ANTIBODY TARGETED LIPOSOMAL SYSTEMS

by

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ABSTRACT

Despite success in the development of clinically useful liposomal anticancer drugs, the advancement of targeted formulations has been limited to only a handful of successful studies in animal models of cancer. This thesis suggests that a fundamental lack of understanding of the biological fate of protein-conjugated liposomes has led to the limited success of targeted carriers. Three critical areas of fundamental importance have been identified to better understand the limited success: i) optimizing the physical and chemical attributes of protein targeted liposomal carriers for *in vivo* applications; ii) evaluation of the biological fate of liposomal carriers; and iii) factors influencing the association of targeting ligands with liposomes. These basic studies identify a number of parameters that will effectively facilitate the development of therapeutically useful targeted liposomes. The initial focus of this investigation was concerned with the rapid aggregation of liposomes that occurs during protein coupling on the liposome surface. By adding polyethylene glycol (PEG)-lipid anchor conjugates onto a liposome, reductions in liposome aggregation were observed. This study demonstrated that incorporation of a 2000 M.W. hydrophilic PEG polymer anchored to a phospholipid in the liposomal membrane at a final concentration of 2 mol% resulted in optimal inhibition of aggregation with no significant inhibition of target site binding and optimal circulation lifetimes. Further investigations focused on passive tumor accumulation of doxorubicin containing liposomes. These studies were completed with the objectives of assessing whether liposomes that reach extravascular spaces within tumors are available for targeting. Finally, experiments focused on the method of coupling antibody to a liposome, either through primary amines or carbohydrates. Results concluded that the conjugation of antibody *via* carbohydrates resulted in extended circulation lifetimes compared with antibody-conjugated through primary amines. Additional characterization raised concerns as to the stability of the antibody on the liposome *in vivo* and suggested that the extended plasma levels observed with the carbohydrate conjugated antibodies

was a result of antibody instability on the liposome. These studies, therefore, suggest that the method of conjugation of antibody is vital to the circulation lifetimes of antibody-conjugated liposomes and will most certainly be of major importance to the accumulation of liposomes to target sites.

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ABBREVIATIONS

AUC	area under the curve
BSA	bovine serum albumin
CD	cluster of differentiation
CHE	cholesteryl hexadecyl ether
Chol	cholesterol
DCC	*N*-*N*'-dicyclohexylcarbodiimide
DMEM	Dulbecco's modified eagle medium
DNaseI	deoxuribonuclease I
DNP	dinitrophenol
Dox	doxorubicin
DSPE	distearoyl phosphatidylethanolamine
DSPC	distearoyl phosphatidylcholine
DPPC	dipalmitoyl phosphatidylcholine
DTT	dithiothretiol
EDTA	ethylenediaminetetra-acetic acid
EGFR	epidermal growth factor receptor
EPC	egg phosphatidylcholine
F(ab')	fragment, antigen-binding
$F(ab')_2$	fragment, antigen-binding
FBS	fetal bovine serum
Fc	fragment, crystalline
FITC	fluorescein isothiocyanate
FPLC	fast performance liquid chromatography
g	centrifugal force
G_{M1}	monosialoganglioside G_{M1}
HBS	HEPES buffered saline
HBSS	Hank's balanced salt solution
HEPES	*N*-2-hydroxyethylpiperazine-*N*-2-ethane-sulphonic acid
HPLC	high pressure liquid chromatography
IgG	immunoglobulin G
i.p.	intraperitoneal
i.v.	intravenous
LUV	large unilamellar vesicles
MAC-1	anti-macrophage antibody
MePEG	monomethoxypoly(ethylene glycol)
MePEG-S-POPE	monomethoxypoly(ethylene glycol) modified *via* a succinate linkage to POPE
MLV	multilamellar vesicles
mol	mole
MPB-DPPE	*N*-(4-(p-maleimidophenyl)butyryl)dipalmitoyl phosphatidyl ethanol-amine
MTD	maximum tolerated dose
MW	molecular weight
NEM	*N*-ethylmaleimide
NHS	*N*-hydroxysuccinimide
PBS	phosphate buffered saline
PE	phosphatidylethanolamine
PEG	poly(ethylene glycol)
PEG-PE	poly(ethylene glycol)-modified phosphatidylethanolamine
POPE	palmitoylolyel phosphatidylethanolamine

PDPH	3-(2-pyridyldithiol)propionyl hydrazide
QELS	quasielastic light scattering
RES	reiculo-endothelial system
s.c.	subcutaneous
SD	standard deviation
SPDP	*N*-succinimidyl 3-(2-pyridyldithio)propionate
SUV	small unilamellar vesicles
TAM	tumor associated macrophages

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DEDICATION

To
My Grandparents,
Mother
and Natashia

CHAPTER 1

INTRODUCTION

1.1 Overview: targeted drug carriers

Treating human disease with targeted drugs will have major impacts on increasing disease free survival and improving patient quality of life. The treatment of illnesses such as cancer with targeted drugs, however, has been an elusive goal. The targeting of drugs using liposomes has been hampered by several biological processes, discussed in Section 1.5, which have played critical roles in the limited success of targeting. As a result, we are faced with difficult challenges to develop practical solutions to overcome these obstacles. Future success of targeted liposomes will require further characterization of the physical and chemical attributes of ligand-liposome complexes including a better understanding of how these attributes dictate biological behavior in general and target site delivery and binding in particular. This introduction will discuss the goals and therapeutic objectives for developing target liposomes.

1.2 Applications

The development of successful targeting agents will have dramatic impacts for a number of medical applications, particularly the diagnostic capabilities of imaging agents (Roselli, et al 1996) and improving the specificity of highly toxic drugs used to treat neoplastic diseases (Gregoriadis, 1982). It is believed that by far the greatest benefits will occur with the use of chemotherapeutics to treat cancer. Currently general, protocols for the treatment of cancer rely on administering chemotherapeutic agents as either single agents or in combination with other drugs. The effective doses for these agents are, in most instances, administered at the drug's maximum tolerated dose. The unfortunate impact of treating at such high doses is side effects

which can be life-threatening. The combination of drug toxicity and poor therapeutic activity at non-toxic doses means that most chemotherapeutic agents exhibit very narrow therapeutic windows. However, having the ability to localize drug to a specific target site should provide a major advancement for anti-cancer drug therapy, as it will allow for more effective treatments to be given at doses that are better tolerated.

The success of nontargeted liposomes for localizing drug to target sites is apparent from the number of formulations in advanced clinical trials and the fact that they are used to treat a number of diseases (Table 1.1). In particular, market approval for two liposomal formulations, Doxil™ and DaunoXome™, has been achieved for the treatment of AIDS related Kaposi sarcoma. The main characteristics which have contributed to this success are a consequence of the ability of liposomes to decrease drug toxicities, to increase drug circulation lifetimes, and to inadvertently accumulate in sites of disease and infection (Bakker-Woudenberg et al., 1992; O'Sullivan et al., 1988; Richardson et al., 1979; Gabizon and Papahadjopoulos, 1988). We believe the latter is a key reason why liposomes are an excellent choice for targeted delivery vehicles. Studies in Chapter 4 and from others, however, suggest that the incorporation of ligands onto liposomes for targeting purposes reduces the natural ability of the liposomes to access target sites (Matzku et al., 1990; Torchilin et al., 1992; Longman et al., 1995b).

1.3 Goal

The pharmacological research goal of developing targeted liposomes is to increase target site accessibility and, ultimately, target diseased cells outside of the vasculature. In this context targeted liposomes will require access to the target and an ability to interact with and bind target

Table 1.1

Liposome drug formulation achievements

State of development	Disease treated	Therapeutic agent	Benefit
Approved for human use	Systemic fungal infection	Amphotericin B (AmBisome)	Decreased nephrotoxicity; increased efficacy
Approved for human use	Aids related Kaposi sarcoma	Doxorubicin (Doxil)	Decreased cardiotoxicity and alopecia; increased efficacy
Approved for human use	Aids related Kaposi sarcoma	Daunorubicin (DaunoXome)	Decreased cardiotoxicity and alopecia; increased efficacy
Approved for human use	Tumor imaging	^{111}Indium (VesCan)	Preferential accumulation in tumor
Phase II/III	Cancer-immunotherapy	Muramyltripeptide	Target to macrophages
Phase II	Gram-negative infection	Gentamicin	Decreased nephrotoxicity; increased efficacy
Phase II	Cancer immunotherapy	DNA (HLA antigen)	Gene transfer
Phase II	Cancer	Mitoxantrone	Decreased acute toxicity and myelosuppression
Phase I	Asthma	Salbutamol aerosol	Decreased tachycardia; prolonged release
Phase I	Malaria	Subunit peptide vaccine	Well tolerated adjuvant

cells. Binding will be dependent upon the presence of a ligand which has a high affinity or specificity for a marker (receptor) on the surface of target cells. The most accessible target cell populations reside in the vasculature compartment and include lymphocytes and erythrocytes (Suzuki et al., 1991; Singh et al., 1993). Although therapeutically important goals can be defined for targeting cells within the vascular compartment, the focus of this review will be on the more

challenging goal of targeting to extravascular sites. Success in achieving extravascular targeted drug delivery has been hampered for reasons which include liposome circulation lifetimes, accessibility to the target site (Matzku et al., 1990) and limitations in the manufacturing procedures used to prepare antibody-conjugated liposomes. Efforts directed to overcome these hurdles will be summarized in this Chapter, which focuses on the use of antibody ligands and liposomal delivery systems for the treatment of solid tumors. This Chapter will also speculate on the development of strategies to improve or control the pharmacodynamic behavior of the targeted liposome, such that the attributes required for proper targeted liposome function are maintained. The consequences regarding repetitive dosing and immunogenicity of such liposome formulations will also be considered.

1.4 Definition of specificity and target specific delivery

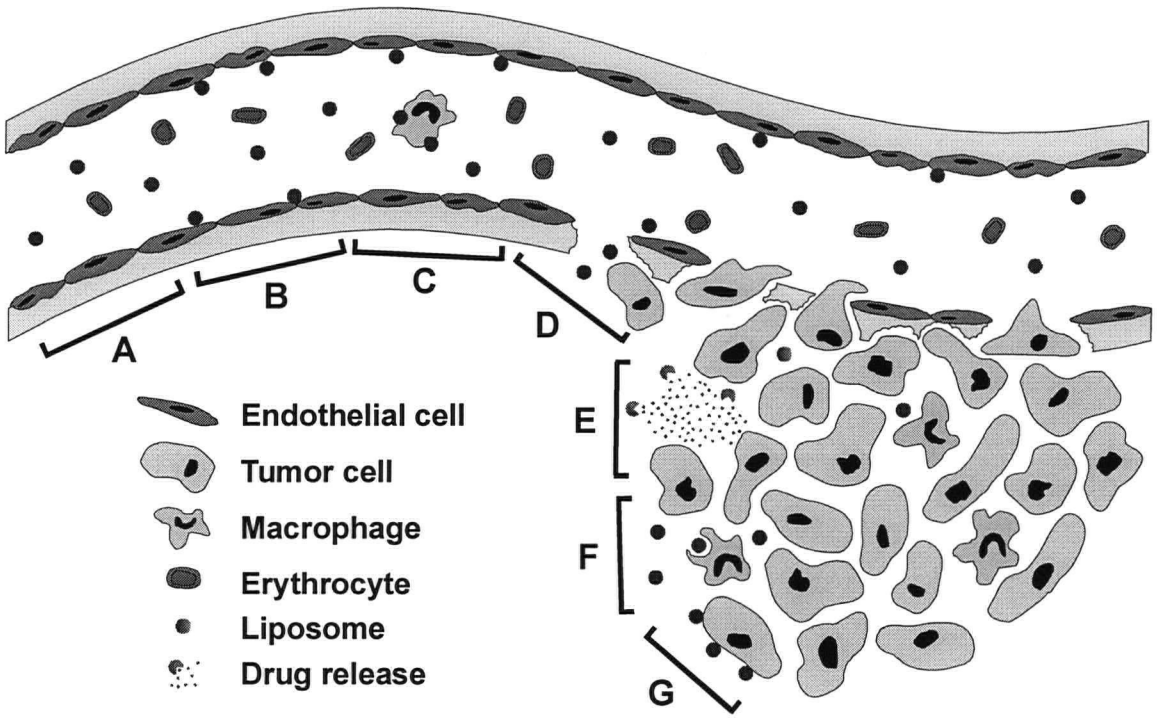
1.4.1 Passive versus active targeting

For reasons that are difficult to define, nontargeted liposomal systems have been reasonably effective at accessing target sites (referred to as passive targeting). In particular, liposomes with long circulation lifetimes, are known to accumulate in extravascular disease sites including sites of infection, inflammation, and tumors (Bakker-Woudenberg et al., 1992; O'Sullivan et al., 1988; Richardson et al., 1979; Gabizon and Papahadjopoulos, 1988). Improvements in drug localization are achieved, therefore, in the absence of any targeting information as a consequence of passive delivery of drug loaded liposomes (Figure 1.1D). Liposome accumulation can result in 3 to 100-times more drug delivery to a target site compared to the injection of the same dose of free drug. However, the majority of localized nontargeted liposomes do not interact with target cells directly (Harasym et al., 1997). It is suggested that therapeutic activity is a

Figure 1.1

Liposome target site accumulation

To allow for passive accumulation to the target site appropriately designed liposomal carriers must be retained in the blood compartment for extended time periods (A). While in the blood compartment liposomes interact with the cells lining the endothelium (B) or with specific target cells (C). Passive targeting is dependent on the presence of altered vascular endothelium, alterations that permit extravasation of circulating macromolecules (D). Following extravasation liposomes can release drug while residing in the interstitial space (E) or can be taken up by tumor associated macrophages (F). The potential to achieve specific interactions with target cells (active targeting) through use of targeting ligands is also feasible (G).



consequence of drug release from liposomes within the disease site, a process that does not require direct binding or association with diseased cells (Figure 1.1E).

The facilitation of binding of the drug carrier or liposome to target cells, through the use of ligands to increase localization of drug and target cell killing is referred to as active targeting (Figure 1.1G). Specificity of actively targeted liposomes is dependent upon the ligand's affinity

for a target cell marker. As with passive targeting it is anticipated that long circulation lifetimes will be required in order to maintain target site accumulation of these carriers (i.e. passive targeting capabilities), an essential requirement for target cell access.

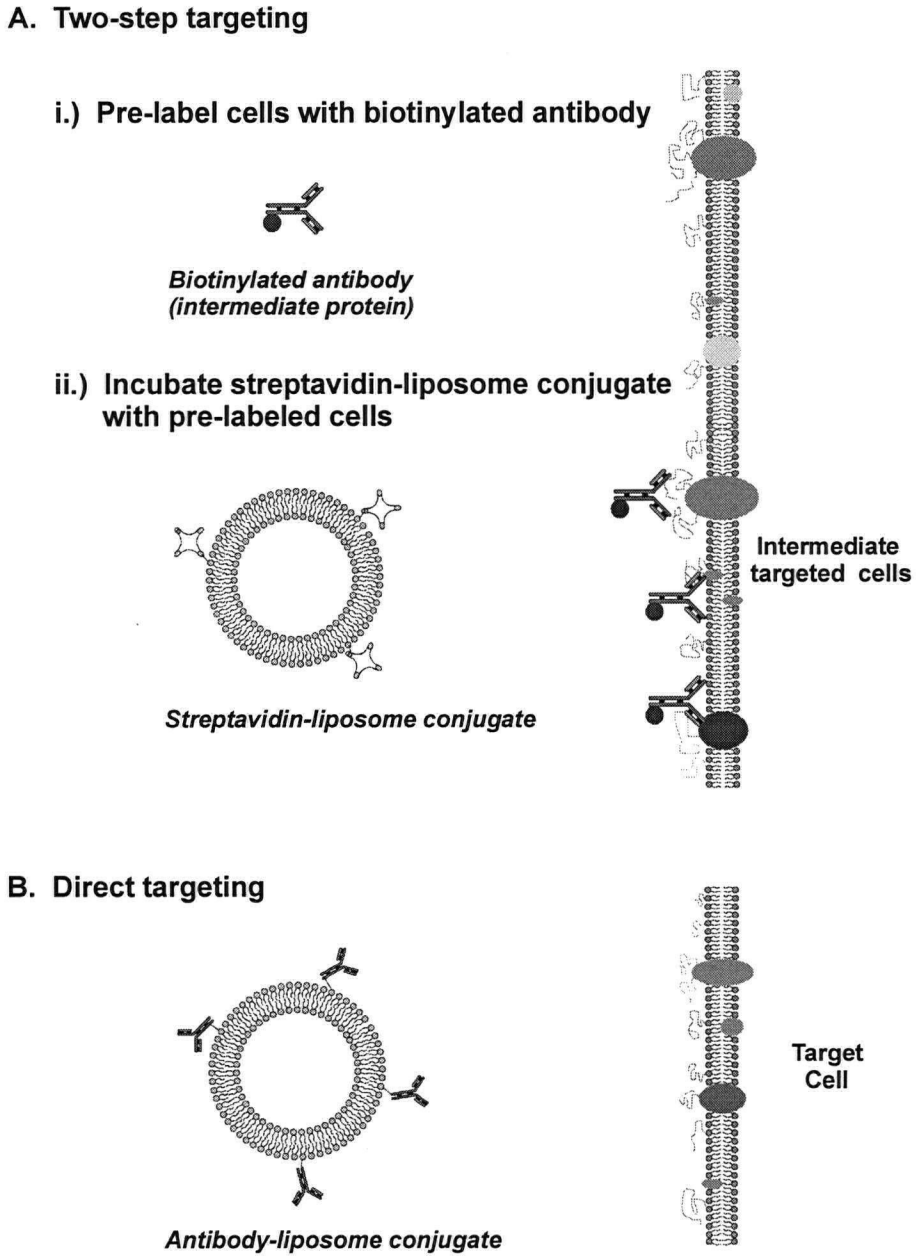
1.4.2 Active targeting: two step approach

One method of incorporating target specificity in a liposome is accomplished by the use of a nonspecific intermediate, or bridging protein, to associate the drug loaded liposomes with target cells. This intermediate has specificity for the target cells and an affinity for a component on the liposome. This approach is best exemplified by antibodies, modified with biotin and liposomes which have covalently attached avidin (or streptavidin), a biotin binding protein (Figure 1.2A). The use of an intermediate protein for targeting liposomes has been referred to as a two step approach, as it requires the administration of the intermediate protein and liposome separately. Customarily, an intermediate protein (one or more may be administered to enhance association) is administered first to allow accumulation and binding to the target cells. At a pre-determined time, when the maximum target cell to blood ratio is achieved, the liposomes are administered. The liposomes can then bind the intermediate protein labeled target cells. Several advantages of two step targeting approaches include i) the use of simple lipids and/or well defined liposomes to achieve targeting, ii) no need for coupling procedures to be optimized for each targeting protein used, iii) characterization of binding/labeling of target cells in the absence of liposomes, and iv) the lack of altered liposome biodistribution resulting from the attachment of large targeting ligands. Two step approaches are not necessarily viable options because of difficulties in developing clinical studies where two active components must be independently characterized pharmacokinetically. Further, the advantage of using small ligands on liposomes was thought to result in reduced immunogenicity. Recent evidence, however, indicates that even small ligands

Figure 1.2

Targeting of protein coated liposomes to target cells

Two approaches to targeting are indicated. In the two-step targeting approach (A) the first-step (i) prelabels target cells with biotinylated antibodies specific for a ligand or several ligands on the target cell. At a predetermined time point, sufficient to allow for plasma elimination of free antibody, streptavidin conjugated liposomes specific for biotin on the antibody prelabeled cells are administered. In the second approach (B) direct targeting, antibodies directly attached to the liposome are used to target cells.



(such as biotin) can be immunogenic (Philips and Dahman, 1995). Finally, the targeting intermediate used can only be directed against cell surface markers that are recycled at a slow rate, either through shedding or internalization. Therefore, it is likely that binding will occur in the absence of efficient intracellular delivery.

1.4.3 Active targeting: direct approach

The second approach to achieve specificity, is by preparing liposomes with targeting ligands directly conjugated onto the membrane surface (Figure 1.2B). Several types of ligands can be incorporated such as peptides, glycoproteins, or vitamins. The latter are used mainly for their smaller size and ease in which they can be attached to liposomes (Lee and Low, 1995). The main focus of such research has, however, been on the use of antibodies. Antibodies are commonly used as they are inexpensive, easy to produce in large quantities, well characterized for pre-clinical and clinical use, have a high affinity for their antigens, and are available against a wide variety of unique, over-expressed, antigenic determinants located on cells. While many different ligands, other than antibodies, can be conjugated to a liposome, coupling techniques rely on a limited number of covalent or noncovalent reactions. Covalent strategies rely use a variety of bifunctional cross-linkers and typically involve modification of the ligand and subsequent conjugation to preformed liposomes containing a modified and reactive phosphatidylethanolamine (PE). Noncovalent strategies involve ligand binding mediated through biotin-streptavidin or similar interactions involving proteins that can bind the Fc domain of IgGs, such as protein G or protein A. Other approaches have used detergent dialysis to incorporate antibodies that have been modified with hydrophobic groups such as fatty acyl chains (Pinnaduwage and Huang, 1992). The latter approach has since been simplified by the development of lipid tagged single chain antibodies. This approach is advantageous as it avoids

the chemical reactions typically required when attaching antibodies to preformed liposomes (Laukkanen et al., 1993).

1.5 The problem

Considering the variety of approaches that have been developed to achieve target cell specific delivery of liposomes, it is curious as to why over 20 years worth of scientific research has not lead to the development of a single therapeutically and clinically viable targeted liposomal drug. Improved understanding in several areas such as careful selection of target sites, as immunoliposomes have difficulty passing from the blood compartment to other extravascular sites, and improvements in protein coupling technology will further the development of protein conjugated systems. In the absence of improved technology, the methods used to prepare targeted liposomes are, however, appropriate for establishing proof-of-concept. To date such proof-of-concept data has been limited to only a few studies demonstrating improved therapy for targeted formulations directed against diseases in the lung (Hughes et al., 1989; Ahmad et al., 1993).

There are two significant concerns associated with the use of liposomes for targeting cells in extravascular sites. First, the targeted liposomes are often rapidly eliminated from the circulation. Rapid plasma elimination decreases passive accumulation of targeted liposomes in the diseased tissue, resulting in low target site access and low target cell contact (Matzku et al., 1990). It is anticipated that if successful increases in plasma levels are achieved then increases in accumulation within target sites will result. The second concern relates to the potential of immunoliposomes to interact with the target cells after entering an extravascular site. In order for this to be achieved one must, i) ensure stable targeting ligand/liposome interactions and ii)

demonstrate targeting ligand binding to the target cell. In order to develop methods/approaches to target liposomes, a review of the methods presently used to attach targeting ligands to liposomes is useful. This information is particularly helpful when considering the biological factors that can reduce/block the potential for these systems to be used systemically. Although the previous sections provide a general description of targeting methods, it is necessary to provide details about commonly employed linker methods prior to assessing how coupling conditions affect liposome structure and biological behavior. In general, these coupling procedures add moieties that elicit immune reactions, engender liposome-liposome crosslinking and decrease affinity of the targeting ligand for its receptor. In turn, these effects result in more rapid liposome clearance, poor target site accessibility, and poor target site binding.

1.6 Methods of target ligand incorporation into liposomes

As mentioned previously, two methods predominate for attaching ligands onto a liposomal surface, i) covalent conjugation, which is the direct coupling of a ligand onto the liposome and ii) noncovalent conjugation, which relies on the interactions of an intermediate to bind the targeting ligand to the liposome.

1.6.1 Noncovalent conjugation

In order to obtain a versatile targeting methodology one can consider the use of liposomes with nonspecific binding ligands, which can subsequently be used to attach site-directing targeting ligands. Liposomes with biotin-modified lipids can be easily prepared and used to attach a variety of avidin/streptavidin linked targeting proteins. The primary advantage of this technique is that many different site-directing ligands can be used with a single well defined liposome

preparation. Nonspecific protein based binding ligands can be attached to liposomes by use of covalent conjugation procedures (described below). Examples of these proteins include avidin, streptavidin, protein A, or protein G. The site-directed targeting ligand, an antibody or biotin modified antibody, can then be noncovalently bound to the liposome *via* interactions with the nonspecific binding ligand. The use of protein A or protein G, however, restricts the targeting ligand to antibodies as site-directing ligands.

Well characterized examples of noncovalent conjugation procedures include the use of biotinylated ligands bound to streptavidin-conjugated liposomes (Rosenberg et al., 1987; Loughrey et al., 1993; Longman et al., 1995) or antibody bound to protein A bearing liposomes (Machy and Leserman, 1984; Gray et al., 1988). Liposomes that have been conjugated to protein A noncovalently bind antibody *via* the interaction between protein A and the Fc portion of certain IgG classes (Langone, 1978; Ey et al., 1978). Protein A conjugated liposomes are known to bind *in vitro* to cells preincubated with cell-specific antibodies, but not to cells preincubated without antibody (Leserman et al., 1980; Machy and Leserman, 1984; Gray et al., 1988). Besides allowing for the use of different ligands, another advantage of this approach includes minimal reductions in binding affinity of the noncovalently bound ligands. As indicated in Section 1.6.2, covalent conjugation techniques can often significantly reduce the binding affinity of the modified protein (Leserman et al., 1980). As well it is established that noncovalent bonds are stable both *in vitro* (Leserman et al., 1980) and *in vivo* (Aragnol and Leserman, 1986) and it should be noted that one of the most successful demonstrations of the potential therapeutic benefits of antibody targeting (Ahmad et al., 1993) used liposomes prepared through noncovalent procedures (biotin/avidin/biotin protein bridging).

1.6.2 Covalent conjugation

Covalent crosslinking reagents, first described over 15 years ago, are most frequently used in preparing protein conjugated liposomes. Two strategies are generally used. In both strategies the ligand is conjugated to a hydrophobic lipid anchor, typically a PE which provides an accessible reactive amine (Sinha and Karush, 1979; Jansons and Mallet, 1981; Mori and Huang, 1993). These procedures rely on attaching the ligand to the liposome *via* the amphipathic attributes of the lipid molecule. The two strategies differ depending on when the ligand is conjugated to the lipid anchor (Table 1.2). The first strategy involves the conjugation of a ligand to PE before liposome formation. The ligand-lipid conjugate is then added to a lipid mixture consisting of mixed micelles of detergent and lipid. Liposomes are then produced by removal of detergent by dialysis. This approach can also be used to incorporate antibodies that have been modified with fatty acids (Huang et al., 1980). An advantage of this technique is that no harsh organic solvents are required (typically used to prepare liposomes) and this helps to preserve the integrity of the ligand (Leserman and Machy, 1987). Further, detergent dialysis can result in excellent levels of ligand incorporation into the liposome (coupling efficiencies of 85-90%; Huang et al., 1980). The main disadvantage with this approach is that there is no control over ligand orientation, resulting in the incorporation of ligand within the inner as well as outer lipid monolayer. It is also, more difficult to control lipid composition, regulate liposome permeability, and achieve high trapping efficiencies in the aqueous interior when using detergent dialysis to prepare liposomes (Allen et al., 1980; Schwendener, 1986; Schwarz et al., 1988).

The second strategy involves attachment of the targeting ligand onto preformed liposomes. This can be achieved by using symmetric bifunctional cross-linking reagents such as glutaraldehyde, suberimidate, (Torchilin et al., 1978 and 1979) and carbodiimide (Endoh et al., 1981) which

Table 1.2

Covalent coupling techniques

Coupling Agent	Type of ligand	References
Ligand coupling before liposome formation		
N-(5-dimethylamino-napthylene-1-sulfonyl)-*L*-lysine coupled to PE	F(ab') fragment (-SH groups)	Sinha and Karush, 1979
N-hydroxyl succinimide (NHS) ester of palmitic acid	IgG ($-NH_2$ groups)	Huang et al., 1980
Carbodiimide derivatized PE	Citraconylated $F(ab')_2$ (-CHO groups)	Jansons and Mallet, 1981
Ligand coupling after liposome formation		
Carbodiimide derivatized PE	IgG (-CHO groups)	Endoh et al., 1981
Glutaraldehyde or dimethyl-suberimidate derivatized PE	IgG ($-NH_2$ groups)	Torchilin et al., 1979
Lactosylceramide oxidized by periodate	$F(ab')_2$ ($-NH_2$ groups)	Heath et al., 1980
SPDP-modified PE	SPDP-modified antibodies F(ab') (-SH groups)	Leserman et al., 1980a Martin et al., 1981
SMPB-modified PE	SPDP-modified antibodies SATA-modified antibodies F(ab') (-SH groups)	Matthay et al., 1984 Derksen and Scherphof, 1985 Martin and Papahadjopoulos, 1982

permit bond formation between amino groups on the ligand and the PE in the liposome. The use of these reagents, however, tends to result in homopolymerization of ligands or liposomes. This leads to ligand aggregation and intervesicular crosslinking (Endoh et al., 1981). To avoid homopolymerization, asymmetric or heterobifunctional cross-linking reagents such as *N*-

succinimidyl 3-(2-pyridyldithio) propionic acid (SPDP) (Carlsson et al., 1978; Leserman et al., 1980) or *N*-succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB) have been characterized (Martin and Papahadjopoulos, 1982; Derksen and Scherphof, 1985). These bifunctional reagents are used to first modify the amino groups on the PE in organic solvent. The resulting modified lipid is then used in the preparation of liposomes containing either the reactive thiopyridine or maleimidophenyl butyrate groups. These groups can then be used to couple ligands with accessible sulfhydryl groups. Endogenous sulfhydryl groups, or free sulfhydryl groups added to a native ligand by modifying agents such as *N*-succinimidyl *S*-acetylthioacetate (SATA) (Hashimoto et al., 1986), *S*-acetylmercaptosuccinic anhydride (SAMSA) (Leserman et al., 1981) or SPDP, may also be used for coupling (Carlsson et al., 1978). It should be noted that the bond formed from the non-reversible alkylating agent SMPB is more stable than the disulfide coupled ligand formed with SPDP (Martin and Paphadjopoulos, 1982). The reactive groups that have been commonly used for covalent conjugation are discussed in greater detail in Section 1.6.2.1.

The advantages of "grafting" ligands onto liposomes *via* this second strategy are based on the fact that preformed liposomes can be prepared to exhibit homogeneous size distributions (Hope et al., 1985) and that the liposomes can be designed for efficient encapsulation of therapeutic agents (Mayer et al., 1986; Madden et al., 1990). However, it is unclear whether the covalent coupling strategies will be pharmaceutically viable. Pharmaceutical viability issues relate to the fact that i) the chemical reactions are reasonably inefficient, ii) the reactive lipids are labile, iii) the chemical reactions require careful control of pH which may impact the liposome formulation, and iv) the procedures are very tedious. Taken together, these issues make it difficult to envision how a formulation could ever be designed to meet the defined manufacturing regulations required by organizations such as the FDA. The coupling protocols developed to date, however,

should be adequate for establishing "proof-of-concept" data demonstrating improved target cell specific binding following extravasation.

