" of liposomes by incorporation of PEG polymer coatings. This enhances circulation lifetimes and increases delivery of drugs to sites of disease. It has been conjectured that reduction of blood protein adsorption to liposomes is the primary mechanism leading to longer circulation

lifetimes of these systems, however this remains to be confirmed *in vivo*. These studies employed three representative lipid compositions to demonstrate that incorporation of PEG induces a nonspecific reduction of blood protein bound to vesicles in the circulation of mice. This reduced blood protein adsorption correlated with the increased circulation lifetimes of the vesicles. Dose-dependent changes in circulation lifetime, similar to those observed for conventional lipid compositions, were noted.

A third factor which can dramatically alter liposome elimination behaviour is the generation of immune responses - particularly against the surface-coupled antibodies or ligands of targeted liposomes. This results in rapid elimination of subsequent administrations, therefore limiting potential applications. Liposomes are known to interact with cells responsible for antigen processing and presentation, antibody production and cell-mediated immunity. Therefore, it was investigated whether the toxic effects of encapsulated drugs on cells of the immune system could solve this problem. Liposome elimination and humoral immune response were monitored for repeated administrations of doxorubicin encapsulated in liposomes with ovalbumin covalently coupled to the surface. The results showed that, at high drug-to-lipid ratios low doses of encapsulated doxorubicin prevented humoral immunity against repeated administration of ovalbumin-proteoliposomes. Immunosuppression was specific for the ovalbumin bound to drug-loaded vesicles at low drug doses. This suggests a selective suppression of immunity against the target ligand for low doses of doxorubicin-loaded targeted liposomes which could prove advantageous for safe repeated administration.

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ABBREVIATIONS

Apo	apolipoprotein
ACA	anti-cardiolipin antibodies
APC	antigen presenting cell
β_2-GPI	β2-glycoprotein-1 or apolipoprotein H
BCA	bicinchoninic acid
BSA	bovine serum albumin
C	complement component designation
^{13}C-NMR	carbon-13 nuclear magnetic resonance
[^{3}H]-CHE	[^{3}H]-cholesteryl hexadecyl ether
CHOL	cholesterol
CL	cardiolipin
CPRG	chlorophenol red-β-D-galactopyranoside
CRP	C-reactive protein
DAPC	diarachidonyl phosphatidylcholine
DCP	dicetyl phosphate
DNA	deoxyribonucleic acid
DODAC	N,N-dioleoyl-N,N-dimethylammonium chloride
DOPA	dioleoyl phosphatidic acid
DOPE	dioleoyl phosphatidylethanolamine
DOPS	dioleoyl phosphatidylserine
DOTAP	1,2-bis(oleoyloxy)-3-(trimethylammonio)propane
DOX	doxorubicin
DPPC	dipalmitoyl phosphatidylcholine
DSPC	distearoyl phosphatidylcholine
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPC	egg phosphatidylcholine
F(ab')	antigen binding portion of antibody
Fc	antibody receptor- and complement-binding portion of antibody
FATMLV	frozen-and-thawed MLV
G_{M1}	monosialoganglioside G_{M1}
H_{II}	hexagonal phase lipid
HBS	HEPES-buffered saline solution
HDL	high density lipoprotein
HEL	hen egg lysozyme
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane-sulphonic acid
IgG	immunoglobulin G
IgM	immunoglobulin M
i.v.	intravenous
LDL	low density lipoprotein
LPS	gram-negative bacterial lipopolysaccharide

LUV	large unilamellar vesicle
LUVET	LUVs prepared by extrusion
MAC	membrane attack complex (complement)
MCR	mouse complement receptor
mg/kg	mg of compound (lipid or drug) / kg body weight of animal
MHC I	major histocompatibility complex class I
MHC II	major histocompatibility complex class II
min	minutes
MLV	multilamellar vesicle
mol %	mole percent
MPB-DSPE	N-(4-(P-maleimidophenyl)butyryl) DSPE
MPS	mononuclear phagocyte system
MTX	methotrexate
PA	phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
P_B value	protein binding value (g protein/mol lipid)
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEG or ^{2000}PEG	poly(ethylene glycol) (^{2000}PEG = 2000 Da molecular weight)
PEG-Cer	poly(ethylene glycol)-modified ceramide
PEG-Cer	1-O-(monomethoxypolyethyleneglycol succinoyl)-ceramide
C_{20} or C_{14}	indicates a ceramide chain of 20 or 14 carbons
PEG-DSPE	N-(monomethoxypolyethyleneglycol succinoyl)-DSPE
PG	phosphatidylglycerol
PI	phosphatidylinositol
QLS	quasielastic light scattering
RES	reticuloendothelial system
SA	stearylamine
SDS	sodium dodecyl sulphate
SM	sphingomyelin
SPDP	N-succinimidyl 2-(2-pyridyldithio) propionate
SSL	sterically stabilized liposome
SUV	small unilamellar vesicle
$T_{1/2}$	half-life
T_C	critical temperature (phase transition temperature)
Tris	tris(hydroxymethyl)aminomethane
Tween-20	polyoxyethylene (20) sorbitan
VEGF	vascular endothelial growth factor
VLDL	very low density lipoprotein

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TO MY LOVING AND SUPPORTIVE WIFE, MAY

AND HER NOT SO LOVING DOG, SCRUFFY

CHAPTER 1: INTRODUCTION

1.1 Interactions and Applications of Liposome Drug Carriers - An Overview

Shortly after their discovery, the potential use of liposomes in the delivery of bioactive agents was recognized (Bangham, 1968; Sessa and Weissman, 1968; Gregoriadis, 1973). Efforts in this area have now culminated in numerous drug carrier systems being approved for human use or undergoing clinical trials; including antifungal treatments with liposomal amphotericin B (Lopez-Berestein et al, 1985; Sculier et al, 1988; Zoubek et al, 1992), vaccine carriers against influenza (Powers et al, 1995) and malaria (Alving and Richards, 1990), tumour imaging using indium[111] (Presant et al, 1988; Kubo et al, 1993), cancer immunotherapy using muramyl tripeptide (Murray et al, 1989), and anticancer systems containing drugs such as doxorubicin (Mayer et al, 1990a and 1990b; Gabizon et al, 1994; Harrison et al, 1995; Working and Dayan, 1996). Liposomal systems have also been applied to the treatment of inherited genetic disorders, through attempts to transfer DNA into cells in complexes with cationic liposomes (reviewed by Gao and Huang, 1995; McLachlan et al, 1996; Liu et al, 1997). The advantages of encapsulating drugs within liposomes include reduced toxicity (Herman et al, 1983; Rahman et al, 1986a), increased uptake in diseased tissues (Bakker-Woudenberg et al, 1992; Wu et al, 1993; Bally et al, 1994; Sakakibara et al, 1996), natural targeting of RES tissues for drug delivery (Fidler et al, 1986; Daemen et al, 1988), and improved efficacy over free drug (reviewed by Ostro and Cullis, 1989; Papahadjopoulos, 1993).

Liposomes began as multilamellar systems with low levels of entrapped drug which were rapidly cleared from the blood. Preparations using extrusion techniques produced homogenous

unilamellar systems with well defined characteristics (Hope *et al*, 1985). Active trapping of drugs in response to pH gradients has achieved drug loading efficiencies close to 100 % (Mayer *et al*, 1986b, 1990a, and 1990b). The short circulation lifetimes originally experienced have been dramatically extended, first by varying liposome size and composition and then incorporating G_{M1} and poly(ethylene glycol) (PEG) polymers to produce "sterically stabilized" liposomes (SSLs). Conventional and sterically stabilized liposomes with improved circulation lifetimes (Gabizon and Papahadjopoulos, 1988; Allen and Chonn, 1989), and substantial drug delivery to sites of disease (Williams *et al*, 1986; Gabizon and Papahadjopoulos, 1988; Bakker-Woudenberg *et al*, 1992; Sakakibara *et al*, 1996), have now progressed to a variety of human trials. Current efforts are focused on specifically targeting drug carriers to diseased cells and tissues. Initial results using surface-attached antibodies and other ligand molecules have shown great potential *in vitro* (Longman *et al*, 1995; Goren *et al*, 1996; Vingerhoeds *et al*, 1996) and led to successful targeting *in vivo* to vascular targets (Maruyama *et al*, 1990; Ahmad *et al*, 1993; Emanual *et al*, 1996). However, targeting extravascular cells has failed to improve efficacy over non-targeted "sterically stabilized" liposomes (Goren *et al*, 1996; Vingerhoeds *et al*, 1996).

It is important to have long liposome circulation lifetimes to achieve preferential delivery to disease sites. This has been observed with tumours (Proffitt *et al*, 1983; Forssen at al, 1992; Bally *et al*, 1994), sites of inflammation (Williams *et al*, 1986), and sites of infection (Bakker-Woudenberg *et al*, 1992). High blood levels of liposomes increase delivery to these areas through a "passive" targeting mechanism which arises from increased capillary permeability accompanying disease. Three factors which can influence this by increasing liposome circulation lifetimes are lipid dose, PEG-polymer incorporation, and control of immune response to liposomes.

Understanding how these three factors influence circulation lifetimes is the object of this thesis.

1.2 Lipids and Membranes

1.2.1 Chemistry and physics of lipids

1.2.1.1 Phosphoglycerides

A phospholipid molecule is made up of two components: a polar headgroup and nonpolar or hydrophobic acyl chains which are attached through a central glycerol backbone. The chemical nature of the headgroup and tails determines the characteristics of each lipid molecule (sample phospholipid structures are shown in Figure 1.1). The primary characteristics of lipids are their phase preference and phase transition temperatures (T_C). The phase transition temperature is the temperature at which a particular lipid in a bilayer changes from a "frozen" gel or solid state to a liquid-crystalline phase, and depends primarily on the acyl chain length and saturation (reviewed by Cullis and Hope, 1991; Fenske *et al*, 1995). Long chain, saturated phospholipids generally have higher phase transition temperatures than those with shorter, unsaturated acyl chains. The gel state is characterized by a rigid, ordered acyl chain organization, and shorter or unsaturated acyl chains disrupt this organization lowering the transition temperature. At higher temperatures, the more fluid liquid-crystalline state exists due to increased movement of the membrane components: rotation about the long molecular axis, lateral diffusion, and trans-gauche isomerization.

Phospholipid shapes provide a qualitative basis for explaining lipid polymorphism, or the formation of different phases or macroscopic structures on hydration (reviewed by Lafleur *et al*,

Figure 1.1 Structures of common phospholipids

R_H
|
O
|
O=P—O⁻   Lipid Headgroups (R_H)
|
O
|
CH₂—CH—CH₂   Glycerol Backbone
| |
O   O
| |
O=C   C=O
| |
R_1 R_2 Acyl Chains (R_1 and R_2)
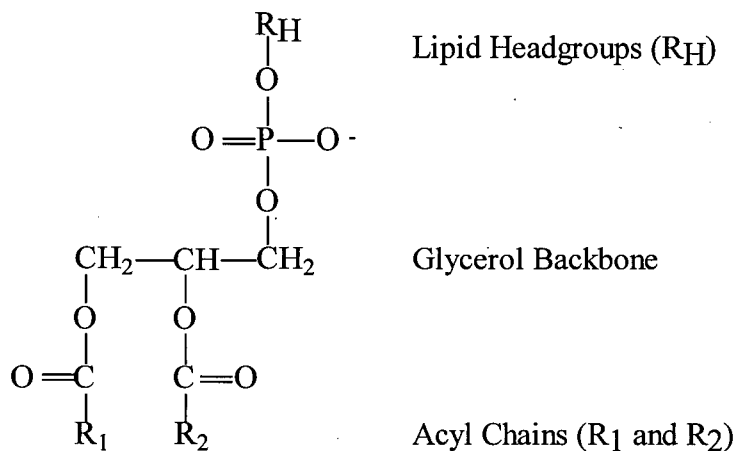
Lipid Headgroups (R_H) :

Phosphatidylcholine (PC)	$R_H=$	—CH₂CH₂N⁺(CH₃)₃
Phosphatidylethanolamine (PE)	$R_H=$	—CH₂CH₂N⁺H₃
Phosphatidic Acid (PA)	$R_H=$	—H
Phosphatidylglycerol (PG)	$R_H=$	—CH₂CH(OH)CH₂OH
Phosphatidylserine (PS)	$R_H=$	—CH₂CH—N⁺H₃ COO⁻

Phosphatidylinositol (PI) $R_H=$
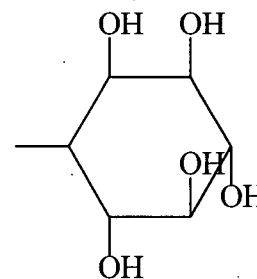
Acyl Chains (R_1 and R_2) :

Saturated Fatty Acids

Lauric (12:0)	—(CH₂)₁₀CH₃
Myristic (14:0)	—(CH₂)₁₂CH₃
Palmitic (16:0)	—(CH₂)₁₄CH₃
Stearic (18:0)	—(CH₂)₁₆CH₃

Unsaturated Fatty Acids

Palmitoleic (16:1$^{\Delta 9}$)	—(CH₂)₇CH=CH(CH₂)₅CH₃
Oleic (18:1$^{\Delta 9}$)	—(CH₂)₇CH=CH(CH₂)₇CH₃
Linoleic (18:2$^{\Delta 9,12}$)	—(CH₂)₇CH=CHCH₂CH=CH(CH₂)₄CH₃

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Figure 1.2 Lipid polymorphism (Lipid Shape Hypothesis)
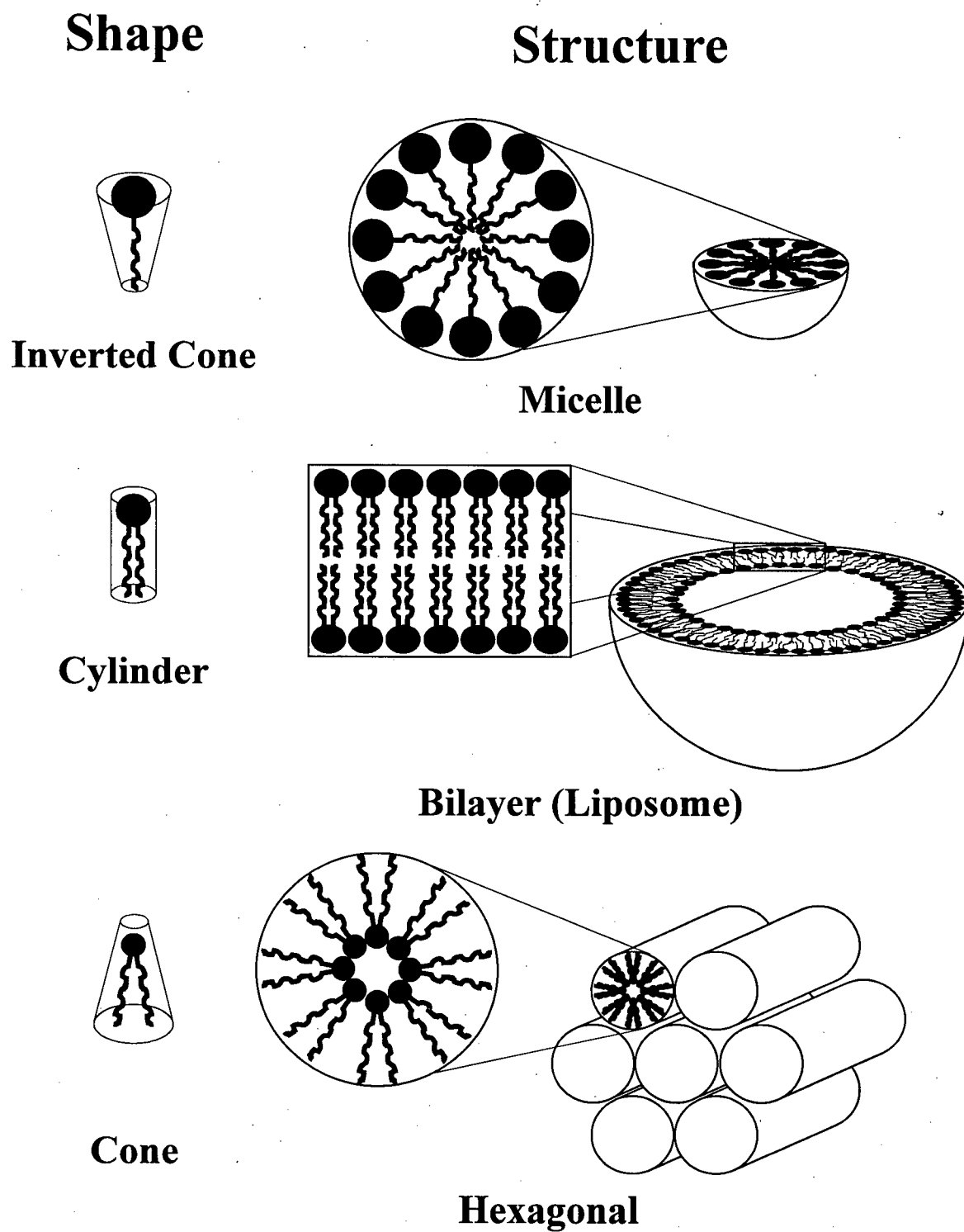
1990; Cullis and Hope, 1991). Lipids can adopt three common phases on hydration (see Figure 1.2) which form depending on the relative shapes of the lipids present. The lipid shape hypothesis is based on the relative size of the lipid headgroup and the acyl chain area. A cone shape lipid (including many "detergent" structures like lysophospholipids) possesses a larger headgroup than acyl chain area, and so will naturally form a micellar phase. Formation of a hexagonal or H_{II} phase requires lipids with a larger acyl chain cross-sectional area than headgroup region (inverted cones), such as unsaturated phosphatidylethanolamine (PE), phosphatidylserine (PS) below pH 4, and cardiolipin (CL) or phosphatidic acid (PA) in the presence of calcium ions. Most lipids, including phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI), cardiolipin (CL), and sphingomyelin (SM) are cylindrical in shape and form bilayer structures, these are representative of lipids commonly used in the production of liposomes.

1.2.1.2 Cholesterol

Cholesterol is an amphipathic lipid molecule, whose polar 3-hydroxyl group is directed with the phospholipid headgroups towards the aqueous solution and rigid ring structure is buried within the hydrophobic interior with the phospholipid acyl chains (see Figure 1.3). Cholesterol has the ability to keep the lipid membrane in an intermediate phase. It decreases the rigidity or order of gel phases and increases the order of the liquid-crystalline phases (Oldfield and Chapman, 1972; Demel and de Kruijff, 1976). Addition of increasing amounts of cholesterol in the bilayer will reduce and then completely eliminate the sharp phase transition normally

Figure 1.3 Structure and membrane orientation of cholesterol

A

Cholesterol

Phospholipid (DSPC)

B

Inner Monolayer

Outer Monolayer
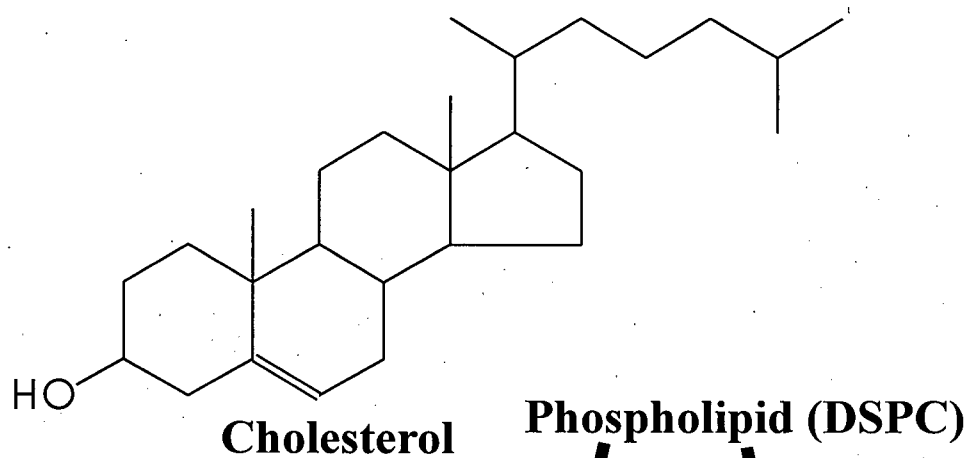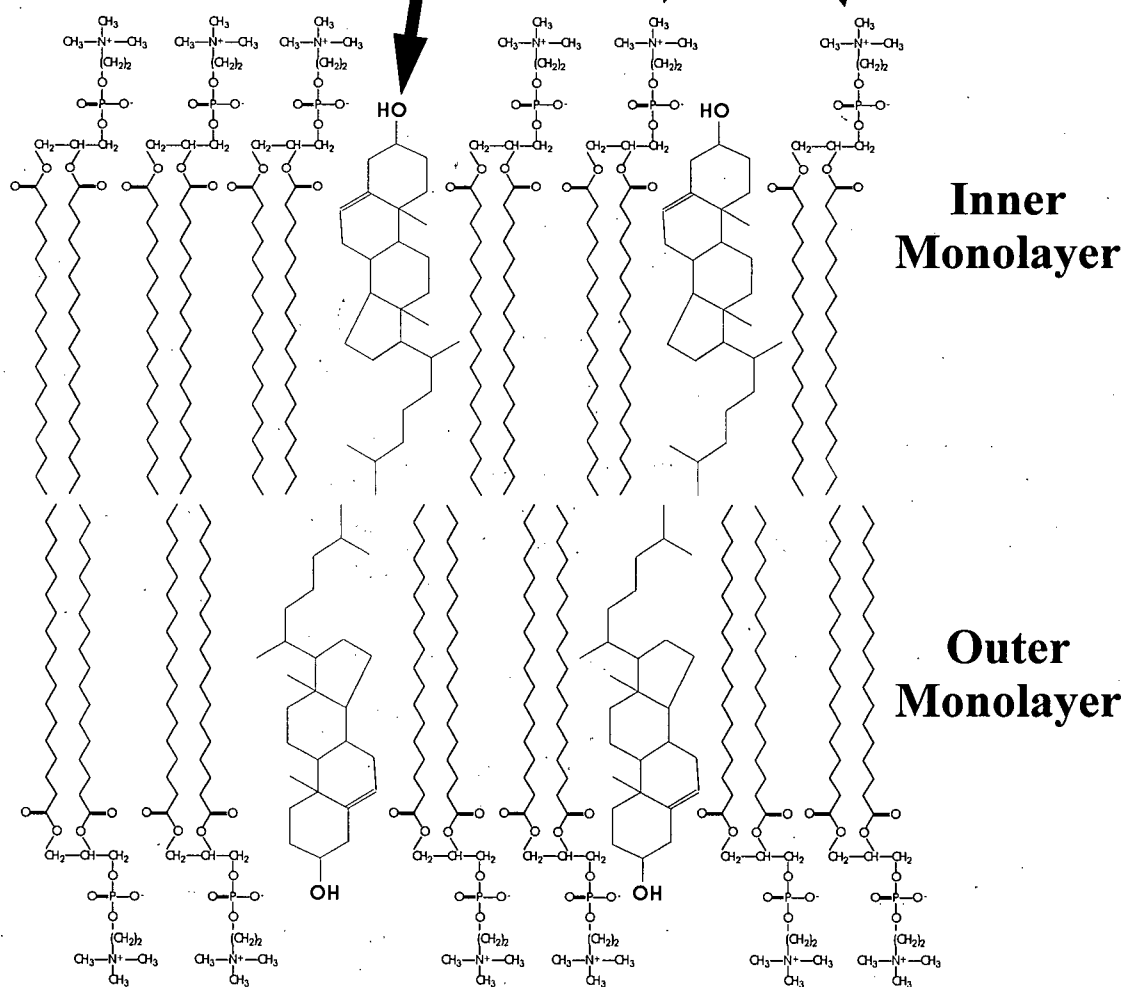
observed for single component phospholipid bilayers (Hubbell and McConnel, 1971; Demel and de Kruijff, 1976; Linseisen *et al*, 1993). The general rule for bilayer permeability is that increased order results in decreased leakage (Fenske *et al*, 1995), thus the addition of cholesterol to liquid-crystalline bilayers decreases their permeability, while it increases the permeability of gel phase membranes (Bittman and Blau, 1972; reviewed by Yeagle, 1985). Incorporation of cholesterol in lipid bilayers has substantially improved the efficiency of liposome drug formulations by dramatically reducing solute permeability (Demel *et al*, 1972; Papahadjopoulos *et al*, 1973), reducing interactions with plasma proteins (Moghimi and Patel, 1988; Semple *et al*, 1996), and increasing thecirculation lifetimes of these vesicles (Senior and Gregoriadis, 1982a; Semple *et al*, 1996).

1.2.2 Liposome preparation

Amphipathic lipid molecules dispersed in an aqueous solution spontaneously form lipid bilayer structures known as liposomes. The hydrophobic acyl chains arrange themselves towards the interior of lipid membranes in the most energetically favourable organization. This bilayer organization includes liposomes of a range of size and lamellarity, characteristics by which they are classified.

1.2.2.1 Multilamellar vesicles (MLVs)

Multilamellar vesicles (MLVs) are bilayer structures of a wide size range (1000 - 10,000

nm) with variable lamellarity (see Figure 1.4), which can possess as little as 10% of their lipid in the outermost bilayer (Mayer *et al*, 1985a). The formation of multilamellar vesicles requires only that dry lipid be mixed in an aqueous solution heated above the phase transition temperature of the lipid components (Bangham *et al*, 1965). Mixed lipid vesicles are more uniform in composition if lipids are first dried to a lipid film from a solution in appropriate organic solvents. These vesicles have very low trapped volumes, which can be increased by sequential freezing and thawing which decreases the number of internal lamellae (producing frozen and thawed MLVs or FATMLVs) (Mayer *et al*, 1985a).

1.2.2.2 Small unilamellar vesicles (SUVs)

Small unilamellar vesicles possess a single bilayer of lipid and are generally less than 50 nm in diameter, the lowest limits of size for phospholipid vesicles, these also possess low trap volumes (see Figure 1.4). They can be produced from MLVs using French press (Barenholz *et al*, 1979) or sonication procedures (Huang, 1969). The small radius of SUVs generates considerable strain due to extreme curvature, resulting in unstable vesicle preparations which are subject to fusion forming larger structures (Lichtenberg *et al*, 1981; Wong *et al*, 1982). This instability, low trap volume and increased lipoprotein induced leakage (Scherphof and Morselt, 1984), make SUVs undesirable for use as drug delivery vehicles.

1.2.2.3 Large unilamellar vesicles (LUVs)

The most common vesicle systems used for drug delivery are large unilamellar vesicles (LUVs) (see Figure 1.4). LUVs range from 50-200 nm in diameter, are stable, have high trapped volumes, and relatively long blood circulation lifetimes - all desirable features for *in vivo* delivery of drugs. Techniques to prepare LUVs include reverse phase evaporation (Szoka and Papahadjopoulos, 1978), detergent dialysis (Mimms *et al*, 1981; Madden, 1986), ethanol injection (Chen and Schullery, 1979), ether infusion (Deamer and Bangham, 1976) and more directly using an extruder device (Hope *et al*, 1985). Of all of these techniques, extrusion is most convenient because it is rapid, consistent, and does not lead to problems with residual detergents or organic solvents. The simplicity of the extrusion technique is its greatest strength, MLVs or FATMLVs are repeatedly forced through polycarbonate filters of a defined pore size at relatively high nitrogen pressure, at temperatures above the phase transition temperature of the lipids (Szoka and Papahadjopoulos, 1980; Hope *et al*, 1985; Mayer *et al*, 1986b). This produces large unilamellar vesicles which are uniform, close to the pore size of the filter, and possess a single lipid bilayer. This technique functions over a wide range of lipid composition and concentrations, producing well-defined vesicles ideal for the preparation and study of drug delivery systems *in vivo*.

1.2.3 Drug encapsulation

1.2.3.1 Passive entrapment

Water soluble agents can be encapsulated in liposomes by including them in the aqueous media used to form the liposomes (Szoka and Papahadjopoulos, 1980). Drugs such as doxorubicin

Figure 1.4 Classification of liposomes

MLV   LUV   SUV
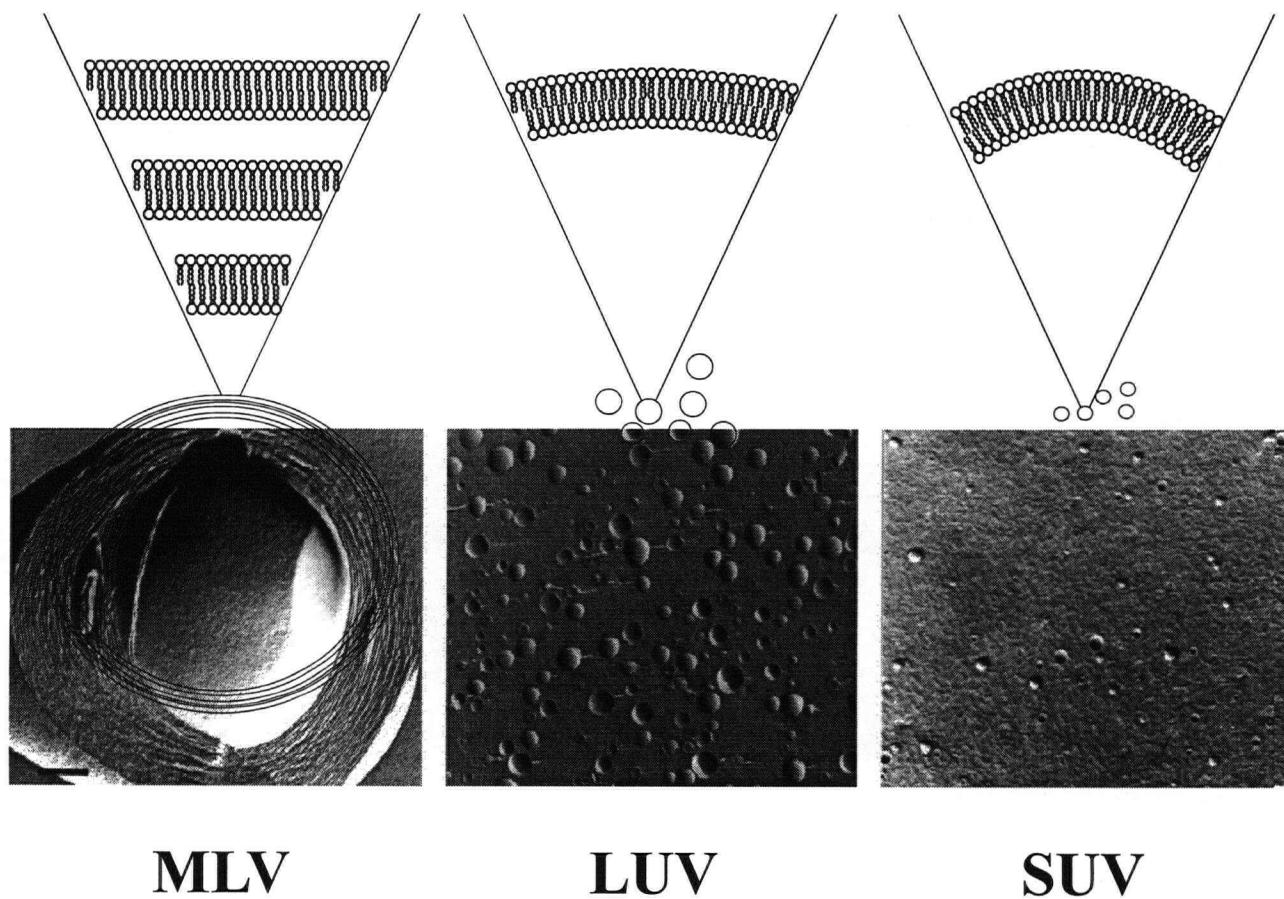
leak out rapidly after loading (Juliano and Stamp, 1975; Mayer et al, 1985b). The efficiency of encapsulation and extent of subsequent leakage was dependent on the particular drug and the composition and size of vesicles used. Hydrophobic molecules can also be loaded during liposome formation as certain lipophilic molecules can directly insert into the bilayer. This can result in trapping efficiencies approaching 100%, but these drugs are often rapidly transferred out exchanging into other lipid membranes upon injection, reducing their potential application (Madden et al, 1990).

1.2.3.2 Active entrapment

The most common system of "active" loading of compounds into liposomes involves the use of a pH-gradient across preformed bilayers to entrap lipophilic drugs with protonatable groups (weak bases) and takes advantage of the ability of the neutral forms of these drugs to rapidly permeate through lipid bilayers. Within the acidic liposome interior the neutral drug molecules are protonated after passage through the bilayer, resulting in charged drug molecules which are trapped inside (see Figure 1.5). A pH gradient with a basic interior can be similarly used to entrap weakly acidic agents (Eastman et al, 1991). Drugs successfully entrapped using this technique have included antineoplastics, local anesthetics, antiarhythmics, antidepressants and antimalarial agents (surveyed by Madden et al, 1990). The loading of doxorubicin was noted to be far more efficient than the residual proton gradient would predict, additionally leakage was considerably slower than the loss of the pH gradient, suggesting a reduction in the soluble aqueous fraction of the loaded drug (Mayer et al, 1990a; Mayer et al, 1990b; Harrigan et al,

1993). Subsequent studies indicated that over 95% of the encapsulated doxorubicin partitioned into the inner monolayer of 100 nm liposomes (represented by BH^+ adjacent to the membrane in Figure 1.5) (Harrigan et al, 1993). This membrane-associated drug would not participate in the soluble drug equilibrium, and would therefore explain the discrepancies between observed results and theoretical behaviour for this drug.

The active entrapment of drugs has also been accomplished using other ion gradient systems such as a valinomycin-dependent K^+ diffusion gradient with a negative intraliposomal potential (Mayer et al, 1985b, 1985c, and 1986a).

1.2.3.3 Doxorubicin - Biological Effects and Liposome Encapsulation

Doxorubicin (Adriamycin) is an anthracycline antibiotic isolated from *Streptomyces peucetius* (see Figure 1.6). It has a wide spectrum of antitumour activity allowing treatment of acute leukemia, non-Hodgkin's lymphoma, breast cancer, Hodgkin's disease, lung carcinomas, and sarcomas (Carter,1975; Young et al, 1981). Acute toxicities to free drug are similar to other anticancer drugs (including nausea, vomiting, diarrhea, stomatitis). The dose-limiting toxicity is myelosuppression (Middleman et al, 1971; Wang et al, 1971), but the cumulative therapy-limiting toxicity is irreversible cardiomyopathy which can result in congestive heart failure in patients who receive a cumulative drug dose exceeding 550 mg/m^2 (Lefrak et al, 1973; Rhinehart et al, 1974). The toxic and antitumour effects of doxorubicin have been attributed to a number of intracellular actions, including inhibition of topoisomerase II and RNA polymerase II (Chuang and Chuang, 1979), intercalation into chromosomal DNA and formation of iron

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Figure 1.5 pH gradient drug encapsulation

Outside Liposome
pH = 7.5

Inside Liposome
pH = 4.0

$$BH^+ \rightleftharpoons BH^+ \qquad BH^+ \rightleftharpoons BH^+$$

$$\Downarrow \qquad\qquad\qquad\qquad\qquad\qquad \Downarrow$$

$$B \longrightarrow B$$
$$+ \qquad\qquad\qquad\qquad\qquad\qquad +$$
$$H^+ \qquad\qquad\qquad\qquad\qquad\qquad H^+$$
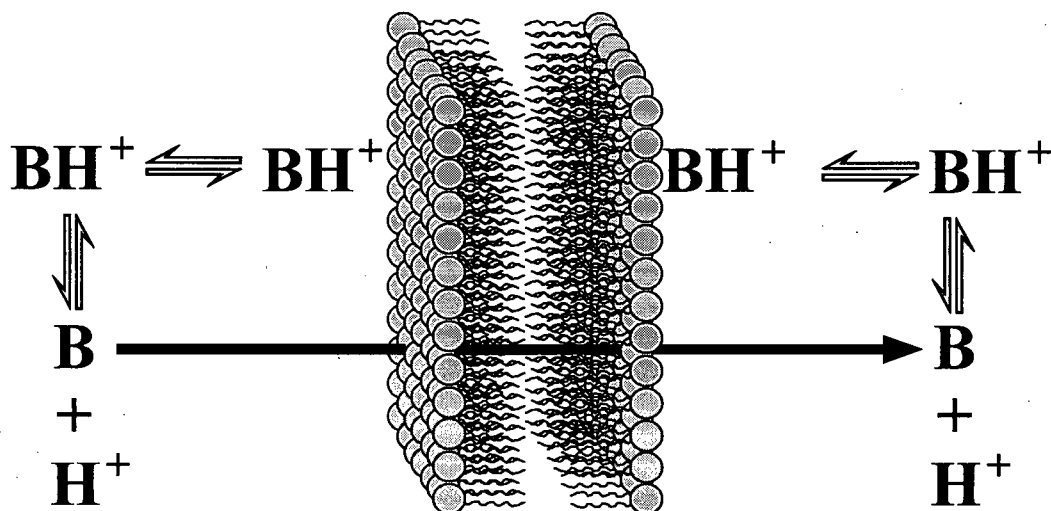
$$K\,a = \frac{[\,B\,]_o[\,H^+]_o}{[\,B\,H^+]_o} \qquad\qquad K\,a = \frac{[\,B\,]_i\,[\,H^+]_i}{[\,B\,H^+]_i}$$

At equilibrium, if : $\qquad [B]_o = [B]_i$

Then : $\qquad \dfrac{[BH^+]_i}{[BH^+]_o} = \dfrac{[H^+]_i}{[H^+]_o}$

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complexes (Neidle *et al*, 1983), and provoking errors in transcription and the formation of reactive oxygen species (free radicals) which can directly damage proteins, lipids and DNA or induce apoptosis (Ling *et al*, 1993). These toxic effects of doxorubicin are most prominent in rapidly dividing cells (such as tumour cells), but are also evident in non-dividing cells (such as Kupffer cells)(Barranco, 1984).

