1999

Characterization of liposome manufacturing using extrusion

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UMI

**CHARACTERIZATION OF LIPOSOME MANUFACTURING
USING EXTRUSION**

A Thesis

Presented to

The Faculty of the Department of Chemical Engineering

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

By

Seema Sood

December 1999

UMI Number: 1397751

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ABSTRACT

CHARACTERIZATION OF LIPOSOME MANUFACTURING USING EXTRUSION

By Seema Sood

The process parameters involved in extrusion were varied to study the effect on size and size distribution of liposomes. Six parameters were varied: cholesterol composition, MPEG-DSPE composition, pressure, buffer, and first and second extrusion pore sizes.

Dynamic light scattering (DLS), Capillary Hydrodynamic Fractionation (CHDF), and Zimm Plots were used to analyze size. DLS determined the mean size of the liposomes, CHDF gave the size distribution, and Zimm plots were used to measure molecular weight.

Cholesterol composition and pore size had the greatest effect on mean size. Pressure, buffer, and MPEG-DSPE showed a weak effect.

Extrusion history did not affect mean size but it did affect the size distribution. Thus, two sets of extrusions did result in less polydispersity but did not affect mean size. Results showed that five passes were sufficient to produce a constant size distribution.

Zimm plot analyses showed molecular weights of experimental liposomes were similar to those in literature.

ACKNOWLEDGMENTS

I would like to express my profound gratitude to my research advisor, Dr. Ananth Annapragada, for coming up with this project and making it possible for me to continue and complete the research at Sequus Pharmaceuticals. I am also thankful for his instruction and support. I wish to thank Dr. Melanie McNeil for her assistance and encouragement during the revision of the manuscript and Dr. Art Diaz for his guidance.

I would like to thank my parents and brothers for their continued love and devotion that has been integral to my pursuit of a master's degree. Also, I am grateful to my husband's parents for their helpful advice and encouragement to reach my goals. Finally, I would like to thank my loving husband for his constant support, never-ending patience, and giving me the strength to fulfill my dreams.

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1.1 Introduction

Liposomes are self-closed structures composed of amphipathic lipid molecules (Lasic and Needham, 1995). These molecules exhibit both hydrophilic and hydrophobic properties and therefore self-assemble in aqueous media to form bilayers. These bilayers self-close into structures of various shapes (usually spherical or spheroidal) to further minimize the free energy of the system. A schematic of a typical liposome is shown in Figure 1. The wall of the liposome is composed of one or more (unilamellar or multilamellar vesicles) lipid bilayers. One useful property of liposomes is their ability to encapsulate aqueous media, making them very similar in structure to a biological cell. Every cell is surrounded by a membrane that has the same basic lipid bilayer structure as that exhibited by liposomes. As with the cell, the lipid bilayer acts as a chemical, electrical, and mechanical barrier that sequesters an internal aqueous solution and separates it from the external surroundings. Liposome size can range over three orders of magnitude, from 10^{-9} to 10^{-6} meters (Lasic and Needham, 1995).

The technological utility of liposomes results from their unique ability to imitate a cellular environment and encapsulate an aqueous medium. Due to their ability to encapsulate aqueous media, they can carry molecules which are to be isolated from the environment outside the liposomal wall. An example is therapeutic drug molecules that are toxic to vital organs in the free drug form and need to be isolated and delivered to specific sites.

Figure 1. Schematic representation of a liposome.
Circles represent the hydrophilic head.
Lines represent the hydrophobic tail.

Upon injection into the body, liposomes are recognized by the reticulo-endothelial system (RES), the system responsible for detecting and removing foreign substances from the body, and removed from circulation fairly rapidly, within a few minutes to a few hours (Lasic and Martin, 1995). Addition of a small percentage of a lipid-polymer conjugate, such as MPEG-DSPE¹, to the phospholipids has the effect of increasing the circulation time of liposomes in the blood. The polyethylene glycol (PEG) molecule and its derivatives attached to a bilayer inhibit recognition by the phagocytic cells of the RES thus increasing the circulation time of the liposomes. Liposomes which have the glycolipids incorporated into the bilayer are called Stealth^{®2} Liposomes for their ability to avoid detection and uptake by the RES. The circulation half-life for Stealth[®] liposomes is 44 hours (Lasic and Martin, 1995). Figure 2 illustrates how MPEG-DSPE is anchored in the bilayer of a stealth liposome.

1.2 Liposomes in drug delivery

In addition to evading the RES, there is a need for a system that can deliver highly toxic drugs to specific sites. Liposomes may be used for this purpose. Theoretically, the benefits of delivering drugs by liposomes are:

¹ Methoxy polyethylene glycol distearoyl phosphatidylethanolamine (Lasic, 1993).

² Stealth[®] is a registered trademark of Sequus Pharmaceuticals, Inc.

Figure 2. Illustration of a stealth liposome and MPEG-DSPE. The phospholipid portion of the MPEG-DSPE, DSPE, makes up the bilayer. The MPEG attached to the DSPE extends out of the bilayer.

- liposomes may cross biomembranes and facilitate the transport of drugs through normally-impermeable barriers. Many drugs with low aqueous solubility can be encapsulated in liposomes. Minoxidil and Amphotericin B are examples of such drugs (Lasic, 1993).
- liposomes can be designed to interact with specific tissues, improving drug selectivity and reducing toxicity. Liposomes can extravasate beyond the endothelial barrier. Although the exact mechanism for drug delivery by liposomes is unknown at present, it seems likely that liposomes provide a local depot source for the drug. The drug can then diffuse further into the tumor mass and affect many tumor cells in the vicinity. This also reduces toxicity to sensitive tissues such as liver, kidneys, and spleen (Lasic, 1993).
- chemically and metabolically labile drugs can be protected by the liposome from deactivation. Encapsulation in liposomes of Cytosine Arabinose, an anticancer drug, greatly reduces the chemical decomposition of the fragile drug (Lasic, 1993). An example of a liposomally encapsulated drug is Doxorubicin Hydrochloride (Lasic, 1993).

The size of liposomes is an important factor in their drug delivery performance. Large liposomes are detected by the RES and quickly excreted (Woodle and Lasic, 1992). Small liposomes have a curvature of the lipid bilayer which imposes stress on the bilayer wall. The wall is then susceptible to rupturing. The optimum size range for

liposomes is 80 nm to 200 nm since these particles are small enough to reduce RES detection and large enough to have minimal strain caused by the curvature.

1.3 Scope of work

Various methods exist for the production of liposomes, each method satisfying some of the desired characteristics of a liposomal preparation. These methods and characteristics will be discussed in detail in later sections. A flow diagram outlining the general steps involved in liposome production is presented in Figure 3. The focus of this research will be on the extrusion method and the parameters involved in this method. The proposed research will investigate the dependence of liposome structures and properties during extrusion on lipid composition, pressure, buffer, and membrane pore size. Size and population distribution will be characterized using the analytical techniques of dynamic light scattering, static light scattering, and capillary hydrodynamic fractionation.

Process Flow Diagram

Figure 3. Liposome production and characterization process.

1. Lipid dissolution is the process of dissolving the dry lipids into a solvent.
2. In lipid hydration, the buffer is added to the dissolved lipids to form liposomes of heterogeneous size.
3. Extrusion consists of passing the hydrated lipid mixture through a series of membrane filters. This reduces the size of liposomes.
4. The size analysis step consists of measuring the size and size distribution of liposomes resulting from extrusion.

LITERATURE REVIEW

2.1 Preparation Methods

There are many methods of liposome preparation reported in the literature. These methods are briefly reviewed below, along with characterization methods for determining particle size distribution and other properties of liposomes.

2.1.1 Simple Hydration

The first liposomes were prepared by letting dry lipids swell in aqueous salt solutions (Bangham et al., 1965). This resulted in multilamellar vesicles (MLV) heterogeneous in size (400-3500 nm) and shape. The major drawback of the MLV preparation method is the relatively low volume of entrapped aqueous space per mole of lipid, 2.41 l/mole (Szoka and Papahadjopoulos, 1981). This is because most of the lipid is participating in the internal lamellae which also limits the internal space available for encapsulation (Szoka and Papahadjopoulos, 1978).

The captured volume per mole of lipid as calculated by Lasic (1993) is

$$V = 6 \times 10^{23} a (r - \delta)^3 / (3(r^2 + (r - \delta)^2)) \quad (\text{Equation 1})$$

where $a = 0.65 \text{ nm}^2$ and 0.22 nm^2 , the area of a phospholipid molecule and a cholesterol molecule, respectively

$\delta = 4 \text{ nm}$, the thickness of the bilayer

r = radius in nanometers

Figure 4 relates the captured volume as a function of size, lamellarity, and lipid composition as calculated by the above formula. For a unilamellar vesicle of 200 nm in

Figure 4. Captured volume for unilamellar, bilamellar, and trilamellar particles at $a = 0.461 \text{ nm}^2$ and 0.516 nm^2 .

diameter, the captured volume is 4.24-4.75 l/mole. A bilamellar vesicle of the same size has a captured volume of 1.95-2.17 l/mole, and a trilamellar shows a captured volume of 1.18-1.32 l/mole. The captured volume decreases as the lamellarity increases since the extra bilayers occupy the space that would otherwise be used for encapsulation of the aqueous medium. Trilamellar vesicles 360-400 nm in diameter capture a volume of 2.41 l/mole. These calculations show the liposomes prepared by simple hydration must be multilamellar if they are 400-3500 nm in diameter and have a captured volume of 2.41 l/mole.

Another problem associated with this method is low encapsulation efficiency, the ratio of the amount of material remaining with the vesicles after separation from the unencapsulated material to the amount of material present for encapsulation (Szoka and Papahadjopoulos, 1978).

Encapsulation efficiency is calculated as

$$E_{\text{eff}} = (\text{lipid concentration})(\text{captured volume of the vesicles per mole lipid})$$

Figure 5 shows the percent encapsulation (encapsulation efficiency x 100) of unilamellar, bilamellar, and trilamellar vesicles of various sizes at varying lipid concentration.

Figure 5. Percent encapsulation for lipid concentration of 10, 25, and 50 $\mu\text{moles/ml}$ and uni-, bi-, and trilamellar vesicles at $a = 0.516 \text{ nm}^2$.

2.1.2 Ethanol Injection

In the ethanol injection technique, lipids dissolved in ethanol are injected into a buffer solution to form small unilamellar vesicles (SUV) (Szoka and Papahadjopoulos, 1981). This method results in the same shortcomings as the previous method, only a small fraction of the aqueous volume is encapsulated, 0.29 l/mole for vesicles in the range of 30-100 nm in diameter (Batzri and Korn, 1973). Size was determined by negative stain electron microscopy. Theoretically, unilamellar vesicles in this range

should encapsulate a volume of 0.35-1.5 l/mole with an encapsulation efficiency of 4-18%, as determined by Equations 1 and 2. However, bilamellar and trilamellar vesicles may also be present. The percent encapsulation of the medium for this method as calculated by Batrzi and Korn using a 40 μ mole/ml lipid concentration and captured volume of 0.52 l/mole was 2 %.

