CIRCULATION LIFETIMES AND TUMOR ACCUMULATION OF LIPOSOMAL DRUG DELIVERY SYSTEMS

by

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ABSTRACT

One of the greatest benefits of liposomal encapsulation of therapeutic drugs is the tendency for these carriers to accumulate in sites of disease. In the case of a solid tumor model employed here, it was the goal of this thesis to maximize the drug delivery to this site. This requires an understanding of the major factors governing tumor delivery. Of central importance to liposomal delivery to solid tumors is the circulation lifetime of the carrier. In the first section of experiments, factors influencing the use of poly(ethylene glycol) (PEG)-lipids to increase the circulation lifetime of liposomes was examined. Second, the observation that drug loaded liposomes last significantly longer in the circulation was more fully examined. And finally, the delivery of drug to a murine solid tumor was assessed and the influence of PEG-lipids in the drug carrier and the effect of entrapped drug has on its delivery determined.

PEG-lipid anchor conjugates can prolong the circulation lifetimes of liposomes following intravenous injection, but this can depend upon the nature of the lipid anchor and the chemical link between the PEG and lipid moieties. Incorporation of various PEG-lipids into large unilamellar vesicles (LUVs) composed of distearoylphosphatidylcholine (DSPC) and cholesterol (chol) (DSPC/chol/PEG-lipid, 50:45:5 mol/mol) results in differing liposomal circulation lifetimes in mice. This is shown to be due to differential removal of the hydrophilic coating in vivo that arises from exchange of the entire PEG-lipid conjugate from the liposomal membrane, although chemical breakdown of the PEG-lipid conjugate is also possible. This work establishes that DSPE is a considerably more effective anchor for PEG_{2000} than POPE and that the chemical stability of PEG-PE conjugates is sensitive to the nature of the linkage and exchangeability of the PEG-PE complex. It is suggested here that retention of the PEG coating is of paramount importance for prolonged circulation lifetimes.

The influence of entrapped drug on the circulation lifetimes of liposomal carriers was investigated next. Pre-doses of liposomally entrapped doxorubicin blocked the accumulation of subsequently injected liposomes in the reticuloendothelial system (RES). This effect is termed RES blockade. Liposomal drug doses as low as 2 mg/kg can induce maximum RES blockade within 24 h after administration, and this effect lasts as long as 8 days. Full recovery is only achieved by 14 days. Another commonly employed liposomal anti-cancer drug, vincristine, has effects that are similar in magnitude, but more transient, allowing recovery of the RES within 2 to 4 days. Liposomes incorporating PEG-lipids or ganglioside G_{M1} are proposed to avoid the RES, however it is shown that when loaded with doxorubicin these liposomes also induce RES blockade and do not avoid uptake by the RES. Rather, these lipids engender a decrease in the rate of uptake by cells of the RES.

The final set of experiments consisted of a comparison of tumor accumulation and efficacy properties of doxorubicin entrapped in liposomes incorporating PEG-lipids versus conventional liposomes by monitoring drug pharmacokinetics and tumor accumulation at the maximum tolerated dose (MTD)(60 mg/kg liposomal doxorubicin). The tumor model consisted of mice bearing Lewis Lung carcinoma solid tumors. In contrast to expected behavior, the efficiency of doxorubicin accumulation at the tumor site, evaluated with an area under the curve analysis, was higher for conventional liposomes than for the sterically stabilized liposomes. Both formulations, however, exhibited profound increases of over 500-fold in tumor accumulation of drug as compared to free drug injected at the MTD (20 mg/kg doxorubicin). These studies suggest that optimization of factors nominally leading to longer blood circulation times do not provide therapeutic advantages for liposomal formulation of doxorubicin administered at the MTD. The

dominant factor influencing the circulation lifetime for both liposomal carrier systems appears to be that of entrapped drug, consistent with RES blockade described in this thesis. Improvement in other parameters, such as drug leakage rates, hold greater promise for improving therapeutic properties of liposomal drug carriers.

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ABBREVIATIONS

ApoA-1	apolipoprotein A-1
AUC	area under the curve
chol	cholesterol
CHE	cholesteryl hexadecyl ether
C_{Tmax}	peak tumor drug concentration levels
DCC	N,N'-dicyclohexylcarbodiimide
DOX	doxorubicin
DSPC	distearoyl phosphatidylcholine
EDTA	ethylenediaminetetra-acetic acid
EPC	egg phosphatidylcholine
FATMLV	frozen and thawed LUVs
G_{M1}	monosialoganglioside G_{M1}
GM-CSF	granulocyte-macrophage colony stimulating factor
HAMA	human anti-mouse antibody
HBS	HEPES buffered saline
HDL	high density lipoprotein
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane-sulphonic acid
IL-2	interleukin-2
i.p.	intraperitoneal
i.v.	intravenous
IgG	immunoglobulin G
LLC	Lewis Lung carcinoma
LUV	large unilamellar vesicles
LUVET	LUVs prepared by extrusion
MePEG	monomethoxypoly(ethylene glycol)
MLV	multilamellar vesicles
MPS	mononuclear phagocyte system
MTD	maximum tolerated dose
NHS	N-hydroxysuccinimide
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEG	poly(ethylene glycol)
PEG-PE	poly(ethylene glycol)-modified phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
QELS	quasielastic light scattering
RES	reticulo-endothelial system
SUV	small unilamellar vesicles
TAM	tumor associated macrophage
T_e	targeting efficiency
t_B	tumor bearing
t_F	tumor free
VEGF	vascular endothelial growth factor
VINC	vincristine
VLS	vascular leak syndrome
VPF	vascular permeability factor
VVO	vesiculo-vacuolar organelles

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I must especially thank two people: Marcel Bally, for all his encouragement, advice and ideas over the years; and of course Pieter Cullis, for providing such an outstanding and enjoyable environment in which to work in addition to his encouragement and advice.

To my wife,

Kristi

CHAPTER 1

INTRODUCTION

1.1 Project overview: liposomes as drug carriers

The concept of a "magic bullet" or targeted drug delivery system for the treatment of human disease was first proposed long ago (Ehrlich, 1906), but it is only recently that significant advances toward this goal have been made. In particular, three basic approaches for the delivery of drugs to disease sites hold promise. These include antibody-drug conjugates, and two carrier based approaches, polymers and liposomes. Advantages and disadvantages of these systems are outlined in Table 1.1. While some of the initial problems with antibody-drug conjugates such as altered antibody and drug properties (Sung et al., 1992: Thomas et al., 1989) and immune responses (Khazaeli et al., 1994) can be overcome in particular cases (Trail et al., 1993; Hale et al., 1988), basic limitations remain. These include the amount of drug that can be attached to the antibody (Ghose and Blair, 1987)(and thus the amount of drug which can be delivered in high concentration to the intended target), the need to re-engineer the antibody-drug complex for each drug or antibody employed (Trail et al., 1983), and an inability to protect the active agent from degradative enzymes in the physiological milieu (Weinstein and van Osdol, 1992).

As a result there has been interest in carrier based technologies. These systems include polymer based carriers and lipid based carriers, both of which can overcome the limitations mentioned above. Polymer based carriers, including nanoparticles and microparticles (reviewed in Brannon-Peppas, 1995), are small particulate polymer constructs capable of carrying large

Table 1.1

Comparison of Different Drug Carriers Used for Systemic Delivery

Drug Carrier System	Advantages	Disadvantages
Antibodies	specific targeting; relatively low MW increases access to extravascular site	low drug-carrying capacity; immunogenicity;
Polymer-based	drug stability; low immunogenicity; controlled release; high drug carrying capacity	RES uptake; limited extravascular access; difficult pharmaceutical manufacture; chemical drug modification; potential toxicity
Liposomes	drug stability; low immunogenicity; controlled release; high drug carrying capacity	RES uptake; limited access to extravascular site

amounts of a pharmaceutical agent. Lipid based systems, including liposomes and lipoproteins, have the ability to entrap biologically active agents in an interior compartment. Liposomes, more specifically, have undergone considerable development since their initial characterization in 1965 (Bangham et al., 1965). Liposomes can offer flexibility with regard to choice of entrapped drug(s), and have the potential for sophisticated modifications, including specific attachment of targeting components, controlled drug release properties, and fusogenic capacity as would be required for intracellular delivery.

One of the first benefits noted for liposomal drug systems was the reduction in toxicity over the free drug for very simple "non-targeted" systems consisting only of the drug encapsulated inside lipid vesicles (Rahman, 1982; Olson et al., 1982). This resulted in liposomal formulations of

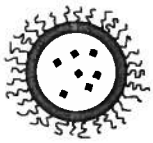
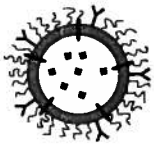
anticancer drugs (Cowens et al., 1993) and antifungal agents (Madden et al., 1990a) that are now completing advanced clinical trials (Cowens et al., 1993; Conley et al., 1993; Gabizon et al., 1994a; Harrison et al., 1995). The reduction in toxicity has been attributed to the elimination of peak plasma concentrations of free drug because most of the drug is sequestered within the carrier and leaks out slowly. Chemotherapeutic agents often exhibit steep dose response curves, but are limited by the toxicity associated with high free drug doses (Minow et al., 1975; Livingston, 1994). Liposomal encapsulation maintains and often increases anti-tumor potency and efficacy (Gabizon et al., 1982; Mayer et al., 1990b;). Furthermore, the reduced toxicity allows higher doses of drug to be administered in the liposomally encapsulated form (Gabizon et al., 1986; Bally et al., 1990b).

The second benefit of liposomal drug carriers results from the increased circulation lifetimes of liposomally encapsulated drug as compared to the free drug. It has been suggested that liposomes can serve as circulating reservoirs for slow release of drug in the blood compartment (Mayer et al., 1990a; Allen et al., 1992). However, one of the biggest benefits of liposomes is the tendency of this carrier to accumulate in sites of disease and act as local reservoirs for release of drug directly into the affected tissue (Huang et al., 1992, Gabizon, 1992, Mayer et al., 1990a). Such "passive targeting" is strongly correlated with longer circulation times of the carrier (Gabizon and Papahadjopoulos, 1988; Gabizon et al. 1990; Gabizon, 1992).

Attempts to further extend the circulation lifetimes and thus the passive targeting of liposomal drug carriers have thus far focused on size and surface properties of the liposomes. Important refinements included (i) incorporation of long-chain saturated lipids for better drug retention and physical and chemical stability, and (ii) reduced liposome size and neutral surface charge for

Table 1.2

Liposome Drug Carrier Technology

Technology	Description	Utility	Stages of Development
first generation: base carrier: 	natural and/or synthetic phospholipids with encapsulated drug	reduced toxicity enhanced efficacy passive targeting within disease site	Phase I, II clinical trials complete Phase III initiated
second generation: "stealth" 	sterically stabilized liposomes (SSL): incorporation PEG-lipids	improved circulation time: improved passive targeting to disease site	approved by FDA in US
third generation: 	sterically stabilized liposome surface associated targeting information (antibodies) thermosensitive? triggered release?	specific target cell delivery improved therapeutic index	experimental

improved circulation times. These first generation systems are in advanced clinical trials in humans for several anti-cancer drugs, including doxorubicin which is now being tested in Phase III trials (Cowens et al., 1993; Conley et al., 1993; Gabizon et al., 1994a; Harrison et al., 1995). Some of these formulations include the so called "sterically stabilized" liposomes, which

incorporate surface modifying agents such as the ganglioside G_{M1} or PEG-lipid conjugates where the polymer poly(ethylene glycol) is covalently attached to the head group of a lipid anchor. Liposomes incorporating these components can have increased circulation lifetimes as compared to formulations which do not incorporate these lipids (reviewed in Allen, 1994). The next generation of liposomal drug carriers include "actively" targeted systems which contain targeting ligands such as antibodies coupled to the liposome surface (Loughrey et al., 1993; Longman et al., 1995).

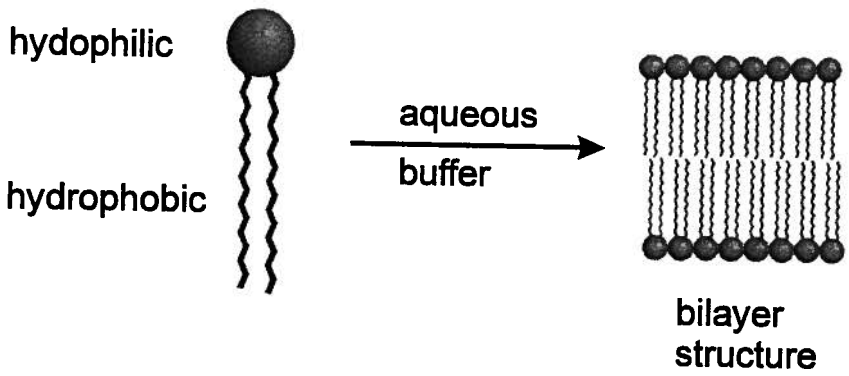
It is proposed here that actively targeted systems must first achieve maximal levels of passive targeting to sites of disease before the benefits of active targeting to particular diseased cells can be realized. Part of this thesis characterizes passive targeting of liposomes in a rodent tumor model, and included the use of sterically stabilized systems and entrapped drug to influence passive drug targeting to a disease site.

1.2 Liposomes

Dispersion of bilayer forming lipids in aqueous media results in the spontaneous formation of liposomes. The multilamellar structure was first described by Bangham et al., (1965) as an onion skin arrangement of concentric lamellae. These lamellae consist of lipids in the bilayer configuration, a structure that arises as a result of the "amphipathic" nature of lipids. The combination of a hydrophilic head group and hydrophobic tails within the same molecule results in an orientation of the lipid head group towards the aqueous environment and the acyl tails toward each other, as depicted in Figure 1.1. Liposomes have been employed as model membranes for studies on the structural and functional roles of lipids in membranes and as

Figure 1.1

Amphipathic lipids in bilayer configuration



matrices for reconstituted membrane proteins. These systems allow investigation of processes such as membrane fusion (Bailey and Cullis, 1994; van Meer et al., 1985), factors leading to complement activation (Devine et al., 1994), protein-lipid interactions, among many other applications. For example, because liposomes form closed spheres with defined interior and exterior aqueous spacesseparated by lipid bilayers, they are excellent tools to study membrane permeability and ion gradient formation (Deamer and Nichols, 1983; 1989; Viero and Cullis, 1990), and the distribution of various molecules in response to these gradients, such as

neurotransmitter uptake and storage (Nichols and Deamer 1976; Bally et al., 1988). The utility of liposomes as drug delivery vehicles will be covered in later sections.

1.2.1 Chemistry and physics of lipids

Most of the liposomal drug delivery systems in use today are composed of a combination of phospholipids and cholesterol. These components are discussed below. Other membrane components will be discussed in the context of more recent advances in this carrier technology.

1.2.1.1 Phospholipids

Phospholipids are composed of various combinations of polar (hydrophilic) headgroups coupled to apolar (hydrophobic) tails via a glycerol-phosphate backbone. A summary of phospholipid structure is shown in Figure 1.2. Each combination of headgroup and acyl chain composition dictates the physical properties of the lipid bilayer. For example, at physiological pH liposomes containing phosphatidyl serine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidic acid (PA) will have a negative surface charge. This charge is an important factor determining serum protein binding (Moghimi and Patel, 1989), which leads to the clearance of liposomes from the circulation as will be discussed later.

Another important parameter for characterizing phospholipids is the temperature of the gel to liquid-crystalline phase transition for bilayers that is principally dependent upon the length and saturation of the acyl chains. In general, longer, more saturated acyl chains give rise to higher phase transition temperatures (T_c). Acyl chain motion is often characterized by an order

Figure 1.2

Structures of common phospholipids

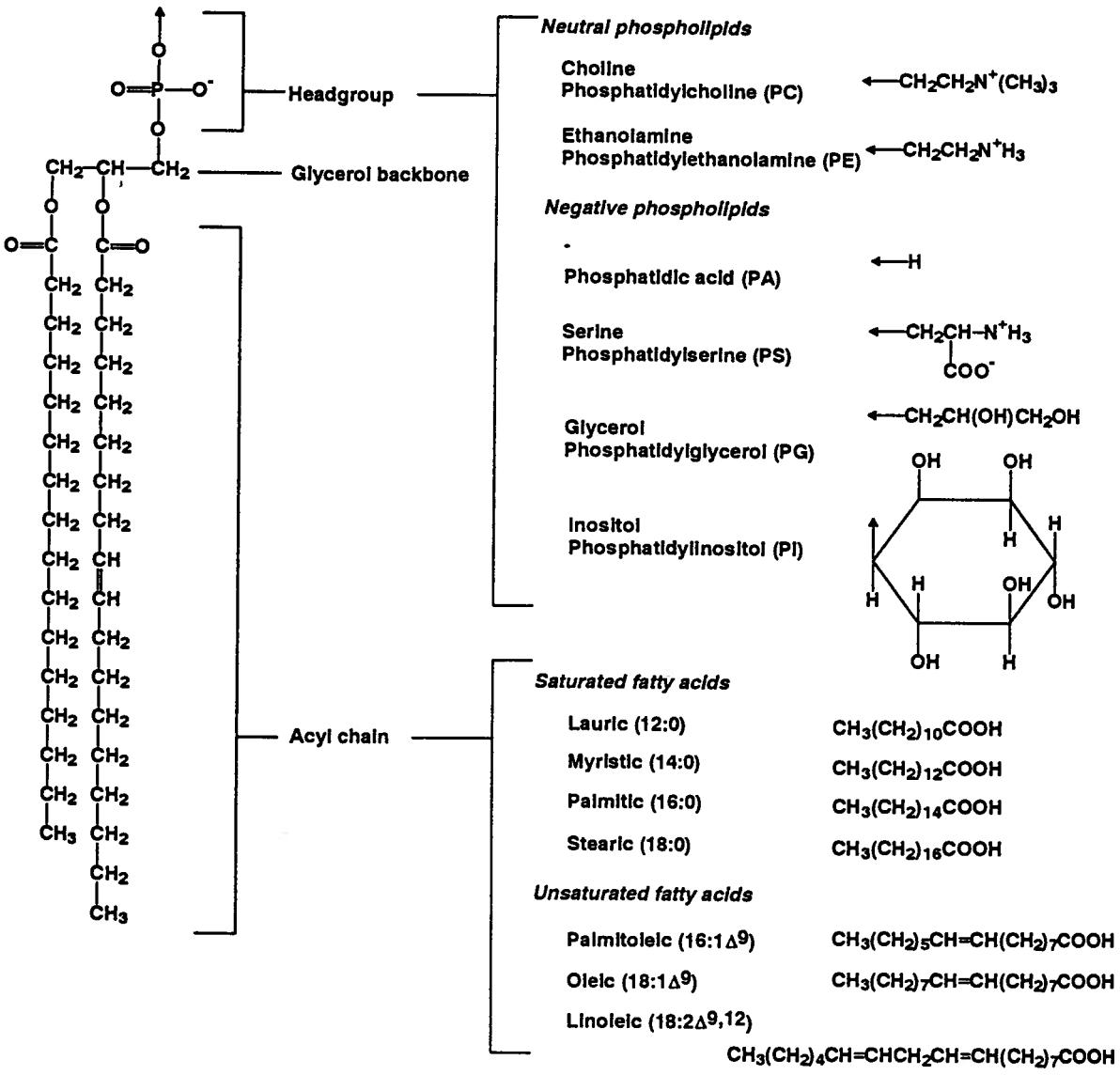


Table 1.3

Transition temperatures (T_c) of various combinations of acyl chain length, degree of saturation, and headgroup moiety

lipid species	transition temperature T_c (°C)
dilauroyl PC (12:0, 12:0)	-1
dimyristoyl PC (14:0, 14:0)	24
dipalmitoyl PC (16:0, 16:0)	41
distearoyl PC (18:0, 18:0)	55
stearoyl, oleoyl PC (18:0, 18:1)	6
stearoyl, linoleoyl PC (18:0, 18:2)	-13
dipalmitoyl PA (16:0, 16:0)	67
dipalmitoyl PE (16:0, 16:0)	63
dipalmitoyl PS (16:0, 16:0)	55
dipalmitoyl PG (16:0, 16:0)	41

parameter "s" where s = 1 for no motion, whereas for rapid isotropic motion s = 0. Below the T_c, the acyl chains have a high "order" (s ~ 1) in that their motion is greatly restricted and the chains pack together in a frozen or "gel" phase. Above the T_c, the acyl chains are less ordered or more "fluid" in nature in what is termed the "liquid-crystalline" phase. Longer acyl chains have increased order whereas unsaturated acyl chains disrupt packing and reduce the acyl chain order of the membrane. In addition, the headgroup can also influence the T_c. These properties are summarized in Table 1.3. In general, membranes are more permeable to a variety of solvents and solutes at or above the T_c than below (Bittman and Blau, 1972). Increased membrane permeability has also been correlated with increased unsaturation or shorter acyl chains (Papahadjopoulos et al., 1973).

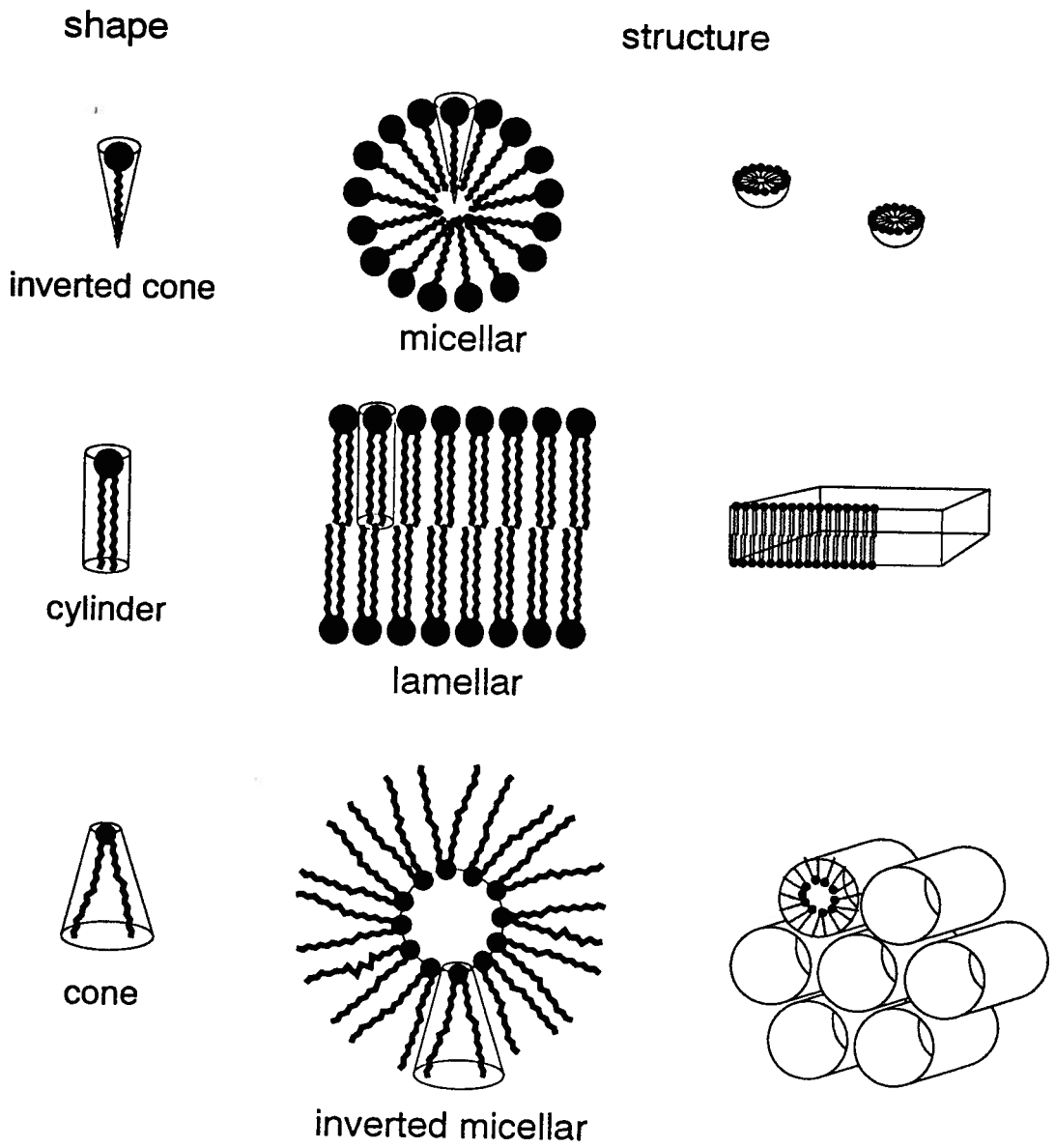
Hydrated phospholipids can adopt a wide variety of structures, the bilayer being only one of them (Figure 1.3). The ability of lipids to adopt structures other than the bilayer is described as lipid polymorphism (Cullis et al., 1986). Some lipids have head groups which occupy large volumes in comparison to their tails, and can be thought of as adopting a cone shape and thus have a tendency to form micelles (see Figure 1.3). These lipids include detergents. Unsaturated species of PE, on the other hand, in general will not form bilayers due to the relatively small neutral headgroup and more flexible acyl chains that are conceptually thought to resemble inverted cones. These lipids tend to form inverted hexagonal or H_{II} phase structures (Cullis and de Kruijff, 1979). Most of the lipids employed for liposomal drug carriers fall into the bilayer forming class, and can be thought of as exhibiting a cylindrical shape. These lipids include phosphatidylcholines, commonly employed as the base phospholipid in liposome compositions for drug delivery.

Other factors can also modulate lipid polymorphism, including the effects that temperature has acyl chain fluidity, and the influence of pH and ionic strength on head group charge (Cullis et al., 1986). As discussed above, more mobile acyl chains increase the relative volume of the hydrophobic portion, whereas charged headgroups effectively increase the volume occupied by the hydrophilic portions. The particular combination of tail and head group relative size determines the relative shape of the molecule as described in Figure 1.3. For example, unsaturated PS has a net negative headgroup charge at physiological pH, however upon lowering the pH, protonation of its carboxyl group results in net neutralization. The neutral headgroup occupies a smaller area due to the reduction in bound water thereby promoting formation of H_{II} phase structures.

Figure 1.3

Lipid polymorphism

Some examples of common shapes and resulting lipid shapes are shown below



1.2.1.2 Cholesterol

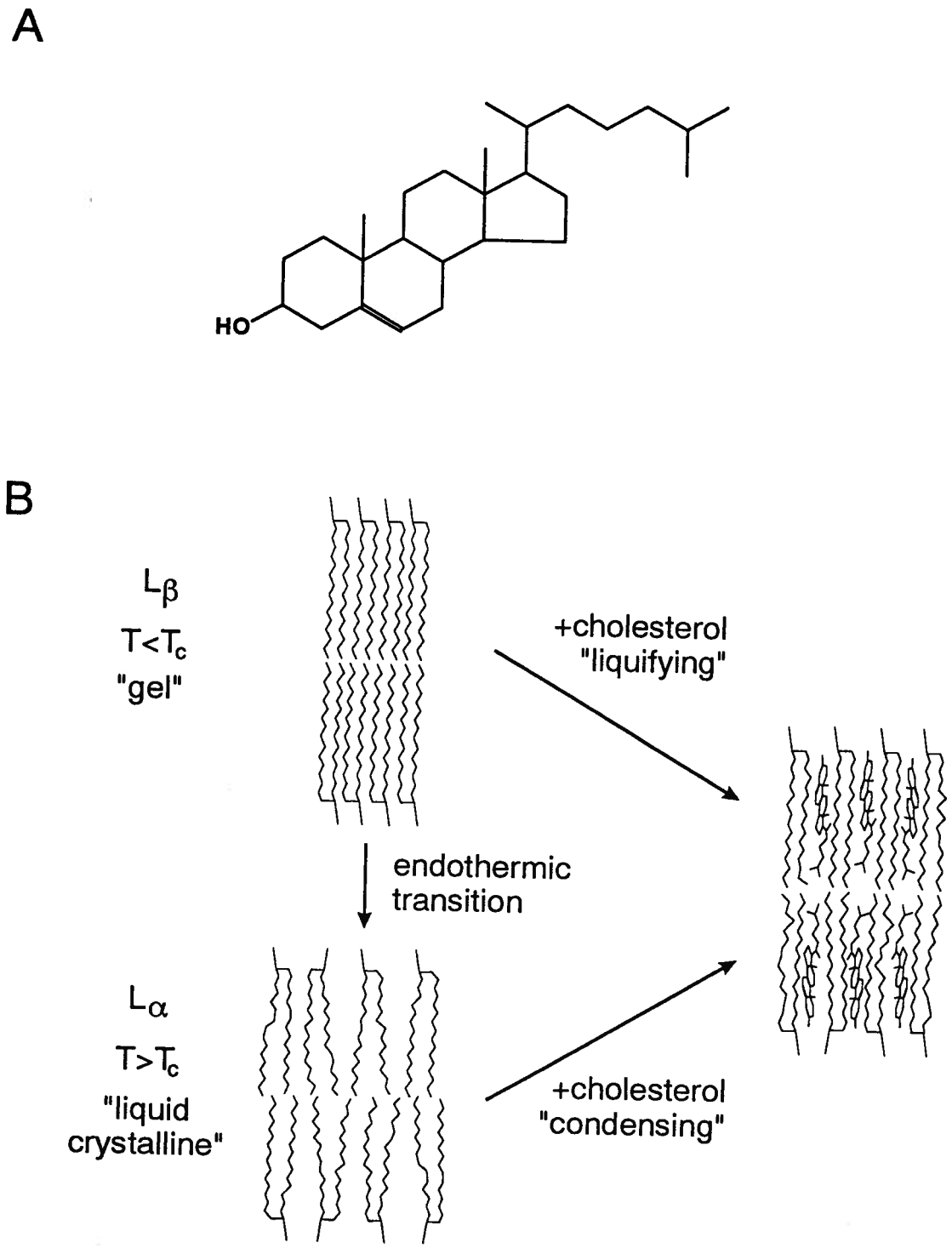
Cholesterol is the major neutral component of almost all eukaryotic biological membranes. Cholesterol is an amphipathic molecule due to the polar 3-β-hydroxyl group that is oriented toward the lipid/water interface but sits buried next to the carbonyl groups of the fatty acyl chains near the head groups, and the rigid steroid ring lying associated with the acyl chains. The more flexible aliphatic tail lies deepest in the membrane. Incorporation of cholesterol decreases the membrane order for phospholipids in the gel phase (i.e. below their T_c) and increases the order of the membrane for lipids in the liquid-crystalline phase (above their T_c; Demel and de Kruijff, 1976). Increasing the cholesterol content above 7 mol % reduces the enthalpy of the gel to liquid-crystalline phase transition, until at 33 mol % and higher, the phase transition can no longer be detected (Hubbell and McConnel, 1971). The addition of cholesterol to both unsaturated and saturated PC membranes above their gel to liquid-crystalline transition temperature decreases membrane permeability, while increasing permeability for membranes composed of saturated PC below the T_c (Bittman and Blau, 1972). In addition, the inclusion of cholesterol helps to stabilize liposomes used for systemic delivery of drugs (Papahadjopoulos et al., 1973), primarily as a result of reduced lipid exchange with lipoproteins (Kirby et al., 1980; Hunt, 1982). This latter role of cholesterol is discussed in detail in Section 1.4.