Entrapment of doxorubicin into liposomes has successfully decreased the cardiotoxicity associated with free drug, while maintaining or improving the antitumour activity (Mayer *et al*, 1990; Papahadjopoulos *et al*, 1991; Sakakibara *et al*, 1996; Parr *et al*, 1997). This improved therapeutic potential of doxorubicin has been attributed to a dramatic shift in the distribution of liposome-encapsulated drug, which leads to a 30-fold reduction in the levels in mouse cardiac tissue and to extended blood circulation lifetimes compared to free drug (Gabizon *et al*, 1982; Mayer *et al*, 1989). Clinical trials of liposomal doxorubicin also support these results, giving no evidence of organ toxicity but indicating that myelosuppression is the dose-limiting effect (Treat *et al*, 1988; Treat *et al*, 1990; Cowens *et al*, 1993). One potential concern with liposomal doxorubicin formulations is the reduced function and eventual elimination of the phagocytic cells of the liver (Kupffer cells) and spleen (fixed macrophages)(Daemen *et al*, 1995, Daemen *et al*, 1997). This could lead to reduced elimination of immune particles, septicemia, and even a local decrease in the tumouricidal activity of the liver (Claassen *et al*, 1986; Phillips *et al*, 1989; Delemarre *et al*, 1990; Heuff *et al*, 1993; Daemen *et al*, 1995).

Experimental study has shown increased tumour localization ("passive targeting") of these liposomal systems resulting from extended circulation lifetimes (Forssen *et al*, 1992; Bally *et al*, 1994). Tumour levels have been increased further using long-circulating "sterically stabilized"

Figure 1.6 Structure of doxorubicin
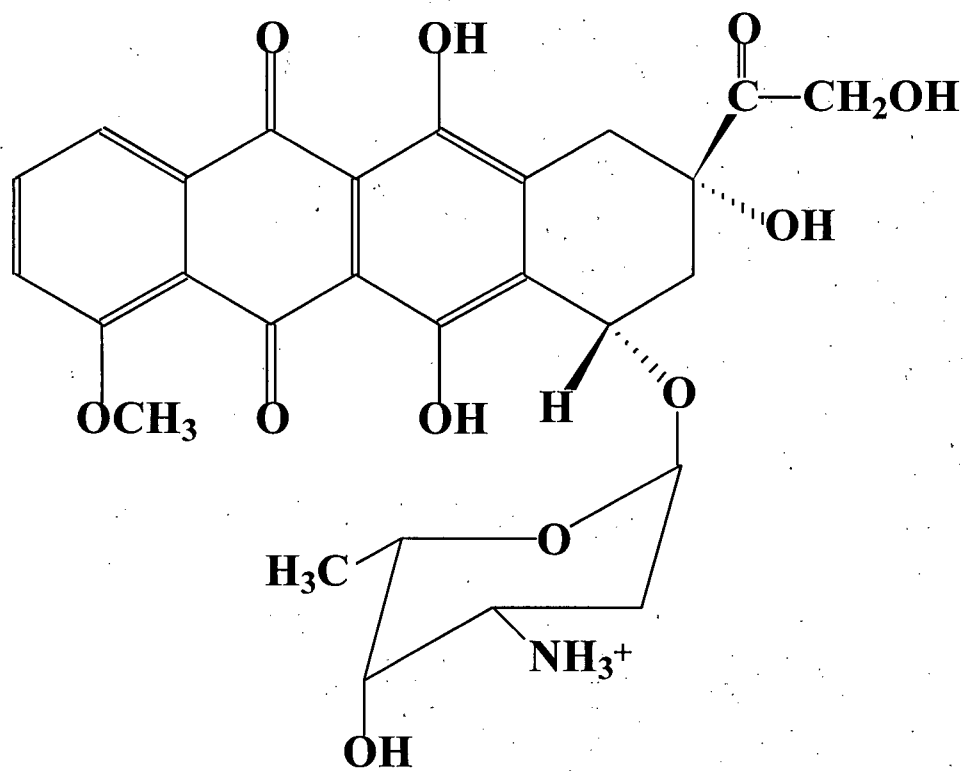
Doxorubicin

liposomes (Gabizon and Papahadjopoulos, 1988; Papahadjopoulos *et al*, 1991; Gabizon, 1992; Huang *et al*, 1992; Wu *et al*, 1993), although one study has suggested that tumour uptake at high doses of liposomal doxorubicin was not further enhanced by steric stabilization of the vesicles (Parr *et al*, 1997).

1.3 Interactions of liposomes with blood proteins

Upon intravenous injection, liposomes rapidly and irreversibly bind a complex coating of blood proteins (Juliano and Lin, 1980; Juliano, 1983). This dramatically alters the membrane surface properties and is crucial in determining subsequent interactions. Understanding this macromolecular adsorption is essential in predicting and understanding the behaviour of liposomes *in vivo*.

1.3.1 Blood proteins and surfaces

The exposure of solid and fluid surfaces to blood results in the rapid binding of a complex profile of proteins, and as early as 1969, it was suggested that no interface can avoid being coated with the most abundant and least stable proteins (Vroman and Adams, 1969b). Surfaces subject to protein binding include fluid-fluid interfaces such as those of cell membranes and liposomes (reviewed by Brash and Horbett, 1995). The forces driving these protein interactions are non-covalent, including hydrogen-bonds, electrostatic and hydrophobic interactions (Brash and Horbett, 1995). Protein size, charge, concentration, structural stability, amphipathicity and

solubility all play significant roles in determining rates and extent of adsorption onto surfaces (Horbett and Brash, 1987; Kochwa *et al*, 1977). At the same time charge, the fluid dynamics, hydrophobicity, and irregularities of the foreign surface can also influence protein binding behaviour (Baier, 1977). These interactions are complex in nature, and results have shown that the interactions of individual serum proteins are also influenced by the presence of other proteins in solution (Brash and Horbett, 1995; Slack and Horbett, 1995). The Vroman Effect postulates a sequence of protein adsorption over time involving binding and then exchange of proteins according to concentration and surface affinity (Vroman and Adams, 1969a; Vroman and Adams, 1969b; Vroman and Adams, 1987). This original work studied the adsorption and subsequent displacement of fibrinogen, but it was later demonstrated that the Vroman Effect is a more general phenomenon that reflects the competitive binding of a range of proteins to a limited number of available binding sites (Slack and Horbett, 1995). In plasma or serum these protein-protein interactions and changes occur so quickly that they cannot even be detected, and so are not important interactions within whole blood, plasma, or serum (Vroman and Adams, 1969; Vroman and Adams, 1987; Slack and Horbett, 1995).

The binding of proteins is not only rapid, but is also irreversible (Brash and Horbett, 1995). This has been attributed to the multiple surface attachments involved in binding a single protein to a surface, as it is highly unlikely that all of the binding sites for one protein would dissociate simultaneously, the adsorbed molecules are not released (Morrissey, 1977; Horbett and Brash, 1987). Over time, "relaxation" or spreading of the surface-adsorbed proteins results in even stronger and more numerous interactions (Brash and Horbett, 1995). Related to this idea of protein "relaxation" is the suggestion that stability can partially determine the affinity of protein

binding. Less stable proteins are proposed to denature faster and more significantly and therefore bind with higher affinity due to greater numbers of potential interaction sites (reviewed by Brash and Horbett, 1995).

It is generally agreed that all protein interactions involve some degree of unfolding, the most recent work suggests that the extent of conformational change experienced is highly specific to the individual protein. Based on circular dichroism spectra of adsorbed proteins, "soft" or easily denatured proteins (like albumin and hemoglobin) extensively change their structure upon adsorption, while the "hard" or more stable proteins (like ribonuclease) experience almost no change (Kondo *et al*, 1991). A variety of different conformations of adsorbed proteins could be present depending on conditions (Horbett and Brash, 1987), and these adsorbed protein could still retain their biological activity (Kochwa *et al*, 1977). Interestingly, the increased mobility of proteins adsorbed at fluid-fluid interfaces (like the surface of cell membranes or liposomes) have been described as having increased rates of reorientation, diffusion and protein conformational changes (Brash and Horbett, 1995).

1.3.2 Blood proteins and liposomes

Liposomes rapidly and irreversibly adsorb a coat of blood proteins upon intravenous injection, which determines their fate by modifying surface properties and through the action of specific proteins (opsonins) which enhance the phagocytic elimination of these vesicles (Juliano and Lin, 1980; Juliano, 1983). Destabilization and leakage of vesicles correlates well with increased protein binding, and is dependent upon lipid charge and composition (Hernandez-

Caselles *et al*, 1993). *In vivo* isolation techniques using spin columns have established a direct relationship between the amount of protein bound *in vivo* and reticuloendothelial system uptake of liposomes. This has been attributed to the presence of opsonins among the bound blood proteins (Chonn *et al*, 1992; Semple *et al*, 1995). These mouse studies have also confirmed the presence of liposome-bound immunoglobulin and complement opsonins, and identified β2-glycoprotein-I bound to rapidly cleared liposomes establishing its potential role in RES uptake (Chonn *et al*, 1992; Chonn *et al*, 1995).

Protein interactions with lipid vesicles have been divided into three distinct types based on the nature of attraction and degree of bilayer penetration (Juliano, 1983):

1) Charge-dependent or electrostatic interactions.

2) Charge-dependent interactions with some penetration of the membrane.

3) Hydrophobic interactions with significant bilayer penetration and disruption.

The liposome characteristics which influence these protein interactions include phospholipid composition, phase transitions, cholesterol content, vesicle size, surface charge, and hydrophobicity. Phospholipid fluidity and packing determines the presence of defects in the membrane surface and it is well accepted that surface defects allow proteins to penetrate and interact with the hydrophobic fatty acyl chains of the bilayer interior (Larrabee, 1979; Schullery *et al*, 1980). Solid liposomes contain phospholipids with saturated, long-chain fatty acids (DSPC, SM) and experience decreased protein interactions which corresponds to their reduced RES uptake and decreased permeability (Gregoriadis and Senior, 1980; Senior and Gregoriadis, 1982b). This

has been attributed to the close spacing of phospholipid headgroups due to reduced acyl chain spreading. Unsaturation results in widely spaced acyl chains, and thus larger gaps between headgroups which allow easier protein insertion. Bilayers in the gel state or with mixed phases of membrane lipid possess irregularities along their surface, especially at phase boundaries, which increase the potential for proteins to penetrate (Scherphof *et al*, 1984). For example, long chain, saturated phospholipid liposomes (DSPC, DPPC, and DAPC) are in a gel state at 37°C and their rough membrane surface has defects which allow high levels of protein binding and very rapid blood elimination (Kirby *et al*, 1980; Semple *et al*, 1996). The incorporation of cholesterol acts to remove the gel-liquid phase transition, increasing the packing density of phospholipids and removing the defects in the bilayer surface which would otherwise allow protein insertion (see Section 1.2.1.2). *In vitro* results have confirmed that cholesterol decreases permeability (Demel *et al*, 1972; Papahadjopoulos *et al*, 1973) and decreases plasma protein binding (Papahadjopoulos *et al*, 1973; Moghimi and Patel, 1988). *In vivo* studies have also shown that protein binding continues to decrease even up to 30 mol % cholesterol, and that this decrease is nonspecific with regard to protein identity (Semple *et al*, 1996). Small vesicles (SUVs) with a high degree of curvature in the bilayer are suggested to possess larger gaps between the lipid headgroups, which similarly provides natural sites for protein interaction (Juliano, 1983; Scherphof *et al*, 1984). Experiments have confirmed this through increased sensitivity of SUVs to lipoprotein-induced damage (Juliano, 1983; Scherphof and Morselt, 1984). The presence of a negative surface charge can either increase or decrease interactions with blood proteins. Liposomes containing negatively charged lipids such as DOPS, DOPA, and CL are rapidly cleared by the RES (Gregoriadis and Neerunjun, 1974; Juliano and Stamp, 1975; Chonn *et al*, 1992), and all experience significant

blood protein interactions, including interactions with known opsonins (Chonn et al, 1992). In contrast, the inclusion of PG and PI led to reduced protein binding and RES uptake (Chonn et al, 1992). Proteins interact more extensively with surfaces of increased hydrophobicity (Senior, 1987; Patel, 1992), and for this reason attempts to reduce protein adsorption have focused on the addition of hydrophilic molecules such as polyethyleneglycol polymers and monosialoganglioside G_{MI}. Evidence of reduced protein interactions has at least partially been attributed to the hydrophilic nature of these components (Senior et al, 1991; Chonn and Cullis, 1992; Blume and Cevc, 1993).

The complex nature of interactions of blood proteins with liposomes in vivo is likely to be very difficult to reproduce in vitro. Work in other areas of blood protein interactions have shown that maintaining accurate protein concentrations and the presence of competing proteins are essential in obtaining an accurate representation of "true" protein adsorption (see Section 1.3.1). Previous work using in vitro serum incubations of liposomes has revealed numerous problems, including evidence against the opsonizing action of serum (Ellens et al, 1982; Juliano, 1982; Dijkstra et al, 1984); a significant dependence on the amount, source, and even individual samples of serum used (Moghimi and Patel, 1989a; Patel, 1992); and variations in attempts to quantify and identify the proteins interacting with liposome formulations (Black and Gregoriadis, 1976, Juliano and Lin, 1980, reviewed by Patel, 1992). Additional consideration must also be given to the evidence of liposome interactions only possible in vivo, including those with a variety of blood cells (reviewed in Section 1.4.1.2) and clotting factors (see Section 1.3.3.6). For liposome interactions, in vivo study appears to be the most suitable indicator of "true" behaviour, suggesting that the conditions of in vitro studies must be carefully considered when interpreting

results.

1.3.3 Interactions with specific blood proteins

1.3.3.1 Lipoproteins

Phospholipid exchange to lipoproteins and membrane disruption especially of negatively charged vesicles has been attributed to interactions with VLDL, LDL, and HDL lipoprotein particles (Chobanian *et al*, 1979; Damen *et al*, 1980; Tall and Green, 1981; Shahrokh and Nichols, 1982). Transfer of phospholipid to LDLs and VLDLs involved less lipid than with HDLs and occurred only at higher liposome doses (Chobanian *et al*, 1979; Shahrokh and Nichols, 1982). The addition of sufficient cholesterol was found to protect vesicle stability, with 37 mol % completely eliminating leakage due to lipoprotein interactions (Demel *et al*, 1972; Guo *et al*, 1980; Juliano, 1983). A variety of apolipoproteins have also been found to adsorb to liposomes *in vitro* and *in vivo*, including Apo AI, AII, AIV, B, CIII, E, and H (Nichols *et al*, 1978; Guo *et al*, 1980; Tall and Green, 1981; Mendez *et al*, 1988; Chonn *et al*, 1995). This provides additional support for the role of the protein components of lipoproteins being significant factors in the interaction of blood components with liposomes. There is also some evidence that these apoproteins undergo conformational changes upon binding (reviewed by Juliano, 1983). The exact mechanism of lipoprotein-induced leakage is still not established, as experimental results have attributed it to both pore formation (Kirby and Gregoriadis, 1981) and the complete destruction of liposome structure (Scherphof and Morselt, 1984).

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1.3.3.2 Immunoglobulin

The involvement of immunoglobulins, especially IgG, in the elimination of foreign particles is well established (Holm *et al*, 1974; Absolom *et al*, 1982; reviewed by Patel, 1992). Recognition by phagocytic cells and subsequent processing occurs through the Fc-portion of the molecule, which has been shown to enhance uptake through receptor-mediated endocytosis via clathrin-coated pits (reviewed by Patel, 1992). Receptors to the Fc moiety have been found on macrophages, granulocytes, lymphocytes and some epithelial cells. Liposomes have been shown to bind native and heat-aggregated immunoglobulin, which has resulted in increased uptake by macrophages and even induced leakage of negatively-charged vesicles (Weissmann *et al*, 1974; Weissmann *et al*, 1975; Juliano and Lin, 1980; Hsu and Juliano, 1982; Senior *et al*, 1986). When liposomes are covalently attached to IgG they also experience increased phagocytosis, as seen with 5-fold increases in the uptake of immunoliposomes by Kupffer cells (Derksen *et al*, 1987; reviewed by Patel, 1992). *In vivo* analysis of the blood proteins bound to a variety of lipid compositions has identified a significant level of IgG bound to liposome compositions experiencing rapid elimination by the RES (Chonn *et al*, 1992; Chonn and Cullis, 1992). This includes primarily negatively charged vesicles containing PA, PS, or CL. No immunoglobulin was bound to neutral PC:CH vesicles or those containing plant PI, PG, or SM and G_{M1}, which all experienced longer circulation lifetimes. These observations suggest the possible role of IgG-mediated elimination of rapidly cleared liposomes *in vivo*.

In addition to nonspecific association of immunoglobulin, there is evidence of specific anti-phospholipid antibodies directed against negatively charged phospholipids. These are often

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referred to as anti-cardiolipin antibodies (ACA), but have been shown to bind all negatively charged lipids (PA, PS, PI, PG, and CL)(Gharavi *et al*, 1987; Pengo *et al*, 1987; McNeil *et al*, 1989). Anti-phospholipid antibodies have been detected in patients with systemic lupus erythematosus, immunological, neoplastic, or infection disorders, as well as apparently healthy individuals (Alving, 1984; Lechner, 1987; Cheng *et al*, 1989). Anti-phospholipid antibody action appears to be dependent on a serum cofactor, which has been identified by molecular weight analysis and partial sequencing to be β_2-glycoprotein-I (apolipoprotein H)(Galli *et al*, 1990; McNeil *et al*, 1990; Cheng, 1991). Immunoblot analysis, purification and sequencing has identified a liposome-bound protein which is the murine equivalent of β_2-glycoprotein-I (β_2GPI). It has been identified *in vivo* as a major protein bound to negatively charged liposomes (PA, CL, and PS)(Chonn *et al*, 1995), suggesting its involvement in rapid phagocytosis, either directly via β_2GPI receptors or through its role as a cofactor in the binding and action of antiphospholipid antibodies.

1.3.3.3 Complement Components

Complement interactions with liposomes have been extensively studied, and have shown significant dependence on liposome characteristics and the species of serum used. Neutral liposomes fail to activate and bind complement regardless of the species studied (Chonn *et al*, 1991b; Devine *et al*, 1994). Use of rat serum as a complement source results in activation of the classical pathway for both positively (SA, DOTAP) and negatively (PA, PG, PI, PS, and CL) charged lipids (Devine *et al*, 1994). While guinea pig or human serum both result in complement

activation via the alternative pathway by positively charged liposomes, the classical pathway still remains the route by which negative lipids act (Cunningham *et al*, 1979; Chonn *et al*, 1991b). In human serum this activation of the classical pathway by negative lipids has been shown to be immunoglobulin-independent (Marjan *et al*, 1994). Additional factors which enhance activation of the classical pathway have included the use of more saturated fatty acyl chains (Shin *et al*, 1978; Chonn *et al*, 1991b; Devine *et al*, 1994), increased membrane cholesterol content (Devine *et al*, 1994), and use of larger vesicles (400 nm versus 50 nm)(Devine *et al*, 1994).

Leakage of contents and decreased stability of liposomes has been suggested to result from complement activation and the subsequent assembly of the membrane attack complex (MAC) in the liposome bilayer (Malinski and Nelsestuen, 1989; Chonn *et al*, 1991b). There is evidence that the components of the attack complex (C5b-C9) are bound to liposomes and form a stable pore within the lipid membrane which results in leakage.

The role of complement components in the opsonization of foreign particulate matter has been well established (reviewed by Patel, 1992), with the primary opsonin being the C3b product of complement activation. The human receptors for complement have also been identified and are located on numerous cell types (see Table 1.1), with CR1 being of primary interest due to its role in C3b-mediated uptake. The CR1 receptor on inactive macrophages encourages adhesion of particles (enhancing uptake by other receptors), while on active macrophages either CR1 or CR3 receptors are sufficient to induce phagocytosis (Griffen, 1988). *In vivo* elimination of liposomes of different compositions have also been shown to correlate with the amount of bound blood protein and specifically the presence of surface adsorbed complement C3b (Chonn *et al*, 1992), and these same rapidly cleared vesicles have shown the greatest ability to activate

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complement *in vitro* (Devine *et al*, 1994). Although species variation of complement exists, studies have shown substantial functional and structural similarities in the complement pathways, essentially concluding that mammal complement systems may be composed of the same constituent proteins, but that minor changes in the structures of the individual proteins exist (Whaley, 1985). More important differences may exist the complement receptors which mediate opsonin (C3) uptake. A review of differences between CR1, CR2 and the mouse receptors MCR1 and MCR2 (mouse complement receptors 1 and 2), has revealed several differences. Sequencing studies indicate that MCR2 is very similar to CR2 in humans, but that MCR1 is unlike CR1, but instead resembles CR2 with additional short consensus repeats which are similar to CR1 (Holgun *et al*, 1990; Molina *et al*, 1990). MCR1 but not CR1 binds C3dg (Molina *et al*, 1992), the intracytoplasmic domain of these two receptors are also different (although MCR1 is similar to CR2), neither MCR1 nor MCR2 are present on mouse platelets despite their binding in mice to complement-coated particles, it is also not clear whether mouse C4 or C4b interact with MCR1 or MCR2 or whether either expresses decay accelerator activity (reviewed by Holers *et al*, 1992). There is another aspect of ligand interactions that differs between human and mouse complement receptors, CR1 and CR2 can co-associate in humans to provide a site of complement binding, while in mice it seems that a single receptor can bind the same forms of C3 observed by the two different receptors in the human (reviewed Holers *et al*, 1992). Despite these differences the high degree of similarity of the early components of the complement cascade and similar mechanisms of the complement activation and opsonization pathways, suggests that the mouse system can be used for general human comparison, but that the known differences must be considered with regard to any specific conclusions drawn.

Table 1.1 Human Complement receptor locations and targets

Receptor	Ligands	Cellular Distribution
CR1	C3b, iC3b, C3c, C4b, C5b	B cells, neutrophils, monocytes, macrophages, erythrocytes, follicular dendritic cells, glomerular epithelial cells, T cells, K cells, eosinophils, basophils, mast cells
CR2	iC3b, C3dg	B cells, follicular dendritic cells, epithelial cells of cervix, epithelial cells of nasopharynx
CR3	iC3b	monocytes, macrophages, neutrophils, NK cells, follicular dendritic cells, eosinophils, basophils, mast cells
CR4	iC3b	neutrophils, monocytes, tissue macrophages

(Dietrich, 1988; Griffen, 1988; Roit *et al*, 1989)

1.3.3.4 C-Reactive Protein

Interactions of C-reactive protein with liposomes, although clearly significant, is highly dependent on the lipid components of the vesicles. Binding was greater to PC-liposomes with positively charged SA and also negatively charged lipid compositions (DCP). Binding was particularly high upon the addition of certain ceramides, but little or no CRP bound to neutral vesicles (Richards *et al*, 1977 and 1979). CRP binding also correlated with complement activation and complement-dependent membrane damage, which were also strongest for positively charged liposomes. The presence of increasing cholesterol (especially over 30 mol %) and increased fatty acyl chain unsaturation increased complement consumption and membrane damage, while longer chain fatty acids decreased these responses (Richards *et al*, 1979). Other results revealed that although cholesterol increased subsequent complement consumption, it actually decreased the quantity of bound CRP (Mold *et al*, 1981; Tsujimuto *et al*, 1981). These responses were

suggested to involve changes in membrane fluidity.

CRP can function as an opsonin (likely requiring complement participation), as demonstrated by increased uptake of MLVs with bound CRP by peritoneal macrophages (Barna *et al*, 1984). CRP-MLVs have also been shown to activate macrophages, enhancing their ability to suppress tumour growth and metastasis (reviewed by Patel, 1992).

1.3.3.5 Fibronectin

Fibronectin is also suggested to have an opsonic role in the elimination of liposomes, macrophage uptake of fibronectin-bound liposomes increased up to 10-fold depending on the vesicle composition (Hsu and Juliano, 1982). Fibronectin-mediated uptake has been observed in blood monocytes, polymorphonuclear leukocytes, peritoneal macrophages, and Kupffer cells (reviewed in Patel, 1992). This uptake has been attributed to interaction with RGD-specific cell receptors for the arginine-glycine-aspartic acid (RGD) type III homology segment in the middle of the fibronectin molecule and cell surface proteoglycan interacting with the heparin binding site of fibronectin (reviewed by Ruoslahti, 1988 and Patel, 1992). Other results have shown that fibronectin causes significant aggregation of liposomes with altered protein structure upon binding (reviewed by Bonte and Juliano, 1986).

1.3.3.6 Clotting Factors

Liposomes, especially those with negatively charged lipids like PS and PA, have been

observed to strongly bind a variety of clotting factors, including Factor VII, VIII, Xa, XII, and prothrombin, possibly even depleting them from plasma (Juliano and Lin, 1980; Juliano, 1983; Bonte and Juliano, 1986; Kemball-Cook and Barrowcliffe, 1992). The effects of liposomes on clotting factors are consistent with the known role of damaged tissue and platelet surfaces and of negatively charged phospholipids in the activation of the coagulation cascade (Juliano and Lin, 1980). The presence of significant but not excessive quantities of PS in liposomes has also been observed to activate the clotting pathway (Zwaal et al, 1977; Juliano, 1983). The involvement of these interactions in determining the biological fate of liposomes is unknown.

1.3.3.7 Albumin

As the most abundant blood protein, it is not unexpected that albumin has been identified interacting with some liposome formulations (Hoekstra and Scherphof, 1979; Juliano and Lin,1980; Comiskey and Heath, 1990; Chonn et al, 1995). The results of albumin binding are not yet certain, but it has been shown to increase vesicle leakage (Kiwada et al, 1988; Comiskey and Heath, 1990). Binding of albumin has also been shown to reduce hepatocyte (Hoekstra and Scherphof, 1979) and lymphocyte uptake of liposomes in vitro (Blumenthal et al, 1977). However, there is evidence that protein contaminants (apo-A-I and C-II) present in commercially available albumin preparations could be responsible for these effects (reviewed by Scherphof et al, 1981). Consequently, the direct role of albumin in liposome protein interactions has not yet been fully elucidated.

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1.3.3.8 α- and β- globulins

An early investigation into the identity of liposome bound proteins identified α_2-macroglobulin as the only protein bound to several positively charged, negatively charged and neutral lipid compositions after plasma incubation and washing (Black and Gregoriadis, 1976). Purified α- and β-globulin fractions bind to liposomes in significant quantities and resulted in decreased hepatocyte uptake *in vitro* (Hoekstra *et al*, 1979). However, the presence of β-globulins have also been shown to increase the uptake of anionic liposomes perfused through rat liver (Tyrrell *et al*, 1977), and more recently increased the uptake of neutral, cholesterol containing MLVs by bone marrow macrophages (Bumrah and Patel, 1994). These results have suggested a particular importance for cell type with regard to the changes caused by the binding of the components of the different globulin fractions.

1.3.3.9 Dysopsonins

The existence of dysopsonins, or proteins which act to reduce uptake by the RES, has been proposed by Moghimi and Patel in their *in vitro* investigations of liposome uptake (Patel *et al*, 1983; Moghimi and Patel, 1989a). Although their identity has not yet been established, they have suggested that potential dysopsonic proteins precipitate in the 0-35% (γ-globulin) and the 50-65 % (crystalline albumin and small molecular weight proteins) range of ammonium sulfate fractions (Moghimi and Patel, 1993). Interactions with dysopsonins were also suggested to explain unusual uptake results obtained for polymer-coated polystyrene microspheres (Moghimi *et al*, 1993) and

G_{M1}-liposomes (Park and Huang, 1993). Dysopsonins could exist, however, the current experimental evidence is inconclusive and additional study is required to confirm their presence and involvement in the fate of liposomes *in vivo*.

1.3.3.10 Tissue Specific Opsonins

Moghimi and Patel (Moghimi and Patel 1988, 1989b, and 1990) have proposed that liposomes with different compositions experience different patterns and rates of uptake because of varying affinity for organ-specific opsonins. In attempts to blockade the RES with liposomes, discrepancies in the uptake of cholesterol-poor and cholesterol-rich liposomes by the spleen were observed (Dave and Patel, 1986). These discrepancies were attributed to certain blood proteins which selectively bound each type of liposome and then favourably targeted one of the two major RES organs, as demonstrated through *in vitro* uptake by Kupffer cells and splenic macrophages (Moghimi and Patel, 1988). The liver-specific opsonin is suggested to be a heat stable protein which is inhibited by the presence of calcium (Moghimi and Patel, 1989b and 1990). The proposed spleen-specific opsonin is heat-labile, possibly involving more than one molecule, and experiences an irreversible loss of activity upon removal of a dialysable factor from serum (Moghimi and Patel, 1989b). Although a significant amount of *in vitro* work has been conducted in the study of these still unidentified opsonins, purification of these serum components and *in vivo* examination is still required to support their existence. Another specific clearance mechanism which does not appear dependent on protein binding involves scavenger receptors, which allow macrophages to recognize specific particles including modified-LDL molecules, apoptotic cells

and negatively charged liposomes through the presence of their negatively charged lipid components (Nishikawa *et al*, 1990; Fukasawa *et al*, 1996). This type of receptor supports the possibility of other mechanisms (like the proposed tissue specific opsonins) which could identify specific lipid components and direct their behaviour *in vivo*, although these other systems have not as yet been conclusively proven.

1.4 Elimination of liposomes from the blood

One of the primary requirements and major barriers in the effective use of liposomal drug carriers is maintaining their presence in the circulation. Even vesicles composed of completely naturally occurring lipids are efficiently removed from the blood primarily by the phagocytes of the reticuloendothelial system. For this reason, considerable research has focused on the nature of liposome-cellular interactions and the mechanisms which control them.

When considering the *in vivo* fate of liposomes, one must consider the physical structure of the capillary walls which limits the extravascular movement of liposomes (see Figure 1.7). Most tissues contain continuous capillaries (including skeletal, cardiac, and smooth muscle), with a continuous layer of endothelial cells and an intact basement membrane. Most endocrine glands, the intestinal villi, and kidney glomerulus contain fenestrated capillaries, with 20-100 nm fenestrae enclosed by a thin membrane. The only natural gaps in the capillary wall to allow extravasation of liposomes are in the discontinuous capillaries of the liver, spleen and bone marrow, which have large (> 100 nm) spaces in the endothelial cell layer, and either a discontinuous or absent basement membrane. In addition to the role played by vascular structure,

a variety of potential routes of extravascular transport exist for molecules and particles in the blood, these include diffusion, transport through cell junctions, through endothelial fenestrae, and vesicular transport mechanisms (see Figure 1.7)(Jain, 1987; Kohn *et al*, 1992).

1.4.1 Liposome interactions with cells

A variety of interactions occur between liposomes and cells (reviewed by Weinstein, 1981 and Pagano *et al*, 1981) including: lipid exchange, adsorption, endocytosis, and fusion (see Figure 1.8). The first three of these are quite common, although fusion appears to be a rare event *in vivo* (New *et al*, 1990; Jones and Chapman, 1995).

1.4.1.1 Interactions with the reticuloendothelial system

The reticuloendothelial system (RES), also referred to as the mononuclear phagocyte system (MPS), is composed of monocytes, macrophages and their precursor cells present in the blood, liver, spleen, lungs, bone marrow, and other sites within the body. Cells of the RES have a wide variety of functions, including the removal of dead, senescent, foreign or altered cells and foreign particles; regulation of other cells functions; processing and presentation of antigens in immune reactions; participation in inflammatory reactions; and destruction of microbes and tumour cells. Considering established RES functions, it is not surprising that liposomes are predominantly taken up by the liver and spleen, and a great deal of work has gone into understanding the mechanisms involved.

Figure 1.7 Capillary endothelial structure and extravasation

The three types of capillary endothelial vascular structure and mechanisms of transport. (adapted from Jain, 1987; Poste, 1984; Parr, 1995).

1 = Direct diffusion across endothelial cells
2 = Lateral membrane diffusion
3 = Interendothelial transport (a = narrow; b = wide)
4 = Endothelial fenestrae (a = closed; b = wide)
5 = Vesicular transport (a = transcytosis; b = vacuolar-vesicular organelles (channels))

Lipophilic Solutes: 1, 2, 3, and 4
Hydrophilic Solutes: 3, 4, and 5
Macromolecules: 3, 4, and 5
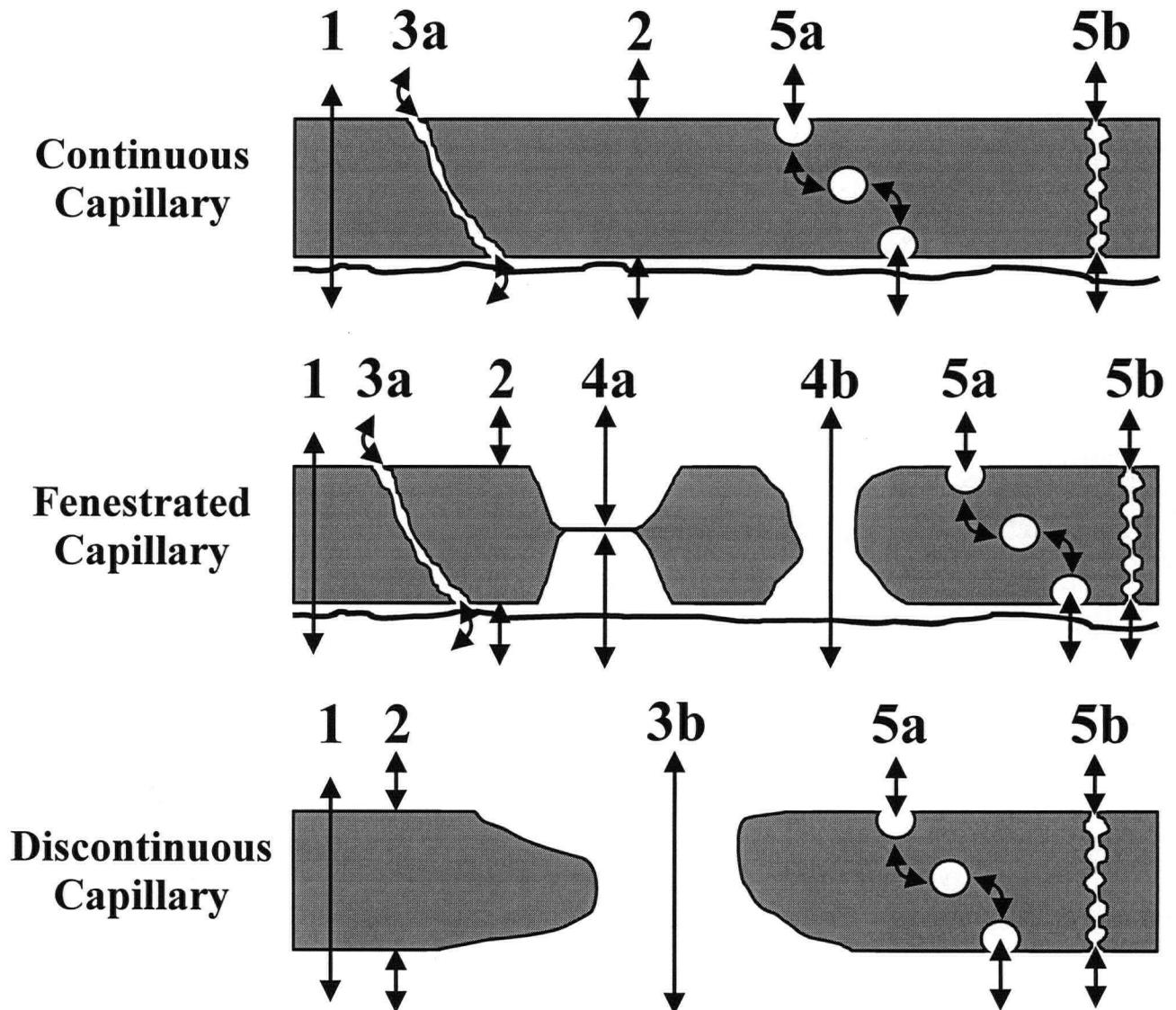
Figure 1.8 Liposome-cell interactions

Endocytosis

Fusion

Fusion with
Lysosome

Degradation in
Phagolysosome

Adsorption

Lipid
Exchange
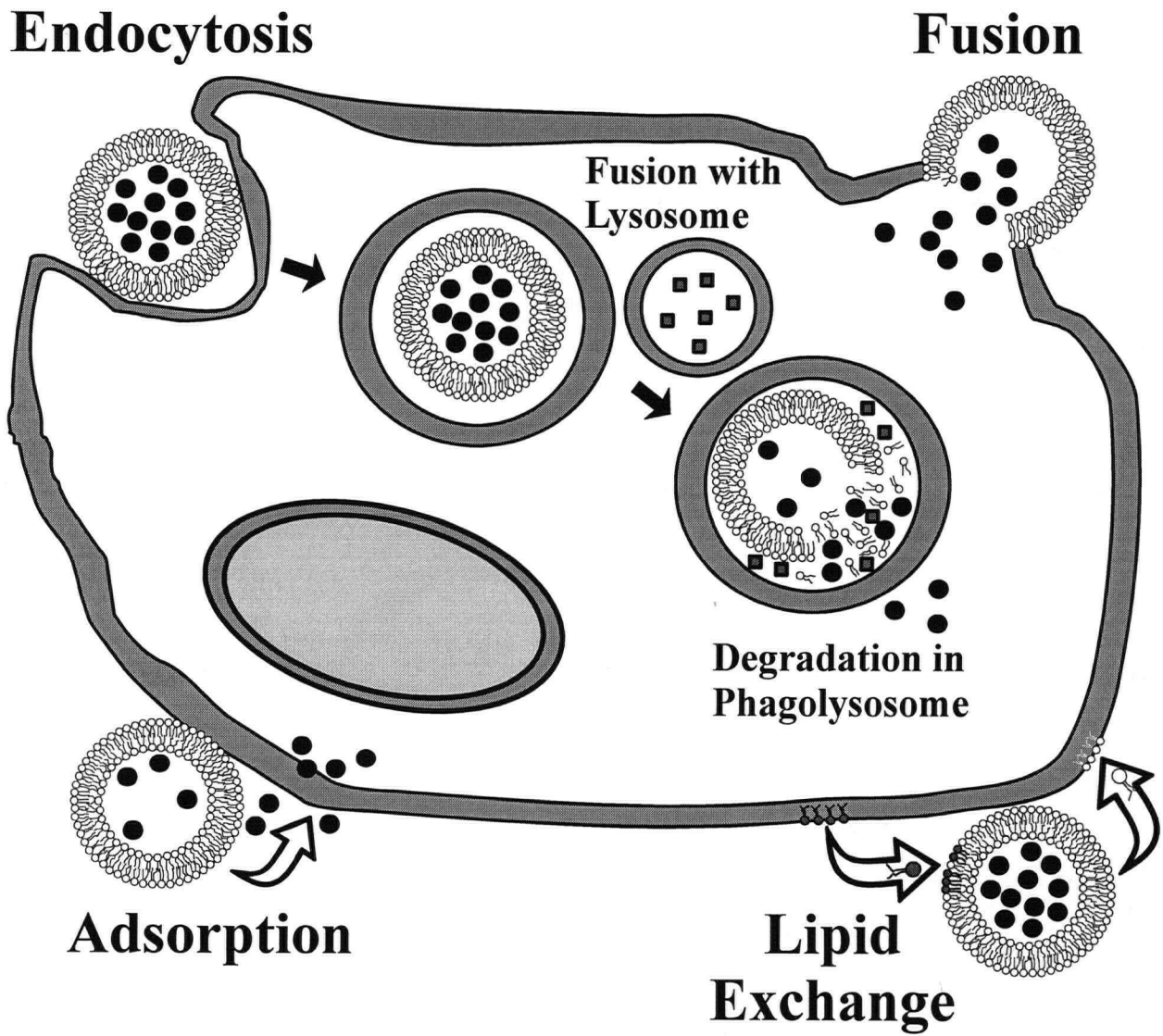
Due to its size and relatively high blood flow the liver is the predominant site of phagocytosis, and a large number of resident tissue macrophages are located within this organ (Weinberg and Athens, 1993). Liver macrophages or Kupffer cells line the sinusoids either attached to or embedded within the endothelium, in an ideal location to encounter foreign particles in the circulation (Weinberg and Athens, 1993). It has been established that phagocytosis of particles is mediated through interactions with Fc and complement receptors among others (reviewed by Weinberg and Athens, 1993). These cells have also been clearly established as the primary site of liposome uptake (Roerdink *et al*, 1981; Poste *et al*, 1982). Participation of parenchymal cell uptake (hepatocytes) has also been indicated, but is limited to smaller vesicles (<100 nm) which can successfully extravasate through pores of the discontinuous endothelial lining of the liver and gain access to these cells (Roerdink *et al*, 1981; Poste *et al*, 1982; Spanjer *et al*, 1986). Hepatocyte uptake of liposomes could be substantial depending on the size and dose of liposomes injected. Elimination of liposomes by the liver has been shown to be dependent upon their interaction with plasma proteins, as plasma-free vesicles were taken up in very limited numbers using a rat liver perfusion model (Kiwada *et al*, 1986 and 1987). In addition, Moghimi and Patel characterized different organ-specific opsonins which they propose are responsible for the variations in uptake by the liver, spleen, and other sites of the reticuloendothelial system (Moghimi and Patel, 1988, 1989a, 1989b, 1990, 1992, and 1996). Although these and other experiments have attempted to identify the proteins responsible for liposome elimination we still do not understand this process in detail (see also Section 1.3).

