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APPLICATIONS OF LIPOSOMES ON ANTI-CANCER AGENTS

by

Yingqing Ran

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF PHARMACEUTICAL SCIENCES

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

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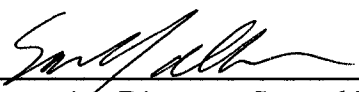
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DEDICATION

To my parents and my husband

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ABSTRACT

Toxicity is a major limitation in clinical use of most anticancer drugs. Liposomes, especially targeted long-circulating liposomes, provide the possibility of delivering drugs specifically to targeted cancer tissues, thus increasing anticancer activity and minimizing toxicity.

2-4'-Amino-3'-methylphenylbenzothiazole (AMPB), a potent anticancer drug, is inappropriate for traditional oral or parenteral formulations because of its severe dose-limiting hepatotoxicity. Several PEG-coated liposomal formulations were developed by using different drug/lipid ratios. Particle size and encapsulation efficiency of each formulation were investigated; the most stable liposomal formulation was selected for animal testing. The formulation with AMPB/egg phosphatidyl choline/cholesterol/1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-polyethylene glycol 2000, PEG2000-DSPE in a 1/5/5/1 molar ratio is the best formulation. This formulation contains 2 mg/ml AMPB with encapsulation efficiency above 95%, average particle size 120-150 nm.

Daunorubicin is a well known anti cancer agent. To minimize its cardiotoxicity, targeted folate-PEG-liposomes were developed in this study. The pH-gradient loading method was used to increase the drug loading efficiency. Above 97% loading efficiency was reached by creating a 3 to 4 unit pH difference across the liposome membrane. The final folate-

PEG-liposomal formulation contained 2.5 mg/ml daunorubicin HCl, with average particle size of 110~120 nm, pH of ~7.4, and a drug/lipid ratio 1/20 (w/w).

The solvent, chloroform, commonly used for liposome preparation, is harmful to humans. Therefore, halothane, a commonly used inhalation anesthetic, was used in this study in place of chloroform to prepare liposomes. AMPB and several other proprietary anticancer agents were formulated in liposomes by using halothane and chloroform. No obvious differences in physicochemical properties were observed between halothane and chloroform mediated liposomes.

CHAPTER 1. BACKGROUND

1.1. STRUCTURE OF LIPOSOMES

Liposomes are vesicles in which an aqueous phase is entirely enclosed by lipid bilayers (usually phospholipids). They can form spontaneously when lipids are dispersed in aqueous media, giving rise to a population of vesicles which may range in size from tens of nanometers to tens of micrometers. In the 1960s, Bangham et al. discovered the similarity between liposomes and biological membranes. This similarity can be increased through extensive chemical modification of the liposome structure. Due to their biocompatibility and ability to incorporate materials in their aqueous compartments and lipid bilayers, liposomes can be used as safe and efficacious vehicles for medical applications.

1.1.1. Chemical Composition

The major structural components of liposomes are phospholipids. Figure1-1 depicts some of the most common phospholipids. Phosphatidyl choline molecules (PC), the most commonly used phospholipids; consist of a glycerol bridge linking a pair of hydrophobic acyl groups, with a hydrophilic polar head. Due to their limited solubility in water, PC molecules align themselves closely in planar bilayers in order to minimize the contact between the aqueous media and the fatty acids chains. The contact is further minimized when the lipid sheets fold on themselves to form closed sealed vesicles. Phosphatidyl

choline forms bilayer sheets instead of micellar structures which are formed by other amphiphatic surfactant molecules. This may result from the double fatty acid chains giving the molecule an overall tubular shape, more suitable for aggregation in planar sheets compared with single non-polar chain surfactants, whose conical shape fits nicely into a spherical micellar structure.

1.1.1.1. Phase Transitions

Liposomal membranes in which phosphatidyl choline is a major component, can exist in different phases, depending upon temperature. The transition from one phase to another can be detected via physical techniques by varying the temperature.

The most common method for determining the phase transition temperature (T_c) is differential scanning calorimetry (DSC). Below the transition temperature, the membrane appears as a tightly ordered 'gel' or 'solid' phase, while it occurs as a liquid-crystal phase above the transition temperature. The phase transition temperature of liposomes is affected by the hydrocarbon chain length and degree of unsaturation, as well as the head groups of different phospholipids composing the membrane. In general, increasing the chain length or the saturation of the chains increases the transition temperature. The fluidity of liposome bilayers can be altered by using phospholipids with different T_c , which can vary from -20 to 90°C depending upon the length and nature (saturated or unsaturated) of the fatty acid chains. The presence of high T_c lipids ($T_c > 37^\circ\text{C}$) makes the liposome bilayers less fluid at physiological temperature and less leaky (i.e. greater encapsulation efficiency). Incorporation of cholesterol into lipid bilayers can also affect

the fluidity of the membrane. At high concentration (>30% molar ratio), cholesterol can totally eliminate phase transition and decrease the membrane fluidity at a temperature above T_c , which makes the liposomes more stable and less leaky after systemic administration. The fluidity of bilayers may also influence their interaction with cells. Liposomes composed of high T_c lipids appear to have a lower uptake by the reticuloendothelial system (RES), compared to those containing low T_c lipids (Gabizon and Papahadjopoulos, 1988).

The prolonged circulation time of liposomes in the blood stream provides more opportunities for encapsulated drugs to reach disease sites such as cancer cells. Besides fluidity, a variety of liposome membrane properties such as permeability, fusion, aggregation, and protein binding are influenced by T_c . Therefore, it is important to understand phase transition in order to improve the stability of liposomes and their behavior in biological systems.

1.1.1.2. Membrane Permeability

Polarity, molecular weight, and charge play important roles in the permeability of molecules across the semi-permeable liposome bilayers. Polar solutes with high molecular weight, such as sugar, pass across the membrane very slowly, while smaller neutral molecules such as water and urea can diffuse quickly. Charged ions differ greatly in their behavior, protons and hydroxyl ions cross liposome membranes fairly fast, while larger ions such as phosphate and sulfate diffuse relatively slowly. The transition

temperature (T_c) is another factor influencing liposome permeability. Most solutes have highest permeability around the transition temperature, in which the membrane has the lowest free energy and is the most stable stage.

1.1.1.3. Surface Charge

The nature and density of charges on the liposome surface are important parameters that influence the mechanism and extent of liposome-cell interaction. Both of these parameters can be altered by changing the lipid composition. Lack of surface charge can reduce the physical stability of small unilamellar liposomes (SUV) by increasing their aggregation (Sharma and Straubinger, 1994). Neutral liposomes do not interact significantly with cells; however, the drug may enter cells after being released from neutral liposomes extracellularly (Sharma et al., 1993a). On the other hand, high electrostatic surface charge could promote liposome-cell interaction. A negative surface charge may not only increase intracellular uptake of liposomes by target cells, it may accelerate their plasma clearance after systemic administration (Gabizon et al. 1990). Negatively charged liposomes may also release their contents either in the circulation or extracellularly after interaction with blood components and tissues. Unlike negatively charged liposomes, cationic liposomes deliver their contents directly to target cells via fusion with cell membranes (Felgner et al., 1994).

1.2. CLASSIFICATION OF LIPOSOMES

1.2.1. On the Basis of Composition

Liposomes are composed of either natural or synthetic phospholipids, and may also contain other bilayer constituents such as cholesterol and hydrophilic polymer conjugated lipids. These chemical components determine physicochemical properties of liposomes, such as membrane permeability, fluidity, charge density, and steric hindrance, which further determine the liposomes' interactions with blood components and other biological tissues after systemic administration.

The nature and extent of liposome-cell interaction determine the mode of intracellular delivery of drugs. Liposomes can be classified into five types based on composition and mechanism of intracellular delivery: (1) *Conventional liposomes (CL)* can be simply formed by dissolving neutral and/or negatively charged phospholipids with or without cholesterol. They can be taken up rapidly by the reticuloendothelial system (RES) and have a short circulation half-life, along with dose-dependent pharmacokinetics (PK). (2) *Long-circulating liposomes (LCL)* are composed of neutral lipids with high T_c , cholesterol, and 5-10% of hydrophilic polymer (e.g. PEG) conjugated phospholipids. Due to their hydrophilic surface coating, LCLs have a low rate of uptake by the RES, long circulating half-life, and dose independent PK. (3) *Immunoliposomes* are CL or LCL with attached antibodies or antibody fragments on the surface. They are subject to receptor-mediated endocytosis and cell-specific binding, and therefore they can be used for specific targeting vehicles. (4) *Cationic liposomes* contain cationic lipids (e.g. DOTAP:

1,2-dioleoyl-3-trimethylammoniumpropane) and have a positive charge. They possibly fuse with the cell or endosome and are suitable for delivery of negatively charged macromolecule (e.g. DNA, RNA, and oligonucleotides). (5) *pH-sensitive liposomes* are subject to coated-pit endocytosis and are therefore suitable for intracellular delivery of weakly basic macromolecules. They fuse with cell or endosome membranes allowing them to release their contents into the cytoplasm.

1.2.2. On the Basis of Size

In addition chemical composition, liposomes can also be characterized by their size and shape, which is primarily influenced by their chemical composition and the method of manufacture. Classification of liposomes according to size is the most common index of characterization in current use. It is typical to divide liposomes into the following categories: (1) *Multilamellar vesicles* (MLVs) usually consist of a population of concentric bilayers covering a wide range of sizes (100-1000 nm), and contain more than one bilayer in the membrane. MLVs are easily formed by gentle shaking of dry phospholipids in water. The lamellarity of MLVs depends on lipid composition among other factors, although it typically varies from five to twenty bilayers. Liposomes with lower numbers of lamellae are sometimes referred to oligo-lamellar liposomes. (2) *Small unilamellar vesicles* (SUVs) are liposomes no greater than 100 nm and are relatively homogeneous. Ultrasonication, high pressure extrusion, and alcohol injection methods are capable of producing vesicles in this smallest size range. (3) *Large unilamellar vesicles*

(LUVs) are single-shelled vesicles with diameters of approximately 500 nm or larger. The first methods developed to prepare such vesicles were calcium-induced fusion of liposomes composed of SUVs, and the ether injection method. (4) *Intermediate-sized unilamellar vesicles* (IUVs) include liposomes measuring 100 nm to 200 nm, although this term has not yet been widely adopted. Liposomes of this size range are easily prepared either by high-pressure extrusion or detergent dialysis. They are important for pharmaceutical applications because intermediate-sized particles provide longer circulation time in the blood stream, and ease of sterilization by membrane filtration.

1.3. METHODS FOR LIPOSOMES PREPARATION

Even though liposomes are easily formed as a result of unfavorable interactions between phospholipids and water, the size, structure, and encapsulation efficiency of liposomes can be manipulated by using different preparation methods. All liposome production techniques include five basic steps (Fig. 1-2): (1) Dissolving the lipids in organic solvents (e.g. chloroform, methanol, and halothane). (2) Drying down the lipids from organic solvents. (3) Dispersing the lipids in aqueous media (i.e. water, normal saline, and buffer). (4) Reducing particle size by sonication, extrusion, or homogenization. (5) Analyzing the final product. The major difference between the various methods of manufacture is the way in which the membrane components are dispersed in the aqueous medium before being allowed to coalesce in the form of bilayer sheets. The liposome

preparation methods have been classified generally according to the mode of dispersion employed: physical dispersion, two-phase dispersion, and detergent solubilization.

1.3.1. Mechanical Dispersion Methods

In this group of methods, to facilitate dispersion, lipids are dried down onto a solid support such as the side of a glass flask. Aqueous solution is then added, followed by mechanical force such as shaking to mix the lipids into a homogeneous solution. Before exposure to the aqueous medium, the lipids in the dried down film are assumed to be oriented in multi-bilayers with separate hydrophilic and hydrophobic regions. After hydration, the lipid films swell and peel off the support in sheets, generally to form multilamellar vesicles. The aqueous volume enclosed within the lipid membrane of the liposomes is usually only 5 to 10% of the total volume used for swelling. Thus mechanical dispersion is not ideal for entrapment of hydrophilic compounds; however, this method provides high efficiency (up to 100%) for lipophilic compounds.

The simplest method of mechanical dispersion is commonly known as “hand-shaking”, since the lipids are sloughed off the side of a glass vessel into the aqueous medium by gentle agitation. In order to increase the entrapment volume, a large round-bottom glass flask is recommended, so that the lipids will be dried down onto as large a surface as possible. Furthermore, adding glass beads to either the aqueous solution or the organic solvent is a very effective aid in suspending lipids.

After hydration of dried lipids, it is possible to continue processing the liposomes in order to modify their size and other characteristics. For many applications, especially for pharmaceutical use, MLVs are too large and heterogeneous to work with. Several techniques have been devised to reduce the particle size and increase homogeneity, including sonication, micro-emulsification, extrusion, and the use of a French pressure cell. Other methods such as freeze-drying, freeze-thawing, or induction of vesiculation by ions or pH change, are designed to increase entrapment volume and/or reduce the lamellarity of the vesicles formed.