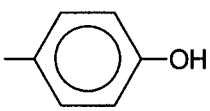
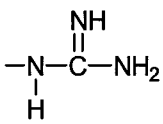
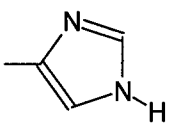
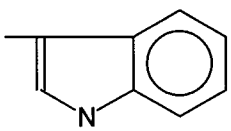
A more recent covalent conjugation technique which has shown limited targeting involves the conjugation of antibodies to the distal end of PEG molecules. This has been achieved with the incorporation of hydrazide, PDPH or sulfhydryl residues at the ends of PEG (Hansen et al., 1995; Allen et al., 1995). However, moving the antibody away from the surface of the liposome still may result in hydrophobic or electrostatic interactions of the antibody with other conjugated antibodies *in vitro* and with plasma proteins *in vivo* resulting in aggregation and rapid plasma elimination, respectively. As well the stability of conjugated antibody to a PEG-DSPE lipid anchor has not been fully addressed.

1.6.2.1 Covalent linker methods: reactive groups

Modification of proteins for cross-linking liposomes can occur *via* a number of reactive functional groups (Table 1.3). For proteins (e.g. peptides and antibodies), amines are the reactive groups most commonly used for conjugation due to their availability. Primary amines are abundantly found on exposed hydrophilic surfaces of proteins, the most reactive being the ε-amine of lysine. Carbohydrates can also be used, particularly for antibodies due to the localization of carbohydrates within the Fc portion of the antibody. Sulfhydryls, available *via* the presence of cysteine residues or generated by the reduction of disulfide bonds, are also commonly used for conjugating antibody fragments (Shahinian and Silvius, 1995; Boeckler et al., 1996). Other less reactive functional groups are available, but are used less frequently due to instability of the cross-linking reagents and difficulties with the chemical reactions (Table 1.3).

Table 1.3

Reactive groups on proteins and peptides used for coupling

Reactive group	Location
Commonly modified reactive groups	
$-NH_2$	Amine groups located on the side chain of lysine residues and at the amine terminus
$-SH$	Sulfhydryl groups are created by mild reduction of intra-chain disulfides or introduced via amine modification reagents such as 2-iminothiolane or SATA
$-C(=O)-OH$	Carboxylic acid groups located on the side chain of glutamic acid and aspartic acid residues and at the carboxyl terminus
$-C(=O)-H$	Aldehyde groups are created by mild oxidation of carbohydrates by periodate
Less commonly modified reactive groups	
$-SH-CH_3$	Methionine
	Tyrosine
	Arginine
	Histidine
	Tryptophan

As stated above, amines are the most utilized reactive groups for conjugation. The use of amines for conjugation can occur; i) directly as the target for an amine reactive cross-linker, ii) following direct modification of the amine function with a reactive moiety attached to a targeting ligand, or more commonly, iii) following modification of the amine function prior to attaching the targeting ligand. The latter is a two-step procedure that is quite versatile. The protein amine function is used to introduce a sulfhydryl group, which is subsequently conjugated to a reactive maleimide modified lipid on a liposome (Figure 1.3B).

Carboxylic acids, when reacted with carbodiimides, can also serve as reactive groups to form amide or hydrazone bonds used in coupling to primary amines or hydrazides. Conjugation with carbodiimides such as EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) are unlike other conjugation reactions in that no cross-bridge is formed between the molecules being coupled. The activated ester intermediate rapidly reduces to a stable amide bond and an isourea side product. The use of carboxylic acids for antibody conjugation is, however, limited as the ester intermediate is unstable in aqueous solutions. Stabilization can be achieved by addition of *N*-hydroxysuccinimide.

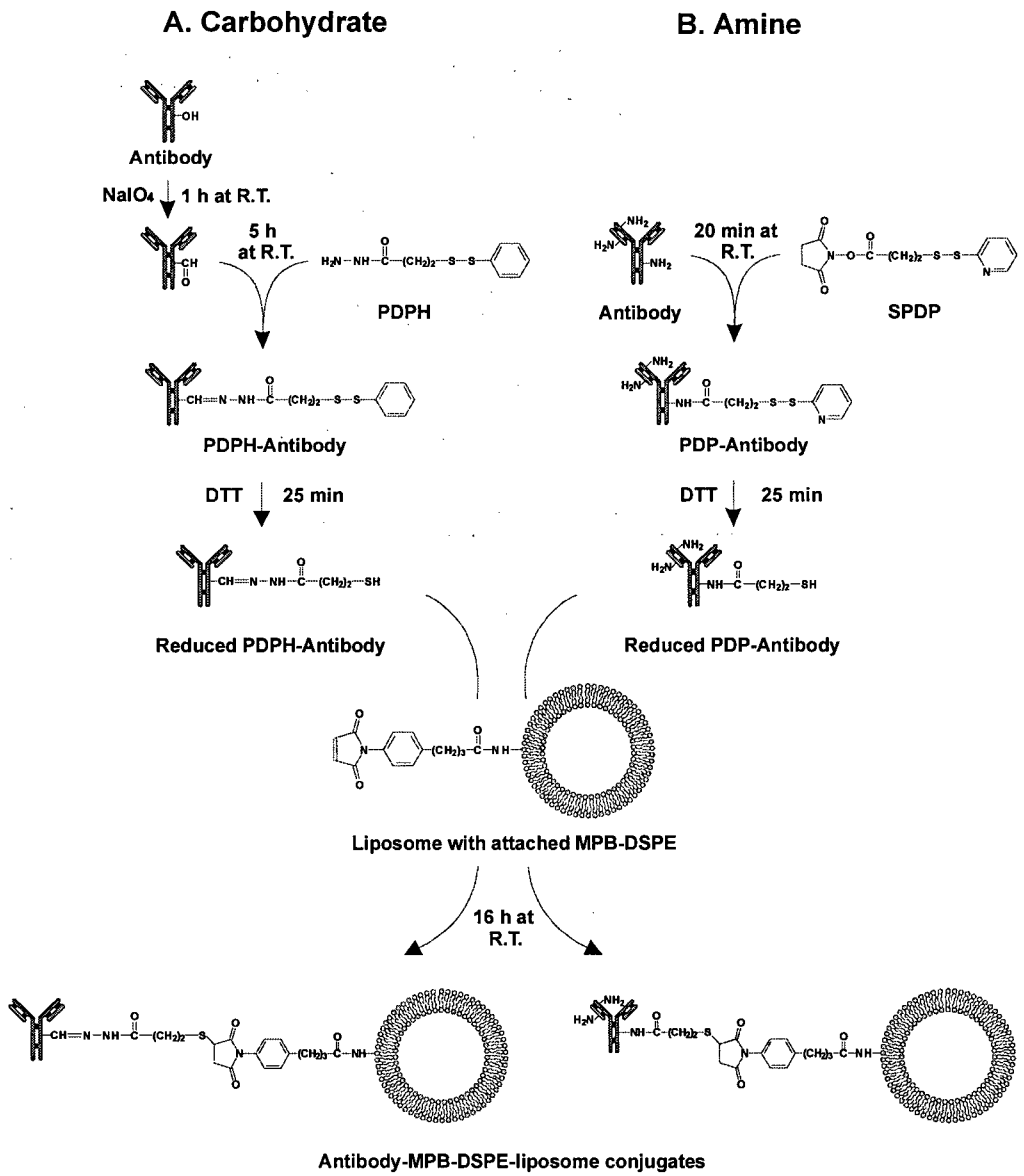
The use of sulfhydryls for conjugation requires mild protein oxidation, as the sulfhydryls in proteins exist largely as cysteine bridges. The oxidation is accomplished with either Traut's reagent to produce free sulfhydryls, SATA and SAMSA to produce protected sulfhydryls, or SPDP and SMPT to produce reactive disulfides.

As carbonyls do not readily exist in proteins, the use of carbonyls as reactive groups also requires mild oxidation of sugar moieties using sodium metaperiodate to convert vicinal hydroxyls to aldehydes or ketones (Figure 1.3A). The aldehydes or ketones are then reacted with

Figure 1.3

Antibody conjugation to liposomes

Two approaches are outlined for conjugating antibodies to maleimide containing liposomes. The first approach (A) couples antibody *via* hydroxyl groups associated with the carbohydrate in the Fc region of the antibody. The second approach (B) couples antibody primarily to the ε-amines of lysine which are distributed through out the antibody. PDPH, 3-(2-pyridyldithiol)propionyl hydrazide; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; MPB-DSPE, *N*-(4-(*p*-maleimidophenyl)butyryl)dipalmitoyl phosphatidylethanolamine; DTT, dithiothreitol.



amines and hydrazides (PDPH) resulting in the formation of a hydrazone bond. The hydrazone is then reacted with maleimide containing liposomes. An advantage of conjugating antibodies through carbohydrates over amines is that minimal decreases in antibody activity should be observed due to the carbohydrates remoteness from the antibodies binding site. In addition, it is anticipated that there will be improved orientation of antibody on the surface of the liposome (Figure 1.4).

In choosing a conjugation reaction it is important to decide which reactive groups will be coupled, availability of the functional group, its reactivity and sensitivity, the use of homobifunctional or heterobifunctional cross-linkers as well as the length and cleavability of the spacer arm. Further, to maintain the native structure of the protein, cross-linking conditions such as pH and ionic strength should also be carefully considered (for a detailed review on protein coupling refer to Wong, 1991).

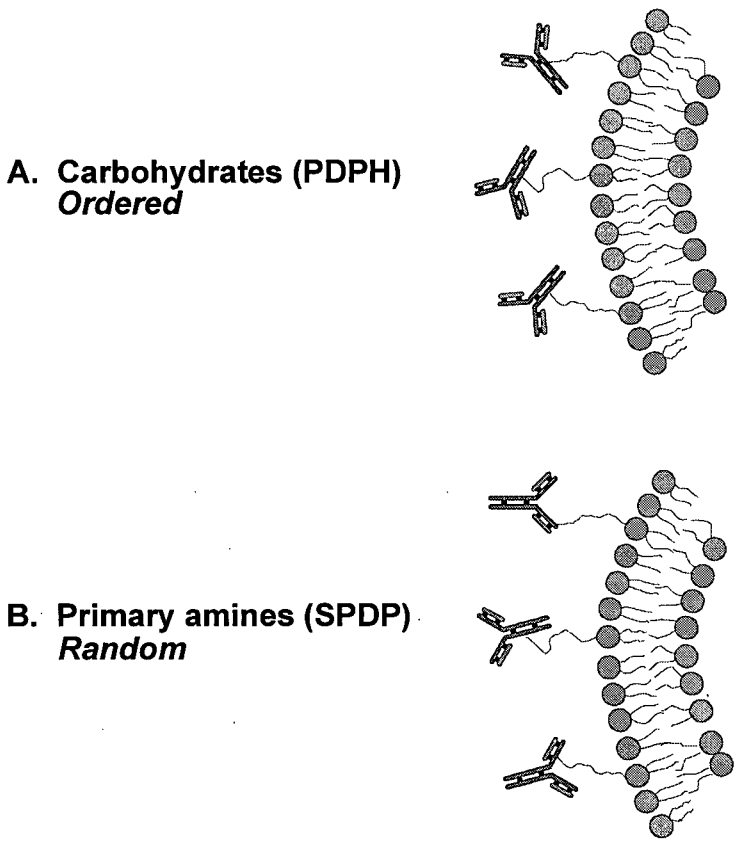
1.7 Biological interactions: effect of target ligand attachment on liposome behavior *in vivo*

It is now well established that modification or conjugation of protein on the liposome surface can have significant effects on the acute biological behavior of the liposome following systemic administration. As well, the presence of attached proteins and reactive groups will lead to an immune response. The effects of greatest interest are those that result in faster liposome elimination. To overcome increased plasma elimination the next Section considers some established methods that have been used to decrease liposome elimination rates and Section 1.7.4 discusses the immunogenicity of protein conjugated liposomes.

Figure 1.4

Antibody alignment

Covalent conjugation as outlined in Figure 1.2 by either carbohydrates (A) or primary amines (B), results in better antibody orientation, for antigen recognition, with the carbohydrate conjugation method.



1.7.1 Maintaining liposomes in the plasma compartment

Several techniques are available for increasing the circulation levels of liposomes, with and without surface associated targeting ligands, including; i) ensuring that the liposome size is small, 50 to 150 nm (Hunt et al., 1979; Juliano and Stamp, 1975; Jackson, 1980), ii) increasing liposome dose beyond that required for saturating the phagocytic cells of the RES, > 100 mg

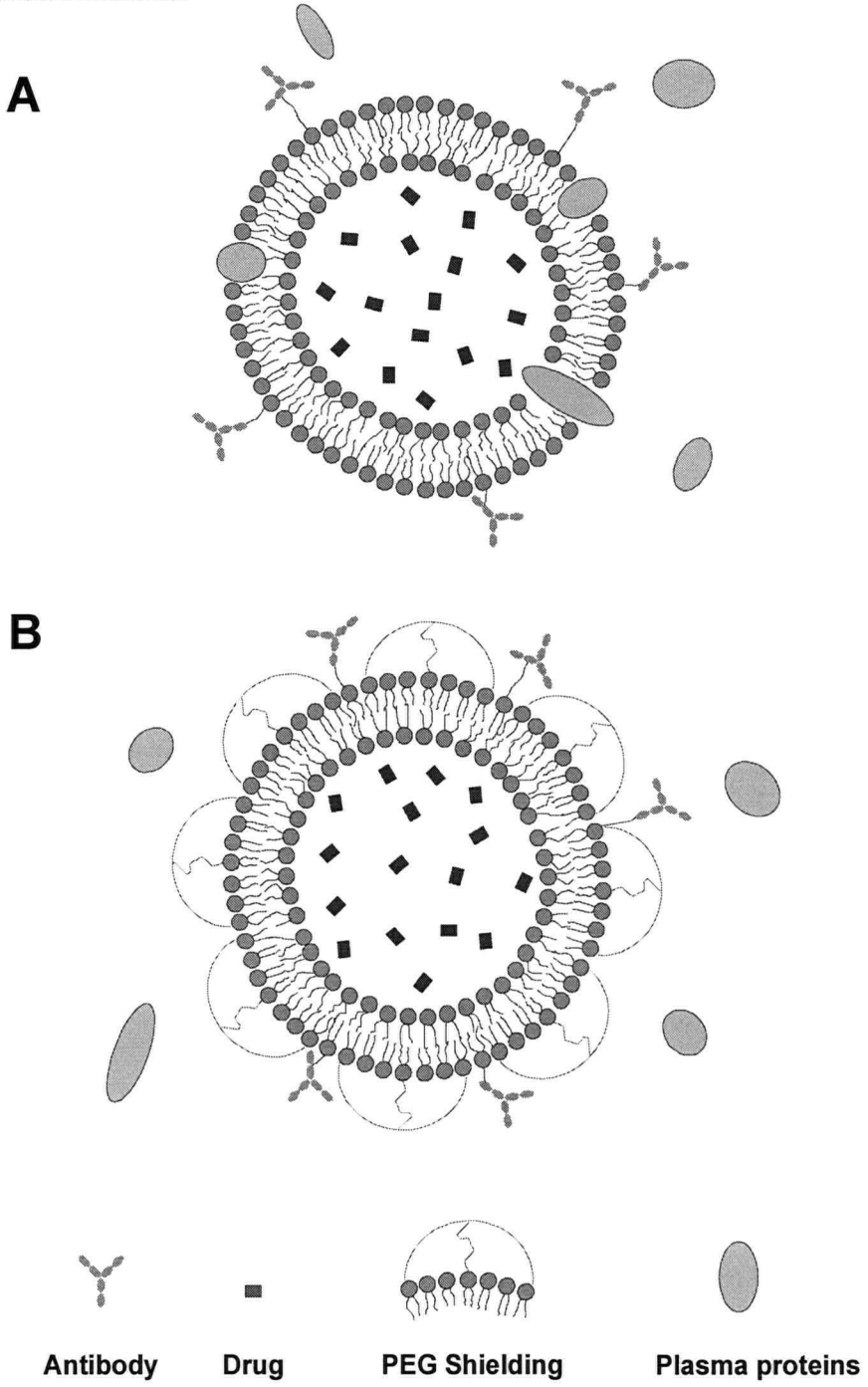
lipid/kg for murine studies (Abra et al., 1980; Sato et al. 1986), iii) incorporation of hydrophilic polymers such as G_{M1} and PEG (Allen et al., 1989; Klibanov et al., 1990; Torchilin et al., 1994, Woodle et al., 1994), and iv) use of selected drugs (liposomal doxorubicin) for RES blockade and reducing liver uptake (Bally et al., 1990). These techniques are all based on overcoming the ability of the phagocytic cells to remove liposomes after i.v. administration. The first two approaches apply principles of saturating the phagocytic cells. The third approach is based on developing surface features which inhibit uptake by phagocytic cells and the last approach relies on the fact that certain liposome encapsulated agents, effectively and specifically inhibit phagocytic activity. The latter two approaches are of greatest interest in the context of developing targeted liposomal anticancer drugs. Although, the use of drug blockade may not be of clinical relevance it is a tool which allows us to evaluate increased plasma liposome levels on target site access

The incorporation of hydrophilic polymers, or other surface structures that provide a steric barrier to serum protein binding (Figure 1.5), significantly increase liposome circulation lifetimes (Blume and Cevc, 1990; Klibanov et al., 1990; Papahadjopolous et al., 1991). The steric barrier is acquired through the incorporation of chemically modified lipids (e.g. poly(ethylene) glycol (PEG) modified-PE or ceramide) or naturally occurring glycolipids (e.g. G_{M1}) into the liposome. The presence of these hydrophilic polymers on the outer monolayer affects reductions in the rate of protein opsonization (Lasic, 1994) and liposome elimination from the circulation (Blume and Cevc, 1990; Klibanov et al., 1990; Papahadjopolous et al., 1991). The reduction in plasma elimination is of key importance, as extended plasma circulation lifetimes allow for increased target site accumulation (Emanual et al., 1996; Mori et al., 1996). As noted in section 1.7.2, PEG modified lipids can inhibit the interaction of a target ligand with its receptor and its use may be

Figure 1.5

Sterically stabilized liposomes

(A) Upon injection plasma proteins associate with the liposomal membrane resulting in opsonization and plasma elimination. (B) The presence of the hydrophilic polymer poly(ethylene glycol), (PEG), inhibits opsonin binding to the liposome surface and effectively increases the plasma circulation lifetimes.



of more value in the preparation of protein coupled liposomes which exhibit defined size distributions rather then enhancement of circulation lifetimes (Harasym et al., 1995).

Inhibition of drug uptake by liver with drug blockade yields a significant increase in liposome plasma levels over extended time periods. Drug blockade can be performed by preinjecting a low dose of liposomal doxorubicin (2 mg doxorubicin/kg) 24 hr prior to the injection of liposomes, resulting in increased liposome circulation lifetimes and 5-fold reductions in liver accumulation (Bally et al., 1990; Parr et al.,1993). The exact mechanism of how this occurs is unknown, but it is speculated that blockade results in the inhibition of Kupffer cell function in the liver, the major RES cell type involved in liposome elimination. An alternative method for achieving RES blockade includes the use of liposomal encapsulated dichloromethylene diphosphonate which specifically eliminates liver macrophage populations (Classen and Van Rooijen, 1986; Van Rooijen, 1989), an affect that can also be achieved by repetitive treatment with high dose (up to 15 mg/kg) liposomal doxorubicin (Daemen et al., 1995).

The two methods, described above, for increasing the levels of liposomes in the circulation involve modification of the liposome. Both methods focus on the general correlation of increasing blood levels to increase extravasation within an extravascular site. An alternative approach, which does not involve increases in liposome circulation, is to modify or adapt target sites for improved extravasation, as was done with the use of substance P (Rosenecker et al., 1996) and angiotensin II (Suzuki et al., 1984). The identification of several highly regulated peptides and proteins (VEGF or VPF), which play vital roles in controlling vascular development and permeability, may also give investigators the ability to modify vascular permeability within defined regions (Senger et al., 1983; Dvorak et al., 1991). It is anticipated that increased target

site access can be achieved by using such peptides or proteins in combination with targeted liposomes.

1.7.2 Methods of maintaining optimal liposome size

Liposome size plays an important role in defining the circulation longevity of liposomes. Liposomes greater than 1 μm in diameter, such as multilamellar vesicles, are eliminated more rapidly from the circulation than unilamellar liposomes of less than 200 nm in diameter (Juliano and Stamp, 1975). Larger vesicles made of phosphatidylcholine and cholesterol have an elimination half-life in mice of less than 15 min, whereas smaller liposomes of the same composition have a half-life increase of 4-times (Juliano and Stamp, 1975). With the incorporation of surface modifying lipids such as PEG-PE or the encapsulation of certain drugs inside liposomes, elimination half-lives of between 12 and 24 h are now routinely reported (Allen et al., 1994). Although liposomes exhibit markedly different elimination profiles, their fate *in vivo* is dictated largely by accumulation in phagocytic cells residing in the liver, spleen, bone marrow, and perhaps skin. The rate at which protein-free liposomes accumulate in these phagocytic cells has been well characterized and it is a simple extension of this previous work to suggest that the development of protein conjugated liposomes which avoid the phagocytic cells of the RES will be necessary to achieve appropriate circulation parameters and target site access.

Early investigations with ligand conjugated liposomes noted that protein conjugated liposomes were more rapidly cleared from the circulation (Papahadjopoulos and Gabizon, 1987) in comparison to liposomes which lacked a surface associated protein. This was attributed to the efficient coupling of proteins which promoted *in vitro* aggregation of the vesicles (Heath et al., 1980) resulting in an increase in polydispersity of the original preparations (Bredehorst, 1986).

This was perhaps best demonstrated by Loughrey et al. (1987, 1990a) who showed that both noncovalent and covalent procedures for attaching targeting ligands to liposomes resulted in vesicle-vesicle crosslinking. Although aggregation can be minimized or reduced for *in vivo* purposes by reducing the concentration of reactants and by limiting the number of reactive groups present on both the liposome and ligand, these steps significantly reduced coupling efficiencies (Jou et al., 1984; Loughrey et al., 1987). Alternatively, the possibility of the conjugated protein/antibody acting as a pre-opsonin or a mediator to increase the opsonization process has also been implicated in increased plasma elimination although no direct evidence has been collected.

Several techniques have been developed to ensure generation of uniformly sized protein coupled liposomes. One method involves the re-extrusion of aggregated liposome conjugates (Loughrey et al., 1990b). Extrusion is a technique used to prepare liposomes by passing hydrated lipid mixtures under medium pressures (< 1000 psi) through membranes of defined size (Hope et al., 1985). When this simple procedure was applied to reaction mixtures that contained aggregated protein-liposome conjugates, the re-extruded liposomes showed narrow size distributions, minimal protein denaturation, and minimal loss of binding activity (Loughrey et al., 1990b). It was noted, however, that after re-extrusion of highly aggregated liposomes there was a tendency for the liposome to reaggregate. It was proposed (Madden, T.D., personal communication) that this reaggregation may have been due to the appearance of exposed hydrophobic domains (acyl chains).

A second, more practical, method developed to control conjugated liposome size involves inhibition of aggregation by incorporating hydrophilic polymers such as poly(ethylene glycol)-modified phospholipids. When PEG-modified lipids were incorporated into liposomes it was

demonstrated that vesicle size can be maintained following coupling reactions (Harasym et al., 1995). It was shown that the presence of PEG_{2000}-modified lipids (at concentrations below 2 mol%) did not impede covalent coupling of thiolated protein, but effectively inhibited liposome-liposome crosslinking. An optimal level of PEG_{2000}-modified lipid was determined empirically, since levels exceeding 5 mol% inhibited protein coupling rates and reduced binding of the protein-liposomes to a defined target molecule *in vitro*. It is important to note that the primary advantage of using PEG-modified lipids is related to maintenance of vesicle size during coupling reactions. The level of PEG lipid used was insufficient to engender enhanced circulation lifetimes.

1.7.3 Plasma elimination and serum protein interactions: studies on protein coated liposomes

It is generally observed that protein (avidin, ovalbumin, streptavidin, neutravidin, or antibodies) conjugated liposomes, even when prepared to exhibit an optimal size (< 200 nm), are more rapidly eliminated (in comparison to protein free liposomes) from the circulation following i.v. administration (Papahadjopoulos and Gabizon, 1987; Loughrey et al., 1990a; Harasym et al., 1995). It has been shown that liposomes with covalently attached rabbit IgG are phagocytosed 5-fold faster by rat liver macrophages than control (protein free) liposomes (Derksen et al., 1987) and the elimination of protein coated liposomes is believed to be achieved primarily by cells of the RES. Studies evaluating the contribution of the various serum factors governing *in vivo* elimination behavior are well established for nontargeted liposomes and it is necessary to understand these interactions if one is to interpret plasma elimination of protein-liposome conjugates.

In the blood compartment, liposomes interact with both lipoproteins and plasma proteins. These interactions affect drug retention and blood elimination characteristics (Chonn et al., 1991, 1992; Schwendener et al., 1991). Within the plasma compartment the interaction of liposomes with high density lipoproteins (HDL) can promote the release of encapsulated agents from the liposome (Kirby et al., 1980). Liposome destabilization and drug release are initiated by a combination of mechanisms involving the transfer of liposomal phospholipid from the liposome to HDL (Krupp et al., 1976; Tall and Small, 1977) and the penetration of ApoA-1 (a protein component of HDL) into the liposome (Wetterau and Jonas, 1983; Klausner et al., 1985). In addition to apolipoproteins, the interaction of complement proteins can also promote leakage of encapsulated contents by activation of the complement cascade (Finkelstein and Weissman, 1979; Devine et al., 1994). Drug release is facilitated by the insertion of complement proteins that lead to the formation of a 10 nm diameter pore-like membrane attack complex (MAC) (Alving and Richards, 1983; Muller-Eberhard, 1986) which can facilitate solute release (Maliniski and Nelsestuen, 1989). Complement proteins are also implicated in opsonization, the process of plasma protein binding which promotes recognition and uptake of foreign particulates by the fixed and free macrophages of the RES (Coleman, 1986; Moghimi and Patel, 1989). The most common opsonins include IgG, fibronectin, and complement proteins (C3 and C3bi). Binding of these proteins leads to liposome elimination by the RES (Coleman, 1986). Relationships between opsonin binding and plasma elimination mediated by the binding of C3 and a newly identified liposome binding protein, apolipoprotein H, have been well characterized (Chonn et al., 1991; 1992).

Liposome interactions with lipoproteins, opsonins and phagocytic cells are highly dependent on lipid composition. Liposomes which avoid these interactions, however, can be designed. What is not well understood is whether targeted carriers can be designed to avoid the problems that

arise as a consequence of protein attachment to liposomes. This is exemplified by antibody conjugated systems where the attached protein can act as a "pre-opsonin" even prior to systemic administration. Optimized protein liposome conjugates will likely consist of an immune suppression strategy as well as strategies that hide the targeting ligand for defined time periods. Exposure of the targeting ligand should only occur when required for target cell interaction.

As indicated above, IgG binding (non-specifically or specifically) is an important component of the opsonization process that marks a liposome for elimination. Mononuclear phagocytes and neutrophils, for example, express receptors for the Fc portions of IgG molecules. These cell surface receptors are known to consist of three subfamilies of related molecules, designated FcγRI, II and III (Abbas et al., 1994). Of these, only the FcγRI (CD64) receptor is a high-affinity receptor for the Fc portion of IgG molecules with a K_d of 10^{-8} to 10^{-9} M. The two remaining receptors are relatively weak with K_d values greater than 10^{-7} M. It is thought that both FcγRI and FcγRII molecules participate in the phagocytosis and signaling in mononuclear phagocytes and neutrophils. Upon IgG binding and coating to antigenic particles (opsonization) it is the bound IgG that is recognized by the FcγR molecules on the phagocytes. Conceivably, a dilemma exists if one is to generate an antibody coated liposome for targeting purposes. This was shown with active and passively immunized mice specific for an antigen (dinitrophenol, DNP) on i.v. administered liposomes (Aragnol and Leserman, 1986). These studies showed increased deposition of the DNP-coated liposomes in the liver. The increased liver accumulation, however, could be diminished by injection of a monoclonal antibody directed against the murine IgG Fc receptor. Further, *in vitro* studies have demonstrated specific binding of antibody opsonized liposomes to a Fc receptor positive phagocytic murine P388D1 cell line (Leserman et al., 1980b). The interactions of the IgG containing liposomes with P388D1 cells were dependent on the Fc region of the antibody since no liposome interaction with these cells occurred when using $F(ab')_2$

or IgA (Leserman et al., 1980b). Coating liposomes with macromolecular ligands capable of interacting with macrophage surface receptors can markedly promote liposome uptake (Hsu and Juliano, 1982). Specifically, formation of an IgG-antigen complex on the liposome surface resulted in a 100-fold enhancement of liposome uptake and coating the liposomes with fibronectin resulted in a 10-fold increase in uptake. Fc receptor recognition can, however, be competitively inhibited. For example, nonspecific binding of liposomes with conjugated IgG to K562 cells was inhibited by addition of soluble human IgG (Bragman et al., 1983). Based on these results it can be suggested that the regulation of Fc availability or blockade of Fc receptors should decease antibody conjugated liposome elimination. Aragnol and Leserman (1986) have characterized strategies involving Fc receptor blockade with the use of anti-Fc receptor antibodies, while Ansell et al. (1996) recently reported on a coupling strategy that facilitates protein orientation where the Fc portion of the IgG is less exposed. The latter can be used to prepare liposomes with extended circulation lifetimes.

1.7.4 Immunogenicity of reactive moieties and associated proteins

Liposomes have been shown to enhance the immunogenicity of a protein, regardless of whether the protein is entrapped or adsorbed on the outer surface (Shek and Sabiston, 1982). Moreover, concerns have been raised about the immunogenicity of the ligands and crosslinkers used to attach targeting ligands. Repeated administration of antibody conjugated liposomes has been shown to elicit an anti-IgG immune response, resulting in significant reductions in blood residence times of targeted liposomes (Phillips and Emili, 1991). This response was directed to both the linker, in this example avidin-biotin, and the targeting ligand (antibody). Investigations studying the immunogenicity of protein conjugated liposomes prepared *via* several sulfhydryl linkers (bromoacetyl, maleimide, dithiopyridine), varying in length from 0.3 to 1.6 nm (compared

to SMPB, SPDP and *N*-succinimidyl bromoacetate-PE), showed that the production of a strong immune response was independent of the length of the linkers or the nature of the reactive moieties (Boeckler et al., 1996).