The spleen acts partially like a filter for the blood and plays a crucial role in the generation of immune response. It contains 20-30% of the lymphocyte population (Timens and Poppema,

1985; Van Kreiken and Velde, 1986) which explains its suggested role as the major site of antibody generation (Borek, 1986). Its structure includes masses of reticular cells and fibers that create a natural blood filter which is ideal for assisting phagocytosis by tissue macrophages and the stimulation of immunity (Rosse, 1987; Athens, 1993). The small size (reduced total number of phagocytic cells) and relatively low blood flow of the spleen, explain its less significant role in particle elimination compared to the liver, despite the fact that on a per cell basis more liposomes are taken up by the spleen. Substantial rapid elimination by the liver prevents high levels of blood particles from getting to the spleen's phagocytic cells. Although the same opsonin-mediated mechanisms of phagocytic clearance are carried out by the tissue macrophages of the spleen, the total amount of organ uptake depends on the total number of phagocytic cells and the amount of blood flow to the organ. The role of the spleen in liposome uptake does become more significant for larger vesicles (Klibanov et al, 1990; Liu et al, 1991; Litzinger and Huang, 1992), because they are retained more effectively in the reticular filter structures (Claassen and Van Rooijen, 1984). Also, any changes which reduce the effectiveness of liver phagocytosis increase the concentration of liposomes in the blood and result in a "spillover effect" which increases spleen uptake (Bradfield, 1974). This change in biodistribution occurs because the amount of liposomes available to the spleen is initially dependent on the amount taken up by the liver, this effect has been observed for increasing lipid dose or predosing with liposomes (Abra et al, 1980; Abra and Hunt, 1981; Dave and Patel, 1986) and the use of sterically stabilized or long-circulating liposomes (Klibanov et al, 1991; Litzinger and Huang, 1992).

1.4.1.2 Interactions with circulating blood cells

In addition to the considerable uptake by the phagocytes of the RES, liposomes in the circulation can interact with other cell types. Uptake of MLVs has been observed for human monocytes and neutrophils, but not human lymphocytes (Weissmann *et al*, 1975; Finkelstein *et al*, 1981; Kuhn, *et al*, 1983). Sonicated vesicles containing dioleoyl lecithin were taken up by human lymphocytes, although this was blocked by serum, suggesting that it would not be relevant *in vivo* (Blumenthal *et al*, 1977). There is also evidence that MLVs containing SA, PS or PG interact with and transiently decrease circulating blood platelets in rats, but not humans (Reinish *et al*, 1988; Loughry et al, 1990). A role for red blood cell interactions with liposomes has been suggested due to the increased stability of liposomes in whole blood of rats and mice (Gregoriadis and Davis, 1979; Kirby *et al*, 1980), although no evidence of stable erythrocyte-liposome complexes has been found. These interactions clearly depend on the exact characteristics of the injected liposomes, but could still have potentially significant physiological implications.

1.4.2 Mechanism for Cellular Uptake of Liposomes

1.4.2.1 Protein-mediated uptake

The elimination of foreign particles from the circulation has long been known to involve interactions with opsonic blood proteins. This same mechanism of elimination has also been supported in the elimination of liposomes (Scherphof *et al*, 1981; Bonte and Juliano, 1986; reviewed by Patel, 1992). The essential role of blood proteins has been confirmed by rat liver perfusion studies which showed little or no liver uptake of vesicles formed by reverse-phase

evaporation in the absence of serum (Kiwada *et al*, 1986 and 1987). Opsonin action can involve specific receptor-mediated binding of liposomes to phagocytes (immunoglobulins, fibronectin, and complement proteins) and nonspecific increases in the adhesiveness of the liposome surfaces with cell surfaces.

1.4.2.2 Protein-independent uptake

Despite the overwhelming evidence in favour of a protein-mediated mechanism of liposome uptake similar to that observed for other foreign blood particulates, advocates of alternative mechanisms still remain. Studies using a variety of cultured cells (including macrophages and monocytes) have shown evidence of serum-independent liposome uptake (Lee *et al*, 1992 and 1993). It is suggested that the direct recognition of particular lipid headgroups mediates uptake by cells, possibly involving scavenger receptors which have been shown to have a role in macrophage phagocytosis of modified-LDL molecules, apoptotic cells and negatively charged liposomes (Nishikawa *et al*, 1990; Fukasawa *et al*, 1996) or other as yet unidentified receptors on phagocytic cells. However, despite these conclusions these investigations were cautious about the limitations of their *in vitro* assay system in representing the *in vivo* situation. Numerous studies of uptake of liposomes by macrophages illustrate discrepancies with *in vivo* uptake behaviour (Lee at al, 1992), and the activity of macrophages has been observed to be highly dependent on their source, environment, and even method of isolation (Patel, 1992). It is also acknowledged that the dynamics of blood flow, interactions with a wide variety of cell types, and adsorption of serum proteins could severely limit the potential for these direct lipid receptor

interactions *in vivo* (Lee *et al*, 1992). Nonetheless, the potential for the observed protein-independent interactions cannot be completely ignored.

Other studies have also attempted to investigate blood protein independent uptake using a single-pass liver perfusion system in order to maintain the liver anatomical structure, blood flow conditions, and eliminate the changing activities upon isolation of macrophages (Lui and Liu, 1996). Results with this system depended on lipid composition and the species of animal tested (Liu *et al*, 1995a, Lui *et al*, 1995b; Hu and Liu, 1996), but suggested the co-existence of serum dependent and independent pathways of liposome uptake (Hu and Liu, 1996). This perfusion system still fails to fully represent the *in vivo* situation, primarily due to the use of serum which liposome studies have shown to be variable in its effectiveness to mimic the opsonization of liposomes by the blood (reviewed by Patel, 1992)(see Section 1.3.2). These investigations therefore show that direct cell interactions with the surface of liposomes are possible, but still fail to indicate whether this behaviour is still relevant *in vivo*.

1.4.3 Changing the fate of liposomes

Liposome uptake by the reticuloendothelial system has been desirable in the delivery of activators or other drugs directed against macrophages (Fidler *et al*, 1986; Deodar, 1988; Daemen *et al*, 1988); the treatment of some intracellular parasites like leishmaniasis which infect cells of the RES (Alving, 1988; Gray and Morgan, 1991), systemic fungal infections (Chopra *et al*, 1992), and in exploiting the immunoadjuvant ability of liposomes (reviewed by Gregoriadis 1990 and 1993; Gupta *et al*, 1993). However, for most applications, retaining a high level of liposomes in

the circulation is essential for effective drug delivery to disease sites. Maintaining high circulation levels allows passive targeting of liposomes to sites of inflammation caused by infection, tumour growth or disease. For this reason, a variety of strategies have been developed to overcome elimination of liposomes by the RES.

1.4.3.1 Liposome characteristics

Initial attempts to change the patterns of biodistribution involved modifying the lipid composition and physical attributes of liposomes. Large vesicles (MLVs)(> 1000 nm) are cleared much faster than small vesicles (< 200 nm)(LUVs), in fact the same dose of smaller vesicles remained in the blood 4 times longer (Juliano and Stamp, 1975). This rapid elimination of larger vesicles has been partially attributed to increased mechanical trapping by the spleen (Klibanov et al, 1990; Litzinger and Huang, 1992) and the lung (Abra et al, 1984). SUVs have shown increased delivery to the bone marrow (Senior et al, 1985), and shifts in the intrahepatic distribution suggest that these smaller liposomes can more readily gain access to hepatocytes (Roerdink et al, 1981). Increasing lipid dose has also increased the relative uptake of liposomes by the spleen, bone marrow, and lung (Abra and Hunt, 1981; Bosworth and Hunt, 1981). This has been attributed to a "spillover effect", where increased levels of lipid are delivered to the other tissues at high levels of liver uptake (discussed in 1.4.1.1).

Lipid composition also plays a critical role in determining liposome behaviour, this has been primarily attributed to changing interactions with blood proteins which regulate RES uptake (Chonn et al, 1992; reviewed by Patel 1992; Semple et al, 1996; and discussed in Section 1.3.2).

Use of saturated, long chain phospholipids (such as DSPC or hydrogenated PI) in the presence of cholesterol decreased the rate of elimination of large and small vesicles by the RES (Gregoriadis and Senior, 1980; Gabizon and Papahadjopoulos, 1988). Increased cholesterol content of liposomes also decreased vesicle uptake by the RES (Kirby et al, 1980; Roerdink et al, 1989; Semple et al, 1996). The inclusion of monosialoganglioside G_{M1} has dramatically decreased the RES elimination of liposomes in mice (Allen and Chonn, 1987). This has been attributed to a nonspecific decrease in liposome adsorption of blood proteins (Chonn and Cullis, 1992), although dysopsonin binding has also been suggested (Park and Huang, 1993). G_{M1}-liposomes experience rapid RES uptake in rats (Liu et al, 1995c), suggested to be caused by G_{M1}-specific antibody-mediated elimination through the activation of complement (Liu et al, 1995d). The same could occur in human applications using these vesicles, as antiglycolipid antibodies in normal and pathologic human serum have been identified and include anti-ganglioside G_{M1} (Kaise et al, 1985; Liu et al, 1995d).

1.4.3.2 Polymer-coated liposomes

The initial successes of G_{M1} and lipid composition fuelled the search for other methods of increasing circulation lifetimes. Among several approaches, coating liposome surfaces with poly(ethyleneglycol) polymers attached to lipid anchors evolved as one of the most successful techniques. Initial results showed a reduced uptake of these sterically stabilized vesicles by the RES, producing the extended circulation lifetimes required for more effective delivery to disease sites (Blume and Cevc, 1990; Allen at al, 1991; Papahadjopoulos et al, 1991). Other polymer

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molecules have included PEG-cholesterol (Allen *et al*, 1991; Vertut-Doi *et al*, 1996), amphiphilic poly(acrylamide) and poly(vinyl pyrrolidone) (Torchilin *et al*, 1994a), oligo- and polysaccharides (Lasic, 1994), and thiolytically cleavable PEG polymers (Kirpotin *et al*, 1996), which have all proven less effective than PEG-PE (reviewed by Lasic, 1994).

The effectiveness of PEG was attributed to steric interference with macromolecular and cellular interactions by the polymer coating (Lasic *et al*, 1991; Blume and Cevc, 1993; Torchilin *et al*, 1994b; Lasic, 1994). In theory, these highly flexible, hydrophilic PEG polymers extend outward from the bilayer creating a dense "statistical cloud" of possible polymer conformations, and sterically preventing the approach of opsonizing proteins or cell surfaces, and through its mobility sweeping away molecules near the lipid surface. This polymer layer was estimated to be 5 nm thick (Lasic, 1994), and even upon strong compression of MLVs which would completely compress conventional liposomes was able to maintain a separation of 4 nm between bilayers containing 4 mol % PEG^{2000}-PE (Needham *et al*, 1992; Zalipsky, 1993). The nature of the PEG layer changes with polymer content, at low levels a "mushroom model" best describes the properties of the PEG layer, while a "brush model" is more appropriate at higher polymer densities (Lasic, 1994; and see Figure 1.9). The "mushroom" conformation of PEG is suggested to exist in a form more like an inverse droplet or pear shape, rather than a uniform hemisphere at the membrane surface, although over longer periods of time a hemisphere shape would develop. In either model, the reduced surface adsorption of protein to PEG-coated surfaces is primarily due to the increased unfavourable free energy (Arakawa and Timasheff, 1985). *In vitro*, there is evidence that increased polymer density and chain length continues to increase repulsive forces (Kuhl *et al*, 1994; Lasic, 1994). However, *in vivo* evidence suggests that relatively short

Figure 1.9 Monomethoxy Poly(ethylene glycol) polymer configurations

A
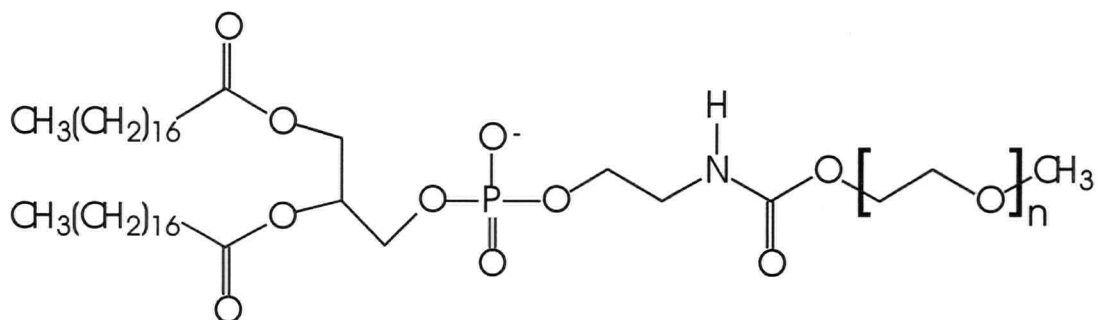
N-(monomethoxy poly(ethylene glycol) succinoyl)-DSPE

B
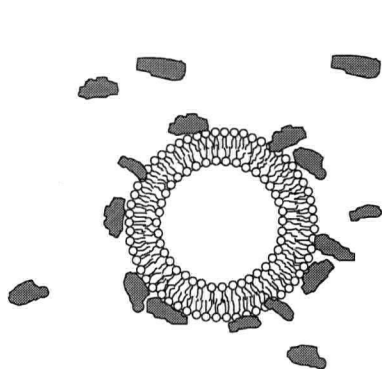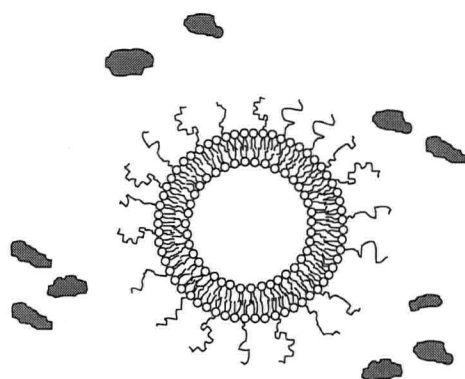
Conventional Liposome   **Sterically Stabilized Liposome**

C
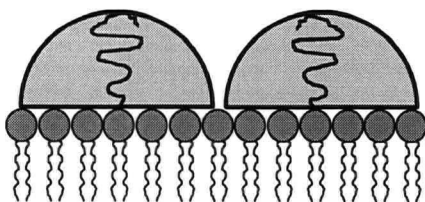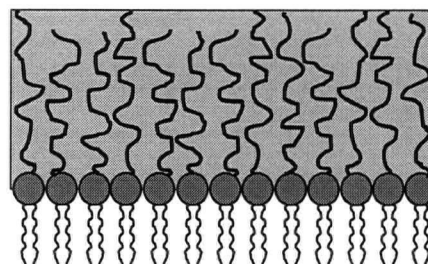
Mushroom Model   **Brush Model**

polymer chains (approximately 2,000 Da) at 5 mol % are optimal for maximizing plasma circulation, supporting additional factors besides these repulsive forces in keeping liposomes in the circulation (Allen *et al*, 1994; reviewed by Lasic, 1994). Initial *in vitro* work has provided some evidence that PEG polymer coatings decrease the serum interactions (Senior *et al*, 1991; Blume and Cevc, 1993), but this remains to be confirmed *in vivo*.

Increased circulation lifetime is only one step in enhancing delivery to disease sites, but studies with sterically stabilized vesicles have also provided evidence of increased delivery to tumours and increased anti-tumour activity (Gabizon and Papahadjopoulos, 1988; Huang *et al*, 1992; Wu *et al*, 1993; Sakakibara *et al*, 1996).

1.4.3.3 Reticuloendothelial system (RES) saturation

Previous results with liposomes with various sizes and lipid compositions have shown increased circulation levels and decreased efficiency of RES uptake at higher doses (Abra and Hunt, 1981; Bosworth and Hunt, 1982; Beaumier *et al*, 1983). This has been attributed to saturation of liver uptake, although later results have suggested the involvement of blood protein depletion (Harashima *et al*, 1993; Oja *et al*, 1996). Regardless of the mechanism responsible, this was the basis of a large number of studies attempting to blockade or saturate the RES with repeated injections of liposomes. Overall, a number of interesting observations came out of these studies (reviewed by Hwang, 1987). First, blockade by liposomes increases the relative delivery to the spleen by up to 3-fold (Abra *et al*, 1980). It was also apparent that liposome size was important, as large liposomes were not as effective at blocking the uptake of small vesicles (Abra

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and Hunt, 1982). This phenomenon has been explained by small liposomes having greater access to hepatocyte uptake (Roerdink *et al*, 1981). The composition of liposomes used to induce blockade is also important, with cholesterol-free proving more effective than cholesterol-rich liposomes at blockading the RES (Dave and Patel, 1986). Regardless of the system studied, this blockade effect was transient, and carbon particle RES uptake studies generally indicate normal levels of phagocytic activity after 24 hours (Ellens *et al*, 1982; Abra and Hunt, 1982).

Overall, despite significant detection of RES blockade using the strategies described above, there was no elevation of liposome delivery to non-RES tissues (Allen *et al*, 1984), merely alterations in the relative uptake and timecourse of uptake by the different RES organs. This suggests that the use of liposomes to blockade RES uptake may not increase delivery to disease sites. However, the disposition of liposomes in tumour-free and tumour-bearing animals has been shown to be significantly different (Bally *et al*, 1994), due to modified vascular structure and inflammation in the areas of tumour growth (Jain, 1988 and 1990). These important differences provide a route by which liposomes can access additional sites of uptake in the body (such as tumours and sites of inflammation). Only monitoring liposome uptake in diseased animals could judge the potential effectiveness of altering liposome biodistribution using these treatments, since in healthy animals the liposomes would have no alternative route of extravasation, and would inevitably only be taken up by various RES tissues. These results show quite clearly that the mechanism of liposome uptake is first limited by the accessibility of different tissues to liposomes circulating in the blood, and that opsonizing interactions and other factors responsible for clearance can also exert their effects within the limits of liposome accessibility.

1.4.3.4 Reticuloendothelial system (RES) poisoning

The presence of functioning macrophages within the reticuloendothelial system is crucial to the elimination of liposomes from the blood. Numerous techniques have been developed which interfere with the actions of phagocytic cells, including the use of silica (Morahan *et al*, 1977), ricin (Simmons *et al*, 1986), anti-macrophage antibodies (Kaminski *et al*, 1986), radiation (Zarling and Tevethia, 1973; Morahan *et al*, 1986), i.v. immunoglobulin (Aragnol and Leserman, 1986; Derksen *et al*, 1987a), gadolinium chloride (Roerdink *et al*, 1981; Vidal *et al*, 1993), carageenan (Shek and Lukovich, 1982), dextran sulfate (McGeorge and Morahan, 1978; Liu *et al*, 1992), and liposomal clodronate (dichloromethylene diphosphonate)(Van Rooijen and Claassen, 1988; Heuff *et al*, 1993; reviewed by Van Rooijen and Sanders, 1994; Buiting *et al*, 1996;). Recent evidence has also implicated liposomal doxorubicin in the suppression of phagocytosis and elimination of phagocytic cells of the liver and spleen (Daemen *et al*, 1995; Daemen *et al*, 1997). The mechanisms of action of macrophage suppression depend on the treatment used.

Doxorubicin has a variety of toxic actions which could act against the non-dividing phagocytic cells of the reticuloendothelial system, including free radical formation or the interference with DNA and the pathways responsible for protein synthesis within macrophages (Barranco, 1984; Cummings *et al*, 1991)(discussed in Section 1.2.3.3). Although the exact mechanism is still a matter of speculation, the use of liposomal doxorubicin as a treatment or pretreatment has successfully extended the circulation lifetimes of liposome formulations (Bally *et al*, 1990; Parr *et al*, 1997). This increased circulation has been attributed to the decreased

function and elimination of macrophages even at subtherapeutic doxorubicin doses (Daemen *et al*, 1995 and 1997).

The fundamental problem with these techniques is that phagocytic cells function in the elimination of foreign particles and generation of immune response, which will be inhibited by their loss of function. In fact, experimental evidence suggests that liposomal doxorubicin treatments result in reduced elimination of bacteria (Rahman *et al*, 1986a; Daemen *et al*, 1995; Daemen *et al*, 1997) and significant suppression of immune response to antigen upon drug treatment (Rahman *et al*, 1986a; Tardi *et al*, 1997). The use of liposomal clodronate has similarly shown indications of reduced immune function including increased tumour growth (Claassen *et al*, 1986; Delemarre *et al*, 1990; Heuff *et al*, 1993; Vreden *et al*, 1993). This suggests that any treatment aimed at reducing macrophage function, has the potential to significantly affect the immune system. Although clinical trials of liposomal doxorubicin have not reported evidence of complications similar to those reported to be possible in animals (Treat *et al*, 1988; Treat *et al*, 1990; Cowens *et al*, 1993), human examples of disease-related RES blockade caused by disseminated histoplasmosis and also microthrombi-induced blockade after artificial heart implantation have led to infectious events including systemic bacterial infection (Wheat *et al*, 1987; Ward *et al*, 1987).

1.4.3.5 Liposome targeting

The numerous strategies to reduce the RES uptake of liposomes have achieved increased blood levels and increased delivery to extravascular disease sites such as tumours (Gabizon and

Papahadjopoulos, 1988; Huang *et al*, 1992; Wu *et al*, 1993; Sakakibara *et al*, 1996). Liposomes have shown significant delivery into sites of infection (Bakker-Woudenberg *et al*, 1992), inflammation (Williams *et al*, 1986), and tumour growth (Proffitt *et al*, 1983; Forssen at al, 1992; Bally *et al*, 1994). This pattern of distribution has been attributed to passive targeting resulting from cytokine induced vascular hyperpermeability and possibly gaps between endothelial cells (Kohn *et al*, 1992). In areas of tumour growth the potential for extravasation is enhanced by the production of vascular permeability factors (like vascular endothelial growth factor, VEGF) and a variety of defects in tumour blood vessels which result in wide interendothelial junctions, fenestrae and transendothelial channels, and vessels with discontinuous or even completely absent basement membranes (Jain, 1987; Dvorak *et al*, 1988; Jain, 1990). Liposome extravasation studies using a transplanted human colon adenocarcinoma have shown pores in tumour vessel which are in the range of 400-600 nm in diameter, providing a route for the extravasation of liposomes at least 400 nm in diameter (Yuan *et al*, 1995).

In order to actively target drug-loaded liposomes against specific disease cells, a variety of ligands have been coupled to the surface of liposomes. These have included glycolipids (Spanjer and Scherphof, 1983), glycoproteins (Utsumi *et al*, 1983), and viral envelope glycoproteins (Chejanovsky and Loyter, 1985). However, the majority of active targeting has focused on antibody-coated immunoliposomes and has shown considerable success *in vitro* (Longman *et al*, 1995; Suzuki *et al*, 1995; Kirpotin *et al*, 1997). *In vivo*, these immunoliposomes have successfully targeted lung cancer cells (Ahmad *et al*, 1993; Emanual *et al*, 1996), erythrocytes (Agrawal *et al*, 1987), T-lymphocytes (Debs *et al*, 1987; Phillips *et al*, 1993), and pulmonary endothelial cells (Maruyama *et al*, 1990). However, targeting cells outside the vascular

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environment have either not been successful (Longman *et al*, 1995; Suzuki *et al*, 1995) or have not improved drug efficacy (Goren *et al*, 1996; Vingerhoeds *et al*, 1996).

The problem of humoral immune response directed against the targeting immunoglobulin molecule (Van Rooijen and Van Nieuwmegen, 1982; Phillips and Emili, 1991; Phillips *et al*, 1994; Phillips and Dahman, 1995) or the linkers used to couple the surface molecules (Phillips and Dahman, 1995; Boeckler *et al*, 1996), may prevent multiple administrations and could result in severe immune reactions. Efforts to reduce this problem have included the development of special "humanized" antibodies or fragments (Mountain and Adair, 1992; Kirpotin *et al*, 1997) and the incorporation of immunotoxic drugs into targeted liposomes to overcome this problem (Shek *et al*, 1986; Tardi *et al*, 1997).

1.5 Immune responses to liposomes

Immune responses to foreign antigens, including liposome coupled-proteins (xenogeneic or allogeneic), possess two primary components - humoral and cell-mediated immunity (reviewed by Roitt *et al*, 1989; Alving, 1992; and Shek, 1995). Humoral immunity involves the production of antibody molecules directed against a specific foreign antigen, through the cooperative interaction of three cell populations, namely the antigen presenting cells (APCs: such as macrophages and dendritic cells), the helper T cells (CD4+), and B cells. The foreign antigen molecules (such as liposome-coupled proteins) are engulfed by APCs, processed through lysosomal proteolysis before being coupled to class II major histocompatibility complex (MHC) molecules. They are subsequently expressed in a complex on the surface of the APCs where they

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initiate the stimulation of helper T cells (CD4+). These helper T cells are stimulated through the antigen-specific T cell receptor binding to the antigen-MHC class II complex and the action of cytokine molecules (IL-1) released from APCs. The activated T helper cells then release cytokines (including IL-2, IL-4, IL-5, and IFN-γ) which along with APCs stimulate the selective recruitment, proliferation, and differentiation of antigen-specific B cells into antibody forming cells. This portion of the immune system acts against exogenous or extracellular antigens which invade the body, and has been shown to be involved in the response against liposomal protein antigens.

Cell-mediated immunity includes the processing of antigen molecules and cell surface presentation complexed with MHC class I molecules, which subsequently stimulate antigen-specific cytotoxic T cells (CD8+). The expression of MHC class I is not cell restricted, which allows T cell cytotoxic action against a wide variety of cell types presenting the appropriate antigen complex on their surface. The action of cytotoxic T cells along with other cytotoxic cell systems (nonspecific natural killer cell action and antibody-mediated killer cell action) primarily involve responses against intracellular foreign molecules such as viruses, parasites and cancer antigens, and as discussed in subsequent sections. Liposome encapsulated proteins have also been shown to stimulate this immune pathway.

1.5.1 Liposomes as adjuvants

Liposome adjuvants were initially developed during attempts to prevent immunity by hiding therapeutically beneficial enzymes within liposomes (Gregoriadis, 1976), the unexpected

increase in immunity compared to free enzymes revealed their potential as an adjuvant system. The ability of liposomes to act as immune adjuvants is now well established, and numerous treatments have emerged to take advantage of this property. Although liposomes themselves are poor immunogens they have been very successful at increasing the humoral and cellular immune response to a variety of associated antigens. These have included successful induction of immunity against bacterial, viral, parasitic, and cancerous antigen molecules (reviewed by Gregoriadis 1990 and 1993; Gupta *et al*, 1993). The lack of adverse effects compared to exisiting adjuvant systems, and the ease of adaptation to a wide range of potential antigen molecules makes liposomes an attractive adjuvant system.

1.5.2 Liposome characteristics and immune response

An important characteristic of liposome adjuvant systems is that the antigen must be physically attached to or encapsulated within the injected liposomes (Shek, 1984). This can be accomplished through a variety of techniques including entrapment of soluble molecules, electrostatic adsorption or covalent coupling to the liposome surface, and even the insertion of lipophilic molecules directly into the bilayer (reviewed by Gregoriadis, 1993). Liposome characteristics that appear to play a role in determining adjuvant ability include bilayer fluidity, number of lamellae or lipid layers, vesicle size, surface charge, and lipid-to-antigen ratio (reviewed by Gregoriadis 1990 and 1993). All of these characteristics have been found to alter adjuvant effectiveness, but the exact conclusions have either been contradicted by subsequent studies or have not yet been further supported, making their results uncertain. One change in

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composition that does cause significant modifications in the immune response is the presence of

Lipid A, a portion of a Gram-negative bacterial lipopolysaccharide (LPS) shown to possess the

adjuvant activity of the larger molecule (Dancey et al, 1977; Cho et al, 1979). The presence of

Lipid A in liposomes dramatically enhances immune responsiveness against associated antigens

and lipid components (Schuster et al, 1979; Tamauchi et al, 1983; Alving, 1986; Verma et al,

1992). It appears to act through the recruitment and activation of macrophages and other APCs

and the prevention of T cell suppressor activity (Verma et al, 1992; and reviewed by Alving et

al, 1993).

1.5.3 Liposomal antigens - mechanisms of immune response

The mechanisms by which adjuvants increase or prolong immunity are believed to involve

changing the fate of antigen and inducing prolonged release from an "antigen depot" at the site

of injection or in local lymph nodes. Adjuvants can also stimulate migration and activation of

macrophages, inducing the release of cytokines which then stimulate immunity (reviewed by

Gregoriadis et al, 1993). The mechanisms by which liposome associated antigens stimulate

increased immunity appear to depend upon the nature of the antigen-liposome association.

1.5.3.1 Encapsulated antigen

Encapsulated antigen formulations produce a classic T cell response of short duration,

characterized by the stimulation of both CD4+ and CD8+ T cells (Fortin et al, 1996) and the

predominant production of IgG (IgG1) antibodies (Shahum and Therien, 1988 and 1994). This indicates that antigen is processed by antigen presenting cells, and subsequently expressed on their surface in association with both MHC I and II molecules (Fortin *et al*, 1996). Macrophages are required for the induction of immunity against encapsulated antigen molecules (although not necessarily for surface-coupled antigen), and the elimination of macrophages has prevented the stimulation of cytotoxic T lymphocytes and the production and activation of antibody producing cells (Shek and Lukovitch, 1982; Szoka *et al*, 1992; Nair *et al*, 1995).

The stimulation of CD4+ T lymphocytes occurs through the pathway for response to exogenous antigens, involving uptake into endosomes with subsequent processing and antigen presentation in association with MHC II. The presented antigen is then recognized by the T helper cell receptor complex, resulting in stimulation of the T cells which are specific for the antigenic epitopes expressed (reviewed by Alving, 1992 and 1995). This T cell stimulation and stimulation of B lymphocytes directly by the presented antigen and indirectly through the actions of helper T cells, results in the activation of the humoral immune response.

The stimulation of cytotoxic (CD8+) T lymphocytes via MHC I presentation of antigen is thought to result from antigen released into the cytoplasm of APCs which then associates with class I MHC molecules (Harding *et al*, 1991; Reddy *et al* , 1991 and 1992; Fortin *et al*, 1996). Zhou and associates (Zhou *et al*, 1994) used immunogold electron microscopy to reveal that antigen is present free in the cytoplasm after delivery encapsulated within liposomes. This work also showed that pH-sensitive liposomes released their contents more readily than pH-resistant lipid compositions, due to disruption of vesicles upon entrance to the acidic endosomes. In contrast to *in vitro* experiments, *in vivo* work shows that both pH-sensitive and pH-resistant

liposomal encapsulated antigen can stimulate cytotoxic T cell responses (Reddy *et al*, 1992). Macrophage processed antigen has been suggested to be transferred to dendritic cells, which directly stimulate cytotoxicity (Reddy *et al*, 1992; Nair *et al*, 1995), supported by experiments following the different migration of antigen and lipid from these systems (Nair *et al*, 1995).

The double stimulation of MHC class I and II has been proposed to shorten the duration of immune response against encapsulated liposomal antigens (Fortin *et al*, 1996), because expression of class I and class II MHC molecules on the same APCs could result in cytotoxic T lymphocyte action directed against the antigen presenting cells themselves.

1.5.3.2 Surface antigen

In contrast to encapsulated antigen, immunological response to surface-coupled molecules occurs at lower doses (Therien *et al*, 1991), lasts significantly longer, and involves the production of IgM, IgG2a, IgG3, and IgG1 antibodies (Shahum and Therien, 1994). Surface antigen stimulates CD4+ but not CD8+ T lymphocytes; probably because the retention of antigen within the endosomes of APCs prevents association with MHC I molecules which are only available to cytoplasmic antigens (Fortin *et al*, 1996). The high primary IgM production and increased IgG3 of antigen booster injections, suggests T-independent B cell activation (Therien *et al*, 1991; Shahum and Therien, 1994).

One of the most significant interactions of liposomal surface antigens is the direct interaction with receptors on immunocompetent lymphocytes (Tadakuma *et al*, 1980; Walden *et al*, 1986a and 1986b; Shek *et al*, 1986; Dal Monte and Szoka, 1989; Su and Van Rooijen, 1989).

It has been shown that high density liposomal surface antigen without MHC or APCs can stimulate T and B lymphocytes (Tadakuma *et al*, 1980; Walden *et al*, 1986a and 1986b; Dal Monte and Szoka, 1989; reviewed by Alving, 1992).

1.5.4 Inhibition of immune response

There are a variety of drugs which suppress immunity (cyclosporine, cyclophosphamide, FK 506, deoxyspergualin), but most cause widespread effects and have short *in vivo* lifetimes. Entrapment of agents into liposomes has previously induced immunosuppressive effects at lower drug doses than free drug, likely due to their altered distribution. Liposomal doxorubicin and clodronate both reduce immune responses to antigen (see Section 1.4.3.4 RES Poisoning), providing evidence that liposome drug delivery can suppress the immune system. Shek and associates suggested that surface antigen-mediated targeting of liposomal immunomodulators might be more effective due to their interactions with antigen presenting cells (Shek *et al*, 1986).

Many of the effects of liposomal cytotoxic and immunosuppressive drugs have been attributed to their effects on macrophages. In fact, much of the work investigating the role of macrophages and other cells in the generation of immune response against liposomal antigens, used liposomal clodronate to eliminate macrophages (Su and Van Rooijen, 1989; Zhou *et al*, 1994; Nair *et al*, 1995). Studies have established that liposomal doxorubicin and clodronate both suppress and eliminate macrophages (Van Roojen and Claassen, 1986; Van Rooijen, 1989; Daemen *et al*, 1995 and 1997). As macrophages play a central role in immunity, especially for liposomal antigens (Shek and Lukovich, 1982; Su and Van Rooijen, 1989; Szoka, 1992), this is

one explanation for their immunosuppressive effects. Liposomal immunomodulators can also achieve very localized immunosuppression, intra-articular administration of liposomal methotrexate has reduced the inflammation of rat antigen-induced arthritis (Williams *et al*, 1996). The increased efficiency and possible localized application of liposomal immunosuppressive agents suggests an increased potential for liposomal systems over free drugs. However, the full potential of these systems cannot be realized until the mechanisms of liposomal immunosuppression are more fully understood.