1.3.1.1. Sonication Methods

In order to reduce the particle size of liposomes, it is necessary to expose MLVs to a high level of energy. This was first achieved through ultrasonic irradiation (Huang, 1968). There are two methods of sonication, probe sonication or bath sonication. Probe sonication is employed for suspensions requiring high energy in a small volume (e.g. high concentration of lipids, or a viscous aqueous solution). Although probe sonication is suitable for reducing the size of large liposomes, the sample volume which can be handled is limited, and due to the high energy input of this method, there is a considerable risk of degradation of the lipids resulting from high temperature. It is important, therefore, that the sonication vessel be cooled efficiently all times.

Bath sonication offers several advantages over probe sonication. Bath sonication is much milder, thus imposing less risk of lipid degradation; larger sample volumes can be utilized; and the field of ultrasonication irradiation is more homogeneous leading to more uniform particle size, which then allows greater reproducibility. It should be noted, however, because the energy is dispersed over a larger area, that the minimum size limit for sonicated vesicles may not be obtainable by bath sonication.

1.3.1.2. Micro-emulsification/ Micro-fluidization Methods

The use of a micro-fluidizer to prepare small MLVs has been reported (Mayhem, 1984). The micro-fluidizer produces very efficient transfer of energy by pumping fluid at high pressure (10,000~15,000 psi) through a 5 μm filter, after which the two streams of fluid are pushed through micro-channels and are forced to collide together at right-angles with a high velocity. After several passes, vesicle size can be reduced to 0.1 to 0.2 μm . Continuing the cycling time tends to bring the size to a steady low value. However, the exact size distribution depends upon the nature of the components of the liposome membrane and the hydration medium. Increasing cholesterol produces larger liposomes, while negative lipids tend to decrease the size of liposomes.

1.3.1.3. Membrane Extrusion Liposomes

An even gentler method of reducing liposome particle size is to pass through a membrane filter of defined pore size (Szoka, et al. 1980). The maximum particle size limit depends upon the exact size of the membrane used. There are two types of membrane filters that can be used: tortuous path and nucleation track. The “tortuous path” type membrane consists of fibers that are criss-crossed over each other to provide a matrix in which channels are formed, with random spaces arising between the fibers. The average diameter of these channels is controlled by the density of the fibers in the matrix. Liposomes which are smaller than the minimum channel diameter can pass through the membrane, while larger ones get stuck. As a result, the filter blocks up easily, even with few liposomes reaching the other side. In contrast, “nucleation track” membranes will allow liposomes to pass through the membrane even if they are larger than the pore diameter. A nucleation track membrane consists of a thin continuous sheet of polymer (e.g. polycarbonate) having straight-sided pore holes of exact diameter created by a laser and chemical etching. Because their pores go straight through one side to the other, nucleation membranes offer much less resistance than tortuous path membranes to material passing through. The flexibility of phospholipid lamellae enables liposomes to change their conformation in order to squeeze through the linear pores of nucleation membrane filters. Liposomes which are much larger than the pore diameter are broken up while passing through the filter, then emerge as smaller particles on the other side of the membrane. After passing liposomes several times through the membrane, the average size of liposomes will be smaller than the membrane pore diameter, while a small portion of

the particles will remain slightly larger than the pores, having managed to squeeze through without breaking up.

1.3.1.4. Freeze-thaw Sonication (FTS) Method

A freeze and thaw process developed by Pick (1981) was used to rupture and re-fuse liposomes, during which time the solute equilibrates between the inside and outside of the vesicles, and liposomes themselves break and re-fuse, increasing markedly in size, so that their entrapment volume increases to 30% of the total volume of the suspension. The starting preparation of liposomes is sonicated followed by freezing in a liquid nitrogen bath and thawing in a water bath. The liposomes are subjected to brief sonication again after thawing. Several cycles of freezing and thawing may be helpful to get homogeneous large unilemmellar vesicles, which are suitable for entrapping water soluble compounds into the aqueous phase of the liposomes.

1.3.2. Solvent Dispersion

Solvent dispersion is another method commonly used to prepare liposomes. In this group of methods, lipids are first dissolved in an organic solvent such as ethanol or ether, which is then brought into contact with aqueous phase. At the interface between organic and aqueous media, the phospholipids align themselves into a monolayer which forms half of the bilayers of the liposomes.

1.3.2.1. Ethanol Injection

Ethanol injection as a means of liposomes preparation was first reported by Batzri and Korn (1973). Solvent dispersion can be achieved by injecting an ethanol solution of phospholipids into aqueous media, thereby producing a high portion of small unilemellar vesicles (~25 nm). The advantages of this method are its simplicity and very low risk of lipid oxidation and degradation. A major drawback, however, is that lipids have limited solubility in ethanol, hence, this approach often results in relatively diluted liposome suspensions, which leads to low encapsulation efficiency of compounds which are water soluble. Another disadvantage is the risk of having residual ethanol in phospholipid membranes, which may produce irritation of the injection site.

1.3.2.2. Ether Injection

Ether injection is similar to ethanol injection except ether is immiscible with aqueous solutions (Deamer and Bangham, 1976). This method of dispersion involves injecting ether slowly into an aqueous solution, allowing ether to be removed by evaporation at room temperature. Since ether is removed at the same rate as it is injected, there is no limit to the final concentration of lipids, which increases the encapsulation efficiency in the aqueous phase of vesicles. The shortcomings of this method involve the long time consumption of each batch preparation of liposomes, also ether is highly flammable, and therefore, careful control is needed for lipid solution injection.

1.4. LIPOSOME APPLICATIONS OF DRUG CARRIERS

The goal of all pharmaceutical therapy is to selectively treat the pathological condition while minimizing side effects. Thus, an ideal drug has high therapeutic efficacy with few or no side effects. For many classes of drugs, this ideal is far from reality. Rational development of drugs has centered on exploiting known differences between the target diseased tissues and normal tissues. Often, the differences in susceptibility to drugs between pathological and normal tissues are quantitative rather than qualitative. This is especially true for cancer chemotherapy, where there is still lack of knowledge of disease-induced differences between cancer and normal cells. The consequence is that most cancer therapies are toxic for both tumor and healthy cells. Liposomes are made of phospholipids, which are nontoxic and biodegradable. Liposomes can be used to increase the rates of desirable to undesirable effects for the following reasons:

- (1) Different types of liposomes have different biological properties, which can be modified in accordance with the goals of specific therapeutic application.
- (2) In general, drugs are entrapped without chemical modification either in solution in the interior aqueous phase of liposomes (hydrophilic drugs) or incorporated in the lipid bilayers of liposomes (lipophilic drugs). Thus, drugs can be protected from the biological environment during transit to the target area, and they are usually released from the liposomes as the form which was entrapped.

- (3) Encapsulation results in dramatic changes in pharmacokinetic properties and biodistribution. Combined with the possibility of producing liposomes capable of targeting specific cell types, development of liposomes for use as drug carriers may yield an enhanced therapeutic index.

In this study, several anti-cancer drugs have been chosen to develop liposomal formulations. Different preparation methods and loading approaches have been investigated and various types of liposomes have been developed. The details and results of these experiments are discussed in the following chapters.

Figure 1-1. Chemical Structures of Some Common Phospholipids.

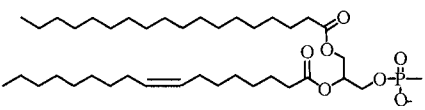
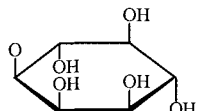
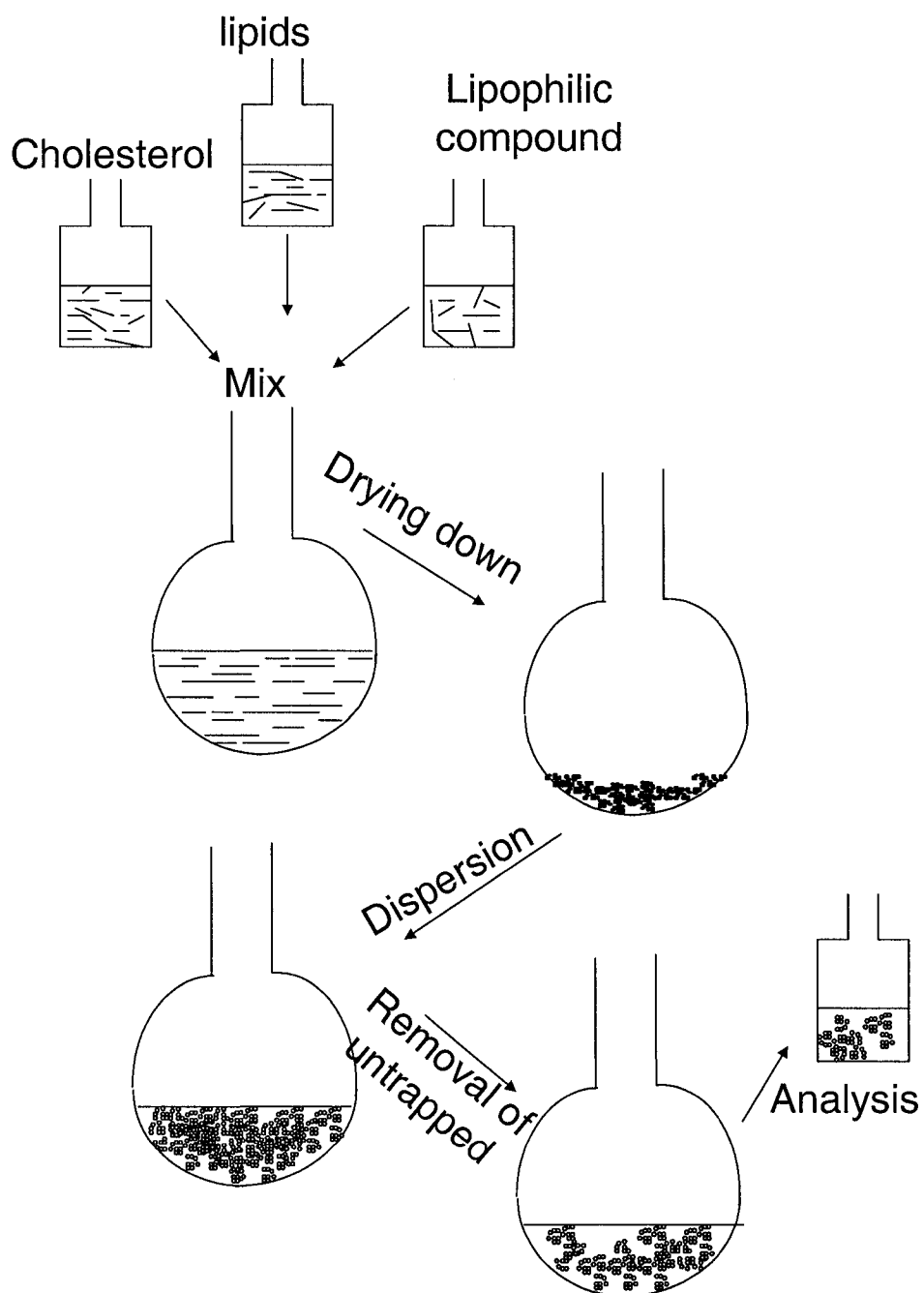
Phosphotidyl moiety	Headgroup	Common name phosphotidyl	Abbreviation
	$\text{O}-\text{CH}_2-\text{CH}_2-\overset{\text{Me}}{\underset{\text{Me}}{\text{N}^+}}$	Choline	PC
	$\text{O}-\text{CH}_2-\text{CH}_2-\overset{+}{\text{N}}\text{H}_3$	ethanolamine	PE
	$\text{O}-\text{CH}_2-\overset{\text{NH}_3^+}{\underset{\text{COO}^-}{\text{CH}}}$	serine	PS
	$\text{O}-\text{CH}_2-\overset{\text{OH}}{\underset{\text{OH}}{\text{CH}}}-\text{CH}_2$	glycerol	PG
	$\text{O}-\text{H}$	acid	PA
		inositol	PI

Figure 1-2. Schematic Describing of the Thin-film Method for Liposome Preparation.

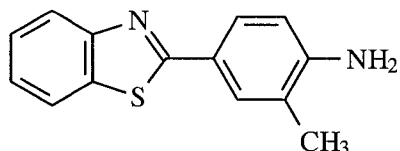


CHAPTER 2. FORMULATION DEVELOPMENT OF PEG-COATED LIPOSOMES CONTAINING THE ANTITUMOR AGENT 2-(4'-AMINO-3'-METHYLPHENYL) BENZOTHAZOLE (AMPB)

2.1. INTRODUCTION

AMPB, 2-(4'-amino-3'-methylphenyl) benzothiazole (Figure 2-1), has shown exceptional potency against cancerous cells of the breast, ovary, lung and kidney (Stevens, et al. 1994, Tabibi, 1998-2000). The drug also displays a severe and dose limiting hepatotoxicity in animal models (Tabibi, 1998-2000)

Figure 2-1. AMPB Chemical Structure.



AMPB has a low solubility in water (0.54 $\mu\text{g/ml}$) and in other commonly used vehicles (Stella, 1997). Its low water solubility and acute hepatotoxicity make the conventional oral or parenteral formulations inappropriate for AMPB. However, polyethylene glycol (PEG) coated liposomes offer an excellent alternative choice. It has been shown that while normal liposomes accumulate in the reticuloendothelial system (RES), a PEG-coating greatly reduces the recognition of liposomes by the RES, and hence prolongs their

systemic circulation (Allen, et al., 1995; Blume and Cevc, 1990; Kilibanov, et al., 1990; Lasic, et al., 1991; Senior, et al., 1991; Woodle and Lasic, 1992; Woodle, 1995). The long-circulating (or stealth) feature is critical in that it gives liposomes sufficient time to extravasate at solid tumors and sites of infection and inflammation where the capillary permeability is high (Maeda, et al., 2003; Muggia, 1999). Reduced RES uptake of PEG-coated liposomes also minimizes drug toxicity to non-target tissues, especially the liver where most phagocytic cells reside.