It is believed that the immunogenicity will be dependent on liposome parameters such as size, surface charge, and associated entrapped therapeutic agent. Further, immunogenicity may not be a factor when the targeted liposomes contain an immunotoxic drug (Shek et al., 1986). It has been demonstrated that the plasma elimination rate increased following repeated administration of ovalbumin liposomes, an effect that can be correlated to generation of anti-ovalbumin IgG (Tardi et al., 1997). However, if these liposomes contained encapsulated doxorubicin the subsequently injected ovalbumin-conjugated liposomes exhibit no change in plasma elimination rate. Importantly, the latter effect was correlated to inhibition of anti-ovalbumin IgG production.

Targeted liposomes prepared with species-specific antibodies have also proven to be immunogenic and induce significant levels of isotype specific antibody after repeated injection (Phillips and Dahman, 1995). In an effort to overcome the immunogenic or HAMA (human anti-mouse antibody) response, highly specialized humanized (Mountain and Adair, 1992; O'Kennedy and Roben, 1991) or chimeric (Morrison et al., 1984; Fleischman, 1985) antibodies have been developed resulting in the rejuvenated interest in antibodies as targeting ligands. Rapid induction of an anti-idiotypic response to unmodified antibodies does occur in syngenic mice following primary immunization with these specialized targeting ligands (Blaser et al., 1983; Lucas et al., 1992, 1993) and it is anticipated that the same anti-idiotypic response will occur with humanized or chimeric antibodies.

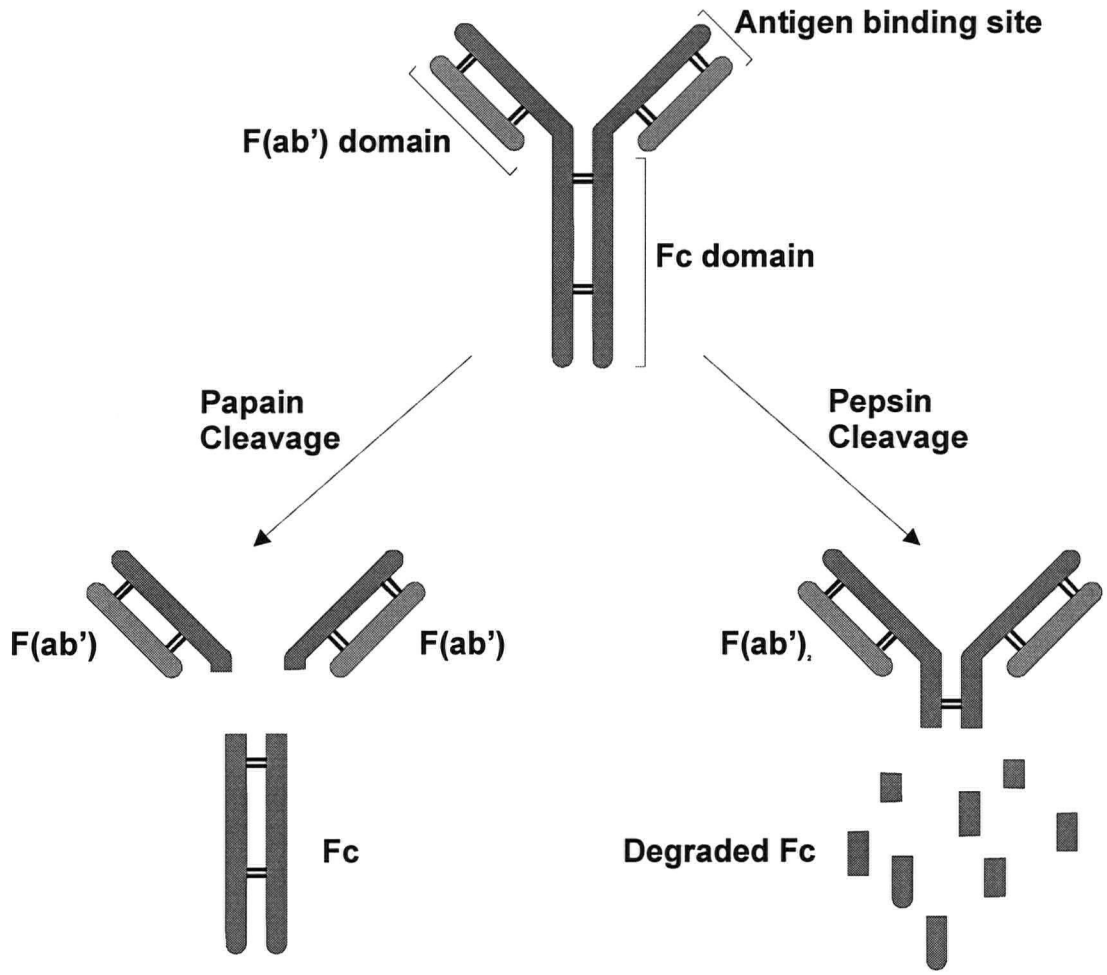
1.7.5 Use of antibody fragments

As previously stated, the most common ligand to conjugate to liposomes has been the antibody. Whole antibody conjugates, however, tend to be rapidly cleared from the circulation as a result of Fc-mediated endocytosis by macrophages (Hsu and Juliano, 1982; Aragnol and Leserman, 1986). The reduction in plasma circulation levels are known to result in decreased target site accumulation. It has been suggested that increases in target site accumulation may be achievable by reducing the bulk of nonantigen binding regions from the antibody. Several investigations have, therefore, used F(ab') (Bredehorst et al., 1986; Shahinian and Silvius, 1995) or $F(ab')_2$ (Singh et al., 1993; Peeters et al., 1988) antibody fragments (Figure 1.6). The coupling of F(ab') fragments to liposomes can be inefficient due to a lack of maleimide-reactive sulfhydryls (Shahinian and Silvius, 1995). Further, use of F(ab') conjugates can result in reductions or loss in binding avidity due to loss of bivalent binding ability (Weinstein et al., 1978; Petrossian and Owicki, 1984; Petrossian, 1993). Fragmentation of whole antibody into $F(ab')_2$ or F(ab') fragments can result in 2-fold and 14-fold reductions in antibody affinity, respectively (Lamarre and Talbot, 1995). Changes in binding avidity as a consequence of antibody fragmentation are not necessarily relevant when considering multiple F(ab') copies attached to each liposome and it is somewhat surprising that better binding avidities have not been observed for F(ab') conjugated liposomes. Better results have been achieved with $F(ab')_2$ conjugates, which can show greater binding than whole antibody conjugates *in vitro* (Weinstein et al., 1978). Results are, however, still mixed as to the best choice of liposomal ligand. Some studies have shown that the rate and strength of binding with whole antibody was greater than those of F(ab'), indicating increased avidity due to the bivalent binding ability of whole antibody (Petrossian and Owicki, 1984). Whereas others have shown that the use of $F(ab')_2$ results in three times greater binding than whole antibody no binding with F(ab') was observed (Weinstein et al., 1978).

Figure 1.6

Antibody fragmentation

For ligand conjugation either whole antibody or antibody fragments can be used. Fragments are produced by enzymatic cleavage of the whole antibody with either papain or pepsin.



$F(ab')_2$ conjugates have been shown to be more immunogenic than whole antibody conjugated liposomes (Phillips et al., 1994). The fact that coupled antibody fragments are more immunogenic is perhaps not surprising considering that damaged antibodies are typically removed rapidly from the circulation. This is an important point to consider, since all protein

conjugation procedures carry the potential to damage the protein and this can result in reductions in binding affinities and/or increased recognition by the immune system.

In most instances the conjugation of an antibody or fragment, F(ab'), to a liposome does not further alter the antibodies specificity (Martin et al., 1981; Nassander et al., 1992). To minimize decreases in antibody affinity it is important that the method of conjugation and the particular antibody used be well characterized during chemical modifications. Since antibodies differ in amino acid sequence and the regions where modifications occur, this characterization is required for each antibody used. Conjugation through primary amines will result in a fraction of antibody being bound through amines within the antigen binding region. It is known that the extent of antibody modification can affect antigen binding ability (Loughrey et al., 1990b) and highly modified antibodies can effect significant changes in antibody structure which can decrease antibody binding affinity. The conjugation of antibody *via* carbohydrate moieties located in the Fc portion of the antibody can potentially minimize effects of the chemical reactions on antibody affinity.

1.8 Objectives

For actively targeted drug loaded liposomes to become clinically useful, certain requirements must be met. First, protein conjugated liposomes must retain the characteristics that defined them as useful drug carrier systems. The presence of a targeting ligand associated with the liposome must not interfere with the liposomes size, stability and drug loading capabilities. Second, targeted liposomes must be able to access the target site and accumulate at significant levels to allow for specific tumor cell association to allow for maximum efficacy. Third, the

circulation longevity must allow for passive accumulation to a target site and the protein coupled onto liposome must remain associated to allow for active targeting.

This thesis investigates several areas to better understand how to successfully target drug loaded liposomes to tumor cells residing in an extravascular site. Chapter 3 incorporates PEG-lipids onto a liposome to inhibit aggregation that occurs upon protein conjugation. In Chapter 4 the cellular association of passively accumulated liposomes at several tumor sites are investigated. Finally, in Chapter 5 the plasma elimination of protein coupled liposomes and the stability of the protein on the liposome are characterized.

CHAPTER 2

PEG-LIPIDS PREVENT AGGREGATION DURING COVALENT CONJUGATION OF PROTEINS TO LIPOSOMES

2.1 Introduction

As outlined in Chapter 1, the use of liposome-based drug carrier systems which accumulate at regions of disease are actively being developed. It is now well established that small liposomes and associated contents accumulate preferentially in sites of infection, inflammation, and cancer following i.v. administration (Gabizon and Papahadjopoulos, 1988; Gabizon, 1992; Wu et al., 1993; Bally et al., 1994). The level of entrapped contents delivered to these diseased sites increases with liposome circulation longevity as well as optimized drug retention characteristics (Wu et al., 1993; Bally et al., 1994; Bowman et al., 1994). Early studies evaluating the pharmacokinetic behavior of liposomes following i.v. administration demonstrated that liposome size was a critical determinant of circulation longevity (Senior et al., 1985; Hwang, 1987). Phosphatidylcholine–cholesterol liposomes exhibiting mean size distributions between 50 and 150 nm, for example, are retained in the circulation for extended time periods (Allen et al., 1985; Chonn et al., 1992). Retention of entrapped contents is dependent on the lipid composition employed as well as the nature of the entrapped material (Bally et al., 1990; Bally et al., 1993; Bowman et al., 1994). Liposomes prepared using phospholipids with long chain saturated fatty acyl chains and cholesterol exhibit improved retention of hydrophilic compounds following i.v. administration.

Research has focused in three areas to develop liposomal drug carriers that have an increased propensity to accumulate in disease sites. The first concerns the use of lipids that engender extended circulation lifetime. Incorporation of the ganglioside G_{M1} or poly(ethylene glycol)-modified phospholipids in liposomes, for example, decreases uptake in the liver and increase

circulating blood levels (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Klibanov et al., 1990). Several studies have shown that these liposomes accumulate efficiently in sites of tumor growth (Gabizon and Papahadjopoulos, 1988; Gabizon, 1992; Wu et al., 1993). The second area of interest concerns the biological elements that mediate movement of liposomes from the blood compartment to an extravascular site. Recent studies have shown that such delivery to tumors occurs through blood vessels that are hyperpermeable to circulating macromolecules (Wu et al., 1993; Bally et al., 1994). Finally, it is reasonable to assume that the extent of accumulation within disease sites, such as tumors, will be dependent on an equilibrium between circulating liposomes and liposomes in the extravascular space. Targeting liposomes to specific elements or cells within the extravascular space should shift the equilibrium in favor of further liposome accumulation at the target site.

Although approaches for attaching targeting proteins to the surface of liposomes are well established (Leserman et al., 1981; Barbet et al., 1981; Martin and Papahadjopoulos, 1982; Loughrey et al., 1990a), the resulting proteoliposomes often do not maintain optimal circulation characteristics. In this Chapter, for example, it is shown that protein–liposome conjugation procedures based on the use of heterobifunctional reagents lead to liposome–liposome crosslinking (i.e. increases in carrier size) which results in dramatically reduced circulation lifetimes. Further, leakage of entrapped contents is also observed (Bredehorst et al., 1986; Loughrey et al., 1990b). Using drug entrapment procedures based on transmembrane pH gradients, where a drug is loaded into preformed liposomes (Bally et al., 1988), eliminates problems associated with drug leakage during coupling. The most significant limitation to the use of these coupling procedures is liposome aggregation.

Aggregation is generally caused by the covalent crosslinking of liposomes *via* a multivalent protein bridge. In addition, noncovalent protein–protein interactions (electrostatic and hydrophobic) can

lead to further aggregation. Although aggregation can be minimized by reducing the concentration of reactants and by limiting the number of reactive groups present on both the liposomes and/or the protein, these steps significantly reduce coupling efficiency (Jou et al., 1984; Bally et al., 1988). The strategy developed in this Chapter is to inhibit covalent crosslinking of liposomes by incorporating poly(ethylene glycol)-modified phospholipids. It is well established that incorporation of hydrophilic polymers in liposomes provides a steric barrier inhibiting surface association of serum proteins (Senior et al., 1991). Further, studies published elsewhere suggest that vesicle size can be maintained following coupling reactions when PEG-modified lipids are incorporated into the liposomes (Ahmad et al., 1993; Allen et al., 1994). In this Chapter it is demonstrated that the efficient conjugation of thiolated avidin to MPB–PE incorporated in liposomes containing PEG-modified phosphatidylethanolamine can be achieved with no aggregation of the liposomes. It is further demonstrated that the biotin binding activity of liposome associated avidin is maintained and that the circulation lifetime of the resulting liposome is significantly improved.

2.2 Materials and methods

2.2.1 Materials

1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar Lipids, and *N*-(4-(*p*-maleimidophenyl)butyryl)dipalmitoyl phosphatidylethanolamine (MPB-DPPE) was synthesized as published previously (Loughrey et al., 1990a). The synthesis and characterization of various MePEG–lipid conjugates has been described elsewhere (Parr et al., 1994), and these lipids are now commercially available through Northern Lipids (Vancouver, B.C.). Avidin-D was obtained from Vector Laboratories and neutravidin from Pierce. Cholesterol (Chol), *N*-ethylmaleimide (NEM), *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), dithiothreitol

(DTT), *N-N'*-dicyclohexylcarbodiimide (DCC), and *N*-hydroxysuccinimide (NHS) were obtained from Sigma. Biotinylated Thy 1.2 antibody was obtained from Cedar Lane Laboratories. Radiolabeled *d*-[carbonyl-^{14}C]biotin and [^{3}H]cholesteryl hexadecyl ether (^{3}H-CHE) were obtained from Amersham. Female CD1 mice were purchased from Charles River Laboratories (Ont.).

2.2.2 Preparation of liposomes

Large unilamellar vesicles were prepared as described by Hope et al. (1985). Lipid mixtures consisting of DSPC, cholesterol, MePEG–S–POPE, and MPB-DPPE were prepared in chloroform and subsequently concentrated to a homogeneous lipid film under a stream of nitrogen gas. The lipid film was then placed under high vacuum for at least 4 h prior to hydration at 65°C with 300 mM citrate pH 4.0. The resulting multilamellar vesicle preparation was frozen and thawed five times (Mayer et al., 1986) before the sample was extruded ten times through stacked 100 nm polycarbonate filters (Nuclepore) employing an extrusion device (Lipex Biomembranes, Vancouver, B.C.) at 65°C. The resulting liposomes were sized by QELS using a Nicomp 270 submicron particle sizer operating at 632.8 nm.

2.2.3 Thiolation of avidin-D

Avidin-D (5 mg/ml in HBS 25 mM Hepes; 150 mM NaCl, pH 7.5) was modified with the amine reactive reagent SPDP according to procedures described for streptavidin (Loughrey et al., 1990a; Loughrey et al., 1990b). Briefly, SPDP (25 mM in methanol, 1–10 mol equiv.) was incubated with avidin-D at room temperature for 30 min. The reaction mixture was then reduced with DTT (25 mM, 10 min) and the thiolated product was isolated by gel filtration on Sephadex G-50 equilibrated with HBS pH 7.5 and used immediately in coupling experiments. The extent of modification of

avidin-D was determined by estimating the protein concentration at 280 nm (molar extinction coefficient at 280 nm of 9.52 x 10^4) prior to the addition of DTT and the 2-thiopyridone concentration at 343 nm (molar extinction coefficient at 343 nm of 7550) 10 min after the addition of DTT.

2.2.4 Coupling of thiolated avidin-D to liposomes

The coupling reaction was performed by incubating thiolated avidin-D with MPB-liposomes at a ratio of 150 μg of protein/μmole lipid (6–7 mM final lipid concentration) at pH 7.5 with stirring at room temperature. Liposomes prepared at pH 4.0 (300 mM citrate) were passed down a sephadex G-50 column equilibrated with HBS (pH 7.5) prior to addition of the thiolated avidin-D. At selected time points coupling was stopped by the addition of β-mercaptoethanol followed by (10 min after β-mercaptoethanol addition) the addition of excess NEM. Samples were then passed down a Sepharose CL-4B column equilibrated with HBS to remove any unassociated protein. The amount of avidin coupled to the liposomes was determined by a modification of the fluorescamine assay for protein (Lai, 1977). Briefly, avidin–liposome conjugates were lysed by addition of 10 mM OGP before addition of 0.2 M borate buffer (pH 9.0) to raise the pH. Fluorescamine (1 mg per 5 ml of anhydrous acetone) was added with immediate vortex mixing. Standards were prepared as above using known quantities of the thiolated avidin and uncoupled liposomes. Fluorescence was then determined at an excitation wavelength of 390 nm and emission wavelength of 480 nm using a Perkin Elmer LS50 luminescence spectrometer.

2.2.5 Doxorubicin encapsulation

Doxorubicin was encapsulated in selected liposome preparations using the transmembrane pH gradient driven loading procedure as described previously (Mayer et al., 1990). The liposome preparation (prepared at pH 4.0 prior to coupling avidin at pH 7.5) was heated to 60–65°C for 10 min prior to addition to a preheated (60°C for 10 min) solution of doxorubicin (5–6 mM in saline). A final drug-to-lipid ratio of 0.2 was typically employed. This mixture was incubated with periodic mixing for 10 min at 60°C. Unencapsulated doxorubicin was removed by passing the sample through a sephadex G-50 column and the doxorubicin-to-lipid ratio was measured as described previously (Bally et al., 1990; Mayer et al., 1990).

2.2.6 Biotin binding activity

The biotin binding activity of the avidin–liposome conjugates was determined as described for streptavidin (Loughrey et al., 1990a; Loughrey et al., 1990b). Briefly, avidin–liposomes (0.5 μmole lipid in 0.5 ml) were incubated with a 10-fold excess of [^{14}C]biotin for 10 min at room temperature. Unbound biotin was removed by gel filtration on a Sepharose CL-4B column equilibrated with HBS. The extent of binding of biotin to a thiolated-avidin standard (100 μg) after gel chromatography on Sephadex G-50 was used as a reference for the calculation of coupling ratios.

2.2.7 Targeting to biotin labeled P388 cells

In vitro quantification of cell-associated lipid after targeting avidin-D and neutravidin-coated LUVs with 2 mol % PEG_{2000}–DSPE to P388 cells were performed as follows. Avidin or neutravidin LUVs (51 and 63 μg/μmole lipid, respectively) incorporating PEG_{2000}–DSPE were prepared as

described above. P388 cells (10^7) were incubated with or without biotinylated anti-mouse Thy 1.2 antibody (10 μg) for 30 min at 4°C. Cells were then washed (3 x 10 min centrifugations at 800 *g*) with PBS prior to addition (2 mM final concentration) of either avidin or neutravidin LUVs. After 30 min incubation at 4°C, the cells were further washed and cell-associated lipid was determined *via* a ^{3}H-CHE lipid marker.

2.2.8 *In vivo* clearance studies

Coated LUVs composed of DSPC/Chol/MePEG$_{2000}$–S–DSPE/MPB–DPPE (52:45:2:1) containing either avidin or neutravidin were prepared as outlined previously. CD1 mice were injected i.v. at 30 mg lipid/kg with one of the above protein-coated LUVs. Whole blood was collected at 1, 4, and 24 h intervals *via* cardiac puncture and collected in EDTA-coated tubes. Plasma was subsequently prepared by centrifuging at 1500 *g* for 10 min. Lipid was then assayed *via* a ^{3}H-CHE lipid marker.

2.3 Results

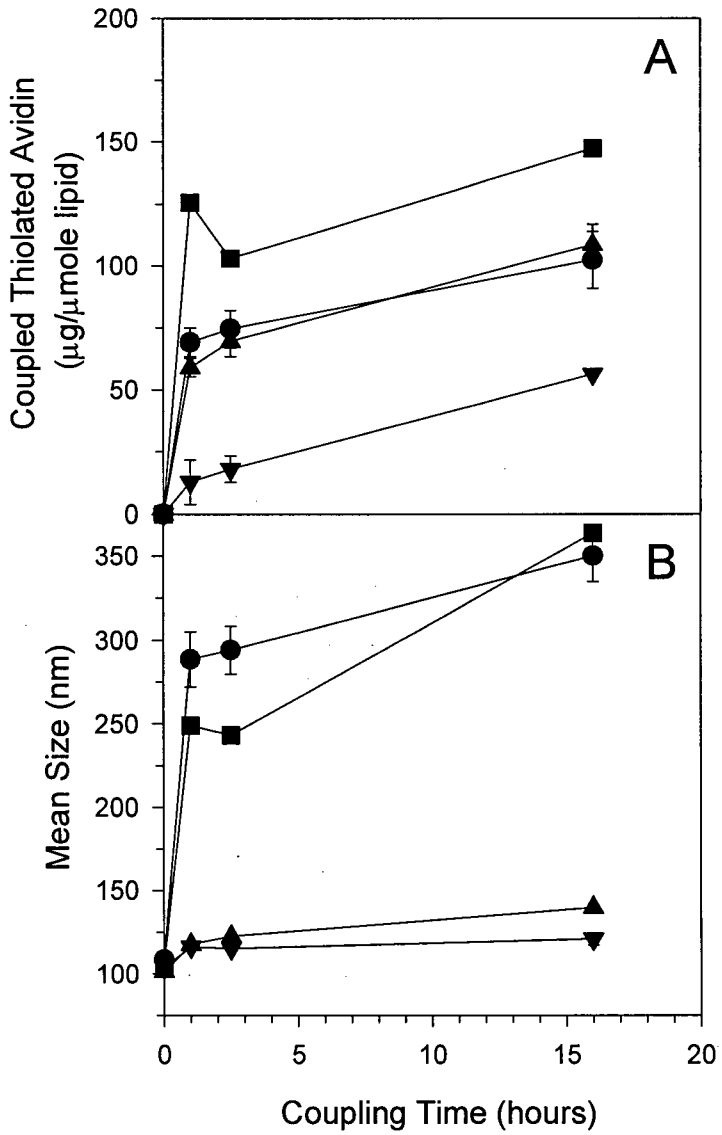
2.3.1 Varying chain lengths and levels of PEG

To determine the influence of different sizes of PEG polymer on the coupling reaction between MPB-liposomes and thiolated avidin, monomethoxy(polyethylene glycol) (MePEG) of three different molecular weights (550, 2000, and 5000) were linked *via* a succinate bond to POPE to form the respective MePEG–lipid conjugates (MePEG$_{550}$–S–POPE, MePEG$_{2000}$–S–POPE, and MePEG$_{5000}$–S–POPE) (Parr et al., 1994). These MePEG–lipid conjugates were then incorporated into MPB-containing liposomes (1 mol % MPB–DPPE/54 mol % DSPC/45 mol % Chol) at a level of 2 mol % of the total lipid. Incubation of these MePEG-coated MPB-containing liposomes with thiolated avidin resulted in the covalent attachment of protein to the liposomes (Figure 2.1). The

Figure 2.1

Effect of different chain lengths of PEG on the coupling reaction of thiolated avidin with MPB-liposomes

MPB-liposomes (DSPC:Chol:MPB-DPPE:MePEG–S–POPE, 52:45:1:2; 6.57 mM) containing no PEG (●), $MePEG_{550}$–S–POPE (■), $MePEG_{2000}$–S–POPE (▲), or $MePEG_{5000}$–S–POPE (▼) were coupled with thiolated avidin (3.6 SH equiv, 150 μg per μmole lipid). Panel A shows the amount of protein (expressed as μg of thiolated avidin per μmole of lipid) coupled to MPB-liposomes as a function of coupling reaction time. Panel B shows the size (nm) of the corresponding proteoliposomes as measured by QELS. At 16 h, LUV size for both $MePEG_{2000}$ and $MePEG_{5000}$ were significantly different from control liposomes ($P < 0.001$ and $P < 0.005$, respectively). Points: mean of three assays. Error bars: SD of at least three experiments.



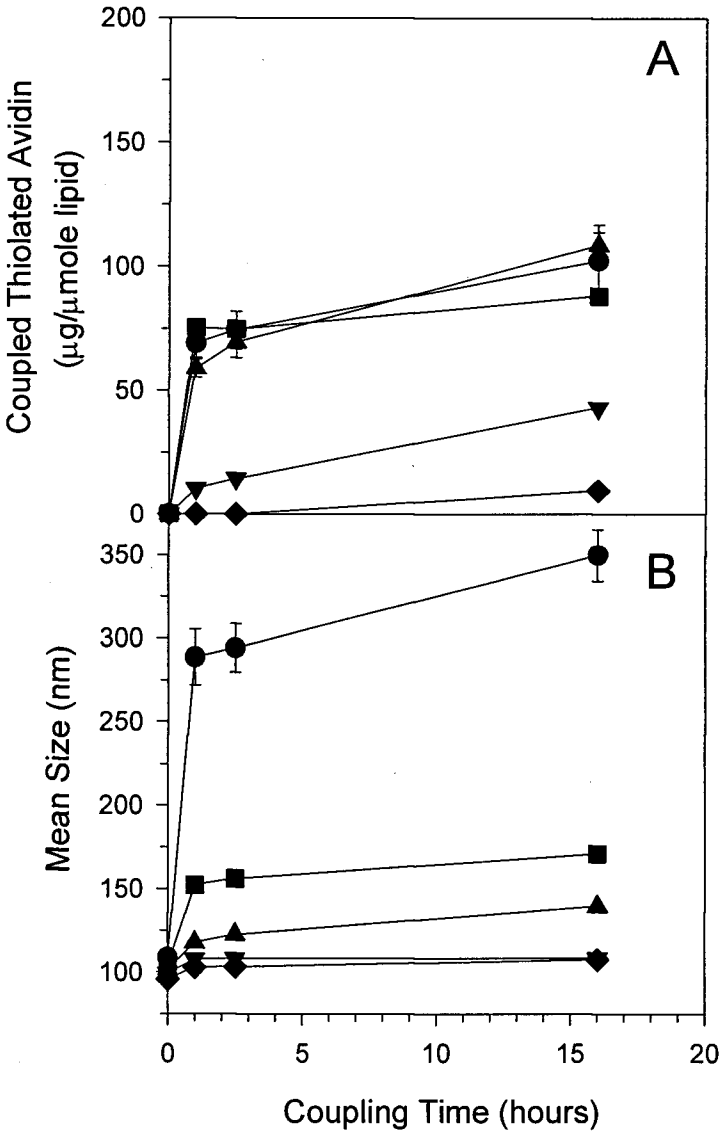
rate and extent of coupling was dependent on the molecular size of the poly(ethylene glycol) incorporated. In the absence of MePEG–S–POPE substantial levels of avidin (70 μg avidin/μmole lipid) were conjugated to the liposomes within 1 h (Figure 2.1A). Subsequently, the coupling reaction occurred at a reduced rate ultimately leading to levels of approximately 100 μg avidin/μmole lipid observed at 16 h. As observed in previous studies (Bredehorst et al., 1986; Loughrey et al., 1990), these liposomes exhibited a dramatic increase in size, indicative of liposome–liposome crosslinking (Figure 2.1B). The time course for size increase was similar to that observed for coupling. As shown in Figure 2.1B, incorporation of $MePEG_{2000}$– and $MePEG_{5000}$–S–POPE substantially reduced time dependent increases in liposome size. While the rate of avidin coupling to liposomes was reduced significantly when 2 mol % $MePEG_{5000}$–S–POPE was present, both the rate and extent of coupling obtained for liposomes with 2 mol % $MePEG_{2000}$–S–POPE was identical to controls.

The results illustrated in Figure 2.1 indicated that a hydrophilic polymer coating imparted by incorporation of 2 mol % $MePEG_{2000}$ was the most suitable for preparation of proteoliposomes in terms of protein-coupling efficiency and effectiveness in inhibiting vesicle aggregation. The next series of experiments were designed to determine whether 2 mol % $MePEG_{2000}$–S–POPE was optimal. Four different levels of $MePEG_{2000}$–S–POPE (1%, 2%, 5%, and 8%) were studied and compared with control liposomes. The different quantities of thiolated avidin that could be coupled to each type of liposome is illustrated in Figure 2.2A. The presence of 5 and 8 mol % $MePEG_{2000}$ significantly reduced the amount of protein that could be conjugated to the surface of the liposomes. In contrast, addition of 1 or 2 mol % $MePEG_{2000}$ did not influence the coupling reaction. For these liposomes, 70 to 80 μg avidin were bound to the liposomes within 1 h after addition of the thiolated protein, representing a coupling efficiency of approximately 50%. As expected, liposomes that did

Figure 2.2

Effect of various concentrations of PEG polymer on the coupling reaction of the thiolated avidin with MPB-liposomes

MPB-liposomes (6.54 mM) containing various concentrations of $MePEG_{2000}$–S–POPE, 0% (●), 1% (■), 2% (▲), 5% (▼), and 8% (◆) were coupled with thiolated avidin (3.9 SH equiv, 150 μg per μmole lipid). Coordinates for panels A and B are the same as in Figure 2.1. At 16 h, all treatment groups were statistically significant from control ($P < 0.05$). Points: mean of three assays. Error bars: SD of at least three experiments.



not efficiently couple protein showed no size increases (Fig. 2.2B). Efficient coupling with only minimal increases in vesicle size was observed for liposomes prepared with 2% $MePEG_{2000}$.

2.3.2 Degree of protein thiolation on coupling

The degree of protein thiolation is also known to have an effect on protein-coupling reactions mediated by MPB-modified lipids. In order to assess whether incorporation of 2 mol % $MePEG_{2000}$–S–POPE inhibited aggregation regardless of the extent of protein thiolation, coupling of modified avidin having approximately 1, 2, 4, and 5 thio equiv was determined (Figure 2.3A, B). As expected, protein association was dependent on the degree of thiolation. The presence of 2–4 thio equiv appeared optimal for efficient coupling with no associated changes in vesicle size. The amount of protein-coupling was significantly enhanced when using avidin with 5 thiol equiv; however, increases in vesicle size were observed for this system even in the presence of 2 mol % $MePEG_{2000}$–S–POPE.