1.6 Objectives

This thesis investigates the importance of three factors which alter the blood elimination behaviour of liposomes. First, the experiments in Chapter 2 investigate the role of blood proteins in regulating the increased circulation lifetimes observed for higher lipid doses. Second, the experiments of Chapter 3 examine *in vivo* the mechanism by which sterically stabilized liposomes experience extended circulation lifetimes. This concerns the relationship between reduced blood protein adsorption and increased circulation lifetimes for PEG-liposomes. Third, the problem of humoral immunity against the surface molecules of targeted liposomes is investigated, specifically the inhibitory effects of encapsulated cytotoxic drug (doxorubicin) on the level of immune response.

CHAPTER 2: INFLUENCE OF DOSE ON LIPOSOME ELIMINATION: CRITICAL ROLE OF BLOOD PROTEINS

2.1 Introduction

As outlined in Chapter 1, the behaviour of conventional liposomes has been well studied. Although a full understanding of the mechanisms of liposome recognition and elimination has not yet been achieved, it has been shown that liposome elimination from the blood is dependent on lipid composition, vesicle size, and lipid dose (Senior, 1986; Hwang, 1987; Gregoriadis, 1988) and this has been attributed to changes in blood protein interactions caused by different vesicle characteristics (reviewed in Section 1.3). The increased half-lives experienced by higher liposome doses (Saba, 1970; Norman, 1974; Abra and Hunt, 1981; Kao and Juliano, 1981; Harashima *et al*, 1993) have been suggested to result from "saturation" of the fixed and free macrophages of the reticuloendothelial system (Abra and Hunt, 1981; Bosworth and Hunt, 1982). These phagocytic cells play a dominant role in the elimination process, so that longer half-lives are observed when their elimination capacity is overwhelmed.

It has been well established that liposomes strongly bind blood proteins, including opsonins which assist RES uptake (reviewed in Section 1.3). Furthermore, liposomes have been shown to bind these blood proteins *in vivo* in amounts inversely related to liposome circulation lifetimes (Chonn *et al*, 1992; Semple *et al*, 1996). Since the binding of blood proteins to liposomes determines recognition and elimination by the RES, it is possible that liposomes at high doses may bind less of the proteins critical to phagocytosis, and this may result in extended circulation

lifetimes. Evidence of the depletion of blood proteins has previously been obtained *in vitro* (Harashima *et al*, 1993), but until now this has not been examined as an *in vivo* explanation for the dose-dependent changes in liposome biodistribution.

This chapter contains an investigation of interactions between liposomes and blood proteins over a range of doses for each of two representative liposome formulations. Egg phosphatidylcholine/ cholesterol/ dioleoylphosphatidic acid (EPC/CHOL/DOPA) is known to bind high levels of blood proteins and is rapidly cleared, and distearoylphosphatidylcholine/cholesterol (DSPC/CHOL), known to bind much less blood protein and remain in the circulation much longer. Our results indicate that the dose-dependent increases in the circulation lifetimes of both liposome formulations are accompanied by corresponding decreases in the amount of protein associated per vesicle, suggesting that the reduced elimination efficiency of high lipid doses can be directly attributed to the decreased levels of blood protein adsorbed onto the liposome surface.

2.2 Materials and Methods

2.2.1 Preparation of Liposomes

Large unilamellar vesicles (LUVs) were prepared at various concentrations by extrusion of freeze-thawed multilamellar vesicles through two stacked 100 nm polycarbonate filters (Nuclepore, Pleasanton, CA, USA) using an extrusion device (Lipex Biomembranes, Vancouver, BC, Canada)(Hope *et al*, 1985). DSPC/CHOL (55:45, mol %) and EPC/CHOL/DOPA (35:45:20, mol %) liposomes were prepared in HEPES buffered saline solution (HBS; 20 mM HEPES, pH 7.4, 145 mM NaCl) at 2-200 mM total lipid concentrations. The average size of these liposomes was 100 ± 30 nm as determined by quasielastic light scattering analysis (QLS) using a Nicomp Model 270 Submicron Particle Sizer (Nicomp Instruments, Santa Barbara, CA, USA). Liposomes were radiolabelled using a lipid marker, $[^3H]$-cholesteryl hexadecyl ether (CHE)(10 μCi/30 μmol of total lipid)(NEN Research Products, Mississauga, Ont, Canada). This label is non-exchangeable, non-metabolizable, and not subject to cholesteryl ester transfer protein activity (Stein *et al*, 1980; Halperin *et al*, 1986; Derksen *et al*, 1987; Bally *et al*, 1990) and has been shown to be similarly stable in CD-1 mice (Chonn, A., Semple, S.C., and Cullis, P.R., unpublished results). Specific activities of liposome preparations were determined by standard liquid scintillation counting methods using a Beckman LS 3801 Liquid Scintillation System (Beckman Instruments, Fullerton, CA, USA), and a colorimetric phosphorous assay (Fiske and Subbarow, 1925). Lipids (DSPC, DOPA and EPC) were purchased from Avanti Polar Lipids (Pelham, AL, USA). Cholesterol (CHOL) was purchased from Sigma Chemical Co., St. Louis,

MO, USA. All lipids were used without further purification.

2.2.2 *In vivo* Mouse Biodistribution Studies

Liposome preparations (200 μL) were administered via the lateral tail vein of female CD-1 mice (20-25 g, Charles River, St. Constant, Que., Canada). At appropriate time points, mice were killed by exposure to carbon dioxide. This allowed rapid sacrificing of the mice without the introduction of anesthetics or other chemical agents, and would only transiently induce minimal decreases in the blood pH, which should not be large enough to alter protein interactions or functions due to the buffering capacity of the blood. Blood was removed by cardiac puncture and collected in ice-cold 1.5 mL microcentrifuge tubes, immediately cooled to 0°C to prevent coagulation and centrifuged to separate plasma from blood cell components (12,000 rpm, 2 min, 4°C)(using a Sorvall MC 12V FA-Micro centrifuge, 1.5 mL rotor)(Sorvall Instruments, Newtown, Conn., U.S.A.). Plasma liposome concentrations were determined from the specific activity of the injected liposomes, using standard liquid scintillation counting techniques. Plasma volume was assumed to be 5% of total body weight. The principle organs of the RES (liver and spleen) were individually collected, weighed, and homogenized (10% homogenates in saline) for 2 min using a Polytron homogenizer (Brinkman Instruments, Rexdale, Ont, Canada). Aliquots of the tissue homogenates (400 μL) were added to 500 μL of Solvable (NEN Research Products, Mississauga, Ontario, Canada) in 7 ml glass scintillation vials and digested for 3 hours at 50°C. Samples were subsequently decolorized for 3 hours at room temperature by the addition of 100 μL of 30% hydrogen peroxide. Finally, 5 ml of Ultima Gold scintillation fluid (Packard Instrument Company,

Meriden, CT, USA) was added to the vials and the levels of [^{3}H]-CHE in the digested tissue samples were determined by standard liquid scintillation analysis with correction for plasma content of organs. Plasma elimination half-lives, when determined, were estimated visually from the plasma concentration versus time graph. All *in vivo* analyses used four mice per time point.

2.2.3 Isolation of Liposomes from Blood Components

Liposome formulations (200 µL) were administered via the lateral tail vein of female CD-1 mice (20-25 g)(Charles River, St. Constant, PQ, Canada). CD-1 mice were selected because they are a outbred animal model with no blood protein deficiencies or other characteristics which would make them inappropriate for general comparison to human response, and also for accurate comparison to previous work studying liposome clearance and blood protein interactions which has used the same animal model. At 2 minutes post injection the mice were killed by exposure to carbon dioxide, and the blood removed by cardiac puncture and collected in ice-cold 1.5 mL microcentrifuge tubes. This short term exposure to higher carbon dioxide levels should have minor effects on the pH of the plasma due to the large buffering capacity of the blood and tissues of the animal. Samples were cooled in an ice-water bath to prevent coagulation and then centrifuged to separate plasma from the blood cell components (12,000 rpm, 2 min., 4°C using a Sorvall MC12V, FA-Micro centrifuge, 1.5 mL rotor)(Sorvall Instruments, Newtown, Conn., U.S.A.). Liposomes were then retrieved from the mouse plasma using an established "spin column" procedure, described in detail elsewhere (Chonn *et al*, 1991a). Briefly, Bio-Gel A-15m, 200-400 mesh (Bio-Rad, Richmond, CA, USA) was equilibrated with HBS and packed in 1.0 mL

Tuberculin syringes with glass wool plugs. Aliquots of mouse plasma (50 μL) were immediately applied to these columns. Column fractions were collected in glass 13 x 100 mm glass tubes by the repetitive addition of 50 μL of HBS to the spin columns followed by centrifugation in a Silencer H-103N Series bench top centrifuge (1,000 rpm for 1 min)(Western Scientific, Vancouver, B.C., Canada). The eluted sample from each centrifugation step was considered to be one fraction. Liposomes eluted in fractions 6 and 7, and were pooled for subsequent use in protein analysis.

2.2.4 Quantification of the Amount of Total Protein Associated with Recovered Liposomes

Liposome-associated proteins were extracted and delipidated according to the procedure described by Wessel and Flugge (Wessel and Flugge, 1984). Delipidation was required to prevent the interference lipid causes in most protein assay results (Kessler and Fanestil, 1986). Protein was quantified using the Micro BCA Protein Assay Reagent Kit (Pierce Chemical Co., Rockford, IL, USA) in 0.5% SDS in Milli-Q (0.22 μm) filtered water. Briefly, 1.0 mL of protein assay working reagent was added to 1.0 mL 0.5% SDS protein solution, and following a 60 min incubation at 60°C, the sample absorbance at 562 nm was compared to a bovine serum albumin standard curve (0-16 μg/mL). Protein binding values (P_B; g protein/mol lipid) were calculated from the lipid concentrations of the recovered liposomes. A minimum of three P_B determinations were made for each independently collected pool of 4 mice, and 3 of these independent samples were obtained for each lipid formulation.

2.2.5 SDS-Polyacrylamide Gel Electrophoretic Analysis of Proteins Associated with Liposomes

Protein analysis was facilitated by SDS-polyacrylamide gel electrophoresis using a Mini PROTEAN II Dual Slab Gel electrophoretic apparatus (Bio-Rad, Richmond, CA, USA). Delipidated *in vivo* protein samples (collected as outlined above) were solubilized in SDS reducing buffer (0.0625 M Tris-HCl, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 0.125% (w/v) bromophenol blue), heated at 95°C, cooled and centrifuged prior to application on the gel. Plasma proteins were separated using Bio-Rad Mini-PROTEAN II Ready Gels, 4-20% polyacrylamide gradient, 0.375 M Tris-HCl, pH 8.8, with a 4% stacking gel (75x75x1.0 mm). The gels were run at 150 V for 60 min. Protein molecular weights were estimated by comparison to High and Low Range Silver Stain SDS-PAGE Standards (Bio-Rad). The proteins were detected using an optimized silver-staining procedure (Heukeshoven and Dernick, 1993). All gel mixtures and buffer solutions were prepared in Milli-Q (0.22 μm filtered) water and degassed. Gel and buffer reagents were purchased from Bio-Rad.

Each sample isolated and presented on the gel represents a pooled sample of 4-5 mice each injected with the same composition and dose of liposomes. In addition, 2-3 pooled samples were isolated for each dose and composition, with the most representative samples being displayed in the final figure. Approximately, 2 μg protein samples were applied to each lane based on expected protein binding values of each sample, the lipid concentration before delipidation (by scintillation counting), and the expected protein content of the solubolized, delipidated protein solution applied to the gel.

Quantitative comparison of the protein bands was carried out using a Strategene Eagleeye II gel densitometer (Stratagene, La Jolla, C.A., U.S.A.), and variation was considered different when individual quantities varied substantially more than 1 standard deviation from the other samples. Results of these comparisons are presented in discussion format in Section 2.3.3.

2.3 Results

2.3.1 Biodistribution of increasing doses of DSPC/CHOL and EPC/CHOL/DOPA liposomes

The first set of experiments was aimed at characterizing the circulation time and tissue distribution of 100 nm diameter LUVs composed of EPC/CHOL/DOPA (35:45:20, mol %) and DSPC/CHOL (55:45, mol %). These two lipid compositions were selected because of their dramatically different circulation elimination characteristics and protein binding behaviour. The effects of increasing lipid dose up to 1000 mg lipid/kg body weight on the circulation elimination and organ biodistribution of the liposomes were examined. As shown in Figure 2.1, the elimination of DSPC/CHOL and EPC/CHOL/DOPA LUVs from the circulation of CD-1 mice is consistent with earlier findings (Chonn *et al*, 1992), in that DSPC/CHOL liposomes experience relatively long circulation lifetimes (Fig. 2.1B), while vesicles containing DOPA are very rapidly cleared (Fig. 2.1A). In both cases longer circulation lifetimes are observed with increased dose. Circulation half-lives for the 1000 mg/kg doses of DSPC/CHOL and EPC/CHOL/DOPA LUVs were 1200 and 80 min, respectively, as compared to 360 min and 4 min for the 100 mg/kg doses.

LUVs which are rapidly cleared from the circulation are accumulated by the RES organs (liver and spleen), as illustrated in Figure 2.2. The lower percentage of liposome uptake observed with high liposome doses suggests saturation of the RES uptake pathways (Fig. 2.2). However, Figure 2.3 shows that despite the decreasing percentage of total liposome uptake observed, the amount of lipid taken up by the principal organs of the RES (liver and spleen) continues to increase even at the highest doses. This pattern is consistent for both short and long-lived lipid

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Figure 2.1 Effect of lipid dose on the elimination of liposomes from the plasma

LUVs (100 nm) containing trace amounts of [^{3}H]-cholesteryl hexadecyl ether were administered intravenously into CD-1 mice. At various times, plasma levels of LUVs were measured by analyzing aliquots of plasma using standard scintillation counting methods. Panel A depicts EPC/CHOL/DOPA (35:34:20, mol %) liposomes of varying dose: 100 mg/kg, (O); 200 mg/kg, (□); 500 mg/kg, (Δ); 1000 mg/kg, (▽). Panel B depicts DSPC/CHOL (55:45 mol %) liposomes of varying dose: 10 mg/kg, (+); 50 mg/kg, (●); 100 mg/kg, (■); 500 mg/kg, (▲); and 1000 mg/kg, (▼). Each data point represents the average plasma recovery and standard error of 4 mice.

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Figure 2.2 Effect of lipid dose on the RES uptake of liposomes

LUVs (100 nm) containing trace amounts of [^{3}H]-cholesteryl hexadecyl ether were administered intravenously into CD-1 mice. At various times, liposome recovery in the liver and spleen was measured by counting aliquots of 10% HBS organ homogenates solubilized using a Solvable digestion procedure, using standard scintillation counting methods. Panel A depicts EPC/CHOL/DOPA (35:45:20, mol %) liposomes of varying dose: 100 mg/kg, (O); 200 mg/kg, (□); 500 mg/kg, (▲); 1000 mg/kg, (▽). Panel B depicts DSPC/CHOL (55:45, mol %) liposomes of varying dose: 10 mg/kg, (+); 50 mg/kg, (●); 100 mg/kg, (■); 500 mg/kg, (▲); and 1000 mg/kg, (▼). Each data point represents the average combined liver and spleen recovery and standard error of 4 mice.

compositions (Fig. 2.3A and 2.3B), and suggests that the reticuloendothelial system is not saturated. Estimates of initial liver uptake rates also indicate a lack of RES saturation, as illustrated in Figure 2.4, which shows that the rates of liver uptake continued to increase with injected lipid dose over the full dose range for both compositions studied. The highest dose sample of DOPA-liposomes showed some loss of linearity of the increasing rate of uptake with dose, which might be the beginning of some saturation effects. This is not true of the lower DOPA-liposome doses or any of the doses of DSPC/CHOL liposomes, suggesting that the dose-dependent changes in plasma circulation, which are observed even at the lowest doses of slowly cleared neutral vesicles, must be attributed to some other factor.

2.3.2 Effect of liposome dose on protein binding values

As previously noted, an inverse relationship exists between the amount of protein bound to liposomes and their circulation lifetimes (Chonn *et al*, 1992; Semple *et al*, 1996). It was therefore of interest to determine whether the high dose liposomes, also followed this trend and bound less blood protein. Liposomes were recovered from the blood at 2 min post-injection, and were subsequently delipidated and analyzed for associated protein using a Micro BCA protein assay. The 2 minute timepoints for isolation and analysis of liposome absorbed blood protein were selected to minimize the possibility of errors in the quantity and profiles of bound proteins by minimizing the plasma elimination of liposomes by the RES (earliest possible collection timepoint). Preferential elimination of liposomes absorbing either greater quantities of blood protein or certain opsonizing blood proteins could result in significant changes in the results of

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analysis of the bound protein at later timepoints. An investigation into these time-dependent changes did indicate certain protein bands in electrophoresis patterns that were not present in later timepoint liposome isolations (Semple *et al*, 1995), although these may have also been altered or removed from the liposomes during their time in the circulation. The 2 minute timepoints allow maximal collection of the injected liposomes and therefore the most representative bound protein sample, within the time constraints of the injection/collection procedures. Figure 2.5 shows the P_B values as a function of lipid dose, for both compositions studied. A decrease in protein binding values was apparent as the dose of each composition increased. When the protein binding values are expressed as a function of the liposome circulation half-lives (Fig. 2.6), the resulting inverse relationship is consistent with earlier observations regarding P_B values (Chonn *et al*, 1992; Semple *et al*, 1996). High liposome doses exhibit lower P_B values and increased circulation lifetimes.

Figure 2.6 also indicates a unique pattern of P_B versus half-life for each liposome composition. Liposome preparations with the same P_B value do not necessarily experience the same elimination kinetics. At a P_B value of 12 g/mol, DOPA-liposomes (1000 mg/kg) possessed a 75 min half-life, while DSPC/CHOL (200 mg/kg) exhibited a half-life of 450 min. Clearly, liposome elimination is not solely dependent on the quantity of protein bound as an identical amount of bound protein to the above lipid compositions did not result in the same circulation half-lives. Previous work has supported the importance of bound protein identity in determining elimination of liposomes from the circulation (Chonn *et al*, 1991a, 1992, and 1995)

The total amount of blood protein available for binding must be limited, a fact supported by the protein binding results observed. The total blood protein (μg total protein) bound to each
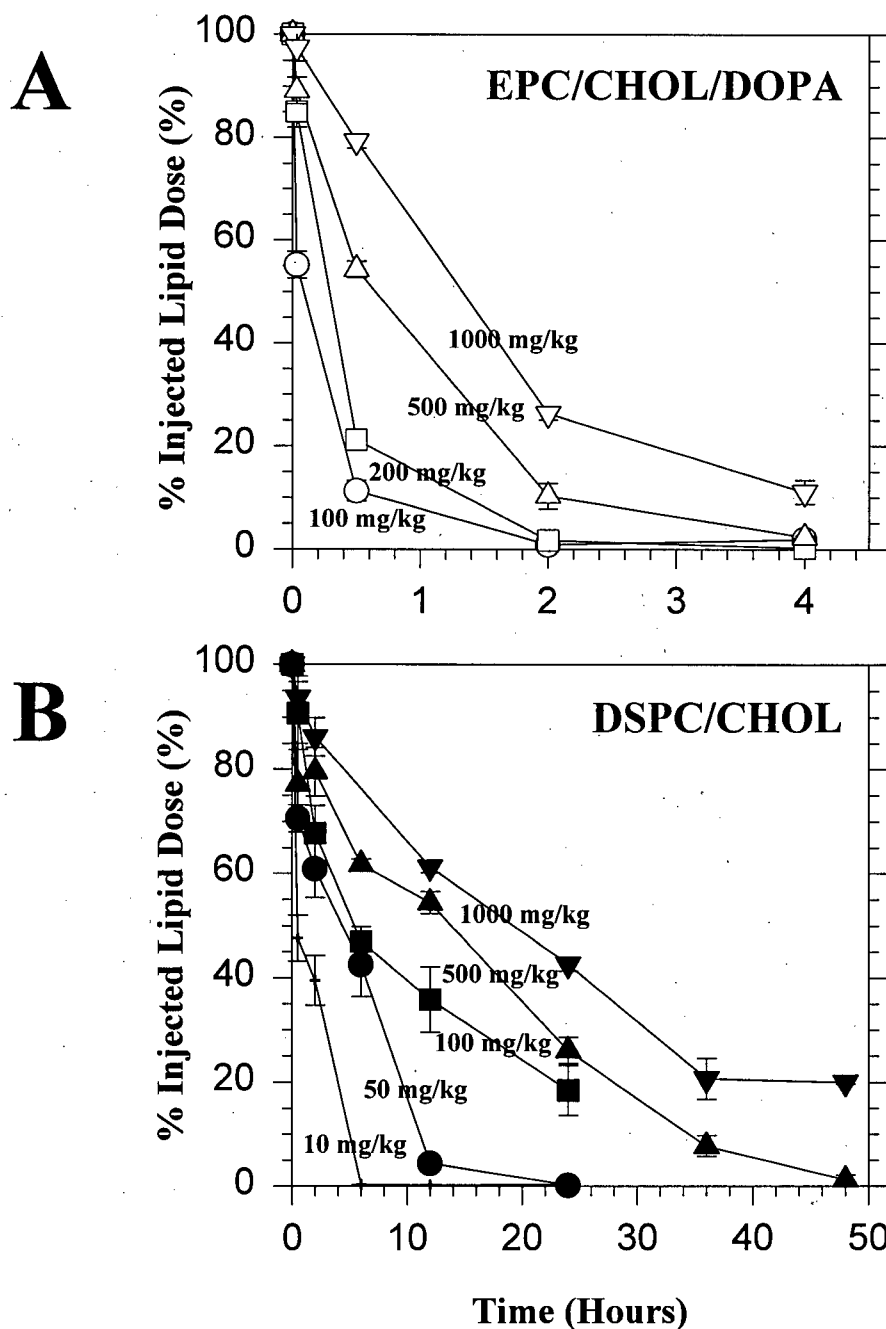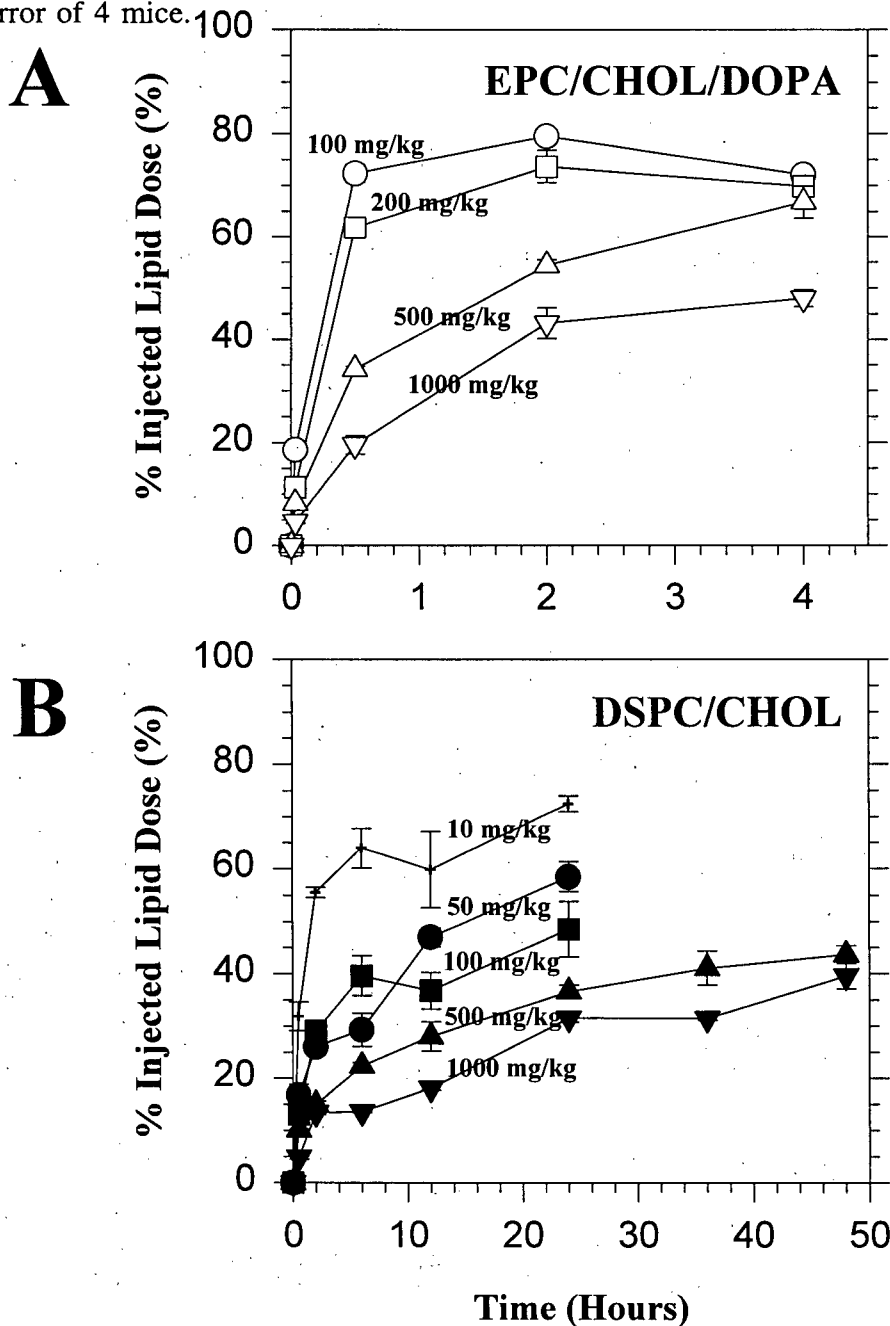
Figure 2.3 Effect of lipid dose on total lipid uptake by the RES

     LUVs (100 nm) containing trace amounts of [^{3}H]-cholesteryl hexadecyl ether were administered intravenously into CD-1 mice. At various times, liposome recovery in the liver and spleen was measured by counting aliquots of 10% HBS organ homogenates solubilized using a Solvable digestion procedure, using standard scintillation counting methods. Panel A depicts EPC/CHOL/DOPA (35:45:20, mol %) liposomes of varying dose: 100 mg/kg, (O); 200 mg/kg, (□); 500 mg/kg, (▲); 1000 mg/kg, (▽). Panel B depicts DSPC/CHOL (55:45, mol %) liposomes of varying dose: 10 mg/kg, (+); 50 mg/kg, (●); 100 mg/kg, (■); 500 mg/kg, (▲); and 1000 mg/kg, (▼). Each data point represents the average combined liver and spleen recovery and standard error of 4 mice.

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Figure 2.4 Effect of lipid dose on the initial rate of liver uptake of liposomes

LUVs (100 nm) containing trace amounts of [^{3}H]-cholesteryl hexadecyl ether were administered intravenously into CD-1 mice. Liposome recovery in the liver was subsequently measured and plotted over time. Estimates of the initial rates of liposome uptake by the liver were determined based on initial slope determinations from the uptake curves (Figure 2.3). Error bars represent the approximated degree of uncertainty associated with the slope determination. Panel A (○) depicts EPC/CHOL/DOPA (35:45:20, mol %) liposomes of varying dose. Panel B (●) depicts DSPC/CHOL (55:45, mol %) liposomes of varying dose.

75
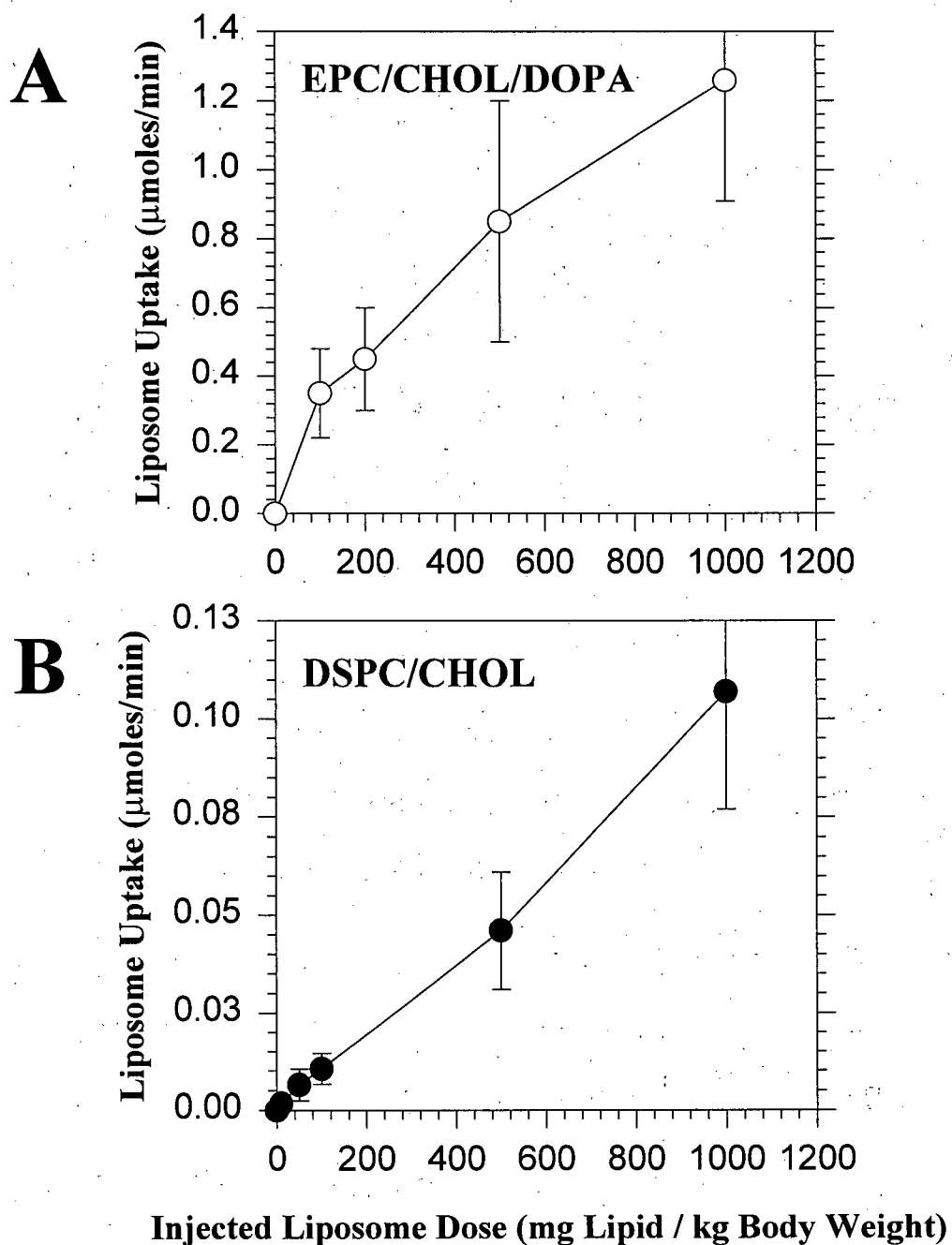
Figure 2.5 Relation between liposome-associated protein and injected lipid dose

Aliquots of recovered liposomes were delipidated, and the extracted proteins quantified using the micro BCA protein assay (see Section 2.2.3-2.2.4). The liposomes were composed of EPC/CHOL/DOPA (35:45:20, mol %) (o) and DSPC/CHOL (55:45, mol %) (●). The data points represent the average and standard error obtained from three independently pooled samples of 4 mice.
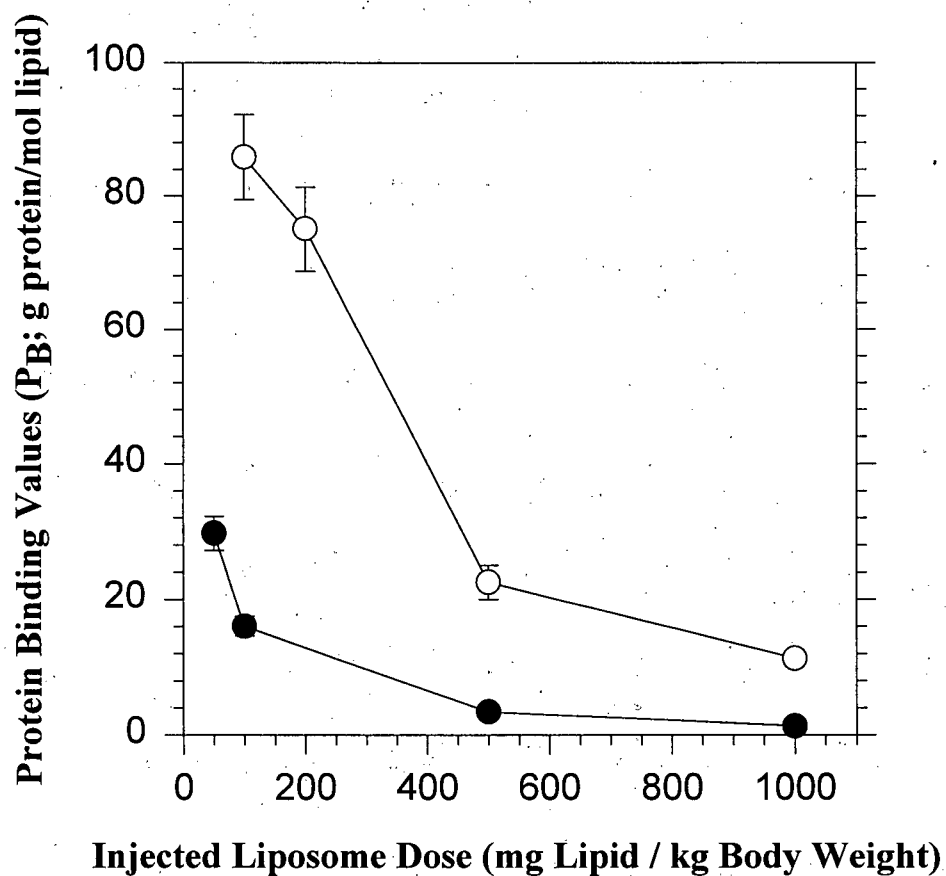
Figure 2.6 Relation between protein bound to liposomes and the circulation half-lives

Aliquots of recovered liposomes were delipidated, and the extracted proteins quantitated using the micro BCA protein assay (see Section 2.2.3-2.2.4). The liposomes were composed of EPC/CHOL/DOPA (35:45:20, mol %): 100 mg/kg, (O); 200 mg/kg, (□); 500 mg/kg, (△); 1000 mg/kg, (▽) and DSPC/CHOL (55:45, mol %) liposomes: 50 mg/kg, (●); 100 mg/kg, (■); 500 mg/kg, (▲); and 1000 mg/kg, (▼). The data points represent the average and standard error obtained from three independently pooled samples of 4 mice. Half-lives were estimated visually from the plasma elimination data of Figure 2.1.

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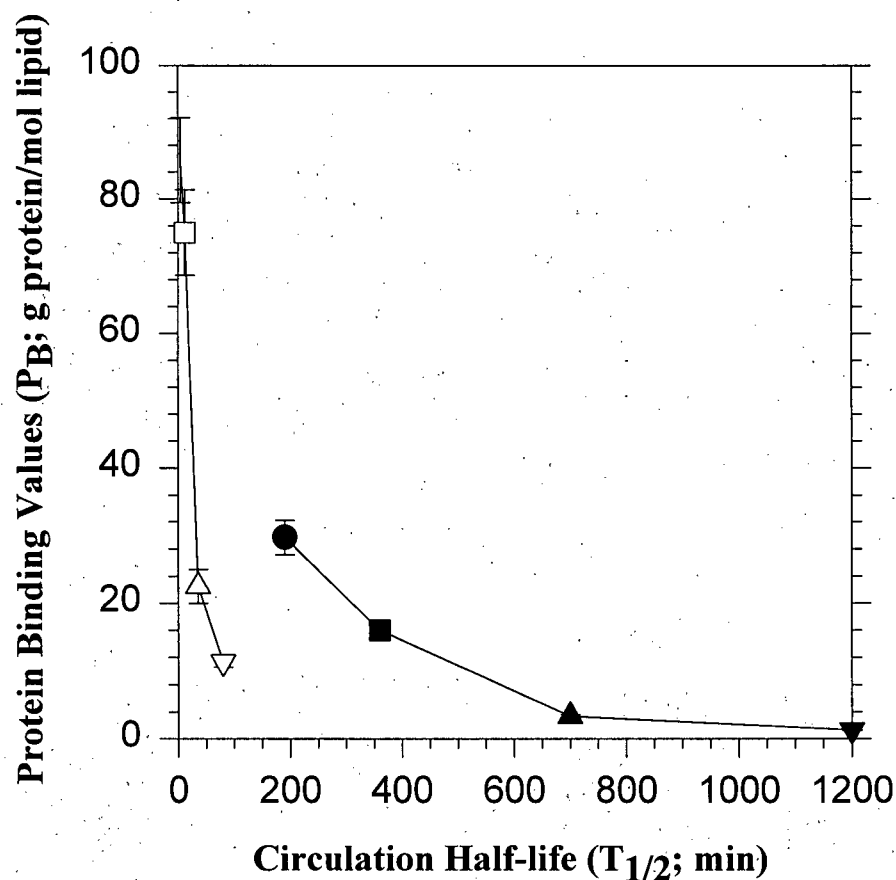
dose of the two compositions is illustrated in Figure 2.7. It is apparent that the maximum amount of blood protein bound to EPC/CHOL/DOPA vesicles is approximately 550 ±70 µg (Fig. 2.7A), whereas DSPC/CHOL vesicles bind a total of 60 ±10 µg (Fig. 2.7B). This indicates that a limited pool of blood protein is available to bind to liposomes of a given lipid composition, regardless of dose. At higher doses, this protein appears to be redistributed over the increased surface area of greater numbers of liposomes, resulting in lower P_B values and reduced RES uptake.