2.1.3 Ether Infusion

The ether infusion technique consists of lipids being dissolved in ether and injected into an aqueous solution of material to be encapsulated under reduced pressure (Deamer and Bangham, 1976). This process results in the formation of large unilamellar vesicles. However, the major fraction of lipid processed by ether infusion forms liposomes that are too small in size. Size distribution of liposomes was heterogeneous (300-500 nm in diameter), as determined by freeze fracture electron microscopy, and captured volume was high, 8-17 l/mole (Szoka and Papahadjopoulos, 1981). Captured volume determined using Equation 1 in this size range is 9.9-17.0 l/mole. Encapsulation efficiency for the ether infusion method was low, 2-3% at 2 μ moles/ml lipid concentration and a captured volume of 8-17 l/mole (Deamer and Bangham, 1976).

2.1.4 Detergent Dialysis

The detergent dialysis technique is used in removing detergent from phospholipid/detergent dispersions using hollow fiber dialysis tubing (Szoka and Papahadjopoulos, 1981). This procedure results in the formation of unilamellar vesicles.

Relatively homogeneous preparations of liposomes whose diameter is in the range of 34-130 nm are formed with this technique as determined by gel filtration. These vesicles also failed to encapsulate aqueous media efficiently, approximately 12 % at a lipid concentration of 50 μ moles/ml (Szoka and Papahadjopoulos, 1981).

2.1.5 Reverse Phase Evaporation

The reverse-phase evaporation method (REV) results in the production of large unilamellar and oligolamellar vesicles with a high space-to-lipid ratio. This procedure consists of dilution of an organic solvent containing phospholipids in an aqueous buffer by evaporating the solvent with an inert gas. A substantial fraction of the aqueous phase is captured within the vesicles, as shown in Table 1. Phosphatidylglycerol/ phosphatidylcholine/ cholesterol vesicles of 200-1000 nm in diameter produced by reverse phase evaporation captured a volume of 8.06 l/mole, multilamellar vesicles (MLV), 400-3500 nm, of the same lipid composition captured a volume of 2.41 l/mole, and SUV of 20-50 nm showed a captured volume of 0.29 l/mole (Szoka and Papahadjopoulos, 1978). This study also determined that vesicles in the size range of 200-1000 nm had the best encapsulation percentage, 24%, from the vesicles considered, and those of size range of 20-50 nm did not encapsulate aqueous media very well, 1.8%. Each preparation consisted of 66 μ moles of lipid per milliliter of aqueous material (Szoka and Papahadjopoulos, 1978).

Table 1. Liposome characteristics using various preparation techniques.

<u>Lipid Composition</u>	<u>Percent Encapsulation</u>	<u>Captured Volume (l/mole)</u>	<u>Diameter (nm)</u>
PG/PC/Chol (1:4:5) REV	24%	8.06	200-1000
PG/PC/Chol (1:4:5) MLV	16.5%	2.41	400-3500
PG/PC/Chol (1:4:5) SUV	1.8%	0.29	20-50

2.2 Size Reduction Methods

2.2.1 Sonication

Sonication is used to reduce the size of multilamellar vesicles to small unilamellar vesicles (SUV) (Olson et al., 1979). Two types of sonicators can be used, a probe sonicator where a high-energy probe is immersed into a dispersion of phospholipids or a bath sonicator where the liposome suspension is placed in a glass vial suspended in a low-energy ultrasonic cleaning bath (Batzri and Korn, 1973). A probe sonicator can reduce the size of the preparation quickly and efficiently but can also contaminate the preparation with the metal tip of the probe. This leads to degradation of the lipids (Szoka and Papahadjopoulos, 1981). It may also damage solute molecules in the medium. Although these difficulties are avoided using low-energy sonication, this procedure varies in its efficiency and requires long periods of sonication. Large quantities of small unilamellar vesicles cannot be prepared at one time. This procedure

can also be destructive to the phospholipids (Batzri and Korn, 1973). Both types of sonication produce vesicles of about 25 nm in diameter with an internal volume of 0.31 l/mole of phospholipid.

2.2.2 Extrusion

The extrusion technique reduces the size of a heterogeneous preparation of liposomes by passing these vesicles through a membrane with a defined pore size (Olson et al., 1979). Multilamellar vesicles extruded by this technique show mean diameters approaching the pore diameter of the membrane through which they are extruded (Olson et al., 1979). In extrusion, the pressure gradient created across any pore generates shearing forces which rupture the vesicle wall. The ruptured bilayer anneals immediately forming smaller vesicles that can eventually pass through the pore. This process is shown in Figure 6. This method is convenient, easily reproducible, does not contaminate the preparation, and does not induce breakdown of the phospholipids, like sonication (Olson, et al., 1979). However, extrusion is only capable of reducing larger liposomes in a preparation but does not control the lower end of the size distribution (Szoka and Papahadjopoulos, 1981).

Polycarbonate track-etch (PCTE) membranes are used as the filters in reducing the size of liposomes during extrusion. The polycarbonate membranes are made by a track-etch process where a polycarbonate film is bombarded with charged particles in a nuclear reactor. As these particles pass through the polycarbonate membrane, they leave sensitized tracks which are etched or dissolved in a strong alkaline solution producing

1. Liposome encounters a smaller size pore.

2. Liposome is forced through pore due to the pressure gradient.

3. Single liposome becomes two smaller liposomes that can pass through the pore.

Figure 6. Illustration of an extrusion process.

cylindrical straight-through pores. Whereas ordinary depth type membrane filters depend upon particle capture by random entrapment within the filter's tortuous pores, in the PCTE membranes, the particle capture takes place on the surface. An illustration of the two filters is shown in Figure 7.

Sequential extrusion through a series of polycarbonate membranes with decreasing size results in liposomes with a new size distribution as shown in Figure 8 (Olson et al., 1979). The liposomes can be passed through membranes of various pore sizes in the range of 3 μm to 0.05 μm . The process produces a smaller mean size and an increase in the captured volume per mole lipid as shown in Figure 8 and Table 2 (Olson et al., 1979). The size distribution of the resulting liposomes depends on the pore size of the membrane. Depending on the lipid concentration, extrusion can be done at low pressures or slightly higher pressures (40-250 psi). Extruding liposomes of 10-12 $\mu\text{mol/ml}$ in concentration is accomplished under relatively low pressure except for the 0.2 μm membrane. This latter membrane size requires a higher pressure, 50 psi, to extrude the vesicles (Olson, et al., 1979). As pore size decreases, greater pressure to force the vesicles through the smaller pores is needed (Gregoriadis, 1984). After undergoing extrusion through a 0.2 μm pore size membrane, the captured volume of liposome vesicles increased from 1.9 l/mol lipid for the unextruded vortexed preparation to 3.8 l/mol lipid for the extruded preparation. Capture efficiency also increased from 11.3% in an unextruded suspension to 22.6% in an extruded suspension. This is due to the fact that extrusion results in unilamellar vesicles which have a greater capture volume than multilamellar vesicles. Table 2 summarizes this data.

(a)

(b)

Figure 7. Illustration of a depth filter (a) and a PCTE membrane (b).

Table 2. Comparison of captured volume of extruded and nonextruded vesicles.

<u>Preparation</u>	<u>Captured Volume</u> <u>(m/mol lipid)</u>	<u>Capture Efficiency</u>
unextruded	1.9	11.3%
0.2 μm membrane	3.8	22.6%

Olson et al. (1979) determined that liposomal preparations varying in their percentage of lipids exhibit different encapsulation efficiencies when prepared under the same initial conditions. It was also determined that the liposomes have similar size and encapsulation efficiencies after extrusion (Olson et al., 1979). Ertel et al. (1993) examined extrusion of vesicles through various pore sizes. It was concluded that vesicles prepared by extrusion through 0.1-0.2 μm pores yielded reproducible preparations with unimodal size distribution as shown in Figure 8 and Figure 9. Average diameters of these vesicles were 100-200 nm. Extrusion through 0.4-0.6 μm pores resulted in preparations with variable, polymodal size distributions. Ertel et al. also concluded that no relationship was observed between extrusion pressure and vesicle diameter per each individual experiment. The data, however, does show a relationship between the membrane pore size and the vesicle diameter. As the pore size decreases, the diameter also decreases. This is demonstrated in Table 3 and Figures 10 and 11. These figures show extrusion through a 600 nm membrane yields vesicles in the range of 454-728 nm. Extrusion through a 400 nm membrane produces a size of 200-450 nm, a 200 nm membrane yields a tighter size distribution of 195-203 nm and a 100 nm membrane

Figure 8. Size distribution of vesicles (Olson et al., 1979).

a) unextruded liposomes b) liposomes extruded through a 1.0 μm membrane
c) extrusion through 0.6 μm d) 0.4 μm e) 0.2 μm

Figure 9. Vesicle population after extrusion through
a) 100 nm b) 200 nm c) 400 nm d) 600 nm membrane
(Ertel et al., 1993).

produces vesicles of 97-106 nm in diameter. Data for extrusion pressure and mean diameter data used in Table 3 and Figure 8 are taken from Table 2 of Ertel et al. (1993).

Table 3. Pressure and diameter as a result of pore size.

<u>Extrusion Pore Size</u> <u>(nm)</u>	<u>Extrusion Pressure</u> <u>(psi)</u>	<u>Mean Diameter</u> <u>(nm)</u>
100	180	106
	250	97
	240	106
200	110	195
	110	203
	140	200
400	50	357
	60	499
	60	205
600	40	728
	40	592
	50	454

Figure 10. Extrusion pore size versus mean diameter.

Figure 11. Extrusion pressure versus vesicle diameter.

2.3 Size Characterization Techniques

2.3.1 Freeze Fracture

In earlier studies, two techniques were predominantly used to study the particle size distribution of liposomes: freeze fracture and negative stain electron microscopy. In freeze fracture, the fracture plane passes through vesicles that are randomly positioned in the frozen section resulting in non-midplane fracture (Szoka and Papahadjopoulos, 1981). A homogeneous population of vesicles will therefore yield a distribution of profile sizes with the largest being equal to the true radius of the vesicle. The assumptions made in the analysis of freeze fracture studies are

- the liposomes are spherically shaped
- the vesicles are randomly distributed in the sample
- the cleavage plane intersects the vesicles in a random fashion

The freeze fracture technique has a serious drawback for estimating the size distributions and mean vesicle size of a heterogeneous population of vesicles (Szoka and Papahadjopoulos, 1981). This technique is biased towards the lower end of the size distribution.

2.3.2 Negative Stain Electron Microscopy

A few difficulties arise with use of the negative stain electron microscopy to analyze the size distributions of a sample. The difficulty in obtaining good negative

stains of liposomes arises in the spreading of the vesicles on the carbon-coated grid (Szoka and Papahadjopoulos, 1981). The vesicles tend to shrink and clump together and irregular shapes of liposomes result during the drying procedure (Olson et al., 1979). As with all microscopic techniques, these techniques have the drawback of poor statistics resulting from the limited field of view as well as limits on the number of particles counted.

