4.1 INTRODUCTION

Liposomes can be formulated by many alternative preparation techniques and using different formulations. Conventional liposomes made from natural lecithin are prone to physical and chemical deterioration, such as aggregation and peroxidation. Egg PC is a mixture of molecular species of PC differing in fatty acyl chains, and it includes a considerable amount of unsaturated fatty acids such as arachidonic (C20:4) and decasohexanoic acids (C22:6) (Grit et al., 1993). These phospholipids with unsaturated acyl chains are subjected to oxidation, which may affect the permeability of the bilayers and the *in vivo* performance of the liposomes (Smolen and Shohet, 1974, Konings, 1984). These acids are more sensitive to oxidative degradation than more saturated forms. Hydrogenated PC or partially hydrogenated PC instead of unsaturated egg PC in the liposome preparation has been widely used to reduce lipid peroxidation. The antioxidant, α-tocopherol was frequently incorporated into the liposomes to inhibit the oxidation of lipid. There are a large variety of methods for preparing liposomes. From a pharmaceutical point of view, the three most important factors to be evaluated before selecting the method of preparation are the trapping efficiency, drug retention property and drug/lipid ratio (Betagiri *et. al.*, 1993).

Trapping efficiency is one of the important parameters in selecting a method of preparation of liposomes. An optimum loading procedure would achieve trapping efficiency of 90% or more. This obviates the need for removal of unentrapped drug because loading doses of 10% or less of free drug can usually be tolerated. The procedures, such as dialysis and passage through exclusion columns, for removal of unentrapped drug are often time-consuming, tedious, expensive and recovery of unentrapped drug is usually difficult.

Many lipid compositions can be employed for liposomal delivery systems; however, stability and cost are important determinants. Thus lipids such as phosphatidylserine (PS), cardiolipin and phosphatidic acid (PA) are not preferred components (as compared to phosphatidyl choline (PC)) because of high costs and often liable nature of these compounds. Similarly, the use

of unsaturated lipids, such as soya PC or naturally occurring PS, phosphatidylethanolamine (PE) and cardiolipin, should be avoided because of the considerable oxidation problems encountered. Thus, given similar loading and retention characteristics, liposomal systems composed of egg PC or hydrogenated varieties of egg or soya PC are more pharmaceutically acceptable.

Considering drug retention, it is unlikely that most drug-liposome formulations can exhibit sufficiently low leakage rates to allow retention times of one year or more. However, if the trapping efficiencies are sufficiently high (e.g. 90% or more), unentrapped drug need not be removed. No leakage of drug would then occur on extended storage, because of the absence of transmembrane drug concentration gradients. The optimum drug/lipid ratio of a liposomal formulation will likely be dictated by the biological efficacy and toxicity of the preparation. From a pharmaceutical point of view, high drug/lipid ratios are obviously more economical.

In summary, optimum liposomal formulations will exhibit drug-trapping efficiencies in excess of 90%, employ relatively saturated lipids such as HSPC and cholesterol and exhibit the highest possible drug/lipid ratio, which is consistent with maintained efficacy of the preparation. Apart from these factors; other factors which need to be considered in selection of the methods of preparation include selection of methods which would avoid the use of organic solvents and detergents (which are difficult to remove), yield well-defined and reproducible liposomes and which are rapid and amenable to scale up procedures. Any special applications of the liposomes to be prepared also may contribute in the selection of the appropriate method.

4.2 EXPERIMENTAL

4.2.1 DRUGS

Cyclosporine (CsA) was gifted by RPG life sciences, Ankleshwar, Vadodara and Leuprolide acetate was gifted from Takeda chemical industries, Osaka, Japan and Calf thymus DNA was a gift from Biotechnology division, M.S.University of Baroda.

4.2.2 REAGENTS

Hydrogenated soya phosphatidyl choline (HSPC), phosphatidylethanolamine (PE), methoxy polyethylene glycol (av. mol. wt. 2000) and methoxy polyethylene glycol (av. mol. wt. 5000) were purchased from Sigma Chemical Co., St.Louis, M.O.; Cholesterol, chloroform (AR grade) and methanol (AR grade) were purchased from S.D.fine chemicals, Boisar, Thane. Cyanuric chloride was purchased from National chemicals, Baroda. Petroleum ether (40°C-60°C, AR grade), diethyl ether (AR grade), acetone, triethylamine, sodium carbonate, silica gel G for TLC, iodine were purchased from Qualigens fine chemicals, Mumbai. DL-α-tocopherol was purchased from E.merck India ltd., Mumbai.

4.2.3 EQUIPMENT

Rotary evaporator with vacuum pump, glass boiling tube (Quick fit neck B-24) and thermostatically controlled water bath and nitrogen purging facility (Superfit Equipments, India), probe sonicator RR-120 (Ralsonics, Mumbai), Remi cooling centrifuge C-24, Remi heating mantle and Remi magnetic stirrer 1MLH (Remi Equipments, Mumbai), Shimadzu UV-1601 UV-Visible spectrophotometer (Shimadzu corporation, Kyoko, Japan), Avance DPX 200 dual probe ^{13}C-NMR (Bruker Inc., Switzerland), Shimadzu Infrared spectrophotometer (Shimadzu corporation, Japan).

4.2.4 SOLUTIONS

Phosphate buffer pH 4.5, Phosphate buffered saline pH 6.4 and 7.4 was prepared as per the method given in Indian pharmacopoeia (1985).

Phosphate buffered saline pH 7.8 (Adler et al., 1992)

0.145M of sodium chloride, 0.003M of Sodium dihydrogen orthophosphate and 0.0035M of Disodium hydrogen phosphate.

16.7% w/v solution of sucrose was prepared by dissolving the appropriate quantity of sucrose in the appropriate quantity of distilled water. Sodium sulphate solutions (0 M to 2 M) were prepared by dissolving the appropriate quantity of anhydrous sodium sulphate in 50ml of 16.7% w/v sucrose solutions.

4.2.5 PREPARATION OF NEUTRAL AND CHARGED LIPOSOMES CONTAINING CYCLOSPORINE

Multi lamellar vesicles (MLVs) of cyclosporine were prepared by the lipid film hydration technique (New, 1990). Neutral (CL), positive (CPL) and negative (CNL) liposomes were prepared by using Hydrogenated Soya Phosphatidyl Choline (HSPC), Cholesterol (Ch), Stearylamine (SA) (positive charge), Distearoyl Phosphatidyl Glycerol (DSPG) (negative charge), by thin film hydration method. The process variables like rotation speed, temperature, vacuum applied and hydration time were optimised by keeping the drug:lipid ratio (1:0.07), Lipid : Chol : charge ratio (1:0.17:0.3) as constant initially and the optimised condition was used through out the study (table 4.1).

Positive charged liposomes containing cyclosporine was optimized for formulation parameters by factorial design. 3^3 factorial design was used to investigate the combined influence of three independent variables in the preparation of cyclosporine liposomes by thin film hydration method as shown in table 4.2. Based on the factorial design, twenty-seven batches of cyclosporine liposomes were prepared by thin film hydration method (New R.R.C, 1990) according to the experimental conditions as shown in the figure 4.1. Drug: Lipid (molar ratio) (X_1), HSPC/Cholesterol (X_2) and pH of the hydration medium (X_3), were selected as the causal factors, whereas percent drug entrapment (PDE) within the liposomes was selected as dependent variable as shown in table 4.3 and 4.4. Potential variables such as lipid: positive charge (1:0.3) and hydration volume (4ml) were kept constant in experimental design. With the optimized process and formulation variables the neutral and negative liposomes were prepared by thin film hydration method. The major process parameters and the formulation parameters were optimised using the percentage drug entrapment as the response parameter. The process and the formulation parameters were optimized to achieve maximum entrapment efficiency. Multilamellar vesicles (MLVs) were prepared by dissolving different molar ratios of lipids, DL-α-tocopherol (0.5 ml of 0.1 % w/v solution in chloroform) and CsA in Chloroform: Methanol solvent mixture (1:1 ratio). The process

and the formulation parameters were optimized to achieve maximum entrapment efficiency. The organic solvent was evaporated at a temperature of 60°C on a rotary flash evaporator to yield a thin, dry lipid film. The lipid film was then hydrated with phosphate buffer at 60°C for 1h to form a stable liposomal CsA formulation. The liposomal suspension so formed was then transferred to a suitable glass container and sonicated for 15 min using a probe sonicator (model – RR-120, Ralsonics, Mumbai) at 60°C. The sonicated dispersion was then allowed to stand undisturbed for about 2 h at room temperature for complete hydration. The multilamellar vesicles formed were sequentially extruded two times through 1μm Nuclepore polycarbonate track-etch membrane filters (Whatman Inc. New Jersey, USA) at 60°C. The process was repeated through two stacked 0.4μm and 0.2μm membranes. The unentrapped drug was removed from the liposomal suspension by centrifugation at 15,000 rpm for 30 minutes at 0°C temperature. A pellet of liposomes and a supernatant containing free drug in a suspended state was obtained. The supernatant was collected and the pellet was resuspended in PBS. The process was repeated thrice to remove the unentrapped drug. The supernatant fractions thus collected were pooled and analysed for drug content. The liposomal suspension was freeze dried overnight at -20°C, lyophilised for 24 hrs using sucrose as cryoprotectant (3 times weight of total lipids used) and stored in vials at 2-8°C. A flowchart depicting the above process is shown in figure 4.1. The coded values and observations of the optimisation process by 3^3 factorial design are tabulated in table 4.2 and 4.3.

With the optimised formulation parameters spray drying process was also tried by JISL Mini Spray Dryer, Mumbai, India. Positive, negative and neutral liposomes were prepared by dissolving different molar ratios of lipids as shown in table 4.9, DL-α-tocopherol (0.5 ml of 0.1 % w/v solution in chloroform) and CsA in Chloroform: Methanol solvent mixture (1:1 ratio). The lipid solution was spray-dried by maintaining the following conditions

Inlet Temperature : 60°C
Outlet Temperature : 40°C
Feed-rate : 10%
Aspirator : 60%

Air-pressure : 2 Kg/cm^2

The spray dried lipid powder was hydrated with Phosphate buffer saline pH7.8 and the process of liposome preparation was carried out as per the flow chart (figure 4.1). No significant difference was observed in the particle size and entrapment efficiency in comparison to the liposomes prepared by thin film hydration method.

Figure 4.1 Flowchart for the preparation of charged liposomes containing cyclosporine

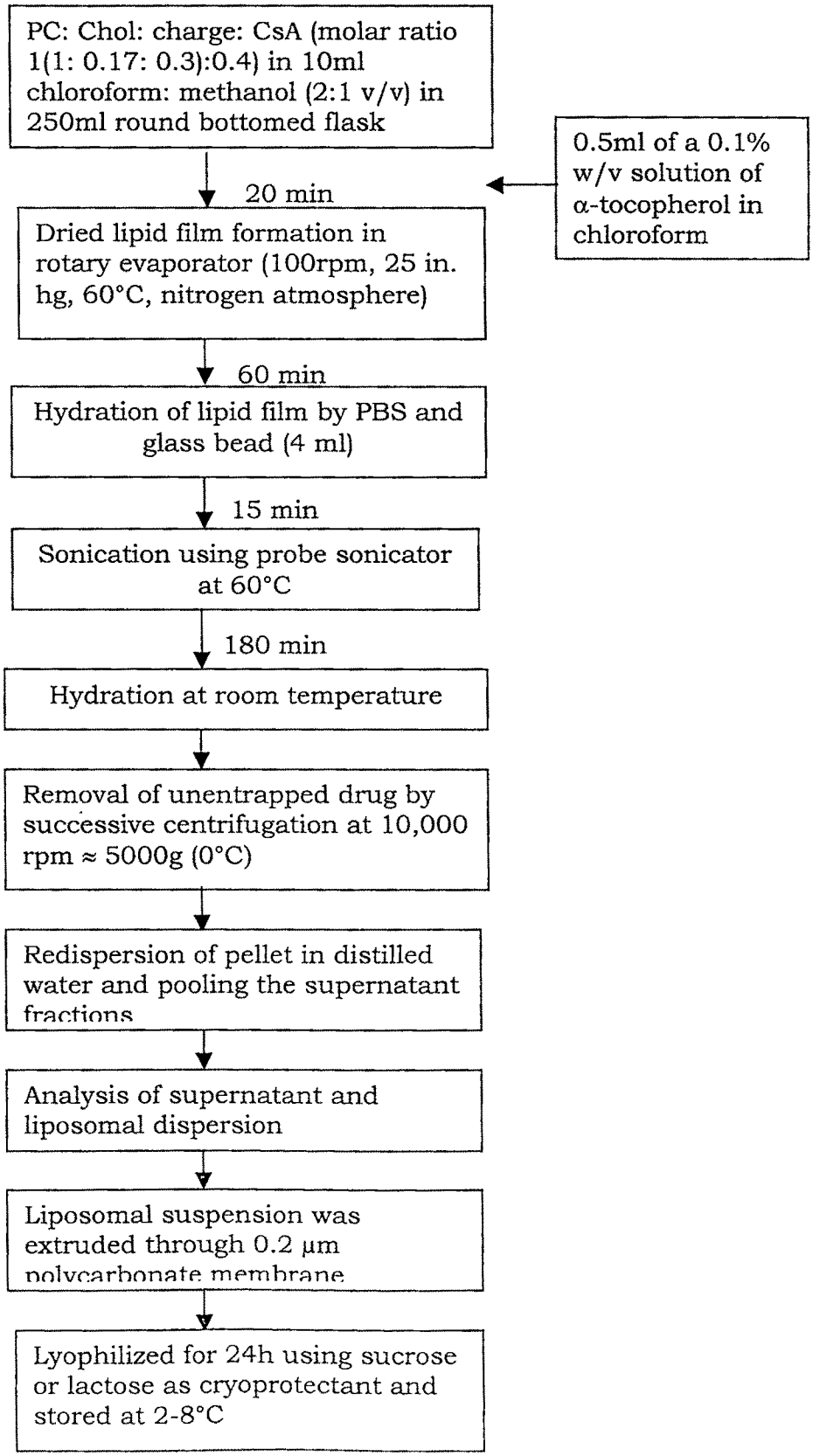


Table 4.1 Effect of process variables for the preparation of charged liposomes containing cyclosporine

Variable	Batch Number	% Entrapment efficiency	Observation and Inferences
Vacuum (Inch.Hg) 10 15 20	CsA (a) CsA (b) CsA (c)	40.23 47.89 20.63	Presence of residual solvent and aggregation of liposomes Uniform thin translucent film with better entrapment Poor drug entrapment, entrapment of air bubbles on lipid film surface
Pre sonication hydration (min) at 60°C 30 60 90	CsA (d) CsA (e) CsA (f)	42.36 47.68 41.65	Hydration is a prerequisite for proper maturation of liposomes yet excessive hydration leads to drug leakage
Post sonication hydration (h) 2	CsA (g)	47.68	Optimum post sonication hydration time is required for regaining physical and conformational stability of the bilayer membrane

Table 4.2 Optimisation of parameters for preparation of liposomes containing cyclosporine

Batch No	Lipid:Drug (molar ratio)	HSPC:Chol	pH of the hydration medium	% Entrapment efficiency ± S.E.M *
CsA1	1:0.07	1:0.0	4.5	30.33±0.96
CsA2	1:0.05	1:0.0	4.5	44.42±1.02
CsA3	1:0.04	1:0.0	4.5	64.80±0.99
CsA4	1:0.07	1:0.05	4.5	42.17±0.87
CsA5	1:0.05	1:0.05	4.5	68.93±1.23
CsA6	1:0.04	1:0.05	4.5	75.43±1.06
CsA7	1:0.07	1:0.17	4.5	43.90±0.48
CsA8	1:0.05	1:0.17	4.5	80.32±0.79
CsA9	1:0.04	1:0.17	4.5	87.63±0.68
CsA10	1:0.07	1:0.0	6.4	39.67±1.23
CsA11	1:0.05	1:0.0	6.4	58.32±1.02
CsA12	1:0.04	1:0.0	6.4	57.86±1.05
CsA13	1:0.07	1:0.05	6.4	69.6±0.99
CsA14	1:0.05	1:0.05	6.4	78.8±0.85
CsA15	1:0.04	1:0.05	6.4	84.23±0.83
CsA16	1:0.07	1:0.17	6.4	75.57±0.93
CsA17	1:0.05	1:0.17	6.4	88.23±0.66
CsA18	1:0.04	1:0.17	6.4	90.25±0.79
CsA19	1:0.07	1:0.0	7.8	35.53±0.69
CsA20	1:0.05	1:0.0	7.8	57.89±1.32
CsA21	1:0.04	1:0.0	7.8	60.46±1.04
CsA22	1:0.07	1:0.05	7.8	68.98±0.95
CsA23	1:0.05	1:0.05	7.8	77.86±1.08
CsA24	1:0.04	1:0.05	7.8	82.32±0.99
CsA25	1:0.07	1:0.17	7.8	75.48±0.82
CsA26	1:0.05	1:0.17	7.8	87.56±0.73
CsA27	1:0.04	1:0.17	7.8	96.61±2.45

*Mean of three batches

Table 4.3 Coded values of the formulation parameters of cyclosporine loaded liposomes by 3^3 factorial design

Coded values	Actual values		
	X_1	X_2	X_3
-1	15	0.0	4.5
0	20	0.05	6.4
1	30	0.17	7.8

X_1 -Drug : Lipid (molar ratio)

X_2 -HSPC: Cholesterol (molar ratio)

X_3 -pH of hydration medium

Table 4.4 3^3 Factorial design layout of cyclosporine loaded liposomes

Batch No	X_1	X_2	X_3	X_1^2	X_2^2	X_3^2	X_1X_2	X_2X_3	X_1X_3	$X_1X_2X_3$	PDE± S.E.M
1	-1	-1	-1	1	1	1	1	1	1	-1	30.33±0.96
2	0	-1	-1	0	1	1	0	1	0	0	44.42±1.02
3	1	-1	-1	1	1	1	-1	1	-1	1	64.80±0.99
4	-1	0	-1	1	0	1	0	0	1	0	42.17±0.87
5	0	0	-1	0	0	1	0	0	0	0	68.93±1.23
6	1	0	-1	1	0	1	0	0	-1	0	75.43±1.06
7	-1	1	-1	1	1	1	-1	-1	1	1	43.90±0.48
8	0	1	-1	0	1	1	0	-1	0	0	80.32±0.79
9	1	1	-1	1	1	1	1	-1	-1	-1	87.63±0.68
10	-1	-1	0	1	1	0	1	0	0	0	39.67±1.23
11	0	-1	0	0	1	0	0	0	0	0	58.32±1.02
12	1	-1	0	1	1	0	-1	0	0	0	57.86±1.05
13	-1	0	0	1	0	0	0	0	0	0	69.6±0.99
14	0	0	0	0	0	0	0	0	0	0	78.8±0.85
15	1	0	0	1	0	0	0	0	0	0	84.23±0.83
16	-1	1	0	1	1	0	-1	0	0	0	75.57±0.93
17	0	1	0	0	1	0	0	0	0	0	88.23±0.66
18	1	1	0	1	1	0	1	0	0	0	90.25±0.79
19	-1	-1	1	1	1	1	1	-1	-1	1	35.53±0.69
20	0	-1	1	0	1	1	0	-1	0	0	57.89±1.32
21	1	-1	1	1	1	1	-1	-1	1	-1	60.46±1.04
22	-1	0	1	1	0	1	0	0	-1	0	68.98±0.95
23	0	0	1	0	0	1	0	0	0	0	77.86±1.08
24	1	0	1	1	0	1	0	0	1	0	82.32±0.99
25	-1	1	1	1	1	1	-1	1	-1	-1	75.48±0.82
26	0	1	1	0	1	1	0	1	0	0	87.56±0.73
27	1	1	1	1	1	1	1	1	1	1	96.61±2.45

n=3

Table 4.5 Model coefficients estimated by multiple linear regression for cyclosporine loaded liposomes by 3^3 factorial design

Factor	Coefficients	Computed t- value	P-value
Intercept	79.74963	30.52809	1.3E-15
X_1	12.13111	10.03171	2.63E-08
X_2	15.34833	12.69216	9.09E-10
X_3	5.82	4.812794	0.000191
X_1^2	-5.76889	-2.75427	0.01411
X_2^2	-6.76722	-3.2309	0.005227
X_3^2	-5.80222	-2.77018	0.013655
X_1X_3	0.1625	0.109719	0.913997
X_2X_3	2.789167	1.883229	0.077979
X_1X_2	-4.33833	-2.92922	0.009826
X_1X_2 X_3	-1.6325	-0.89999	0.381473

* Significant at $p < 0.05$

Table 4.6 Analysis of variance (ANOVA) of full and reduced models of cyclosporine loaded liposomes by 3^3 factorial design

		DF	SS	MS	F	R	R^2	Adj R^2
Regression	FM	10	8516.228	851.62	32.35	0.9762	0.9529	0.9234
	RM	7	8404.237	1200.18	42.53	0.9695	0.9400	0.9179
Error	FM	16	421.16(E1)	26.32	32.35			
	RM	19	536.15(E2)	28.22	42.53			

SSE2 -SSE1 = 536.15 -421.16 = 114.99

No: of parameters omitted = 3

MS of Error (full model) = 26.32

F calculated = (114.99/3)/26.32 = 1.456

Table 4.7 Effect of positive charge on encapsulation efficiency of cyclosporine loaded liposomes

Batch	HSPC:Chol:Charge	% Entrapment efficiency
CsA 1	1:0.17:0.0	93.52 ± 3.22
CsA 2	1:0.17:0.2	93.68±3.02
CsA 3	1:0.17:0.3	96.61 ± 2.45
CsA 4	1:0.17:0.4	96.02±1.68
CsA 5	1:0.17:0.5	96.12±1.23

Table 4.8 Effect of negative charge on encapsulation efficiency of cyclosporine loaded liposomes

Batch	HSPC:Chol:Charge	% Entrapment efficiency
CsA 1	1:0.17:0.0	92.13 ±1.67
CsA 2	1:0.17:0.2	91.09±2.02
CsA 3	1:0.17:0.3	93.52 ± 3.22
CsA 4	1:0.17:0.4	93.03 ±1.37
CsA 5	1:0.17:0.5	93.08 ±1.02

Table 4.9 Entrapment efficiency of optimized batches of freshly prepared liposomal suspensions containing cyclosporine. Each value is the mean of triplicate results

Type of Liposome	Lipid Composition HSPC:Cholesterol:Charge	% Drug Entrapment ± S.E.M.
		Before lyophilisation
Positive liposomes (CPL)	1: 0.17: 0.3 (HSPC:Ch:SA)	96.61 ± 2.45
Negative liposomes (CNL)	1: 0.17: 0.3 (HSPC:Ch:DSPG)	93.52 ± 3.22
Neutral liposomes (CL)	1: 0.17 (HSPC:Ch)	92.13± 1.67

4.2.6 LEUPROLIDE ACETATE AND DNA LIPOSOMES

The use of liposomes as drug carriers requires the liposomal preparations with various clearance rates and biodistribution patterns to better fit the specifics of each particular application. Liposome charge and liposome coating with different polymers, such as PEG, are among the parameters known to strongly affect biological properties of liposomes. It was repeatedly demonstrated that the incorporation of charged phospholipids into liposomes accelerates their clearance, while grafting liposomes with PEG and similar polymers makes liposomes long circulating. Thus the incorporation of Phosphatidyl serine (PS) or dicetyl phosphate (DCP) into PC/Chol liposomes dramatically enhances liposome uptake by the perfused mouse liver. The fact that the negative charge strongly increases the clearance of liposomes. Negatively charged PS was found to abolish the longevity of liposomes prepared of a lipid composition resembling that of erythrocyte membrane. The major mechanism behind the charge facilitated liposome clearance is an interaction of charged phospholipids head groups with certain opsonizing proteins. Liposomes of different charge and composition exhibit different binding centers for plasma proteins. Liposome grafted PEG prevents liposome clearance by neutralizing the surface charge of liposomes and shielding various opsonins. An interesting question arises -does PEG provide the same effect for liposomes of all compositions or does the protective effect depend not only on the thickness of the layer of protecting polymer on the liposome surface. Hence our aim was to compare the liposome biodistribution with negative or positive surface charge additionally coated with a PEG moiety with different molecular weight, in order to investigate the relative role of the liposome charge and the length of the PEG chains attached to liposome on the liposome circulation time and liver accumulation.

