STUDIES OF FACTORS INFLUENCING DRUG EFFLUX RATES FROM LIPOSOMES AND THEIR IMPACT ON ANTITUMOR EFFICACY

By

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Abstract

The anti-tumor efficacy of liposomal formulations of cell cycle dependent anticancer drugs is dependent on the rates at which the drugs are released from the liposomes. Previous work on liposomal formulations of vincristine has shown increasing efficacy for formulations with progressively slower release rates. This thesis examined methods for increasing drug retention *in vitro* and *in vivo* for various antineoplastic agents as well as enhancing the circulation lifetime of the drug delivery system.

In Chapter 2 the effects of very high drug-to-lipid (D/L) ratios on vincristine release rates were investigated, and the antitumor efficacy of these formulations characterized in human xenograft tumor models. It is shown that the half-times ($T_{1/2}$) for vincristine release from egg sphingomyelin/cholesterol liposomes in vivo can be adjusted from $T_{1/2}$ = 6.1 h for a formulation with a D/L of 0.025 (wt/wt) to $T_{1/2}$ = 117 h (extrapolated) for a formulation with a D/L ratio of 0.6 (wt/wt). The increase in drug retention as the D/L ratio is increased is attributed to the presence of drug precipitates in the liposomes. Variations in the D/L ratio did not affect the circulation lifetimes of the liposomal vincristine formulations. The relationship between drug release rates and anti-tumor efficacy was evaluated using a MX-1 human mammary tumor model. It was found that the antitumor activity of the liposomal vincristine formulations increased (tumor growth delay 21 d to greater than 60 d respectively) as the D/L ratio was increased from 0.025 to 0.1 (wt/wt) ($T_{1/2}$= 6.1 – 15.6 h respectively) but decreased (tumor growth delay 29 d) at higher D/L ratios (D/L=0.6, wt/wt, $T_{1/2}$=117 h). Free vincristine exhibited the lowest activity of all formulations examined. These results demonstrate that varying the D/L ratio provides a powerful method for regulating drug release and allows the

generation of liposomal formulations of vincristine with therapeutically optimized drug release rates.

In Chapter 3, it has been demonstrated that the release properties of ciprofloxacin, a drug that does not precipitate following accumulation into large unilamellar vesicles, are not affected by the D/L ratio, whereas the release properties of liposomal doxorubicin are strongly dependent on the D/L ratio. It has also been shown that the crystalline intravesicular doxorubicin precipitates observed as the D/L ratio is raised from 0.05 to 0.46 (wt/wt) adopt increasingly unusual morphologies. Linear crystals are observed at the lower D/L values (0.05), however triangular and rectangular variations are observed as the D/L ratio is increased that induce considerable distortion in the vesicle morphology. It is noted that the trapping efficiency following uptake of external doxorubicin into LUV is reduced from nearly 100% at a D/L ratio of 0.05 (wt/wt) to less than 50% at an (initial) D/L ratio of 0.8 (wt/wt). It is suggested that this arises, at least in part, from membrane-disrupting effects of internal drug crystals as they increase in size

In Chapter 4, it was shown that replacement of egg sphingomyelin (ESM) by dihydrosphingomyelin (DHSM) in sphingomyelin/cholesterol (55/45; mol/mol) large unilamellar vesicles (LUV) results in substantially improved drug retention properties both *in vitro* and *in vivo*. In the case of liposomal formulations of vincristine, for example, the half-times for drug release ($T_{1/2}$) were approximately 2-fold longer for DHSM/Chol LUVs as compared to ESM/Chol LUVs, both *in vitro* and *in vivo*. Further increases in $T_{1/2}$ could be achieved by increasing the D/L ratio of the liposomal vincristine formulations. In addition, DHSM/Chol LUVs also exhibit improved circulation lifetimes in vivo as compared to ESM/Chol LUV. For example, the half-time for LUV clearance

($Tc_{1/2}$) at a low lipid dose (15 μmol lipid/kg, corresponding to 8.4 mg lipid/kg body weight) in mice was 4.3 h for ESM/Col LUVs compared to 6.7 h for DHSM/Chol LUVs. It is shown that the clearance properties of DHSM/Chol LUV are comparable to those observed for poly(ethylene glycol) (PEG)-coated LUV with lipid compositions associated with "Stealth" characteristics. In addition, it is also shown that DHSM/Chol LUV exhibited longer half-times for vincristine release as compared to LUVs with the "Stealth" lipid composition. It is anticipated that DHSM/Chol LUVs will prove useful as drug delivery vehicles due to their excellent drug retention and circulation lifetime properties.

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Abbreviations

BSM	Bovine brain sphingomyelin
CHE	Cholesteryl hexadecyl ether
Chol	Cholesterol
CRYO-TEM	Cryogenic transmission electron microscopy
D/L ratio	Drug-to-lipid ratio
DBPC	Dibehonylphosphatidylcholine
DHSM	Dihydrosphingomyelin
DPPC	Dipalmitoyl phosphatidylcholine
DRM	Detergent resistant membranes
DSPC	Distearoyl phosphatidylcholine
EDTA	Ethylenediaminetetraacetic acid
EPC	Egg phosphatidylcholine
EPR	Enhanced penetration and retention
ESM	Egg sphingomyelin
FBS	Fetal bovine serum
H_{II}	Hexagonal
LUV	Large unilamellar vesicle
MLV	Multi-lamellar vesicle
MSM	Bovine milk sphingomyelin
NMR	Nuclear magnetic resonance
PA	Phosphatidic acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamiine
PEG	Poly (ethylene glycol)
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PS	Phosphatidylserine
RES	Reticular endothelial system
SHE	300mM Sucrose, 20mM HEPES, 3mM EDTA pH 7.4 loading buffer
SM	Sphingomyelin
SUV	Small unilamellar vesicle
T_c	Transition temperature
TX-100	t-octylphenoxypolyethoxyethano

Chapter 1: Introduction

1.1 Liposomes as drug delivery systems: an overview

Liposomes were first characterized in the 1960's and have played an important role in the development of our understanding of the physical and structural properties of biological membranes. This is because liposomes can be used as simple "model membrane" systems in which the properties of individual components of membranes can be studied in detail (Bangham et al. 1965; Bangham 1968). Examples include the role of individual lipids in establishing the permeability barrier of biological membranes (Mandersloot et al. 1975), the ability of lipids to diffuse laterally in the plane of the membrane (Cullis 1976), the importance of particular lipids in membrane protein function using "reconstituted" model membranes containing membrane proteins (Papahadjopoulos et al. 1973; Eytan et al. 1977; Schroeder et al. 1998) among many other applications (Ahmed et al. 1997; Evans and Roger MacKenzie 1999; Ghosh and Bell 2002; Frostell-Karlsson et al. 2005).

Liposomes have also been developed in their own right as drug delivery systems, which is the application used in this thesis. Soon after the discovery of liposomes their potential as drug delivery systems was recognized (Gregoriadis 1973) due to their intrinsic ability to encapsulate bioactive agents in the liposome interior as well as their biocompatible nature. This led quickly to applications of liposomes as delivery vehicles for biologically active molecules such as peptides/proteins, nucleic acids or conventional small molecule drugs (Mukherjee et al. 1978; Gregoriadis 1984; Mayer et al. 1985a; Kabanov et al. 1990).

The utility of liposomes as delivery systems has shown the greatest success for conventional small molecule anticancer drugs, with many liposomal formulations of anticancer drugs (Figure 1.1) either approved for clinical use or in late clinical studies (Allen and Cullis 2004; Hofheinz et al. 2005). For most antineoplastic agents, toxic side effects limit their clinical utility (Ewer et al. 2004). The major clinical anticancer application of liposomes has been for doxorubicin, a drug used for treatment of numerous types of tumors (Sharpe et al. 2002), where a major benefit of liposomal delivery is a reduction in the cardiotoxicity associated with free doxorubicin as liposomes do not accumulate in heart tissue (Forssen and Tokes 1981; Herman et al. 1983; Ewer et al. 2004). In addition, it has been shown that long-circulating liposomal formulations of doxorubicin result in enhanced accumulation of drug at tumor sites as compared to equivalent doses of free drug (Gabizon 1992; Krishna et al. 2001), potentially leading to enhanced activity. This enhanced accumulation at tumor sites has been attributed to the "enhanced penetration and retention" effect (Proffitt et al. 1983; Gabizon 1992) resulting from the more permeable nature of tumor vasculature as compared to normal vasculature (Yuan et al. 1995).

This primary focus of this thesis is on liposomal formulations of the anticancer drug vincristine, which is one of the most commonly employed anticancer drugs used for the treatment of Hodgkin's and non-Hodgkin's lymphomas, acute lymphoblastic leukemia, embryonal, rhabdomyosarcoma, neuroblastoma, breast carcinoma and Wilm's tumor (Carter and Livingston 1976) (Sieber et al. 1976). In contrast to doxorubicin (Minotti et al. 2004), vincristine is a cell cycle specific agent (Owellen et al. 1972; Owellen et al. 1976). As detailed below, there is reason to believe that liposomal delivery systems are best suited for the delivery of cell-cycle specific drugs due their ability to accumulate at tumor sites and pay out the drug slowly (Jackson and Bender

1979; Mayer et al. 1993), thus enhancing the probability that the drug is present at the vulnerable part of the cell cycle.

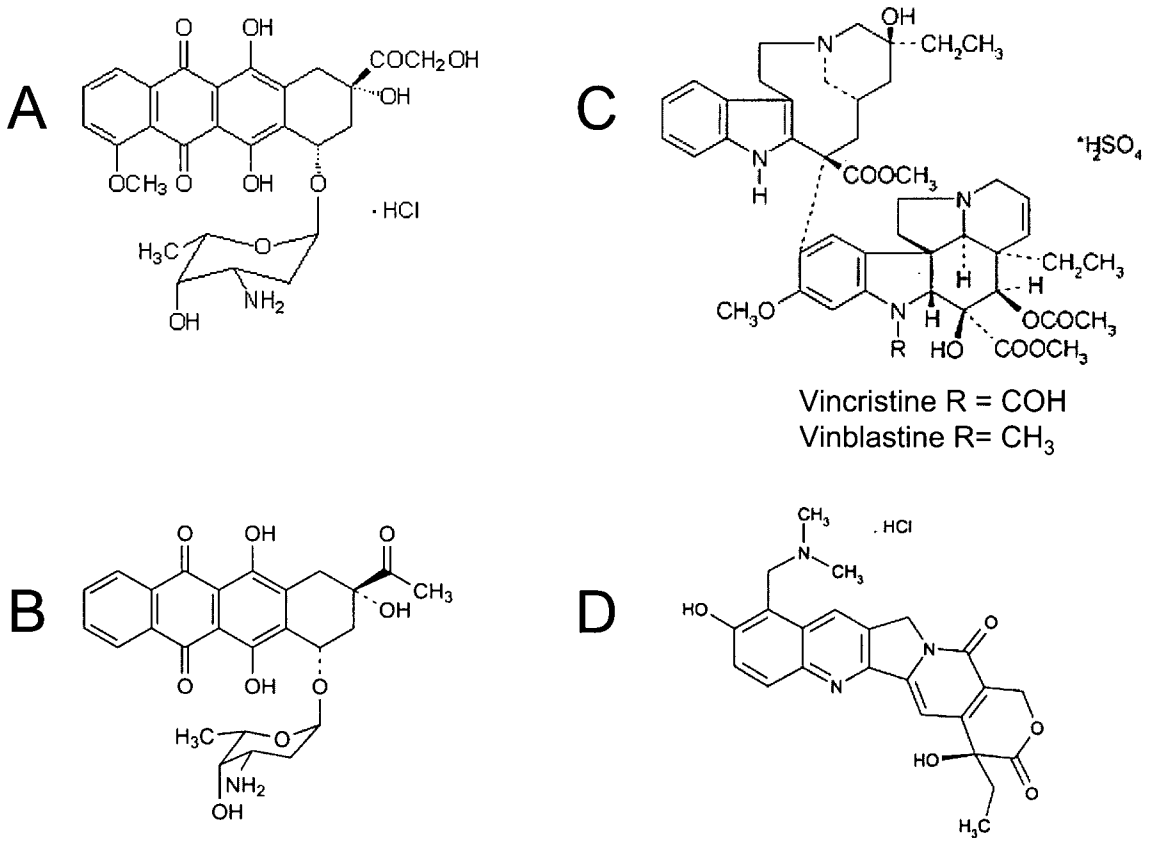


Figure 1.1. Structures of antineoplastic agents that have shown enhanced therapeutic activity when formulated in liposomal delivery systems, A: Doxorubicin, B: Idarubicin, C: Vinca Alkaloids, D: Topotecan.

Vincristine (Figure 1.1) is a vinca alkaloid isolated from Madagascar periwinkle (*Catharanthus roseus*) (Kruczynski et al. 1998; Perry et al. 2004). Vincristine arrests cell growth during metaphase by interrupting mitotic spindle function by binding to the β-subunit of tubulin and preventing GTP hydrolysis (Jordan and Wilson 1990; Jordan et al. 1991; Toso et al. 1993; Lobert et al. 1996; Kavallaris et al. 2001; Ngan et al. 2001). The vinca alkaloids may also interact, either directly or indirectly, with microtubule associated proteins (MAPs) or regulators of microtubule dynamics (Lobert et al. 2000). Alterations to MAP4 have been associated with vincristine resistance in childhood acute

lymphoblastic leukemia, although the exact mechanism remains unclear (Kavallaris et al. 2001).

At minimum effective concentrations of the drug *in vivo*, it has been observed that there is decreased tubulin addition to growing microtubule ends with higher concentrations leading to massive microtubular disassembly (Lobert et al. 1996). Studies have shown that agents that disrupt microtubular dynamics, such as vincristine, can induce apoptosis though the activation of the Ras cascade, although the mechanism that links the disruptions and signaling cascade remains to be elucidated (Wang et al. 1998) .

It is advantageous to expose target cells to vincristine for extended periods of time, thereby allowing more cells to be exposed to the drug during the vulnerable stage in the cell cycle (Figure 1.2). For example, Mayer and coworkers observed that increasing the exposure of L1210 leukemic cells to vincristine from 1 to 72 h resulted in a 10^5-fold decrease in the concentration of vincristine required to yield 50% cytotoxicity, as shown in Table 1.1 (Mayer et al. 1993). This effect has also been observed for patients where increased response rates were observed when vincristine was administered over a 5-day infusion (Jackson et al. 1986a; Jackson et al. 1986b). These studies suggest that the anticancer activity of vincristine can be enhanced by the prolonged exposure of tumor cells to the drug. As shown in Figure 1.2, this increased duration of exposure must be balanced with the need to maintain the concentration of the drug at the tumor site above the minimum effective concentration.

Table 1.1. Effect of exposure time on the *in vitro* cytotoxicity of vincristine against L1210 cells.

The drug concentration required to yield 50% cytotoxicity (IC50) decreases from 12 μM to 0.12 nM as the duration of drug exposure is increased from 1 to 72 h. (Taken from

Exposure time(h)[a]	IC_{50} (nM)[b]
1	12,000
4	2,400
6	2,400
24	7.3
72	0.12

Mayer, Nayar et al. 1993)

[a] Duration of drug exposure starting at t=0 over a total period of 72h

[b] Vincristine concentration required to achieve 50% cytotoxicity

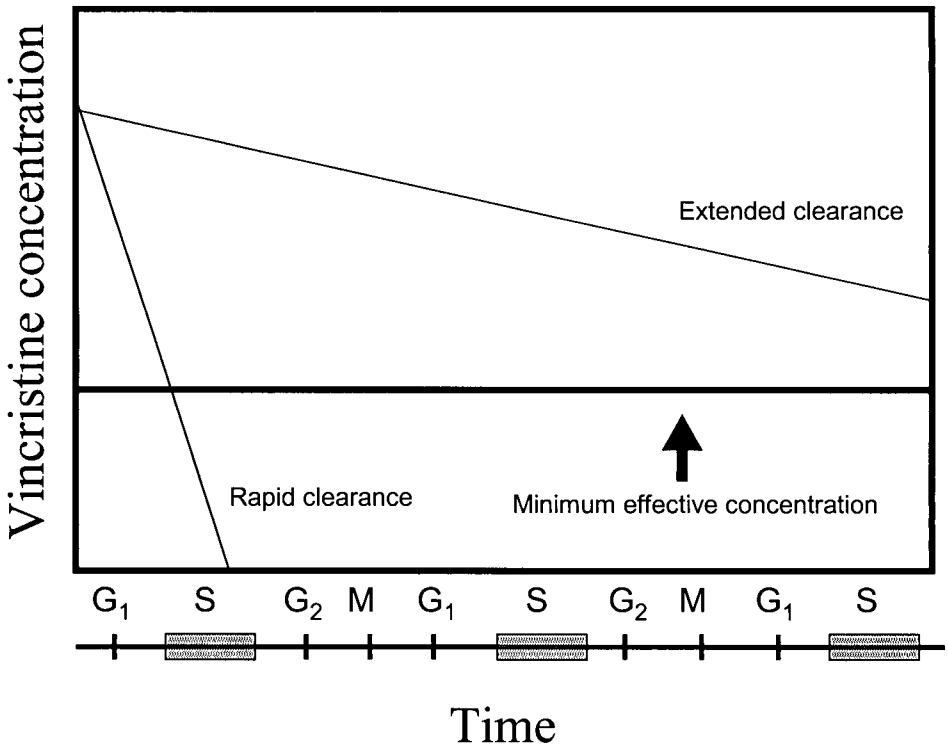


Figure 1.2. Theoretical model for vincristine sensitivity in tumor models. For maximized therapeutic effects the concentration of drug at the tumor site must be maintained above the minimum effective concentration required for antitumor activity for as long as possible. Taken from Boman, 1994 .

In accord with these observations, liposomal formulations of vincristine have demonstrated significantly improved anticancer activity compared to the same doses of free drug (Mayer et al. 1990; Boman et al. 1995; Mayer et al. 1995; Webb et al. 1995; Leonetti et al. 2004). Reduced toxicity was also observed due to decreased bioavailibity in susceptible tissues (Boman et al. 1996). Further, the improvements in efficacy correlate well with increased duration of drug exposure to the tumor, as liposomal formulations of vincristine with the slowest rates of drug release demonstrate the highest levels of activity (Webb et al. 1995; Boman et al. 1998) (Figure 1.3). Many approaches to developing formulations with slower drug release rates have been explored, including reducing the pH of the liposome interior (Boman et al. 1993; Boman et al. 1994), increasing the phosphatidycholine acyl chain length and saturation (Boman et al. 1993) and substituting sphingomyelin for PC (Webb et al. 1995). However, none of these efforts have resulted in liposomal vincristine formulations that exhibit therapeutically optimized rates of drug release. This is because, even at the slowest release rates achieved, the antitumor efficacy of liposomal vincristine in A431 human epidermoid carcinoma xenograft and P388 lymphocytic leukemia models was still increasing (Webb et al. 1995).

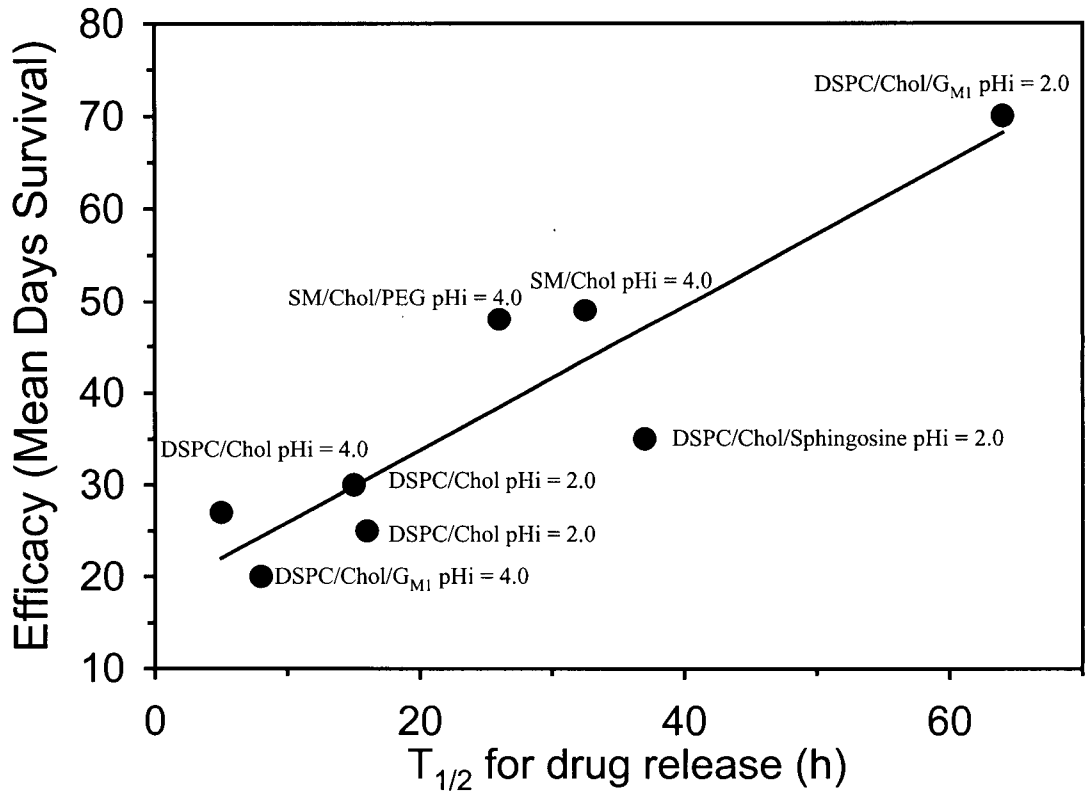


Figure 1.3. Influence of the half-life of vincristine release from liposomes on the antitumor efficacy of liposomal vincristine formulations in P388 tumor bearing BDF1 mice. Adapted from Boman et al. 1998 (pHi, internal pH).

A primary emphasis of this thesis concerns the development of new approaches to achieve liposomal formulations of vincristine that release drug so slowly that therapeutically optimized formulations can be achieved. In addition to achieving this aim, these studies have also led to investigations of the effects of drug crystallization in the liposome interior. In order to provide the appropriate background to this work this introductory chapter will describe lipids and their physical properties, liposome structure, the procedures for loading drugs in liposomes and the *in vivo* properties of liposomes and liposome-drug formulations.

1.2 Lipids

Lipids serve both energy storage and structural roles in eukaryotes. From a structural perspective, the formation of lipid bilayer membranes functions to separate cellular compartments from each other and from the external environment. Membrane lipids are amphipathic, possessing both hydrophobic and hydrophilic regions. In the presence of water the hydrophobic tails are oriented towards each other and the hydrophilic heads face the aqueous environment.

1.2.1 Phospholipids

Phospholipids are the most abundant class of naturally occurring lipids. They contain a glycerol backbone and a phosphate-containing head group. The phospholipids include phosphatidylcholines (PC), phosphatidylethanolamiines (PE), phosphatidylserines (PS), phosphatidic acids (PA) phosphatidylglycerols (PG) and phosphatidylinositols (PI). Structures for these phospholipids are shown in Figure 1.3. The head-group of a phospholipid is important in determining its various properties. Naturally occurring phospholipids can contain either anionic head-groups (PS, PG and PI) or zwitterionic head-groups (PC and PE). Phospholipid diversity can also result from the length and degree saturation of the acyl chains (Table 1.2). Typically, acyl chains range in length from 16-24 hydrocarbons with the most common saturated fatty acids found being palmitic and stearic acid. The most common unsaturated fatty acids are oleic and linoleic acids.

1.2.2 Sphingomyelin

Sphingomyelin, in common with glycosphingolipids, is formed from a sphingosine base. An immediate precursor of sphingomyelin is ceramide which is composed of a fatty acyl chain (C16-26) and sphingosine linked through an amide bond (Yano et al.

1998) (Figure 1.3). Sphingomyelin is the major phospholipid used in this thesis and is found closely associated with cholesterol in the outer monolayer of plasma membranes (Brown and London 1998; Brown and London 2000).

The use of SM in formulations for liposomal delivery systems confers advantages over more the commonly used phospholipids, such as distearoylphosphatidylcholine (DSPC) (Webb et al. 1998a). The amide bond in SM is less susceptible to acid catalyzed hydrolysis than the ester linkages in DSPC and can participate in hydrogen bonding to other lipids within the membrane (Webb et al. 1998a; Ramstedt and Slotte 2002; Mombelli et al. 2003). These properties lead to increased stability in storage, increased drug retention and circulation lifetimes for liposomal drug delivery systems employing sphingomyelin as compared to other phospholipids (Webb et al. 1995; Boman et al. 1998).

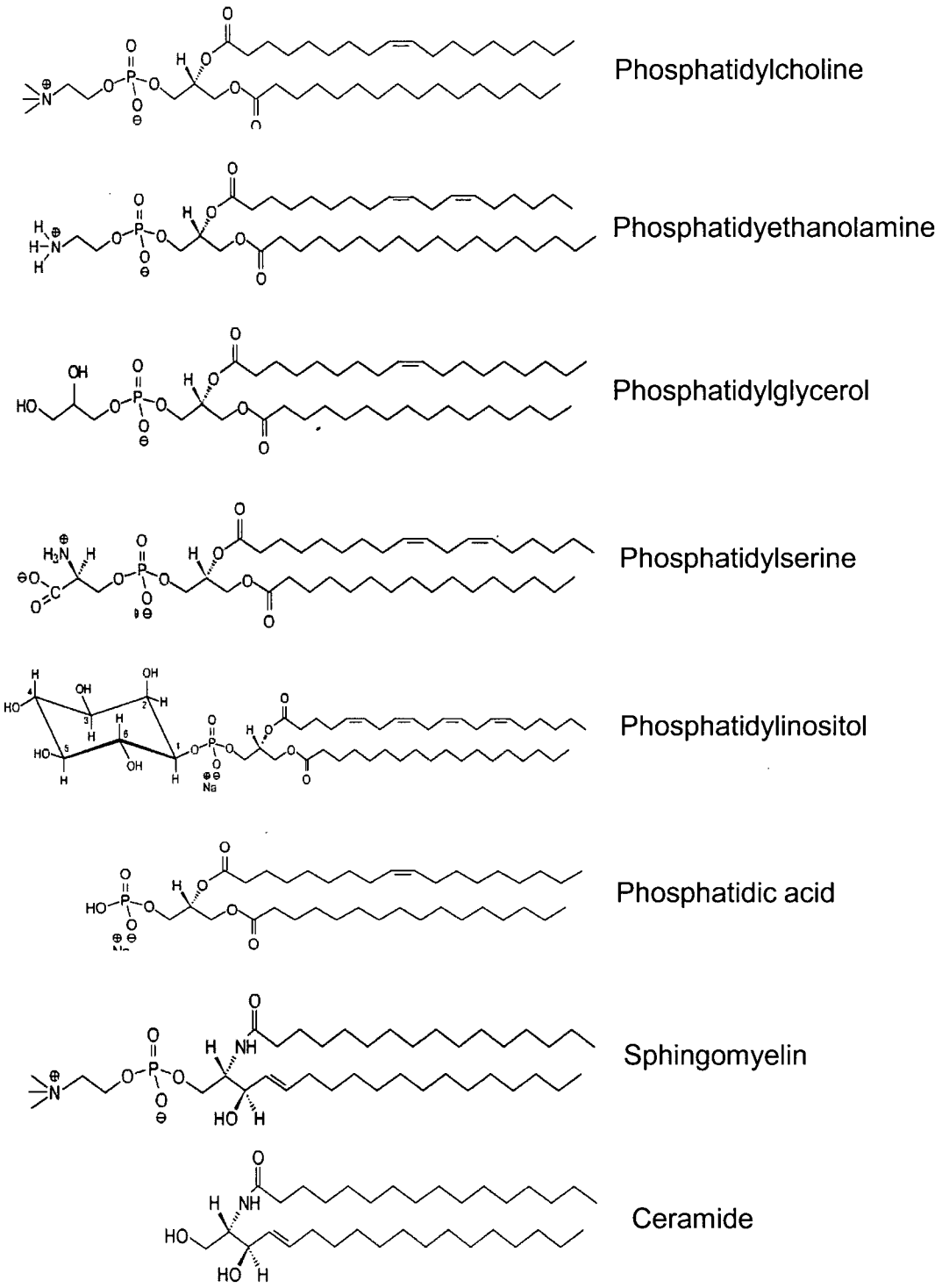


Figure 1.4 Structures of common phospholipids.

Table 1.2. Naturally occurring fatty acids

Common name	Carbon skeleton
Lauric acid	12:0
Myristic acid	14:0
Palmitic acid	16:0
Stearic acid	18:0
Arachidic acid	20:0
Lignoceric acid	24:0
Palmitoleic acid	16:1 (Δ^9)
Oleic acid	18:1 (Δ^9)
Linoleic acid	18:2 ($\Delta^{9,12}$)
Linolenic acid	18:3 ($\Delta^{9,12,15}$)
Arachidonic acid	20:4 ($\Delta^{5,8,11,14}$)

1.2.3 Cholesterol

Cholesterol, a major component of eukaryotic cellular membranes, is a neutral lipid and is comprised of a rigid sterol structure with a polar 3-β-hydroxyl group on one end (Figure 1.4). Cholesterol is found in equimolar concentrations with phospholipids in plasma membranes (Brown and London 2000; Ramstedt and Slotte 2002; Simons and Vaz 2004). It is also a major component of the liposomal delivery systems employed in this thesis, generally composing 45 mol percent of the total lipid within the liposomes.

When included in liposomal formulations, cholesterol functions to modify the physical and pharmacokinetic properties of liposome based drug delivery systems. It has been shown that cholesterol increases the packing densities of phospholipid molecules resulting in reduced encapsulated solute leakage. The inclusion of cholesterol also leads to a decrease in liposomal uptake by cultured Kupffer cells *in vitro* (Roerdink et al. 1989) and reduced liposomal clearance *in vivo* (Patel et al. 1983). The modifications cholesterol confers to liposomal delivery systems are highly beneficial as it is critical for *in vivo* drug delivery systems to have long circulation lifetimes while maintaining their encapsulated pharmaceutically relevant payload.

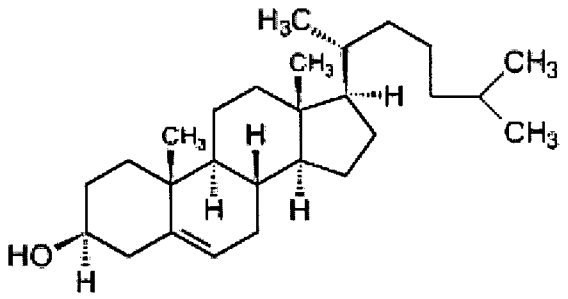


Figure 1.5 Structure of cholesterol.

1.3 Physical properties of lipids

Both the chemical structure and the physical environment that a lipid is in governs its molecular behavior. Lipids in an aqueous environment adopt particular organizations or phases. The different phases that lipids can adopt include the lipid bilayer, the most common phase as well as a number of non-bilayer phases, including the hexagonal phase (H_{II}) or isotropic phases. This ability to adopt different phases is referred to as lipid polymorphism and can be influenced by the presence of other lipids, the temperature or the pH among other factors.

1.3.1 Gel to liquid crystalline phase transition

Lipid bilayers can exist in two distinct states; the first is a gel or frozen state which is ordered and rigid, the second, a liquid-crystalline state, is disordered and fluid (Reiss-Husson and Luzzati 1967; Lee 1977) (Figure 1.5). In the gel state all the carbon-carbon bonds are in the extended, all-trans conformation, while in the liquid crystalline state some are in the gauche conformation. Due to the rigidity of the membrane, lateral diffusion occurs much more slowly in the gel state than in the liquid-crystalline (Cullis 1976). For example, dipalmitoylphosphatidycholine in the liquid-crystalline state exhibits a lateral diffusion rate of $D_t \approx 2 \times 10^{-8}$ cm^2/sec, whereas in the gel state, $D_t < 10^{-9}$ cm^2/sec

(Cullis 1976). Employing the relation $x^2 = 4D_t t$, where x is the distance diffused in the time t, it can be illustrated that in 1 second a DPPC molecule in a liquid-crystalline state will diffuse approximately 2.8×10^{-6} m, whereas in the gel state the distance diffused is less than 0.3×10^{-6} m.