Liposomes are often evaluated with regard to their particle size and encapsulation efficiency. The size of the liposome vesicles is particularly important, as it relates to liposome transport and localization in the body. It is found that liposome vesicles in the 80 to 150 nm size range circulate the longest (Litzinger, et al., 1994 and Liu, et al., 1992). Other studies indicate that the maximum size for tumor accumulation is less than 200 nm (Litzinger, et al., 1994; Liu, et al., 1992).

This study aims to develop AMPB-containing PEG-coated liposome, and to evaluate their physical stability with regard to particle size and encapsulation efficiency. The proposed formulation employs egg phosphatidylcholine (EPC), cholesterol (Chol), and PEG2000-DSPE. PEG2000-DSPE, or 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-polyethylene glycol 2000, is a PEG-phospholipid and is often used for liposome surface coating. EPC is widely reported in pharmaceutical liposomes due to its natural abundance, ease in purification, and neutrality. Chol is also added along with EPC and at

a one to one molar ratio, as it rigidifies the lipid bilayer and hence increases liposome physical stability (Cullis, et al., 1989; Gregoriadis, 1976; Woodle and Lasic, 1992).

DSPE2000 is used in its commonly accepted range of 5-10% of the total molar lipids (Allen and Hansen, 1991; Blume and Cevc, 1990; Sadzuka and Hirota, 1997).

The objective is to prepare an intravenous (i.v.) formulation with high dose loading, small particle size (< 200 nm) and good stable physical stability.

2.2. EXPERIMENTAL

2.2.1. Materials

2-(4'-Amino-3'-methylphenyl) benzothiazole (AMPB, or NSC 674495, MW: 240.32) was provided by the National Cancer Institute (Bethesda, MD). The drug is yellow, crystalline solid with a melting point of 195.9°C. The lipid chemicals egg phosphatidylcholine (EPC, MW: 760), phosphatidylglycerol (PG, MW: 771), 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-polyethylene glycol 2000 (PEG 2000-DSPE, MW: 2,788) were purchased from Avanti Polar Lipids, Inc. Cholesterol (Chol, MW: 386.6) was purchased from Sigma. Normal saline (0.9% NaCl) was purchased from Baxter Healthcare Corp. All other chemicals and organic solvents of analytical grade were purchased from either Sigma or Aldrich (Saint Louis, MO).

2.2.2. HPLC Assay

A Beckman Gold HPLC system with a model # 168 detector was used for all assays. A Pinnacle octyl amine (C8) column (5 μm , Dimension: 150 \times 4.6 mm, Cat. No.: 9183565, Restek Corporation) was used to detect AMPB at a wavelength of 254 nm. The HPLC mobile phase was composed of acetonitrile (ACN), and an aqueous solution that contained 0.1% trifluoroacetic acid (TFA). The gradient elution program started with 70% aqueous solution and 30% ACN. The injection volume was 50 μl , and the flow rate was 1.0 ml/min. The retention time of AMPB is approximately 9.6 \pm 0.1 minutes at ambient temperature. The diode array detector connected to the HPLC provided the verification of the peak homogeneity. Each run took approximately 20 minutes. A calibration plot of peak area versus concentration was found to be linear in the range of 1–100 $\mu\text{g/ml}$. None of the lipids interfered with the AMPB peak.

2.2.3. Liposome Preparation

Two different preparation methods (thin-film hydration and solvent injection) were employed. For the thin-film method, all the ingredients were dissolved in chloroform/methanol (2/1, v/v), completely dried in a rotary evaporator and then reconstituted with normal saline. For the solvent injection method, all the ingredients were dissolved in ether and slowly injected (0.5 ml/min) into 55 $^{\circ}\text{C}$ normal saline under

moderate agitation. The ether was removed during the process and the final volume was adjusted.

2.2.3.1. Thin-film Hydration Method

The thin-film hydration procedure is illustrated by the following preparation of 2 mg/ml AMPB PEG-coated liposomes:

- a) Prepare the solution of drug and lipids with the following ratio: 10/158/80/114 mg for AMPB/EPC/Chol/DSPE-PEG2000 (molar ratio: 1/5/5/1), in 5 ml of chloroform/methanol solvent mixture (2:1 v/v).
- b) Introduce the above solution into a 250-ml round bottom flask with a ground glass neck: Attach the flask to a rotary evaporator (Rotavapor[®], Brinkmann Instruments Inc), immerse the flask in a 37-40°C water bath and evacuate with a rotating speed of approx. 60 rpm.
- c) Continue until all the liquid is evaporated and a dry lipid film is deposited on the walls of the flask; continue for 15 minutes after the dry residue first appears;
- d) Place the flask in a vacuum desiccator for 8 hours to completely remove the residual solvents. After releasing the vacuum and removing samples from the desiccator, add approx. 4.5 ml of saline and shake gently in the water-bath of 50°C for 20 minutes. A homogeneous milky suspension free of visible particles is formed.

- e) After transferring the liposomal suspension, adjust the volume to 5 ml by adding 0.9% saline. Allow the suspension to stand for 2 hours at room temperature in order to complete the swelling process. The final liposome solution contains 2 mg/ml AMPB.

2.2.3.2. Solvent Injection Method

The solvent injection is illustrated by the following preparation of 2 mg/ml AMPB liposomes:

- a) Prepare the mixture of drug and lipids with the following ratio: 10/158/80 mg for AMPB/EPC/Chol (molar ratio: 1/5/5), in 10 ml ether and place the solvent solution into a sterile syringe.
- b) Prepare approximately 4.5 ml of 0.9% saline into a sterile, polypropylene tube (50 ml) and warm to 55°C in a water bath.
- c) Attach the tube to the vortex device, and turn on; use moderate to high speed (without splashing) to agitate the solution. It is critical that the vortexing be performed without the production of bubbles. With constant vortexing, slowly inject the ether solutions, at approx. 0.5 ml/min, into water. Vortex for another 10 minutes.
- d) Following production, the liposome solution is maintained for 20 minutes at a temperature of 55°C to anneal the liposome structure followed by 8 hours inside the ventilation hood for complete removal of solvent residuals. The final

solution is adjusted by 0.9% saline to 5 ml so that the AMPB dose loading is 2 mg/ml.

2.2.4. Reduction of Vesicle Size

The size reduction was performed by hand-homogenization, water-bath sonication, and mini-extrusion using polycarbonate membranes of certain pore size (Avanti Polar Lipids, Inc.), a microfluidizer with a interaction chamber pressurized at 10,000 psi (model 110T, Microfluidics Corporation), and a probe sonicator (model SDT1810, Tekmar Company), and a Homogenizer (Model EmulsiFlex-C5, Avestin, Canada).

2.2.5. Optical Microscope Observation

An optical microscope was used to observe the liposome particle size and the AMPB encapsulation. Microliters of the liposome solution were pipetted on glass microscope slides and observed by using an optical microscope (Leica Mikroskopie & Systeme GMBH Wetzler, Germany) at different magnifications (20-63 times). Particle diameter measurement was made possible with the aid of a calibrated eyepiece scale that detects particle 0.3 μm and larger.

2.2.6. Particle Size Measurement

The Zetasizer 1000 (Malvern Instruments, UK) was used to measure the liposome particle size in the range 3-1000 nm. The size measurement is the analysis of the

time dependence of intensity fluctuations in scattered laser light due to the Brownian motion of particles in suspension. Since small particles diffuse more rapidly than large particles, the rate of fluctuation of scattered light intensity varies accordingly. Thus the translational diffusion coefficient (D) can be measured, which in turn can be used to determine the mean hydrodynamic radius \bar{r} of the particles using the Stokes-Einstein equation as

$$D = \frac{RT}{6\pi\eta rN}$$

Where R is the gas constant, T is the absolute temperature, N is Avogadro's number, and η is the viscosity of the medium.

The instrument settings were as following: temperature: 25°C; scattering angle: 90°; the aperture: 400 μm ; count rate (kilo counts/sec): 100 \pm 50; analysis mode: auto/continuous; running time: automatic (range 120-3,000 seconds). Samples were diluted with de-ionized water if necessary. All samples were sonicated approximately 30 seconds before measurement. The reading was the average mean particle size (nm).

2.2.7. Drug Encapsulation Efficiency Determination

The extruded liposomes (size: approx. 100-130 nm) were diluted 40 times by 0.9% saline solution. The diluted solutions were gently shaken for approx. 20 seconds and were free from any visible aggregation and precipitation.

Duplicate 1 ml samples were placed in centrifuge tubes specially made for ultracentrifugation (NalgeneTM, Nalge Company). The instrument (L8-55 ultracentrifuge, Beckman) was used at 50,000 rpm and 10°C for 2 hours in a temperature pre-equilibrated head. After separation from the lipid pallet, the supernatant was measured by HPLC to determine the released drug concentration. The drug concentration in the liposome solution before centrifugation and the concentration of the supernatant allowed the percentage of the drug encapsulated by the liposomes to be calculated.

An alternative was to measure the drug content in the lipid pallet after centrifugation. The pallet was first dissolved in water followed by sonication at 37°C for 10 minutes to fully disperse the pallet. The suspension was then diluted to proper concentrations for HPLC analysis. It was found that the drug amount measured by the combined amount from the supernatant and the lipid pallet was consistent with the drug content in the samples before ultra-centrifugation. Since the measurement of the supernatant was easier, it was preferred and was used in this study.

2.3. RESULTS AND DISCUSSION

2.3.1. Evaluation of Preparation Methods

Table 2-1 compares the results of the two preparation methods, i.e., the thin-film hydration method and solvent injection method, on two test liposomes.

The thin-film hydration method is simple and widely used for liposome preparation. The chloroform/methanol mixture is the commonly used solvent system, as it provides good solubility for lipids and can facilitate lipids to form a nice thin film on the wall of the round bottom flask. Egg phosphatidylcholine has high solubility (140 mg/ml) in chloroform, while its solubility decreases two-fold (to 70 mg/ml) in ether. The AMPB solubility is 7.5 mg/ml in chloroform and 1.2 mg/ml in ether, which indicates that chloroform is a better solvent to prepare AMPB liposomes. However, chloroform is a hazardous solvent, and any residue is undesirable for the safety reasons. Halothane, an inhalation anesthetic and a safer solvent, has been used in place of chloroform in liposome preparation for two anti-cancer agents (Ran and Yalkowsky, 2003; Jain and Yalkowsky, 2003).

The solvent injection method was reported in a few case studies (New, 1997). This type of method is simple and is known to treat lipids gently. Our study employed both ether (b.p.: 34.5°C) and a mixture of ether and dichloromethane (b.p.: 40°C). The low solubility of both drug and lipids in ether requires a large volume of solvent to be applied. As seen in Table 2-1, this prolongs the time for solvent

removal. The highly volatile and flammable ether also posed safety problems. Even though the use of mixed ether and dichloromethane reduced the amount of ether used and eliminated the AMPB crystal (Table 2-1), it tremendously increased the time for solvent injection and solvent removal. This indicates that a better solvent is needed before the method could be put in use.

Though ethanol (b.p.: 78.5°C) is commonly used for solvent injection, it is often used for preparing low concentrations of lipids and for hydrophilic drugs. In addition, some ethanol would remain in the final product, which is a severe restriction to its use.

Therefore, the thin-film hydration method was considered to be more suitable and was investigated in the following AMPB liposome (MLV) preparations.

2.3.2. Evaluation of Lipid Components and Lipid/AMPB Ratio

This study was designed to determine the basic lipid components and the AMPB to lipid ratio for further PEG-coated AMPB liposomal studies

Table 2-2 summarizes the preparation method and microscopic observation of the AMPB-containing liposomes. These liposomes were freshly prepared, with 2 mg/ml AMPB without size reduction. Microscope observation revealed that they

were multilamellar vesicles (MLV) with a 0.5-10 μm diameter range. Table 2-2 shows that formulations with higher lipids to drug ratios (i.e. formulation 1-3) have no crystal particles. The lack of AMPB crystal in the liposomes is an indication of high encapsulation efficiency. With the same ratio, the thin-film hydration method provides better encapsulation efficiency than the solvent (ether) injection method. Reduction of the multilamellarity of liposomes 1-3 by hand homogenizer yielded large unilamellar vesicles (LUV) (approx. 250-260 nm). Physical stability studies showed that the particle sizes of all three liposomes remained largely unchanged at 25°C (from 250 to 275 nm) and at 37°C (from 250 to 290 nm) over a 7-day period. AMPB/EPC/Chol with molar ratios 1/8/8 and 1/5/5 were chosen for further PEG-liposomes preparation by adding PEG2000-DSPE.

2.3.3. Evaluation of Size Reduction Techniques

Table 2-3 lists the comparison results for a sample multilamellar liposome by various sizing techniques. It shows that both hand homogenizer and water-bath sonicator were less efficient and produce a wide distribution of particle sizes. These two methods, along with probe sonication, were not able to reduce the particle size to less than 200 nm. On the other hand, mini-extrusion, homogenization and microfluidization are able to produce liposomes with small sizes (mostly <200 nm) in a narrow range. The microfluidizer is most attractive for handling large volume samples (>20 ml), while the mini-extruder is good for the small volumes (<5 ml) which are employed in this study.