4.2.6.1 PREPARATION OF CONVENTIONAL LIPOSOMES CONTAINING LEUPROLIDE ACETATE

Conventional (LL) leuprolide acetate liposomes were prepared by the reverse phase evaporation technique as shown in figure 4.2. 3^3 factorial design was used to investigate the combined influence of three independent variables in

the preparation of conventional (LL) leuprolide acetate liposomes by reverse phase evaporation method. The process variables like rotation speed, temperature, vacuum applied and hydration time were optimised initially and the optimised condition was used through out the study. Based on the factorial design, twenty-seven batches of leuprolide acetate liposomes were prepared by reverse phase evaporation method (New R.R.C, 1990) according to the experimental conditions as shown in the figure 4.2. Conventional liposomes containing leuprolide acetate was optimized by factorial design. The process and the formulation parameters were optimized to achieve maximum entrapment efficiency. The lipid mixtures in chloroform solution were taken in glass boiling tube (Quick fit neck B-24). Aqueous solution of leuprolide acetate was injected rapidly into the lipid mixture through 23 gauge hypodermic needle. The tube was closed with a glass stopper and sonicated for 10 minutes in a bath sonicator (Model V33, Frequency 22 KHz, 120W, Vibronics Pvt. Ltd., Mumbai, India.) It was then attached directly into the rotary evaporator at 60°C; the organic solvent was removed under vacuum in two stages: evaporated at 400mmHg until a gel was formed. Vacuum was released and the tube was subjected to vigorous mechanical agitation by vortex mixer for 5 minutes, followed by evaporation of the traces of the organic solvent at 600mmHg until a homogeneous suspension was obtained. The preparation was diluted with the continuous phase (Phosphate buffer saline pH 7.4) and the vesicles formed were sequentially extruded two stacked 0.2μm Nuclepore polycarbonate track-etch membrane filters (Whatman Inc. New Jersey, USA) at 60°C. The vesicles were centrifuged at 15,000 rpm for 30 min, the supernatant was removed and the pellet was resuspended in PBS.

The major process parameters were optimised using the percentage drug entrapment as the response parameter. The coded values and observations of the optimization process by 3^3 factorial design are tabulated in table 4.10 and 4.11 and subjected to Artificial neural network (ANN) and Multiple linear regression (MLR). Twenty seven formulations were prepared based on 3^3 factorial design. The volume of aqueous phase (X_1), HSPC/DSPG [negative charge] (X_2), and HSPC/Cholesterol (X_3) were selected as the causal factors. Potential variables such as concentration of lipid: drug and

hydration medium were kept constant in experimental design. The PDE (dependent variable) and the transformed values of independent variables were subjected to multiple regression analysis to establish a second order polynomial equation (full model). To simplify the polynomial equation, F-statistic was applied to reduce polynomial equation (reduced model) by neglecting non-significant ($P<0.05$) terms as shown in table 4.12 and 4.13. A set of PDE and causal factors was used as tutorial data for ANN and fed into a computer. The feed forward back propagation (bp) method was optimized. Figure 4.4 shows a representative plot of r^2 values for an ANN model prediction performance as a function of number of nodes in the hidden layers. In this case, an ANN with 9 nodes in the hidden layer resulted in slope and r^2 values that are closest to 1.0. The reduced polynomial equation was used to plot three two-dimensional contour plots as shown in figure 4.5 at fixed levels of -1, 0 and 1 of the variable X_3 to obtain various combination values of the two other independent variables (X_1 and X_2) at predetermined PDE. The root mean square value of the trained ANN model by feed forward bp method was 0.0000354, which indicated that the optimal model was reached. The ANN model and MLR were validated for accurate prediction of PDE. The optimization methods developed by both ANN and MLR were validated by preparing another six liposomal formulations. The predetermined PDE (from ANN and MLR) and the experimental data were compared with predicted data by paired "t" test, no statistically significant difference was observed as shown in table 4.14. ANN showed less error compared to MLR.

Figure 4.2 Flowchart for the preparation of conventional liposomes containing Leuprolide acetate.

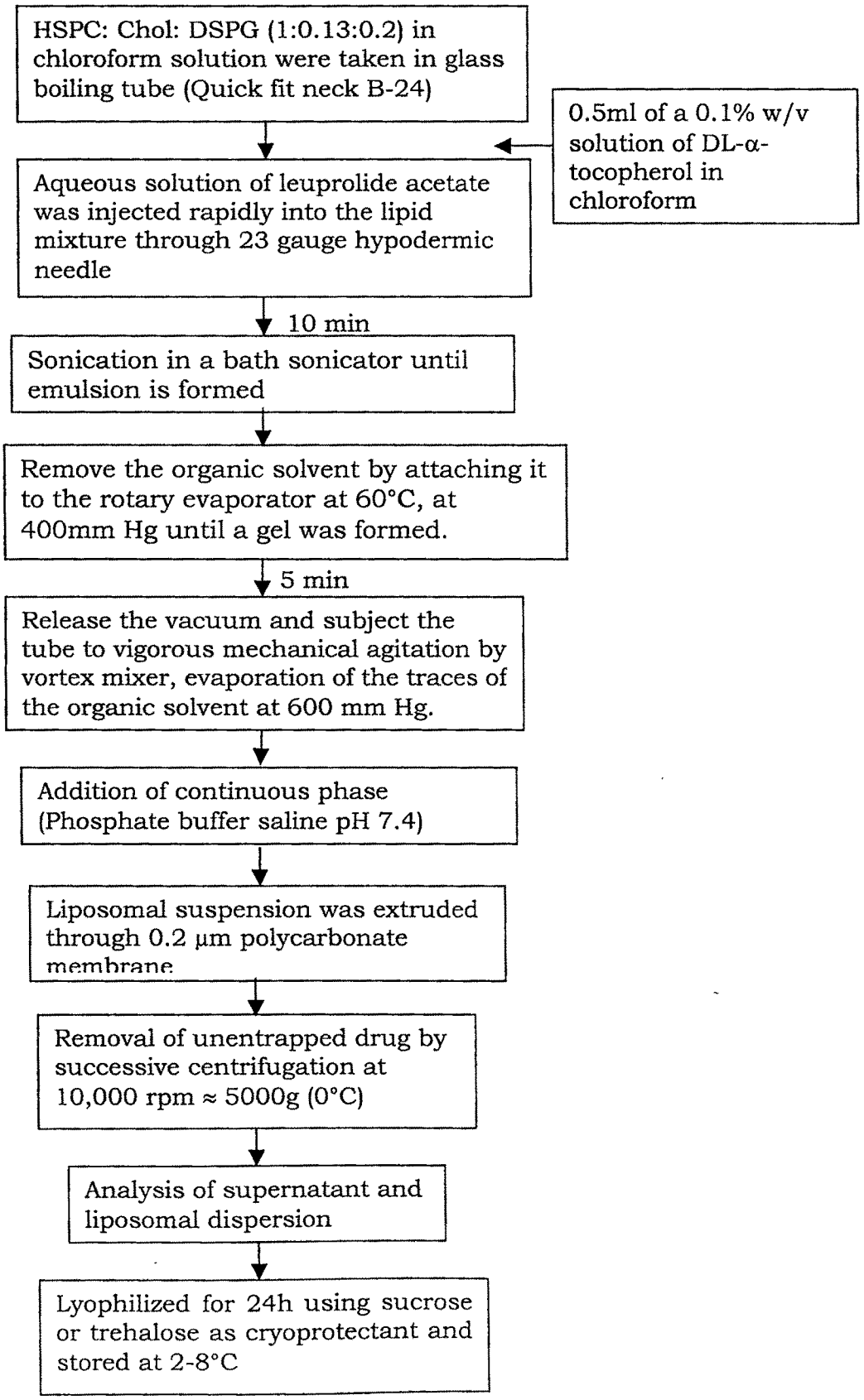


TABLE 4.10 Coded values of the formulation parameters of conventional (LL) leuprolide acetate liposomes by 3^3 factorial design

Coded values	Actual values		
	X_1	X_2	X_3
-1	0.25	0.05	0.13
0	0.5	0.2	0.2
1	1.0	0.5	0.5

X_1 -Volume of aqueous phase (ml)

X_2 -HSPC: DSPG [Negative charge](molar ratio)

X_3 -HSPC: Cholesterol (molar ratio)

TABLE 4.11 3^3 Full factorial design of conventional (LL) leuprolide acetate liposomes

Batch No.	X_1	X_2	X_3	X_1^2	X_2^2	X_3^2	X_1X_3	X_2X_3	X_1X_2	$X_1X_2X_3$	Y (PDE)* (± S.E.M)
1	-1	-1	-1	1	1	1	1	1	1	-1	48.94 (0.236)
2	0	-1	-1	0	1	1	0	1	0	0	48.25(0.352)
3	1	-1	-1	1	1	1	-1	1	-1	1	46.80(0.186)
4	-1	0	-1	1	0	1	1	0	0	0	60.80(0.050)
5	0	0	-1	0	0	1	0	0	0	0	56.28(0.036)
6	1	0	-1	1	0	1	-1	0	0	0	50.43(0.086)
7	-1	1	-1	1	1	1	1	-1	-1	1	30.32(0.074)
8	0	1	-1	0	1	1	0	-1	0	0	24.67(0.044)
9	1	1	-1	1	1	1	-1	-1	1	-1	20.86(0.102)
10	-1	-1	0	1	1	0	0	0	1	0	42.25(0.136)
11	0	-1	0	0	1	0	0	0	0	0	42.56(0.032)
12	1	-1	0	1	1	0	0	0	-1	0	40.23(0.122)
13	-1	0	0	1	0	0	0	0	0	0	55.82(0.075)
14	0	0	0	0	0	0	0	0	0	0	50.32(0.089)
15	1	0	0	1	0	0	0	0	0	0	43.86(0.032)
16	-1	1	0	1	1	0	0	0	-1	0	26.83(0.066)
17	0	1	0	0	1	0	0	0	0	0	20.32(0.056)
18	1	1	0	1	1	0	0	0	1	0	19.23(0.108
19	-1	-1	1	1	1	1	-1	-1	1	1	34.98(0.205)
20	0	-1	1	0	1	1	0	-1	0	0	38.86(0.049)
21	1	-1	1	1	1	1	1	-1	-1	-1	36.23(0.036)
22	-1	0	1	1	0	1	-1	0	0	0	50.23(0.102)
23	0	0	1	0	0	1	0	0	0	0	47.89(0.112)
24	1	0	1	1	0	1	1	0	0	0	43.56(0.031)
25	-1	1	1	1	1	1	-1	1	-1	-1	22.42(0.098)
26	0	1	1	0	1	1	0	1	0	0	17.68(0.133)
27	1	1	1	1	1	1	1	1	1	1	16.20(0.056)

*Mean of three batches

Table 4.12 Model coefficients estimated by multiple linear regression of leuprolide acetate loaded liposomes by 3^3 factorial design

Factor	Coefficients	Computed t- value	P-value
Intercept	50.69185	55.17267	1.10E-19*
X_1	-3.06611	-7.20904	2.08E-06*
X_2	-10.0317	-23.5864	7.42E-14*
X_3	-4.40556	-10.3583	1.68E-08*
X_1^2	-0.20389	-0.27677	0.785499
X_2^2	-18.9306	-25.6976	1.95E-14*
X_3^2	0.697778	0.947209	0.35762
X_1X_3	0.860833	1.652581	0.117901
X_2X_3	1.1975	2.298895	0.035322*
X_1X_2	-1.6975	-3.25877	0.004929*
X_1X_2 X_3	-0.01875	-0.02939	0.976917

* Significant at $p < 0.05$

Table 4.13 Analysis of variance (ANOVA) of full and reduced models of leuprolide acetate loaded liposomes by 3^3 factorial design

		DF	SS	MS	F	R	R^2	Adj R^2
Regression	FM	10	4544.045	454.4045	139.56	0.994	0.989	0.982
	RM	6	4531.979	755.3298	235.44	0.993	0.986	0.982
Error	FM	16	52.097(E1)	3.256	139.56			
	RM	20	64.163(E2)	3.208	235.44			

SSE2 -SSE1 = 64.163-52.097 = 12.066

No: of parameters omitted = 4

MS of Error (full model) = 3.256

F calculated = (12.066/4)/3.256 = 0.926

Figure 4.3 The feed forward back-propagation network.

X_1, volume of aqueous phase, X2, HSPC: DSPG, X_3, HSPC: Chol, Y, Percent drug entrapment (PDE); H_1-H_9, nodes of the hidden layer; W_{11}, connection from first input node to the first hidden node; W_{11}, connection from the first hidden node to the output node; W_{93}, connection from the third input node to the ninth hidden node; W_{19}, connection from the ninth hidden node to the output node.

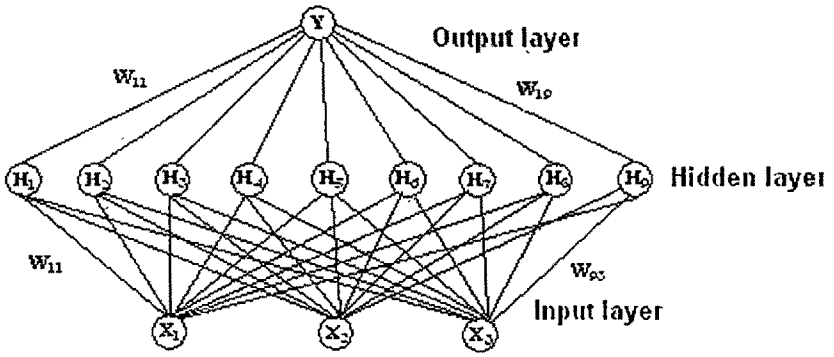


Figure 4.4 Squared correlation coefficients (r^2) for 27 formulations as a function of the number of hidden nodes using ANN

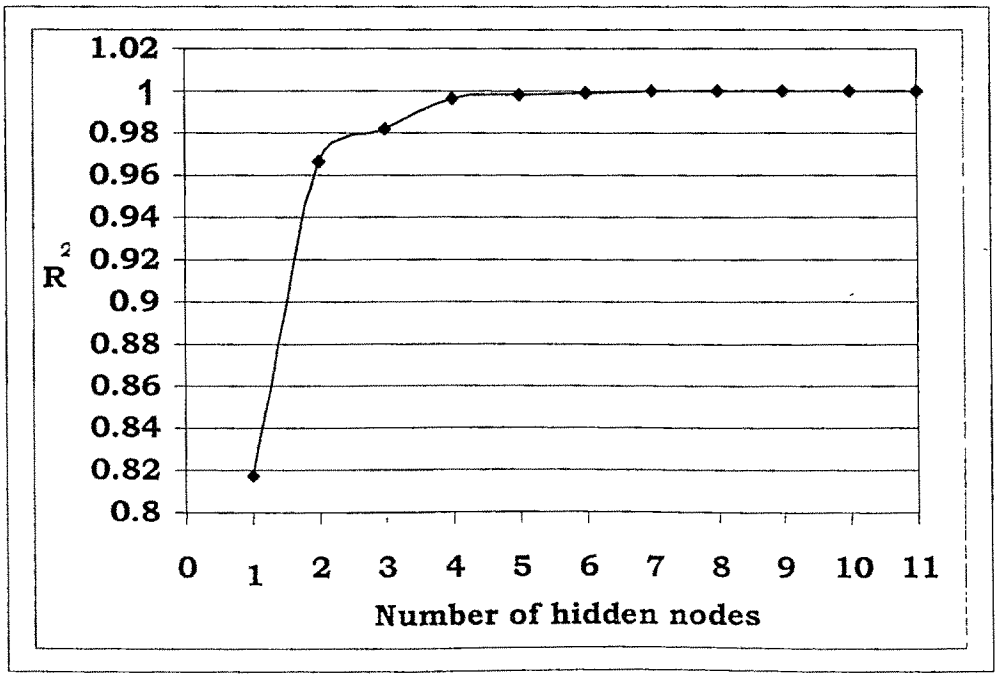


Figure 4.5 Contour plots of leuprolide acetate loaded liposomes by 3^3 factorial design (A) at -1 level of variable X_3, (B) at 0 level of variable X_3, (C) at 1 level of variable X_3

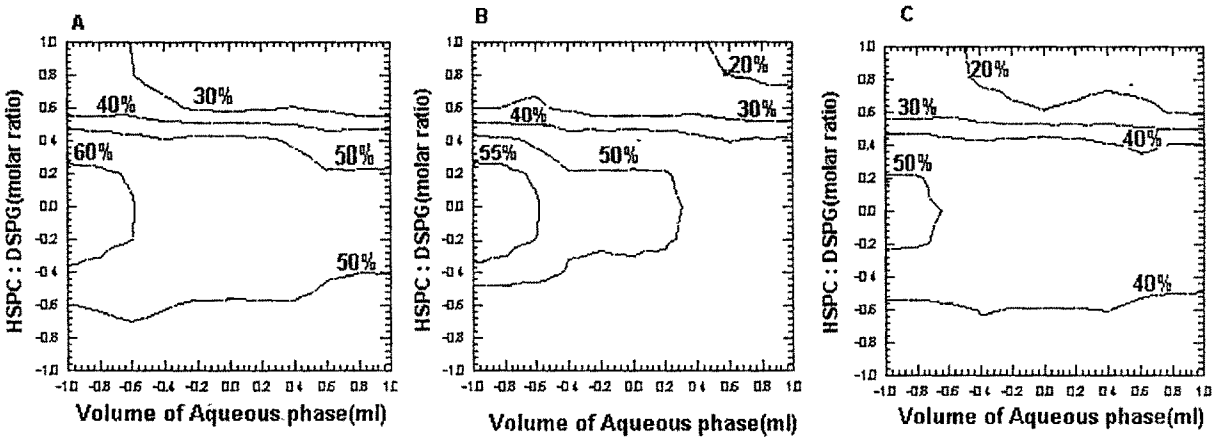


Table 4.14 Test data set for validating ANN and MLR model of leuprolide acetate loaded liposomes by 3^3 factorial design

Formulation	Volume of aqueous phase (ml)	HSPC:DSPG (molar ratio)	HSPC:Chol (molar ratio)	Experimental PDE	Predicted PDE (ANN Bp)	Predicted PDE (MLR from contour)
1	1	1:0.26	1:0.13	48.23	48.739	50.42
2	0.25	1:0.14	1:0.2	53.96	54.606	54.7
3	0.45	1:0.14	1:0.5	47.36	47.94	46.85
4	0.8	1:0.05	1:0.2	40.01	39.898	40.44
5	0.3	1:0.17	1:0.5	48.73	48.889	50.19
6	0.35	1:0.08	1:0.13	50.23	49.637	49.07
t'calculated				0.3611	0.3451	
t'tabulated				2.5706		
Normalised error				0.0211	0.0658	

Table 4.15 Entrapment efficiency of optimized batches freshly prepared liposomal suspension containing leuprolide acetate. Each value is the mean of triplicate results

Type of Liposome	Lipid Composition HSPC:Cholesterol:DSPG :mPEG-PE	% Drug Entrapment ± S.E.M.
Conventional liposomes (LL)	1: 0.13: 0.2:0	47.12±2.64
Sterically stabilized liposomes coated with mPEG5000-PE (SLL5000)	1: 1:0.15:0.15	37.8±3.46
Sterically stabilized liposomes coated with mPEG2000-PE (SLL2000)	1: 1:0.15:0.15	33.03±4.02

4.2.6.3 Preparation of conventional liposomes containing DNA

Freeze dried empty liposomes (FDELs) technique as shown in figure 4.6 (Yachi et al., 1996; Ishiwata et al., 2000) was used for the incorporation of DNA into liposomes. In brief, 1.5ml of small unilamellar vesicles prepared by the above method composed of 94.5mgm of HSPC, 60mgm of anionic DSPG (anionic lipid) and 40.5mgm of Chol (molar ratio 1:0.63:0.88) containing DNA (0.77%w/w of lipid). The major process parameters were optimised using the percentage drug entrapment as the response parameter. With the optimized process parameters vesicles composed of 94.5mgm of HSPC, 50mgm of cationic DOTAP and 40.5mgm of Chol (molar ratio 1:0.9:0.13) containing DNA (0.77%w/w of lipid) were also prepared. A flowchart depicting the above process is shown in figure 4.6. The observations of the optimization process are tabulated in table 4.16. Comparison of these liposomes was made with sterically stabilized liposomes containing DNA using the electrolyte induced flocculation test described later in this chapter. The results of this test for conventional liposomes containing DNA are shown in figure 4.8 and table 4.18.