2.3.3 Capillary Hydrodynamic Fractionation

The capillary hydrodynamic fractionation technique determines size and size distribution of particles traveling in an aqueous carrier fluid. Separation of particle size is caused by flow. This is due to two effects:

- The radial velocity profile developed by the fluid moving through the capillary tube allows the particles to move at different speeds.
- The inability of larger particles to approach the capillary wall as closely as smaller particles, which causes them to sample fluid streamlines of higher velocity, moving on the average at speeds greater than the smaller particles (Silebi and DosRamos, 1989).

The velocity of colloidal particles through the capillary is determined by measuring the average residence time of the particles injected into the flowing eluant. It is calculated from the ratio of capillary length to average particle residence time. The velocity of particles relative to eluant can be expressed by the separation factor, R_f , where

$$R_f = \frac{\text{Velocity of colloidal particles through tube}}{\text{Velocity of eluant}} \quad (\text{Equation 2})$$

CHDF is limited to a particle size range up to 1000 nm. Particles larger than 1000nm cannot be analyzed with this technique due to the small inner diameter of the capillary as indicated by the manufacturer, Matec Applied Sciences.

2.3.4 Dynamic Light Scattering

Another technique for determining size is light scattering. Particles in solution scatter light from an incident beam in all directions of space. The total scattered field at a given point in space fluctuates in time. Dynamic light scattering (DLS) methods monitor the time-dependence of the intensity of light scattered by the medium. The rate at which this intensity fluctuates about its average value in turn depends upon the rate at which the scattering elements move in solution. The intensity of scattered light is dependent upon the spatial arrangement of the scattering centers at any instant in time. The particles are undergoing constant motion because of collisions with the solvent molecules. The instantaneous value of the intensity fluctuates in time about the average intensity, where the rate at which these spontaneous fluctuations decay to the equilibrium value is directly dependent upon the dynamics of the molecules. The fluctuations are measured using the autocorrelation function.

$$G(\tau) = 1 + \exp(-2Dq^2\tau) \quad (\text{Equation 3})$$

where D = diffusion coefficient
 $q = (4\pi/\lambda)\sin(\theta/2)$ = the scattering vector
 λ = wavelength
 τ = time

The scattering elements are characterized by diffusion coefficients (Pecora, 1985). The diffusion coefficients are in turn used in the Stokes-Einstein equation to determine size. The Stokes-Einstein equation is given as

$$D = k_B T / (6\pi\eta r) \quad (\text{Equation 4})$$

where D = diffusion constant
 k_B = Boltzmann's constant
 T = temperature
 η = viscosity
 r = radius

2.3.5 Static Light Scattering

Static light scattering measures the angular dependence of scattered light intensity. According to the Raleigh-Gans-Debye theory (Van Zanten and Monbouquette, 1994), the angular variation is a function of particle geometry alone. The RGD approximation is valid when

$$2kL |n_p - n_s| \ll 1 \quad (\text{Equation 5})$$

where L = characteristic particle size (radius)
 n_p = refractive index of scatterer
 n_s = refractive index of solvent
 $k = 2(\pi)/\lambda_0$
 λ_0 = wavelength of incident radiation in vacuo

This condition is satisfied when $L \ll \lambda_0$. The case of $n_p \approx n_s$ also satisfies the RGD condition but results in negligible scattered intensity and is therefore uninteresting. The RGD condition guarantees homogeneity of the incident field over the sample volume.

The dependence of scattered intensity on angle is given by

$$P(\theta) = 1 - q^2 R^2/3 + 2q^4 R^4/45 + \dots \quad (\text{Van Zanten and Monbouquette, 1994})$$

(Equation 6)

where $q = 4\pi n_s/\lambda_0 (\sin (\theta/2))$
 n_s = the refractive index of the solvent
 λ_0 = the wavelength of the incident radiation
 R = radius of particle

Monodisperse solid particles or thin walled hollow particles have very similar scattering properties, summarized in Figure 10. Experimentally, the scattered intensities are measured over a range of angles and plotted as $P(\theta)/P(q_0)$ vs θ . Deviations from the form of the plots in Figure 12 suggest a deviation from monodisperse behavior. Agreement with the plots in Figure 12 suggest a monodisperse system. The best fit to the above equation is used to evaluate the mean size of particles.

Figure 12. Angular dependence of light scattering by vesicles as described by the Rayleigh-Gans-Debye model. The range of liposomes used in this model is 41 nm to 111.3 nm in diameter.

2.3.6 Zimm Plots

The weight-average molecular weight of liposomes can be determined from absolute intensity measurements, also known as Zimm plots. Scattered light intensities are measured at several angles for each of several solution concentrations as well as for the pure solvent at each angle. Subtracting the solvent scattering from the solution value yields the excess intensity scattered by the polymer (Brookhaven Instruments).

Concentration and angular measurements are plotted on an x-y axis resulting in a grid-like plot. Molecular weight of the polymer can be determined from extrapolation of the angular and concentration measurements. Extrapolating to zero angle and zero concentration yields the inverse of the weight-average molecular weight as the y-intercept (Van Zanten and Monbouquette, 1991).

The equation of interest is given as

$$H^*c/R(\theta) = [1/M_w] [1 + (R_g^2 q^2)/3] + 2A_2 \cdot c \quad (\text{Equation 7})$$

$$\text{With } H = 4\pi^2 n_o^2 (dn/dc)^2 / (N_o \lambda_o^4) \quad (\text{Equation 8})$$

- n_o = refractive index
- dn/dc = specific refractive index dispersion
- c = suspension concentration
- N_o = Avogadro's number
- λ_o = wavelength in vacuum of the laser
- θ = the scattering angle
- R = Rayleigh Ratio
- M_w = weight-average molecular weight
- q = $(4\pi n_o / \lambda_o) \sin(\theta/2)$, the scattering wave vector
- A_2 = second virial coefficient

An illustration of a Zimm plot is given in Figure 13. The theoretical molecular weight of liposomes is dependent on the molecular weight of the lipids used and is calculated as follows:

Figure 13. Illustration of a Zimm plot.

$$MW_{\text{liposomes}} = \frac{4\pi(r^2 + (r-\delta)^2)}{A} (MW_{\text{lipids}}) \quad (\text{Equation 9})$$

where $a = 0.65 \text{ nm}^2$ and 0.22 nm^2 , the area of a phospholipid molecule and a cholesterol molecule, respectively
 $\delta = 4 \text{ nm}$, the thickness of the bilayer
 r = radius in nanometers.

2.4 Summary

Particle size distribution is an important parameter for liposomes. For example, it clearly influences the distribution of liposomes within the body when they are administered parenterally (Woodle and Lasic, 1992). Yet, the control of particle size distribution within a suspension is a difficult problem. Many methods that may result in a homogeneous size distribution have been examined. However, each method has its shortcomings. Examining effects of certain process parameters on liposome size may be helpful in obtaining a controlled size distribution. Results are presented in the literature regarding the significance of extrusion to the captured volume and particle size to captured volume. However, the effect of other process parameters on liposome efficiency has not been examined, for instance, lipid composition, extrusion pressure, buffer system, and pore size. As more of these parameters are investigated, a better understanding of the behavior of liposomes will be achieved.

Despite the existence of many methods, there is no single method capable of accurate and reliable size distribution analysis of liposome samples. Therefore,

comparison and compilations of results from many methods are necessary to fully characterize the size and size distribution of liposomes within a particular preparation. Capillary hydrodynamic fractionation and light scattering are useful analytical techniques used to determine size and distribution.

EXPERIMENTAL SECTION

3.1 Introduction and Objective

The objective of this study was to investigate the relationship of process parameters on size and size distribution of liposomes during extrusion. Extrusion is the method of choice for precise size control in the industry, hence the interest in it. A mechanistic understanding of extrusion is valuable in developing a more consistent, more reproducible, and more reliable process.

The method of investigation consisted of preparing liposomes of various characteristics by lipid dissolution and lipid hydration, reducing size by extrusion, and analyzing the size and distribution of the extruded liposomes. The size was analyzed by capillary hydrodynamic fractionation (CHDF) and dynamic light scattering (DLS), and molecular weight was determined using Zimm Plots.

3.2 Materials

The phospholipids were purchased from Genzyme (Cambridge, MA) and Solvay DuPhar (Hilversum, Netherlands). Cholesterol was purchased from Croda Inc. (Parsippany, NJ). Saline (0.9% NaCl) was purchased from Baxter Healthcare Corp. (Deerfield, IL). Nucleopore polycarbonate filters were purchased from Nucleopore Corp. (Pleasanton, CA). LTI-500 anionic surfactant for the CHDF, prepared especially for Sequus Pharmaceuticals, Inc., was purchased from Matec Applied Sciences (Northborough, MA). The ethanol used was reagent grade. MilliQ grade water from a Millipore apparatus (model# MSPB00174, 18.2M Ω) was used.

3.3 Experimental Procedure

The experimental plan consisted of varying a number of factors during liposome production. Six factors were selected from the ones involved in this process, the rest were held constant. These factors were expected to be most relevant to the extrusion process. The selected factors were pressure, buffer, percent cholesterol in the lipid composition, the percent of MPEG-DSPE used, pore size of the membrane used in the first extrusion set, and pore size of the membrane used in the second extrusion set. The factors held constant were temperature, solvent, and number of passes.

These factors were selected for the following reasons:

The range of MPEG-DSPE in which the stealth properties appear is 1.5% to 5.1% of total lipid composition (Lasic and Martin, 1995).

Cholesterol is added to increase the stability of liposomes in the presence of plasma proteins. It also increases encapsulation efficiency (Lasic and Martin, 1995). A cholesterol content of 44.3 mol% is used in industry. A content of 22 mol% is used for informational purposes.

The buffers chosen are saline and ammonium sulfate for their physical stability and relevance to industrial processes.

Pressures of 80 psi and 95 psi are chosen since these are the pressures used industrially.

Ethanol is chosen as a solvent for its relatively low toxicity. The solubility of lipid components in ethanol is relatively high. It is also miscible with water.

After a number of passes at a given membrane pore size, the particle sizes are supposed to be predominantly unilamellar, therefore the liposomes will undergo 10 passes per extrusion membrane (Lasic, 1993).

Amphiphilic molecules within a liposome bilayer can be in a solid state, in which hydrocarbon chains exhibit good packing or in a liquid crystalline state in which these chains are more disordered. The temperature of this solid-liquid crystal phase transition is an important physical characteristic of the membrane. Systems exhibiting transitions (5°C to 60°C) demonstrate a complex behavior (Lasic, 1993). The selected temperature of 65°C is above the phase transition temperature range for the lipids used. This is also the temperature used industrially.

Table 4 gives a list of factors selected. Table 5 gives a list of factors held constant.

Table 4. Factors used in the experimental matrix.

<u>Factor</u>	<u>Level 1</u>	<u>Level 2</u>	<u>Selection Criteria</u>	<u>Measured Response</u>
1 st extrusion Pore Size	0.4 μm	0.2 μm	Effect on vesicle size	Mean size
2 nd extrusion Pore Size	0.2 μm	0.1 μm	Effect of smaller pore size	Mean size
Lipid Composition	44.3mol% Cholesterol	22 mol% Cholesterol	44.3% used in industry; 22% is for informational purposes	Mean size
Buffer	Saline	Ammonium Sulfate	Physically stable and used industrially	Mean size
Pressure	80 psi	95 psi	Pressures used industrially	Mean size
MPEG- DSPE	5.1 mol%	1.5 mol%	Range of Stealth [®] properties	Mean size

Table 5. The factors held constant.