The EmulsiFlex C-5 homogenizer has the high efficiency and is able to handle relatively small volume (>7 ml) samples.

Combining water bath sonication with microfluidization or homogenization was used in this study and was shown to generate a narrow range of particles of liposomes.

2.3.4. Evaluation of PEG-coated Liposomes

Six PEG-coated liposomes with various lipids and drug ratios were prepared by the thin-film method and are listed in Table 2-4. Liposomes A and B were directly based on liposomes 3 and 5 of Table 2-2. The added PEG2000-DSPE increased the lipid concentration and also increased liposome encapsulation ability, as no drug crystals were observed. It also suggests that there might be a potential for a drug loading increase with the given lipid composition. Further testing on liposomes C, D, E and F showed that liposomes C might have reached the limit of encapsulation, as there were problems with preparations D and E due to lower solubility of the solids in the solvent system, and drug crystals were observed in liposomes F.

As there is a natural tendency for liposome vesicles to fuse and/or aggregate, the particle size becomes an important indicator of liposome physical stability and is closely related to drug therapeutic activity. The initial particle sizes were in the range of 112-138 nm after size reduction. Figures 2-2 and 2-3 summarize the results of particle size fluctuation at different temperatures, 4 and 37°C, over time. At 4°C, the particle size of all the five

formulations was stable for at least 50 days. At 37°C, high lipid concentrations tend to have more pronounced effect on the particle size (liposomes D, E) as compared to low lipid concentrations (liposomes B, C). High temperature (37°C) also tended to increase the particle size growth.

Apart from particle size, encapsulation efficiency is also an important parameter of liposome physical stability. It is a ratio of the amount of drug entrapped inside the liposomes, plus the amount incorporated between the lipid bilayers, over the total amount of drug in the liposome solution. The extruded liposomes (A-E) were evaluated for their encapsulation efficiency at 37°C (Table 2-4). The initial encapsulation efficiency was high for all five liposomes, indicating that the lipid concentrations used are sufficient for entrapping drug molecules in the lipid bilayers. The encapsulation efficiency gradually decreased over time. After 9 days, they all dropped to mid-90% with no significant difference among the formulations.

Formulation B, which has high encapsulation efficiency and small particle size in a narrow range (Table 2-6), was shown to be the best among the five formulations after six-month stability study. Formulation B has AMPB/EPC/Chol/PEG2000-DSPE in a molar ratio 1/5/5/1 and the concentration of AMPB of 2 mg/ml. Based on this study, it is the optimal drug/lipid ratio which provides both high encapsulation efficiency above 95% and particle size less than 150

nm after six months storage at 4°C. The other formulations with higher or lower drug/lipid ratio either have less size stability or a tendency for AMPB leakage over time.

2.4. CONCLUSIONS

The thin-film method, along with homogenization or microfluidization, is effective for preparation of liposomes. The drug/lipid ratio is an important factor determining the physical stability of liposome formulations. High lipid concentrations can entrap more non-polar drugs like AMPB in the bilayers, while they tend to have difficulty hydrating and producing larger particle size liposomes. It is critical to choose the right drug/lipid ratio in order to get good liposome formulations for a specific compound. Based on this study, formulation B with AMPB/EPC/Chol/PEG2000-DSPE 2000 molar ratio of 1/5/5/1 are the most stable PEG-coated liposomes. Samples were made and sent to the NCI for animal PK studies.

2.5. ACKNOWLEDGEMENTS

This work was performed under contract CM-77109 from the National Cancer Institute.

Table 2-1. Evaluation of Liposome Preparation Methods*

Preparation method	Solvent used	Crystals**	Comments
Thin-film hydration	CHCl ₃ /MeOH (2/1, v/v, 5 ml)	-	8 hrs in a desiccator before hydration
Solvent injection	Ether (15 ml)	+	30 min for solvent injection, 16 hrs in ventilation hood
Solvent injection	Ether/CH ₂ Cl ₂ (1/1, v/v, 12 ml)	+	35 min for solvent injection, 24 hrs in ventilation hood
Solvent injection	Ether/CH ₂ Cl ₂ (1/1, v/v, 18 ml)	-	50 min for solvent injection, 30 hrs in ventilation hood

*The composition of prepared liposomes: 2/32/16/23 mg/ml for AMPB/EPC/Chol/PEG2000-DSPE and the molar ratio was 1/5/5/1. The final solution was 5 ml for each preparation.

** AMPB crystals were seen from the optical microscope.

The symbol '-' denotes no crystals observed, '+' slight amount of crystals and '++' large amount.

Table 2-2. Microscope Observation of Multilamellar AMPB Liposomes

Label	AMPB/EPC/Chol/PG (molar ratio)	Preparation Method*	Crystals observed**
1	1 / 10 / 10 / 1	TFH	NC
2	1 / 8 / 7.2 / 0.8	TFH	NC
3	1 / 8 / 8 / 0	TFH	NC
4	1 / 5 / 5 / 1	TFH	SC
5	1 / 5 / 5 / 0	TFH	SC
6	1 / 3 / 3 / 0	TFH	LC
7	1 / 5 / 0 / 0	TFH	LC
8	1 / 5 / 5 / 0	SI	LC

*TFH = thin-film hydration method, SI= solvent injection method

**NC = no crystal observed, SC = slight amount of crystal, LC = large amount of crystal

Table 2-3. Evaluation of Sizing Methods on Multilemellar Liposomes*

Device	Condition	Particle size (nm)	Efficiency	Reproducibility	Applicable volume
Hand homogenizer	10 passes	258-333	low	low	> 10 mL
Microfluidizer	5 passes	170-204	high	moderate	> 20 mL
Homogenizer	5 passes	165-190	high	high	> 7 mL
Water-bath sonicator	30 min	296-387	low	low	all volumes
Probe sonicator	20 min	230-275	low	low	2-10 mL
Mini-extruder	10 passes	110-136	low	high	< 5 mL

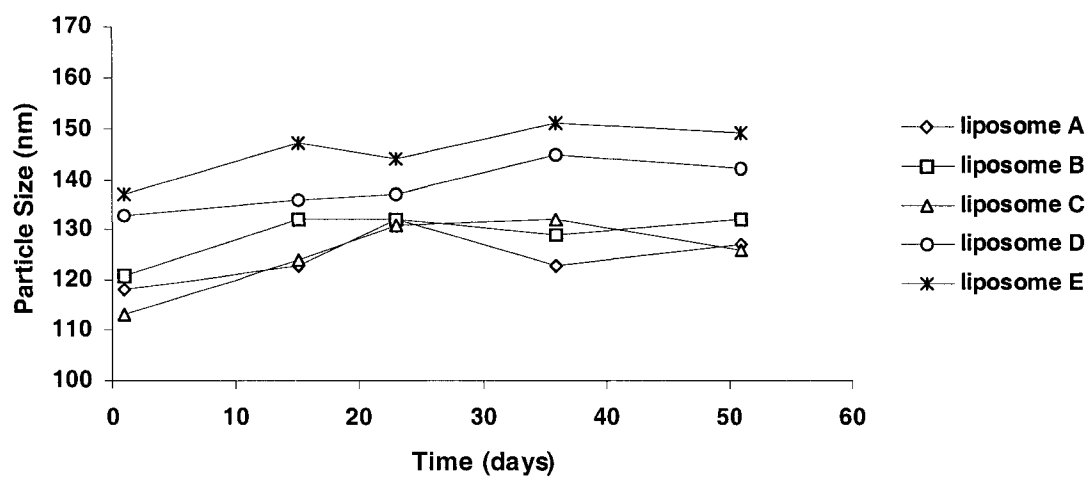
Table 2-4. Composition and Microscope Observation of Multilamellar AMPB Liposomes

Label	AMPB/EPC/Chol/PEG- DSPE2000 (mg/ml)	AMPB/EPC/Chol/PEG- DSPE2000 (molar ratio)	Observation *
A	2 / 51 / 26 / 23	1 / 8 / 8 / 1	NC
B	2 / 32 / 16 / 23	1 / 5 / 5 / 1	NC
C	4 / 32 / 16 / 23	2 / 5 / 5 / 1	NC
D	4 / 51 / 26 / 23	2 / 8 / 8 / 1	NC
E	8 / 51 / 26 / 23	4 / 8 / 8 / 1	NC
F	10 / 32 / 16 / 23	5 / 5 / 5 / 1	LC

* NC = no crystal observed, SC = slight amount of crystal, LC = large amount of crystal

Composition for the test liposome: 2/51/26/23 mg/ml for AMPB/EPC/Chol/PEG2000-DSPE (molar ratio: 1/8/8/1); 2-3 samples were used for size measurement; after certain passes and certain time period as recorded above, the decrease in liposome particle size seemed to have reached limits, as further repetition or prolonged time did not produce meaningful size changes; for hand homogenization and microfluidization, the test liposome samples were diluted 10× and 20× by normal saline, respectively, due to small original volumes.

Figure 2-2. Particle Size Studies for Liposomes* and E at 4°C.



*A: AMPB/EPC/Chol/ PEG200-DSPE: 2/51/26/23 mg/ml

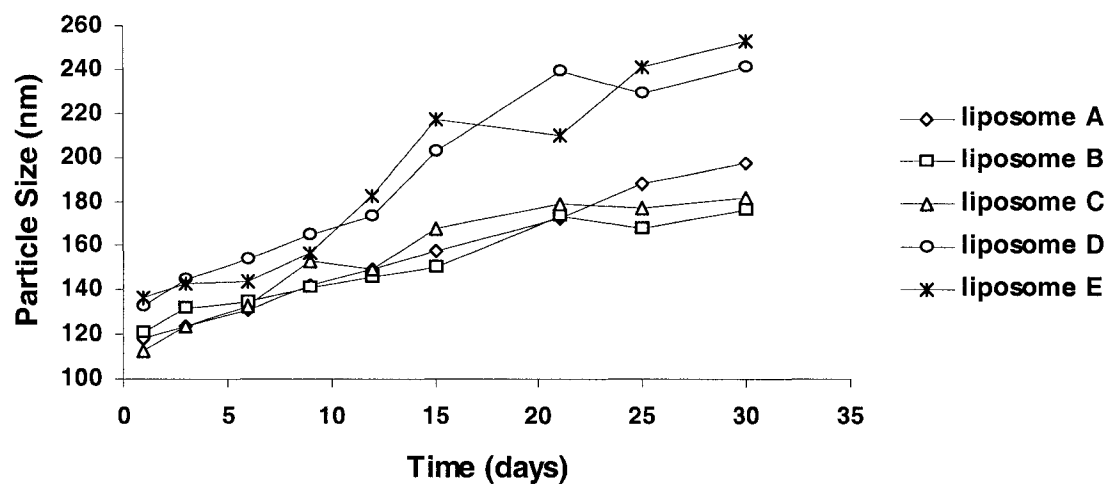
B: AMPB/EPC/Chol/ PEG200-DSPE: 2/32/16/23 mg/ml

C: AMPB/EPC/Chol/ PEG200-DSPE: 4/32/16/23 mg/ml

D: AMPB/EPC/Chol/ PEG200-DSPE: 4/51/26/23 mg/m

E: AMPB/EPC/Chol/ PEG200-DSPE: 8/51/26/23 mg/ml

Figure 2 -3. Particle Size Studies for Liposomes A, B, C, D and E at 37°C.



A: AMPB/EPC/Chol/ PEG200-DSPE: 2/51/26/23 mg/ml

B: AMPB/EPC/Chol/ PEG200-DSPE: 2/32/16/23 mg/ml

C: AMPB/EPC/Chol/ PEG200-DSPE: 4/32/16/23 mg/ml

D: AMPB/EPC/Chol/ PEG200-DSPE: 4/51/26/23 mg/ml

E: AMPB/EPC/Chol/ PEG200-DSPE: 8/51/26/23 mg/ml

Table 2-5. Encapsulation Efficiency (%)* of Liposomes A, B, C, D and E at 37°C

Label**	Day 1	Day 3	Day 6	Day 9
A	97.7	97.3	96.4	93.2
B	98.6	95.5	94.8	92.1
C	96.7	97.3	95.8	94.4
D	99.1	100.0	95.2	94.3
E	100.0	97.2	96.2	96.3

*encapsulation efficiency = (drug in liposomes – drug in supernatant)/ (drug in liposomes)×100%, and the liposomes were before centrifugation.

**A: AMPB/EPC/Chol/ PEG200-DSPE: 2/51/26/23 mg/ml

B: AMPB/EPC/Chol/ PEG200-DSPE: 2/32/16/23 mg/ml

C: AMPB/EPC/Chol/ PEG200-DSPE: 4/32/16/23 mg/ml

D: AMPB/EPC/Chol/ PEG200-DSPE: 4/51/26/23 mg/ml

E: AMPB/EPC/Chol/ PEG200-DSPE: 8/51/26/23 mg/ml

Table 2-6. Physical Stability of Formulation B* at 4°C

Time (day)	0	6	15	30	60	90	180
Size (nm)	123	124	127	129	132	137	150
EE (%)	99.4	99.2	98.7	97.9	96.5	96.2	95.8

* Formulation B: AMPB/EPC/Chol/PEG2000-DSPE: 2/32/16/23 mg/ml

CHAPTER 3. FOLATE-PEG-LIPOSOMES AS A TARGETED DRUG DELIVERY SYSTEM: APPLICATION TO DAUNORUBICIN

3.1. INTRODUCTION

Liposomes as vehicles for intravenous drug delivery have received a lot of attention in recent years because of their unique desirable qualities (Litzinger and Huang, 1992).

