PHARMACOKINETIC CHARACTERIZATION OF TUMOR INFLUX AND EFFLUX OF LIPOSOMES BY RAPID REMOVAL OF CIRCULATING LIPOSOMES

by

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ABSTRACT

Studies in this thesis were aimed at developing a versatile strategy to study tumor influx and efflux pharmacokinetics of liposomes, and comparing these kinetic parameters between conventional and sterically stabilized lipid formulations under specified conditions.

This research effort was initiated following recently published studies that indicate the lack of benefits associated with steric stabilization of liposomal drug formulations. In these studies, an improvement in the plasma lipid pharmacokinetics was not translated into improved pharmacokinetics and pharmacodynamics in solid tumors. It was thought that a better understanding for liposomal accumulation in tumors can be gained by characterizing the individual influx and efflux kinetics of liposomes that contribute to the net flux, or accumulation, in solid tumors.

Avidin-induced cross-linking of biotinylated liposomes was utilized to rapidly deplete liposomes from the blood compartment, thereby allowing liposome tumor efflux and influx rates to be determined. In the conventional [1,2 distearoyl-sn-glycero-3-phosphocholine (DSPC)/cholesterol (Chol) (55:45 mol ratio)] liposomal system (100 nm in diameter), incorporation of 0.5 mol% N-((6 (Biotinoyl)amino)hexanoyl)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (Biotin-X-DSPE) into the formulation and administration of 50 μg neutravidin resulted in greater than 90% of plasma liposomal lipid (10 mg/kg and 100 mg/kg) removed within 1 hour in female CD-1 mice. This rapid

removal was achievable at 1, 4 and 8 hours post-injection of liposomes. Using the LS180 human colon carcinoma solid tumor xenograft model in SCID/RAG2 mice, the rapid lipid removal permitted characterization and determination of tumor influx and efflux rate constants, which were estimated to be 0.022 $hour^{-1}$ and 0.041 $hour^{-1}$ respectively when neutravidin was injected 4 hours after liposome injection. Therefore, it appears that DSPC/Chol liposomal accumulation, in LS180 solid tumor is dictated primarily by plasma liposome concentrations and liposome rate constant is higher for efflux than influx into the tumor.

Rapid elimination (>90% plasma lipid removed within 1 hour) of sterically stabilized or PEGylated [DSPC/Chol/1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[Poly(ethyleneglycol)2000] (PEG_{2000}-DSPE) 5%] liposome systems (100 nm in diameter, at 10 mg/kg and 100 mg/kg lipid dose) in female balb/c mice was achieved with 100 μg of avidin and a double biotinylated system formulated with 0.5 mol% biotin-X-DSPE and 0.5 mol% N-[w-(Biotinoylamino)poly(ethyleneglycol)2000]1,2-distearoyl-sn-glycero-3-phosphoethanolamine (Biotin-PEG_{2000}-DSPE). Using the LS180 solid tumor xenograft model in SCID/RAG2 mice, the rapid lipid removal led to characterization of tumor influx and efflux rate constants, which were estimated to be 0.062 $hour^{-1}$ and 0.011 $hour^{-1}$ respectively when avidin was injected 4 hours after liposome injection. Therefore, it appears that DSPC/Chol/PEG_{2000}-DSPE 5% liposomal accumulation, in LS180 solid tumor is dictated primarily by plasma liposome concentrations and liposome tumor influx rate constant is greater than efflux rate constant.

Comparisons between conventional and sterically stabilized formulation in our studies show that PEGylation results in favourable tumor influx and efflux pharmacokinetics. The question related to the conflicting data of PEGylation arising from the few studies still remains unanswered. Nevertheless, the present thesis introduces a novel methodology that allows characterization of the detailed tumor accumulation properties of liposomes, and the application has the potential to be fully utilized to characterize the impact of various liposome parameters such as dose, composition, and size on the tumor accumulation kinetics.

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ABBREVIATIONS

AUC	Area Under the Curve
Biotin-X-DSPE	N-((6 (Biotinoyl)amino)hexanoyl)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine
Biotin-PEG_{2000}-DSPE	N-[w-(Biotinoylamino)poly(ethyleneglycol)2000]1,2-distearoyl-sn-glycero-3-phosphoethanolamine
CHE	cholesteryl hexadecyl ether
Chol	cholesterol
DSPC	1,2 distearoyl-sn-glycero-3-phosphocholine
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane-sulphonic acid
HBS	HEPES buffered saline
i.v.	intravenous
k_1	tumor influx rate constant
k_{-1}	tumor efflux rate constant
LUV	large unilamellar vesicles
MLV	multilamellar vesicles
mol	mole
MPS	mononuclear phagocyte system
PEG_{2000}-DSPE	poly(ethyleneglycol)2000
r^2	coefficient of determination
RES	reticuloendothelial system
s.c.	subcutaneous
S.D.	standard deviation
SUV	small unilamellar vesicles
Tc	gel-liquid crystalline phase transition phase
Vd	volume of distribution

CHAPTER 1

INTRODUCTION

1.1 Overall Objectives of the Project

The objectives of the present thesis were 1) to develop a versatile method to rapidly eliminate liposomes from the circulation, 2) to utilize the rapid clearance strategy to study and characterize the pharmacokinetics of tumor influx and efflux of liposomes, and 3) to compare the influx and efflux kinetics between conventional and sterically stabilized lipid formulations

1.2 Liposomes

The structure of aqueous dispersions of lipids, or liposomes was first documented by A. Bangham (Bangham *et al.*, 1965). Many lipids adopt a bilayer configuration consisting of concentric lipid bilayers separated by aqueous channels. The resulting configuration arises due to the amphipathic nature of the lipid; the hydrophilic head group and hydrophobic tail of the lipid molecule orient themselves such that the head groups face the aqueous environment and the fatty acyl chains are oriented toward one another. In general, liposomes are made up of phospholipids and cholesterol.

1.2.1 Lipids: Building Blocks of Liposomes

1.2.1.1 Phospholipids

Phospholipids can exhibit different headgroups (hydrophilic regions) and different acyl chain compositions (hydrophobic regions) which dictate the physical properties of the lipid (Seelig, 1978; Cullis and de Kruijff, 1979). The presence of negatively charged phospholipids will control the membrane surface charge of the resulting liposomes. Liposomes with phosphatidylserine and phosphatidic acid are rapidly eliminated from the circulation following intravenous (i.v.) administration, in part because of serum protein binding effects attributed to the negative charge at physiological pH (Moghimi and Patel, 1989). In addition to defining surface charge, the headgroup can play a critical role in determining whether an isolated phospholipid can assume a bilayer configuration after hydration. For example, phosphatidylethanolamines with two unsaturated acyl chains will assume a structure referred to as the hexagonal phase instead of a bilayer structure (Cullis and de Kruijff, 1979). This phase is characterized by cylinders of lipids in an "inverted" (headgroups oriented inwardly towards aqueous channels) organization. Depending on physiological conditions such as temperature and pH, other phospholipids can also adopt non-bilayer structures.

In addition to the importance of phospholipid head group charge, the acyl chain composition of the phospholipid can affect the characteristics of the liposomes. A key property of phospholipids is the temperature of the gel to liquid-crystalline phase

transition (Tc) which is dependent on the length and saturation of the acyl chains (McElhaney, 1982; Wang *et al.*, 1997; Huang *et al.*, 1993). Below the Tc, the acyl chains have a high degree of order and the chains are packed in a frozen or "gel" phase where motion of the chains is restricted. At temperatures above the Tc, the acyl chains are more fluid and less ordered in a "liquid-crystalline" phase. Typically, longer, more saturated acyl chains have increased order and higher Tc whereas shorter unsaturated acyl chains disrupt packing and reduce the acyl chain order of the membrane. Therefore, in general, increased unsaturation or shorter acyl chains have been correlated with increased membrane permeability (Papahadjopoulos *et al.*, 1973) and membranes are more permeable to a variety of solutes at or above the Tc (Bittman and Blau, 1972).

1.2.1.2 Cholesterol

In addition to phospholipids, another commonly used lipid in the preparation of liposomes is cholesterol. It is the major neutral lipid component in the plasma membrane of eukaryotic cells and also possesses amphipathic characteristics due to the presence of the polar 3-B-hydroxyl group at one end of the rigid steroid ring. Within a phospholipid bilayer, cholesterol orients itself with the hydroxyl group toward the lipid/water interface and the rigid steroid ring associated with the acyl chains (Huang, 1977). The consequences of incorporating cholesterol in a phospholipid bilayer include: 1) a decrease in the enthalpy of the gel to liquid-crystalline transition (Hubbell and McConnel, 1971); 2) a decrease in the membrane permeability (Demel and de Kruyff, 1976; Fielding and Abra, 1992); and 3) stabilization of liposomes for systemic delivery of

drugs and thus an increase in the circulation lifetime of the carrier (Kirby *et al.*, 1980; Patel *et al.*, 1983). The *in vivo* stabilizing role of cholesterol may be attributed to the reduction of serum protein binding observed at cholesterol levels greater than 30 mol% in the liposome formulation (Patel *et al.*, 1983; Semple *et al.*, 1996)

1.2.2 Liposome Preparation

In order to form a liposome, the component lipids must be able to adopt a bilayer structure upon hydration. In general, liposomes are classified in three categories: multilamellar liposomes (MLVs), large unilamellar liposomes (LUVs) and small unilamellar liposomes (SUVs). The classification is primarily assigned on the basis of size and lamellarity (number of bilayers present within a liposome) characteristics.

1.2.2.1 Multilamellar Vesicles (MLVs)

Upon hydration of lipids, MLVs are formed. These liposomes have an appearance similar to an onion, in that they consist of numerous concentric phospholipid bilayers. They are heterogeneous in size and lamellarity, typically exhibiting diameters ranging from 1-10 microns. MLVs have shown to be of limited value for pharmaceutical applications since they are rapidly eliminated from the plasma following injection due to their large size (Rahman *et al.*, 1982). In addition, MLVs tend to have a low trapped volume due to close packing of the bilayers, thereby limiting the amount of drugs to be encapsulated (Perkins *et al.*, 1988).

1.2.2.2 Unilamellar Vesicles (SUVs and LUVs)

Small unilamellar vesicles (SUVs) range in size from 25-50 nm in diameter and are produced by sonicating MLVs or by forcing MLVs under high pressure through small openings (Barenholz *et al.*, 1979). Vesicles of this size have extremely low trapped volumes (Barenholz *et al.*, 1979) and also, the acute radius of curvature of these vesicles can create instability, resulting in spontaneous fusion to form larger structures (Parente and Lentz, 1984). These properties thus make SUVs less useful as drug carriers.

LUVs exhibit a mean diameter of between 50 and 400 nm and the majority of the vesicles consist of one bilayer enclosing an aqueous space. The most frequently utilized procedure in preparation of LUVs involves extruding MLVs through stacked polycarbonate filters with defined pore size under high pressure (Olson *et al.*, 1979; Hope *et al.*, 1985; Mayer *et al.*, 1986). LUVs are most suitable for pharmaceutical applications due to their relatively high trapped volumes and favourable pharmacokinetic characteristics.

1.3 Rationale for Liposome Use in Delivery of Chemotherapeutics to Solid Tumors

A number of properties make liposomes versatile drug carriers for many agents. They are made up of lipids which have low intrinsic toxicity, are biodegradable, and of quite low antigenicity, and they can be formulated in a large range of sizes and chemical composition. In addition, their unique structural properties allow drugs to be entrapped

in the internal aqueous phase or in the lipid bilayers, depending on their degree of hydrophilicity.

One attractive application of liposomes is for cancer chemotherapy, since most anticancer drugs are quite toxic to normal as well as to tumor cells. If the anticancer drug is delivered exclusively to the cancer cells, the clinically effective dosage can be administered without the production of toxic side effects. Liposomes have the ability to alter the pharmacokinetics of their associated drugs, resulting in dramatic effects on both the efficacy and toxicity of the drugs (Gabizon *et al.*, 1982; 1990; Herman *et al.*, 1983; Profitt *et al.*, 1983; Cullis *et al.*, 1987; Mayer *et al.*, 1990; 1993; Papahadjopoulos *et al.*, 1991; Bally *et al.*, 1994; Gabizon and Martin, 1997; Northfelt *et al.*, 1997; Vail *et al.*, 1997).

Regions of solid tumor growth have capillaries with increased permeability because of the disease process (eg. angiogenesis) (Folkman, 1985; Dvorak, 1988; 1991). The gaps in the endothelial layers can range in size from 30nm for fenestrated capillaries to >500nm in disease sites (Jain, 1987; Dvorak, 1991; Yuan *et al.*, 1995; Gabizon and Martin, 1997). Therefore, drug-containing liposomes, which are usually formulated in size of 100nm, are able to localize in these regions more than in normal tissues, which have intact capillaries that are essentially impermeable to liposomes (Mayer *et al.*, 1989; Wu *et al.*, 1993; Yuan *et al.*, 1994). Once the liposomal drug has localized in a solid tumor, the mechanism of action for most liposomal drugs appears to be associated with a gradual release of the drug from the liposomes, followed by uptake of the free drug by

the tumor cells (Mayer *et al.*, 1989; Horowitz *et al.*, 1992; Vaage *et al.*, 1994, 1997; Yuan *et al.*, 1994; Siegal *et al.*, 1995; Lim *et al.*, 1997). Because liposomes and their associated drugs are confined principally to the vasculature (central compartment) and tissues with increased vascular permeability, the volume of distribution (Vd) is dramatically lower than that for unencapsulated drug, which is widely distributed in the body (Cowen *et al.*, 1993; Gabizon *et al.*, 1993; 1994; Working and Dayan, 1996). The reduction in Vd results in decreased distribution of the liposomal drugs to tissues with normal (i.e. nonleaky) vasculature. The reduction thus gives rise to a lower toxicity profile of the liposomal drug relative to the free drug.

1.4 Pharmacokinetics of Liposomes

The circulation lifetime of liposomes is determined by parameters such as their size and composition of the formulation (Abra and Hunt, 1981; Allen *et al.*, 1989, 1992; Mayer *et al.*, 1989; Allen and Stuart, 1999). For example, smaller liposomes have slower removal rates than larger liposomes (Abra and Hunt, 1981; Hwang, 1987; Senior *et al.*, 1987; Allen and Stuart, 1999). This is why liposomes are often prepared in the range of 100 nm in diameter which leads to long circulation lifetime and thus gives the liposomes sufficient time to extravasate through the more leaky tumor neovasculature (Senior, 1987; Allen *et al.*, 1989). In the present thesis, the words "clearance", "elimination" and "removal" are used interchangeably and the word "clearance" is not to be interpreted as the usual pharmacokinetic terminology. The principal site of clearance of liposomes and their associated drugs from the blood is the mononuclear phagocyte system (MPS), also

often referred to as the reticuloendothelial system (RES), comprised principally of Kupffer cells in the liver and fixed macrophages in the spleen, although bone marrow and lymph node uptake also occur (Gregoriadis, 1976; Allen and Cleland, 1980; Hwang, 1987; Chonn *et al.*, 1992; Allen *et al.*, 1995; Liu *et al.*, 1997). In general, the liver exhibits the largest capacity for liposome uptake; however, the spleen accumulates the highest tissue concentration of liposomes (liposomal lipid/gram tissue). The extent of liposome accumulation in the lungs is typically below 1% of the injected dose. After intravenous administration, plasma proteins (opsonins) are adsorbed onto the surface of liposomes, triggering recognition and liposome uptake by RES through receptors such as the complement C3b receptor, the Fc receptor and others (Aragnol and Leserman, 1986; Chonn *et al.*, 1992). The RES is an obstacle to effective drug delivery due to the resulting low circulation times of certain liposomal drugs, thus reducing the chance for the carriers and drugs to accumulate in non-RES tissues such as tumors (Briele *et al.*, 1990; Presant *et al.*, 1994). This is particularly the case for conventional liposomes (Patel *et al.*, 1983; Gabizon *et al.*, 1997).

1.4.1 Conventional Liposomes

Conventional liposomes are typically composed of only phospholipids (neutral and/or negatively charged) and/or cholesterol. They are defined to be those with "naked" phospholipid surfaces, not containing other surface-grafted components which function to sterically protect the liposome surface (Bally *et al.*, 1998). Depending on lipid composition, conventional liposomes may display increased leakage of entrapped drugs

in the circulation and a relatively short blood circulation time. Up to 70% of a dose of conventional liposomes may be found in RES tissues within a few minutes of injection (Gabizon, 1994; Gabizon *et al.*, 1997; Allen and Stuart, 1999). The pharmacokinetics of conventional liposomes can also be nonlinear, being characterized by a pronounced dependence on dose (Abra and Hunt, 1991; Allen and Hansen, 1991). Clearance of these liposomes decreases with increasing dose. The clearance of conventional liposomes at low doses, after i.v. administration, is biphasic (Juliano and Stamp, 1978; Juliano *et al.*, 1978), and with increasing dose the initial phase of clearance gradually disappears and the clearance becomes primarily log-linear at high doses with the slow phase of clearance dominating the pharmacokinetics (Allen and Hansen, 1991). The pharmacokinetics can be described mathematically by a two-compartment open model where the rapid initial phase of clearance is thought to reflect distribution into the MPS (Allen and Stuart, 1999). Disappearance of the initial phase with increasing dose reflects saturation of MPS uptake mechanisms (Abra and Hunt, 1981; Bosworth *et al.*; 1982; Allen and Hansen, 1991). The second slower phase of elimination from the blood is hypothesized to reflect recycling of MPS binding sites and/or recruitment of new MPS cells (Allen and Hansen, 1991; Allen and Stuart, 1999).