<u>Variable</u>	<u>Value</u>	<u>Selection Criteria</u>
Temperature	65°C	Temperature used industrially for chosen lipids
Solvent	Ethanol	Low toxicity High solubility of lipids Miscible with water
Number of Passes	10	Reputed passes needed to reach a constant size

3.4 Design of Experiments

An experimental matrix was created using the fractional factorial design. This design estimates the main effects and interactions with maximum precision. Since six factors are selected, a 2^{6-1} factorial design is employed. This design accommodates six factors, each at two levels, yielding 32 experiments.

In this experimental design, 24 experiments were carried out. Experiments with the same first extrusion and second extrusion membrane size, 0.2 μm , were eliminated because they did not show the effects of changing membrane size on the size and distribution of the liposome suspension. Those experiments would result in 20 passes at one pore size rather than 10 passes at one membrane size. Industrial processes use two extrusion sets with different membrane sizes for each extrusion, hence the reason for its

use in these experiments. Thus, the eight experiments consisting of the same first and second extrusion membrane pore size were eliminated resulting in 24 experiments in the matrix.

Table 8 consists of the complete design of experiments for the factors considered.

Table 8. An experimental matrix of the selected factors.

<u>Exp</u>	<u>Pressure</u> <u>(psi)</u>	<u>Buffer</u>	<u>1st</u> <u>Extrusion</u> <u>Pore Size</u> <u>(μm)</u>	<u>Lipid</u> <u>Comp</u> <u>(mol%)</u>	<u>MPEG-</u> <u>DSPE</u> <u>(mol%)</u>	<u>2nd</u> <u>Extrusion</u> <u>Pore Size</u> <u>(μm)</u>
1	80	Saline	0.4	44.3	5.1	0.2
2	95	Saline	0.4	44.3	5.1	0.1
3	80	(NH ₄) ₂ SO ₄	0.4	44.3	5.1	0.1
4	95	(NH ₄) ₂ SO ₄	0.4	44.3	5.1	0.2
5	80	Saline	0.2	44.3	5.1	0.1
6	95	(NH ₄) ₂ SO ₄	0.2	44.3	5.1	0.1
7	80	Saline	0.4	22	5.1	0.1
8	95	Saline	0.4	22	5.1	0.2
9	80	(NH ₄) ₂ SO ₄	0.4	22	5.1	0.2
10	95	(NH ₄) ₂ SO ₄	0.4	22	5.1	0.1
11	95	Saline	0.2	22	5.1	0.1
12	80	(NH ₄) ₂ SO ₄	0.2	22	5.1	0.1
13	80	Saline	0.4	44.3	1.5	0.1
14	95	Saline	0.4	44.3	1.5	0.2
15	80	(NH ₄) ₂ SO ₄	0.4	44.3	1.5	0.2
16	95	(NH ₄) ₂ SO ₄	0.4	44.3	1.5	0.1
17	95	Saline	0.2	44.3	1.5	0.1
18	80	(NH ₄) ₂ SO ₄	0.2	44.3	1.5	0.1
19	80	Saline	0.4	22	1.5	0.2
20	95	Saline	0.4	22	1.5	0.1
21	80	(NH ₄) ₂ SO ₄	0.4	22	1.5	0.1
22	95	(NH ₄) ₂ SO ₄	0.4	22	1.5	0.2
23	80	Saline	0.2	22	1.5	0.1
24	95	(NH ₄) ₂ SO ₄	0.2	22	1.5	0.1

The number of passes used during each experiment was held constant. The liposome suspension was extruded a total of ten times using the specified pore size membrane. Samples were collected after every pass and the size and size distribution analyzed by CHDF and DLS. For static light scattering, the liposomes were analyzed after the last pass of the experiment, pass 20. Molecular weight measurements were also taken after the last pass.

3.5 Analytical Methods

Size is characterized using dynamic light scattering, capillary hydrodynamic fraction, and Zimm plots. All three of these methods are effective in determining particle sizes. The liposomal suspension could not be clearly analyzed by static light scattering. Therefore, this technique was eliminated from the analysis and results will not be shown.

3.5.1 Dynamic Light Scattering

The sample contained in a 5-ml glass culture tube (VWR Scientific, West Chester, PA) was placed in a thermally jacketed sample holder. The light source, an Argon ion laser of wavelength 514 nm (Lexel Laser Inc., Fremont, CA) was focused onto a sample and scattered light was detected at 90° to the incident beam by a photomultiplier tube.

For sizing, 5 µl of sample were diluted in 4 ml of saline prefiltered through a 0.2 µm filter. Three separate preparations were made of each sample. Each preparation was analyzed twice for a total of six analyses of the sample. The duration of each analysis was four minutes. The resulting data from each sample went through a selection

process. Three criteria were used to accept or reject an analysis: the magnitude of the deviations from the autocorrelation function, the baseline error of the measurement, and the number of data points deleted to obtain a distribution without artifacts. The analysis that showed the smallest magnitude of deviations from the autocorrelation function, the lowest baseline error, and the least number of data points deleted for a distribution was selected to represent the size distribution of that sample.

3.5.2 Static Light Scattering

The static light scattering measurements were carried out with the same apparatus used for dynamic light scattering. The light source was an Argon ion laser with a wavelength of 514 nm. The intensity measurements were conducted at eight different angles from 30° to 155°. Statistical analysis and parameter estimation were conducted with Brookhaven Instruments Goniometer alignment software (Brookhaven Instruments, Holtsville, NY).

3.5.3 Zimm Plots

The same apparatus as dynamic and static light scattering was used for Zimm plots. Ten microliters of sample at each concentration were diluted in 15 ml of saline prefiltered through a 0.2 μm filter. The intensity was measured at various angles. The resulting intensity measurements were plotted against the angular measurements to determine weight-average molecular weight.

3.5.4 Capillary Hydrodynamic Fractionation

A fresh surfactant solution was prepared for sample analysis. One hundred milliliters of LTI-500 carrier fluid were combined with 900 ml of Milli-Q water to get a 1-liter of solution of aqueous surfactant in a 1:10 dilution. This solution was then filtered using a vacuum filtration apparatus with a Millipore 0.45 μm pore size filter (HVLP) (Nucleopore Corp., Pleasanton, CA). This solution was the mobile phase for the instrument. It was also the solution in which the sample was diluted. Five hundred microliters of the sample were added to 500 μl of the eluant in a 2 ml flip top glass vial (National Scientific, Lawrenceville, GA).

The surfactant solution flows through the injection valve of an autosampler (Hitachi Instruments, model L-7250) where the sample was loaded and injected into the continuously flowing surfactant solution. This eluant was split into two streams at the exit of the injection valve. The largest portion was sent to a split-flow capillary while the other portion flowed into the fused silica capillary tube where the size fractionation of the colloidal particles takes place. The eluant exiting from the capillary was mixed with fresh surfactant (which acts as scavenger or “make-up” fluid) delivered by a constant flow rate pump (Waters 510 HPLC pump) equipped with a pulse dampener. A UV light detector (Matec Applied Sciences, Inc., Northborough, MA) was used to continuously monitor the turbidity of the resulting mixture as a means of determining the residence time of colloidal species eluting from the capillary tube. The exiting sample was collected in fractions using a Bio-Rad fraction collector and 5 ml glass culture tubes (VWR Scientific, West Chester, PA). Sample splitting at the injection valve and addition

of “make-up” fluid at the detector were used to achieve the small sample volumes and low flow rates required due to the small capillary inner volume.

Uniform polystyrene latex particles (Matec Applied Sciences, Northborough, MA) were used as reference standards. These standards have a very narrow size distribution. The sizes of latex particles used were in the range of 39 nm-605 nm.

RESULTS AND DISCUSSION

4.1 Introduction

Experiments were carried out between July 1998 and January 1999.

Typically, liposomes from experiments consisting of the same cholesterol, MPEG-DSPE composition, and buffer were prepared at one time – each set resulting in a block of three experiments. The exception occurred initially when experiments 1-9 were prepared individually. However, experiments 1-6 were carried out using the wrong cholesterol composition, 38.3 mol% instead of 44.3 mol%, requiring these experiments to be repeated. Since some other experiments had been completed and results indicated that liposome size was not significantly affected by buffer type, it was decided not to repeat experiments 3, 4, and 6 in order to save time. Hence, results of experiments 3, 4, and 6 are not shown. The remaining experiments to be repeated, 1, 2, and 5, contained the same amount of cholesterol, MPEG-DSPE, and buffer type (saline) and thus, were prepared at one time. Therefore, only experiments 7, 8, and 9 were prepared individually. The rest were prepared in sets of three as described above.

The results are presented according to the effects of the process parameters on liposome mean size analyzed using DLS, on size distribution analyzed using CHDF, and on molecular weight analyzed using Zimm plots.

The new matrix of experiments including the mean sizes of liposomes per each experiment is listed in Table 7.

Table 7. DLS mean sizes of liposomes at each experiment.

<u>Exp</u>	<u>Chol</u>	<u>MPEG</u>	<u>Buffer</u>	<u>Pressure</u>	<u>0.4μm</u>	<u>0.2μm</u>	<u>0.1μm</u>
1	44.3%	5.1%	Saline	80	201.6	176	
2	44.3%	5.1%	Saline	95	195.6		134.7
5	44.3%	5.1%	Saline	80		177.8	139
7	22%	5.1%	Saline	80	159.5		116.4
8	22%	5.1%	Saline	95	155.7	136.3	
9	22%	5.1%	AS	80	164.9	132.2	
10	22%	5.1%	AS	95	180.3		116.8
11	22%	5.1%	Saline	95		136.2	109.4
12	22%	5.1%	AS	80		144.4	119.6
13	44.3%	1.5%	Saline	80	205.9		142.3
14	44.3%	1.5%	Saline	95	208.8	181.2	
15	44.3%	1.5%	AS	80	215.6	188	
16	44.3%	1.5%	AS	95	211		145.6
17	44.3%	1.5%	Saline	95		180.3	138.8
18	44.3%	1.5%	AS	80		192.5	149.8
19	22%	1.5%	Saline	80	177.9	157.3	
20	22%	1.5%	Saline	95	175.3		123
21	22%	1.5%	AS	80	182.3		130.5
22	22%	1.5%	AS	95	185.8	162.8	
23	22%	1.5%	Saline	80		160.5	122.7
24	22%	1.5%	AS	95		161.4	130.8

4.2 Mean Size Analysis by DLS

4.2.1 Membrane History

Liposomes were either extruded through a 0.4 μm membrane followed by a 0.2 μm , a 0.4 μm membrane followed by a 0.1 μm , or a 0.2 μm membrane followed by a 0.1 μm . The mean size of liposomes after extrusion through a certain pore size is shown in Figures 14, 15, and 16. The mean size range in these figures is also listed next to each pore size extruded through. Figures 14 and 15 show mean sizes of liposomes initially extruded through a 0.4 μm membrane. As expected, both ranges are similar. Figures 15 and 16 show the mean liposome size after a second extrusion (10 passes) through a 0.1 μm membrane. Again, the ranges are similar although in Figure 15 the liposomes were initially extruded through a 0.4 μm membrane and in Figure 16 a 0.2 μm membrane. Figures 14 and 16 show the results of extrusion through a 0.2 μm membrane. In Figure 14, 0.2 μm membrane is used for the second set of extrusions – the first set of extrusions was through a 0.4 μm membrane 10 times. In Figure 16, the 0.2 μm membrane is used for the first set of extrusions. The mean size range for both extrusion sets is nearly the same. These results show that history of extrusion does not have a relationship to the mean size. It appears the mean size range is defined by the pore size of the current membrane, not by previous extrusions or starting size of the populations before using this membrane. Liposomes can be hydrated and unextruded or previously extruded, the mean size range will depend on the extrusion membrane size used.