Estimates of the bound protein coverage of the liposome surface were made using a model of 100 nm vesicles with trap volumes of 2.7 µL / µmol lipid and 7.6×10^{12} vesicles / µmole lipid (Cullis and Hope, 1991). Blood proteins were considered for calculations to be 66 kDa molecular weight and approximately 5 nm x 5 nm in size (molecular weight of albumin and estimates for the size of small globular proteins). The protein binding values resulted in a very low surface coverage of the liposomes by protein. An approximate minimum of 0.03% of the available surface area was covered at the 1.2 g protein/mol lipid for 1000 mg/kg DSPC/CHOL liposomes and close to 2.5% was the maximum surface coverage at protein binding values in the highest range (close to 100 g/mol), such as those observed for the lowest doses of DOPA-liposomes. It is clear, according to these estimates, that even the highest levels of bound protein result in very little surface coverage of the lipid vesicles studied (with the assumptions made here). A more unfolded protein structure as suggested by theory could cover substantially more surface area and probably be more indicative of the *in vivo* situation.

2.3.3 Influence of bound protein composition on elimination

Figure 2.8 shows the silver stained SDS-PAGE profiles of *in vivo* protein samples obtained from liposomes composed of EPC/CHOL/DOPA and DSPC/CHOL isolated at 2 min post-injection. As explained in the previous section, this early timepoint was to ensure maximal recovery of the injected liposomes and therefore provide the most representative sample of bound blood proteins. Comparison of protein composition was simplified by examining equal protein quantities (2 μg protein / lane). In the process of visualizing the most proteins bound to the negatively charged DOPA-liposomes, the gel was overloaded and the high molecular weight blood proteins known to bind these liposomes were not visible as can be observed by the lack of staining in the top portion of gel A of Figure 2.8 (some high molecular weight bands were observed in Chonn *et al*, 1991a and 1992 and in Figure 3.8 of this thesis).

Quantification of the differences and similarities of the isolated protein samples was done as accurately as possible using the relative density determinations of the Strategene Eagleeye II gel densitometer, while also attempting to correct for discrepancies in the amounts of total protein applied to the different lanes of the gel (also based on density measurements of the gel). Results of quantitative comparison of the protein bands showed variation of several protein bands, although the majority of protein bands that were clearly visible for analysis, contained similar quantities of protein within the limitations of this analysis.

Generally, the patterns of protein binding do not appear consistent with the patterns observed for normal mouse serum (representative of a normal plasma protein separation pattern, despite missing clotting factors). The banding pattern of normal mouse serum is more clearly represented in Figure 3.8 with larger amounts of protein loaded onto the gel. The changing relative amounts of blood protein in the liposome isolates compared to the normal mouse serum

samples suggests that blood proteins have variable affinities for interactions with liposomes, binding is not simply concentration dependent. The different patterns for EPC/CHOL/DOPA versus DSPC/CHOL show clear differences in protein interactions which depend upon membrane lipid composition, this has been previously confirmed for a variety of other compositions of liposomes (Chonn et al, 1991a, 1991b, 1992, and 1995; Semple et al, 1995). These differences and protein quantity have both been suggested to explain the dramatic differences in plasma half-lives observed among liposome compositions. One unusual observation, which has been previously noted for neutral, positive, and some negative liposome lipid compositions (Juliano and Lin, 1980; Chonn et al, 1991a) is the presence of numerous very high molecular weight proteins or protein-complexes (> 200 kDa) larger than most blood proteins, which are again observed with the DSPC/CHOL vesicles isolated here. These have yet to be identified, but due to the reducing conditions and presence of detergent should indicate single proteins or subunits (such as α_2-macroglobulin (185 kDa subunits), fibronectin (200 to 250 kDa subunits), or Factor V (330 kDa)) and not simply protein aggregates of some kind. It is unlikely although possible, that some proteins may be incompletely reduced resulting in numerous other considerably larger protein molecules which may correspond to these bands. Gel A and gel B of Figure 2.8 suggests that the pattern of protein binding changes very little with dose, for liposomes of the same lipid composition. These differences are discussed in detail below.

Examining first the DOPA-containing liposomes of gel A. A distinct band exists at approximately 45 kDa which has separated very well in the 100 mg/kg lane, but not the other two samples. Although it does appear to be present in all three samples, despite these differences in the degree of separation densitometer measurements indicate that it is present in significantly

higher levels associated with the 100 mg/kg dose sample (> 1 standard deviation from the mean of all three sample doses). The major band present at 66 kDa is present at greater levels in the 100 mg/kg sample, and less in the 1000 mg/kg sample. This is also true of a protein band present around 16 kDa, which is even visibly darker in the 100 mg/kg sample. Although, some variation was found in the three bands mentioned above, this primarily involved slightly higher levels associated with the lowest dose liposome sample. There were no bands present for one and not another lipid dose of the DOPA-liposomes, and the level of variation could be a least partially accounted for by errors in the isolation, quantification, and separation of the samples. Overall, suggesting a substantial level of similarity between these three doses of DOPA-liposomes.

The neutral DSPC-containing liposomes produced similar results upon comparison of an even greater number of separated gel protein bands. Visibly different bands at approximately 40 and 90 kDa were confirmed by density to be associated at significantly higher levels with the 100 mg/kg lipid dose (> 1 standard deviation from the mean of the samples). Another band very close to 30 kDa was found to be associated in reduced quantities with the 1000 mg/kg lipid dose of DSPC/CHOL liposomes. Lastly, an unusual band located close to 16 kDa was found associated at high levels with both 100 and 1000 mg/kg samples, but not as significantly with the 500 mg/kg lipid dose. Again, these few variable bands were by no means representative of the majority of isolated proteins bands able to be compared on these gels and no protein bands were unique to specific doses, suggesting that these differences were attributable more to experimental variation and error sources rather than true significant changes in the identities of proteins bound to the same composition of liposomes at different lipid dose.

2.3.4 Elimination of subsequent liposome injections after protein depletion

The depletion of blood proteins due to irreversible, rapid protein interactions (Juliano and Lin, 1980; Juliano, 1983) with an initial liposome injection has an immediate and lasting effect on the elimination of subsequent liposome injections. The extended lifetimes of these later liposome injections suggests that they may not be binding the normal levels of blood proteins which are responsible for their clearance. Although the protein bound to the subsequent injections cannot be determined directly, due to the presence of multiple populations of liposomes in the circulation. As indicated in Figure 2.9, plasma levels of the second injection are increased while RES uptake is reduced, and these effects last longer than the circulation lifetimes of the predose sample. By 4 hours the DOPA-predose is almost completely removed from the blood, and the circulation of subsequent liposome injections remains altered even after 8 hours. This supports the previous conclusion of blood protein depletion indicated by the predose studies, and would explain the long lasting reduction in RES liposome removal which persists for some time after an initial liposome injection. Similar results have been observed for injections of DSPC/CHOL predose liposomes (data not shown), which indicates that even low protein binding liposomes can have some influence on the subsequent clearance of some liposome formulations. RES function has been previously reported to return to previous levels by 24 hours, indicating only a transient reduction in the elimination of foreign particles (see Section 1.4.3.3).

Figure 2.7 Relation between total protein bound to circulating liposomes and the injected dose

Aliquots of recovered liposomes were delipidated, and the extracted proteins quantitated using the micro BCA protein assay (see Section 2.2.3-2.2.4). Based on the P_B values obtained, the total protein bound to each of the injected lipid samples was determined and plotted versus the injected lipid dose. Panel A (○) depicts liposomes composed of EPC/CHOL/DOPA (35:45:20, mol %). Panel B (●) depicts liposomes composed of DSPC/CHOL (55:45, mol %). The data points represent the average and standard error obtained from three independently pooled samples of 4 mice.
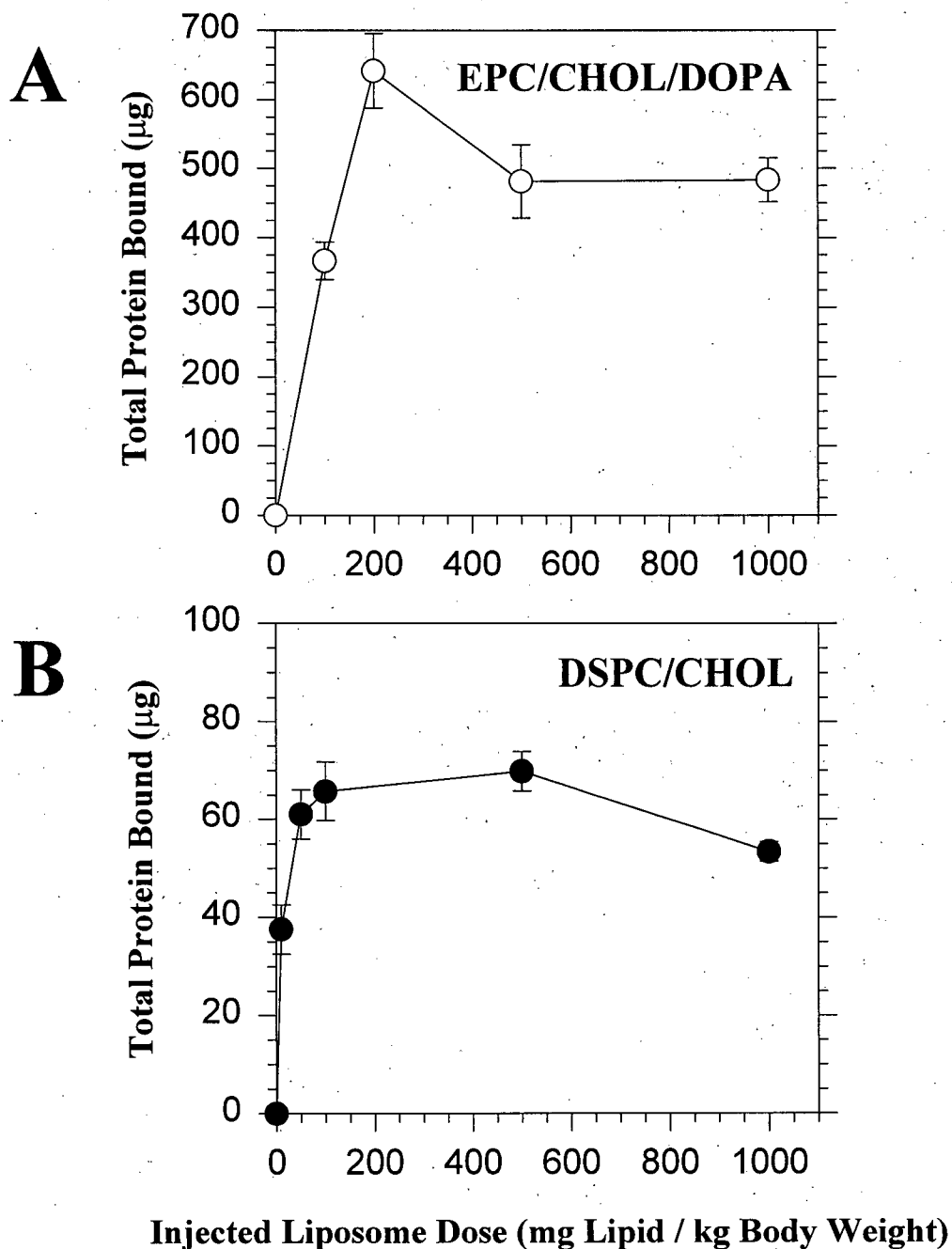
Figure 2.8 Silver-stained reducing SDS-PAGE gels of proteins associated with liposomes recovered from the circulation of CD-1 mice at 2 min post-injection

The proteins associated with the liposomes were separated electrophoretically on 4-20% SDS-polyacrylamide gels and visualized by silver stain. Each lane of Panel A separates 2 μg of protein isolated from EPC/CHOL/DOPA (35:45:20, mol %) liposomes injected at the following doses: 100 mg/kg (lane 1), 500 mg/kg (lane 2), and 1000 mg/kg (lane 3). Panel B separates 2 μg protein samples isolated from DSPC/CHOL (55:45, mol %) liposomes injected at the following doses: 100 mg/kg (lane 1), 500 mg/kg (lane 2), and 1000 mg/kg (lane 3). Lane M contains silver-stained SDS-PAGE molecular weight standards from Bio-Rad (myosin, 200,000; β-galactosidase, 116,250; phosphorylase b, 97,400; serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; trypsin inhibitor, 21,500; and lysozyme, 14,400). Lane S contains normal mouse serum.
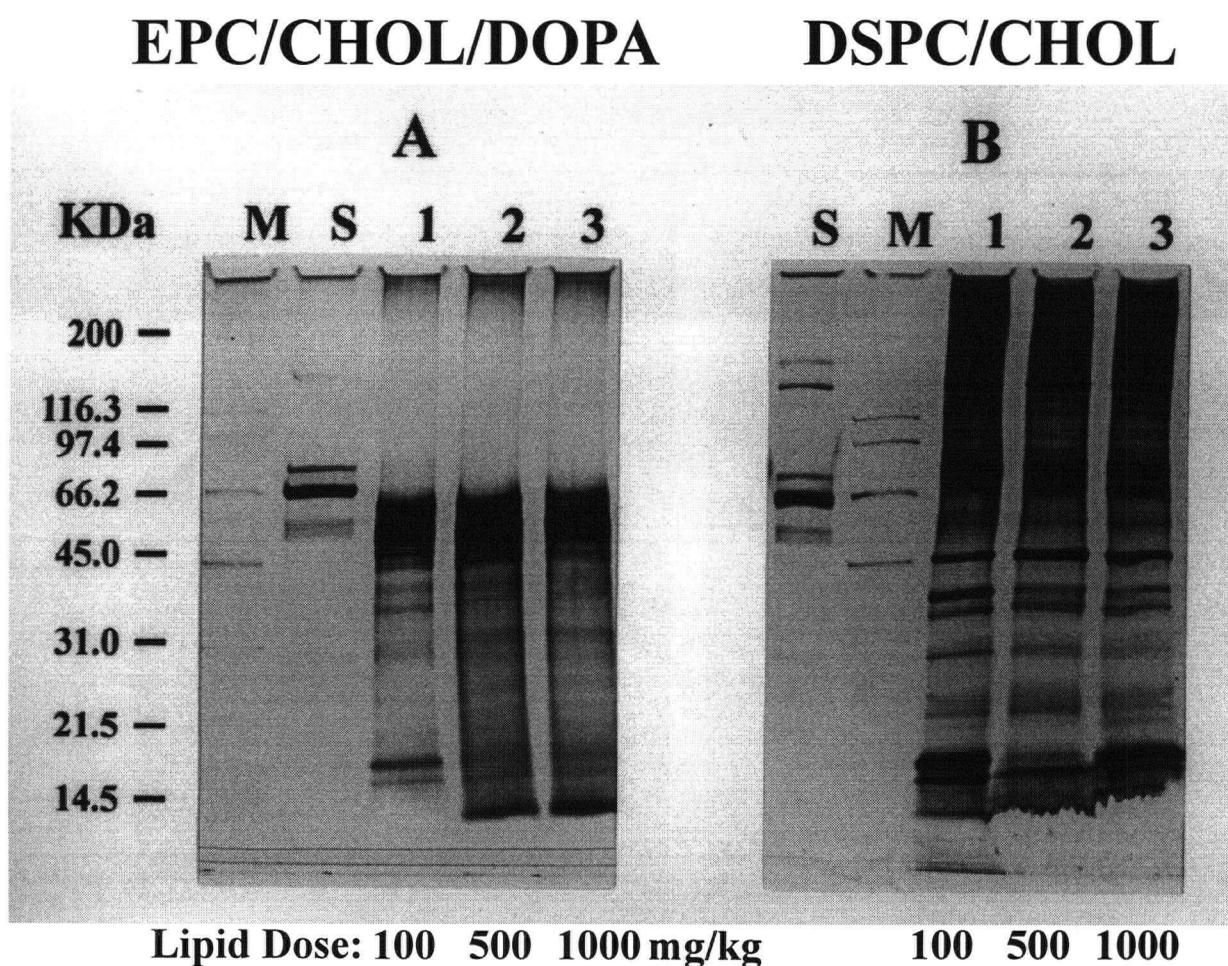
Figure 2.9 Effect of PC/CHOL/DOPA predosing on plasma elimination and liver uptake of subsequent PC/CHOL/DOPA liposomes

100 mg/kg PC:CHOL:DOPA (35:45:20, mol%) LUVs (100 nm) containing trace amounts of [^{3}H]-cholesteryl hexadecyl ether were administered intravenously into CD-1 mice at set timepoints after injection of a 1000 mg/kg PC/CHOL/DOPA liposome predose. At various times, recovery of the labelled liposomes in plasma and liver were measured by standard scintillation counting methods (see Section 2.2.1-2.2.2). Panel A depicts plasma elimination and Panel B depicts uptake by the liver. The predoses were injected at 0.5 hr (■), 4.0 hr (▲), and 8.0 hr (▼) before the test injections. The control is a 100 mg/kg PC/CHOL/DOPA without a predose (○). Each data point represents the average plasma or liver recovery and standard error from 4 mice.

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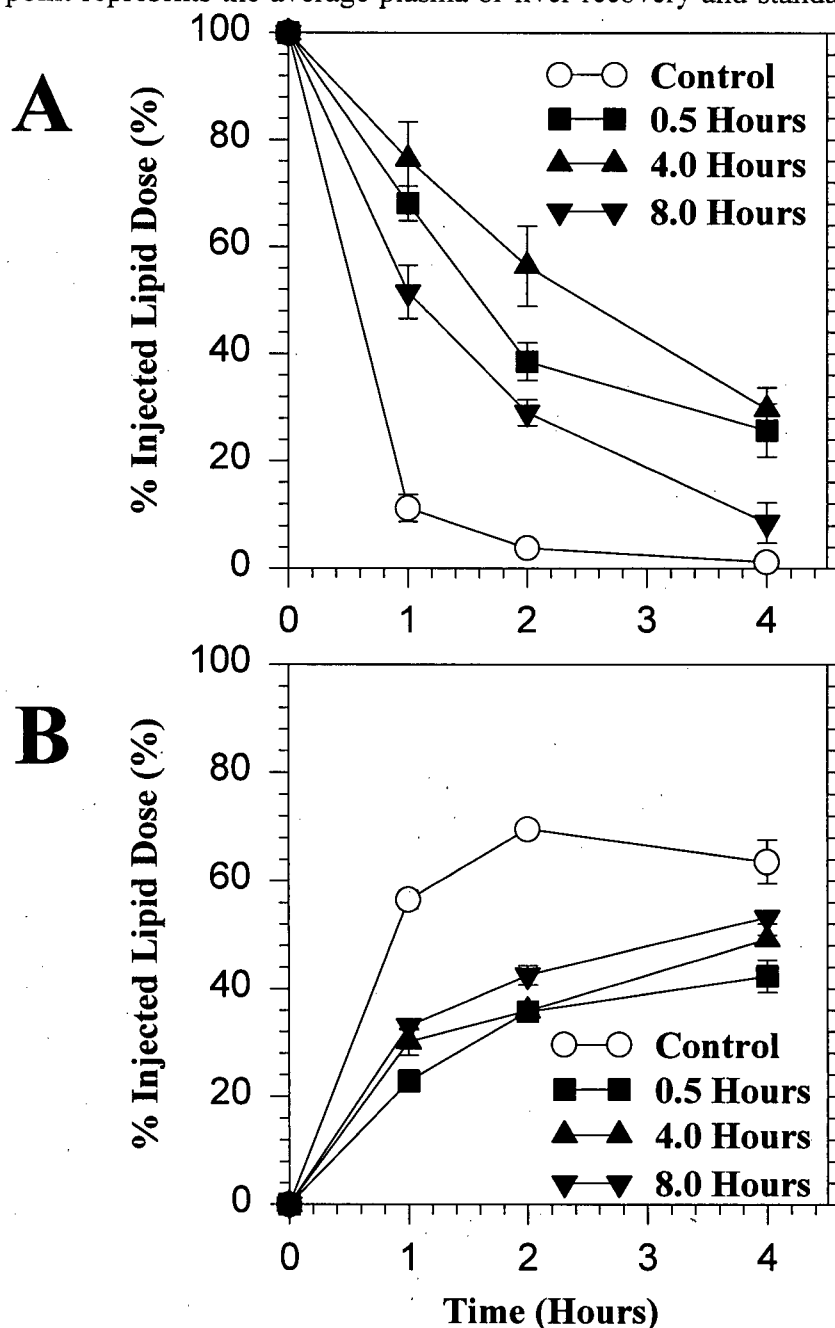
2.4 Discussion

In agreement with earlier work (Abra and Hunt, 1981; Kao and Juliano, 1981; Harashima *et al*, 1993), these results indicate increased circulation half-lives for liposomes of increased lipid dose. However, it was widely believed that this dose effect resulted from saturation of the RES (Abra and Hunt, 1981; Bosworth and Hunt, 1982). As demonstrated in this chapter, increased lipid dose does not clearly show saturation of uptake but does show decreased protein binding, which produces a similar inverse relationship to that previously observed between P_B values and circulation half-lives (Chonn *et al*, 1992; Semple *et al*, 1996). This suggests that the prolonged circulation lifetimes observed with increasing liposome dose are the direct result of the decreased protein binding. The results also suggest the existence of a specific quantity and identity of blood proteins which bind to liposomes of a given composition, these proteins are completely bound to low liposome doses and are diluted over the large surface area of increasing doses. Comparison of protein profiles bound to liposomes indicates very few significant variations among the different doses of the same liposome composition, supporting the suggestion of blood protein dilution or depletion with increasing lipid dose. There were dramatic differences between the protein profiles of DSPC/CHOL and EPC/CHOL/DOPA, which agrees with previous results (Chonn *et al*, 1991a, 1991b, and 1992). This further supports their conclusions that these differences in protein identity (as well as quantity) are a major factor in the dramatic differences in the elimination of liposomes of different composition.

Comparisons between DSPC/CHOL and EPC/CHOL/DOPA liposomes at equivalent doses make it quite evident that both the total lipid accumulation and the rate of lipid uptake by the

RES, are up to 10 times greater for the DOPA-containing systems (Fig. 2.3 and 2.4). If the two different liposome compositions both follow a similar pathway of elimination from the blood as their equal size and the established fate of liposomes suggests, then the DOPA systems reflect the minimum capacity for the RES to take up liposomes. DSPC/CHOL uptake is only a small fraction of this capacity, suggesting that RES saturation cannot possibly account for the increased lifetimes of higher doses of DSPC/CHOL liposomes. In addition, neither lipid composition reaches a maximum rate or level of lipid uptake over the dose range studied. The only possible evidence of saturation is at the highest dose of the most rapidly cleared DOPA-liposomes where rate of uptake appears no longer linear with dose but still continues to increase (Fig. 2.4A), which makes saturation an inadequate mechanism for explaining the significant changes in behaviour of liposomes that are visible at doses well below 1000 mg/kg. Therefore the quantity and type of adsorbed blood protein and not RES saturation must be of important in determining the RES phagocytic ability towards these different liposome doses.

A number of recent investigations have focused on the importance of blood protein-liposome interactions (reviewed in Section 1.3). Blood opsonins have long been known to be involved in the phagocytosis of foreign particulate matter (Jenkin and Rawley, 1961; Saba, 1970), and Chonn and associates have demonstrated that blood protein associated with liposomes is inversely related to the circulation half-lives (Chonn et al, 1992; Semple et al, 1996). When dose-dependent circulation half-lives are examined, the results presented here also reflect a similar inverse relationship with the quantity of protein bound. Analysis of plasma protein binding over this dose range reveals an 8-12 fold increased protein binding at the lowest doses of liposomes studied, for both DSPC/CHOL and EPC/CHOL/DOPA liposomes (Fig. 2.5). Just as low protein

binding lipid compositions have previously been associated with enhanced blood circulation (Chonn *et al*, 1992; Semple *et al*, 1996), high lipid doses with low P_B values also experienced extended lifetimes. This is likely because low P_B values result in a decreased probability that the liposomes can be recognized and ingested by the RES, as the blood opsonins on the liposomal surface are "diluted" over a much greater surface area at high lipid doses. A mechanism of clearance completely dependent on specific opsonin-receptor uptake should also be saturable by ligand at some point, and there is no evidence of substantial saturation except possibly at the highest dose of DOPA-liposomes (discussed above). This suggests several possibilities, first the receptors for opsonins may not yet be saturated by these levels of ligand interactions. Second, alternative protein-independent uptake mechanisms or those involving nonspecific protein enhancement of uptake (such as increased hydrophobicity or surface charge interactions) could also be occurring (see Section 1.4.2.2). Also, alternative sites of uptake could become more significant or accessible at high concentrations of liposomes, such as increased hepatocyte uptake (discussed Section 1.4.1.1). The kinetics of liposome clearance has previously been suggested to be composed of both saturable and nonsaturable uptake pathways, which have not been fully elucidated (Hwang, 1987). In reality the situation likely involves multiple pathways of uptake or routes by which phagocytes and other cells in numerous body sites can interact with and take up liposomes, of which protein-mediated uptake is a pathway of major importance.

The estimates of how much of the liposome surface is covered by these blood proteins (see Section 2.3.2) indicate that a very sparse monolayer of protein is likely to exist on the liposome surface. At the lower levels of bound protein such as for DSPC/CHOL vesicles as little as 0.03% of the surface area was covered, while the highest levels of binding to low doses of DOPA-

liposomes still only bound close to 2.5% of the available liposome surface area. Clearly, it seems that unless large amounts of unfolding and denaturation occur at the lipid surface there would still be a great deal of uncovered lipid surface available to interact *in vivo*. Evidence on the structure of surface adsorbed blood proteins (reviewed in Section 1.3.1), suggests that the proteins experience variable degrees of unfolding and are stabilized on surfaces by multiple attachment sites. The loss of protein globular structure might explain the calculated low liposome surface coverage of bound proteins, as this assumed a small globular structure. Unfolded proteins could cover substantially greater areas of lipid surface and result in a protein layer that is far more significant than indicated by this initial approximation. This unfolding of proteins would also explain the strong and rapid nature of the interactions between blood proteins and liposomes (discussed in Section 1.3.2), as it would allow multiple attachment sites, possible insertion of hydrophobic protein areas through gaps between lipid headgroups into the hydrophobic core of the lipid membrane, numerous ionic interactions with charged lipid headgroups and other interactions which have been suggested to explain the strong nature of liposome interactions with blood proteins (reviewed in Sections 1.3.2). Currently, there is very little direct information confirming the exact nature of liposome-blood protein interactions, therefore, it is impossible to estimate the degree to which unfolding of proteins during binding could increase the surface area of the blood protein layer. Although, studies of blood proteins with solid and fluid surfaces support the interactions suggested above and reviewed in the introduction (see Section 1.3.2).

An important result of the work in this chapter is demonstrating the apparent existence of specific and finite pools of plasma proteins that are available for binding to liposomes with different lipid compositions. Previous work has clearly illustrated that lipid composition strongly

influences protein binding, both in total amount of protein bound and in terms of protein profiles. This has been attributed to a variety of lipid membrane characteristics including charge, specific lipid headgroup interactions, and packing density of the membrane (reviewed in Section 1.3.2). By varying only liposome dose, the results presented here show that the total quantity of liposome-bound blood protein reaches a plateau at relatively low lipid doses (Fig. 2.7). Regardless of the mechanisms of protein adsorption and whether these vary with lipid composition, the increased surface area of higher doses of the same membrane composition should allow for additional blood protein to bind due to the greater availability of potential binding sites. As the higher doses do not continue to bind greater total amounts of blood protein, additional protein which binds must no longer be available - the binding proteins must be depleted. Furthermore, the composition of this mixture of liposome-associated protein did not vary substantially with dose as indicated by the profiles of adsorbed blood proteins separated by electrophoresis (Fig. 2.8). However, one must be cautious as some blood constituents (such as C3 and β_2-GPI) have been demonstrated to be associated with dramatic changes in liposome elimination, but would not substantially change the observed profile of adsorbed proteins. Together, these studies suggest that distinct blood protein pool is available to bind to liposomes of a specific lipid composition. The DOPA-containing liposomes interact strongly with blood proteins, and the associated protein composition has been shown to be enriched in known blood opsonins (Chonn et al, 1992). Complement and immunoglobulin are particularly important opsonizing proteins and have been shown to be associated with the increased elimination of liposomes from the circulation (Chonn et al, 1992), as lipid compositions which bound these clearance proteins experienced substantially increased plasma elimination compared with those lipid compositions which do not. DSPC/CHOL

liposomes are low protein binding systems, and do not bind known opsonins with the same affinity. It should be noted that the maximum amount of blood protein bound, at any liposome composition or dose, was 0.6 mg/mL. This is approximately 1% of the total plasma protein, 60-80 mg/mL, calculated based on a plasma protein concentration of 6-8 g/dL (Jordan *et al*, 1992) and assuming a plasma volume of 5% of body weight. Although this represents only 1% of total blood protein, LUVs interact with a range of blood protein components (albumin, β_2-GPI, IgG, and C3)(reviewed in Section 1.3.3). Some of these blood proteins can be depleted even at these low levels of bound protein, and this is supported by the elimination and protein binding changes with lipid dose. The proposed liposome protein binding and depletion can also explain the pronounced effect on the behaviour of subsequent liposome injections. After administering an injection of liposomes, enhanced circulation levels and reduced RES uptake of subsequent injections occurs and persists well after removal of the first injection from the circulation. This provides some support for the transient depletion of blood proteins which could explain the changes in the elimination of subsequent liposome injections in treated mice.

In summary, the studies described within this chapter illustrate the importance of liposome-bound blood proteins in mediating liposome elimination from the blood circulation and confirms the importance of membrane lipid composition in regulating changes in these blood protein interactions. Different doses of the same liposomes lead to different blood protein binding properties, consistent with the hypothesis that the amount of bound protein is a major factor in dictating the liposome elimination properties. Low P_B values were associated with higher doses of liposomes which exhibited extended circulation times, suggesting that blood proteins (including opsonins) were depleted. The RES "saturation" phenomena suggested by previous studies, does

not appear to play a major role in the clearance of liposomes over the dose range studied. In all samples except the highest dose of the most rapidly cleared liposomes, the rates of RES uptake continued to increase linearly with increased lipid dose, suggesting a lack of saturation. The importance of blood protein binding is further substantiated by data supporting the existence of a limited identity and quantity of blood proteins that seems available for binding to any dose of a particular liposome composition. The depletion of this blood protein pool over the higher liposome surface areas of increased liposome doses, suggests a simple mechanism which can explain the increased circulation half-lives with dose. It also agrees with a primarily protein-mediated mechanism of liposome elimination from the blood which is supported by extensive research (Scherphof *et al*, 1981; Bonte and Juliano, 1986; Chonn *et al*, 1991a, 1992; Patel, 1992).

CHAPTER 3: POLY(ETHYLENE GLYCOL) NONSPECIFICALLY DECREASES LIPOSOME BLOOD PROTEIN ADSORPTION

3.1 Introduction

The incorporation of PEG-polymers into liposomes has been shown to extend circulation lifetimes, potentially increasing efficacy by enhancing the delivery of drug to sites of disease (see Section 1.4.3.2). This has been primarily attributed to the flexible and hydrophilic PEG molecules extending outward from the bilayer creating a dense statistical cloud of possible mobile polymer conformations. This mobile polymer layer has been suggested to sterically prevent the approach of opsonizing proteins or cell surfaces reducing the protein-mediated RES elimination of these vesicles (Lasic *et al*, 1991; Torchilin *et al*, 1994b; Lasic, 1994). Liposome elimination studies have firmly established a crucial role for blood proteins in the regulation of RES uptake, showing relationships to both the quantity of surface bound protein and the presence of specific opsonins (reviewed in Section 1.3; Chonn *et al*, 1992; Chonn *et al*, 1995; Semple *et al*, 1996; Oja *et al*, 1996). However, the only experimental evidence that PEG decreases blood protein binding is indirect measurements of the surface character of membranes containing PEG using an aqueous two-phase partitioning technique (Senior *et al*, 1991) and reduced binding in an *in vitro* serum incubation (Blume and Cevc, 1993).

The experiments described within this chapter determine the effect of PEG on liposome-blood protein adsorption *in vivo*, by isolating intravenously injected liposomes with and without PEG-polymer from the blood of CD-1 mice using established spin column techniques (Chonn *et*

al, 1991a). Analysis of the bound protein content and composition, as well as plasma elimination of these formulations, should determine any change in protein interaction and its significance in altering the liposome elimination from the blood. The results clearly show that regardless of lipid composition, the addition of PEG-polymer decreases the level of blood protein bound to the surface of liposomes, which at least partially explains the substantial increases in plasma circulation levels observed for these sterically stabilized vesicles. However, it is just as clear that protein binding is not totally blocked, and that the most of the proteins isolated with conventional liposomes appear to be adsorbed at similar levels after the incorporation of PEG-polymer.

3.2 Materials and Methods

3.2.1 Preparation of liposomes

Large unilamellar vesicles (LUVs) were prepared as outlined in Chapter 2. DSPC/CHOL (55:45, mol %) and EPC/CHOL/DOPA (35:45:20 mol %) with and without PEG-DSPE as outlined in each figure legends were prepared in HEPES-buffered saline solution (HBS; 20 mM Hepes, pH 7.4, 145 mM NaCl) at 20-200 mM total lipid concentrations. DOPE/DODAC (85:15, mol %) liposomes with and without PEG-DSPE or PEG-Cer(C_{20}) were hydrated overnight in distilled water at 5 mg/mL total lipid concentration before extrusion at room temperature. This produced homogenous vesicles of all of an average size of approximately 100 nm ± 30 nm as determined by quasielastic light scattering analysis (QLS) using a Nicomp Model 270 Submicron Particle Sizer (Nicomp Instruments, Santa Barbara, CA, USA). DOPA and EPC were purchased from Avanti Polar Lipids (Pelham, AL, USA), while DSPC, DOPE, and [2000]PEG-DSPE were purchased from Northern Lipids (Vancouver, BC, Canada). DODAC and PEG-Cer (C_{20}) respectively were generously provided by Dr. Steven Ansell and Dr. Louis Choi of Inex Pharmaceuticals (Vancouver, BC, Canada). This PEG-DSPE is known to be stable on liposomes (stable anchor to lipid) for the time periods studied in these experiments in buffer, plasma or in vivo (Steven Ansell, unpublished results). Cholesterol (CHOL) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). All lipids were used without further purification.

3.2.2 *In vivo* mouse biodistribution studies

In vivo biodistribution studies were carried out as outlined in Chapter 2 (see Section 2.2.2). Liposomes were administered according to the doses and compositions outlined in individual figure legends.

3.2.3 Isolation of liposomes from blood components

Liposomes were isolated from the various components from the blood following the procedures outlines in Chapter 2 (see Section 2.2.3).

3.2.4 Quantification of the amount of total protein associated with recovered liposomes

Blood protein associated with *in vivo* isolated liposome samples, was determined as outlined in Chapter 2 (see Section 2.2.4).

3.2.5 SDS-Polyacrylamide gel electrophoretic analysis of proteins associated with isolated *in vivo* liposomes

In vivo liposomes were isolated as outlined in Chapter 2 (see Section 2.2.3-2.2.4), the SDS-PAGE electrophoresis and silver staining was carried out as outlined in Chapter 2 (see Section 2.2.5). The only procedural changes were that gels were run for 60 min at 130 V, and each lane was loaded with 3 μg of protein. Approximately, 3 μg protein samples were applied to each lane based on expected protein binding values of each sample, the lipid concentration before

delipidation (by scintillation counting), and the expected protein content of the solubolized, delipidated protein solution applied to the gel.

Each sample isolated and presented on the gel represents a pooled sample of 4-5 mice each injected with the same composition and dose of liposomes. In addition, a minimum of 2 pooled samples were isolated for each dose and composition, with two representative samples being displayed in the repeated lanes of Figure 3.8.

Quantitative comparison of the protein bands was carried out using a Strategene Eagleeye II gel densitometer (Stratagene, La Jolla, C.A., U.S.A.), and variation was considered significant when individual quantities varied substantially more than 1 standard deviation from the other samples. Significant results of these comparisons are presented in discussion format in Section 3.3.4.

3.3 Results

3.3.1 PEG-incorporation inhibits protein binding to neutral and charged LUVs

Investigations of blood protein binding *in vivo* to LUVs without a PEG coating have shown a direct relationship between the amount of blood protein bound and the rate of elimination by the reticuloendothelial system (Chonn *et al*, 1992; Semple *et al*, 1996). The experiments within this chapter were carried out to determine whether this relationship is also true for liposomes with surface associated poly(ethylene glycol). A spin column procedure has been developed for this purpose (Chonn *et al*, 1991a), and successfully applied in investigating the *in vivo* behaviour of a range of conventional liposome formulations (Chonn *et al*, 1992; Semple *et al*, 1996).

In order to get an accurate representation of liposome behaviour, we have chosen to study LUVs with three lipid compositions. Uncharged LUVs composed of DSPC/CHOL (55:45, mol %) are slowly cleared and bind relatively small quantities of blood protein (Chonn *et al*, 1992). This is similar to formulations being used for the delivery of a variety of drugs. Net negatively charged EPC/CHOL/DOPA (35:45:20, mol %) LUVs are rapidly cleared and bind large quantities of blood protein including known opsonins (Chonn *et al*, 1992). Lastly, the cationic LUVs composed of DOPE/DODAC (85:15, mol %) are representative of preparations currently being considered in the area of gene therapy. The protein binding behaviour of these lipids has not yet been studied, although their rapid blood elimination (Mori *et al*, 1997) and positive charge suggests that they experience significant interactions with blood proteins.

Figure 3.1 depicts the *in vivo* protein binding behaviour of the neutral (DSPC/CHOL) (Fig.