1.4.2 Sterically Stabilized Liposomes

The development of sterically stabilized liposomes increases the versatility of liposomes as drug carriers and enhances the therapeutic index of some encapsulated agents (Allen *et al.*, 1991, 1992, 1995; Woodle and Lasic, 1992; Oku, 1994; Torchilin *et al.*, 1994, 1995;

Needham *et al.*, 1999) . A common sterically stabilized formulation involves grafting of amphipathic polyethyleneglycol (PEG) to the liposomal surface, which then confers steric stabilization of the liposomes and reduces recognition and uptake by the RES (Allen *et al.*, 1991; Gabizon and Martin, 1997; Parr *et al.*, 1997; Needham *et al.*, 1999). The molecular weight of PEG for achieving long circulation times is in the range of 1000 to 5000 Da (2000 Da PEG is considered optimum), at concentrations ranging from 3-10 mol% (5-7 mol% is considered optimum) of phospholipids (Lasic *et al.*, 1991; Allen, 1994). The pharmacokinetics differs from that of conventional liposomes, being primarily log-linear (monophasic) and dose-independent over a wide dose range (Allen *et al.*, 1995). The elimination profile is described at first approximation by a single compartment open model (Allen *et al.*, 1995). The presence of PEG is hypothesized to attract a water shell to the surface, resulting in a decrease in the adsorption of protein opsonins onto the liposomes (Senior *et al.*, 1991; Torchilin *et al.*, 1995). This in turn results in a decrease in both the rate and extent of uptake by the RES, and results in the prolonged circulation and reduced clearance of the liposomes. The longer half-life in the circulation means that the liposomes will have more opportunity to traverse the tumor capillaries, increasing tumor accumulation of the drug and thus its therapeutic effect (Vaage *et al.*, 1993; William *et al.*, 1993; Yuan *et al.*, 1994; Siegal *et al.*, 1995; Gabizon *et al.*, 1996; Goren *et al.*, 1996; Sakakibara *et al.*, 1996). The reduction in the Vd compared to unencapsulated drug and conventional formulation is also more pronounced with PEGylated system (Gabizon *et al.*, 1993, 1994). In light of the reduction in Vd and sudden peak plasma level, reduced toxicity is also observed for many sterically stabilized liposome drug formulations.

1.5 Steric Stabilization Dilemma

Although steric stabilization technology has clearly been able to provide increased circulation lifetimes of liposomal anticancer agents, the design of liposomes that will exhibit maximal extravasation to tumor sites is an area of some controversy. Many studies have shown that PEGylation provides higher tumor accumulation and therapeutic effect compared to conventional (PEG-free) formulations. It has generally been assumed that prolonged circulation times of the liposomes will lead to increased accumulation of liposomes in the extravascular disease sites. However, conflicting results from recent studies question the beneficial effects from PEGylation (Mayer *et al.*, 1997; Parr *et al.*, 1997; Hong *et al.*, 1999). In these studies, high plasma levels of PEGylated liposomes did not give rise to an increased extravasation and accumulation in solid tumor tissue, and therapeutic effect was only comparable to conventional formulations. These observations raise the need to more carefully characterize the pharmacokinetics behind liposomal accumulation in tumors, since it appears that inclusion of PEG may decrease the efficiency of liposome extravasation from the blood into tumor tissue, or alternatively increase the rate of egress from the tumor (Du *et al.* 1997; Parr *et al.*, 1997; Hong *et al.*, 1999; Mayer *et al.*, 2001).

1.6 Rationale and Hypothesis of Thesis Project

Liposomes have been widely employed as carrier systems for anti-cancer drugs and other agents. It is well established that liposomes accumulate preferentially in disease sites,

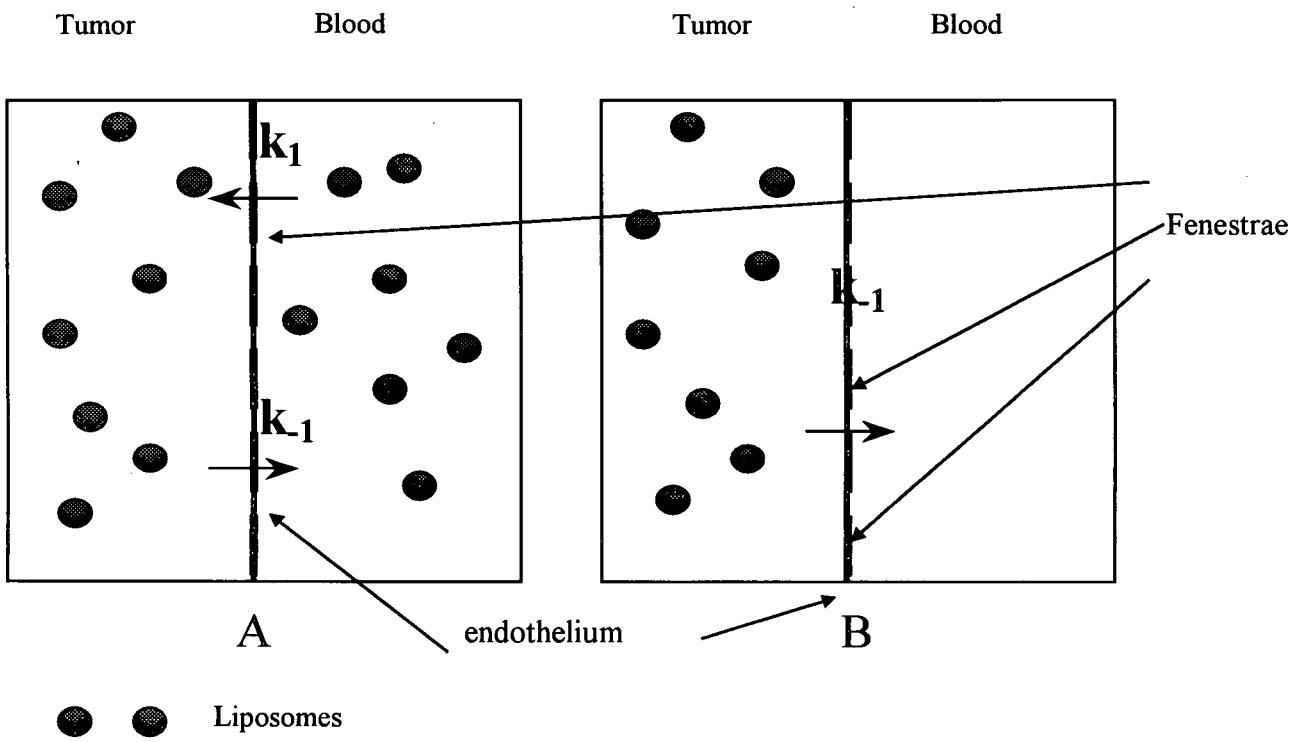
including tumors (Profitt *et al.*, 1983; Mayer *et al*, 1990; Allen *et al.*, 1991) due to increases in tumor blood vessel permeability (Folkman, 1985; Dvorak *et al.*, 1991). However, little is known about the detailed kinetics associated with liposome accumulation in tumors. The aim of our work is to develop methods to study tumor influx and efflux of liposomes since accumulation levels in tumors reflect a combination of these two processes. These processes are simultaneously taking place during tumor accumulation (Figure 1.1); therefore, it is difficult to study each one independently. However, we postulate that rapid removal of circulating liposomes can allow investigation of tumor efflux alone as it eliminates the otherwise interfering influx factor (Figure 1.1). We propose that the accumulated liposomes will leave the tumor tissue upon clearance of circulating liposomes in a first order manner and once these liposomes re-enter the blood compartment, they will be rapidly diluted and consequently their contribution to tumor influx will be negligible. It may be expected that the tumor accumulation pharmacokinetic behaviour of liposomes will depend on parameters such as dose, size, and lipid composition since liposomes of different parameters may enter and exit the tumor at different rates. Consequently, we propose that understanding the influence of individual liposome properties on tumor influx and efflux kinetics will greatly enhance our ability to optimize formulations designed for specific tumor applications.

In order to achieve rapid removal of circulating liposomes, the biotin-avidin interaction will be exploited (Green, 1963; Gitlin *et al.*, 1987). Biotin is a water-soluble vitamin that is found in various tissues which can bind to any one of the four available binding sites

Figure 1.1

Influx and efflux contribution of liposomal accumulation

During tumor accumulation of liposomes, both influx and efflux take place (A), but rapid clearance of circulating liposomes allows the isolation of a single kinetic variable-tumor efflux (B).



$$\frac{d[lipo_{tumor}]}{dt} = k_1[lipo_{blood}] - k_{-1}[lipo_{tumor}]$$

$$\lim[lipo_{blood}] \rightarrow 0,\ k_1 \rightarrow 0$$

$$\frac{d[lipo_{tumor}]}{dt} = -k_{-1}[lipo_{tumor}]$$

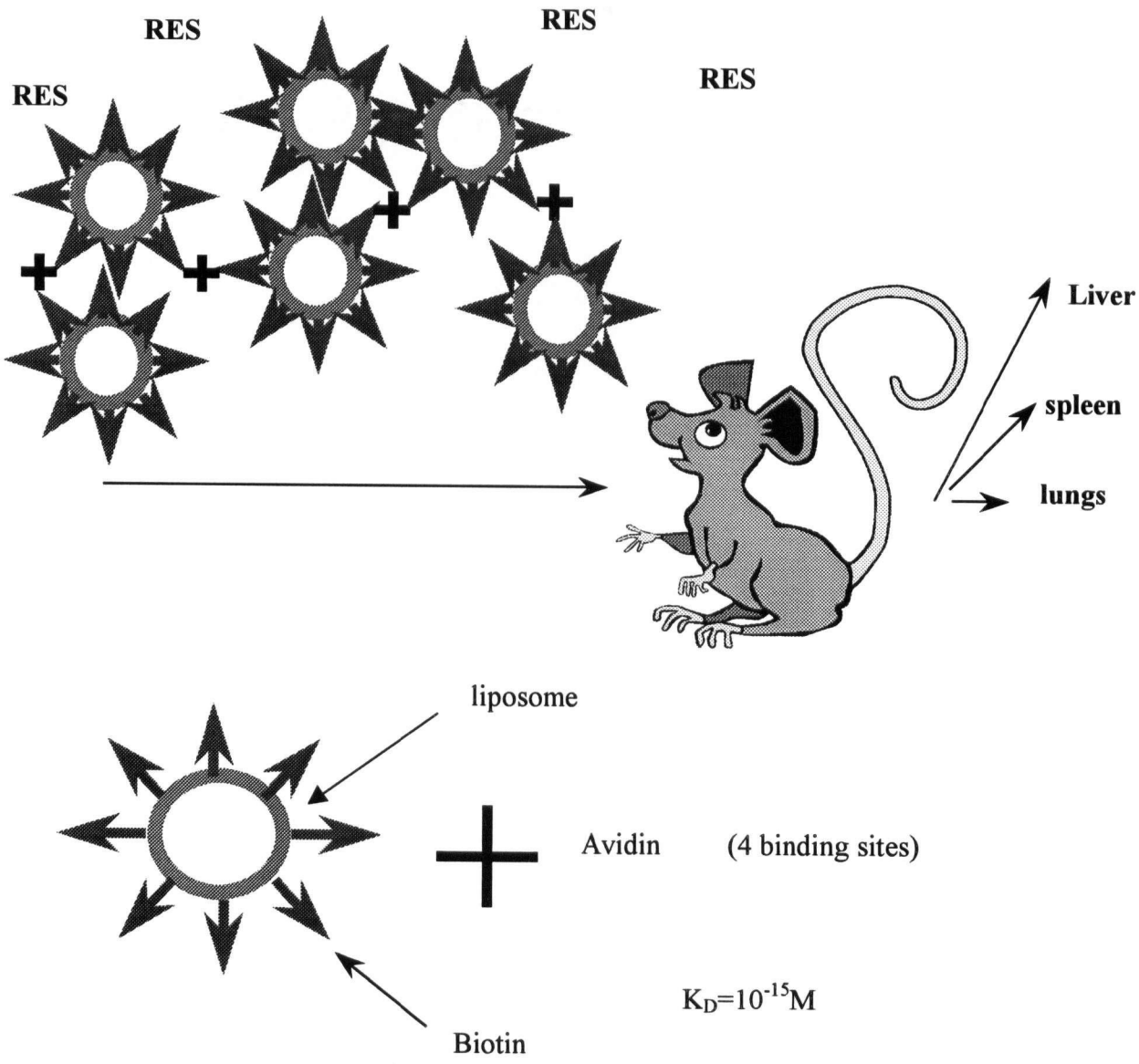
on avidin, a protein commonly found in raw egg white (Green, 1963; Gitlin *et al.*, 1987). The binding interaction between biotin and avidin is virtually irreversible, and is characterized by strong association affinity ($K_d=10^{-15}$ M) (Green, 1963; Gitlin *et al.*, 1987). By incorporating biotin-derivatized lipids into the formulation and exposing the biotin moiety on the surface of liposomes, we anticipated that liposomal aggregates would form when avidin is administered (Figure 1.2).

The large aggregates should induce rapid clearance of circulating liposomes, since liposomal clearance follows size-dependent pharmacokinetics, as explained in section 4 of this chapter with larger aggregates being removed more rapidly than smaller ones. This approach has actually been previously utilized to improve radio-imaging for rapid diagnostic purposes (Ogihara-Umeda *et al.*, 1993, 1997). However, we will attempt to apply the rapid clearance system in the LS180 human colon carcinoma solid tumor xenograft model to characterize tumor accumulation kinetics of liposomes once rapid clearance is achieved. The two key components of this project are as follows:

1) Appropriately formulated biotin-containing liposomes will be rapidly cleared from the blood compartment upon systemic administration of avidin
2) The rapid clearance method can be used to characterize tumor efflux behaviour, and to determine tumor influx and efflux rate constants of liposomes

Figure 1.2

Uptake of aggregated liposomes to organs of the reticuloendothelial system



Two formulations were examined in the present study: 1) conventional, PEG-free liposome and 2) PEGylated liposome. Due to conflicting results from recent studies that question the benefits of PEGylation on liposomal tumor accumulation, we hypothesize that PEG grafted to the liposomal surface may result in less favourable kinetics of tumor influx and/or efflux of the formulation compared to conventional PEG-free liposomes (eg. reduced influx).

1.7 Summary of Specific Research Objectives

The following steps were taken in order to meet the overall objectives of the research project carried out in this thesis:

1. *To develop a versatile biotinylated liposome system that maintains high affinity avidin-binding properties in vitro and in vivo*

   Biotinylated liposomes were prepared using the extrusion method, and were formulated by incorporating the biotin-derivatized lipids in the formulation. The formulation was tested to determine if it binds to avidin *in vitro*. It was then taken to *in vivo* experiments where its administration to mice was followed by introduction of avidin. *In vitro* and *in vivo* observations were compared.

2. *To achieve rapid plasma clearance of biotinylated liposomes of different doses, different liposomal accumulation times and different formulations*

Rapid clearance was defined as mean clearance of greater than 90% of liposomes from the central blood compartment within 1 hour after avidin administration. The combination of the amount of biotin to be incorporated into the formulation and the dose of avidin to be administered was examined in order to generate a system that results in rapid clearance of the liposomes from the circulation. Different variables were also studied in order to evaluate the versatility of the application. Both conventional and PEGylated formulations were studied. Under each formulation, different doses of lipids were tested (up to 100 mg/kg) in *in vivo* studies. In addition, various liposomal accumulation times were evaluated where liposomes will be allowed to accumulate at different times before avidin is introduced.

3. *To characterize tumor efflux behavior of liposomes in tumor-bearing mouse models and to determine tumor influx and efflux rate constants*

Once the rapid clearance was established, we applied it to characterize tumor accumulation behaviour in the LS180 tumor-bearing mouse model. Avidin was injected at a specified time after liposomal accumulation in tumor. Liposomes were cleared rapidly from the circulation, and tumor efflux behaviour was subsequently monitored over time. An efflux profile or graph was obtained, and appropriate equations and mathematical calculations were applied to determine tumor efflux rate constants. This enabled the influx rate constants to be estimated

based on the observed plasma liposome concentrations, tumor liposome accumulation levels and the calculated liposome efflux rate constant.

4. *To compare tumor accumulation pharmacokinetics of conventional and PEGylated lipid formulations.*

All the above were carried out with PEGylated liposome formulations in order to establish whether increased tumor accumulation typically observed for such sterically stabilized liposomes arises from altered tumor influx and/or efflux rate constants compared to conventional, PEG-free liposomes.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

1,2 Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL). N-((6 (Biotinoyl)amino)hexanoyl)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (Biotin-X-DSPE) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[Poly(ethyleneglycol)2000] (PEG_{2000}-DSPE) and N-[w-(Biotinoylamino)poly(ethyleneglycol)2000]1,2-distearoyl-sn-glycero-3-phosphoethanolamine (Biotin-PEG_{2000}-DSPE) were obtained from Northern Lipids (Vancouver, BC). [^{3}H] cholesteryl hexadecyl ether (CHE), a lipid marker that is not exchanged or metabolized *in vivo* (Stein *et al.*, 1980) was from Amersham (Oakville, ON). N-2-hydroxyethylpiperazine-N-2-ethane-sulphonic acid (HEPES), hydrogen peroxide and streptavidin magnetic beads (S2415) were purchased from Sigma Chemical Company (St. Louis, MO). Neutravidin and avidin were obtained from Pierce Chemical (Rockford, IL). Solvable™ was from NEN Research Products (Dupont Canada, Mississauga, ON). LS180 cells (a human colon carcinoma cell line) were purchased from the ATCC (Manassas, VA) and maintained in culture. Female CD-1 and balb/c mice (20-23g) were purchased from Charles River Laboratories (St. Constant, PC). Male SCID/RAG2 (25-26g) mice were bred at the British Columbia Cancer Agency Animal Breeding Facility (Vancouver, Canada).