1.2.2 Liposome preparation

Categories of liposomes include multilamellar vesicles (MLVs), large unilamellar vesicles (LUVs), and small unilamellar vesicles (SUVs) as depicted in Figure 1.5 and depend upon liposome size and the number of bilayers (lamellarity) contained within each vesicle.

Figure 1.4

The structure of cholesterol (*A*) and its effect on the structure of lipid bilayers (*B*)



1.2.2.1 Multilamellar vesicles (MLVs)

Dispersion of a lipid powder or a dry lipid film by mechanical agitation (e.g. vortex mixing) in aqueous solution results in the spontaneous formation of MLVs composed of concentric bilayers. The resulting vesicles are heterogeneous in size, typically ranging in size from 1-10 μm, and can be multilamellar to the extent that less than 10% of the total lipid is present in the outermost bilayer (Mayer et al., 1985).

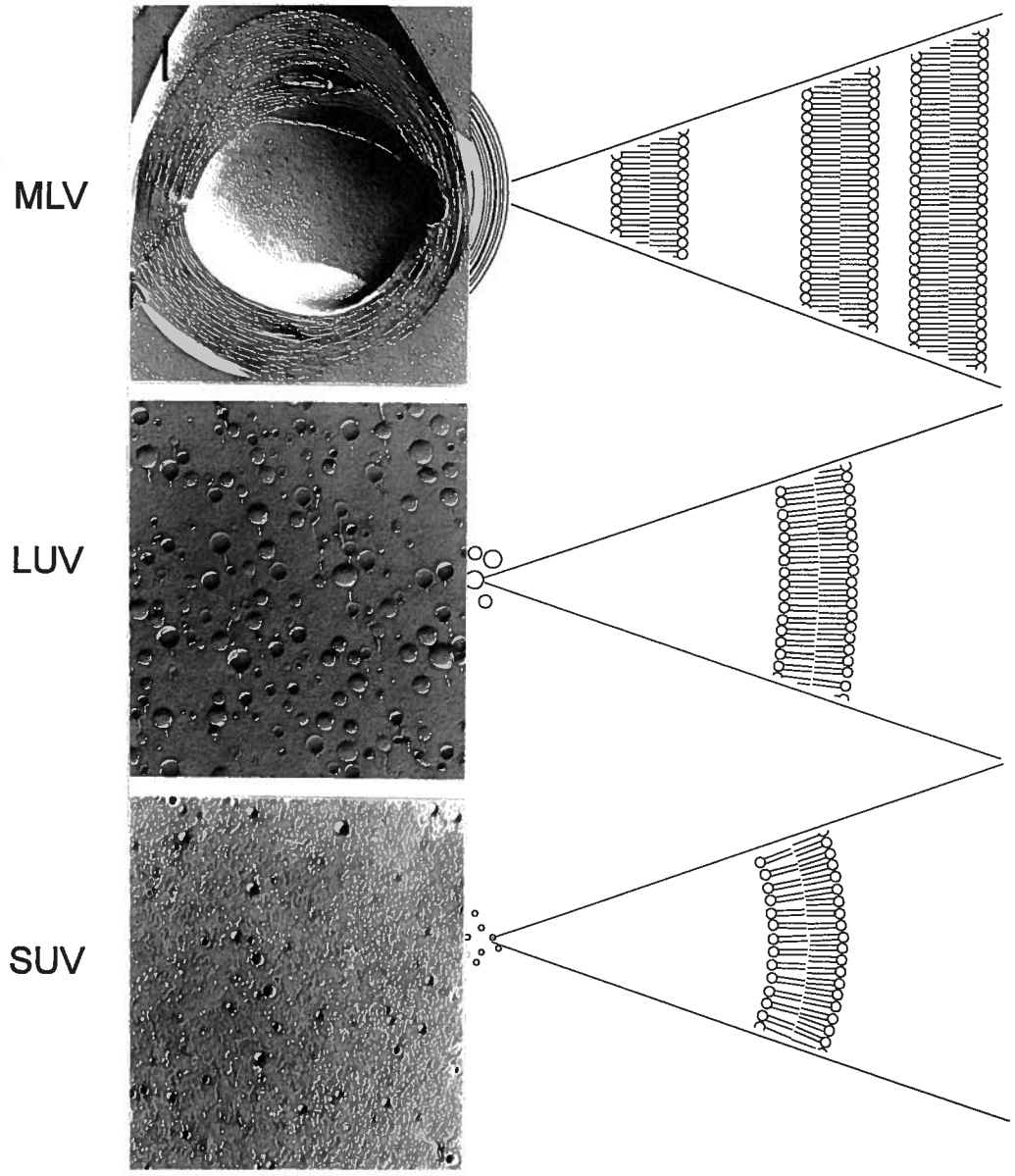
These MLVs typically have low aqueous trapped volumes (of the order of 0.5 μl/μmol lipid) due to the close packing of adjacent lamellae. Trapped volumes can be increased by incorporation of charged lipids that lead to charge repulsion between bilayers and increased interbilayer spacing (Hope et al., 1986). Alternatively, repeated cycles of freezing and thawing can also increase the interbilayer spacing allowing trapped volumes of approximately 7 μl/μmol lipid to be achieved. These MLVs are often referred to as frozen and thawed MLVs (FATMLVs; Mayer et al., 1985).

Reverse phase evaporation (Szoka and Papahadjopoulos, 1978; 1980) is an important alternative method for the production of MLVs with large trapped volumes and increased intralamellar spacing. Briefly, the lipid is first dissolved in organic solvent. Then, as the solvent is diluted or evaporated away in the presence of aqueous buffer, the lipids move from the organic to aqueous phase forming vesicles in the process. While MLVs prepared by this method can have high trapped volumes (up to 10 μl/μmol), this procedure can be limited by low lipid solubility of certain lipids in the organic solvent as well as difficulties in removing residual solvent from the final aqueous preparation.

Figure 1.5

Liposome morphology

Freeze-fracture electron microscopy of multilamellar vesicles (MLVs), large unilamellar vesicles (LUVs), and small unilamellar vesicles (SUVs) prepared from egg phosphatidylcholine (EPC). (The bar in the upper left hand corner represents 200 nm)



1.2.2.2 Small unilamellar vesicles (SUVs) large unilamellar vesicles (LUV)

Unilamellar vesicles (SUVs and LUVs) are grouped by size, usually 25-50 nm, and 50-200 nm, respectively. SUVs can be produced directly from MLVs by several methods, including sonication (Huang, 1969) and French press (Barenholz et al., 1979). The limiting factor in terms of size appears to be the maximum curvature that the bilayer can assume as a result of packing constraints generated by the small radius. SUVs can have a outer to inner monolayer lipid ratio as high as 2:1, and typically have low trapped volumes (~0.2 μl/μmol; Huang, 1969; Barenholz et al., 1979). SUVs are often unstable because of the thermodynamically unfavorable stresses resulting from their small radii and can spontaneously fuse into larger structures over time with the loss of their entrapped contents (Wong et al., 1982). These properties tend to make SUVs poor choices for drug carriers.

LUVs are more useful as drug carriers because the higher trapped volumes result in the potential for greater drug encapsulation and the greater membrane stability results in longer circulation lifetime and stability of the carrier within the circulation. There are several means for producing LUVs. Detergent dialysis (Mimms et al., 1981) involves solubilization of either dried lipid or pre-formed vesicles in buffer containing detergent. The resulting mixed micelles are then dialyzed against buffer to remove the detergent resulting in the formation of LUVs. The size and trapped volumes depend upon the detergent, the rate and method of detergent removal, and the lipid composition (Madden, 1986). It is a relatively gentle method compatible with protein integrity and thus is often used in studies involving the reconstitution of membrane proteins (Helenius et al., 1977). A problem often associated with this method, however, is the presence of

residual detergent. Other methods include ethanol injection (Chen and Schullery, 1979), ether infusion (Deamer and Bangham, 1976), and reverse phase evaporation (Szoka and Papahadjopoulos, 1978) which are similar to that described for MLVs in that lipid dissolved in an organic solvent are hydrated in aqueous buffer and then the organic solvent is slowly removed. In these methods more precise control of organic solvent removal can result in large unilamellar vesicles. Problems regarding residual solvents or detergents limit applications of these procedures for production of liposomal drug carriers.

One of the most general methods for the production of LUVs involves extrusion. This typically relies on forcing either MLVs or FATMLVs through polycarbonate filters of a defined pore size (Szoka and Papahadjopoulos, 1980; Hope et al., 1985; Mayer et al., 1986). The resulting vesicles have well defined sizes (close to the pore size) and are largely unilamellar if the filter pore size is less than 200 nm. The technique requires that the extrusion step be carried out repeatedly (typically 10×) at a temperature above the transition temperature (T_c) of the phospholipid component. Some advantages of this method are that it allows high lipid concentrations to be used, a wide variety of bilayer forming lipid compositions can be prepared, and it is simple, rapid, and reproducible. LUV sizes around 100 nm prepared by this method have trapped volumes of 1-3 µl/µmol. These LUVs are sometimes referred to as LUVETs (LUVs by extrusion technique). They are useful as drug carriers since the procedure is amenable to pharmaceutical production, allowing control of quality and sterility and does not require residual solvent or detergent.

1.2.3 Drug encapsulation

There are two basic strategies for encapsulating drugs within liposomes. In passive trapping techniques, the drug is combined with the lipid during liposome formation. For aqueous agents, encapsulation depends upon the trapped aqueous volume of the liposome, whereas for lipophilic compounds, encapsulation efficiency usually depends on the capacity of the bilayer to solubilize the agent while maintaining vesicle structure. A second basic trapping method is termed active trapping and involves the encapsulation of the drug in pre-formed vesicles exhibiting transmembrane ion gradients. Many anti-cancer drugs are now encapsulated using procedures based on active loading.

1.2.3.1 Passive entrapment

Hydrophobic drugs can be passively trapped by including the agent in the original lipid mixture prior to liposome generation. Amphotericin B is one example of a drug which is entrapped in this manner (Madden et al., 1990a). Depending on the packing constraints and the lipid characteristics determining compatibility with drug incorporation into the membrane, this technique can have a high efficiency of incorporation. However, drugs of this class often exhibit appreciable exchange rates into other membranes and thus in vivo the drug can often rapidly leave the carrier (Madden et al., 1990a).

The passive entrapment of water soluble drugs into liposomes is similar to hydrophobic drugs in that both drug and lipid are combined in the original preparation prior to vesicle formation but depends on the aqueous trapped volume of the vesicle preparation. For SUVs with low trapped

volumes (0.2 µl/µmol), efficiency of entrapment is typically around 1% (Szoka and Papahadjopoulos, 1980). For FATMLVs and LUVs with trapped volumes ranging from 1-10 µl/µmol combined with high lipid concentrations of the order of 400 mg lipid/ml, efficiencies of up to 80% can be achieved (Mayer et al., 1985). The retention of water soluble drugs depends on both the membrane and drug. For relatively membrane impermeable drugs such as methotrexate and cytosine, the half-lives for retention in EPC/chol vesicles are 50 and 18 hours, respectively (Madden et al., 1990b). On the other hand, passively entrapped doxorubicin, which has lipophilic qualities, has a retention half-life of less than 1 hour in identical vesicles (Juliano and Stamp, 1975). As discussed earlier, the incorporation of longer chain saturated acyl chains in the lipid species combined with cholesterol can dramatically improve the retention of these drugs. For both lipid and aqueous entrapment procedures, the use of MLVs also usually results in improved retention because of increased numbers of lamellar barriers to pass to reach the external medium.

1.2.3.2 Active (ΔpH) entrapment

Active loading procedures are designed to load drug into the interior of pre-formed liposomes. Drugs that can be entrapped by this method are typically lipophilic cations or anions with an ionizable amino or carboxyl function, respectively. For example, drugs that are weak bases, such as doxorubicin or vincristine, will accumulate inside liposomes in response to a proton gradient, ΔpH, where the liposomes have an acidic interior. The mechanism for this accumulation is shown in Figure 1.6. In the external environment a proportion of the weak base exists as the neutral form along with the charged (protonated) form of the drug. In the neutral form the drug is more membrane permeable (Addanki et al., 1968; Rottenberg, 1979) and able to cross the bilayer

readily. However, on arrival in the acidic interior, the weak base becomes protonated. The charged species is not significantly membrane permeable, and is trapped within the interior.

The equilibrium transbilayer distribution of this weak base can be described by a simple derivation. The equilibrium constant (K_a) of a weak base B is:

$$K_a = H^+ \bullet B/BH^+$$

where H^+ is the proton activity, B is the activity of the neutral base, and BH^+ is the activity of the protonated base. To a first approximation activity can be replaced by concentration. The relative concentration of the neutral and protonated weak base at a given pH are therefore described by the Henderson-Hasselbach equation:

$$pH = pK_a + \log\{[B]/[BH^+]\}$$

If the dissociation constants for a weak base are the same on both the $inside_i$ and $outside_o$ of the vesicle membrane, then:

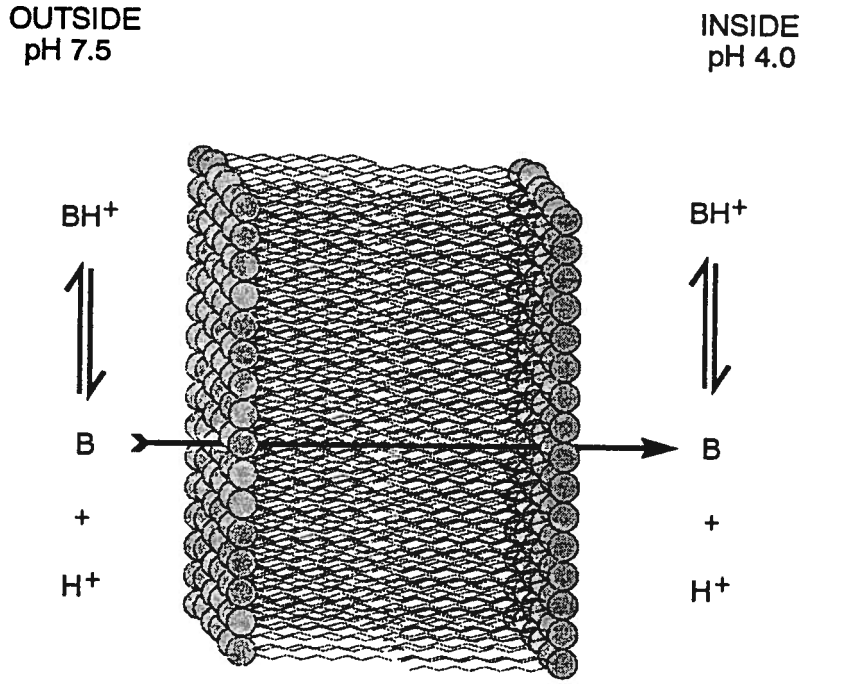
$$K_a = [H^+]_i[B]_i/[BH^+]_i = [H^+]_o[B]_o/[BH^+]_o$$

As noted previously, in general the uncharged species of an ionizable compound tends to be more membrane permeable than the corresponding charged species. As a result, at equilibrium the concentration of the neutral species will be the same on both sides of the membrane. The

Figure 1.6

Active drug entrapment in liposomes

The equilibrium redistribution of a lipophilic amine (weak base) in response to a pH gradient (ΔpH) across the liposome membrane. Only the neutral form of the molecule is significantly membrane permeable.



$$K_a = \frac{[B]_o[H^+]_o}{[BH^+]_o} \qquad K_a = \frac{[B]_i[H^+]_i}{[BH^+]_i}$$

At equilibrium, if:

$$[B]_o = [B]_i$$

Then:

$$\frac{[BH^+]_i}{[BH^+]_o} = \frac{[H^+]_i}{[H^+]_o}$$

above equation can therefore be simplified and the equilibrium distribution will then reflect the pH gradient according to:

$$[BH^+]_i/[BH^+]_o = [H^+]_i/[H^+]_o$$

This indicates that for a ΔpH of 3 units, for example, a weak base may be accumulated to an interior concentration 1000 fold higher than in the exterior medium. For 100 nm diameter LUVs composed of EPC or EPC and cholesterol, doxorubicin active trapping efficiencies can approach 100% at drug to lipid mol ratios of 0.2 or lower (Madden et al., 1990b). In an analogous fashion, weak acids can also be accumulated into liposomes with oppositely directed pH gradients (interior basic; Eastman et al., 1991).

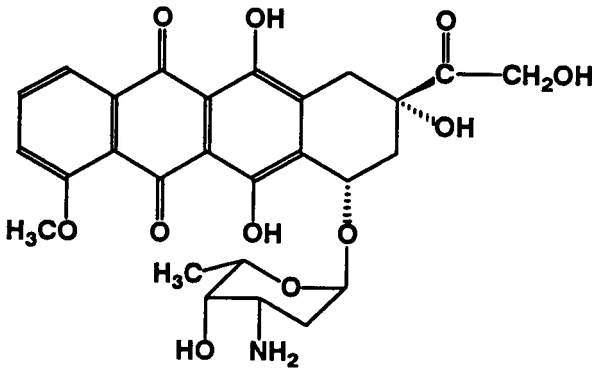
The generation of a pH gradient across the LUV membrane is straightforward and simply involves the exchange of the external buffer for a buffer of a different pH. Methods include size exclusion chromatography or simple titration of the external buffer to a new pH. In some cases dialysis of the external buffer may also be useful. pH gradients for several PC membrane species appear to be stable for hours or days even at elevated temperatures (Harrigan et al., 1992), while the addition of cholesterol further retards the decay of the imposed ΔpH (Madden et al., 1990b). The maximum ΔpH that can be stably maintained in EPC/cholesterol LUVs is ~3.7 units (corresponding to a membrane potential $\Delta\psi$ of approximately 220 mV; Harrigan et al., 1992).

Many other drugs are also lipophilic weak base amines and thus the mechanism of ΔpH entrapment is of general utility. A survey of drugs (Madden et al., 1990b) illustrates the

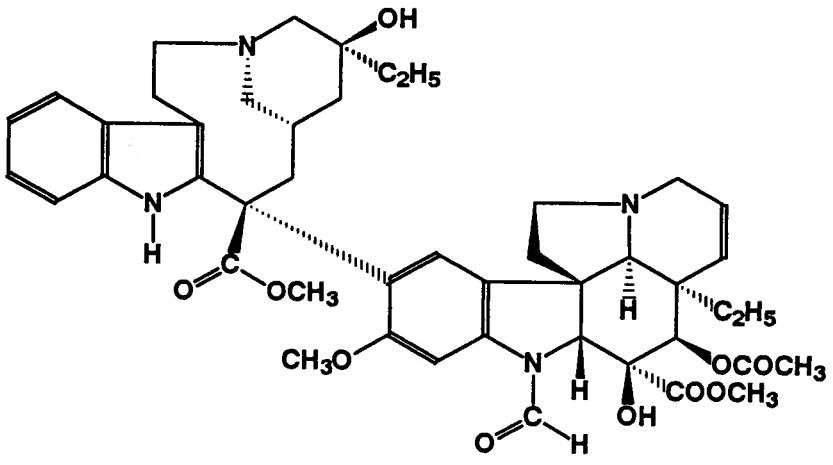
Figure 1.7

Structure of doxorubicin and vincristine.

DOXORUBICIN



VINCRISTINE



generality of ΔpH dependent entrapment, but while most redistribute in response to a transmembrane proton gradient, the level and stability of accumulation varies considerably. Poor uptake or subsequent release of most drugs was shown to be primarily due to loss of the pH gradient. For example, vincristine leakage appears to be directly related to eventual dissipation of the ΔpH (Boman et al., 1993). For other drugs, however, leakage was correlated with significant membrane permeability of the ionized species (Madden et al., 1990b). For doxorubicin, the initial accumulation is higher than that predicted by the Henderson-Hasselbach equation and drug retention is longer than the decay of the ΔpH. This is likely due to a reduced soluble aqueous fraction (Mayer et al., 1990b; Harrigan et al., 1993). A variation of the ΔpH loading method involves the use of entrapped ammonium sulfate (Haran et al., 1993) to generate a ΔpH gradient that subsequently mediates doxorubicin encapsulation.

1.3 In vivo behavior of intravenously injected liposomes

The use of liposomes as intravenous drug carriers requires a detailed understanding of the two major factors controlling their pharmacokinetic and pharmacodynamic behavior in vivo. First, interaction of liposomes in vivo involves the binding of plasma proteins and lipoproteins. Second, this then determines the subsequent interaction with the major clearance mechanism for liposomes, the reticuloendothelial system (RES).

1.3.1 Interactions of liposomes with plasma proteins

The interaction of liposomes with lipoproteins can cause the release of encapsulated agents due to lipid exchange, resulting in dissolution of the carrier (Kirby et al. 1980). The apolipoprotein

ApoA-1, found predominantly in the high density lipoprotein (HDL) fraction, plays a major role in this process which involves insertion of the protein into the lipid bilayer (Klausner et al., 1985). Another source of leakage involves interactions with complement proteins that can lead to activation of the complement cascade (Devine et al., 1994). Activation of the complement pathway can result in the formation of a membrane attack complex, a pore forming channel 10 nm in diameter, and resulting loss of interior contents or lysis of the membrane (Silverman et al., 1984).

Complement protein binding also represents one form of opsonization, a particularly important process regulating liposome elimination. Opsonization is the binding of plasma proteins that promote the uptake of foreign particulate by the fixed and free macrophages of the RES, also known as the mononuclear phagocytic system (MPS)(Coleman, 1986; Moghimi and Patel, 1989). Opsonins include IgG, fibronectin, and certain complement proteins (C3, C3bi). While some opsonins are thought to be only surface bound, such as C3 or IgG, others may have hydrophobic interactions with the liposome membrane. Subsequent RES uptake depends on recognition of membrane associated opsonins by specific receptors on the macrophage surface (Coleman, 1986), and it has been shown that the total amount of bound protein on the liposome surface is directly related to the clearance rate (Chonn et al., 1991; 1992).

1.3.2 Interactions with the reticuloendothelial system

The removal of foreign particulate matter such as opsonized liposomes from the circulation is carried out by the reticuloendothelial system (RES). The RES is primarily composed of the resident macrophages of the liver (Kupffer cells), spleen, lungs, and bone marrow. It has been

more recently recognized that circulating monocytes also play a role in this clearance mechanism (Senior, 1987), hence the total system is also sometimes referred to as the mononuclear phagocyte system (MPS).

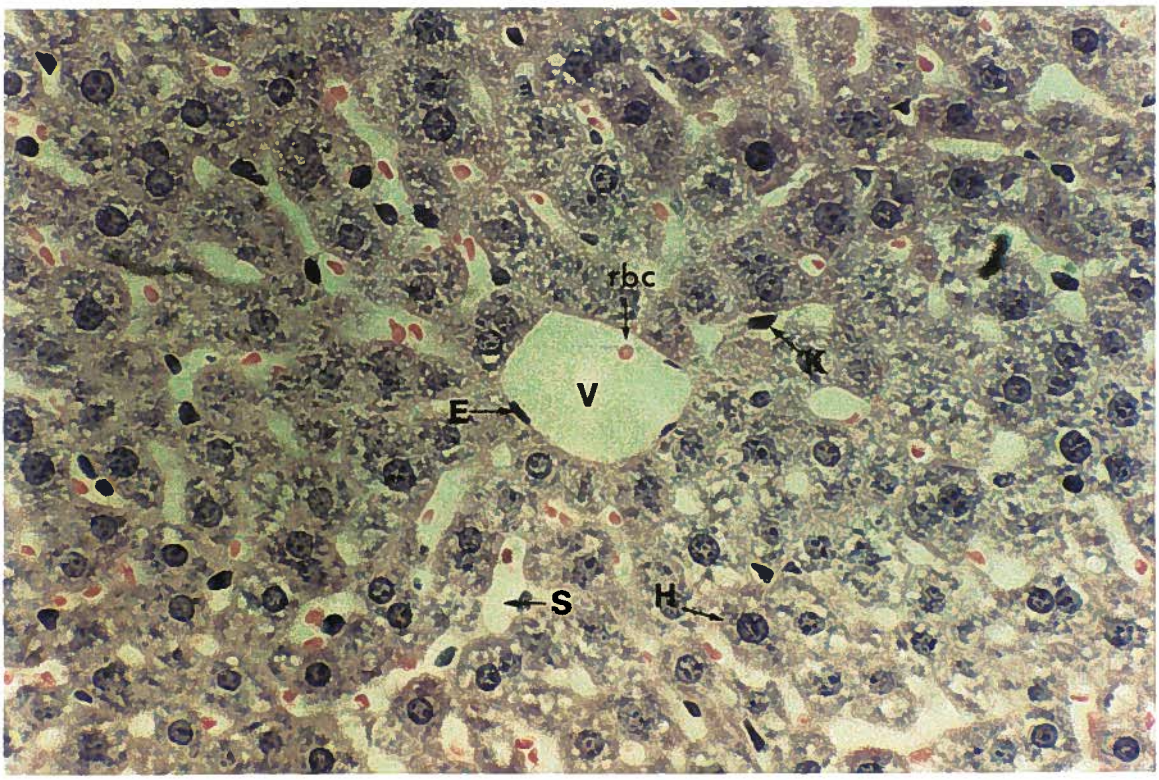
The vast majority of liposomes cleared from the circulation are found in the liver, and to a lesser extent in the spleen. In the liver, the specialized Kupffer cells lie within the sinusoids (Figure 1.8), and phagocytose a wide variety of particles from bacteria and other foreign pathogens as well as defined particulates such as latex beads (Pratten and Lloyd, 1986), liposomes (Roerdink et al, 1981), carbon particles (Kampschmidt et al., 1966), and other macromolecules. The primary role of this specialized macrophage is to capture and then degrade or detoxify foreign agents in the blood.

In some cases, liposome uptake by the RES is useful, such as when this cell population is the intended biologic target. Liposomal delivery of macrophage activators, for example, may be of benefit for increasing the tumoricidal activity of macrophages (Fidler et al., 1982). Many intracellular parasites and bacterial infections use phagocytic cells as hosts, and liposomal delivery of specific drugs to eliminate these intracellular parasites have also shown promise (Alving, 1988). Finally, liposomes have proven to be particularly potent immune adjuvants for certain antigens, as these macrophages are also involved in antigen processing and presentation (Alving, 1987). However, the RES presents a major obstacle if alternate target sites are being considered. Strategies to avoid the RES, primarily involving the modification of liposomes to reduce opsonization, show promise in terms of reducing RES accumulation.

Figure 1.8

Liver cross section

Liver section taken from a normal CD-1 mouse. Identified on the figure are (V), central vein; (H), hepatocyte; (b), red blood cell; (E), endothelial cell; (S), sinusoid; (K), Kupffer cell (hematoxylin and eosin, 5 μm thick paraffin)



1.4 Properties of liposomes influencing circulation lifetimes

The primary properties governing circulation lifetimes of liposomes are size, composition, charge, and dose. Large liposomes (>1 μm diameter) are cleared more rapidly than small liposomes (<200 nm). For example, large MLVs have typical half-lives of less than 15 minutes, whereas LUVs (30-80 nm) of identical lipid composition and dose exhibit half-lives over 4 fold longer (Juliano and Stamp, 1975). In general large liposomes are taken up by the Kupffer, however the spleen phagocytes and sinusoidal structure also play roles in the uptake of large liposomes (Litzinger and Huang, 1992). Liposomes that are very small (SUVs), however, are often also cleared quickly from the circulation. It is thought that SUVs have membrane defects arising because of the high radius of curvature that may act as sites for opsonin binding. There is also evidence that many small pores exist in the liver sinusoids that allow small liposomes to extravasate and thus facilitate binding and uptake by liver hepatocytes (Roerdink et al., 1981).

The administered lipid dose also plays an important role in determining the circulation lifetime of injected liposomes. It is often observed that, in terms of percent of the injected dose, higher doses result in higher circulation levels and lower liver uptake at a given time point (Poste et al., 1984). This is thought to be due to saturation of the liver macrophages that then results in spillover into the spleen, followed by further spillover into the lung and bone marrow. In studies with saturating pre-doses of MLVs (1100 mg/kg in mice), Abra et al. (1980) for example were able to demonstrate over 5-fold depression in liver uptake and a 3 to 4-fold increase in spleen uptake of a second identical injection 1 hour after the pre-dose. More recent work, however, has focused on the possibility that large doses of liposomes may actually deplete certain opsonins (Oja et al., 1995).

The clearance behavior of liposomes is also strongly influenced by lipid composition. As mentioned earlier, surface charge plays a large role in the clearance of liposomes by influencing the extent of opsonization. While neutral surfaces usually exhibit low levels of opsonization and slow RES uptake, negative liposome surfaces accumulate significantly more opsonins and are cleared faster from the circulation (Chonn et al., 1991, 1992). In vitro studies measuring macrophage uptake levels of MLVs with 30 mol % PS, PG, or PI indicated that 25, 18, and 15-fold greater levels of lipid uptake occur compared to similar vesicles composed of the corresponding neutral PC (Shroit et al., 1986). Available evidence suggests that positively charged liposomes are also cleared considerably faster than neutral liposomes (Senior et al., 1991a).

The role of lipid composition effects on serum protein binding and RES uptake extends to acyl chain composition. In general unsaturated acyl chains result in more fluid lipid bilayers and may, therefore, facilitate association of some opsonins. Long chain saturated acyl chains result in more rigid lipid bilayers and thus resist protein insertion. However, this rigidity can be detrimental. In the case of liposomes composed only of highly saturated long acyl chain phospholipids, circulation lifetimes can actually be reduced if the lipid gel-to-crystalline phase transition temperature is higher than physiological temperature. The inclusion of cholesterol in this case greatly increases circulation lifetimes, primarily as a result of removal of packing defects that occur in gel state systems (Kirby et al., 1980; Hunt, 1982).

The process of opsonization appears to be the first critical step in liposome clearance. Attempts to reduce opsonization and maximize drug retention, therefore, focus on using LUVs composed of long chain saturated neutral lipids such as DSPC in combination with cholesterol.

1.4.1 Poly(ethylene glycol)-lipids (PEG-lipids)

The realization that plasma proteins interact with liposome surfaces has led to further modification of surface properties. The incorporation of the ganglioside G_{M1} into the lipid mixture at 10 mol % (Allen and Chonn, 1987) was found to greatly increase the circulation lifetimes of the liposomal carrier. There was a concomitant reduction in RES uptake resulting in a higher blood-to-RES ratio at a given time point. Part of the original rationale for the incorporation of this naturally occurring lipid component was that its sugar residues would provide a surface resembling that of cells. It has since been shown that the reduced clearance is primarily the result of reduced opsonin binding (Chonn et al., 1991; 1992) The failure of similar gangliosides to effectively prolong circulation lifetimes, however, has led to some debate as to the function of G_{M1} and other gangliosides in the liposome membrane (Allen et al., 1994). One suggestion is that G_{M1} may promote the binding of specific dysopsonins, plasma binding proteins which would either label the liposome surface as not foreign, or simply prevent the binding of clearance-promoting opsonins (Park and Huang, 1993). Unfortunately one of the major drawbacks to the use of G_{M1} in drug delivery applications is its cost. The results obtained with G_{M1} did spark renewed interest in the development of liposomes exhibiting enhanced circulation lifetimes and has led to other means of altering the liposome surface characteristics.