3.1A) and negatively charged (EPC/CHOL/DOPA) (Fig. 3.1B) liposome compositions upon incorporation of PEG-DSPE. The results demonstrate a decreased amount of blood protein associated with the isolated liposomes possessing PEG, which agrees with the expected results due to reports of improved circulation lifetimes for these vesicles (reviewed in Section 1.4.3.2) and the proposed mechanism of reduced protein-dependent liposome elimination. Just as clearly, it indicates that further increasing the molar percentage of PEG-DSPE does not induce additional reductions in the protein adsorbed to liposomes. It is interesting to note that DOPA-liposomes with their naturally high affinity for protein required a more dense PEG-coating before the reduction in protein adsorption occurred, 5 mol % PEG compared to 2 mol % for the neutral formulation. Another significant observation is that DOPA-liposomes still bound 50 g protein/mole lipid, a substantial quantity of blood protein compared to neutral lipid compositions, despite the presence of an effective PEG-polymer coating. This suggests that the PEG "barrier" does not block all protein interactions.

For cationic LUVs (Fig. 3.2) 90% of protein binding was prevented by the incorporation of 5 mol % of either of the two PEG-polymers shown (PEG-DSPE or PEG-Cer(C_{20})). As with the rapidly cleared DOPA-formulation, these liposomes also experienced significant protein interactions even in the presence of PEG-polymer, adsorbing more than 100 g protein/mol lipid despite the presence of PEG. Therefore, despite the presence of PEG-polymer coatings it appears that liposomes still bind variable blood protein quantities which vary with their lipid composition.

Figure 3.1 Relation between liposome-associated protein *in vivo* and the % PEG-DSPE

     Aliquots of recovered *in vivo* liposomes (100 nm LUVs) were delipidated, and the extracted proteins quantified using the Micro BCA protein assay (See Section 3.2). Panel A depicts the protein binding values obtained for formulations of DSPC/CHOL/PEG-DSPE (55-x:45:x, mol %) injected at a lipid dose of 50 mg/kg. Panel B depicts the protein binding values obtained for formulations of EPC/CHOL/DOPA/PEG-DSPE (35-x:45:20:x, mol%) injected at a lipid dose of 100 mg/kg. Each data point represents the average and standard error obtained from 2 or more independently pooled samples of 4 mice.
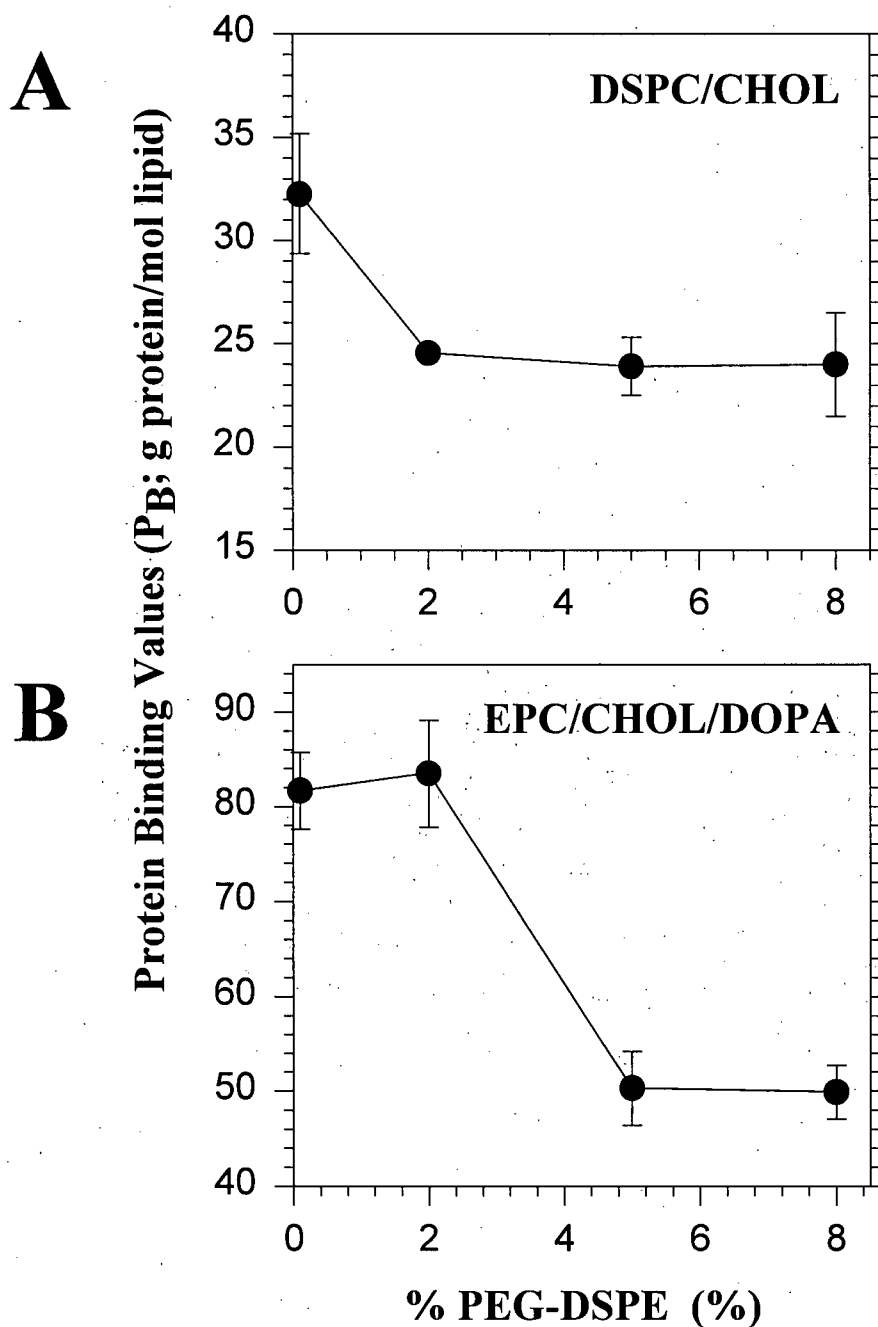
Figure 3.2 Relation between liposome-associated protein *in vivo* and the % PEG-DSPE for cationic liposomes

Aliquots of recovered *in vivo* liposomes (100 nm LUVs) were delipidated, and the extracted proteins quantified using the micro BCA protein assay (See Section 3.2). Solid symbols represent formulations of DOPE/DODAC/PEG-Cer(C_{20}) (85-x:15:x, mol %), while open symbols represent formulations of DOPE/DODAC/PEG-DSPE (85-x:15:x, mol %) injected at a lipid dose of 40 mg/kg. Each data point represents the average and standard error obtained from 2 independently pooled samples of 4 mice.
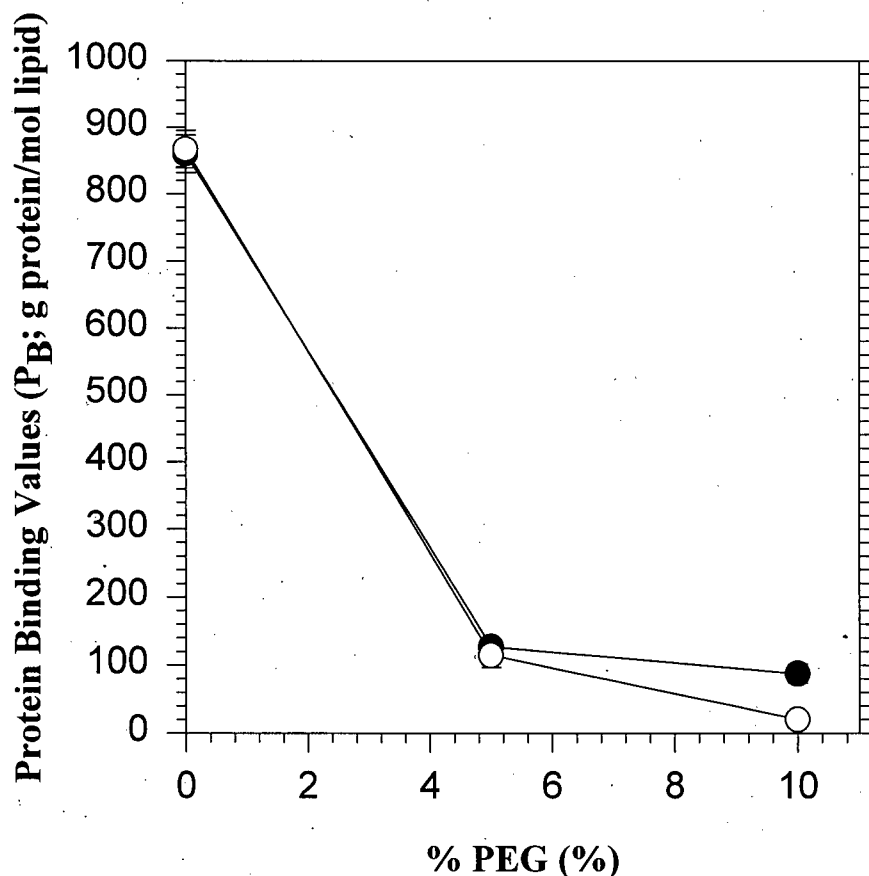
3.3.2 Plasma elimination of PEG-liposomes correlates with the amount of bound protein

Previous investigations have shown an inverse relationship between the amount of bound protein and the circulation half-lives of liposomes (Chonn *et al*, 1992; Oja *et al*, 1996; Semple *et al*, 1996). If PEG-liposomes are also eliminated by a similar protein-dependent uptake mechanism, then the reduction in protein binding caused by PEG incorporation should correspond to increases in plasma lipid levels. LUV circulation levels were monitored in CD-1 mice in order to determine whether this was true. Single timepoints which would accurately reflect the differences in plasma concentration were chosen for each lipid composition, and 5 mol % PEG-DSPE was used to ensure a sufficiently dense polymer coating for both the negative and neutral lipid compositions.

Figure 3.3 shows the effect of incorporating 5 mol % PEG-DSPE on the plasma levels of DSPC/CHOL liposomes. At 4 hours, the PEG formulation demonstrates a plasma lipid concentration 1.6 times that of the control (Fig. 3.3A), and corresponds to a 25% reduction in the level of bound protein (Fig. 3.3B). Examination of EPC/CHOL/DOPA liposomes using 30 minute timepoints, demonstrated that the plasma lipid level increased by a factor of 3 compared to the control without PEG (Fig. 3.4A), while these PEG-liposomes experienced more than a 40% reduction in protein binding (Fig. 3.4B). Previous observations on the elimination of cationic liposomes with and without PEG showed 59.1 % and 0.8 % plasma lipid at 1 hour respectively (Mori *et al*, 1997), a more than 70 fold increase which relates well with the 90% reduction in protein adsorbed to DOPE/DODAC/PEG-DSPE liposomes (see Figure 3.2).

In attempting to relate the half-lives and protein binding values of PEG-liposomes with

those of equivalent conventional liposome formulations, one could estimate a half-life of more than 1000 min. generated for 100 mg/kg DSPC/CHOL/PEG liposomes (Fig. 3.7), which is significantly higher than the 300 min. that would be predicted by the protein binding versus half-life relationship graphed in the previous chapter for DSPC/CHOL liposomes (from the data of Fig. 2.6). Similarly, the EPC/CHOL/DOPA liposomes appear to possess a half-life close to the 30 min. timepoint (Fig. 3.4), while estimates from the previous chapter would suggest a half-life close to 20 minutes based on the level of protein binding (from the data of Fig. 2.6). This more than 3 fold and 1.5 fold increase in expected half-lives for DSPC and DOPA PEG-liposomes respectively, can be explained by the additional mechanisms through which PEG in proposed to interfere with liposome elimination from the blood. Specifically, that PEG in addition to decreasing blood protein adsorption, will also decrease the interactions between bound opsonins and their receptors on phagocytic cells and decrease any direct liposome interactions with phagocytic cells (reviewed in Section 1.4.2.2 and 1.4.3.2). This would explain the dramatic increases in half-life with only moderate decreases in the level of bound protein, such as those reflected in the greater than predicted half-lives of PEG-liposomes (based solely on protein binding quantity).

One must be careful in comparing protein binding quantities of different lipid compositions, because of the established differences in protein identities associated with different liposome compositions (including known opsonins)(Chonn *et al*, 1991a, 1992; Semple *et al*, 1996). However, the relative effects of PEG on liposome circulation does seem to be different for different compositions, with substantial increases in circulation half-lives of neutral liposomes with relatively small changes in the quantity of protein bound (compared to the changes of

DOPA-liposomes upon PEG incorporation). The reason for these differences is not clear, but it could involve changes in the relative importance of protein-mediated and alternative mechanisms of liposome uptake (discussed in Section 1.4.2) or even the established differences in the adsorption of specific proteins which mediate uptake which are influenced differently by the PEG coating.

3.3.3 PEG-liposomes experience dose-dependent plasma elimination and protein binding

In Chapter 2 conventional liposome formulations are observed to deplete liposome-binding blood proteins, resulting in less protein per liposome at higher doses, and a corresponding increase in the blood circulation lifetimes. Allen and associates have suggested that PEG-liposome uptake is independent of dose (Allen and Hansen, 1991), showing highly variable patterns of plasma elimination of different dose formulations. This implies that incorporation of PEG-polymer results in completely different liposome behaviour than that observed for conventional lipid vesicles. Our results show protein-dependent uptake of PEG-liposomes, which is similar to that of conventional lipid vesicles. The results of DSPC/CHOL/PEG, EPC/CHOL/DOPA/PEG, (Figures 3.3 and 3.4) as well as the additional data on the DOPE/DODAC/PEG liposomes (Section 3.3.2) all showed increases in plasma circulation levels that reflected the decreased amount of liposome adsorbed blood protein. These results showed a general inverse relationship between liposome protein binding and plasma elimination lifetimes that has been confirmed for a variety of PEG-free liposome formulations (Chonn *et al*, 1992; Semple *et al*, 1996), and suggests a protein-dependent mechanism eliminating these sterically stabilized vesicles. Therefore,

Figure 3.3 Effect of 5 mol % PEG-DSPE on the plasma elimination and protein binding values of DSPC/CHOL vesicles

DSPC/CHOL/PEG-DSPE (55-x:45:x, mol %) LUVs (100 nm) containing trace amounts of [^{3}H]-cholesteryl hexadecyl ether were administered intravenously into CD-1 mice at a lipid dose of 50 mg/kg. Panel A depicts the plasma levels of LUV's at 4 hours, measured by analyzing aliquots of plasma using standard scintillation counting methods. Each data point represents the average and standard error from 4 mice. Panel B depicts the protein binding values obtained for these formulations as determined by analyzing aliquots of recovered *in vivo* liposomes (see Section 3.2). Shaded regions indicate samples containing 5 mol % PEG-DSPE. Each data point represents the average and standard error obtained from 2 or more independently pooled samples of 4 mice.

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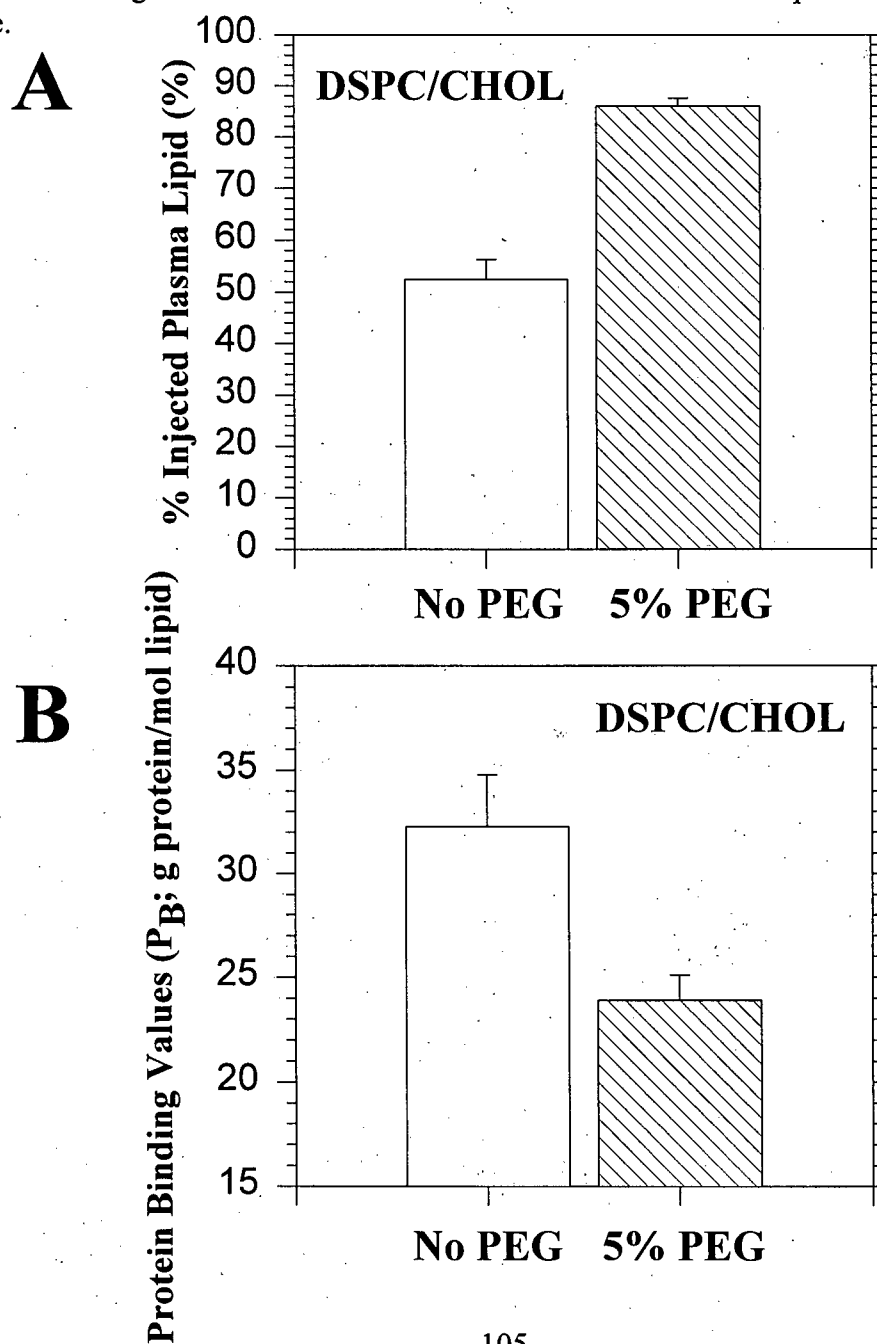
Figure 3.4 Effect of 5 mol % PEG-DSPE on the plasma elimination and protein binding values of EPC/CHOL/DOPA vesicles

EPC/CHOL/DOPA/PEG-DSPE (55-x:45:20:x, mol %) LUVs (100 nm) containing trace amounts of [^{3}H]-cholesteryl hexadecyl ether were administered intravenously into CD-1 mice at a lipid dose of 100 mg/kg. Panel A depicts the plasma levels of LUV's at 30 minutes, measured by analyzing aliquots of plasma using standard scintillation counting methods. Each data point represents the average and standard error from 4 mice. Panel B depicts the protein binding values obtained for these formulations as determined by analyzing aliquots of recovered *in vivo* liposomes (see Section 3.2). Shaded regions indicate samples containing 5 mol % PEG-DSPE. Each data point represents the average and standard error obtained from 2 or more independently pooled samples of 4 mice.
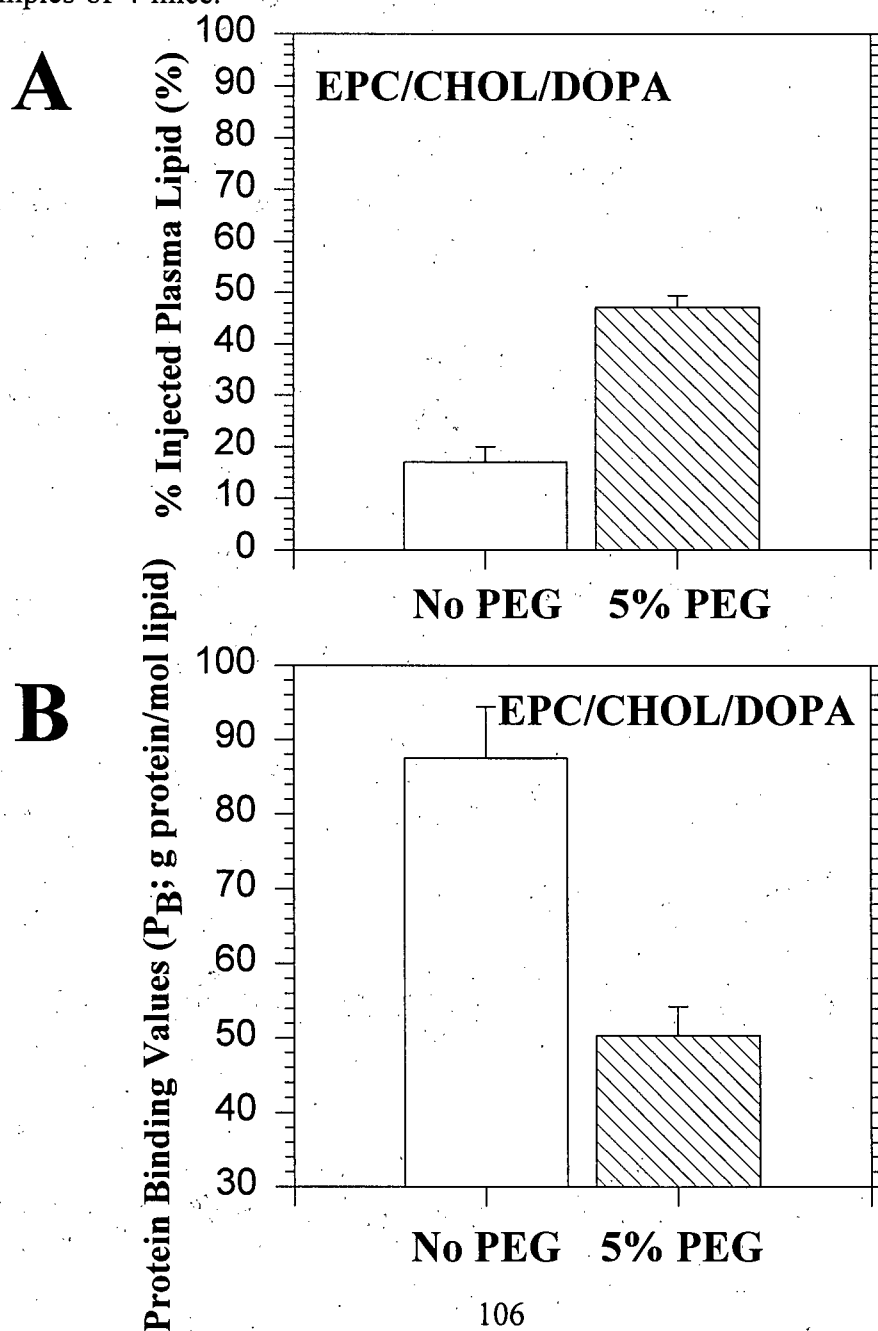
it was of interest to re-examine whether the *in vivo* behaviour of these PEG-systems was dependent on lipid dose. To determine this, the plasma elimination and protein binding of negatively charged (EPC/CHOL/DOPA) and neutral (DSPC/CHOL) liposome formulations were determined with and without 5 mol % PEG-DSPE over a range of lipid dose.

Figure 3.5 indicates that protein binding is reduced with increasing dose both in the presence and absence of PEG-polymer for negatively-charged liposomes. Additionally, DOPA-liposomes display a clear increase in plasma circulation levels with dose (Fig. 3.5B), which agrees with the decreasing trend in protein binding (Fig. 3.5A).

Neutral PEG-liposomes also experience similar changes in both protein binding and plasma lipid levels (Fig 3.6A and 3.6B). Small differences in the plasma lipid levels at 4 hours made it difficult to conclude dose-dependent blood elimination. However, an additional examination over a larger dose range and more timepoints, confirmed the dose-dependent changes in plasma elimination of neutral PEG-liposomes (Fig. 3.7). ANOVA tests confirmed variation in plasma elimination among different doses at each timepoint ($P < 0.05$), and Tukey-Kramer tests confirmed that although similar dose injections did not significantly differ (at $P < 0.05$) there was significant variation between the plasma elimination of low (5 and 10 mg/kg) and high (100 and 250 mg/kg) doses ($P < 0.05$). Additional studies over the full 500 mg/kg dose range examined for the negatively-charged PEG-liposomes, would likely even further support this trend. This contradicts the previous conclusion of completely dose-independent behaviour for PEG-liposomes (Allen and Hansen, 1991), whose different plasma elimination results may be partially attributed to the use of a less reliable radioactive liposome marker. The dose-dependence observed for either composition of PEG-liposomes is not as pronounced as that of the corresponding PEG-free

liposome formulation of the previous chapter. This once again can also be explained due to the multiple potential beneficial effects of PEG-polymers on the bilayer surface, including interference with ligand-receptor uptake and direct cell-liposome interactions. The dose-dependent effects of chapter 2 relied on the dilution of opsonizing blood proteins on the liposomes to reduce uptake by the phagocytic cells. If the additional mechanisms of PEG action also hinder the phagocytic pathways of liposomes, then the dose-dependent changes observed in this chapter should be less pronounced to reflect this decreased efficiency of uptake for all PEG-liposomes regardless of dose.

3.3.4 PEG-incorporation nonspecifically reduces the protein bound to liposomes

Previous investigations have shown that the composition of liposome-bound protein is important in determining blood elimination (Chonn *et al*, 1992), it was therefore important to compare the profiles of protein which bound to liposomes after addition of PEG-DSPE. SDS-PAGE electrophoretic separation of *in vivo* isolated protein samples allowed accurate determination of the protein profiles. The comparison of protein composition was simplified by examining equal protein quantities (3 μg / lane) from each liposome formulation. Figure 3.8, shows two independently isolated samples of neutral (lanes 1-2) and negatively charged (lanes 3-4) liposome compositions, with either 0 mol % PEG-DSPE (lanes 1 and 3) and 5 mol % PEG-DSPE (lanes 2 and 4).

As observed in Figure 2.8, the patterns of protein binding are not consistent with the patterns observed for normal mouse serum (representative of a normal plasma protein separation

pattern). These differences suggest that blood proteins have variable affinities for interactions with liposomes, as individual bands do not appear in proportion to there blood concentrations. Different patterns are clearly observed for EPC/CHOL/DOPA (gel A) versus DSPC/CHOL liposomes (gel B)(as in Figure 2.8), supporting the role of membrane lipid composition in determining liposome-blood protein interactions (Chonn et al, 1991a, 1991b, 1992, and 1995; Semple et al, 1995). However, the comparison of PEG-free to PEG-liposome associated proteins shows a substantial degree of similarity in the isolated profiles. Despite numerous similarities, there are some differences between PEG and PEG-free absorbed blood protein profiles, which are discussed in detail.

Quantification of the differences and similarities of the isolated protein samples was done as accurately as possible using the relative density determinations of the Strategene Eagleeye II gel densitometer, while also attempting to correct for discrepancies in the amounts of total protein applied to the different lanes of the gel (also based on density measurements of the gel). For instance in gel A of Figure 3.8 it is clear that the left hand lane #1, EPC/CHOL/DOPA, was loaded with additional protein despite attempts to load identical quantities of total protein in each lane, resulting in some visible differences when compared to the righthand lane #1. These are corrected by the density estimations.

Results of quantitative comparison of the protein bands showed significant variation of a number of protein bands. First, examination of DOPA-containing liposomal proteins revealed bands at 200, 170, 150, and 70 kDa which are slightly higher for one of the two PEG-containing isolated protein samples than the chosen acceptable degree or variability of 1 standard deviation from the mean of the 4 DOPA-samples shown. The 0 mol % PEG samples of DOPA-liposomes

were both associated with slightly larger amounts of a 45 and 50 kDa band of protein. Also, despite the overloading of protein in the lefthand lane #1, this 0 mol % PEG sample also possesses a visibly more substantial level of a 40 kDa protein than all other DOPA-liposome samples. One of the two 5 mol % PEG liposome samples was isolated with very significantly lower quantities of a 66 kDa while the other had slightly lower levels of a 22 kDa protein band. Overall, although there are several proteins bands with significant variability, the lack of consistency of these changes for duplicate samples suggests that these differences might be attributed to variability in isolation, gel separation or other quantitative sources of error. The numerous other protein bands which showed no significant variation, and the lack of proteins which were actually unique to either the 0 mol % or 5 mol % EPC/CHOL/DOPA liposome preparations suggests that the majority of bound proteins are the same with or without the presence of PEG-DSPE. This supports a mechanism of reduced protein binding which acts relatively nonspecifically with respect to protein identity.

Examination of the proteins isolated from DSPC-liposomes (gel B of Figure 3.8) showed several bands with densities that showed some variability greater than 1 standard deviation from the mean of the densities for the specific bands. Protein bands at approximately 170, 70, 66, 45, 43 and 30 kDa showed marginally increased association with either one or both of the two PEG-containing neutral liposomes, over the same liposomes with 0 mol % PEG-DSPE. In contrast, DSPC/CHOL vesicles without PEG, were associated with slightly higher levels of approximately 150 and 160 kDa molecular weight proteins. As observed in the previous DOPA-analyses, these variations are relatively small changes in the relative densities of proteins associated with both 0 mol % and 5 mol % PEG-DSPE DSPC/CHOL lipid compositions. The only evidence of a truly

unique protein band (a change in the actual composition of the bound protein profile) is one band associated with 5 mol % PEG DSPC-liposomes (gel B) at approximately 155 kDa which appears in the profile of both PEG samples, but neither of the PEG-free liposomal protein samples (in the lanes numbered 3). This band appears by it's density to also be a very minor component of the entire protein profile, although that does not discount it's potential significance in altering clearance, as some minor protein components appear to have significant effects on liposome clearance (β_2-GP-I, complement C3)(Chonn et al, 1992; Chonn et al, 1995). There is also some general darkening of the very high molecular weight region (>200 kDa) of the gel of the lane 3 samples of 0 mol % PEG/DSPC/CHOL, compared to the PEG-vesicles, although no distinct bands are visible. As before, the changes observed after the addition of PEG are primarily changes in the relative densities of protein bands that fall just outside of the range set up as significant for these examinations (one standard deviation), except for one protein unique to PEG-vesicles. The possibility of error in the isolation, quantification, and gel separation can at least partially explain these density differences. Overall, this suggested very little clear evidence of changes in the composition of proteins associated with liposomes due to the incorporation of PEG-DSPE in to the bilayer.

Overall, the reduction in protein binding observed for PEG-liposomes apparently results from a relatively nonspecific or general decrease in the ability of all blood proteins to bind to a bilayer surface coated with PEG-polymer molecules. Only some variation in relative densities of protein bands seemed to fall outside the selected range of acceptable variation. This is in contrast to other changes between different lipid compositions, for example between DSPC/CHOL and EPC/CHOL/DOPA which show substantial visual variation of the identities and amounts of

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associated protein bands (as seen on Figure 2.8 and 3.8)

Figure 3.5 Effect of lipid dose on the protein binding values and plasma elimination of EPC/CHOL/DOPA vesicles with and without 5 mol % PEG-DSPE

EPC/CHOL/DOPA/PEG-DSPE (55-x:45:20:x, mol %) LUVs (100 nm) containing trace amounts of [^{3}H]-cholesteryl hexadecyl ether were administered intravenously into CD-1 mice over a lipid dose of 100 to 500 mg/kg. Panel A depicts the protein binding values obtained for these formulations as determined by analyzing aliquots of recovered *in vivo* liposomes (see Section 3.2). Each data point represents the average and standard error obtained from 2 or more independently pooled samples of 4 mice. Panel B depicts the plasma levels of LUVs at 4 hours, measured by analyzing aliquots of plasma using standard scintillation counting methods. Each data point represents the average and standard error of 4 mice. Solid symbols represent formulations containing 5 mol % PEG-DSPE, while open symbol samples contain none.

113
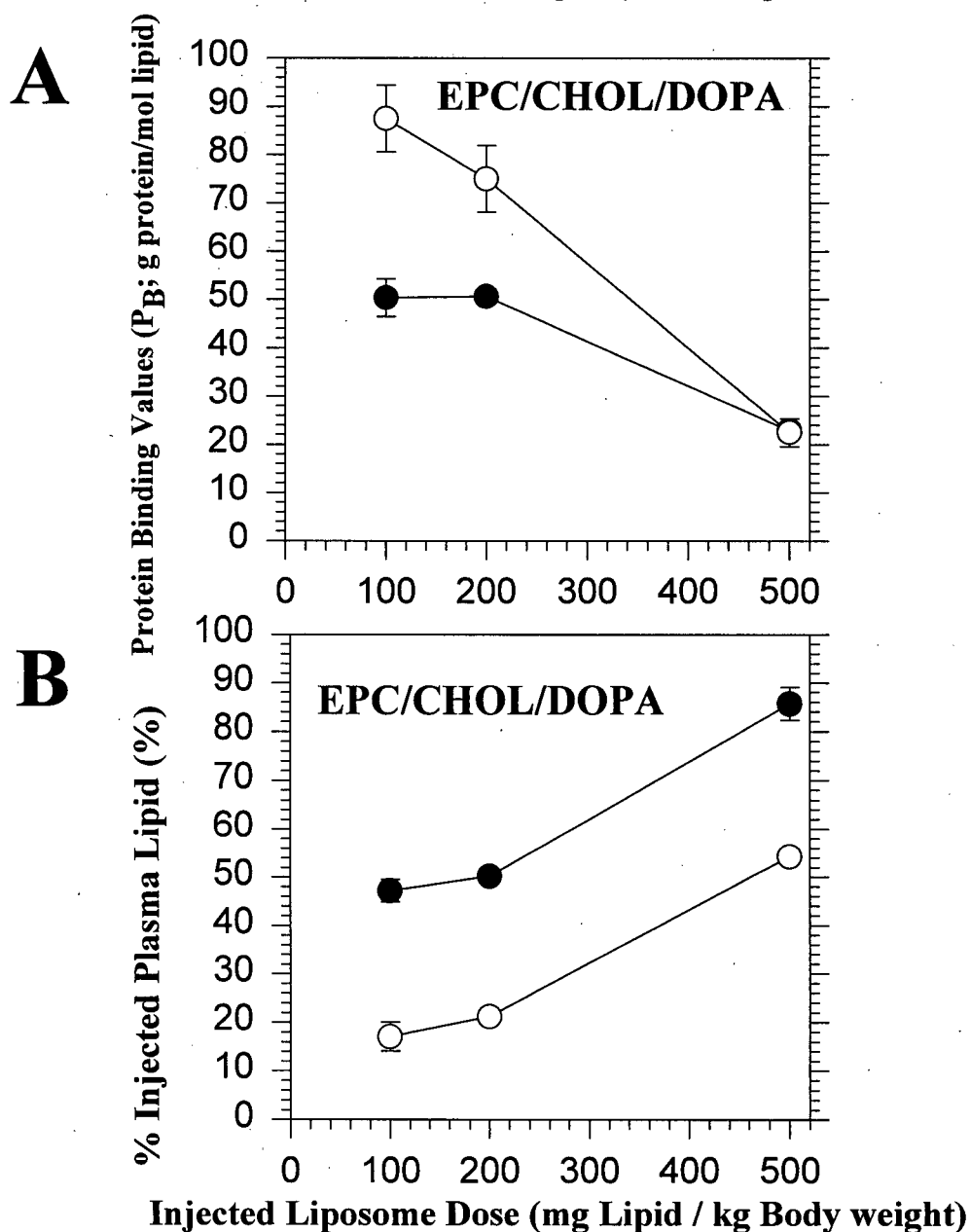
Figure 3.6 Effect of lipid dose and the protein binding values and plasma elimination of DSPC/CHOL vesicles with and without 5 mol % PEG-DSPE

DSPC/CHOL/PEG-DSPE (55-x:45:x, mol %) LUVs (100 nm) containing trace amounts of [^{3}H]-cholesteryl hexadecyl ether were administered intravenously into CD-1 mice over a lipid dose of 10 to 100 mg/kg. Panel A depicts the protein binding values obtained for these formulations as determined by analyzing aliquots of recovered *in vivo* liposomes (see Section 3.2). Each data point represents the average and standard error obtained from 2 or more independently pooled samples of 4 mice. Panel B depicts the plasma levels of LUV's at 4 hours, measured by analyzing aliquots of plasma using standard scintillation counting methods. Each data point represents the average and standard error of 4 mice. Solid symbols represent formulations containing 5 mol % PEG-DSPE, while open symbol samples contain none.