2.2 Preparation of Liposomes

Biotinylated liposomes were prepared using extrusion technology (Hope *et al.*, 1985). Lipids were dissolved in chloroform, and trace amount of [^{3}H] cholesteryl hexadecyl ether was added before drying down the mixture to a homogenous lipid film under a stream of nitrogen gas. This resulting lipid film was placed under high vacuum overnight to remove any residual chloroform. Subsequently, the lipid film was hydrated in 20 mM HEPES/150 mM NaCl buffer (pH 7.5) (HBS) at 65 °C to a final lipid concentration of 100 mg/ml to form multilamellar vesicles. The resulting mixture was frozen in liquid nitrogen and thawed five times (Mayer *et al.*, 1986) followed by extrusion through two stacked 0.08 μm pore size polycarbonate filters (Nuclepore, Pleasanton, CA) using an extrusion device (Northern Lipids, Vancouver BC). The size of the liposomes was determined by quasielastic light scattering using a Nicomp 270 submicron particle sizer (Pacific Scientific, Santa Barbara, CA) operating at 632.8 nm. The mean diameter of these liposomes was 100 nm±10 nm.

2.3 *In Vitro* Aggregation

Liposomes (1 mg) of 100 nm in diameter and neutravidin or avidin (25 μg) were incubated in a total volume of 750 μl HBS in 1.5-ml eppendorf tubes for 45 minutes at 37°C. Aliquots were removed and the size of liposomes was determined by quasielastic light scattering at 5, 15, 30, and 45 minutes.

2.4 Streptavidin Magnetic Bead Test

The commercially available beads are coated with streptavidin which is a protein produced by the bacterium *Streptomyces avidinii* and has a structure similar to avidin and exhibits lower non-specific binding (Chaiet and Wolf, 1964). Liposomes (500 nmol) were incubated with 100 µl of streptavidin coated magnetic beads (S2415) for 15 minutes in a total volume of 200 µl at room temperature. The resulting mixture was placed under a magnetic field tilted at 45° for 2 minutes. The supernatant of unbound liposomes was withdrawn, leaving the bound liposomes with the streptavidin magnetic beads. The mixture was washed again with 100 µl HBS, and the steps were repeated twice. In the final step when the supernatant was withdrawn, 100 µl of HBS was added to the mixture of beads and bound liposomes. The entire mixture was added with 5 ml Pico-Fluor 40 and radioactivity was measured using a Canberra Packard 1900 scintillation counter. The amount of bound liposomes was expressed as a fraction of the bound liposomes to the original amount added.

2.5 Plasma Pharmacokinetics and Tissue Distribution of Liposomes

Liposomes, labelled with [^{3}H] CHE were injected i.v. with various lipid doses in 200 µl via the lateral tail vein. At designated times, three mice per time point were terminated with CO_2 asphyxiation and whole blood was collected *via* cardiac puncture and placed into EDTA-coated tubes (Microtainers; Becton Dickinson, Lincoln Park, NJ). Plasma was isolated following centrifugation at 4 °C for 15 minutes at 1000 x g. Aliquoted

plasma samples (100 μl) were mixed with 5 ml Pico-Fluor 40 (Packard, Meriden, CT) scintillation fluid for radioactivity counting.

Isolated tissues including liver, spleen, lungs and tumors were harvested and washed in saline and placed into pre-weighed glass tubes. Tissue weights were determined prior to freezing the samples at -70 °C. Appropriate volumes of distilled water were added to tissues to achieve a 30% homogenate (w/v) by homogenizing the mixture with a Polytron tissue homogenizer (Kinematica, Lucerne, Switzerland). Aliquots of the homogenate (200 μl) were added with 500 μl Solvable™ and the mixture was incubated at 50 °C for 3 hours. After cooling to room temperature, 50 μl of 200 mM EDTA, 200 μl of 30% hydrogen peroxide and 25 μl of 10 N HCl were added. The mixture was then incubated for 1 hour at room temperature before adding 5 ml scintillation fluid for radioactive counting. All tissue lipid levels were corrected for lipid in the plasma compartment by using pre-determined plasma volume correction factors (Bally *et al.*, 1993).

2.6 Liposome Elimination from Plasma *via* Neutravidin or Avidin Administration

In order to achieve rapid removal of biotinylated liposomes, the effects of different doses of neutravidin/avidin on their pharmacokinetics were studied. In the absence of neutravidin/avidin, the elimination profile of biotinylated liposomes at a dose of 100 mg/kg was initially studied in female CD-1 or balb/c mice over a 24-hour period. Liposomes were quantitated in plasma and various organs at 1, 4 and 24 hours.

To examine the effects of neutravidin/avidin on the biodistribution changes of biotinylated liposomes, various neutravidin/avidin doses were injected into the tail vein at different times after liposome injection. Plasma and various organs were then sampled at the indicated times and liposomal levels were measured.

2.7 Tumor Accumulation and Plasma Elimination Studies of Biotinylated Liposomes with and without Neutravidin or Avidin

Male SCID/RAG-2 mice were inoculated bilaterally with 1 x 10^6 LS180 cells subcutaneously on the hind regions of the back. Once the tumors reached an estimated mass of 0.5 g, mice were injected with 100 mg/kg dose of biotinylated liposomes. Tumor and plasma liposomal levels were determined at 1, 2, 4, 8 and 24 hours after injection of liposomes. To characterize the effects of neutravidin/avidin on tumor and plasma levels of liposomes, neutravidin/avidin was administered to mice 4 hours post-injection of liposomes. Liposome levels in both tumor and plasma were then quantified at 1, 2, 4, 8 and 24 hours after administration of neutravidin/avidin. Tumor efflux behavior was then monitored, and efflux and influx rate constants were calculated using pharmacokinetic equations and integral calculus.

2.8 Statistical Analysis

ANOVA was performed with the statistical software package (Jmp-In. 4.0, Cary, NC) on the results obtained after administration of the three neutravidin doses and on the results

of comparing avidin-induced elimination for three different biotinylated PEGylated formulations. Differences were considered significant at $p<0.05$.

CHAPTER 3

OPTIMIZATION OF LIPOSOME CLEARANCE DESIGN TO CHARACTERIZE TUMOR INFLUX AND EFFLUX PHARMACOKINETICS OF CONVENTIONAL LIPOSOMES

3.1 Introduction

This chapter discusses the development of a rapid clearance system for the conventional liposome formulation composed of DSPC and Chol lipids. The DSPC/Chol/Biotin-X-DSPE (54.5:45:0.5 mol %) liposomal formulation was examined in this chapter. Compared to prior studies that exclusively utilized biotin-DSPE and avidin for their applications (Ogihara-Umeda *et al.*, 1993, 1997), biotin-X-DSPE and neutravidin were selected in our studies. Biotin-X-DSPE lipid contains an amino-hexanoyl spacer that allows biotin to be extended further away from surface of the liposomes, thus increasing exposure of the biotin moiety and significantly enhancing its apparent affinity to avidin (Redelmeier *et al.*, 1995). Neutravidin was chosen as opposed to avidin because it has less non-specific binding. The extremely high isoelectric point of avidin, as well as the presence of carbohydrate on the protein, both contribute to non-specific binding (Hiller *et al*, 1987). We speculated that a high non-specific binding may mean a decrease in protein availability for binding to biotinylated liposomes since it will non-selectively bind to other molecules in the body as well. In addition, it may cause untoward reactions when it is bound to other molecules that are necessary for the normal functioning of the

body. These two modifications (biotin-X-DSPE and neutravidin) were made in the hope of achieving more rapid clearance of liposomes from the central blood compartment.

The successful system that rapidly eliminates liposomes from the circulation was also evaluated for its versatility in relation to different lipid doses and neutravidin administration times or alternatively, lipid accumulation times. Subsequently, it was applied to characterize the tumor influx and efflux parameters qualitatively and quantitatively in the LS180 human colon carcinoma solid tumor xenograft model.

3.2 Results

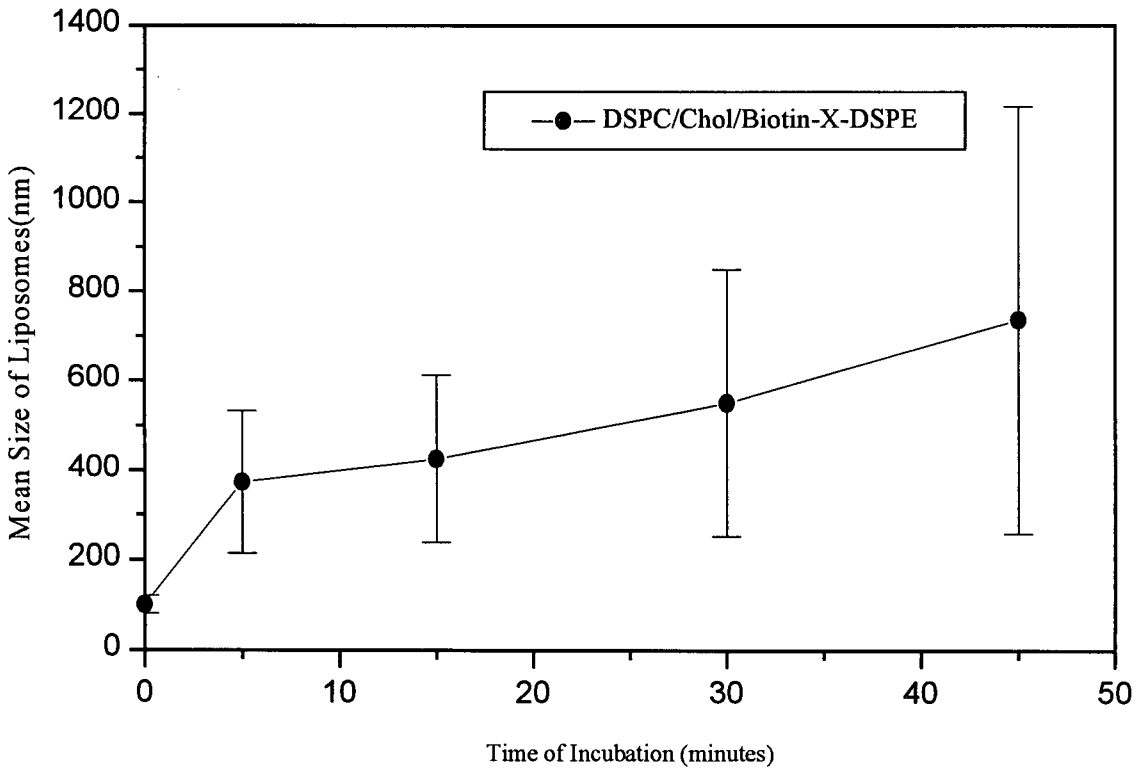
3.2.1 Aggregation by Neutravidin *In Vitro*

The aggregation test was initially performed in order to ensure the incorporation of the biotinylated lipids in the liposome formulation and the possible formation of aggregates with neutravidin. As depicted in Figure 3.1, increase in liposome size was observed when neutravidin was added to biotin-derivatized liposomes. The size of liposomes increased to a mean value of approximately 720 nm after 45 minutes of incubation. It is important to understand that the mean size of liposomes merely serves as an indicator of aggregate formation and is not to be used as an accurate measurement of the aggregate size. Since aggregation is a random process, homogenous formation of aggregates cannot be expected. This can be self-evident by observing the dramatic increase in standard

Figure 3.1

Aggregate formation of DSPC/Chol/Biotin-X-DSPE liposomes with neutravidin at 37 °C over 45 minutes

Liposomes (1 mg) and neutravidin (25 μg) were incubated in a total volume of 750 μl HBS at 37 °C over 45 minutes. Aggregates were sized by quasielastic light scattering at 5, 15, 30 and 45 minutes using a Nicomp 270-submicron particle sizer. Error bars represent the S.D. of the size of liposomes detected by the particle sizer.



deviation (S.D. of up to 500nm at t=45min) of the mean diameter over time, indicating the heterogeneity and vast number of different aggregate sizes present.

3.2.2 Effects of Various Doses of Neutravidin on Plasma Elimination and Tissue Distribution of Biotinylated Liposomes

The elimination profile of the biotinylated liposomes in female CD-1 mice without neutravidin was examined over a 24-hour period (Figure 3.2), and it was demonstrated that the incorporation of biotin-derivatized lipids did not significantly alter the inherent plasma pharmacokinetics of conventional non-biotinylated liposomal formulation (data not shown). The lipid levels dropped from approximately 135 μg/100 μl plasma at t=1 hour to about 85 μg/100 μl plasma at t=4 hours and then slowly to about 20 μg/100 μl plasma at t=24 hours. About 15% of lipids relative to t=1 hour remained in the plasma at t=24 hours. Accumulation of the cleared liposomes was also observed in the various RES organs in a pattern virtually identical to non-biotinylated liposomes (Figure 3.3).

After 1 hour of liposome injection, neutravidin was administered to induce rapid clearance. All three doses resulted in at least about 95% clearance of liposomes from the plasma by 1 hour after injection of the protein (Figure 3.2). The lipid levels dropped from a mean of about 135 μg/100 μl plasma at t=1 hour to a mean of less than 6 μg/100 μl plasma at t=2 hour. The cleared liposomes were observed to accumulate in the RES organs (Figure 3.4). The effects appear to be dose-dependent, with 200 μg giving rise to a mean plasma elimination in 1 hour of approximately 99%, 98% with 100 μg and

Figure 3.2

Plasma elimination of DSPC/Chol/Biotin-X-DSPE liposomes with and without neutravidin

The elimination of the biotinylated liposomes (100 mg/kg and 100 nm in diameter) was studied over a 24-hour period in female CD-1 mice. Neutravidin was also administered (50 μg, 100 μg, 200 μg) at 1 hour after liposome injection, and plasma lipid levels were sampled after a further 1 hour. Each point represents the mean +/- S.D. for 3 animals.

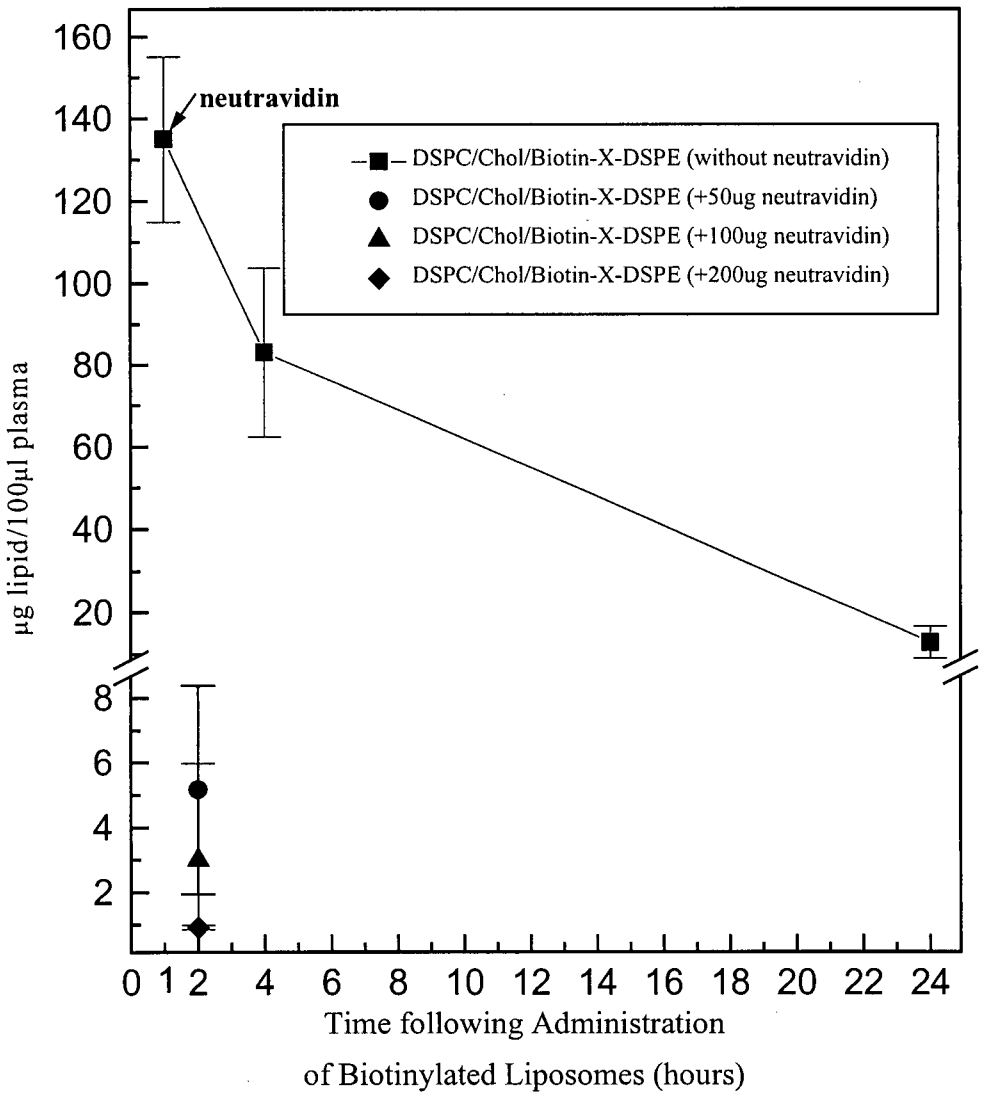


Figure 3.3

Biodistribution of DSPC/Chol/Biotin-X-DSPE liposomes over 24 hours

Biotinylated liposomes (100 mg/kg and 100 nm in diameter) were injected into female CD-1 mice and liver, spleen and lungs were quantified for lipid levels at t=1, 4, and 24 hours. Each bar represents the mean +/- S.D. for 3 animals.