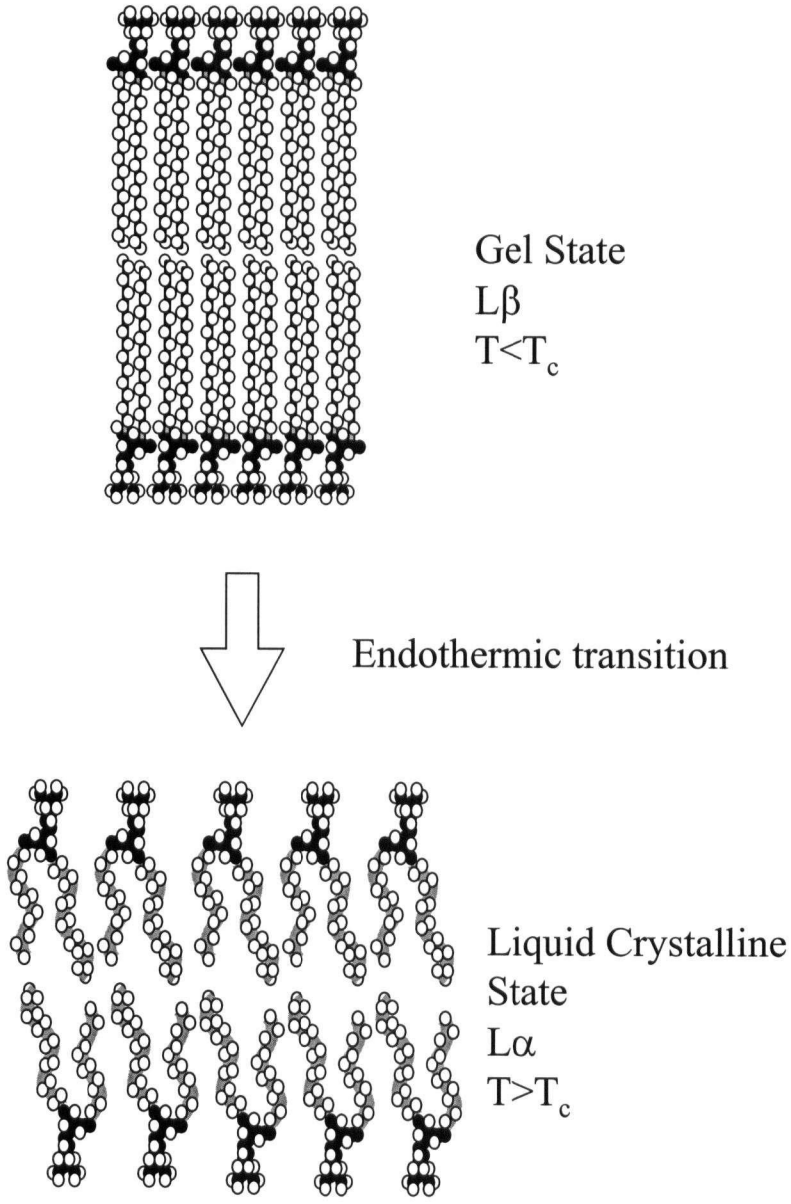


Figure 1.6 The phase transition of a lipid bilayer from the gel state (Lβ) to the liquid crystalline state (Lα) upon reaching the transition temperature of the lipid (T_c)

The phase transition temperature (T_c) is the specific temperature at which a lipid membrane transforms between these two states. Increasing the length of the fatty acyl chain and increasing their degree of saturation raises this transition temperature (Jacobs et al. 1975; Mason and O'Leary 1990). The transition can be monitored using nuclear magnetic resonance (NMR), electron spin resonance and fluorescence (Cullis et al. 1996). The transition between the gel and liquid-crystalline state can also be detected via calorimetric techniques, where the heat absorbed as the sample undergoes an endothermic phase transition is measured. The phase transition temperatures of various phospholipids are shown in table (Table 1.3). Cholesterol has a modifying effect on the phase transition when it is included in membranes. When added to liposomal system composed of saturated phosphatidylcholine, a progressive decrease in the enthalpy of the gel-liquid crystalline phase transition is observed. At approximately 30mol% or higher, the transition can no longer be detected (Houslay and Stanley 1982). An example of this progression for dipalmitoyl- PC can be seen in Figure 1.6. Cholesterol increases the "order" in the acyl chains of PCs which are in the liquid-crystalline state and decreases the order for PCs which are in the gel state (Demel and De Kruyff 1976). Also, cholesterol can have a condensing effect with PCs, where the volume of a mixture of liquid-crystalline PC and cholesterol is less than the volume of the two components separately (Hyslop et al. 1990).

Table 1.3. Transition temperatures (Tc) of various combinations of acyl chain length, degree of saturation and headgroup moiety. Taken from Cullis and Hope, 1985

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
Stearoly, 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

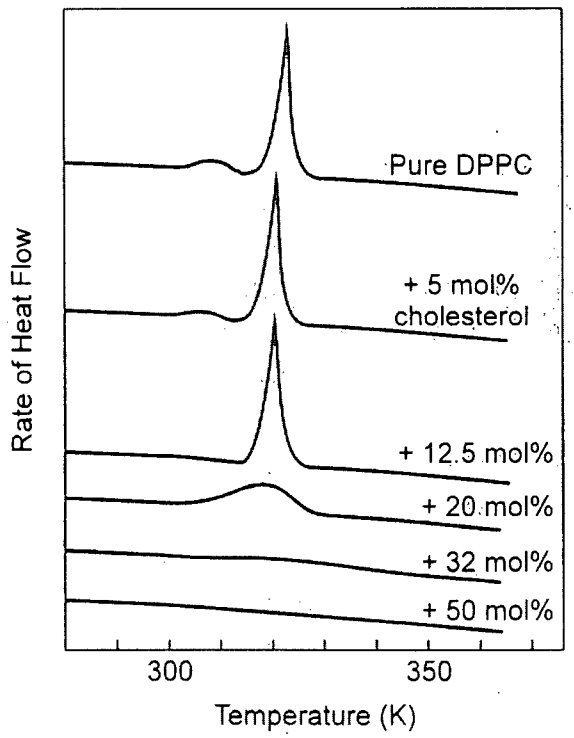


Figure 1.7. Phospholipid gel-liquid-crystalline phase transition and the effect of cholesterol. The influence of cholesterol on the phase transition can be observed for dipalmitoyl-PC, the enthalpy of the phase transition (represented by the area under the endotherm) is dramatically reduced. At greater than 30 mol% cholesterol, the lipid phase transition is effectively eliminated. Taken from Cullis, Fenske et al. 1996.

1.3.2 Lipid polymorphism

In the liquid crystalline state, lipids can adopt a number of different lipid polymorphic phases, including the bilayer (lamellar) phase, the inverted hexagonal (H_{II}) phase and other non-bilayer phases known as cubic phases as shown in Figure 1.8 (Lasic 1998; Ulrich 2002). The polymorphic state which a lipid adopts is dependent on its shape as shown in Figure 1.8 (Israelachvili et al. 1980; Cullis et al. 1986; Ulrich 2002). Lipids such as detergents possess head groups that occupy large areas in comparison to their tails. These lipids can be thought of as having an " inverted cone" shape with a tendency to form micelles. Lipids that adopt the bilayer phase possess head groups that have cross-sectional areas similar to that of their tails, and exhibit a cylindrical shape. An example of this class of lipids would be the PCs. Finally lipids such as unsaturated species of PE exhibit a cone shape due to their relatively small neutral head groups and more flexible acyl chains and adopt an inverted hexagonal (H_{II}) phase structure.

Lipid polymorphism can also be modulated by a number of other factors (Cullis et al. 1986). Increasing temperatures leads to increasing mobility of the acyl chains, thus increasing the relative cross-sectional areas of the hydrophobic section, ultimately favoring the formation of the H_{II} phase. The pH and ionic strength can alter the charge of the head group, with charged head groups effectively increasing the cross sectional area occupied by the hydrophilic portion of the lipid. An example of this would be the formation of inverted hexagonal phases from bilayers of PS when the pH is reduced to below 4. The low pH results in the protonation (neutralization) of the carboxyl group of PS, resulting in the head group occupying a smaller volume and change from a cylindrical shaped lipid to an inverted cone shaped lipid, thus promoting the formations of the H_{II} phase.

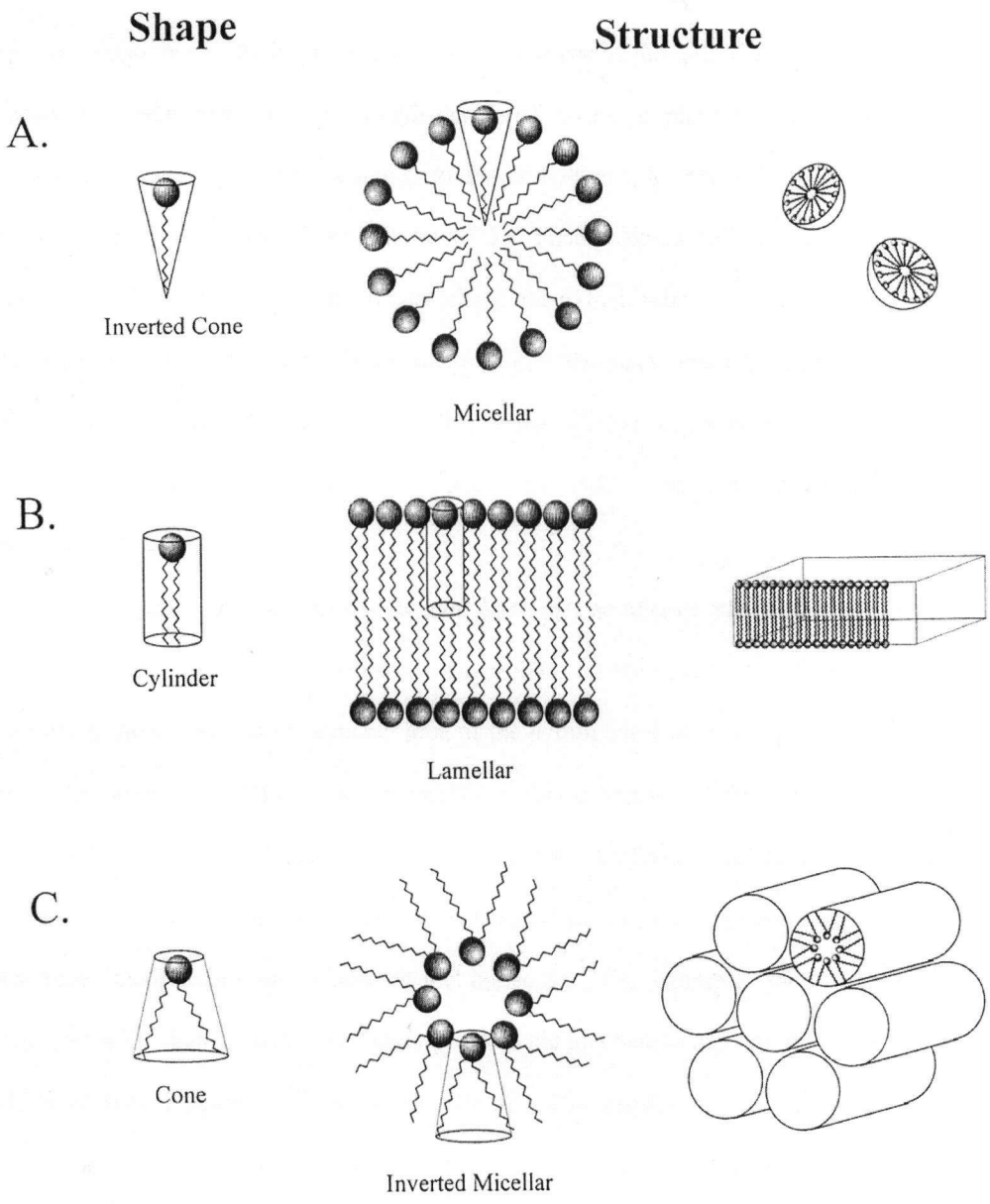


Figure 1.8. The polymorphic phases of lipids. Common lipid shapes and resulting structures. (A) inverted cone and micellar structures, (B) cylindrical and lamellar/bilayer structure and (C) cone and inverted micellar/hexagonal structures. Taken from Parr, 1995

1.3.3 Membrane permeability

One of the most critical roles of a lipid bilayer is to function as a barrier between the external and internal environment, consequently the permeability and factors that influence the permeability of the bilayer, with respect to water, ions and small molecules, are of great interest for both the study of biological systems and for lipid based drug delivery vehicles.

The study of membrane permeability and the permeability coefficient of a given lipid system and how that parameter relates to the movement of material across the membrane must begin with a basic overview of *Fick's law*. *Fick's law* states that the diffusion rate (number of molecules per unit of time, dn/dt) of a given substance across a membrane is directly proportionally to the area A of the membrane and the concentration difference $\Delta C(t)$ across that membrane. Hence $dn/dt \propto A\Delta C(t)$ or rewritten as $dn/dt = -PA\,\Delta C(t)$, where P is the permeability coefficient and t is the time. In the case of a liposomal system with radius R and an initial internal concentration $C_l(0)$ it can be shown that $\Delta C(t) = C_l(0)\exp(-3Pt/R)$. If it presumed that the external volume is much greater than the internal trapped volume, $\Delta C(t) \cong C_l(t)$ (where $C_l(t)$ is the internal concentration at time t) and the previous relationship can be simplified to $C_l(t) = C_l(0)\exp(-3Pt/R)$. Using a model case of a 100 nm diameter vesicle, the time it takes for one-half of the encapsulated material ($T_{1/2}$) to release can be calculated to be 0.1 s for $P = 10^{-5}$ cm/s and 3.2 h for $P = 10^{-10}$ cm/s.

Water permeates rapidly through bilayer membranes that are in the liquid-crystalline state with permeability coefficients in the range of 10^{-2} to 10^{-4} cm/s. Movement of water across the membrane can be easily measured by observing the swelling/shrinkage of model membrane systems in response to osmotic gradients (Blok

et al. 1976). Utilizing this technique studies have shown that bilayers with increasing quantities of cholesterol or increasing saturation of the lipid component have decreased water permeability. In general, lipid components that lead to increased order and tighter packing of the membrane lead to decreased membrane permeability. Permeability of non-electrolytes (uncharged polar solutes) appears to behave in a similar fashion to that of water, being dependent on the order of the lipid membrane.

Lipid bilayers are generally considered to have low permeability for sodium and potassium ions with permeability coefficients less than 10^{-10} cm/s, whereas H^+ and OH^- ions are much more permeable with permeability coefficients in the range of 10^{-4} cm/s (Deamer 1981). The charge of the head group can also influence ion permeability (Cullis and Hope 1985). For example a negatively charge membrane will repel anions and attract cations to the lipid-water interface.

Factors that influence the membrane permeability for water and ions also influence the membrane permeability of small lipophilic weak bases, such as many antineoplastic drugs. Studies have shown that increases in acyl chain length from DMPC (C_{14}) to DBPC (C_{22}) for PC/Chol liposomes increased retention of vincristine *in vitro* at 37°C from 1 h to 12 h respectively (Boman et al. 1993).

1.4 Liposomes

Liposomes, first characterized in 1965 by Bangham and coworkers (Bangham et al. 1965), are composed of one or more lipid bilayers forming an enclosed vesicle which encapsulates a distinct internal aqueous volume. The ability to retain encapsulated material and the circulation lifetimes of liposomes are highly dependent on the lamellarity, size and lipid composition of the liposome (Mayer et al. 1986b).

1.4.1 Multilamellar vesicles (MLVs)

Bilayer forming membrane lipids spontaneously form large multilamellar vesicles (MLVs) (Figure 1.7) when vortex mixed in an aqueous environment (Bangham et al. 1965; Cullis and Hope 1985). MLVs are generally heterogeneous in size, ranging from 1-10 μm in diameter, and can exhibit unequal solute distributions across the bilayers where the concentration of trapped solute is lower than in the external medium (Mayer et al. 1985b). MLVs are at a disadvantage for experimental and practical usage due to their large heterogeneous size distribution as well as their variable lamellarity (Cullis and Hope 1985). These properties preclude quantitative studies on the permeability of bilayers to various solutes and limits MLVs as *in vivo* drug delivery vehicles, as large systems are rapidly cleared from the circulation by the reticular endothelial system (RES) (Juliano and Stamp 1975). To overcome these limitations techniques were developed to produce liposomal delivery systems with defined lamellarity and size.

1.4.2 Small unilamellar vesicles (SUVs)

Easily prepared homogeneous small unilamellar vesicles (SUVs) with a diameter between 25 and 50 nanometers can be produced via sonication of MLVs or by forcing MLVs though a narrow orifice under very high pressure (Huang 1969; Barenholzt et al. 1979; Cullis and Hope 1985). SUVs have two major disadvantages as drug delivery systems. The first is the high degree of membrane curvature that SUVs posses (Figure 1.7), making these systems unstable and fusogenic, ultimately forming larger systems (Cullis and Hope 1985). An additional disadvantage of SUVs is their very small trapped volumes (<0.2 μl/μmol phospholipid) (Cullis and Hope 1985). To overcome these disadvantages, techniques have been employed to produce unilamellar vesicles of larger sizes.

1.4.3 Large unilamellar vesicles (LUVs)

There are several techniques to produce large unilamellar vesicles with diameters between 50 and 200nm and trapped volumes (aqueous volume within the liposomes) between 1-3 μl/μmol. The most utilized method for producing LUVs is to repeatedly extrude MLVs through polycarbonate filters of appropriate sizes (Olson et al. 1979; Hope et al. 1985). Utilizing this procedure a homogenous population of LUVs can be rapidly produced with diameters ranging from 50 to 200 nm (Figure 1.7, 1.8). This technique is ideal for the production of *in vivo* drug delivery systems as the carrier liposomes can be made rapidly, solvents and detergents are not required, high trapping efficiencies are observed, homogenous populations are easily obtained and the size of the liposome can be readily selected (Hope et al. 1985).

Other methods of producing LUVs include ethanol injection (Kremer et al. 1977), ether infusion (Deamer and Bangham 1976), reverse phase evaporation (Szoka and Papahadjopoulos 1978) and detergent dialysis (Mimms et al. 1981). These procedures involve solubilizing an appropriate lipid mixture in an organic solvent or detergent; followed by injection into an aqueous buffer. The solvent or detergent must then be removed via distillation, dilution or dialysis. These techniques have a number of disadvantages with respect to the extrusion technique, mainly these techniques are time consuming, may produce a heterogeneous population or residual solvent or detergent maybe present (Parente and Lentz 1984). Due to these disadvantages, the extrusion technique was utilized to produce all LUVs used for all studies in this thesis.

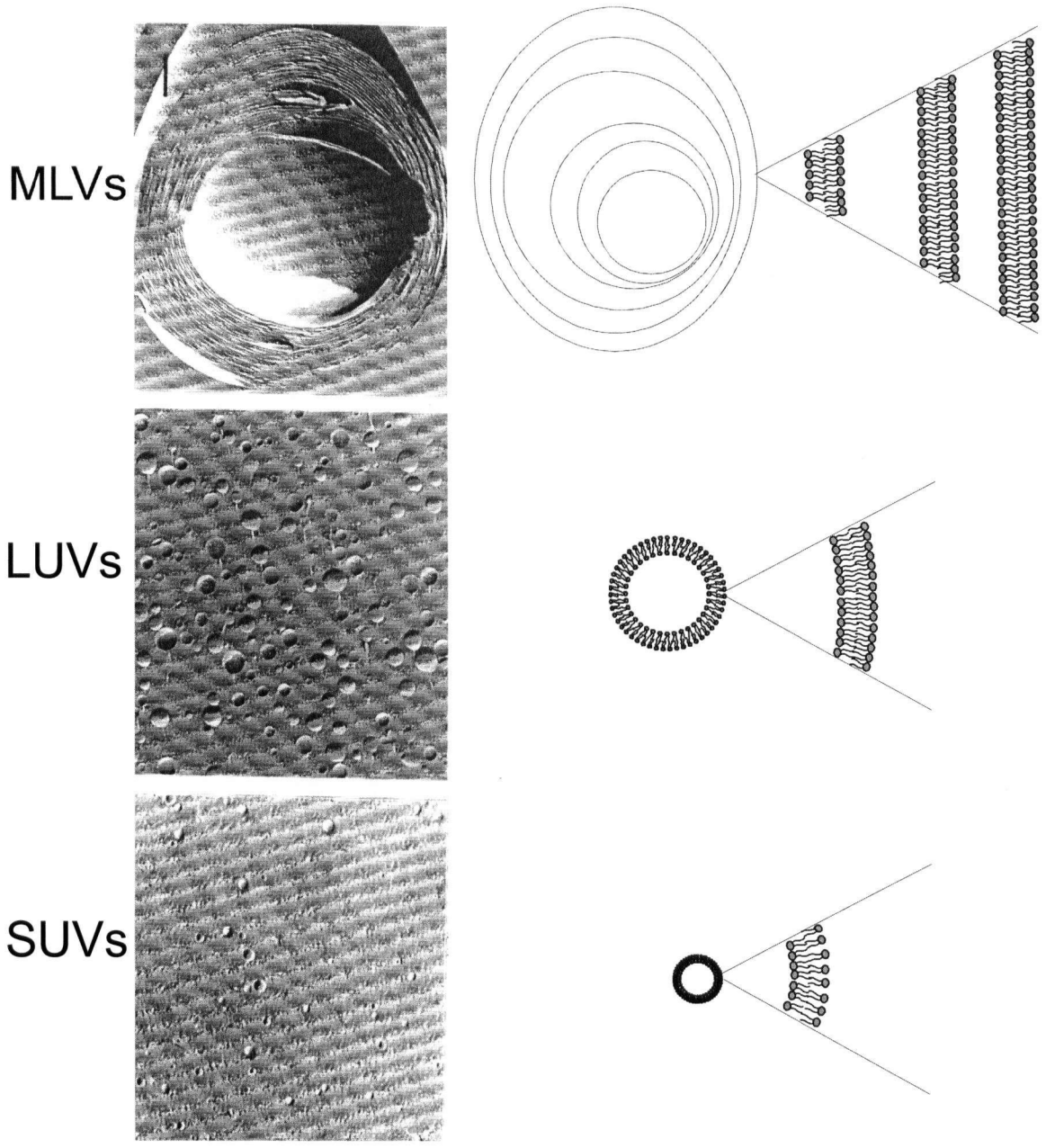


Figure 1.9. Freeze fracture electron micrographs and schematic representations of differing liposome morphology for multilamellar vesicles (MLVs), large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs). Taken from Parr, 1995.

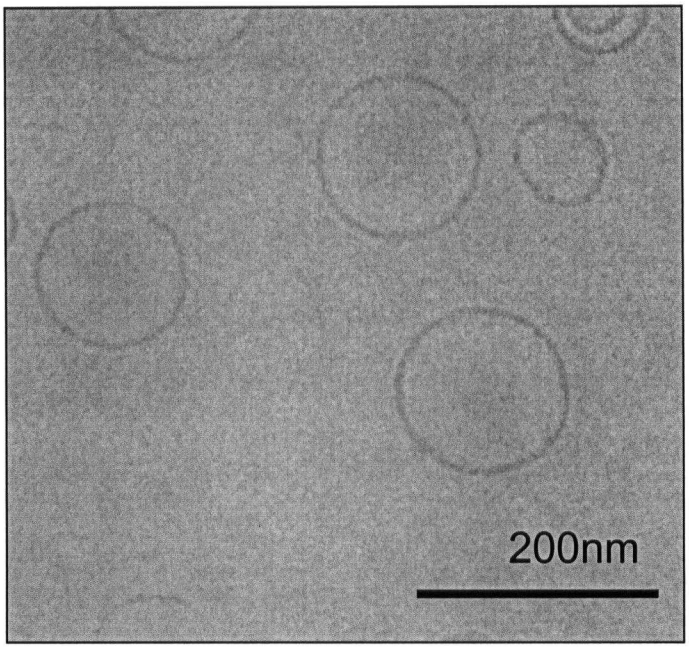


Figure 1.10. Cryo transmission electron micrograph of DSPC/Chol LUVs produced by extruding MLVs though two 100nm polycarbonate filters 10 times.

1.5 Drug encapsulation

To encapsulate antineoplastic agents into liposomes, two distinct techniques can be used. The first is passive encapsulation, where the drug is combined with the lipid during liposome production. For drugs that are soluble in aqueous environments, the encapsulation efficiency is dependent on the trapped volume. For lipophilic compounds, the encapsulation efficiency is dependent on the ability of the membrane to solubilize the drug while maintaining the liposome structure. A second approach is to "actively" encapsulate drugs that are week bases into liposomes in response to a transmembrane ion gradient.

1.5.1 Passive encapsulation

One example of passive encapsulation of a hydrophobic drug is liposomal formulations of the antifungal agent amphotericin B (Madden et al. 1990b). Although this

technique can produce high encapsulation efficiencies for this drug, the efficiency is dependent on the lipid characteristics and packing constraints of the membrane. It has also been demonstrated that the encapsulated drug can have high rates of exchange into other membranes, resulting in poor retention properties *in vivo* (Madden et al. 1990b).

Procedures to passively encapsulate water-soluble drugs are essentially the same as those used for hydrophobic drugs. Lipids and drugs are mixed prior to the formation of vesicles, but encapsulation is dependent on the aqueous trapped volume. For SUVs possessing trapped volumes of less than 0.2 μl/μmol, the trapping efficiency is generally below 1% (Szoka and Papahadjopoulos 1980). For LUVs, trapping efficiencies can approach 80% when high lipid concentrations (~400 mg lipid/ml) are combined with trapped volumes ranging between 1-10 μl/μmol (Mayer et al. 1985b). The ability of liposomes to retain water-soluble drugs that are passively encapsulated is dependent on the liposomal membrane and the drug. Drugs that have low membrane permeability, such as methotrexate, should display improved retention *in vitro* and *in vivo* compared to lipophilic drugs such as doxorubicin (Drummond et al. 1999). As mentioned above, these poor retention properties can be improved by utilizing lipids with longer acyl chains and/or by incorporating cholesterol into the membrane.

1.5.2 Active encapsulation

To overcome the low encapsulation efficiencies and poor *in vivo* retention observed with passive encapsulation techniques for lipophilic drugs, active encapsulation methods, where the drug is loaded in response to a transmembrane pH gradient, were developed. These techniques involve loading lipophilic drugs that contain ionizable amino groups, into preformed liposomes that have an acidic interior relative to

the surrounding external medium (Mayer et al. 1986b). Examples of pharmaceutically relevant compounds that can be loaded this way include vincristine, doxorubicin, vinorelbine, topotecan and ciprofloxacin (Madden et al. 1990a). A schematic of this process is shown in Figure 1.11. The drug in the external environment exists as either a neutral or a charged (protonated) species. The neutral species of the drug is much more membrane permeable and readily able to pass through the liposomal membrane reaching the acidic interior (relative to the external environment) (Cullis et al. 1997). Upon reaching the internal acidic interior the neutral species becomes protonated and is trapped within the liposome (Cullis et al. 1997).

A simple derivation can be used to describe the equilibrium transmembrane bilayer distribution of a weak base, such as vincristine. The equilibrium constant (K_a) of a weak base (B) is:

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

where H^+ is the proton concentration, B is the concentration of the neutral base and BH^+ is the concentration of the protonated base.

Assuming the dissociation constant for the weak base is the same regardless if the drug in inside$_i$ or outside$_o$ the liposome, then:

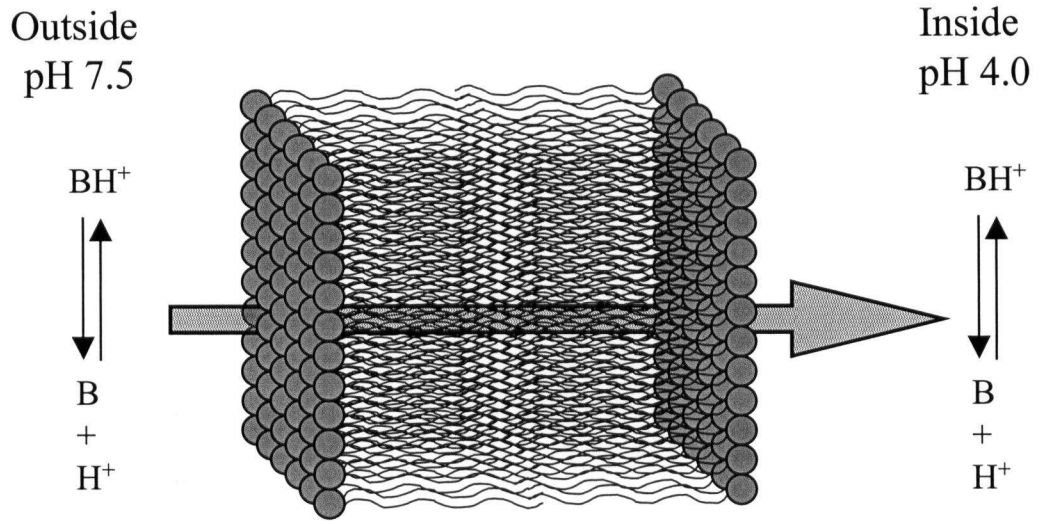
$$K_a = [H^+]_i[B]_i/[BH^+]_i = [H^+]_o[B]_o/[BH^+]_o$$

As the neutral species of the drug is membrane permeable, the concentrations of the that species, at equilibrium, will be the same on either side of the liposomal membrane and the above equation can be simplified, to give:

$$[BH^+]_i / [BH^+]_o = [H^+]_{li} /[H^+]_o$$

This relationship indicates that at a pH gradient of 3 units for example, the interior concentration of the charged species will be 1000 times higher than the exterior.

Transmembrane pH gradients can be generated employing a number of different techniques (Figure 1.12).



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

At equilibrium, if:

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

Then :

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

Figure 1.11. Active drug encapsulation in liposomes. The equilibrium redistribution of a lipophilic amine (weak base) in response to a pH gradient (ΔpH) across the liposomal membrane. Only the neutral form of the molecule is significantly membrane permeable. Taken from Parr, 1995

1.5.2.1 Active loading – buffered systems

The most straight foward method of active loading in response to a pH gradient across a liposomal membrane involves the formation and extrusion of LUVs in a low pH buffer (i.e. citrate buffer, pH 4.0) with a pH gradient generated though subsequent external buffer exchange (i.e. HEPES at pH 7.5) using dialysis or size exclusion column chromatography (Figure 1.12A) (Mayer et al. 1986a). This method of loading has successfully achieved loading efficiencies ranging from 90-100% at drug-to-lipid ratios to 0.1 (Mayer et al. 1986a).

1.5.2.2 Active loading - amine gradient

Transmembrane pH gradient can also be generated though the use of liposomes that maintain an ammonium sulfate, or other amine, transmembrane gradient (Haran et al. 1993). This ammonium sulfate gradient generates a pH gradient, which subsequently drives drug uptake (Figure 1.12B).

1.5.2.3 Active loading - ionophore method

Work by Fenske and coworkers has demonstrated a technique to generate transmembrane pH gradients in LUVs using a calcium ionophore (A23187) and transmembrane gradients of divalent cations, such as magnesium (Figure 1.12C) (Fenske et al. 1998). As the divalent cations travel down the concentration gradient, moving outward, the interior of the liposome is acidified. Fenske and coworkers have demonstrated that this loading technique is capable of loading vincristine and ciprofloxacin at high efficiencies (95-100%) to drug-to-lipid ratios of 0.2 (wt/wt)(Fenske et al. 1998). Higher drug-to-lipid ratios are achievable with this technique however the encapsulation efficiency is reduced (Abraham et al. 2004a; Johnston et al. 2006). An additional benefit of this technique is the observed dependence between the release

rate and the drug-to-lipid ratio, not seen for other loading methods, such as active loading with a citrate buffered pH gradient, for drugs such as the vinca alkaloids (Zhigaltsev et al. 2005; Johnston et al. 2006) and topotecan (Abraham et al. 2004a). The $MgSO_4$/ionophore loading method was used for all studies in this thesis, as high drug-to-lipid ratios (> 0.5 wt/wt) could be achieved and a dependence between drug-to-lipid ratio and release rate could be used to optimize formulations of liposomal vincristine.