2.3.3 Comparison of different M.W. PEG coatings

Further investigations on the importance of the effect of molecular size of the polymer chain were conducted by comparing the coupling reaction of thiolated avidin with MPB-liposomes containing 8% $MePEG_{550}$–S–POPE or 2% $MePEG_{2000}$–S–POPE. It can be estimated that these liposomal preparations should exhibit similar numbers of PEG units on the surface of the liposomes. However, as illustrated in Figure 2.4A, the initial rates of protein conjugation are quite different. With 8% $MePEG_{550}$, the higher density of the shorter PEG_{550} molecules on the liposomal surface initially inhibited the coupling reaction with thiolated avidin. Alternatively, MPB-liposomes with

Figure 2.3

Effect of the degree of protein thiolation on the coupling reaction of thiolated avidin with pegylated liposomes

Pegylated liposomes (DSPC:Chol:MPB-DPPE:MePEG$_{2000}$–S–POPE, 53:45:2; 6.49 mM) were coupled with thiolated avidin (150 μg per μmole lipid) at 1.2 SH equiv (●), 1.9 SH equiv (■), 4.5 SH equiv (▲), and 5.3 SH equiv (▼). Coordinates for panels A and B are the same as in Figure 2.1. Points: mean of three assays. Error bars: SD of at least three experiments.

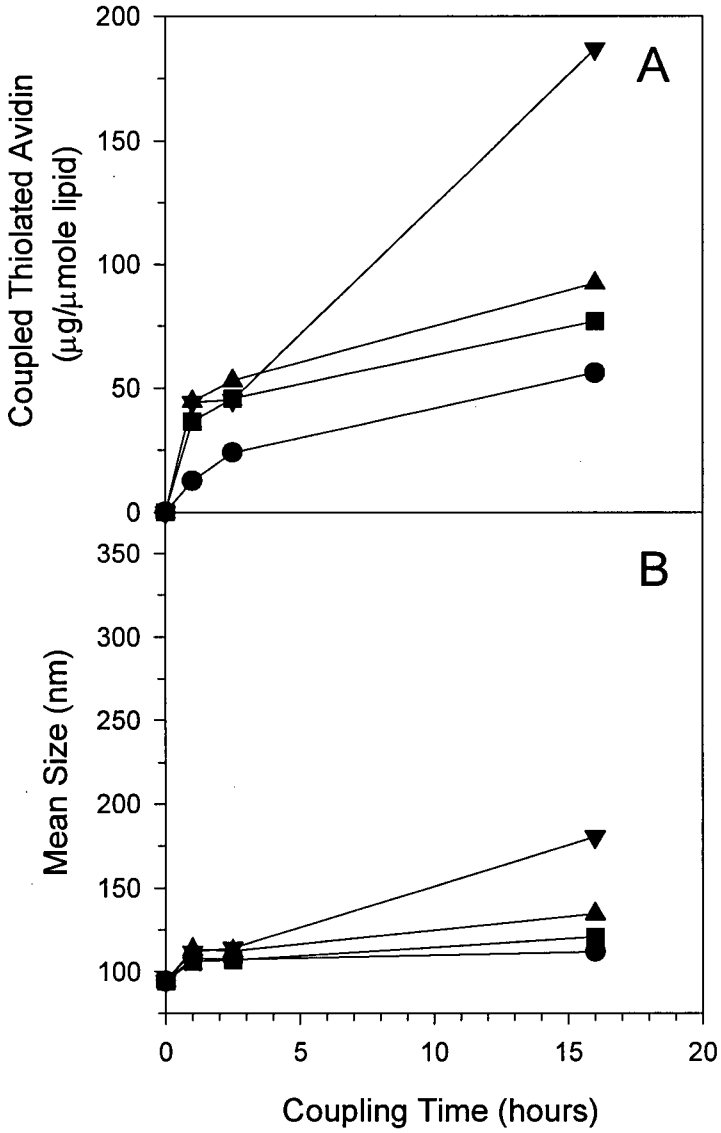
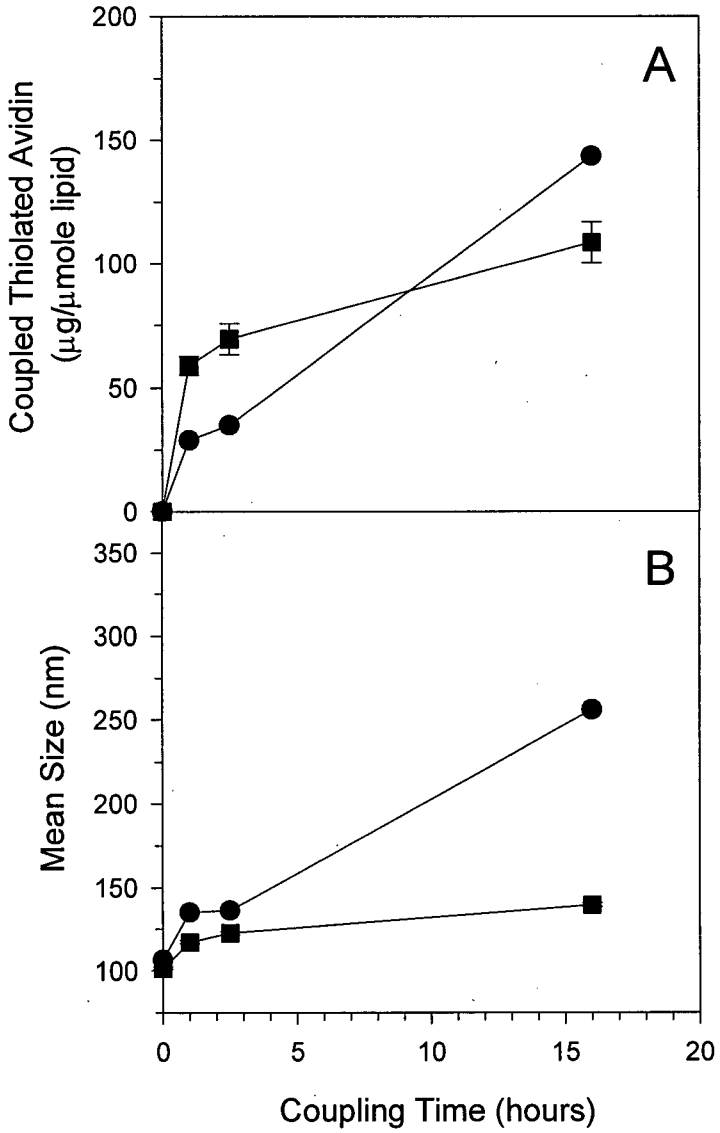


Figure 2.4

Effect of different polymer coatings on the coupling reaction of the thiolated avidin with MPB-liposomes

MPB-liposomes (6.15 mM) containing 8% PEG_{550}–S–POPE (●) or 2% $MePEG_{2000}$–S–POPE (■) were coupled with thiolated avidin (4.2 SH equiv, 150 μg per μmole lipid). Coordinates for panels A and B are as in Figure 2.1. Points: mean of three assays. Error bars: SD of at least three experiments.



2% $MePEG_{2000}$–S–POPE did not exhibit any noticeable barrier to chemical coupling of thiolated avidin (Figure 2.4A). As indicated before, the presence of this lipid did provide a substantial barrier in terms of inhibition of intervesicular crosslinking and liposome aggregation (Figure 2.4B). As the coupling reaction proceeded further (16 h), the steric stabilization effect of the longer chain $MePEG_{2000}$ was apparent.

2.3.4 Comparison of different levels of PEG_{5000}

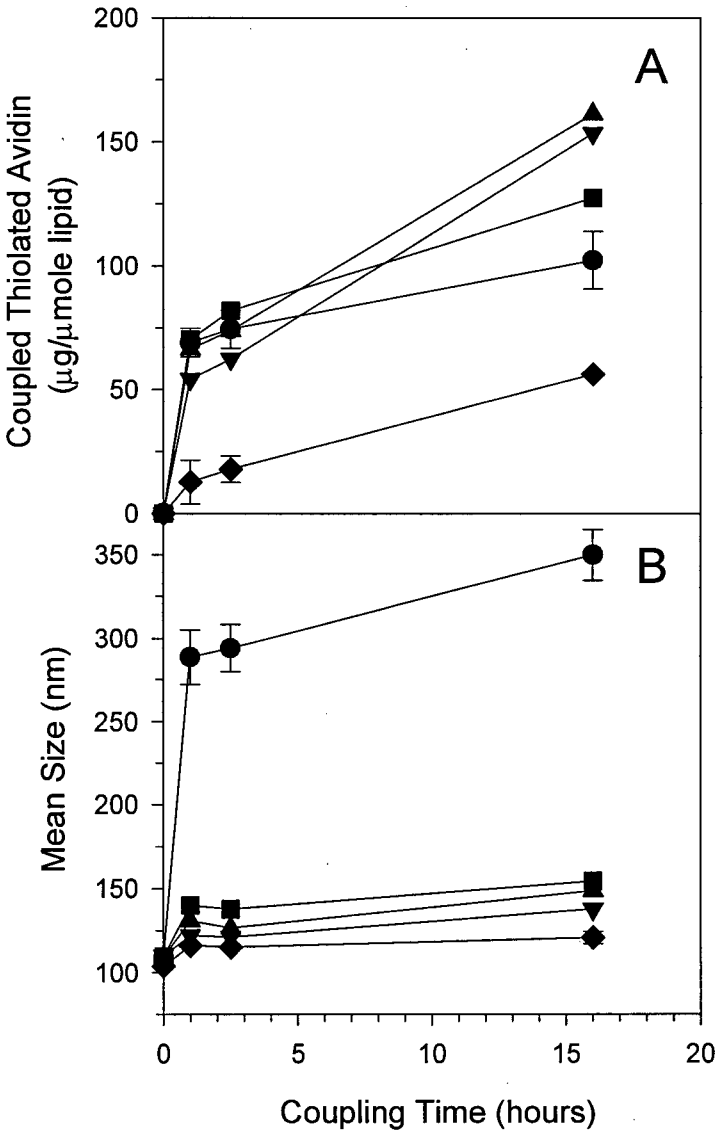
The effect of incorporating decreased quantities of the longer $MePEG_{5000}$–S–POPE on the coupling reaction between MPB-liposomes and thiolated avidin is illustrated in Figure 2.5. Results indicate that incorporation of 1.2, 0.8, and 0.4 mol % $MePEG_{5000}$–S–POPE effectively inhibits aggregation of the avidin-liposome conjugates (Figure 2.5B) without hindering the protein-coupling efficiency (Figure 2.5A). It should be noted that studies were initiated to determine whether G_{M1}, a ganglioside that is similar to PEG-modified lipids in that it can prolong the *in vivo* circulation lifetime of liposomes, prevents coupling-induced aggregation. The results (not shown) indicate that at levels of 10 mol % G_{M1}, the coupling reaction was not effected. Specifically, the rate and extent of coupling were identical to control liposomes and there is a coupling dependent increase in vesicle size.

The results presented thus far demonstrate that incorporation of either $MePEG_{2000}$ or $MePEG_{5000}$ at appropriate levels inhibits vesicle–vesicle crosslinking that occurs when coupling thiolated protein to MPB-PE-containing liposomes. Optimal coupling, in terms of reaction rates and coupling efficiency, are achieved when using 2 mol % $MePEG_{2000}$–S–POPE or 0.8 mol % $MePEG_{5000}$–S–POPE. It is important to demonstrate, however, that the resulting liposomal preparations exhibit

Figure 2.5

Effect of various concentrations of PEG_{5000} polymer on the coupling reaction of thiolated avidin with MPB-liposomes

MPB-liposomes (6.2 mM) containing various concentrations of $MePEG_{5000}$–S–POPE, 0% (●), 0.4% (■), 0.8% (▲), 1.2% (▼), and 2% (◆) were coupled with thiolated avidin (3.6 SH equiv 150 μg per μmole lipid). Coordinates for panels A and B are as in Figure 2.1. At 16 h, all treatment groups were statistically significant from control ($P < 0.05$). Points: mean of three assays. Error bars: SD of at least three experiments.



appropriate characteristics required for *in vivo* drug delivery applications. For this reason the following experiments characterized four important parameters, namely the drug loading characteristics, the biotin binding capacity, the *in vitro* cell-targeting efficiencies, and the *in vivo* plasma clearance behavior of the avidin-coated liposomes. The drug loading characteristics of avidin-D coated liposomes was assessed using the transmembrane pH gradient mediated loading procedure to encapsulate the anticancer drug doxorubicin (Bally et al., 1988; 1994). The pH gradient was established by preparing liposomes at pH 4.0 (300 mM citrate buffer) prior to adjusting the external pH to 7.5 (as required for the protein-coupling reaction). Efficient doxorubicin loading was achieved for the avidin-D coated liposomes prepared with $MePEG_{2000}$–S–POPE, where greater than 95% of the added doxorubicin (a drug to lipid weight ratio of 0.2) was encapsulated within 5 min at a incubation temperature of 65°C. The resulting liposomes retain drug over storage periods (at 4°C) in excess of 48 h.

2.3.5 Biotin binding activity: quantification of cell associated lipid

As shown in Figure 2.6, the biotin binding capacity of liposomes that have bound avidin-D in the presence of incorporated $MePEG_{2000}$–S–POPE was well retained at levels below 5 mol %. At levels of 8 mol % $MePEG_{2000}$–S–POPE, a level shown to inhibit avidin-D coupling, there was a greater than 50% loss of biotin binding activity of the surface-associated avidin-D. A further indication of the biotin binding capacity of avidin-D coupled liposomes with 2 mol % $MePEG_{2000}$ is illustrated in Figure 2.7. Briefly, avidin-D liposomes were targeted *in vitro* to P388 cells (a murine lymphocytic leukemia cell line) prelabeled with biotinylated anti-Thy 1.2 antibody. This convenient two-step targeting approach has been used previously to assess the binding of streptavidin coated liposomes to this cell line (Longman et al., 1994). The results demonstrate that a 3-fold increase in liposome-cell association is achieved when incubating avidin-D coated

Figure 2.6

Biotin binding activity of proteoliposomes formed by the coupling of thiolated avidin with various levels of $MePEG_{2000}$

Avidin-liposomes (1 μmole/ml; 3.9 SH equiv; 150 μg per) μmole lipid) containing $MePEG_{2000}$–S–POPE were incubated with [^{14}C]biotin (10-fold excess for 10 min at room temperature. Unbound biotin was removed on a sepharose CL-4B column and the extent of biotin binding was evaluated against a thiolated-avidin standard as a reference for the calculation of coupling ratios.

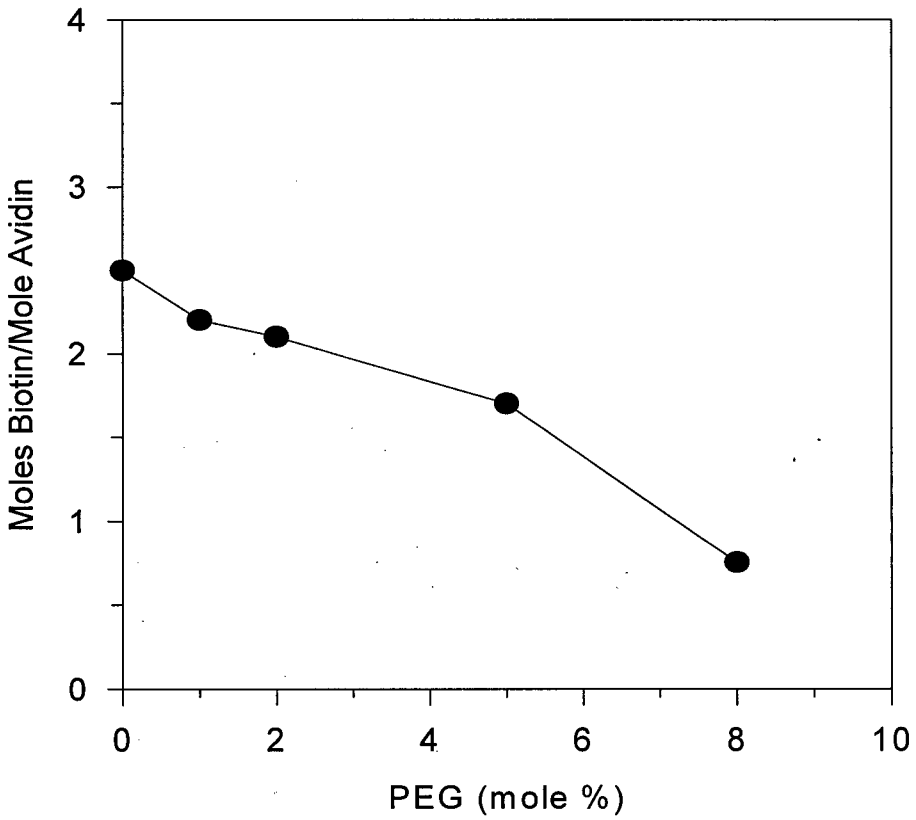
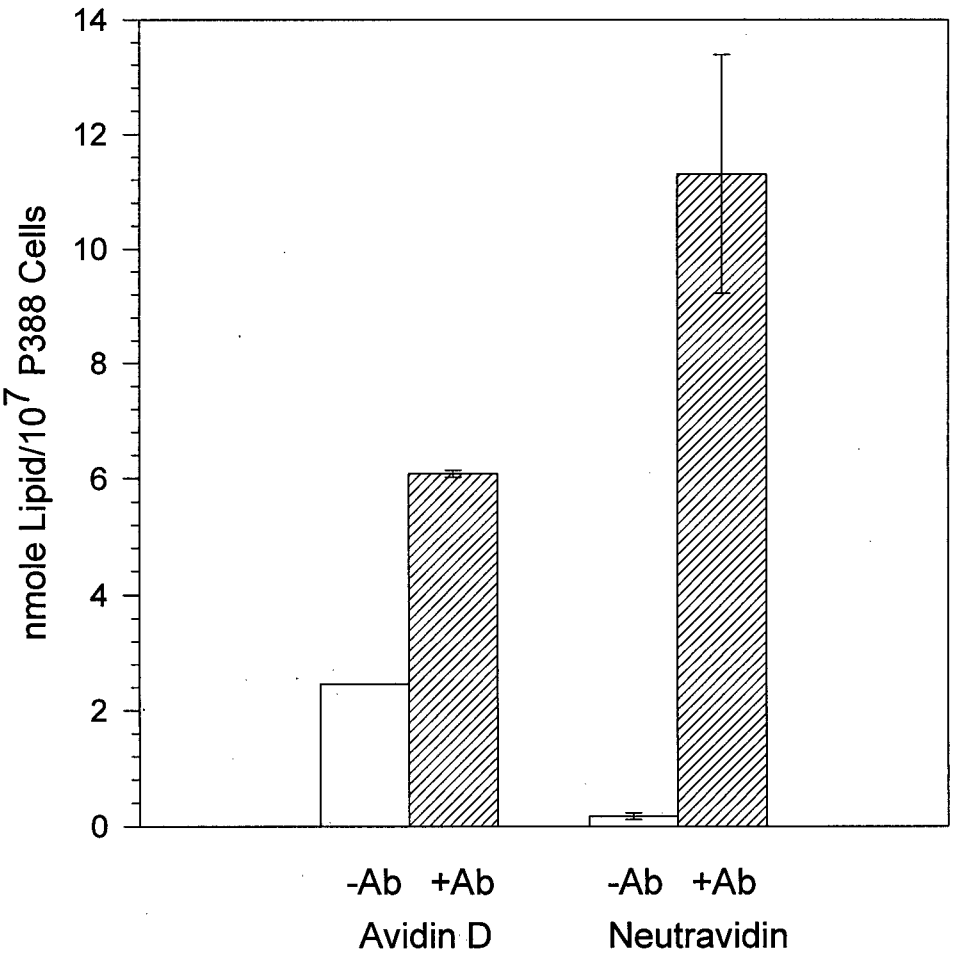


Figure 2.7

Quantification of cell associated lipid after targeting avidin-D and neutravidin-coated LUVs incorporated with 2 mol % PEG_{2000} to P388 cells *in vitro*

Avidin-D or neutravidin LUVs (51 and 63 μg per μmole lipid, respectively) incorporating PEG_{2000}–DSPE were prepared as described in Materials and Methods. P388 cells (10^7) incubated with (hatched) or without (empty bars) biotinylated anti-mouse Thy 1.2 antibody (10 μg) for 30 min at 4 °C followed by a further 30 min incubation with avidin or neutravidin LUVs (2 mM final concentration). Cell-associated lipid was determined by a ^{3}H-CHE lipid marker. Neutravidin + Ab was significantly different from avidin + Ab, $P < 0.05$. Points: mean of three assays. Error bars: SD of at least three experiments.



liposomes with biotin-labeled P388 cells when compared with incubations with unlabelled P388. The increase in liposome targeting achieved is far less than that observed previously for liposomes with bound streptavidin (Longman et al., 1994). The avidin-D coated liposomes exhibit significantly higher background (nonspecific) binding to P388 cells than streptavidin liposomes (results not shown), and this is believed to be a consequence of the carbohydrate groups present on avidin-D. The influence of the carbohydrate moiety on nonspecific cell association is illustrated in Figure 2.7. A deglycosylated version of avidin, referred to as neutravidin, coupled to liposomes using procedures identical to those used for avidin-D resulted in a liposome preparation with vastly improved specificity. It should be noted that the level of protein bound to these liposomes was 51 and 63 μg/μmole for avidin-D and neutravidin, respectively.

It was anticipated that the carbohydrate groups on avidin-D would promote liposome clearance following i.v. administration of the protein-coated liposomes with 2% $MePEG_{2000}$. For this reason the deglycosylated version of avidin (neutravidin) was also selected for preliminary *in vivo* clearance studies. A further modification in the liposomes used for *in vivo* studies involved the use of $MePEG_{2000}$ linked to DSPE rather than POPE. Results demonstrate that, *in vivo*, the POPE based PEG_{2000} lipid conjugates rapidly exchange out of the liposomal membrane (Parr et al., 1994). This study also demonstrated that DSPE-modified-PEG lipids were most appropriate for *in vivo* applications on the basis of exchangeability and stability. The DSPE–PEG lipid-containing liposomes also exhibit similar protein-coupling characteristics as observed for POPE–PEG systems. *In vivo* plasma clearance studies (in female CD1 mice), therefore, determined the circulation lifetime of protein-free, avidin-D, and neutravidin-coated liposomes with 2% $MePEG_{2000}$–S–DSPE.

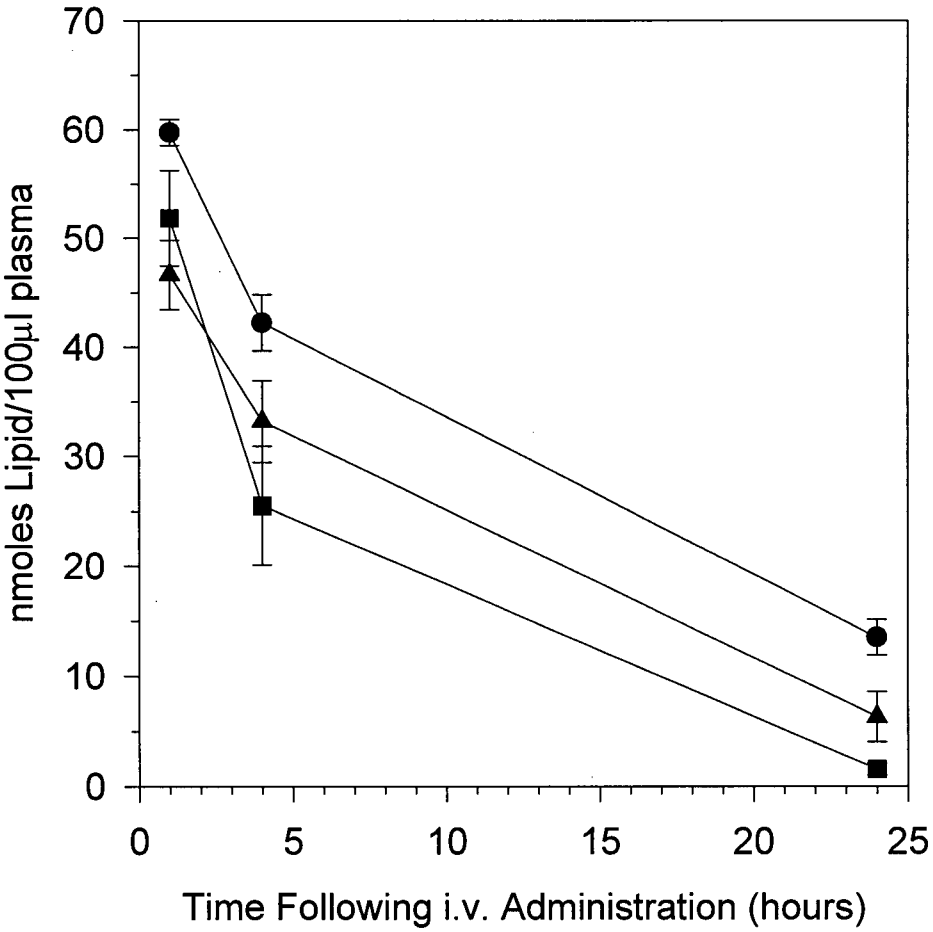
2.3.6 Clearance characteristics of protein coated liposomes

The results shown in Figure 2.8 were obtained after i.v. administration of liposomes at a lipid dose of 30 mg/kg. All liposomal preparations were similar in size prior to administration, where protein-free, avidin-D, and neutravidin liposomes exhibited mean diameters (as measured by QELS) of 109 nm, 106 nm, 119 nm, respectively. Liposomal lipid levels were determined using [^{3}H]cholesteryl hexadecylether as a nonexchangeable lipid marker. Protein-free liposomes were maintained in the plasma compartment at levels greater than either of the protein-coated liposomes. The results suggest that avidin-D liposomes are removed from the circulation faster than neutravidin-coated liposomes (half-lives for avidin-coated, neutravidin-coated, and protein-free LUVs of ~2.5, 3, and 11 h, respectively). It should be noted that previous studies have already shown that in the absence of size reduction, protein-coated liposomes are rapidly cleared following i.v. administration (Loughrey et al., 1990b). More specifically, streptavidin liposomes prepared by the covalent coupling procedure described here, but in the absence of PEG lipids exhibited circulation half-lives of less than 30 min (Loughrey et al., 1990b). These results suggest that the methodology (i.e. the incorporation of lipid anchored PEG) developed here will be appropriate for *in vivo* targeting of liposomal carriers. Detailed characterization of the utility of $MePEG_{2000}$–S–DSPE containing liposomes for preparation and targeting (*in vitro* and *in vivo*) of avidin-coated and IgG-coated liposomes will be provided in Chapter 4.

Figure 2.8

***In vivo* clearance of LUVs containing either no protein, avidin or neutravidin**

Female CD1 mice were injected *via* a lateral tail vein at a dose of 30 mg lipid/kg of protein-coated LUVs composed of DSPC/Chol/MePEG$_{2000}$-S-DSPE/MPB–DPPE (52:45:2:1) containing either no protein (●), avidin (■) or neutravidin (▲) (0, 51 and 63 μg/μmole lipid, respectively). Whole blood was collected at the indicated time points *via* cardiac puncture and plasma was prepared as outlined in Materials and Methods. Theoretical levels of lipid at t = 0 are approximately 100 nmoles lipid/100 μl plasma based on 20 g mice. At 24 h avidin and neutravidin were statistically significant from control ($P < 0.001$ and $P < 0.05$, respectively) and from each other, $P < 0.01$. Points: mean of three assays. Error bars: SD of at least three experiments.



2.4 Discussion

One of the most significant problems associated with the preparation and use of protein-coated liposomes for targeting purposes concerns coupling induced liposome–liposome crosslinking. The resulting liposome aggregates release entrapped contents (Bredehorst et al., 1986) and are very rapidly cleared from the circulation. The most versatile approach for attaching proteins to liposomes is based on the use of heterobifunctional reagents. The use of these coupling reagents for attaching protein to liposomes was first documented in early 1980 by the work of Leserman et al. (1981) and Martin and Papahadjopoulos (1982). However, this coupling technology has not yet resulted in a liposomal formulation that can specifically target defined cell populations *in vivo*. This Chapter focused on developing methodologies that result in a protein-coated liposome preparation more appropriate for *in vivo* targeting applications. The studies described investigate the use of PEG-modified lipids to inhibit liposome crosslinking, and clearly demonstrate that efficient protein-coupling to liposomes can be achieved with little or no change in liposome size when PEG-modified lipids are incorporated in the liposomes prior to coupling. The importance of providing an appropriate balance between steric inhibition of liposome–liposome crosslinking while maintaining efficient protein-coupling reactions is discussed below.

Two closely related factors are important for designing a hydrophilic-polymer coating on the surface of liposomes used for protein coupling. A balance must be reached between the polymer length of the PEG used and the density of the polymeric coating. For coupling of thiolated avidin to MPB-liposomes, either 2 mol % $MePEG_{2000}$ or 0.4–0.8 mol % $MePEG_{5000}$ on the liposomal surface is optimal for the formation of nonaggregated avidin-D liposome conjugates (see Figure 2.3 and Figure 2.5). As the amount of $MePEG_{2000}$ or $MePEG_{5000}$ is increased to 5 or 2 mol %, respectively, there is a significant reduction in protein-coupling efficiencies. Lower levels are insufficient to

prevent liposome crosslinking. In contrast, results with the lower molecular weight PEG ($MePEG_{550}$) suggest that this chain length is not capable of preventing liposome crosslinking at any concentration employed. Yet at levels of 8 mol % $MePEG_{550}$ there is an initial inhibition of protein coupling (Figure 2.4). Clearly, the higher density of the polymer coating presents a large steric barrier impeding close contact of the thiolated avidin molecule with the liposome surface. This is reflected in the initial lower amounts of protein coupled. However, once covalently conjugated, the thiolated avidin is not adequately shielded by the smaller $MePEG_{550}$ chain on the liposome surface and interliposomal crosslinking becomes more prevalent resulting in a significant increase in size of the proteoliposome.

The presence of appropriate levels of either $MePEG_{2000}$ (2%) or $MePEG_{5000}$ (0.4–0.8%) on the surface of MPB-liposomes did not impede covalent coupling of thiolated avidin; however liposome–liposome crosslinking was inhibited. These results are consistent with studies reported by Klibanov et al. (1991) who demonstrated that incorporation of PEG-modified lipids into liposomes prevented streptavidin induced aggregation of biotin-labeled liposomes. It is of interest to note that the ganglioside G_{M1}, a lipid that behaves comparably to PEG-modified lipids in terms of inhibiting protein binding, reducing RES uptake and engendering long circulation lifetimes (Allen and Chonn, 1987), does not inhibit either protein-coupling reactions or protein-coupling induced liposome aggregation.