Figure 4.6 Flowchart for the preparation of conventional liposomes containing DNA

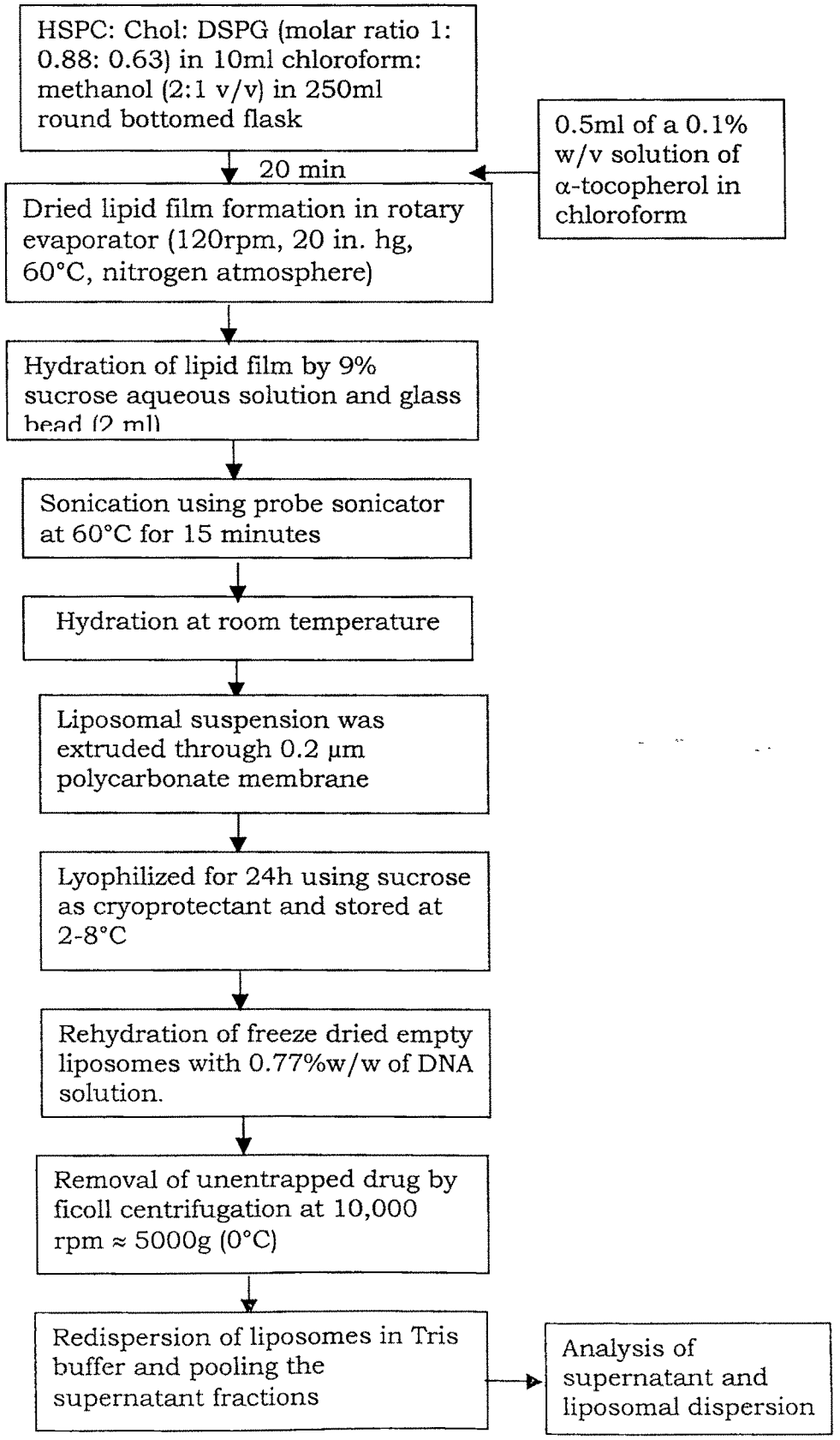


Table 4.16 Optimisation of parameters for the preparation of conventional liposomes containing DNA

Batch No.	Molar ratio (HSPC:DSPG:Chol)	DNA % w/w of lipid	Hydration medium volume (ml)	Hydration time (h)	% Entrapment efficiency (± S.E.M)*
DL-1	1:0.2:0.3	0.55	1.5	15	22.32±0.62
DL-2	1:0.2:0.3	0.77	1.5	15	26.35±0.89
DL-3	1:0.3:0.3	0.55	1.5	15	24.62±0.79
DL-4	1:0.3:0.3	0.77	1.5	15	27.96±1.02
DL -5	1:0.3:0.88	0.55	1.5	15	33.65±1.02
DL -6	1:0.3:0.88	0.77	1.5	15	35.26±1.23
DL -7	1:0.63:0	0.55	1.5	15	36.56±0.99
DL -8	1:0.63:0	0.77	1.5	15	38.36±0.82
DL -9	1:0.63:0.3	0.55	1.5	15	40.63±1.18
DL -10	1:0.63:0.3	0.77	1.5	15	42.36±1.22
DL -11	1:0.63:0.88	0.55	1.5	15	47.86±1.05
DL -12	1:0.63:0.88	0.77	1.5	15	49.56±0.96
DL -13	1:0.63:0.88	0.77	1.5	30	49.02±0.68
DL -14	1:0.63:0.88	0.77	2	15	47.23±0.79
DL -15	1:0.63:0.88	0.77	2	30	47.03±0.95

*Mean of three batches

4.2.7 ELECTROLYTE INDUCED FLOCCULATION TEST

Sodium sulphate solutions ranging from 0 M to 2.0 M were prepared in 16.7 % sucrose solution. An appropriate volume of liposome formulation, which gives a final concentration of 1 mg/ml of lipid, was taken and the volume was made up to 5 ml using the sodium sulphate solutions of various concentrations. The resulting dispersions were mixed and the absorbances were measured within 5 min at 400 nm on Shimadzu 1601 UV- Visible Spectrophotometer against respective blank. The results of this test for conventional liposomes containing leuprolide acetate and DNA are shown in figures 4.7 & 4.8 and tables 4.17 & 4.18.

Table 4.17 Results of electrolyte induced flocculation test on conventional liposomes containing Leuprolide acetate

Mean concentration of sodium sulphate (in M)	Mean absorbance ± S.E.M at 400nm
0.0	0.521±0.01
0.4	0.538±0.014
0.8	0.552±0.016
1.2	0.559±0.020
1.6	0.580±0.008
2.0	0.612±0.018

Figure 4.7 Results of electrolyte induced flocculation test on conventional liposomes containing Leuprolide acetate

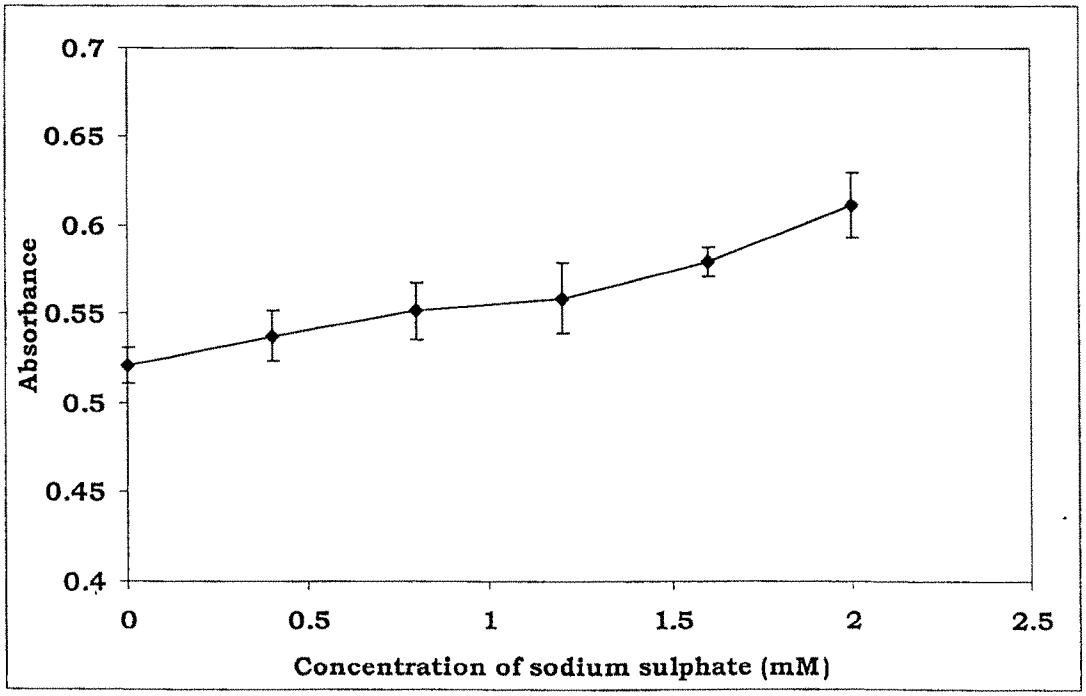
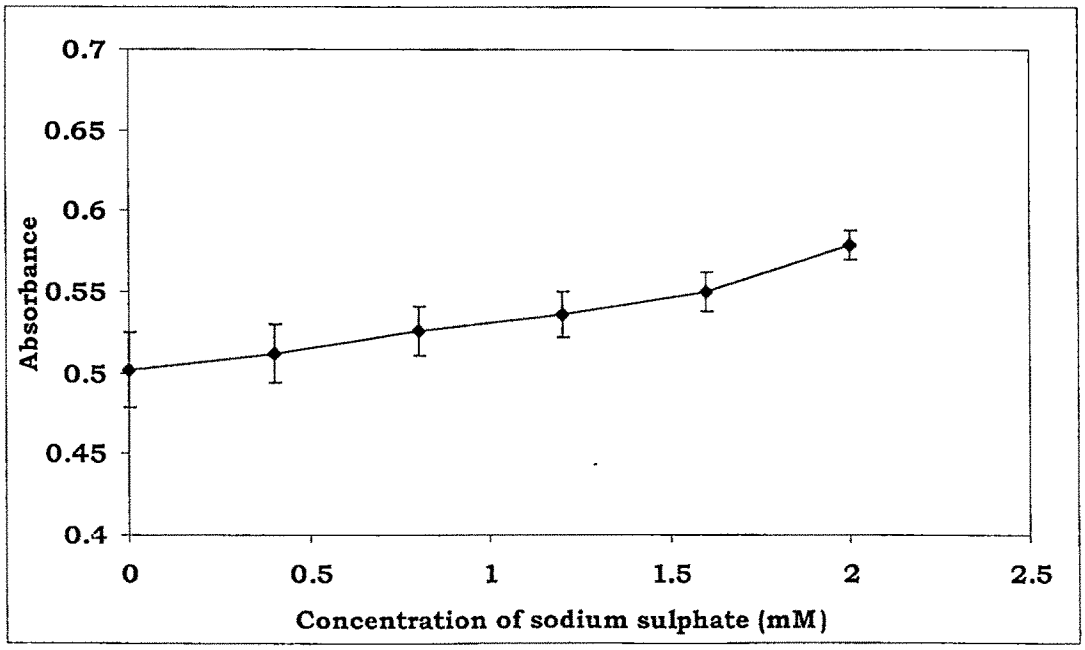


Table 4.18 Results of electrolyte induced flocculation test on conventional liposomes containing DNA

Mean concentration of sodium sulphate (in M)	Mean absorbance ± S.E.M at 400nm
0.0	0.502±0.023
0.4	0.512±0.018
0.8	0.526±0.015
1.2	0.536±0.014
1.6	0.550±0.012
2.0	0.579±0.009

Figure 4.8 Results of electrolyte induced flocculation test on conventional liposomes containing DNA



4.2.8 PREPARATION OF STERICALLY STABILISED LIPOSOMES CONTAINING LEUPROLIDE ACETATE AND DNA

4.2.8.1 Introduction

Over the last years, attempts have been made to create long circulating liposomes for the sustained drug release along the same lines, tried mainly by inclusion of various negatively charged bilayer components like glycolipids, phosphatidylinositol and monosialoganglioside or by the surface modification of the carrier liposomes with sialo glycopeptides (Blume et.al., 1990).

While inclusion of gangliosides in liposomes appears to increase the Blood/RES ratio of liposomally associated drug, it is not clear as to what surface property of the vesicle this can be attributed, since any increase in hydrophilicity will be accompanied by a net surface negative charge due to sialic acid component of the gangliosides. Furthermore, incorporation of relatively small amounts of gangliosides destabilizes the liposomal membrane to different extents.

In a new approach, the surface polarity of small neutral liposomes which quantitatively retain aqueous solutes can be increased, without causing inter-vesicle cross linking and without conferring net surface charge, by covalently linking methoxy polyethylene glycol (mPEG) to the bilayer surface. mPEG is preferred as the starting material because one of the two terminal hydroxyl groups of PEG is locked as the methoxy ether, leaving the other hydroxyl group free for derivatization PEG and mPEG have previously been coupled to protein, the conjugates showing an increased half life in blood (Senior et. al., 1990).

Polyethylene glycols of different chain lengths have been attached to the liposome for longer circulation time in blood. Intermediate molecular weights from 1500 to 5000 daltons at 5% to 10% mol in the bilayer give rise to the longest blood circulation times. Although many different lipids have been used, the only lipid used for attachment of longer PEG chains was phosphatidylethanolamine with different chain lengths and degrees of saturation because of the reactivity of the amino group. The reactivity of

this group is further catalysed by deprotonation by triethylamine or triethanolamine.

Different researchers used different coupling strategies; all adopted from the protein modification fields. All of them start with mPEG and phosphatidyl ethanolamine, three different synthetic routes were employed using succinyl, cyanuric chloride and carbamate derivatives yielding ester, secondary amine and urethane linkage respectively. Preliminary results using several other polymers did not match the prolongation of the blood circulation as achieved by PEG as they do not combine the same hydrophilicity and flexibility behaviour as PEG or the presence of dipolar interactions (Lasic, 1998).

4.2.8.2 Synthesis of methoxy polyethylene glycol 5000 activated with cyanuric chloride (mPEG5000-CC)

The method of Abuchowski (Abuchowski et. al., 1977) was modified for preparing methoxy polyethylene glycol 5000 (mPEG5000) activated with cyanuric chloride. 25g of mPEG5000, 2.75g of cyanuric chloride (molar ratio 1:3) and 5g of anhydrous potassium carbonate was taken in a 250ml round bottomed flask. To the contents 200ml of benzene was added and the flask was fitted with a calcium chloride guard tube. The contents were then filtered and the compound was precipitated by adding 300ml of petroleum ether (40°C - 60°C) slowly with stirring. The compound was then purified by successive precipitation from benzene using petroleum ether (40°C - 60°C), the process was monitored by quantitative ultraviolet spectroscopy for ascertaining the absence of impurities viz. cyanuric chloride. Absorptivity scans over the ultraviolet wavelength range of cyanuric chloride, methoxypolyethylene glycol 5000 and mPEG5000-CC in methanol taken on a Shimadzu 1601 UV-Visible spectrophotometer as shown in figures 4.9, 4.10 and 4.11 respectively. The identity of mPEG5000 activated with cyanuric chloride was ascertained by taking its mid infrared spectrum on a Shimadzu FTIR-8300 spectrophotometer (figure 4.12) and by taking its ^{13}C NMR spectrum on Avance DPX 200 dual probe NMR instrument (figure 4.13).

4.2.8.3 Synthesis of methoxy polyethylene glycol 5000 activated with cyanuric chloride-phosphatidylethanolamine conjugate (mPEG5000-CC-PE)

The method suggested by Blume and Cevc (Blume and Cevc, 1990) was followed preparing the conjugate of phosphatidylethanolamine (PE) with mPEG5000 activated with cyanuric chloride (mPEG5000-CC). The entire reaction scheme is shown in figure 4.14. PE (0.35mM) and triethylamine (1.2mM) were dissolved in 10ml of chloroform: methanol (5: 1 by volume) and added to a solution of mPEG5000-CC (0.4mM) in 50ml of chloroform: methanol (1:5 by volume). The mixture was stirred magnetically under a calcium chloride guard tube for 5 days at room temperature. The course of the reaction was monitored by thin layer chromatography on silica gel plates using chloroform: methanol: water:: 65: 25: 4 (v/v) as mobile phase (New, 1990a). At the end of the reaction, the compound was recovered by precipitation using solvent ether, dried using a rotary flash evaporator and the solid so collected was redissolved in chloroform: methanol (2: 1 by volume) and stored at less than 0°C until further use. The absorptivity scan of phosphatidyl ethanolamine in methanol is shown as figure 4.15. The ultraviolet, mid infrared region and ^{13}C-NMR spectra of the compound (mPEG5000-CC-PE) were taken using the same instruments as detailed in the section 4.2.9.2 and are shown as figures 4.16, 4.17 and 4.18 respectively.

Figure 4.9 Ultraviolet absorptivity scan of cyanuric chloride (CC) in methanol (1mg/ml)

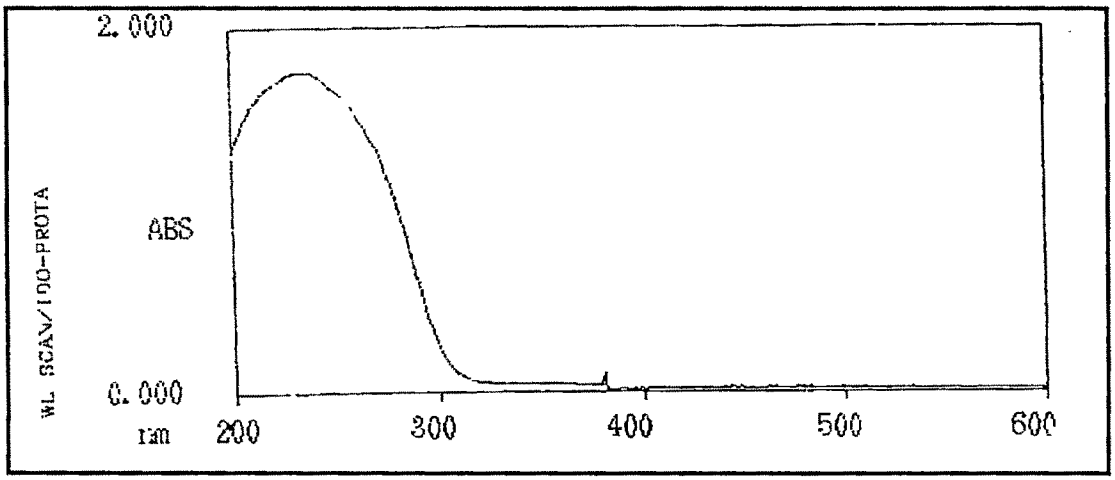


Figure 4.10 Ultraviolet absorptivity scan of methoxy polyethylene glycol 5000 (mPEG5000) in methanol (1mg/ml)