Since the history of the pore sizes previously extruded through is irrelevant to mean size, the data for the first and second set of 0.2 μm extrusions will be lumped

Figure 14. Liposomes extruded through a 0.4 um membrane for the first set of extrusions and a 0.2 um membrane for the second set.

Figure 15. Liposomes extruded through a 0.4 um membrane for the first set of extrusions and a 0.1 um membrane for the second set.

Figure 16. Liposomes extruded through a 0.2 um membrane for the first set of extrusions and a 0.1 um membrane for the second set.

together in subsequent figures. This will also be done for the 0.1 μm extrusions which followed either a 0.4 μm or a 0.2 μm first extrusion set.

4.2.2 Strength of Parameters

The effect of each parameter on liposome mean size is determined by the difference in mean size when the parameter is varied from one level to the other. Figures 17-20 show this effect for cholesterol, pressure, buffer and MPEG-DSPE. Since liposomes from all experiments had to be extruded, the pore size effect was always superimposed on the effect of the other factors. In Figures 17-20, as well as subsequent figures, the response (y-axis) is plotted versus the extrusion pore size (x-axis) to show the effect of a factor. Typically, the response is plotted versus a factor in the experimental design. In these experiments, the mean size response is coupled to the extrusion pore size; therefore, data is plotted against extrusion pore size.

Figure 17 shows a change in cholesterol composition results in a significant change in liposome mean size. This factor has a strong effect on mean size. In Figures 18 and 19, varying the buffer and pressure results in small changes in mean size due to the levels of a factor. These parameters have a weak effect on particle size. The effect of MPEG-DSPE is not as strong as that of cholesterol, but it is not as weak as that of pressure and buffer, as indicated in Figure 20. However, a closer examination of MPEG-DSPE by DLS in later sections will show a weak effect for this parameter.

Figure 17. Comparing overall effect of 44.3 mol% versus 22 mol% cholesterol.

Figure 18. Comparing overall effect at 80 psi versus 95 psi extrusion pressure.

Figure 19. Comparing effect of saline versus ammonium sulfate as buffer.

Figure 20. Comparing effect of 5.1 mol% versus 1.5 mol% as the MPEG-DSPE composition used.

4.2.3 Pore Size

Figure 21 compares the mean size of liposomes extruded through a 0.4 μm or 0.2 μm membrane for the first set of extrusions. Figure 22 shows data for the second set of extrusions through a 0.2 μm or 0.1 μm membrane. As indicated by both figures, extruding through the smaller membrane results in the smaller mean size.

The pore size of the polycarbonate membrane has an effect on the mean size of liposomes. Extrusion through a membrane with large size pores results in liposomes smaller than the diameter of the pore whereas extrusion through smaller pores yields liposomes larger than or approaching the diameter of the pore size. Extrusion through a 0.4 μm membrane yields liposomes approximately one-half the diameter of the pore, 215.6-155.7 nm. Liposomes extruded through a 0.2 μm membrane result in diameters smaller than 200nm, 192.5-132.2 nm. Liposomes extruded through a 0.1 μm membrane have mean sizes approaching the diameter of the pore, 149-109.4 nm, as also concluded by Olson et al. (1979). These results are in agreement with the results reported by Mayer et al. (1986).

The effect of extrusion through a 0.4 μm and a 0.2 μm size membrane at each pass is seen in Figures 23 and 24. Although pass number is not a varying factor in the experimental design - it is held constant at 10 passes per each extrusion set - enough data were collected to show mean sizes at each pass. It is apparent the data fall into two envelopes, one for liposomes extruded through a 0.4 μm membrane and one for liposomes extruded through a 0.2 μm membrane. Experiments of liposomes extruded

Figure 21. Mean size of liposomes extruded through a 0.4 um or 0.2 um membrane for the first set of extrusions.

Figure 22. Mean size of liposomes extruded through a 0.2 um or 0.1 um membrane for the second set of extrusions.

Figure 23. Mean size as a function of pore size at 44.3 mol% cholesterol and 1.5 mol% MPEG.

Figure 24. Mean size as a function of pore size at 22 mol% cholesterol and 5.1 mol% MPEG.

through a 0.4 μm membrane show larger mean sizes than experiments extruded through a 0.2 μm membrane.

E14 and E17, in Figure 23, differ only in the membrane size used for the first set of extrusions, 0.4 μm and 0.2 μm , respectively. Liposomes from both experiments consist of the same conditions except pore size: 44.3 mol% cholesterol, 1.5 mol% MPEG-DSPE, saline, and an extrusion pressure of 80 psi. Experiments with the same parameters are indicated with the same marker shape. E14 shows a larger mean size at every pass than E17. E15 and E18 also differ only in the membrane size used for the first set of extrusions. Both experiments consist of 44.3 mol% cholesterol, 1.5 mol% MPEG-DSPE, saline and extrusion at 95 psi. The liposomes of E15 show larger mean sizes at every pass than liposomes of E18. The mean size results for these experiments fall in the 99.95% confidence interval as obtained from the p-values in a t-distribution test. Figure 24 also shows mean sizes as a function of pass number. The same trend is seen here: particles extruded through a 0.4 μm membrane result in larger mean sizes at every pass than those extruded through a 0.2 μm membrane. A 0.4 μm membrane has larger pores and therefore results in larger liposome sizes after extrusion. The smaller pores of a 0.2 μm membrane yield smaller liposomes. Results of the 0.1 μm membrane could not be compared with 0.4 μm or 0.2 μm membranes due to more than one parameter varying. The pressure or buffer also varied with the pore size.

4.2.4 Cholesterol

Liposomes prepared with 44.3 mol% cholesterol range from 215.6-134.7 nm in size. Liposomes composed of 22 mol% cholesterol range from 185.8-109.4 nm in size.

Figure 25 shows mean sizes of liposomes at varying cholesterol and pressure. This figure shows two experiments for each set of cholesterol and pressure conditions. The experimental design allows two experiments with two varying factors to be compared. All other factors are constant. This comparison can be made because one of the varying factors has a weak effect on mean size while the other has a strong effect. In Figure 25, pressure is a weak factor while cholesterol is a strong factor, as determined in an earlier section. It is assumed the weak factor does not mask the effect of the stronger factor. Therefore, the effect of cholesterol can be examined at both levels although pressure is also varying. Other factors can also be examined in a similar manner. This assumption, however, does not eliminate the need for one-variable tests. In this case, this assumption allows certain trends to be discussed clearly. It is apparent in Figure 25 that extrusion results in two groups of particles based on cholesterol composition. Liposomes with the higher cholesterol content exhibit larger mean sizes than those with lower cholesterol content. This trend is present after extrusion through all membrane sizes.

Also evident in Figure 25 is the effect of extrusion pressure on mean size. A small difference in mean size is observed between liposomes extruded at 80 psi and 95 psi, 3.8-7 nm. Therefore, it is reasonable to conclude that pressure has a weak effect on liposome mean size.

Figure 25. Comparing effect at varying cholesterol and pressure at 1.5 mol% MPEG and saline.

Figure 26 shows mean sizes at each pass for liposomes with varying cholesterol composition. E1 and E7 in this figure consist of 80 psi, saline, 5.1 mol% MPEG-DSPE, and extrusion through a 0.4 μm membrane. E2 and E8 consist of 95 psi, saline, 5.1 mol% MPEG-DSPE, and extrusion through a 0.4 μm membrane. The data falls into two sets of curves, one for 44.3 mol% cholesterol and one for 22 mol% cholesterol. The data representing liposomes with the greater cholesterol content show larger mean sizes than that of liposomes with the smaller cholesterol content. This trend continues with extrusion through all pore sizes. The mean size results fall in the 99.95 % confidence interval as indicated by the p-values. Figure 27 shows the same trend with different parameters.

4.2.5 Buffer

Liposomes formed in ammonium sulfate exhibit mean sizes of 215.6-116.8 nm. Liposomes formed in saline have mean sizes of 205.9-109.4 nm. The difference in mean size due to the buffer is 6.8-11.3 nm.

Figure 28 shows the effect of buffer on the mean size of liposomes at varying pressure. Extrusion results in two groups of liposomes: the ammonium sulfate group and the saline group. The mean size range after extrusion through a 0.4 μm membrane is 185.8-175.3 nm. The groups of points representing liposomes with saline and ammonium sulfate are close together with a difference of 10.5 nm between the largest and the smallest mean size. Liposomes prepared in ammonium sulfate result in larger sizes than liposomes prepared in saline.

Figure 26. Effect of 44.3 mol% versus 22 mol% cholesterol at each pass at 5.1 mol% MPEG-DSPE, 0.4 μ m.

Figure 27. Mean size at each pass of liposomes consisting of 44.3 mol% versus 22 mol% cholesterol, 1.5 mol% MPEG-DSPE, 0.4 μ m.

Figure 28. Effect of buffer on pore size with varying pressure at 22 mol% cholesterol and 1.5 mol% MPEG.

Also seen in Figure 28 is the effect of extrusion pressure. Extrusion through the 0.1 μm membrane results in two groups with pressure points within the same buffer superimposed on one another. There is no difference in mean size between liposomes extruded at 80 psi and 95 psi. This shows that pressure exerts a weak effect on the mean size of liposomes.

The mean size of liposomes prepared in saline and ammonium sulfate and extruded at 80 psi and 95 psi at each pass is seen in Figure 29. The mean sizes of all experiments considered intertwine throughout the passes. Since there is not a consistent trend, this is an indication of the weak effects of these two factors on mean size.

4.2.6 Pressure

Liposomes extruded at a pressure of 80 psi result in larger sizes, 215.6-116.4 nm, than those extruded at 95 psi, 211-109.4 nm. As seen in Figures 25, 28, and 29, the effect of pressure on the mean size of liposomes is weak.

4.2.7 MPEG-DSPE

The mean particle size for liposomes composed of 5.1 mol% MPEG-DSPE is in the range of 201.6-109.4 nm. Liposomes consisting of 1.5 mol% MPEG-DSPE are in the range of 215.6-122.7 nm. Figure 30 represents the mean particle size of liposomes at the two MPEG-DSPE compositions with varying pressures. Extrusion through a 0.4 μm membrane results in larger mean sizes for liposomes prepared with 1.5 mol% MPEG-DSPE. The difference in the mean size of liposomes at these compositions is 19.6 nm.

Figure 29. Mean size at each pass for liposomes at various pressures and buffers.

Figure 30. Mean size versus pore size at varying MPEG and pressures with 22 mol% cholesterol and saline.

