Chapter 4

Preparation of Liposomes

4.1 INTRODUCTION

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, acidic (negatively charged) lipids such as phosphatidyl serine (PS), cardiolipin and Phosphatidic acid (PA) are not preferred components as compared to phosphatidyl choline (PC) because of high costs and the often labile nature of these compounds. Similarly, the use of highly 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 acceptable pharmaceutically.

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 inexpensive and relatively saturated lipids such as egg PC 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

Methotrexate was gifted by Sun Pharma Advanced Research Centre, Vadodara and Tamoxifen citrate and Cytarabine were gifted by Dabur Research Foundation, Ghaziabad.

4.2.2 REAGENTS

Egg Phosphatidyl choline, phosphatidylethanolamine (PE), methoxy polyethylene glycol (av. mol. wt. 2000) methoxy polyethylene glycol (av. mol. wt. 5000) and methoxy polyethylene glycol (av. mol. wt. 5000) imidazoline carbonyl 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 and iodine were purchased from Qualigens fine chemicals, Mumbai. DL-α-tocopherol was purchased from E.merck India ltd., Mumbai.

4.2.3 APPARATUS

Rotary evaporator with vacuum pump 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, Japan), Avance DPX 200 dual probe ^{13}C-NMR (Bruker Inc., Switzerland), Shimadzu Infrared spectrophotometer (Shimadzu corporation, Japan).

4.2.4 SOLUTIONS

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 (0M to 2M) 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 CONVENTIONAL LIPOSOMES CONTAINING METHOTREXATE

Multi lamellar vesicles (MLVs) of methotrexate were prepared by the lipid film hydration technique (New, 1990). Briefly, 83mg of egg phosphatidyl choline, 17mg of cholesterol and 5mg of methotrexate (molar ratio 1:0.4: 0.1) were dissolved in a mixture of chloroform and methanol (ratio 2:1 by volume) in a 250ml round bottom flask. To this, 0.5ml of 0.1% w/v DL- DL-α-tocopherol solution in chloroform was added. The flask was rotated in the rotary flash evaporator at 100rpm for 20min in a thermostatically controlled water bath at 37°C under vacuum (600mm of mercury) with nitrogen as bleed. The thin dry lipid film formed was hydrated using 2ml of distilled water and the flask was rotated once again, at the same speed as before at ambient temperature for 30min. The liposomal suspension so formed was then transferred to a suitable glass container and sonicated for 30min using a probe sonicator (model –RR-120, Ralsonics, Mumbai) in an ice bath for heat dissipation. The sonicated dispersion was then allowed to stand undisturbed for about 2h at room temperature for the annealing to be completed. The unentrapped drug was removed from the liposomal suspension by centrifugation at 10,000rpm for 30min 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 resuspended in fresh distilled water. The process was repeated thrice to remove the unentrapped drug. The supernatant fractions thus collected were pooled and analysed for drug content. The multilamellar vesicles formed were sequentially extruded through 2-stacked Nuclepore polycarbonate track-etch membrane filters (Whatman Inc. New Jersey, USA) of 1μm and 0.2μm pore size for 5 times at 40°C. The liposomal suspension was freeze dried overnight at -20°C, lyophilised for 24h 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 major process parameters were optimised using the percentage drug entrapment as the response parameter. The observations of the optimization process are tabulated in Table 4.1. Comparison of these liposomes was made with sterically stabilized liposomes containing methotrexate using the electrolyte induced flocculation test described later in this chapter. The results of this test for conventional liposomes containing methotrexate are shown in Figure 4.2 and Table 4.2.

Table 4.1 Optimization of parameters for preparation of conventional liposomes containing methotrexate

Batch No.	Molar ratio (PC: Chol: MTX)	Hydration volume (ml)	Hydration time (min)	Sonication time (min)	Drug entrapment (% ± S.E.)*
ML1	1: 0.2: 0.1	1	15	15	70.1 ± 1.49
ML2	1: 0.2: 0.1	1	30	15	72.6 ± 1.68
ML3	1: 0.2: 0.1	1	60	30	68.4 ± 0.57
ML4	1: 0.2: 0.1	2	15	30	75.3 ± 1.65
ML5	1: 0.2: 0.1	2	30	30	75.6 ± 0.87
ML6	1: 0.2: 0.1	3	30	30	78.2 ± 0.36
ML7	1: 0.4:0.1	1	15	15	84.2 ± 1.58
ML8	1: 0.4:0.1	1	30	15	89.1 ± 0.89
ML9	1: 0.4:0.1	1	60	15	87.5 ± 1.54
ML10	1: 0.4:0.1	2	15	30	90.2 ± 0.23
ML11	1: 0.4:0.1	2	30	30	95.8 ± 1.42
ML12	1: 0.4:0.1	3	30	30	95.2 ± 1.56
ML13	1: 0.6: 0.1	2	15	30	84.6 ± 1.01
ML14	1: 0.6: 0.1	2	30	15	88.2 ± 1.66
ML15	1: 0.6: 0.1	3	30	30	89.0 ± 0.41

*n=3

Figure 4.1 Flowchart for the preparation of conventional liposomes containing methotrexate