There are two major limitations for conventional liposomes to be widespread as drug carriers *in vivo*. First, unmodified liposomes are removed by macrophages of the reticuloendothelial system (RES) within a few hours of administration (Allen, et al. 1991; Papahajopoulos, et al. 1991). This problem has been largely solved by optimization of liposome size and composition, and by protecting the surface of liposomes with hydrophilic polymers such as gangliosides (monosialoganglioside GM1) or polyoxyethylene glycol (PEG) (Bally, et al. 1990; Klivanov, et al. 1991).

Monosialoganglioside or PEG-coated liposomes have shown long circulation time before removal by the RES system. Second, the inability of targeting liposomes to the tissue of choice such as tumors limits their therapeutic efficiency. Immunoliposomes, which have monoclonal antibodies or antibody fragments attached to PEG-coated liposomes, have been designed for specific targeting (Carrion, et al. 2001). However, the instability and antibody-mediated immunogenicity of immunoliposomes are obstacles to their effectiveness as specific target delivery carriers. Naturally occurring small ligands such as vitamins, hormones and growth factors which bind to cell surface receptors may also used

as a “homing device”. They have some advantages over antibodies, such as lack of immunogenicity, high affinity for their receptors, and low cost.

A low molecular weight ligand, folic acid, has been employed as a targeting agent because the high affinity folate receptors (FR) are greatly over expressed in various types of cancer cells and are generally absent in most normal tissues. Choroid plexus and placenta, are exceptions and a low level expression is in lung, thyroid and kidneys (Whiteman, et al. 1992).

The folate receptor also known as the high affinity membrane folate-binding protein is a glycosylphosphatidylinositol-linked membrane glycoprotein with an apparent molecular weight of 38-40 kDa (Antony, 1996). Two membrane-bound isoforms of FR have been identified in humans and designated α and β . The FR α isoform has a dissociation constant (K_d) for folic acid of ~ 0.1 nM, which is approximately ten fold lower than its K_d for reduced folates (e.g., 5-methyltetrahydrofolate) (Wang, 1992)

In order to gain access to the tumor cell surface via the circulatory system, the water solubility of the anticancer agent must be high; however, to penetrate the cancer cell plasma membrane upon arrival at the cell surface, lipid solubility must be high. These two properties are often difficult to design into the same drug molecule. However, this limitation can be overcome by encapsulating desired drugs into folate-liposomes. Since folate-liposomes and the encapsulated drugs can enter cancer cells by receptor-mediated

endocytosis (Antony et al., 1985; Lee and Low, 1994), the normal permeability barriers that limit drug entry into cells are bypassed, allowing even very hydrophilic drugs/macromolecules to enter folate receptor-bearing cells as readily as their low molecular weight hydrophobic counterparts. Therefore, folate-liposomes as targeted drug delivery systems, provide selectivity to cancer cells as well as minimization of toxicity to normal cells.

Even though daunorubicin (DNR) is one of the most effective anticancer agents, its clinical application is limited by its deleterious side effects, including myelosuppression, gastrointestinal toxicity, and especially cardiotoxicity (Minow, 1975). To avoid these complications, the use of liposomes as carriers for DNR has recently been explored both in animals and in humans. In fact Daunoxome[®] NDR liposomes are on the market the advantage of liposomal DNR over free DNR is its improved pharmacokinetic performance including increased AUC, C_{max} and decreased V_d (Bally, et al. 1990). Liposomes also have the tendency to extravasate and preferentially accumulate in tumor tissues with leaky endothelia and reduced lymphatic drainage (Gabizon, et al. 1994). The attractiveness of liposomes as carriers was further enhanced by long-circulating liposomes coated with polyethylene glycol (PEG). The PEG coating inhibits recognition of the liposomes by the phagocytes of the reticuloendothelial system (RES) (Papahadjopoulos, et al., 1991 and Allen, et al. 1991). Lee and Low (1995) found that liposomes can be efficiently targeted to receptor-bearing tumor cells when conjugated to folate via long PEG-spacer. An NCI contract lab has proposed that folate conjugated

PEG-liposomes show improved therapeutic efficacy of doxorubicin (DOX) in the mouse model. This study aimed to develop folate-PEG-liposomes for daunorubicin and provide samples to NCI for further PK studies.

3.2. EXPERIMENTAL

3.2.1. Materials

Folic acid (MW: 441.40), N-hydroxysuccinimide (NHS, MW: 115.09), triethylamine (MW: 101.19), and dimethyl sulfoxide (DMSO, MW: 78.13) were purchased from Sigma; N,N'-dicyclohexylcarbodiimide (DCC) was purchased from Fluka; dioleoylphosphatidylethanolamine (DOPE, MW: 760), dioleoytrimethylammonium propane (DOTAP, MW:698.55), distearoylphosphatidylcholine (DSPC), distearoylphosphatidylethanolamine polyethyleneglycol 2000 (PEG2000-DSPE, MW: 2788) were purchased from Avanti Polar Lipids. Cholesterol and other chemicals were purchased from Sigma Chemical. Daunorubicin HCl and Folate-PEG 3350-DSPE (MW: 4605.58) were provided by the NCI.

3.2.2. Methods

3.2.2.1. Synthesis of Folate-NHS

- a) Folic acid (1 g) was dissolved in 20 ml dry DMSO (dried with CaH_2 and distilled under reduced pressure) and 500 μl triethylamine.

- b) 0.94 g DCC and 0.52 g NHS were added to the solution. The mixture was stirred overnight at room temperature in dark.
- c) The yellow solution was filtered with filter paper #1 to remove the by-products and unreacted materials. 15.5ml of clear yellow solution was obtained.
- d) DMSO was removed under reduced pressure with heating to 60-70°C and the yellow powder was precipitated by ether.
- e) Send 500 µl Folate-NHS DMSO solution to MS lab.

The reaction scheme was described in Figure 3-1.

3.2.2.2 Synthesis Folate –DOPE

- a) DOPE (50 mg) was dissolved in 5 ml dry chloroform.
- b) 130 µmol (0.89ml) F-NHS in DMSO and 130 µmol (18.07 µl) triethylamine were added to DOPE solution and stirred for about 4 hours at RT.
- c) CHCl₃ (2ml) was added to the solution and about 7 ml PBS (pH = 7.4) was used to wash the products 3 times in glass centrifuge tubes which were spun at 8000 rpm for 20 min each time to extract folate-DOPE in chloroform.
- d) TLC was run with the system chloroform:methanol:acetic acid = 50:50:5, A color reaction was observed by dipping the plate to the solution (KMnO₄ 3g, K₂CO₃ 20 g and 5ml 5% NaOH in 300ml water). 95% DOPE (R_f=0.7-0.8) was converted to folate-DOPE (R_f=0.9-0.95).
- e) Adjust the final concentration to 3.5mg lipid/ml CHCl₃. Store in N₂ flow at -20°C.
- f) Send 500 µl Folate-DOPE for mass spectrum analysis.

The reaction scheme of folate-DOPE was shown in Figure 3-2.

3.2.2.3. Preparation of Folate-liposome by Using Folate-DOPE

- a) Mix 10 μmol (6.99 mg) dioleoyltrimethylammonium-propane (DOTAP), 10 μmol (7.6mg) DOPE and 1.3 mg (54 μl , 1 μmol) folate-DOPE (DOTAP: DOPE: Folate-DOPE=10:10:1 molar ratio) to 3ml chloroform in a 15 ml round-bottom flask.
- b) Attach the flask to a rotary evaporator (Rotavapor-R 110, Brinkmann Instrument Inc).
- c) Immerse the flask into a 40°C water bath and evaporate the chloroform with a gentle rotating speed (about 60 rpm).
- d) Place the flask in a vacuum desiccator for more than 5 hours to remove the residual solvents; after releasing the vacuum and removing the flask from the desiccator, add 8 mL distilled water and shake gently in the water –bath 40-50°C for 15 to 20 minutes. A yellow homogeneous milky solution free of visible particles was formed.
- e) Adjust the final volume to 10 mL by adding the distilled water.
- f) Pass through the liposome solution membrane mini-extruder (Avanti Polar Lipids, Inc) 10 times.

3.2.2.4. Preparation of Folate-liposome by Using Folate --PEG3350-DSPE

- a) Dissolve DSPC/Cholesterol/PEG2000-DSPE as molar ratio (12/7/1) and DSPC/Cholesterol/PEG2000-DSPE/Folate-PEG3350-DSPE as molar ratio (60/32/7.5/0.5) in 20 ml chloroform/MeOH (2/1 v/v) in a round bottom flask.
- b) Remove chloroform from the flask by rotary evaporation until thin-film lipids form at the bottom of the flask.
- c) Leave the flask in a desiccator for at least eight hours to get rid of the residue of chloroform.
- d) Hydrate the lipids in 300 mM Na citrate and 150 mg/ml sucrose (pH= 3.5 adjusted by HCl) by putting the flask in a rotary evaporator at atmosphere pressure and at 60 rpm (40-50°C) for half an hour; sonication can also be used to facilitate hydration and suspension.
- e) Adjust the final volume to be 25 ml.
- f) Submit the lipid suspension to 6 cycles of freezing (liquid nitrogen) and thawing (45-50°C water-bath).
- g) Pass through the liposomes through EmulsiFlex C5 about ten times at 5000 to 10,000 psi.
- h) Measure the particle size with a Zetasizer 1000.

3.2.2.5. Particle Size Measurement

The Zetasizer 1000 (Malvern Instruments, UK) was used to measure the liposome particle size in the range of 1 to 1000 nm. The instrument settings were as follows: temperature, 25°C; scattering angle, 90°C; aperture, 400 μm ; count rate (kilocounts/sec): 100 \pm 50; analysis mode, auto/continuous. Samples were diluted with filtered deionized water if necessary. The reading was the average mean size particle size (nm).

3.2.2.6. HPLC Assay

An Agilent 1100 G1315B diode array and an Agilent C8 4.6x 150 mm (SN USRK030017) column were used to detect the concentration of Daunorubicin HCl in formulations at wavelength 480 nm. A gradient method was developed with mobile phase starting with 80 % (0.1% trifluoroacetic acid in water) plus 15% CAN, over 11 minutes, the mobile phase ratio was changed to be 50% acetonitrile and 50% buffer. The retention time was 9.5 \pm 0.2 min for the drug and the total running time was 14.2 min. Standard is in a range of 5 to 120 $\mu\text{g}/\text{ml}$.

3.2.2.7. Loading DNR in Folate-PEG-Liposomes

Two drug and lipids ratios (1/5.6, 1/20 w/w) were used in this study.

- a) Dissolve 59.58 mg DNR HCl in 2.5 ml 5mM HCl.
- b) Add 2.5 ml 2 N NaOH and 0.5 ml 1N NaOH to 19 mL placebo Folate-PEG-liposomes (pH \sim 4) to adjust the pH to 7.45.

- c) Mix DNR/HCl well with liposome from step two by vortexing Incubate the mixture for 15 min in a 60°C water bath (vortex 10 sec every two min).
- d) The final suspension was translucent dark red and the pH was 7.36.

3.2.2.8. Determine Loading Efficiency of DNR HCl

- a) Equilibrate SP-10 column (Amersham Bioscience) by adding 25 ml of normal saline.
- b) Add 2 ml of DNR liposomes on the column (8.3 ml bed volume, 2.5 ml void volume).
- c) Collect elute fractions (1 ml each time).
- d) Discard the first 2.5ml (void volume).
- e) Most concentrated liposomes are the following 2 ml solution (fraction 1, 2, 3), they have Tyndall beam effects compared to free DNR fractions.
- f) Free DNR came out about 9 ml.
- g) Add 100 µl samples from fraction 1 and 2 in 2 ml 0.1% Triton X-100/ISPB, pH was 7.24.
- h) Determine the DNR concentration by measuring OD at 475 nm on a UV spectrometer.
- i) The loading efficiency was calculated as the percentage of DNR entrapped in liposomes (add up DNR in all fractions of liposomes) in the total amount of DNR loaded to the liposomes (DNR in liposomes and free DNR).
- j) The loading efficiency is 95 %.

3.2.2.9. Remove Unencapsulated Free DNR HCl

- a) Flush X14S-300-04N dry polysulfone fibers (Spectrum Labs, Rancho Dominguez, CA) with 60 ml of pure alcohol.
- b) Wash the fibers with 60 ml of distilled water to remove alcohol.
- c) Wash the fibers with 40 ml of phosphate buffer (pH 7.4).
- d) Add 10 ml of folate liposomes containing DNR to the inlet syringe, push slowly 1ml/min to let the samples run through the fibers, collect the free drug from the middle syringe and the liposomes were collected at the end of the fibers by another syringe.
- e) Repeat step d twice.
- f) Wash the fibers with 10 ml of phosphate buffer.
- g) Add another 10 ml of sample and repeat steps d and e.
- h) Pass the liposomes through 0.22 μm filter to sterilize them.