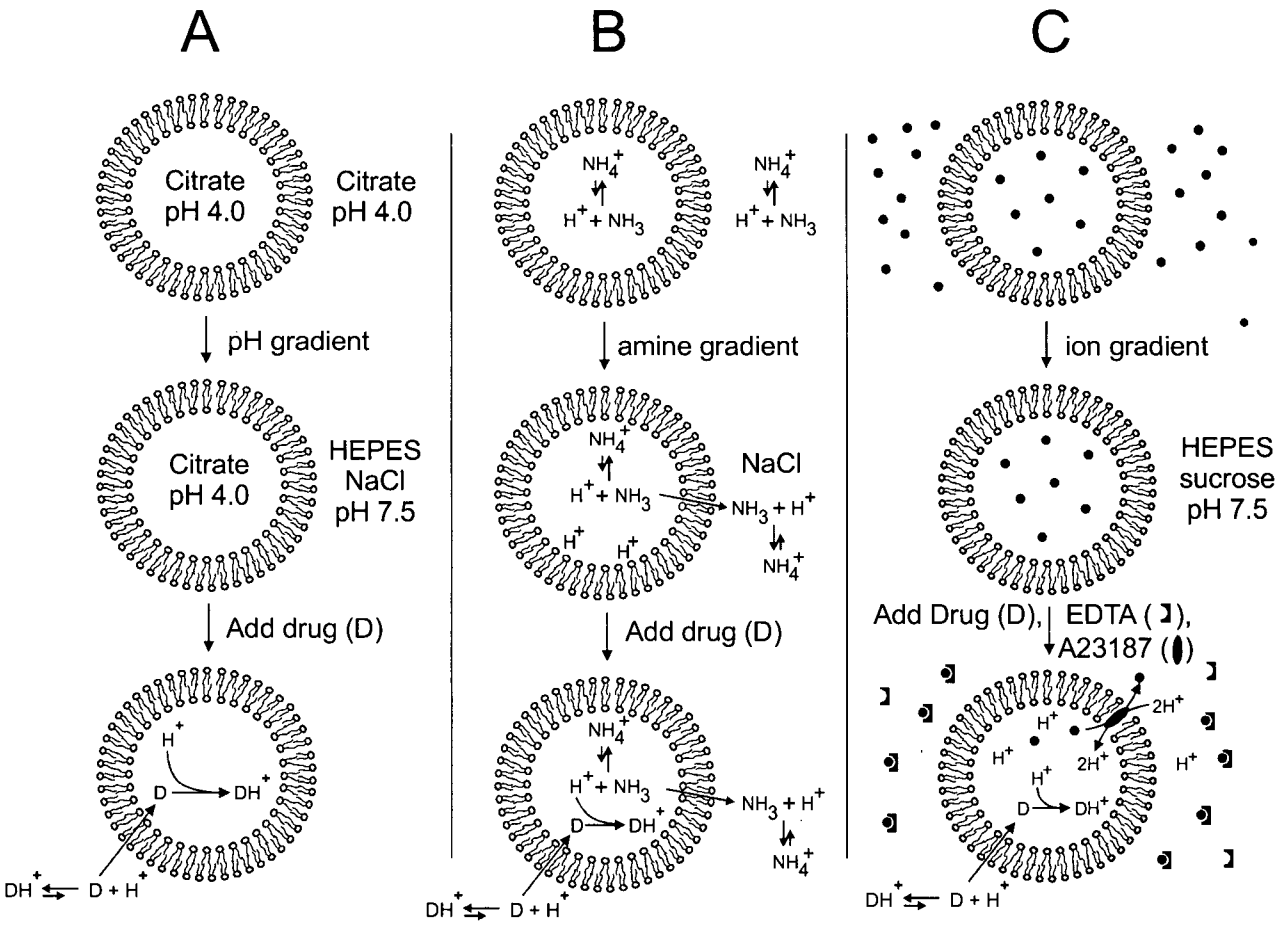


Figure1.12. Schematic of active drug loading in response to a transmembrane pH gradient. Panel A represents a pH gradient generated via a buffer exchange, panel B via an amine gradient and panel C the generation of a pH gradient using an ionophore.

1.5.3 Drug precipitation

With active encapsulation techniques allowing for greater encapsulation efficiencies and higher drug-to-lipid ratios, the internal concentration of an encapsulated drug can be greater than its maximum solubility in solution. This high efficiency of encapsulation and increased drug-to-lipid ratio has lead to the precipitation of certain drugs within liposomal formulations. Examples of liposomal encapsulated drugs that for precipitates are doxorubicin (Li et al. 1998), mitoxantrone (Almgren et al. 2000) and topotecan (Abraham et al. 2004a) all of which form clearly defined linear bundle-like structures.

Doxorubicin's solubility in water is less than 60mM (Li et al. 1998). At a drug-to-lipid ratio of 0.5 (wt/wt) in 100nm vesicles, doxorubicin can reach concentrations greater than 600mM. Li and coworkers have extensively investigated the nature of doxorubicin precipitates within liposomes and have proposed that the drug forms fibers composed of stacked doxorubicin molecules (Li et al. 1998). These fibers are grouped into hexagonal arranged bundles giving the appearance of repeating striations (Li et al. 1998). Other examples of drugs that can form precipitates when formulated in liposomes are the vinca alkaloids (vincristine and vinorelbine), which tend to form amorphous precipitates (Semple et al. 2005; Zhigaltsev et al. 2005; Johnston et al. 2006).

The precipitation of encapsulated drug has also been proposed as the mechanism by which increasing drug-to-lipid ratios leads to increased drug retention for liposomal formulations of drugs such as topotecan, vincristine and vinorelbine (Abraham et al. 2004b; Semple et al. 2005; Zhigaltsev et al. 2005; Johnston et al. 2006). This concept is further discussed in greater detail in Chapters 2 and 3 and it is evident that inducing drug precipitation is a powerful technique for prolonging drug release.

1.6 *In vivo* fate of liposomes

For the effective use of liposomes as *in vivo* drug delivery systems, an understanding of the *in vivo* fate of liposomes and their interactions with components of the immune system, circulation and accumulation at sites of disease is critical.

1.6.1 Liposomal interactions with blood components

Liposome based drug delivery system interactions with blood components are critical to their pharmacokinetic behavior and ability to retain the encapsulated drug. Drug leakage arises from the breakdown of a transmembrane pH gradient and/or destabilization of the liposomal membrane. It has been demonstrated that high-density lipoproteins (HDL) interact with liposomes, resulting in liposome lipid transfer to serum lipoproteins, potentially through the actions of phospholipid transfer protein (Krupp et al. 1976; Scherphof et al. 1978; Chobanian et al. 1979; Tall and Green 1981). This process of lipid transfer as well as the interaction of the ApoA-I protein (a component of HDL) can result in the destabilization of the liposomal membrane and encapsulated solute leakage (Klausner et al. 1985).

In addition to destabilization caused by liposomal interaction with HDL, it has also been demonstrated that interactions with complement components can result in liposome destabilization and release of encapsulated solute (Malinski and Nelsestuen 1989; Chonn et al. 1991). Chonn and coworkers have demonstrated that liposomes that are neutrally charged do not bind complement, regardless of the species used to formulate the liposomal membrane (Chonn et al. 1991; Devine et al. 1994). Liposomes that carry a surface charge do activate complement in human serum, negatively charged liposomes activate complement through a immunoglobulin independent (Marjan et al. 1994) classical pathway, where positively charged liposomes activate

complement through the alternative pathway (Shin et al. 1978; Chonn et al. 1991). It is believed that insertion of complement components into the liposomal membrane results in the formation of a 10nm diameter pore-like membrane attack complex (MAC), resulting in solute leakage (Malinski and Nelsestuen 1989; Chonn et al. 1991). Studies have also shown that incorporation of complement into liposomal membranes may also result in the loss of phospholipid components, further destabilizing the liposomal delivery system (Shin et al. 1978)

Not only do components of the blood cause destabilization of the liposome resulting in encapsulated drug leakage, they can also function as opsonins, playing a critical role in liposomal clearance (Patel 1992a; Patel 1992b). Opsonized liposomes are cleared by the free and fixed macrophages of the reticular endothelial system (RES). RES uptake depends on the recognition of the surface bound opsonin by macrophages (Coleman 1986) and the clearance rate is dependent on the total amount of bound protein (Chonn et al. 1991; Chonn et al. 1992). Opsonins can include complement components (C3 fragments – C3B, iC3b), immunoglobulins, β2-glygoprotien I and dysopsonins (Ishida et al. 2002). Opsonins can either be surface bound or interacting with the hydrophobic region of the liposomal membrane. Non-opsonic blood proteins may also play a role in marking circulating liposomes for clearance, with interactions with the liposomal membrane inducing conformational changes resulting in their recognition by phagocytic cell receptors or functioning as ligands for circulating opsonins.

The most prominent protein opsonin associated with liposomes is a 50kDa protein identified by Chonn and coworkers (Chonn et al. 1992). Interestingly this 50kDa protein has similar electrophoretic mobility as that of apolipoprotein H, which has been

shown to function as a cofactor for antiphospholipid antibody binding (Galli et al. 1990; McNeil et al. 1990)

1.6.2 Liposomes and the immune system

The immune system is comprised of two distinct components, humoral and cellular based immunity (Goldsby et al. 2000). The cellular immune response is non-specific and is mediated by cytotoxic T cells that are stimulated by antigens presented on cell surfaces when complexed with MHC class I molecules. The actions of cytotoxic T cells and other cytotoxic cell systems (natural killer cells and antibody mediated cell cytotoxicity) is primarily directed against virally infected cells, parasites and cells displaying cancer specific antigens. The humoral immune system is the component of the immune system responsible for the generation of antibodies that are specific to epitopes on a foreign antigen. Generation of antibodies is through the cooperation between 3 types of immune cells, antigen presenting cells (macrophages and dendritic cells), helper T-cells (CD4+) and B-cells.

Liposomes generally elicit a poor immune response, however it has been demonstrated that liposomes may enhance both a cellular and humoral immune response when they are coated with protein or contain genetic material. Additionally, studies have demonstrated that liposomes coated with polyethylene glycol demonstrate enhanced clearance on repeated dosing (Dams et al. 2000; Ishida et al. 2003a; Ishida et al. 2003b). Cheng and coworkers have shown that PEG conjugated proteins are capable of eliciting the generation of IgM antibodies specific to PEG, resulting in accelerated conjugate clearance (Cheng et al. 1999). In a similar fashion, IgM anti-PEG antibodies may be responsible for the enhanced clearance of PEGylated liposomes on repeated doses. Research by others suggested that the accelerated clearance is due to the opsoniztion of the PEGylated liposomes by a heat-labile serum factor or factors with

clearance facilitated by hepatosplenic macrophages (Laverman et al. 2001). Although the mechanism(s) for the accelerated clearance of PEGylated liposomes upon repeated injections remains unclear, the fact that it occurs at all possess a potential problem for the use of PEGylated liposomal drug delivery systems.

1.6.3 Interactions with reticular endothelial system

The reticular endothelial system is responsible for removing foreign material from the circulation and is made up of macrophages resident in the liver, spleen, bone marrow and lungs (also referred to as the first pass organs) as well as circulating monocytes (Goldsby et al. 2000). The majority of circulating liposomes are cleared by specialized Kuppffer cells (Roerdink et al. 1981) (Moghimi and Hunter 2001) that lie within the sinusoids of the liver and function to phagocytose and detoxify foreign material from the circulation. Liposome clearance, to a lesser extent, has also been observed in the spleen. Clearance by Kuppffer cells can be beneficial if liposomal drug delivery systems are being used to treat bacterial or intracellular parasitic infections that involve phagocytic cells, such as *Leishmania donovani* the causative agent of visceral leishmaniasis, (Mukherjee et al. 2004). However, if liposomal delivery systems are being used to treat systemic diseases or distal tumor sites, uptake and clearance by the RES remains a major obstacle.

1.6.4 Long circulating liposomal systems

It is essential that liposomal anticancer drug delivery systems designed for systemic treatment are long circulating systems that avoid uptake and clearance by organs of the RES. The primary factors that influence liposome circulation times are size, dose, and lipid composition (Juliano and Stamp 1975; Poste et al. 1984; Semple et al. 1998).

1.6.4.1 Liposome size

Research has shown that large liposomes (>1000 nm), such as MLVs, are cleared more rapidly (4X) than smaller liposomes (<200nm), such as LUVs, even if both liposomal formulations have the identical lipid composition and dose (Juliano and Stamp 1975). The accelerated clearance of large liposomes has been partially attributed to the mechanical trapping by the spleen and lung. Some studies have also suggested that larger liposomes are prone to higher degrees of opsonization and are more readily cleared by liver phagocytes (Ishida et al. 2002). Although smaller liposomes demonstrate longer circulation lifetimes, very small liposomes (SUVs) are actually cleared faster than LUVs. As mentioned previously (1.3.2) SUVs have a degree of membrane curvature that may facilitate opsonin binding and ultimately enhanced clearance. Their small size may also facilitate SUV extravasation and entry into small pores of liver sinusoids thereby facilitating binding and uptake by liver hepatocytes (Roerdink et al. 1981).

1.6.4.2 Lipid dose

Lipid dose also has a critical role in the pharmacokinetic behavior of liposomal delivery systems *in vivo* (Semple et al. 1998). Studies have shown that increased lipid doses lead to increased circulation lifetimes, when measured in terms of the percent of initial dose remaining at a specific time point (Oja et al. 1996). This phenomenon may be a result of larger lipid doses depleting opsonins responsible for marking liposomes for clearance (Oja et al. 1996) or the saturation of free and fixed macrophages of the RES. Using this knowledge it may be possible to pre-dose with empty liposomes, thereby depleting the circulating blood opsonins responsible for marking the injected drug loaded liposomes for clearance (Figure 1.13).

1.6.4.3 Lipid composition

Finally, lipid composition has a major influence on the pharmacokinetics of the liposomal drug carrier. The surface charge the liposome carries, due to its lipid composition, has a dramatic affect on the level of opsonization (Hernandez-Caselles et al. 1993; Oku et al. 1996). Studies have demonstrated that liposomes that have negatively charged membrane surfaces accumulate opsonins faster (Hernandez-Caselles et al. 1993) and are cleared more rapidly than those that have a neutral surface charge (Chonn et al. 1992). Studies using MLVs containing 30 mol% of negatively charged phosphatidylserines (PS) demonstrated a 25 fold increase in macrophage uptake, compared to MLVs composed entirely of phosphatidylcholines (Schroit et al. 1986). Evidence has also shown that positively charged liposomes behave in a similar manner to negatively charged ones, with respect to the clearance and opsonin binding. Senior and coworkers established that increasing the cationic content of SUVs with a variety of positively charged lipid increased plasma protein interactions and plasma turbidity (Senior et al. 1991).

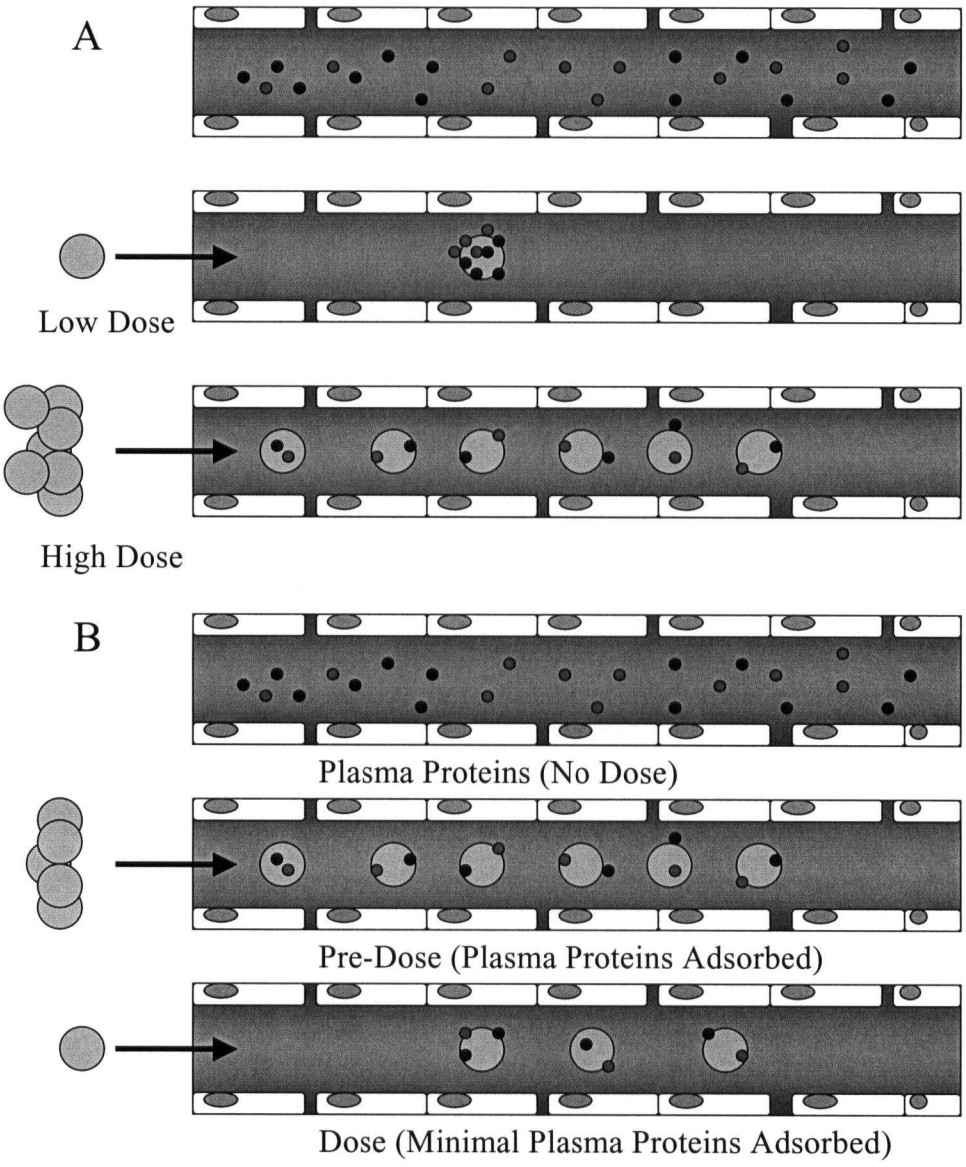


Figure 1.13. (A) Depletion of plasma opsonins. (B) Influence of predosing on plasma protein binding to a subsequent lipid dose.

Opsonin binding and clearance by the RES is also influenced by the length and degree of saturation of the acyl chain of lipids that make up the membrane (Senior 1987). Generally, liposomes containing lipids with longer saturated acyl chains will have bilayers that are more tightly packed and rigid (Gregoriadis and Senior 1982; Senior and Gregoriadis 1982a; Senior and Gregoriadis 1982b). These rigid membranes resist protein insertion, ultimately leading to increased circulation lifetimes. However, if liposomes are composed entirely of lipids containing long, saturated acyl chains, the gel to liquid phase transition temperature maybe above the physiological temperature, which may result in packing defects being present on the surface of the liposome in the circulation (Chonn et al. 1994). These packing defects may be target sites for opsonin insertion and can result in rapid clearance (Chonn et al. 1994). As mentioned in section 1.2.3, inclusion of cholesterol can increase the circulation time of these systems through its ability to remove packing defects in gel state systems (Kirby et al. 1980a; Kirby et al. 1980b; Hunt 1982).

As opsonization is the critical first step to liposome clearance *in vivo*, strategies have been developed to reduce opsonin binding, thereby increasing circulation lifetimes, drug retention and ultimately antitumor efficacy. Initially improvements in both circulation and efficacy have been observed when long chain saturated phosphatidycholines or sphingomyelins and cholesterol are use to formulate delivery systems for anticancer drugs. Additional strategies have been to include a poly (ethylene-glycol) conjugated lipids (PEG-lipid).

1.6.4.4 PEGylated liposomal systems

Initial attempts to further enhance liposomal circulation lifetimes involved the addition of ganglioside G_{M1} to liposomes at 10 mol% (Allen and Chong 1987). The rationale for including G_{M1} was that liposomes would more readily resemble cell

surfaces, due to the polysacaride component of the lipid, and would have reduced opsonization. This was the case and increased circulation lifetimes and blood-to-RES ratios were observed for liposomes that were formulated with G_{M1} (Allen and Chong 1987). Chonn and coworkers attributed the enhanced circulation lifetimes to decreased opsonin binding (Chonn et al. 1992). Attempts to utilize other gangliosides failed to produce similar results and debate over the function of G_{M1} in liposomal membranes ensued (Allen et al. 1994). One theory, put forward by Park and Huang, suggests that G_{M1} binds a specific set of dysopsonins that could potentially label the liposomes as self or G_{M1} could prevent further binding of proteins that function as ligands for clearance-promoting opsonins (Park and Huang 1993). Regardless of the exact role of G_{M1} in promoting enhanced liposome circulation lifetimes, the use of G_{M1} is limited as the cost is prohibitive, however these results have encouraged the development of liposomes that demonstrate enhanced pharmacokinetic profiles through modifications to their surface.

A wealth of data clearly indicates that particles that are covered with polyethylene glycol or other polymeric structures display *in vivo* circulation lifetimes that surpass those of uncoated particles (Allen 1994; Francis et al. 1996). The first liposomes to have PEG modified surfaces contained PEG conjugated to phosphatidylethanolamine and displayed substantially improved circulation lifetimes (Harris et al. 2001; Moghimi and Szebeni 2003). PEG is a flexible polymer of repeating ethylene glycol units and is believed to enhance circulation lifetimes by covering the liposome in a steric barrier thus preventing protein binding/interaction and reducing recognition of cells of the RES (Figure 1.14). Clinically available PEGylated formulations of doxorubicin (Doxil/Caylex) have shown much success and are now used in the clinic to treat ovarian cancer (Rose 2005).

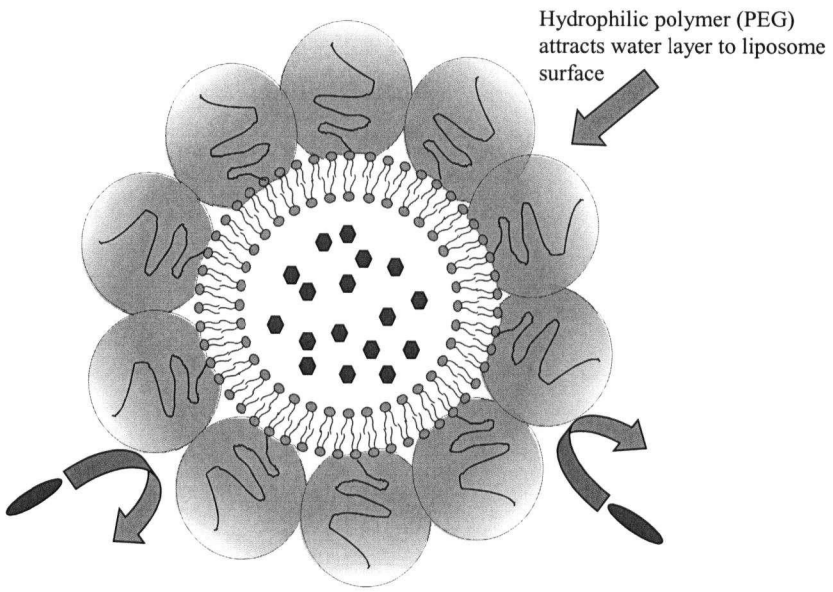


Figure 1.14. Long circulating PEGylated liposome (not to scale) containing encapsulated drug. The water layer functions as a barrier to opsonin binding enhancing circulation lifetime and promoted membrane integrity by preventing phospholipid exchange with other membranes.

1.6.5 Tumor accumulation of liposomes

In addition to liposomal delivery systems enhancing efficacy by reducing toxicity and extending circulation lifetimes of antineoplastic therapeutic agents, liposomal delivery systems also allow the targeting of these agents, either actively or passively, to sites of disease.

1.6.5.1 Passive tumor accumulation of liposomes

Liposomal delivery systems allow for passive accumulation or targeting of anticancer drugs to sites of inflammation or disease (tumor sites) due to the hyperpermeable vasculature present (Gabizon 1992). It has been observed that liposomal delivery systems allow for up to 10 times more liposomal drug to accumulate at a tumor site than an equivalent dose of the free drug (Krishna et al. 2001). In normal vasculature, the permeability of blood capillaries is tightly controlled, with small

molecules able to freely pass through the vessel walls whereas macromolecules, such as liposomes, remain in the circulation (Baldwin 2000). At sites of inflammation, the vasculature becomes more permeable in response of proinflammatory cytokines such as IL6, and allows for the extravasation of macromolecules such as liposomes (Kohn et al. 1992). The permeability of tumor vasculature, however, is not well controlled and allows the extravasation of macromolecules due to defects in the neovasculature such as fenestrations, widened interendothelial junctions and blood channels that have little or no endothelial linings (Dvorak et al. 1988; Kohn et al. 1992). Yuan and coworkers demonstrated that these pores are permeable to liposomes of up to 400nm in diameter and have a cut-off between 400-600nm (Yuan et al. 1995). The hyperpermeable neo-vasculature in tumors is due to tumor cells secreting growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and thymidine phosphorylase (TP) in response to their hypoxic environments (Shweiki et al. 1992; Fox 1997; Sivridis et al. 2003). VEGF is a proangiogenic cytokine and over is expressed in many malignancies (De et al. 2005), it allows for the leakage of macromolecules and nutrients into a growing tumor mass and also results in the formation of provisional stroma, ultimately causing angiogenesis and the in-growth of new blood vessels into the tumor mass (Blood and Zetter 1990).

In addition to having permeable vasculature, solid tumors also have increased microvessel density relative to normal tissues and the degree of microvessel density is predictive of patient survival (Sivridis et al. 2003). Tumor vasculature is structurally defective with poorly defined artery/vein structure containing many dead ends, loops and poor drainage in comparison to other tissues, thereby allowing increased duration of exposure and accumulation of circulating liposomes in the tumor mass.

1.6.5.2 Active tumor targeting

Active targeting involves the use of specific ligands that are attached to the external surface of the liposomal delivery vehicle allowing for the targeting of specific cell populations. These targeting ligands can include antibodies, antibody fragments, peptides or carbohydrates (Medina et al. 2004). Conventional liposomes that are coated with targeting antibodies or antibody fragments, attached covalently to a short anchor (Figure 1.15A), are cleared more rapidly by cells of the RES (Maruyama 2002), however as mentioned above, coating these immuno-liposomes with PEG enhances their circulation lifetimes (section 1.5.4). These PEG-lipid derivatives can also function as an anchor for the targeting ligand, and systems that contain this type of anchor have demonstrated the highest levels of target binding (Figure 1.15B) (Maruyama 2002).

Targeted liposomes have shown enhanced efficacy in a number of *in vivo* tumor xenograft models. Park and coworkers demonstrated that PEGyated liposomal doxorubicin with anti-HER2 monoclonal antibody Fab' fragments or short chains of the variable region of the antibody (scFVs) demonstrated enhanced efficacy in a number of tumor models that overexpress HER2, while maintaining the reduced toxicity associated with liposomal delivery systems (Park et al. 2002). It has also been demonstrated by Sugano and coworkers that liposomal doxorubicin targeted using Fab' fragments of monoclonal antibodies directed against the human β1 integrins reduced metastases and tumor growth in orthotopically established human lung tumor xenografts in severe combined immunodeficient (SCID) mice (Sugano et al. 2000).

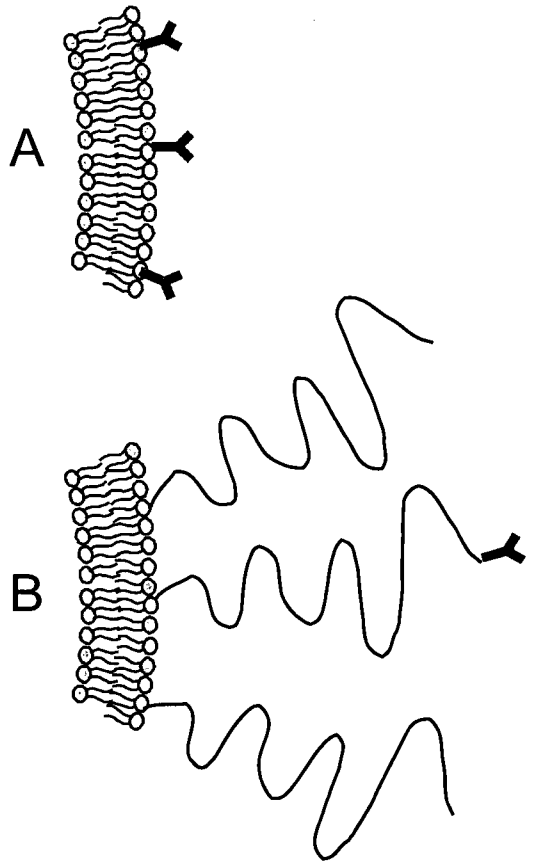


Figure 1.15. Schematic of antibodies immobilized on the surface of liposomes. (A) Antibodies covalently linked, via short anchor, to a phospholipid. (B) Antibodies attached to the distal end of a PEG molecule, referred to as pendant-type PEG-immunoliposome.

1.7 Objectives

The objective of this thesis is to develop methods to regulate the half-life for drug release by liposomal formulations of antineoplastic agents, such as vincristine, with an ultimate aim of producing formulations with optimized antitumor efficacy.

In the second Chapter of the thesis, it was discovered that the D/L ratios for liposomal vincristine dramatically influenced the half-life for drug release as well as the efficacy of liposomal formulations of vincristine in a human breast cancer xenograft model. Release rates for vincristine are so long that decreases in efficacy are observed thereby allowing therapeutically optimized rates of drug release to be determined.

In Chapter 3 an explanation for this phenomenon, involving the precipitation of encapsulated drug is presented. This chapter also details the potential of the drug-to-lipid ratio method for regulating drug release rates for other liposomal drugs.

Experiments detailed in the fourth chapter show that liposomal vincristine formulations containing dihydrosphingomyelin (DHSM) display improved drug retention *in vitro* and *in vivo* compared to delivery systems formulated with egg sphingomyelin. Also, it is demonstrated that *in vivo* circulation lifetimes of liposomal formulations containing DHSM are comparable to "stealth" systems that posses a poly (ethelene-glycol) coating.

Chapter 2: Therapeutically Optimized Rates of Drug Release can be Achieved by Varying the Drug-to-lipid Ratio in Liposomal Vincristine Formulations

2.1 INTRODUCTION

Liposomal formulations of anticancer drugs can result in increased activity and reduced toxicity compared to the free drug (Balazsovits et al. 1989; Oh et al. 1995; Lim et al. 1997; Grunaug et al. 1998; Sadava et al. 2002). The benefits of liposomal encapsulation are, however, dependent on the rates of drug release from the liposomes, leading to the need to develop formulations that exhibit therapeutically optimized drug release rates. Such therapeutically optimized release rates have not been developed for any liposomal formulation of any drug to date. The major focus of research efforts has been on developing liposomal formulations that are small (diameter ~ 100 nm) and long circulating in order to take advantage of the "enhanced penetration and retention" (EPR) effect (Yuan et al. 1995) leading to preferential accumulation of the liposomes at tumor sites and sites and infection and inflammation due to the hyperpermeable nature of the vasculature in these regions (Drummond et al. 1999). Within the context of these formulations there is a clear need to keep the drug encapsulated during the time it takes to achieve appreciable tumor accumulation of the carrier, which implies a half-time for drug release ($T_{1/2}$) of hours or longer. However, there is an equally clear need for the drug to be released on arrival at the tumor site at rates sufficient to provide enough bio-available drug to inhibit tumor growth.