It has been known for some time that polymer coated nanoparticles and other polymeric coated spheres circulate longer than uncoated particles (reviewed in Brannon-Peppas, 1995). There is a wealth of physical chemistry data which describes the steric stabilization effect and reduced protein binding and this information has recently been applied to drug carriers. This technology has also led to the use of polymers directly coupled to proteins and drugs for intravenous injection, most often using polyethylene glycol (PEG; Abuchowski et al., 1977; Delgado et al., 1992,). First reports on the use of PEG-phosphatidylethanolamine conjugates to modify the surface of conventional liposomes revealed that circulation lifetimes of these drug carriers could also be substantially improved (Blume and Cevc, 1990, Klibanov et al., 1990; Papahadjopoulos et al., 1991).

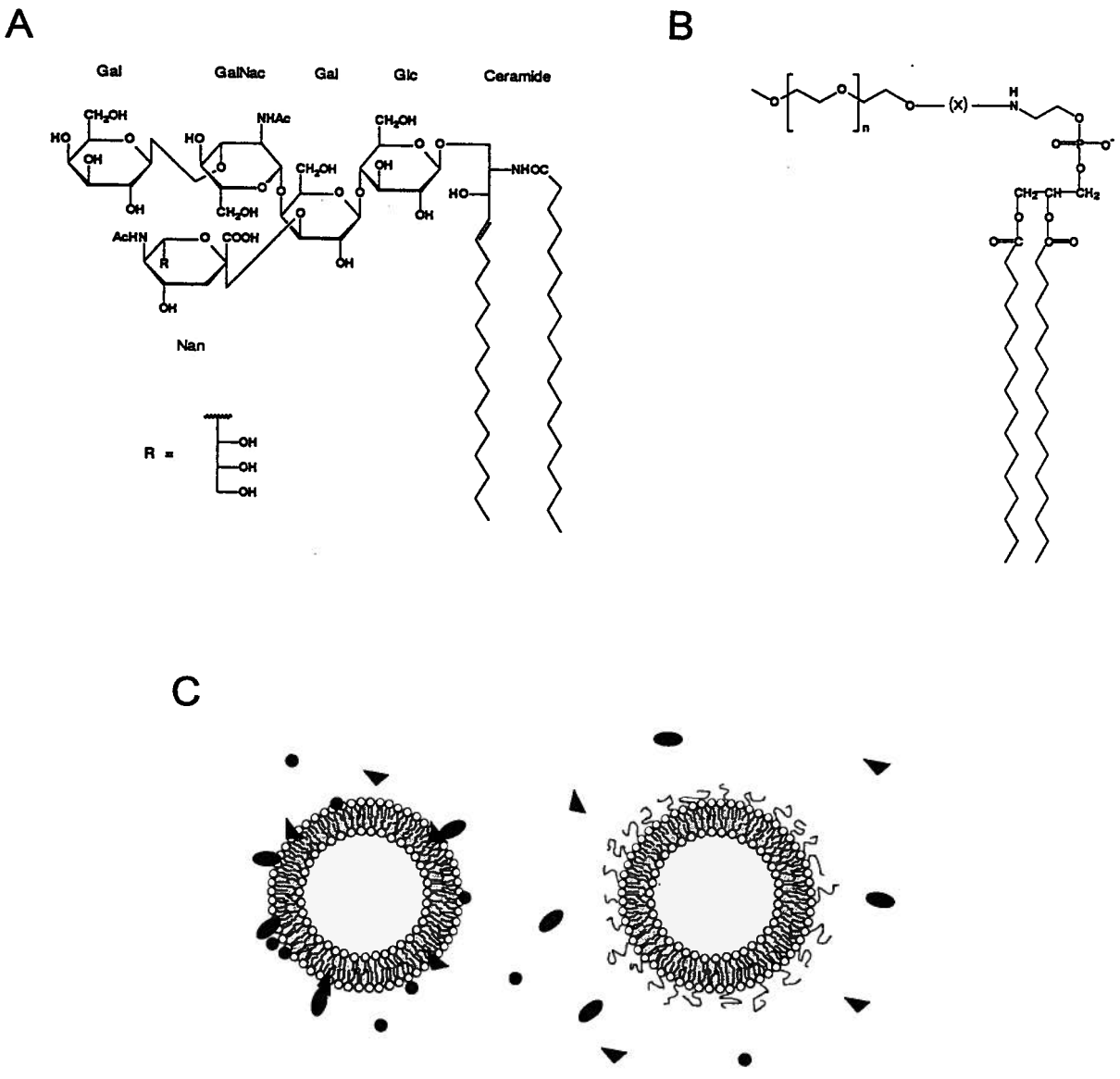
PEG is a flexible hydrophilic polymer of repeating ethylene glycol units (-[O-CH_2-CH_2]$_n$-) and is usually coupled to the headgroup of PE via well established chemistries involving reactions with the primary amine of PE. Although there are methods to attach PEG to the surface of pre-formed liposomes resulting in a polymeric coating only on the outside surface (Senior et al., 1991b), these tend to be difficult and the preferred method of incorporation is the inclusion of PEG-PE in the original mixture. Thus, after liposome formation, the polymer coats both the inner and outer monolayer.

A PEG coating on liposomes increases the circulation lifetimes. This is thought to be due to reduced recognition by cells of the RES. More detailed studies on the surface characteristics of liposomal PEG-PE have yielded a refined view of the polymeric coating as a steric barrier, inhibiting the close approach of other surfaces or plasma proteins (Lasic, 1994). These liposomes are now commonly referred to as sterically stabilized liposomes (SSL).

Figure 1.9

Sterically stabilized liposomes (SSLs)

Shown are the structures of *A*, ganglioside G_{M1}, *B*, PEG-PE. G_{M1} (MW 1544) is composed of a lipid anchor attached to a hydrophilic branched sugar residue. PEG-PE is typically composed of a phosphatidylethanolamine lipid anchor to which is attached (via a short linker group -X-) to the repeating $(-OCH_2CH_2-)_n$ hydrophilic polymer poly(ethylene glycol)(PEG). For an average molecular weight of the PEG polymer of 2000, n is approximately 45. *C* depicts the binding and inhibition of opsonin binding to the liposome surface in the absence or presence of a sterically stabilized liposome surface.



Other polymers, thought to have the same relative hydrophilicity and steric stabilization as PEG, fail to perform as well as PEG in terms of extending circulation lifetimes (Lasic, 1994). It is now hypothesized that PEG is sufficiently flexible when coupled to the liposome surface that inhibition of protein binding stems from a combination of both steric and dynamic properties of the polymer (Lasic, 1994). PEG is envisioned to provide not only an initial barrier to close approach but also a sweeping action to inhibit protein binding near the liposome surface. Lower levels of PEG provide a "mushroom" conformation that can adopt an extended "brush" conformation at higher PEG contents. In either case, the inhibition of protein binding is largely due to the increased unfavorable surface free energy provided by the PEG coating (Arakawa and Timasheff, 1985). The optimal PEG concentrations in terms of circulation lifetimes appears to be approximately 5 mol % PEG-PE for PEG with an average molecular weight for the polymer of 2000 (Allen et al., 1994).

In summary, the principal effect of PEG on the liposome surface is a major reduction in the interaction of liposomes with plasma proteins. This leads to a stabilization effect (avoiding destabilizing apolipoproteins) and a reduction in the total amount of opsonin binding (Senior et al., 1991b; Blume and Cevc, 1990). There may also be a reduction in the binding of some specific opsonins (Allen et al., 1994). It is speculated that the PEG coating may also reduce cellular uptake directly such that even if there is protein bound, the coating may still inhibit receptor mediated binding at the level of the macrophage (Allen et al., 1994).

1.4.2 RES blockade: the effect of entrapped drug

Another possible strategy for increasing the circulation lifetime of injected liposomes is to alter the cells of the RES itself. As mentioned previously, there have been attempts to reduce the activity of the RES by administering saturating pre-doses of liposomes (Abra and Hunt, 1981) or immunoglobulin (Coleman, 1986). In this regard, it has been noted that liposomal drug carriers containing doxorubicin exhibit significantly longer circulation lifetimes over the identical empty carrier (Bally et al., 1990a). This is almost entirely due to reduced liver uptake. It was recognized many years ago that there was the potential for harmful effects with liposomally entrapped cytotoxic drugs because of accumulation in the RES or other non-target tissue (Patel, 1984). Both mouse and human studies, however, have suggested that there are no specific liver toxicities as measured by histology or biochemical functions following i.v. administration of liposomal doxorubicin (Cowens et al., 1993). The question remains, however, whether RES toxicity is responsible for the altered pattern of clearance seen with liposomal doxorubicin. Drugs such as liposomal dichloromethylene diphosphonate eliminate macrophages by delivering drugs designed to be toxic to phagocytic cells directly to the macrophage population (Classen and Van Rooijen, 1986; Van Rooijen, 1989). Doxorubicin has a number of modes of action which might be expected to affect Kupffer cells, including DNA intercalation and topoisomerase II interference, free radical formation, covalent binding to DNA, and various cell binding effects (Cummings et al., 1991). Non dividing cells including Kupffer cells might be killed by doxorubicin as a result of destructive free radical formation or interference with the normal DNA to RNA to protein pathway. Vincristine on the other hand binds to microtubules (Owellen et al.,

1972; 1976). Microtubules are important for normal cellular structure and rearrangement and for membrane trafficing which is important in phagocytic proceses.

1.5 Targeting to sites of tumor growth

1.5.1 Passive versus active targeting

Liposomes with long circulation lifetimes result in increased accumulation in extravascular sites of disease including sites of infection, inflammation, and tumors (Gabizon and Papahadjopoulos, 1988; Gabizon et al. 1990; Gabizon, 1992). This targeting ability is termed passive targeting, and constitutes targeting because up to 10-100 time more liposomal drug can be directed to the disease site as compared to injection of the same amount of free drug. Active targeting, on the other hand, involves the attachment of specific ligands such as antigen specific antibodies onto the liposome surface and thus target to specific cell populations. It should be noted that active targeting also requires long circulation times and excellent passive targeting capabilities to allow penetration to the disease site.

It is usually observed however that antibody coated liposomes are cleared more rapidly than their non-targeted counter parts. Even when immune reactions to the antibody are minimized via host compatibility or use in immunocompromised animals such as SCID mice, clearance is usually faster than non-targeted systems, but with the addition of a PEG coating, circulation times can approach that of carriers which are not coated with antibodies (Loughrey et al., 1993). It would be expected that a surface bound antibody would have target binding problems. The problem of target binding can also be overcome by coupling of the antibody to the end of the PEG chain. A major effect that the PEG coating has on circulation lifetimes of the targeted systems is that it

reduces the size of the liposome aggregates which often form during the antibody coupling procedure (Harasym et al., 1995). As mentioned earlier, size plays a critical role in determining liposome circulation lifetimes.

Thus for both non-targeted and targeted systems, the accumulation at a disease site depends upon the circulation lifetime. However the primary barrier to drug delivery remains access to that site. Even for targeted systems, this remains a two step process, the first being the passive accumulation within the site. Once there, "targeting" can occur by altering the distribution of the liposomes within that site (Longman et al., 1995). The process by which the specific barriers are crossed during movement from the blood to a defined interstitial space is referred to as extravasation.

1.5.2.1 Normal vasculature

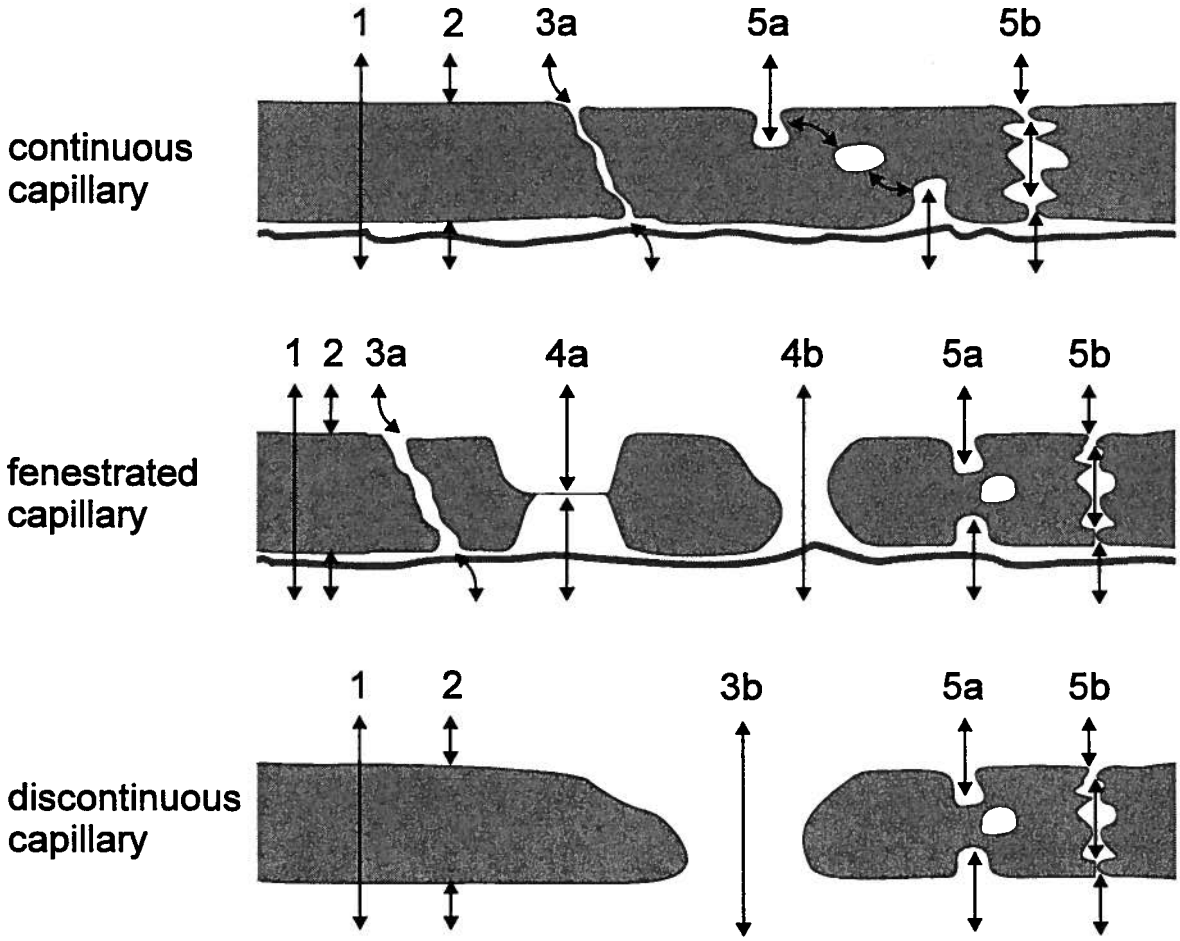
In the normal vasculature, blood capillaries act as the primary sites of nutrient exchange between the blood and tissue. The movement of material from the blood to extravascular sites is dependent on the capillary blood vessel structure. In general there are three classifications (Poste et al., 1984; Jain, 1987; see Figure 1.10) defined by the endothelium and basement membrane structure. Continuous capillaries are found in tissues such as muscle, connective tissue, and skin. They are composed of a continuous lining of endothelial cells and have an uninterrupted subendothelial layer of basement membrane. Fenestrated capillaries, found in many glands, the gastrointestinal tract, and renal glomerous has an endothelial layer interrupted by fenestrae 30 to 80 nm in diameter sealed by a membranous diaphragm. The basement membrane is usually continuous. The last class of capillary structure is called discontinuous or sinusoidal because of

Figure 1.10

Capillary endothelial vascular structure and modes of extravascular transport

The three major classes of capillary endothelial vascular structure (continuous, fenestrated, and discontinuous) are shown with the various modes of transport possible across them (adapted from Jain, 1987; Poste 1984) (1), direct diffusion across endothelial cell; (2) lateral membrane diffusion; (3), interendothelial transport - (a) narrow, (b) wide; (4) endothelial fenestrae - (a) closed, (b) wide; (5), vesicular transport - (a) transcytosis, (b) vacuolar-vesicular organelles (VVOs)(channels).

Transport pathways: lipophilic solutes: (1), (2), (3), (4); hydrophilic solutes, macromolecules (3), (4), (5).



its prevalence in the sinusoids of the liver, spleen, and bone marrow. The endothelial layer has large openings (>100 nm) between or even within cells. The basement membrane is either absent (liver) or interrupted and fragmented (spleen, bone marrow).

The permeability of normal vascular structure is tightly controlled. Whereas small molecules can, in general, pass freely through normal capillaries and other vessels with intact interendothelial junctions, macromolecules are retained in the circulation. Extravasation of circulating macromolecules can occur via vesicular transport or related mechanisms (Kohn et al., 1992). During inflammation, macromolecular leakage is increased. This may involve contraction of the postcapillary endothelial cells, creating gaps that macromolecules such as liposomes can penetrate (Kohn et al., 1992).

The various modes of transport across these classes of vessels are also illustrated in Figure 1.10. These include the direct diffusion of some molecules across the endothelial cell, lateral membrane diffusion, movement through the interendothelial junctions (both tight and wide), across the endothelial fenestrae, and via vesicular mechanisms (Jain, 1987; Kohn et al., 1992).

1.5.2.2 Tumor vasculature

In general, the vascular lining of blood vessels within a tumor are hyperpermeable to circulating macromolecules. This is due, in large part, to larger defects in the endothelial layer such as fenestrations, widened interendothelial junctions, and in some cases blood channels that have little or no endothelial lining at all (Dvorak et al., 1988; Kohn et al., 1992). Most of the leakage is thought to occur across post-capillary venules and even large veins. Interestingly enough,

these vessels often have continuous endothelium with closed junctions (Dvorak et al., 1988; Kohn et al., 1992). The mechanism of transport in these cases is increased vesicular structures called vesiculo-vacuolar organelles (VVOs) that may actually be more stable and long lived structures than previously assumed.

One of the mechanisms responsible for increased vascular permeability in tumors is due to secretion by tumor cells of vascular permeability factor (VPF; Dvorak et al., 1991), also known as vascular endothelial growth factor (VEGF). VPF is important in the development of growing tumors by allowing the leakage of various macromolecules and nutrients into the tumor tissue. The leakage of plasma clotting proteins into a newly formed tumor region due to increased permeability of nearby host blood vessels allows the formation of provisional stroma. This then initiates the process of angiogenesis, the ingrowth of blood vessels necessary for continued solid tumor growth and development via perfusion and paracrine support (Blood and Zetter, 1990).

1.5.2.3 Other solid tumor properties

Most solid tumors appear to be more highly vascularized than the surrounding host tissue. However, despite the abundance of relatively large blood vessels, tumor blood flow is slower than normal tissues. This is due to the tortuous blood vessel pathways, including dead ends and cut off loops (Jain 1987; 1988). The general organization also consists of poorly defined artery/vein subsystems. Therefore, the amount of blood and associated circulating liposomes flowing through the tumor per unit time is very low.

A second barrier to macromolecular delivery is the high interstitial pressure (Jain, 1988). In normal tissues, the pressure is 0 mm Hg due to the easy drainage of extravascular fluid via the lymphatic system. In solid tumor tissue, however, the lymphatic system is usually absent and drainage is therefore very poor except at the periphery/host tissue interface. This results in very high interstitial pressures, found in some mouse solid tumor models to be upwards of 10-30 mm Hg.

The actual physical basis for transport across the vascular barrier falls under two basic mechanisms (Jain, 1987). The transport of large macromolecules is governed by convection, the flow of fluid from vessel to interstitium, while small molecules are governed by diffusion, dependent upon concentration differences for that molecule for the blood versus interstitium. Interstitial pressures are often as high as the post-capillary venous pressure, where most of the available sites are for extravasation of large macromolecules. The minimal pressure differences give rise to minimal convection and therefore limited extravasation of macromolecules.

1.6 Objectives

Of central importance to liposomes as drug carriers is their circulation lifetime. This thesis is divided into three areas of investigation. First, it is well established that the incorporation of PEG-lipids into liposomes can significantly prolong their circulation lifetime. However, several key questions remain regarding important factors influencing their retention and stability in the liposomal carrier. Second, the effect that entrapped drug can have on clearance characteristics have not been adequately addressed. This is investigated using sensitive assays to monitor the function of the RES. And third, the delivery of liposomal drug to a murine tumor model is

examined comparing a conventional liposomal drug carrier to one which has incorporated PEG-lipid to optimize its circulation time.

CHAPTER 2

THE USE OF PEG-LIPIDS TO IMPROVE THE CIRCULATION LIFETIMES OF LIPOSOMES

2.1 Introduction

As outlined in Chapter 1, the use of liposomes as systemic drug delivery vehicles requires long circulation lifetimes. The incorporation of monomethoxy poly(ethylene glycol)-phosphatidylethanolamine (MePEG-PE) conjugates into liposomal systems has been shown to significantly extend the circulation lifetimes of intravenously injected liposomes (Klibanov et al., 1990; Blume and Cevc, 1990; Senior et al., 1991b; Allen et al., 1991a; Allen and Hansen, 1991). However, the extent to which the PEG coating remains associated with the liposome after injection into the circulation has not yet been adequately addressed in the literature. Therefore, the studies outlined in this chapter investigate factors influencing the retention and chemical stability of poly(ethylene glycol)-lipid conjugates incorporated into large unilamellar vesicles.

The lipid moiety of the molecule must obviously be sufficiently lipophilic to firmly anchor the hydrophilic coat to the surface. In this regard, liposomally incorporated PEG-cholesterol or PEG-monostearate are relatively ineffective at improving the circulation lifetimes of intravenously injected liposomes (Allen et al., 1991a). Presumably, the hydrophobic moiety in these compounds is an ineffective anchor and thus the hydrophilic coat is rapidly lost from injected LUVs. Reports on the anchoring properties of diacyl phosphatidylethanolamine anchors have been conflicting. It has been suggested that the lipid moiety has little effect on the circulation characteristics of LUVs incorporating PEG-lipids (Woodle et al. 1992), while others indicate that

the lipid anchor is a important factor (Allen et al., 1991a). In addition, the chemical stability of MePEG-PEs in vivo has not received detailed attention.

This chapter examines the influence of the lipid anchor and linkage chemistry on the ability of MePEG-PE to improve circulation lifetimes of LUV systems. It is shown that the anchoring capacity of PE anchors is extremely sensitive to the acyl chain composition, where distearoyl PE species are considerably more effective anchors than palmitoyl oleoyl species. Second, depending on the type of linkage between the PEG and the PE, breakdown can occur either on the LUV surface or after release of PEG-PE from the LUV. These factors should be considered when discussing the usefulness or mechanisms of PEG-PEs incorporated into liposomes.

2.2 Materials and methods

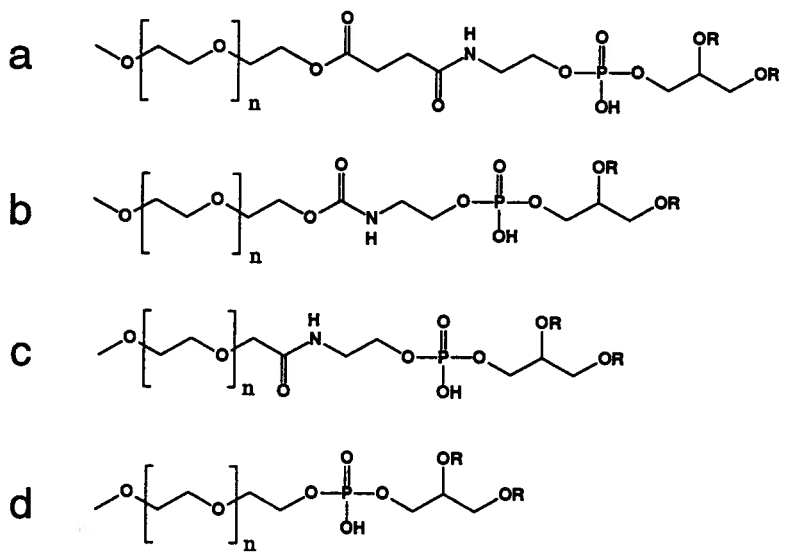
2.2.1 Monomethoxypoly(ethylene glycol)-lipid (MePEG-lipid) synthesis

The overall chemical structures of the of the various MePEG-lipids synthesized are shown in Figure 2.1, which include MePEG linked to phosphatidylethanolamine via succinate (MePEG-S-PE), carbamate (MePEG-C-PE) and amide (MePEG-A-PE) linkages, and directly to phosphatidic acid (MePEG-PA). All PEG-lipids were isolated as a single component on TLC, with similar R_f values, and showed ^1H NMR resonances characteristic of the MePEG and lipid groups. $MePEG_{2000}$-S-POPE and $MePEG_{2000}$-S-DSPE were synthesized as follows. Monomethoxypoly(ethylene glycol) ($MePEG_{2000}$-OH) (10 g) was treated in pyridine with ten equivalents of succinic anhydride (0.5 g) at room temperature for two days. The solution was diluted with water, acidified, extracted with methylene chloride, and the organic extracts were dried over magnesium sulfate, filtered, and the solvent removed. The resulting residue was

Figure 2.1

Summary of PEG-lipid conjugate chemical structures

(a), succinate linkage: MePEG-S-PE; (b), carbamate linkage: MePEG-C-PE; (c), amide linkage: MePEG-A-PE; (d), direct linkage: MePEG-PA. (see Materials and Methods for detailed descriptions of the compounds synthesized)



subjected to silica gel column chromatography in methylene chloride/methanol (96/4 v/v) and $MePEG_{2000}$-succinate isolated. The dry $MePEG_{2000}$-S (1.5 equivalents), DCC (1.2 equivalents), and NHS (1.6 equivalents) were dissolved in chloroform, stirred for an hour and filtered. Dry 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE) or 1,2-distearoyl phosphatidylethanolamine (DSPE) (1.0 equivalents) was dissolved in warm chloroform and added to the filtrate, then 5 equivalents triethylamine was added and the reaction mixture stirred for half an hour. Combined organic extracts were dried over anhydrous magnesium sulphate, filtered and the solvent removed under reduced pressure. The residue was subjected to silica gel column chromatography. Fractions collected were analyzed by TLC using methanol/chloroform

as the solvent (15/85 v/v) and visualized by exposure to iodine. Fractions containing pure $MePEG_{2000}$-S-POPE or $MePEG_{2000}$-S-DSPE were combined, taken up into distilled water, centrifuged at 1500 × g for 30 min. and the supernatants dialyzed against distilled water overnight. The resultant solutions were lyophilized to yield a white powder.

The synthesis of $MePEG_{2000}$-[^{14}C]S-POPE was carried out as described for $MePEG_{2000}$-S-POPE with the exception that succinic anhydride-1,4-[^{14}C] was reacted first before addition of excess succinic anhydride (non-radioactive). The product was isolated by preparative TLC using two successive plates run in methanol/chloroform (15/85 v/v). The $MePEG_{2000}$-S-POPE component was extracted from the appropriate scraped bands with methanol and then methanol/water (1:1) to yield $MePEG_{2000}$-[^{14}C]S-POPE with specific activity 1.74 mCi/mmol. The extract was dispersed in water, centrifuged at 1500 × g for 30 minutes, frozen and lyophilized.

The synthesis of $MePEG_{2000}$-S-[^{3}H]DSPE first required making $MePEG_{2000}$-S-DOPE similarly to the procedure described for $MePEG_{2000}$-S-POPE. The $MePEG_{2000}$-S-DOPE and Pd-C were then added with methanol to a vial which was sealed and flushed with nitrogen. Sodium borotritide (490 mCi/mmol) was injected and the mixture stirred for an hour. Sodium borohydride was added and the solution stirred for three hours. After carefully opening in a fume hood, the suspension was acidified with a drop of hydrochloric acid (10%), more methanol added, and then centrifuged. The supernatant was filtered through celite, diluted with water and extracted with methylene chloride. The organic fractions were dried over magnesium sulfate, filtered, and the $MePEG_{2000}$-S-[^{3}H]DSPE was purified by preparative TLC as described above (99% of the remaining radioactivity was located in the $MePEG_{2000}$-S-DSPE component, specific activity 140 mCi/mmol).

To synthesize [^{3}H]MePEG$_{2000}$-S-DSPE, a sample of [^{3}H]MePEG$_{2000}$-OH (Amersham, custom synthesis) was used as the starting material with the remainder of the synthetic procedure followed that of MePEG$_{2000}$-S-DSPE. Purification was carried out using preparative TLC plates as described above to yield [^{3}H]MePEG$_{2000}$-S-DSPE (specific activity 44 mCi/mmol). [^{3}H]MePEG$_{5000}$-S-POPE (specific activity 156 mCi/mmol) was synthesized similarly to [^{3}H]MePEG$_{2000}$-S-DSPE.

MePEG$_{2000}$-S-(1-palmitoyl-2-(4-pyrenyl)-butyroyl)PE was made by first synthesizing MePEG$_{2000}$-S-(1-palmitoyl-2-hydroxy)-phosphatidylethanolamine as the starting species using the procedure described for MePEG$_{2000}$-S-POPE. A solution of 3 equiv. pyrenebutyric acid and DCC (1.5 equiv.) in alcohol free chloroform was allowed to stir at room temperature for 1 h, filtered and then 1.0 equiv. (100 mg) of the lyso-PEG-PE added to the reaction mixture with 10 mg 4-dimethylaminopyridine. After work up, the product was isolated by column chromatography and preparative thin layer chromatography as before to yield a pure fluorescent compound, MePEG$_{2000}$-S-(1-palmitoyl-2-(4-pyrenyl)-butyroyl)PE (MePEG$_{2000}$-S-PPBPE).

MePEG$_{2000}$-C-POPE was made according to the following procedure. Dry MePEG$_{2000}$-OH (10 g) was dissolved in chloroform/toluene (50/2 v/v), reacted with 3 equiv. triphosgene and the product precipitated by addition of ether. The precipitate, MePEG$_{2000}$-chloroformate (MePEG$_{2000}$-C-Cl), was filtered and dried under vacuum. The MePEG$_{2000}$-C-Cl (1.0 g) and dry POPE (1 equiv.) were dissolved in chloroform and treated with 5 equiv. triethylamine. The solvent was removed under vacuum and the residue dissolved in water. The aqueous solution was acidified and extracted with methylene chloride. The combined organic fractions were dried

over magnesium sulphate, filtered, solvent removed and the residue subjected to column chromatography using silica gel and methanol/methylene chloride to yield pure $MePEG_{2000}$-carbamate-POPE ($MePEG_{2000}$-C-POPE). The product was dispersed in water, centrifuged at 1500 × g for 30 min. and the supernatant lyophilized to yield a white powder.