Liposomes extruded through a 0.1 μm membrane differ by 6.3 nm due to MPEG-DSPE composition.

Figure 31 compares mean sizes at each pass of experiments consisting of 1.5 mol% and 5.1 mol% MPEG-DSPE. Although these data show liposomes consisting of 1.5 mol% MPEG-DSPE result in larger mean sizes than 5.1 mol%, similar to Figure 30, it must be noted the p-values for this set of data have a wide range, 0.005-0.30. Figure 32 shows the opposite effect of Figure 31. Liposomes consisting of 5.1 mol% MPEG-DSPE are larger in mean size than those consisting of 1.5 mol% MPEG-DSPE. The p-values of these experiments are 0.0005. Figures 30, 31 and 32 present contradicting results of the effect of MPEG-DSPE on mean size. Therefore, based on these results, it is difficult to conclude the effect of MPEG-DSPE. However, since the data do not support each other, the effect is likely to be weak.

4.2.8 DLS Conclusions

As evident from the mean size data, pore size and cholesterol composition have a strong effect on the mean size of liposomes whereas buffer and pressure have a weak effect. Furthermore, it is apparent the smaller pore size in both sets of extrusions, 0.2 μm and 0.1 μm , yields smaller liposomes. A cholesterol composition of 22 mol% produces smaller vesicles. Although a strong effect of pore size is expected since extrusion is a physical process dependent on the pore size of the membrane used, it is interesting to note that the material composition of liposomes, cholesterol, also has a strong effect on the mean size. Liposomes prepared in saline result in smaller mean sizes than those

Figure 31. Effect of 1.5 mol% versus 5.1 mol% MPEG-DSPE at 22 mol% cholesterol.

Figure 32. Effect of 1.5 mol% versus 5.1 mol% MPEG-DSPE at 44.3 mol% cholesterol.

prepared in ammonium sulfate. Extrusion at a pressure of 95 psi produces smaller liposomes.

The necessity for two sets of extrusions to reduce liposome size is not clear from the DLS data. This will be explained in later sections.

4.3 CHDF Analysis

The CHDF distributions were not analyzed in the typical main-effects fashion as the DLS mean size data were. For the purpose of this work, it was more instructive to examine individual distributions for the effect of a factor. In this section, the individual characteristics will be discussed. However, one-variable experiments are necessary to see the full effect of a factor.

4.3.1 Pore Size History

The CHDF distributions show the range of particle sizes present in the liposomal suspension. In contrast to the results obtained in DLS analyses, CHDF data show a difference between liposomes extruded through a 0.2 μm membrane for the first versus the second extrusion set. Figures 33 and 34 show this effect. In Figure 33, a 0.2 μm membrane is used for the second set of extrusions. Few passes are needed to achieve a tight distribution. The hydrated liposomes have previously been reduced by extrusion through the 0.4 μm membrane. When a 0.2 μm pore size membrane is used for the first set of extrusions, as in Figure 34, the hydrated mixture of heterogeneous liposomes must

Figure 33. E1 with 0.2 um membrane used for the second set of extrusions.

Figure 34. E5 with a 0.2 um membrane used for the first set of extrusions.

be reduced first. Subsequent passes yield a tightening of the distribution but more passes are needed. By the fourth pass, the distributions are similar for both cases.

Using a 0.2 μm membrane for the second set of extrusions, minor change is observed in the distributions after the second pass, as seen in Figure 33. It can be concluded the effect of 10 passes through a 0.4 μm membrane during the first extrusion set is equivalent to two passes through a 0.2 μm membrane in the first extrusion set while all other conditions are constant. This is seen when comparing the distribution of the 1st pass in Figure 34 to the distribution of the 2nd pass in Figure 33. First and second set extrusions through a 0.1 μm membrane also show a similar effect due to membrane history.

4.3.2 Pore Size

In the following figures all but one of the factors are held constant and the resulting distributions are compared. Since only one experiment is used in each case, the effect at each factor is not fully determined but trends can be discussed. Figure 35 represents the size distribution of liposomes extruded through 10 passes in the first extrusion set for E1 and E5. The liposomes in E1 and E5 were extruded through 0.4 μm and 0.2 μm membranes, respectively. Liposomes from both experiments consist of 44.3 mol% cholesterol, 5.1 mol% MPEG-DSPE, and an extrusion pressure of 80 psi. Distributions of both experiments exhibit a strong peak in the small size end at approximately 40 nm. However, the size distribution for particles extruded through a 0.4 μm membrane (40-500 nm) is broader in the larger size end than for particles extruded through a 0.2 μm

Figure 35. CHDF distributions comparing extrusion through 0.4 μm and 0.2 μm .

membrane (40-275 nm). DLS mean sizes are 201.6 nm for E1 and 177.8 nm for E5 and are represented by a circle above each distribution. The decrease in maximum size in the distribution of particles extruded through a 0.2 μm membrane results in a tighter distribution, hence a lower mean size. The particles in the low-size end contribute little to the mean size.

Figure 36 compares pass 10 of two different experiments, E19 (0.4 μm) and E23 (0.2 μm). The distributions of both experiments consist of a strong peak at the small size end, approximately 40 nm. The distribution of E19 (40-375 nm) is broader than the distribution of E23 (40-275 nm). The DLS mean sizes are 177.9 nm for E19 and 160.5 nm for E23. The decrease in maximum size in the distribution of the particles extruded through a 0.2 μm membrane again results in a tighter distribution, thus a lower mean size.

The CHDF distributions show data consistent with the DLS mean size data of membrane size having a strong effect on the mean size of liposomes. The change in mean size experienced by liposomes extruded through the various membranes is explained by the distributions. Both analyses indicate a larger mean size for liposomes extruded through a 0.4 μm membrane than a 0.2 μm membrane. However, the CHDF distribution explains the larger mean size results from the presence of liposomes in the large size range for experiments extruded through the 0.4 μm membrane.

Figure 36. CHDF distributions comparing extrusion through 0.4 um and 0.2um membrane.

4.3.3 Cholesterol

Figure 37 describes the effect of cholesterol after the 10th pass of the first extrusion on the size distribution of liposomes. Particles consisting of 44.3 mol% cholesterol in E13 are compared with particles consisting of 22 mol% cholesterol in E19. All other parameters are the same, 1.5 mol% MPEG-DSPE, saline, extrusion through a 0.4 μ m membrane at a pressure of 80 psi. The distributions of both experiments are similar. However, the distribution for liposomes consisting of 44.3 mol% cholesterol (40-600 nm) is broader than the distribution of liposomes consisting of 22 mol% cholesterol (40-400 nm). The DLS mean size for E13 is 205.9 nm and E19 is 177.9 nm. The particles present in the range from 400-600 nm in E13 contribute to the larger mean size.

The distribution of E1 consisting of liposomes of 44.3 mol% cholesterol is compared to the distribution of E7 consisting of 22 mol% cholesterol in Figure 38. However, these distributions do not show any similarities. The peak present at the small size end in E1 is not present in E7. The distribution of E1 is broader than the distribution of E7. Particles are present in the 400-500 nm range in E1 that do not exist in E7. It is the presence of these particles that contributes to the increase in mean size of liposomes in E1. The particles in the small size range of 40 nm contribute little to the mean size. This will be explained in greater detail in the next section. Absence of the large size population in E7 results in a smaller mean size and a tighter distribution. The DLS mean sizes of E1 and E7 are 201.6 nm and 159.5 nm, respectively.

Figure 37. CHDF distributions comparing liposomes consisting of 44.3 mol% and 22 mol% cholesterol.

Figure 38. CHDF distributions comparing liposomes consisting of 44.3 mol% and 22 mol% cholesterol.

4.3.4 Buffer

The spread of the populations of liposomes prepared in saline (E20) and ammonium sulfate (E22) is shown in Figure 39. Distributions of liposomes prepared in both buffers exhibit a strong peak at 40 nm. The distribution of liposomes in ammonium sulfate (40-400 nm) is broader than the distribution of liposomes in saline (40-350 nm). The DLS mean size of E20 and E22 is 175.3 nm and 185.8 nm, respectively. The small population in the 350-400 nm range contributes to the increase in mean size of liposomes prepared in ammonium sulfate albeit only slightly.

Population distribution and mean size behavior can be explained by the Mie Scattering theory. Figure 40 predicts the intensity contribution for various particle diameters (Stock and Ray, 1987). Using the 90° scattering angle curve, the measuring angle used in DLS and CHDF, the intensity can be estimated for liposomes in the distribution. Particles in the range of 325-400 nm and approximately 40-50 nm have minimal intensity contribution to the population, on the order of 10^{-3} , as indicated by the valley in the curve. Therefore, the contribution to the mean size is also minimal. The maximum intensity contribution results from particles 150-250 nm and 400-500 nm in diameter, 8×10^{-1} to 1×10^{-2} , the peak in the curve.

Looking again at Figure 39, particles present in the 375-400 nm range in ammonium sulfate have minimal intensity contribution to the overall population. Hence, the DLS mean sizes are similar although the distributions are not.

Figure 41 also shows the distribution of liposomes prepared in ammonium sulfate (E19) and saline (E21). The DLS mean size for liposomes prepared in ammonium sulfate

Figure 39. CHDF distributions comparing saline and ammonium sulfate.

Figure 40. Predictions of intensity as a function of particle diameter for three scattering angles according to Mie theory (Stock and Ray, 1987).

Figure 41. CHDF distributions comparing liposomes prepared in saline (E19) and ammonium sulfate (E21) at 22 mol% cholesterol, 1.5 mol% MPEG, 80 psi, 0.4 μm .

and saline are 177.9 and 182.3nm, respectively. Liposomes prepared in saline exhibit larger mean size than those prepared in ammonium sulfate. The distribution of E19 (40-375) is also broader than the distribution for E21 (40-350nm). These distributions don't follow the trend of liposomes prepared in ammonium sulfate resulting in larger mean sizes than saline. This may be due to experimental error or an error in analysis. It also may be due to the fact that another weak parameter, pressure, is varying as well as buffer. However, the difference in mean size between liposomes prepared in saline and ammonium sulfate is very small compared to the difference in mean size due to the membrane size or cholesterol composition.

The results from the DLS mean size analysis suggest effect of buffer on particle size is weak. The difference in size due to the buffers is approximately 5-10 nm. The distributions of saline and ammonium sulfate are changing only subtly (25-50 nm). In general, they support the results from the DLS mean size analysis since the effect of both variables on mean size is nearly the same.

4.3.5 Pressure

Figure 42 represents size distributions of experiments at 80 psi (E19) and 95 psi (E2). The distribution of liposomes extruded at 80 psi (40-375 nm) is slightly broader at the larger size end than that at 95 psi (40-350 nm). The DLS mean size of E19 is 177.9 nm and E20 is 175.3 nm. Absence of the area from 350-375 nm results in a mean size drop of 2.6 nm. This drop is not very significant because the particles fall in the 300-400 nm range, where the intensity contribution is minimal.

Figure 42. CHDF distributions comparing liposomes extruded at 80 psi and 95 psi with 22 mol% cholesterol and 1.5 mol% MPEG.