Previous work has shown that the efficacy of liposomal formulations of cell cycle-specific drugs such as vincristine is particularly sensitive to drug release rates. For example, in studies employing the L1210 and P388 murine leukemia models, or the

A431 human squamous cell carcinoma model, formulations of vincristine with progressively slower release rates converted the drug from being essentially inactive to one that produced 100% cures in mice (Mayer et al. 1993; Webb et al. 1995). This dependence on release rates is consistent with the fact that prolonged exposure of cells to cell cycle-specific agents results in greater cell killing *in vitro* and enhanced antitumor activity *in vivo* (Bruce et al. 1969; Jackson and Bender 1979; Horton et al. 1988; Burris et al. 1992; Gomi et al. 1992; Georgiadis et al. 1997). Thus prolonged vincristine release from liposomes accumulated at the tumor site and the resulting extended tumor cell exposure would be expected to result in enhanced antitumor activity. However, formulations with release rates so slow that efficacy is compromised have not yet been achieved, preventing identification of truly optimized release rates.

Considerable efforts have been made to develop liposomal vincristine formulations with improved retention and efficacy properties. These include increasing acyl chain length and saturation (Boman et al. 1993), using sphingomyelin (SM) instead of phosphatidylcholine (Mayer et al. 1989), and using lower internal pH environments in the liposome (Tomita et al. 1989; Ning et al. 1994; Zellmer and Cevc 1996). An SM/Chol liposome formulation (Webb et al. 1995) was identified as having the best retention properties achievable ($T_{1/2}$ *in vivo* of ~ 24h) using this technology, and this formulation has shown enhanced efficacy profiles both in animal models and in humans (Mayer et al. 1993; Boman et al. 1996; Sarris et al. 2000; Guthlein et al. 2002; Leonetti et al. 2004).

In recent work it has been shown that the release rates of vincristine and other drugs from liposomes are highly sensitive to the drug-to-lipid (D/L) ratio employed in the formulation (Zhigaltsev et al. 2005) . In the case of vincristine an increase in the D/L ratio from 0.1 to 0.3 (wt/wt) results in an increase in the half-time for drug release of

more than a factor of two. Here we explore the possibility that, at higher D/L values achievable through the use of an ionophore-based drug accumulation technique (Fenske et al. 1998), rates of drug release can be so slow that a therapeutically optimized release rate can be identified. In addition, studies to determine the mechanism whereby increased D/L values lead to improved retention have also been performed; with particular attention paid to the possibility that high D/L values lead to precipitation of the drug in the liposome interior.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Egg sphingomyelin (ESM) was purchased from Northern Lipids (Vancouver, BC, Canada) and was used without further purification. Vincristine sulfate was obtained from Fine Chemicals (Cape Town, South Africa). Cholesterol (Chol) was obtained from Sigma (St. Louis, MO, USA) and used without further purification. [^{3}H]-Cholesterylhexadecyl ether (CHE) was obtained from Perkin Elmer Life Sciences (Boston, MA, USA). [^{14}C]- vincristine sulfate was obtained from Chemsyn Laboratories (Lenexa, Ka, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

2.2.2 Liposome preparation

Lipids (ESM and Chol) and trace amounts of [^{3}H]-CHE were co-dissolved at appropriate molar ratios (55/45 mol%) in ethanol. Multilamellar vesicle (MLV) suspensions were generated after the addition of a 300 mM aqueous solution of magnesium sulfate, yielding a final ethanol concentration of 10% (v/v). Large unilamellar vesicles (LUVs) were generated by extrusion of MLVs through two stacked Nuclepore polycarbonate filters with a pore size of 100nm (10 passes) using an extrusion device obtained from Northern Lipids (Vancouver, BC, Canada) (M.J. Hope 1985; Mayer et al. 1986). A transmembrane ion gradient was established through the removal of the external 300 mM magnesium sulfate by dialysis against SEH loading buffer (300 mM sucrose, 3mM EDTA, 20mM HEPES, pH 7.4). The mean diameter of LUVs was determined by dynamic light scattering using a NICOMP 370 particle sizer (Nicomp Particle Sizing Inc., Santa Barbara, CA) and found to be 110 $\pm$ 25 nm. Phospholipid concentrations were determined using established techniques (Fiske and Subbarow

1925) and the specific activity of the liposomes was determined using a Beckman LS3801 scintillation counter (Fullerton, CA, USA).

2.2.3 Vincristine encapsulation in liposomes

Vincristine was encapsulated into LUVs using an ionophore-mediated drug loading procedure, as described previously (Fenske et al. 1998). Briefly, vincristine sulfate and trace amounts of [^{14}C]-vincristine sulfate were added to LUVs (5 mM final lipid concentration) at appropriate drug-to-lipid ratios (wt/wt), and subsequently pre-incubated at 65 °C prior to the addition of the calcium ionophore A23187. The LUV/drug/ionophore mixture was then incubated at 65°C for 90 minutes to provide optimal drug loading conditions. Non-encapsulated vincristine was removed using dialysis against SEH loading buffer (Fenske et al. 1998) prior to quantification of liposome entrapped drug using dual channel counting on a Beckman LS 3801 scintillation counter. Formulations used for *in vivo* studies received additional dialysis into 300mM sucrose prior to quantification of liposome-entrapped drug.

2.2.4 *In vitro* release of vincristine

In vitro drug release assays were performed to make quantitative comparisons of drug leakage between formulations of varying drug-to-lipid ratios. Initially, *in vitro* release assays for liposomal vincristine were conducted using ammonium chloride to degrade the pH gradient (Maurer et al. 1998). Drug loaded vesicles were diluted with release buffer (2 mM ammonium chloride, 300 mM sucrose, 20 mM HEPES, 3 mM EDTA, pH 7.4) to a lipid concentration of 1.25 mM. The diluted liposomal drug was then placed into dialysis tubing (12-14K M.Wt. cut off) and dialyzed against release buffer at 50°C. This temperature was chosen to provide an optimal and convenient *in vitro* drug leakage rate, generally 20-30% after 60 minutes for a drug-to-lipid ratio of 0.05 (wt/wt).

Leakage of vincristine from the loaded LUVs was assayed by the removal of aliquots for spin column analysis and quantification using dual label liquid scintillation counting (Mayer et al. 1990; Fenske et al. 1998). Vincristine release from liposomes was also assessed using fetal bovine serum. Liposomal drug in SEH loading buffer was added to fetal bovine serum (FBS; Invitrogen, San Diego, CA) to give a final total lipid concentration of 1.25 mM and a final FBS concentration of 50% (v/v). Samples were incubated at 50°C. Vincristine release was quantified over time through the removal of aliquots for spin column analysis and dual label liquid scintillation counting. The halftimes for drug release ($T_{1/2}$) were calculated from exponential best fits to the release profiles as the time at which the internal drug concentration was half the initial concentration. Error bars were calculated from the 95% confidence intervals associated with the exponential best fits.

2.2.5 Mice

Female, 6-8 week old outbred ICR mice were obtained from Harlan (Indianapolis, IN) and were used for pharmacokinetic and drug leakage studies. Female, 6-8 week old, athymic Crl:CD-1®- nuBR mice were obtained from Charles River Laboratories (Quebec, Canada) and used for the antitumor efficacy studies. All mice were quarantined for at least two weeks prior to use. Immunocompromised animals were maintained under sterile conditions, with a controlled temperature (22 ± 1°C) and humidity (60 ± 10%) environment. Lighting was maintained on automatic 12 h light/dark cycles. Animal studies were conducted in compliance with the guidelines established by the Canadian Council on Animal Care (CCAC).

2.2.6 *In vivo* pharmacokinetics

Vincristine loaded LUVs were prepared at various drug-to-lipid (D/L) ratios and contained 300mM sucrose as the external buffer. In most instances, drug and lipid concentrations were adjusted to 0.2 mg/ml vincristine and 4 mg/ml total lipid, resulting in drug and lipid doses of 2 and 40 mg/kg, respectively, in mice. Empty ESM/Chol liposomes were included in liposomal vincristine formulations prepared with higher D/L ratios to ensure that mice were injected with an equivalent total lipid dose. In some instances, formulations with different D/L ratios were injected into mice at equivalent lipid doses (no empty liposomes) to determine whether formulations with high drug-to-lipid ratios exhibited altered pharmacokinetics. Mice were injected via a lateral tail vein and, at appropriate time points, were anesthetized (ketamine/xylazine) and blood was collected via cardiac puncture into EDTA Microtainer tubers. Blood was then centrifuged at 400×g for 15 minutes and plasma was collected and de-colorized for lipid and drug determination by dual label liquid scintillation counting as described previously (Fenske et al. 1998).

2.2.7 *In vivo* efficacy studies

MX-1 human mammary adenocarcinoma was obtained from the Division of Cancer Treatment and Diagnosis (DCTD) Tumor Repository (Frederick, MD), maintained by serial passage *in vivo* and implanted by trocar into the dorsal flank of nude mice. Experiments were performed between the fourth and tenth serial passage in nude mice. Treatments were initiated in all models when tumor volumes were 200-300 mm^3 (14 days post tumor implantation). Animals were dosed according to body weight (10 ml/kg body weight) and received a single intravenous (i.v.) injection of one of several formulations of liposomal vincristine. Liposomal vincristine formulations were diluted to achieve a total lipid dose of 66 mg/kg and a drug dose of 1.5 mg/kg body

weight. As with the pharmacokinetic studies, empty liposomes were included, as appropriate to ensure mice received equivalent total lipid doses for all drug-to-lipid ratios. Tumors were measured at least three times per week with calipers and tumor volume (mm^3) was calculated using the formula: (length x $width^2$)/2, where width was the smaller of the two perpendicular measurements (Fiebig and Burger 2002). Tumor growth delay (T-C); the median difference in time (days) for treated and control tumors to reach 1000 mm^3, was evaluated for each treatment group.

2.2.8 Cryo-transmission electron microscopy

Cryogenic transmission electron microscopy (cryo-TEM) was performed on empty and drug-loaded ESM/Chol liposomes using a Zeiss EM 902A Transmission Electron Microscope (LEO Electron Microscopy, Oberkochen, Germany) operated at 80kV in the zero loss bright-field mode. Digital images were recorded under low dose conditions with a BioVision Pro-SM Slow Scan CCD camera (Proscan GmbH, Scheuring, Germany) and analySIS software (Soft Imaging System, GmbH, Münster, Germany). In order to visualize maximum detail, an underfocus of 1-2 μm was used to enhance the image contrast. Sample preparation was performed at 25°C and approximately 99% relative humidity within a climate chamber. A small drop (ca 2 ml) of sample was deposited on a copper grid covered with a perforated polymer film coated with carbon on both sides. Excess liquid was removed by blotting with filter paper, leaving a thin film of the solution on the grid. Immediately after blotting, the sample was vitrified by plunging the grid into liquid ethane held at -182°C. Samples were maintained below –165°C and protected against atmospheric conditions during both transfer to the TEM and examination. Images at 100,000× total magnification were captured for each sample.

2.3 RESULTS

2.3.1 Vincristine can be loaded into liposomes to high drug-to-lipid ratios employing the ionophore loading technique

Previous work (Zhigaltsev et al. 2005) has shown that vincristine loaded into LUVs using an ionophore loading technique exhibits improved retention at higher D/L values. For example, increasing the D/L value from 0.1 to 0.3 (wt/wt) resulted in an increase in the half-time for retention by more than a factor of two. Initial work was therefore devoted to characterizing this effect further and, in particular, extending the range of D/L ratios that could be achieved. ESM/Chol (55/45 mol%) LUVs were prepared containing 300 mM $MgSO_4$ as described in Methods and incubated in the presence of increasing amounts of vincristine corresponding to D/L ratios, if all the drug was accumulated, of up to 1.5. The LUVs were then dialyzed at room temperature for 2 h to remove untrapped drug and the D/L ratio measured as described in Methods. As shown in Figure 2.1, the efficiency of the accumulation process in the presence of the ionophore A23817 decreased at the higher vincristine concentrations, however D/L ratios as high as one (wt/wt) were achieved with approximately 50% trapping efficiency at the highest external vincristine concentrations employed.

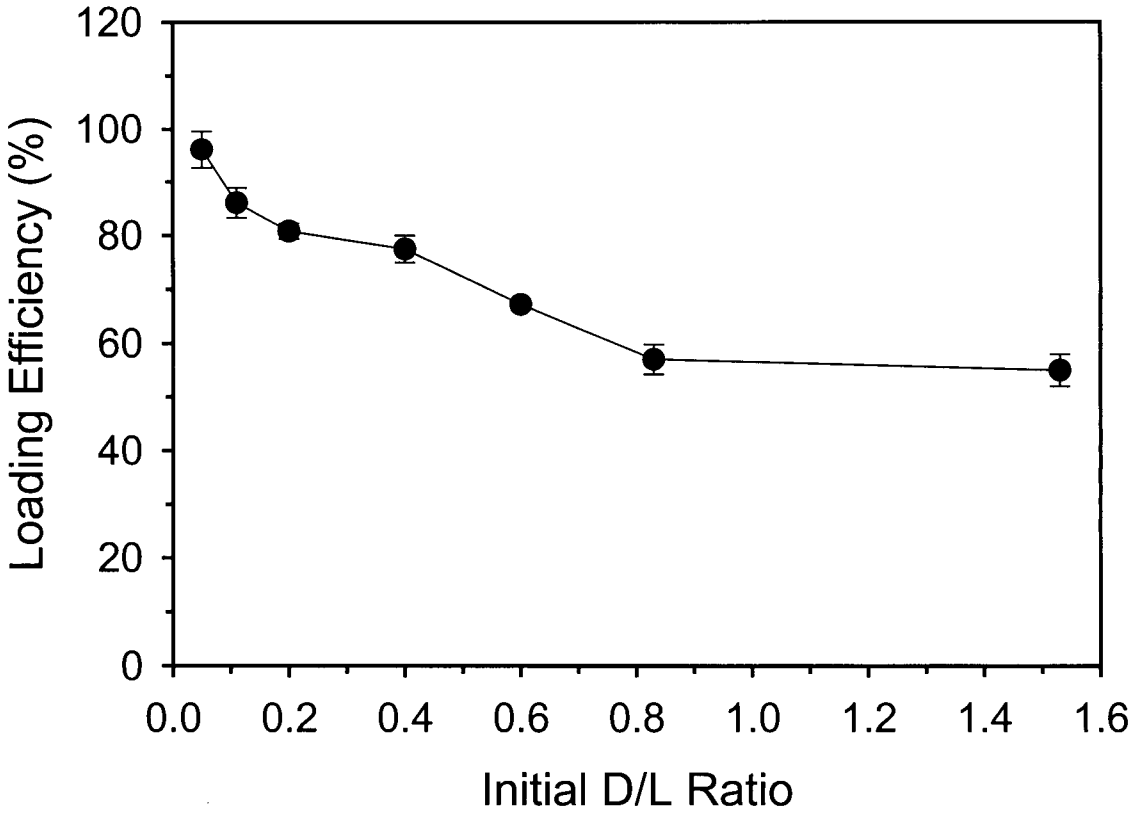


Figure 2.1. Loading efficiencies for formulations of liposomal vincristine at increasing initial drug-to-lipid ratios. Vincristine was loaded using the ionophore method, described in Methods, into ESM/Chol liposomes (55/45 mol%). Data points represent mean loading efficiencies (± standard deviations) calculated from 3 samples.

2.3.2 The half-time of vincristine release from LUV is linearly dependent on the drug-to-lipid ratio *in vitro* and *in vivo*

The next set of experiments were aimed at characterizing the release characteristics of ESM/Chol LUVs loaded to achieve D/L ratios of up to 0.6 (wt/wt). Two *in vitro* assays were employed that resulted in release rates occurring on experimentally convenient timescales. The first assay involved incubation of drug loaded LUVs in the presence of 2 mM NH_4Cl at 50°C, where the presence of the NH_4Cl raises the interior pH of the LUVs, converting more of the encapsulated drug to the neutral, membrane permeable form. The second assay involved incubation in the presence of 50% fetal bovine serum at 50°C. Increased leakage in this case arises from adsorption of serum proteins to the LUV, thus increasing the permeability of the lipid bilayer. The high temperature was employed because incubation at 37°C did not result in sufficiently rapid release characteristics. As shown in Figure 2.2, both assays showed dramatic reductions in release rates as the D/L ratio is increased, corresponding to increases in the half-time of release of more than 10-fold as the D/L ratio is increased from 0.05 to 0.6 (wt/wt).

The drug retention properties of liposomal vincristine formulations with different D/L ratios were also evaluated *in vivo*. Outbred ICR mice were injected intravenously with liposomal vincristine formulations with D/L ratios of up to 0.33 at a dose corresponding to 2 mg/kg vincristine, which is the maximum tolerated dose of the free drug (Mayer et al. 1993). Since the total lipid dose can strongly affect the pharmacokinetic properties of liposomes (Oja et al. 1996), empty SM/Chol liposomes were included with drug-loaded formulations having higher D/L ratios to ensure that mice received an equivalent lipid dose of 40 mg/kg in all situations. Blood was collected

at appropriate time points and assayed for total vincristine and total lipid. Note that free vincristine is rapidly removed the circulation with the half-time for clearance of free vincristine less than 5 min (Webb et al. 1995) and thus the total amount of vincristine in the blood overwhelmingly reflects liposome-encapsulated drug (Webb et al. 1998a). As shown in Figure 2.3, a remarkable increase in the retention properties of the LUVs is observed as the D/L ratio is increased, corresponding to an increase in $T_{1/2}$ from ~ 6 h for a D/L ratio of 0.025 to more than 60 h for a D/L ratio of 0.33.

If it is assumed that the efflux of vincristine from LUVs obeys Fick's law, which states that the efflux rate is proportional to the area of the LUV membrane and the concentration gradient of vincristine across the membrane, then it is straightforward to show that $[D_i(t)] = [D_i(o)]\exp(-kt)$, where $[D_i(t)]$ is the concentration of drug inside the LUV at time t, $[D_i(o)]$ is the interior concentration of drug at time 0 and k is the rate constant associated with the release process. It follows that $[D_i(t)]/[D_i(o)] = \exp(-kt)$ and thus that the % released over time should be independent of the initial interior drug concentration, which is in direct contradiction to the results shown in Figures 1 and 2. In an effort to understand the mechanism involved, the $T_{1/2}$ values calculated from the release data of Figures 2.2 (incubation in the presence of ammonium chloride) and 2.3 were plotted as a function of initial D/L ratio. As shown in Figure 2.4, a linear relationship is observed, which is consistent with a large proportion of the encapsulated drug being in a precipitated (non-soluble) form (see Discussion). A linear correlation between D/L ratio and the half-time of drug retention was also observed when drug leakage was induced by addition of fetal bovine serum.

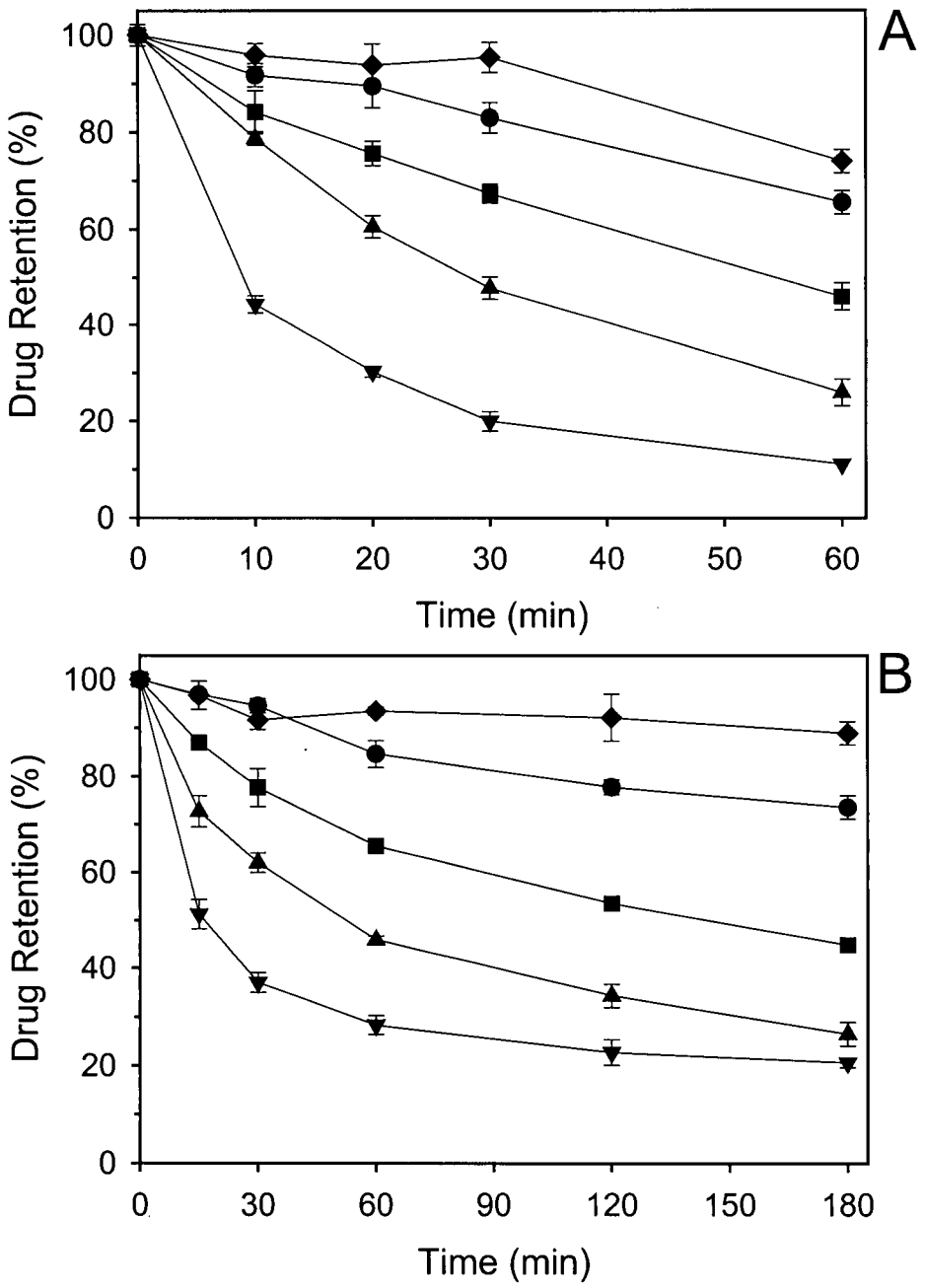


Figure 2.2. Vincristine retention in ESM/Chol (55/45, mol%) liposomes *in vitro*. Panel A: retention in 2mM ammonium chloride at 50°C; formulations had drug-to-lipid ratios (wt/wt) of 0.05 (▼), 0.1 (▲), 0.2 (■), 0.35 (●) and 0.64 (◆). Panel B: retention in 50% FBS at 50°C; formulations had drug-to-lipid ratios (wt/wt) of 0.05 (▼), 0.1 (▲), 0.2 (■), 0.4 (●) and 0.7 (◆). The lipid concentration in the release assays was 1.25 mM total lipid. Data points represent mean drug retention (± standard deviations) calculated from 3 samples and are representative of at least 3 separate experiments.

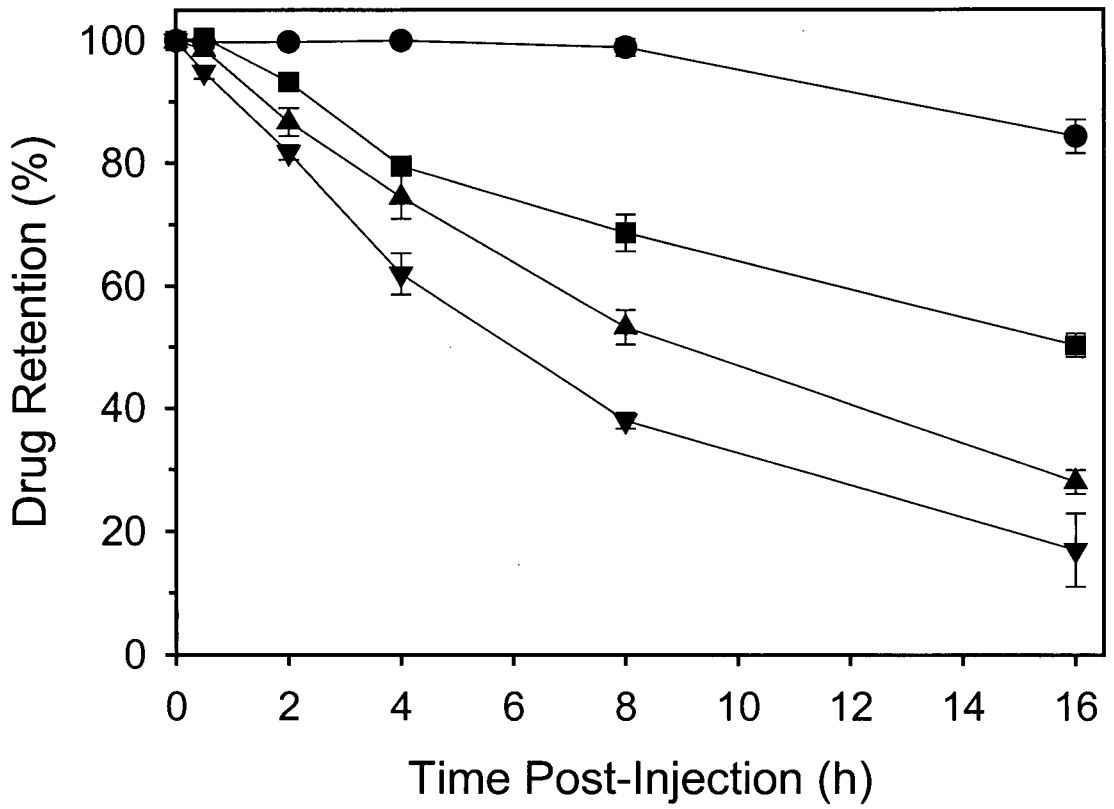


Figure 2.3. Vincristine retention of ESM/Chol (55/45, mol%) liposomes *in vivo*. ICR mice were injected intravenously with ESM/Chol formulations of vincristine having drug-to-lipid ratios (wt/wt) 0.025 (▼), 0.05 (▲), 0.1 (■) and 0.33 (●). All mice received lipid doses at 40mg/kg total lipid and vincristine at 2mg/kg drug, with the exception of the 0.025 D/L ratio sample which was dosed at 1 mg/kg vincristine. Rate constants for release were calculated to be 0.1128 ($T_{1/2}$=6.1 h), 0.079 ($T_{1/2}$=8.7 h), 0.0443 ($T_{1/2}$=15.6 h) and 0.0106 h^{-1} ($T_{1/2}$=65 h) for D/L ratios of 0.025, 0.05, 0.1 and 0.33 respectively. Data points represent mean values (± standard deviations) calculated from 3 mice.

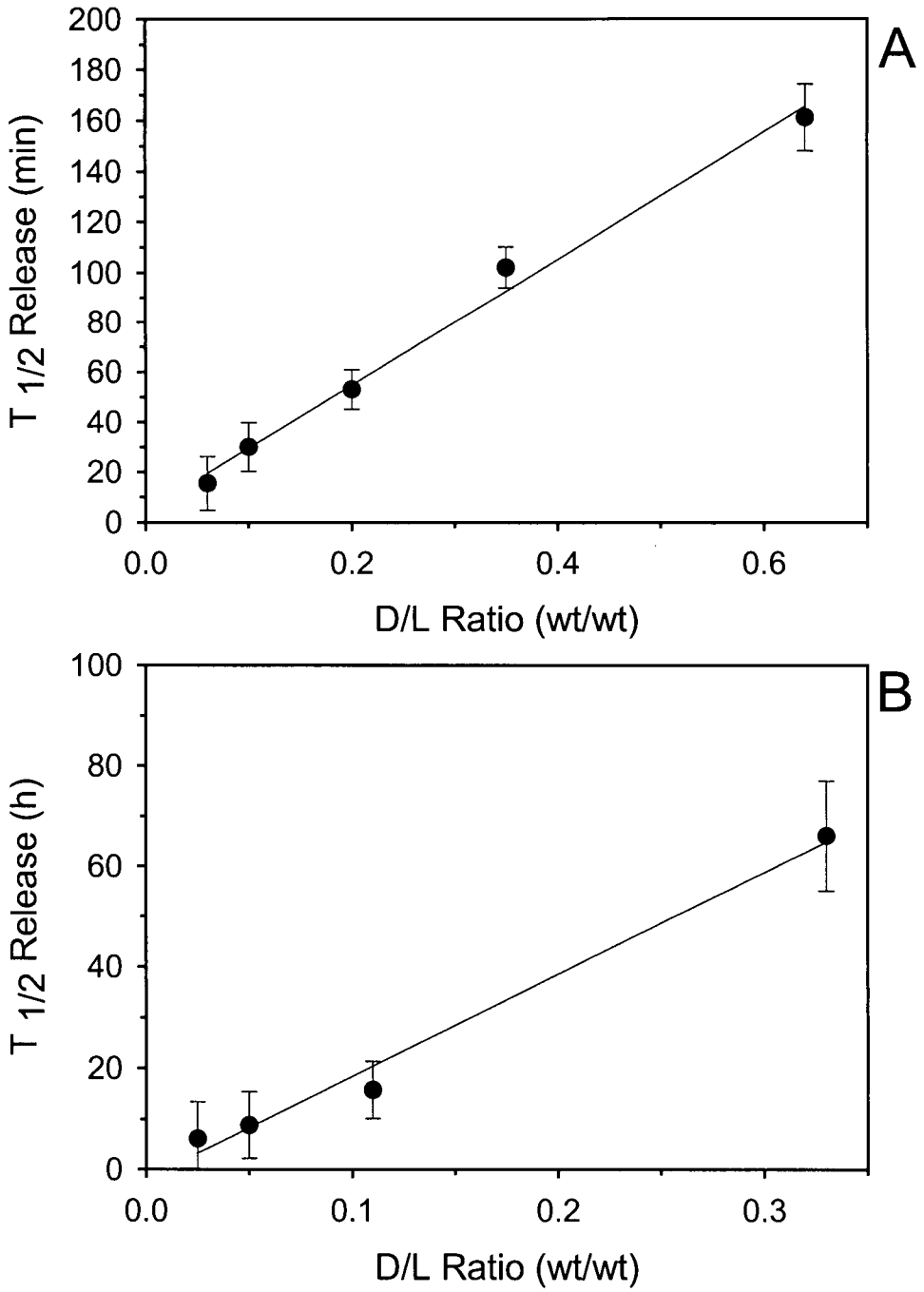


Figure 2.4. Correlation of drug-to-lipid ratio and vincristine release half-life in ammonium chloride (Panel A) (R^2=0.991) and in female ICR mice (Panel B) (R^2=0.994). Half-lives were calculated as described in Methods, and were 15, 30, 53, 102 and 161 min for D/L ratios of 0.06, 0.1, 0.2, 0.35 and 0.64 *in vitro* and 6.1, 8.7, 15.6 and 65 h for D/L ratios of 0.025, 0.05, 0.1 and 0.33 *in vivo*. Error bars were calculated from the 95% confidence interval associated with the best-fit analysis.