$MePEG_{2000}$-A-POPE and $MePEG_{2000}$-A-DSPE were synthesized as follows. $MePEG_{2000}$-acetic acid was synthesized by an adaption of the procedure in Sessler et al. (1992). Briefly, $MePEG_{2000}$-OH (10 g) was added to a solution of sodium dichromate (1.5 equiv.) in dilute sulfuric acid (10%) and the solution stirred at room temperature overnight. The solution was extracted with methylene chloride and the combined organic extracts washed with sodium hydroxide solution (1 M). The organic fraction was dried over magnesium sulphate, filtered and the solvent removed under reduced pressure. The residue was dissolved in a minimum of chloroform and precipitated with ether. The precipitate was filtered and dried, yielding $MePEG_{2000}$-acetic acid as a pale blue powder (colour due to complexed chromium). $MePEG_{2000}$-A-POPE and $MePEG_{2000}$-A-DSPE were then prepared using the same procedure as the succinate analogs, substituting $MePEG_{2000}$-acetic acid for $MePEG_{2000}$-succinate.

To synthesize $MePEG_{2000}$-POPA, a mixture of 1-palmitoyl-2-oleoyl-phosphatidic acid (POPA)(1 equiv.), $MePEG_{2000}$-OH (1.0 g, 1.1 equiv.) and 2,6,6-triisopropylbenzenesulphorylchloride (TIPBSC)(3 equiv.) was suspended in dry pyridine. The reaction mixture was protected from light and allowed to stir overnight. Water was added and the mixture allowed to stir for a further three hours. The solution was diluted with water, acidified and extracted with methylene chloride. After removal of the solvent, the residue was dispersed in water, filtered, centrifuged and the supernatant lyophilized. The resultant powder was subjected to column chromatography

using silica gel and methanol/chloroform. Pure fractions were combined, taken up in water, centrifuged and lyophilized to yield a white powder.

All lipids utilized in the above synthetic procedures were from Avanti Polar Lipids. Unless indicated otherwise, all other materials were from Sigma. Column chromatography was carried out using silica gel 60 (70-230 Mesh ASTM) (Merck). Analytical TLC employed aluminum backed silica gel 60 - F_{254}, 0.2 mm thick (Merck) and preparative TLC employed glass backed silica gel 60, 0.5 mm thick (Merck).

2.2.2 Preparation of large unilamellar vesicles (LUVs)

The production of LUVs was carried out as previously described (Hope et al., 1985). Briefly, lipid mixtures composed of distearoylphosphatidylcholine (DSPC) and cholesterol (55:45 mol/mol), DSPC, cholesterol and MePEG-PE (50:45:5), or DSPC, cholesterol and G_{M1} (45:45:10), each with trace amounts of [^{14}C] or [^{3}H]cholesteryl hexadecylether (CHE) as a non-metabolizable and non-exchangeable liposome marker (Derksen et al., 1987) were freeze-dried from benzene/methanol solution and hydrated in physiological sterile HEPES buffered saline (HBS) (20 mM HEPES, 150 mM NaCl, pH 7.4). The sample was freeze-thawed five times and then extruded at 65°C ten times through two stacked 100 nm pore size polycarbonate filters (Costar/Nuclepore, Canada) employing an Extruder (Lipex Biomembranes, Canada). Liposome size ranged from 95 to 115 nm as determined by quasi-elastic light scattering on a NICOMP Model 270 submicron particle sizer. The resultant LUVs were loaded onto a conventional Bio-Gel A-15m (200-400 mesh)(Bio-Rad, Canada) 10 × 1 cm column equilibrated with HBS to remove unincorporated MePEG-PE, and the pooled liposome peak diluted with HBS to an

appropriate concentration. All initial liposome preparations were checked for concentration by determination of phosphorous (Fiske and Subbarow, 1925) using a Shimadzu UV-visible recording spectrophotometer at 815 nm, and thereafter by scintillation counting using a Beckman LS3801 with Pico-Fluor 40 scintillation fluid (Packard). [^{14}C] and [^{3}H]CHE was from NEN/DuPont, DSPC from Avanti Polar Lipids, and cholesterol and other chemicals were from Sigma.

2.2.3 Exchange studies

The MePEG-PE to liposome radiolabel ratios for 5 mM (total lipid) preparations incorporating either $MePEG_{2000}$-[^{14}C]S-POPE, [^{3}H]$MePEG_{2000}$-S-DSPE, or $MePEG_{2000}$-S-[^{3}H]DSPE were taken to determine the starting ratio for the exchange studies. Then 500 µl of the liposome preparation was diluted in 500 µl of either HBS or normal mouse serum (Cedar Lane, Canada). For $MePEG_{2000}$-S-POPE, an additional incubation involving 500 µl of liposomes with 200 µl of mouse serum and 300 µl of HBS was carried out. These mixtures were incubated at 37°C and at various times, two 50 µl aliquots were removed and passed down 1 ml Bio-Gel A-15m spin columns to separate liposomes in the void volume from serum and unincorporated MePEG-PE radiolabel. (The use of these spin columns to separate liposomes from serum components has been previously described (Chonn et al., 1991)). The peak two liposome fractions from both columns were counted and the MePEG-PE to liposome radiolabel ratios were determined by a corrected DPM dual label determination.

2.2.4 Chemical stability studies

Five mg of the MePEG-lipid indicated was dissolved first in 200 µl of water, and then incubated in 1000 µl normal mouse serum at 37°C. Additional incubations involving 500 µl of liposomes (20 mM total lipid) composed of DSPC/cholesterol/MePEG-lipid in 500 µl serum at 37°C were also carried out. At various times, aliquots from the micellar or liposomal incubations were removed and lipid components extracted by the following procedure. 80 µl of sample was added to 920 µl of water. To this was added 2.1 ml of methanol and 1.0 ml of chloroform. After mixing, 1.0 ml of water was added, vortexed, and then an additional 2.0 ml of chloroform was added. After thorough vortexing, the sample was allowed to sit for 10 min. before centrifuging at 1500 × g for 1 hour. The organic layer was isolated, concentrated, and then spotted on 0.25 mm thick, silica gel 60, 5 × 10 cm TLC glass plates (Merck). The solvent system used to develop the plates was chloroform/methanol (85:15 vol/vol), and were visualized with iodine vapor. Where [^{3}H]MePEG$_{5000}$-S-POPE was used, 0.5 cm sections of each running lane were scraped and extracted with 3 × 1.0 ml chloroform/methanol/water (50:40:10 vol/vol). The extract was placed in scintillation vials, solvent removed, 5.0 ml scintillation fluid added, and left overnight before counting. Where MePEG$_{2000}$-S-PPBPE was used, fluorescence associated with scraped sections was extracted as above, solvent removed, and resuspended in 4.0 ml of 0.5% (w/vol) sodium cholate detergent. Fluorescence was read on a Perkin Elmer LS50 luminescence spectrometer operating at 600 V using an excitation wavelength of 339 nm (2.5 mm slit width), emission wavelength of 377 nm (2.5 mm slit width), and filter set at 350 nm. TLC standards used to aid identification included the appropriate free MePEG-OHs, extracted serum, free 4-(1-pyrenyl)butyric acid, and the appropriate MePEG-lipids.

2.2.5 Biodistribution and circulation longevity studies

The LUV preparations employing trace [^{3}H] or [^{14}C]CHE as liposome markers were injected via lateral tail vein in a volume of 200 μl (1 μmol total lipid) into 25 g CD-1 mice (Charles River, Canada). At various times, the mice were sacrificed and blood withdrawn by cardiac puncture and collected in microtainer tubes with EDTA (Becton-Dickinson, Canada). After centrifuging at 1500 × g for ten minutes, the plasma was isolated and showed no hemolysis. Two 100 μl samples from each mouse were counted directly in 5.0 ml scintillation fluid. The percentage recovery of liposomes remaining in circulation was based on a plasma volume of 4.55% of individual mouse body weight. Liver and spleen tissue were homogenized by Polytron to 20% and 10% in saline, respectively. 200 μl of tissue homogenate was solubilized with 500 μl Solvable (NEN/DuPont) for 2 hours at 60°C, after which the samples were cooled and treated overnight with 200 μl hydrogen peroxide. Five ml scintillation fluid was then added before counting. Liver and spleen associated liposomes are expressed as percent injected dose per tissue (total organ weight). Where in vivo exchange studies were carried out, two 50 μl aliquots of plasma were passed down spin columns as described above and the MePEG-lipid to liposome ratio determined.

2.3 Results

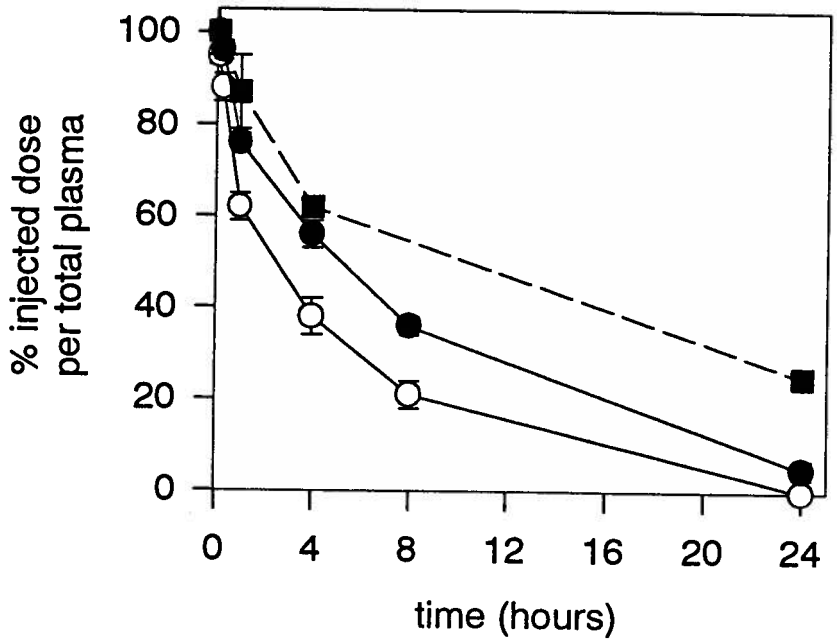
2.3.1 PEG_{2000}-S-POPE is lost from the liposome surface both in vivo and in vitro

The first series of experiments were designed to ascertain the influence of $MePEG_{2000}$-S-POPE on the circulation lifetimes of 100 nm diameter DSPC/cholesterol (55:45 mol/mol) LUVs in mice. As shown in Figure 2.2, the incorporation of 5 mol % $MePEG_{2000}$-S-POPE results in a

Figure 2.2

Circulation lifetime of DSPC/cholesterol/MePEG$_{2000}$-S-POPE liposomes

Large unilamellar vesicles composed of DSPC/cholesterol (55:45) (O), DSPC/cholesterol/MePEG$_{2000}$-S-POPE (50:45:5) (●), (or DSPC/cholesterol/MePEG$_{2000}$-S-DSPE (50:45:5) (■), dashed line, taken from Figure 2.6*A*) were injected via the lateral tail vein into 25 g CD-1 mice (5 mM total lipid in 200 µl, 1 µmol total lipid/mouse). At various times, the mice were sacrificed and plasma isolated. The marker [^{3}H]CHE was used to determine liposome recovery ([^{14}C]CHE for (■)). Results shown represent the mean of four animals ± S.E. of the mean.



relatively modest increase in LUV circulation lifetimes. At 24 h, the LUV preparation incorporating PEG-POPE is almost completely cleared. This may be contrasted with previous reports (Allen et al., 1991a) that incorporation of 5 mol % PEG-PE can result in up to 30% of DSPC/cholesterol LUVs remaining in the circulation at 24 h, and the third curve where liposomes incorporating $MePEG_{2000}$-S-DSPE have greater circulation levels (over 20% at 24 h). In order to determine whether this could be due to the loss of the PEG coating arising from interactions with serum protein factors, the DSPC/cholesterol LUVs (labeled with [^{3}H]CHE as a non-exchangeable liposome marker) incorporating $MePEG_{2000}$-[^{14}C]S-POPE were incubated with normal mouse serum at 37°C and the retention of radiolabel monitored. As shown in Figure 2.3*A*, this incubation results in rapid loss of the MePEG-PE radiolabel. While the MePEG-PE content of the LUVs is relatively unaffected when incubated in HBS alone, the MePEG-PE radiolabel rapidly drops when incubated in the presence of either 20% or 50% serum. After 24 h in 50% serum, the MePEG-PE to liposome marker ratio has dropped to nearly 50% of its initial value, suggesting that practically all of the MePEG-PE radiolabel in the outer monolayer has been removed. This is supported by the results of Figure 2.3*B*, which shows the spin column profile for the 24 h time point. There is an exact overlap of the MePEG-PE and liposomal peaks, and good separation from the MePEG-PE no longer associated with the liposomes. Integration of the two peaks yields a 52:48 ratio for liposome associated to free MePEG-PE. After an additional 6 days at room temperature in the 20% incubation, this ratio was also nearly 50%. Given our data, it is not unreasonable to assume that little more than 50% of the label is available for exchange. In addition, studies with $MePEG_{1900}$-carbamate-DSPE at 4 mol % indicate that the PEG extends outward from the surface in a brush formation approximately 5 nm (Needham et al., 1992). Although direct evidence is lacking, it has been suggested that the distribution should

Figure 2.3 (following page)

Loss of PEG coating from the surface of the LUV

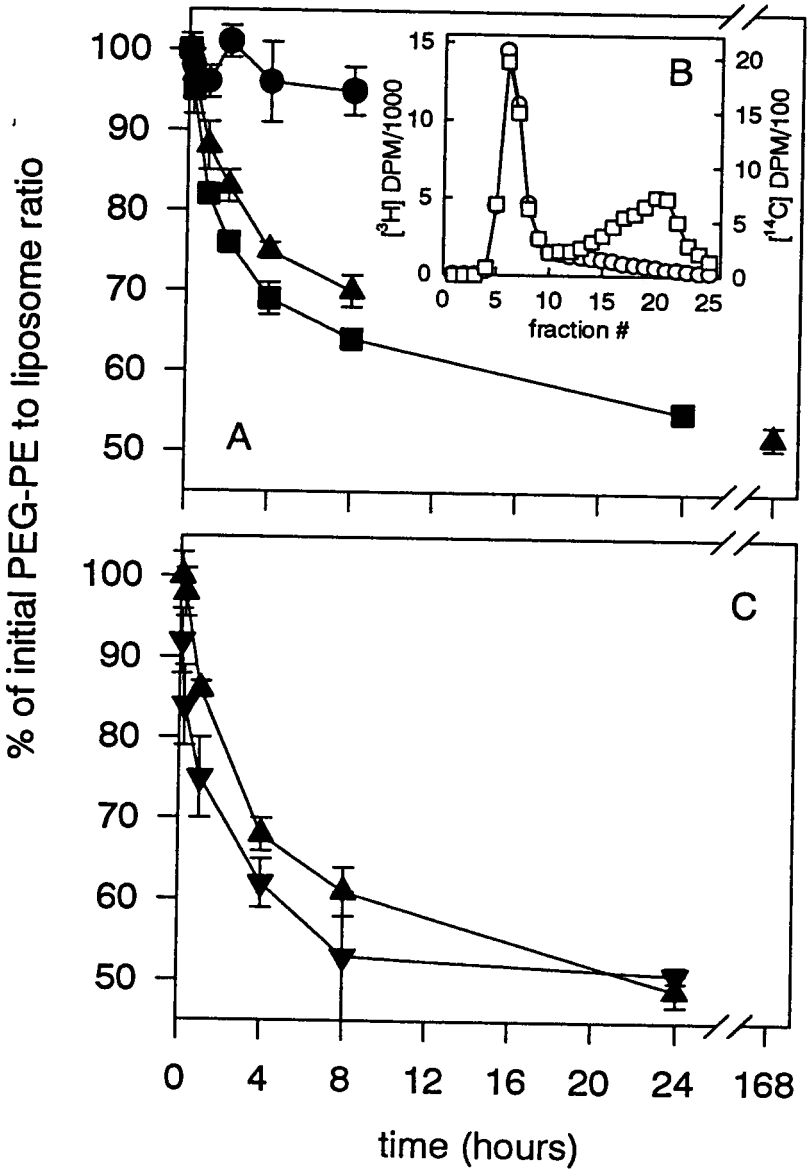
A, in vitro incubation of DSPC/cholesterol/$MePEG_{2000}$-[^{14}C]S-POPE (50:45:5 mol/mol) large unilamellar vesicles in normal mouse serum at 37°C. 500 µl of the LUV preparation (5 mM total lipid) was incubated in the presence of 500 µl HBS (●), 200 µl serum plus 300 µl HBS (▲), or 500 µl serum (■), representing 0%, 20%, and 50% serum concentrations, respectively. At various times, two 50 µl aliquots of the incubation mixture were removed and liposomes separated from the free PEG-PE and serum components by loading onto a 1 ml Bio-Gel A-15m spin column. The [^{14}C] (PEG-PE) to [^{3}H] (CHE, liposome marker) ratios were obtained from the peak two liposome fractions from both columns and expressed as a percentage (± S.D.) of the initial ratio before incubation.

B, Bio-Gel A-15m (200-400 mesh) spin column profile of DSPC/cholesterol/$MePEG_{2000}$-[^{14}C]S-POPE LUVs in 50% serum at 37°C for 24 hours. (○), the [^{3}H] DPM (liposome) label, and (□), the [^{14}C] DPM (PEG-PE) label measured for 30 µl of each collected fraction.

C, the in vivo loss of the PEG-PE coating from injected liposomes. The [^{14}C] (PEG-PE) to [^{3}H] (CHE) ratio was determined for liposomes recovered from mice injected with DSPC/chol/PEG-PE liposomes both before (▲) and after (▼) separation of liposomes from plasma components via spin column. Results represent the means of [^{14}C] (PEG-PE) to [^{3}H] (CHE) ratios obtained from the four mice before, or after the peak two liposome fractions from two columns for each mouse plasma sample, and expressed as a percentage (± S.D.) of the initial ratio before injection.

Figure 2.3

Loss of PEG coating from the surface of the LUV



be approximately equal between the two leaflets of the bilayer as long as the radius of the vesicle is large (greater than a factor of 10) relative to the length of the polymer (Woodle and Lasic, 1992) as is the case here.

The in vivo exchange results presented in Figure 2.3*C* confirm that the MePEG-PE is lost from the surface of the injected LUVs. Here, plasma samples were taken at various times from mice which had received [^{3}H]CHE labeled liposomes incorporating $MePEG_{2000}$-[^{14}C]S-POPE and were counted to compare the MePEG-PE to liposome ratios both before and after separation of the liposomes from the plasma. In agreement with the in vitro data, this ratio drops significantly with time indicating rapid loss of the MePEG-PE. The differences in the ratios for unseparated and separated liposomes can be attributed to MePEG-PE which is no longer associated with the liposomes but which continues to circulate for a short period of time and to the short time required to isolate plasma and elute the spin columns. The level of MePEG-PE still associated with the liposome approaches 50% by 8 h, and by 24 h has completely leveled off. The faster rate of loss of this component from the outer monolayer in vivo versus in vitro is expected given the greater plasma/serum to liposome ratio in vivo. It would also appear that the rate of loss of MePEG-PE is faster than the clearance rate of the LUVs initially containing this component. If only approximately 50% of the label is available for exchange, label loss leads clearance by a significant amount. For example, at 1 hour post injection 76% of the vesicles remain in the blood (from Figure 2.2), whereas after serum removal, 75% of the total label remains. This indicates that 50% of the available exterior label has been lost and 24% of the vesicles have been cleared. At 4 hours, by the same argument, 75% of the available outer label has been removed while 45% of liposomes have been cleared. Thus, at these early time points outer label removal leads clearance by 25 to 30%. It is also of interest to compare the clearance rate to the control. One can

see that when at least some proportion of the PEG coating remains, these liposomes have a slower clearance rate than the control. However, beyond some critical value of PEG loss between 4 and 8 hours after which there is very little PEG remaining on the surface, the clearance of these liposomes quickly approaches that of the control.

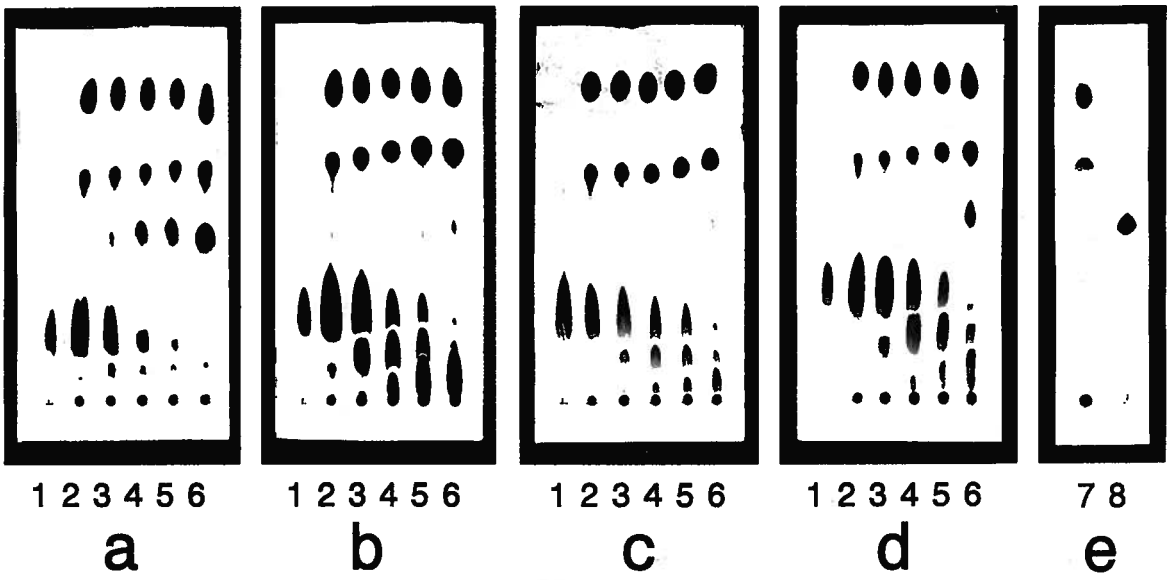
2.3.2 Chemical stability of various linker groups in the MePEG-PE conjugate

There are two possible mechanisms for the loss of the MePEG-PE radiolabel from the LUVs in vitro and in vivo. These are cleavage of the MePEG moiety from the lipid anchor or exchange of the entire MePEG-PE out of the LUV. Chemical breakdown was monitored by thin layer chromatography (TLC) after incubation of MePEG-PE micelles in normal mouse serum. The data of Figure 2.4 shows the effect of different chemical linkages between MePEG and the lipid anchor on the chemical stability of MePEG-PE. All four versions degrade significantly after exposure to serum at 37°C. The succinate linked version rapidly generates a compound which corresponds to free MePEG, while the other three (carbamate, amide and direct linked) versions show very little of this product. The breakdown of the succinate ester linkage is perhaps not unexpected given a similar phenomenon observed in the corresponding MePEG-protein conjugates (Zalipsky et al., 1992), however, this has not been previously reported for PEG-lipid conjugates. In addition, all four versions show the appearance of a product which is slightly more polar than the starting MePEG-lipid, which in turn is broken down to yield a product with further increased polarity by 24 h. When these four compounds were incorporated into liposomes, incubation in serum yielded the same patterns of breakdown products, but the rates at which these products are formed was significantly slower (results not shown).

Figure 2.4

Thin layer chromatography of the results following incubation of micellar PEG-PE in serum at 37°C

(a), $MePEG_{2000}$-S-POPE; (b), $MePEG_{2000}$-C-POPE; (c), $MePEG_{2000}$-A-POPE; (d), $MePEG_{2000}$-POPA. 5.0 mg of the PEG-PE indicated was first dissolved in 200 µl of water, then incubated with an additional 1000 µl of normal mouse serum at 37°C. At various times, 80 µl aliquots were withdrawn, extracted, and concentrated. The samples were run on 0.25 mm silica plates developed with chloroform/methanol (85:15 vol/vol) and spots visualized with iodine vapor. Lanes were: (1), before incubation; (2), 5 min. incubation; (3), 1 h; (4), 4 h; (5), 8 h; (6), 24 h. (e) shows TLC standards normal mouse serum extract (7) and free $MePEG_{2000}$-OH (8).



Additional experiments were performed to characterize the breakdown products, utilizing [^{3}H]MePEG$_{5000}$-S-POPE and MePEG$_{2000}$-S-(1-palmitoyl-2-(4-pyrenyl)-butyroyl)PE (MePEG$_{2000}$-S-PPBPE). These experiments confimed that free MePEG-OH was being generated in addition to lyso-MePEG-lipid compounds. Furthermore, heat inactivation of serum (65°C for 10 min) or addition of 5 mM EGTA was found to reduce the rate of breakdown by approximately 80%. All versions of the MePEG-lipid conjugates remain stable in HBS (pH 7.4) over 24 h, although the succinate version did show some slow breakdown (approximately 10 % over 24 h). Lower pH values (pH 2) resulted in the appearance of lyso compounds within several hours due to acid catalyzed hydrolysis at the sn-1 and sn-2 positions (Derksen et al., 1987).

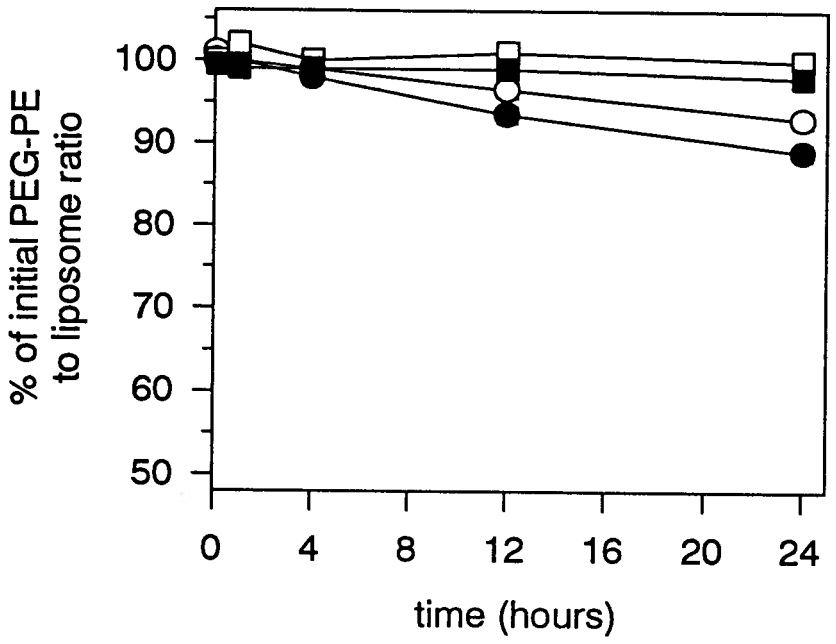
2.3.3 MePEG$_{2000}$-DSPE is retained in DSPC/cholesterol LUVs and exhibits enhanced chemical stability

The chemical breakdown of MePEG-lipids can occur either on the surface of the liposome or after exchange of the whole molecule out of the LUV. In order to reduce the possibility of exchange, MePEG$_{2000}$-PEs were synthesized with a DSPE anchor which may be expected to result in improved retention. The exchange of either [^{3}H]MePEG$_{2000}$-S-DSPE or MePEG$_{2000}$-S-[^{3}H]DSPE from [^{14}C]CHE labeled LUVs in serum or HBS at 37°C is illustrated in Figure 2.5. The acyl chain label remains associated with the liposome in both HBS and in serum. However, the MePEG leaves the liposome to a measurable extent, resulting in approximately 7% loss in HBS over 24 h. In serum, the loss of this label is somewhat greater, up to approximately 12% loss over 24 h, although this rate is reduced compared to MePEG$_{2000}$-S-POPE. Thus, the results of Figure 2.5 demonstrate that a primary factor for retention of the MePEG coating is the lipid anchor and that these compounds are relatively chemically stable if they remain associated with

Figure 2.5

In vitro incubation in normal mouse serum at 37°C of DSPC/cholesterol large unilamellar vesicles incorporating 5 mol % $MePEG_{2000}$-S-DSPE

500 µl of the LUV preparations (5 mM total lipid) incorporating [^{3}H]$MePEG_{2000}$-S-DSPE (circles) or $MePEG_{2000}$-S-[^{3}H]DSPE (squares) was incubated in the presence of 500 µl HBS (open symbols), or 500 µl serum (closed symbols). At various times, two 50 µl aliquots of the incubation mixture were removed and liposomes separated from free components by loading onto a 1 ml Bio-Gel A-15m spin column. The [^{3}H] (PEG-PE) to [^{14}C] (CHE) ratios were obtained from the peak two liposome fractions from both columns and expressed as a percentage (± S.D.) of the initial ratio before incubation.



the liposome. However, it also appears that slow hydrolysis of the succinate bond can occur on the liposome surface leaving the lipid anchor behind.

The ability of $MePEG_{2000}$-S-DSPE, when incorporated into DSPC/cholesterol LUVs, to prolong the circulation lifetime is significantly improved over that observed for $MePEG_{2000}$-S-POPE. Using [^{14}C]CHE labeled liposomes incorporating [^{3}H]$MePEG_{2000}$-S-DSPE or $MePEG_{2000}$-S-[^{3}H]DSPE, approximately 20% of the injected dose remains in the circulation at 24 h as shown in Figure 2.6, with the two preparations exhibiting very similar clearance behavior. When the [^{3}H] to [^{14}C] ratio was checked at various times both before and after separation of liposomes from plasma components (Figure 2.6*B* and 2.6*C*), both the MePEG and acyl moiety labels of the $MePEG_{2000}$-S- DSPE are shown to remain associated with the liposome. It may, however, be more accurate to say that the liposomes recovered, which are representative of those still in circulation, have retained most of their PEG coating. It is probable that liposomes which have lost their protective coating would have been rapidly cleared.

2.3.4 Biodistributions of DSPC/cholesterol LUVs containing different species of $MePEG_{2000}$-PE

The final series of experiments were performed to characterize the biodistribution at 24 h for DSPC/cholesterol LUVs incorporating various species of $MePEG_{2000}$-PE varying in acyl chain composition or PEG-PE chemistry at a liposome dose level of 1 μmol total lipid per mouse. The results are shown in Table 2.1. In the absence of a PEG-PE coating, DSPC/cholesterol LUVs are almost completely removed from the circulation at 24 h (less than 1% of the injected dose remains), with high levels accumulated in the liver and spleen. The incorporation of 10 mol % G_{M1} significantly increases the circulation levels to approximately 11% of the injected dose

Figure 2.6 (following page)

Circulation lifetime of DSPC/cholesterol/MePEG$_{2000}$-S-DSPE liposomes and in vivo exchange of MePEG$_{2000}$-S-DSPE from injected liposomes

A, circulation lifetime. Large unilamellar vesicles composed of DSPC/cholesterol/[^{3}H]MePEG$_{2000}$-S-DSPE (50:45:5) (□) or DSPC/cholesterol/MePEG$_{2000}$-S-[^{3}H]DSPE (50:45:5) (■) were injected via lateral tail vein into 25 g CD-1 mice (5 mM total lipid in 200 µl, 1 µmol total lipid/mouse). At various times, the mice were sacrificed and plasma isolated. The marker [^{14}H]CHE was used to determine liposome recovery. Results shown represent the mean of four animals ± S.E. of the mean.