3.2.2.10. Determine Drug Concentration in Liposomes

- a) Dilute 100 μL liposomes after diafiltration 441 times with 50% acetonitrile and 50% water solution.
- b) Make triplicates of the above.
- c) Inject to HPLC, detect at 480 nm.
- d) Dilute 100 μl liposomes after diafiltration 441 times with 0.1% tritonX-100 in phosphate buffer.

3.3. RESULTS AND DISCUSSION

3.3.1. Mass Spectrum

The mass spectrum of NHS-Folate (Figure 3-2a) showed M: 538.31 and MH^+ : 539.31 and the calculated MW for Folate-NHS is 538. The spectrum of Folate-DOPE (Figure 3-2b) showed that M: 1166.89; MH^+ : 1167.93; MNa^+ : 1189.87 and the calculated MW for Folate-DOPE is 1167.

3.3.2. Particle Size of Cationic Folate-liposomes

The average particle size of different liposomal formulations is described in Table 3-1. Mini-extrusion and homogenization have been shown to be effective in reducing the particle size of liposomes. The particle size can be reduced to less than 200 nm by passing through a mini-extruder or homogenizer five to ten times.

3.3.3. Loading Efficiency

The pH gradient method (Figure 3-3) was used to increase the loading efficiency of DNR HCl into liposomes (Lee and Low, 1995). A transmembrane pH gradient was created to enhance the loading efficiency. The inside of the liposomes has a low pH of 3.5 to 4 while the outside bulk solution has a higher pH of 7.5 to 8. Daunorubicin HCl molecules exist as neutral and unionized species outside of liposomes at pH 8 due to its basic pK_a of 8.5. They can pass through the liposome membrane passively due to their lipophilicity. The neutral molecules of DNR HCl, which reach the inside the liposomes, become

protonated as the result of the much lower pH inside liposomes. Those ionized molecules are difficult to pass through the membrane; therefore the concentration of DNR HCl can be increased over the concentration outside of liposomes.

The transmembrane pH gradient is one of the major factors determining the loading efficiency of a weak base (e.g. daunorubicin) or weak acid. The pH difference creates the driving force for active loading. Apart from the pH difference, the lipid/ drug ratio also plays an important role in the loading efficiency and amount of DNR HCl encapsulated inside of liposomes. Table 3-2 shows that a higher lipid/drug ratio along with a transmembrane pH gradient can increase loading efficiency.

3.3.4. Summary of the Folate-PEG-Liposomes for DNR HCl

The folate-PEG-liposome formulation contained 2.45 mg/mL DNR HCl with a drug to lipid ratio of 1 to 20 (w/w). The average particle size was 120-130 nm and the pH of the final liposome formulation was around 7.4. Three batches were made and sent to the NCI for animal study. Pan, Huang, and Lee (2002) developed folate-PEG-liposomes for doxorubicin. They found out that uptake of folate-L-DOX by the FR (+) KB cells was about 50 times greater than that of L-DOX. In addition, the folate receptor (FR) targeted f-L-DOX has been found to be more effective in tumor growth inhibition and survival prolongation than the nontargeted L-DOX in an FR (+) murine tumor xenograft model. The FR targeted folate-PEG-liposomes containing DNR developed in this study may have a similar advantage over nontargeted L-DNR.

Figure 3-1. Reaction Scheme of NHS-Folate.

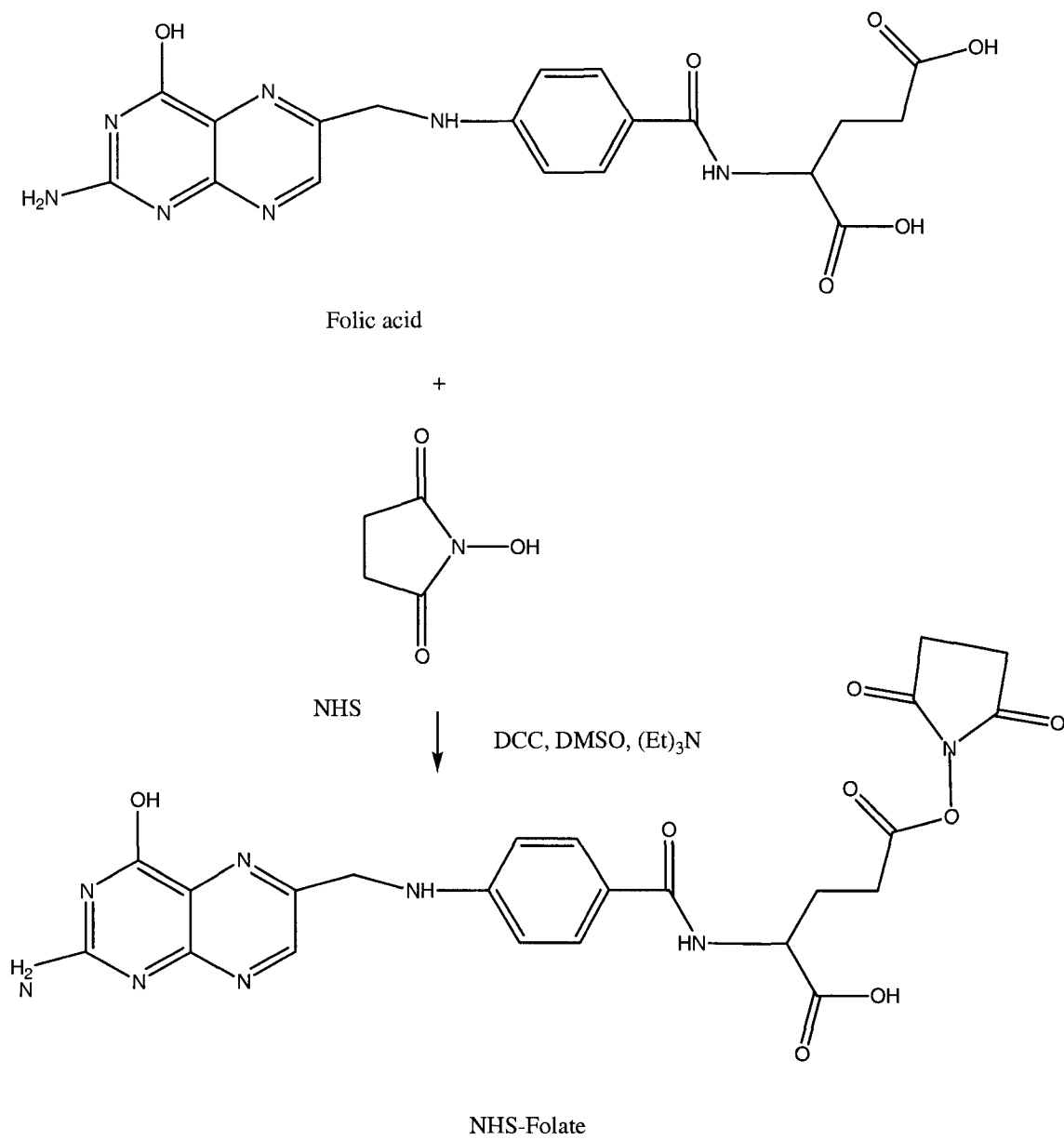


Figure 3-2a. Reaction Scheme of Folate-DOPE.

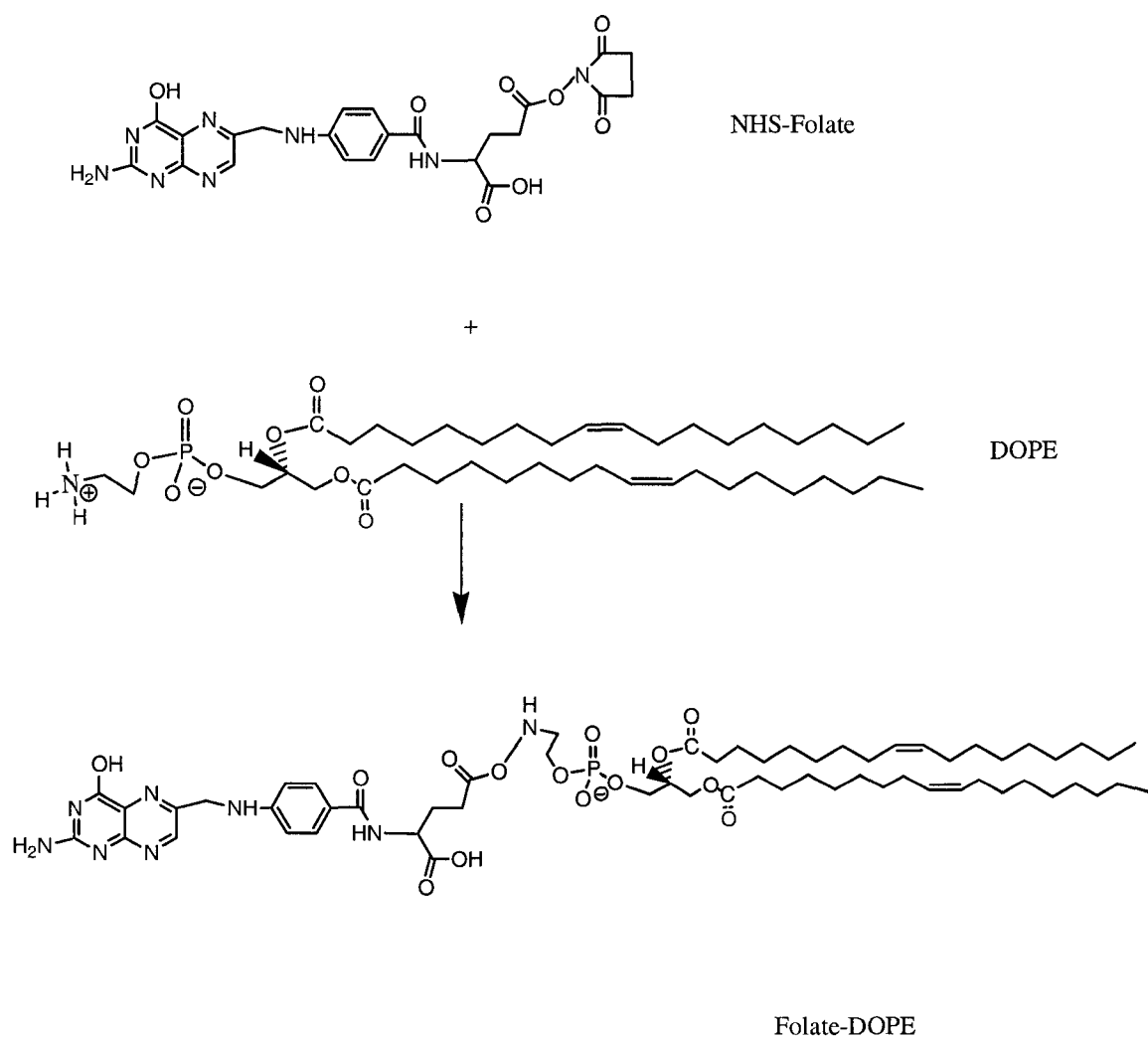
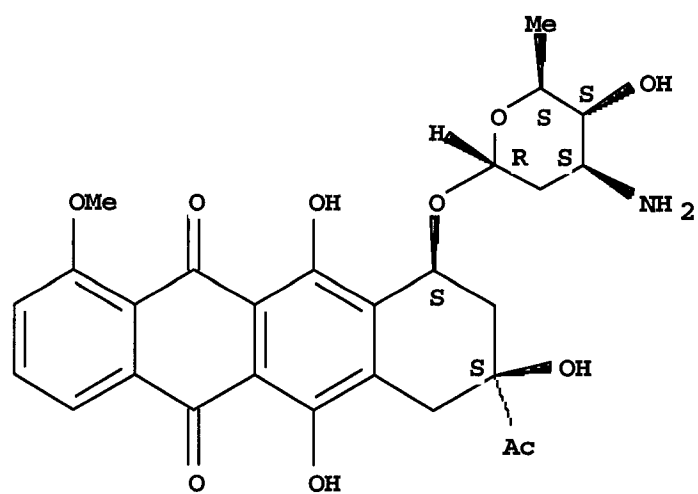


Figure 3-3. Chemical Structure of Daunorubicin HCl.



• HCl

Figure 3-4a. The Mass Spectrum of NHS-Folate.