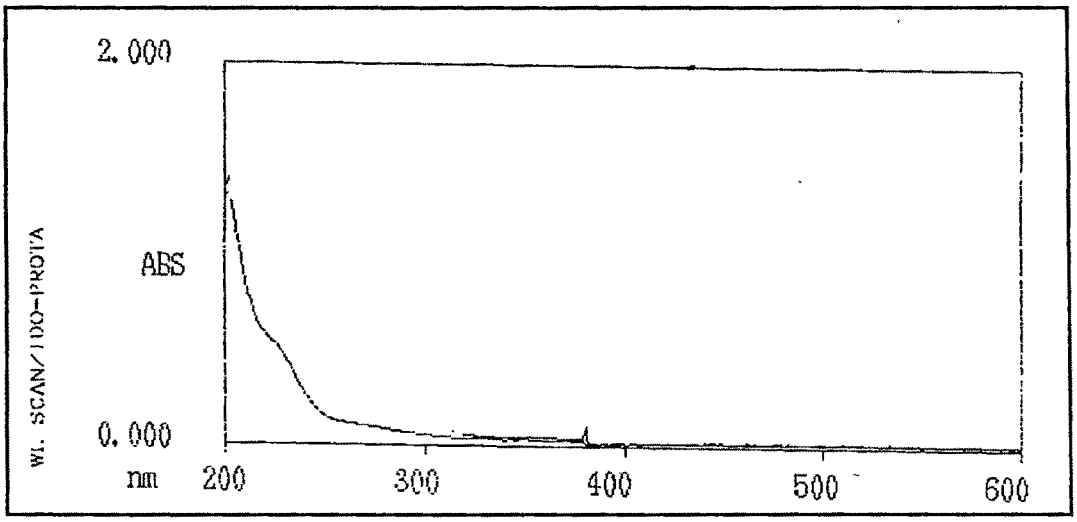
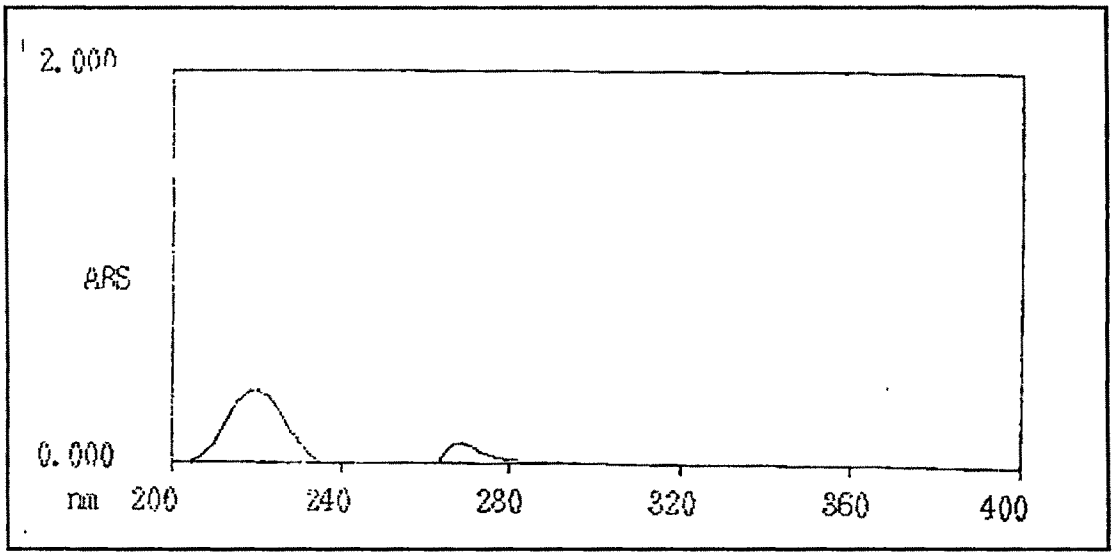


Figure 4.11 Ultraviolet absorptivity scan of methoxy polyethylene glycol 5000 activated with cyanuric chloride (mPEG5000-CC) in methanol (1mg/ml)



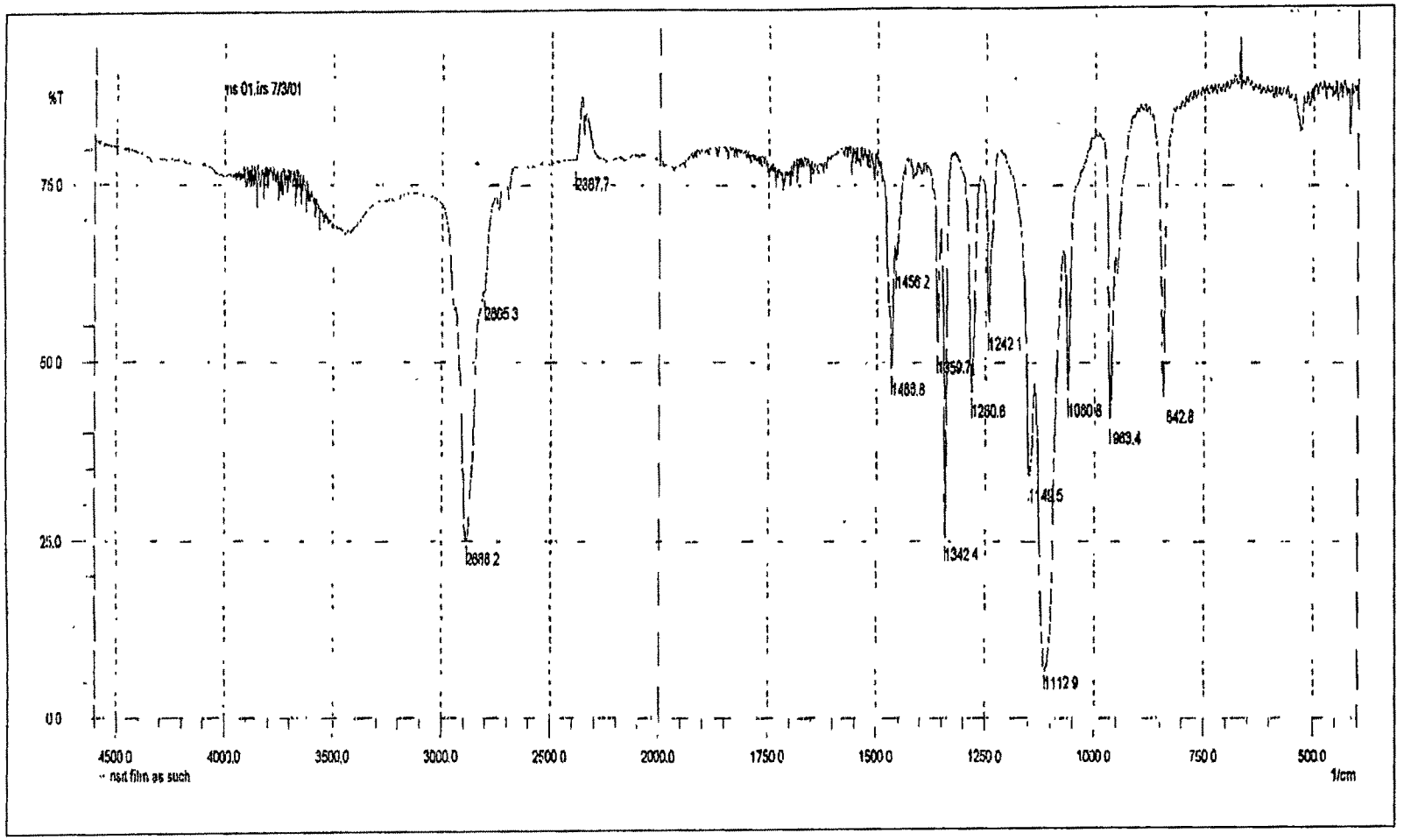


Figure 4.12 Mid infra-red spectrum of methoxy polyethylene glycol 5000 activated with cyanuric chloride (mPEG5000-CC)

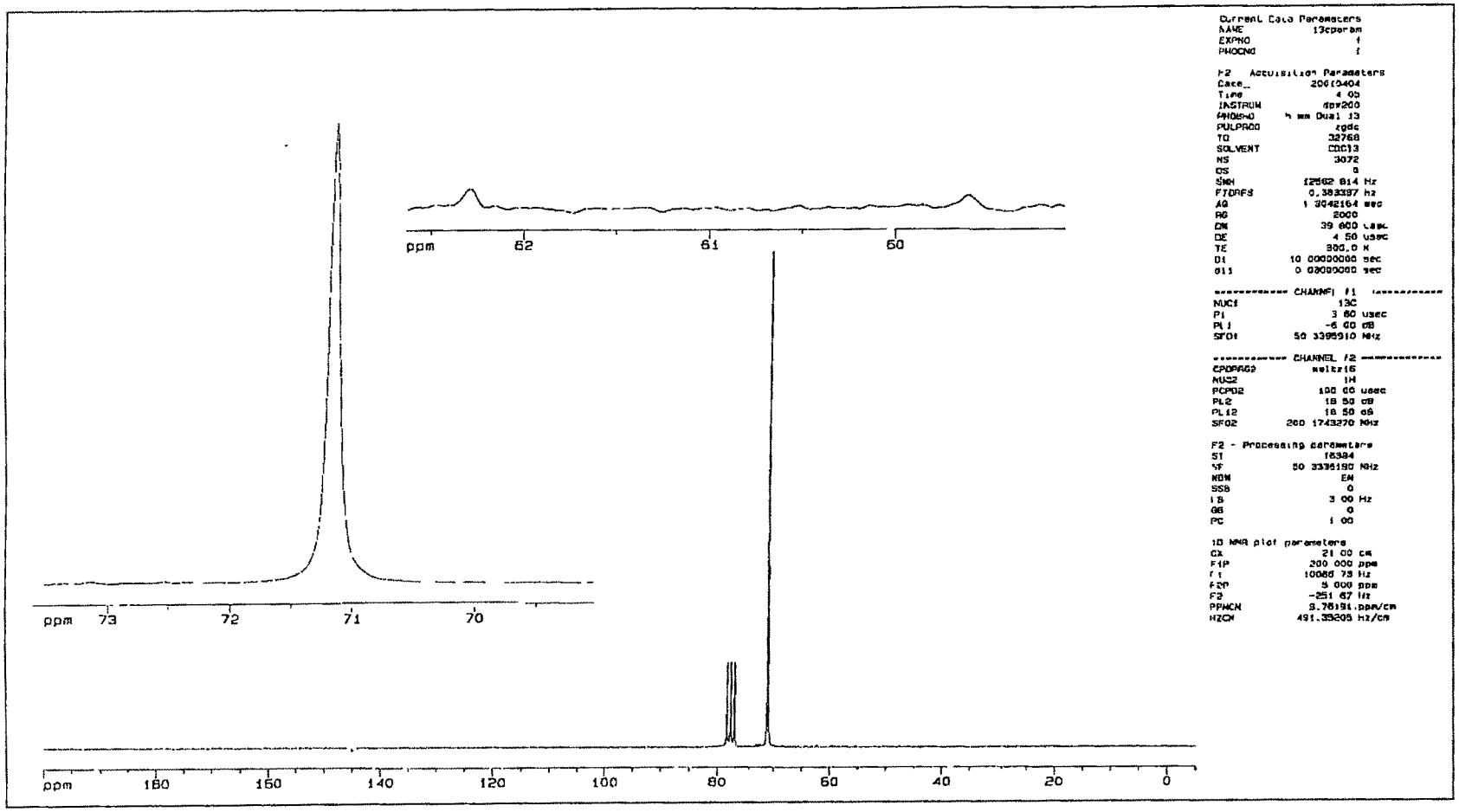
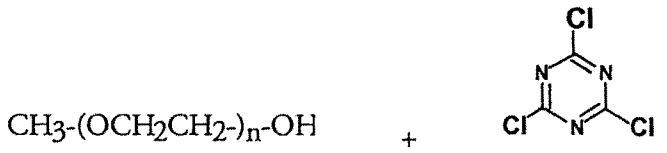


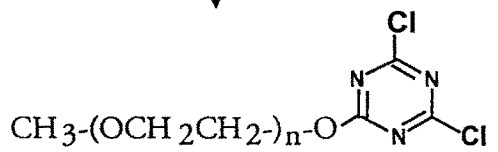
Figure 4.13 ^{13}C-NMR spectrum of methoxy polyethylene glycol 5000 activated with cyanuric chloride (mPEG5000-CC)

Figure 4.14 Reaction scheme for the synthesis of methoxy polyethylene glycol 5000 activated with cyanuric chloride-phosphatidylethanolamine conjugate (mPEG5000-CC-PE)

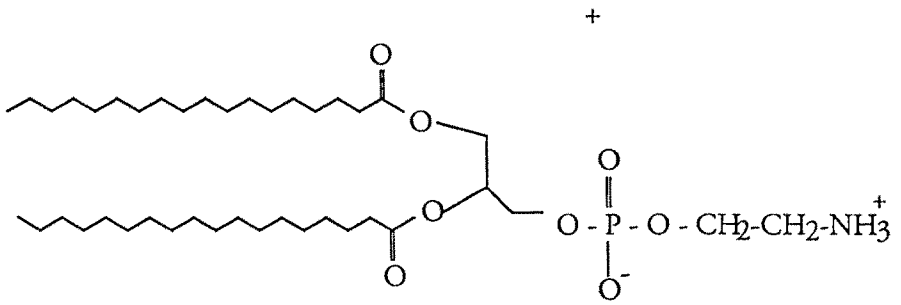


Methoxy Polyethylene glycol (Average mol. wt. 5000, mPEG5000)

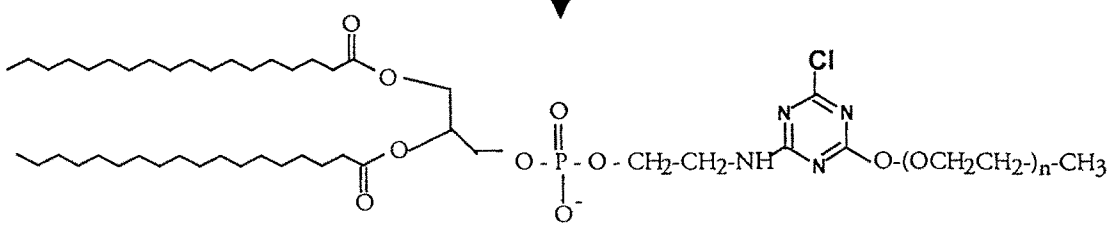
2,4,6-trichloro-s-traizine / cyanuric chloride (CC)



methoxy polyethylene glycol 5000 activated with cyanuric chloride (mPEG5000-CC)



Phosphatidylethanolamine



mPEG5000-CC-PE

Figure 4.15 Ultraviolet absorptivity scan of Phosphatidyl ethanolamine-(PE) in methanol (1 mg/ml)

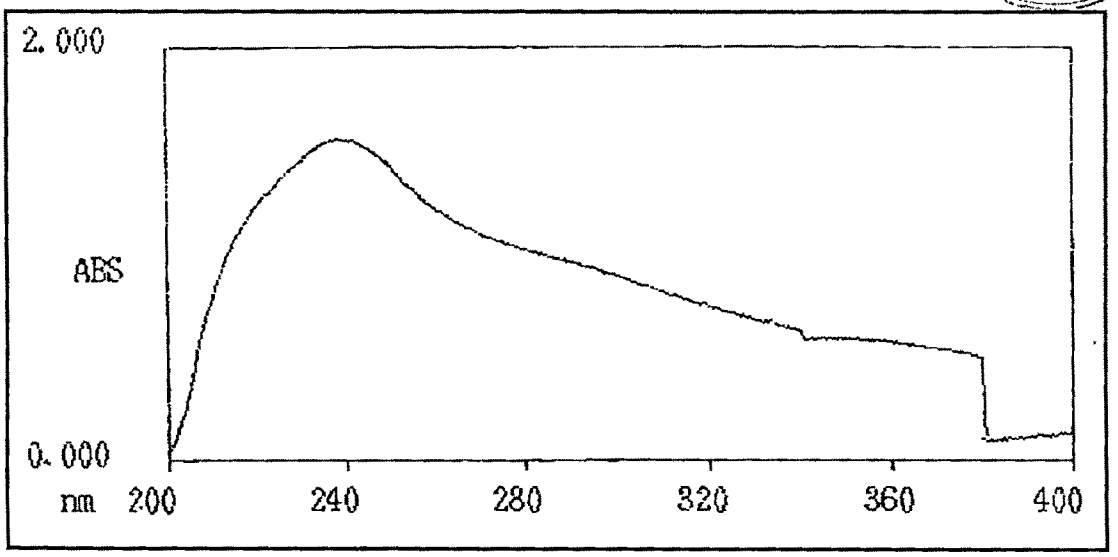
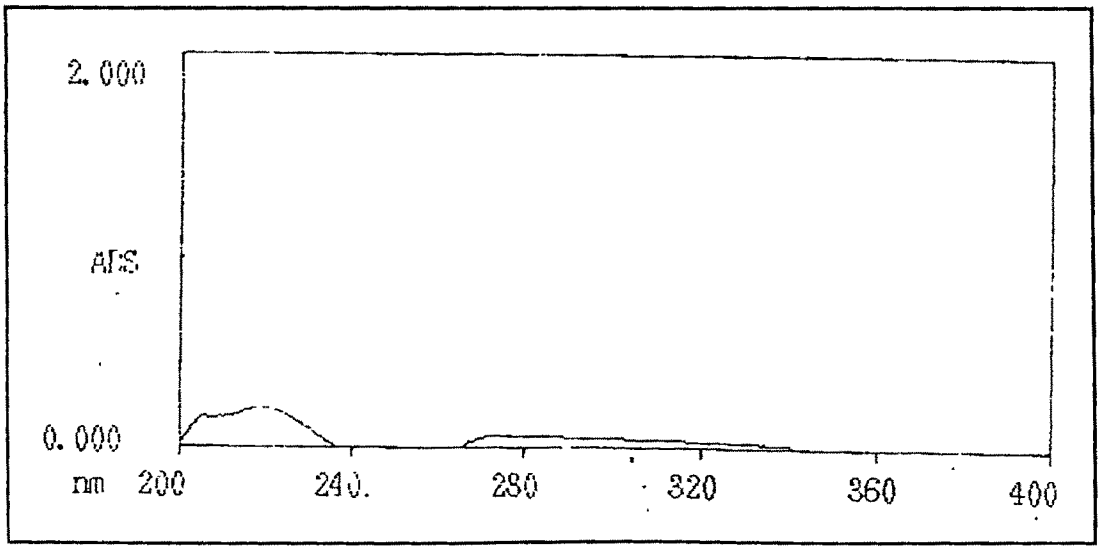


Figure 4.16 Ultraviolet absoptivity scan of methoxy polyethylene glycol 5000 activated with cyanuric chloride – phosphatidyl ethanolamine conjugate (mPEG5000-CC-PE) in methanol (5mg/ml)



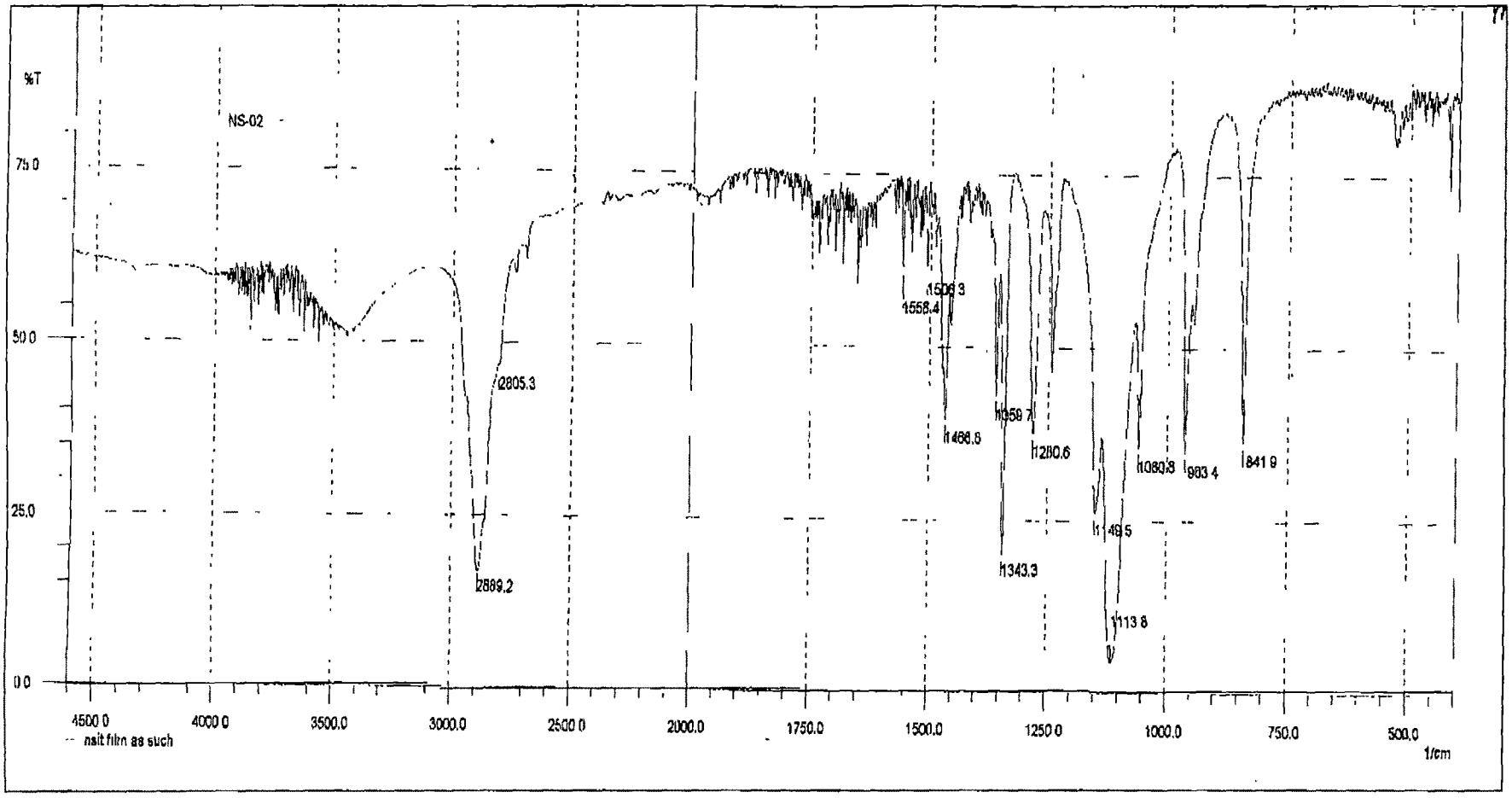


Figure 4.17 Mid infra-red spectrum of methoxy polyethylene glycol 5000 activated with cyanuric chloride – phosphatidyl ethanolamine conjugate (mPEG5000-CC-PE)