This Chapter demonstrates that the presence of $MePEG_{2000}$ in proteoliposomes should facilitate development of liposomes for *in vivo* targeting applications. Such liposomes must maintain an ability to efficiently encapsulate and retain drugs such as doxorubicin following covalent attachment of the selected targeting protein, and still be able to bind the target antigen. As

demonstrated here, the use of $MePEG_{2000}$ as a polymer coating at levels of 2 mol % did not affect binding to a target-cell population labeled with a biotinylated antibody (Figure 2.7). A further consideration, as is discussed in Chapter 3, is the importance that the proteoliposome exhibit a pharmacological behavior comparable to a liposome with no surface-associated protein. This will, in part, be dependent on the nature of the associated protein. For example, discussed in Chapter 4, antibodies may promote liposome clearance due to Fc-mediated clearance (Aragnol and Leserman, 1986; Longman et al., 1994). Alternatively, as shown here, glycoproteins attached to liposomes reduce circulation lifetime even in the presence of 2 mol % $MePEG_{2000}$. Long circulation lifetimes are particularly important when liposome targeting is attempted *in vivo* following i.v. administration of proteoliposomes. It has been suggested that in order to maximize liposome movement from the blood compartment to an extravascular site, the liposomes must exhibit an enhanced circulation lifetime (Wu et al., 1993; Bally et al., 1994).

Optimal levels of PEG density (required for enhanced circulation longevity) and attached targeting ligand (required for specificity) must be established for effective *in vivo* targeting to be achieved. Although it has been demonstrated that the incorporation of PEG at 5 mol % is very effective in terms of increasing circulation lifetimes of liposome formulations (Klibanov et al., 1990), studies presented here indicate that this level of PEG–lipid significantly reduced the quantity of protein coupled to the liposome surface (Figure 2.2). Further, the biotin binding capacity to covalently attached avidin–liposomes with 5 mol % PEG was effectively reduced. This would suggest that the ability of these liposomes to bind a biotinylated-antibody would be reduced or, alternatively, the ability of the liposomes to bind an antigen expressed on a cell could be inhibited. By incorporating PEG at lower mole percentages greater levels of protein can be coupled to the surface of the liposome (Figure 2.1) and adequate target specific binding can occur (Figure 2.6); however, this is achieved at the risk of reduced circulation lifetimes. Thus the appropriate balance of protein

content and PEG density in *in vivo* applications is a feature of targeted liposomal systems that will have to be empirically derived through experimentation.

In summary, the presence of hydrophilic polymers such as PEG on the surface of liposomes provides a general and practical method for controlling liposome size during the covalent conjugation of proteins to liposomes. A balance between the molecular size of the MePEG chain and the concentration of the polymer on the liposomal surface has been determined to allow efficient protein coupling with little or no liposome crosslinking. The resulting liposomes exhibit characteristics suitable for development of *in vivo* targeting approaches.

CHAPTER 3

PASSIVE INTRATUMOR ACCUMULATION OF i.v. ADMINISTERED LIPOSOMES

3.1 Introduction

The principal benefit achieved following administration of anticancer drugs encapsulated in liposomes is carrier-mediated delivery of drug to the site of disease (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; O'Sullivan et al., 1988; Gabizon, 1992). Considering the important role of carrier-mediated drug delivery in defining therapeutic activity (Mayer et al., 1994), it can be reasoned that high drug-to-lipid ratios and enhanced drug retention characteristics are required to ensure that the greatest quantity of drug is delivered *via* liposomes to extravascular sites. The problem with this rationale is that drug must be released from the liposomes in order to exert its effect (Mayer et al.., 1994). In this regard liposomes can function as delivery systems, where cell-specific transfer is the goal, and good drug retention characteristics are required. Alternatively, liposomes can act as sustained release systems, where optimal drug release characteristics govern therapeutic activity. For nontargeted liposomal carriers it is anticipated that the latter is the most important function, and it is important to establish a balance between drug release from liposomes that have extravasated and retention of drug during the time period required for extravasation to occur.

The primary objective of this Chapter was to define the intratumor distribution of drug following intravenous (i.v.) administration of an egg phosphatidylcholine/cholesterol (EPC/Chol) liposomal doxorubicin formulation. EPC/Chol liposomes were chosen due to the abundance of both *in vitro* and *in vivo* data available as this formulation is in advanced clinical trials. The parameters assessed included measurements of drug and liposomal lipid in the plasma as well as within sites of tumor

growth. EPC/Chol was selected because it has been established that drug release from these liposomes is significantly faster than from liposomes prepared with saturated phosphatidylcholine species such as DSPC and DPPC (Bally et al., 1990; Kanter et al., 1993). Further, previous studies have shown that the therapeutic activity of these latter formulations, when administered at equivalent doses to the EPC/Chol formulation, is significantly less (Mayer et al., 1989). It is anticipated that enhanced activity is a consequence of enhanced drug release from the EPC/Chol formulation and it is suggest that this should be reflected in improved drug bioavailability and increases in drug binding to tumor-associated cells.

The second objective of these studies was to define the intratumor distribution of liposomal lipid following i.v. administration of an EPC/Chol liposomal doxorubicin formulation. It can be speculated that along with carrier-mediated increases in regional drug localization, further optimization in therapeutic activity would be achieved using carriers that specifically bind to tumor cells (targeting). Here, the long-term aim is to define formulation characteristics that facilitate direct interaction of liposomal carriers with cells within the tumor. If, however, targeting is to be of potential therapeutic value it is essential to develop a carrier with targeting features that do not interfere with its tendency to move from the blood compartment to an extravascular site (Wu et al., 1993; Bally et al., 1994). Within this context it is just as important to determine the availability of extravasated liposomes for targeting, since nonspecific association of liposomes with nontumor cells such as tumor-associated macrophages or endothelial cells of the tumor vasculature will interfere with the potential for liposomes to efficiently bind the specific tumor cell population.

3.2 Materials and methods

3.2.1 Materials

Liposomal doxorubicin was provided as a kit by The Liposome Company, (Princeton, N.J.). It included EPC/Chol liposomes (100 mg/ml, 120 nm mean diameter as determined by quasielastic light scattering, QELS), 0.1 *M* sodium carbonate, and doxorubicin (adriamycin, 10 mg vial with 60 mg lactose and 1 mg methylparaben). EPC was purchased from Avanti Polar Lipids (Birmingham, Ala.). Cholesterol and deoxyribonuclease I (DNase I) were obtained from Sigma (St. Louis, Mo.). Radiolabeled [^{3}H]-cholesteryl hexadecyl ether (^{3}H-CHE) was purchased from Amersham (Oakville, Ont.) and doxorubicin from Adria Laboratories (Mississauga, Ont.). FITC-labeled rat antimouse CD11b (MAC-1) antibody was purchased from PharMingen (San Diego, Calif.) and Tissue-Tek OCT compound (10% polyvinyl alcohol, 4% polyethylene glycol, and 86% nonreactive ingredients) from Miles (Elkhart, Ind.). RPMI-1640 and Hank's balanced salt solution (HBSS) were purchased from StemCell Technologies (Vancouver, B.C.). Fetal bovine serum (FBS) and trypsin were from ICN Biomedicals (Mississauga, Ont.). Pico-Fluor 40 was from Canberra Packard (Meriden, Ct.) and microtainer EDTA-coated tubes from Becton Dickinson (Mississauga, Ont.).

3.2.2 Preparation of liposomal doxorubicin

EPC/Chol (55:45, mole) liposomal doxorubicin was prepared as outlined in directions included as part of a kit that consisted of EPC/Chol liposomes (100 mg/ml solution in 300 mM citrate buffer, pH 4.0), sodium carbonate (53 mg/ml), adriamycin (10 mg doxorubicin), and 0.9% sodium chloride. The reconstitution procedure consisted of three steps. First, 1.9 ml liposome solution was mixed with 1.2 ml sodium carbonate. Second, 4.4 ml 0.9% sodium chloride was

added to the 10 mg vial of doxorubicin. Finally, 0.6 ml liposomes (EPC/Chol/sodium carbonate) was injected into the vial of doxorubicin. To encapsulate doxorubicin the mixture was immediately heated in a water bath at 60°C for 10 min with occasional mixing. When radiolabeled liposomes were required for pharmacokinetic studies, 200 µl of the radiolabeled liposomes (see below) was added to the 2.1-ml vial of EPC/Chol liposomes prior to initiation of the reconstitution procedure. The final lipid concentration for this vial was less than 0.5% more than the original liposome supply.

3.2.3 Production of tracer liposomes

To follow liposome distribution following i.v. administration, labeled (^{3}H-CHE) EPC/Chol liposomes (100 nm) were used. These liposomes were prepared in a manner identical to that used for the preparation of the kit. The methods involve extrusion of multilamellar vesicles through polycarbonate filters exhibiting 100-nm pores as described by Hope et al. (1985). Lipid mixtures consisting of EPC and cholesterol (55:45 mol%) were dissolved in chloroform with the addition of 100 µCi ^{3}H-CHE/10 mg total lipid and concentrated to a homogeneous lipid film under a stream of nitrogen gas. The lipid film was placed under high vacuum for at least 4 h prior to hydration at room temperature with 50 µl 300 mM citrate buffer, pH 4.0. Premade EPC/Chol liposomes (950 µl, obtained from the kit described above) were added to the resulting dispersion to a final lipid concentration of 105 mg/ml. The sample was frozen and thawed five times (Mayer et al., 1986) before extruding ten times through three stacked 100-nm polycarbonate filters (Poretics, Livermore, Calif.) employing an extrusion device (Lipex Biomembranes, Vancouver, B.C.). The mean liposome size was determined using a Pacific Scientific Nicomp 270 submicron particle sizer operating at a laser wavelength of 632.8 nm (Santa Barbara, Calif.). The liposomes exhibited a mean size distribution identical to that of the

clinical supplies. The resulting radiolabeled liposomes were filter sterilized through a 0.22 μm filter and placed into a sterile vial.

3.2.4 Plasma elimination and tissue distribution studies

Female BDF1 mice (18–22 g) were obtained from Charles River Canada (St. Constant, Que.). Mice were weighed and organized into appropriate groups one day prior to initiation of the study. For treatments, mice were given a single bolus lateral tail vein injection of the indicated doxorubicin formulations. At 1, 4 and 24 h after administration, mice from each group were sacrificed by CO_2 asphyxiation. Blood was immediately removed by cardiac puncture and collected into an EDTA-coated microtainer tube. The samples were centrifuged at 500 *g* in a clinical bench-top centrifuge for 10 min. Plasma was removed and placed into an Eppendorf tube prior to analysis of lipid and/or doxorubicin (see below). Where indicated, tissue samples were collected in preweighed glass tubes. The weight of tissue was then determined prior to freezing at –70°C. Frozen tissue samples were homogenized in distilled water with a Kinematica Polytron tissue homogenizer (Switzerland) to a 10% homogenate (w/v). If required, tumors were also prepared for fluorescent microscopy (see below).

3.2.5 Quantitation of liposomal lipid and doxorubicin

Liposomal lipid was quantified by employing the nonexchangeable and nonmetabolizable lipid marker, ^{3}H-CHE (Bally et al., 1990). Tracer liposomes were mixed with EPC/Chol liposomes to a known specific activity prior to injection. Upon animal sacrificed, at the indicated time-points, selected tissues were isolated and prepared as 10% homogenates, and 200 μl aliquots of homogenate were assayed as previously outlined (Mayer et al., 1989; Bally et al., 1990). For

plasma samples, 100–200 μl was added directly to Pico-Fluor 40 scintillate. Samples were subsequently assayed for radioactivity using a Canberra Packard 1900TR Liquid Scintillation Counter (Meriden, Ct.). Background radioactivity was determined using samples derived from control tissues and plasma obtained from untreated mice.

Doxorubicin was quantified using a modified extraction assay as previously outlined (Mayer et al., 1989; Bally et al., 1990). A standard doxorubicin curve was prepared in control tissue homogenates employing an identical extraction procedure to that described above. Drug levels were estimated on the basis of doxorubicin fluorescent equivalents, but analysis of selected plasma samples by HPLC indicated that >90% of the fluorescence was derived from doxorubicin. The extraction efficiency for each sample was dependent on the tissue type, being typically more than 85%. Distinguishing encapsulated doxorubicin from nonencapsulated doxorubicin was not undertaken as this is extremely difficult to do so, however, it is assumed that the total levels of nonencapsulated doxorubicin with EPC/Chol liposomes are small (Thies et al., 1990).

3.2.6 Tumors

Lewis lung carcinoma and B16/BL6 melanoma were from the NCI Tumor Repository (Fredrick, Md.). The L1210 lymphocytic leukemia was from the ATCC (Rockville, Md.). Subcutaneous injection of Lewis lung cells typically required 10 days growth and B16/BL6 cells required 2 weeks growth before an optimal tumor size of 1–2 mm^3 was reached. The ascitic L1210 cells, injected intraperitoneally (i.p.), required 1 week of growth before experiments were initiated.

3.2.7 Tumor fixation and staining

Isolated solid tumors were collected in phosphate-buffered saline (PBS) at 4°C. Tumors were subsequently fixed with a 3% paraformaldehyde solution in PBS at 4°C for 30 min. Tumors were washed with PBS and immersed in increasing concentrations of sucrose for 20 min: 10% sucrose PBS, 15% sucrose PBS, and 15% sucrose PBS containing OCT compound (1/1 v/v). The processed tissue was embedded in OCT compound and frozen in liquid nitrogen. Sections (5 μm) were prepared on a Leica Frigocut 2800 E microtome (Germany), placed on a slide, and used for antibody staining. The sections were washed three times in PBS and nonspecific antigens were blocked with 0.02% bovine serum albumin (BSA) for 30 min. FITC-labeled MAC-1 (macrophage-specific antibody) was added at a 1:100 dilution and incubated for 30 min at room temperature in a humid chamber. The sections were washed with distilled water to remove salt and mounted on a microscope slide. Phase contrast and fluorescent microscopy were performed with a Leitz Dialux microscope and Orthomat microscope camera (Germany).

3.2.8 Peritoneal lavage and removal of adherent cells

Mice bearing ascitic tumors were asphyxiated with CO_2 and the peritoneum was carefully exposed (without rupturing the membrane) to reveal the peritoneal cavity. HBSS (5 ml) was subsequently injected along the midline into the peritoneal cavity with a 28 G needle and the peritoneum was gently massaged with a pair of curved forceps. Peritoneal fluid was removed with a 20 G needle and transferred to a 15-ml tube (polypropylene) and centrifuged at 500 *g* for 10 min to pellet the cells. The supernatant was discarded and the peritoneal cells were resuspended in indicator-free HBSS supplemented with 10% FBS. A portion of the isolated cells was analyzed by flow cytometry and a portion was assayed for liposomal lipid and doxorubicin

as indicated previously. The remaining sample was transferred to culture flasks and incubated for 2 h at 37°C in a CO_2 incubator. Nonadherent cells were collected, counted, and washed extensively with medium and analyzed by flow cytometry. The levels of doxorubicin and liposomal lipid associated with these nonadherent cells were determined as outlined previously.

3.2.9 Preparation of cell suspensions from solid tumors

Solid tumors were excised and chopped finely in a culture dish with a razor blade. Tumor pieces were collected in a polypropylene tube and 5 ml PBS added. The tube was capped, shaken lightly and the tumor subjected to a modified trypsin digestion as outlined originally by Hill and Stanley (1975). Dispase (20 mg/ml) and collagenase (4 mg/ml) were prepared in PBS and 0.3 ml of each solution was added to the chopped tumor. Tumor tissues were then incubated in an oven, rotated at 37°C for 45 min and 0.3 ml of DNase I (3 mg/ml) was added. The tumor solution was transferred to a new polypropylene tube through an 80-μm sterile filter and centrifuged at 500 *g* for 6 min. The resulting pellet was resuspended in 4 ml RPMI-1640 containing 15% FBS to generate a single-cell suspension. This was diluted twice at a dilution of 1:9 in RPMI-1640. Cell counts and viability (trypan blue) were then determined using a hemocytometer.

3.3 Results

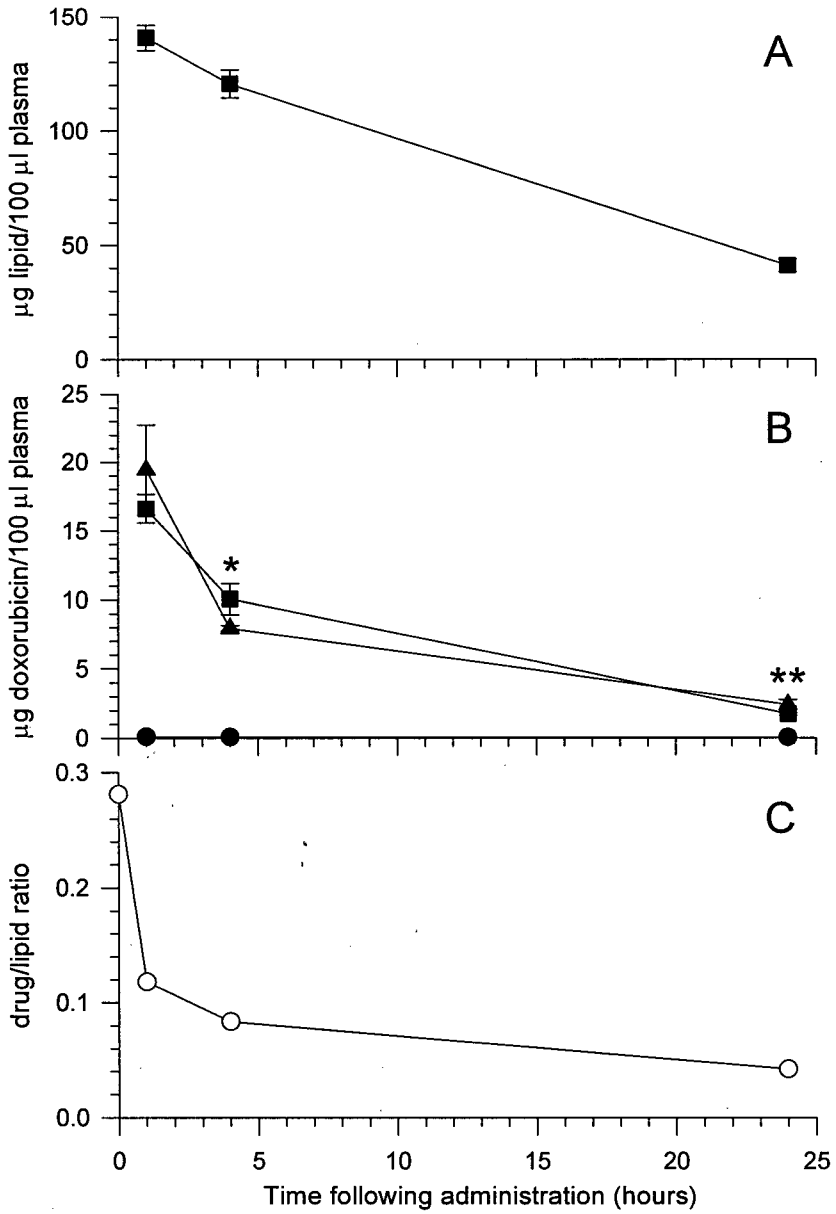
3.3.1 Plasma elimination characteristics of drug loaded liposomes

The plasma elimination characteristics of drug and liposomal lipid following i.v. administration of EPC/Chol liposomal doxorubicin are shown in Figure 3.1. Liposomal lipid levels in the plasma indicated that 90, 77, and 23% of the injected liposomal lipid was retained in the blood

Figure 3.1

Plasma clearance of EPC/Chol liposomal doxorubicin (kit) versus radiolabeled liposomes (tracer)

Liposomes were injected i.v. into the lateral tail vein of female BDF1 mice at a drug dose of 20 mg/kg and a lipid dose of 73.5 mg/kg (drug to lipid ratio 0.27). At the indicated time points, mice (four per group) were sacrificed and plasma was assayed for lipid (**A**) and drug (**B**). Drug to lipid ratios are plotted in **C**. (● free doxorubicin, ■ tracer liposomes, ▲ EPC/Chol liposomes). Theoretical values of drug and lipid at $t = 0$ are ~40 μg doxorubicin/100 μl plasma and ~140 μg lipid/100 μl plasma based on 20-g mice. Data are means ± SD of four assays; where error bars are not visible, they are smaller than the size of the symbol. Asterisks indicate no significant difference between data points (* $P < 0.02$, ** $P < 0.05$).



compartment 1, 4, and 24 h after administration, respectively (Figure 3.1A). Plasma levels of drug obtained after i.v. administration of doxorubicin in EPC/Chol liposomes were significantly greater than those achieved following administration of free drug (Figure 3.1B, $P < 0.05$ at all time-points). In combination, the data shown in Figure 3.1A and B allowed an estimation of drug retention for these liposomes (Figure 3.1C), which indicate that 58% of the encapsulated drug was released from the circulating liposomes within 1 h of administration (consistent with previous reports (Bally et al., 1990; Mayer et al., 1994). Doxorubicin elimination rates were essentially identical when the drug was encapsulated in the liposomes prepared with and without added tracer liposomes (Figure 3.1B).

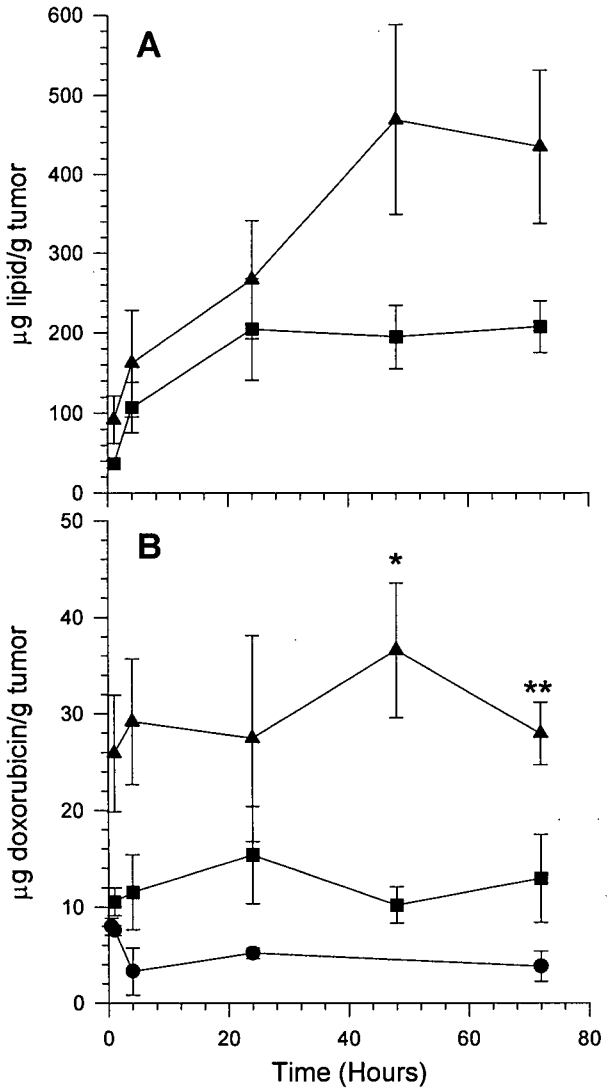
3.3.2 Drug and lipid biodistribution within the Lewis lung carcinoma

Drug and liposomal lipid biodistribution studies within a Lewis lung carcinoma tumor were initiated 10 days after tumor cell inoculation (Figure 3.2). A time- and dose-dependent increase in liposomal lipid delivery to the established tumor was evident (Figure 3.2A), demonstrating that liposome accumulation in extravascular sites was a slow process (as seen in other studies Bally et al., 1994). Drug accumulation in the tumor following administration of free and liposomal doxorubicin (Figure 3.2B) did not appear to be time-dependent. Values for the AUC (area under the plasma concentration-time curve) following administration of liposomal drug at 20 and 40 mg/kg showed that total exposure within the tumor was increased 2.5-fold from an AUC of 870 to 2200 $\mu g\ g^{-1}$ hr, respectively. Drug levels measured at 4 h were comparable to levels measured at 72 h; however, a dose-dependent increase in drug levels was achieved as the dose of liposomal doxorubicin was increased from 20 to 40 mg/kg. Furthermore, the level of drug achieved within the tumor following injection of liposomal doxorubicin was two- to threefold greater than that achieved following administration of a comparable dose of free drug.

Figure 3.2

Doxorubicin and lipid accumulation within the Lewis lung tumor

Female BDF1 mice (four per group) were injected with 3 x 10^5 Lewis lung cells subcutaneously in the flank. Ten days following tumor cell inoculation mice were treated i.v. with free doxorubicin (20 mg/kg), EPC/Chol liposomes (20 mg/kg), or EPC/Chol liposomes (40 mg/kg). Mice were sacrificed 24 h following treatment and assayed for lipid (**A**) and drug (**B**) (● free doxorubicin, 20 mg/kg; ■ EPC/Chol liposomes, 20 mg/kg; ▲ EPC/Chol liposomes, 40 mg/kg). Data are means ± SD of three assays; where error bars are not visible, they are smaller than the size of the symbol. The doxorubicin levels following liposomal treatment at 20 mg/kg and 40 mg/kg were significantly different (* $P < 0.007$, ** $P < 0.02$).



3.3.3 Cellular association of liposomal doxorubicin with Lewis lung carcinoma cells

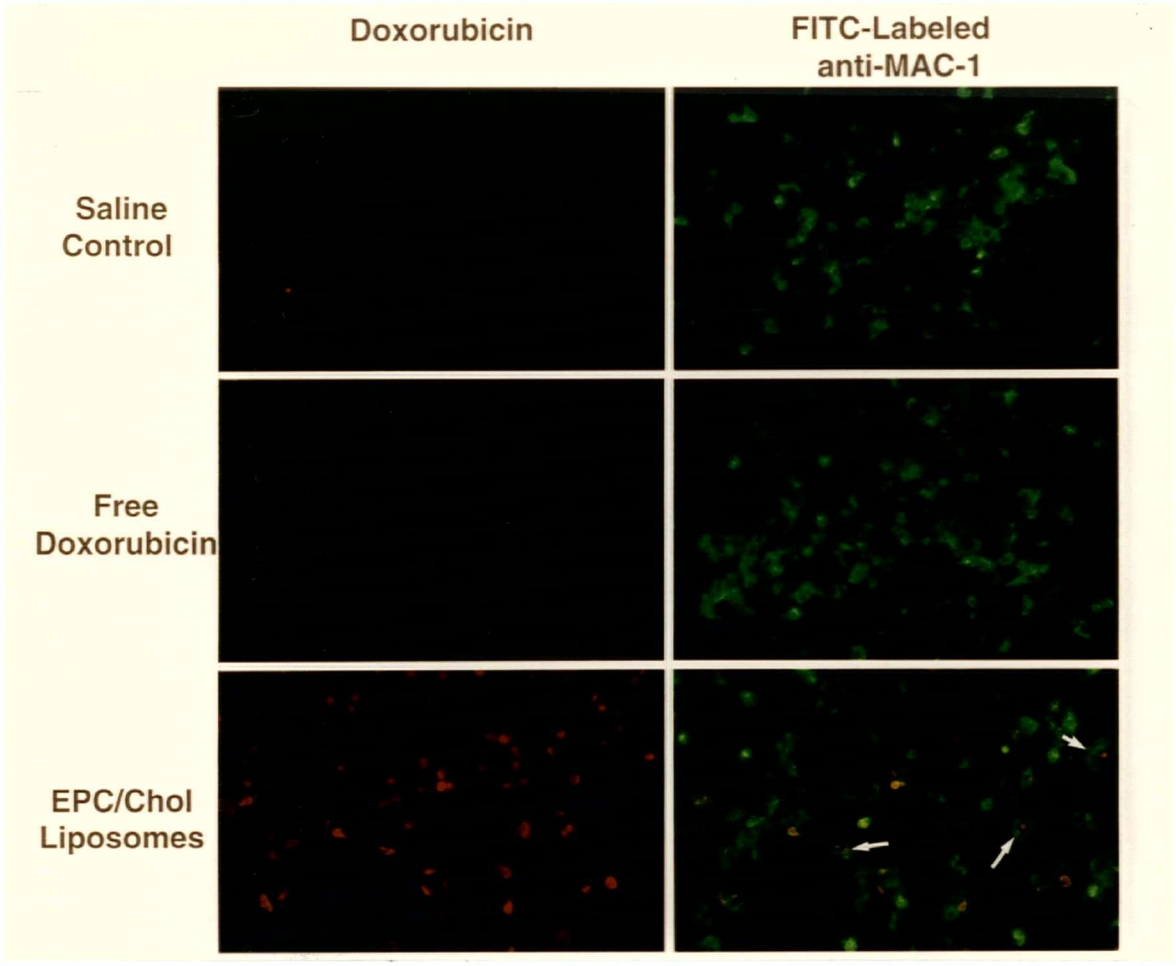
To assess the cellular association of liposomal doxorubicin with Lewis lung carcinoma cells, two fluorescent microscopy approaches were used. First, since doxorubicin is a fluorescent drug, fluorescent microscopy was used to evaluate its distribution using a 530–560 nm bandpass filter. Second, fluorescent microscopy was also used to identify MAC-1-positive cells (macrophages, monocytes, and neutrophils) with an FITC-labeled anti-MAC-1 antibody (CD11b). The results (Figure 3.3) are consistent with the data shown in Figure 3.2 and show that drug delivery within the tumor was greater for the liposomal doxorubicin formulation when compared with free drug at the equivalent dose (20 mg/kg). In addition, doxorubicin fluorescence was frequently associated with MAC-1-positive cells (indicated by FITC-labeled anti-MAC-1 antibodies, some of which are indicated by arrows as examples). A small proportion of the doxorubicin, however, was associated with MAC-1-negative cells (areas of high doxorubicin fluorescence with no MAC-1 FITC fluorescence).

A second approach used to assess cell delivery to the Lewis lung tumor was based on the preparation of tumor-derived single-cell suspensions (see Methods). The cell suspensions typically had a viability of less than 50% and were devoid of MAC-1-positive cells (as assessed by flow cytometry and fluorescent microscopy). Loss of MAC-1-positive cells may have been a consequence of protease-mediated loss of the antigen from the isolated cells or, alternatively, may have been lost as a consequence of adherence to the plastic-ware used during the processing and washing of the isolated cells. To estimate drug levels in tumor cells it can be assumed that a 1-g tumor consists of 10^9 cells (Tannock, 1989). On this basis the data in Figure 3.2B indicate that, for mice given the liposomal drug at a drug dose of 40 mg/kg, 150 ng of doxorubicin would be associated with 10^7 cells provided that 100% of the tumor-associated drug is cell-associated.