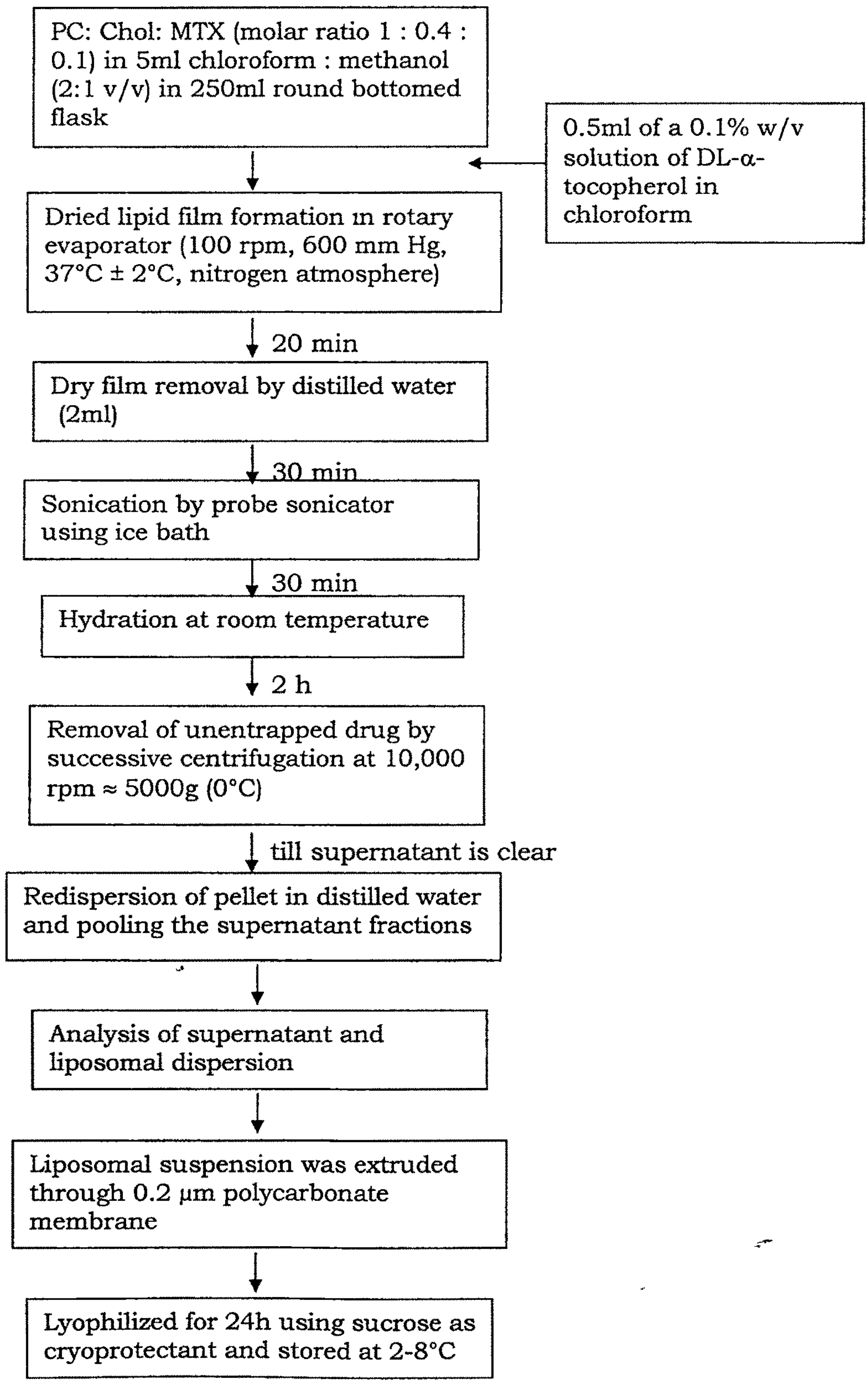
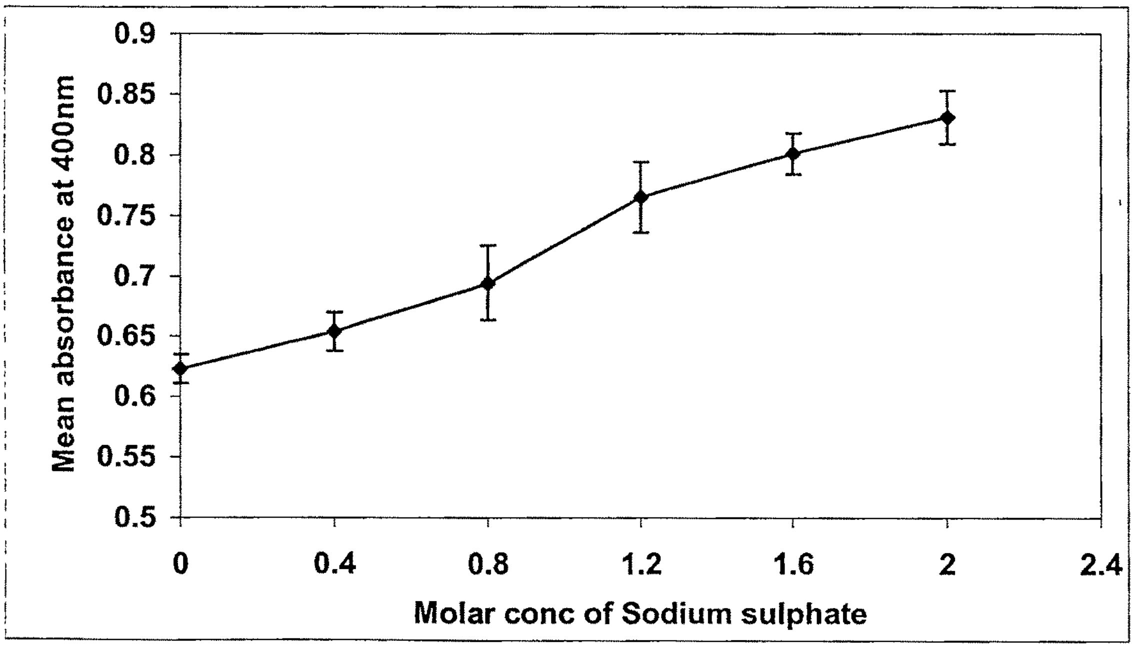