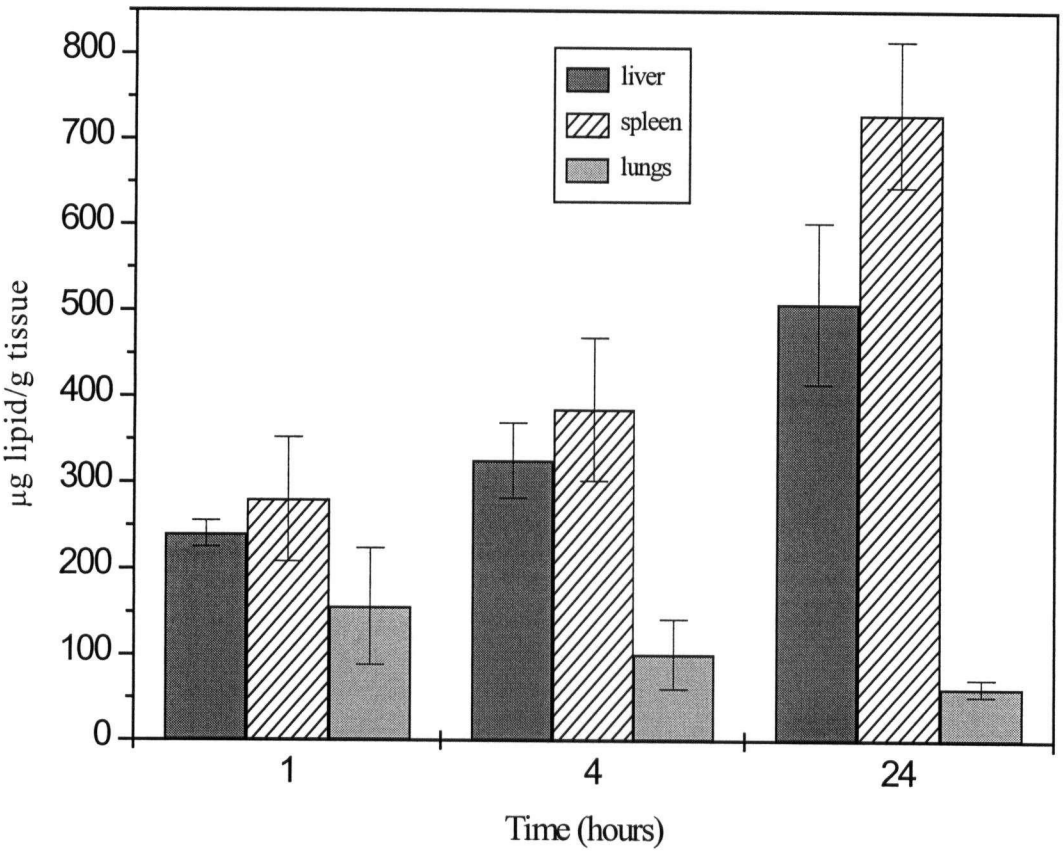
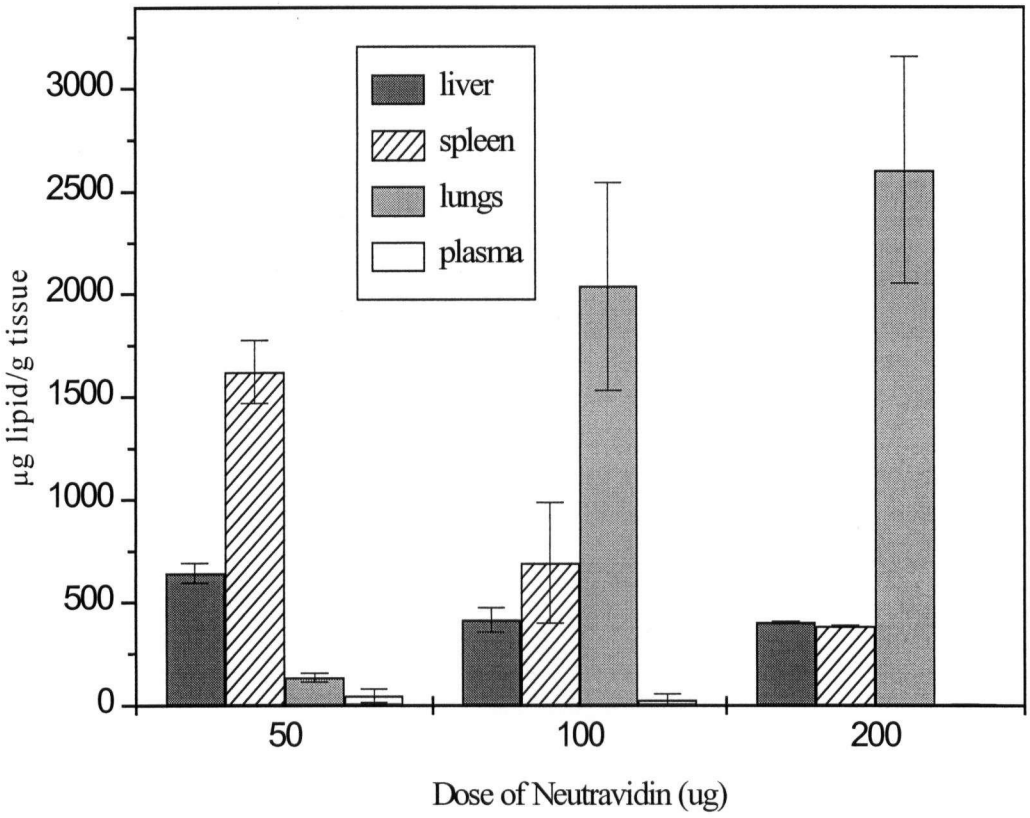


Figure 3.4

Biodistribution of DSPC/Chol/Biotin-X-DSPE liposomes with and without neutravidin

Biotinylated liposomes (100 mg/kg and 100 nm in diameter) were injected into female CD-1 mice, various doses of neutravidin were administered (50 μg, 100 μg, 200 μg) after 1 hour. Liver, spleen, lungs and plasma were quantified for lipid levels after a further 1 hour. Each bar represents the mean +/- S.D. for 3 animals.



95% with the 50 μg dose. The differences, however, were not statistically significant (ANOVA, $p>0.05$). In addition, compared to the mice with 50 μg neutravidin, the mice in the 100 μg and 200 μg groups were sluggish, tired and were not as active throughout the 1-hour period. This could be attributed to the high accumulation of liposomes in the lungs as depicted in Figure 3.4. In the groups administered with the 100 μg and 200 μg dose of neutravidin, the mean lipid levels in the lungs were about 2000 μg/g and 2700 μg/g respectively, compared to a mean lipid level in the lungs of only about 150 μg/g in the 50 μg neutravidin group. Therefore, 50 μg dose of neutravidin was chosen for subsequent experiments in order to avoid untoward reactions.

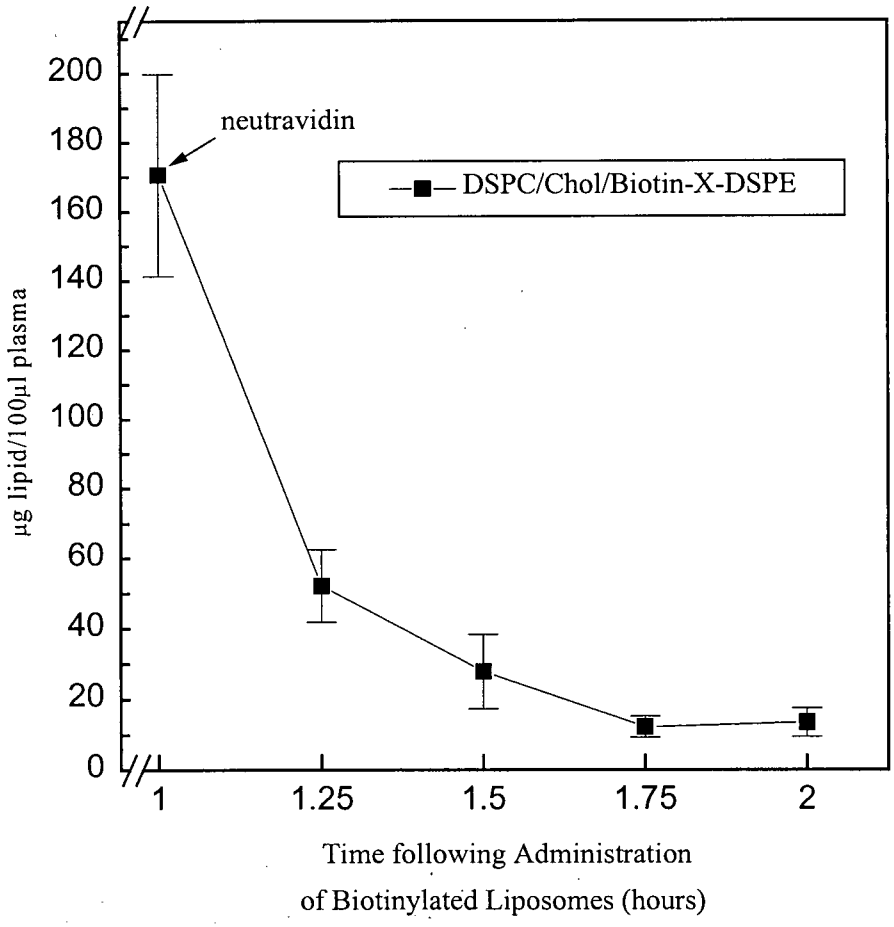
3.2.3 Characterization of the Rapid Elimination of Biotinylated Liposomes with Neutravidin

Female CD-1 mice were sacrificed at 15, 30, 45 and 60 minutes relative to neutravidin administration in order to evaluate whether the effects of neutravidin on removal of the liposomes in the 1-hour period was a fast or slow process. The removal within the 1-hour time period was a rapid process as shown in Figure 3.5, and appeared to follow a monophasic elimination characterized by an exponential decline in the early time points rather than a linear decline or an exponential decline in latter time points. More than 70% of lipids was cleared within 15 minutes of neutravidin administration.

Figure 3.5

Plasma elimination of DSPC/Chol/Biotin-X-DSPE liposomes with neutravidin over 1 hour

Biotinylated liposomes (100 mg/kg and 100 nm in diameter) were injected into female CD-1 mice, 50 μg of neutravidin was administered 1 hour after liposomal injection. Plasma lipid levels were quantified 15, 30, 45 and 60 minutes post-injection of neutravidin. Each data point represents the mean +/- S.D. for 3 animals.



3.2.4 Effects of Neutravidin on Liposomal Dose and Administration Times

In addition to examining the effects of neutravidin on 100 mg/kg dose of lipid, 10 mg/kg of lipid was also studied in order to evaluate the versatility and applicability of the rapid removal design. As shown in Table 3.1, administration of 50 μg neutravidin also resulted in greater than 95% of a 10 mg/kg dose lipid being eliminated from plasma in 1 hour. The effects of administering neutravidin at 4 and 8 hours after lipid injection were also assessed. Rapid removal was also seen at these time points, since greater than 95% of liposomal lipid was cleared within half an hour (Table 3.1). The versatility of the removal design allows potential characterization of tumor influx and efflux kinetics of different lipid doses at various time points. The following section describes the characterization of the tumor accumulation on efflux of DSPC/Chol liposomes at 100 mg/kg dose of liposomal lipid at 4 hours after liposome injection.

3.2.5 Liposomal Pharmacokinetics in Tumor-bearing Mice and Characterization of Tumor Efflux and Influx

Liposomal levels were measured in established LS180 solid tumors and plasma over a 24-hour period after a single i.v. injection of free biotinylated liposomes (100 mg/kg). In Figure 3.6, plasma liposome levels are shown in panel A and tumor liposome levels are shown in panel B. The liposomes appeared to follow a biphasic elimination from the plasma. The levels declined from about 1800 μg/g at t=1 hour to 1300 μg/g at t=4 hours and then slowly dropped to about 500 μg/g at t=24 hours. Liposomal levels in tumor

Table 3.1

Percent removal of plasma liposomal lipid (DSPC/Chol/Biotin-X-DSPE liposomes) in female CD-1 mice after administration of 50 μg neutravidin

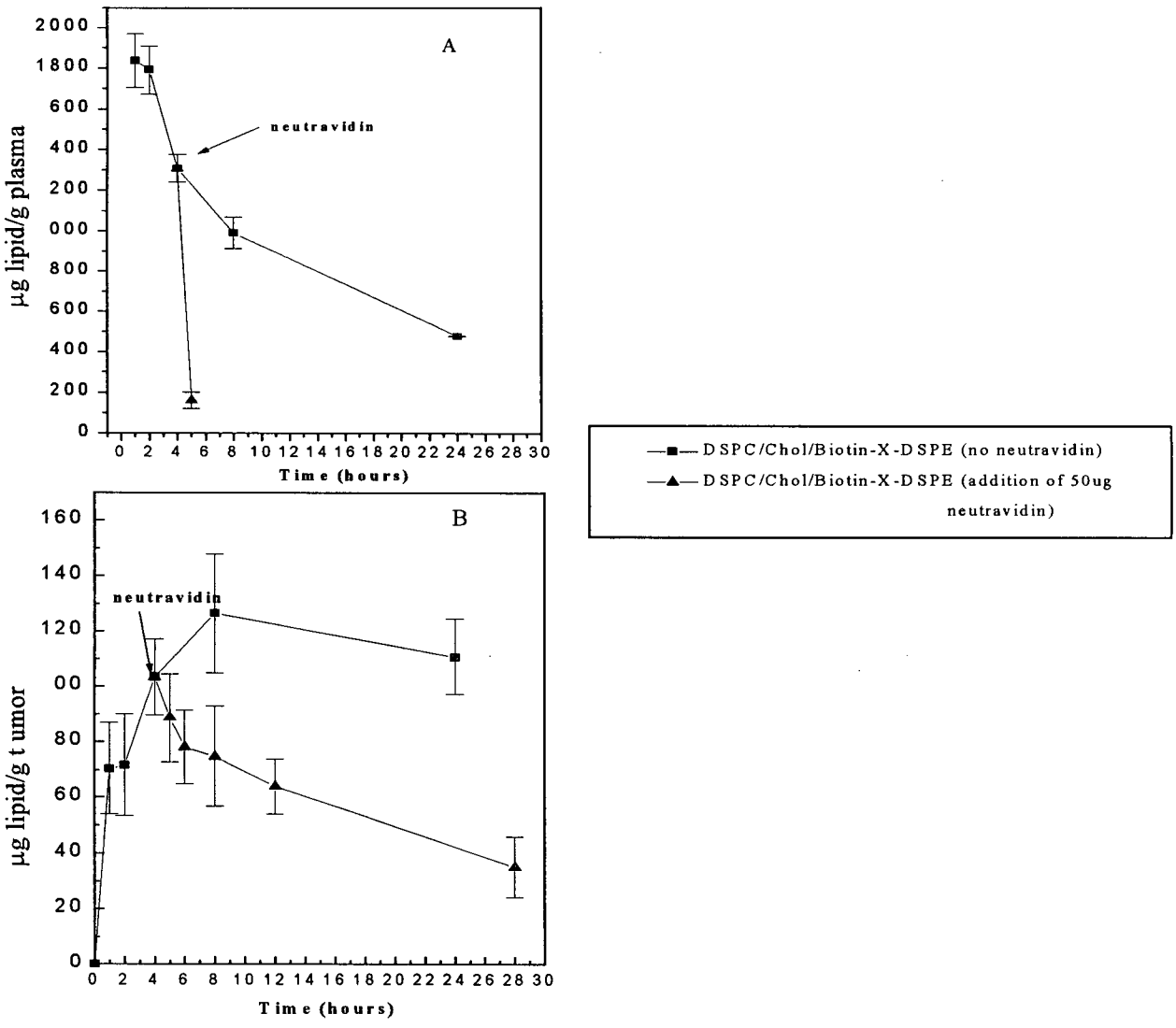
Neutravidin-induced Liposomal Plasma Removal (% of control)		
Lipid Dose (mg/kg)	Time (h)	% Removal (S.D.)
100	1[a]	95 (1)
	4[b]	98 (2)
	8[b]	96 (1)
10	1[a]	97 (1)

[a]Plasma levels were taken one hour after neutravidin injection
[b]Plasma levels were taken half an hour after neutravidin injection

Figure 3.6

Plasma elimination and tumor accumulation of DSPC/Chol/Biotin-X-DSPE liposomes with and without administration of neutravidin

Biotinylated liposomes (100 mg/kg and 100 nm in diameter) were injected into SCID/RAG2 mice bearing LS180 solid tumor. Panel A and B represent plasma and tumor lipid levels respectively. Tumor and plasma lipid levels were determined at 1, 2, 4, 8 and 24 hours. 50 μg neutravidin was administered at 4 hours after liposome injection, and tumor and plasma lipid levels were determined at 1, 2, 4, 8 and 24 hours after neutravidin administration. Data points represent average data and S.D. from 6 tumors. Plasma lipid levels were below the limit of quantitation at 6, 8 12, and 28 hours.