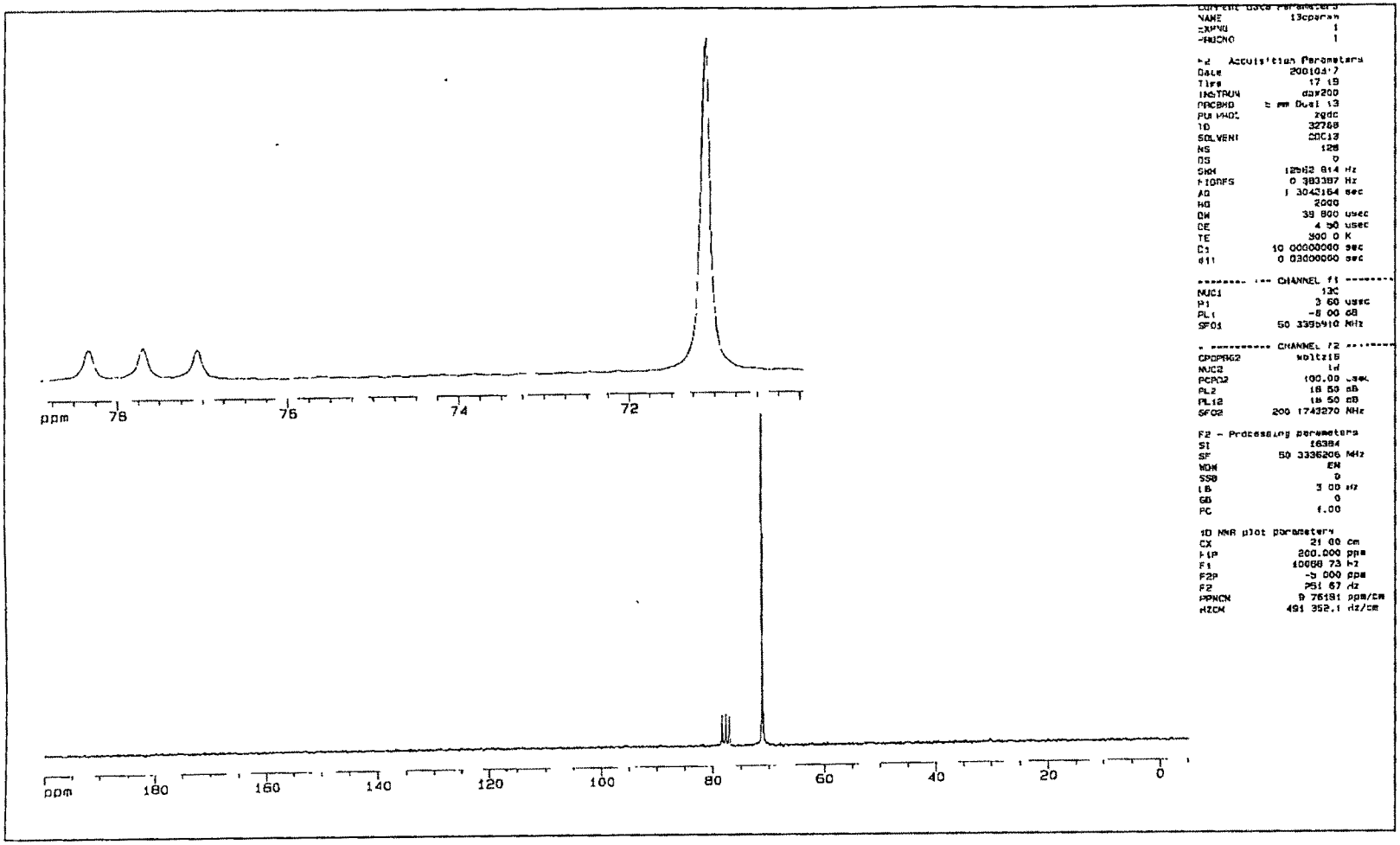


Figure 4.18 ^{13}C-NMR spectrum of methoxy polyethylene glycol 5000 activated with cyanuric chloride -phosphatidyl ethanolamine conjugate (mPEG5000-CC-PE)

4.2.8.4 Synthesis of methoxy polyethylene glycol 2000 activated with cyanuric chloride (mPEG2000-CC)

The method of Abuchowski (Abuchowski et. al., 1977) was modified for preparing methoxy polyethylene glycol 2000 (mPEG2000) activated with cyanuric chloride. 10g of mPEG2000, 2.75g of cyanuric chloride (molar ratio 1:3) and 5g of anhydrous potassium carbonate was taken in a 250ml round bottomed flask. To the contents 200ml of benzene was added and the flask was fitted with a calcium chloride guard tube. The contents were then filtered and the compound was precipitated by adding 300ml of petroleum ether (40°C - 60°C) slowly with stirring. The compound was then purified by successive precipitation from benzene using petroleum ether (40°C - 60°C), the process was monitored by quantitative ultraviolet spectroscopy for ascertaining the absence of impurities viz. cyanuric chloride. Absorptivity scans over the ultraviolet wavelength range of methoxy polyethylene glycol2000 and mPEG-2000-CC in methanol taken on a Shimadzu 1601 UV-Visible spectrophotometer are shown in figures 4.19 and 4.20 respectively. The identity of mPEG2000 activated with cyanuric chloride was ascertained by taking its mid infrared spectrum on a Shimadzu FTIR-8300 spectrophotometer (figure 4.21).

4.2.8.5 Synthesis of methoxy polyethylene glycol 2000 activated with cyanuric chloride-phosphatidylethanolamine conjugate (mPEG2000-CC-PE)

The method suggested by Blume and Cevc (Blume and Cevc, 1990) was followed preparing the conjugate of phosphatidylethanolamine (PE) with mPEG2000 activated with cyanuric chloride (mPEG2000-CC). The entire reaction scheme is shown in figure 4.22. PE (0.35mM) and triethylamine (1.2mM) were dissolved in 10ml of chloroform: methanol (5: 1 by volume) and added to a solution of mPEG2000-CC (0.4mM) in 50ml of chloroform: methanol (1:5 by volume). The mixture was stirred magnetically under a calcium chloride guard tube for 5 days at room temperature. The course of the reaction was monitored by thin layer chromatography on silica gel plates using chloroform: methanol: water:: 65: 25: 4 (v/v) as mobile phase (New, 1990a). At the end of the reaction, the compound was recovered by

precipitation using solvent ether, dried using a rotary flash evaporator and the solid so collected was redissolved in chloroform: methanol (2: 1 by volume) and stored at less than 0°C until further use. The ultraviolet, mid infrared region and ^{13}C-NMR spectra of the compound were taken using the same instruments as detailed in the section 4.2.9.2 and are shown as figures 4.23, 4.24 and 4.25 respectively.

4.2.8.6 Preparation of liposomes using mPEG5000-CC-PE and mPEG2000-CC-PE

Tables 4.19, 4.20, 4.21, 4.22 and 4.23 as well as figures 4.27, 4.28, 4.30, 4.31 and 4.32 present the results of the optimisation of the amount of mPEG5000-CC-PE and mPEG2000-CC-PE required for steric stabilization of liposomes containing leuprolide acetate and DNA respectively. The method of preparation of these liposomes are similar to that used to prepare conventional liposomes containing the drugs as described earlier, with the sole modification that an appropriate amount of mPEG5000-CC-PE or mPEG2000-CC-PE was added to the lipid solution before it was subjected to liposome preparation. The amount of mPEG5000-CC-PE and mPEG2000-CC-PE added was compensated by removal of an equimolar amount of PC from the system. The amount of mPEG5000-CC-PE required for steric stabilization was optimised by subjecting triplicate batches of liposomes, formed with different amounts of the mPEG5000-CC-PE and mPEG2000-CC-PE, to the electrolyte induced flocculation test, which is described earlier in this chapter. The preparation methods are shown as flowcharts (figure 4.26 and figure 4.29 respectively for liposomes containing leuprolide acetate and DNA).

Figure 4.19 Ultra violet absorptivity scan of methoxyl polyethylene glycol 2000 in methanol (1mg/ml)

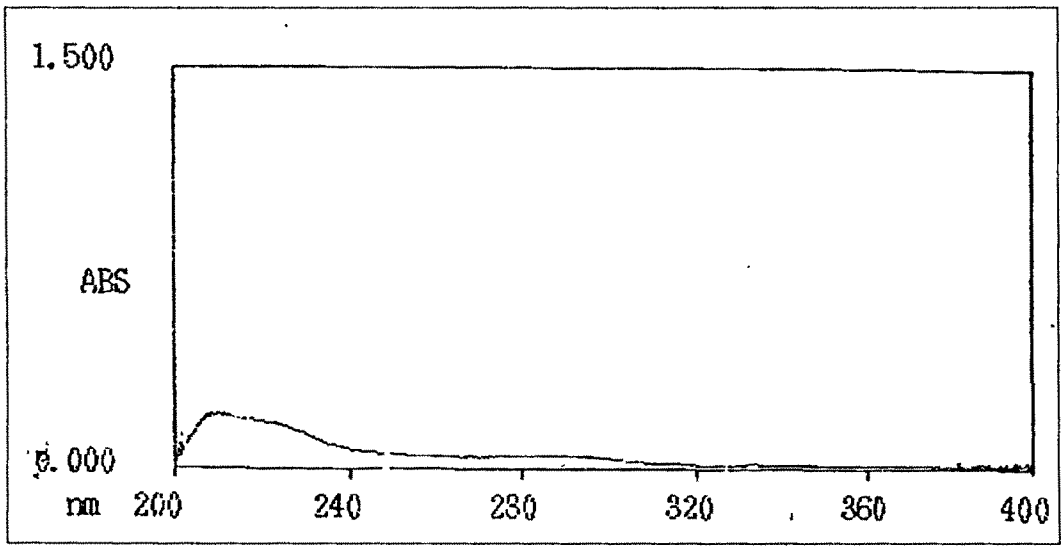
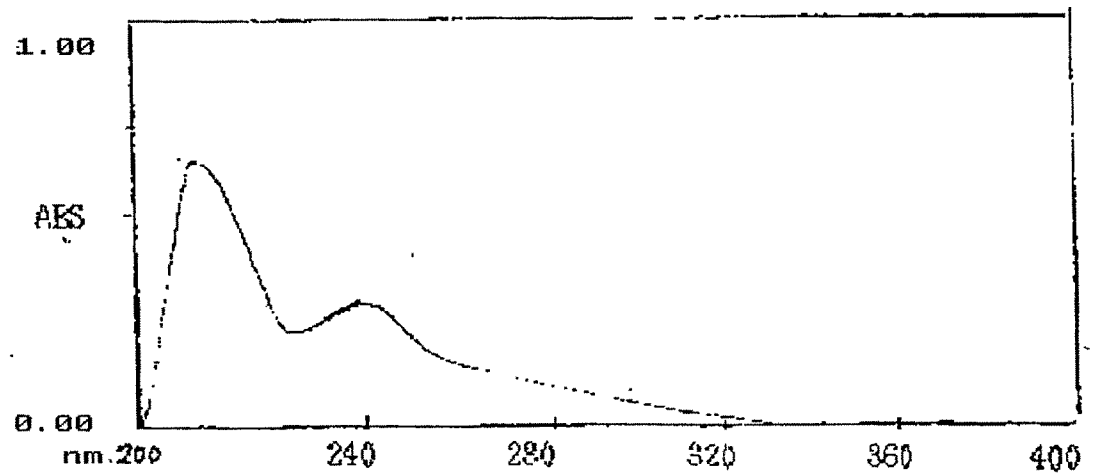


Figure 4.20 Ultraviolet absorptivity scan of methoxy polyethylene glycol 2000 activated with cyanuric chloride (mPEG2000-CC) in methanol (1mg/ml)



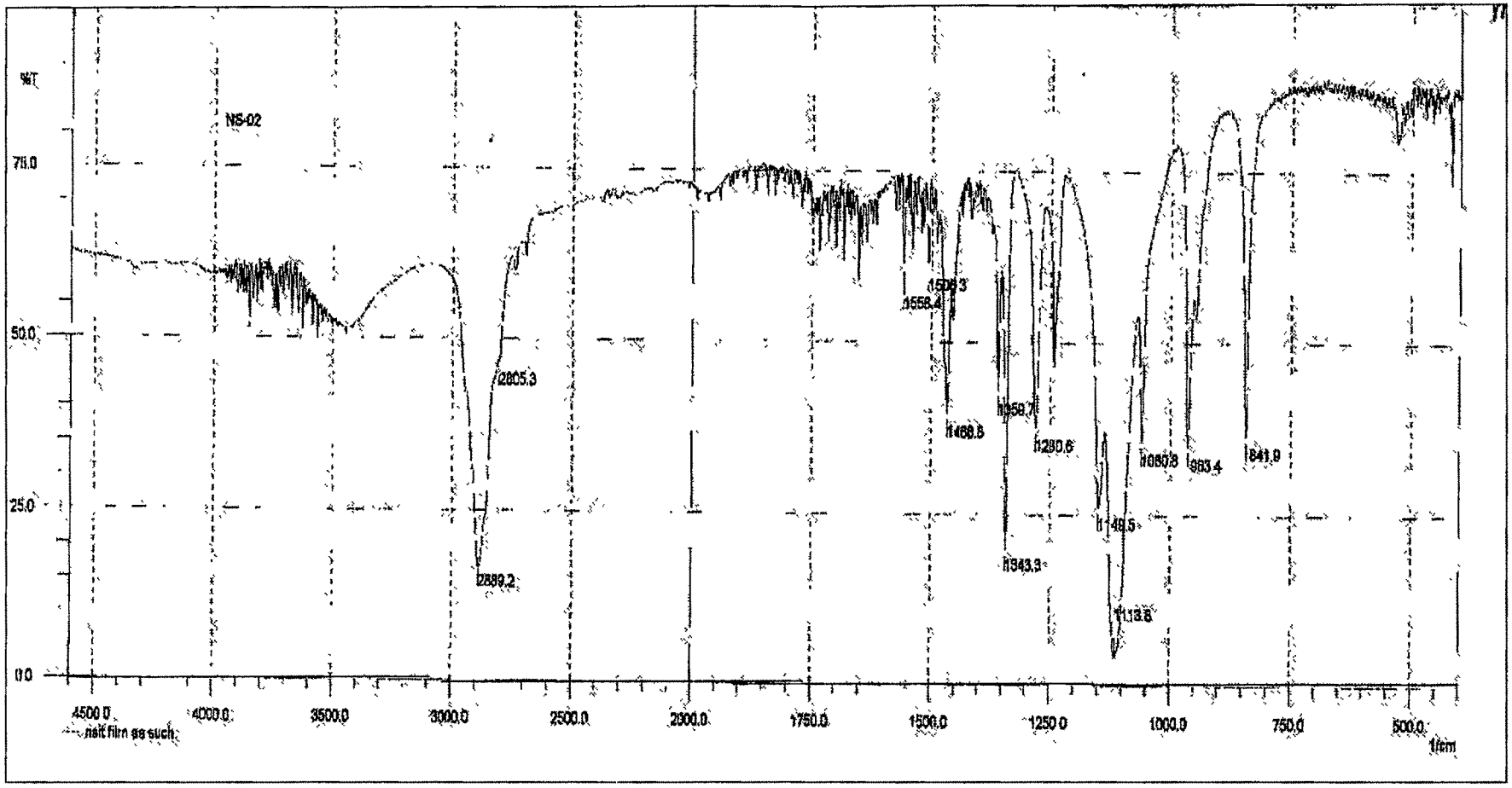
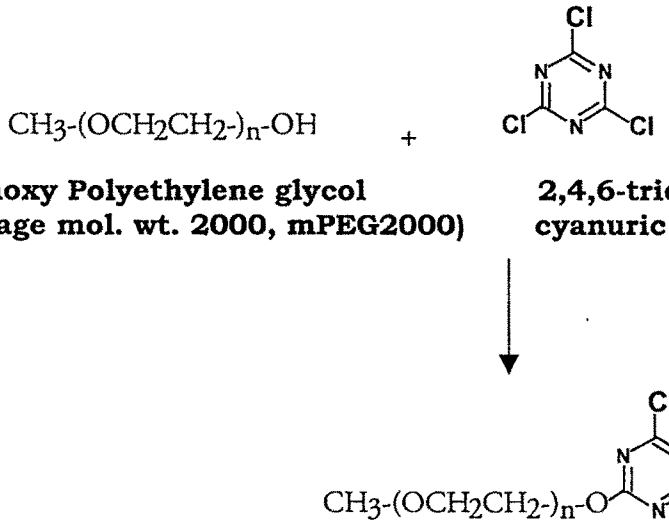
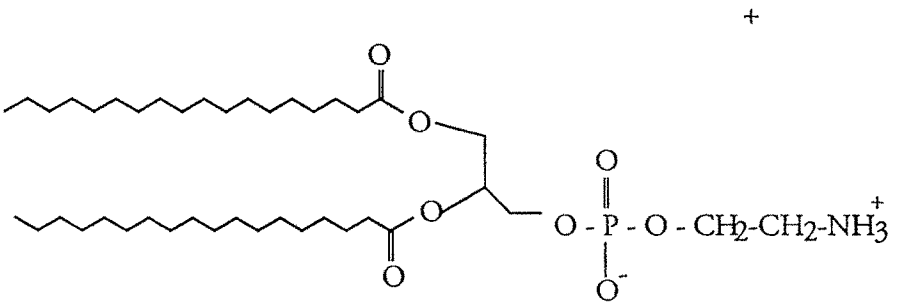


Figure 4.17 Mid infra-red spectrum of methoxy polyethylene glycol 5000 activated with cyanuric chloride – phosphatidyl ethanolamine conjugate (mPEG5000-CC-PE)

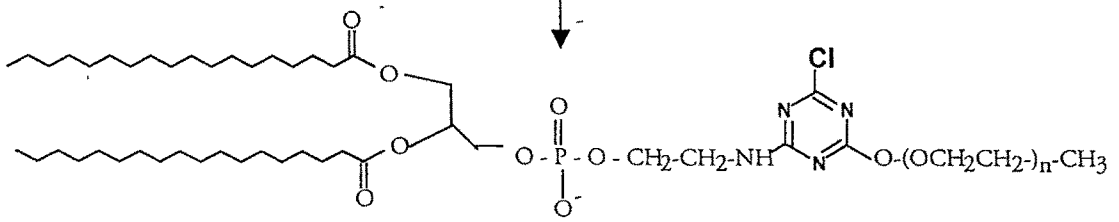
Figure 4.22 Reaction scheme of synthesis of methoxy polyethylene glycol 2000 activated with cyanuric chloride-phosphatidylethanolamine conjugate (mPEG2000-CC-PE)



methoxy polyethylene glycol 2000 activated with cyanuric chloride (mPEG2000-CC)

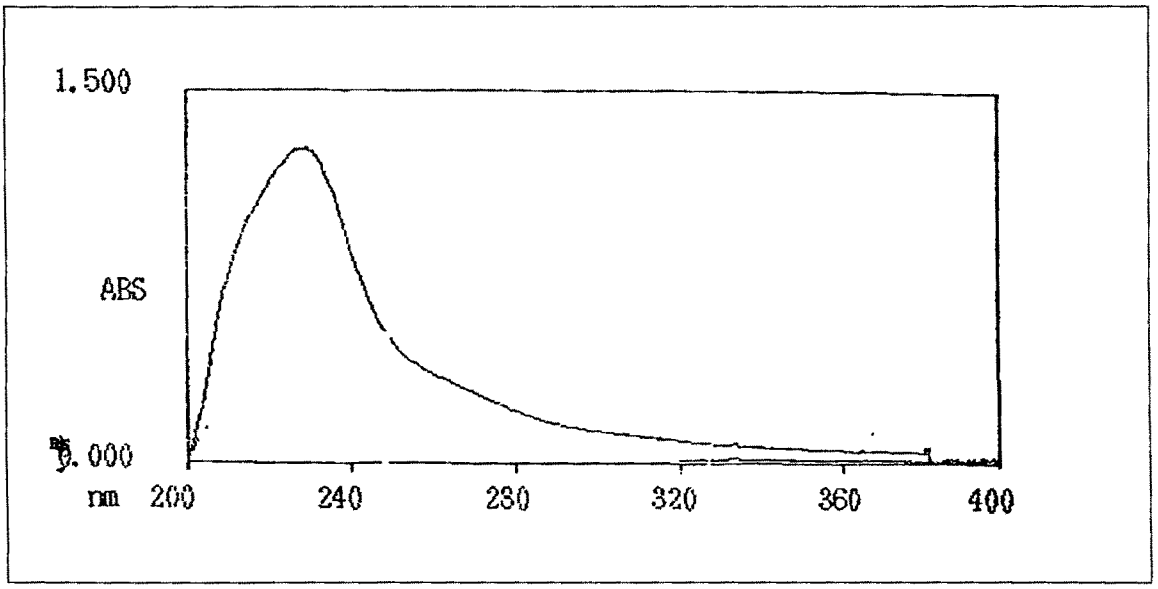


Phosphatidylethanolamine



mPEG2000-CC-PE

Figure 4.23 Ultraviolet absorptivity scan of methoxy polyethylene glycol 2000 coupled with cyanuric chloride -phosphatidyl ethanolamine conjugate (mPEG2000-CC-PE) in methanol (1mg/ml)