B, the [^{3}H] to [^{14}C] ratio for plasma isolated from *A* before separation of liposomes from plasma components via spin column. (□), [^{3}H]MePEG$_{2000}$-S-DSPE, and (■), MePEG$_{2000}$-S-[^{3}H]DSPE preparations. Results represent the means of ratios obtained from the four mice and expressed as a percentage (± S.D.) of the initial ratio before injection.

C, the [^{3}H] to [^{14}C] ratio for plasma isolated from (a) after separation of liposomes from plasma components via spin column. (□), [^{3}H]MePEG$_{2000}$-S-DSPE, and (■), MePEG$_{2000}$-S-[^{3}H]DSPE preparations. Results represent the means of ratios of the peak two liposome fractions from two spin columns from four mice, and expressed as a percentage (± S.D.) of the initial ratio before injection.

Figure 2.6

Circulation lifetime of DSPC/cholesterol/MePEG$_{2000}$-S-DSPE liposomes and in vivo exchange of MePEG$_{2000}$-S-DSPE from injected liposomes

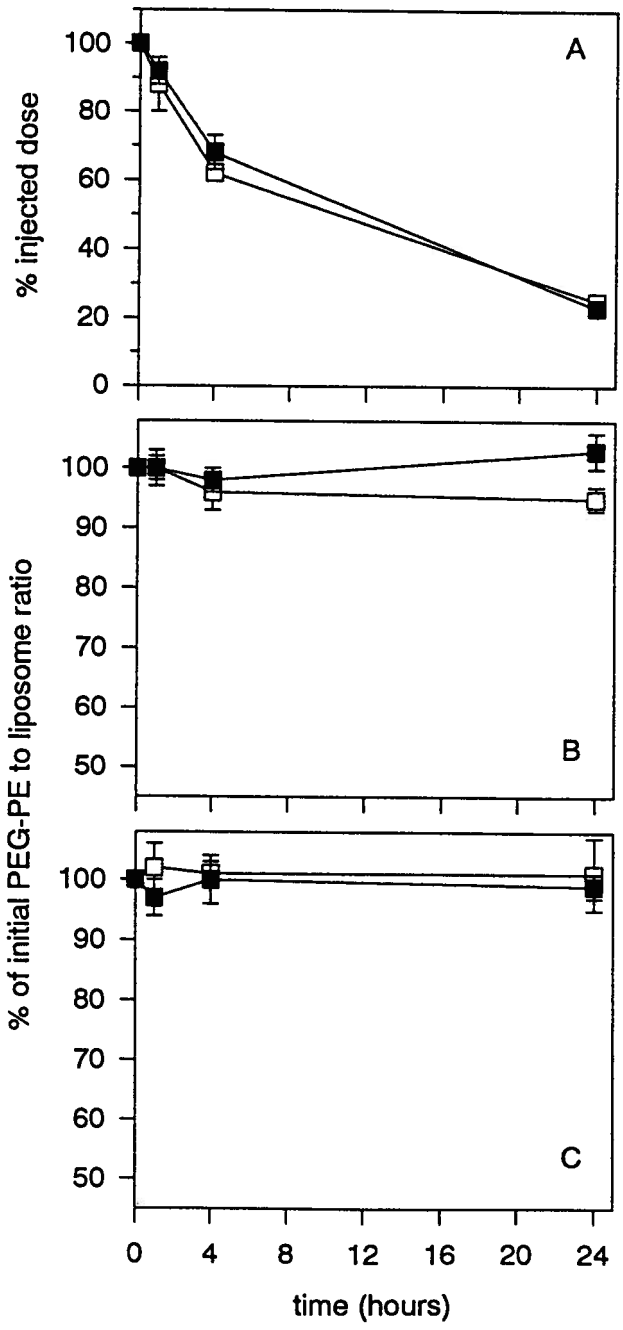


Table 2.1

Biodistribution of DSPC/cholesterol large unilamellar vesicles incorporating G_{M1} or PEG-PE one day after i.v. injection

The 5 mM 100 nm LUV preparations were injected via lateral tail vein in a volume of 200 µl (1 µmol total lipid) into 25 g CD-1 mice. At 24 h, the mice were sacrificed and plasma, liver, and spleen isolated. The percentage recovery of liposomes remaining in circulation was based on a plasma volume of 4.55% of individual mouse body weight. Liposomes associated with liver and spleen tissues were determined based on total organ weight. Each preparation employed trace [^{3}H]CHE as a liposome marker, and the results represent the mean of four animals ± S.E. of the mean.

Liposome composition (molar ratio)		% of injected dose recovered per total tissue		
		blood	liver	spleen
DSPC/chol	(55:45)	0.20 ± 0.10	56.4 ± 3.3	7.76 ± 1.50
DSPC/chol/G_{M1}	(45:45:10)	11.3 ± 0.5	25.4 ± 0.4	1.90 ± 0.09
DSPC/chol/$MePEG_{2000}$-S-POPE	(50:45:5)	3.31 ± 1.15	42.2 ± 2.8	3.85 ± 0.45
DSPC/chol/$MePEG_{2000}$-A-POPE	(50:45:5)	2.57 ± 0.92	42.6 ± 2.8	3.49 ± 0.49
DSPC/chol/$MePEG_{2000}$-S-DSPE	(50:45:5)	16.3 ± 1.1	24.9 ± 2.6	1.60 ± 0.10
DSPC/chol/$MePEG_{2000}$-A-DSPE	(50:45:5)	18.0 ± 0.9	18.8 ± 1.4	1.41 ± 0.07

remaining while decreasing the amount found in the liver and spleen at 24 h by factors of 2 and 4 respectively. Incorporation of 5 mol % $MePEG_{2000}$-S-POPE and $MePEG_{2000}$-A-POPE have smaller effects than G_{M1} in altering the LUV biodistribution. However, the presence of either $MePEG_{2000}$-S-DSPE or $MePEG_{2000}$-A-DSPE greatly increased the circulation levels present at 24 h to almost 20%, higher than achieved with G_{M1}, while the accumulation by the liver and spleen is reduced to an equal or better extent as G_{M1}. While there is little difference in biodistribution behavior between the succinate and amide versions for MePEG-POPE, the data for DSPE anchored species suggest that the amide linkage may be slightly superior in both improved circulation lifetimes and reduced liver and spleen uptake.

2.4 Discussion

The use of liposomes as systemic drug delivery vehicles depends upon their ability to remain in circulation for extended periods of time. The incorporation of PEG-lipids clearly allows extended circulation lifetimes to be achieved. However, the results presented here emphasize two major points. First, relatively subtle changes in the acyl chain composition of the PE anchor can significantly influence retention of the PEG-PE in the outer monolayer of the liposome. Second, significant chemical breakdown of PEG-PE conjugates may occur, particularly after the PEG-PE is lost from the LUV surface.

The influence of acyl chain composition on PEG-PE retention and related clearance behavior is particularly profound. As shown here, when $MePEG_{2000}$-S-POPE is incorporated into DSPC/cholesterol LUVs, the circulation lifetime is only modestly increased. This increase is less than that reported for $MePEG_{2000}$-DSPE (Allen et al., 1991a; Woodle et al., 1992), but comparable to other studies using $MePEG_{2000}$-DOPE (Mori et al., 1991). The poor performance of PEG-POPE is due to rapid removal of the exterior PEG coating, with a half-time of approximately two hours in vitro (50% mouse serum at 37°C) and approximately one hour in vivo. This may be compared with the rate of clearance of the injected LUVs, which exhibit a half-life in the circulation of approximately five hours. The fact that loss of the hydrophilic coating precedes liposome clearance suggests that loss of the PEG coating hastens clearance. As shown here, the loss of the PEG-POPE coating is primarily due to exchange of the entire PEG-POPE molecule out of the external monolayer.

The use of DSPE as the lipid anchor in place of POPE results in a dramatic improvement on the retention of the PEG coating. When $MePEG_{2000}$-S-DSPE is incorporated into LUVs and incubated in 50% mouse serum, approximately 90% of the PEG-PE remains associated with the LUVs after 24 h. The DSPE anchor also exhibits markedly superior properties in vivo. The circulation half-life of LUVs incorporating $MePEG_{2000}$-S-DSPE is approximately 10 h, with over 20% of the injected dose remaining in circulation at 24 h. In addition, the LUVs recovered from the circulation even up to 24 h show no exchange or breakdown of the $MePEG_{2000}$-S-DSPE, although any liposomes which have lost their PEG coating would likely have been cleared from the circulation.

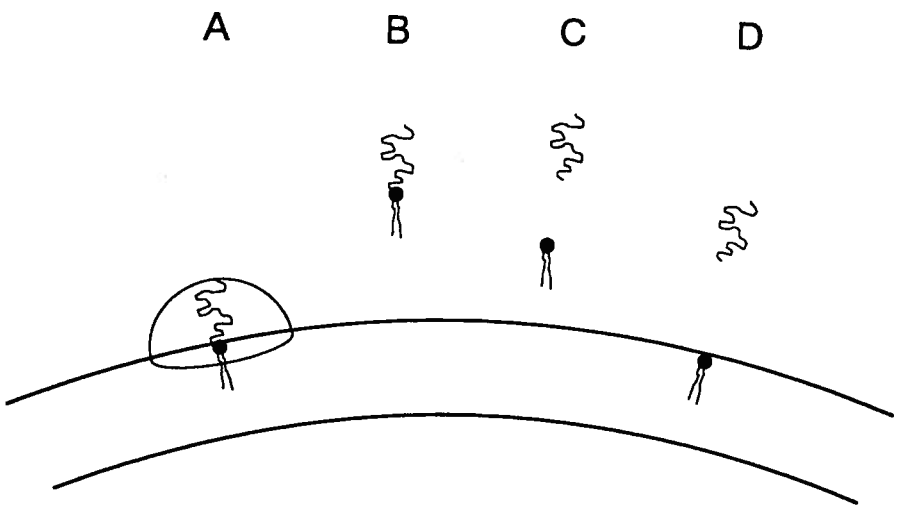
Previous work (Silvius and Zuckermann, 1993) examining the intervesicular exchangeability of several PEG-lipids in vitro showed that transfer of saturated diacyl conjugates of $MePEG_{2000}$ decreased exponentially with increasing chain length. In addition, transfer of POPE derivatives of $MePEG_{2000}$ and $MePEG_{5000}$ was found to be 30 to 40 fold slower than the corresponding DMPE derivatives. Thus, the increase in LUV retention in vitro and in vivo between POPE and DSPE anchors observed here is not unreasonable.

The chemical stability studies on pure (micellar) PEG-lipids indicate that the succinate linkage is labile in mouse serum, generating free MePEG-OH by one hour with complete hydrolysis of this linkage within 24 hours. A variety of other linkages proved to be more stable in this respect, including carbamate, amide, and direct linkages. The results presented here also indicate that the succinate bond is protected by retention of the PEG-lipid in the LUV. For $MePEG_{2000}$-S-DSPE, a slow loss of the PEG headgroup as compared to the lipid anchor (which is completely retained) indicates that it is possible to remove the PEG from the LUV surface and leave the lipid anchor

Figure 2.7

Models for PEG-PE exchange and breakdown

A, intact PEG-PE remaining on liposome surface providing steric stabilization; *B*, exchange of the entire molecule from the membrane as in PEG-POPE; *C*, breakdown of chemical linkage (succinate) after exchange, or; *D* possible breakdown of chemical linkage (succinate) on surface of liposome membrane when PEG-PE is well anchored.



behind, however, this effect is small compared to loss of the POPE anchored version which is almost completely removed from the outer monolayer within several hours.

The rationale for the use of PEG-lipids is their ability to significantly reduce the rate of clearance of liposomes from the circulation. At a practical level, the results presented here demonstrate that

chemical stability and lipid anchoring ability are determining factors for the ability of PEG-lipids to provide improved circulation lifetimes for LUVs in vivo. While it has been reported that the nature of the anchor of PEG-lipids influences the circulation lifetimes of injected liposomes (Allen et al., 1991a) and that the LUV lipid composition can also affect the circulation lifetimes of liposomes incorporating PEG-PEs (Maruyama et al., 1992; Litzinger and Huang, 1992), others suggest that different PEG anchors are equivalent and that the lipid composition may be varied with little effect if PEG-PEs are incorporated (Woodle et al., 1992). Our results conclusively demonstrate the importance of the PEG-lipid anchor.

In summary, the lipid anchor is a primary factor in the retention of a PEG polymeric coating for LUVs. Chemical breakdown of the conjugate largely occurs after exchange out of the LUV but can occur on the LUV surface depending upon the PEG-lipid linkage. The use of a strong membrane anchor (DSPE) and chemically stable conjugate bond (amide) results in retention of the polymeric coating and greatly enhanced circulation lifetimes. It is concluded that since the major rationalization for the use of PEG-lipids in liposomes is the prolonged circulation lifetimes and hence the greater chance of accumulation in targets other than the RES, by whatever detailed mechanisms PEG-PE is proposed to work, this requires that the PEG coating is retained and should be a consideration in any practical discussion of the use of PEG-lipids.

CHAPTER 3

CHARACTERIZATION OF RES BLOCKADE WITH DOXORUBICIN AND VINCRISTINE

3.1 Introduction

The studies in this chapter are based on previous work from this laboratory concerning the characterization and biodistribution of doxorubicin-loaded liposomes (Bally et al., 1990a). It was shown that the RES function was strongly influenced by liposome containing doxorubicin as such liposome were cleared from the circulation at a slower rate than empty liposomes. Further, it was found that pre-dosing with liposomes containing doxorubicin resulted in greatly extended circulation lifetimes of a subsequent injection of empty liposomes. These results were attributed to an ability of doxorubicin-loaded liposomes to impair or "blockade" RES function. RES "blockade" provides an alternative means to achieve long circulation lifetimes and does not necessarily require the use of lipids such as those described in Chapter 2.

The experiments performed here are divided into two sections. It has been suggested that stealth or sterically stabilized liposomes exhibit an ability to avoid uptake by the phagocytic cells of the reticuloendothelial system (RES) found predominantly in the liver and spleen (Allen and Chonn, 1987; Klibanov et al., 1990; Lasic et al., 1991; Oku et al., 1992). In the first part of this study the validity of this hypothesis is examined. Specifically, the influence of G_{M1} on the ability of a pre-dose of liposomes containing doxorubicin to blockade RES function, as expressed by the extended circulation lifetimes exhibited by a subsequent injection of empty liposomes, is examined. One of two possible results would be expected. If G_{M1}-containing liposomes with

entrapped doxorubicin do in fact avoid the RES, the liver and spleen function should not be affected and the subsequent injection of empty liposome should be cleared normally. On the other hand, if G_{M1}-containing liposomes do not avoid the RES, one would expect to see an impaired ability of the RES to clear liposomes and thus extended circulation lifetimes for subsequent injections.

The second component to the studies described in this chapter concern potential adverse side effects due to the use of procedures involving RES blockade. Because the RES is the primary site for foreign particulate clearance, it has long been recognized that many potential harmful effects may result following the delivery of cytotoxic liposomal agents. The liver macrophages in particular play a key role in host defense mechanisms (Phillips, 1989, Toth and Thomas, 1992). The effect that entrapped doxorubicin has on the RES is further examined here. Preliminary studies indicated that liposomally delivered doxorubicin had a maximum effect at 24 h after injection, and that very little drug was needed to achieve optimal blockade. These studies were extended to more fully characterize the RES blockade technique, including the amount of liposomally delivered doxorubicin necessary to achieve blockade as well as the duration of the effect. In addition a different anticancer agent, vincristine, was also studied in terms of inducing RES blockade. This agent was selected as an appropriate alternative since, unlike doxorubicin which is a cytotoxic drug, vincristine is a cytostatic agent known to act by inhibiting formation of microtubules required for formation of the spindle apparatus in dividing cells (Owellen et al., 1972, 1976). It is anticipated that this cell-cycle specific agent will not directly affect the viability of mature macrophages such as Kupffer cells. Vincristine, will, however, affect the function of these cells by inhibition of cellular filament formation required for membrane trafficking and cellular rearrangement as seen in phagocytic processes. It is shown that either

liposomal drug can induce RES blockade even at very low doses, although the duration is considerably shorter for vincristine. These findings are discussed in terms of the different potential modes of action of these two drugs.

3.2 Materials and methods

3.2.1 Liposome preparation

Liposomes were prepared as described in Chapter 2. Briefly, lipid mixtures in chloroform were dried to a film under a stream of nitrogen gas, then further dried under high vacuum for a minimum of 4 h. For the pre-dose composition, the lipid was hydrated with 300 mM citric acid (pH 4.0), frozen and thawed five times, and then extruded at 65°C ten times through three stacked polycarbonate filters (Nuclepore, Canada) of 100 nm pore size employing and extrusion device (Lipex Biomembranes, Canada). An approximate. 100 nm mean diameter for the resultant LUVs was determined employing a NICOMP 370 particle sizer. A transmembrane pH gradient was established by passing the LUV preparations down a Sephadex G-50 column equilibrated with 150 mM sodium carbonate buffer (pH 7.5) and collecting the LUVs in the void volume.

For the empty LUVs employed for the subsequent injection, the lipid film was hydrated in HBS (20 mM HEPES, 150 mM NaCl, pH 7.4) and extruded as described above.

3.2.2 Drug loading

Doxorubicin (Adria Laboratories) and vincristine (Lyphomed Canada) were loaded into LUVs as described previously (Mayer et al., 1986; Mayer et al., 1990a; 1990b). Aliquots of preheated

drug in saline were added to pre-heated liposomes at 65°C to achieve the indicated drug/lipid ratios (mol/mol) and incubated at this temperature for a further 10 min. Entrapment efficiencies were in excess of 95%.

3.2.3 Animal biodistribution studies

Female BDF-1 (for the first series of experiments) and CD-1 (for the second) mice (20-23 g, Charles River, Canada) were injected with the specified dose of empty and drug-loaded liposomes via the lateral tail vein. Pre-doses consisted of 100 nm diameter LUVs composed of DSPC/cholesterol (55:45, mol/mol), DSPC/cholesterol/G_{M1} (45:45:10), or DSPC/cholesterol/$MePEG_{2000}$-A-DSPE (50:45:5) with or without entrapped doxorubicin or vincristine. These LUVs were injected at a dose of 0.33 μmol lipid per mouse (10 mg/kg lipid dose for DSPC/cholesterol) delivered in a volume of 200 μl. A trace amount of [^{3}H]cholesteryl hexadecylether (NEN, Canada) was used as a non-exchangeable lipid marker (Derksen et al., 1987) for determining the biodistribution of this pre-dose at 24 h. Blood was collected by heart puncture and placed in EDTA-treated microtainers (Becton-Dickinson, Canada). Plasma was prepared by centrifuging (200 × g) the blood samples for 10 min. followed by liquid scintillation counting of 200 μl samples to determine radioactivity. Liver and spleen were removed whole from the animal carcass and weighed. 20% or 10% homogenates in water were prepared for liver and spleen, respectively. 200 μl of this was then digested with 500 μl Solvable (NEN/DuPont) for 1 h at 60°C, cooled, bleached with 200 μl H_2O_2 (30%), and tissue-associated radioactivity determined by liquid scintillation counting. For the biodistributions of the subsequent injection, the pre-doses described above (but not containing any radiolabeled lipid marker) were given, and then a subsequent injection of empty liposomes was administered 24 h later. This later injection

was composed of 100 nm diameter DSPC/cholesterol (55:45) LUVs with a trace amount of [^{3}H]cholesteryl hexadecylether, injected at a dose of 3.3 μmol lipid per mouse (100 mg/kg lipid dose) delivered in 200 μl. One control group of mice received no pre-dose. The mice were sacrificed 24 h later and the liposome biodistribution in blood, liver, and spleen was determined. Biodistribution results were analyzed using a two-tailed Student's t-test.

DSPC was obtained from Avanti Polar Lipids, and cholesterol, G_{M1}, and all other chemicals were obtained from Sigma. $MePEG_{2000}$-A-DSPE was synthesized as described in Chapter 2.

3.2.4 Liver histology

Groups of 4 CD-1 mice were injected with pre-doses (0.33 μmol lipid per mouse) consisting of DSPC/cholesterol LUVs with entrapped doxorubicin (0.2 mol:mol drug:lipid ratio) or vincristine (0.05). Four days later the mice were sacrificed and liver removed. Cryostat sections were prepared by washing the excised liver lobes in PBS at 4°C and then fixed in 3% parafomaldehyde in PBS. They were then washed passed through graded concentrations of sucrose, and embedded in OCT and frozen in liquid nitrogen. Sectioning (5 μm thick) was carried out at -20°C using a Frigocut 2800N Reichert-Jung Leica microtome. These sections were then washed in PBS, stained with Carazzis hematoxylin, washed with water, 1.5% $NaHCO_3$, and water again, and then mounted for viewing. Sections were scored for Kupffer cells over 15 randomly selected fields (40×, Leitz Dialux microscope) over a range of liver sections for each treatment group. Photomicrographs were obtained using a Orthomat microscope camera. All images were recorded on Fuji color ASA400 negative film.

Liver uptake of colloidal carbon (du Souich et al., 1981) was also carried out. A commercially available India Ink (Koh-I-Noor)(80 mg/ml) was diluted 20 fold and 200 µl injected i.v. 24 h after the injection of pre-doses described for the histology experiment above. Four hours after the injection of colloidal carbon the mice were sacrificed and liver cryostat sections were prepared as described above.

3.3 Results

3.3.1 The presence of G_{M1} in liposomes with entrapped doxorubicin does not prevent RES blockade

The biodistribution of the pre-dose at 24 h after injection is shown in Figure 3.1. In the blood, the liposomes which did not contain G_{M1} are present in very low levels (<1% of injected dose) whereas those containing G_{M1} are present at levels corresponding to approximately 25% of the injected dose at 24 h. The difference is largely accounted for by reduced liver and (to a smaller extent) spleen uptake for the G_{M1} containing formulation. Both DSPC/cholesterol liposomes and G_{M1}-containing liposomes exhibit significantly reduced liver uptake when the liposomes contain entrapped doxorubicin ($p<0.05$ for both groups). This results in greatly increased uptake in the spleen for the DSPC/cholesterol LUVs which did not contain G_{M1}. However this effect is not seen for G_{M1}-containing liposomes where the two spleen panels are not significantly different ($p > 0.05$). The biodistribution 24 h after the subsequent injection of empty DSPC/cholesterol LUVs is shown in Figure 3.2. The biodistribution observed in a group of mice which received no pre-dose is shown in the left bars. This pattern of blood clearance and liver and spleen uptake is taken as a primary control. Pre-doses of liposomes which did not contain drug results in no significant difference in the uptake into the liver and spleen from their respective controls (p values all > 0.05) and indicates that pre-injection of a low dose of lipid alone does not alter the

Figure 3.1

Biodistribution of the pre-dose containing liposomal doxorubicin

Large unilamellar vesicles of various compositions were injected via lateral tail vein at a dose of 0.33 μmol lipid/mouse. At 24 h, the mice were sacrificed and tissue samples indicated were measured for liposomal lipid levels. Where employed, doxorubicin was entrapped at a drug:lipid ratio of 0.2 (mol:mol). Values shown represent the mean of results from 8 animals ± S.E. of the mean.

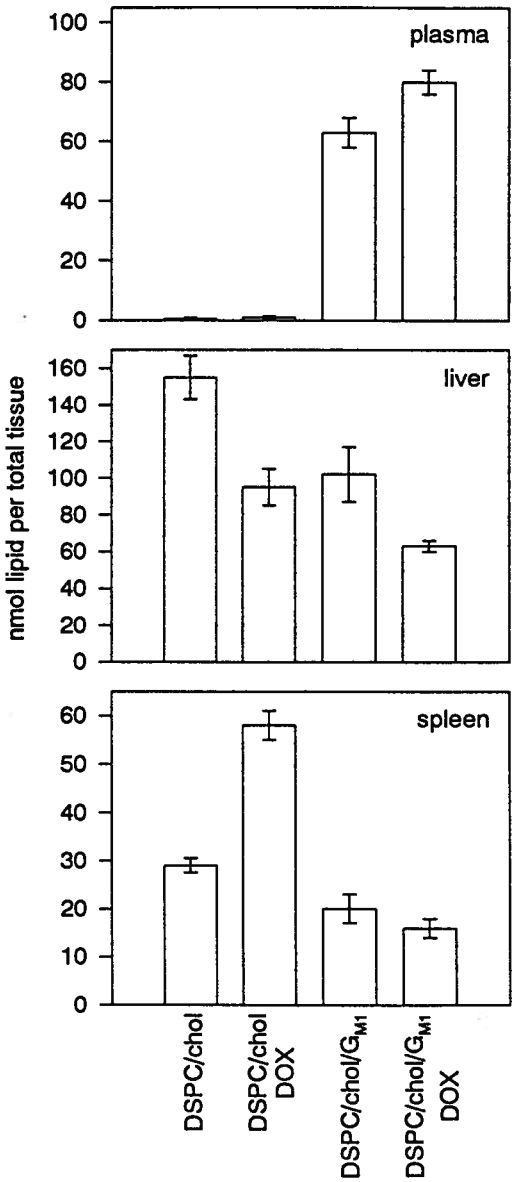
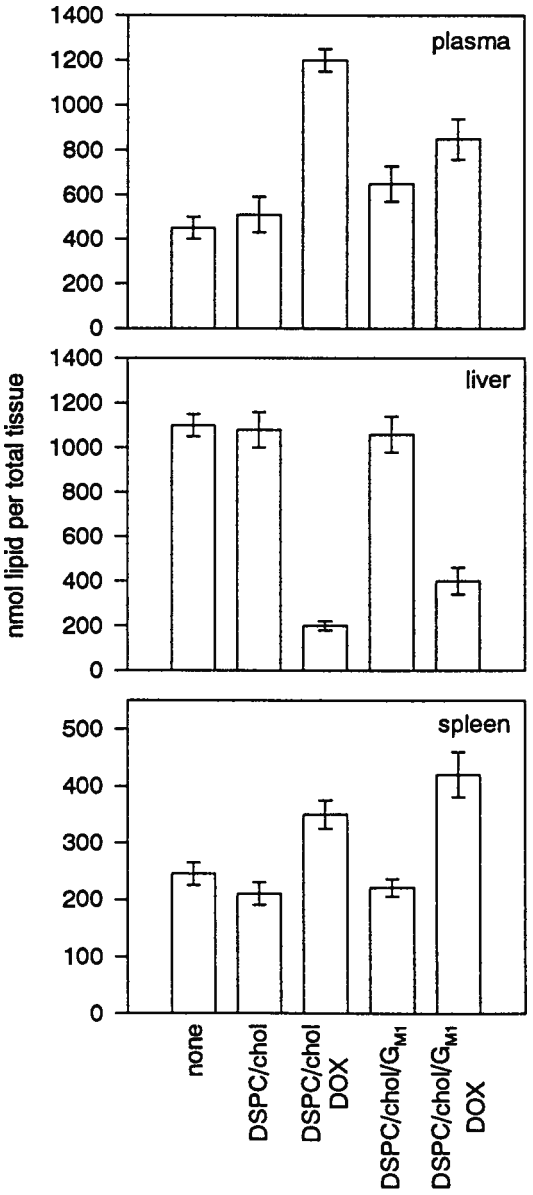


Figure 3.2

Biodistribution of the subsequent injection of empty liposomes

At 24 h after the injection of the pre-doses indicated at bottom (as in Figure 3.1), LUVs composed of empty DSPC/cholesterol were injected at a dose of 3.3 μmol lipid per mouse. Twenty four hours after this the mice were sacrificed and lipid levels of this subsequent injection in the tissues determined. Values represent the mean of 8 animals ± S.E. of the mean.



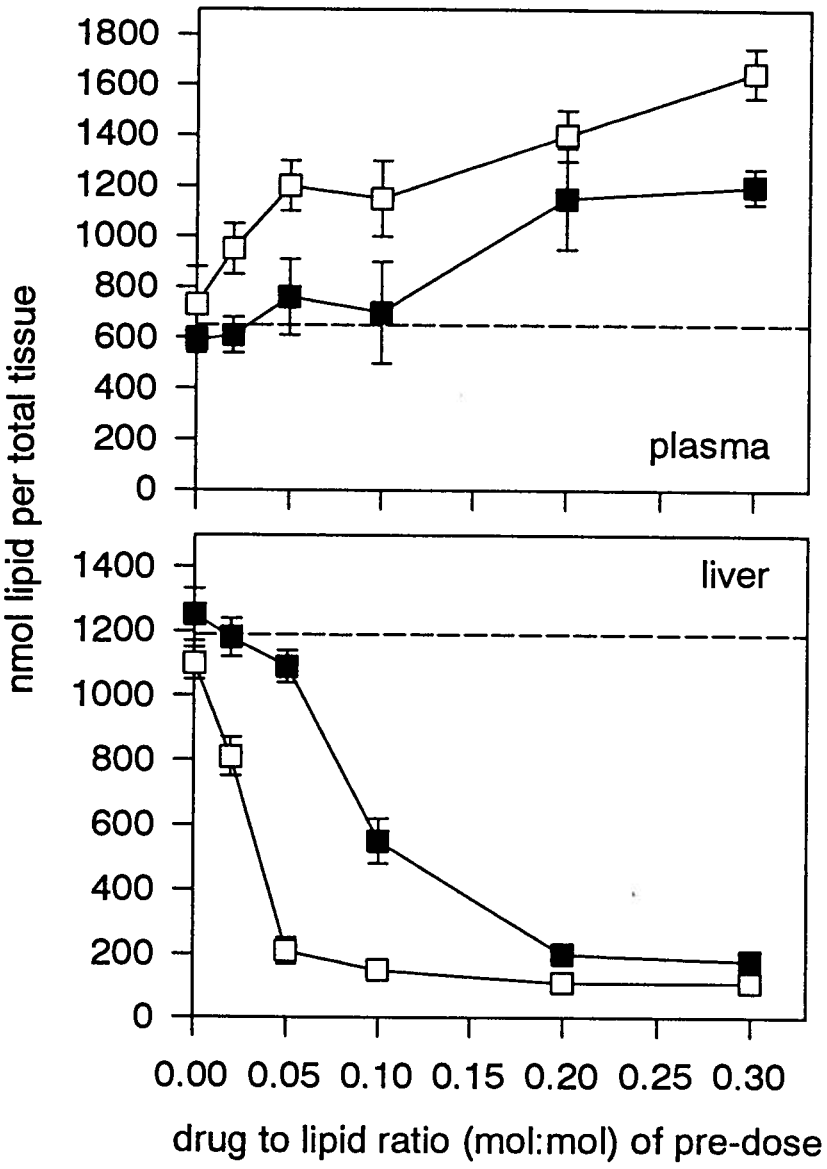
pattern of uptake into these tissues. A slight increase in blood levels of the subsequent injection for the pre-dose containing empty G_{M1} is liposomes is observed. However, pre-injection of drug-loaded liposomes which did not contain G_{M1} substantially blocks liver uptake of the later injection of empty liposomes, resulting in elevated blood levels and spleen uptake. Pre-doses of doxorubicin-loaded G_{M1}-containing LUVs also results in dramatic blockade of liver uptake as well as elevated spleen uptake. The reduction of liver uptake is slightly less than that observed in the absence of G_{M1}. Interestingly, there is no significant difference for blood levels of G_{M1}-containing LUVs. It is important to note that these results represent the effect of liposomally entrapped doxorubicin; the administration of free doxorubicin prior to injection of empty liposomes has been shown not to alter the liposome clearance (Bally et al., 1990a).