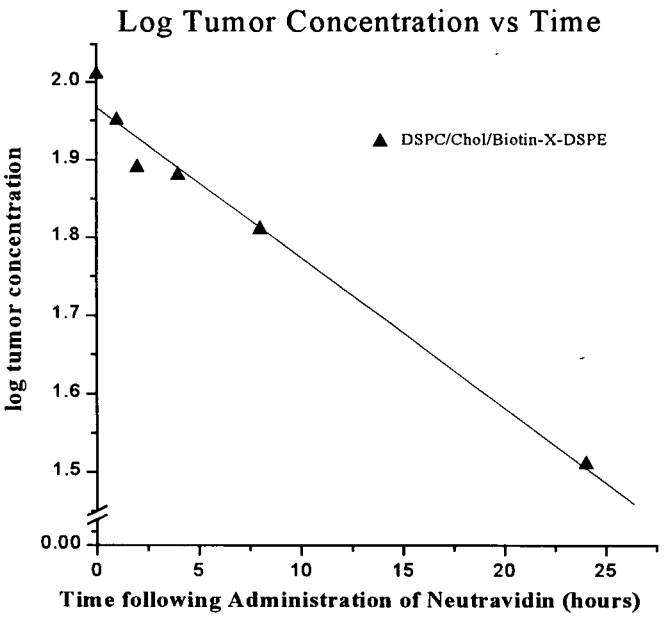


increased to about 100 μg/g at t=4 hours, then peaked at about 120 μg/g at t=8 hours. The tumor levels appeared to plateau after this time as the levels fell to about 110 μg/g at t=24 hours. At 4 hours post-injection of liposomes, neutravidin was administered. A rapid decrease in plasma lipid levels was induced and lipid levels decreased from a mean of about 1300 μg/g at t=4 hours to about 150 μg/g 1 hour later. The lipid levels remained below the limit of quantitation (about 5 μg of lipids/100 μl plasma) over the subsequent 23-hour period, as indicated from the absence of data from t=6 hours to t=28 hours in Figure 3.6, panel A. Tumor levels were also observed to gradually drop over 24 hours after neutravidin addition. The levels decreased from 100 μg/g at t=4 hours to about 30 μg/g at t=28 hours. The efflux curve was fitted with the Microcal Origin 5™ software package, utilizing the least squares principle to determine that the efflux curve followed first-order kinetics (Figure 3.7, left panel). Using the best line of fit, an r^2 of 0.98 was obtained for the line. The efflux rate constant (k_{-1}) was estimated by fitting the curve and calculating it using first-order equations (Figure 3.7, left panel). Based on the assumptions that at any given point in time, the rate of change of tumor lipid levels is equal to the difference between the products of the concentrations in the individual pharmacokinetic compartments and their respective rate constants (see Figure 1.1), the influx rate constant (k_1) can be calculated using integral calculus (see Figure 3.7, right panel). The tumor influx (k_1) and efflux (k_{-1}) rate constants were determined to be 0.022 $hour^{-1}$ and 0.041 $hour^{-1}$ respectively after liposome accumulation over 4 hours.

Figure 3.7

Calculations of efflux and influx rate constants

Efflux



Efflux curve (from Figure 3.6B) is approximated with a first-order kinetic equation. It is fitted and graphed.

$Logy=Logy_o-k_{-1}t/2.303$; y=tumor concentration (μg lipid/g)

and y_o=tumor concentration at t=0

k_{-1}=tumor efflux rate constant

Slope=-0.0178hour^{-1}

k_{-1}=0.041hour^{-1}

Influx

$dy/dt=k_1[X]-k_{-1}[y]$

know [x] at t=4 hours

so can treat [x]=C (constant)

integrating both sides:

$dy/(k_1C-k_{-1}y) = dt$

gives

$y=k_1/k_{-1}*C(1-e^{-k_{-1}t})$

at t=4 hrs, y=106.4μg/g

C=1307μg/g

k_{-1}=0.041hour^{-1}

therefore, **k_1=0.022hour^{-1}**

[y]=tumor lipid concentration (μg/g)

[x]=plasma lipid concentration (μg/g)

k_1=influx rate constant (hour^{-1})

k_{-1}=efflux rate constant (hour^{-1})

3.3 Discussion

The avidin-induced strategy for removal of circulating liposomes has been attempted in other laboratories to improve target to background ratios for imaging and diagnostic purposes (Ogihara-Umeda *et al.*, 1993, 1997). With some modifications from previous investigations, our aim was to optimize the liposome clearance design in order to study the pharmacokinetics of liposome tumor efflux and influx. It is important to elucidate these variables since accumulation levels in the tumor (in both absolute and dynamic terms), is actually dependent on the combination of these processes. A low C_{max} (absolute) may indicate a high efflux and/or slow influx, and vice versa. Similarly, a slow accumulation rate or high t_{max} (dynamic) could be attributed to a similar pharmacokinetic behaviour. In addition, plasma levels were believed to be correlated with tumor level and therapeutic efficacy; however, recent studies with conflicting evidence suggested that plasma levels may not correlate with these variables (Mayer *et al.*, 1997; Parr *et al.*, 1997; Hong *et al.*, 1999). In these studies, PEGylated liposomes show neither higher tumor lipid Area Under the Curve (AUC) values nor better therapeutic profiles compared to the conventional formulation, even though they displayed higher plasma lipid AUC values.

The above observations led us to postulate the possibility that the observation is attributed to unfavorable pharmacokinetics of influx and/or efflux, such that any single liposome molecule may be unable to remain sufficiently long in the tumor. That is, despite high plasma levels, any single liposome molecule may have difficulty entering

into tumor tissues or easily leaves the tissues even if extravasated. This phenomenon may be able to explain the relatively low tumor AUC even when the plasma AUC is high. We believe that plasma levels are neither the most accurate predictor nor the ultimate governing factor for tumor levels or therapeutic effects, and that a more mechanistic or defined pharmacokinetic characterization is necessary.

At any point in time during tumor accumulation, both processes of influx and efflux occur simultaneously, making it difficult to determine the individual variables. Rapid removal of lipids, however, permits the isolation and thus the characterization of a single kinetic variable--tumor efflux, by eliminating the otherwise interfering influx factor that is due to accumulating liposomes from plasma (Figure 1.1). Unlike other studies that simply aim to obtain a relative improvement in clearance (Ogihara-Umeda *et al.*, 1993; 1997), rapid clearance is crucial in the present study as we do not want tumor influx to obscure efflux monitoring since any unremoved liposomes in the circulation have the opportunity to extravasate back to the tumor. It is shown from our results that plasma levels remain below the limit of quantitation throughout the efflux process after neutravidin injection, and thus there is minimal interference from the influx process.

Rapid clearance of liposomes from the central blood compartment was achieved utilizing biotin-avidin technology. The interaction between biotin and avidin allows aggregate formation of biotinylated liposomes due to 1) the irreversible binding between biotin and avidin, and 2) the multi-binding capacity of avidin (Green, 1963; Gitlin *et al.*, 1987). These two properties between biotin and avidin are crucial for rapid clearance. Stable

aggregates allow continual and rapid removal of the liposomes, as opposed to a reversible and unstable interaction that compromises the integrity of the aggregates and thus may reduce such clearance. The presence of multiple binding sites on the avidin also enables cross-linkage and lattice formation of high complexities. The formation of stable and huge aggregates allows the liposomes to be easily recognized and thus rapidly taken up by the RES, resulting in low circulating liposome levels.

The present chapter describes the development of a methodology that allows rapid elimination of conventional DSPC/Chol liposomes (100 nm) and subsequent characterization of their tumor influx and efflux pharmacokinetics in the LS180 solid tumor-bearing mouse model. Biotinylated liposomes were prepared by incorporating biotin-conjugated phospholipid into the DSPC/Chol lipid composition. The incorporation of the conjugated lipid to the composition, accessibility of biotin moieties on the liposome surface to neutravidin and formation of aggregates were confirmed by the *in vitro* aggregation test. By incorporating 0.5 mol% of biotin-X-DSPE in the formulation and administering 50 μg of neutravidin *in vivo*, greater than 90% of the liposomes were removed from the plasma within 1 hour in female CD-1 mice. The dose of choice was 50 μg since it was sufficient to induce rapid clearance without causing untoward side effects such as lung accumulation that was observed from higher doses (Figure 3.4). Successful rapid elimination was achieved with both low (10 mg/kg) and high lipid doses (100 mg/kg), suggesting the broad spectrum of application this system can work in terms of lipid doses (Table 3.1). This method was also successfully applied at 1, 4 and 8 hours

post-injection of liposomes (Table 3.1), allowing the potential for characterizing tumor kinetic behaviour of efflux and influx at different time points.

At 4 hours after tumor accumulation, tumor efflux of liposomes was characterized using the LS180 solid tumor model (Figure 3.6). By itself, the efflux profile provides certain degree of information and can be used graphically for comparisons, for example, by overlapping two different efflux curves of interest and qualitatively making comparisons between them. A more quantitative measure is the tumor influx (k_1) and efflux (k_{-1}) rate constants which were also determined in this study. The influx and efflux rate constants were 0.022 $hour^{-1}$ and 0.041 $hour^{-1}$ respectively at 4 hours after tumor accumulation (Figure 3.7). It is important to note that these values are only an approximation of the true values considering the standard deviations involved and the potential interference from liposomes remaining in the circulation during characterization of efflux process. They do, nevertheless, provide certain degree of information about influx and efflux, and serve as comparators for other models in the future. It is also important to note that the data only pertain to t=4 hours specifically and should not be extrapolated to other time points. However, if neutravidin were administered at different times after tumor accumulation, different efflux curves and thus different rate constants may be obtained, and the information provided can be very useful as it provides insight into the pharmacokinetic behavior or differences at various times throughout the accumulation process. In the present study, it appears that DSPC/Chol liposomal accumulation in the tumor is dictated primarily by plasma liposome concentrations and liposomal rate constant is higher for efflux than influx into the tumor. The relative high efflux/low

influx rate constants could be explained by the high hydrostatic pressure that is usually built up inside tumors (Buocher and Jain, 1992; Jain, 1993). The pressure makes it more difficult for liposomes to extravasate, and easier for them to efflux out if extravasated.

The research described in the following chapters looks at optimizing plasma elimination of sterically stabilized formulations and utilizing it to characterize their influx and efflux kinetics. Due to steric stabilization that leads to barriers in optimizing clearance, a chapter is devoted in exploring and understanding the problems underlying the development of such a system, and discussing the strategies and experimental design in overcoming these barriers.

CHAPTER 4

LIPOSOMAL DEVELOPMENT OF DOUBLE BIOTINYLATED SYSTEM FOR RAPID CLEARANCE OF STERICALLY STABILIZED LIPOSOMES

4.1 Introduction

In addition to applying the technology to characterize the kinetics of the conventional DSPC/Chol liposomes, we intended to use the same strategy to determine the tumor accumulation kinetics for other types of liposomes, especially sterically stabilized liposomes composed of DSPC/Chol/PEG_{2000}-DSPE (55:40:5 mol%) or PEGylated liposomes. We incorporated 0.5% of biotin-X-DSPE into the DSPC/Chol/PEG_{2000}-DSPE formulation and administered 50 μg neutravidin *in vivo* in order to achieve rapid elimination for these liposomes. However, only 8% of liposomal lipids were removed within 1 hour. Increasing the content of biotinylation from 0.5 to 5% did not significantly increase the elimination rate (data not shown). This chapter discusses the methodology and steps that were carried out to develop a system that could optimize clearance for the sterically stabilized liposomal formulation. It explores the various components of design including modifications of the formulation and increases in neutravidin doses. Since both *in vitro* and *in vivo* experiments were performed, we also examined the relationship between *in vitro* behaviour and *in vivo* clearance kinetics.

4.2 Results

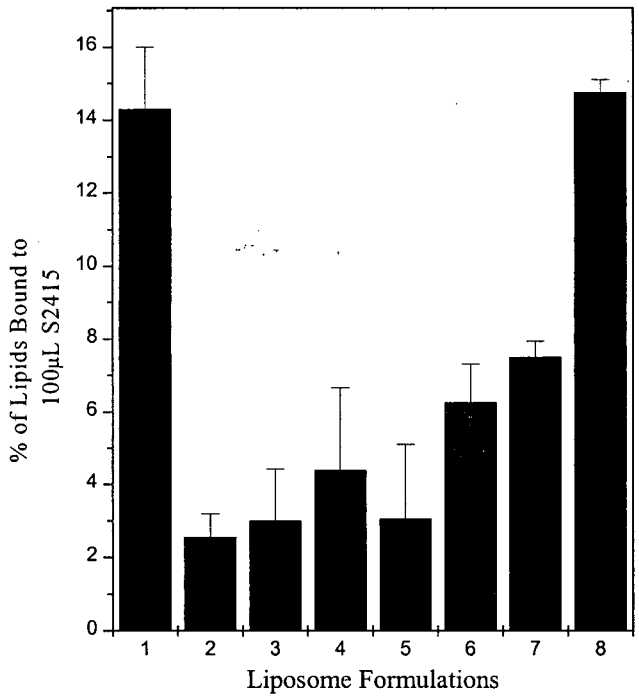
4.2.1 Binding of PEGylated Liposomes to Streptavidin *In Vitro*

Various formulations of PEGylated liposomes were subject to the streptavidin magnetic bead test to assess their *in vitro* binding capacity before introducing them to *in vivo* studies. The binding results for the conventional formulation is included for comparison purposes as shown in panel 1 of Figure 4.1. Two types of biotinylated liposomal formulations were tested—the single and double biotinylated system. The differences between the formulations were based on the types of biotin-derivatized lipids incorporated. The single system simply contained either a biotin-X-DSPE lipid or a biotin conjugated to a longer lipid chain such as PEG_{3400}-DSPE whereas the double system contained both the short and the longer biotin-derivatized lipids. The rationale behind the development of the double biotinylated system for clearance optimization will be discussed in detail under section 3 of this chapter. As shown in Figure 4.1, the double biotinylated liposomal system gave rise to higher binding than the single biotinylated formulation. In the single biotinylated system with 5% PEGylation, the *in vitro* binding was about 3% (Figure 4.1, panels 2,3 and 5), whereas the double biotinylated system resulted in binding that ranged from 6 to15% (Figure 4.1, panels 6-8). Biotin-PEG_{2000} conjugated lipid (Figure 4.1, panels 8) incorporated into the PEGylated formulation resulted in superior *in vitro* binding than incorporation of biotin-PEG_{3400} conjugated lipid (Figure 4.1, panels 6,7) as it is evident by its comparative binding with the conventional formulation (Figure 4.1, about 15% in panel 1).

Figure 4.1

In vitro binding profile of various liposome formulations to S2415

500 nmol of different liposomal formulations were incubated with 100 µl of S2415 in a total volume of 200 µl for 15 minutes. The mixture is subject to a magnetic field and various steps as described under Streptavidin Magnetic Bead Test in Chapter 2 in order to determine the % of lipids bound. Error bars represent S.D. for 3 samples.



1. DSPC/Chol/Biotin-X-DSPE 0.5%
2. DSPC/Chol/Biotin-X-DSPE 0.5%/DSPE-PEG_{2000} 5%
3. DSPC/Chol/Biotin-X-DSPE 5%/DSPE-PEG_{2000} 5%
4. DSPC/Chol/Biotin-PEG_{3400}-DSPE 0.5%
5. DSPC/Chol/Biotin-PEG_{3400}-DSPE 0.5%/DSPE-PEG_{2000} 5%
6. DSPC/Chol/Biotin-PEG_{3400}-DSPE 0.5%/Biotin-X-DSPE 0.5%/ DSPE-PEG_{2000} 5%
7. DSPC/Chol/Biotin-PEG_{3400}-DSPE 0.5%/Biotin-X-DSPE 0.1%/ DSPE-PEG_{2000} 5%
8. DSPC/Chol/Biotin-PEG_{2000}-ceramide 0.5%/Biotin-X-DSPE 0.1%/ DSPE-PEG_{2000} 5%

4.2.2 Effects of Various Doses of Neutravidin on Plasma Elimination of Double Biotinylated Liposomal Formulations: Comparisons Between *In Vitro* and *In Vivo* Behaviour

The streptavidin magnetic bead test was exploited to streamline or screen potential candidates for *in vivo* experiments. The three different double biotinylated liposomal formulations (100 mg/kg) which were shown to demonstrate more favourable *in vitro* characteristics were studied in combination with different doses of neutravidin in female CD-1 mice. Neutravidin doses injected into each mouse include 50, 100 and 200 μg. As shown in Table 4.1, none of the combinations could induce close to 90% clearance. Data from the i*n vitro* aggregation test (at 5 minutes of incubation) were included for comparison. Although some combinations gave rise to comparable or even greater aggregate size than the conventional system *in vitro* [eg. DSPC/Chol/Biotin-X-DSPE0.5%/Biotin-PEG_{3400}-DSPE0.5%/PEG_{2000}-DSPE5% (DCBBP) with 100 μg and DSPC/Chol/Biotin-X-DSPE0.1%/Biotin-PEG_{3400}-DSPE0.5%/PEG_{2000}-DSPE5% (DCB1BP) with 100 μg show mean aggregate size of about 400 nm vs. 300 nm obtained for the DSPC/Chol conventional liposomes with 50 μg neutravidin], high reduction in plasma lipid levels was not observed *in vivo*. It appears that *in vivo* clearance kinetics cannot be predicted simply on the basis of *in vitro* data.