Figure 3.3

Fluorescence microscopy of Lewis lung tumors from BDF1 mice treated with free doxorubicin and EPC/Chol liposomal doxorubicin

Female mice (three per group) were injected subcutaneously with 3 x 10^5 Lewis lung cells. Mice were treated i.v. 10 days following tumor cell inoculation with a saline control, free doxorubicin (20 mg/kg) or EPC/Chol liposomal doxorubicin (20 mg/kg). Mice were sacrificed 1 h after treatment and isolated tumors prepared for microscopy. Macrophages were identified using an FITC-labeled anti-MAC 1 antibody and doxorubicin-containing cells were identified using a rhodamine filter. *Arrows* indicate some of the cells positive for doxorubicin and MAC-1 as examples.



If this value is related to a concentration, assuming a 1-g tumor approximates 1 ml, then the liposomes were delivering ~15000 ng drug/ml. For Lewis lung cells in culture IC_{90} values for doxorubicin are ~500 ng/ml (<1000 n*M*), therefore, it would be anticipated that there is sufficient drug present to exert cytotoxic effects. Following the preparation of the single-cell suspension, however, the level of cell-associated doxorubicin (assay of 800 μl of 10^7 cells/ml) was below the detection limits (1 ng/800 μl) of the assay. There was no measurable level of liposomal lipid found in this isolated cell population.

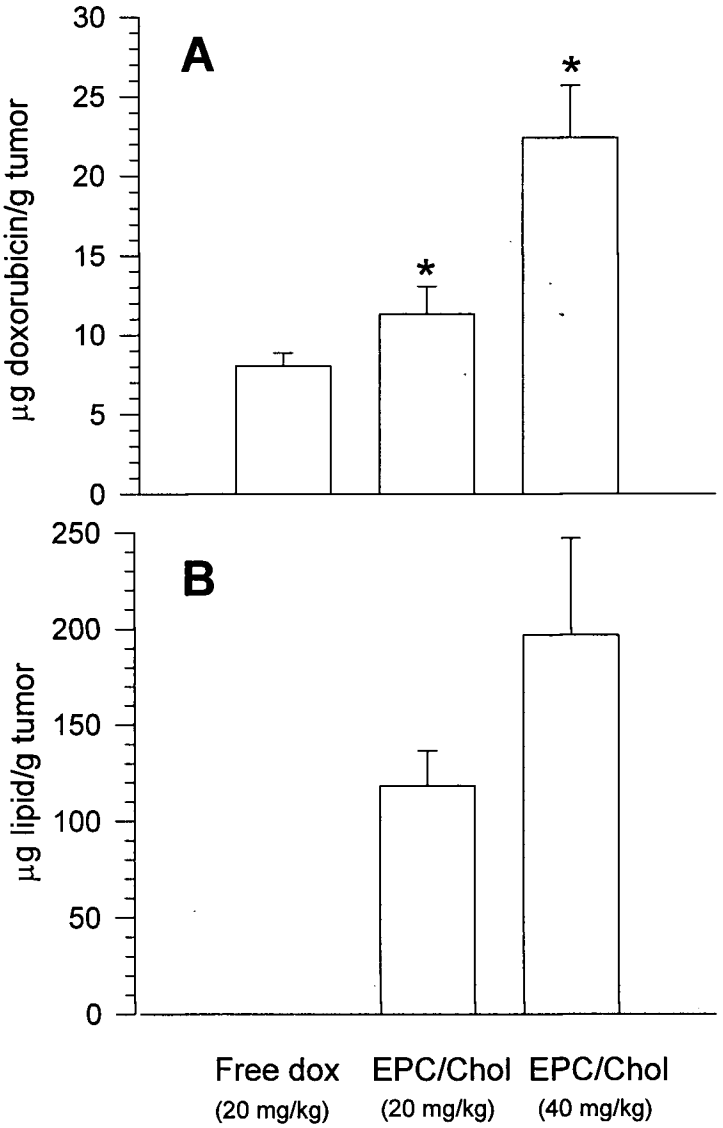
3.3.4 Doxorubicin and lipid accumulation in a murine B16/BL6 solid tumor

A similar analysis of doxorubicin and liposomal lipid accumulation in an established murine B16/BL6 solid tumor was also completed. The results, shown in Figure 3.4, are based on a single time-point, 24 h. As anticipated, both liposomal doxorubicin (Figure 3.4A) and lipid (Figure 3.4B) accumulated in this tumor. When the tumor levels of free and liposomal drug are compared, following administration of equivalent drug doses, 40% more drug was obtained in tumors from mice treated with the liposomal formulation. At the maximum tolerated dose (MTD, 40 mg/kg) of EPC/Chol liposomal doxorubicin, there was a greater than two-fold increase in tumor-associated drug compared with free drug-treated mice. This is in contrast to the tenfold increase observed using the Lewis lung carcinoma model. As noted here, doxorubicin delivery following administration of the liposomal drug is associated with accumulation of liposomal lipid (Figure 3.4B). Cell suspensions prepared through a nonenzymatic approach (mincing the tumor with scissors prior to filtration through 80-μm filters) were also evaluated with the B16/BL6 tumors. As with the Lewis lung tumors, the resulting cells were washed, resuspended at a concentration of 10^7 cells/ml, and the level of cell-associated doxorubicin and lipid was

Figure 3.4

Levels of B16/BL6 tumor associated liposomal lipid and doxorubicin following administration of free drug and EPC/Chol liposomal doxorubicin

Female BDF1 mice (four per group) were injected with 2 x 10^5 B16/BL6 cells subcutaneously in the flank. Mice were treated i.v. 2 weeks following tumor cell inoculation, with free doxorubicin or the indicated dose of EPC/Chol liposomal doxorubicin. Mice were sacrificed 24 hr following treatment and levels of drug (**A**) and lipid (**B**) were assayed. Data points are the means of 12 assays from three experiments ± SD. *Asterisks* indicate a significant difference from free drug (* $P < 0.01$).



measured. The drug and liposomal lipid levels associated with the isolated cells were below assay detection limits.

3.3.5 Extravasation of liposomes in mice bearing the ascitic L1210 tumor

A comparative study of the extravasation phenomenon seen with liposomes in mice bearing the murine ascitic L1210 tumor is illustrated in Figure 3.5. Results show that cells residing in the peritoneal cavity were exposed to five to ten times more drug at 24 h when doxorubicin was administered in liposomal form (Figure 3.5A). Cell-associated doxorubicin accounted for approximately 50% of the drug measured in the peritoneal cavity of mice given the liposomal formulation. Importantly, following administration of free doxorubicin, 100% of the drug was localized within the peritoneal cells, a result that is consistent with previous studies (Bally et al., 1990). Within the limitations of the two doses of liposomal doxorubicin studied (20 and 40 mg/kg) there appears to be a dose-dependent increase in doxorubicin delivery to cells residing in the peritoneal cavity. The results shown in Figure 3.5B suggest that doxorubicin delivery to the peritoneal cavity was mediated, in part, by the liposomal carrier. An estimation of the drug-to-lipid ratio (wt/wt) in the isolated peritoneal cells gave a value of 0.09–0.11 which was comparable to that measured in liposomes within the plasma compartment at this time-point (see Figure 3.1C).

The level of macrophage-associated drug, measured using a procedure in which functional peritoneal macrophages were removed by adherence to plastic in culture, is shown in Figure 3.6. It should be noted that in the absence of an ascitic tumor 4–6 x 10^6 peritoneal cells can be recovered following peritoneal lavage. Typically, 30–40% of these cells will be easily distinguished by flow cytometry techniques as macrophages on the basis of forward and side

Figure 3.5

Accumulation of EPC/Chol liposomal doxorubicin and free doxorubicin in the peritoneum of L1210 tumor-bearing mice 24 h after i.v. administration

Female BDF1 mice (four per group) were injected i.p. with 1 x 10^5 L1210 cells, and 24 h later were treated either with free drug or EPC/Chol liposomal doxorubicin at the indicated doses. Mice were sacrificed 24 h after treatment and isolated peritoneal cells were assayed for drug (**A**) and lipid (**B**). Data are means ± SD of four assays. *Asterisks* indicate a significant difference from free drug (* $P < 0.01$).

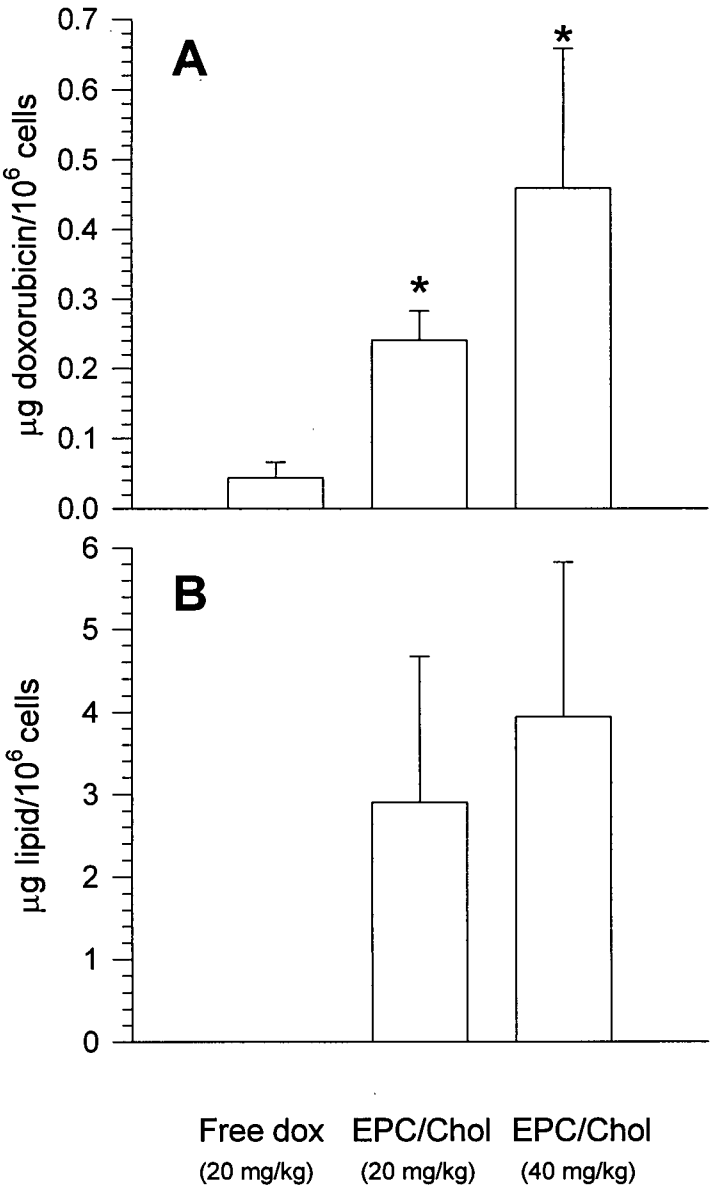
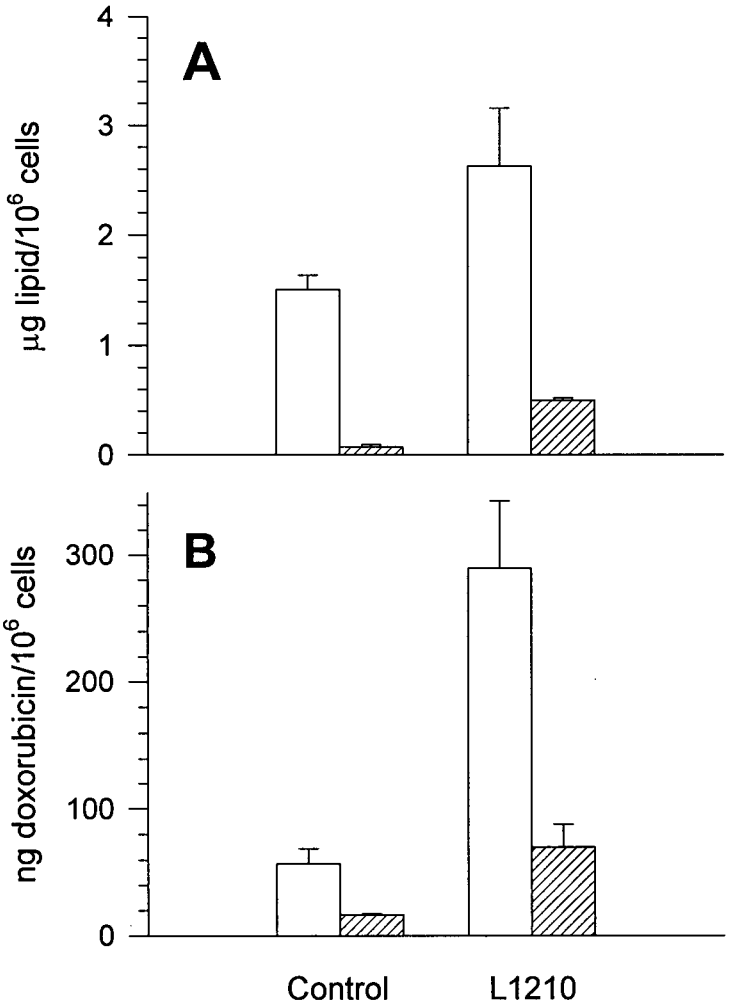


Figure 3.6

Drug and lipid association to adherent peritoneal cells before and after adherent cell removal

Control (no tumor) and L1210 tumor-bearing mice (four per group) were injected i.v. with EPC/Chol liposomal doxorubicin (20 mg/kg) 24 h prior to isolation of peritoneal cells. Adherent cells were removed from peritoneal cells *via* a 2-h incubation in a culture flask. The association of lipid (**A**) and drug (**B**) to the adherent and nonadherent cells was determined as outlined in Materials and methods (*open bars* before adherent cell removal, hatched bars, after adherent cell removal). Data are means ± SD of four assays.



light scattering characteristics. In the presence of tumors, however, the number of cells isolated by peritoneal lavage increases to more than 2 X 10^7. It was determined that cultured L1210 cells cannot be distinguished from peritoneal macrophages on the basis of size and granularity characteristics. The next approach, therefore, involved evaluating the level of macrophage-associated drug using a procedure in which functional peritoneal macrophages were removed by adherence to plastic in culture. For cells isolated from control mice, more than 95% of the cell-associated lipid was removed by the adherence step (Figure 3.6A). In addition, a 70% reduction in cell-associated doxorubicin was observed compared with cells not depleted of macrophages (Figure 3.6B). When adherent cells were removed from peritoneal cells isolated from L1210 tumor-bearing mice there was an 81% and 76% reduction in cell-associated liposomal lipid and doxorubicin, respectively. These results demonstrate that the majority of cell-associated doxorubicin in this ascitic tumor resides in adherent cells which are probably resident and/or induced macrophages.

3.4 Discussion

In this Chapter studies have been designed to characterize the target site distribution of an EPC/Chol liposomal doxorubicin formulation. Several reported studies have shown that liposomes administered i.v. exhibit a time-dependent movement from the blood compartment to extravascular sites (Gabizon and Papahadjopoulos, 1988; O'Sullivan et al., 1988; Bakker-Woudenberg et al., 1992; Gabizon, 1992) and in particular the peritoneal cavity (Bally et al., 1994). Although these studies show that drug delivery to sites of tumor growth can be enhanced significantly through the use of liposomal drug carriers, it has yet to be established (preclinically or clinically) whether controlled drug release and/or use of factors that enhance tumor delivery would lead to enhanced therapeutic activity. There is little evidence supporting the concept that

the therapeutically active agent is liposome-associated doxorubicin. In contrast, it is likely that drug released from liposomes, in the circulation, in the interstitial space of progressing tumors, or from liposomes that have been degraded within the lysosomal compartment of phagocytic cells (Storm et al., 1988), is the biologically active agent. The studies in this Chapter were developed to gain a better understanding of the mechanism of action of an EPC/Chol liposomal anticancer drug. Two factors of interest are discussed, drug release and the intracellular distribution of liposome-associated drug following extravasation in regions of tumor growth.

It can be suggested that, for both ascitic and solid tumor models, improved therapy may be a consequence of enhanced drug exposure at the site of tumor growth achieved through the use of a liposomal carrier. The use of an ascitic tumor model for assessing potential therapeutic mechanisms of a liposomal anticancer drug as shown in Figures 3.5 and 3.6 is simple but easily criticized as ascitic tumors do not structurally resemble solid tumors in several important ways. First, unlike solid tumors, ascitic tumors do not exhibit angiogenesis. Second, in contrast to solid tumors there is an absence of a defined matrix structure allowing the tumor cells and associated host-derived cells in the ascitic tumor to progress in the fluid environment. Third, macromolecules that have extravasated into the site will have an increased probability of direct interaction with the cell populations present. Therefore, it was not surprising that the levels of cell-associated drug (and liposomal lipid) were apparently greater when the ascitic model was used in comparison with either solid tumor. However, under these ideal conditions (for an *in vivo* model) by far the major amount of all drug delivered to the tumor is either non-cell-associated or bound in macrophages. Even vast differences in the pathology between the solid tumors showed no improvements in drug or lipid accumulation. The murine melanoma has a vasculature that is characterized by the presence of blood channels (Warren, 1979) and lacks a

well-defined intratumor matrix. In contrast, the Lewis lung carcinoma is well vascularized and is known to produce significant quantities of VEGF (vascular endothelial cell growth factor).

Since it can be shown using tumors (not expressing a multidrug-resistant phenotype) that free doxorubicin efficiently accesses and is effectively retained by progressing tumors (Broxterman et al., 1995), improvements in tumor cell exposure to drug may best be achieved with liposomes that release their contents (EPC/Chol) at well-defined rates or under controlled conditions. For mice bearing Lewis lung carcinoma, the EPC/Chol liposomal doxorubicin formulation mediates an increase in tumor-associated drug levels; however, the levels obtained are independent of the time evaluated after administration. These increased levels may have been achieved by the liposomes acting as sustained-release systems for free drug, allowing more drug to accumulate in the tumor than from single doses of free drug, or by the accumulation of drug in the tumor as a combination of free drug uptake plus uptake of drug still retained in the liposomes. Either way, the drug accumulation profiles were contrary to what has been observed with saturated liposomal doxorubicin formulations which produce a time-dependent drug accumulation (Mayer et al., 1989; 1990; Bally et al., 1994). It should also be noted that the levels of drug achieved in the Lewis lung tumor following i.v. administration of the EPC/Chol liposomal formulation were less than those observed for saturated liposomal carrier systems. Since the EPC/Chol liposomes release their contents into the circulation over a reasonable time frame (70% drug loss over 24 h), free doxorubicin accumulation within the tumor can progress in the absence of efficient liposome accumulation. Clearly, identifying the role of controlled drug release from liposomes into the circulation prior to liposome-mediated drug accumulation in tumors is essential. It can be suggested that the steady-state drug levels observed are a consequence of using liposomes that exhibit poor drug retention characteristics. Importantly, peak free drug levels are avoided when drug is encapsulated in this liposomal carrier system which contributes to the well-established

reduction in cardiotoxicity for the EPC/Chol liposomal formulation (Balazsovits et al., 1989; Kanter et al., 1993; Gill et al., 1995).

Increased drug release, however, is incompatible with drug carrier systems designed to achieve maximum drug levels in the tumor, where drug retention and liposome circulation longevity are key optimization parameters. Although others have shown that liposomes can leave blood vessels and enter extravascular sites, the rate of extravasation is slow. This is primarily a consequence of collodial size (Nagy et al., 1989; Litzenger et al., 1994). Liposomal anticancer agents displaying optimal activity typically exhibit a size distribution between 80 and 160 nm (Mayer et al., 1989). It is anticipated that such structures could only cross blood vessel walls that exhibit fenestrations or pores, although movement *via* transcytotic vesicles cannot be excluded (Huang et al., 1993). Regardless of the mechanism of liposome extravasation, optimal drug delivery will be achieved with liposomes that retain drug effectively during the time period required for extravasation to occur. If liposomes are retained in the circulation for extended time periods then the probability for the carrier to extravasate will increase.

The results presented in this Chapter demonstrate that both liposomes and associated drug accumulate at sites of solid tumor growth. The extent of accumulation was dependent on tumor type and for liposomal lipid this accumulation process was time dependent. Since the liposomal carriers used in this Chapter are known to release drug, the kinetics (see Figure 3.2) of drug accumulation were different and the level of drug obtained was relatively constant over a 3-day time course. Even though there were significant drug and liposomal lipid levels in these tumors, analysis of single-cell suspensions derived from these tumors suggested that there was little cell-associated material.

Optimization of liposome drug retention and extravasation properties must proceed, together with the development of mechanisms for efficient release of encapsulated drug from regionally localized liposomes. An elegant example of such an approach, based on temperature-induced destabilization of the liposomal carrier, has recently been published (Huang et al., 1994; Unezaki et al., 1994). Alternatively, it will be important to develop approaches that will promote binding and intracellular delivery of regionally localized liposomal drugs to specific cells within the site. These results suggest that it may be possible to achieve tumor cell-specific delivery. A significant proportion of regionally localized liposomal carriers reside within the tumor's interstitial space and, provided liposome movement is not restricted (i.e. the liposomes are not bound to matrix components), the target cell specific delivery of tumor cells may be possible. For this reason, studies in Chapter 4 have focused on maximizing the tendency for liposomes, with surface associated targeting ligands, to access regions of tumor growth. Subsequent, studies will need to evaluate the intratumor distribution of liposomal drugs exhibiting optimal binding characteristics with the objective of demonstrating target cell specific delivery.

CHAPTER 4

ELIMINATION OF ANTIBODY CONJUGATED LIPOSOMES FROM PLASMA AND THE STABILITY OF ANTIBODIES ON ANTIBODY CONJUGATED LIPOSOMES

4.1 Introduction

With the development of monoclonal antibodies and the identification of overexpressed epitopes on cancer cells, targeted drug delivery has become a highly sought after advancement for chemotherapy of cancer. Research efforts focused on drug targeting over the last 15 years have led to the development of several agents with potential clinical utility. For example, antibody conjugates have been employed as imaging agents (Laufer et al., 1990; Stickney et al., 1991; Juweid et al., 1996) and as antineoplastic drugs (Hellstrom et al., 1996; Stone et al., 1996). Further, effective targeting of lipid based drug carriers has been demonstrated in animal models (Ahmad et al., 1993; Maruyama et al., 1995). Despite these successes, therapeutically effective antibody-drug carrier systems are proving to be a significant research challenge (Kosmas et al., 1989; Kummer and Staerz, 1993). This is primarily a consequence of biological barriers (Juweid et al., 1992; Jain, 1994; Yuan et al., 1995) rather than technological or pharmaceutical limitations. Although there have been significant advances in designing both targeting agents and drug carriers, the technology has not provided substantial improvements in drug specificity or therapeutic activity.

Several biological barriers have been identified that account for the problems experienced in targeting applications: i) immunogenicity (Phillips and Dahmen, 1995; Boeckler et al., 1996), ii) rapid elimination from the circulation (Senior et al., 1986; Torchilin et al., 1992), iii) obstructed movement from the blood compartment to an extravascular site (Wu et al., 1993; Litzenger et al.,

1994), and iv) subsequent limited diffusion within the extravascular site (Rosenecker et al., 1996). All of these barriers serve to limit target site accumulation of the targeted carrier. Advances in the development of humanized antibodies overcome some of the problems related to immunogenicity (Winter and Harris, 1993; Khazaeli et al., 1994). However, antibodies do not efficiently localize in disease sites (Goldenberg, 1993). It is suggested that inefficient disease site localization of antibody is a consequence of poor extravasation and disease site penetration properties (Sung et al., 1992; Shockley et al., 1992). In dramatic contrast, the benefits achieved through the use of nontargeted drug carriers, such as liposomes, consist primarily of efficient passive accumulation in disease sites (Gabizon et al., 1992, 1994; Huang et al., 1992, 1994; Yuan et al., 1994). For example, as much as 10% of the injected liposome dose can accumulate in established tumors (Nassander et al., 1992), with little or no measurable accumulation in nonspecific tissues (muscle, kidney, lung, and heart). As with systemically administered foreign proteins and nanoparticles, liposomes do accumulate in the liver, spleen, and bone marrow. The rate and extent of accumulation in specific regions (tumor) relative to nonspecific regions (liver), however, is dependent on the physical/chemical properties of the liposomal carrier. These attributes can be changed to promote retention in the blood compartment, increasing accumulation in tumors or other disease sites while avoiding accumulation in the nonspecific regions.

The basis for the limited success of antibody-liposome conjugates to accumulate within extravascular targets is not clearly understood. It is implied that poor target site accessibility is a consequence of the protein attached to the liposome surface. The influences of antibody coupling on the pharmacodistribution characteristics of liposomes is characterized in this Chapter. This Chapter identifies several factors which work to limit target site accumulation. Importantly, results summarized demonstrate that antibodies covalently attached to the liposome

can readily dissociate from the carrier following interaction with a target ligand and after systemic administration. Protein dissociation from the liposome is not surprising considering recent data demonstrating exchange of lipids following conjugation to hydrophilic polymers (Parr et al., 1994), therefore, antibody attachment to a selected lipid anchor with minimal tendencies to dissociate from the carrier should be selected.

4.2 Materials and methods

4.2.1 Preparation of liposomes

Large unilamellar vesicles consisting of DSPC/Chol/PEG_{2000}-DSPE/MPB-DSPE (52:45:2:1; mol %) or DSPC/Chol/PEG_{2000}-DSPE (53:45:2; mol %) (all lipids obtained from Northern Lipids, Inc., Vancouver, BC) were prepared as previously described by Hope et al. (1985). Lipids were dissolved in chloroform in the presence of 1 μCi ^{3}H-CHE (NEN-Dupont, Boston, MA)/10 μmole lipid and subsequently dried to a homogeneous lipid film under a stream of nitrogen gas. Lipid films were hydrated at 65°C with HBS (25 mM Hepes, 150 mM NaCl, pH 7.5). Resulting multilamellar vesicle preparations were frozen and thawed five times (Mayer et al., 1986) before extruding ten times at 65°C through three stacked 100-nm polycarbonate filters (Poretics Corp., Mississauga, ON) employing an extrusion device (Lipex Biomembranes, Vancouver, BC). The resulting liposomes were sized by QELS (quasi-elastic light scattering) using a Pacific Scientific Nicomp 270 submicron particle sizer (Mono Research Laboratories, Brampton, ON) operating at 632.8 nm.

4.2.2 Tumors and hybridoma cell lines

The human adenocarcinoma, A431, and the hybridoma cell line 528 (produces the anti-EGFR monoclonal antibody) were obtained from the American Type Culture Collection (Rockville, MD). The A431 cells were maintained in Dulbecco's modified Eagle's medium (StemCell Technologies, Vancouver, BC) with 4.5 g/L glucose and 10% fetal bovine serum (ICN Biomedicals, Aurora, OH). For tumor initiation 1 x 10^6 cells were injected s.c. in each flank to generate bilateral tumors. Cells typically required 10 days growth before they tumors reached optimal size of 1-2 mm^3. The 528 cell line (anti-EGFR antibody producer) was maintained in culture with RPMI 1640 media (StemCell Technologies, Vancouver, BC) supplemented with 10% horse serum (ICN Biomedicals, Aurora, OH).

4.2.3 Antibody ascites preparation and purification

Female adult SCID/RAG-2 mice (BC Cancer Agency, Vancouver, BC) were primed with 0.5 ml of pristane (Sigma, St. Louis, MO) injected into the peritoneal cavity. After 7 days, 5 x 10^6 hybridoma cells (528) were inoculated i.p.. At weekly intervals ascitic fluid was tapped with an 18-gauge needle attached to a 5 ml syringe. The ascitic fluid was then incubated at 37°C for 1 h and transferred to 4°C overnight. The fluid was subsequently centrifuged at 3000 x *g* for 10 min and the supernatant collected. Anti-EGFR antibody was then purified on an UltraLink Protein G affinity column (Pierce, Rockford, IL).

4.2.4 Amine thiolation of the anti-EGFR antibody

A stock solution of the amine reactive reagent SPDP (Sigma, St. Louis, MO) at a concentration of 12.5 mM (3.9 mg/ml) was prepared in ethanol. Eighty µl (1000 nmoles) was then added to 920 µl of HBS to give a solution of 1 nmole SPDP/µl. Anti-EGFR antibody (3.5 mg/ml in HBS) was then modified with SPDP at a SPDP/protein mole ratio of 5:1 according to procedures described for streptavidin (Loughrey et al., 1990a). After 25 min the thiolated product was isolated by gel filtration on Sephadex G-50 (Sigma, St. Louis, MO) equilibrated with sodium acetate buffer (100 mM sodium acetate, 50 mM NaCl, pH 4.5). Fractions with an A_{280} larger than 1 were pooled and concentrated to 1 ml or less using Centricon 30 concentrators at 1500 x *g* for 20 min on a bench top centrifuge. The reaction mixture was then reduced with DTT (Sigma, St. Louis, MO) at a final concentration of 25 mM for 20 min and the thiolated product was isolated by gel filtration on Sephadex G-50 equilibrated with HBS. Fractions were again concentrated to 1 ml or less, as previously described, and used immediately in coupling experiments. The extent of modification of antibody was determined by estimating the protein concentration at 280 nm (molar extinction coefficient at 280 nm of 9.52×10^4) prior to the addition of DTT and the 2-thiopyridone concentration at 343 nm (molar extinction coefficient at 343 nm of 8.08×10^3) 25 min after the addition of DTT.

4.2.5 Carbohydrate thiolation of the anti-EGFR antibody

Anti-EGFR antibody (3.5 mg/ml in HBS) was oxidized with sodium metaperiodate (Pierce, Rockford, IL) at 1 mg/ml dissolved in 0.2 ml dH_20 at room temperature for 1 h. The antibody was isolated and buffer exchanged by gel filtration on Sephadex G-50 equilibrated with sodium acetate buffer. Fractions with an A_{280} larger than 1 were pooled and combined with 0.1 M PDPH in ethanol

(40 μl/ml of IgG solution) for 5 h at room temperature. The antibody was isolated by gel filtration on Sephadex G-50 equilibrated with sodium acetate buffer and treated with DTT (25 mM) for 20 min. The thiolated product was isolated by gel filtration on Sephadex G-50 equilibrated with HBS. Fractions were concentrated and used immediately in coupling experiments. The extent of modification was determined as outlined above for amine thiolation.

4.2.6 Coupling of thiolated anti-EGFR antibody to liposomes

The coupling reaction was performed by incubating thiolated antibody at room temperature with MPB-liposomes at a ratio of 75 μg of antibody per μmole lipid (10 mM final lipid concentration) in HBS. After 16 h, coupling was stopped by passing the liposome/antibody solution down a Sepharose CL-4B (Sigma, St. Louis, MO) column equilibrated with HBS to remove unconjugated antibody. Fractions with the highest lipid content were pooled and assayed for lipid (^{3}H-CHE) and protein content (BCA protein assay; Pierce, St. Louis, MO). Coupling of amine modified antibodies typically resulted in 15 to 25 μg antibody/μmole lipid whereas the coupling of carbohydrate modified antibodies resulted in 6 to 16 μg antibody/μmole lipid.