These studies were extended to determine the minimum dose of doxorubicin required in both control and G_{M1}-containing liposome to induce significant RES blockade. These studies were performed by injection of the same lipid pre-doses which contained varying amounts of doxorubicin, and then determining the biodistribution of a subsequent injection of empty liposomes. The drug/lipid ratios were 0.00, 0.02, 0.05, 0.10, 0.20, and 0.30 (mol/mol). The mean values obtained for mice which received no pre-dose are indicated by the dashed lines. In both the blood and liver, the effect of entrapped doxorubicin in liposomes without G_{M1} on the biodistribution of the subsequent injection are readily apparent even at very low drug levels (drug/lipid ratios < 0.02 mol/mol), whereas for the G_{M1}-containing pre-dose, higher drug doses (drug/lipid ratios of 0.10 mol/mol) are required to induce the same effect. It should be noted that these dose levels are very small in comparison to the dose required to result in therapeutic benefit. A drug/lipid ratio of 20 would be required to achieve the maximum tolerated doxorubicin dose of 20 mg/kg (Mayer et al., 1990b; Bally et al., 1990b). for example. Thus at

Figure 3.3

Dose titration of entrapped doxorubicin in the pre-dose: biodistribution of the subsequent injection

Pre-doses with entrapped doxorubicin at drug:lipid ratios indicated were injected at a dose of 0.33 μmol lipid per mouse and then at 24 h, a 3.3 μmol lipid per mouse injection of DSPC/chol liposomes was given. 24 h after this lipid levels of the subsequent injection were determined. Pre-dose compositions were (□), DSPC/chol, and (■)DSPC/chol/G_{M1}, with entrapped doxorubicin as indicated by the drug:lipid ratio. Dashed line indicates results from mice which received no pre-dose. Values shown represent the mean of results from 4 animals ± S.E. of the mean.



any reasonable dose of doxorubicin in G_{M1}-containing liposomes, strong RES blockade would be expected. In addition to the ability of G_{M1}-containing LUVs with entrapped doxorubicin to blockade liver uptake, a further point of interest concerns the different uptake behavior of the liver and spleen. An ability to dramatically block liver uptake by a small pre-dose of liposomally entrapped doxorubicin is consistent with specific uptake of liposomes by Kupffer cells (Roerdink et al., 1981; Senior, 1987) and argues against any non-specific mechanism. Similarly, the increases in spleen uptake seen as a result of liver blockade imply that liposome uptake in the spleen by fixed macrophages plays a relatively minor role in liposome clearance. Rather, these results suggest a non-specific filter model (Liu et al., 1991; 1992) where the spleen accumulates liposomes not cleared by the liver.

3.3.2 Characterization of RES blockade with entrapped doxorubicin and vincristine

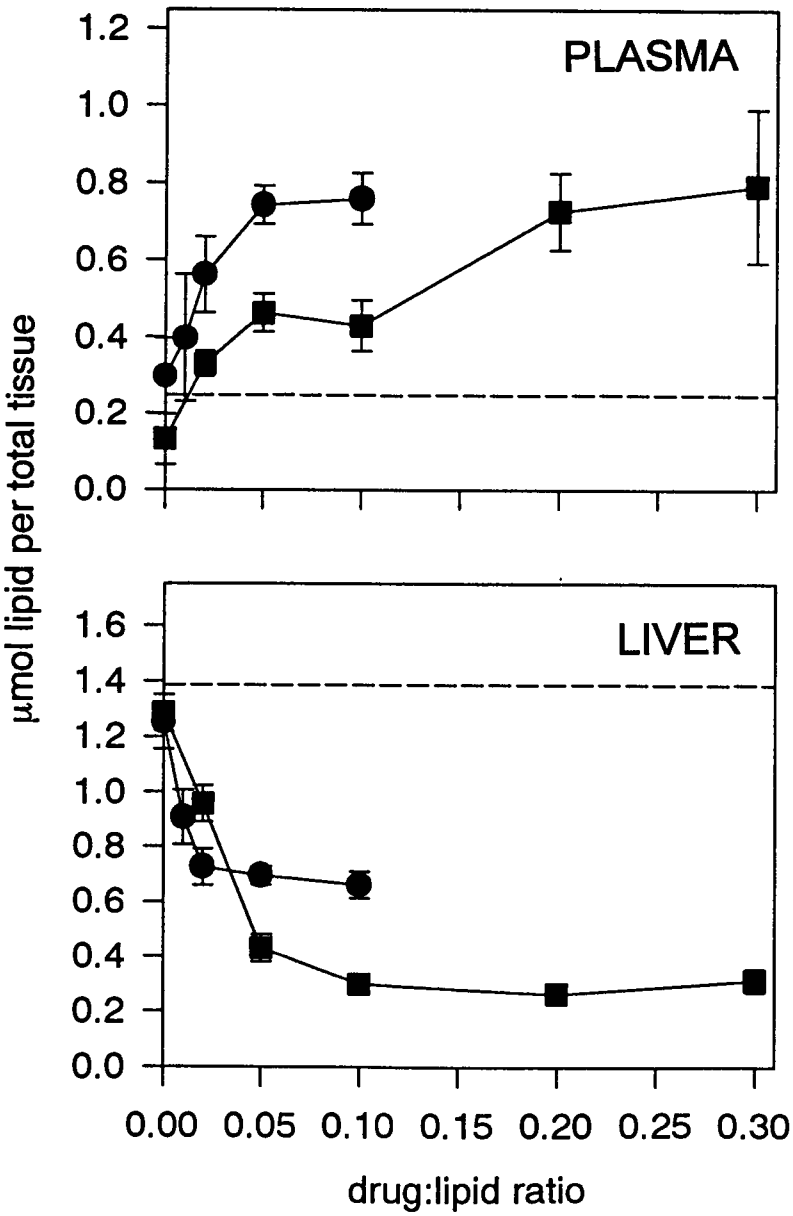
Figure 3.4 shows a dose titration of liposomally entrapped drug in the pre-dose and the clearance of the subsequent test injection. The results for doxorubicin are similar to that from the previous section (Figure 3.3) where a maximum liver depression is observed at drug to lipid ratios of approximately 0.05 (nominally 0.5 mg/kg DOX). As indicated in Section 3.3.1, there is an almost 5 fold depression in liposomal lipid uptake in livers of these pre-treated animals. The absolute levels, however, differ between the BDF-1 mice used previously and the CD-1 mice used here.

Dose titration of entrapped vincristine at this time point (24 h) shows similar dose titration effects as entrapped doxorubicin. Following administration of a second dose of liposomes (100 mg/kg) one observes increases in plasma liposomal lipid levels and decreases in liver levels.

Figure 3.4

Dose titration of entrapped doxorubicin or vincristine in the pre-dose: biodistribution of the subsequent injection

Pre-doses consisted of 0.33 μmol lipid per mouse (10 mg/kg) DSPC/chol with entrapped drug at various drug:lipid ratios indicated at bottom. 24 h later the subsequent injection (empty DSPC/chol, 3.3 μmol lipid per mouse; 100 mg/kg) was given, and then the biodistribution of the subsequent injection 24 h after this was then determined. (top plasma, bottom liver). The dashed line represents results from mice that received no pre-dose. Pre-dose compositions contained liposomally entrapped (●), VINC or (■) DOX. Values shown represent the mean of results from 4 animals ± S.E. of the mean.



Maximum effects are achieved when the drug to lipid ratio is greater than 0.025. The depression in liver uptake levels off past the 0.02 drug:lipid ratio and only represents a 2-3 fold decrease. Interestingly, a higher elevation of plasma levels at lower drug doses is seen with liposomal vincristine.

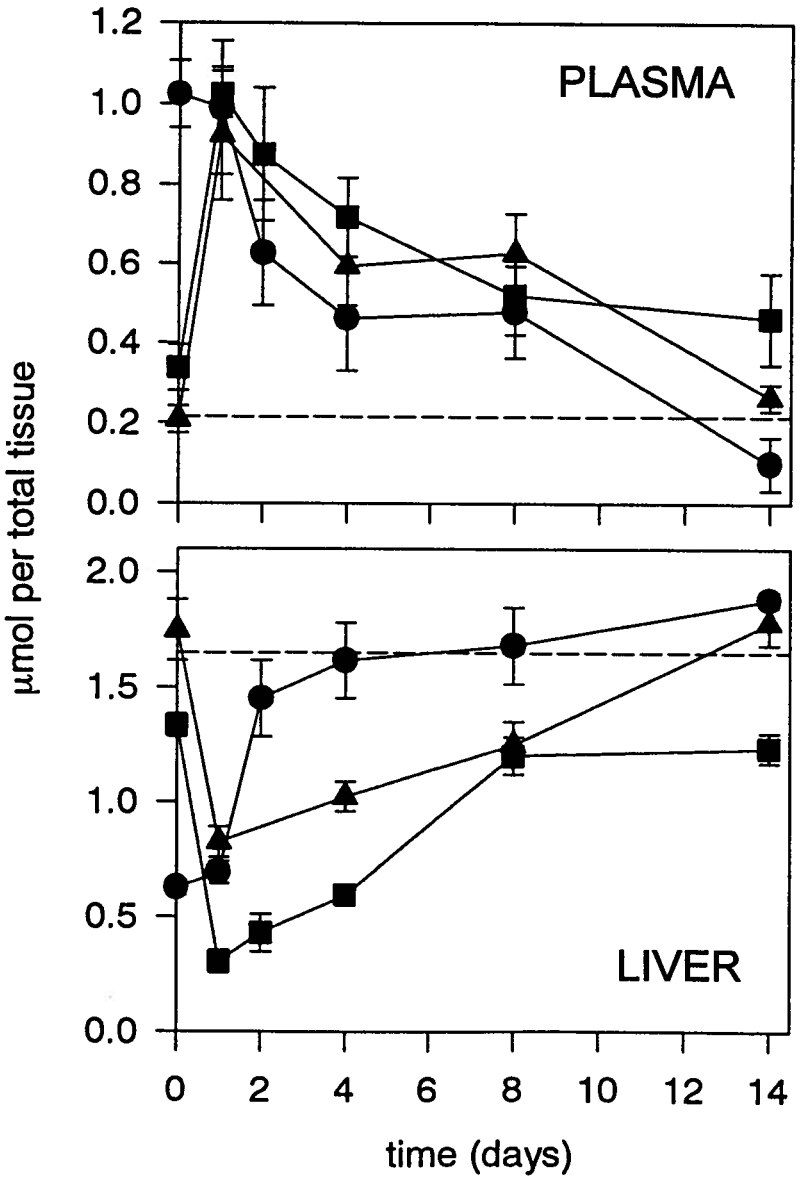
A time course for RES recovery is shown in Figure 3.5, in which the timing between the pre-doses and subsequent injection is varied. For this experiment, the drug to lipid ratio corresponding to the maximum effect from the dose titration data was used (0.2 mol:mol for doxorubicin and 0.05 mol:mol for vincristine). When the pre-dose and subsequent injection were given simultaneously (day 0), this dose of doxorubicin had little effect on the clearance of the subsequent injection. However for vincristine, there was an immediate liver depression and greatly increased plasma levels. By one day, the doxorubicin pre-dose produced maximum depression of liposomal lipid uptake in liver (now under the same conditions as that for Figure 3.4). Liposomal vincristine pre-injection also resulted in a suppression that was comparable to that shown in Figure 3.4 on day 1. For vincristine treated animals, normal liver uptake was restored by day 2, whereas doxorubicin treated animals did not recover until approximately day 8 to 14.

Additional data shown in this figure for the pre-dose compositions includes a pre-dose consisting of DSPC/cholesterol/$MePEG_{2000}$-A-DSPE liposomes with entrapped doxorubicin (0.2 drug:lipid ratio), analogous to the G_{M1} liposomes used as a pre-dose composition in the previous section. The timing of recovery of clearance behavior closely parallels that for the conventional liposomal doxorubicin treated animals, although the effect is attenuated overall. There is similar plasma elevation throughout the time course ($p > 0.05$), and the liver is also similar, although

Figure 3.5

Time course of recovery of RES blockade achieved following i.v. administration of liposome entrapped doxorubicin or vincristine

Pre-doses consisted of 0.33 μmol lipid per mouse (10 mg/kg) DSPC/chol with entrapped drug at a drug:lipid ratio of 0.05 for VINC and 0.2 for DOX. At various times later, as indicated by the bottom scale, the subsequent injection was given (empty DSPC/chol, 3.3 μmol lipid per mouse; 100 mg/kg), and then the biodistribution of this subsequent injection 24 h after this was then determined. (top plasma, bottom liver). The dashed line represents results from mice that received no pre-dose. Pre-doses contained liposomally entrapped (●), VINC or (■) DOX as described above. The results for doxorubicin entrapped within a DSPC/chol/PEG pre-dose (0.2 mol:mol drug to lipid ratio are also shown (▲). Values shown represent the mean of results from 4 animals ± S.E. of the mean.



significantly reduced in magnitude ($p < 0.05$ at 1, 4 and 14 days compared to the conventional liposomal DOX liver blockade) and possibly recovery period.

3.3.3 Liver histology after RES blockade

Liver cryostat sections taken 4 days after administration of liposomal doxorubicin or vincristine (Figure 3.6) were scored for Kupffer cells and, surprisingly, revealed no differences in the number of Kupffer cells identified via morphological and staining characteristics between control and drug treated animals. A second approach to assess RES status involved carbon clearance. No quantitative differences in the number of cells shown to accumulate colloidal carbon one day after the pre-dose treatments were found (Figure 3.7). Qualitative differences may, however, exist. Control liver sections, for example, revealed a close association between the colloidal carbon and Kupffer cells (panel *A*), whereas in the liposomal doxorubicin treated animals the carbon appeared to be somewhat more associated with hepatocytes (panel *B*). For the vincristine treated animals, carbon distribution appeared both qualitatively and quantitatively similar to the controls (panel *C*).

3.4 Discussion

Longer circulation lifetimes are observed for doxorubicin loaded liposomes in general (Bally et al., 1990a). In order to better elucidate the mechanism behind this phenomenon, experiments were performed here in which pre-doses of various amount of liposomal doxorubicin could be given and their effect on the RES determined by testing with a subsequent test injection of empty liposomes. In the first part of this chapter it was established that pre-doses without entrapped

Figure 3.6

Cryostat sections of livers obtained from normal, liposomal doxorubicin treated, and liposomal vincristine treated animals

Four days after a pre-dose of liposomal drug, the mice were sacrificed and liver sections fixed, frozen and stained with hematoxylin. (*A*) normal liver, (*B*) liposomal DOX treated and (*C*) liposomal VINC treated animal livers sections. Only those sinusoidal cells which were large, irregular (length to width ratio of 2 or greater), and darkly staining were scored as Kupffer cells.

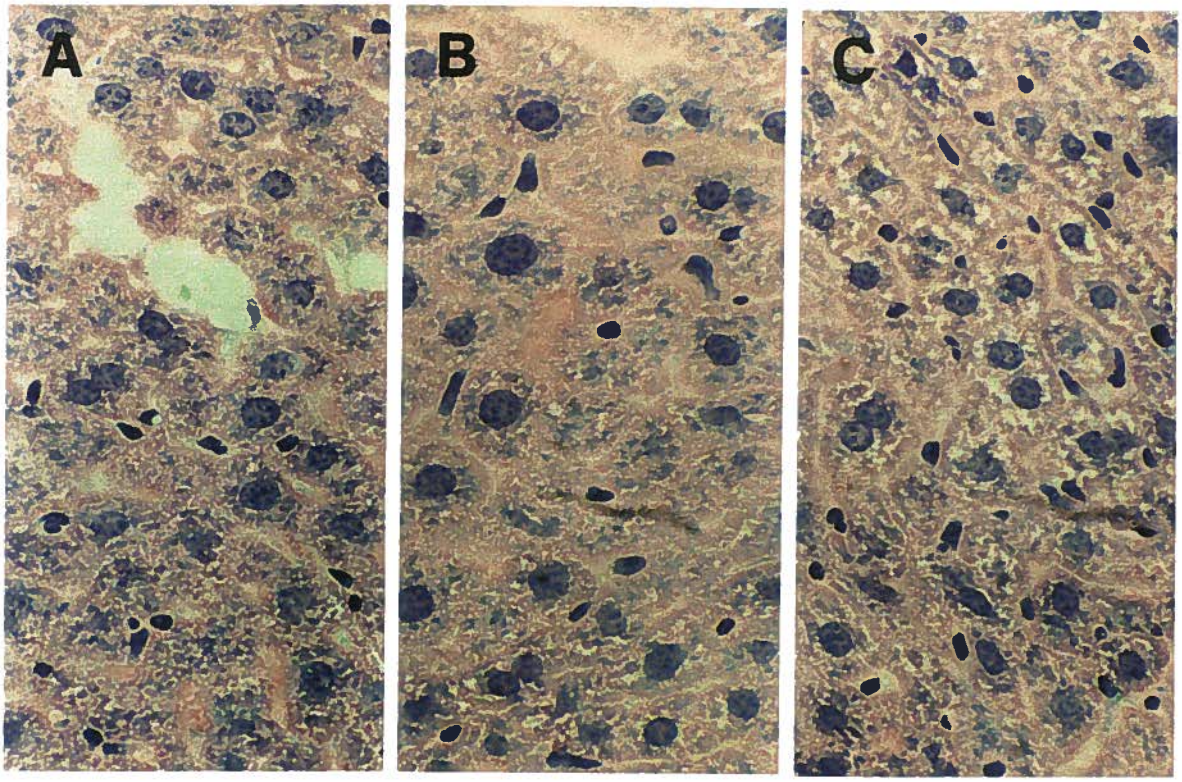
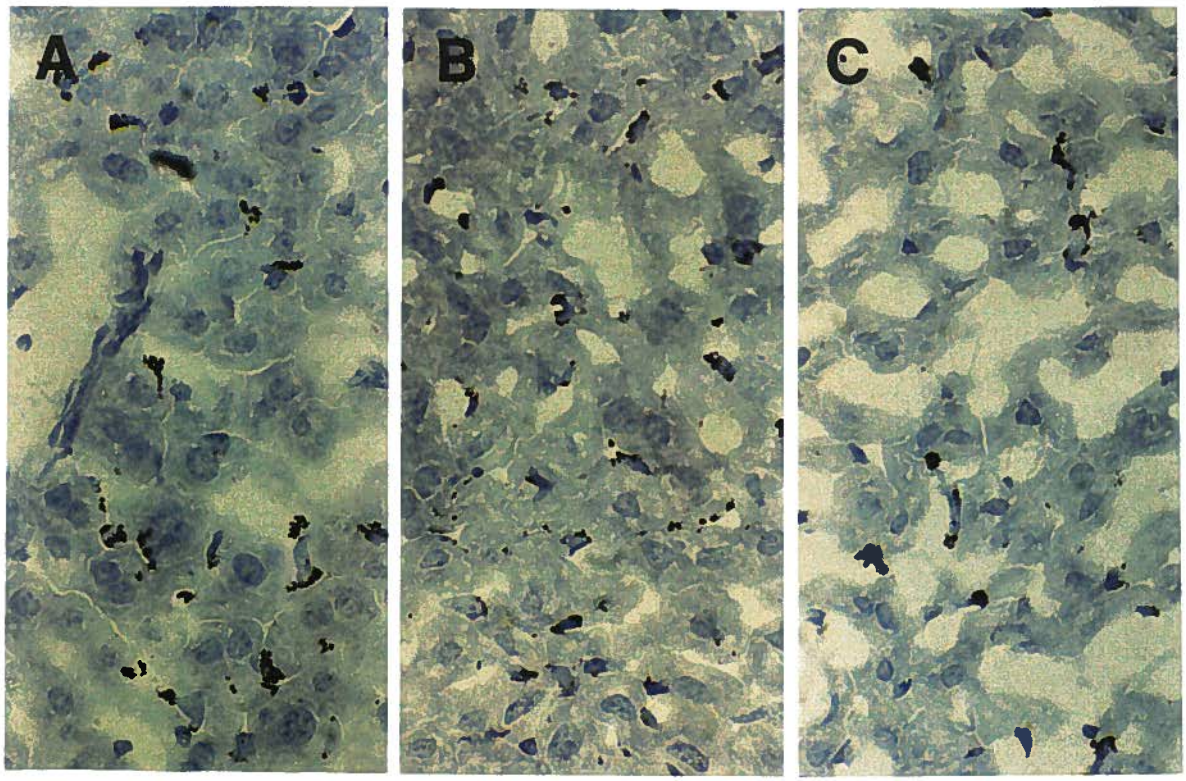


Figure 3.7

Cryostat sections of normal liver, liposomal DOX treated and liposomal VINC treated animals after injection of colloidal carbon

24 h after a pre-dose of liposomal drug, 40 mg/kg colloidal carbon was injected and then 4 h after this the mice were sacrificed and liver sections fixed, frozen and stained with hematoxylin. Colloidal carbon appears as sharply defined, aggregated black particles. Liver sections were from (*A*) normal, (*B*) liposomal DOX treated, and (*C*) liposomal VINC treated animals



drug had no effect on RES uptake of a subsequent injection and thus allowed us to isolate the effect of entrapped drug. Pre-doses containing doxorubicin were found to greatly impair the RES, and even when the pre-dose contained G_{MI} RES blockade was readily apparent. In the second part of the chapter, additional data suggested that vincristine, another liposomal drug under current development, could also induce RES blockade, although a time course suggested that the effect was more transient. Animals pre-dosed with liposomal vincristine recovered within several days whereas those treated with liposomal doxorubicin took over 8 days to recover to normal liver liposome clearance behavior. Additional experiments with PEG-PE containing liposomes confirmed that such longer circulation lifetime formulations do not strictly avoid the RES as evidenced by the similar albeit attenuated RES blockade.

Based on the time course data it was originally assumed that even at the extremely low levels of liposomal doxorubicin being employed, the liver macrophages were being killed. The 8-14 day recovery period is consistent with the turnover times for the Kupffer cell population (Crofton et al., 1975). Modes of action for doxorubicin include DNA intercalation and topoisomerase II interference, free radical formation, covalent binding to DNA, and various cell binding effects (Cummings et al., 1991). While most effective against rapidly dividing cells such as tumor cells, non dividing cells including Kupffer cells are also killed by doxorubicin as a result of some or all of the above modes of action (Barranco, 1984).

Vincristine on the other hand is more specific in its mode of action in that it binds to the growing end of microtubules and prevents their assembly (Owellen et al., 1972, 1976). Given that recovery of the capacity of the liver to accumulate liposomes was rapid (within 1-2 days), it seems reasonable that vincristine inhibits phagocytosis only for the time that the drug is present

and does not kill Kupffer cells. While phagocytosis of larger (> 1 μm) particles is dependent on actin filaments, microtubules appear involved in the uptake of smaller (< 0.9 μm) particles, with a gradual continuum of different filament involvement between the two extremes (Pratten and Lloyd, 1986; Toyohara and Inaba, 1989).

When the livers obtained from animals pre-treated with liposomal doxorubicin or vincristine were examined, no reduction in the number of Kupffer cells was observed. The 4 day time point was selected for these studies because depression of liposome uptake in the liver after doxorubicin pre-treatment was maximal. This also eliminated potential problems due to cells that were killed but not yet eliminated from the tissue at earlier time points. The conclusion from this data is that liposomal doxorubicin, although functionally shutting down mechanisms responsible for liposome uptake in liver, is not eliminating Kupffer cells. Daemen et al. (1995) have done very similar work in rats and shown that a single injection of 5 mg/kg liposomal doxorubicin can impair foreign particle clearance mechanisms of the liver but requires multiple injections to show actual elimination of Kupffer cells.

The results summarized above were confirmed using data derived following colloidal carbon injection 24 hours after the liposomal drug pre-doses were administered. Even though both treatment groups showed maximum liver depression of liposome uptake at the time point selected, there was little quantitative difference between the control and treatment groups for colloidal carbon uptake. This raises the intriguing possibility that the "RES" blockade approach described here targeted a specific uptake mechanism or, alternatively, a particular sub-population of cells. The liver macrophage population can be divided into several sub-populations based on size and maturity and thus a variety of functions (Daemen et al., 1989; Hoedemakers et al.,

1993). In general, large liver macrophages are more active for phagocytosis than smaller cells (Daemen et al., 1989), although elimination or inhibition of large macrophages can induce activation and thus a shift to the smaller population (Lazar et al., 1989).

Commercial colloidal carbon suspensions are reported to have particle sizes of the order of 60-70 nm (Miyata et al., 1994), although sizes exceeding several hundred nanometers were measured for the carbon suspensions used here, possibly due to aggregation. Particles that are 60-70 nm would have access to the liver parenchyma and can be taken up by the endothelial cells lining the liver sinusoids (Roerdink et al., 1981). It is possible that Kupffer cell activity has been shut down, resulting in a shift in the distribution of particulates to these other areas of the liver. In addition, the differences between the duration of RES blockade for doxorubicin versus vincristine may be related to the retention properties of the carriers within the Kupffer cells. Doxorubicin is known to be very stable and leak slowly from engulfed liposomes and thus the interior of macrophages may act as a slow release reservoir (Storm et al., 1988). Vincristine is expected to leak out rapidly from both the liposome and the cell (Mayer et al., 1990a; Boman et al., 1994). This is likely why co-injection of the pre-dose and subsequent test injection together resulted in immediate liver depression in the case of vincristine but not for doxorubicin.

The results presented in this chapter show that very low doses of liposomal doxorubicin or liposomal vincristine can have large effects on the liposome clearance mechanisms of RES, even when the carriers are composed of "RES avoiding" liposomes. This effect becomes particularly important in terms of understanding the behavior of liposomal anticancer drugs when used at therapeutically active levels as described in the following chapter. While the experiments here did not demonstrate Kupffer cell elimination when using low (< 10 mg/kg) pre-doses of

liposomal drugs, higher or multiple doses certainly will (Daemen et al., 1995). At 30 times the dose employed here, the effect that entrapped doxorubicin has on the circulation lifetime of the liposomal carrier is shown in Chapter 4 to be even more dominant than that of the lipid composition.

CHAPTER 4

TUMOR ACCUMULATION OF CONVENTIONAL AND STERICALLY STABILIZED LIPOSOMAL DOXORUBICIN

4.1 Introduction

Therapeutic responses obtained following administration of anti-cancer drugs are dependent on tumor physiology and tumor cell heterogeneity. These drugs must access the target cell populations at levels sufficient to cause cytotoxic effects and should be effective in all the differing microenvironments present with tumors. In humans, strategies designed to maximize the anti-tumor activity of chemotherapeutic agents must also contend with a heterogeneous combination of proliferating cells that are in various phases of the cell cycle, proliferating at widely different rates, growing in different tissues and capable of adapting rapidly to the chemotherapeutic stresses exerted on them. In practical terms this means that chemotherapy typically involves the use of multiple drugs that exert antitumor activity via different mechanisms. It also means that the maximum dose intensity of antineoplastic agents should be employed (Livingston, 1994). Tumor cells must be exposed to the highest levels of drug for the longest time periods if maximum therapeutic effects are to be realized (Lin, 1994).

Efforts to maximize the dose intensity of chemotherapeutics are limited by the non-specific toxic side effects exhibited by these drugs. Doxorubicin, one of the most commonly employed anti-cancer drugs, provides a good example in that it is a potent myelosuppressive agent (Gabizon et al., 1986; Bally et al., 1990b; Bonadonna et al., 1970). Therapeutic doses must be limited to schedules and amounts that do not dangerously compromise regeneration of blood cells or cells of the immune system. In addition, doxorubicin exhibits a dose limiting cardiotoxicity (Rhinehart et al., 1974; Minow et al., 1975) limiting the total drug dose to approximately 450 mg/m^2. Myelosuppression can be counteracted using the hematopoietic growth factor

granulocyte-macrophage colony stimulating factor (GM-CSF; Vose and Armitage, 1995). Cardiotoxicity on the other hand can be reduced by administering the drug by infusion or by utilizing this drug in a liposomally encapsulated form (Gabizon et al., 1982; Olson et al., 1982). It has been shown that the therapeutic activity of the liposomal drug is greater than or equal to free doxorubicin in a variety of pre-clinical and clinical studies (Conley et al., 1993; Cowens et al., 1993).

Pharmacokinetic studies to establish the mechanisms whereby liposomes improve the therapeutic profile of doxorubicin have focused in two areas. First, there is good evidence from pre-clinical studies that the reduced cardiotoxicity of liposomal formulations is a consequence of reduced drug accumulation in cardiac tissue (Gabizon et al., 1982; Herman et al., 1983). Second, therapeutic activity arises from liposome mediated increases in drug circulation lifetimes which results in improved drug delivery to tumor sites (Gabizon et al., 1990). Higher lipid doses can also lead to increased liposome circulation lifetimes (Abra and Hunt, 1981) and may be expected to facilitate increased accumulation of the carrier at solid tumor sites, provided that the carrier retains encapsulated drug following i.v. administration.

The studies summarized here evaluate the accumulation of drug at the tumor site for free doxorubicin and liposomal doxorubicin when administered at the maximum tolerated dose via a single bolus intravenous injection in mice. The major aim was to characterize the potential therapeutic advantage of liposomes containing PEG polymers, which exhibit longer circulation lifetimes (Blume and Cevc, 1990; Allen et al., 1991a). It is demonstrated that incorporation of PEG lipids in the liposomal doxorubicin formulation did not lead to improved tumor delivery or enhanced therapeutic activity under these conditions.

4.2 Materials and methods

4.2.1 Preparation of liposomes and doxorubicin loading

The production of 100 nm large unilamellar vesicles (LUVs) was carried out in general as previously described (Hope et al., 1985). Dry lipid mixtures composed of DSPC/chol (55/45 mol/mol) or DSPC/chol/PEG-DSPE (55/45/5) each with trace amounts of [^{3}H]CHE (NEN/Dupont) as a non-metabolizable and non-exchangeable liposome marker (Derksen et al., 1987) were dissolved in chloroform. In a warm water bath (50°C) this was reduced to a minimum volume under at stream of nitrogen gas and in order to avoid precipitation of cholesterol, quickly placed under high vacuum and dried for a further 4 hours. This procedure results in a homogeneous expanded lipid foam with a significant total surface area which facilitates complete lipid hydration. Multilamellar vesicles (MLVs) were produced first, hydrating the dried lipid in 300 mM citrate buffer (pH 4.0) followed by vigorous vortexing, warming, and five freeze-thaw cycles. The MLVs were then extruded ten times through two stacked 100 nm pore size polycarbonate filters (Costar/Nuclepore, Canada) employing an extrusion device (Lipex Biomembranes, Canada) equilibrated at 65°C. The resulting LUVs had a mean diameter of 100 ± 15 nm, as determined by QELS on a NICOMP Model 270 submicron particle sizer operating at a wavelength of 632.8 nm. No differences were observed between systems prepared with and without PEG-modified lipids. The lipid concentration of each liposome preparation was determined by a phosphorous assay (Fiske and Subbarow, 1925), where the colored product was measured spectrophotometrically at 815 nm using a Shimadzu UV-visible recording spectrophotometer. This measurement was used to derive a specific activity for the radiolabeled liposomes (DPM/μmol total lipid), and thereafter liposomal lipid concentrations were estimated by scintillation counting using a Beckman LS 3801 instrument.