Lower doses (50 mg/kg vs.100 mg/kg) of liposomal lipid (DCBBP) were investigated to determine whether rapid liposomal removal from the plasma could be established and whether neutravidin-induced clearance of PEGylated formulation was lipid

Table 4.1

Different PEG-containing lipid formulations of the double biotinylated system were tested with various doses of neutravidin *in vivo*

Effects of neutravidin on clearance were measured by injecting it into female CD-1 mice 1 hour after administration of liposomes (100 mg/kg and 100 nm in diameter) and evaluating the plasma lipid level 1 hour later. Data from the *in vivo* aggregation test at 5 minutes of incubation are also included.

Lipid formulations	Neutravidin added (μg)	Clearance at 1 hour post-injection of neutravidin (%)	*In vitro* aggregates (mean +/- S.D.) (nm)
DCBBP	50	~2-8	231+/-104
	100	~40-60	413+/-188
	200	~40	202+/-78
DCB1BP	50	*	154+/-62
	100	~20-50	431+/-216
	200	~30	232+/-54
DCB1BCP	50	~30	160+/-55
	100	~30	147+/-51
DCB#	50	>90	315+/-130

DCBBP: DSPC/Chol/Biotin-X-DSPE 0.5%/Biotin-PEG_{3400}-DSPE 0.5%/DSPE-PEG_{2000} 5%

DCB1BP: DSPC/Chol/Biotin-X-DSPE 0.1%/Biotin-PEG_{3400}-DSPE 0.5%/DSPE-PEG_{2000} 5%

DCB1BCP: DSPC/Chol/Biotin-X-DSPE 0.1%/Biotin-PEG_{2000}-ceramide 0.5%/DSPE-PEG_{2000} 5%

DCB: DSPC/Chol/Biotin-X-DSPE 0.5%

* Experiment not performed as low clearance would be anticipated
# Its inclusion is for comparison purpose.

dose-dependent. It was found that injection of 100 μg neutravidin could not achieve 90% clearance in 1 hour even when lower liposome doses were employed (data not shown).

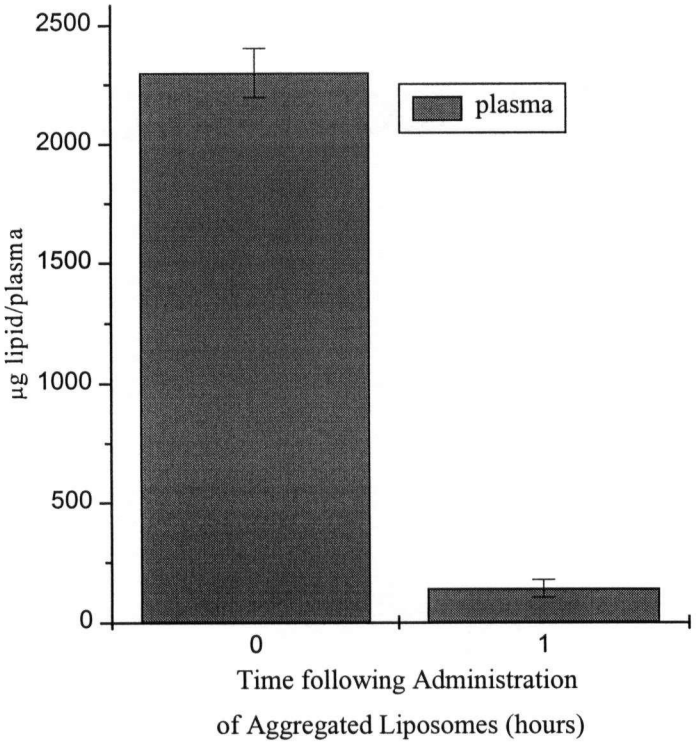
4.2.3 Clearance of Pre-aggregated Liposomes

The challenges thus far associated with successfully developing a system to rapidly eliminate PEGylated liposomes from the circulation started to raise doubt as to the possibility of achieving this goal. To address this question and to rule out this possibility, we attempted to inject pre-aggregated liposomes *in vivo* to see if they would be rapidly removed from plasma. DSPC/Chol/Biotin-X-DSPE0.5%/Biotin-PEG_{3400}-DSPE0.5%/DSPE-PEG_{2000}5% liposomes (100 mg/kg) that were incubated with 100 μg neutravidin to achieve mean aggregate size of 1000 nm after 10 minutes were administered i.v. and plasma was analyzed 1 hour later for liposomal lipid content. Figure 4.2 shows that greater than 95% of liposomal lipid was removed in 1 hour under these circumstances.

Figure 4.2

Plasma elimination of pre-aggregated liposomes in 1 hour

DSPC/Chol/Biotin-X-DSPE0.5%/Biotin-PEG_{3400}-DSPE0.5%/DSPE-PEG_{2000}5% liposomes (100 mg/kg) were incubated with 100 μg of neutravidin over 10 minutes to achieve mean aggregate size of about 1000 nm. Liposome aggregates were then immediately injected into female CD-1 mice and plasma lipid level was evaluated 1 hour later. Each bar represents the mean +/- S.D. for 3 animals.

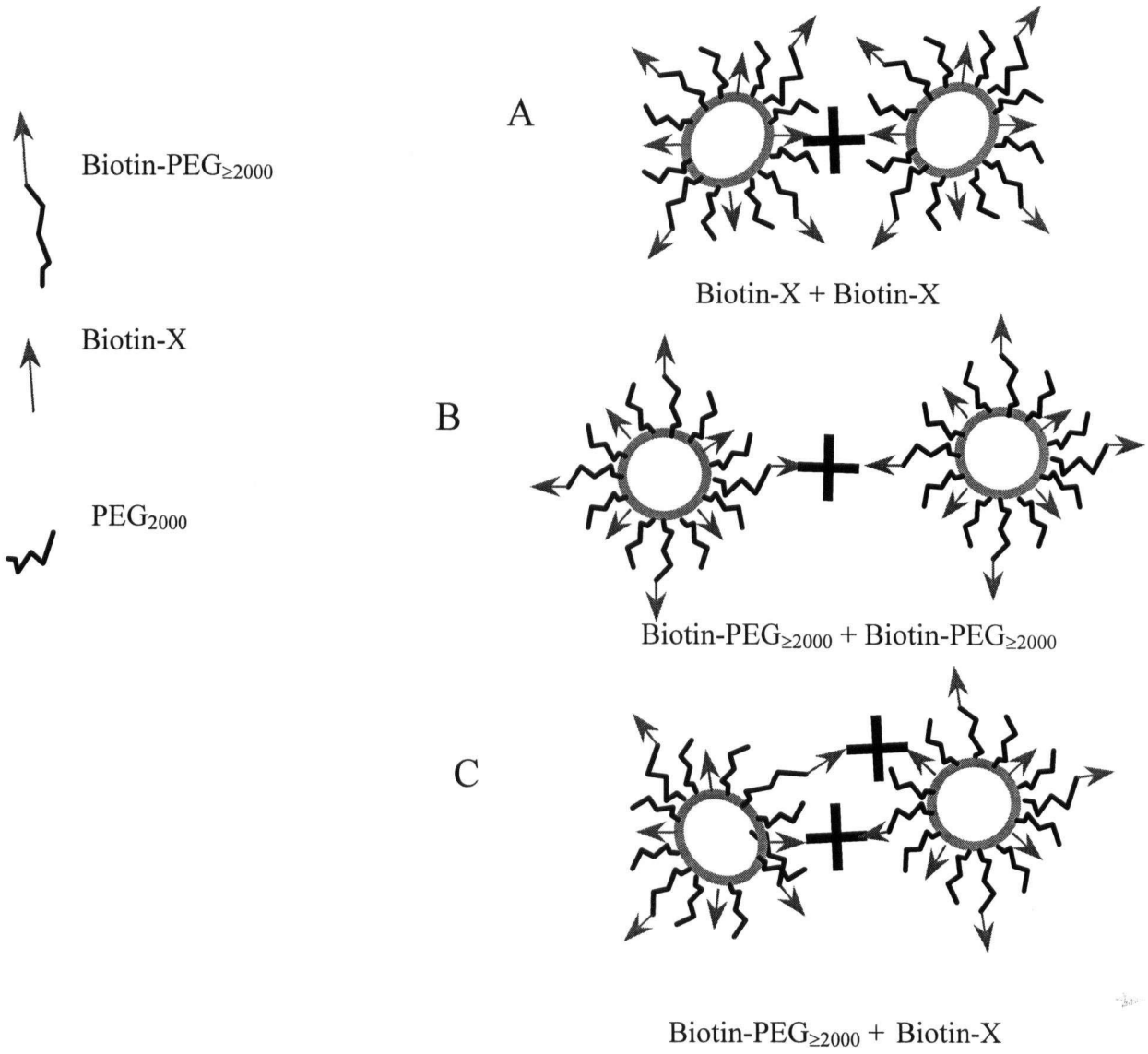


4.3 Discussion

The presence of PEG has been shown to reduce interactions of proteins with liposomes to prolong the longevity of the formulation (Lasic *et al.*, 1991; Needham *et al.*, 1992, 1999; Blume and Cevc, 1993; Allen, 1994). At the same time, this introduces barriers in optimizing clearance design for the purposes of this thesis project as the hydrophilic polymer prevents the exposure of the biotin moiety to avidin, presents steric hindrance to binding of approaching liposomes to form aggregates and introduces lower affinity for RES due to reduced protein interactions (Chonn *et al.*, 1992; Corley and Loughrey, 1994; Yuda *et al.*, 1996; Needham and Kim, 2000). The failure to develop a rapid clearance method with 0.5% and 5% mol biotin-X-DSPE led us to initially screen different formulations for favourable binding using the streptavidin magnetic bead test prior to carrying out *in vivo* studies. It was hoped that using formulations with biotin conjugated to PEG lipid could increase the accessibility of biotin to avidin. However, *in vitro* studies showed that low binding still occurred (Figure 4.1, panel 5). The double biotinylated formulation was developed in the hope of achieving rapid clearance due to an increase in binding variation (Figure 4.3). In addition to potential binding between the biotin-X-DSPE lipids or between biotin-PEG lipids, an intercalating interaction can be expected as well due to binding of the shorter biotin-linked lipid with the longer biotin-linked lipid (Figure 4.3 C). In the streptavidin magnetic bead test, the double biotinylated system was shown to demonstrate superior binding levels than the single biotinylated system and even comparable *in vitro* binding to the conventional system when biotin-PEG_{2000} conjugated lipid was used (Figure 4.1, panel 8). However, administration of even up to

Figure 4.3

Postulated binding variations of the double biotinylated lipid formulations



200μg neutravidin could not achieve rapid clearance with any of the double biotinylated systems. The *in vitro* aggregate test also shows that aggregate formation was possible at the *in vitro* level, but even though some formulations demonstrated comparable or even greater aggregate size when compared to conventional liposomes, rapid elimination was not attained. These results indicate that *in vitro* data (from both the streptavidin magnetic bead and aggregation test) cannot be used to predict *in vivo* behaviour.

The unsuccessful attempts to obtain neutravidin-induced rapid clearance could be attributed to three possibilities. First, the aggregates that are formed *in vivo* may not be large enough to induce rapid removal. Second, population effects may be an alternative explanation, as not all liposome species may aggregate and a heterogeneous distribution may leave unaggregated species in the circulation. Finally, even if all liposomes aggregate, steric stabilization may even prevent the clearance of aggregated liposomes. In an effort to understand the underlying problems, the clearance of pre-aggregated liposomes study was done and it demonstrated that as long as large stable aggregates can be induced *in vivo*, rapid clearance of PEGylated liposomes should be achievable.

In the next chapter, a successful rapid clearance design for PEGylated liposomes is described. Following the same steps that are carried out for the conventional system, the next chapter describes the experiments leading to characterization of the tumor accumulation kinetics for the PEGylated liposome formulation.

CHAPTER 5

DEVELOPMENT OF RAPID CLEARANCE OF DOUBLE BIOTINYLATED SYSTEM TO CHARACTERIZE TUMOR INFLUX AND EFFLUX PHARMACOKINETICS OF STERICALLY STABILIZED LIPOSOMES

5.1 Introduction

It was seen from the previous chapter that rapid clearance of PEGylated (DSPC/Chol/PEG_{2000}-DSPE at 55:40:5 mol%) liposomes was not achievable. Even though the design of the double biotinylated system led to improvement in the *in vitro* and *in vivo* effects associated with neutravidin binding and clearance induction, a rapid removal similar to that obtained for the conventional DSPC/Chol formulation was not possible. By making slight modifications to the double biotinylated formulations and introducing avidin instead of neutravidin *in vivo*, rapid clearance and subsequent characterization of tumor influx and efflux pharmacokinetics of PEGylated liposomes were finally achieved and these results are described in this chapter.

5.2 Results

5.2.1 Optimizing the Design for Rapid Clearance of Sterically Stabilized Liposomes Using Double Biotinylated System and Avidin

Greater than 95% of PEGylated liposomes formulated with 0.5% biotin-X-DSPE and 0.5% biotin-PEG_{2000}-DSPE were removed within 1 hour after administration of 100 μg avidin (Figure 5.1). Two other formulations (PEGylated formulation incorporated with 1%biotin-X-DSPE, and PEGylated formulation containing 1% biotin-PEG_{2000}-DSPE) were included for comparisons. The elimination of the double biotinylated formulation was greater than that achieved with the single biotinylated system where administration of 100 μg avidin resulted in only about 35% of plasma lipids removed within 1 hour for the PEGylated formulation with 1%biotin-X-DSPE, and about 90% removed for the PEGylated formulation with 1% biotin-PEG_{2000}-DSPE. The results suggest superior effects exerted by double biotinylation since all three formulations have the same level of biotin binding sites (1%). The differences between the two types of biotinylation were statistically significant ($p<0.05$).

5.2.2 Aggregation by Avidin *In vitro*

The *in vitro* aggregation test was performed to ensure the incorporation of the biotinylated lipids in the double biotinylated formulation and the possible formation of aggregates with avidin. As depicted in Figure 5.2, increase in liposome size was observed. The increase associated with the PEGylated system is less than the conventional system. The aggregate size increased to about a mean value of 200nm upon incubation for 45 minutes as opposed to the conventional DSPC/Chol system which increased up to 700nm.

Figure 5.1

Plasma elimination of three different biotinylated formulations 1 hour after treatment with avidin

The three different biotinylated formulations (100 mg/kg and 100 nm in diameter) were injected into female balb/c mice. 100 μg of avidin was administered after 1 hour. Lipid levels were then quantified after a further 1 hour. Results are expressed as percentage of lipids removed within the 1 hour period. Each bar represents the mean +/- S.D. for 3 animals.