4.2.7 Doxorubicin encapsulation

Doxorubicin (Adria Laboratories, Mississauga, ON) was encapsulated using the transmembrane pH gradient driven loading procedure as described by Mayer et al. (1990). Liposomes with an internal pH 4 (300 mM citrate) external pH 7.5 (HBS) and doxorubicin at a concentration of 5-6 mM in saline, were heated to 60°C. After 10 min, liposomes were added to the doxorubicin at a final drug-to-lipid ratio of 0.2 and incubated with periodic mixing for 10 min. Unencapsulated doxorubicin

was removed on a Sephadex G-50 column in HBS and the doxorubicin-to-lipid ratio was measured as described by Bally et al. (1990).

4.2.8 Plasma clearance and tissue distribution studies

Antibody coated and uncoated liposomes were prepared as outlined earlier. Female SCID/RAG-2 mice weighing 18 to 22 g were typically used. The animal room environment and photoperiod were controlled at 19-25°C, 30-70% humidity and with a 12 h light cycle. One day prior to initiation of the study, mice were weighed and organized into appropriate groups. Mice were given a single bolus lateral tail vein injection of the indicated liposome formulations at a lipid dose of 100 mg/Kg. At 1 and 4 h after administration, the tail was nicked and blood was collected in a 25 µl capillary tube (Fisher Scientific, Nepean, ON) prerinsed with 5% EDTA. The samples were placed in a 500 µl microcentrifuge tube and then centrifuged at 500 x *g* for 10 min. The pellets were washed 2x with 400 µl of Hanks buffered saline (StemCell Technologies, Vancouver, BC) and the supernatants were combined and assayed for lipid. Twenty-four h after liposome administration, mice from each group were terminated by CO_2 asphyxiation. Blood was immediately removed by cardiac puncture and collected into EDTA coated microtainer tubes (Becton Dickinson, Franklin Lakes, NJ). The samples were centrifuged at 500 x *g* in a clinical bench top centrifuge for 10 min. Plasma was isolated and analyzed for lipid (see Quantification of liposomal lipid). Where indicated, tissue samples were collected in preweighed glass tubes. Tissue weight was recorded and samples stored at -70 °C until further analysis. Frozen tissue samples were homogenized in distilled water with a Polytron tissue homogenizer (Brinkman Instruments, Mississauga, ON) to a 10% homogenate (w/v). If required, tumors were also prepared for fluorescent microscopy (see tumor fixation).

4.2.9 Quantification of liposomal lipid

Liposomal lipid was quantified by employing the nonexchangeable and nonmetabolizable lipid marker, ^{3}H-CHE (Stein et al., 1980). Upon animal termination, blood and selected tissues were assayed for lipid content. The homogenized samples (200 µl) were mixed with 0.5 ml of Solvable (NEN-Dupont, Boston, MA) and incubated in a 50°C water bath for 3 h. Subsequently, the samples were cooled and the following solutions were added: 50 µl of 200 mM EDTA, 200 µl of 30% H_2O_2 and 25 µl of 10 N HCl. After 1 h at room temperature, 6 ml of Pico-Fluor 40 scintillation fluid (Packard, Meriden, CT) was added. The samples were vortexed and assessed for radioactivity using a Packard 1900TR Liquid Scintillation Counter (Packard, Meriden, CT). Background radioactivity was determined using samples derived from control tissues obtained from untreated mice. For plasma samples, 50-100 µl of plasma was added directly to the Pico-Fluor 40 and radioactivity determined. For column fractions, 5 µl samples were added to 7 ml scintillation vials and vortexed with 6 ml of Pico-Fluor 40 scintillation cocktail and assessed for radioactivity.

4.2.10 Tumor fixation and staining

Isolated solid tumors were collected in PBS at 4°C. Tumors were subsequently fixed with a 3% paraformaldehyde solution in PBS at 4°C for 30 min. Tumors were washed with PBS and immersed in increasing sucrose concentrations for 20 min: 10% sucrose-PBS, 15% sucrose-PBS and 15% sucrose-PBS containing O.C.T. compound (1/1, v/v; Miles Laboratories, Elkhart, IN). The processed tissue was embedded in O.C.T. compound and frozen in liquid nitrogen. Five µm sections were prepared on a Leica Frigocut 2800 E microtome (Willowdale, ON), mounted on a slide and used for antibody staining. The mounted sections were washed three times in PBS and

nonspecific antigens were blocked with 0.02% BSA for 30 min. FITC-labeled goat anti-mouse (Sigma, St. Louis, MO) antibody was added at a 1:100 dilution and incubated for 30 min at room temperature in a humid chamber. Sections were further washed with dH_2O to remove salt. Phase contrast and fluorescent microscopy was then performed with a Leitz Dialux microscope and Orthomat microscope camera (Midland, ON).

4.2.11 Protein G chromatography

All liposome samples were chromatographed as outlined in the protein G kit supplied by Pierce (Rockford, IL).

4.2.12 Western blot analysis

Antibody-conjugated liposomes were separated by SDS-PAGE on a precast 4%-stacked 20% TRIS-HCl gel (Bio-Rad Laboratories, Mississauga, ON) for 45 min. The proteins were then electroblotted to an Immobilon-P transfer membrane (0.45 μm pore size; Millipore, Mississauga, ON) at 100 V for 1 h. Protein was detected using the goat anti-mouse AP (alkaline phosphatase) Immun-Blot Assay Kit (Bio-Rad Laboratories, Hercules, CA).

4.3 Results

4.3.1 Specificity of anti-EGFR antibodies for the A431 tumor

The tumor model chosen to investigate targeting of an antibody-conjugated liposome was a human adenocarcinoma, A431, which overexpresses the epidermal growth factor receptor (EGFR). This tumor was grown s.c. in female SCID/RAG-2 mice. These mice are deficient in

their ability to produce immunoglobulin (deficient recombinase activating gene-2, RAG-2) and provide an opportunity to measure the levels and distribution of parentrally administered IgG using simple techniques which exhibit minimal or no background. This is illustrated by the data shown in Figure 4.1 and Table 4.1, described below. The *in vivo* specificity of the 528 cell line derived anti-EGFR antibody for the adenocarcinoma was determined with an injection of free anti-EGFR antibody into the lateral tail vein of tumor bearing mice. Subsequently, tumors were removed and the presence of the anti-EGFR antibody was detected histologically using a FITC-labeled goat anti-mouse IgG antibody (Figure 4.1). The results indicate that the anti-EGFR antibody has specificity for tumor cells with labeling occurring around blood vessels. There also appears to be a time dependent penetration into the interstitial tissue, as noted when comparing the depth of penetration at 1 h and 24 h.

Other tissues known to express EGFR were also evaluated for anti-EGFR antibody binding. The tissues indicated in Table 4.1 were isolated from untreated mice or from mice which received an injection of free anti-EGFR antibody. Tissues were then stained with a FITC-conjugated secondary antibody. For untreated mice results show intense labeling, as seen in Figure 4.1, of the tumor as well as intermediate labeling of esophagus and intestine. For anti-EGFR antibody treated mice nonspecific labeling of lung, liver, spleen, and kidney were observed which is consistent with nonspecific biodistribution characteristics of i.v. administered antibodies (Burchiel et al., 1982).

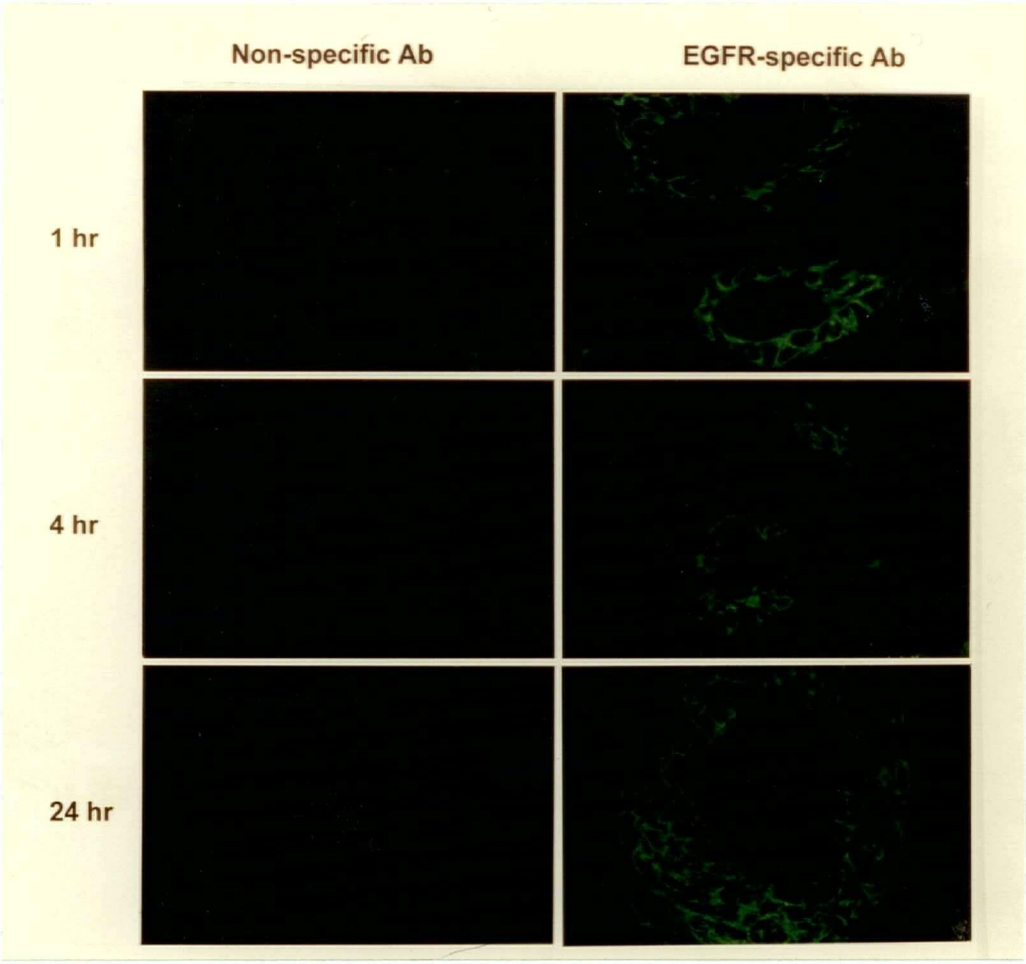
4.3.2 Use of PEG and doxorubicin to decrease plasma elimination

The above model is an ideal system to evaluate target specific delivery *in vivo* because of the proven potential for the anti-EGFR antibody to label tumor cells following i.v. administration.

Figure 4.1

***In vivo* specificity of the anti-EGFR antibody for the A431 human adenocarcinoma**

One-hundred μg of a nonspecific antibody or a specific anti-EGFR antibody were injected *via* the lateral tail vein into tumor bearing (A431) SCID/RAG-2 female mice. At 1, 4, and 24 h tumors were removed and assayed for the presence of mouse antibody as outlined in the Methods.



This antibody binds cells with a high affinity (Kd ≅ 1 nM) and *in vitro* studies show efficient labeling (> 20 fold increase in cell association) of the A431 cells with liposomes that have covalently attached anti-EGFR antibody. Further, published results demonstrate efficient accumulation of liposomes in A431 tumors following i.v. administration (Webb et al., 1995). Therefore, to achieve equivalent labeling of antibody-conjugated liposomes, the liposomes must

Table 4.1

Determination of the labeling of EGFR in tissues extracted from untreated SCID/RAG-2 mice or mice injected with a anti-EGFR antibody.

Tissue	Labeling following recovery of tissue sections from untreated mice[a]	Labeling following i.v. administration of an anti-EGFR antibody[b]
tumor	++++	++
esophagus	++	--
intestine	++	--
lung	--	+++
liver	--	+++
spleen	--	+++
kidney	--	+++

[a] extracted tissues from untreated mice were stained with the EGFR antibody then a FITC-conjugated secondary antibody.

[b] 100 mg of free anti-EGFR antibody injected i.v. *via* the lateral tail vein; 1 h after injection extracted tissues were stained with a FITC-conjugated secondary anti-EGFR antibody.

+ indicates the relative degree of visual fluorescence

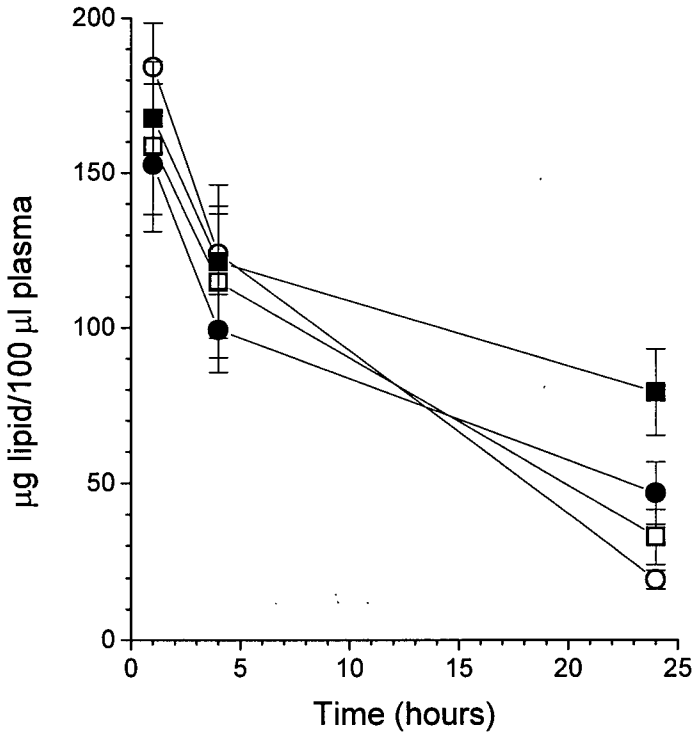
-- no fluorescence detected

show good plasma circulation lifetimes. Results in Figure 4.2 show that the addition of PEG or the addition of encapsulated doxorubicin (dox) increase the lipid levels within the circulation at 24 h. Addition of PEG increases plasma values 1.5-fold and encapsulated dox increases plasma values 2.5-fold. The addition of both PEG and dox act cooperatively to increase plasma values 4-fold. Plasma half-lives were approximately 9 h for liposome/dox, 11.5 h for both liposomes and PEG-liposomes, and 21.5 h for PEG-liposome/dox. The elevated plasma levels in the presence of PEG and dox are a result of inhibition of opsonization by plasma proteins (Woodle et al., 1992 and 1994) and reduced activity of Kupffer cells (macrophages) in the liver (Daemen et al., 1995; Bally et al., 1990), respectively. It is also observed that the encapsulation of dox in liposomes with surface associated protein inhibits the humoral response that arises following i.v. administration (Tardi et al., 1997) and that the addition of 2 mol % poly(ethylene glycol) (PEG)-modified lipid inhibits aggregation upon conjugation of proteins (Harasym et al., 1995).

Figure 4.2

Liposome plasma levels in the presence and absence of both PEG and doxorubicin

DSPC/Chol liposomes (55:45; mol %) with or without 2 mol % PEG or dox (drug-to-lipid ratio of 0.2) were injected i.v. into female SCID/RAG-2 mice at an initial lipid dose of 100 mg/kg (~2 mg lipid/mouse; 0.4 mg drug/mouse). (O) DSPC/Chol, (□) DSPC/Chol/PEG, (●) DSPC/Chol+dox, (■) DSPC/Chol/PEG+dox. At 1 and 4 h, 25 μl of whole blood was collected by a tail nick in EDTA-coated capillary tubes and assayed for lipid as outlined in Methods. At 24 h plasma was collected by heart puncture in EDTA-microtainer tubes. Each data point represents mean ±SD obtained from 7 mice. Plasma concentration for theoretical time zero based on 20 g mice with a plasma volume of 58.4 ml/kg is ~230 μg lipid/100 μl plasma.



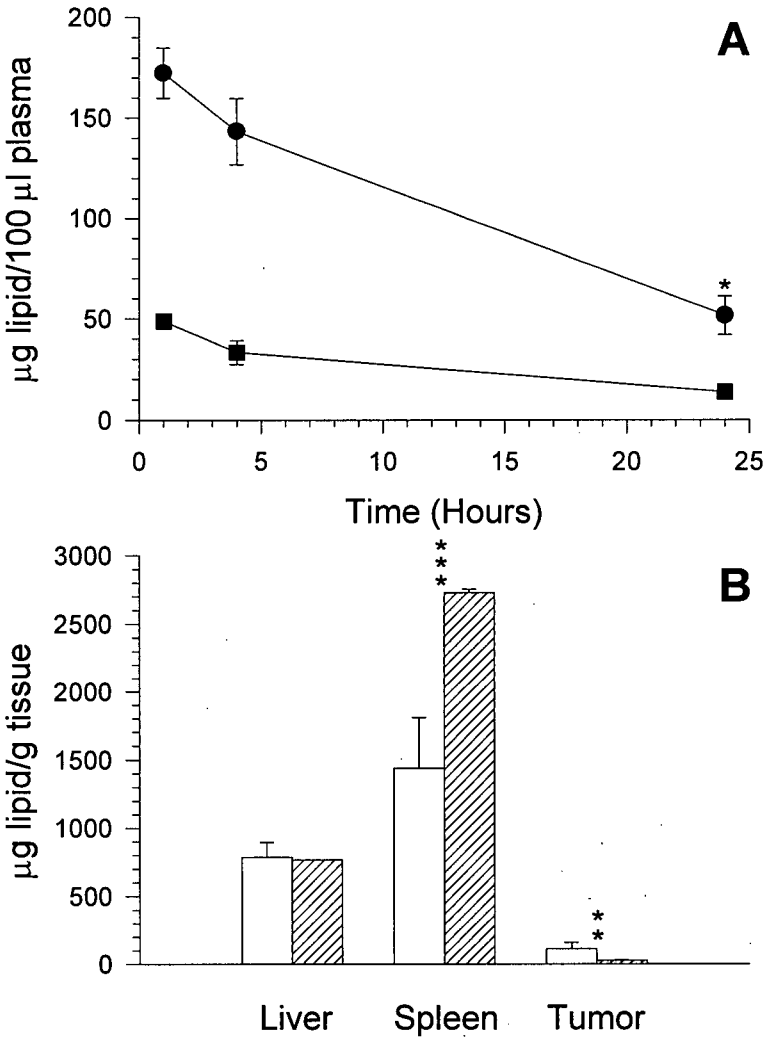
4.3.3 Antibody conjugation increases liposome plasma elimination

Since it is established that the incorporation of PEG is effective at increasing plasma levels of liposomes, the effects of incorporating anti-EGFR antibodies on PEG-liposomes was established between liposomes and antibody-conjugated liposomes. Results in Figure 4.3A indicate that the

Figure 4.3

Plasma and tissue levels of PEG containing liposomes and amine conjugated anti-EGFR liposomes

Liposomes containing either PEG or conjugated to the anti-EGFR antibody through primary amines were injected i.v. into tumor bearing (A431) female SCID/RAG-2 mice at a lipid dose of 100 mg/kg. Panel A, plasma lipid values: (●) liposomes with 2 mol % PEG, (■) amine conjugated anti-EGFR liposomes. At 1 and 4 h blood was collected by a tail nick at 24 h animals were killed and blood collected by cardiac puncture. Panel B, tissue lipid values at 24 h: open bars, DSPC/Chol/PEG; hatched bars, amine conjugated anti-EGFR liposomes. Error bars represent mean ±SD from 4 mice. *, $P<0.02$; **, $P<0.005$; ***, $P<0.04$.



presence of an amine conjugated antibody significantly increases the plasma elimination of liposomes at 1, 4, and 24 h (49, 33, and 14 μg lipid/100 μl plasma, respectively) in comparison to unconjugated PEG-liposomes (170, 140, and 52 μg lipid/100 μl plasma). Plasma elimination profiles indicate that between 0 and 1 h, 80% of the lipid was eliminated following i.v. administration of the amine conjugated anti-EGFR liposomes and between 1 and 24 h, a plasma elimination rate comparable to the elimination rate of unconjugated PEG-liposomes was observed. Liver, spleen, and tumor were also evaluated for liposome accumulation at 24 h (Figure 4.3B). No significant difference in lipid accumulation was observed in liver (both formulations show ~780 μg lipid/g tissue). However, significant differences in uptake of lipid were observed in the spleen (2700 μg lipid/g tissue) and tumor (26 μg lipid/g tissue) from mice injected with conjugated anti-EGFR liposomes when compared to spleen (1400 μg lipid/g tissue) and tumor (110 μg lipid/g tissue) of mice injected with unconjugated PEG-liposomes. For spleen a 2.5-fold increase and tumor a 4-fold decrease were observed. Tumor lipid analysis indicated a significant reduction in tumor levels associated with the amine conjugated anti-EGFR liposomes ($P<0.005$).

4.3.4 Strategies known to decrease liposome plasma elimination

These data clearly identify that antibody conjugation caused increased liposomal clearance, which in turn, resulted in reduced delivery to the tumor. This is consistent with observations correlating increased plasma elimination and reduced uptake in extravascular disease sites. Therefore, strategies known to decrease liposome plasma elimination in an attempt to increase the level of amine conjugated anti-EGFR liposomes in the blood compartment were assessed, the results have been summarized in Figure 4.4 and Table 4.2. RES blockade which temporarily reduces liposome uptake in the liver (Parr et al., 1993; Yachi et al., 1995) was evaluated first.

Figure 4.4

Plasma levels of PEG and amine conjugated anti-EGFR liposomes in the presence of RES or Fc receptor blockade

For RES blockade, twenty-four h prior to injecting liposomes, mice were pretreated with a low dose of liposomal doxorubicin (10 mg/kg lipid, 2 mg/kg drug). For Fc receptor blockade, 1 h prior to liposome injection, mice were pretreated i.v. with 100 μg of a nonspecific antibody or antibody coupled *via* carbohydrates. Panel A, plasma levels of 2 mol % PEG-liposomes. (○) PEG-liposomes, (□) PEG-liposomes with antibody blockade, (Δ) PEG-liposomes with RES blockade. Panel B, plasma levels of amine and carbohydrate conjugated anti-EGFR liposomes. (▲) amine conjugated EGFR-liposomes, (●) amine conjugated EGFR-liposomes with antibody blockade, (■) amine conjugated EGFR-liposomes with RES blockade, and (▼) carbohydrate conjugated EGFR-liposomes. *, $P<0.02$; **, $P<0.001$; ***, $P<0.001$.

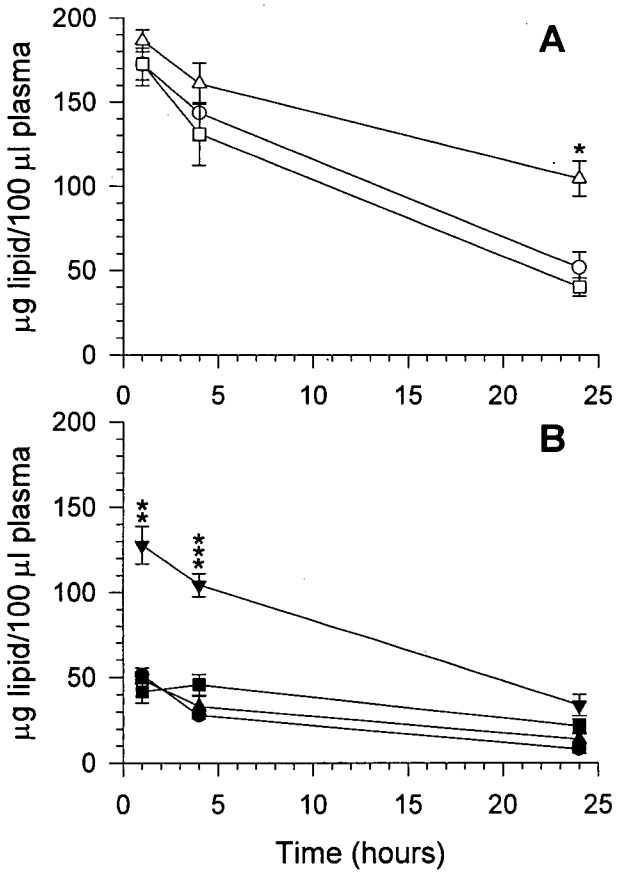


Table 4.2

Tissue lipid levels at 24 h for unconjugated and anti-EGFR conjugated liposomes in the presence or absence of RES or Fc blockade

Sample	Tissue[a]	Controls	RES blockade	Fc blockade	
				Amine coupled	Carbohydrate coupled
Control liposomes	blood	52 ± 10[b]	100 ± 10[b]	40 ± 5.4	—
	liver	790 ± 110[c]	360 ± 98[c]	840 ± 49	—
	spleen	1400 ± 370	3100 ± 280	1500 ± 430	—
	tumor	110 ± 47	160 ± 36	89 ± 19	—
EGFR-liposomes	blood	14 ± 3.3[d]	22 ± 4.1	7.9 ± 2.5	34 ± 6.2[d]
	liver	770 ± 0.21	710 ± 81	760 ± 92	640 ± 98
	spleen	2700 ± 26	3900 ± 740	2100 ± 690	2200 ± 390
	tumor	26 ± 3.3[e,f]	53 ± 11[e]	32 ± 5.4	150 ± 23[f]

[a] blood values μg lipid/100 μl plasma, organ values μg lipid/g tissue
[b,c,d,e,f] $P<0.05$

Figure 4.4A shows that pretreatment of mice with low dose liposomal doxorubicin was effective at doubling the plasma levels of control liposomes from 52 to 110 μg lipid/100 μl plasma at 24 h. When comparing data for the anti-EGFR liposomes in the absence of RES blockade (Figure 4.3) and in the presence of RES blockade (Figure 4.4B and Table 4.2), three important observations can be made. First, RES blockade did not effect a significant increase in blood levels at any time point measured. At 24 h, for example, 14 μg lipid/100 μl plasma and 22 μg lipid/100 μl plasma were measured in the absence and presence of RES blockade, respectively (Table 4.2). Second, the level of lipid accumulation in the liver was not effected by RES blockade (Table 4.2). At 24 h, 770 μg lipid/g tissue and 710 μg lipid/g tissue were measured in the absence and presence of RES blockade. This is in contrast to results with unconjugated liposomes where RES blockade caused a 2-fold increase in circulating levels and a 2-fold decrease in liver levels of liposomal lipid. Third, RES blockade did engender a 2-fold increase in tumor levels (measured at 24 h) of the anti-EGFR liposomes. At this time point 26 μg lipid/g and 53 μg lipid/g tumor were

measured in the absence and presence of RES blockade, respectively. Importantly, the anti-EGFR liposomes exhibited a 2- to 3-fold reduction in tumor accumulation compared to control liposomes (Table 4.2).

A second method attempting to increase plasma levels of anti-EGFR liposomes was based on the possibility that removal from the circulation may be due to binding of conjugated antibody by Fc receptors expressed on various cells (e.g. macrophages). Two approaches were taken in order to address this possibility. First, saturation of Fc receptors on macrophages, a strategy known to effect increases in blood levels and target cell labeling of free antibodies (Leserman et al., 1980; Bragman et al., 1984), was attempted to elevate plasma levels of the amine conjugated anti-EGFR liposomes. Fc blocking was accomplished by predosing mice i.v. with 100 μg of a free, nonspecific, antibody prior to administration of the antibody-conjugated liposomes. Three important observations can be made from these data, also shown in Figure 4.4 and Table 4.2. First, at all time points measured no significant effect on plasma levels were observed with or without Fc blocking (Figure 4.4B). For example, at 24 h, 14 μg lipid/100 μl plasma was measured before Fc blocking and 8 μg lipid/100 μl plasma after blocking (Table 4.2). Second, no difference in liver and spleen lipid levels were observed at 24 h. Before Fc blocking, liver and spleen lipid values measured 770 and 2700 μg lipid/g tissue, respectively. After Fc blocking, liver and spleen lipid values measured 760 and 2100 μg lipid/g tissue, respectively. Third, no improvement in tumor lipid levels was observed at 24 h. Compare, before Fc blocking (26 μg lipid/g tissue) with after Fc blocking (32 μg lipid/g tissue) (Table 4.2). The data indicates that blocking Fc receptors by this strategy had no effect on increasing tumor specificity, as tumor lipid levels remained significantly lower than that which can be achieved using unconjugated liposomes.

A second Fc blocking approach attempted, relied on use of an alternative coupling procedure that appears to provide better orientation of the antibody on the liposome (Ansell et al., 1996). The orientation of an antibody on the surface of a liposome should play a role in its ability to recognize its target antigen as well as on its elimination characteristics, particularly, if Fc mediated clearance is a mechanism responsible for the increased elimination rates observed. Therefore, anti-EGFR antibodies coupled to liposomes involving reactions with carbohydrate groups, rather than amine groups, was evaluated. It was anticipated that conjugation through the carbohydrate would orient the antibody with its antigen binding regions (Fab's) directed away from the surface of the liposome, effectively increasing antigen accessibility. These data are shown in Table 4.2 and Figure 4.4B. Three observations were evident. First, coupling *via* the carbohydrate region showed elevated plasma lipid levels at all time points measured (Figure 4.4B). For example, at 1 h, an increase from 49 to 128 μg lipid/100 μl plasma was observed and at 24 h an increase from 14 to 34 μg lipid/100 μl plasma was observed. The plasma elimination curves, however, still indicated that approximately 30% of the injected dose was eliminated in the first hour after administration. Second, liver and spleen lipid levels were consistent with measured values observed for amine conjugated antibodies (Table 4.2). At 24 h the measured amount of lipid in the liver was, 640 and 760 μg lipid/g for carbohydrate and amine conjugated liposomes, respectively. In the spleen lipid levels were 2200 and 2100 μg lipid/g, respectively. Carbohydrate conjugation did result in a significant 5- to 6-fold lipid increase to tumor tissue. Where 26 μg lipid/g tumor was observed for liposomes prepared using the amine conjugation technique and 150 μg lipid/g tumor for liposomes prepared using the carbohydrate conjugation technique. Antibody content, however, was lower on the carbohydrate conjugated liposomes, 15 versus 6 μg antibody/μmole lipid. The tumor lipid levels were higher than tumor levels observed with control liposomes (110 μg lipid/g tissue) but the differences were not statistically different. The carbohydrate conjugated anti-EGFR liposomes appear to be of greater utility then the amine

conjugated anti-EGFR liposomes which exhibit increased circulation lifetimes and improved tumor localization characteristics.