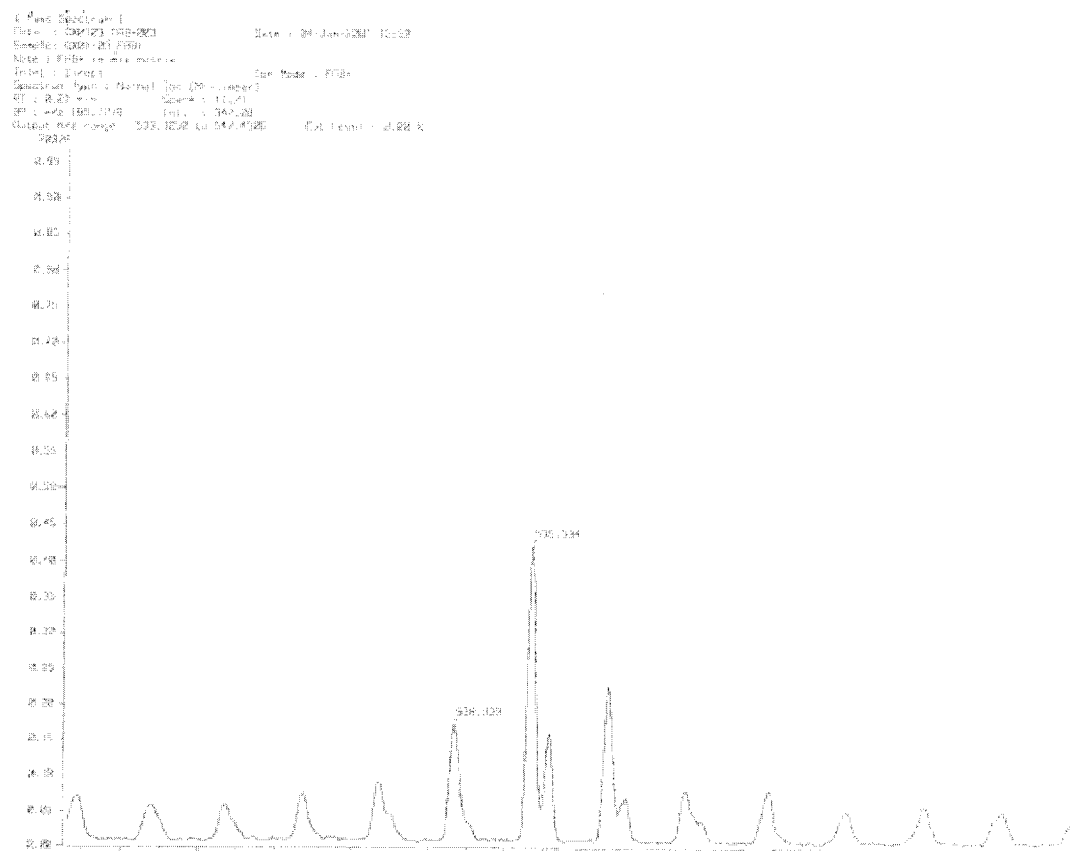


Figure 3-4b. The Mass Spectrum of Folate-DOPE.

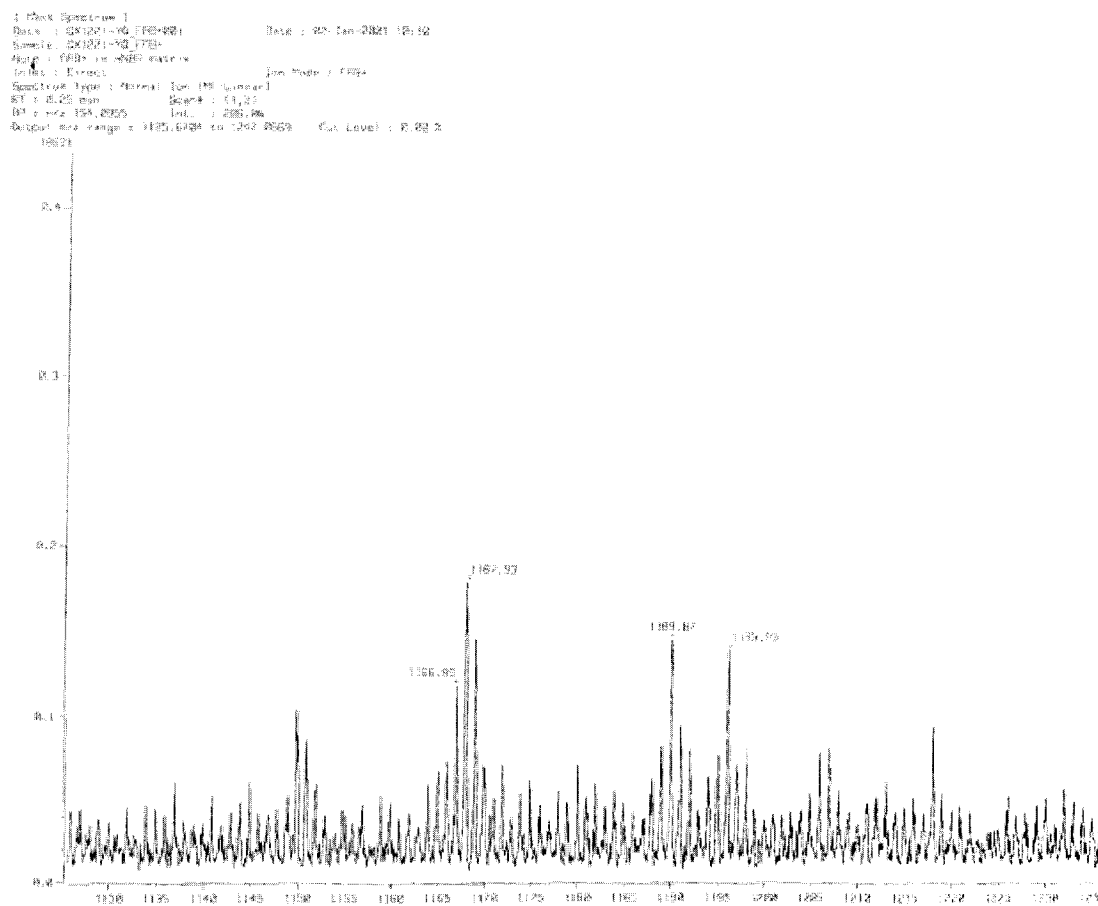


Figure 3-5. Schematic of pH-Loading.

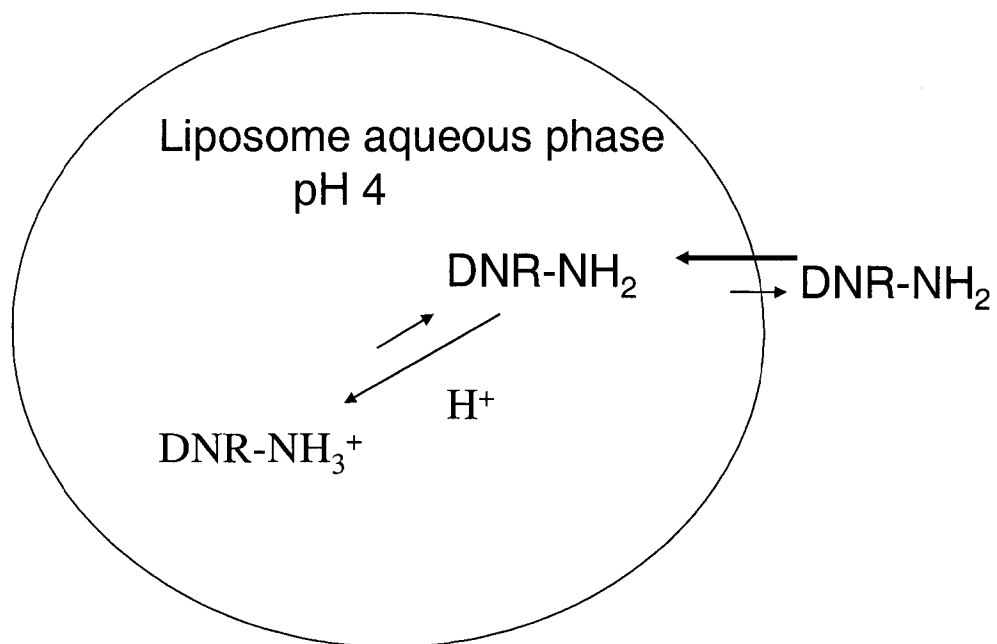


Table 3-1. Particle Size of Liposomes

Lipids Composition	Molar ratio	Average Particle Size
DOTAP/DOPE/Folate-DOPE	10/10/1	190 nm
DSPC/Chol/DSPE-PEG2000	60/32/8	130 nm
DSPC/Chol/DSPE-PEG2000/Folate- DSPE3350-PEG2000	60/32/7.5/0.5	132 nm

Table 3-2. Loading Efficiency of Different Batches of Liposomes

Batches	lipids/drug(wt/wt)	pH(in-out)	loading efficiency
11/14/02	5.6/1	4.9-7.8	NA
01/15/03	5.6/1	4.9-8.7	10%
03/10/03	20/1	3.75-7.2	90%
09/10/03	20/1	3.5-7.5	95%

CHAPTER 4. HALOTHANE, A NOVEL SOLVENT FOR THE PREPARATION OF LIPOSOMES CONTAINING 2-(4'-AMINO-3'-METHYLPHENYL) BENZOTHIAZOLE (AMPB), AN ANTI-CANCER DRUG

4.1. INTRODUCTION

Most anticancer drugs have the tendency to target normal as well as tumor cells.

Liposomes, especially long-circulating liposomes, are promising carriers for delivering drugs designed specifically to target cancer cells (Allen, et al., 1995; Forssen and Willis, 1998; Jia, et al., 1994; Lee and Low, 1994; Papsivo, 1998), thus increasing anticancer activity and minimizing toxicity.

Anticancer activity has been shown by 2-(4'-amino-3'-methylphenyl)benzothiazole (AMPB) (Figure 2-1) against a variety of cancers including breast, ovary, lung, and kidney cancers in animal studies (Stevens, unpublished data, 1994, and Stella, unpublished data, 1997). Unfortunately, however, severe dose-limiting hepatotoxicity of AMPB has also been reported in animals (Stevens, unpublished data, 1994, and Stella, unpublished data, 1997).

AMPB has low water solubility (0.54 µg/ml) (Stella, unpublished data, 1997) and therefore must be manipulated through solubilization techniques in order to increase its solubility. Although traditional approaches such as cosolvency, pH control, micellization,

and complexation could solubilize this drug, its acute hepatotoxicity cannot be reduced using these techniques. To increase the anticancer activity as well as minimize toxicity, long-circulating liposomal formulations have been developed in this study.

The first step in the preparation of liposomes is to dissolve the lipids in organic solvent. The solvent must be volatile and have high solubility for lipids. This requirement has introduced safety issues. Chloroform and ether are the commonly used solvents.

Chloroform was used as an anesthetic during surgery for many years before its harmful effects on the liver and kidney were recognized. Based on animal studies, the Department of Health and Human Services (DHHS), the International Agency for Research on Cancer (IARC), and the Environmental Protection Agency (EPA) have determined that chloroform is possibly carcinogenic to humans. The breakdown products of chloroform in air include hydrogen chloride and phosgene, which are even more toxic than chloroform. Ether, while relatively nontoxic, is highly flammable. Both of these agents have been abandoned as anesthetics for clinical use. Even though only a small amount of residue would exist in liposomal formulations, it is still not acceptable. Because chloroform and ether evaporate very quickly, they would be harmful to the lab workers who were exposed to them. Chloroform is also toxic to aquatic and soil organisms after entering the natural environment, which is a probable consequence of using it as a solvent (Butterworth and Bogdanffy, 1999; Mcculloch, 2003; Templin, et al. 1998).

Halothane, a commonly used inhalation anesthetic (Kennedy, 1996) was used in this study in place of either chloroform or ether to prepare liposomal formulations. The Occupational Safety and Health Administration does not classify halothane as a carcinogen to humans. Thus halothane is preferred to chloroform and ether because it is safer, more volatile, and an excellent solvent for most lipids.

4.2. EXPERIMENTAL

4.2.1. Materials

AMPB, 2-(4'-amino-3'-methylphenyl)benzothiazole, NSC 674495) was provided by the National Cancer Institute, Bethesda, MD. Egg phosphatidylcholine (EPC, mw: 760); 1,2-distearoyl sn-glycero-3-phosphocholine (DSPC, mw: 790.15); and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-polyethylene glycol 2000 (PEG 2000-DSPE, mw: 2748) were all purchased from Avanti Polar Lipids, Inc.(Alabaster, AL). Cholesterol (Chol, mw: 386.6) was purchased from Sigma-Aldrich Corp (St Louis, MO). The United States Pharmacopoeia (USP) normal saline (0.9% NaCl) was purchased from Baxter Healthcare Corp. (Round Lake, IL). Halothane (mw: 197.38; bp: 50°C), chloroform (mw: 119.37, bp: 61.7°C), and all other chemicals and organic solvents were purchased from Sigma-Aldrich Corp (St Louis, MO) and were of analytical grade.

4.2.2. High-Performance Liquid Chromatography Assay

A Beckman Gold high-performance liquid chromatography (HPLC) system with a #168 detector and a Pinnacle octylamine (C8) column (5 μ m, 150 \times 4.6 mm, cat no: 9183565, Restek Corp, Bellefonte, PA) were used for all assays. The wavelength 254 nm was chosen for AMPB detection. The HPLC mobile phase was composed of acetonitrile (CAN) and an aqueous solution that contained 0.1% trifluoroacetic acid (TFA) in water. The gradient method was started with 70% aqueous solution and 30% CAN. The

percentage of CAN was increased at the rate of 2% per minute until reaching 55% at about 12 minutes. The injection volume was 50 μ l, and the flow rate was 1.0 ml/min. The total running time was 13 minutes, and the retention time of AMPB was approximately 9.5 ± 0.3 minutes at ambient temperature. A calibration plot of peak area versus concentration was found to be linear in the range 1 to 100 μ g/ml.

4.2.3. Liposome Preparation: Thin-Film Method

Zhao et al. (2000) determined that the optimal molar ratio of drug and lipids with AMPB/EPC/Chol/ DSPE-PEG 2000 is 1:5:5:1. This ratio and a 2 mg/ml concentration of AMPB were used in this study to investigate halothane as a replacement for chloroform to prepare liposomes.