Table 4.2 Results of electrolyte induced flocculation test on conventional liposomes containing methotrexate

Concentration of sodium sulphate (in M)	Mean absorbance ± S.E. at 400nm
0.0	0.612±0.012
0.4	0.654±0.023
0.8	0.694±0.021
1.2	0.765±0.036
1.6	0.801±0.018
2.0	0.831±0.041

Figure 4.2 Results of electrolyte induced flocculation test on conventional liposomes containing methotrexate



4.2.6 PREPARATION OF CONVENTIONAL LIPOSOMES CONTAINING TAMOXIFEN CITRATE

Multi lamellar vesicles (MLVs) of tamoxifen citrate were prepared by the lipid film hydration technique similar to the preparation of conventional liposomes containing methotrexate except the composition of drug: lipid ratio. In case of conventional liposomes containing tamoxifen citrate, 100mg of egg phosphatidyl choline, 34mg of cholesterol and 5mg of tamoxifen citrate (molar ratio 1:0.67: 0.067) were used for lipid film formation. Other procedure remains the same. A flowchart depicting the above process is shown in Figure 4.3. The major process parameters were optimised using the percentage drug entrapment as the response parameter. The observations of the optimization process are tabulated in Table 4.3. Comparison of these liposomes was made with sterically stabilized liposomes containing tamoxifen citrate using the electrolyte induced flocculation test described later in this chapter. The results of this test for conventional liposomes containing tamoxifen citrate are shown in Figure 4.4 and Table 4.4.

Table 4.3 Optimization of parameters for preparation of conventional liposomes containing tamoxifen citrate

Batch No.	Molar ratio (PC: Chol: TMX)	Hydration volume (ml)	Hydration time (min)	Sonication time (min)	Drug entrapment (% ± S.E.)*
TL1	1: 0.5: 0.1	1	30	15	62.3 ± 1.48
TL2	1: 0.5: 0.1	1	60	30	61.6 ± 1.03
TL3	1: 0.5: 0.1	2	30	15	65.2 ± 1.67
TL4	1: 0.5: 0.1	3	60	30	67.3 ± 1.34
TL5	1: 0.33: 0.067	1	30	15	76.6 ± 1.57
TL6	1: 0.33: 0.067	2	60	30	78.9 ± 0.26
TL7	1: 0.33: 0.067	3	30	15	77.2 ± 1.58
TL8	1: 0.33: 0.067	3	60	30	79.1 ± 1.49
TL9	1: 0.67:0.067	1	30	15	86.5 ± 0.16
TL10	1: 0.67: 0.067	1	60	30	86.2 ± 0.78
TL11	1: 0.67: 0.067	2	30	30	89.0 ± 0.66
TL12	1: 0.67:0.067	3	60	30	88.8 ± 0.29
TL13	1: 0.25: 0.05	1	30	15	86.6 ± 1.01
TL14	1: 0.25: 0.05	1	60	30	87.5 ± 1.11
TL15	1: 0.25: 0.05	2	30	30	89.0 ± 0.91
TL16	1: 0.25: 0.05	2	60	30	88.7 ± 1.49

*n=3

Figure 4.3 Flowchart for the preparation of conventional liposomes containing tamoxifen citrate