114
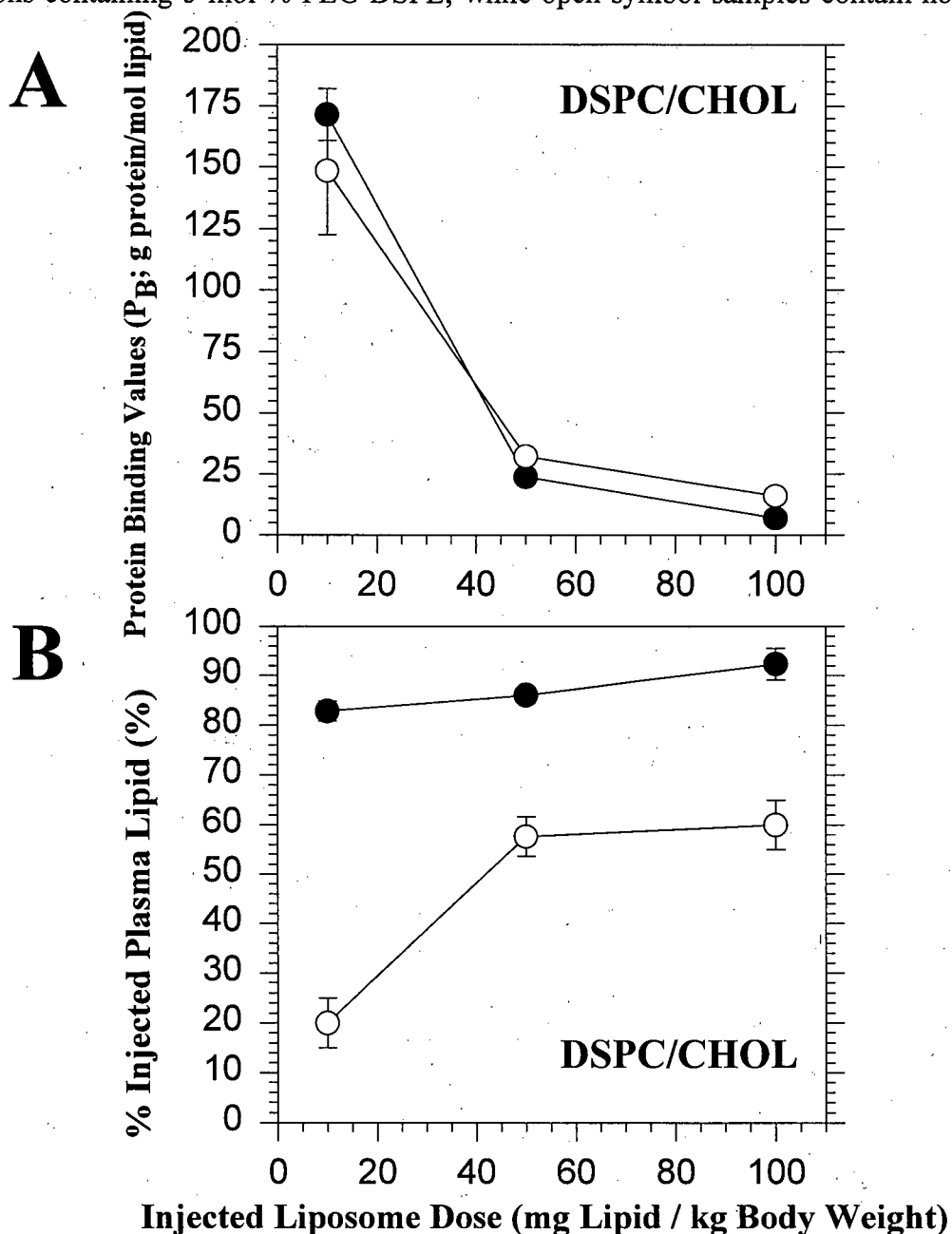
Figure 3.7 Effect of lipid dose on the plasma elimination of DSPC/CHOL vesicles with 5 mol % PEG-DSPE

DSPC/CHOL/PEG-DSPE (50:45:5, mol %) LUVs (100 nm) containing trace amounts of [^{3}H]-cholesteryl hexadecyl ether were administered intravenously into CD-1 mice. At various times, plasma levels of LUVs were measured by analyzing aliquots of plasma using standard scintillation counting methods. The graph depicts LUVs of varying dose: 5 mg/kg (+); 10 mg/kg, (O); 50 mg/kg, (□); 100 mg/kg, (▲); 250 mg/kg, (▽). Each data point represents the average plasma recovery and standard error of 4 mice. Using ANOVA and Tukey-Kramer tests each timepoint was determined to contain significant variation over the lipid dose range studied (P < 0.05).
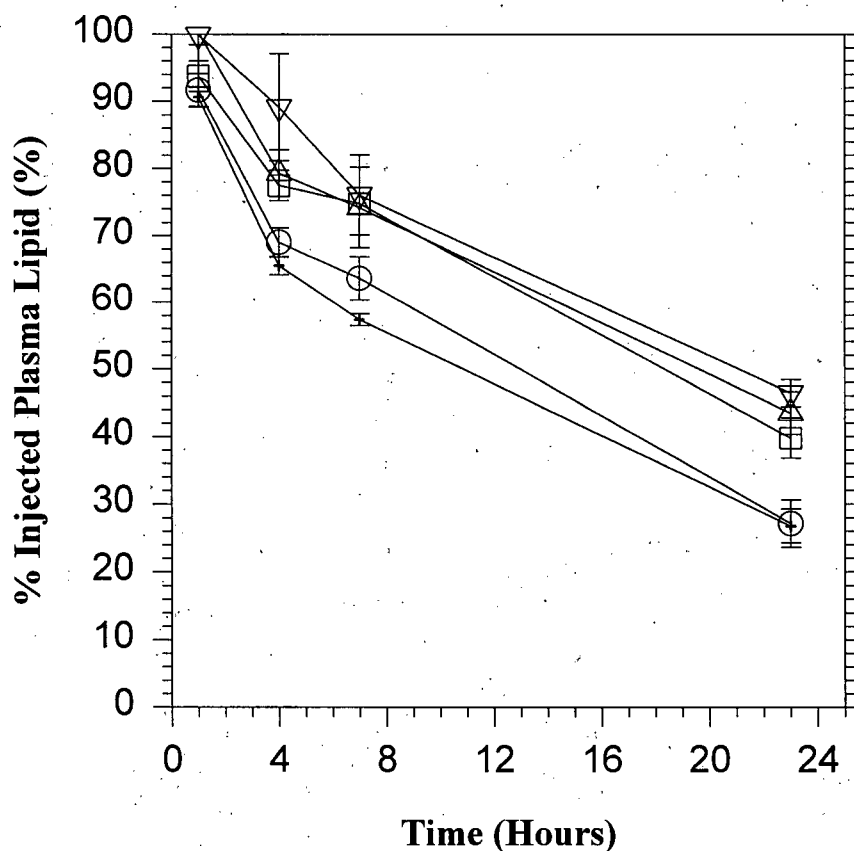
Figure 3.8 Effect of PEG-DSPE on the silver-stained reducing SDS-PAGE gels of proteins associated with liposomes recovered from the circulation of CD-1 mice

The proteins associated with liposomes isolated from CD-1 mice after 2 minutes, were separated electrophoretically on 4-20% SDS polyacrylamide gels and visualized by silver staining. Panel A consists of 3 µg of total protein isolated from EPC/CHOL/DOPA (35:45:20, mol %) liposomes with (lane 1) and without (lane 2) 5 mol % PEG-DSPE injected at 100 mg/kg. Panel B consists of 3 µg protein samples isolated from DSPC/CHOL (55:45, mol %) liposomes with (lane 1) and without (lane 2) 5 mol % PEG-DSPE injected at 100 mg/kg. Lane M contains silver-stained SDS-PAGE molecular weight standards from Bio-Rad (myosin, 200,000; β-galactosidase, 116,250; phosphorylase b, 97,400; serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; trypsin inhibitor, 21,500; and lysozyme, 14,400). Lane S contains normal mouse serum.

116
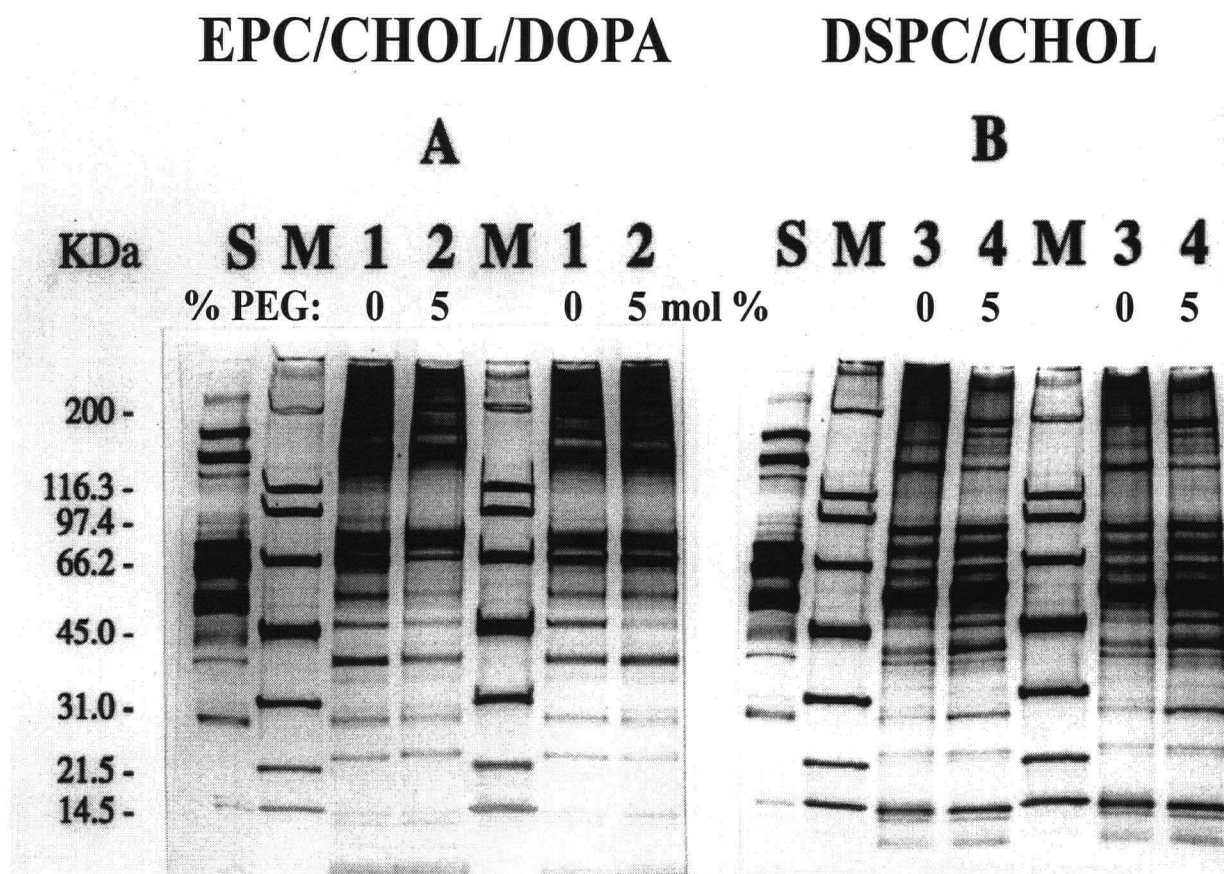
3.4 Discussion

The study of blood protein interactions with liposomes *in vivo* outlined in this chapter shows that incorporation of PEG-polymer significantly decreases protein interactions, presumably by reducing adsorption onto the bilayer surface. This reduction was common to all membrane compositions studied, and is consistent with one of the theoretical mechanisms of PEG action which proposes the formation of a statistical cloud of possible PEG-polymer conformations which sterically hinders the normal interaction of blood proteins with the vesicle surface (see Section 1.4.3.2). The results presented in this chapter support several important conclusions. First, poly(ethylene glycol) polymer coatings decrease protein binding, and that these decreases are associated with increases in the plasma circulation levels of liposomes. Second, PEG does not completely block protein adsorption to liposomes, but instead relatively nonspecifically reduces the proteins which bind to liposomes without the PEG-polymer coating. Although the majority of proteins adsorbed in similar amounts both to liposomes with and without PEG, biologically important proteins involved in liposome elimination could be among the individual proteins that did show quantitative binding changes. Third, PEG-liposomes exhibit dose-dependent decreases in their protein binding which corresponded to increased plasma circulation lifetimes, although these plasma circulation changes with dose were more substantial for negatively charged versus neutral liposomes.

Previous studies have shown that *in vivo* protein binding correlates very well with blood circulation lifetimes (Chonn *et al*, 1992; Semple *et al*, 1996), this same relationship is confirmed for the PEG-formulations studied here. Negatively charged liposomes incorporating 5% PEG experienced substantial decreases in bound protein (40%), which resulted in a 3-fold increase in

plasma lipid concentration relative to controls. Neutral vesicles experienced only moderately decreased protein binding with PEG (25%), and show correspondingly smaller (1.6-fold) increases in plasma lipid compared to controls. Positively charged vesicles followed a similar trend. These results demonstrated increases in circulation lifetimes as the protein interactions were reduced, providing support for a protein-mediated uptake mechanism also existing for PEG-liposomes. It appears that liposomes with naturally higher levels of protein interactions experience more substantial decreased protein binding upon PEG-incorporation. Perhaps lower levels of bound protein correspond to protein layers which cover less liposome surface area, which allows PEG to be present with less disruption of the adsorbed blood protein layer. Another explanation for the different effects of PEG could involve differences in protein populations. Different protein profiles associated with varying lipid compositions could have different binding affinities for membranes, different binding mechanisms, or even different surface areas when adsorbed to the liposome surface - which could all explain variation in the ability of PEG-polymers to disrupt these normal interactions. It is interesting to estimate the surface coverage of the protein layer bound to liposomes. Protein binding values of PEG-liposomes are less than those of the control compositions, however, even the higher levels of protein bound to control liposomes result in a maximum of 2.5% of available surface area covered by protein in high protein binding samples (close to 100 g protein/mol lipid)(see Section 2.3.2). The PEG-free cationic liposomes studied within these experiments are the only liposome compositions to actually be substantially covered by blood protein with approximately 20% of their surface being covered by blood proteins (at protein binding values close to 900 g protein/mol lipid). Accordingly, the absorbed protein layer would likely be a sparse monolayer of blood protein with the majority of surface still made up

of uncovered lipid molecules. These estimates assume that the proteins retain a small globular structure, studies of blood proteins on surfaces have suggested a wide range of unfolding which could substantially vary these calculations of surface coverage. However, even with large amounts of unfolding the majority of the liposome surface could remain free of protein. This supports a model allowing some level of blood protein interaction, despite the PEG-layer, as the bound blood protein might need only a minimal amount of the available lipid surface on which to act.

The results presented here show that PEG-coatings do not create an impenetrable barrier to protein adsorption, on the contrary, the charged PEG-formulations still experienced significant levels of protein binding (50-100 g protein / mol lipid), compared to neutral formulations without PEG. As with conventional LUVs, PEG containing LUVs exhibit dramatic differences in protein binding and plasma circulation depending on the characteristics of the membrane lipids. Rapidly cleared cationic and anionic formulations bind substantially more protein than neutral systems with or without the PEG-coating, and experience more rapid blood elimination. Similar high levels of bound protein have previously been correlated with significant binding of specific opsonins (such as immunoglobulin and complement protein) which was also suggested to explain their rapid elimination (Chonn *et al*, 1992). Dramatic changes in elimination behaviour have been attributed to a combination of protein quantity and identity, and the same appears to be true for these sterically stabilized liposomes. SDS-PAGE separation of equal quantities of isolated liposome-bound proteins showed only some marginal changes in the relative densities of some of the associated protein bands, but no changes in the actual identities of proteins bound upon incorporation of PEG-polymer. This supports a relatively nonspecific mechanism of PEG action, whereby interactions with all bound blood proteins are reduced resulting in the changes in

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liposome behaviour. Although, care must be taken to consider the possible importance of even minor protein bands which could correspond to proteins known to be crucial to the clearance of liposomes.

PEG-liposomes also exhibit dose-dependent changes in protein binding and plasma lipid elimination, especially for the negatively-charged DOPA-formulations. This is consistent with the behaviour of conventional liposome formulations, outlined in Chapter 2. This contradicts an earlier suggestion that sterically stabilized vesicles experience dose-independent elimination behaviour (Allen and Hansen, 1991). These studies indicate that the bound protein is reduced at higher lipid doses, at least partially explaining the less efficient plasma elimination of these high dose samples. In Chapter 2 it is suggested that this is caused by depletion of blood proteins which are diluted or spread out over the larger lipid surface area of high liposome doses. PEG-liposomes of different doses also experience these changes, however, the change in circulation with dose appears much less significant for PEG-containing DSPC/CHOL vesicles. This suggests that the low amounts of protein associated with neutral liposomes (especially those proteins which specifically mediate clearance), combined with the additional mechanisms by which PEG would interfere with liposome elimination, result in very little specific opsonin-mediated uptake possible for these neutral PEG-liposomes. This could even suggest that at low enough levels of opsonizing proteins on liposomes, the additional actions of PEG could almost eliminate the interactions of bound opsonins with their phagocytic cell receptors - explaining the minor alterations in clearance observed with dose for PEG-containing neutral liposomes.

In summary, the results presented here support the conclusion that PEG-liposomes experience a nonspecific decrease in the binding of blood proteins which is at least partially

responsible for their reduced RES uptake. As noted earlier (Section 3.3.2) these amounts of protein bound to PEG-liposomes do not directly follow the graphed results of Chapter 2 (Fig. 2.6) for the corresponding PEG-free formulations. Instead longer circulation half-lives are produced than would be suggested by the amount of protein alone. It must be stressed that the decreased protein binding with PEG incorporation is not the sole mechanism by which the circulation lifetimes of these vesicles are enhanced. As discussed in the introduction (see Section 1.4.3.2), the polymer coating can also interfere with the interactions between liposome-bound opsonizing proteins and their phagocytic cell receptors. In addition, the same mobile polymer molecules can interfere with any direct interactions between the coated liposomes and phagocytic cells membranes, including additional protein-independent uptake pathways suggested in the introduction (see Section 1.4.2.2). These mechanisms can also help explain the substantial success of these polymer-coated liposomes in avoiding elimination from the blood, but still do not take away from the obvious importance of reduced blood protein interactions in enhancing the circulation lifetimes of PEG-liposomes.

CHAPTER 4: ENTRAPPED DOXORUBICIN INHIBITS IMMUNE RESPONSES TO LIPOSOME ASSOCIATED ANTIGENS

4.1 Introduction

As discussed in Chapter 1, the latest generation of liposomal drug delivery systems has focused on delivery to specific target cells through antibodies and other ligands coupled to the membrane surface (see Section 1.4.3.5). The earliest techniques for coupling antibodies to liposomes resulted in rapidly cleared, unstable formulations (Aragnol and Leserman, 1986; Debs et al, 1987). The later addition of PEG-polymers improved stability and reduced the RES uptake of targeted systems (Loughrey et al, 1993), although it also interfered with the binding of ligands to their target receptors (Klibanov et al, 1991; Mori et al, 1991). Despite these difficulties, there have been successful in vitro and in vivo delivery of liposomes and their contents to target cells (Longman et al, 1995; Suzuki et al, 1995; Goren et al, 1996; Vingerhoeds et al, 1996).

One of the major remaining obstacles in the application of targeted liposome delivery systems concerns immune response to the surface-coupled molecules. The natural adjuvant property of liposomes has been recognized since the 1970's (Gregoriadis, 1976), with the most significant humoral immune response generated against vesicles with protein covalently coupled to the surface (Therien et al, 1991; Shahum and Therien, 1994). This generation of humoral immune response will result in rapid elimination of these targeted drug delivery systems from the circulation, creating a major problem in their potential applications in the treatment of disease. Encapsulation of cytotoxic drugs (such as doxorubicin), has been suggested as a possible solution

to immune responses against surface-coupled molecules, due to their toxic effects on cells of the immune system which interferes with antigen processing and presentation (Shek *et al*, 1986; Van Rooijen and Sanders, 1994; Tardi *et al*, 1997). The role of macrophages in the generation of immune response to liposomal antigens has been well documented (Shek and Lukovich, 1982; Su and Van Rooijen 1989; Szoka, 1992), and liposomal doxorubicin is known to be toxic to liver Kupffer cells and splenic macrophages (Daemen *et al*, 1995 and 1997). The administration of liposomal doxorubicin should therefore directly inhibit immune responses involving these cells, by inhibiting the antigen processing and presenting functions of these cell populations. Additional evidence has documented direct interactions between liposomal surface proteins and antigen-specific lymphocyte subpopulations (reviewed in Section 1.5.3.2), providing a potentially more efficient route for the suppression of the humoral immune response by protein-coupled liposomes containing cytotoxic drug (Shek *et al*, 1986). The primary objective of these experiments was to characterize the dose limitations and the possible antigen selectivity of the immune suppression induced by doxorubicin encapsulated in proteoliposomes, for the purpose of evaluating the potential of and problems for targeting liposome encapsulated doxorubicin.

4.2 Materials and Methods

4.2.1 Materials

DSPC (1,2-distearoyl-sn-glycero-3-phosphocholine) and MPB-DSPE (N-(4-(P-maleimidophenyl) butyryl) distearoyl phosphatidylethanolamine) were purchased from Northern Lipids (Vancouver, BC, Canada). PEG-DSPE was generously provided by Dr. Steven Ansell of Inex Pharmaceuticals (Vancouver, BC, Canada). Lipid marker, [^{3}H]-cholesteryl hexadecyl ether (CHE) was provided by NEN Research Products, Mississauga, ON, Canada. Doxorubicin was obtained from Adrian Laboratories Inc. (location, location, USA). Imject® Ovalbumin, biotinylated goat anti-mouse IgG, and streptavidin-linked β-galactosidase were purchased from Pierce Chemical Company (Rockford, IL, USA). Female CD-1 mice were obtained from Charles River (St. Constant, PQ, Canada). Cholesterol (CHOL), SPDP (N-succinimidyl 2-(2-pyridyldithio) propionate), dithiothreitol (DTT), HEPES (N-2-hydroxyethylpiperazine-N-2-ethane-sulphonic acid), citrate, CL-4B Sepharose, G-50 Sephadex, hen egg lysozyme (HEL), bovine serum albumin and most other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

4.2.2 Preparation of liposomes

Large unilamellar vesicles (LUVs) were prepared as outlined in Chapter 2. DSPC/CHOL/MPB-DSPE/PEG-DSPE (52:45:1:2 mol %) were prepared in 300 mM citrate, pH 4.0, at 70 mM total lipid concentration. This produced homogenous vesicles of all of an average

size of approximately 100 nm ± 30 nm as determined by QLS. When used for biodistribution, liposomes were radiolabelled using a lipid marker, [^{3}H]-cholesteryl hexadecyl ether (CHE) (1 µCi / 3 µmol of total lipid). For all other samples a trace amount of this label (0.01 µCi / µmol) was used to monitor lipid concentration changes. A pH gradient for drug-loading was generated by exchanging the external buffer with HEPES-buffered saline solution (HBS; 20 mM Hepes, pH 7.5, 145 mM NaCl) by gel filtration on pre-packed sephadex G-25 minicolumns (PD-10 columns)(Pierce Chemical Company).

4.2.3 Protein coupling to liposomes

Imject® Ovalbumin (10 mg/mL in 0.9% saline) was coupled to the amine cross-linker SPDP as described previously by Loughrey *et al* (Loughrey *et al*, 1990a and 1990b)(see Figure 4.1). Ovalbumin was selected because it is highly immunogenic, possesses well studied antigenic epitopes and immune response behaviour, and has been prepared in a purified, stable form ideal for these coupling procedures. Briefly, SPDP was dissolved in ethanol and diluted with HBS just prior to addition to the ovalbumin solution in a 5-fold molar excess. After a 30 minute reaction at room temperature, the unreacted SPDP was removed using a G-50 Sephadex column equilibrated with HBS (pH 7.5). The sample was then reduced with DTT (25 mM for 30 min at room temperature). The thiolated modified-ovalbumin was isolated on a G-50 sephadex column just prior to coupling to liposomes at a ratio of 90 µg protein / µmole lipid (15 mM final lipid concentration), for 18 hours at room temperature under nitrogen. Free protein was separated from the protein-coupled liposomes by passage down a Sepharose CL-4B column (HBS, pH 7.5).

Figure 4.1 Procedure for protein conjugation to liposomes

SPDP Modification of Protein

30 minutes
Room Temperature

MPB-DSPE

DTT for 30 minutes
Room Temperature

Liposome-MPB

Reduced PDP-Protein

18 hours
Room Temperature

Protein-MPB-DSPE Liposome Conjugate

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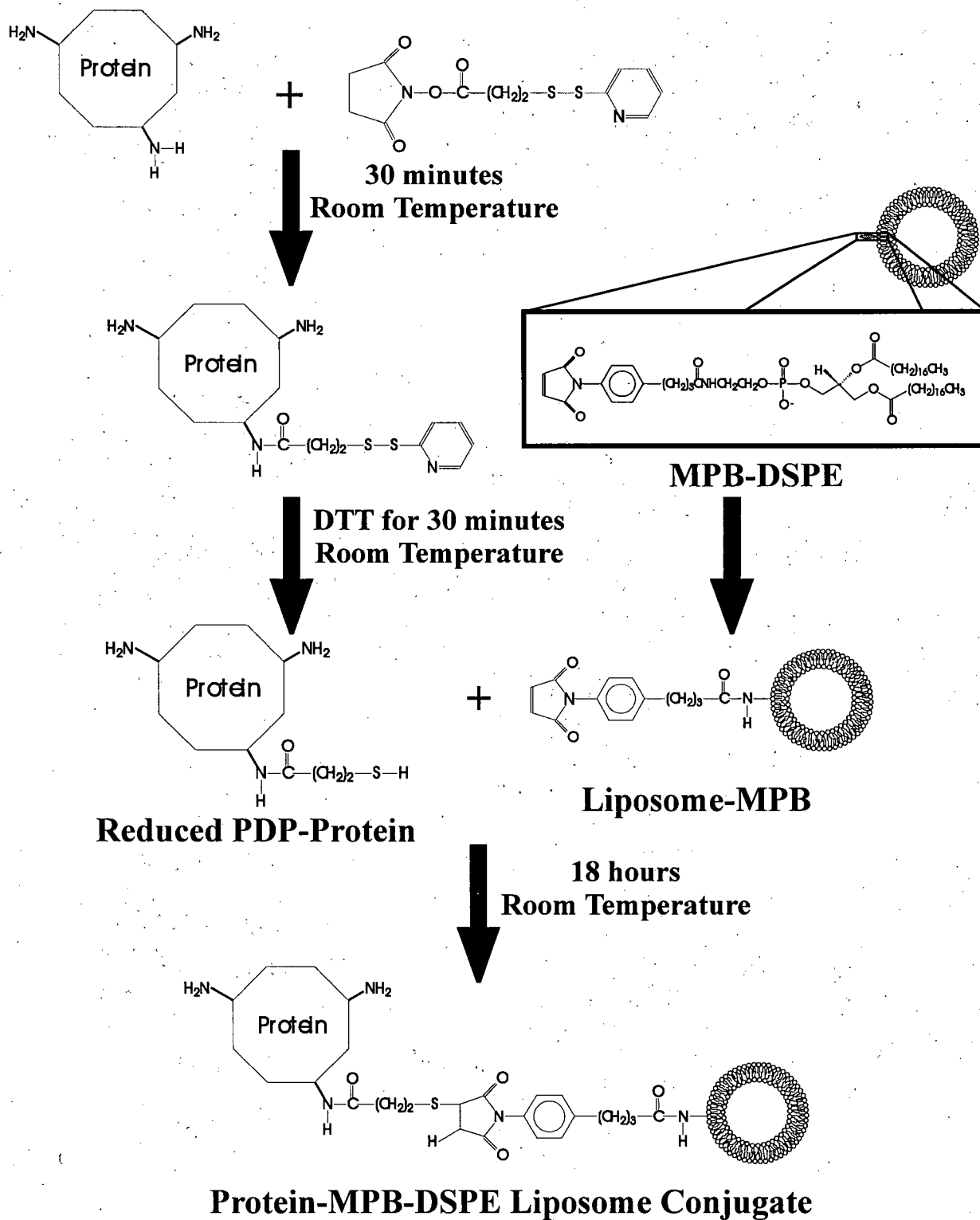
Quantification of ovalbumin coupling was carried out using the Pierce Micro BCA protein assay in the presence of 1% Triton-X-100 to disrupt the liposomes. The ovalbumin coupling procedure consistently resulted in 30-40 µg protein / µmol lipid. Coupling of hen egg lysozyme (HEL) followed the same procedures outlined above and consistently produced approximately 40 µg protein / µmol lipid. The choice of HEL was also made due to its highly antigenic nature and well studied immunogenic behaviour of the protein in animal systems.

4.2.4 pH gradient doxorubicin encapsulation

For indicated samples doxorubicin was encapsulated using a transmembrane pH gradient as previously described by Mayer and associates (Mayer *et al*, 1986b, 1990a and 1990b). Ovalbumin-coupled liposomes with an internal pH of 4.0 (citrate buffer) and an external pH of 7.5 (HBS) were heated to 65°C (5 min) before mixing and incubating with a doxorubicin solution for 10 minutes (10 mg/mL in 0.9% saline, 65°C). The samples were measured to achieve specific drug-to-lipid ratios (usually 0.2 mol/mol). This procedure results in >95% trapping efficiencies, eliminating the need to separate free doxorubicin (Mayer *et al*, 1990a). For ovalbumin-liposome formulations not requiring doxorubicin, the same procedure was carried out using a drug-free 0.9% saline solution.

4.2.5 *In vivo* mouse biodistribution studies

Liposome formulations (200 µL) were administered via the lateral tail vein of female CD-1

mice as outlined in Chapter 2 (see Section 2.2.1). Lipid doses (except where specified in figure legends) contained 50 μg of coupled protein, equivalent to a lipid dose of approximately 45 mg lipid / kg body weight and a doxorubicin dose of 8 mg/kg. All time points were taken four hours after the designated injections, with mice being killed by exposure to carbon dioxide. Plasma liposome levels were measured using the procedures outlined in Chapter 2 (see Section 2.2.2), except that blood was collected in EDTA-coated plasma collection tubes (Microtainer Tubes)(Bectin-Dickinson). All *in vivo* analysis used four mice per time point and the standard error as error bars.

4.2.6 Immunization studies with ovalbumin-coupled liposomes

All immunization experiments used liposome formulations as described above, with samples possessing [^{3}H]-CHE only when monitoring biodistributions. Unless otherwise designated in figure legends, experiments involved i.v. injection of specified formulations for two consecutive weeks (priming and secondary stimulation). Plasma elimination studies examined biodistributions of the second weekly injections at four hour timepoints. Immune response to the coupled protein was determined by ELISA detection of antigen-specific IgG 7 days after the second weekly injection. Additional experiments on the prevention, specificity, or elimination of immune response used procedures outlined above with the dosing and injection schedules described in figure legends and the appropriate Results sections.

4.2.7 ELISA assay for antigen-specific immunoglobulin

96 well micro-assay plates (Bectin Dickinson, Franklin Lakes, N.J., U.S.A.) were coated with 50 μL / well of 40 μg / mL ovalbumin and incubated at 4°C overnight (HEL was similarly examined in specificity studies). The plates were washed twice with PBS-Tween 20 (phosphate buffered saline, Ca^{+2} and Mg^{+2} free, 0.1% (vol/vol) Tween 20), before blocking for 1 hour with 2% bovine serum albumin (in PBS) at 37°C. After rinsing with PBS-Tween 20 and then 1% BSA (in PBS), 100 μL samples of sample plasma dilutions in PBS were allowed to incubate at room temperature for 1 hour. Plates were subsequently rinsed three-times with PBS-Tween 20, before addition of 100 μL aliquots of 0.5 μg/mL biotinylated goat anti-mouse IgG (1% BSA, PBS)(30 min, room temperature). Plates were again rinsed three times with PBS-Tween 20, before addition of 100 μL of 500 mU / mL of streptavidin-linked β-galactosidase (1% BSA, PBS) (30 min, room temperature). Plate-associated β-galactosidase was determined by 100 μL of 2 mg / mL substrate, CPRG (chlorophenol red-β-D-galactopyranoside). After 20 min the production of chlorophenol red was monitored by determining the absorbance (relative to controls) at 570 nm on a Titertek Multiscan plate reader (Titertek Instruments Inc., Huntsville, A.L., U.S.A.). Controls consisted of plasma from untreated female CD-1 mice (see diagram of procedure Figure 4.2).

Figure 4.2 ELISA assay procedure for detection of ovalbumin and lysozyme

● = Ovalbumin or Lysozyme Protein

= Protein-Specific IgG from Mouse Plasma

= Biotinylated Goat Anti-Mouse IgG

= Streptavidin-Linked β-Galactosidase
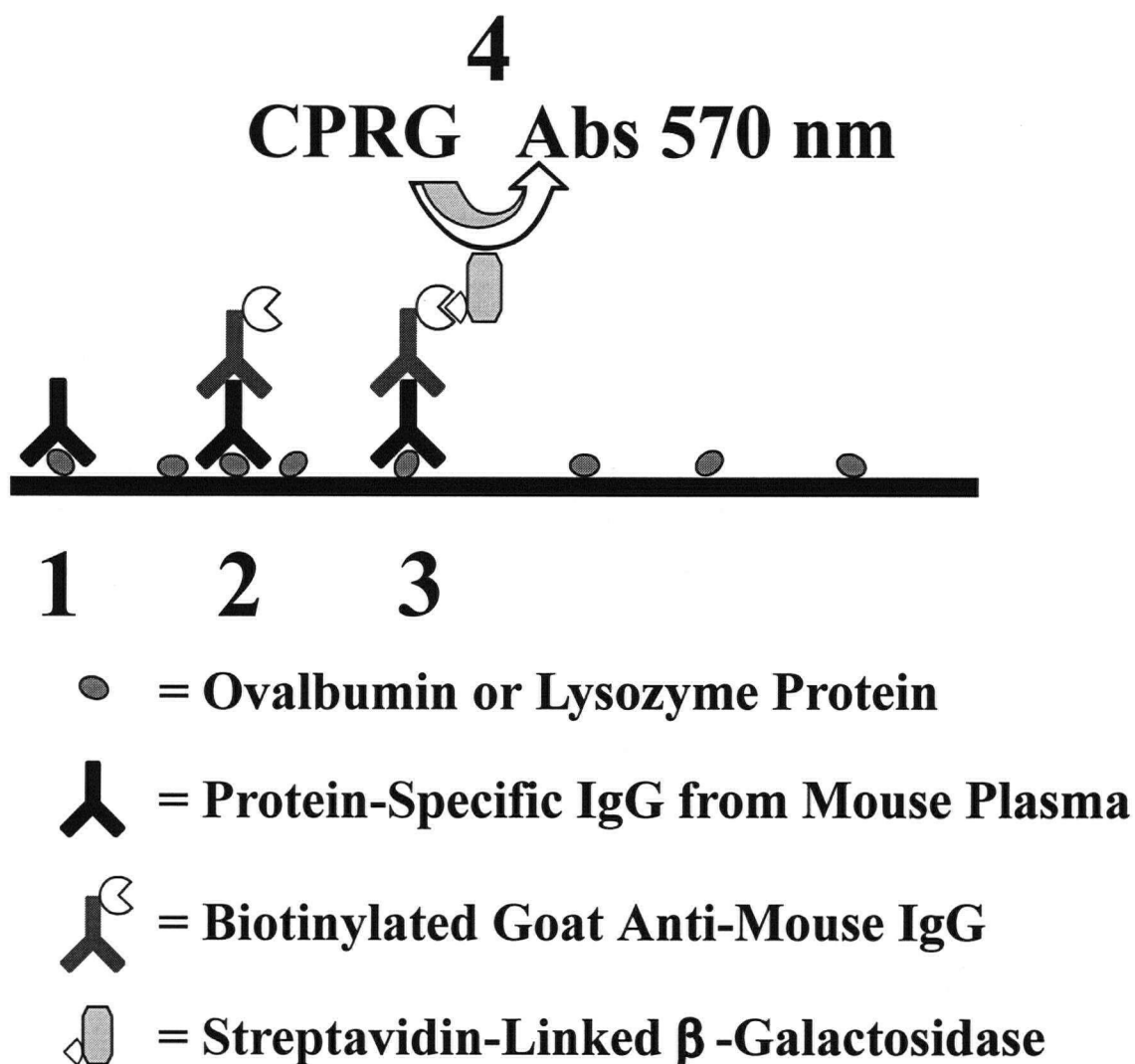
1- Mouse Plasma Incubation

2- Biotinylated Goat Anti-Mouse IgG Added

3- Streptavidin-Linked β-Galactosidase Added

4- CPRG Reaction Monitored (570 nm Absorbance)

4.3 Results

4.3.1 Entrapped doxorubicin eliminates the humoral immune response against liposomal-ovalbumin

Investigations using liposomes with surface-bound molecules have revealed the generation of humoral immunity. In the experiments presented here, we use liposomes coupled to the highly immunogenic protein ovalbumin to study the effects of cytotoxic drugs such as doxorubicin on this immune response. These proteoliposomes are models for targeted liposome delivery systems, and will assist in evaluating the potential problems of immune response against targeted liposomes containing this or similar cytotoxic drugs.

Figure 4.3 clearly shows that i.v. administration of empty ovalbumin proteoliposomes induces a significant immune response against ovalbumin and rapid elimination of subsequent injections. All subsequent proteoliposome administrations were completely cleared from the blood predominantly by the organs of the RES before the 4 hour timepoint as shown by the 50 µg ovalbumin treatments (Fig. 4.3A), with similar results for 5 µg ovalbumin injections (results not shown). Complete elimination from the blood has been observed as quickly as 15 minutes (Tardi *et al*, 1997), suggesting that high levels of ovalbumin-specific immunoglobulin mediate the immediate uptake of these vesicles. Production of ovalbumin-specific IgG (detected by ELISA) correlated with the rapid elimination from the blood, with significant levels of ovalbumin-specific antibody detected 7 days after the second weekly injection (Fig. 4.3B). The results suggest an "all-or-none" humoral immune response, with only 0.5 µg ovalbumin proteoliposome treatments

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low enough to fail to stimulate humoral immunity. This suggests that differences due to the lipid dose are not substantial factors in the induction of immunity and subsequent clearance of these protein-coupled liposomes. The level of detected immune response and immune-mediated clearance behaviour of the subsequent injections was not related to injected lipid or antigen dose over the range used for all subsequent trials. Minor changes in the biodistribution of liposomes related to the presence of small amounts of non-opsonizing protein conjugated to the liposome surface or small size changes among liposome populations should not be major factors in determining the results of these experiments, due primarily to the increased levels of specific antibody-mediated clearance mechanisms. Importantly, encapsulation of doxorubicin within these proteoliposomes at a drug-to-lipid ratio of 0.2 and a total drug dose of 8 mg/kg (the maximum tolerated dose of doxorubicin in encapsulated dose of doxorubicin encapsulated in DSPC-cholesterol LUVs is in excess of 50 mg/kg (Mayer *et al*, 1990a)) demonstrated complete elimination of the immune response to ovalbumin (Fig. 4.3B), and restored the long circulation lifetimes of subsequent injections (Fig 4.3A).