PicoFluor 40 scintillation fluid (Packard) was used as a high efficiency scintillation cocktail. Distearoylphosphatidylcholine (DSPC) was from Avanti Polar Lipids, cholesterol (chol) and other chemicals were from Sigma, and poly(ethylene glycol)-distearoylphosphatidylethanolamine (PEG-A-DSPE)(PEG-PE) was synthesized as previously described (see Chapter 2).

Doxorubicin was encapsulated using the transmembrane pH gradient loading procedure (Mayer et al., 1986). To establish a pH gradient across the LUVs for doxorubicin loading, the resultant LUVs were dialyzed (12-14000 molecular weight cut off, Spectrapor) against 150 mM $NaHCO_3$ buffer, pH 7.5 for several hours to remove most of the external citrate and raise the external pH to 7.5. Subsequently, preheated (65°C) aliquots of these LUVs and doxorubicin (doxorubicin-HCl, Adria Laboratories dissolved in saline) were combined in a 0.2 mole drug to lipid ratio. These samples were incubated for an additional 10 min. at 65°C, resulting in over 95% trapping efficiency. "Empty" liposomes were prepared using a parallel procedure, with saline as a replacement for doxorubicin.

4.2.2 Animal and tumor models

All mice used in this study were 20-22g female BDF-1 mice (Charles River, Canada). The Lewis Lung carcinoma (LLC) was obtained from the National Cancer Institute Tumor Repository (Bethesda, Maryland) as a frozen tumor fragment from stock number G50132. Tumor cell suspensions were prepared by mechanical then enzymatic (Dispase/Collagenase/Dnase treatment) processing of excised tissue and used for experiments on passage number 2 to 5. In each passage, 3×10^5 cells in a volume of 50 µl were implanted subcutaneously (s.c.) in each of

the mouse flanks (bilateral tumors). Tumors were left to progress to a estimated 0.2 to 0.4 g size before initiation of pharmacology or therapeutic studies. At this time the doubling time of the tumor was approximately 3 days. All drug and liposome injections were delivered intravenously (i.v) through the lateral tail vein in a volume of 200 µl. At various times after injection the mice were anesthetized by i.p. administration of ketamine/xylazine (160 mg/kg, 20 mg/kg, MTC Pharmaceuticals, Canada). Blood was collected via cardiac puncture, placed in microtainer tubes with EDTA (Becton Dickinson, Canada) and centrifuged at 1500 × g for 10 min. to isolate plasma. Tissues were carefully removed, washed, blotted to remove attached blood, weighed and homogenized with a Polytron to a 20% (liver, tumor) and 10% (spleen) homogenate (wt:vol) in saline.

4.2.3 Assays for liposomal lipid and doxorubicin

To determine lipid levels, 100 µl plasma and 200 µl tissue homogenate were solubilized with 500 µl Solvable (NEN/Dupont) for 2 hours at 60°C. Subsequently the samples were cooled and treated overnight with 200 µl H_2O_2 before addition of 5 ml scintillation fluid. The samples were counted to determine [^{3}H]CHE. For doxorubicin levels, 100 µl plasma and 200 µl tissue homogenate were diluted with water up to 800 µl. Then 100 µl of 10% SDS and 100 µl of 10 mM H_2SO_4 were added. After these samples were vortexed well 2 ml of chloroform/isopropylalcohol (1:1 v/v) was added prior to additional mixing. The resulting samples were frozen overnight, thawed, and centrifuged for 10 min. at 1000 × g. The organic (lower) phase was removed and the amount of associated doxorubicin fluorescent equivalents was measured with a Perkin-Elmer fluorimeter (excitation/emission at 500/550 nm). Doxorubicin standards (0 to 20 nmol) were prepared for each set of assays and these were

prepared after mixing appropriate volumes of the standard with tissue homogenates derived from organs isolated from untreated mice. All tissues drug and lipid levels were corrected for drug and lipid in the plasma compartment of these tissues using published plasma volume correction factors (Bally et al., 1993).

4.2.4 Acute toxicity evaluation

Tumor free mice were used to test the doxorubicin mediated acute toxicity and to establish the maximum tolerated dose (MTD) for both free and liposomal drug formulations. Previous work from our laboratory indicated that in BDF-1 mice free doxorubicin has a MTD of between 20 and 25 mg/kg while conventional liposomal doxorubicin has a MTD above 60 mg/kg. Therefore 0.66 μmol DOX-HCl dissolved in saline as the free drug and 2.00 μmol DOX-HCl entrapped within 10 μmol lipid (LUV) was administered (i.v) per mouse. For an additional comparison, 0.66 μmol DOX entrapped within 3.3 μmol lipid was evaluated. Toxicity was measured qualitatively through evaluations of mean body weight lose and survival up to 40 days after treatment. These studies were done in accordance to Canadian Council on Animal Care (CCAC) Guidelines and it should be noted that only one of 25 animals used in this experiment suffered a drug related death and no animals had to be terminated as a result of unacceptable suffering.

4.2.5 Plasma elimination and tumor accumulation

In order to demonstrate the influence of lipid dose on elimination of liposomal lipid from the blood compartment a dose titration of liposomal lipid was completed up to the amount of lipid required to deliver the MTD of associated drug. For the dose titration, increasing doses of empty

and drug loaded (0.2 drug:lipid ratio) liposomes were administered i.v in tumor free mice. The mice were sacrificed 24 hours after injection and the level of liposomal lipid in plasma and selected organs were determined as described above.

Additional plasma elimination and tissue distribution studies were completed in BDF-1 mice bearing Lewis Lung tumors. All mice receiving liposomal doxorubicin were given 2 μmol doxorubicin/10 μmol total lipid (approximately 60 mg doxorubicin/kg) and sacrificed at 1, 4, 24, 48, 96 and 168 hours. Free doxorubicin treated mice were given (i.v.) 0.66 μmol doxorubicin (approximately 20 mg drug/kg) and sacrificed at 15 min., 1, 4, 24, 48 and 72 hours. Lipid and drug levels in plasma and tissues were determined as described above.

4.2.6 Tumor histology

Selected tumors derived from animals treated as described were carefully excised and fixed in 3% paraformaldehyde in PBS. After passing through graded concentrations of sucrose (0% to 15%), the tissue was imbedded in O.C.T. (Tissue-Tek, Miles Inc., USA), frozen in liquid nitrogen, and then 5 μm cryostat sections were prepared using a Frigocut 2800E microtome from Leica. Cryostat sections were washed, blocked with BSA and then stained with an anti-MAC-1 FITC-antibody conjugate (Pharmingen, CA). A Leitz Dialux fluorescence microscope (at 40 × magnification) was used to evaluate FITC fluorescence of the sections (430-490 nm cut off filter) and doxorubicin fluorescence (530-560 nm cut off filter) with fluorescent photomicrographs obtained using a Orthomat microscope camera. Normal phase contrast photomicrographs of the sections were also obtained. All images were recorded on Fuji color ASA400 negative film.

4.2.7 Tumor growth inhibition

Animals bearing tumors of between 0.2 and 0.4 g were treated with free and both liposomal doxorubicin formulations at doses of 0.66 and 2.00 μmol drug per mouse (approximately 20 and 60 mg/kg), respectively. Tumor size was determined at various times after a single drug dose using a caliper to estimate length and width. Tumor mass (g) was calculated using the following formula (Mayer et al., 1990b):

$$\frac{(a) \times (b)^2}{2}$$

where a = length and b = width measurements in cm. Mice were terminated on the nearest whole day when tumor mass equaled or exceeded 1.5 g. According to the CCAC approved protocol used animals must be terminated when total tumor mass exceeds 20% body weight or when tumors become ulcerated.

4.2.8 Statistical analysis

Differences between results obtained after administration of the two liposomal formulations of doxorubicin and free drug were determined using an ANOVA analysis. Comparisons were made for various common time points incorporating all sets of collected data for that time point using the Post Hoc Comparison of Means, Scheffé test. Differences were considered significant at a $p<0.05$ criterion.

4.3 Results

4.3.1 Estimation of Maximum Tolerated Doses

Dose range studies in tumor-free BDF-1 mice indicated that the maximum tolerated dose (MTD) for free and liposomal doxorubicin was approximately 20 mg/kg and 60 mg/kg, respectively. Both liposomal formulations of doxorubicin were tolerated up to 60 mg/kg, however there was significant loss of body weight at these high doses (Table 4.1). A nadir equivalent to almost a 25% decrease in mean body weight was observed between days 8 and 10 after drug administration. Recovery of normal body weight was achieved by day 18 and all mice survived this drug dose with the exception of 1 (out of 5) that died in the group treated with doxorubicin encapsulated in the liposomes containing PEG-PE. At necropsy (day 40), there were no signs of gross pathological abnormalities. Free drug treated animals (20 mg/kg) exhibited a mean body weight loss of 10 to 12% (observed from day 4 through to day 10). Weight loss data observed after a comparable drug dose (20 mg/kg) given in either liposomal formulations showed reduced nadir weight loss and faster recovery to normal body weight (day 7) consistent with the well established liposome mediated reduction in doxorubicin toxicity (Balazsovits et al., 1989; Gabizon et al., 1994a). Based on weight loss toxicity and long-term (40 day) survival, doses of 20 mg/kg for free and 60 mg/kg liposomal doxorubicin were used for the plasma elimination and tumor accumulation studies summarized below.

4.3.2 Influence of dose escalation on plasma liposomal lipid levels

In general, the circulating blood levels of liposomal lipid increase as the dose of liposomes increases (Mauk and Gamble, 1979; Abra and Hunt, 1981). In the case of PEG-PE containing liposomes dose independent pharmacokinetic characteristics are observed (Allen and Hansen,

Table 4.1

Toxicity/weight loss in response to the maximum tolerated dose for free and liposomal doxorubicin

Female BDF-1 mice (20-22 g) were administered various doses of free or liposomally encapsulated doxorubicin delivered i.v. in 200 µl volume via the lateral tail vein and weight loss (mean for 4 mice per group) was recorded daily over several weeks.

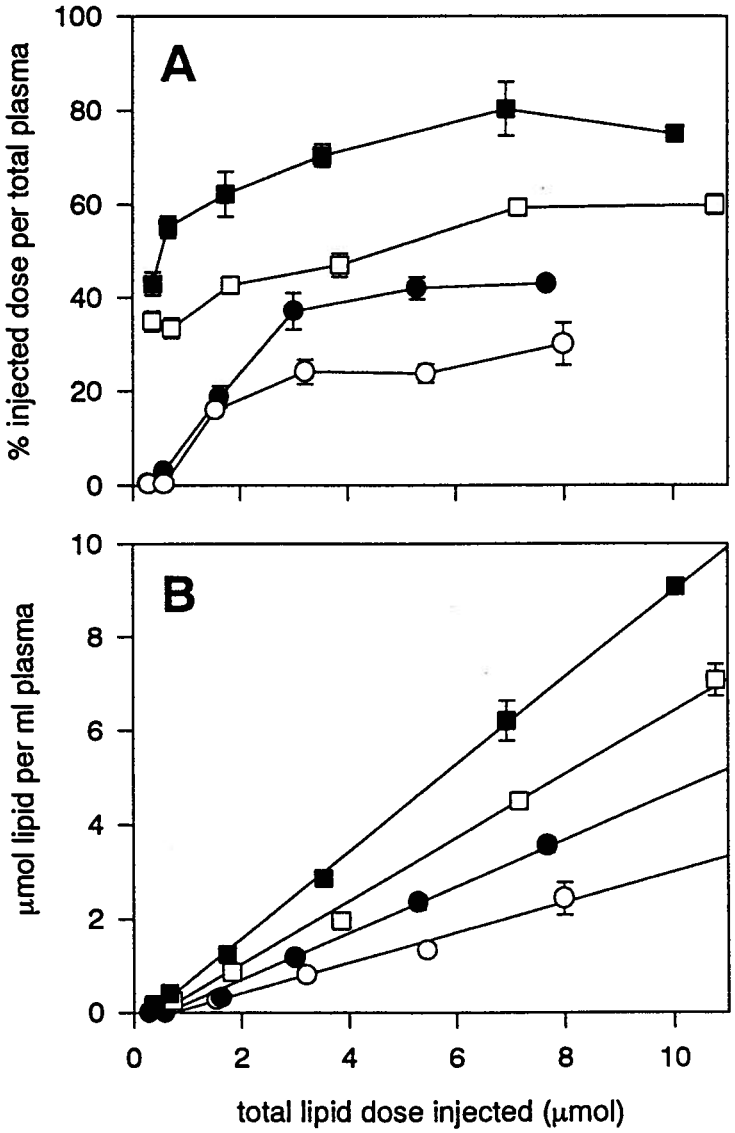
drug dose µmol per mouse	formulation	nadir percent weight loss (day)	estimated MTD mg/kg
0.66	free drug	12% (5)	>20
2.0	DSPC/chol	23% (9)	>60
2.0	DSPC/chol/PEG-PE	25% (9)	>60
0.66	DSPC/chol	8% (3)	
0.66	DSPC/chol/PEG-PE	7% (5)	

1991; Huang et al., 1992), while lower doses of conventional liposomes are cleared from the circulation more rapidly than higher doses (Mauk and Gamble, 1979). These effects are illustrated in Figure 4.1, which shows the lipid dose remaining in the plasma at 24 h as a function of the total lipid dose. These results illustrate two important features of drug loaded liposomes and PEG-PE containing liposomes. First, the addition of PEG-modified lipids greatly improves circulation lifetimes for both the empty and doxorubicin loaded systems. At doses under 1 µmol lipid/mouse, typically 40% of the injected dose is present in the plasma 24 h after administration of PEG-liposomes, whereas less than 5% of the injected dose is observed in plasma 24 h after administration of conventional liposomes (Figure 4.1*A*). As the lipid dose increases the differences between the PEG-containing and conventional liposomes are still significant, but these differences are reduced from a 10-fold (observed below the 1 µmol lipid per mouse dose) to less than a 3-fold increase (observed above the 2 µmol lipid per mouse dose). The results

Figure 4.1

Dose titration of the liposomal carrier

Various doses of empty or drug loaded liposomes (0.2 drug:lipid ratio) were administered in 200 µl volume i.v. into tumor free mice. *A*, plasma recovery at 24 h expressed as a percent of the injected dose per total plasma; *B*, the same results expressed as an absolute lipid concentration. (○), DSPC/cholesterol; (●) DSPC/cholesterol, DOX; (□) DSPC/cholesterol/PEG-PE; (■) DSPC/cholesterol/PEG-PE, DOX. Results shown represent the mean of four animals ± S.E. per group.



shown in Figure 4.1*B* clearly demonstrate a linear relationship between administered lipid dose and the levels of lipid in the circulation at 24 h, regardless of the liposomal formulation used (correlation coefficients (r^2) for the conventional formulations were 0.89 and 0.97 for empty and loaded systems, and 0.98 and 0.99 for the PEG liposomes, empty and loaded, respectively). In total, these results are consistent with dose independent and dose dependent pharmacokinetic behavior for PEG-containing liposomes and conventional liposomes, respectively. The second point from data in Figure 4.1 is that entrapped doxorubicin significantly increases the plasma blood levels obtained 24 h after i.v. administration of PEG-PE containing liposomes or conventional liposomes. This effect has been noted previously in Chapter 3.

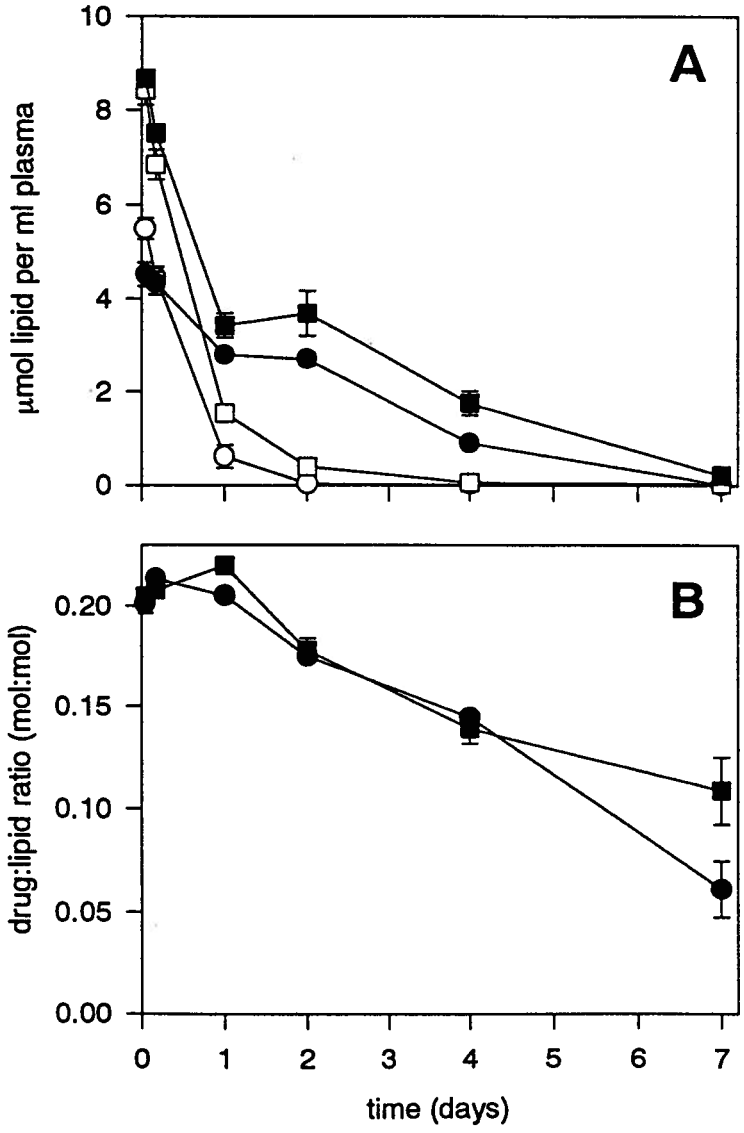
4.3.3 Drug elimination from plasma and tumor accumulation in BDF-1 mice bearing Lewis Lung tumors

A comprehensive examination of drug and liposome circulation lifetime following i.v administration was completed in BDF-1 mice bearing Lewis Lung tumors (Figures 4.2 and 4.3). Several trends are evident for elimination of the liposomal carriers from the blood compartment (Figure 4.2*A*) for the tumor bearing mice. First, at 24 h and later, the dominant factor dictating enhanced circulating blood levels of liposomes is the presence of entrapped doxorubicin. The drug loaded liposomes, for both PEG-containing and conventional liposomes, are consistently at higher concentrations in the blood than the respective empty systems, resulting in 3- to 10-fold increases in the plasma concentrations of liposomal lipid. It should be noted that for both doxorubicin loaded liposomal carriers there was an approximately equivalent reduction in liposome uptake by the liver (data not shown). The plasma liposomal lipid levels obtained after 24 h are still significantly greater ($p<0.05$) for the PEG-containing liposomes. The differences

Figure 4.2

Pharmacokinetic analysis of liposome clearance in tumor bearing mice

Mice were administered 10 μmol total lipid per mouse of either conventional or PEG-containing liposomes with or without entrapped doxorubicin (2 μmol drug). Mice were sacrificed at 1, 4, 24 h, 2, 4, and 7 d, and lipid and drug plasma concentrations determined. *A*, total time course of liposomal clearance. *B*, drug to lipid ratio for the conventional vs. the PEG liposomal systems. (○), DSPC/chol; (●), DSPC/chol + DOX; (□) DSPC/chol/PEG-PE; (■), DSPC/chol/PEG-PE + DOX. Results shown represent the mean of four animals ±S.E. per group.



between the two liposomal preparations, however, are reduced substantially as compared to the behavior in tumor free animals.

A second important observation evident from the results in Figure 4.2*A* is related to tumor induced increases in liposome elimination from the circulation. For the conventional formulations, nearly half of the injected dose is eliminated from the circulation within 1 hour. Comparison of the 24 hour data in Figure 4.2*A* with that from equivalent dose data shown in Figure 4.1 indicates that liposomal blood levels at this time point are approximately two or four fold lower in tumor bearing mice than in tumor free mice given empty liposomes and doxorubicin loaded liposomes, respectively. This contrast with previous studies in our laboratory using BDF-1 mice with solid tumors derived following s.c. injection of B16/BL6, L1210, P388 and FSA cells, which typically show equivalent pharmacokinetic behavior in tumor and non-tumor bearing animals. The differences between the pharmacokinetic behavior for tumor bearing versus tumor free mice in terms of absolute amounts of lipid are shown in Table 4.2. For the conventional liposomes, all of the circulating lipid lost due to the presence of tumor could be accounted for through increased liposome uptake by the liver, spleen, and solid tumors, whereas for the PEG-PE containing systems, over 75% could be accounted for by increased uptake in these three tissues.

Drug retention by liposomes in the blood compartment can be measured by assaying lipid and drug concentrations in plasma over time. This analysis (Figure 4.2*B*) shows that both conventional and PEG- liposomes retain encapsulated drug with half-times for drug release of five days or longer. This is consistent with previous reports for doxorubicin retention in DSPC/cholesterol liposomes (Bally et al., 1990a). There was no measurable change in the drug

Table 4.2.

Comparison of liposomal biodistribution in Lewis Lung solid tumor bearing versus tumor free BDF-1 mice after i.v. administration of equivalent doses of lipid (10 μmol per mouse)

tumor bearing t_B versus tumor free t_F (μmol)[1]	DSPC/chol		DSPC/chol/PEG-PE	
	empty	DOX	empty	DOX
plasma (t_B-t_F)	-2.4	-1.6	-4.3	-3.8
tissues[2] (t_B-t_F)	2.4	1.5	3.2	2.9
NET t_B-t_F	0.0	-0.1	-1.1	-0.9

[1] total lipid uptake for tumor bearing minus tumor free mice (μmol)
[2] sum of liver, spleen, tumor tissue total uptakes

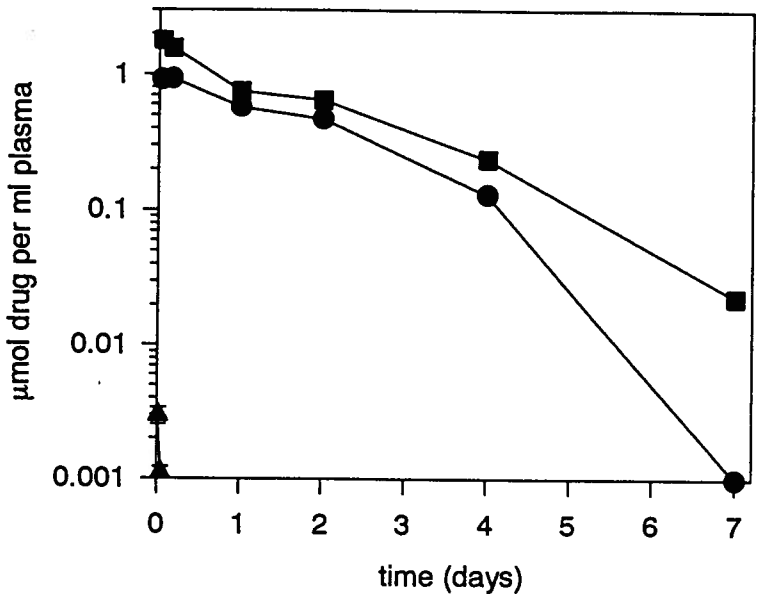
to lipid ratio measured over the initial 24h period after i.v. injection. After 2 and 4 days in circulation, the drug to lipid ratio is approximately 90% and 70% of the value measured prior to injection. The doxorubicin leakage rate after 24 h can thus be estimated to be approximately 0.75 nmol/μmol lipid/hour for both liposomal formulations studied.

The plasma concentrations of doxorubicin obtained after injection of free and liposomal systems are shown in Figure 4.3. For the liposomal drugs, drug elimination rates were similar to elimination rates for liposomal lipid, a reflection of the slow drug release rates. Measurements made one and two days after injection indicate circulating drug levels of greater than 0.5 μmol of drug per ml plasma (equivalent to 25% of the injected drug). An estimation of doxorubicin area under the curve (AUC,) for the blood compartment indicates a 1.5 fold increase in AUC when the drug is given encapsulated in PEG-liposomes (AUC = 78 $\mu mol \cdot ml^{-1} \cdot h$) versus conventional liposomes (50 $\mu mol \cdot ml^{-1} \cdot h$). Figure 4.3 also includes results obtained following administration

Figure 4.3

Pharmacokinetic analysis of drug clearance in tumor bearing mice

Drug plasma concentrations from Figure 4.2 were plotted. Also shown is the clearance of 0.66 μmol free drug per mouse. (▲), 0.66 μmol free DOX; (●), 2 μmol DOX in DSPC/chol; (■), 2 μmol DOX in DSPC/chol/PEG-PE. Results shown represent the mean of four animals ±S.E. per group.



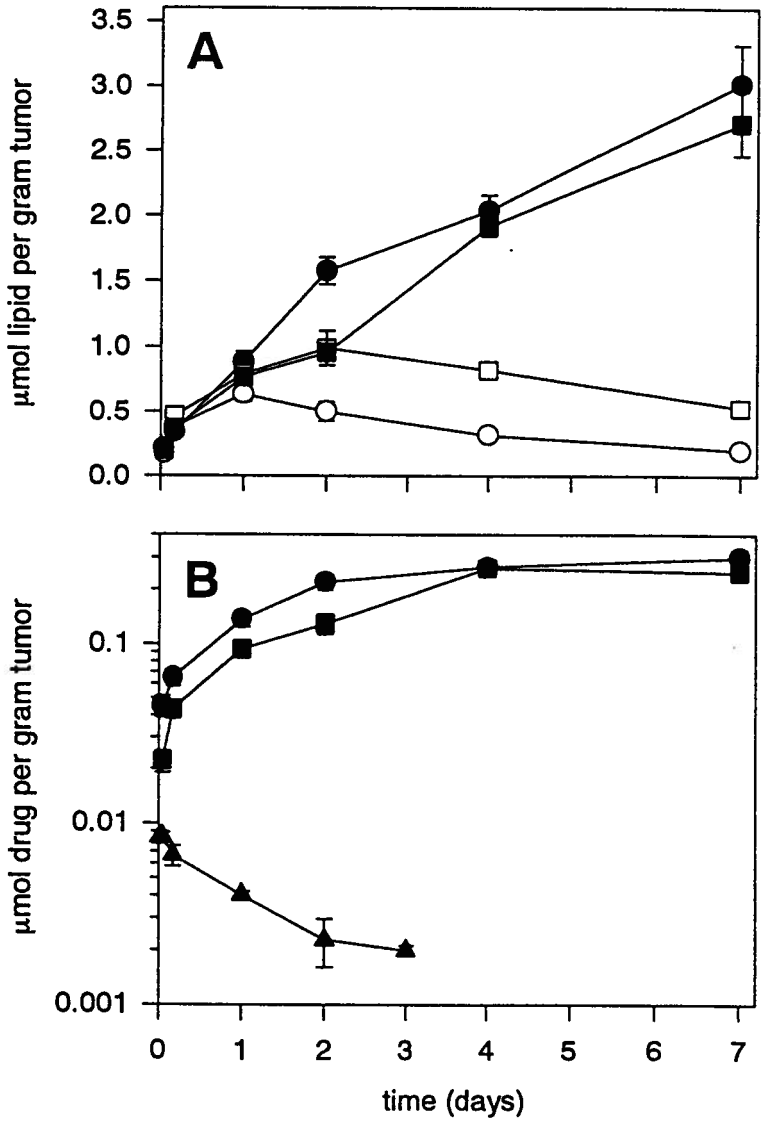
of free drug at the MTD. In the absence of a carrier, plasma doxorubicin levels fall below detectable limits within hours. Assuming that the plasma volume of a 20-22 g mouse is 1 ml and an injected drug dose of 0.66 μmol doxorubicin per animal, it is estimated that greater than 99% of the injected free drug was eliminated from the circulation within 15 minutes after injection. The AUC for free drug was estimated to be 87 $nmol \cdot ml^{-1} \cdot h$ which is approximately 600 and 900 fold less than that obtained for doxorubicin given in conventional and PEG-containing liposomes, respectively.

In order to ascertain whether the increased blood levels of PEG-PE containing liposomes increased tumor accumulation the drug and liposomal lipid levels were compared in Lewis Lung tumors for 7 days following i.v. administration for PEG-PE and conventional liposomal doxorubicin. These data, shown in Figure 4.4, indicate that there is similar tissue uptake for both drug loaded and "empty" liposomal carriers during the initial 24 h after i.v. administration. The empty conventional liposomes reach a peak level in tumor tissue of 0.6 μmol lipid/g tissue. This value was achieved 24 h after administration. Following administration of "empty" PEG-containing liposomes the peak lipid concentration is achieved 48 h after administration and a value of 1.0 μmol lipid/g tissue was obtained. A gradual decline in peak values is primarily a consequence of continued tumor growth. The amount of liposomal lipid delivered per tumor was equivalent to approximately 5% and 8% of the injected lipid dose for conventional liposomes and PEG-containing liposomes, respectively. In the case of empty liposomes the level of liposomal lipid achieved per g tumor was significantly greater ($p < 0.05$ at time points beyond 48h) when using PEG-containing liposomes. This is consistent with the fact that the circulation lifetimes of the PEG-containing liposomes are greater than that of conventional liposomes (see Figure 4.2*A*). For the drug loaded systems however, tumor levels of liposomal lipid (μmol

Figure 4.4

Tumor loading of liposome and drug loading in the murine Lewis Lung solid tumor model

A, Liposome accumulation in the Lewis Lung solid tumor.(O), DSPC/chol; (●), DSPC/chol + DOX; (□) DSPC/chol/PEG-PE; (■), DSPC/chol/PEG-PE + DOX. *B*, drug accumulation. (▲), free DOX; (●), DOX in DSPC/chol; (■), DOX in DSPC/chol/PEG-PE. Results shown represent the mean of four animals ±S.E. per group.



lipid/g) increased over the 7 day time course. This apparent increase in liposome delivery is primarily a consequence of doxorubicin mediated regression of the solid tumors. Although a maximum lipid concentration cannot be estimated from the data in Figure 4.4, levels of liposomal lipid achieved in the tumor exceed 2.5 μmol lipid/g. There is little difference between the PEG-containing and conventional formulations when the carriers contain encapsulated doxorubicin.