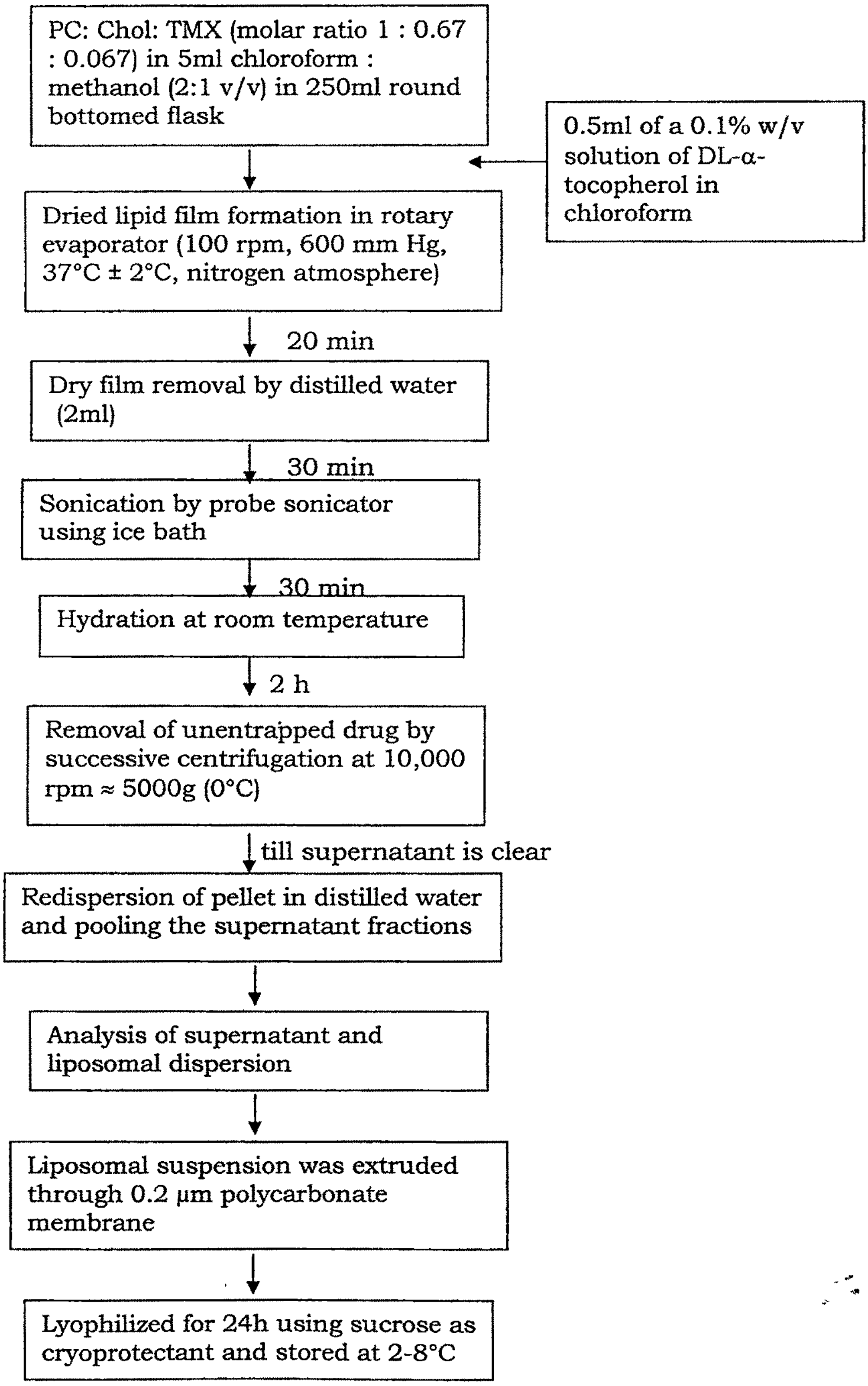
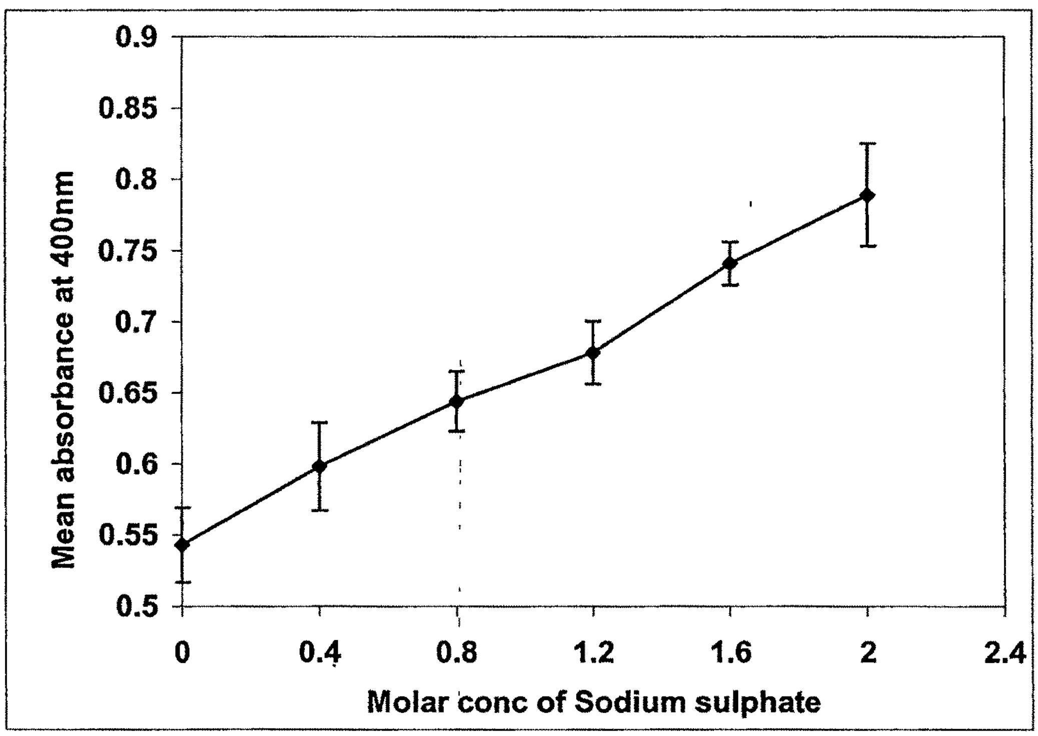