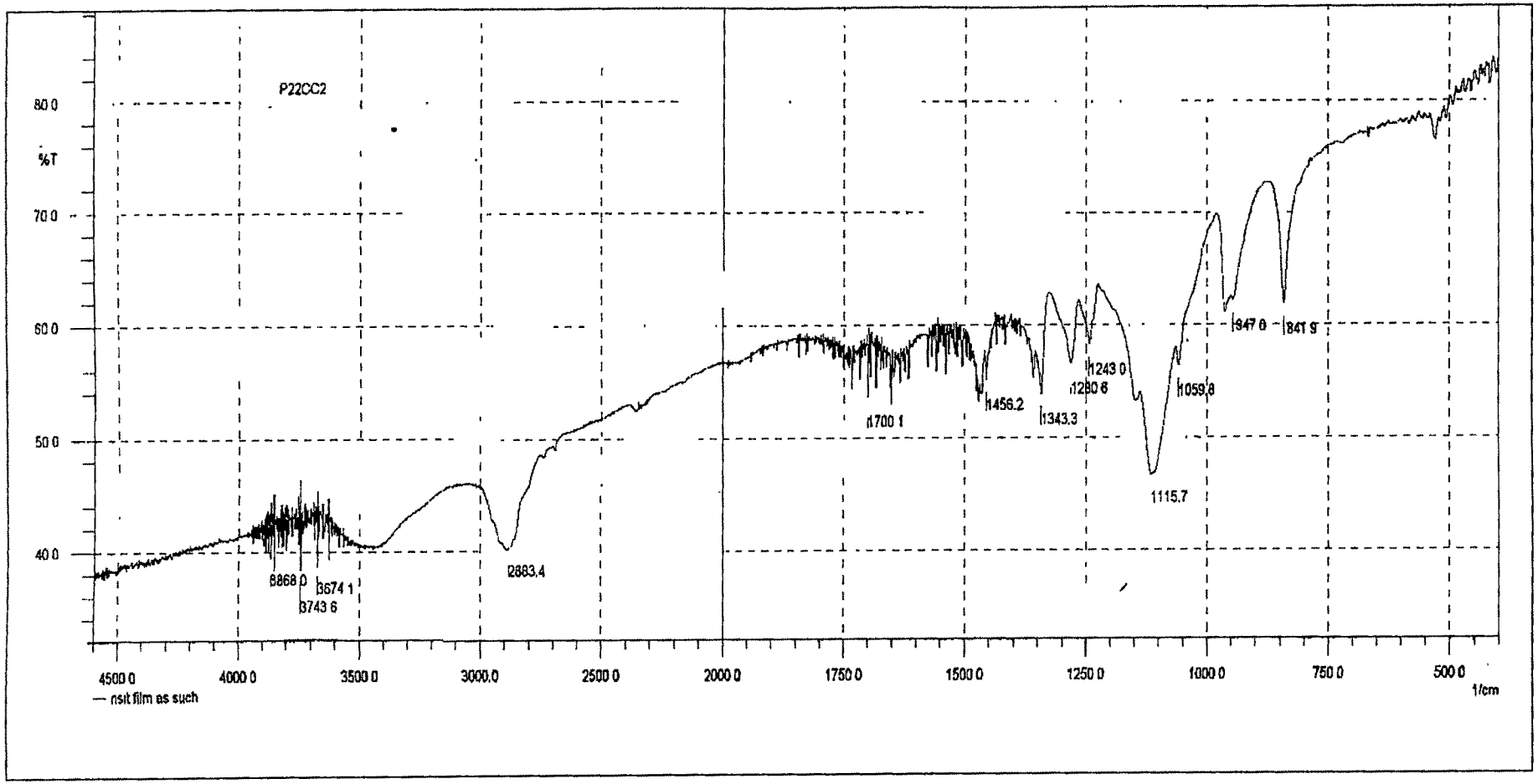


Figure 4.24 Mid infra-red spectrum of methoxy polyethylene glycol 2000 activated with cyanuric chloride – phosphatidyl ethanolamine conjugate (mPEG2000-CC-PE)

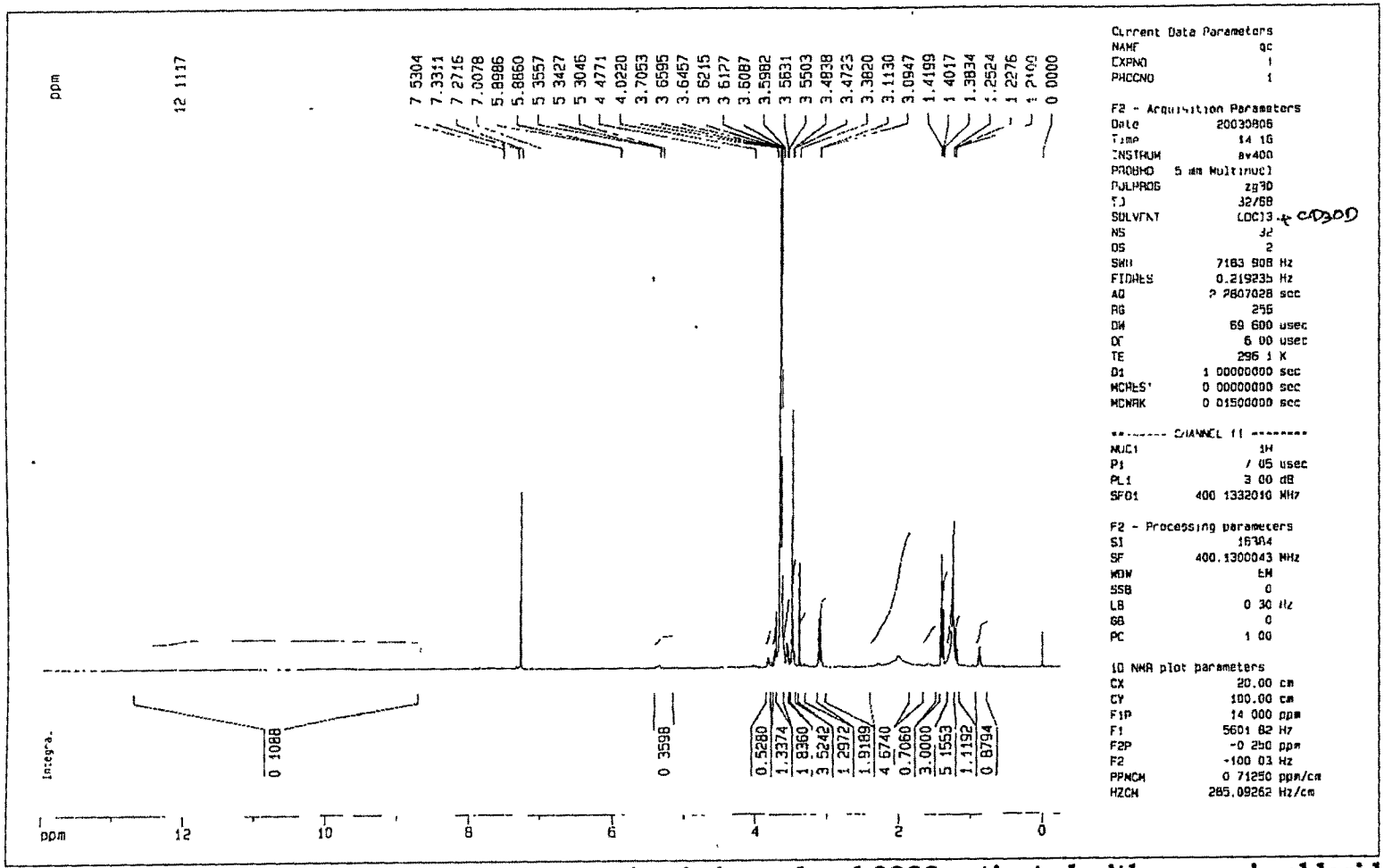


Figure 4.25 ^{13}C-NMR spectrum of methoxy polyethylene glycol 2000 activated with cyanuric chloride – phosphatidyl ethanolamine conjugate (mPEG2000-CC-PE)

Figure 4.26 Flowchart for the preparation of sterically stabilized liposomes containing Leuprolide acetate

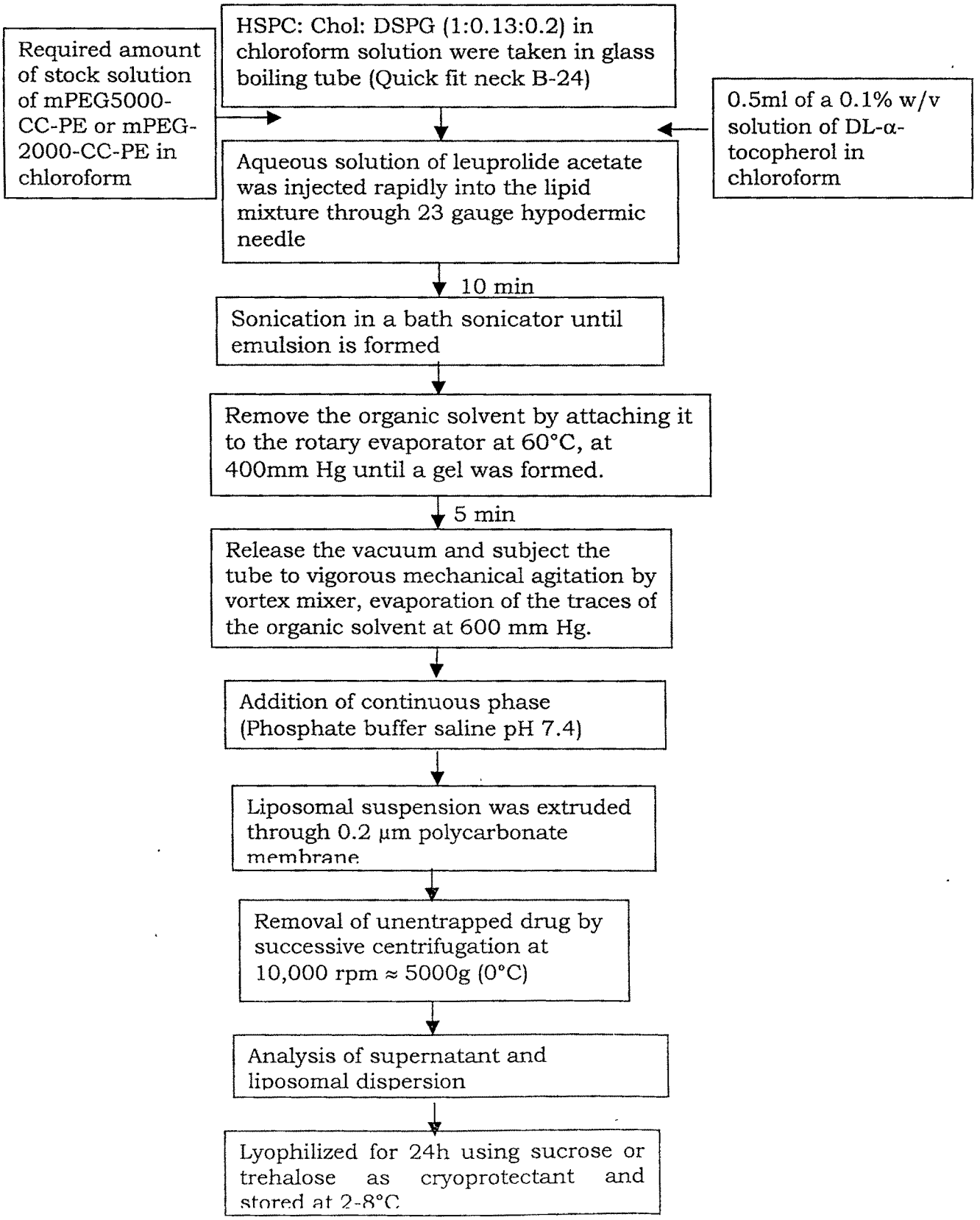


Table 4.19 Optimization of mPEG-5000-CC-PE concentration, required for steric stabilization of Leuprolide acetate containing liposomes, using electrolyte induced flocculation test

Mean concentration of sodium sulphate (in M)	Mean absorbance ± S.E. at 400nm of Leuprolide acetate liposomes containing mPEG2000-CC-PE			
	1 mol%	2 mol%	4mol%	6mol%
0.0	0.521±0.012	0.520±0.009	0.519±0.018	0.520±0.009
0.4	0.530±0.013	0.528±0.011	0.526±0.017	0.521±0.002
0.8	0.548±0.008	0.537±0.020	0.530±0.020	0.526±0.020
1.2	0.551±0.009	0.546±0.020	0.540±0.022	0.530±0.013
1.6	0.565±0.021	0.559±0.013	0.550±0.012	0.539±0.015
2.0	0.590±0.017	0.586±0.019	0.579±0.011	0.542±0.016

Figure 4.27 Optimization of mPEG-5000-CC-PE concentration, required for steric stabilization of leuprolide acetate containing liposomes, using electrolyte induced flocculation test

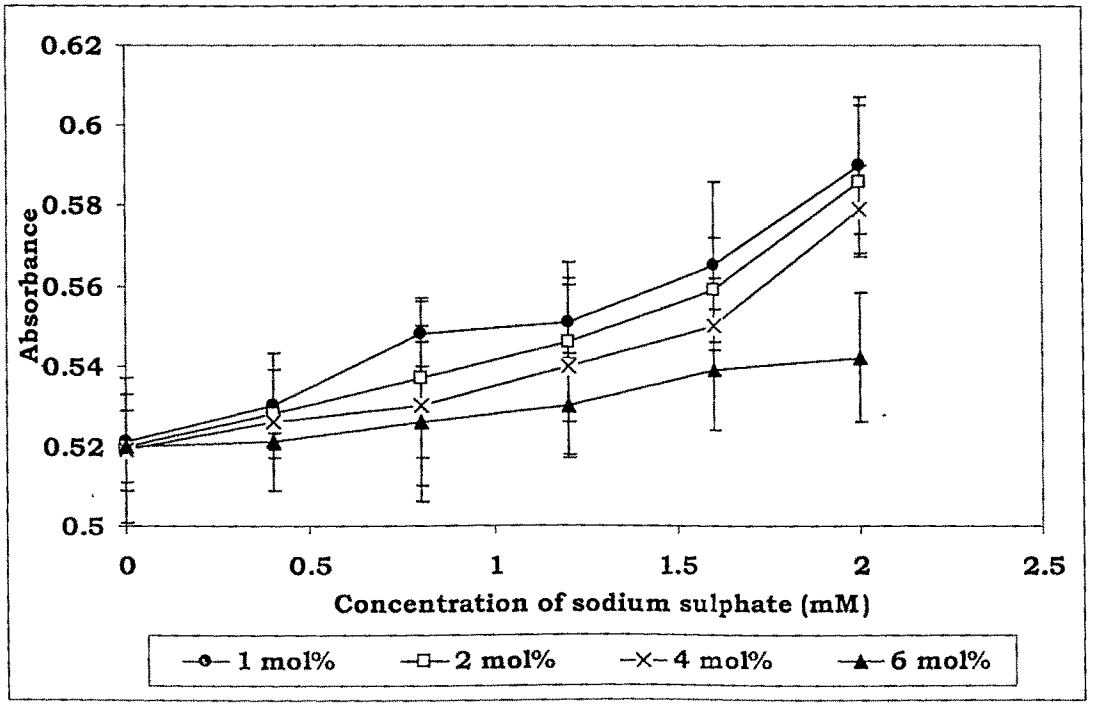


Table 4.20 Optimization of mPEG-2000-CC-PE concentration, required for steric stabilization of leuprolide acetate containing liposomes, using electrolyte induced flocculation test

Mean concentration of sodium sulphate (in M)	Mean absorbance ± S.E. at 400nm of Leuprolide acetate liposomes containing mPEG2000-CC-PE			
	1 mol%	2 mol%	4mol%	6mol%
0.0	0.517±0.015	0.515±0.011	0.519±0.016	0.515±0.008
0.4	0.528±0.012	0.526±0.008	0.522±0.020	0.520±0.012
0.8	0.538±0.013	0.530±0.021	0.529±0.016	0.526±0.007
1.2	0.546±0.020	0.543±0.023	0.536±0.018	0.530±0.012
1.6	0.558±0.021	0.556±0.011	0.542±0.017	0.532±0.011
2.0	0.578±0.022	0.570±0.012	0.560±0.012	0.532±0.013

Figure 4.28 Optimization of mPEG-5000-CC-PE concentration, required for steric stabilization of leuprolide acetate containing liposomes, using electrolyte induced flocculation test

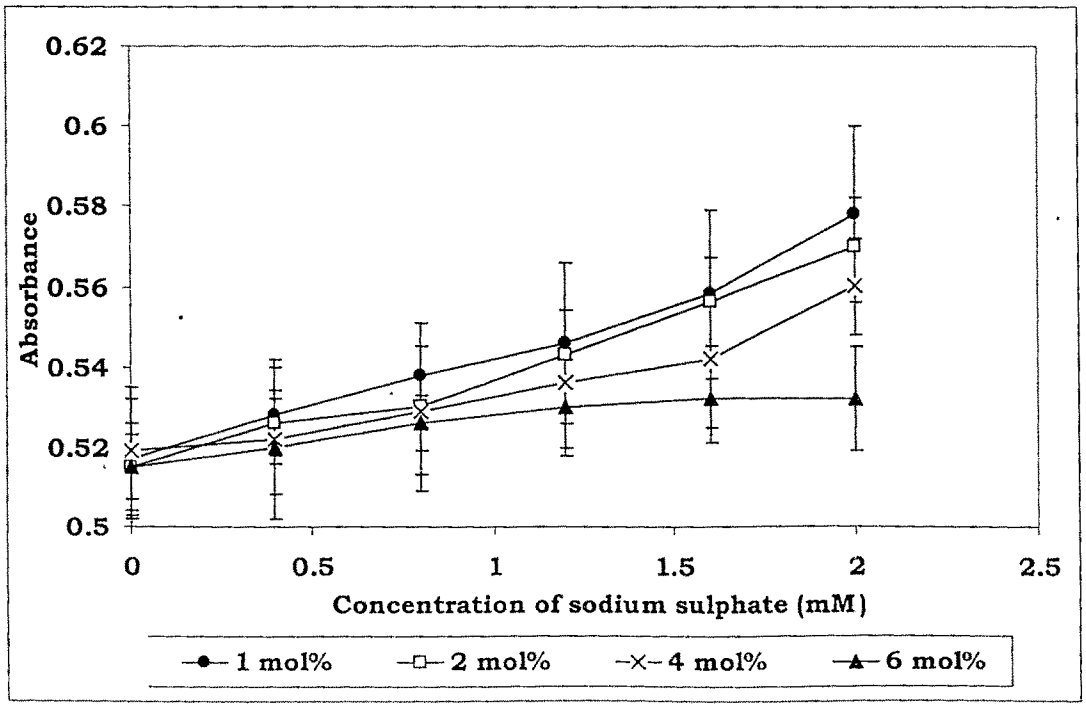


Figure 4.29 Flowchart for the preparation of sterically stabilized liposomes containing DNA

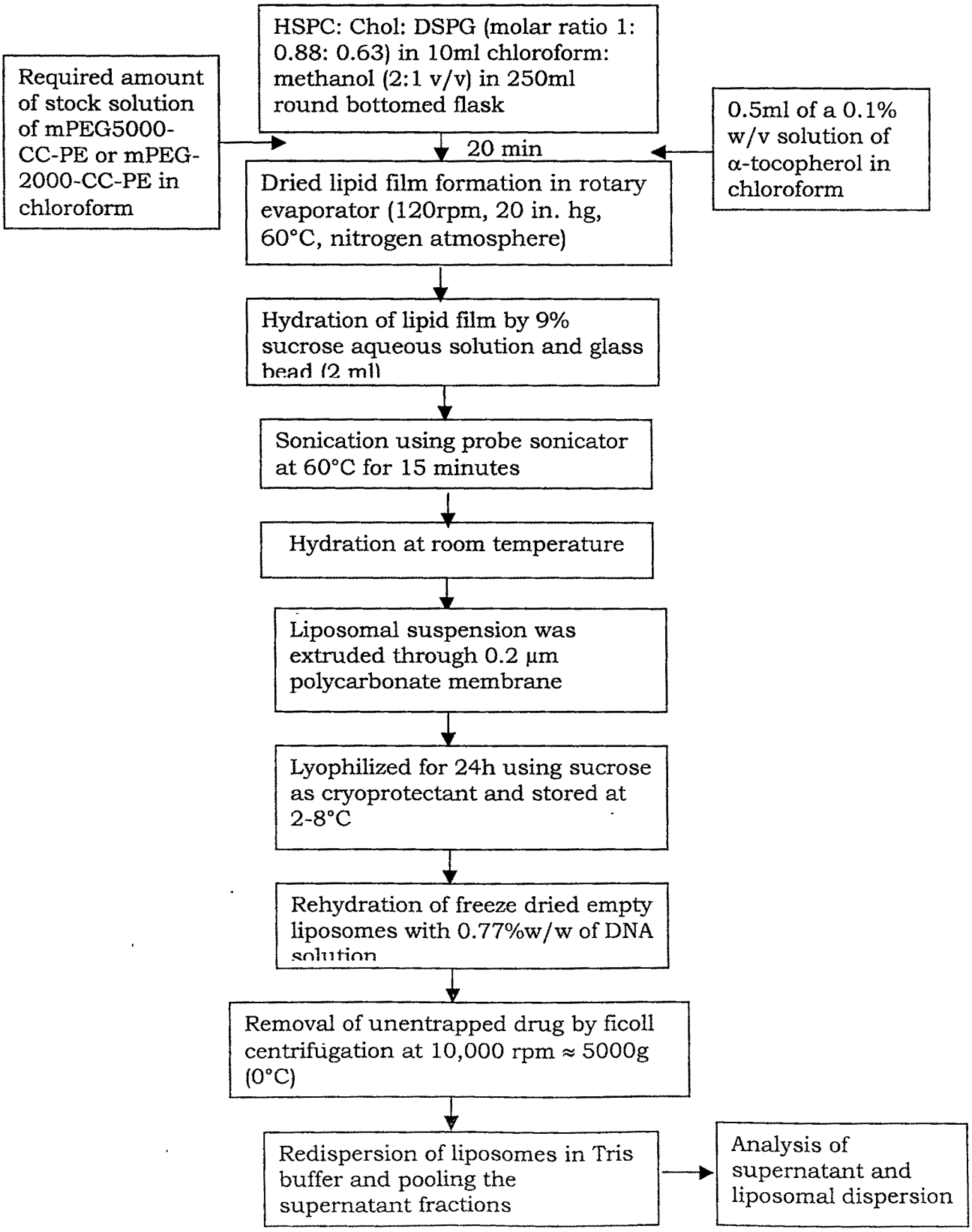


Table 4.21 Optimization of mPEG5000-CC-PE concentration, required for steric stabilization of DNA containing liposomes, using electrolyte induced flocculation test