For pressure, the DLS mean size analysis generally shows an extrusion at 80 psi results in larger mean sizes than 95 psi. The difference in mean sizes with varying pressure is approximately 6-10 nm. The CHDF distributions consistently show 80 psi having a slightly broader distribution than 95 psi. The size difference in the distributions is 25 nm. Both of these analyses indicate pressure has a weak effect on particle size.

Hunter and Frisken (1998) report that the size of the vesicles decreases significantly as the extrusion pressure increases. Results from present research show that while this is usually true, it is not always true. Perhaps this is due to the small difference in extrusion pressures used in the present research. A more significant difference in pressures may not show the same results.

4.3.6 MPEG-DSPE

Figure 43 compares the CHDF distributions of liposomes at both MPEG-DSPE compositions. The distribution of E2 consisting of 5.1 mol% MPEG-DSPE is slightly broader than the distribution of E14 consisting of 1.5 mol% MPEG-DSPE. The two distributions are otherwise similar. However, the DLS mean size is larger for liposomes consisting of 1.5 mol% MPEG-DSPE, 208.8 nm, than for 5.1 mol% MPEG-DSPE, 195.6 nm. In this case, the DLS data do not support the CHDF distribution data.

Figure 44 shows the CHDF distributions of liposomes consisting of 5.1 mol% and 1.5 mol% MPEG-DSPE. The distribution of liposomes consisting of 5.1 mol% is slightly broader than that of 1.5 mol% MPEG-DSPE. The DLS mean sizes are 155.7 nm and

Figure 43. CHDF distributions for 5.1 mol% versus 1.5% MPEG.

Figure 44. CHDF distributions for 5.1 mol% versus 1.5% MPEG.

175.3 nm for 5.1 mol% and 1.5 mol%, respectively. Again, the CHDF distribution data do not support the DLS mean size data.

According to DLS analyses, liposomes with 1.5 mol% MPEG-DSPE are larger in size than liposomes with 5.1 mol% MPEG-DSPE. The CHDF distributions show a slightly broader distribution for liposomes with 5.1 mol% data. Since, the CHDF distributions are similar in size and the difference in DLS mean sizes is small and the data from these two techniques do not support each other, it is reasonable to conclude the effect of MPEG-DSPE on liposome size is weak.

4.3.7 Distribution Trends at Each Pass

Figures 45 and 46 represent the CHDF distributions for a complete experiment, E1. Five passes were analyzed, passes 1-4 and pass 10. Minor change was observed in the distributions from pass 4 to pass 10 that passes 5-9 were omitted in the analysis. This is also demonstrated in the distributions. The distribution for pass 4 is very similar to the distribution for pass 10. It was assumed there was little change between these two passes.

The first pass of E1 shows a broad distribution. Particles are in the range of 40 – 500 nm and a population also exists at 800-1000 nm. The second pass results in nearly eliminating the largest population at 800-1000 nm. This also results in a greater volume of the population at 40-500 nm. In passes 3 and 4, the largest size population is slowly eliminated. Pass 10 shows a tighter population at the large size end although the distributions of pass 4 and 10 are similar.

Figure 45. CHDF distributions representing the first extrusion set in E1 with 44.3 mol% cholesterol, 5.1 mol% MPEG, Saline, 80 psi and extrusion through a 0.4 μm membrane.

Figure 46. CHDF distributions representing the second extrusion set in E1. Extrusion through a 0.2 μm membrane.

At early stages of extrusion, each pass has a strong effect of reducing size. However, after a certain number of passes, the change in the distributions is minimal. The distributions of passes 3, 4, and 10 look similar at both the 0.4 μ m pore size and 0.2 μ m pore size.

The first pass through a 0.2 μ m membrane results in considerable tightening of the distribution from pass 10 of a 0.4 μ m membrane. The resulting distribution is tighter by 100 nm in the larger size end. Passes 2-4 show little change in the overall distribution. Thereafter, little change is observed between pass 4 and pass 10. It appears most of the size reduction occurs at the change to a smaller size membrane. These distributions show the need to use two separate membrane sizes for greatest size reduction. Although, this is the industry standard, this conclusion is clearer with the use of the CHDF distributions. In fact, using DLS alone, this conclusion would not be reached. As shown in the DLS, the mean size does not seem to be affected by extrusion history. These distributions also indicate that 10 passes are not necessary for size reduction of the suspension. Since there is little change from pass 4 to pass 10, five passes may be sufficient for the desired size reduction.

4.3.8 Micelles

There is a very strong and persistent population present in nearly all of the distributions at approximately 40 nm, as seen in Figures 45 and 46. This population is not very strong after the first pass through a 0.4 μ m membrane. However, after the second pass, this peak is present in every pass at the same position and with the same

intensity. Particles of this size are usually micelles. These particles may or may not be a product of extrusion. It is difficult to determine if these micelles are a result of liposome hydration or if they assemble during extrusion. Unextruded liposomes cannot be analyzed by the CHDF due to size limitations. When analyzed by DLS, particles up to 10,000 nm are detected. Due to the bias of DLS towards larger particles, it is difficult to determine if smaller particles were present before extrusion or are solely a product of extrusion.

As explained earlier, particles in this size range contribute little to the overall mean size of the suspension. Although the CHDF data indicate a large volume of these particles in nearly every pass, the mean size change as a result of these particles is minimal.

4.3.9 CHDF Conclusions

The CHDF analysis is necessary in understanding the population distribution of a liposomal suspension. It is also useful in explaining the erratic behavior observed in the DLS analysis at times. The CHDF data in this section indicate the DLS mean size information alone may not be sufficient to draw an accurate conclusion regarding the effect of a factor. The DLS technique gives no indication of a monodisperse or polydisperse system. A polydisperse population may have the same mean size as a monodisperse population. Although DLS is the industry standard for size determination, knowledge of the mean size as well as the population distribution gives greater confidence to the measured results.

4.4 Molecular Weight

The weight-average molecular weight was determined from Zimm plot measurements. Only the last pass of an experiment, pass 10 of the second extrusion set, was analyzed since a monodisperse population was expected after this pass. Due to having only one molecular weight measurement for each experiment, the effect of pore size history could not be examined as with the DLS and CHDF analyses. Since enough data was not collected, the typical main-effects analysis was not done with this molecular weight data. Nonetheless, a few trends can be observed with this data. The information collected can be used to compare molecular weight results from these experiments to those in literature.

4.4.1 Observed Trends

The molecular weight of the liposomes was in the range of 9.36×10^6 to 8.07×10^7 g/mole. The Zimm plots indicated a polydisperse system. Extrusion, even through 20 passes, did not result in a system homogeneous in size. CHDF distributions also show this polydispersity with the wide range in populations. The distribution data was used to obtain molecular weight of liposomes. The molecular weight obtained at each experiment from the CHDF distribution was higher than the Zimm plot measurements. Molecular weight of liposomes as calculated by the CHDF analysis was in the range of 2.7×10^7 to 1.26×10^8 g/mole.

Figure 47 shows the expected molecular weights of liposomes with the lipid concentrations used in the experiments. These molecular weights were calculated using

Figure 47. The ideal molecular weight of the liposomes for each of the lipid concentrations used in the experiments.

Equation 9, as presented in Chapter 2. These molecular weights represent the ideal, monodisperse system. The molecular weights measured by the Zimm plots should lie on these curves. However, the measured weights deviate from the ideal curves in nearly all instances, as seen in Figure 48. This deviation shows the system is polydisperse. It must also be noted that the experimental liposomes follow the trend of the ideal curves although they have lower molecular weights.

Figure 49 compares the CHDF data with the Zimm plot data. As with the Zimm plot data, the calculated molecular weight from the CHDF data do not fall on the ideal curves. In the case of the CHDF, this is expected since the distributions showed a wide range of populations present in the system. Furthermore, the CHDF data and the Zimm plot data from the same experiments do not match. Yet at larger radii, the data from these two analyses fall in the same molecular weight range and show some overlap. These weights are circled in Figure 49. All five data points represent particles extruded through a 0.4 μm membrane for the first set of extrusions and 0.2 μm membrane for the second set of extrusions--the larger size membrane for both extrusion sets. Extrusion through larger membranes may also contribute to the higher molecular weight for the Zimm data. A group of points with low molecular weight and low radii are also present in the measured data. All particles in this group were extruded through 0.1 μm membrane as the second set of extrusion. They also share the same cholesterol composition, 22 mol%, the composition resulting in the lower mean size and tighter distributions, as indicated by DLS and CHDF analyses, respectively.

Figure 48. Molecular weight measurements by Zimm plots compared to the ideal molecular weight calculations.

Figure 49. Comparing ideal molecular weight calculations, Zimm plot measurements, and the CHDF calculations.

Since particles extruded through a 0.2 μm membrane for the second extrusion set show overlapping in Zimm plot data and CHDF data, this may indicate accuracy of the CHDF system in detecting particles at this range. Particles extruded through a 0.1 μm membrane show low molecular weights and smaller radii. This may indicate the CHDF distributions lose accuracy at the low end. Smaller particles, such as micelles, may escape detection by the system thus resulting in skewed distributions and lower molecular weights.

4.4.2 Results Compared to Literature

Van Zanten and Monbouquette (1991) report a weight-average molecular weight of 26.7×10^6 to 45.4×10^6 g/mole for monodisperse liposomes 65-90 nm in diameter. Data from present experiments show weight-average molecular weights of 9.3×10^6 to 80.7×10^6 g/mole for liposomes 109-188 nm in diameter. These measurements do not correspond to results reported by Van Zanten and Monbouquette. Since the experimental vesicles are larger in diameter than those reported by Van Zanten and Monbouquette, a larger weight-average molecular weight is expected. Figure 50 shows a plot of molecular weight of liposomes at various radii for results reported by Van Zanten and Monbouquette and those of the current research. Molecular weights reported by Van Zanten and Monbouquette follow a clear trend. Molecular weights belonging to present experiments are shifted towards the larger radii but do follow the pattern of vesicles studied by Van Zanten and Monbouquette with some deviations. The reason for the shift

Figure 50. Comparing Zimm measurements of experimental liposomes to those of Van Zanten and Monbouquette (1991) in literature.

in molecular weight may be attributed to the polydispersity of the system which may skew the molecular weight measurements. The lipid compositions may also have an effect. Van Zanten and Monbouquette used phosphatidylcholine and sodium cholate for their vesicles. Phosphatidylcholine, phosphatidylethanolamine, and cholesterol were used to form liposomes in the present research.

4.4.3 Zimm Plot Summary

At this time, data collected from Zimm plot measurements do not give sufficient information of the molecular weight phenomena to draw a sound conclusion regarding the behavior of liposomes due to the varying process parameters in this research. However, a few trends have been observed with this data. The Zimm plot measurements support data from DLS and CHDF analyses. The largest weight-average molecular weights measured by the Zimm plots belong to liposomes with 44.3 mol% cholesterol and were extruded through the larger size membranes. This corresponds to the largest mean sizes in DLS analyses and the broadest distributions in CHDF analyses. Liposomes with the lowest weights consist of 22 mol% cholesterol and were extruded through the 0.1 μm membrane as also observed with CHDF and DLS. Moreover, the results from present experiments show some similarities with those of Van Zanten and Monbouquette (1991). Furthermore, the polydispersity of the system is clear. However, to obtain more information regarding the wall thickness and the mean size of the population, a monodisperse system is needed. More work must be done to obtain a monodisperse suspension to reach conclusions regarding mean size and wall thickness.