2.3.3 Addition of "empty" liposomes allows equivalent clearance rates to be achieved for LUV with different D/L ratios

At low lipid doses, LUVs are increasingly removed from the circulation (Oja et al. 1996). Thus, in order for LUV loaded with drug at very high D/L ratios to exhibit long circulation lifetimes, it may be necessary to add "empty" liposomes to achieve the lipid dose required for long circulation lifetimes in combination with reasonable drug dose levels. For example, the maximum tolerated dose of vincristine is approximately 2 mg/kg in mice. If this dose were given in LUVs containing vincristine at a D/L ratio of one (wt/wt), this would correspond to a lipid dose of also 2 mg/kg, which is well below the lipid dose level at which dose-independent pharmacokinetics are observed for most LUV compositions (Oja et al. 1996). However, as shown in Figure 2.5, by adding empty liposomes to liposomal vincristine formulations with increasing D/L ratios to achieve constant lipid doses of 40 mg/kg, the circulation lifetimes following intravenous administration *in vivo* achieved are constant, with a half-time for clearance of approximately 8 h. Further, in order to show directly that high drug contents do not affect clearance behavior, the clearance behavior of LUVs with a D/L ratio of 0.4 (wt/wt) with no empty liposomes, administered at a vincristine dose of 20 mg/kg (corresponding to a lipid dose of 50 mg/kg), was also examined. The clearance behavior of these systems was no different than other formulations (Figure 2.5).

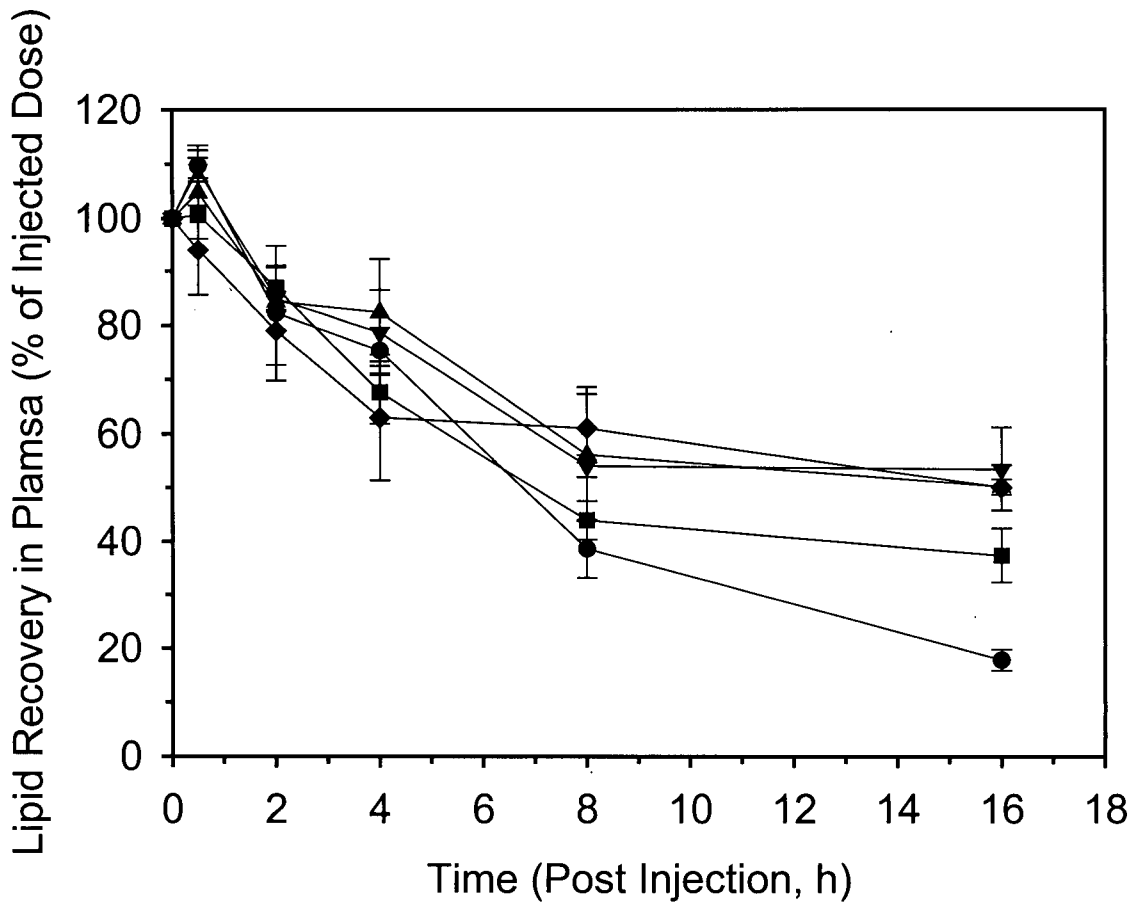


Figure 2.5. Plasma lipid recovery (% of injected dose) of ESM/Chol (55/45, mol%) liposomes *in vivo*. ICR mice were injected intravenously with ESM/Chol formulations of vincristine having drug-to-lipid ratios (wt/wt) 0.025 (▼), 0.05 (▲), 0.1 (■), 0.33 (●) and 0.4 (◆). All mice received lipid doses at 40mg/kg total lipid and vincristine at 2mg/kg drug, with the exception of the 0.025 D/L ratio sample which was dosed at 1 mg/kg vincristine and the 0.4 D/L ratio sample which was dosed at 20mg/kg vincristine and 50 mg/kg total lipid. Data points represent mean values (± standard deviations) calculated from 3 mice.

2.3.4 Liposomes containing high levels of vincristine show internal morphology consistent with drug precipitation

Structural and morphological changes in liposomes, attributed to precipitation of encapsulated drug, have been observed employing cryo-transmission electron microscopy (cryo-TEM) when certain drugs, such as doxorubicin, are loaded into liposomes (Haran et al. 1993). In addition, previous studies have indicated that precipitation of encapsulated drug may enhance *in vitro* and *in vivo* drug retention (Abraham et al. 2004). Our previous cryo-EM studies on liposomal vincristine formulations (Zhigaltsev et al. 2005) have shown that increasing internal electron densities are observed as the D/L ratio is increased to 0.3, however no unambiguous evidence for drug precipitates was observed. Efforts were therefore made to produce liposomal vincristine formulations with the highest D/L ratios achievable in order to visualize potential internal structures. As shown above (Figure 2.1), D/L ratios as high as 1 (wt/wt) can be achieved using the ionophore technique. Cryo-TEM studies were therefore conducted to include LUVs with these very high vincristine contents. As shown in Figure 2.6 a progressive increase in intravesicular electron density as the D/L (wt/wt) ratio is increased to 1.0 is observed (Figure 2.6, panels A-E). This increase in electron density could represent an amorphous or gel-like precipitate at the lower vincristine contents. However, at the D/L ratio of 1.03 (wt/wt), a large proportion of the liposomes contain electron dense granular structures within the vesicles (Figure 4, panel F). These structures, which are unlike the more linear precipitates observed for drugs such as doxorubicin and topotecan (Lasic et al. 1995; Abraham et al. 2004), represent the first direct evidence that vincristine can form internal precipitates when loaded into LUVs. Previous studies have shown a somewhat similar stippled, diffuse morphology in the liposome core when doxorubicin is loaded into $MnSO_4$ containing liposomes , with Chui

and coworkers suggesting the morphology could be due to either an amorphous precipitate of doxorubicin or the precipitation of manganese dihydroxide. In the study presented here, the precipitation magnesium, in the form of magnesium dihydroxide, is unlikely to be responsible for the electron dense granular structures seen at higher D/L ratios in figure 2.6 as the majority of internal magnesium is removed by the ionophore as the pH gradient is generated.

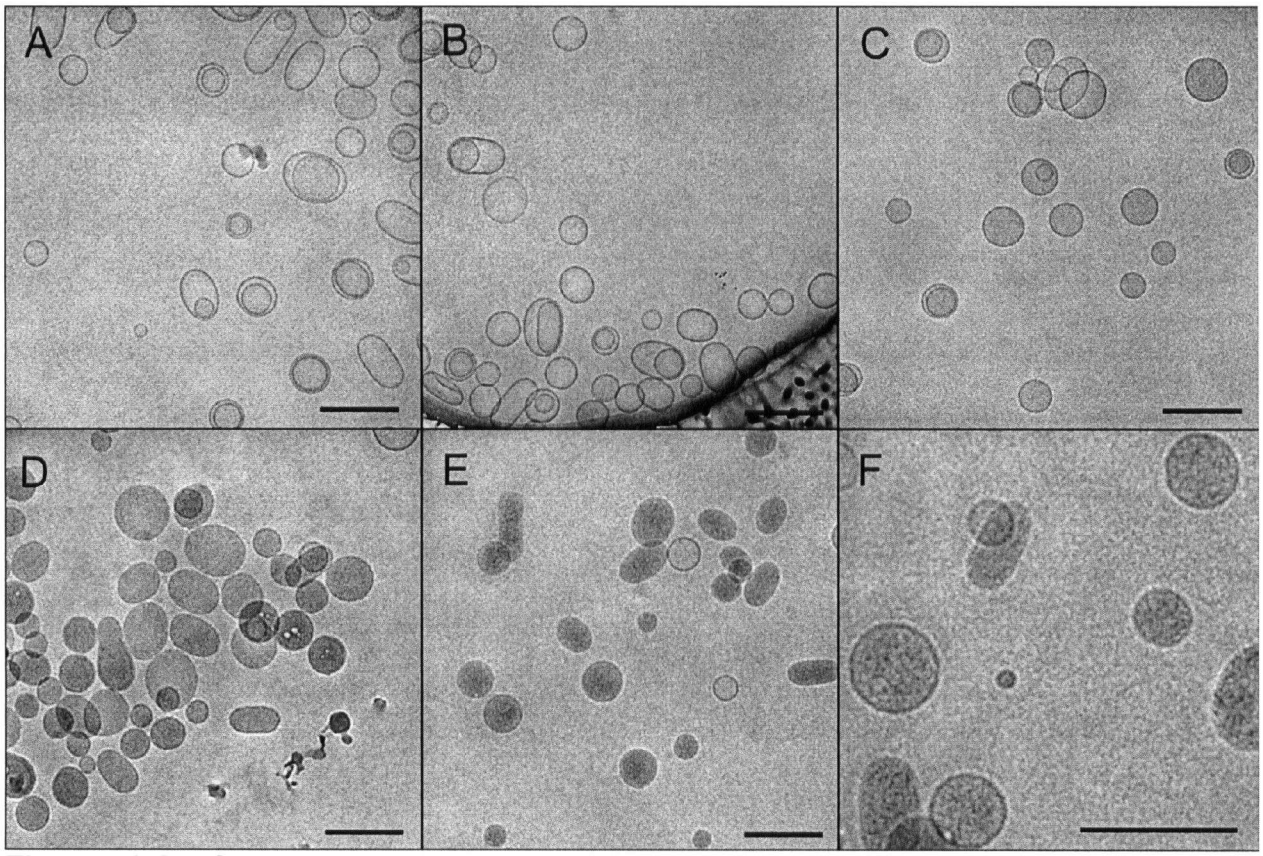


Figure 2.6. Cryo-transmission electron microscopy of ESM/Chol (55/45, mol%) liposomes containing vincristine at different drug-to-lipid ratios. Liposomes containing 300 mM internal magnesium were loaded with vincristine using the ionophore method as described in Methods. Panels represent empty liposomes (A), and D/L ratios (wt/wt) of 0.06 (B), 0.27 (C), 0.6 (D) and 1.03 (E). Panel F represents an enlarged section of Panel E and shows granulated structures within the liposomes at higher D/L ratios. The bar in panel A represents 200 nm and all micrographs (A-E) are shown at the same magnification. Each panel is a representative image taken from at least 5 images per D/L ratio.

2.3.5 A therapeutically optimized drug release rate can be achieved for liposomal vincristine

Early studies on liposomal vincristine demonstrated that the anti-tumor efficacy in a variety of tumor models was very sensitive to the drug release rate. In particular, the efficacy of liposomal vincristine in the L1210 (Mayer et al. 1993), P388 (Mayer et al. 1990; Boman et al. 1994) and A431 (Webb et al. 1995) tumor models was significantly improved over free drug and showed further improvement as the rate of release of vincristine was decreased. Obviously, if the rate of drug release is sufficiently slow, reduced activity would be expected, however such slow release rates were not achievable with the technology then available. The formulations with the slowest rates of release consisted of SM/Chol (55:45) loaded at a D/L ratio of 0.1 in response to a pH gradient established by entrapped phosphate buffer, which demonstrated a $T_{1/2}$ for drug release of approximately 24 h *in vivo* (Webb et al. 1995). This formulation is currently in advanced clinical trial for treatment of non-Hodgkin's lymphoma (Winter 2005).

In order to demonstrate that a therapeutically optimized drug release rate does exist, at least for one tumor model, the antitumor efficacy of liposomal vincristine was evaluated in the MX-1 human breast cancer model employing formulations with a variety of D/L ratios and corresponding large variations in the rates of drug release. Formulations with D/L ratios of 0.025 ($T_{1/2}$=6.1 h), 0.05 ($T_{1/2}$=8.7 h), 0.1 ($T_{1/2}$=15.6 h) and 0.6 ($T_{1/2}$=117 h, as extrapolated from the results of Fig 4b), were employed with the hope of bracketing the optimum release rate.

Tumors were induced in the flanks of female animals, allowed to grow for 14 days (150-200 mm^3) and then the mice were treated with a single intravenous dose of liposomal vincristine (1.5 mg/kg) using empty liposomes to maintain a constant lipid dose as the D/L ratio was increased. All treatment groups demonstrated reversible

weight loss compared with untreated animals, and mice that received the high D/L ratio formulations, which displayed progressively slower drug release rates, generally exhibited less weight loss (Figure 2.7). All treatment groups, including vincristine alone, showed initial tumor regressions at the vincristine dose used (1.5 mg/kg), followed by tumor regrowth in most groups. As shown in Figure 2.8, in all cases, liposomal vincristine showed superior efficacy over vincristine alone, regardless of the D/L ratio. Further, significant differences in efficacy between liposomal formulations were observed depending on the D/L ratio used. The tumor growth delay T-C, which is the time taken for tumors in the treatment group to reach a tumor volume of 1000 mm^3 minus the time taken for the control group, is a commonly used parameter to measure relative efficacy of different formulations. As noted in Figure 2.9 T-C values increased from 21 days at the lowest D/L ratio (0.025, wt/wt, $T_{1/2}$=6.1 h) to greater than 60 days at a 0.1 D/L ratio ($T_{1/2}$=15.6 h), at which time all animals were tumor free. Importantly, further increases in the D/L ratio up to 0.6 (wt/wt) ($T_{1/2}$ release=117 h), resulted in a decrease in activity (T-C= 29 days). At this D/L ratio, drug retention is long and the reduced activity observed suggests that drug release is sufficiently slow that antitumor activity is compromised.

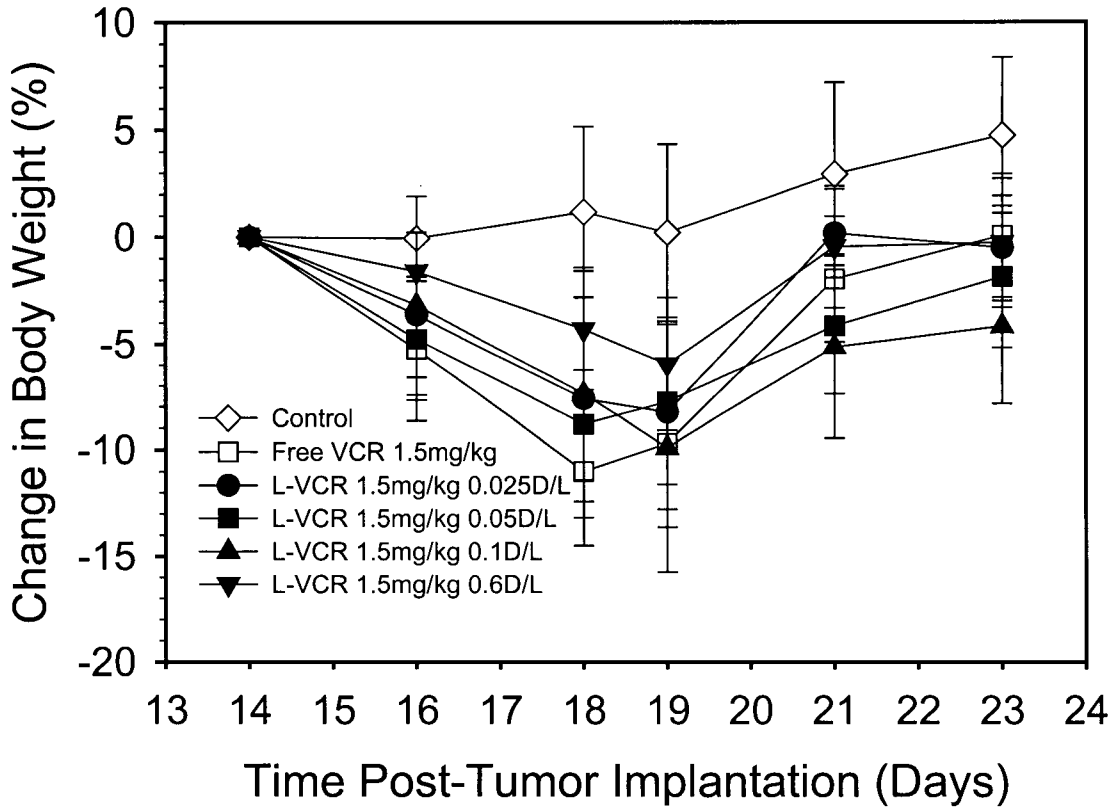


Figure 2.7 Change in body weight of female ICR mice bearing MX-1 tumors in response to liposomal vincristine with differing $T_{1/2}$ values for vincristine drug release. Once tumors were appropriately sized (~150-200 mm^3), mice were treated with 300 mM sucrose (◇), 1.5 mg/kg free vincristine alone (□), or various formulations of liposomal vincristine (SM/Chol, 55/45, mol%) at a dose level of 1.5mg/kg vincristine and 66 mg/kg total lipid, with D/L ratios (wt/wt) of 0.025 (●, $T_{1/2}$=6.1 h), 0.05 (■, $T_{1/2}$=8.7 h), 0.1 (▲, $T_{1/2}$=15.6 h) and 0.6 (▼, extrapolated $T_{1/2}$=117 h). Empty ESM/Chol liposomes were used at the higher D/L ratios to keep the total lipid dose at 66 mg/kg. All mice began treatment on day 14, data points represent mean values (± standard deviations) calculated from 5 mice.

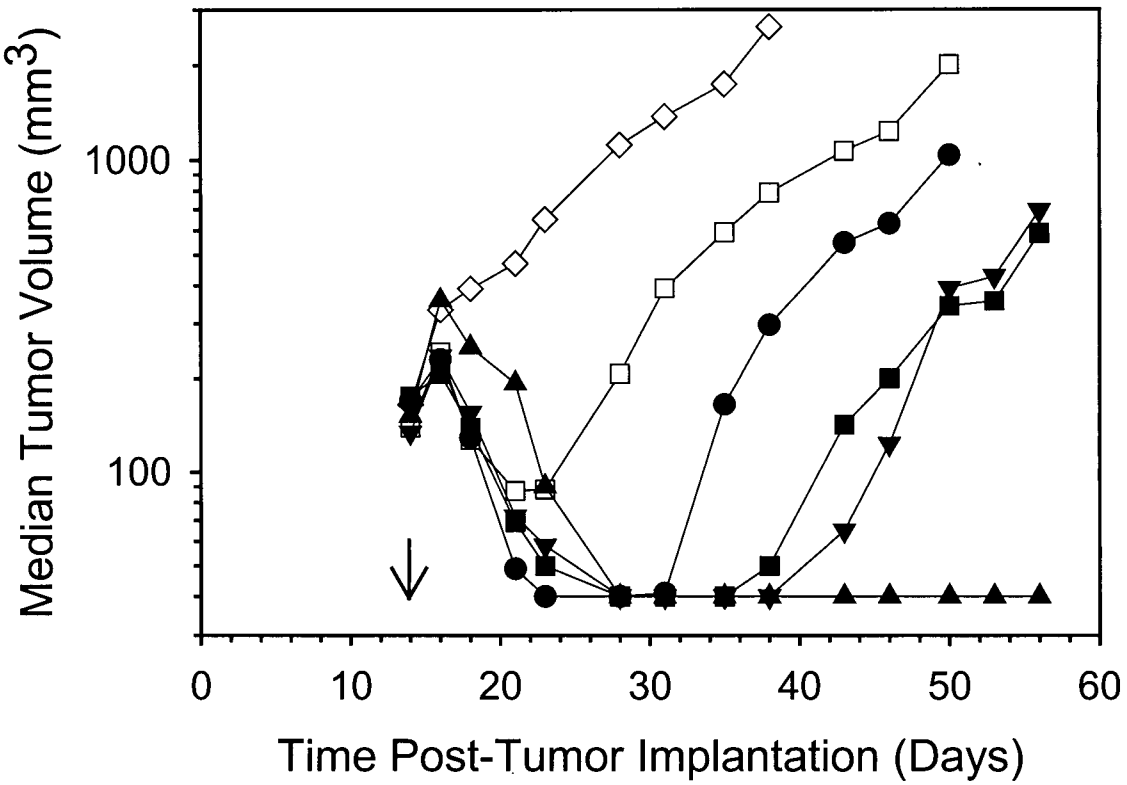


Figure 2.8 Therapeutic effects of liposomal vincristine formulations with different D/L ratios (and correspondingly different drug release rates) in the MX-1 human mammary xenograft model. MX-1 tumors were implanted in the dorsal flank of nude mice as described in Methods. Once tumors were appropriately sized (~150-200 mm^3), mice were treated with 300 mM sucrose (◇), 1.5 mg/kg free vincristine alone (□), or various formulations of liposomal vincristine (ESM/Chol, 55/45, mol%) at a dose level of 1.5mg/kg vincristine and 66 mg/kg total lipid, with D/L ratios (wt/wt) of 0.025 (●, $T_{1/2}$=6.1 h), 0.05 (■, $T_{1/2}$=8.7 h), 0.1 (▲, $T_{1/2}$=15.6 h) and 0.6 (▼, extrapolated $T_{1/2}$=117 h). Empty SM/Chol liposomes were used at the higher D/L ratios to keep the total lipid dose at 66 mg/kg. All mice began treatment on day 14 (arrow), median tumor volumes are displayed (data points represent median values from 5 mice).

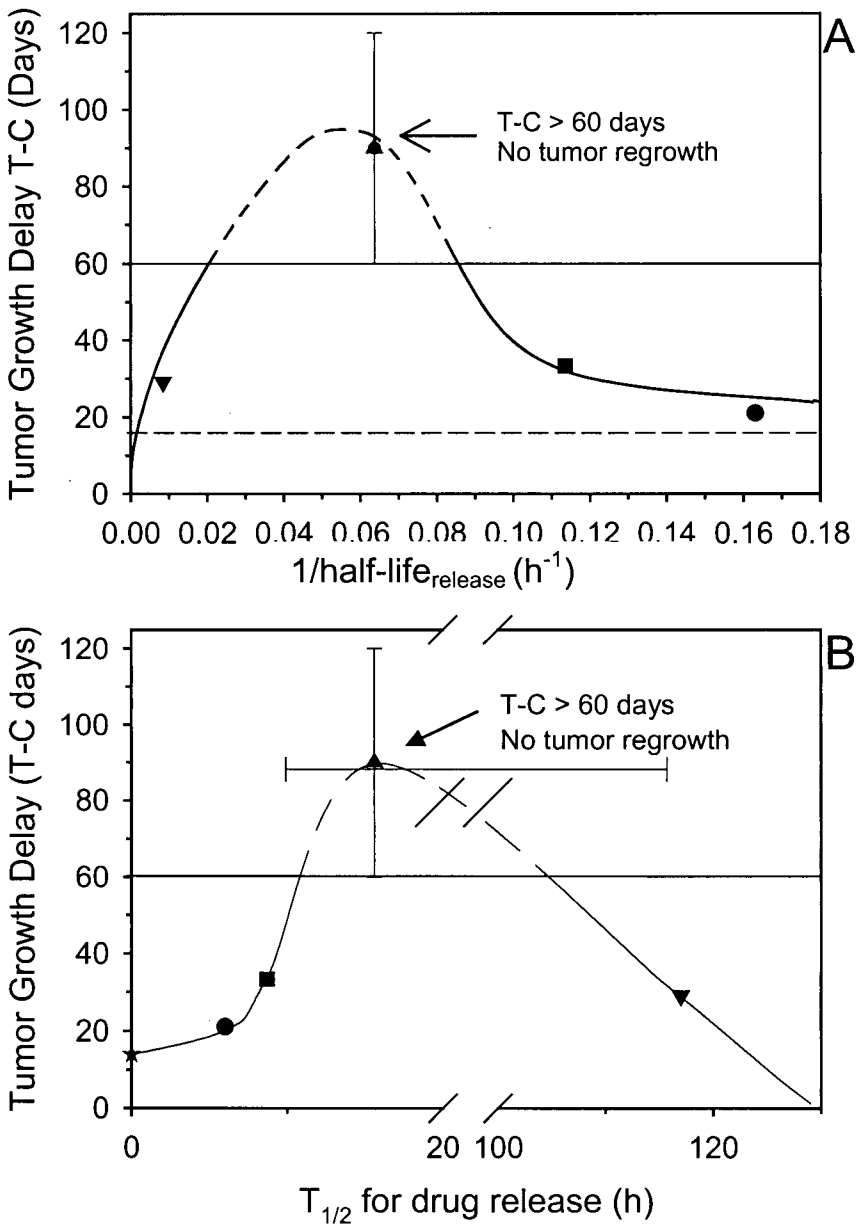


Figure 2.9 Therapeutically optimized rates of drug release for liposomal vincristine in MX-1 human mammary xenograft model are in the range of 0.067 h^{-1} ($T_{1/2}$=15.6 h). These figures depict tumor growth delay (T-C, the time taken for tumors in the treatment group to reach a tumor volume of 1000mm^3 minus the time taken for the control group) as calculated from the tumor growth profiles observed in Figure 2.8, as a function of the rate of vincristine release ($T_{1/2}^{-1}$ panel A) and the $T_{1/2}$ for vincristine release (panel B). The symbols represent T-C for each of the different D/L ratio (wt/wt) formulations: 0.025 (▼), 0.05 (▲), 0.1 (■) and 0.6 (●). The dashed line (panel A) represents the tumor growth delay for a single intravenous dose of vincristine at 1.5 mg/kg (★, panel B). Note that for the point corresponding to a drug release rate of 0.067 h^{-1}, $T_{1/2}$ = 15.6 h, T-C is much greater than 60 d as there was no tumor re-growth at this release rate. For didactic purposes the T-C has been arbitrarily set at 90 d for this release rate.

2.4 Discussion

This study demonstrates that the release rates of vincristine from liposomes can be regulated by varying the drug-to-lipid ratio and that, at high D/L ratios in SM/Chol liposomes, therapeutically optimized rates of release can be achieved. There are four major points of interest. The first concerns the extremely high D/L ratios that are demonstrated here for vincristine, which are considerably higher than achieved previously. The second concerns the mechanism whereby changes in the D/L ratio can modulate drug release rates. The third concerns the great variations in activity that are observed for liposomal formulations of vincristine with different release rates and how this may relate to the drug mechanism of action. A final point concerns whether optimized release rates determined in specific cancer models are relevant to liposomal formulations used clinically.

The results reported here demonstrate that vincristine can be loaded into LUVs, employing the ionophore loading procedure, to achieve D/L ratios as high as one, which is close to the maximum that is theoretically possible (the vincristine levels that could theoretically be achieved in exchange for 300mM Mg^{2+} entrapped in a 100 nm diameter LUVs corresponds to a D/L ratio of ~1.1). Such high D/L ratios have not been reported previously for any drug or drug loading protocol. For example, the highest levels of doxorubicin reported (Mayer et al. 1989; Mayer et al. 1990) correspond to D/L ratios of approximately 0.3 (wt/wt), for vincristine to 0.3 (wt/wt) (Zhigaltsev et al. 2005), for topotecan to 0.3 (wt/wt) (Abraham et al. 2004a) and vinorelbine to 0.3 (wt/wt) (Semple et al. 2005). Aside from the obvious utility of systems with very high D/L ratios for achieving extremely slow release times *in vivo* (the $T_{1/2}$ for a liposomal vincristine system with a D/L ratio of one can be extrapolated to be approximately 8 days *in vivo*),

these systems are of interest because of the extremely high potency of each liposomal entity. For example, a D/L ratio of one corresponds to approximately 84,000 molecules of vincristine per 100 nm diameter liposome (see Appendix 1). Although it is difficult to accurately determine the number of vincristine molecules required to kill a cell, an estimate can be achieved from the IC_{50} values (concentrations of drug required to inhibit cell growth *in vitro* by 50%) that have been reported in the literature. In particular, for exposure times of 24-48 h, IC_{50} values in the range of 10 nM have been observed for L1210 murine leukemia cells (Mayer et al. 1993) and for a variety of human tumor cell lines (Kruczynski et al. 1998). These studies employed approximately 10^5 cells per well. Although neither of these studies reported the actual volume of 10 nM vincristine that was added to the wells, a volume of 200 μl would be a reasonable assumption. It may then be calculated that the IC_{50} corresponds to approximately 10^7 molecules of vincristine per cell. Thus even in the unlikely event that all of the vincristine in the solution partitions into the cells and is required to inhibit cell growth, this would be supplied by only 120 liposomes with a D/L ratio of 1. In the more realistic scenario where the drug partitions into the cells according to its octanol-water partition coefficient (log P=2.14) (Greig et al. 1990), the concentration in the cells would be approximately 1.4 μM, corresponding to approximately 440,000 vincristine molecules per 10 μM diameter cell, requiring delivery of only five liposomes per cell. Such highly potent liposomes may well be of utility in targeting applications, particularly when the number of cell surface receptors that are targeted is limited.

The mechanism whereby higher D/L ratios lead to reduced release rates is of obvious interest. As indicated under Results, if drug release is governed by the usual Fick's law relationship, the efflux rates should be proportional to the total amount of

encapsulated drug and the percent of drug released over time should be independent of the initial interior drug concentration, which is inconsistent with the observed behavior. The results can, however, be accounted for if the large bulk of the encapsulated drug exists in a precipitated form at equilibrium with a small proportion of the drug in the soluble form. Under these conditions the efflux rate will be proportional to the concentration of the soluble form of the drug, which will remain constant until the precipitated form is dissolved (Figure 2.10). Thus the half-time ($T_{1/2}$) for drug release will be proportional to the amount of drug in the precipitated form. If the bulk of the drug is in the precipitated form over the range of D/L ratios examined, the half-times for drug release from the various formulations will then be directly proportional to the initial D/L ratio, as observed experimentally.

The proposal that vincristine precipitates in the liposome interior is consistent with the results presented here, particularly the cryo-EM studies on formulations with the highest D/L ratios, where well defined interior structures became apparent at D/L ratios of 1. At lower D/L ratios the liposome interior becomes increasingly electron dense, however precipitated particulates are difficult to discern. It is likely that the increasing electron density reflects the presence of precipitated vincristine in an amorphous form, given the linear dependence of the release rate on the D/L ratio over the entire range of D/L ratios examined. The appearance of the precipitate contrasts with that observed for liposomal formulations of other anti-cancer drugs such as doxorubicin (Li et al. 1998), mitoxantrone (Almgren et al. 2000) and topotecan (Abraham et al. 2004a), where well defined internalized precipitates are observed at much lower D/L ratios. In the case of topotecan (Abraham et al. 2004) it was noted that a formulation with a D/L ratio of 0.2 exhibited internal precipitates and improved retention compared to formulations with lower D/L ratios, consistent with the results presented here for vincristine.