Figure 4.3 Dose titration of ovalbumin-proteoliposomes with and without entrapped doxorubicin

Female CD-1 mice were injected i.v. for three consecutive weeks with varying doses of ovalbumin-coupled liposomes up to 50 µg of protein. Formulations were either empty or contained doxorubicin encapsulated at a 0.2 drug-to-lipid ratio (mol/mol), producing a maximum lipid dose of 45 mg/kg containing 8 mg/kg of drug. Panel A depicts the plasma levels of injected lipid 4 hours after each weekly injection of 45 mg/kg lipid doses of empty and DOX-loaded liposomes, determined using standard scintillation counting to detect the nonexchangeable, nonmetabolizable radiolabelled lipid marker ([³H]-CHE) incorporated in the lipid bilayer. Panel B shows the relative production of ovalbumin-specific IgG (absorbance 570 nm) as determined by ELISA assays of plasma isolated 7 days after the second weekly injection of each formulation. Solid symbols represent doxorubicin-loaded and open symbols indicate empty-liposomes. All data points represent the mean and standard error of 4 mice. * indicates no measurable plasma lipid in the second and third week of empty ovalbumin-coupled liposomes.

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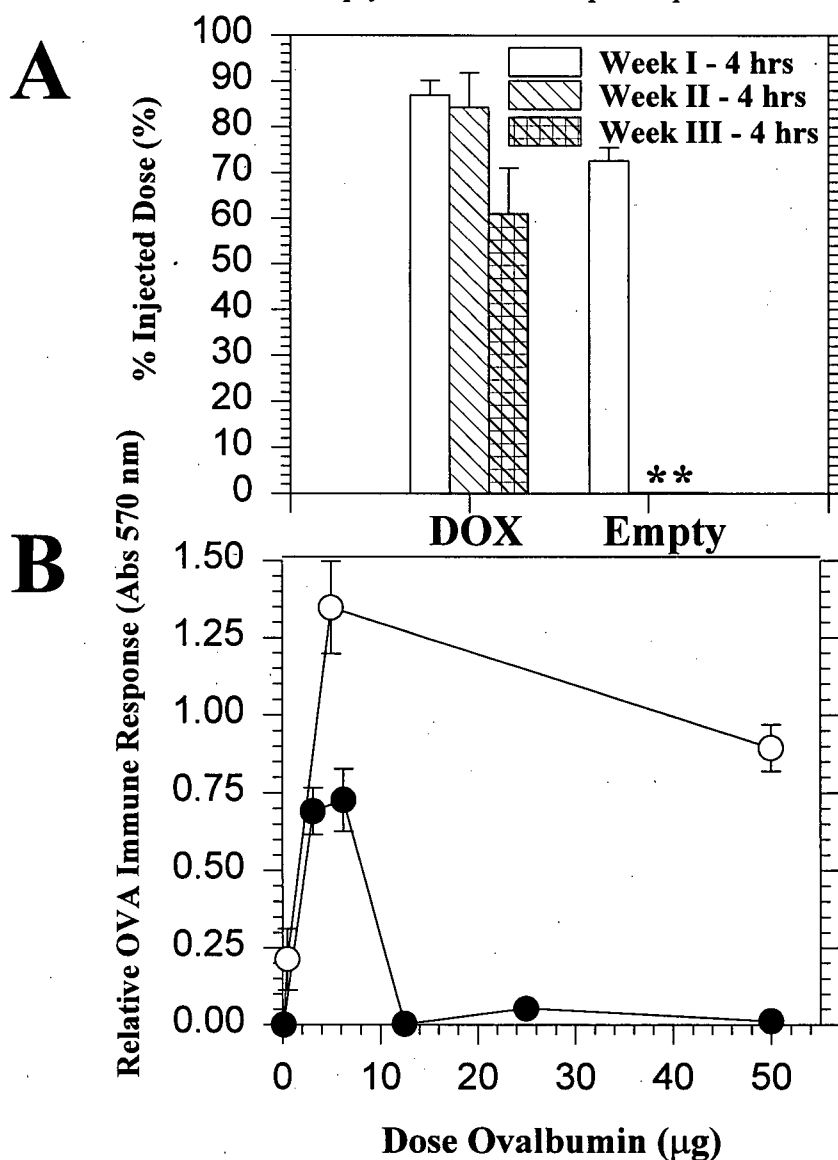
4.3.2 Influence of drug-to-lipid ratio on the immune response against liposomal-ovalbumin

Different drug doses within the LUVs would be expected to change the level of immune response to the liposome-coupled surface molecules. In order to determine the lowest dose at which doxorubicin within proteoliposomes eliminates the immune response, two injections of doxorubicin proteoliposomes at decreasing drug-to-lipid ratios were administered 7 days apart to the same groups of CD-1 mice. Initial experiments demonstrated that two injections were sufficient to manifest the changes in immune response and liposome behaviour, therefore each mouse in these trials received only two injections. The total lipid dose was kept constant at approximately 45 mg lipid / kg body weight, equivalent to 50 μg of coupled ovalbumin, so that the decreasing drug-to-lipid ratio resulted in decreased total drug dose. The results, summarized in Figure 4.4, show that lowering the drug-to-lipid ratio to a 0.1 drug-to-lipid ratio (equal to a 4 mg / kg total drug dose) still successfully suppressed the ovalbumin-specific immunity generated by second injection (Fig. 4.4). This resulted in high plasma lipid concentrations for the second injections (results not shown). At lower drug-to-lipid ratios, the immune response and rapid elimination are unaffected. Drug-to-lipid ratios lower than 0.1 did not result in suppression of the ovalbumin-induced immune response.

4.3.3 Influence of total doxorubicin dose on the immune response against liposomal-ovalbumin

Results of the previous section indicate that a high drug-to-lipid ratio makes encapsulated

doxorubicin more effective against immune response generation. In order to determine the lowest total drug dose that would result in suppression of the immune response, we administered two weekly injections of doxorubicin-proteoliposomes at a constant 0.2 drug:lipid ratio, but decreased the total drug dose by administering lower lipid doses. Figure 4.5, shows that high drug:lipid ratio preparations were more effective at eliminating immune responses than equivalent doses of doxorubicin encapsulated at lower drug-to-lipid ratios (Fig. 4.4). Only doxorubicin doses of 1 mg/kg or less failed to suppress the production of anti-ovalbumin IgG (Fig. 4.5). This indicates that delivery of highly concentrated doxorubicin within liposomes (high drug-to-lipid ratios) was twice as effective at inhibiting antibody production against the liposomal surface protein as lower drug-to-lipid ratio preparations, based on the minimum drug dose observed to suppress immune response in the above trials (4 and 2 mg/kg doxorubicin, respectively).

Liposomal doxorubicin has been shown to eliminate phagocytic cells, which are known to act as antigen presenting cells for the surface-coupled proteins (Daemen *et al*, 1995 and 1997). This might result in the inhibition of immune response through the negative effects of liposome encapsulated doxorubicin on the antigen processing and presentation functions of these cells. The significant uptake of liposomes and proteoliposomes by the macrophages of the liver and spleen (Roerdink *et al*, 1981; Poste *et al*, 1982; Shek and Lukovich, 1982; Su and Van Rooijen, 1989), suggests that protein-free liposomal doxorubicin could be just as effective as proteoliposomes with encapsulated drug, as both are accumulated within these phagocytic cells of the RES. To test this possibility, two weekly treatments of doxorubicin entrapped in protein-free liposomes were administered simultaneously with empty ovalbumin proteoliposomes. The resulting plasma elimination behaviour and ELISA immune response data showed that only the highest dose of

Figure 4.4 Effect of drug-to-lipid ratio on the immune response to doxorubicin-encapsulated ovalbumin-liposomes

Female CD-1 mice were injected i.v. for two consecutive weeks with doxorubicin entrapped in ovalbumin-coupled liposomes at a constant lipid dose of 45 mg lipid / kg body weight (50 µg ovalbumin). Plasma samples were isolated 7 days after the second weekly injection for immune response determination. The figure depicts the relative production ovalbumin-specific IgG after injection of formulations encapsulating doxorubicin at increasing drug-to-lipid ratios up to a maximum of 0.2 (mol/mol), producing a maximum drug dose of 8 mg/kg. All data points represent the mean and standard error of 4 mice.

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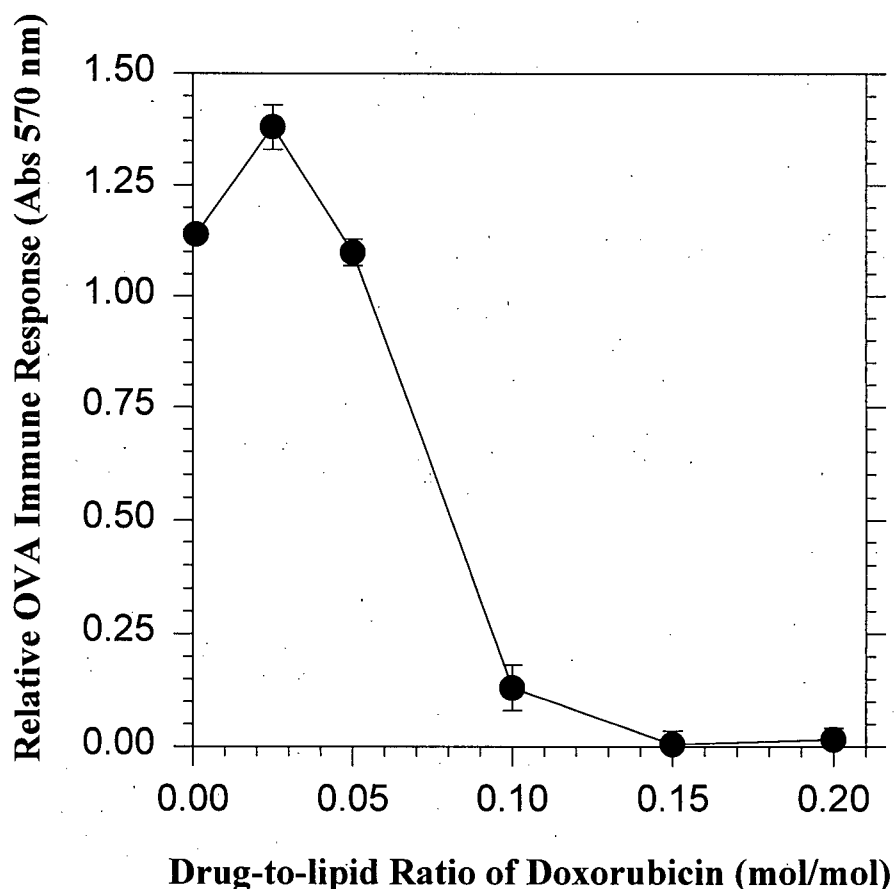
Figure 4.5 Effect of doxorubicin dose on the immune response to DOX-ovalbumin-liposomes

Female CD-1 mice were injected i.v. for two consecutive weeks with doxorubicin entrapped in ovalbumin-coupled liposomes at varying lipid doses up to 45 mg lipid / kg body weight (50 µg ovalbumin). Plasma samples were isolated 7 days after the second weekly injection for immune response determination. The graph shows the relative production of ovalbumin-specific IgG (absorbance 570 nm) after injection of increasing doxorubicin doses at a constant 0.2 drug-to-lipid ratio, up to a maximum of 8 mg/kg doxorubicin. All data points represent the mean and standard error of 4 mice.
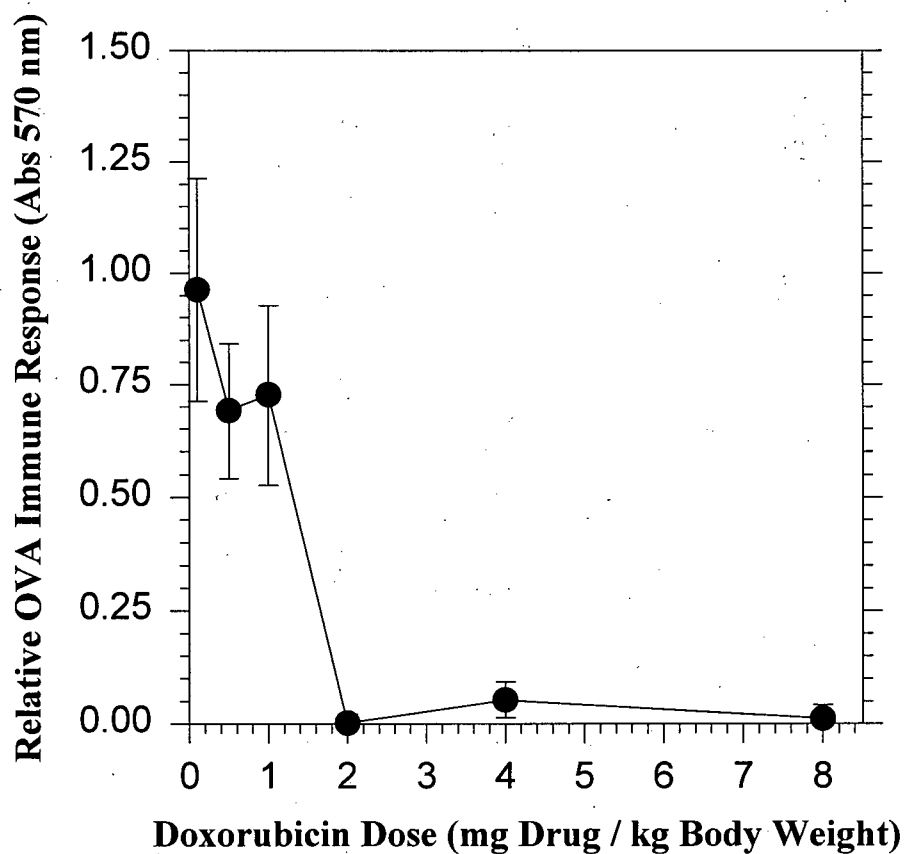
8 mg/kg doxorubicin administered in protein-free liposomes prevented the immune response to and rapid elimination of co-administered ovalbumin proteoliposomes (Fig. 4.6A and 4.6B). This suggests that although high doses of protein-free liposomal-doxorubicin could act generally against phagocytic cells in the prevention of immune response, the success of low doses of doxorubicin-loaded proteoliposomes suggests that they interact through a more selective or efficient mechanism.

4.3.4 Entrapped doxorubicin can selectively inhibit immune response to a liposomal-protein

It has been suggested that liposomes with surface-coupled protein can interact directly with lymphocytes specific to the coupled surface antigen (possibly inducing T-cell independent immunity)(Tadakuma *et al*, 1980; Walden *et al*, 1986a and 1986b; Shek *et al*, 1986; Su and Van Rooijen, 1989; Therien *et al*, 1991; Shahum and Therien, 1994), a similar mechanism might allow doxorubicin in proteoliposomes to selectively suppress the activity of certain lymphocyte subpopulations and induce a more specific rather than general suppression of immunity. In addition, normal T-cell dependent pathways of immune activation would likely also be involved in the generation of immune response. To determine whether the doxorubicin proteoliposome-induced immune suppression was at all selective for a specific protein (e.g. ovalbumin) and did not reflect blockade of the entire immune system, the humoral immune response to an additional liposomal protein was studied in drug-treated animals. Immune response to liposomal-hen egg lysozyme (HEL) was monitored in animals co-administered doxorubicin-loaded ovalbumin-liposomes encapsulated at a 0.2 drug-to-lipid ratio, to determine whether the drug-treated immune

system could respond to an unrelated liposomal-antigen which did not contain encapsulated drug. As demonstrated in Figure 4.7, there is a significant level of humoral immunity generated against injected liposomal-HEL, despite administration of doxorubicin encapsulated in ovalbumin-liposomes at doses which successfully block an immune response to ovalbumin. Even an 8 mg/kg dose of doxorubicin administered in ovalbumin-liposomes failed to block the majority of anti-HEL antibody production against the liposomal-HEL. This indicates some degree of selectivity in the immune system inhibition for the specific protein associated with the drug. Macrophage depletion resulting from these levels of injected doxorubicin can be compared to those of Daemen *et al*, where 3 weekly i.v. injections of 5 mg/kg doxorubicin in PEG-containing liposomes reduced the phagocytic capacity of rat liver macrophages by 44%, without a substantial change in the actual number of macrophages (Daemen *et al*, 1997). This moderate degree of macrophage suppression from such treatments could allow normal mechanisms of elimination of and immune response to HEL-liposomes which do not contain doxorubicin, possibly at least in part explaining the successful although slightly reduced production of anti-HEL immunoglobulin and the rapid elimination of HEL-liposomes from the plasma.

Figure 4.6 Ovalbumin proteoliposome immune response and plasma elimination with simultaneous injection of doxorubicin within protein-free liposomes

Female CD-1 mice were injected i.v. for two consecutive weeks with 50 µg of empty ovalbumin-liposomes were injected simultaneously with varying doses of 0.2 drug-to-lipid ratio liposomal-doxorubicin (DSPC/CHOL/PEG-DSPE; 53:45:2, mol %), up to a maximum drug dose of 8 mg/kg. Panel A depicts the plasma levels of ovalbumin-liposomes 4 hours after the second weekly injection, determined using standard scintillation counting to detect the nonexchangeable, nonmetabolizable radiolabelled lipid marker ($[^3H]$-CHE) incorporated in the lipid bilayer. Panel B shows the relative production of ovalbumin-specific IgG (absorbance 570 nm) as determined by ELISA assays of plasma isolated 7 days after the second weekly injection of each formulation. All data points represent the mean and standard error of 4 mice.
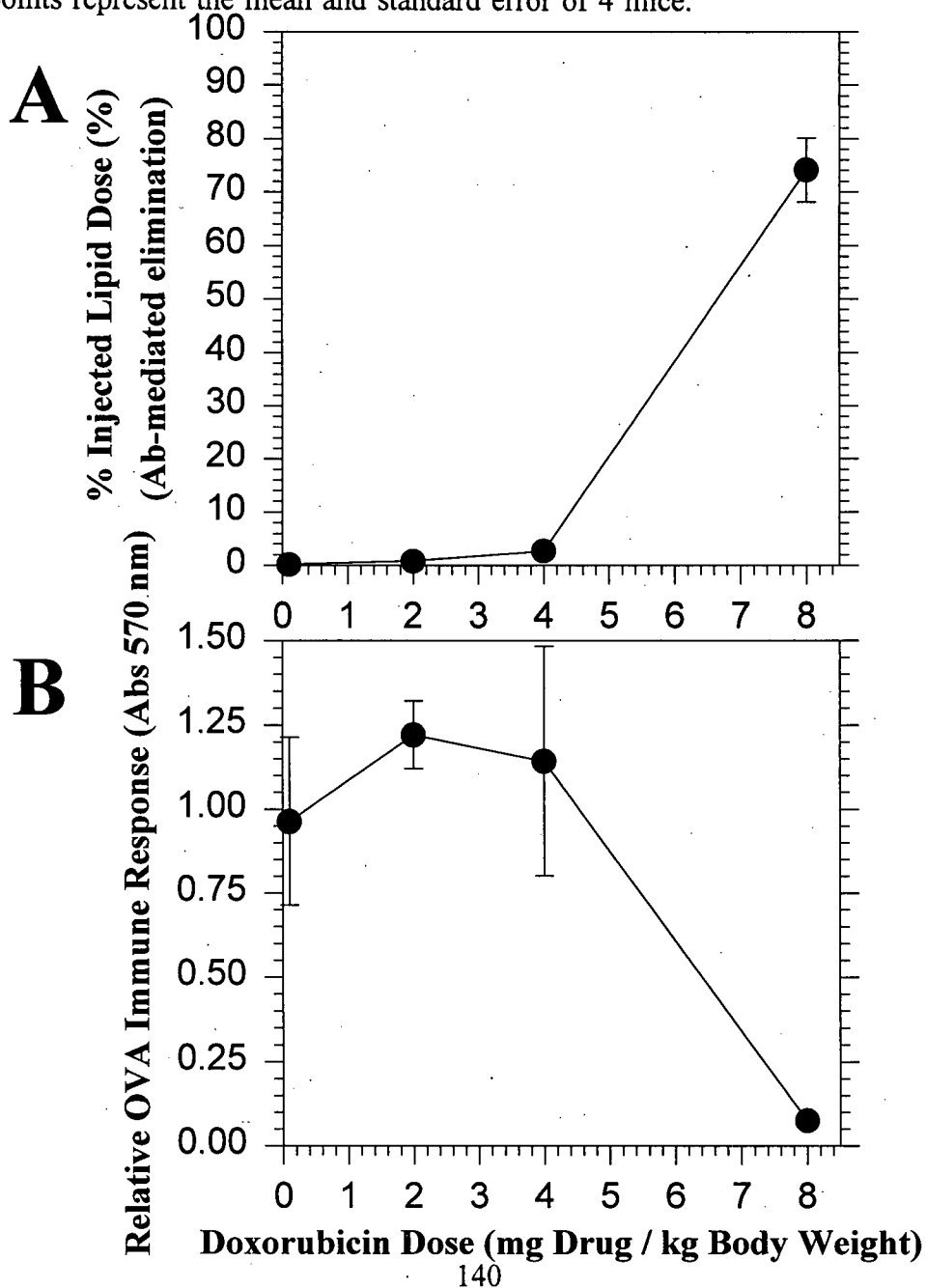
Figure 4.7 Immune response to HEL proteoliposomes with simultaneous injection of doxorubicin within ovalbumin-liposomes

Female CD-1 mice were injected i.v. for two consecutive weeks with 50 μg of empty HEL-liposomes simultaneously with varying doses of 0.2 drug-to-lipid ratio doxorubicin in ovalbumin-coupled liposomes, up to a maximum drug dose of 8 mg/kg. The figure shows the relative production of HEL-specific IgG (absorbance 570 nm) as determined by ELISA assays of plasma isolated 7 days after the second weekly injection of each formulation. All data points represent the mean and standard error of 4 mice.
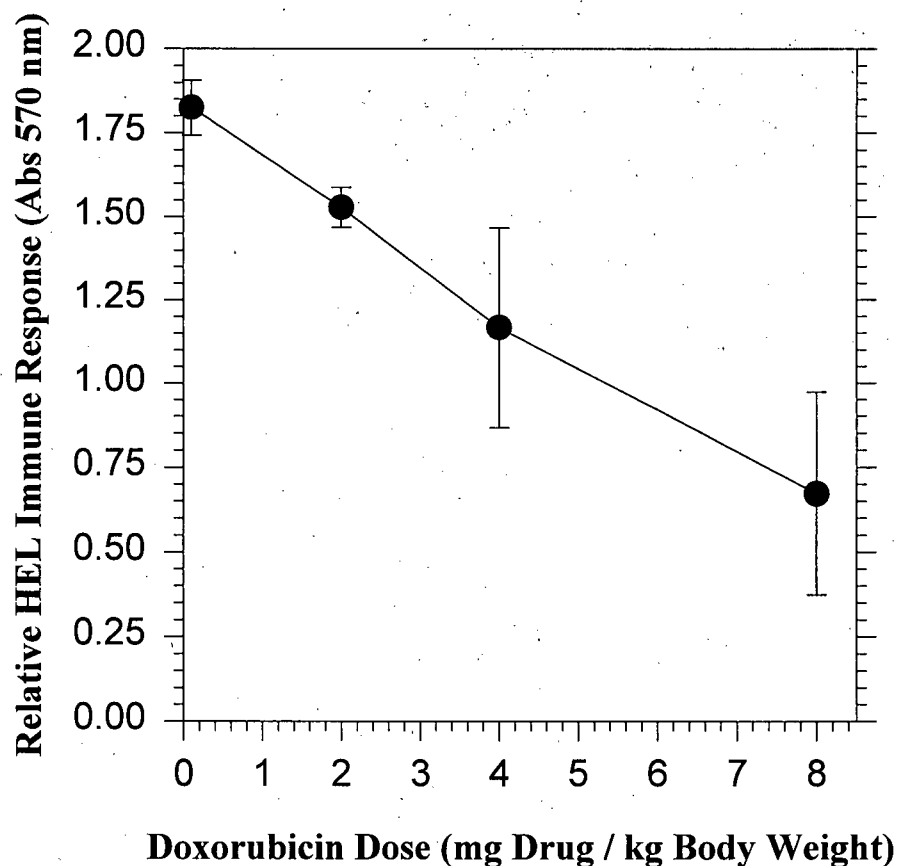
4.4 Discussion

The results presented in this chapter show that humoral immunity to liposomal proteins can be overcome by the incorporation of cytotoxic drugs such as doxorubicin. This indicates that doxorubicin can be effectively administered in targeted liposome systems without the generation of humoral immunity against the surface molecules. First, repeated administration of low doses of doxorubicin at high drug-to-lipid ratios within proteoliposomes successfully prevents the production of protein-specific antibodies. Second, at low drug doses this immune suppression is more specific to the protein coupled to the doxorubicin-loaded proteoliposomes. Although their was some suppression of anti-HEL immunity (especially at high drug doses)(Fig. 4.7), the suppression of the anti-OVA response by the same treatment was complete even at low doxorubicin doses (Fig. 4.5).

Repeated injections of ovalbumin-liposomes induce high levels of detectable humoral immune response against the surface protein, causing later injections to exhibit rapid elimination from the circulation as they are taken up by the organs of the RES. As shown in the dose titration experiments, low doxorubicin doses can prevent this immune response. Complete suppression of ovalbumin immune response could be achieved by 2 mg/kg doxorubicin administered at the highest tested drug-to-lipid ratio of 0.2:1 (mol:mol)(Figure 4.5). This dose of doxorubicin encapsulated at lower drug-to-lipid ratios resulted in immune response and rapid elimination of subsequent injections, indicating the need to maintain a high concentration of encapsulated doxorubicin.

The generated immune suppression was more specific to the protein actually coupled to

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the drug-loaded liposomes. Doxorubicin loaded ovalbumin-liposome doses as high as 8 mg/kg (at a 0.2 drug-to-lipid ratio (mol:mol)) still failed to completely block an immune response to co-administered drug-free hen egg lysozyme-liposomes, despite the response to ovalbumin being completely lost at only a 2 mg/kg drug dose. However, the production of anti-HEL immunoglobulin was lowered by the higher dose drug treatments, suggesting that higher doxorubicin doses possess more general immunosuppressive effects. Daemen et al showed that comparable levels of injected liposomal-doxorubicin reduced the phagocytic capacity of rat liver macrophages by 44% without substantially changing the number of recovered cells when injected at 7-day intervals (Daemen et al, 1997). The possible effects on other antigen presenting cell populations are unknown, but cells engulfing or directly interacting with these vesicles would likely also experience inhibitory effects due to the entrapped doxorubicin. This partial macrophage suppression and the possible T-independent nature of immune response to surface-coupled liposomal proteins could explain the continued phagocytic elimination of and humoral immune response to drug-free HEL-liposomes. While interactions such as those with specific lymphocyte populations demonstrated by others (see Sections 1.5.3.2 and 4.3.4), could explain the more efficient suppression of immunity to and reduced immunoglobulin-mediated elimination of liposomal-ovalbumin containing doxorubicin. Although unlikely, due to the identical nature of the liposomes, lack of extreme differences in the physical nature of the proteins, and lack of known functional role of these proteins in mediating the elimination of liposomes, there is the remote possibility that the proteins themselves somehow altered the cellular interactions with populations or even subpopulations of phagocytic cells. Macrophage subpopulations have been suggested to have different phagocytic capacities (Daemen et al, 1995), and so could also have

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varying antigen presenting ability. However, a substantial alteration in the phagocyte interactions would be necessary to significantly alter the explanations of the results observed.

This immunosuppression must involve liposome interactions with cells of the immune system, this is supported by established interactions with macrophages, dendritic cells, and lymphocytes (reviewed in Chapter 1). Macrophages of the liver and spleen are one possibility. They are established as the predominant cells of liposome uptake, are also involved in the processing and presentation of liposomal proteins, and are killed or inactivated *in vivo* by liposome entrapped doxorubicin (Daemen *et al*, 1995 and 1997). The increased effectiveness of proteoliposomes over protein-free liposomes suggests that surface-coupled proteins might mediate direct interactions with lymphocytes, possibly even the subpopulations specific for their antigenic epitopes. These specific interactions are supported by considerable evidence using liposomes with high concentrations of surface antigen (see Section 1.5.3.2). Additional experiments outlined under Future Directions (Section 5.2), are required to elucidate the exact nature of the interactions and mechanism of immune suppression by which doxorubicin proteoliposomes act, and also to provide a more complete understanding of the potential uses for liposomal systems in the manipulation of immune response.

In summary, the results presented here show that doses of liposome encapsulated doxorubicin as low as 2 mg/kg (at a 0.2 drug-to-lipid ratio), can be repeatedly administered with surface-coupled ovalbumin without the generation of a humoral immune response. Low doses of doxorubicin selectively inhibit humoral immunity, while high drug doses induce more general immune suppression. The more general effects of high drug doses could involve elimination of the phagocytic cells required for efficient antigen processing and presentation. However, lower

drug doses appear to involve some degree of selective interactions involving their surface protein molecules, which could include direct lymphocyte interactions, as outlined above. Overall, the results presented within this chapter suggest that encapsulated doxorubicin, and therefore possibly other cytotoxic drugs, could be successfully used in targeted liposome delivery systems due to their inhibitory effects on target molecule immunity, and also could potentially be useful in the generation of more selective immune suppression by cytotoxic drug treatments.

CHAPTER 5: SUMMARIZING DISCUSSION

5.1 Summary of results

This thesis has investigated three factors which influence the elimination of liposomes from the circulation - lipid dose, PEG-polymer surface coating, and immune response to liposome surface molecules.

In Chapter 2, it is shown that the increase in circulation lifetime resulting from increasing liposome doses can be attributed to depletion of blood proteins, and not RES saturation as previously thought. This chapter investigated two lipid compositions which are representative of the extremes of liposome elimination behaviour *in vivo*, to study the relationship between *in vivo* liposome protein binding and the corresponding pharmacokinetics and biodistributions of these formulations administered over a full range of lipid doses. For both compositions, the results show a substantial increase in circulation half-life and reduction in the relative RES uptake for increasing lipid dose. However, these results provide no evidence that the RES uptake capacity is saturated, and that saturation cannot explain the changes which occur in the plasma circulation even at the lowest doses. Even at the highest lipid doses of rapidly cleared liposomes, the quantity and rate of RES lipid uptake continued to increase, although some loss of linearity was observed in the increasing rate for this sample. Longer circulation lifetimes of increasing lipid doses can, however, be directly related to the reduced protein binding values of the higher liposome doses. Characterization of the quantity and identity of liposome bound protein suggested the existence of distinct pools of blood proteins which are available to bind liposomes of each lipid composition. At higher doses, the decreased protein binding values indicate a dilution of this

same protein pool over the greater surface area of a larger number of liposomes. These results suggest that a specific pool of opsonins bind to liposomes of a given lipid composition and mediate their elimination from the circulation. The nonspecific decrease in protein bound to liposomes given at higher doses would include these elimination proteins, resulting in the decreased RES uptake affinity and corresponding increased circulation half-lives.

In Chapter 3, it is shown that incorporation of poly(ethylene glycol) polymers into liposomes results in a relatively nonspecific decrease in blood protein binding to liposomes *in vivo*. These studies examined three representative lipid formulations to investigate the effects of PEG in different types of lipid bilayers. Decreased protein binding in the presence of a PEG coating was observed for all three compositions, and in all cases increased plasma circulation levels accompanied decreased blood protein adsorption. The results also indicate protein binding levels of liposomes which bind the higher levels of blood protein are more substantially decreased by PEG-incorporation (negative and positive liposomes), although the circulation half-lives appear more substantially increased by PEG incorporation into the neutral, low protein-binding vesicles. The polymer layer does not completely block protein interactions with the membrane however, as some formulations still bound substantial levels of blood protein even with the PEG-coating. This is consistent with the fact that the PEG-containing liposomes show varying degrees of dose-dependent plasma elimination behaviour. The increased plasma circulation levels and corresponding decreased protein binding values are similar to the trends observed for liposomes without PEG in Chapter 2, suggesting similar elimination mechanisms. However, the significant enhancements in circulation half-lives are more than would be predicted based on the quantitative protein data for corresponding PEG-free liposomes. This suggests that alternative mechanisms of

PEG action in liposome elimination, such as interference with receptor binding or direct cell surface associations, also play a role in the increasing circulation lifetimes of PEG-liposomes.

Chapter 4 shows that encapsulation of doxorubicin even at low total drug doses can effectively prevent humoral immune responses against liposomes with surface-coupled molecules, such as antibody- or ligand-targeted drug delivery formulations. This indicates that doxorubicin-loaded protein-targeted liposomes can be repeatedly administered, without the complications of an immune response. Maximum efficiency of immune suppression requires a high drug-to-lipid ratio, and also that the doxorubicin be encapsulated in the liposomes possessing the surface antigens. At low total doxorubicin doses this suppression was selective for the particular protein on the drug-loaded liposomes, but at higher drug doses this antigen-selectivity was lost. This indicates that low doses of these drug-loaded formulations will not completely block the activity of the entire immune system, but that repeated administration at higher doses could lead to more general immune suppression. This immune system inhibition could result from suppression or elimination of macrophages and/or lymphocytes depending on the drug dose administered; however, further experimentation is required to understand the exact nature of these interactions.

5.2 Discussion and Future Directions

Three areas of future studies follow on from the results presented within this thesis. First, it is clear that blood protein interactions can explain many aspects of the elimination behaviour of liposomes. Previous results have demonstrated the influence of lipid composition on protein binding (Chonn *et al*, 1992; Semple *et al*, 1996), and here the reduction of bound protein with increasing lipid dose has been directly related to increased liposome circulation lifetimes. These results dispute previous claims of RES saturation which could not be demonstrated even at the highest doses of most rapidly cleared vesicles (reviewed in Section 1.4.3.3), and provide further support for a protein-regulated elimination mechanism controlling liposome elimination behaviour.

Future work should identify the blood proteins involved in liposome elimination. Although some proteins have been identified (C3, IgG, and β_2GPI), others appear to exist, including the tissue specific opsonins and dysopsonins proposed by Moghimi and Patel (reviewed in Sections 1.3.3.9 and 1.3.3.10). Identification and purification of proteins which increase or decrease the elimination of liposomes could allow their use in preventing or specifically targeting organ uptake by their incorporation into liposomes. Alternatively, transiently decreasing the levels of blood proteins responsible for liposome elimination could also be used to increase the circulation lifetimes of injected liposomes.

Second, the use of PEG-modified liposomes demonstrated that the reduced RES uptake and increased plasma circulation of liposomes incorporating PEG-polymers is directly related to their decreased *in vivo* liposome protein adsorption. The results of Chapter 3 also extended the relationship between the plasma circulation and protein binding of conventional liposomes to

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include polymer-coated vesicles. In contrast to previous results, PEG-liposomes were shown to exhibit dose-dependent plasma elimination and RES uptake kinetics, which were shown to correlate with protein binding in a pattern very similar to that established for the conventional vesicles of Chapter 2. Overall, these studies clearly demonstrate that PEG reduces blood protein binding and slows RES uptake, while at the same time similarities to the trends of conventional liposome elimination behaviour suggest that PEG- and conventional-liposomes are subject to similar blood protein interactions and elimination mechanisms.

Future investigations of PEG liposomes concern their use in targeted liposome delivery systems. Aggregation and rapid elimination is eliminated when targeted liposomes contain PEG-polymer, providing the extended circulation lifetimes required to achieve *in vivo* targeting. Quantitative measurement of blood protein-binding could allow a method of evaluating the ability of these systems to maintain long circulation lifetimes. In addition, fusogenic liposomes which contain PEG-polymer coupled to lipid anchor molecules which exchange out of the liposome membrane, are being considered as a trigger for destabilizing liposomes with fusogenic properties to achieve delayed release of liposome contents (Holland *et al*, 1996; Madden, T. D. *et al*, unpublished results). Such a system could also be combined with a target molecule to attempt to direct fusion with specific cells. Understanding the quantity and identity of blood protein interactions could help explain the biological fate of these liposomes, and assist in development of these fusogenic systems. This is an especially important aspect of study in light of recent results showing that serum proteins inhibit fusion of these systems (Bailey and Cullis, 1997).

Finally, the study of immune response to liposomes using a model ovalbumin-coupled system, showed that targeted liposomes containing doxorubicin can be administered repeatedly

in mice without inducing problems of humoral immunity directed against the surface-coupled moieties. Low total doses of encapsulated doxorubicin at high drug-to-lipid ratios were effective at selectively suppressing an immune response against these proteoliposomes, whereas an immune response was observed for co-administered (drug-free) liposomal surface antigens. Possible mechanisms of this immune suppression could involve inhibition or elimination of macrophages or B lymphocytes, depending on the drug dose.

Future possibilities in the area of liposomes and immunity are extensive. The results directly indicate that targeted liposomes containing doxorubicin can be administered without immune response complications, and this would be expected to apply to other cytotoxic or immunosuppressive drugs. An understanding of the nature of this immune system suppression may identify additional applications for use in immunosuppression. *In vitro* experiments monitoring antigen-stimulated proliferation of isolated immune system cell populations from antigen-stimulated and drug-treated animals could identify the cell types and interactions involved in the suppression of immune response induced by these drug-loaded proteoliposomes. The results presented in Chapter 4 suggest the possibility of selectively suppressing immunity against a specific target moiety. If possible this could expand the potential applications of targeted delivery to include drugs which are not immunosuppressive, otherwise it would be limited to agents such as doxorubicin which suppress the humoral immune response. Other areas of investigation include further uses of liposomes for the delivery of immunomodulators. As outlined in Chapter 1, considerable efforts have already focused on liposomes as adjuvant systems, but the delivery of immune system inhibitors via lipid vesicles is in its infancy. Liposomes, especially those which successfully target appropriate circulating target cells, could dramatically enhance the

immunosuppressive specificity of immune system regulation.

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