CONCLUSIONS

All factors tested had an effect on the particle size. The membrane pore size and the cholesterol composition had strong effects. Buffer, pressure, and MPEG-DSPE composition showed weak effects on the mean size.

Liposomes containing 44.3mol% cholesterol were larger in mean size than those containing 22 mol% cholesterol. Liposomes consisting of 1.5 mol% MPEG-DSPE composition were larger than liposomes consisting of 5.1 mol% MPEG-DSPE. Using ammonium sulfate as the buffer resulted in larger liposome sizes than saline. The extrusion pressure of 80 psi generally resulted in larger sizes than 95 psi.

Extrusion of liposomes through a 0.4 μm membrane resulted in the largest particles. Extrusion through a 0.1 μm membrane produced the smallest particles. Particles resulting from extrusion through a 0.2 μm membrane were smaller than ones produced through a 0.4 μm membrane but larger than those resulting from a 0.1 μm membrane. Extrusion through large membrane pore sizes yielded liposomes smaller in diameter than the diameter of the pore. Extrusion through smaller pore sizes yielded liposomes larger than the pore diameter.

Extruding liposomes through a smaller size membrane resulted in eliminating large size peaks that remained with extrusion through a larger size membrane. A strong peak was present in nearly 90% of the runs, with the size generally in the range of 30-50nm. These particles were probably micelles. Extrusion through the first pass resulted in the largest size distribution range, 0-1000nm. The second pass eliminated the largest

particles. The area of action, where the size reduction occurred, was at the large-size end of the distribution. Larger particle peaks were eliminated with subsequent passes. The particles resulting from extrusion through 0.2 μm pore as the first set of extrusion were smaller than those from 0.4 μm pore. The distributions of liposomes extruded through 0.2 μm membrane as the first set of extrusions were similar at pass 4 and 10 to liposomes extruded through a 0.2 μm membrane as the second set of extrusions. The mean sizes were also similar. However, more passes were needed to reach the same distribution when a 0.2 μm membrane was used as the first extrusion membrane rather than the second. Most of the size reduction takes place during the first set of extrusions. Little change was seen in the distributions from pass 4 to pass 10. Therefore, for future extrusions, fewer than 10 passes will be needed to achieve constant distributions and mean size through a membrane. It may be reasonable to conclude that more than 5 passes may not be necessary.

Based on the results of the experiments, an ideal liposomal suspension with a tight distribution and a small mean size will consist of the following criteria:

Extrusion through a smaller size membrane, 0.2 μm followed by 0.1 μm

A cholesterol composition of 22 mol%

Saline as the buffer

An extrusion pressure of 95 psi

MPEG-DSPE composition of 1.5 mol%

Five passes per extrusion set

Zimm plot data indicated a polydisperse system of particles for all experiments.

This data can be separated into three groups—

- 1) particles extruded through a 0.4 μm membrane followed by a 0.2 μm membrane.

These particles show an overlap in Zimm measurements and CHDF data.

- 2) particles extruded through a 0.1 μm membrane during the second extrusion set and consisting 22 mol% cholesterol.
- 3) particles consisting of 44.3 mol% cholesterol and extruded at the various extrusion membrane combinations.

Zimm plot data supported CHDF and DLS analyses.

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APPENDIX A

DLS Mean Size at Each Pass

		Experiment Number					
Pass #		1	2	3	4	5	6
First Extrusion Set	1	269.6	266.7	No data	No data	197.9	No data
	2	223.4	223.5	No data	No data	192	No data
	3	219.5		No data	No data	185.3	No data
	4	211.8	204.4	No data	No data	185.1	No data
	10	201.6	195.6	No data	No data	177.8	No data
Second Extrusion Set	1	195.6	157.6	No data	No data	160.1	No data
	2	188.4	145.4	No data	No data	150.2	No data
	3	189.3	143.4	No data	No data	146.1	No data
	4	180.4	147.2	No data	No data	142	No data
	10	176	134.7	No data	No data	139	No data

		Experiment Number					
Pass #		7	8	9	10	11	12
First Extrusion Set	1	230.6	220.6	217.6	220.6	178.7	188.4
	2	1913	181.5	197.5	198.7	158.6	166.7
	3	178.3	169.4	184.2	186.9	151.8	164
	4	172.8	165.2	184	181.4	145.1	159.7
	10	159.5	155.7	164.9	180.3	136.2	144.4
Second Extrusion Set	1	142.1	151.9	152.1	142.8	125.2	134
	2	130.4	145.3	147.3	131.7	116.6	128.9
	3	125.7	145.2	141.9	130	118.7	126.1
	4	122.1	145.8	139	127	115.5	128
	10	116.4	136.3	132.2	116.8	109.4	119.6

		Experiment Number					
Pass #		13	14	15	16	17	18
First Extrusion Set	1	217.8	238.6	250.3	270.2	199.6	222.5
	2	205.5	223.2	250.4	235.7	187.3	203.6
	3	214.8	215.4	230.2	239.1	190.8	197.6
	4	212.4	212.1	224	221.6	187.9	200.3
	10	205.9	208.8	215.6	211	180.3	192.5
Second Extrusion Set	1	183.4	197.8	200.4	171	156.1	165.7
	2	148.1	190	203.3	160.3	152.4	158.7
	3	151.8	188.7	196.6	151.7	147.9	152.8
	4	146.3	186.5	193.9	151	146.6	149.7
	10	142.3	181.2	188	145.6	138.8	149.8

		Experiment Number					
Pass #		19	20	21	22	23	24
First Extrusion Set	1	237.7	223.3	270.3	235.2	186.4	194.5
	2	200.9	203.8	209.3	216.7	178.8	180.5
	3	190.7	189.6	201.6	194.3	165.9	174.3
	4	187.7	187.5	195.2	187.3	167.9	169.9
	10	177.9	175.3	182.3	185.8	160.5	161.4
Second Extrusion Set	1	175.4	142.3	145.2	173.4	147.8	148.3
	2	169.8	136.4	141.3	173.3	137.5	143.4
	3	166.8	131.1	137.5	170.7	134.4	139.9
	4	167.2	130.5	135.9	168.2	132.6	137
	10	157.3	123	130.5	162.8	122.7	130.8

APPENDIX B

CHDF raw data for Experiment 1 showing diameter of liposome and the weight % present at each diameter.

Experiment 1	Diameter	Weight %
First extrusion set	1067.346	98.092
0.4um	1058.748	74.944
Pass #1	1050.115	51.013
	1041.424	30.541
	1032.651	16.028
	1023.764	7.469
	1014.724	3.220
	1005.487	1.419
	996.000	0.870
	986.200	1.124
	976.014	2.239
	965.359	4.528
	954.141	8.273
	942.255	13.314
	929.590	18.736
	916.029	23.035
	901.457	24.803
	885.770	23.495
	868.882	19.727
	850.742	14.874
	831.342	10.320
	810.738	6.884
	789.051	4.719
	766.477	3.571
	743.281	3.091
	719.782	3.001
	696.333	3.123
	673.294	3.351
	650.999	3.612
	629.737	3.858
	609.732	4.058
	591.132	4.185

Experiment 1	Diameter	Weight %
	574.013	4.226
	558.386	4.189
	544.207	4.098
	531.393	3.994
	519.836	3.964
	509.413	4.198
	499.998	5.076
	491.466	7.295
	483.701	11.981
	476.596	20.558
	470.055	34.112
	463.994	52.292
	458.341	72.458
	453.032	89.981
	448.015	100.000
	443.244	99.748
	438.681	89.927
	434.294	74.195
	430.058	57.210
	425.949	42.603
	421.949	32.032
	418.044	25.453
	414.219	21.985
	410.465	20.655
	406.772	20.736
	403.134	21.782
	399.544	23.543
	395.998	25.873
	392.490	28.668
	389.017	31.822
	385.578	35.203
	382.168	38.639
	378.785	41.927
	375.429	44.864
	372.098	47.291
	368.790	49.144
	365.504	50.462
	362.240	51.369

Experiment 1	Diameter	Weight %
	358.996	52.007
	355.772	52.463
	352.567	52.720
	349.381	52.658
	346.213	52.123
	343.064	51.020
	339.931	49.385
	336.817	47.381
	333.719	45.239
	330.637	43.185
	327.572	41.372
	324.524	39.863
	321.491	38.645
	318.474	37.662
	315.473	36.856
	312.488	36.189
	309.517	35.644
	306.562	35.208
	303.622	34.862
	300.697	34.582
	297.786	34.366
	294.890	34.233
	292.009	34.219
	289.142	34.347
	286.289	34.611
	283.450	34.975
	280.626	35.393
	277.815	35.826
	275.018	36.251
	272.234	36.650
	269.464	36.993
	266.708	37.235
	263.965	37.316
	261.235	37.193
	258.518	36.859
	255.814	36.360
	253.124	35.783
	250.446	35.223

Experiment 1	Diameter	Weight %
	247.780	34.756
	245.128	34.420
	242.487	34.224
	239.860	34.162
	237.244	34.233
	234.641	34.424
	232.050	34.697
	229.471	34.983
	226.904	35.208
	224.349	35.320
	221.806	35.314
	219.274	35.218
	216.754	35.066
	214.246	34.876
	211.748	34.646
	209.263	34.365
	206.788	34.030
	204.325	33.639
	201.873	33.184
	199.432	32.637
	197.001	31.967
	194.582	31.164
	192.173	30.256
	189.775	29.295
	187.388	28.325
	185.011	27.369
	182.645	26.423
	180.289	25.477
	177.943	24.530
	175.608	23.596
	173.282	22.705
	170.967	21.890
	168.662	21.173
	166.367	20.552
	164.081	20.003
	161.805	19.486
	159.539	18.967
	157.283	18.437

Experiment 1	Diameter	Weight %
	155.036	17.917
	152.799	17.445
	150.571	17.060
	148.353	16.783
	146.143	16.611
	143.943	16.517
	141.753	16.460
	139.571	16.401
	137.398	16.301
	135.234	16.133
	133.079	15.886
	130.933	15.566
	128.796	15.192
	126.667	14.791
	124.547	14.386
	122.435	13.990
	120.332	13.612
	118.238	13.258
	116.151	12.940
	114.073	12.669
	112.003	12.452
	109.942	12.287
	107.888	12.159
	105.843	12.046
	103.805	11.922
	101.775	11.760
	99.754	11.544
	97.740	11.267
	95.733	10.945
	93.735	10.608
	91.744	10.302
	89.760	10.062
	87.784	9.908
	85.815	9.839
	83.854	9.843
	81.900	9.911
	79.953	10.043
	78.014	10.239

Experiment 1.

Graph based on the weight % and diameter information for extrusion through the first pass using a 0.4 μm membrane.

The same procedure is followed in each experiment for extrusion through subsequent passes.

CHDF data for experiments 1, 2, 5, 7-24 at passes 1-4, 10, 11-14, 20 is provided in the enclosed diskettes.