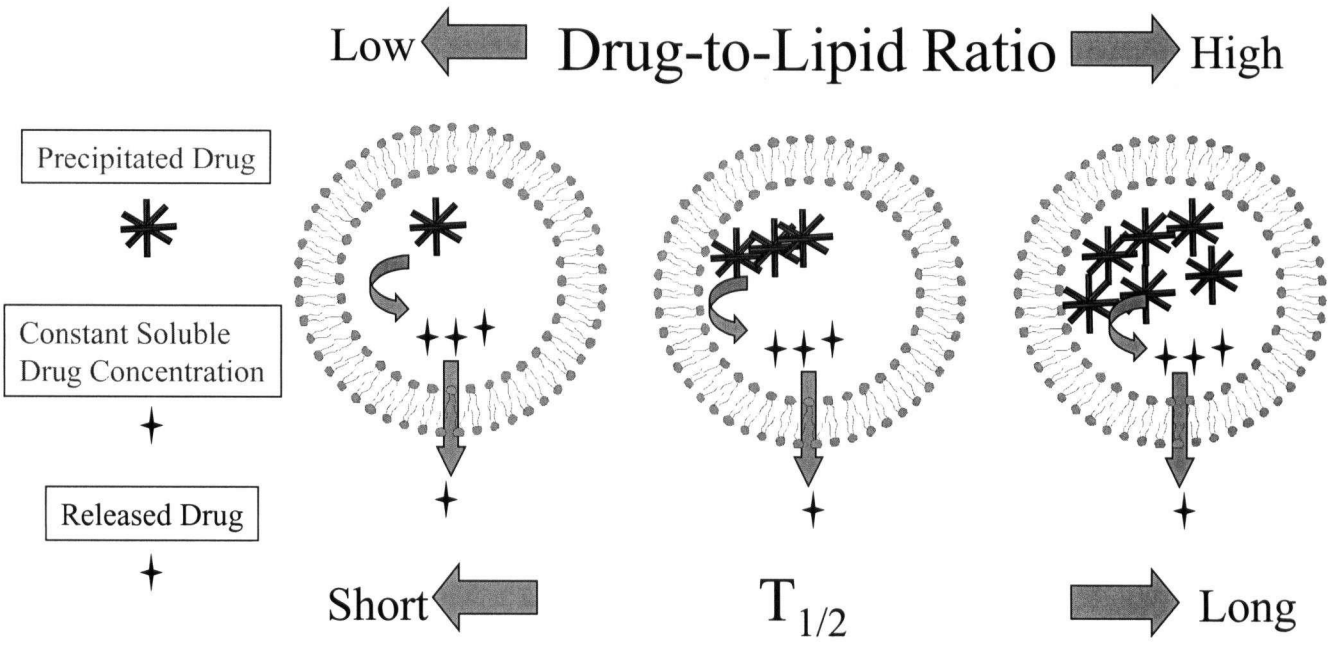


Figure 2.10 Schematic representation of precipitated drug functioning as a reservoir, replenishing a constant concentration of soluble drug as drug leakage is induced. As a result of this behavior the half-time for drug release would be expected to be linearly dependent of the D/L ratio.

It has long been recognized that the efficacy of liposomal formulations of vincristine is highly sensitive to the drug release rate, a point that is reinforced by the results presented here employing the MX-1 human breast cancer model. Early studies using the L1210 and P388 murine leukemia models (Boman et al. 1993; Boman et al. 1994), and the A431 human squamous carcinoma model (Webb et al. 1995) demonstrated that formulations with slow release rates essentially converted the drug from being inactive to one that produced 100% cures. This behaviour contrasts with that of other drugs, such as doxorubicin, where release rates have a much smaller effect on activity in the L1210 model (Mayer et al. 1989) or the orthotopic 4T1 murine mammary carcinoma model (Charrois and Allen 2004). In the latter study the maximum tumor growth delays were less than 50% even though the doxorubicin halftimes for release were varied over

a range of approximately 7h to 10 days. It has been proposed (Boman et al. 1998) that the reason for this difference concerns the fact that vincristine is a cell cycle specific drug (Jordan et al. 1991; van Der Heijden et al. 2004), whereas doxorubicin is not (Gardner 2000). Extended drug release at tumor sites would be expected to have the greatest benefit for cell cycle specific agents, such as vincristine, where strong relationships between tumor cell exposure times and enhanced cell killing have been established (Bruce et al. 1969; Jackson and Bender 1979; Horton et al. 1988; Burris et al. 1992; Gomi et al. 1992; Georgiadis et al. 1997).

The sensitivity of the activity of liposomal formulations of vincristine to the release rate of the drug naturally leads to the question of what is the therapeutically optimized rate of drug release. As indicated in Figure 2.9, the efficacy of formulations with different payout rates would be expected to exhibit little or no activity if the drug is released extremely slowly and activity similar to that observed for the free drug if the drug is released very quickly after administration. The fact that at rates of release intermediate between these two extremes enhanced activity compared to the free drug is observed implies that a therapeutically optimized rate of drug release exists. As indicated under Results, the efficacy of previous formulations of liposomal vincristine improved as the rate of drug release was decreased, however rates of drug release that were so slow that activity was compromised could not be achieved and thus truly optimized rates of drug release could not be identified. As demonstrated here, a formulation with a D/L ratio of 0.6 (wt/wt) with an extrapolated *in vivo* $T_{1/2}$ of 117 h exhibits reduced efficacy compared to a formulation with a D/L ratio of 0.1 (wt/wt) with a $T_{1/2}$ of 15.6 h, implying that the optimized rate of drug release corresponds to a $T_{1/2}$ intermediate between 16 h and 117 h. It may be concluded that a formulation with a D/L ratio between 0.1 and 0.6

will exhibit therapeutically optimized properties in the MX-1 human xenograft tumor model.

The final point of discussion concerns the relation between optimized rates of release observed in a human xenograft model and formulations with release rates that are clinically optimal. Human tumors grow over a period of months to years, whereas murine and human tumor xenograft models in mice grow over days, weeks or months. Cell growth rates and tumor doubling times of the various preclinical models would be expected to influence the determination of optimal drug release rates. It is expected that determination of optimal release rates in tumor models will, however, have utility. For example, it is likely that similar levels of free drug are required to achieve tumor cell killing both in xenograft models and in humans. The use of animal models to determine the slowest release rates compatible with activity may therefore be of direct relevance to the clinical situation, provided that similar levels of total (encapsulated) drug are delivered to the tumor site in both the model and in humans. Future work will focus on the influence of tumor cell growth rates and repeat dosing schedules on release rates leading to optimized therapeutic properties.

In summary the results presented here show that vincristine can be loaded into liposomes to achieve very high D/L values, that by varying the D/L values the rates of drug release from the liposomes can be varied over a wide range, that this behavior is consistent with drug precipitation in the LUV interior and that by varying the D/L ratio formulations with therapeutically optimized rates of drug release can be developed. It is expected that such procedures will be applicable to the development of therapeutically optimized liposomal formulations of other cell cycle specific drugs such as topotecan.

Chapter 3: Influence of Drug-to-Lipid Ratio on Drug Release Properties and Liposome Integrity in Liposomal Doxorubicin Formulations

3.1 Introduction

The utility of liposomal carrier systems in the treatment of malignancies has been clearly demonstrated in numerous preclinical and clinical studies (Balazsovits et al. 1989; Grunaug et al. 1998; Lim et al. 2000; Hofheinz et al. 2005). The enhanced efficacy observed with liposomal systems containing antineoplastic agents can be attributed to a number of factors including reduced toxic side effects (Forssen and Tokes 1981; Herman et al. 1983), accumulation at tumor sites (Gabizon 1992), protection of encapsulated drug from degradation (Abraham et al. 2004a) and the ability to regulate drug release (Webb et al. 1995; Boman et al. 1998). Regulation of drug release is most critical for those agents that function in a cell cycle specific manner, such as the vinca alkaloids (Bruce et al. 1969; Horton et al. 1988). Many approaches have been employed in the formulation of liposomal vincristine systems that demonstrate differing drug release rates, including the use of different phospholipids, altering fatty acyl chain length and saturation of the lipids (Boman et al. 1993), the inclusion of cholesterol (Mayer et al. 1989) and using temperature or pH-sensitive lipids (Tomita et al. 1989; Ning et al. 1994; Zellmer and Cevc 1996). Recently studies have shown that inducing vincristine precipitation by loading liposomes at high drug-lipid ratios is also an effective means of regulating liposomal drug release, leading to optimized rates of drug release in human tumor models (Johnston et al.). *In vivo* half-lives of release could be regulated in ESM/Chol liposomal formulations from hours

(6.2h) to days (117h) by varying the drug-to-lipid ratio between 0.05 (wt/wt) and 0.6 (wt/wt). This phenomenon has been attributed to precipitation of the encapsulated vincristine at increasing drug-to-lipid ratios (Johnston et al. 2006).

The precipitation of certain drugs within liposomal formulations has been observed previously. Doxorubicin (Li et al. 1998), mitoxantrone (Almgren et al. 2000) and topotecan (Abraham et al. 2004a) form clearly defined linear bundle-like structures. Li and coworkers have extensively investigated the nature of doxorubicin precipitates within liposomes and have proposed that the drug forms fibers composed of stacked doxorubicin molecules (Li et al. 1998). These fibers are grouped into hexagonal arranged bundles aligned along the line of sight at rotation multiples of 60°, giving the appearance of repeating striations on the fiber bundle (Li et al. 1998). The vinca alkaloids (vincristine and vinorelbine) tend to form amorphous precipitates (Semple et al. 2005; Zhigaltsev et al. 2005; Johnston et al. 2006).

It has been noted for vincristine (Johnston et al. 2006) and topotecan (Abraham et al. 2004a), that increasing the drug-to-lipid ratio leads to increasing drug precipitation which correlates with decreasing release rates of encapsulated drug. However, it was also observed that increasing initial drug-to-lipid ratios also leads to decreased loading efficiencies. Increasing the initial drug-to-lipid ratio from 0.3 (wt/wt) to 0.4 (wt/wt) decreased the loading efficiency by 30 percent when using an ionophore ($MnSO_4$/A23187) loading method (Abraham et al. 2004a). For liposomal vincristine systems, a 45 percent decrease in loading efficiency was observed as the initial drug-to-lipid ratio was increased from 0.05 (wt/wt) to 1.6 (wt/wt) (Johnston et al. 2006).

In this chapter I investigate the potential of modulating drug-to-lipid ratio as a method of regulating encapsulated drug release for doxorubicin and further examine

potential causes of the reduced loading efficiency observed when loading drugs at high drug-to-lipid ratios.

3.2 Materials and Methods

3.2.1 Materials

Distearoylphosphatidylcholine (DSPC) and cholesterol (Chol) were obtained from Sigma (St. Louis, MO, USA) and egg sphingomyelin (ESM) was purchased from Northern Lipids (Vancouver, BC, Canada), all were used without further purification. [^{3}H]-Cholesterylhexadecyl ether (CHE) was obtained from Perkin Elmer Life Sciences (Boston, MA, USA). Vincristine sulfate was obtained from Fine Chemicals (Cape Town, South Africa) and ciprofloxacin hydrochloride was obtained from Bayer (Leverkusen, Germany). [^{14}C]-vincristine sulfate was obtained from Chemsyn Laboratories (Lenexa, Ka, USA) and [^{14}C]-Ciprofloxacin hydrochloride was a gift from Inex Pharmaceuticals Corporation (Burnaby, BC, Canada). [^{14}C]-sucrose was obtained from Amersham Biosciences (Arlington Heights, IL, USA). Doxorubicin hydrochloride and all other chemicals were obtained from Sigma (St. Louis, MO, USA).

3.2.2 Liposome preparation

Lipids (ESM, DSPC or Chol) and trace amounts of [^{3}H]-CHE were co-dissolved at appropriate molar ratios in ethanol. Multilamellar vesicle (MLV) suspensions were generated after the addition of a 300 mM aqueous solution of magnesium sulfate, yielding a final ethanol concentration of 10% (v/v). Large unilamellar vesicles (LUVs) were generated by extrusion of MLVs through two stacked Nuclepore polycarbonate filters with a pore size of 100nm (10 passes) using an extrusion device obtained from Northern Lipids (Vancouver, BC, Canada) (M.J. Hope 1985; Mayer et al. 1986). A transmembrane ion gradient was established through the removal of the external 300 mM magnesium sulfate, and remaining ethanol, by dialysis against SEH loading buffer (300 mM sucrose, 3mM EDTA, 20mM HEPES, pH 7.4). The mean diameter of LUVs

was determined by dynamic light scattering using a NICOMP 370 particle sizer (Nicomp Particle Sizing Inc., Santa Barbara, CA) and found to be 110 ± 25 nm. Phospholipid concentrations were determined using established techniques (Fiske and Subbarow 1925) and the specific activity of the liposomes was determined using a Beckman LS3801 scintillation counter (Fullerton, CA, USA).

3.2.3 Drug encapsulation in liposomes

Doxorubicin, vincristine or ciprofloxacin were encapsulated using an ionophore-mediated drug loading procedure, as described previously (Fenske et al. 1998). Briefly, vincristine sulfate, doxorubicin hydrochloride or ciprofloxacin hydrochloride were added to LUVs (2.5 mM final lipid concentration) at appropriate drug-to-lipid ratios (wt/wt), and subsequently pre-incubated at 65°C prior to the addition of the calcium ionophore A23187. Trace amounts of [^{14}C]-vincristine or [^{14}C]-ciprofloxacin were included where appropriate. The LUV/drug/ionophore mixture was then incubated at 65°C for up to 90 min. Non-encapsulated drug was removed using dialysis against SEH loading buffer (Fenske et al. 1998) for samples that were used for drug leakage assays or 1ml sephadex G50 chromatography spin columns for samples that were used to measure loading only. Quantification of liposome entrapped drug was achieved via dual label scintillation counting on a Beckman LS 3801 scintillation counter for vincristine and ciprofloxacin loaded liposomes. Quantification of doxorubicin-loaded liposomes was via [^{3}H]-liquid scintillation counting and a doxorubicin absorbance assay. Briefly, this spectrophotometric assay involved the addition of Triton X-100 to liposomal doxorubicin to produce a 1% (v/v) final concentration of detergent. The liposomal drug/detergent was heated to 90°C for 30 s prior to reading at 480 nm on a UV160U Shimadzu spectrophotometer (Kyoto, Japan) and compared to a doxorubicin standard curve.

3.2.4. *In vitro* drug release

In vitro drug release assays were performed to make quantitative comparisons of drug leakage between formulations of varying drug-to-lipid ratios and were conducted using ammonium chloride to enhance the drug release rate (Maurer et al. 1998). Drug loaded vesicles were diluted with release buffer (2 mM ammonium chloride, 300 mM sucrose, 20 mM HEPES, 3 mM EDTA, pH 7.4) to a lipid concentration of 1.25 mM. The diluted liposomal drug was then placed into dialysis tubing (12-14K MW cut off) and dialyzed against release buffer at 50°C. This temperature was chosen to provide an optimal and convenient *in vitro* drug leakage rate, generally 20-30% after 60 min for a drug-to-lipid ratio of 0.05 (wt/wt). Leakage of doxorubicin or ciprofloxacin from the loaded LUVs was assayed by the removal of aliquots for spin column analysis with lipid quantified using liquid scintillation counting and doxorubicin with the spectrophotometric assay described above (Mayer et al. 1990; Fenske et al. 1998). Lipid and remaining encapsulated ciprofloxacin was quantified via dual label scintillation counting. The halftime for drug release ($T_{1/2}$) was calculated from the release profiles as the time at which the internal drug concentration was half the initial concentration at time zero.

3.2.5 Cryo-transmission electron microscopy

Cryogenic transmission electron microscopy (cryo-TEM) was performed on empty and drug-loaded SM/Chol liposomes using a Zeiss EM 902A Transmission Electron Microscope (LEO Electron Microscopy, Oberkochen, Germany) operated at 80kV in the zero loss bright-field mode. Digital images were recorded under low dose conditions with a BioVision Pro-SM Slow Scan CCD camera (Proscan GmbH, Scheuring, Germany) and analySIS software (Soft Imaging System, GmbH, Münster, Germany). In order to visualize maximum detail, an underfocus of 1-2 µm was used to

enhance the image contrast. Sample preparation was performed at 25°C and approximately 99% relative humidity within a climate chamber. A small drop (ca 2 ml) of sample was deposited on a copper grid covered with a perforated polymer film coated with carbon on both sides. Excess liquid was removed by blotting with filter paper, leaving a thin film of the solution on the grid. Immediately after blotting, the sample was vitrified by plunging the grid into liquid ethane held at -182°C. Samples were maintained below –165°C and protected against atmospheric conditions during both transfer to the TEM and examination. Images at 100,000× total magnification were captured for each sample.

3.2.6 Assessing membrane integrity following drug accumulation

To assess the potential of doxorubicin precipitation and fiber bundle formation to rupture liposomes and reduce loading efficiency, DSPC/Chol (55/45 mol%) liposomes were produced as described previously containing a trace amount of [^{14}C]-sucrose (3.38 μM). Doxorubicin was loaded using the ionophore method at a total lipid concentration at 2.5mM. Unloaded doxorubicin and any released [^{14}C]-sucrose were removed employing spin column chromatography. Quantification of remaining encapsulated [^{14}C]-sucrose and [^{3}H] labeled CHE was performed using dual label scintillation counting on a Beckman LS 3801 scintillation counter.

3.3 Results

3.3.1 The half-time of doxorubicin release from LUVs is linearly dependent on the drug-to-lipid ratio

The retention properties of doxorubicin in DSPC/Chol (55/45 mol%) liposomes at various D/L ratios were assessed using an *in vitro* release assay and are shown in Figure 3.1. A dramatic enhancement of drug retention was observed as D/L ratios were increased from 0.047 (wt/wt) to approximately 0.39 (wt/wt) for liposomal doxorubicin. Over this range, the release half-life increased from approximately 38 min to 239 min, representing a more than 6-fold increase in drug retention. In an effort to confirm the mechanisms previously put forward to explain this phenomenon, the $T_{1/2}$ values calculated from the release data were plotted as a function of initial D/L ratio. As shown in Figure 3.2, a linear relationship is observed between the D/L ratio and the $T_{1/2}$ values (R^2 = 0.999) which is consistent with a large proportion of the encapsulated drug being in a precipitated (non-soluble) form (Johnston et al. 2006).

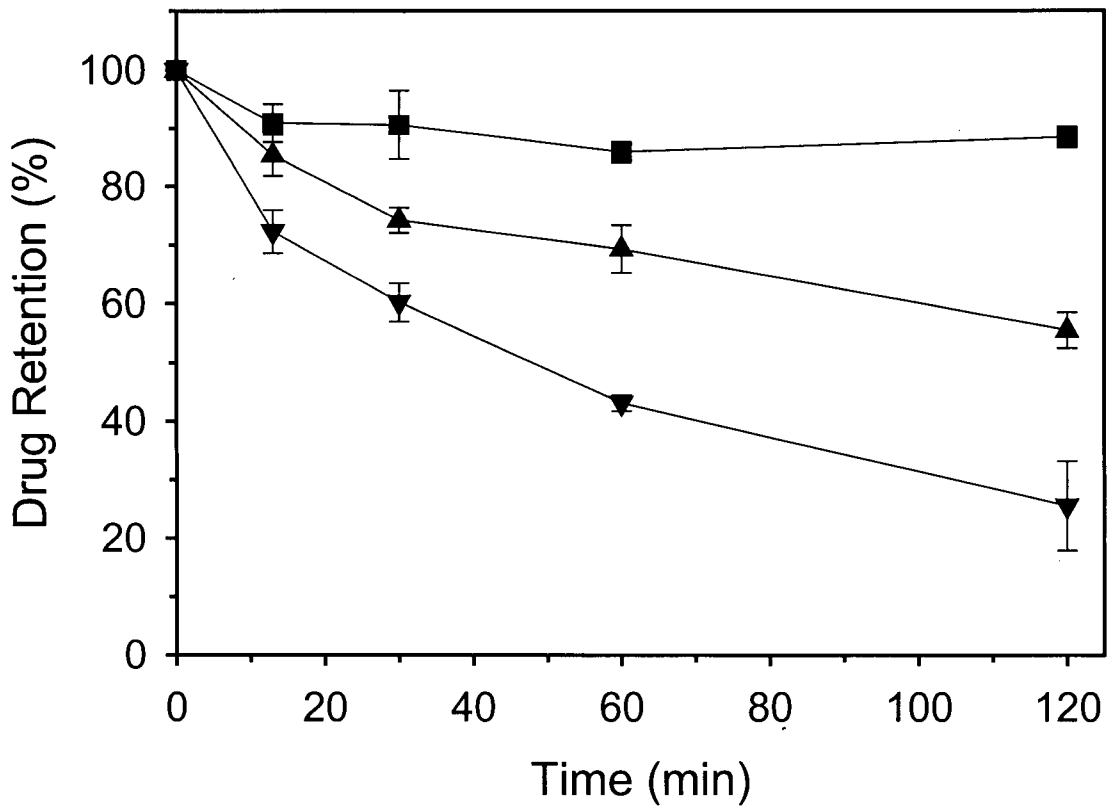


Figure 3.1. *In vitro* drug retention for liposomal formulations of doxorubicin (DSPC/Chol-55/45 mol%) in 2mM ammonium chloride at drug-to-lipid ratios (wt/wt) of 0.047 (▼), 0.1 (▲) and 0.39 (■). Data points represent mean drug retention (± standard deviations) calculated from 3 samples.

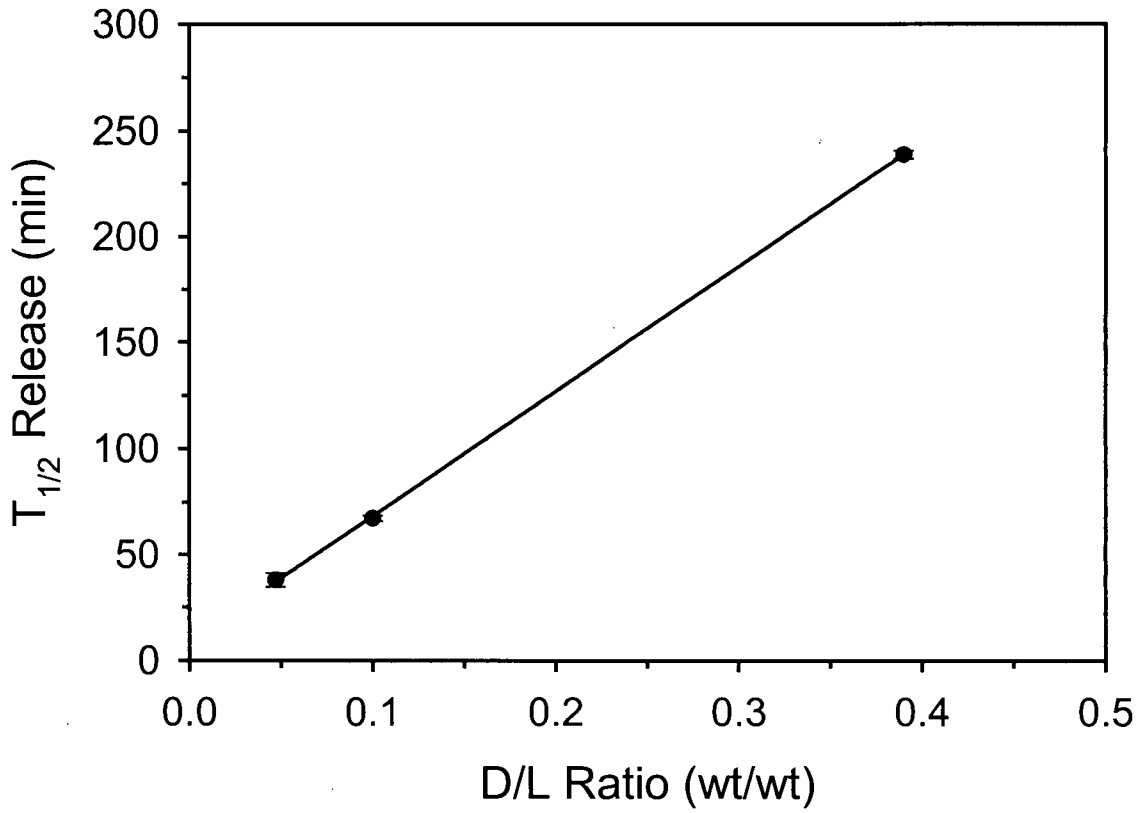


Figure 3.2. Correlation of drug-to-lipid ratio and doxorubicin release half-life in ammonium chloride (R^2=0.999) Half-lives were interpolated as described in Methods, and were 38, 67 and 239 min for D/L ratios of 0.047, 0.1and 0.39 in vitro. Error bars represent the 95% confidence interval for the linear regression.

3.3.2 Liposomes containing high levels of doxorubicin show internal morphology consistent with drug precipitation

As stated in the Introduction, previous cryo-transmission electron microscopy (cryo-TEM) studies of liposomal doxorubicin formulations have demonstrated the existence of linear precipitates of encapsulated drug resulting in a "coffee bean" shaped liposomal morphology. Therefore it is no surprise that when samples here were examined with cryo-TEM, increasingly linear structures and deformed liposomal morphology were observed with increasing D/L ratio. Representative images (n=5 per D/L ratio) from the cryo-TEM studies are shown in Figure 3.3. What was unexpected however was the appearance of triangular and occasionally rectangular structures as the D/L ratio was increased Fig. 3.3, panel D. Further, at the highest D/L ratio examined (D/L=0.46 wt/wt) the defined linear structures were less apparent. At these D/L ratios the internal precipitate is similar in appearance to liposomal vincristine formulations at high drug-to-lipid ratios (Johnston et al. 2006) with the precipitated doxorubicin appearing to coalesce into an amorphous precipitate with no clearly defined structural organization (Figure 3.3, panel E). However, a greater degree of liposomal deformation is observed with liposomal doxorubicin as compared to liposomal vincristine.

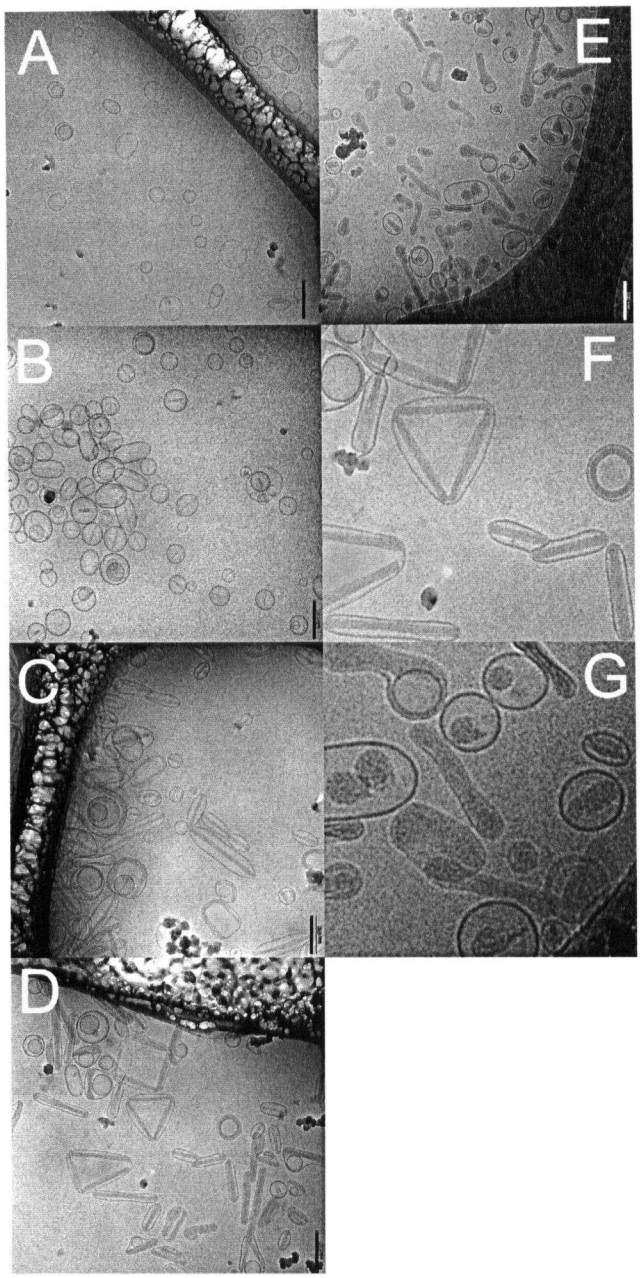


Figure 3.3. Cryo-transmission electron microscopy of DSPC/Chol (55/45 mol%) liposomes loaded with doxorubicin at different drug-to-lipid ratios. Liposomes containing 300mM internal magnesium were loaded with vincristine using the ionophore method as described in Section 4.3.2. Panels represent empty liposomes (A) and D/L ratios (wt/wt) of 0.05 (B), 0.18 (C), 0.37 (D) and 0.46 (E). Panels F and G represent enlarged sections of panels D and E respectively and show the large linear precipitates and the coalescing of those precipitates at higher D/L ratios. The bar in panels A to E represents 200nm and all micrographs (A-E) and all micrographs (A-e) are shown at the same magnification. Each panel is a representative image from at least 5 images per D/L ratio.

3.3.3 Drug precipitation is necessary for release half-life to be dependent on drug-to-lipid ratio

Certain drugs, such as ciprofloxacin, do not precipitate inside liposomes even when loaded to extremely high D/L ratios (Maurer et al. 1998). For such drugs increases in the D/L ratio would not be expected to result in reduced rates of drug leakage. In order to show that this is the case the release properties of ciprofloxacin were investigated over a D/L range from 0.08 to 0.27 (wt/wt). As shown in Figure 3.4 increasing the drug-to-lipid ratio over this range did not affect the halftime for drug release from ESM/Chol (55/45 mol%) LUV. The *in vitro* assay relying on the presence of 2mM ammonium chloride (as described in Methods) to achieve release rates over an experimentally convenient timeframe was used in these experiments.

3.3.4 Formulations with high doxorubicin-to-lipid ratios exhibit reduced trapping efficiencies and leakage of internal buffer during the loading process

The morphological changes observed for doxorubicin-loaded liposomes as the D/L ratio is increased (Figure 3.3) lead to questions regarding the influence of internal precipitated drug on the integrity of the surrounding liposome membrane. In particular, it appears possible that the drug crystals could disrupt the liposome bilayer at the higher D/L values. If this were the case the trapping efficiencies (expressed as the percentage of the initial external drug that is loaded into the liposomes) would be expected to decrease at the higher D/L values due to leakage of drug or Mg^{2+} arising from membrane disruption. As shown in Figure 3.5 for doxorubicin, loading efficiencies are reduced from nearly 100% at an initial external drug-to-lipid ratio of 0.05 (wt/wt) to less than 70% at an initial external D/L ratio of 0.8 (wt/wt).