Three batches of liposomes were made for stability study, and the thin-film method was used in their preparation (Shew and Deamer, 1985). Lipids and AMPB were dissolved in chloroform or halothane. The organic solvents were then removed by a rotary evaporator under reduced pressure. A thin film of lipid was observed after the solvents were removed. At least 8 hours in a desiccator under house vacuum was needed to completely remove the residues of organic solvents. Normal saline was then added to hydrate the lipid thin film. The hydration was followed by sonication in a 45°C water bath. A yellowish homogeneous suspension appeared after hydration. The final volume of the

liposomal suspension was adjusted to make the desired concentration of AMPB (e.g. 2 mg/ml).

4.2.4. Optical Microscope Observation

An optical microscope (Leica Microsystems Switzerland Ltd., Heerbrugg, Switzerland) was used to observe the particle size and shapes before particle size reduction. Particle diameters above 0.3 μm were determined by using a calibrated eyepiece scale.

Photographs were taken using a SPOT camera (Spot Image Corp. Chantilly, VA)

4.2.5. Particle Size Reduction

A laboratory homogenizer (EmulsiFlex-C5, Avestin, Ottawa, ON, Canada) was used to reduce the particle size of the liposomes. Multiple passes (5-10k psi) were often needed to obtain the desired particle size (~200 nm).

4.2.6. Transmission Electron Microscope Observation

A transmission electron microscope (Philips Electron Optics, model 420, Eindhoven, The Netherlands) was used to observe the size and lamellarity of liposomes after size reduction by homogenization. Liposome suspensions were stained by 2% phosphotungstic acid/sodium hydroxide (pH 6.2) and dried on carbon-coated grids for observation.

4.2.7. Particle Size Measurement

The Zetasizer 1000 (Malvern Instruments, Worcestershire, UK) was used to measure the final particle size of liposomes in the range of 1-1000 nm. The instrument settings were as follows: temperature, 25°C; scattering angle, 90°; aperture, 400 μm ; count rate, 150 \pm 50 kilo counts/sec); analysis mode, auto/continuous; running time, automatic (120-3000 seconds). Samples were diluted with deionized water in triplicate. The mean particle size from each of the 3 batches was averaged to get the average mean particle size.

4.2.8. Drug Encapsulation Efficiency Determination

The liposomes (approximately 200-300 nm) were diluted 10 times by normal saline. The diluted solutions were gently vortexed for about 20 seconds and were free from any visible aggregation and precipitation.

Duplicate 1-ml samples from each batch were placed in ultracentrifugation tubes (Nalge Nunc International, Rochester, NY). The instrument (L8-55 ultracentrifuge, Beckman Coulter, Inc. Allendale, NJ) was set at 50,000 rpm and 10°C for 3 hours with a temperature pre-equilibrated head. Separated phases (supernatant and pellets) in the centrifuge tubes were observed after ultracentrifugation. The supernatant was pipetted out and the drug concentration (C_s) in it was determined by HPLC. The pellet was washed with normal saline 3 times before it was well hydrated and dispersed by adding 1 ml of

normal saline and sonicated in a water-bath sonicator for several minutes. Dilution was conducted by adding pure ACN, and the drug concentration (C_p) in the pellet was determined by HPLC. The encapsulation efficiency (EE) equals the concentration of drug in the pellet divided by the total concentration of drug in the formulations, as described by the following equation:

$$EE = \left(\frac{C_p}{C_p + C_s} \right) \times 100\%$$

4.3. RESULTS AND DISCUSSION

Liposomal formulations were prepared using chloroform, chloroform/methanol (2/1, vol/vol) and halothane/methanol (2/1, vol/vol). No obvious differences of liposomal formulations mediated by halothane and chloroform were observed under the microscope. The maltisian structures, which are due to the multi-bilayers of liposomes produced by the thin-film method before particle size reduction, were observed in all formulations (Figure 4-1). This was supported by transmission electron microscope (TEM) observation (Figure 4-2). Multilamellar structures were observed for all formulations under TEM and the particle size for liposomes after homogenization ranged from 100 to 200 nm. Encapsulation of AMPB between the lipid bilayers of liposomes (Figures 4-2A, B, C) had little effect on the shape of the liposomes except to produce a thicker membrane compared with liposomes without the drug (Figure 4-2D).

The data indicate that all 3 liposomal formulations have high encapsulation efficiency, are stable for at least 3 months at 4°C, and show a similar trend of encapsulation efficiency decreasing over time. Also, the 3 formulations show a similar increase in particle size with time (Figure 4-3).

The slight difference in the particle size of the liposomes may be because of the multiple steps of hydration and homogenization. After the solvents (halothane or chloroform) were removed, lipids formed a thin film at the bottom of the round-bottom flask. At the same

experimental conditions (temperature and pressure), halothane is easier to remove. The lipid thin films mediated by halothane are more homogeneous after drying than are the chloroform-mediated films and the halothane-mediated thin films are also easier to hydrate using sonication.

Since AMPB is a lipophilic compound, most of the drug was encapsulated within the bilayers of the liposomes. After reaching equilibrium partitioning, liposomes encapsulating AMPB are quite stable. More than 90% of the drug remains inside the liposomes after 3 months of storage at 4°C. The tendency of slight leakage of the drug is probably due to lipid oxidative degradation, which may also influence the packing of the lipids in the liposomes. Loss of residual solvent by evaporation may also play a role in reducing encapsulation.

Additional AMPB liposomal formulations containing different ratios of DSPC, EPC, cholesterol, and DSPE-PEG 2000 were prepared by using halothane and chloroform, respectively. Furthermore, liposomal formulations of fenretinide (a lipophilic compound), a hydrophilic prodrug of AMPB, and some proprietary compounds provided by the National Cancer Institute were prepared. In all cases, no significant differences in the physicochemical properties (particle size, size distribution, drug encapsulation efficiency) were observed between halothane-mediated and chloroform-mediated liposomes. These studies indicate that halothane, a safer solvent, is equivalent to chloroform as a vehicle for liposome preparation.

4.4. CONCLUSIONS

Chloroform and ether are currently the solvents of choice despite the fact that chloroform is a known carcinogen and ether is highly flammable. Halothane is volatile yet nonflammable and is currently used as an inhalation anesthetic; hence it is safe.

This study uses halothane in place of chloroform or ether to prepare liposomal formulations for the antitumor drug 2-(4'-amino-3'-methylphenyl) benzothiazole (AMPB or NSC 674495). The preliminary data show that there are no significant physicochemical property (particle size, encapsulation efficiency, and vesicle shapes) differences between the halothane- and chloroform-mediated formulations. Similar results have been observed for other anticancer drugs tested.

Based on its success with AMPB, halothane may be a promising replacement for chloroform and ether in preparation of liposomes for other anti-cancer drugs.

4.5. ACKNOWLEDGEMENTS

This work was performed under contract NCI-CM-77109 from the Pharmaceutical Resources Branch, National Cancer Institute (NCI), Bethesda, MD 20892.

Figure 4-1. Structure of Liposomes Containing AMPB (2 mg/ml) under Light Microscope ($\times 400$).

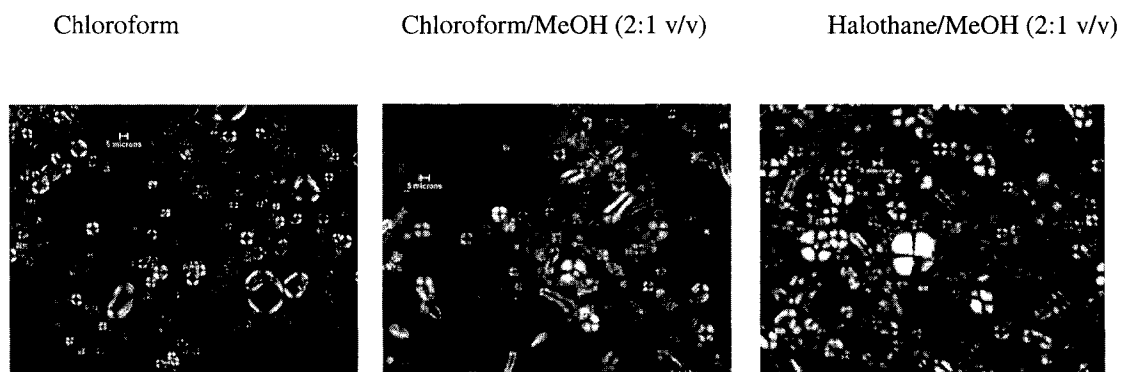


Figure 4-2. TEM Micrograph of Liposomes after Homogenization ($\times 164k$).

(A) liposomes with AMPB by using chloroform; (B) liposomes with AMPB by using chloroform/MeOH; (C) liposomes with AMPB by using halothane/MeOH; (D) liposomes without AMPB by using halothane.

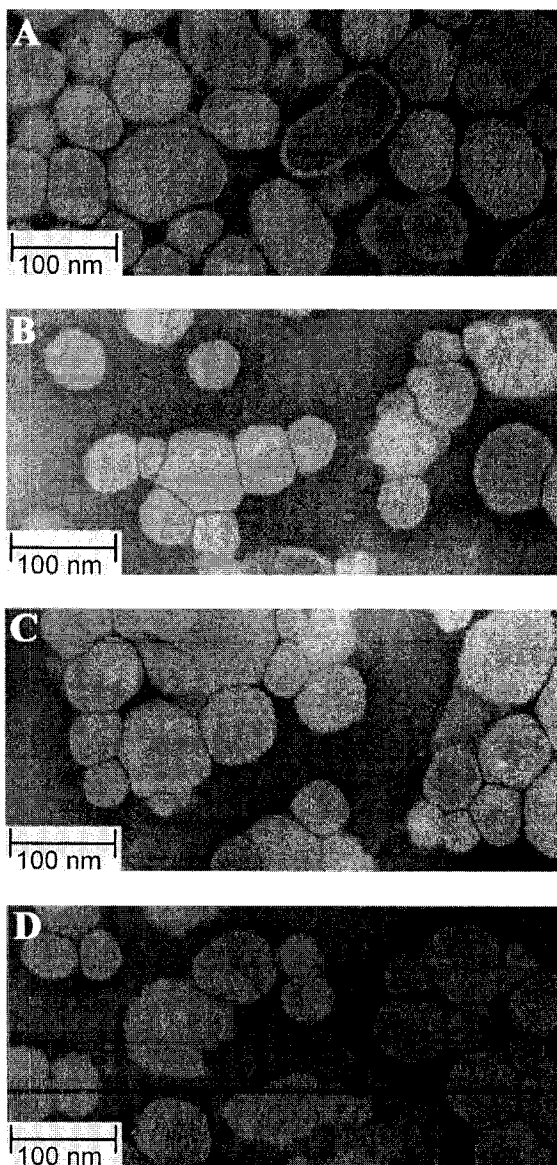
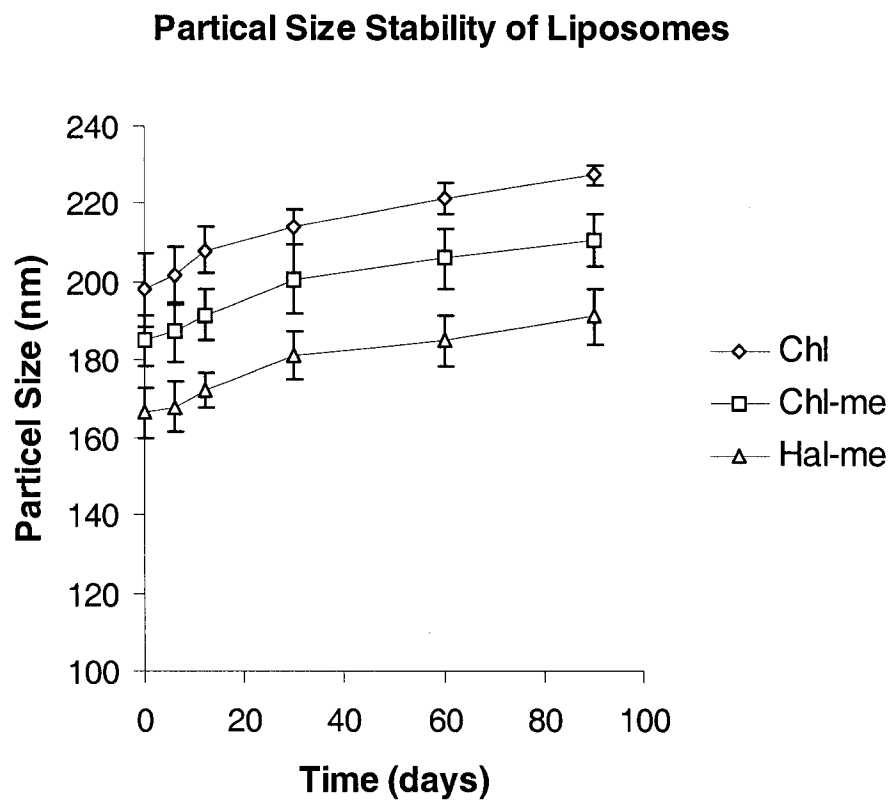


Figure 4-3. Particle Size Stability of Three Liposomal Formulations of AMPB at 4°C.

(n = 3; error bars represent standard deviation of the mean).



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