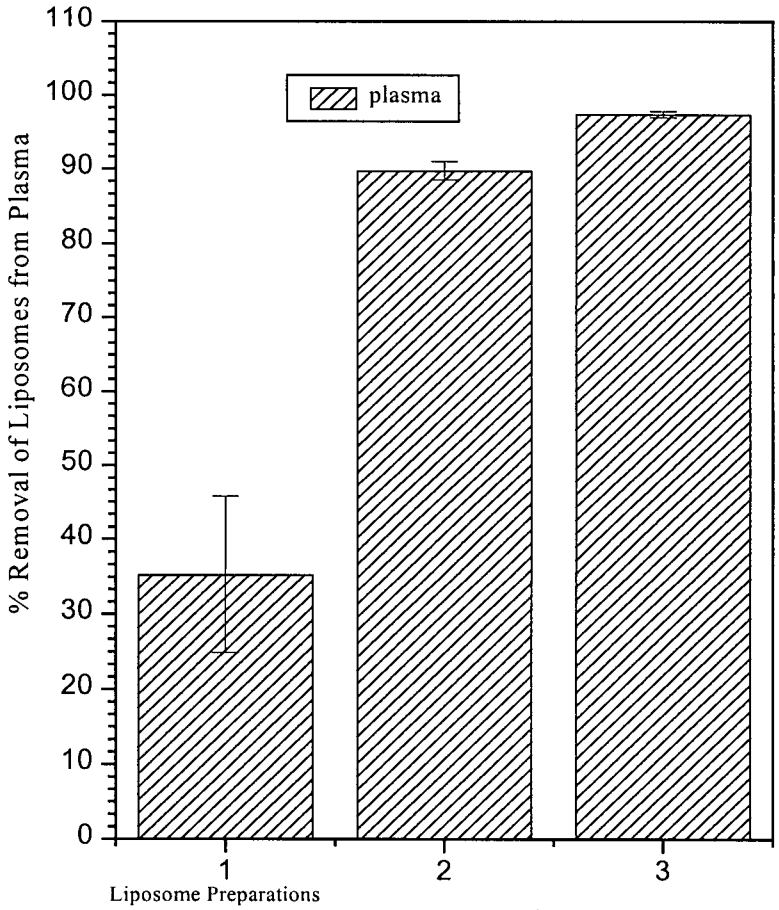
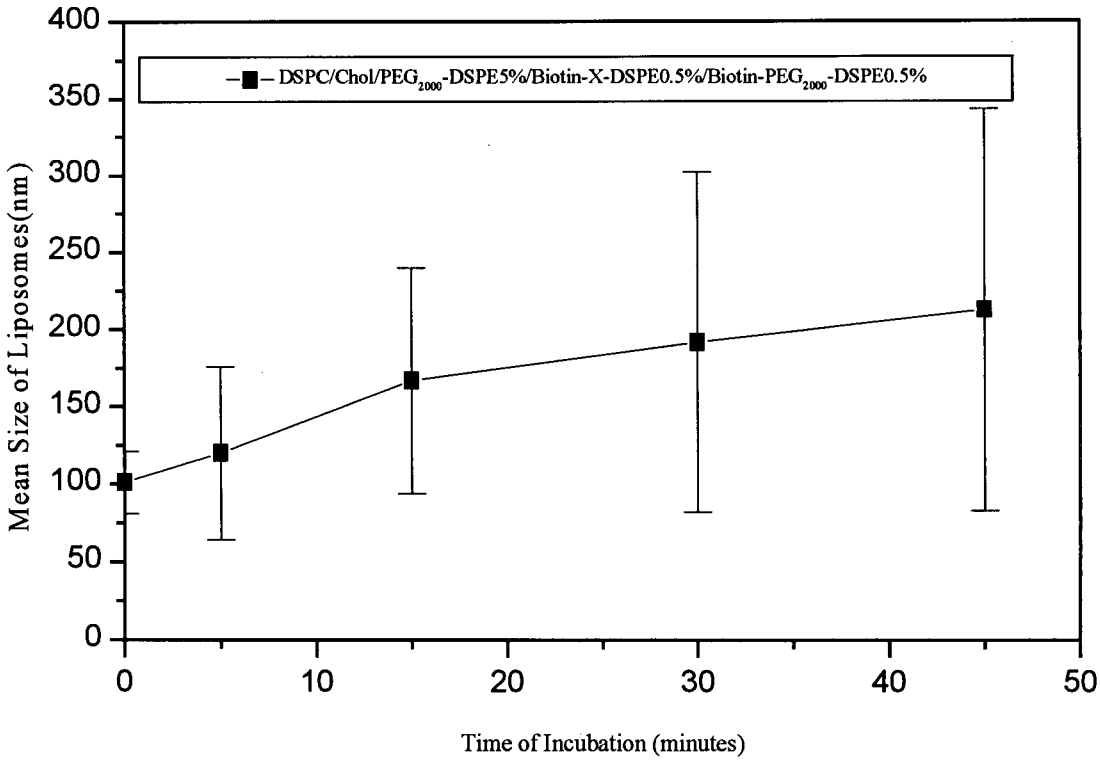


Figure 5.2

Aggregate formation of DSPC/Chol/PEG_{2000}-DSPE5%/Biotin-X-DSPE0.5%/Biotin-PEG_{2000}-DSPE0.5% liposomes with avidin at 37 °C over 45 minutes

Liposomes (1 mg) and avidin (50 μg) were incubated in a total volume of 750 μl HBS at 37 °C over 45 minutes. Aggregates were sized by quasielastic light scattering at 5, 15, 30 and 45 minutes using a Nicomp 270-submicron particle sizer. Error bars represent the S.D. of the size of liposomes detected by the particle sizer.



5.2.3 Plasma Pharmacokinetics and Tissue Distribution of Double Biotinylated Liposomes with and without Avidin

The elimination profile of the double biotinylated DSPC/Chol/PEG_{2000}-DSPE liposomes in female balb/c without avidin was examined over a 24-hour period (Figure 5.3) and it was demonstrated that the incorporation of biotin-derivatized lipids did not significantly alter the inherent plasma pharmacokinetics of non-biotinylated PEGylated formulation (data not shown). The formulation displayed log-linear plasma elimination kinetics, with approximately 50% of lipids relative to levels at t=1 hour still remaining in plasma at 24 hours (Figure 5.3). Accumulation of these liposomes was observed in the RES organs in a pattern virtually identical to non-biotinylated PEGylated liposomes (data not shown). At t=1 hour, administration of 100 μg avidin resulted in a rapid removal of liposomes from the plasma compartment to the RES organs within 1 hour post avidin injection as seen in Figure 5.4. For example, administration of the avidin at t=1 hour resulted in an increase of liver lipid levels from about 200 μg/g to 1800 μg/g in 1 hour, and an increase of spleen lipid levels from about 750 μg/g to 3500 μg/g. Similar to the DSPC/Chol conventional liposome system with 50 μg neutravidin, the majority of the removed liposomes were observed in the liver and spleen. However, unlike the DSPC/Chol system with 100 and 200 μg neutravidin, there was minimal accumulation in the lungs when 100 μg of avidin was injected to mice previously administered with the PEGylated liposome formulation.

Figure 5.3

Plasma elimination of DSPC/Chol/PEG_{2000}-DSPE5%/Biotin-X-DSPE0.5%/Biotin-PEG_{2000}-DSPE0.5% liposomes over 24 hours

The elimination of the double biotinylated liposomes (100 mg/kg and 100 nm in diameter) was studied over a 24-hour period in female balb/c mice. Each point represents the mean +/- S.D. for 3 animals.

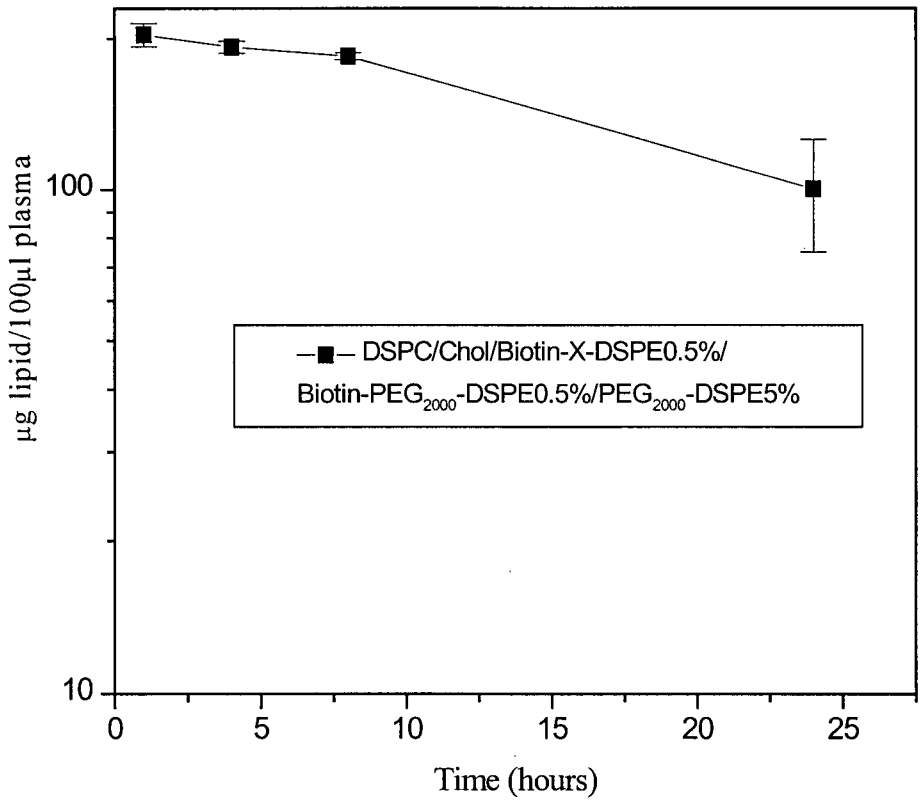
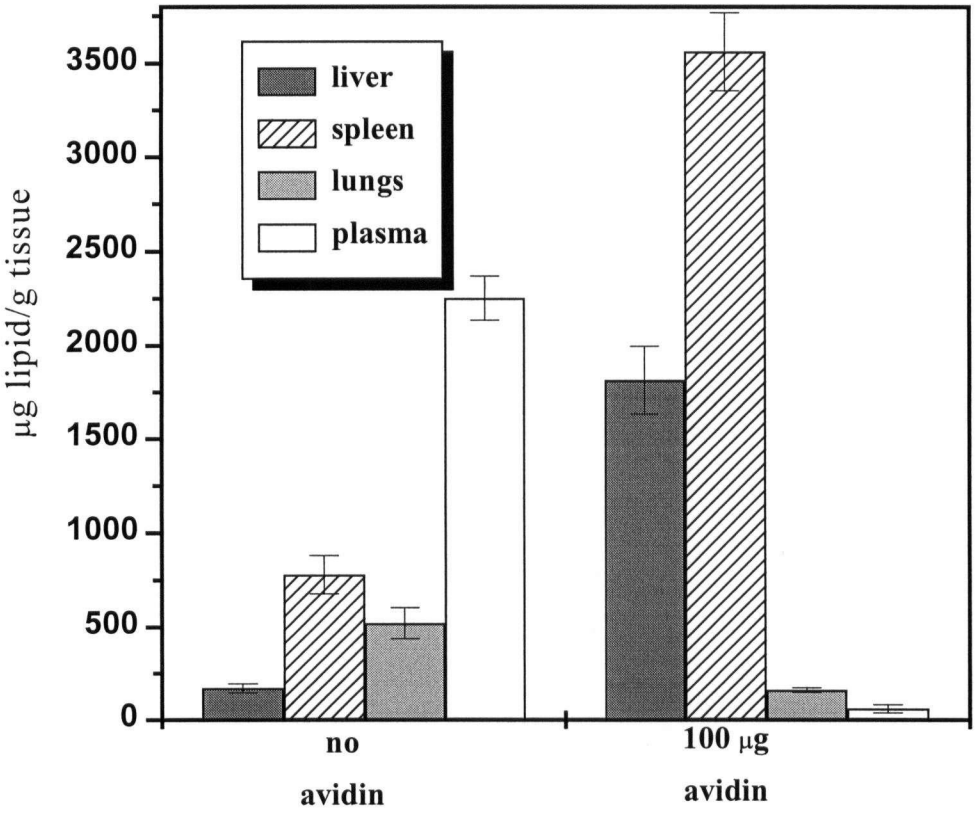


Figure 5.4

Biodistribution of DSPC/Chol/PEG_{2000}-DSPE5%/Biotin-X-DSPE0.5%/Biotin-PEG_{2000}-DSPE0.5% liposomes with and without avidin

Biotinylated liposomes (100 mg/kg and 100 nm in diameter) were injected into female balb/c mice, 100 μg avidin were administered after 1 hour. Liver, spleen, lungs and plasma were quantified for lipid levels after a further 1 hour. Lipid levels at t=1 hour without avidin were included for comparisons. Each bar represents the mean +/- S.D. for 3 animals.



5.2.4 Characterization of the Rapid Elimination of Biotinylated Liposomes with Avidin

Female balb/c mice were sacrificed at 15, 30, 45 and 60 minutes relative to avidin administration in order to evaluate the kinetics of PEGylated liposome removal from plasma after avidin administration. The removal within the 1-hour time period was a rapid process as shown in Figure 5.5, where approximately 90% of the lipids was removed within 15 minutes of avidin administration and the levels remained minimal (<5%) in the subsequent time points.

5.2.5 Effects of Avidin on Liposomal Dose and Administration Times

In addition to examining the plasma clearance effects of avidin on the 100 mg/kg dose of lipids, 10 mg/kg of liposomal lipid dose was also studied in order to evaluate the versatility and lipid dose dependency of the rapid plasma clearance system for sterically stabilized liposomes. As shown in Table 5.1, administration of 100 μg avidin also resulted in greater than 95% clearance of liposomal lipids from plasma within 1 hour of avidin injection for that lipid dose. The administration of avidin at 4 and 8 hours after lipid injection also resulted in rapid liposome removal where approximately 90% of liposomes were cleared within half an hour when avidin was administered at these time points (Table 5.1). The elimination was not as rapid as observed for DSPC/Chol liposomes at the later time points. For example, approximately 88% of PEGylated liposomes were removed within half an hour at t=8 hours whereas about 96% of lipid removal was achieved with the conventional system (Table 3.1).

Figure 5.5

Plasma elimination of DSPC/Chol/PEG_{2000}-DSPE5%/Biotin-X-DSPE0.5%/Biotin-PEG_{2000}-DSPE0.5% liposomes with avidin over 1 hour

Biotinylated liposomes (100 mg/kg and 100 nm in diameter) were injected into female balb/c mice, 100 μg of avidin was administered 1 hour after liposome injection. Plasma lipid levels were quantified 15, 30, 45 and 60 minutes post-injection of avidin. Each data point represents the mean +/- S.D. for 3 animals.

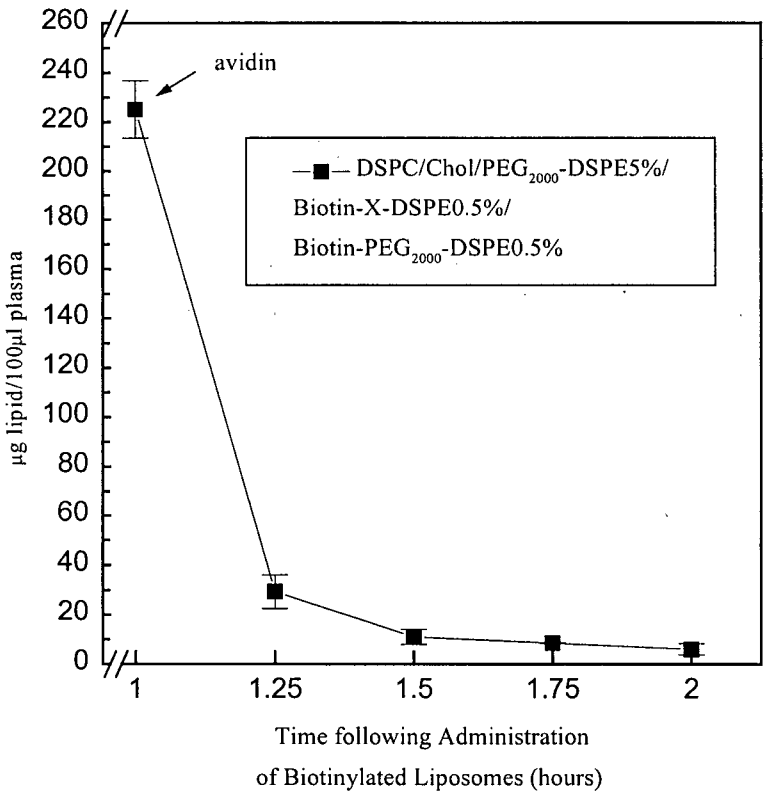


Table 5.1

Percent removal of plasma liposomal lipid (DSPC/Chol/PEG_{2000}-DSPE5%/Biotin-X-DSPE0.5%/Biotin-PEG_{2000}-DSPE0.5% liposomes) in female balb/c mice after administration of 100 μg avidin

Avidin-induced Liposomal Plasma Removal (% of control)		
Lipid Dose (mg/kg)	Time (hours)	% Clearance (S.D.)
100	1[a]	97(<1)
	4[b]	96 (1)
	8[b]	88 (3)
10	1[a]	97 (1)

[a]Plasma levels were taken one hour after avidin injection
[b]Plasma levels were taken half an hour after avidin injection

5.2.6 Liposomal Pharmacokinetics in Tumor-bearing Mice and Characterization of Tumor Efflux and Influx

Liposomal levels were measured in established LS180 solid tumors and plasma over a 24-hour period after a single i.v. injection of free biotinylated liposomes (100 mg/kg). In Figure 5.6, plasma liposome levels are shown in panel A and tumor liposome levels are shown in panel B. The plasma lipid levels declined from about 2250 μg/g at t=1 hour to 1625 μg/g at t=4 hours and then slowly dropped to about 800 μg/g at t=24 hours. Liposomal levels in tumor increased to about 400 μg/g at t=4 hours, then peaked at approximately 650 μg/g at t=8 hours. The tumor levels then fell to about 400 μg/g at t=24 hours. At 4 hours post-injection of liposomes, 100 μg avidin was administered. A rapid decrease in plasma lipid levels was induced and lipid levels decreased from a mean of about 1625 μg/g at t=4 hours to about 40 μg/g within 1 hour after. The plasma lipid levels remained below the limit of quantitation (about 2 μg of lipids/100 μl plasma) over the subsequent 23-hour period as indicated from the absence of data from t=6 hours to t=28 hours in Figure 5.6, panel A. Tumor levels were also observed to gradually drop over 24 hours after avidin addition. The tumor concentrations stayed relatively constant from t=4 hours to 6 hours as they remained to be about 400 μg/g. Then the levels dropped to about 350 μg/g at t=8 hours and remained steady for the subsequent 20 hours since there was no change in the levels within standard deviations, at t=28 hours. Due to the difficulty involved in mathematically characterizing the efflux curve, it was simply approximated with first-order kinetics (Figure 5.7, left panel) for the purpose of

Figure 5.6

Plasma elimination and tumor accumulation of DSPC/Chol/PEG$_{2000}$-DSPE5%/Biotin-X-DSPE0.5%/Biotin-PEG$_{2000}$-DSPE0.5% liposomes with and without administration of avidin

Biotinylated liposomes (100mg/kg and 100nm in diameter) were injected into SCID/RAG2 mice bearing LS180 solid tumors. Panel A and B represent plasma and tumor lipid levels respectively. Tumor and plasma lipid levels were determined at 1, 2, 4, 8 and 24 hours. 100 μg avidin was administered at 4 hours after liposome injection, and tumor and plasma lipid levels were determined at 1, 2, 4, 8 and 24 hours after avidin administration. Data points represent average data and S.D. from 6 tumors. Plasma lipid levels were below the limit of quantitation at 6, 8 12, and 28 hours.