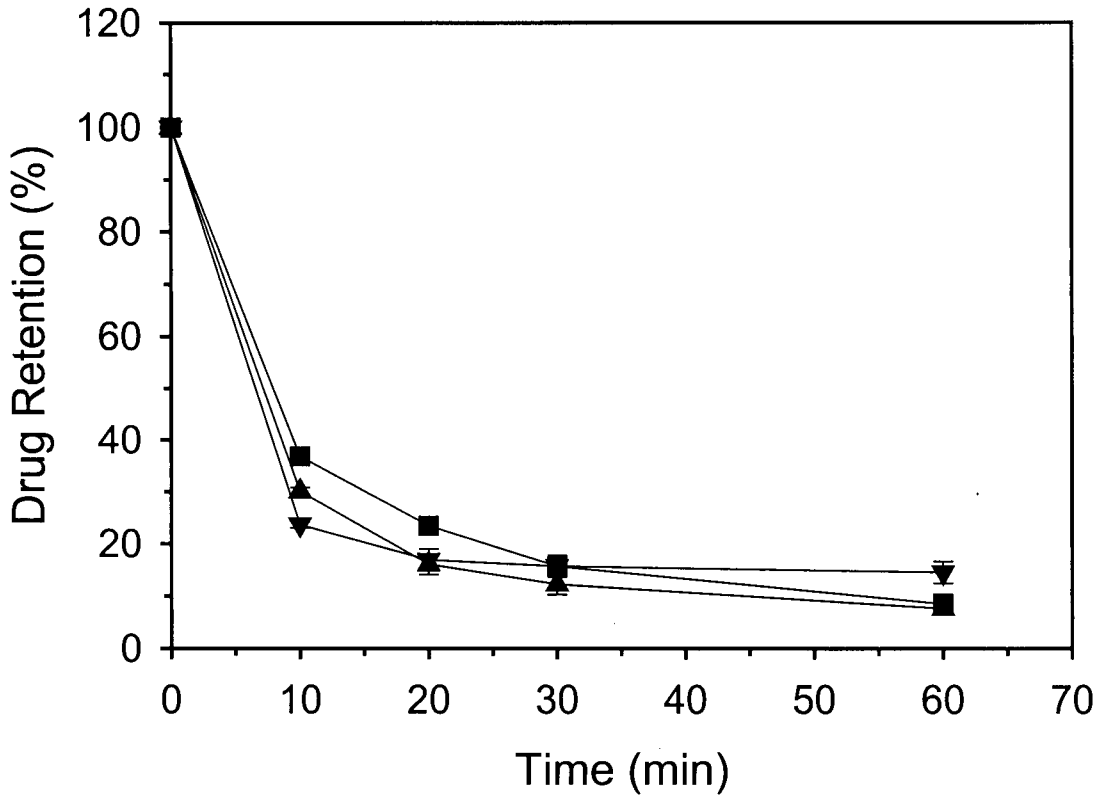


Figure 3.4. *In vitro* drug retention for liposomal formulations of liposomal ciprofloxacin (ESM/Chol-55/45 mol%) in 2mM ammonium chloride at drug-to-lipid ratios (wt/wt) of 0.08 (▼), 0.15 (▲) and 0.27 (■). Data points represent mean drug retention (± standard deviations) calculated from 3 samples

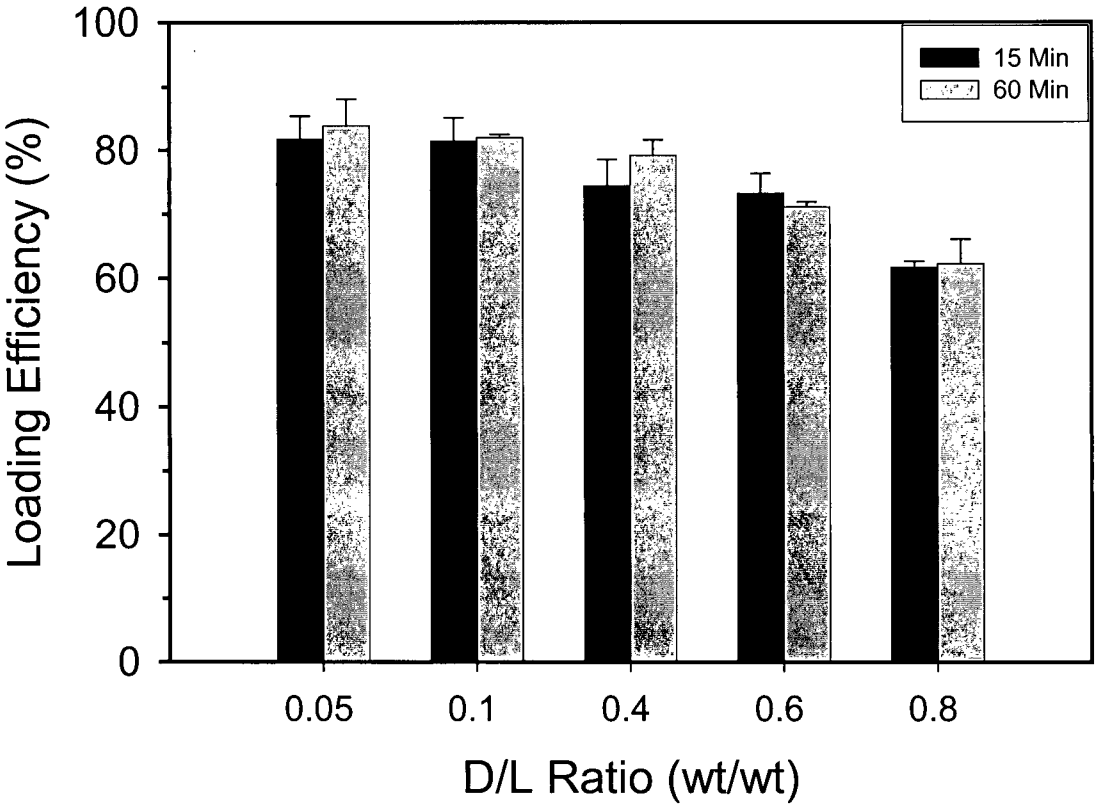


Figure 3.5. Loading efficiencies for liposomal formulations at increasing initial drug-to-lipid ratios for doxorubicin loaded using the ionophore method into liposomes containing 300mM magnesium (DSPC/Chol 55/45 mol%). Data points represent mean loading efficiencies (± standard deviations) calculated from 3 samples.

Reduced trapping efficiencies at high D/L ratios might be explained by a number of facts other than membrane disruption, including the possibility that the ion gradient giving rise to the pH gradient driving the uptake process is exhausted by the influx of high levels of (neutral) doxorubicin molecules, each of which is protonated on arrival in the liposome interior. In order to ascertain whether membrane disruption could account, at least in part, for the reduced trapping efficiencies observed at high D/L ratios, the effect of doxorubicin loading on release of a liposomally entrapped solute was investigated. Specifically, DSPC/Chol (55/45 mol%) liposomes were formulated containing trace amounts of [^{14}C] labeled sucrose and trace amounts of labeled CHE as described in Methods (section 3.2.2). Doxorubicin was loaded into these liposomes employing the ionophore technique and exterior drug (and released sucrose) was removed following loading by spin column chromatography. Measures of the labeled CHE to [^{14}C] labeled sucrose ratio then allowed the % release of entrapped solutes, as assayed by the release of sucrose, to be calculated. As shown in Figure 3.6A as the initial D/L ratio is increased from 0.2 to 0.4 more than 50% of the liposomally-encapsulated sucrose was released during the loading process.

It was of interest to extend this approach to vincristine loading, a drug that can be loaded to achieve D/L ratios as high as one, but which does not give rise to the linear internal crystalline precipitates that are observed for doxorubicin (Johnston et al. 2006). Rather, an amorphous internal precipitate is observed, with some punctate precipitates at the highest D/L values. As shown in Figure 3.6B, leakage of sucrose was also observed as vincristine was loaded at increasing initial drug-to-lipid ratios. However, the sucrose leakage was more gradual and less extensive than for doxorubicin, with only 33% sucrose release at the highest initial vincristine D/L ratio of 0.8.

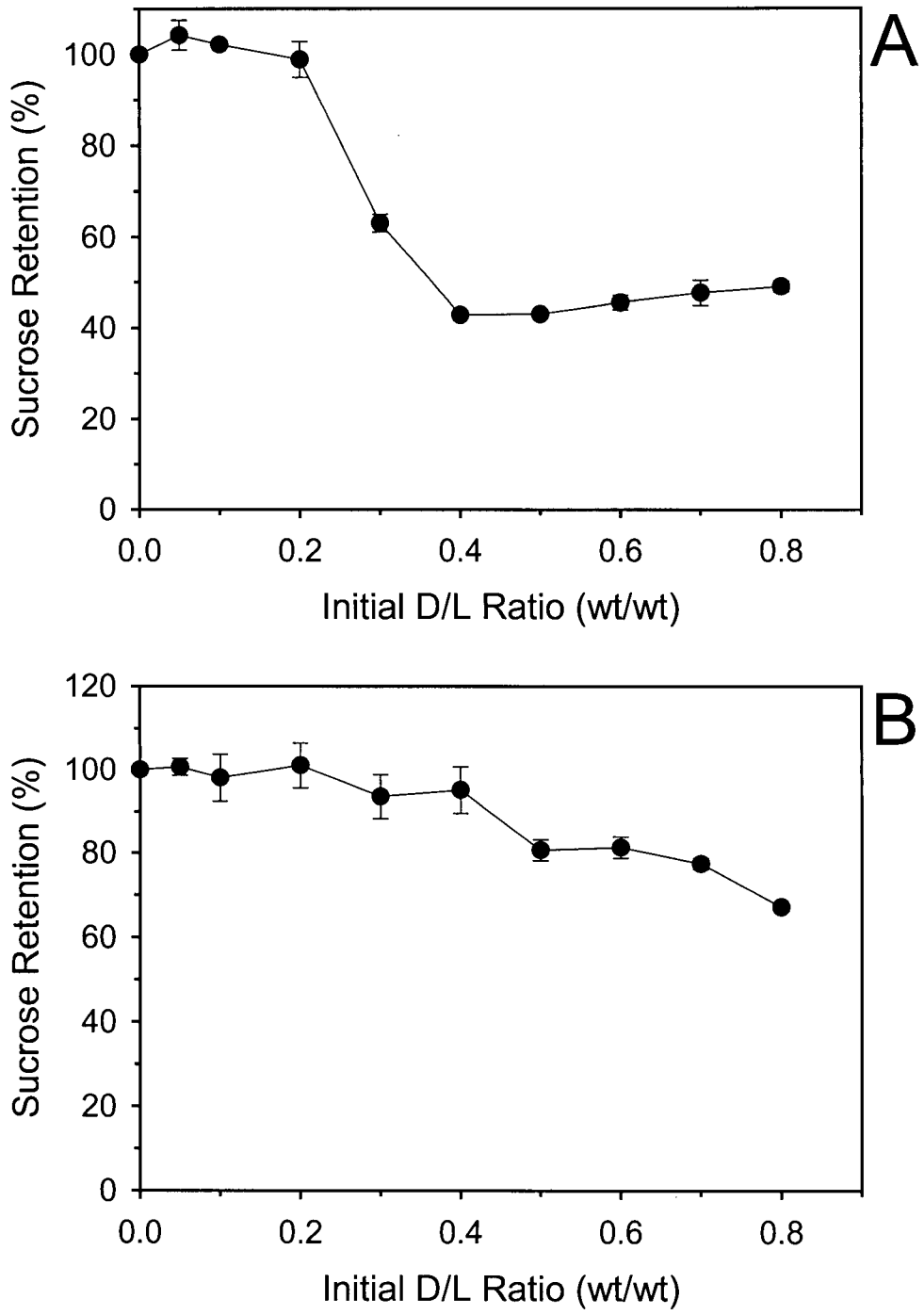


Figure 3.6. Release of encapsulated sucrose from DSPC/Chol (55/45mol %) liposomes when doxorubicin (A) and vincristine (B) are loaded at increasing initial D/L ratios (wt/wt). Samples were loaded at a total lipid concentration of 2.5mM at 65 °C with aliquots removed at appropriate time points and subjected spin column chromatography and dual label liquid scintillation counting.

3.4 Discussion

This study demonstrates that varying the drug-to-lipid ratio, as a method for regulating drug release and ultimately obtaining therapeutically optimized liposomal drug formulations, is also applicable to liposomal drugs other than vincristine. There are three main points of interest. The first concerns the utility of varying the drug-to-lipid ratio to regulate doxorubicin release from DSPC/Chol (55/45 mol%) liposomes. The second involves the mechanism by which variations in the drug-to-lipid ration affects drug release. A final point concerns the reasons why reduced trapping efficiencies are seen when drugs are encapsulated at high drug-to-lipid ratios.

The results reported here demonstrate that varying the drug-to-lipid ratio provides an effective means for regulating drug release from liposomal doxorubicin formulations. As noted previously for vincristine (Johnston et al. 2006), the relative release rates determined employing the NH_4Cl-based *in vitro* assay accurately reflect the relative release rates *in vivo*. It is therefore likely that the ability to increase the halftimes for drug release *in vitro* by a factor greater than 6 by changing the D/L ratio from 0.047 (wt/wt) to 0.38 (wt/wt) will result in a corresponding increase in the halftimes for release *in vivo*. Studies conducted by Parr and coworkers indicated that a DSPC/Chol liposomal formulation of doxorubicin at a D/L ratio of 0.18 (wt/wt) possessed a $T_{1/2}$ release for the encapsulated drug of approximately 5.9 days in Lewis Lung Carcinoma bearing BDF1 mice (Parr et al. 1997). Based on the data presented here, doubling the D/L ratio to 0.36 should increase the half-life of drug release to nearly 12 days. However, it should be noted that liposomal doxorubicin formulations that demonstrate increased drug retention may not result in increased anti-tumor activity (as was observed for vincristine (Johnston et al. 2006)) due to the cell cycle independent

activity of doxorubicin (Minotti et al. 2004). In this regard previous studies demonstrate that increasing the half-life of release from approximately 7 h to over 10 days results in only a 50% increase in antitumor efficacy (Charrois and Allen 2004).

The mechanism by which the drug-to-lipid ratio influences the release rate is of particular interest. If the efflux of drug from the liposome is governed by Fick's law and the traditional view of drug release, the efflux should be proportional to the drug encapsulated within the liposome and drug release, as a function of time, should be independent of the internal drug concentration. As shown here, this is the behavior exhibited by liposomal formulations of ciprofloxacin, a drug that does not precipitate in the liposome interior (Maurer et al. 1998). However, as shown previously for vincristine (Zhigaltsev et al. 2005; Johnston et al. 2006) and topotecan (Abraham et al. 2004a) and shown here for doxorubicin, the halftime for drug release is highly dependent on the D/L ratio. As indicated elsewhere (Zhigaltsev et al. 2005; Johnston et al. 2006), this behavior is consistent with the presence of drug precipitates in the liposome interior (Zhigaltsev et al. 2005; Johnston et al. 2006), a feature that has been observed for all three drugs. Assuming that soluble and precipitated forms of the drug are in equilibrium, the efflux rate will then be proportional to the concentration of the soluble form of the drug, which will remain constant until the precipitated form is dissolved. Thus the time taken for drug release will be proportional to the amount of drug in the precipitated form, as observed experimentally.

As seen here and in previous studies (Abraham et al. 2004a; Johnston et al. 2006) a decrease in trapping efficiency is observed as the drug is loaded to increasing drug-to-lipid ratios. A possible explanation for this is the physical disruption of the liposome bilayer by the growing drug precipitate resulting in either the leakage of encapsulated drug and/or degradation of the Mg2+ ion gradient giving rise to the pH

gradient driving drug loading. Here we demonstrate that entrapped sucrose is released as doxorubicin is loaded at increasing drug-to-lipid ratios, indicating that the growing crystals lead to significant membrane disruption at higher D/L ratios above 0.2 (wt/wt). Leakage of sucrose was also observed when liposomal vincristine was loaded at increasing drug-to-lipid ratios but the extent of leakage is much lower. This may arise due to differences in the structures of precipitated doxorubicin and vincristine. In particular, the linear precipitates of doxorubicin seen at all drug-to-lipid ratios (Figure 3.3) result in a very small radius of membrane curvature when they contact the liposome membrane, possibly resulting in reduced stability for that region of the bilayer. This could have at least two effects. First, at some point the surface tension experienced by the lipid bilayer could exceed some critical value, leading to membrane failure and contents leakage. Alternatively, regions of high membrane curvature have been associated with regions that are susceptible to membrane fusion events. Lipid membranes that display high membrane curvature have been shown to fuse more readily (Wilschut et al. 1981; Nir et al. 1982). Structures that could correspond to an intra-liposomal fusion event are present in the cryo-TEM micrographs presented here (see Figure 3.3, Panel F arrow), and may explain why some larger vesicles are seen at higher doxorubicin-to-lipid ratios. Vincristine on the other hand forms amorphous or gel-like precipitates with little liposomal deformation, even at drug-to-lipid ratios of 1 (wt/wt). This lack of membrane deformation may explain why vincristine loaded at increasing drug-to-lipid ratios does not induce membrane leakage to the same extent as doxorubicin.

In summary the results presented here show that doxorubicin can be loaded into liposomes to achieve D/L values as high as 0.46 (wt/wt), that by varying the D/L values the rates of drug release from the liposomes can be varied over a wide range and that

this behavior is consistent with drug precipitation in the LUV interior. It is also shown that drug precipitation results in remarkable internal structures that lead to deformation and disruption of the liposomal membrane at D/L ratios above 0.2 (wt/wt).

Chapter 4: Characterization of the Drug Retention and Pharmacokinetic Properties of Liposomes Containing Dihydrosphingomyelin

4.1 Introduction

The therapeutic benefits of liposomal formulations of anticancer drugs include decreased toxicity, increased antitumor efficacy and improved sensitization of drug resistant tumors as compared to equivalent doses of the free drug (Balazsovits et al. 1989; Oh et al. 1995; Lim et al. 1997; Grunaug et al. 1998; Leonetti et al. 2004). Two important parameters that affect the therapeutic benefits of liposomal delivery are the circulation lifetimes of the carriers following intravenous (i.v.) injection and the rate of drug release from the liposomes. Long circulation lifetimes lead to enhanced accumulation of liposomes in disease sites such as tumors (Yuan et al. 1995; Drummond et al. 1999) and slow drug release rates are of particular benefit for cell-cycle specific drugs such as vincristine (Mayer et al. 1993; Webb et al. 1995). Extended circulation lifetimes can be achieved using pegylated liposomes (Allen et al. 1991), high lipid doses (Oja et al. 1996) or by using lipids such as sphingomyelin (Webb et al. 1995). Many methods have been employed to regulate drug release, including varying the lipid composition of the liposome carrier (Mayer et al. 1989), using temperature and/or pH-sensitive lipids (Tomita et al. 1989; Ning et al. 1994; Zellmer and Cevc 1996), increasing the drug-to-lipid ratio (Abraham et al. 2004a; Johnston et al. 2006) and altering the fatty acyl chain length and saturation of the lipids (Boman et al. 1993).

In previous work it has been shown that the substitution of egg sphingomyelin for phospholipids such as distearoylphosphatidylcholine (DSPC) in LUVs results in improved drug retention properties and longer circulation lifetime properties (Webb et al.

1995). It has also been shown that LUVs containing more saturated PC exhibit better drug retention and longer circulation lifetimes as compared to LUVs composed of less saturated PCs (Boman et al. 1993). In this work we characterize the effects of hydrogenating ESM in the anticipation that further improvements in drug retention and circulation properties could be achieved employing these more saturated variants of SM. These studies employ vincristine as a representative drug that is relatively "leaky" and difficult to retain in liposomal systems. It is shown that LUVs containing hydrogenated ESM (HSM) exhibit improved vincristine retention and longer circulated lifetimes following intravenous injection as compared to ESM LUVs.

4.2 Materials and Methods

4.2.1 Materials

Egg sphingomyelin (ESM), bovine milk sphingomyelin (MSM), hydrogenated soybean phosphatidylcholine (HSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly(ethyleneglycol)2000] (DSPE-PEG_{2000}) were obtained from Northern Lipids Inc.(Vancouver, B.C.) and bovine brain sphingomyelin (BSM) was purchased from Avanti Polar Lipids (Alabaster, Al, USA). These lipids were used without further purification. Vincristine sulfate was obtained from Fine Chemicals (Cape Town, South Africa). Cholesterol (Chol) was obtained from Sigma (St. Louis, MO, USA) and used without further purification. [^{3}H]-Cholesterylhexadecyl ether (CHE) was obtained from Perkin Elmer Life Sciences (Boston, MA, USA). [^{14}C]- vincristine sulfate was obtained from Chemsyn Laboratories (Lenexa, Ka, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

4.2.2 Synthesis of dihydrosphingomyelin (DHSM)

Synthesis of dihydrosphingomyelin was conducted at Inex Pharmaceuticals. Briefly, twenty-five grams of ESM was dissolved in 250ml of ethanol and 2.5g of a 10% palladium/carbon catalyst was added, the lipid and catalyst were then placed under an argon atmosphere The mixture was heated to 40°C and stirred for 2 hours while hydrogen was passed through the system. Residual hydrogen was removed with an argon flush and any active catalyst was quenched with the addition of cyclohexane. The product was filtered through diatomaceous earth to remove the catalyst, and then subsequently the solvent was removed. Purification of the HSM was accomplished by dissolving the crude product in ethanol and adding acetone (1:1 v/v) followed by filtration, this precipitation was repeated until the desired purity was obtained. The final

product was 99%+ pure based on high performance liquid chromatography (HPLC) with NMR analysis showed no sign of the non-hydrogenated analog.

4.2.3 Differential scanning calorimetry

Differential scanning calorimetry was carried out on MLVs composed of the various sphingomyelins, in distilled water, with a Calorimetry Sciences Corporation MC-DSC 4100 instrument (Lindon, UT). Prior to loading into DSC cells, samples were brought to room temperature and degassed for five min under vacuum with stirring. The samples were then vortexed to ensure homogeneity and scanning with a range of 20 to 60°C at a scan rate of 5°C/h was immediately carried out.

4.2.4 Liposome preparation

Lipids and trace amounts of [^{3}H]-CHE were co-dissolved at appropriate molar ratios in ethanol. Multilamellar vesicle (MLV) suspensions were generated after the addition of a 300 mM aqueous solution of magnesium sulfate for liposomes used for drug loading or buffer composed of 20mM HEPES, 145mM NaCl pH 7.5 for unloaded liposomes used in pharmacokinetic studies, yielding a final ethanol concentration of 10% (v/v). Large unilamellar vesicles (LUVs) were generated by extrusion of MLVs through two stacked Nuclepore polycarbonate filters with a pore size of 100nm (10 passes) using an extrusion device obtained from Northern Lipids (Vancouver, BC, Canada) (M.J. Hope 1985; Mayer et al. 1986). For drug loading a transmembrane ion gradient was established through the removal of the external 300 mM magnesium sulfate by dialysis against SEH loading buffer (300 mM sucrose, 3mM EDTA, 20mM HEPES, pH 7.4). Liposomes used in comparative pharmacokinetic studies, where no loaded drug was necessary, were dialyzed against 20mM HEPES, 145mM NaCl pH 7.5 to remove the remaining ethanol. The mean LUV diameter was determined by dynamic light scattering using a NICOMP 370 particle sizer (Nicomp Particle Sizing Inc., Santa

Barbara, CA) and found to be 100 to 130 nm. Phospholipid concentrations were determined using established techniques (Fiske and Subbarow 1925) and the specific activity of the liposomes was determined using a Beckman LS3801 scintillation counter (Fullerton, CA, USA).

4.2.5 Vincristine encapsulation in LUV

Vincristine was encapsulated into LUVs using the ionophore-mediated drug loading procedure described previously (Fenske et al. 1998). Briefly, vincristine sulfate and trace amounts of [^{14}C]-vincristine sulfate were added to LUVs (5 mM final lipid concentration) at appropriate drug-to-lipid ratios (wt/wt), and subsequently incubated at 65°C prior to the addition of the calcium ionophore A23187. The LUV/drug/ionophore mixture was then incubated at 65°C for 90 min to provide optimal drug loading conditions. Non-encapsulated vincristine was removed using dialysis against SEH loading buffer (Fenske et al. 1998) prior to quantification of liposome entrapped drug using dual channel counting on a Beckman LS 3801 scintillation counter. Formulations used for *in vivo* studies were first dialyzed against 300mM sucrose.

4.2.6 Assays for *in vitro* release of vincristine

In vitro drug release assays giving rise to release rates on experimentally convenient timescales were utilized to make quantitative comparisons of drug leakage between vincristine-loaded LUV of varying drug-to-lipid ratios. *In vitro* release assays for liposomal vincristine were conducted using ammonium chloride to degrade the pH gradient (Maurer et al. 1998). Drug loaded vesicles were diluted with release buffer (2 mM ammonium chloride, 300 mM sucrose, 20 mM HEPES, 3 mM EDTA, pH 7.4) to a lipid concentration of 1.25 mM. The diluted liposomal drug was then placed into dialysis tubing (12-14K M.Wt. cut off) and dialyzed against release buffer at 50°C. This

temperature was chosen to provide an optimal and convenient *in vitro* drug leakage rate, generally 50-60% after 60 minutes for a drug-to-lipid ratio of 0.03(mol/mol). Leakage of vincristine from the loaded LUVs was assayed by the removal of aliquots for spin column analysis and quantification using dual label liquid scintillation counting (Mayer et al. 1990; Fenske et al. 1998). The halftimes for drug release ($T_{1/2}$) were calculated from exponential best fits to the release profiles as the time at which the internal drug concentration was half the initial concentration.

4.2.7 Mice

Female, 6-8 week old outbred ICR mice were obtained from Harlan (Indianapolis, IN) and were used for pharmacokinetic and drug payout studies. All mice were quarantined for at least two weeks prior to use and kept within a controlled temperature (22 ± 1°C) and humidity (60 ± 10%) environment. Lighting was maintained on automatic 12 h light/dark cycles. Animal studies were conducted in compliance with the guidelines established by the Canadian Council on Animal Care (CCAC).

4.2.8 *In vivo* pharmacokinetics

Vincristine loaded LUV were prepared at various drug-to-lipid (D/L) ratios and contained 300mM sucrose as the external buffer. In most instances, drug and lipid concentrations were adjusted to 0.2 mg/ml vincristine and 4 mg/ml total lipid, resulting in doses of 2 mg/kg drug and 40 mg/kg lipid. Empty SM/Chol liposomes were included in liposomal vincristine formulations prepared with higher D/L ratios to ensure that mice were injected with an equivalent total lipid dose. Mice were injected via a lateral tail vein and, at appropriate time points, were anesthetized (ketamine/xylazine) and blood was collected via cardiac puncture into EDTA microtainer tubers. Blood was then centrifuged at 400×g for 15 min and plasma was collected and de-colorized for lipid and drug

determination by dual label liquid scintillation counting as described previously (Fenske et al. 1998).

To assess the pharmacokinetics of three long circulating liposomal formulations (ESM/Chol 55/45 mol%; DHSM/Chol 55/45 mol%; HSPC/Chol/DSPE-PEG_{2000} 57.2/37.6/5.2 mol%), unilamellar liposomes without loaded drug were prepared with an external buffer composed of 20mM HEPES, 145mM NaCl pH 7.5. Mice were injected via a lateral tail vein at lipid doses of either 150 μmol/kg or 15 μmol/kg and, at appropriate time points, were anesthetized (ketamine/xylazine overdose) and blood was collected via cardiac puncture into EDTA microtainer tubes. Blood was then centrifuged at 400×g for 15 minutes and plasma was collected and de-colorized for lipid determination by liquid scintillation counting as described previously (Fenske et al. 1998).

4.3 Results

4.3.1 LUVs containing more saturated species of SM exhibit slower vincristine release rates

The first set of experiments was aimed at characterizing the influence of SM unsaturation on vincristine release rates from LUVs. Four species of SM were employed, namely bovine brain SM (BSM), bovine milk SM (MSM), ESM and DHSM. LUVs composed of SM/Chol (55:45) were made as indicated under Methods and loaded with vincristine to achieve a drug-to-lipid (D/L) ratio of 0.06 (mol/mol) employing the ionophore loading procedure. In order to achieve vincristine release rates on an experimentally convenient timescale an *in vitro* release assay consisting of incubation of drug-loaded LUVs in the presence of 2mM ammonium chloride at 50°C was employed. The vincristine release profiles for each liposomal formulation are shown in Figure 4.1. As may be noted, LUVs containing either BSM or MSM release vincristine very rapidly, with halftimes for drug release ($T_{1/2}$) of less than 10 min under the assay conditions employed. LUVs composed of ESM/Chol exhibit improved retention properties (T1/2 ~ 29 min) whereas DHSM/Chol LUVs exhibit the slowest drug payout characteristics (T1/2 ~ 61 min).

It may be noted that the relative drug efflux rates from LUVs containing the different species of SM correlate with the degree of unsaturation of the SM (Table 1). More unsaturated lipid species also exhibit lower gel-to-liquid crystalline transition temperatures (Tc) values (Cullis et al. 1996) and the Tc values of the SM species employed here were determined employing differential scanning calorimetry. As shown in Table 4.1 the transition temperatures vary from 34°C to 46°C as the saturation index of SM increases.

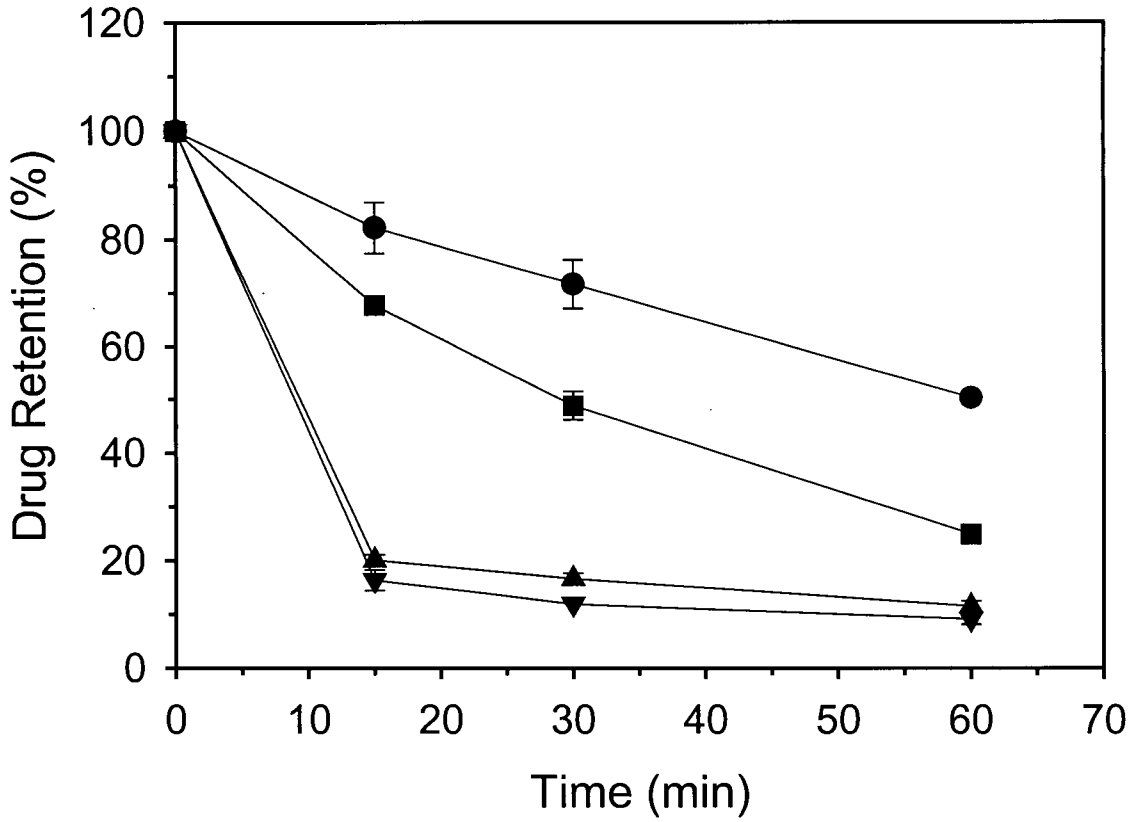


Figure 4.1. Vincristine *in vitro* retention of liposomal preparations (0.06, mol/mol) in 2mM ammonium chloride at 50°C, composed of various sphingomyelin sources. Bovine milk sphingomyelin (▼), bovine brain sphingomyelin (▲), egg sphingomyelin (■) and hydrogenated egg sphingomyelin (●). The lipid concentration in the release assays was 1.25 mM total lipid. Data points represent mean drug retention (± standard deviations) calculated from 3 samples.

Table 4.1. Comparison of lipid saturation, phase transition temperature and half-life of vincristine release for liposomes of differing sphingomyelin composition.