Table 4.4 Results of electrolyte induced flocculation test on conventional liposomes containing tamoxifen citrate

Concentration of sodium sulphate (in M)	Mean absorbance ± S.E. at 400 nm
0.0	0.543 ± 0.026
0.4	0.598 ± 0.031
0.8	0.644 ± 0.021
1.2	0.678 ± 0.022
1.6	0.741 ± 0.015
2.0	0.789 ± 0.036

Figure 4.4 Results of electrolyte induced flocculation test on conventional liposomes containing tamoxifen citrate



4.2.7 PREPARATION OF CONVENTIONAL LIPOSOMES CONTAINING CYTARABINE

3^3 factorial design was used to investigate the combined influence of three independent variables in the preparation of Cytarabine liposomes by thin film hydration method (Subramanian and Murthy, 2003). 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. The formulation variables, Drug (Cytarabine) / Lipid [Phosphatidyl choline (PC) and Cholesterol (Chol)] molar ratio (X_1), PC / Chol in percent ratio of total lipids (X_2) and the volume of hydration medium (X_3) were found to be contributing a lot to the percent drug entrapment. Hence these three parameters were selected as the independent variables, whereas percent drug entrapment (PDE) within the liposomes was selected as dependent variable.

Based on the factorial design, twenty-seven batches of cytarabine liposomes were prepared by thin film hydration method (New R.R.C, 1990) according to the experimental conditions as shown in the Table 4.5. PC, Chol and DL - α - tocopherol (0.5ml of 0.1% w/v solution in chloroform) were dissolved in 5ml of chloroform and methanol (2:1 by volume ratio) in a 250ml round bottom flask. The flask was rotated in the rotary flash evaporator at 100rpm for 20min in a thermostatically controlled water bath at 37°C under vacuum (600mm of mercury) with nitrogen as bleed. To the thin dry lipid film formed, drug solution (5mg of drug dissolved in distilled water [hydration medium]) was added and the flask was rotated again at the same speed and temperature as before but without vacuum for 30min for lipid film removal and dispersion. The liposomal suspension so formed was then transferred to a suitable glass container and sonicated for 30min using a probe sonicator (model –RR-120, Ralsonics, Mumbai) in an ice bath for heat dissipation. The sonicated dispersion was then allowed to stand undisturbed for about 2h at room temperature for the annealing to be completed. The unentrapped drug was removed from the liposomal suspension by centrifugation at 10,000rpm for 30min 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 resuspended in fresh distilled water. The process was repeated thrice to remove the unentrapped drug. The supernatant fractions thus collected were pooled and analysed for drug content. The multilamellar vesicles formed were sequentially extruded through 2-stacked Nuclepore polycarbonate track-etch membrane filters (Whatman Inc. New Jersey, USA) of 1 μm and 0.2 μm pore size for 5 times at 40°C. The liposomal suspension was freeze dried overnight at -20°C, lyophilised for 24h 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 4.5. The major process parameters were optimised using the percentage drug entrapment as the response parameter. The mean PDEs of all the twenty-seven batches are shown in Table 4.5. Comparison of these liposomes was made with sterically stabilized liposomes containing cytarabine using the electrolyte induced flocculation test described below. The results of this test for conventional liposomes containing cytarabine are shown in Figure 4.6 and Table 4.6.

4.2.8 ELECTROLYTE INDUCED FLOCCULATION TEST

Sodium sulphate solutions ranging from 0M to 2.0M were prepared in 16.7% sucrose solution. An appropriate volume of liposome formulation, which gives a final concentration of 1mg/ml of lipid, was taken and the volume was made up to 5ml using the sodium sulphate solutions of various concentrations. The resulting dispersions were mixed and the absorbances were measured within 5min at 400nm on Shimadzu 1601 UV- Visible Spectrophotometer against respective blank (Subramanian and Murthy, in Press).

Table 4.5 Optimization of preparation of conventional liposomes containing cytarabine