Drug levels achieved within the tumors are shown in Figure 4.4*B* and results obtained following administration of free drug (20 mg/kg) have been included for comparison. For both liposomal drug formulations, peak solid tumor concentrations of drug (C_{Tmax}) are achieved after 48 h. The combined effects of tumor regression and drug release from liposomes within the tumor leads to this plateau. Drug levels delivered up to 48 h via conventional liposomes are slightly higher than that delivered via PEG-liposomes, with no significant difference past this time point. Using the AUC (μmol doxorubicin/g tissue - time curve) as an estimate of tumor drug exposure, conventional liposomes (AUC_T of 38 $\mu mol \cdot g^{-1} \cdot h$) expose the tumor to slightly more doxorubicin than PEG-containing liposomes (AUC_T of 31 $\mu mol \cdot g^{-1} \cdot h$). This suggests that more efficient drug delivery to tumors is obtained for conventional liposomes.

The peak level of drug obtained in tumors was reached 4 days after i.v. administration of both conventional and PEG-containing liposomal formulations, when values of 250 nmol per g were measured. This represents approximately 140 μg equivalents of doxorubicin per g tumor. In contrast, peak drug levels are achieved within 15 min. after administration of free drug and these levels (10 nmol per g) were 25-fold lower than those obtained following administration of the liposomal drug. In animals receiving liposomal doxorubicin formulations there was a progressive

decline in the drug to lipid ratios measured within the tumor. For conventional liposomes, drug to lipid ratios (mol:mol) dropped from 0.2 at the earliest time points to 0.13 and 0.10 at 4 and 7 days, respectively. Similar results were obtained in tumors from animals given PEG-liposomal doxorubicin, where ratios of 0.14 and 0.09 were obtained at 4 and 7 days, respectively. These tumor drug to lipid ratios are comparable to those measured in the circulation (Figure 4.2*B*). Based on these changes in drug to lipid ratio, it can be estimated that liposomes within the tumor are releasing drug at 0.60 to 0.65 nmol drug/μmol lipid/h, rates which are comparable to those in the circulation.

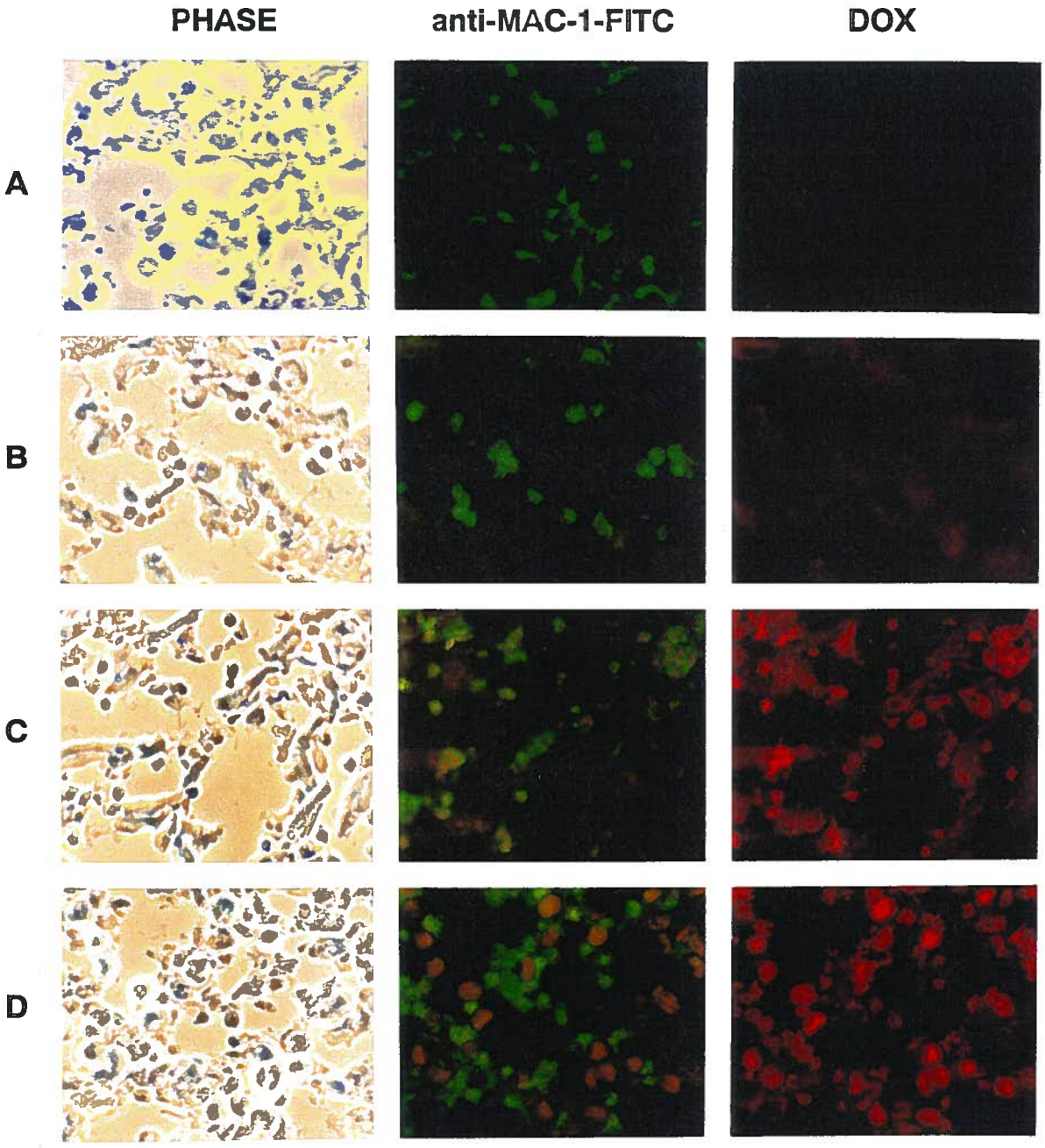
4.3.4 Tumor histology

In order to gain a more precise understanding of the fate of doxorubicin localized within the tumor, histological studies were initiated to qualitatively assess the distribution of doxorubicin. In addition, these studies attempted to correlate drug distribution with the distribution of tumor associated macrophages (TAMs)(detected using an anti-MAC-1 antibody). The results, shown in Figure 4.5, illustrate selected regions within tumors obtained from control and doxorubicin treated animals as a function of time after i.v. administration. Doxorubicin, a fluorescent drug, appears red when viewed using a fluorescent microscope equipped with a rhodamine filter. TAMs were labeled with an FITC conjugated anti-MAC-1 antibody and appear green under a fluorescene filter. From the phase contrast photomicrographs of control tumors, it is evident that the Lewis Lung solid tumor is a loose, poorly formed network of cells. Although not shown in the photomicrographs incorporated in Figure 4.5, these tumors have a large necrotic center and this is surrounded by more densely packed cells near the outer surface of the tumor. FITC MAC-1 positive cells are interspersed within the outer region of the tumor. It should also be noted that

Figure 4.5 (following page)

Lewis Lung solid tumor histology after administration of either free or liposomal doxorubicin

Cryostat sections were visualized (40 ×) under either phase contrast or fluorescence microscopy and selected slides shown. The top labeling identifies mode of visualization: Phase, phase contrast; anti-MAC-1-FITC, fluorescence to identify MAC-1 positive cells; DOX, doxorubicin fluorescence. The various treatments were: *A*, control tumor; *B*, free doxorubicin (0.66 μmol/mouse) at 24 h; *C*, 2.00 μmol/mouse doxorubicin in DSPC/chol at 48 h; *D*, 2.00 μmol doxorubicin in DSPC/chol/PEG-PE at 48 h.


PHASE
anti-MAC-1-FITC
DOX
A
B
C
D

the distribution of MAC-1 positive cells is variable, where some regions were almost devoid of TAMs. This is a clear reflection of the microenvironment heterogeneity with this particular solid tumor. Sections derived from tumors treated with free doxorubicin exhibited a weak and diffuse doxorubicin fluorescence that was difficult to differentiate from auto fluorescence. Sections derived from tumors treated with conventional liposomal doxorubicin exhibited strong doxorubicin fluorescence over the entire time period evaluated. Many of the MAC-1 positive cells also appeared to contain doxorubicin, presumably a consequence of liposome uptake by TAMs. In contrast, sections derived from tumors treated with PEG-liposomal doxorubicin exhibit a poor correlation between drug fluorescence and FITC positive TAMs. These results suggest that doxorubicin delivery achieved with conventional liposome and PEG-liposomes, although quantitatively similar, may result in different intratumor drug distributions. It should again be emphasized however that doxorubicin distribution within tumors from animals treated with either liposomal drug formulations was highly variable. The photomicrographs presented in Figure 4.5 were selected for the presence of both doxorubicin and TAMs.

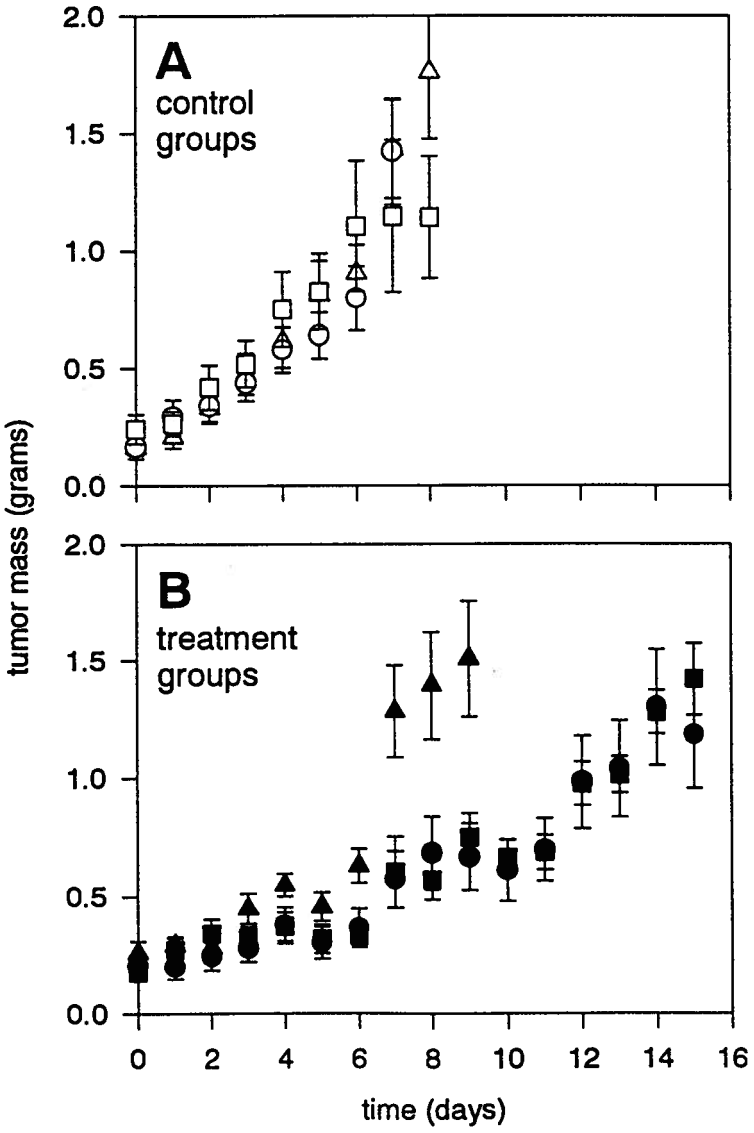
4.3.5 Inhibition of tumor growth by liposomal doxorubicin

The antitumor activity of free doxorubicin and both liposomal doxorubicin formulations (given at the MTD) was determined. Tumor size was followed after a single bolus injection of drug, where treatment was initiated when the tumors were 0.2 to 0.4 g. These data are illustrated in Figure 4.6. The control group (receiving no treatment) grew rapidly, reaching more than 1.5 g within eight days. The administration of empty liposomes (10 μmol total lipid) for either conventional or PEG systems had no significant effect on tumor growth. Treatment with free drug resulted in a slight delay in tumor growth, where the time required for the tumor to double

Figure 4.6

Doxorubicin mediated Lewis Lung solid tumor growth inhibition

Tumor bearing mice were given various treatments and tumor mass was estimated daily using caliper measurements. Control groups: (Δ), normal control; (○), 10 μmol empty DSPC/chol; (□), 10 μmol empty DSPC/chol/PEG-PE. Treatment groups: (▲), 0.66 μmol free DOX; (●), 2 μmol DOX in 10 μmol DSPC/chol; (■), 2 μmol DOX in 10 μmol DSPC/chol/PEG-PE. Results shown represent the mean of four animals ± S.E. per group.



in size increased from 3 days in control animals to 4 to 6 days in free drug treated animals. After this time point the tumors progressed rapidly to achieve a tumor mass of 1.5 g by day 9 (similar to control animals). Conventional and PEG liposomal doxorubicin were more effective than free drug, increasing the tumor doubling time to approximately 7 days in treated groups. The tumors continued to grow and within 16 days after treatment the tumor mass approached 1.5 g. The results indicate that there is little difference between the two liposomal systems in terms of therapeutic activity.

4.4 Discussion

Maximization of dose intensity is important for effective cancer chemotherapy. Accordingly, studies presented here employ MTD values of 20 mg/kg (0.66 μmol per mouse) for the free drug and 60 mg/kg (2.0 μmol per mouse) for both the conventional and sterically stabilized liposomal doxorubicin. For liposomal systems, a consequence of working at high drug levels is a higher lipid dose. At a drug to lipid ratio of 0.2 (mol:mol), for example, a dose of 2 μmol drug per mouse corresponds to a lipid dose of 10 μmol lipid. As can be seen in Figure 4.1, increasing the lipid dose up to this level produces a steady increase in the plasma concentration of the liposomal carrier. This, in turn, may be expected to lead to higher tumor levels, as enhanced circulation lifetime is positively correlated with accumulation of these carriers in tumors (Gabizon et al., 1990). This result has been confirmed here for empty liposomes where PEG-PE containing liposomes accumulate in the tumor to a higher level than conventional liposomes. However, when these systems are loaded with drug, similar levels of liposome delivery to the tumor are observed for both carrier systems.

The observation that plasma clearance of liposomes is faster in tumor bearing mice has been noted elsewhere for a subcutaneous S180 tumor model (Oku et al., 1992). The difference between the tumor free vs. tumor bearing mice plasma levels observed here is largely accounted for by increased uptake in the liver, spleen, and tumor. Metastatic solid tumors such as the Lewis Lung carcinoma shed large amounts of cells and other debris (Butler and Gullino, 1975; Glaves, 1983), and it has been suggested that the release of this material into the circulation can stimulate the RES (Thomas et al., 1995). In addition, solid tumors can either directly or indirectly stimulate the release of TNF-α or other lymphokines such as IL-2 (Nagarkatti et al., 1990; Thomas et al., 1995). Such molecules are implicated in vascular leak syndrome (VLS; Fujita et al., 1991; Deehan et al., 1994). Although no evidence for increased tissue plasma volumes was found, a slightly increased liver size and a greatly increased spleen size was observed, an effect that has also been noted for IL-2 induced VLS (Fujita et al., 1991).

There are two major ways in which liposomal drug carriers can be used, as a circulating reservoir for free drug or as a vehicle to deliver drug to the tumor site. For some anti-cancer drugs, frequent or continuous administration can produce the best therapeutic effect, and therefore the principle of encapsulating drugs in slow release liposomes has practical significance (Mayer et al., 1990a; Allen et al., 1992; Vaage et al., 1993). However, there is considerable evidence that the mechanism of solid tumor delivery involves extravasation of the intact liposome to the tumor site followed by slow release of drug. Thus, anti-tumor effects may be attributed largely to release from liposomes that localize in the tumor as opposed to systemic release of drug (Huang et al., 1992). In fact, studies have shown that intact liposomes can be found within the interstitial space between tumor cells (Gabizon, 1992, Huang et al. 1992). The lipid and drug data presented here is consistent with this mode of doxorubicin delivery to tumor cells.

A central question that arises from the concept that intact liposomes access sites outside the blood compartment concerns mechanisms of transfer. Several routes for transendothelial transfer of liposomes have been suggested, including both open channels and some forms of transcytosis (Huang et al., 1993). Recent analysis for sterically stabilized liposomes suggests that the main route is through gaps in the endothelial layer and not via vesicular transport or leukocyte mediated extravasation (Yuan et al., 1994), and the data presented here can be interpreted similarly. Doxorubicin delivered via conventional liposomes is positively correlated with macrophages within the tumor, whereas the correlation between these TAMs and doxorubicin for the PEG-PE containing liposome mediated delivery is not as strong. This is consistent with a reduced affinity of PEG-PE containing liposomes with liver macrophages noted elsewhere (Allen et al., 1991b).

The results presented here indicate that at higher drug dose levels the PEG coating may not result in improved drug delivery to solid tumors, as shown here for the Lewis Lung solid tumor model. This contrasts with previous results which indicate that sterically stabilized liposomes have increased microvascular permeabilities compared to conventional liposomes (Wu et al., 1993). These studies (Wu et al., 1993) were compromised, however, by the fact that the plasma concentrations of the sterically stabilized systems were considerably higher than the conventional systems. For longer time courses and comparable plasma concentrations, the results presented here show little difference in either the kinetics or total amount of drug accumulation in a solid tumor. The movement of drug from the plasma compartment to the tumor site can be described employing a drug targeting efficiency parameter T_e relating the AUC in the circulation to the tumor AUC ($T_e = AUC_T/AUC_P$). The conventional liposomes gave T_e values (0.76) which

were almost a factor of 2 higher than that for the PEG-PE containing liposomes (0.40). In contrast, the free drug exhibited a T_e of 3.0. The greater efficiency of tumor targeting for the free drug is consistent with increased small molecule penetration within solid tumors when compared to penetration of larger particles such as liposomes (Yuan et al., 1994; Jain, 1987).

The peak drug concentrations (C_{Tmax}) obtained here for tumor drug loading are extremely impressive. For both liposomal formulations, peak drug levels were achieved after 4 days, closely paralleling liposome lipid uptake, consistent with a model of intact liposome extravasation (Gabizon et al., 1994b). The C_{Tmax} values obtained in this study were approximately 250 nmol doxorubicin per g tumor, or roughly 140 μg doxorubicin equivalents per g tumor. This is far higher than achieved in previous studies, and dramatically illustrates the effect of dosing at the MTD rather than lower dose levels. Previous tumor drug loading values have been of the order of 20 nmol per g solid tumor (Mayer et al., 1990b; Gabizon, 1992; Maruyama et al., 1993) to 40 nmol per g (Ning et al., 1994). While the levels of drug obtained in the experiments here do result in a significant reduction in tumor growth in a rapidly growing and aggressive solid tumor model, these results also point to the problem of drug bioavailability. The rate of drug release in the tumor microenvironment (0.60 to 0.65 nmol drug/μmol lipid/h), which corresponds to a half-time for release of over 5 days, is likely too slow to be of therapeutic consequence to the tumor. The development of techniques which result in increased rates of drug release, combined with the techniques employed here for delivering maximal levels of liposomal drug to tumors could potentially be of great therapeutic advantage.

In summary, the results of this work establish that by increasing the liposomal carrier dose up to the MTD for the encapsulated drug, increasingly higher plasma concentrations, longer

circulation lifetimes, and very high levels of tumor associated drug can be obtained. Inclusion of PEG-PE in the liposomal composition does not improve tumor delivery of drug under these conditions. Maximum tumor drug levels (C_{Tmax}) were similar for both conventional and PEG-containing formulations, however limited drug release was evident, supported by limited therapeutic effects. Given the extremely high levels of drug delivery to the tumor achieved here, it is proposed that techniques leading to the triggered release of liposome contents may lead to major improvements in therapeutic outcome.

CHAPTER 5

SUMMARIZING DISCUSSION

5.1 Summary of results

This thesis incorporates a progression of studies designed to more fully understand certain important parameters for the use of liposomes as drug delivery vehicles, covering three basic areas of investigation. These included the use of PEG lipids incorporated into the liposome composition in order to prolong the circulation lifetime of the carrier in the circulation, the effect that entrapped drug has on the RES and the circulation lifetime, and finally the use of liposomal carriers to deliver drug to a solid tumor site as affected by these two areas of investigation. In Chapter 2 it is shown that poly(ethylene glycol)(PEG)-lipid anchor conjugates can prolong the circulation lifetimes of liposomes following intravenous injection. This chapter investigated the influence of the lipid anchor and the nature of the chemical link between the PEG and lipid moieties on circulation lifetime. It is shown that incorporation of N-(monomethoxy poly(ethylene glycol)$_{2000}$-succinyl)-1-palmitoyl-2-oleoyl-phosphatidylethanolamine (MePEG$_{2000}$-S-POPE) into large unilamellar vesicles (LUVs) composed of distearoylphosphatidylcholine (DSPC) and cholesterol (DSPC/cholesterol/MePEG$_{2000}$-S-POPE, 50:45:5 mol/mol) results in only small increases in the circulation lifetimes as observed in mice. This is shown to be due to rapid removal of the hydrophilic coating in vivo, which likely arises from exchange of the entire PEG-lipid conjugate from the liposomal membrane, although chemical breakdown of the PEG-lipid conjugate is also possible. The chemical stability of four different linkages was tested, including succinate, carbamate and amide linkages between MePEG derivatives and the amino head group of PE, as well as a direct link to the phosphate head group of phosphatidic acid (PA). The succinate linkage was found to be the most labile.

The anchoring capability of DSPE as compared to POPE in PEG-PE conjugates was also examined. It is shown that incorporation of $MePEG_{2000}$-S-DSPE conjugates into DSPC/cholesterol LUVs results in little loss of the PEG coating in vivo, long circulation lifetimes and reduced chemical breakdown of the PEG-lipid conjugate. This work establishes that DSPE is a considerably more effective anchor for PEG_{2000} than POPE and that the chemical stability of PEG-PE conjugates is sensitive to the nature of the linkage and exchangeability of the PEG-PE complex. It is therefore suggested that retention of the PEG coating is of paramount importance for prolonged circulation lifetimes.

In Chapter 3, it is shown that the incorporation of ganglioside G_{M1} or phosphatidylethanolamine-poly(ethylene glycol) conjugates into liposomes can result in extended circulation lifetimes in vivo. However, pre-doses of LUVs which incorporate either G_{M1} or PEG-PE, with entrapped doxorubicin, block the accumulation of subsequently injected empty distearoylphosphatidylcholine/cholesterol liposomes in liver. It is therefore concluded that liposomes exhibiting extended circulation lifetimes can induce RES blockade and do not strictly avoid uptake by liver phagocytes. Further characterization of RES blockade revealed that extremely low doses of doxorubicin could induce a maximum blockade effect, and that the inhibition of liver uptake can last past 8 days, only approaching full recovery by 14 days. Another commonly employed liposomal anticancer drug, vincristine, had effects which were more transient, although significant RES blockade could also be demonstrated.

The final set of experiments presented a comparison of tumor accumulation and efficacy properties of doxorubicin entrapped in "sterically stabilized" liposomes and conventional liposomes. The conventional liposomes were composed of distearoyl phosphatidylcholine and

cholesterol, whereas sterically stabilized liposomes contained in addition 5 mol % of polyethylene glycol coupled to phosphatidylethanolamine. Drug pharmacokinetics and tumor accumulation at the maximum tolerated dose (MTD)(60 mg/kg liposomal doxorubicin) were monitored in mice bearing Lewis Lung carcinoma solid tumors. In contrast to expected behavior, the efficiency of doxorubicin accumulation at the tumor site, measured employing an area under the curve analysis, was higher for the conventional liposomes than for the sterically stabilized liposomes. Both formulations exhibited profound increases of over 500-fold in tumor accumulation of drug as compared to injection of the MTD of free doxorubicin (20 mg/kg). These studies suggest that optimization of factors nominally leading to longer blood circulation times do not provide therapeutic advantages for liposomal formulation of doxorubicin administered at the MTD. Improvement in other parameters, such as drug leakage rates, hold greater promise for improving therapeutic properties.

5.2 Discussion

The results presented here highlight several important conclusions for drug delivery and intravenously injected carriers in general. First, it is important to establish that the properties of the carrier in vivo must be well understood. This is particularly evident for liposomes incorporating PEG-lipids. The studies outlined here were the first to address the possibility that the PEG coating could be lost. Second, most liposomal drug carrier work has been carried out using empty liposomes. It is not necessarily true that results obtained for empty carriers can be directly translated or implied for drug loaded systems. As shown in Chapters 3 and 4, entrapped drug can have a profound impact on the in vivo clearance behavior of liposomes. Furthermore, it is important to assess tumor loading or other goals in terms of a variety of therapeutic conditions.

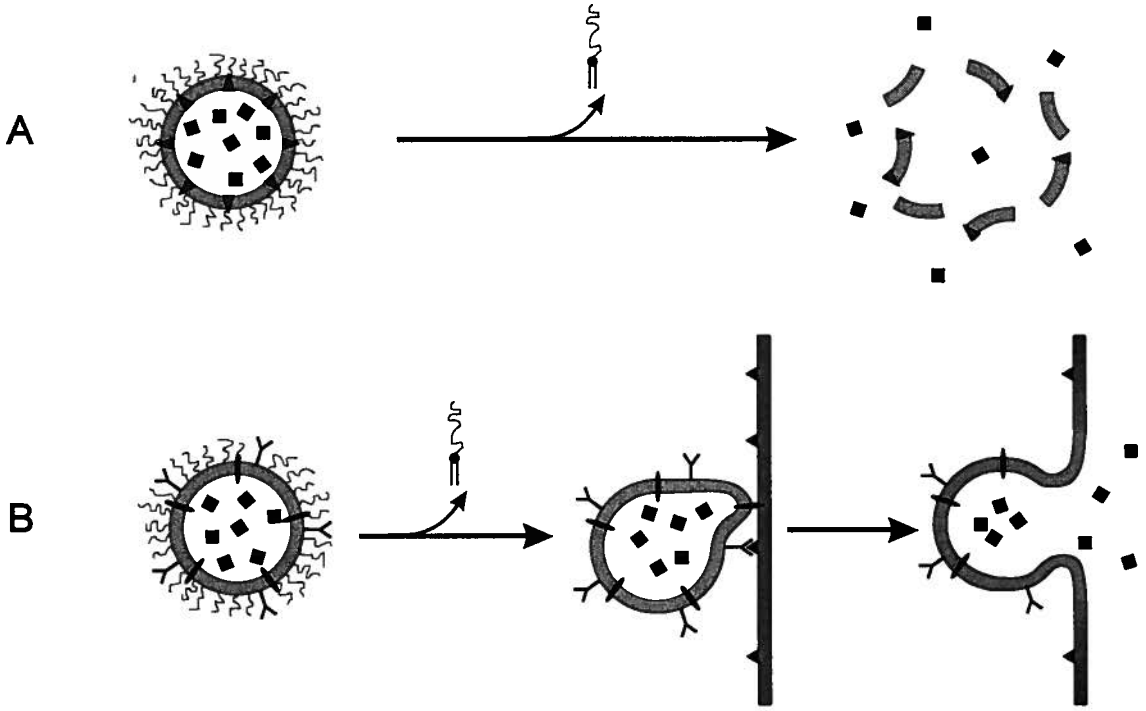
PEG-liposomes may accumulate to a significantly greater extent in solid tumors compared to conventional liposomes at extremely low dose ranges, however at the maximum tolerated dose of therapeutically interesting liposomal anticancer drugs, there may be little difference in drug delivery to the tumor. Perhaps of greater general interest, further application of the work contained in this thesis may be applicable to the generation of a new class of lipid based carrier systems. This is discussed below.

The information gained from the studies of various parameters in the construction of PEG-lipids may have implications for a variety of applications. For example, it may be possible to incorporate PEG-lipids in liposomes which are designed to exchange out of the membrane after some pre-set time. Chapter 4 demonstrated that not only can extremely high levels of liposomal drug be delivered to a solid tumor, but this delivery largely occurs within the first 2 days after intravenous injection. Furthermore, the limited therapeutic effects observed were likely a result of drug release that was too slow to be of toxic consequence to the tumor cells. Whereas the PEG coating was of limited benefit under conditions maximizing dose intensity, its subsequent loss from the membrane after tumor delivery may be used to destabilize the membrane. The incorporation of lipids such as unsaturated PEs which normally form the inverted hexagonal H_{II} phase can be stabilized in a bilayer configuration by the presence of selected lipids (Cullis et al, 1986), such as PEG-PE. However, upon loss of the PEG-lipid component, such a system may be induced to undergo bilayer destabilization with the rapid loss of entrapped contents. Such a scheme is depicted in Figure 5.1*A*. Work by other members of this laboratory stemming directly from the information gained from Chapter 2 has recently shown promise in this regard in vitro for doxorubicin loaded systems.

Figure 5.1

Potential uses for exchangeable PEG-lipids

In *A*, the loss of the PEG-lipid component destabilizes the membrane resulting in rapid release of encapsulated agent. In *B*, loss of the PEG-lipid component triggers fusion with the target membrane.



Another possible role for such meta-stable liposomes is that of fusion with a target membrane (Figure 5.1*B*). Such systems could potentially be very useful for delivering their contents directly into a target cell. The control of membrane fusion in vitro is fairly straight forward, however for systemic delivery such control is exceedingly difficult. With the timed loss of a PEG-lipid component, such a system would have the benefit of extended circulation lifetime allowing target accumulation followed by the subsequent release of fusion inhibition. Chapter 4 suggested that a PEG coating may alter the distribution of liposomes within a solid tumor away from the

macrophage population. Combined with targeted system to further enhance the association of the liposomal carrier with the tumor cell, the fusion of such a carrier with the cell should have potentially huge therapeutic benefit. Preliminary data from our laboratory with erythrocyte ghosts again suggests that this approach may be feasible.

The potential future application of RES blockade as described here is questionable. Recent experiments have shown that pre-dosing with low doses of liposomal doxorubicin can not only significantly increase plasma levels of a subsequent injection as shown here, but also that this results in increased accumulation in a remote site not normally accessed by liposomes which would otherwise have shorter circulation lifetimes (Longman et al., 1995). It was interesting to note, however, that the addition of PEG-PE to the subsequent injection was not additive in terms of circulation lifetime increases or disease site accumulation as was expected based on the data from RES blockade or PEG-PE liposomal strategies separately. This is in general agreement with our findings in Chapter 4 that when plasma levels are already significantly elevated, the addition of PEG to the liposome does not significantly improve tumor targeting.

There are several potential problems with RES blockade, most notably the potential for cell death, although doxorubicin and vincristine at the doses employed in Chapter 3 do not appear to kill a major portion of Kupffer cell population. This is probably not true for higher doses such were employed in Chapter 4. Although clinical trials with liposomal doxorubicin have reported no major poisoning of important liver functions (Cowens et al., 1993), the impairment of Kupffer cell phagocytosis is only recently being considered (Daemen et al., 1995). It is possible that other liposomally delivered agents may be used to temporarily shut down the RES without significant harm to this cell population.

Finally, the last chapter demonstrated that maximization of dose intensity can result in the delivery of massive amounts of potentially therapeutic drug but results in minimal therapeutic effect. This illustrates the problem of bioavailability. This kind of approach combined with a release mechanism (thermosensitive liposomes, or triggered release as discussed above) could have huge therapeutic effects.

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