Lipid Sample	Saturate/Unsaturated Ratio	T_c, °C	T ½ release, minutes
Bovine Milk Sphingomyelin	5.8*	34.2	6.4
Bovine Brain Sphingomyelin	27.33*	37.8	7.7
Egg Sphingomyelin	34.7*	40.8	28.9
Dihydrosphingomyelin	Completely saturated	46.0	60.8

* As noted from (Avanti Polar Lipids)

4.3.2 Increasing the drug-to-lipid ratio results in reduced leakage rates in DHSM/Chol LUVs

The preceding results indicate that vincristine release from DHSM/Chol LUVs is approximately 2 times slower than from ESM LUVs. Previous studies have shown that the release rate of vincristine from ESM/Chol LUVs is also highly sensitive to the D/L ratio employed, with formulations with higher D/L values exhibiting much slower drug release rates (Abraham et al. 2004a; Johnston et al. 2006). In order to demonstrate that similar behavior is also observed for DHSM-containing systems, the *in vitro* release properties of DHSM/Chol LUVs loaded to D/L ratios of 0.0265 to 0.14 (mol/mol) were compared. As shown in Figure 4.2, the $T_{1/2}$ for vincristine release increased from 29 min to more than 300 min as the D/L ratio was increased from 0.03 to 0.158 (mol/mol).

4.3.3 The use of DHSM and high D/L ratios produces additive reductions in vincristine leakage *in vivo*

The next set of studies was aimed at delineating the *in vivo* release properties of DHSM/Chol LUVs loaded with vincristine. As shown in Figure 4.3 for LUVs loaded with vincristine at a D/L ratio of 0.034 (mol/mol) and administered to outbred ICR mice at a drug dose level of 2 mg/kg, the substitution of DHSM for ESM in SM/Chol resulted in an increase in the $T_{1/2}$ for drug release from 8.9 to 16.6 h. Similarly, increasing the D/L ratio from 0.034 to 0.235 (mol/mol) in the DHSM formulation resulted in an increase in $T_{1/2}$ from 16.6 to 282 h (Figure 4.3). Because lipid dose can affect the clearance of liposomes from the circulation (Oja et al. 1996), empty DHSM/Chol liposomes were included with the loaded liposomes formulated at the high D/L ratio to achieve a constant lipid dose of 71 μmol/kg (40 mg/kg) for both formulations.

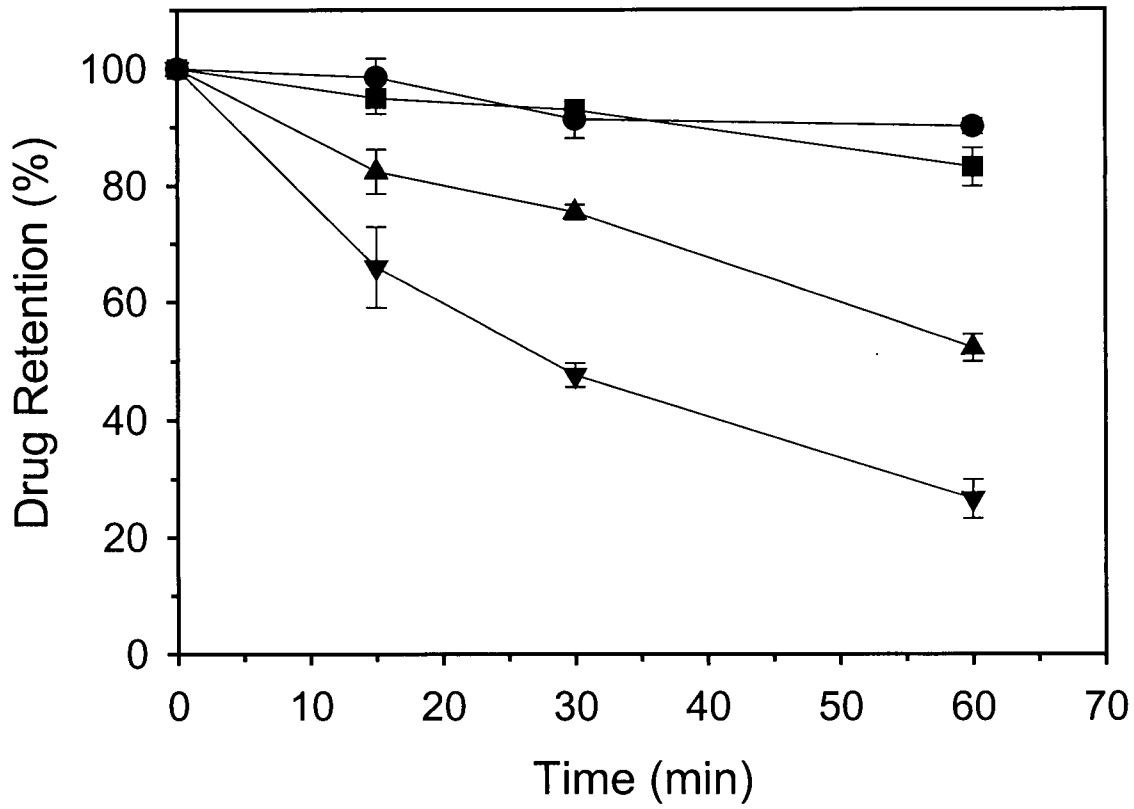


Figure 4.2. Vincristine *in vitro* retention for DHSM/Chol liposomal preparations in 2mM ammonium chloride at 50°C as determined for drug to lipid ratios (mol/mol) of 0.03(▼), 0.055 (▲), 0.099 (■) and 0.158 (●). The lipid concentration in the release assays was 1.25 mM total lipid. Data points represent mean drug retention (± standard deviations) calculated from 3 samples.

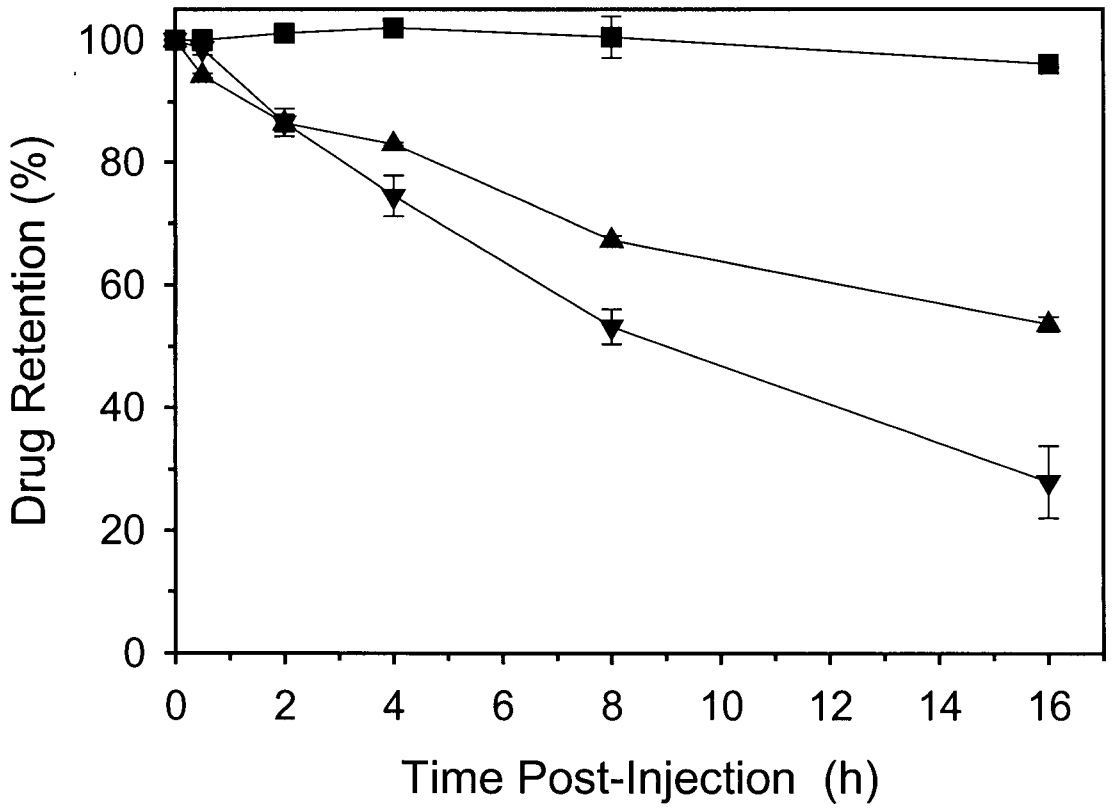


Figure 4.3. Vincristine retention in circulating liposomes composed dihydrosphingomyelin/cholesterol (55/45 mol%) and egg sphingomyelin/cholesterol (55/45 mol%) in mice. Drug-to-lipid ratios (mol/mol) of liposomal formulations of vincristine composed of DHSM/Chol were 0.034 (▲), 0.235 (■) and ESM/Chol at 0.035 (▼). Mice were dosed at 71 μmol/kg (40 mg/kg) total lipid and 2.4 μmol/kg (2 mg/kg) drug, data points represent mean drug retention (± standard deviations) calculated from 3 mice.

4.3.4 DHSM/Chol LUVs exhibit circulation lifetimes comparable with "Stealth" LUVs

In addition to drug retention properties, the circulation lifetime of liposomes following i.v. administration is an important parameter affecting the performance of liposome-drug formulations. This is because long-circulating systems accumulate more effectively at sites of disease such as tumor sites (Gabizon 1992), leading to improved therapeutic benefits. Liposomes of various lipid compositions usually exhibit dose dependent pharmacokinetics, with relatively rapid clearance observed at low lipid doses and much slower clearance at higher dose levels (Semple et al. 1998). The gold standard for LUVs with long circulation lifetimes is the so-called "Stealth" formulation (Cattel et al. 2003) that contains the PEG-lipid DSPE-PEG_{2000}, hydrogenated soy PC (HSPC) and cholesterol and exhibits *in vivo* blood clearance properties that are relatively dose-independent (Allen and Hansen 1991). The circulation lifetimes of liposomes composed of ESM/Chol (55/45 mol%), DHSM/Chol (55/45 mol%) and the "Stealth" composition (HSPC/Chol/DSPE-PEG_{2000}; 57.2/37.6/5.2 mol%) were therefore examined in female ICR mice by Inex Pharmaceuticals. Two lipid doses were employed. The low lipid dose corresponded to 15 μmol phospholipid/kg (8.4 mg lipid/kg for the SM-containing LUVs; 10.2 mg lipid/kg for the "Stealth" formulation) and the high dose to 150 μmol phospholipid/kg (84 mg lipid/kg for the SM-containing LUVs; 102 mg lipid/kg for the "Stealth" formulation). As shown in Figure 4.4, at the high lipid doses, all the formulations exhibited circulation half-times ($Tc_{1/2}$) values in the range 10-12 h. However, at the low dose levels, the DHSM/Chol LUVs and the "Stealth" LUVs exhibited comparable $Tc_{1/2}$ values in the range of 7-9 h, whereas the ESM/Chol formulation exhibited faster clearance properties ($Tc_{1/2}$ = 4.3 h).

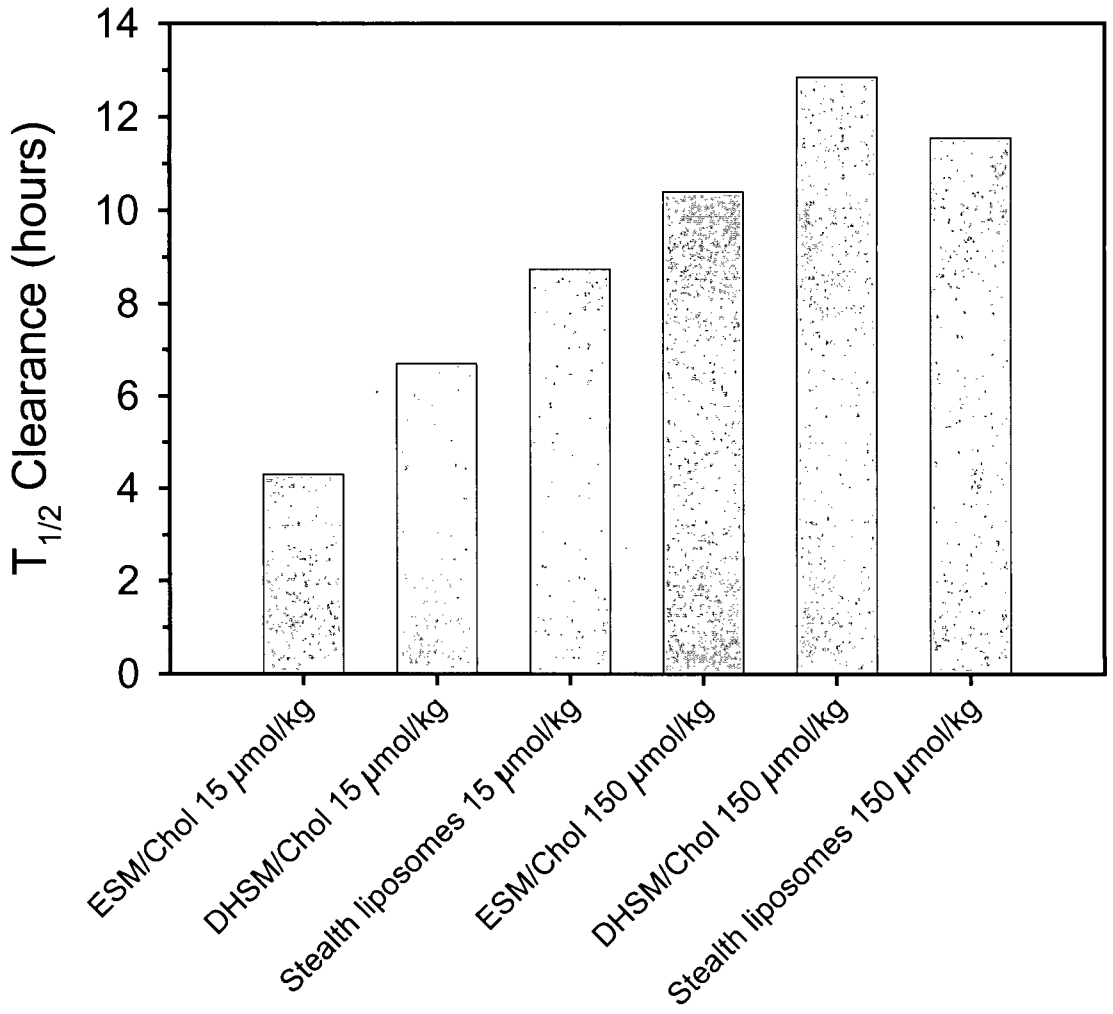


Figure 4.4. Lipid recovery in plasma from circulating liposomes formulated of ESM/Chol (55/45 mol%), DHSM/Chol (55/45 mol%) or stealth (HSPC/CH/DSPE-PEG_{2000} 57.2/37.6/5.2 mo%) liposomal formulations. Mice were dosed at either 15 µmol/kg or 150 µmol/kg, data points represent mean lipid recovery in plasma (± standard deviations) calculated from 2 separate from experiments.

4.3.5 DHSM/Chol LUVs exhibit significantly improved drug retention properties compared to "Stealth" LUVs

It is of obvious interest to compare the drug retention properties of "Stealth" LUVs and DHSM/Chol LUVs, particularly given the observation that the presence of PEG-containing lipids such as DSPE-PEG_{2000} in LUVs increases the rate of drug release *in vivo* (Webb et al. 1998b). This effect is illustrated in Figure 4.5A, where it is shown that the presence of 5.2 mol% DSPE-PEG_{2000} in ESM/Chol LUVs reduces the half-time for vincristine release from 29 min to 11.1 min employing the *in vitro* release assay where the drug release rates have been increased to experimentally convenient time frames by addition of 2 mM NH_4Cl. Previous work employing this assay has shown that the relative release rates determined between LUVs with different lipid compositions reflect the relative release rates observed *in vivo* (Johnston et al. 2006). The relative release rates between DHSM/Chol LUVs and "Stealth" LUVs are illustrated in Figure 4.5B, from which it may be seen that DHSM/Chol LUVs exhibit $T_{1/2}$ values for vincristine release that are considerably longer than those achieved by "Stealth" LUVs.

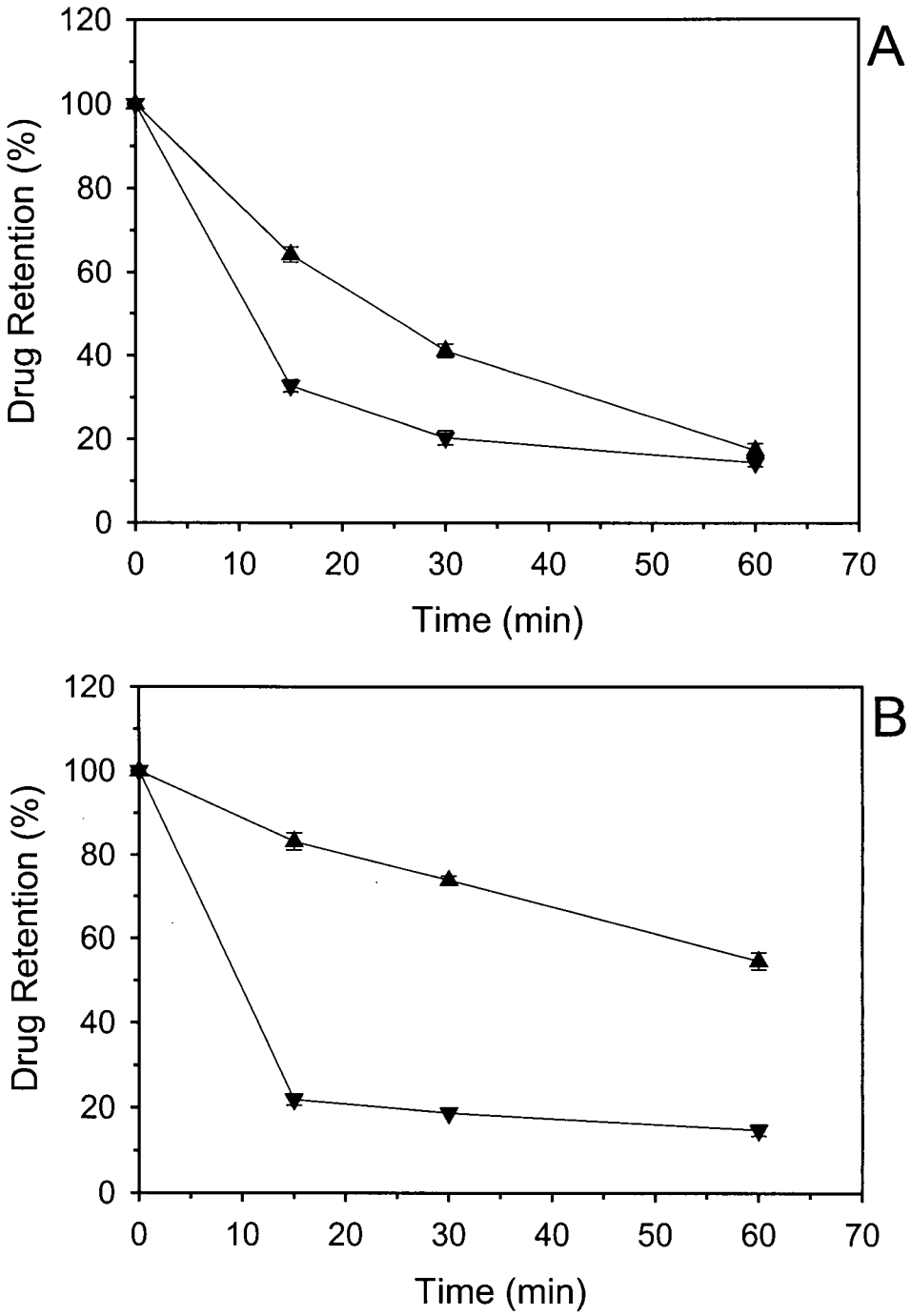


Figure 4.5. Comparison of vincristine *in vitro* retention for PEGylated and non-PEGylated liposomes in 2mM ammonium chloride at 50°C. Panel A; ESM/Chol (▲) and ESM/Chol containing 5.2 mol% DSPE-PEG$_{(2000)}$ (▼) at D/L ratios (mol/mol) 0.063 and 0.067 respectively. Panel B; DHSM/Chol (▲) and a PEGylated "stealth" liposome formulation (▼) at D/L ratios (mol/mol) of 0.06 and 0.042 respectively.

4.4 Discussion

The results of this study indicate that DHSM is a promising lipid for use in liposomal drug delivery applications due to its superior drug retention and circulation lifetime properties. Particular areas of interest concern the reasons why DHSM LUVs should exhibit such properties, the potential effects of the increased retention on the efficacy of liposomal drug formulations and the biocompatibility of liposomal systems composed of DHSM.

The improved drug retention properties of DHSM as compared to ESM indicate that the DHSM/Chol lipid bilayer provides an improved permeability barrier as compared to that provided by ESM/Chol bilayers. This is consistent with the fact that, as shown here and elsewhere (Kuikka et al. 2001), DHSM exhibits a higher gel-to-liquid crystalline transition temperature than SM, reflecting tighter intermolecular packing of DHSM as compared to SM. It is also consistent with the finding that DHSM exhibits a greater affinity for cholesterol than SM (Li et al. 1985; Epand 2003). The improvements in drug retention are significant, corresponding to two-fold increases in the half-times for drug release for DHSM/Chol LUVs loaded with vincristine as compared to ESM/Chol LUVs. In this context it is interesting that substitution of DSPC for DPPC in PC/Chol LUVs has little effect on vincristine release properties (Boman 1994). DSPC has a transition temperature of 54°C as compared to 42°C for DPPC. This large difference in transition temperature may be contrasted to the small difference in transition temperature between DHSM and ESM (~5°C), for which large gains in drug retention properties are observed. The transition temperatures of component lipids are important because, as demonstrated elsewhere (Boman et al. 1993), liposomes containing diarachidoynoyl PC (20:0/20:0 PC) or dibehenoyl PC (22:0/22:0 PC), which have transition temperatures of

75°C and 80°C respectively, are very difficult to extrude to form LUVs, and LUVs formed from these lipids tend to aggregate extensively (Boman et al. 1993). This contrasts with the behavior of the DHSM-containing LUVs, which are relatively straightforward to extrude and do not exhibit significant aggregation following extrusion.

Previous work has shown that certain drugs such as vincristine (Boman et al. 1993), vinorelbine (Semple et al. 2005) and ciprofloxacin (Maurer et al. 1998) are difficult to retain in LUVs composed of PC or ESM. The importance of reducing drug release rates is well known for vincristine, for which it has been shown that the anti-tumor efficacy of nine liposomal vincristine formulations with differing release and clearance rates correlates strongly with the circulation half-life of the drug (Boman et al. 1998). This relationship indicates that, for formulations with equivalent circulation lifetimes, the anti-tumor efficacy is directly proportional to the half-time for drug release *in vivo*. As a result it would be expected that a DHSM/Chol vincristine formulation could exhibit improved efficacy properties compared to LUVs with the "Stealth" composition.

The improved circulation lifetime properties of DHSM/Chol LUVs compared to ESM/Chol LUVs is consistent with the tight packing of the DHSM/Chol lipid bilayer noted above. Previous work has shown that the clearance rate of LUVs from the circulation is critically dependent on the amount of blood protein adsorbed to the LUVs surface (Chonn et al. 1992). The ability of proteins to adsorb onto lipid bilayer membranes is, in turn, dependent on the packing properties of the component lipids (in addition to other factors such as surface charge) (Semple et al. 1996), where proteins adsorb more readily to bilayers containing more unsaturated lipids. The ability of the PEG coating on "Stealth" LUVs to extend circulation lifetimes by inhibiting the binding of serum proteins has also been noted (Senior et al. 1991). It is also possible that DHSM-

containing LUVs have reduced adsorbed protein levels compared to SM systems due to reduced surface potentials of DHSM membranes (Kuikka et al. 2001).

A final point of discussion concerns the biocompatibility of DHSM/Chol LUVs, and, in particular, whether DHSM-containing systems could elicit toxic side effects. In this regard it has been shown that DHSM in multilamellar liposomes is readily degraded following i.v. injection (Schneider and Kennedy 1968), although, as indicated by the results presented here, such degradation does not result in undue drug leakage from DHSM/Chol LUVs over 24 h *in vivo*. It should also be noted that DHSM is a naturally occurring species of SM. For example, over 50% of the phospholipid associated with the human occular lens is DHSM (Li et al. 1985) and approximately 10% of the SM in cell plasma membranes is DHSM (Ramstedt et al. 1999). It is therefore unlikely that DHSM/Chol LUVs should exhibit any additional toxicity over SM/Chol LUVs.

In summary the results of this investigation show that DHSM exhibits improved properties of drug retention and circulation lifetime following i.v. injection as compared to SM-containing LUVs. Further, DHSM/Chol LUVs exhibit superior drug retention properties and comparable circulation lifetime properties as "Stealth" LUVs. As a result, it is likely that DHSM/Chol LUVs will be of considerable utility for the *in vivo* delivery of drugs that are usually hard to retain in liposomal delivery systems.

Chapter 5: Summary

In this thesis I have presented methods for regulating release of vincristine from liposomes, that for the first time demonstrates drug retention half-lives that are so great that *in vivo* efficacy is actual reduced at extreme retention half-lives. First, it was shown in Chapter 2 that increasing the drug-to-lipid ratio (wt/wt) for liposomal vincristine leads to a proportional increase in drug retention both *in vitro* and *in vivo*. Additionally, I have established that increasing drug-to-lipid ratio leads to a detrimental decrease in loading efficiency as the initial drug-to-lipid ratio is escalated. These results contradicted the conventional view of drug release, which states the half-life of release should be independent of the initial internal liposomal concentration. It was proposed that drug precipitation could account for these observations and this hypothesis was supported by cryo-transmission electron microscopy that showed gel-like or amorphous precipitates of vincristine at drug-to-lipid ratios of approximately 1.0 (wt/wt). Subsequent to these studies it was demonstrated that regulated release, brought about through varying the drug-to-lipid ratio, had a dramatic influence on the efficacy of liposomal vincristine in a human MX-1 xenograft model. It was determined that the therapeutically optimized halftime of drug release was in the range of 15.6 h.

Next, in Chapter 3 I determined that the drug-lipid ratio technique for regulating release of liposomal vincristine was also suitable for other liposomal drug formulations with the caveat that the encapsulated drug must be able to precipitate within the liposome. Additionally, it was shown that drug precipitation for liposomal formulations of vincristine and doxorubicin may cause a reduction in liposomal membrane integrity which could be responsible for the reduced loading efficiencies observed as the initial drug-to-lipid ratio is increased.

Finally in Chapter 4 I investigated the use of dihydrosphingomyelin (DHSM) as a method of prolonging drug retention. It was determined that liposomes formulated with hydrogenated sphingomyelin displayed enhanced drug retention both *in vitro* and *in vivo*. It was also shown that DHSM liposomes had similar circulation life times to PEGylated liposomes at equivalent lipid doses.

The studies presented here can function as a springboard for future research that can be taken in several directions. First, as both the release rate of encapsulated drug and the tumor doubling time will influence the level exposure of active chemotherapeutic agent to tumor cells it will be important to examine both a range of release rates for liposomal vincristine and doxorubicin and a range of tumor doubling times using a variety of *in vivo* efficacy models. Utilizing a variety of tumor lines will also provide broader activity profiles when determining an optimized liposomal formulation and will avoid model selection bias when altering formulation release rates.

Next, the techniques developed in this thesis for regulating drug release and optimizing liposomal drug formulations could be applied to other cell cycle specific anticancer drugs such as vinorelbine or topotecan. For vinorelbine, the maximum retention half-life *in vivo* that has been established is only 11 h at a drug-to-lipid ratio of 0.3 (wt/wt) (Semple et al. 2005). This poor retention as compared to vincristine at similar drug-to-lipid ratios ($T_{1/2}$ 117 h, D/L ratio 0.33 wt/wt), could be potentially due to vinorelbine's increased lipophilicity relative to other vinca alkaloids (Rahmani-Jourdheuil et al. 1994; Zhou et al. 1994; Zhigaltsev et al. 2005). From the studies by Semple and coworkers it is unknown if the maximum release of 11 h achieved is optimal. To identify the optimal release rate, increased vinorelbine retention would be required and could accomplished by loading at higher drug to lipid ratios or formulating with Dihydrosphingomyelin.

A similar scenario could be envisaged for topotecan (9-dimethylaminomethyl-10-hydroxy-camptothecin), a water soluble derivative of an alkaloid extracted from the stem wood of *Camptotheca acuminate* (Kong et al. 2003). Topotecan functions during S phase through reversibly stabilizing covalent complexes between the nuclear enzyme topoisomerase I and DNA, (Hsiang and Liu 1988; Hsiang et al. 1988; Kaufmann et al. 1997; Evans et al. 2004). This leads to irreversible double strand cytotoxic breaks in DNA leading to G2 cycle arrest and apoptosis (Evans et al. 2004). Due to topotecan's cell cycle dependent activity it would be expected that optimization of the release of the drug from liposomal formulations would provide enhanced *in vivo* efficacy. Abraham and coworkers have demonstrated that drug-to-lipid ratio influences the half-life of release *in vitro* for DSPC/Chol formulations of topotecan (Abraham et al. 2005). Other studies have shown that liposomal topotecan shows a 20 and 10-fold increase in anti-tumor efficacy over free drug in breast cancer and non small cell lung carcinoma models respectively (Semple, unpublished data), although drug release rates have not been optimized. As with vincristine studies correlating release rates and tumor growth rates need to be conducted using D/L ratio and DHSM to achieve optimized release rates. Because the vinca alkaloids, doxorubicin and topotecan have different mechanisms of action, comparisons between the three might give a better insight into mechanisms by which liposomal delivery systems enhance efficacy of conventional anticancer drugs and which type and class of drug would most benefit from liposomal delivery systems and optimization of release rate.

Finally, the ability to encapsulate large numbers of drug molecules at high drug-to-lipid ratios (D/L ratio > 1.0), techniques presented in this thesis might be of high utility to targeted drug delivery vehicles. This applies particularly to situations where the cell

surface receptors are limited. An example might be the $\alpha_v\beta_3$ integrin associated with tumor neovasculature that is internalized upon binding of an RGD containing ligand (Holig et al. 2004). Liposomes containing a cyclic RGD ligand and loaded with 5-fluorocil have already shown antitumor activity against B16F10 melanoma tumor bearing BALB/c mice (Dubey et al. 2004). It probable that a liposomal system possessing the same or similar targeting ligand and a powerful antineoplastic agent at extreme drug-to-lipid ratios may prove highly potent. As mentioned in Chapter 1 (section 1.6), targeting liposomal delivery systems can be accomplished through the use of antibodies, antibody fragments or peptide targeting ligands.

In conclusion, it has been shown in this thesis that drug release rates, both *in vitro* and *in vivo*, can be effectively regulated through varying the drug-to-lipid ratio or incorporation of dihydrosphingomyelin into liposomal vincristine formulations. Additionally, it is demonstrated that drug release rates of a cell cycle dependent antineoplastic agent can have a dramatic affect on efficacy for a human breast cancer xenograft tumor model. It is also shown that the drug-to-lipid ratio technique is effect for other liposomal formulations of anticancer drugs that precipitate in the liposome interior. The studies presented here will be of use in designing therapeutically optimized liposomal formulations for the treatment of malignancies.

Appendix 1

It is straightforward to show that the total number of vincristine molecules in an SM/Cholesterol liposome is given by the relation

$$N_v = \frac{8\pi R^2 (M_s/M_v + M_c/L_r M_v) W_r}{A_s(1 + A_c/L_r A_s)}$$

where:
N_v = # of vincristine molecules per liposome
R = radius of liposome
M_s = molecular weight of sphingomyelin
M_v = molecular weight of vincristine
M_c = molecular weight of cholesterol
L_r = mole ratio of sphingomyelin to cholesterol
W_r = weight ratio of vincristine to lipid
A_s = area per molecule of sphingomyelin
A_c = area per molecule of cholesterol

Using R= 50 nm, M_s= 703 (Avanti Polar Lipids), M_v=824 (Sigma 2005), M_c= 387 (Avanti Polar Lipids), L_r= 55/45, A_s= 0.6 nm^2 (Shipley et al. 1974), A_c=0.4 nm^2 (Grechishnikova et al. 1999) then for W_r=1 N_v= 83,700.

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