Batch No.	Drug: Lipid (molar ratio) X_1	PC: Chol (% of total lipids) X2	Hydration volume(ml) X_3	Drug entrapment (% ± S.E.)*
CL1	1: 7	50: 50	1	43.5 ± 1.46
CL2	1: 10	50: 50	1	52.1 ± 2.24
CL3	1: 13	50: 50	1	77.8 ± 2.14
CL4	1: 7	60: 40	1	63.1 ± 1.50
CL5	1: 10	60: 40	1	59.6 ± 2.10
CL6	1: 13	60: 40	1	78.6 ± 1.04
CL7	1: 7	70: 30	1	70.5 ± 2.11
CL8	1: 10	70: 30	1	64.1 ± 1.10
CL9	1: 13	70: 30	1	80.4 ± 2.15
CL10	1: 7	50: 50	2	70.4 ± 2.50
CL11	1: 10	50: 50	2	66.9 ± 1.10
CL12	1: 13	50: 50	2	77.2 ± 2.34
CL13	1: 7	60: 40	2	70.6 ± 2.40
CL14	1: 10	60: 40	2	69.2 ± 1.70
CL15	1: 13	60: 40	2	83.5 ± 1.60
CL16	1: 7	70: 30	2	71.3 ± 1.50
CL17	1: 10	70: 30	2	67.8 ± 1.13
CL18	1: 13	70: 30	2	77.4 ± 2.11
CL19	1: 7	50: 50	3	70.1 ± 1.11
CL20	1: 10	50: 50	3	64.2 ± 1.30
CL21	1: 13	50: 50	3	59.2 ± 2.19
CL22	1: 7	60: 40	3	64.5 ± 2.20
CL23	1: 10	60: 40	3	58.7 ± 1.62
CL24	1: 13	60: 40	3	75.4 ± 1.13
CL25	1: 7	70: 30	3	59.7 ± 1.32
CL26	1: 10	70: 30	3	60.2 ±.2.54
CL27	1: 13	70: 30	3	71.3 ± 1.68

* n = 3

Figure 4.5 Flowchart for the preparation of conventional liposomes containing cytarabine

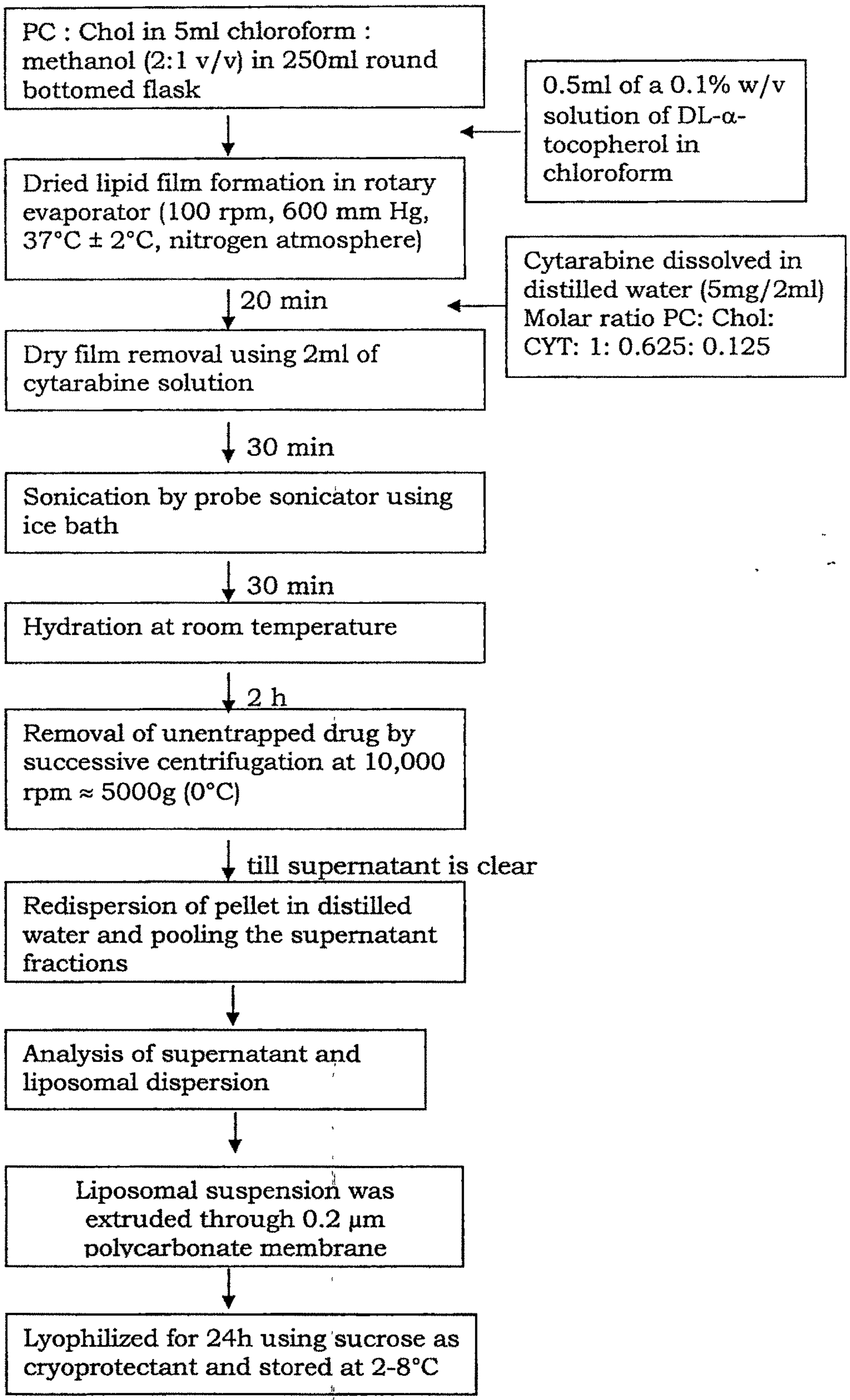
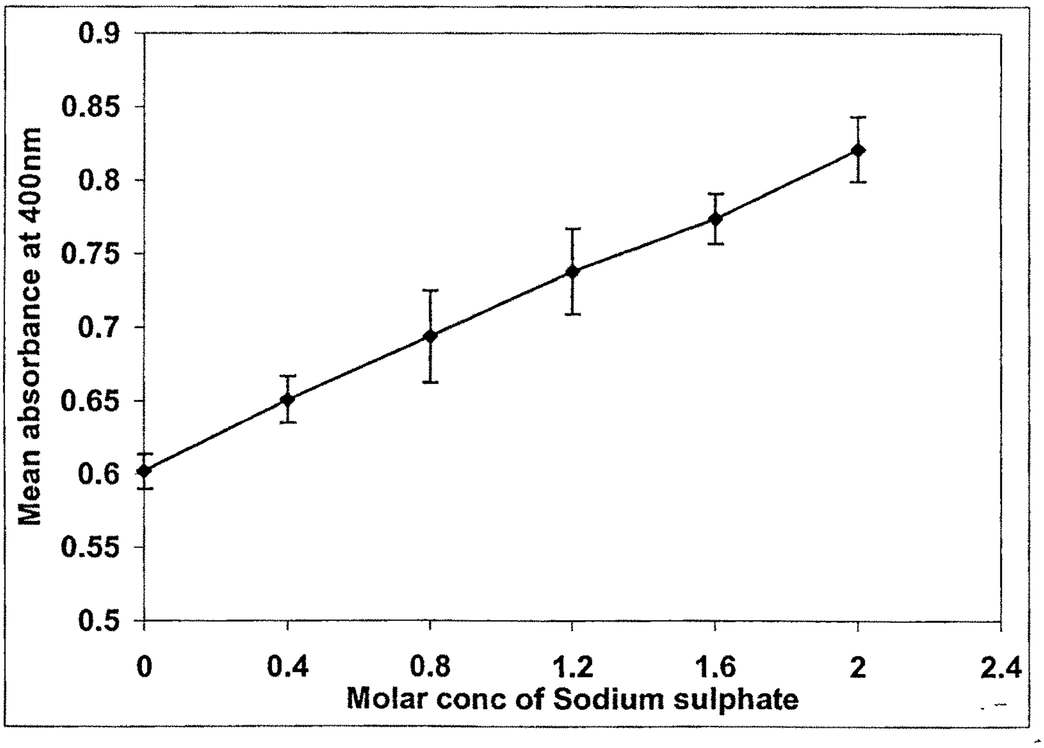