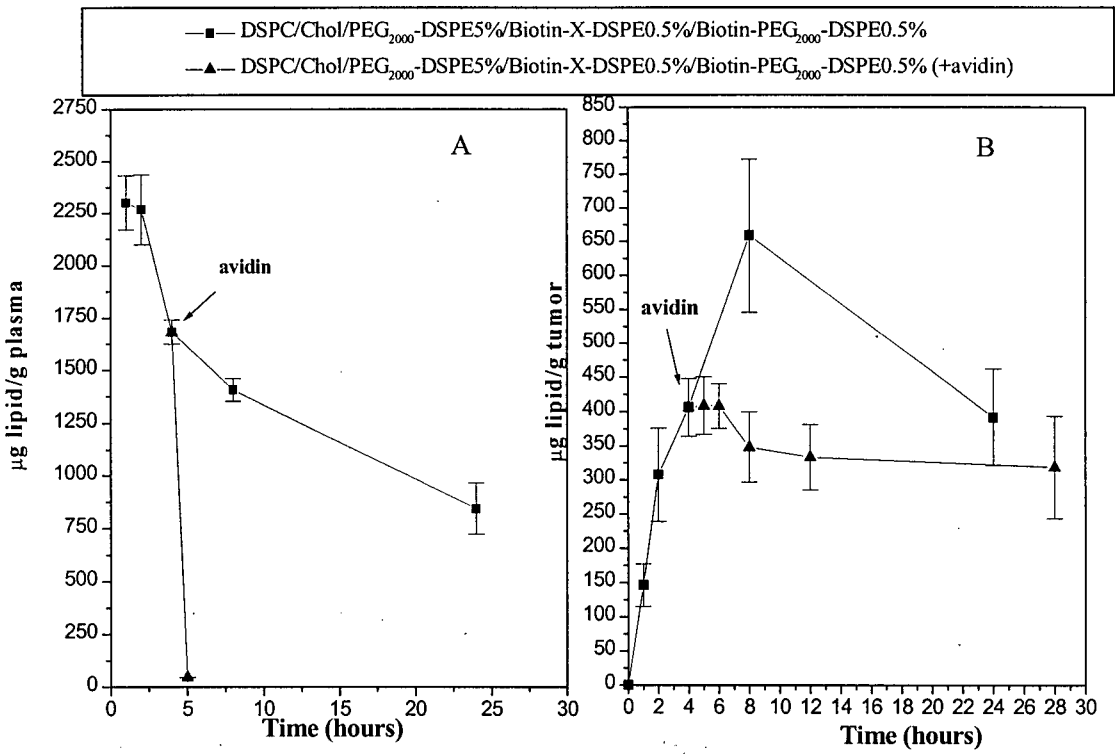
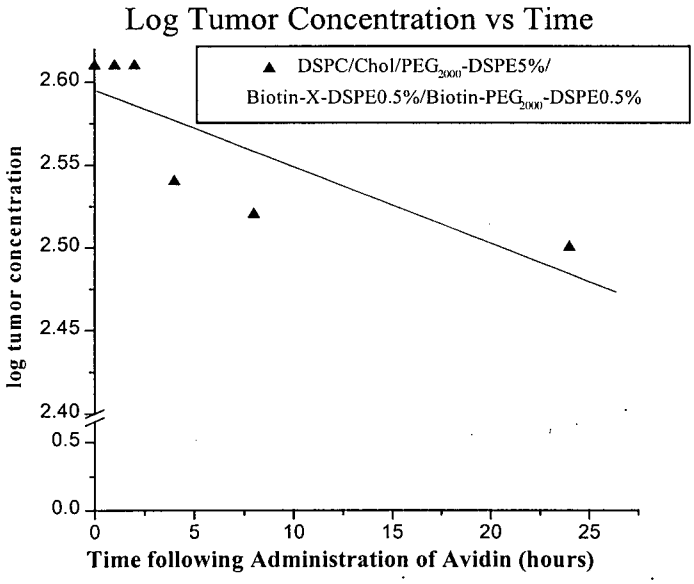


Figure 5.7

Calculations of efflux and influx rate constants

Efflux



Efflux curve (from Figure 5.6B) is approximated with a first-order kinetic equation. It is fitted and graphed.

$Logy=Logy_0-k_{-1}t/2.303$; y=tumor concentration (μg lipid/g) and y_0=tumor concentration at t=0

k_{-1}=tumor efflux rate constant

Slope=$-0.0046hour^{-1}$

$k_{-1}=0.011hour^{-1}$

Influx

$dy/dt=k_1[X]-k_{-1}[y]$

know [x] at t=4 h

so can treat [x]=C (constant)

integrating both sides:

$dy/(k_1C-k_{-1}y) = dt$

gives

$y=k_1/k_{-1}*C(1-e^{-k_{-1}t})$

at t=4 hrs, y=406μg/g

C=1683μg/g

$k_{-1}=0.011hour^{-1}$

therefore, **$k_1=0.062hour^{-1}$**

[y]=tumor lipid concentration (μg/g)

[x]=plasma lipid concentration (μg/g)

k_1=influx rate constant ($hour^{-1}$)

k_{-1}=efflux rate constant ($hour^{-1}$)

comparing with the DSPC/Chol liposome system. Based on the assumptions that at any given point in time, the rate of change of tumor lipid levels is equal to the difference between the products of the concentrations in the individual pharmacokinetic compartments and their respective rate constants (Figure 1.1), the influx rate constant (k_1) can be calculated using integral calculus (Figure 5.7, right panel). The tumor influx (k_1) and efflux (k_{-1}) rate constants were determined to be 0.062 $hour^{-1}$ and 0.011 $hour^{-1}$ respectively after liposome accumulation over 4 hours.

5.3 Discussion

Contrary to our speculation, avidin appears to be a more effective agent for rapid clearance of liposomes from plasma than neutravidin. This may be attributed to advantages associated with glycosylation and non-specific binding (Bruch and White, 1982). The higher non-specific binding of avidin allows binding to other molecules *in vivo*, thereby increasing the effective size of the liposomes and enhancing RES recognition. Carbohydrate moieties on avidin (e.g. mannose, glucose) also may be recognized by receptors in the liver *in vivo*, which may also contribute to increased RES uptake and rapid liposome clearance (Haltiwanger *et al.*, 1986; Kuiper *et al.*, 1994; Kawakami *et al.*, 2000).

The double biotinylated system also appears to be a superior formulation over the single biotinylated system as shown in Figure 5.1, supporting the potential proposed

mechanisms of increased binding variations (Figure 4.3). The increase in binding variations potentially leads to the formation of liposome aggregates that are greater in number and larger than the single biotinylated system, resulting in the observed increase in the removal of the double biotinylated DSPC/Chol/PEG_{2000}-DSPE or PEGylated liposomes (Figure 5.1).

Even though the PEGylated formulation was biotinylated, it displayed log-linear plasma kinetics (Figure 5.3) similar to that described in a lot of previous literature (Allen *et al.*, 1991, 1995; Woodle and Lasic, 1992; Allen and Stuart, 1999; Drummond *et al.*, 1999). The hydrophilic polymer reduces recognition by the RES and thereby prolongs the longevity of the liposomes (Lasic *et al.*, 1991; Needham *et al.*, 1992, 1999; Blume and Cevc, 1993; Allen, 1994). Regardless of the steric stabilization that contributes to the challenges seen in developing a rapid system as described in chapter 4, the combination of avidin administration and double biotinylation allows a rapid elimination of the PEGylated liposomes from the circulation (Figure 5.1). The successful development of the system to eliminate more than 95% of PEGylated liposomes in 1 hour gives us the opportunity to develop and assess the versatility of the system and can eventually lead to characterization of influx and efflux kinetics. It is noteworthy that administration of 100 μg avidin did not lead to an increase in accumulation of liposomes in the lungs (Figure 5.4) whereas a high accumulation was observed with 100 μg neutravidin in the DSPC/Chol liposome system (Figure 3.4). One possibility is that avidin-induced aggregates of the double biotinylated PEGylated system might not be as large as that obtained from the neutravidin-induced conventional liposomal aggregates, but were large

enough to induce rapid clearance. The question still remains as to whether the rapid elimination is dictated solely by a size phenomenon or is mediated by other alternative pathways such as the non-specific protein binding and glycosylation associated with avidin or a combination of both. As can be seen from Table 5.1, the elimination appears to be slower at later time points when compared to the conventional system (Table 3.1). This observation may be attributed to the reduction of biotin activity due to biotinidase activity *in vivo* (Thoma and Peterson, 1954; Laverman *et al.*, 2000) with the effects of slow removal being amplified by steric stabilization. Nevertheless, the application can still be utilized at these time points provided that the influx contributed by levels in the half-hour time period on the estimation of the overall efflux kinetics over the 24-hour time period is negligible.

Avidin-induced lipid removal was a rapid process since greater than 90% of lipid was removed in 15 minutes as depicted in Figure 5.5. The rapid elimination allows characterization of influx and efflux pharmacokinetics and suggests that sterically stabilized liposomal accumulation is dictated primarily by plasma concentrations and the liposome influx rate constant is about 6-fold higher than efflux rate constant. Influx and efflux rate constants were estimated to be 0.062 $hour^{-1}$ and 0.011 $hour^{-1}$ respectively after liposome accumulation over 4 hours and the results differ from that obtained for the DSPC/Chol liposomes.

5.3.1 Comparison between Conventional and PEGylated System

The studies presented here show that PEGylation of liposomes enhances circulation longevity and tumor accumulation when compared to conventional DSPC/Chol liposomes. The tumor C_{max} of the PEGylated formulation was approximately 5 times that obtained for DSPC/Chol liposomes (650 vs. 120 μg/g) in the LS180 tumor-bearing mouse model. These studies also indicate that PEGylation enhances the influx (0.062 $hour^{-1}$ vs 0.022 $hour^{-1}$) and lowers the efflux (0.011 $hour^{-1}$ vs 0.041 $hour^{-1}$) kinetics when compared to the DSPC/Chol liposomes at t=4 hours in this tumor model. The high plasma concentration may be responsible for the observed high influx due to enhanced accumulation through mass action (Wu *et al.*, 1993; Yuan *et al.*, 1994; Parr *et al.*, 1997; Mayer *et al.*, 2001). The increased influx may lead to liposomes penetrating deep into the tumor interstitium, making it more difficult for them to leave the tumor tissues into the circulation upon elimination of the influx variable. This may explain the slow efflux kinetics observed for the PEGylated liposomes. In our studies, the PEGylated liposomal levels remained relatively steady from t=4 to 6 hours when avidin is introduced at t=4 hours, and the levels remained relatively steady from 8 to 28 hours (Figure 5.6). The tumor liposomal lipid levels dropped from 400 μg/g to only about 350 μg/g over 24 hours post-administration of avidin whereas the level dropped more than 3-fold (from 100 μg/g to 30 μg/g) over 24 hours for DSPC/Chol liposomes.

It should be kept in mind that any interpretation generated from the results is only valid in relation to the specified conditions under which the current studies were carried out (e.g. type of tumor model--LS180, time of clearance application--4 hours post-injection of liposomes, etc.). In addition, the influx and efflux kinetics of liposomes can be different

depending on whether the liposome carriers are empty or drug-loaded . The present thesis only investigated on the kinetics of empty or free liposomes. It has been shown from previous studies that encapsulation of certain agents can influence the plasma elimination pharmacokinetics of the liposome carriers (Bally *et al.*, 1990; Daemen *et al.*, 1995). For example, the blood residence time of DSPC/Chol liposomes with entrapped doxorubicin was shown to be significantly longer than for identically prepared empty liposomes (Bally *et al.*, 1990). The percentage of the dose retained in the circulation at 24 hours increased 2-3-fold when the liposomes contain entrapped doxorubicin (Bally *et al.*, 1990). Drug-induced inhibition of RES activity is believed to account for the slower clearance of drug-loaded carriers (Bally *et al.*, 1990; Daemen *et al.*, 1995). This inhibition, or "RES blockade," is believed to reflect cytotoxicity against phagocytic cells responsible for clearance (Bally *et al.*, 1990; Daemen *et al.*, 1995). The effects of "RES blockade" from certain drug-loaded liposomes may lead to an increase in the tumor influx and/or a decrease in the efflux kinetics when compared to empty or free liposomes due to increased plasma lipid concentrations. Thus, generalized conclusions regarding the effects of steric stabilization on liposomal influx and efflux pharmacokinetics should be tempered unless they were elucidated under various other experimental conditions.

CHAPTER 6

SUMMARIZING DISCUSSION AND IMPLICATIONS

Liposomes have been utilized extensively as drug delivery vehicles for anticancer agents and there are now several such products approved for widespread use in cancer patients (e.g. Doxil™ and DaunoXome™). As this technology has developed, an increasing focus has been placed on designing formulations with extended circulation lifetimes. This is due to the fact that small (100 nm) liposomes preferentially extravasate across the leaky vasculature of solid tumors and accumulate in the tumors. It was reasoned that improved disease site delivery could be achieved by increasing plasma liposome concentrations which would provide increased solid tumor accumulation simply through mass-action relationships. However, it is likely that alterations in liposome physical properties such as surface grafting of hydrophilic polymers (eg. PEG) may alter both influx and efflux rates that contribute to the net flux, or accumulation, of liposomes in solid tumors. Furthermore, it is very possible that liposome influx and efflux rates may be differentially affected by such formulation changes. In this study, avidin cross-linking of liposomes was utilized to rapidly deplete liposomes from the blood compartment, thereby allowing liposome tumor efflux and influx rates to be determined. This methodology was assessed to establish its utility in characterizing the detailed tumor accumulation properties of liposomal drug delivery systems.

Conflicting results and interpretation still exist regarding the effects of steric stabilization. Many studies show that an increase in the circulation times of liposomes can lead to

increased solid tumor accumulation and improved therapeutic effect. However, inclusion of lipids such as PEG may decrease the efficiency of liposome extravasation from the blood into tumor tissue (slow influx) due to steric hindrance or alternatively, increase the rate of egress (high efflux) from the tumor as proposed in some studies (Du *et al.* 1997; Parr *et al.*, 1997; Hong *et al.*, 1999; Mayer *et al.*, 2001). In spite of the conflicting data presented in these studies, one has to bear in mind that these are probably the only ones to date that show an absence of beneficial effects from PEG, comparing to numerous already-published studies that did show positive results. The conflicting data do raise the need to develop a method that allows the detailed characterization of the mechanisms underlying liposomal accumulation in solid tumors.

The results presented in this thesis demonstrate the utility of the plasma quenching method in understanding the mechanisms underlying accumulation of liposomes in solid tumors. It can potentially serve as a very useful and important tool for characterizing and comparing tumor accumulation kinetics of numerous combinations of liposome parameters and tumor models. The pharmacokinetics of different lipid formulations, different doses and even different liposome sizes in various tumor models can be studied. In addition, characterization of kinetics can be applied to tumor progression where different time periods after tumor cell inoculation can be examined. It is likely that pharmacokinetic differences exist between, for example, early and late solid tumours. This can be conceptualized by understanding the physiological differences in tumor sizes where smaller tumor may mean a decrease in interstitial pressure, leading to reduced efflux or enhanced influx. Less angiogenesis in early tumor growth may be translated

into reduced influx or reduced efflux since more blood vessels into tumor tissues allow greater liposomal delivery, and probably easier for extravasated liposomes to exit back out due to accessibility of vasculature.

The approach studied here allows a better understanding of the mechanisms underlying accumulation of liposomes in solid tumors. The information can point out specific problems of a particular liposome formulation in a specific tumor type (eg. poor accumulation due to high efflux of a formulation). One can then address the problems accordingly either by making manipulations to formulation to overcome the specific problems (eg. to decrease efflux) or resorting to an alternative formulation that is shown to exhibit better pharmacokinetics in that specific tumor. A database could be built for the latter purpose. The information gathered from numerous such studies on different liposome parameters in the many types of tumor models can be compiled into a single database. In the future, this database could be used to assist drug developers in selecting the most appropriate formulations or regimen based on the intended cancer indication.

Our studies do not give a definitive answer to our hypothesis (chapter 1.6), and the exact mechanisms of PEG on the pharmacokinetics and pharmacodynamics of liposomal drug delivery systems still remain to be elucidated and further investigations are needed including the pharmacokinetic characterization of drug-loaded liposomes. Nevertheless, the present thesis describes a novel and versatile method to study qualitatively and quantitatively the individual contribution of influx and efflux on the net tumor

accumulation of liposomes, and provides supporting evidence that steric stabilization enhances the pharmacokinetics of liposomes in solid tumor accumulation.

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