4.3.5 Fractionation of antibody conjugated liposomes on a protein G column

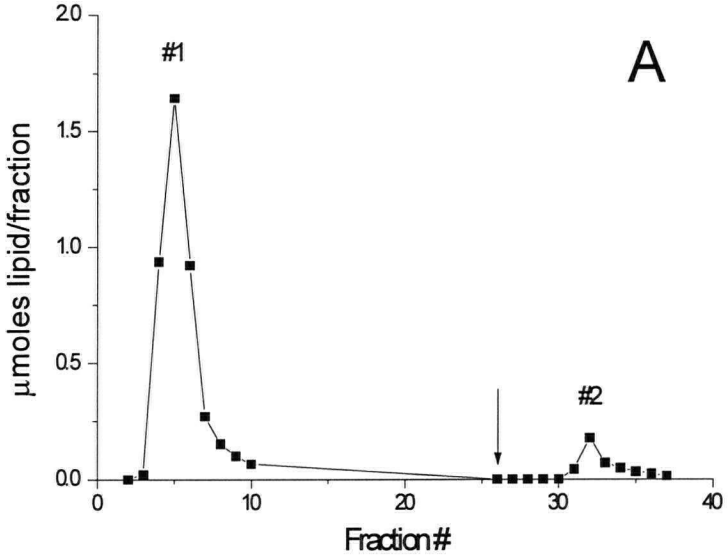
Since a large portion of both the amine and the carbohydrate conjugated liposomes are eliminated rapidly (within 1 h) after i.v. injection the possibility that the coupling procedures resulted in the formation of heterogeneous populations of antibody conjugated liposomes, one population that is eliminated rapidly and one that is eliminated slowly, were addressed. This was initially evaluated by fractionating the amine and carbohydrate conjugated liposomes on protein G columns. The affinity column separates those liposomes with exposed Fc domains from those with Fc portions buried near the surface of the liposome. Although the exact mechanism of protein G binding is unknown, the ability of protein G to bind antibodies occurs through the Fc region (Eliasson et al., 1988). It was anticipated that column retention would be inhibited when antibody is coupled by the carbohydrate groups.

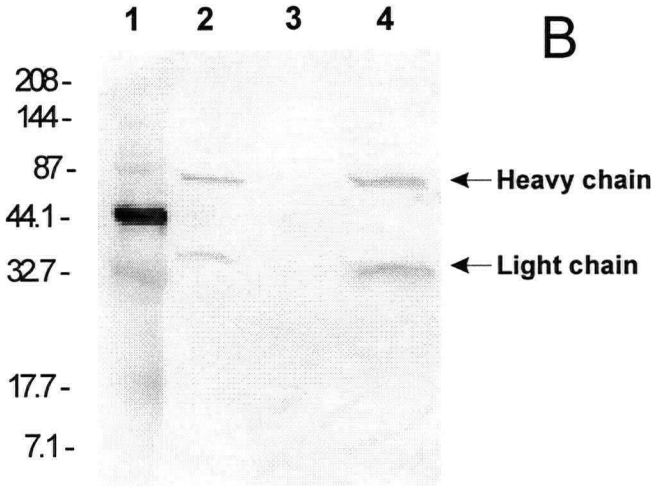
For amine conjugated anti-EGFR liposomes, the column profile in Figure 4.5A shows that 90% (peak #1) of the applied liposomes were not bound and only 10% of the liposomes were retained (peak #2). This suggests that the majority of the conjugated antibodies on the liposome were maintained in a configuration such that the antibody liposome complex could not bind the protein G column. However, analysis of the protein levels in each peak by protein assays (data not shown) and Western blot analysis (Figure 4.5B) indicated that antibody was associated with only the retained fractions (lane 4). The measured protein-to-lipid ratio in peak #2 (150 μg/μmole lipid) was significantly greater than measured from the sample (15 μg/μmole lipid) prior to addition onto the protein G column. Since repeated passage of newly conjugated anti-EGFR

Figure 4.5

Protein G column profile for amine conjugated anti-EGFR liposomes and Western blot analysis of the two peaks obtained from the column

Liposomes (5 μmoles) with anti-EGFR antibodies conjugated *via* a primary amine were passed down a 2 ml protein G column equilibrated with 0.1 M sodium acetate, pH 5.0 and eluted at the indicated fraction (arrow) with 75 mM glycine pH 2.5. Panel A: elution profile. Approximately, 750 μl fractions. Panel B: lane 1: 10 μl of a kaleidoscope M.W. standard; lane 2: amine conjugated anti-EGFR liposome before application to column (8 nmoles); lane 3: protein G peak #1 of amine conjugated liposomes (8 nmoles); lane 4: protein G peak #2 of amine conjugated liposomes (2 nmoles).





liposomes (prepared by either the amine or carbohydrate coupling techniques) down a nonaffinity column (sepharose CL-4B) had no impact on the measured protein-to-lipid ratio, it must be concluded that either an antibody rich liposome population is generated during the coupling reaction or, alternatively, that the antibody-lipid conjugate dissociated from the liposome following binding to protein G. The later conclusion is supported by recent results demonstrating that lipids conjugated to hydrophilic polymers of between 2000 and 5000 MW can readily exchange out of liposomes (Parr et al., 1994; Holland et al., 1996).

For carbohydrate conjugated anti-EGFR liposomes, protein G column profiles also show two distinct liposome peaks: peak #1 (nonbound liposomes) contain 55% of the applied lipid; peak #2 (retained liposomes) contain 45% of the applied lipid (Figure 4.6A). Unlike primary amine conjugated liposomes, however, analysis for protein by a protein assay (data not shown) and Western blot analysis (Figure 4.6B), indicated that antibody was associated with both peak #1 (lane 3) and peak #2 (lane 4). The fact that carbohydrate conjugated anti-EGFR liposomes were eluted off the protein G column with attached antibody is likely a consequence of improved antibody orientation, with the Fc portions unavailable for interaction with the protein G column due to carbohydrate conjugation. An extension to this conclusion is that dissociation of antibody from the liposomes prepared using the amine based conjugation procedure is a consequence of Fc binding.

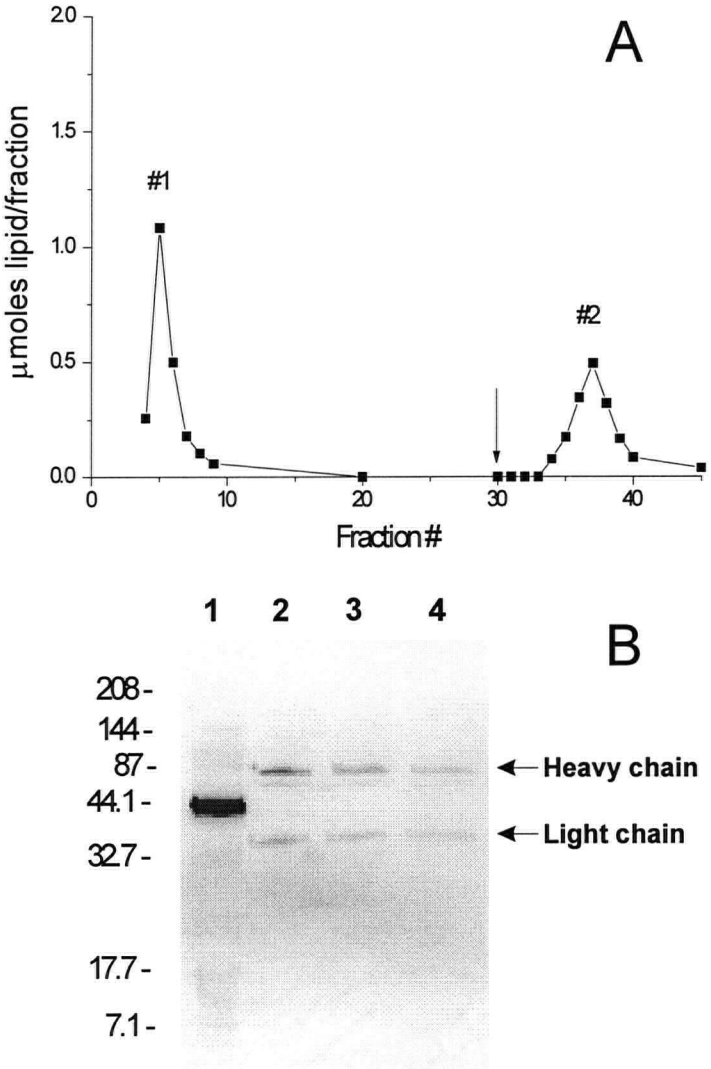
4.3.6 Antibody association with liposomes following i.v. administration and incubation in plasma

Antibody dissociation and, conversely, the levels of antibody associated with liposomes following i.v. administration were also characterized. Western blot analysis, assaying plasma

Figure 4.6

Protein G column profile for carbohydrate conjugated anti-EGFR liposomes and Western blot analysis of the two peaks obtained from the column

Column details as in Figure 4.5. Panel A: elution profile. Approximately, 650 µl fractions. Panel B: lane 1: 10 µl of a kaleidoscope M.W. standard; lane 2: carbohydrate conjugated anti-EGFR liposome before application to column (8 nmoles); lane 3: protein G peak #1 of carbohydrate conjugated liposomes (8 nmoles); lane 4: protein G peak #2 of carbohydrate conjugated liposomes (2 nmoles).



obtained 30 minutes after i.v. administration of both amine and carbohydrate conjugated antibody liposomes, show the presence of antibody with amine conjugated liposomes but not with carbohydrate conjugated liposomes (Figure 4.7; lane 3 and lane 2, respectively). These results are perplexing even though the analysis was completed using equal volumes of plasma where the lipid concentration was typically 2- to 3-fold greater for the carbohydrate conjugated liposomes than the amine conjugated liposomes (Figure 4.4A). Further analysis by fast performance liquid chromatography (FPLC), a technique developed to fractionate lipoprotein fractions and serum proteins from liposomes (personal communication, Edward Choice), of the antibody conjugates incubated in plasma for 30 min concluded that the conjugated antibodies remain associated with the liposome fractions in the presence of plasma (Figure 4.8). The removal of antibody from the carbohydrate conjugated liposomes *in vivo* but not in the presence of plasma is puzzling. An original contention, from the protein G columns, was that the plasma elimination profiles were the result of either two vesicle populations formed during coupling or dissociation. The results presented here favor antibody dissociation upon binding and i.v. administration.

Figure 4.7

Western blot analysis of carbohydrate and amine conjugated liposomes 30 min after injection

Liposomes at a lipid dose of 100 mg/kg were injected in the lateral tail vein of SCID/RAG-2 mice. At 30 min blood was collected by cardiac puncture. Lane 1: 10 µl of a kaleidoscope M.W. standard; lane 2: carbohydrate conjugated anti-EGFR liposomes (1.5 nmoles lipid); lane 3: amine conjugated anti-EGFR liposomes (0.4 nmoles lipid); lane 4: control SCID/RAG-2 plasma (20 µl of a 7.5x dilution); lane 5: amine conjugated anti-EGFR liposomes before injection (8 nmoles lipid).

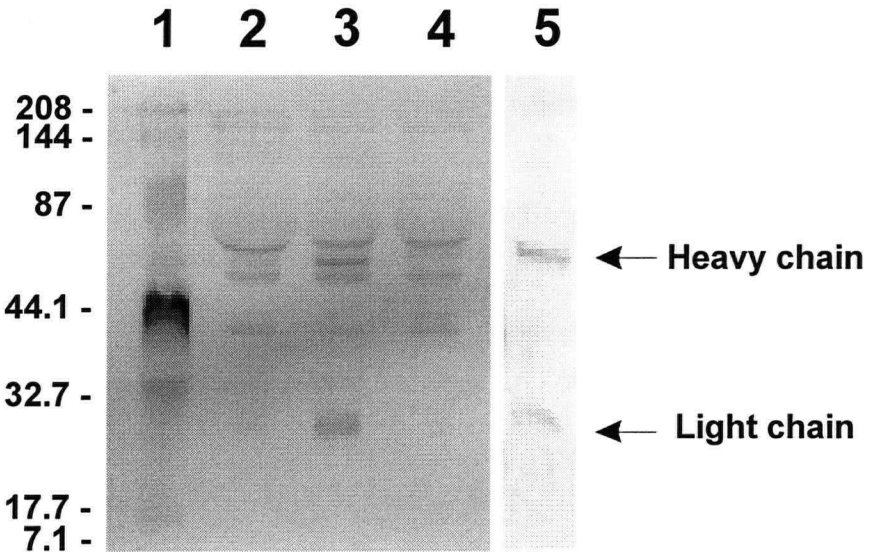
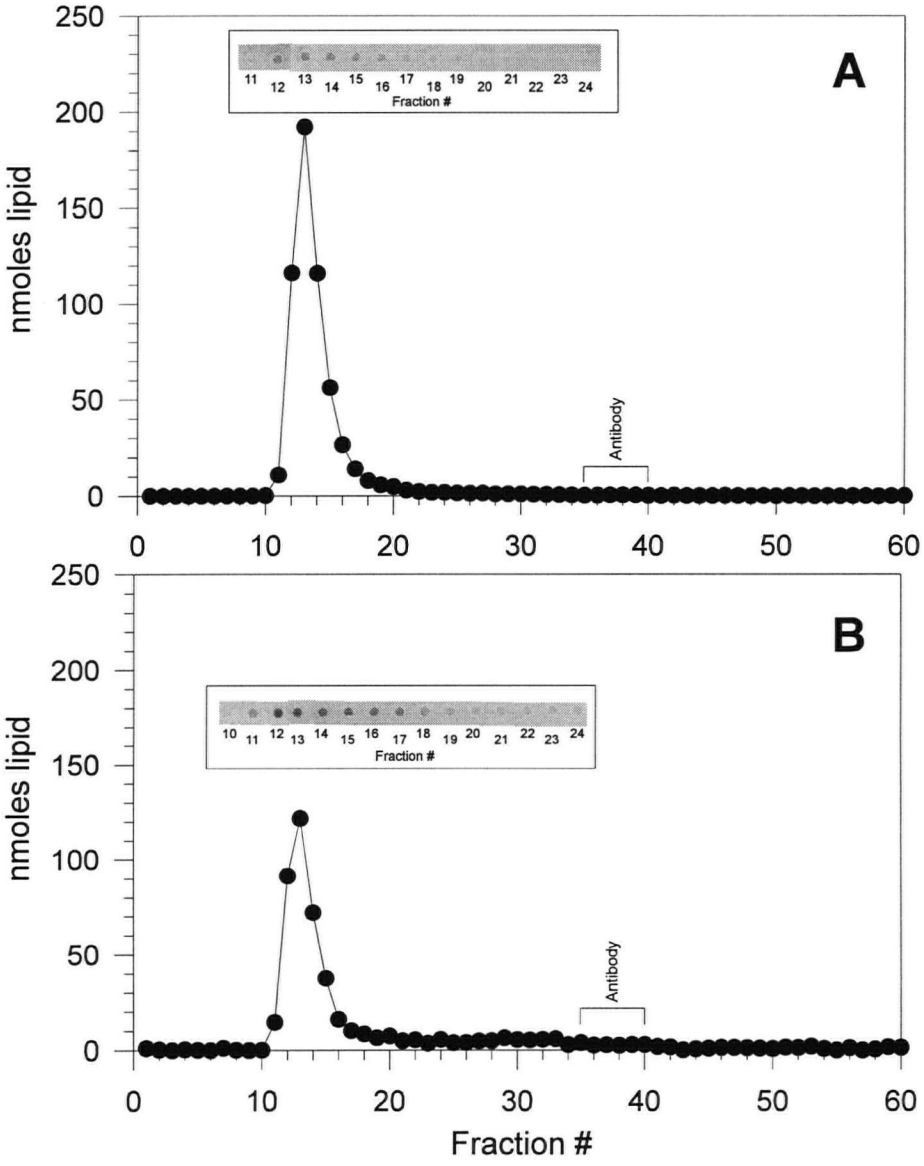


Figure 4.8

Elution profiles of amine and carbohydrate conjugated EGFR-antibodies incubated in plasma for 30 min and separated *via* FPLC

Either amine or carbohydrate conjugated liposomes (16.7 mM) were diluted to 2 mM lipid with SCID/RAG-2 plasma and incubated for 30 min at 37°C. Subsequently, 250 µl of each incubation mixture was separated from plasma proteins and lipoproteins *via* a FPLC. One ml fractions were collected and assayed for lipid (^{3}H-CHE) and for antibody (dot blot analysis using a modified procedure from the Western blot analysis; inserts). (A) Elution profile of PDPH (carbohydrate) conjugated antibody. (B) Elution profile of SPDP (amine) conjugated antibody. Elution position of free antibody as indicated.



4.4 Discussion

Applications for targeted liposomal anticancer agents are based on the premise that the liposome will shift the association of an encapsulated drug, such as doxorubicin, from the plasma or interstitial compartment to the target (tumor cells). Alterations in the target specific delivery are dependent on the use of a ligand (antibody) specific for either overexpressed, or novel tumor specific, antigens. To date, investigators evaluating targeting have been confronted with many challenges. It is suggested that these challenges are mainly attributed to biological characteristics that prevent optimal extravasation in a site of tumor growth (Wu et al., 1993; Litzenger et al., 1994) as well as poor binding avidity (Klibanov et al., 1985). These properties are not, however, a consequence of inefficient protein coupling techniques or due to changes in physical (size) characteristics of the carrier. The results presented in this Chapter strongly suggest that problems associated with *in vivo* targeting are a consequence of coupling induced generation of different vesicle populations as well as dissociation of the antibody from the liposome. This would, in turn, result in elimination behaviors that are different from control liposomes, reduced target site delivery and reduced binding avidity *in vivo*.

Indirect evidence supporting the contention that the conjugated antibodies can dissociate from liposomes include recent data demonstrating exchange of PEG-modified lipids from liposomes. This exchange rate is dependent on the lipid anchor, where saturated DSPE conjugates are retained better than unsaturated DOPE-PEG conjugates (Parr et al., 1994). As well, Silvius and Zuckermann have shown that conjugation of large hydrophilic molecules (10,000-70,000 KDa) to diacyl or alkyl lipids with 14- to 18-carbon chains will experience intermembrane transfer on a time scale of several hours or less (Silvius and Zuckermann, 1993). Finally, studies by Uster et al. (1996) have demonstrated that PEG lipids added to the outside of liposomes can efficiently

exchange into the liposomal membranes. It seems reasonable, therefore, that larger molecules, such as an antibody (150,000 KDa), coupled to diacyl lipid anchors could also exchange.

The direct evidence supporting these claims is based on results that show removal of conjugated antibody from liposomes following elution through a protein G column (Figure 4.5) and following i.v. administration (Figure 4.7). Regardless of whether amine or carbohydrate based conjugation procedures were used, two peaks were observed following elution through a protein G column, a nonbound and a retained peak. It is believed that for primary amine conjugated proteins, the column binds to conjugated antibody through the Fc portion with subsequent dissociation of antibodies from the liposome. This is less evident for carbohydrate based coupling procedures, where column binding is less effective due to reduced exposure of the Fc region on the antibody.

The plasma elimination profile, tumor localization characteristics, incubation in plasma, and results from the protein G column suggested that carbohydrate-conjugation would be optimal for *in vivo* targeting applications. It was, therefore, disappointing that *in vivo* data suggested loss of antibody from the liposome prepared using the carbohydrate conjugation procedure. It is unclear what would mediate dissociation of the antibody, however, the possibility that modification of the carbohydrate groups on IgG results in immune recognition and binding can not be discounted. Binding of the antibody in this case, may promote dissociation of the protein from the liposome.

It has been suggested that for effective *in vivo* targeting, it will be necessary to have a high-affinity antibody conjugated to a liposome as well as a high concentration of target antigen (Klibanov et al., 1985). What remains to be clearly defined are the numbers of antibodies needed

for sufficient target cell labeling. Evidence presented in this Chapter suggests that lability of the liposome coupled antibody may have a role in decreased antibody liposome targeting and hence directly effect target binding avidity/affinity. It is concluded that poor targeting of either conjugated antibody liposome formulation is likely due to poor antibody stability with the liposome and it is hypothesized that in order to increase the efficiency of antibody liposome targeting, issues related to the stability of the antibody on the liposome must be addressed.

CHAPTER 5

SUMMARIZING DISCUSSION

5.1 Summary of results

The objective of the studies in this thesis were developed to more fully understand, optimize and define important parameters required for protein conjugated liposomal targeting. Three areas relevant for targeting were investigated. Initially, it was shown that the use of PEG lipids was an essential requirement for the prevention of aggregation during covalent protein conjugation to a liposome surface. Secondly, the tumor biodistribution of an accumulated nontargeted liposome was resolved, to evaluate liposome distribution within the tumor environment. Thirdly, the effects of conjugating antibody to a liposome with regards to circulation longevity and antibody stability were evaluated.

In Chapter 2, a procedure for coupling proteins to liposomes that resulted in little or no change in liposome size was demonstrated. This was achieved by incorporating appropriate levels of poly(ethylene glycol)- modified lipids into the liposome. The studies employed thiolated avidin-D coupled to liposomes containing the thio-reactive lipid *N*-(4-(*p*-maleimidophenyl)butyryl)dipalmitoyl phosphatidylethanolamine (1 mol % of total lipid) and various amounts of MePEG–S–POPE (monomethoxypoly(ethylene glycol) linked to phosphatidylethanolamine *via* a succinate linkage). The influence of PEG chain length and density was also assessed. The presence of PEG on the surface of liposomes was shown to provide an effective method of inhibiting aggregation and the corresponding increase in liposome size during the covalent coupling of avidin-D. A balance between the size of the PEG used and the amount of PEG–lipid incorporated into the liposome had to be achieved in order to maintain efficient

coupling. Optimal coupling efficiencies in combination with minimal aggregation effects were achieved using 2 mol % $MePEG_{2000}$–S–POPE (PEG of 2000 MW) or 0.8 mol % $MePEG_{5000}$–S–POPE (PEG of 5000 MW). At these levels, the presence of PEG did not affect the biotin binding activity of the covalently attached avidin. The ability of the resulting liposomes to specifically target to biotinylated cells was also demonstrated.

In Chapter 3 a pharmacological evaluation of an egg phosphatidylcholine/cholesterol (55:45 mole ratio, EPC/Chol) liposome doxorubicin formulation was evaluated. The objectives were to define liposomal lipid and drug distributions within sites of tumor growth following intravenous (i.v.) administration to female BDF1 mice bearing either Lewis lung carcinoma, B16/BL6 melanoma, or L1210 ascitic tumors. This was achieved by injecting mice i.v. with EPC/Chol liposomal doxorubicin and determining plasma and tumor levels of lipid and drug at 1, 4, and 24 h with radiolabeled lipid and fluorimetry or fluorescence microscopy, respectively. In addition, single-cell suspensions of the Lewis lung and B16/BL6 tumors were prepared and the presence of macrophages were determined with an FITC-labeled rat antimouse CD11b (MAC-1) antibody. For mice bearing the Lewis lung solid tumors, there was a time-dependent accumulation of liposomal lipid, with a plateau of approximately 500 μg lipid/g tumor at 48 h. In contrast, the apparent plateau (μg doxorubicin/g tumor) for doxorubicin was achieved at 1 h and remained constant over a 72-h time course. In comparison with free drug administered at the maximum tolerated dose (MTD, 20 mg/kg) doxorubicin levels in tumors were two- to threefold greater when the drug was administered in liposomal form. The increase in drug delivery was comparable for both solid tumors. With animals bearing the L1210 ascitic tumor, drug exposure was as much as ten times greater (in comparison with free drug) when doxorubicin was administered in liposomes. An evaluation of single-cell suspensions prepared from the two solid tumors suggested that more than 98% of the tumor-associated drug and liposomal lipid was not

tumor cell-associated. Histological studies with the Lewis lung carcinoma, however, revealed that a proportion of the drug did colocalize with tumor-associated macrophages. Analysis of cells obtained from mice bearing ascitic tumors showed that more than 80% of the cell-associated drug could be removed by procedures designed to remove adherent cells. The results summarized suggest that drug concentrations within a solid tumor, such as the Lewis lung carcinoma, are constant over time when the drug is given in a "leaky" EPC/Chol formulation. The results also suggest that liposomal lipid within sites of tumor growth was primarily localized within the interstitial spaces or tumor-associated macrophages.

The final set of experiments presented in Chapter 4 evaluated the stability of antibody/liposome conjugates in an effort to identify a protein coupling strategy that would enhance target specific delivery to solid tumors. The *in vivo* model consisted of a human tumor (A431 adenocarcinoma) grown s.c. in SCID/RAG-2 mice. This tumor cell line overexpresses the epidermal growth factor receptor (EGFR) and provided a good model for assessing binding of anti-EGFR antibody/liposomes following i.v. administration. In this study, the anti-EGFR antibody was covalently coupled to liposomes *via* linkages through modified amine ($-NH_2$) or carbohydrate (–CHO) moieties on the antibody. The plasma elimination and biodistribution characteristics of these antibody-coupled liposomes were evaluated following i.v. administration to A431 tumor bearing SCID/RAG-2 mice. The results showed that liposomes with surface associated antibodies were eliminated from the plasma more rapidly than control (protein free) liposomes. Specifically, 50% of the injected dose was eliminated from the plasma within 6 h for control liposomes and less than 1 h and 3 h for liposomes with antibodies attached *via* the $-NH_2$ and –CHO moieties, respectively. There was no improvement in tumor delivery as a consequence of antibody attachment. Both protein conjugated systems exhibited biphasic plasma elimination profiles and the profiles were not affected by techniques (e.g. blockade of the reticuloendothelial

system) that would typically engender a decrease in liposome elimination. These data suggested that the plasma elimination behavior of the antibody-coupled systems was dictated by mechanisms different then those involved in elimination of control liposomes. The possibility that two populations of liposomes were present following the protein coupling reaction was also investigated. This was assessed by separating antibody coupled liposomes on a protein G affinity column. The column results suggest that binding of antibody to the column promotes antibody dissociation from the liposome. In addition, the amount of antibody associated with liposomes recovered from the plasma following i.v. administration was measured by Western blot analysis. The results from these analyses identified that reduced antibody levels were detected in plasma samples which contain significant levels of liposomal lipid. FPLC analysis, of conjugated antibodies indicated that the antibody conjugated liposomes were stable in plasma. Together, these results indicated that covalently attached antibodies readily dissociate from liposomes upon binding and in an *in vivo* environment.

5.2 Discussion

Regardless of the targeting ligand used the results presented here highlight several important conclusions for targeted drug delivery of liposomes that must be achieved for these systems to become therapeutically useful. First, targeted liposomes must have reasonable plasma circulation times to allow access to a tumor site. Ample evidence demonstrates that liposomes accumulate in disease sites and in sites of infection and inflammation (Bakker-Woudenberg et al., 1992; O'Sullivan et al., 1988; Richardson et al., 1979; Gabizon and Papahadjopoulos, 1988), an effect primarily attributed to the presence of leaky vasculature in regions of necroses and neovascularization (Dvorak et al., 1988; Kohn et al., 1992). It is not clear whether extravasation is a consequence of active or passive transport mechanisms. Evidence indicates that the transport

of macromolecules *via* vesiculo-vacuolar-organelles can occur (Dvorak et al., 1988), but transport of targeted liposomes by this mechanism has not been observed to date. With regards to tumors, targeted liposomes must at the very least exhibit accumulation properties comparable to nontargeted liposomes.

Second, conjugated liposomes that have accessed the tumor interstitium must have the potential to bind a defined target cell. Nontargeted liposomes that localize within the tumor interstitium do not appear to interact with tumor cells (Matzku et al., 1990; Harasym et al., 1997). Therefore, the potential to achieve redistribution of these liposomes through targeting strategies exists. To date, however, no one has demonstrated *in vivo* specific targeting of a liposome to a tumor cell in an extravascular site. For this reason it has been argued that targeting strategies should focus on readily accessible targets such as those in the vascular compartment.

Several physical factors will play key roles in inhibiting liposome targeting following extravasation, factors such as size, nature of the vascular barrier, tumor structure, and binding site barriers. As was described in Chapter 1, methods are available to achieve uniformly small targeted liposomes. The other two factors, however, have been more challenging obstacles to overcome. The binding site barrier, nicely described by Fujimori for antibodies (1989), will likely prevent liposome diffusion within solid tumors. Further, high intratumor pressures may also limit liposome access to tumors (Jain and Baxter, 1988; Hori et al., 1992; Less et al., 1992).

Regardless of mechanisms mediating extravasation, it is known from animal studies that antibodies typically localize in tumors at levels not exceeding 0.1% of injected dose (Sung et al., 1992) while liposome accumulation is much greater, resulting in as much as 5 to 10% of the injected dose localizing in an extravascular site (Ogihara et al., 1986; Patel et al., 1985; Proffitt

et al., 1983; Gabizon et al., 1990). The movement of macromolecules, antibodies and presumably liposomes across the vascular barrier is likely governed by two physical forces, convection and diffusion (Jain and Baxter, 1988). Large molecule transfer is governed by convection or the flow of fluid from the lumen to the interstitium. Small molecule transfer is governed by diffusion or the movement from areas of high to low concentration (Jain, 1987). The leaky tumor neovasculature allows the two passive forces to work together to achieve reasonably efficient accumulation of conventional and PEG modified liposomes. What also needs to be defined are methods to increase targeted liposome circulation levels, to allow for these passive forces to augment tumor accumulation.

Beyond the targeting strategy proposed it needs to be recognized that the production of liposomes, with attached targeting ligands, requires an extensive number of modification steps. The feasibility of manufacturing a one step antibody-targeted liposome formulation under Good-Manufacturing-Practices (GMP) regulations of sterility and quality control is, therefore, difficult to envision. Over the last two decades liposome targeting strategies have been based on slight variations of old themes. This has not proven to be successful and identification of new technologies and new approaches to targeting must be promoted. Membrane spanning peptides to increase the association of ligand to liposome or ligand conjugation to glycosylphosphatidylinositol (GPI) anchors are approaches that should be investigated. The latter, as GPI anchored proteins are significant contributors to the anchoring of diverse populations of proteins in eukaryotes, with functions ranging from enzymes, adhesion molecules, activation antigens, and as differentiation markers (Brown and Waneck, 1992). The elimination of linker molecule chemistries by direct lipid or peptide conjugates will also aid targeted delivery systems by simplifying production and formulation methods. Clearly, studies focusing on small

molecules such as single chain antibodies, vitamins, and peptides linked to lipids are also of great interest.

Currently, major improvements in increasing extravasation of targeted liposomes to extravascular targets have not been achieved. Since efficient extravasation of targeted liposomes may be difficult to achieve, targeting to sites of neovascularization with subsequent release of encapsulated therapeutic agents which enter the tumor interstitium may provide a reasonable interim strategy. "Triggered" release of high concentrations of encapsulated drug upon antibody binding to vascular endothelial cells at a tumor site may effect significant increases in drug efficacy. Considering the excitement generated in the area of targeting ligands directed to adhesion molecules on vascular endothelial cells and exposed basement membranes, this strategy may be timely.

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