Table 4.6 Results of electrolyte induced flocculation test on conventional liposomes containing cytarabine

Concentration of sodium sulphate (in M)	Mean absorbance ± S.E. at 400nm
0.0	0.602 ± 0.012
0.4	0.651 ± 0.016
0.8	0.694 ± 0.031
1.2	0.738 ± 0.029
1.6	0.774 ± 0.017
2.0	0.821 ± 0.022

Figure 4.6 Results of electrolyte induced flocculation test on conventional liposomes containing cytarabine



4.2.9 PREPARATION OF STERICALLY STABILIZED LIPOSOMES CONTAINING METHOTREXATE, TAMOXIFEN CITRATE AND CYTARABINE USING POLY ETHYLENE GLYCOL DERIVATIZED PHOSPHATIDYL ETHANOLAMINE (MPEG-PE)

4.2.9.1 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, phosphatidyl inositol 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 that quantitatively retain aqueous solutes can be increased, without causing inter-vesicle cross-linking and without conferring net surface charge. This is done 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).

4.2.9.2 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 were 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 stirred magnetically for 24h at room temperature. 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 glycol5000 and mPEG5000-CC in methanol taken on a Shimadzu 1601 UV-Visible spectrophotometer as shown in Figures 4.7, 4.8 and 4.9 respectively. The identity of mPEG5000 activated with cyanuric chloride was ascertained by taking its mid infrared spectrum on a Shimadzu FTIR-8300 spectrophotometer (Figure 4.10) and by taking its

^{13}C-NMR spectrum on Avance DPX 200 dual probe NMR instrument (Figure 4.11).

4.2.9.3 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 4.12. 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 5days 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 4.13. 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 4.14, 4.15 and 4.16 respectively.

4.2.9.4 Preparation of liposomes using mPEG5000-CC-PE

Tables 4.7, 4.8 and 4.9 as well as Figures 4.18, 4.20 and 4.22 present the results of the optimization of the amount of mPEG5000-CC-PE required for steric stabilization of liposomes containing methotrexate, tamoxifen citrate and cytarabine 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 was added to the lipid solution before it was subjected to evaporation for lipid film formation. The amount of mPEG5000-

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, to the electrolyte induced flocculation test which is described earlier in this chapter. The preparation methods are shown as flowcharts (Figures 4.17, 4.19 and 4.21 respectively for liposomes containing methotrexate, tamoxifen citrate and cytarabine).