Mean concentration of sodium sulphate (in M)	Mean absorbance ± S.E. at 400nm of DNA liposomes containing mPEG5000-CC-PE			
	1 mol%	2 mol%	3mol%	5mol%
0.0	0.578±0.014	0.572±0.01	0.570±0.016	0.570±0.019
0.4	0.592±0.016	0.590±0.013	0.585±0.017	0.578±0.018
0.8	0.598±0.015	0.585±0.021	0.582±0.012	0.580±0.001
1.2	0.605±0.012	0.598±0.009	0.592±0.014	0.585±0.015
1.6	0.612±0.013	0.601±0.008	0.600±0.021	0.595±0.014
2.0	0.632±0.021	0.622±0.015	0.613±0.019	0.598±0.020

Figure 4.30 Optimization of mPEG5000-CC-PE concentration, required for steric stabilization of DNA containing liposomes, using electrolyte induced flocculation test

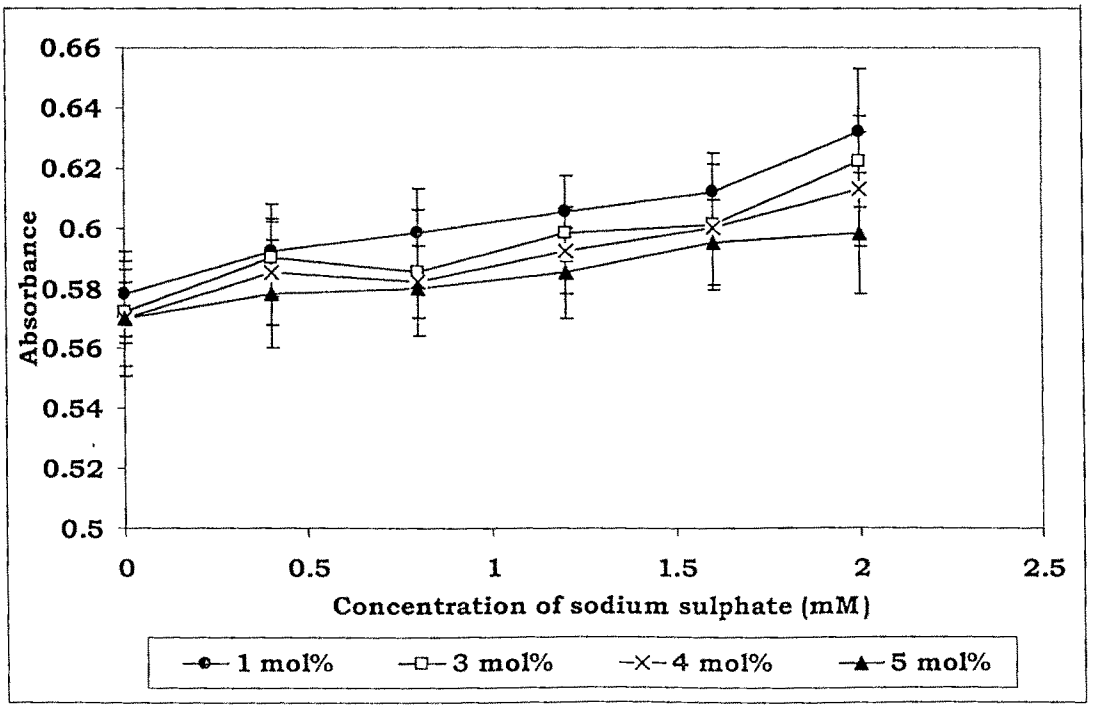


Table 4.22 Optimization of mPEG2000-CC-PE concentration, required for steric stabilization of DNA containing liposomes, using electrolyte induced flocculation test

Mean concentration of sodium sulphate (in M)	Mean absorbance ± S.E. at 400nm of DNA liposomes containing mPEG2000-CC-PE			
	1 mol%	2 mol%	3mol%	5mol%
0.0	0.521±0.012	0.510±0.016	0.512±0.016	0.510±0.01
0.4	0.530±0.02	0.530±0.018	0.525±0.020	0.520±0.015
0.8	0.546±0.015	0.539±0.019	0.528±0.018	0.518±0.012
1.2	0.559±0.021	0.549±0.01	0.526±0.017	0.520±0.015
1.6	0.582±0.009	0.578±0.012	0.535±0.013	0.525±0.017
2.0	0.595±0.012	0.586±0.009	0.538±0.011	0.530±0.008

Figure 4.31 Optimization of mPEG2000-CC-PE concentration, required for steric stabilization of DNA containing liposomes, using electrolyte induced flocculation test

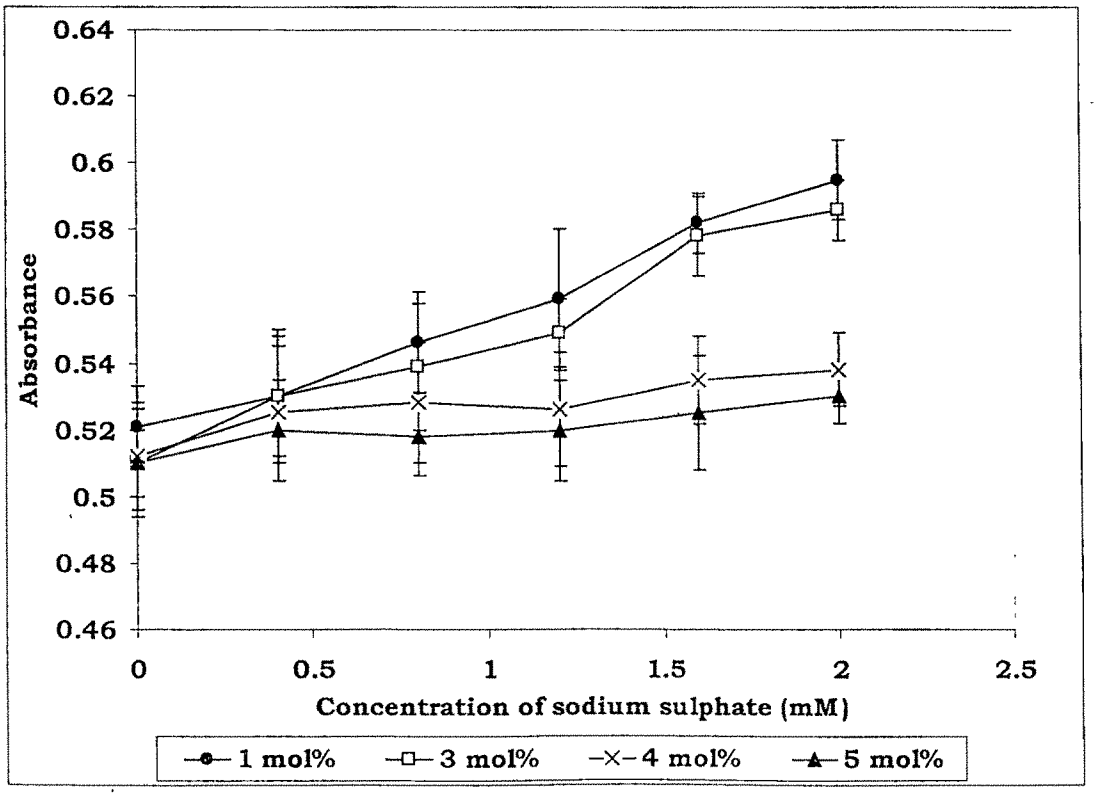


Table 4.23 Optimization of mPEG2000-CC-PE concentration, required for steric stabilization of DNA containing liposomes, using electrolyte induced flocculation test

Mean concentration of sodium sulphate (in M)	Mean absorbance ± S.E. at 400nm of DNA liposomes containing mPEG2000-CC-PE			
	1 mol%	2 mol%	3mol%	5mol%
0.0	0.515±0.012	0.512±0.018	0.515±0.008	0.516±0.012
0.4	0.53±0.023	0.525±0.015	0.520±0.019	0.518±0.019
0.8	0.54±0.002	0.532±0.013	0.529±0.020	0.520±0.014
1.2	0.546±0.011	0.54±0.02	0.532±0.016	0.526±0.021
1.6	0.559±0.016	0.544±0.010	0.540±0.014	0.530±0.022
2.0	0.568±0.01	0.555±0.009	0.548±0.015	0.536±0.021

Figure 4.32 Optimization of mPEG2000-CC-PE concentration, required for steric stabilization of DNA containing liposomes, using electrolyte induced flocculation test

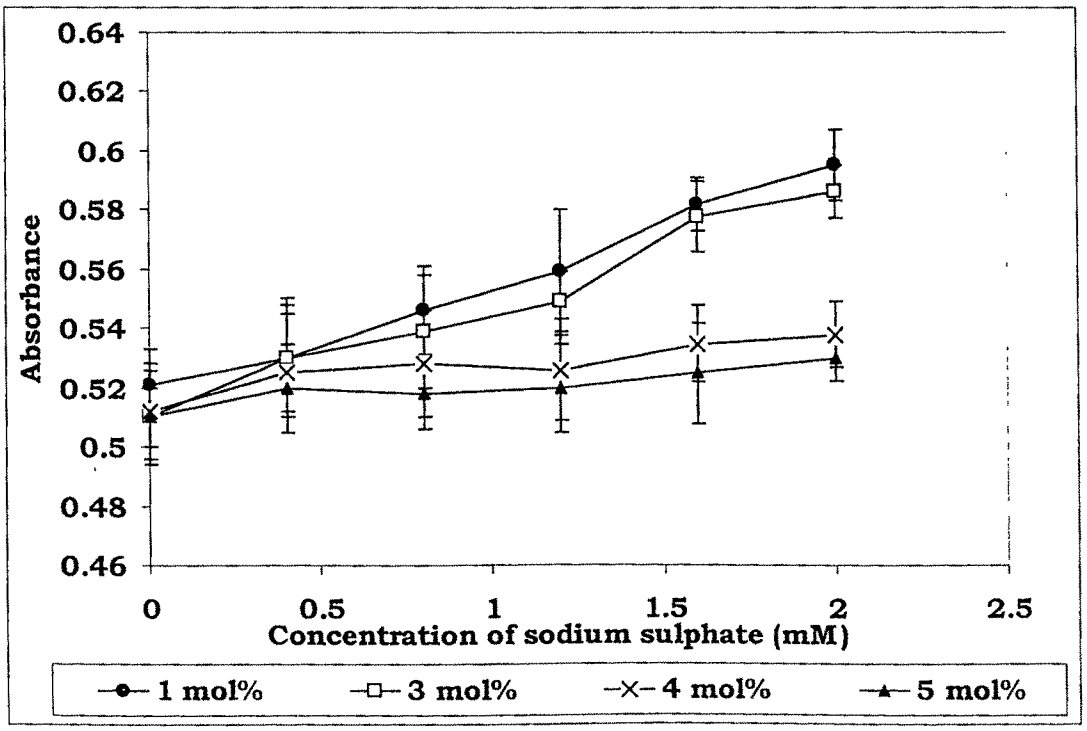


Table 4.24 Entrapment efficiency of optimized batches of freshly prepared liposomal suspension of DNA containing liposomes. Each value is the mean of triplicate results.

Type of Liposome	Lipid Composition	% Drug Entrapment ± S.E.M.
Conventional anionic liposomes (DL)	HSPC:Chol:DSPG 1:0.88:0.63	49.56±0.96
Sterically stabilized anionic liposomes coated with mPEG-PE 5000 (SDL5000)	HSPC:Chol:DSPG: mPEG5000 1:0.88:0.63:0.13	45.34±1.46
Sterically stabilized anionic liposomes coated with mPEG-PE 2000 (SDL2000)	HSPC:Chol:DSPG: mPEG2000 1:0.63:0.88:0.13	46.23±0.89
Conventional cationic liposomes (CDL)	HSPC:Chol:DOTAP 1:0.3:0.9	78.02±1.02
Sterically stabilized cationic liposomes coated with mPEG-PE 2000 (CSDL2000)	HSPC:Chol:DOTAP: mPEG2000 1:0.3:0.9:0.13	75.62±1.45

4.3 RESULTS AND DISCUSSION

An attempt was made

1. To prepare charged liposomes encapsulating cyclosporine with the aim of increasing the circulation half-life and reducing the nephrotoxicity associated with this drug.
2. To prepare sterically stabilized liposomes containing Leuprolide acetate and DNA with the objective of increasing the blood circulation time.

4.3.1 OPTIMISATION OF THE PREPARATION OF CHARGED LIPOSOMES OF CYCLOSPORINE

Liposome formulation containing cyclosporine was prepared with high entrapment efficiency by optimization of the formulation variables by 3^3 factorial design. The process variables like temperature, vacuum applied, sonication time and hydration time were optimized and kept constant and we found that these variables does not contribute much towards the change in drug entrapment. Positive, negative and neutral liposomes were prepared by using Hydrogenated Soya Phosphatidyl Choline (HSPC), Cholesterol (Ch), Stearylamine (SA) (positive charge), Distearoyl Phosphatidyl Glycerol (DSPG) (negative charge) by thin film hydration.

4.3.1.1 Influence of formulation process parameters

Process variables, viz. vacuum, hydration medium, hydration time, speed of rotation of flask sonication etc. were optimized to prepare lipid vesicles of CsA as shown in table 4.1. PBS (pH 7.8) was found to be the best hydrating medium that ensured better drug stability. The rotational speed of the flask demonstrated discernible influence on the thickness and uniformity of the lipid film. The speed of 120rpm yielded a uniform and thin-lipid film resulting in the preparation of desired vesicular characteristics upon hydration, while lower and higher rate of rotation resulted in preparation with noticeable aggregated non-vesicular lipid artifacts. The vacuum used for drying of film was raised from 10 to 20 in. of Hg. Vacuum of 10 in. of Hg was found to be insufficient for the complete removal of the solvents and resulted in aggregation of liposomes on hydration. The vacuum of 20 in. of

Hg. resulted in rapid evaporation of solvents, leading to entrapment of air bubbles on lipid film surface. Hydration of these films resulted in liposomes with poor drug entrapment. At an optimal vacuum of 15 in. of Hg, the lipid films were translucent and on hydration gave a high entrapment in liposomes. Upon evaporation of the lipid solution under the above conditions the dry residue appeared first in about 5 - 10min. The uniform, transparent film formed subsequently was allowed to dry for a further 10 - 15min time period. Drying for longer period of time rendered flaky film, which could not dispersed easily by hydration. A possible reason for this type of behaviour may be that minute traces of solvent are required for proper hydration and dispersion. This solvent subsequently gets removed from the system after dispersion.

Hydration is a prerequisite for proper maturation of liposomes yet excessive hydration leads to drug leakage. The drying step was followed by hydration by the addition of PBS pH 7.8, which assisted in film removal and dispersion. For the complete removal of the film, which remained adhering to the walls of the flask, a few glass beads (0.3cm diameter) was introduced into the flask and rotated gently to effect film removal and uniform dispersion. The proper hydration of the film for orientation of the HSPC molecules and intimate packing of lamellae (annealing) is necessary and hydration temperature (near phase transition temperature) and time was found to be essential parameters influencing it. The vesicles were first prepared by hydrating the lipid film for 45 minutes to 1 h at 55°C to 60°C followed by sonication for 15 minutes in a probe sonicator. Hydrated lipid film in suspension form was sonicated using a probe sonicator (at 60°C). The probe sonicator was found suitable over bath sonicator for small sample size in imparting the energy required for particle size reduction (up to ≅1μm). The time of sonication was optimised based on the particle size requirement and is discussed later in this chapter. The suspension after sonication was allowed to stand undisturbed at room temperature for a specified time period for annealing. The sonicated dispersion was then allowed to stand undisturbed for about 2 h at room temperature for complete hydration and extrusion through polycarbonate filters to achieve a particle size of less than 200nm.

4.3.1.2 Conditions for preparation of cyclosporine containing liposomes. Influence of formulation component variables

The formulation variables, Drug / Lipid [Phosphatidyl choline (HSPC) and Cholesterol (Chol)] molar ratio, HSPC / Chol (molar ratio) and the pH of hydration medium which have been predicted to play a significant role in enhancing the percent drug entrapment (PDE) are taken as variable parameters and the formulation variable such as HSPC: positive charge (SA) was kept constant throughout the design. 3^3 factorial design was used to study the main and interaction effects of the variables on the PDE. Based on the factorial design, twenty-seven batches of cyclosporine loaded liposomes were prepared. The liposome batches were evaluated for its drug entrapment within the liposomal vesicles. Mathematical modeling was carried out to obtain a second order polynomial equation (full model, equation 1) (Anthony et al, 1996).

A substantial high drug entrapment achieved in liposomes prepared by lipid film hydration method was 96.61% at X_1 (1: 0.04), X_2 (1:0.17) and X_3 (7.8). The PDE (dependent variable) obtained at various levels of three independent variables (X_1, X_2 and X_3) were subjected to multiple regression to yield a second order polynomial equation (full model, equation 1).

$$Y = 79.75 + 12.13\ X_1 + 15.35X_2 + 5.82\ X_3 - 5.77X_1^2 - 6.77X_2^2 - 5.80\ X_3^2 - 4.34\ X_1X_2 + 2.79\ X_2X_3 + 0.16\ X_1X_3 - 1.63\ X_1X_2X_3 \quad (1)$$

The main effects of X_1, X_2 and X_3 represent the average result of changing one variable at a time from its low to high value. The interactions (X_1X_2, X_1X_3, X_2X_3 and $X_1X_2X_3$) show how the PDE changes when two or more variables were simultaneously changed. The PDE values for the twenty-seven batches showed a wide variation from 30.33% to 96.6 % (table 4.3). Small values of the coefficients of the terms X_1X_2, X_2X_3 and $X_1X_2X_3$ in equation 1 are regarded as least contributing in the preparation of cyclosporine loaded liposomes by lipid film hydration method. Hence, these terms are neglected from the full model considering non-significance and a reduced polynomial equation (equation 2) obtained following multiple regression of PDE and very significant terms ($p<0.01$) of equation 1.

$$Y = 79.75 + 12.13\,X_1 + 15.35X_2 + 5.82\,X_3 - 5.77X_1^2 - 6.77X_2^2 - 5.80\,X_3^2 - 4.34\,X_1X_2 \quad (2)$$

The significance of each coefficient of the equation 1 was determined by student 't' test and p-value, which showed that the quadratic main effects of Drug / Lipid ratio (p value = 0.000000026), HSPC / Chol ratio (p value = 0.0000000009) and pH of hydration medium (p value = 0.00019) are found to be extremely significant. The second order main effects of all the three variables are significant, as is evident from their p-values. The interaction between X_1X_3 is found to be very significant with the p values of 0.004 respectively.

ANOVA between the full and reduced model was performed. F-Statistic of the results of ANOVA of full and reduced model confirmed omission of non-significant terms of equation 1. Since the calculated F value (1.086) is less than the tabled F value (3.25) (α = 0.05, V_1 = 3 and V_2 = 16), it was concluded that the neglected terms do not significantly contribute in the prediction of PDE. . When the coefficients of the three independent variables in equation 1 were compared, the value for the variable X_1 (b_1 = 12.13) and X_2 (b_2 = 15.35) was found to be maximum and hence both the variables, drug: lipid ratio (X_1) and HSPC: Chol (X_2) was considered to be a major contributing variable for PDE of cyclosporine liposomes. The fisher F test with a very low probability value ($P_{model} > F$ = 0.000001) demonstrates a very high significance for the regression model. The fisher F test with a very low probability value ($P_{model} > F$ = 0.000001) demonstrates a very high significance for the regression model. The goodness of fit of the model was checked by the determination coefficient (R^2). In this case, the values of the determination coefficients (R^2 = 0.9529 for full model and 0.9400 for reduced model) indicated that over 90 % of the total variations are explained by the model. The values of adjusted determination coefficients (adj R^2 = 0.9234 for full model and 0.9179 for reduced model) are also very high which indicates a high significance of the model. All the above considerations indicate an excellent adequacy of the regression model (Adinarayana et. al., 2002; Box et. al., 1978; Cochran and Cox, 1992; Yee et. al., 1993).

Thus from the above study, it was found that the entrapment of cyclosporine (CsA) in liposomes primarily based on the combination of the lipids and the ratio between the drug and the lipids, lipid and the cholesterol. CsA binds strongly to phospholipids like PC, thereby assisting its entrapment within liposomes and contributing to the rigidity of the bilayer. The requirement of mechanical stability and rigidity was fulfilled by incorporating cholesterol, which is well documented as being able to

a. Decrease the fluidity or micro viscosity of the bilayer by filling empty spaces among the phospholipid molecules, anchoring them more strongly into the structure.
b. Reduce the permeability of the membrane to water-soluble molecules due to the above effect
c. Stabilize the membrane in the presence of biological fluids such as plasma (Lasic et. al., 1998).

With the optimized process and formulation parameters neutral and negative charged liposomes were prepared in the similar manner as positive charged liposomes. The lipid: drug ratio used in all the formulations was 1: 0.04 (by molar ratio). The inclusion of charge into the lipid layers could avoid the aggregation and fusion of vesicles to maintain their integrity and uniformity. In addition to the charge, an antioxidant DL- α -tocopherol (0.5 ml of 0.1 % w/v solution in chloroform) was added to each formulation to minimize the oxidative degradation of phospholipids leading to stability problems. At a lower level of cholesterol the entrapment was found to be less, with increase in cholesterol at the molar ratio of 1: 0.17 high entrapment with better stability was achieved. The entrapment was reduced with further increase in cholesterol content. This deciphers the fact that cholesterol level beyond a certain level starts disrupting the bilayered membrane leading to the drug leakage and loss of drug entrapment levels (Redziniak and Perrier, 1996). Further in order to study the effect of charge on the liposomes the varying amount of DSPG and SA (1:0.3, 1:0.4 and 1:0.5 [molar ratio of HSPC: charge] was added and it was found that increased concentration of charges does not affect the entrapment efficiency and size characteristics. α-tocopherol was used as a lipophilic antioxidant in the concentration of 0.5 % w/w of PC to

inhibit the oxidation of PC. α-tocopherol is an established antioxidant for PC (Hunt and Tsang, 1981) and is allowed parenterally in concentrations ranging from 0.05% to 0.5% (Boylan et. al., 1996), an important requirement because these liposomes were intended for parenteral administration.

4.3.2 OPTIMISATION OF THE PREPARATION OF LEUPROLIDE ACETATE LIPOSOMES

By using 3^3 factorial design, twenty seven batches of leuprolide acetate liposomes were prepared by REV method by varying three independent variables, volume of aqueous phase (X_1), HSPC: DSPG [molar ratio (X_2)] and HSPC: Chol [molar ratio (X_3)]. The percent drug entrapment (PDE), which was taken as dependent variable was determined and the results are recorded (table 4.11). A substantially better drug entrapment achieved in liposomes prepared by REV method was 60.8% at -1 level of X_1 (0.25ml), 0 level of X_2 (1: 0.2) and -1 level of X_3 (1:0.13).

4.3.2.1 Multiple linear regression

The PDE (dependent variable) obtained at various levels of three independent variables (X_1, X_2 and X_3) were subjected to multiple regression to yield a second order polynomial equation (full model).

$$Y = 50.69 - 3.07\ X_1 - 10.03\ X_2 - 4.41\ X_3 - 0.20\ X_1^2 - 18.93\ X_2^2 + 0.698\ X_3^2 + 0.861 X_1X_3 + 1.2\ X_2X_3 - 1.698\ X_1X_2 - 0.0188\ X_1\ X_2X_3 \quad (3)$$

Multiple linear regression indicated a strong correlation between the PDE and each of the experimental variables. It also indicated strong interactions between these variables, which are represented by the interaction terms in the fitting equation.

The main effects of X_1, X_2 and X_3 represent the average result of changing one variable at a time from its low to high value. The interactions (X_1X_2, $X1X_3$, $X_2\ X_3$ and $X_1\ X_2\ X_3$) show how the PDE changes when two or more variables were simultaneously changed. The PDE values for the twenty-seven batches showed a wide variation from 16.2 to 60.8 % (table 4.11). This is reflected by the wide range of coefficients of the terms of equation 2 representing the individual and combined variables. Small values of the coefficients of the terms X_1^2, X_3^2, X_1X_3, $X_1\ X_2\ X_3$ in equation 3 were regarded

as least contributing variables in the preparation of leuprolide acetate liposomes by REV technique. Hence, these terms were neglected from the full model considering non-significance and a reduced polynomial equation (equation 4) obtained following multiple regression of PDE and significant terms ($p<0.05$) of equation 3.

$$Y = 50.69 - 3.07 X_1 - 10.03 X_2 - 4.41 X_3 - 18.93 X_2^2 - 1.698 X_1X_2 + 1.2 X_2X_3 \quad (4)$$

The significance of each coefficient of the equation 3 was determined by student't' test and p-value, which are listed in table 4.12. The larger the magnitude of the t value and the smaller the p value, the more significant is the corresponding coefficient. This implies that the quadratic main effects of volume of aqueous phase (X_1), HSPC: DSPG (X_2) and HSPC: Chol (X_3) are found to be very significant. The second order main effects of HSPC: DSPG are significant, as is evident from their p-values. The interaction between X_2X_3 and X_1X_2 are found to be significant from their p-values (table 4.12).

The results of ANOVA of the second order polynomial equation are given in table 4.13. F-Statistics of the results of ANOVA of full and reduced model confirmed omission of non-significant terms of equation 4. Since the calculated F value (0.926) is less than the tabulated F value (3.256) (α = 0.05, V_1=4 and V_2 = 16), it was concluded that the neglected terms do not significantly contribute in the prediction of PDE. When the coefficients of the three independent variables in equation 3 were compared, the value for the variable X_2 (b_2 = -10.0317) was found to be maximum and hence the variable X_2 was considered to be a major contributing variable for PDE of leuprolide acetate liposomes. The fisher F test with a very low probability value demonstrates a very high significance for the regression model. The goodness of fit of the model was checked by the determination coefficient (R^2). In this case, the values of the determination coefficients (R^2 = 0.989 for full model and 0.986 for reduced model) indicated that over 90 % of the total variations are explained by the model. The values of adjusted determination coefficients (adj R^2 = 0.982 for full model and 0.982 for reduced model) are also very high which indicates a high significance of the model. A higher value of correlation coefficients (R = 0.994 for full model and 0.993 for reduced model) signifies an excellent correlation between the independent variables.

4.3.2.2 Contour plots

Figure 4.5A shows the contour plot drawn at -1 level of X_3 (1:0.13) for a prefixed PDE value of 30%, 40%, 50%, and 60%. The plots were found to be linear for 30 %, 40% and 50% but for 60 % PDE the plots were found to be non-linear having upward and downward segment signify non-linear relationship between volume of aqueous phase (X_1) vs. HSPC/DSPG (X_2) variables. It was determined from the contour that maximum PDE (60%) could be obtained with X_1 range at – 1 level to – 0.6 level (0.25ml to 0.35ml) and with X_2 range at - 0.2 levels to 0.2 level (1:0.17 to 1: 0.26) when X_3 (1:0.13) was used.

Figure 4.5 B shows the contour plot drawn at 0 level of X_3 (1:0.2) for a prefixed PDE value of 20%, 30%, 40%, 50% and 55%. The plots were found to be linear for 20 %, 30% and 40% but for 50 % and 55% PDE the plots were found to be non-linear having upward and downward segment signify non-linear relationship between X_1 and X_2 variables. It was determined from the contour that maximum PDE (55%) could be obtained with X_1 range at – 1 level to – 0.6 level and with X_2 range at – 0.2 level to 0.2 level.

Figure 4.5 C shows the contour plot drawn at 1 level of X_3 (1:0.5) for a prefixed PDE value of 20%, 30%, 40%, and 50%. The plots were found to be linear for 20 %, 30% and 40% but for 50 % PDE the plots were found to be non-linear having upward and downward segment signify non-linear relationship between X_1 and X_2 variables. It was determined from the contour that maximum PDE (50%) could be obtained with X_1 range at – 1 level to – 0.6 level and with X_2 range at – 0.2 level to 0.2 level.

4.3.2.3 ANN structure

A multi-layer feed forward back-propagation network using Levelberg-Marquardt's learning rule was used to predict PDE of the liposomal formulations. Three causal factors corresponding to different levels of the volume of aqueous phase (X_1), HSPC/DSPG [negative charge] (X_2), and HSPC/Cholesterol (X_3) were used as each unit of the input layer in the ANN. PDE were used as output layer. The output layer was composed of one response variable, Y, Percent drug entrapment (PDE). A set of PDE and causal factors was used as tutorial data for ANN and fed into a computer.

Several training sessions were conducted with different numbers of nodes of hidden layer and training times in order to determine the optimal ANN structure. For selecting the number of hidden nodes, we started of with 1 hidden node and we gradually increased the number of nodes until a network of least mean squared error was attained. Increase in number of nodes led to decrease in least mean squared error. The learning period was completed when minimum root mean square (RMS) was reached.

$$RMS = [\sum (y_i^p - y_i^m)^2 / n]^{1/2} \quad (5)$$

Where y_i^p is experimental (observed) response, y_i^m is calculated (predicted) response, and n is number of experiments. The RMS reached after the training was 0.0000354, which is found to be minimum. Further increase in hidden nodes produced high error, when the network was validated with another set of test data (table 4.14). Figure 4.3 shows a representative plot of r^2 values for an ANN model prediction performance as a function of number of nodes in the hidden layers. In this case, an ANN with 9 nodes in the hidden layer resulted in slope and r^2 values that are closest to 1.0.The student t'test carried out between the predicted results (t value = 0.3611) from the ANN and the experimental results showed no statistically significant difference between them. The normalized error (NE) between the predicted and experimental response variables was employed as an evaluation standard between ANN and MLR. The NE value observed with the optimal ANN structure was 0.0211, while it was 0.0658 in case of second order polynomial equation (MLR).

4.3.2.4 Comparison of ANN and MLR

Both ANN and MLR visualized similar results and their predictions regarding the PDE coincided very well. To check the accuracy of these predictions, we prepared experimentally six liposomal formulations by random selection of causal factors. Experimental results were comparable to the predicted results (table 4.14). Data analysed using paired students t' test revealed that there was no statistically significant difference between the experimental results and the predicted results of ANN (t = 0.3611) and MLR (t = 0.3451). A close look of both ANN and MLR reveals following facts. The normalized error obtained from ANN was less, compared to the multiple

regression analysis shows the higher accuracy in prediction. ANN can easily handle more input variables and extremely helpful when the numbers of experiments are more, but in case of MLR a large number of input variables lead to a polynomial with many coefficients that involves tedious computation. Another major advantage with ANN is the flexibility to work with the theoretical data for better prediction, but MLR does not accommodate theoretical or historical data.

4.3.3 OPTIMIZATION OF THE PREPARATION OF CONVENTIONAL LIPOSOMES OF DNA

To begin with, liposome formulation was optimised before proceeding for steric stabilization. Accordingly conventional anionic and cationic liposomes containing DNA were prepared initially followed by introduction of the agents, responsible for steric stabilization, into these formulations. The freeze dried empty liposome was selected for the preparation of vesicles of DNA as it is simple and reproducible. HSPC was taken as the chief compound of the liposomal membrane due to its comparative ease of availability and low cost over the other high melting lipids such as distearoyl phosphatidyl choline and dipalmitoyl phosphatidyl choline. A 250ml round bottomed flask was selected based on the optimum surface area requirement for the volume of lipid mixture used in the batch. A mixture of chloroform and methanol in a ratio of 2:1 by volume was used to dissolve the lipid mixture because solubility of these lipids is more in this solvent blend in comparison to their solubility in the individual solvents (New 1990). 10ml of this solvent system was found suitable to dissolve the lipid mixture based on the rate of formation of uniform film of satisfactory thickness. The speed of rotation of the rotary evaporator was kept at a maximum of 120rpm because rapid rotation increases the surface area for evaporation of the solvent thereby reducing the time required for the process (New, 1990). Uniformity of heating during evaporation by use of a water bath at 60°C was found to be a critical factor for uniform transparent film formation. This is evident from the fact that the film obtained at 37°C ± 2°C showed opaque patches due to non-uniformity of evaporation. Such films were also found to be difficult to hydrate completely. When the water

bath was used, at higher temperatures (≅ 50°C ± 2°C) even though gave uniform films, showed opaque deposits at certain places. Temperatures in the vicinity of 60°C ± 2°C gave even films which were transparent in all regions. Vacuum was maintained at about 20 inch of Hg by means of nitrogen introduction via a bleed valve. Lower values of vacuum (≅5 inch of mercury) increased the time of dry film formation.

Upon evaporation of the lipid solution under the above conditions the dry residue appeared first in about 5 - 10min. The uniform, transparent film formed subsequently was allowed to dry for a further 10 - 15min time period under lower vacuum. Drying for longer period of time rendered flaky film, which could not dispersed easily by hydration. A possible reason for this type of behaviour may be that minute traces of solvent are required for proper hydration and dispersion. This solvent subsequently gets removed from the system after dispersion.

The drying step was followed by hydration by the addition of 9% sucrose aqueous solution, which, apart from acting as hydration medium, also assisted in film removal and dispersion. In cases where some film remained adhering to the walls of the flask, a few glass beads (0.3cm diameter) was introduced into the flask and rotated gently to effect film removal and uniform dispersion. Hydrated lipid film in suspension form was sonicated using a probe sonicator. The probe sonicator was found suitable over bath sonicator for small sample size in imparting the energy required for particle size reduction (up to (≅1μm) at 60°C. The time of sonication was optimised for 15minutes. The suspension after sonication was allowed to stand undisturbed at room temperature for a specified time period for annealing. The liposomal suspension was then extruded through 0.2μm polycarbonate filters to achieve a desired particle size of less than 200nm. The liposomal suspension was then freeze dried at -20°C overnight and lyophilised for 24h. To the freeze dried empty liposomal powder, required weight ratio of DNA (0.77%w/w) was added and incubated for 15-30minutes at 60°C and the separation of unentrapped drug from the liposomes was carried out by ficoll gradient centrifugation at 20,000rpm for 30min at 0°C. Results indicated that ficoll gradient centrifugation was quite effective for separation

of free DNA from their respective liposomes. The results of the optimized batches of DNA containing liposomes with better entrapment efficiency were shown in table 4.24.

4.3.3.1 Conditions for preparation of DNA containing liposomes

The following conditions were optimised for DNA containing liposomes.

A. Composition of the lipid mixture

Trials were initiated for the preparation of conventional (anionic) liposomes (DL-1 –DL-15) with a molar ratio of 1:0.2:0.3 PC: DSPG: Chol and DNA (0.55%w/w of lipid) (DL-1, Entrapment efficiency = 22.32% ± 0.62, table 4.16). Trials were initiated with a molar ratio of 1:0.2:0.3 PC: DSPG: Chol and DNA (0.77%w/w of lipid) (DL-2, Entrapment efficiency = 26.35% ± 0.89, table 4.16). Increase in the weight ratio of DNA significantly enhanced the percentage of DNA entrapped (Calculated $t^{\prime}$= 6.43, tabulated t'= 2.78, at 4 degree of freedom, P= 0.001). Increase in the proportion of cholesterol in the lipid mixture (DL-6, Entrapment efficiency = 35.26% ± 1.23, table 4.16) significantly enhanced the percentage of DNA entrapped (Calculated $t^{\prime}$= 10.16, tabulated t' = 2.78, 4 degree of freedom, P= 0.0002). Being amphipathic molecule, cholesterol inserts into the membrane with its hydroxyl group oriented towards the aqueous surface and the aliphatic chain aligned parallel to the acyl chains in the centre of the bilayer. The 3-β hydroxyl group of cholesterol is positioned level with very little vertical freedom of movement. The presence of the rigid steroid nucleus along side the first ten or so carbons of the phospholipid chain has the effect of reducing the freedom of motion of these carbons while, at the same time, creating space for a wide range of movement for the remaining carbons towards the terminal end of the chain (New, 1990b). The films formed in the further increase in the cholesterol content were not smooth even when the temperature of the bath was increased and hence higher cholesterol content was not tried further.

B. Hydration Volume

Increase in the hydration volume from 1.5ml (DL-13, entrapment efficiency = 49.02% ± 0.68, table 4.16) to 2ml (DL-15, drug entrapment = 47.03% ± 0.95, table 4.16) led to a significant decrease in the entrapment efficiency as

evaluated by student t' test (Calculated t' = 2.9, tabulated t' = 2.78, at 4 degree of freedom and p=0.02)

C. Hydration time

Increase in the hydration time after the addition of aqueous DNA solution to the freeze dried empty liposomes from 15 min (DL14, entrapment efficiency = 47.23% ± 0.79, table 4.16) to 30 min (DL15, drug entrapment = 47.03 ± 0.95, table 4.16) did not show any significant increase in the entrapment efficiency as evaluated by student t' test (Calculated t' = 0.32, tabulated t' = 2.78, at 4 degree of freedom, α=0.05)

4.3.4 STERICALLY STABILIZED LIPOSOMES OF LEUPROLIDE ACETATE AND DNA USING PEG DERIVATIVES

Sterically stabilized liposomes of leuprolide acetate and DNA were then prepared with an aim of altering the pharmacokinetics of peptide and DNA in the body. Methoxy polyethylene glycol 5000 (mPEG5000) activated with cyanuric chloride-phosphatidyl ethanolamine conjugate (mPEG5000-CC-PE) has been investigated as a sterically stabilizing agent. Figure 4.14 outlines the synthesis of this agent. Potassium carbonate was used in place of sodium carbonate suggested by Abuchowski and coworkers (Abuchowski et. al., 1977) due to its greater water uptake capacity. A molar ratio of 1:3 of mPEG5000 to cyanuric chloride was found to give the desired product (methoxy polyethylene glycol 5000 activated with cyanuric chloride, mPEG-CC) as evidenced by quantitative UV spectroscopy, IR and ^{13}C-NMR spectra (figures 4.9-4.13 respectively). Reaction of this product with phosphatidyl ethanolamine using the method suggested by Blume and Cevc (Blume and Cevc, 1990) gave the agent methoxy polyethylene glycol 5000 activated with cyanuric chloride-phosphatidyl ethanolamine conjugate, mPEG5000-CC-PE. The UV, IR and ^{13}C-NMR spectra of this product are shown in figures 4.16-4.18 respectively. Quantitative UV spectroscopy confirmed the synthesis of this reagent. The IR and ^{13}C-NMR show evidence of the -CH_2 group of the mPEG5000. Other groups are not prominent primarily because of the very small proportion of these groups in the molecule. The concentration of polymer necessary for steric stabilization was optimized using the electrolyte induced flocculation test. This is a standard test to

investigate whether the dispersed system is sterically stabilized or not. The physical stability of a dispersed system is mainly dependant upon the competitive forces of attraction (van der Waals forces) and repulsion (either electrostatic repulsive forces or steric stabilizing barrier or both) (Lin et. al., 1994). In addition to the electrostatic and van der Waals forces, a number of other interactions (depletion and steric interactions) could play an important role in colloid stability (Tadros and Vincent, 1983). Steric stabilization occurs due to the presence of steric barriers from the adsorbed non ionic molecules on particles that prevent the particles from coming close enough to allow van der Waals attractive forces between the particles to dominate (Tadros, 1986).

The conventional liposomes are predominantly electro statically stabilized. Addition of electrolyte will be sufficient to compress the electrostatic double layer surrounding the liposomes and results in the aggregation leading to flocculation with a corresponding increase in optical turbidity. But if the liposomes (steric stabilized liposomes) are mainly stabilized by hydrated steric stabilizing barriers which is produced by the surface modification due to the polymer incorporated, the system should be stable even if the electrostatic double layers have been compressed. The flocculation might occur even in steric stabilized liposomes after addition of certain amount of electrolyte, due to dehydration of the hydrated steric stabilized barriers. Thus, if the optical turbidity of the liposomal dispersion is measured at 400 nm after adding different concentrations of electrolyte, the change in optical turbidity can be used to ascertain whether the liposomes are sterically stabilized or not. The lipid concentration was kept at approximately 1mg/ml and sucrose, a density-neutralizing agent, at a concentration of 16.7% w/v, was included in the electrolyte solution to prevent settling of liposomes. The scattering of the samples increase by the inverse 4^{th} power of the wavelength of the incident light, hence a lower wavelength (400 nm) was used for measurements (Betagiri et al, 1993).

In all the cases, 1-4 % by molar ratio of lipid was insufficient to provide protection against electrolyte induced flocculation probably due to insufficient coverage at the surface of the prepared liposomes. 5% - 6% on molar basis of this reagent was found to be sufficient for providing steric

stability to the liposomes of leuprolide acetate and DNA (Tables 4.19 and 4.21 and figures 4.27 and 4.30 respectively). This, once again, points to the prominence of liposome composition in steric stabilization studies.

Methoxy polyethylene glycol 2000 (mPEG2000) activated with cyanuric chloride-phosphatidyl ethanolamine conjugate (mPEG2000-CC-PE) has been investigated as a sterically stabilizing agent. Figure 4.22 outlines the synthesis of this agent. Potassium carbonate was used in place of sodium carbonate suggested by Abuchowski and coworkers (Abuchowski et. al., 1977) due to its greater water uptake capacity. A molar ratio of 1:3 of mPEG5000 to cyanuric chloride was found to give the desired product (methoxy polyethylene glycol 2000 activated with cyanuric chloride, mPEG-CC) as evidenced by quantitative UV spectroscopy and IR spectra (figures 4.20 and 4.21 respectively). Reaction of this product with phosphatidyl ethanolamine using the method suggested by Blume and Cevc (Blume and Cevc, 1990) gave the agent methoxy polyethylene glycol 2000 activated with cyanuric chloride-phosphatidyl ethanolamine conjugate, mPEG2000-CC-PE. The UV, IR and ^{13}C-NMR spectra of this product are shown in figures 4.23-4.25 respectively. Quantitative UV spectroscopy confirmed the synthesis of this reagent. The IR and ^{13}C-NMR show evidence of the -CH_2 group of the mPEG2000. Other groups are not prominent primarily because of the very small proportion of these groups in the molecule. In all the cases, 1-4% molar ratio of lipid was insufficient to provide protection against electrolyte induced flocculation probably due to insufficient coverage at the surface of the prepared liposomes. 5% - 6% on molar basis of this reagent was found to provide steric stability to the liposomes of leuprolide acetate and DNA (Tables 4.20, 4.22 and 4.23 and Figures 4.28, 4.31 and 4.32 respectively). The flow chart for the preparation of steric stabilized liposomes containing leuprolide acetate and DNA are shown in figures 4.26 and 4.29 respectively. The results of the optimized batches of leuprolide acetate and DNA containing sterically stabilized liposomes with better entrapment efficiency were shown in table 4.15 and 4.24. The effect of the steric stabilizing agents on entrapment efficiency will be discussed in Chapter 5, Characterization of liposomes.

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