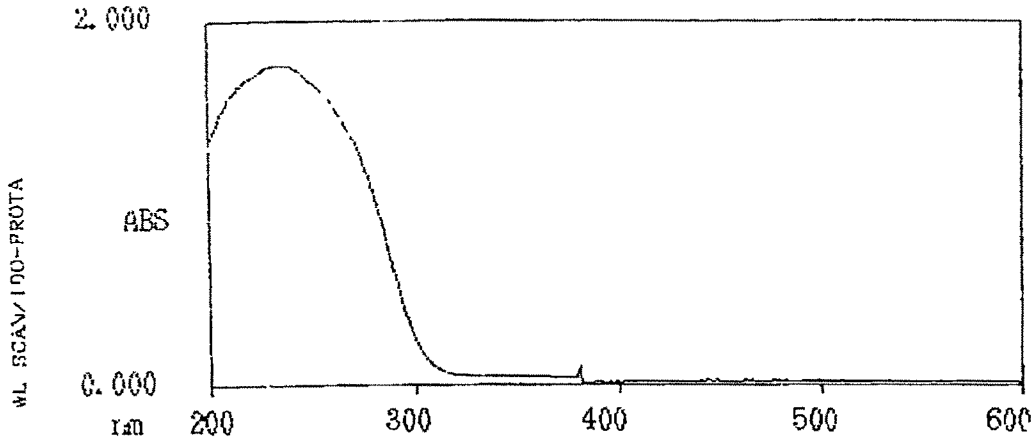


Figure 4.7 Ultraviolet absorptivity scan of cyanuric chloride (CC) in methanol (1mg/ml)

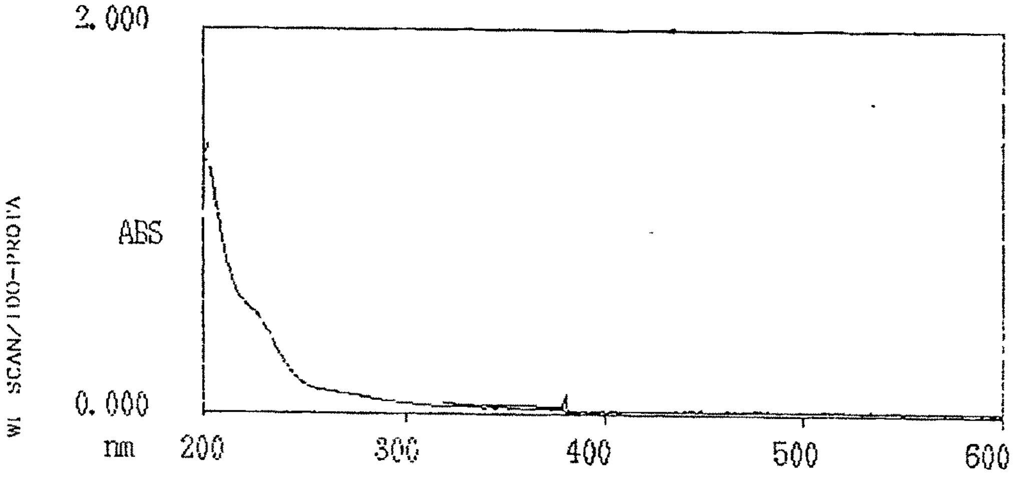


Figure 4.8 **Ultraviolet absorptivity scan of methoxy polyethylene glycol 5000 (mPEG5000) in methanol (1mg/ml)**

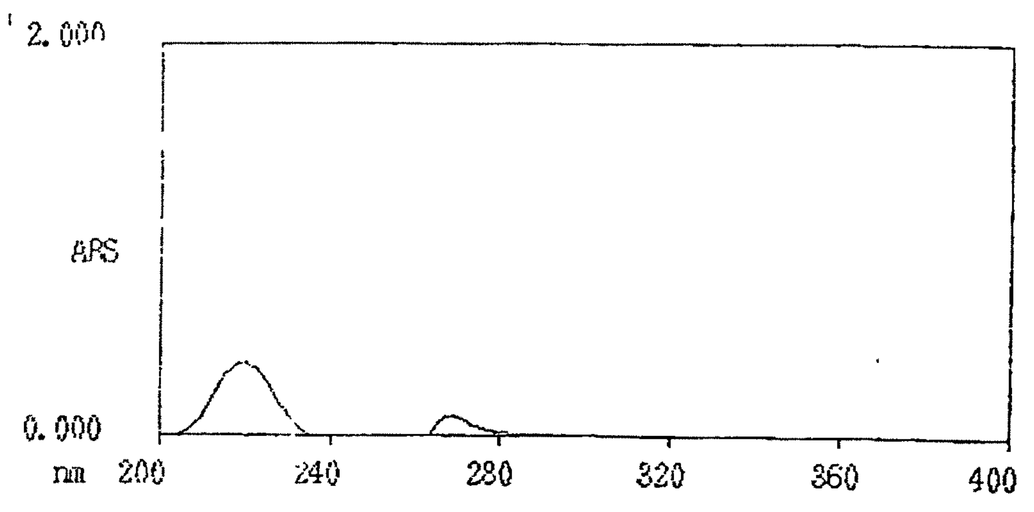


Figure 4.9 **Ultraviolet absorptivity scan of methoxy polyethylene glycol 5000 activated with cyanuric chloride (mPEG5000-CC) in methanol (1mg/ml)**

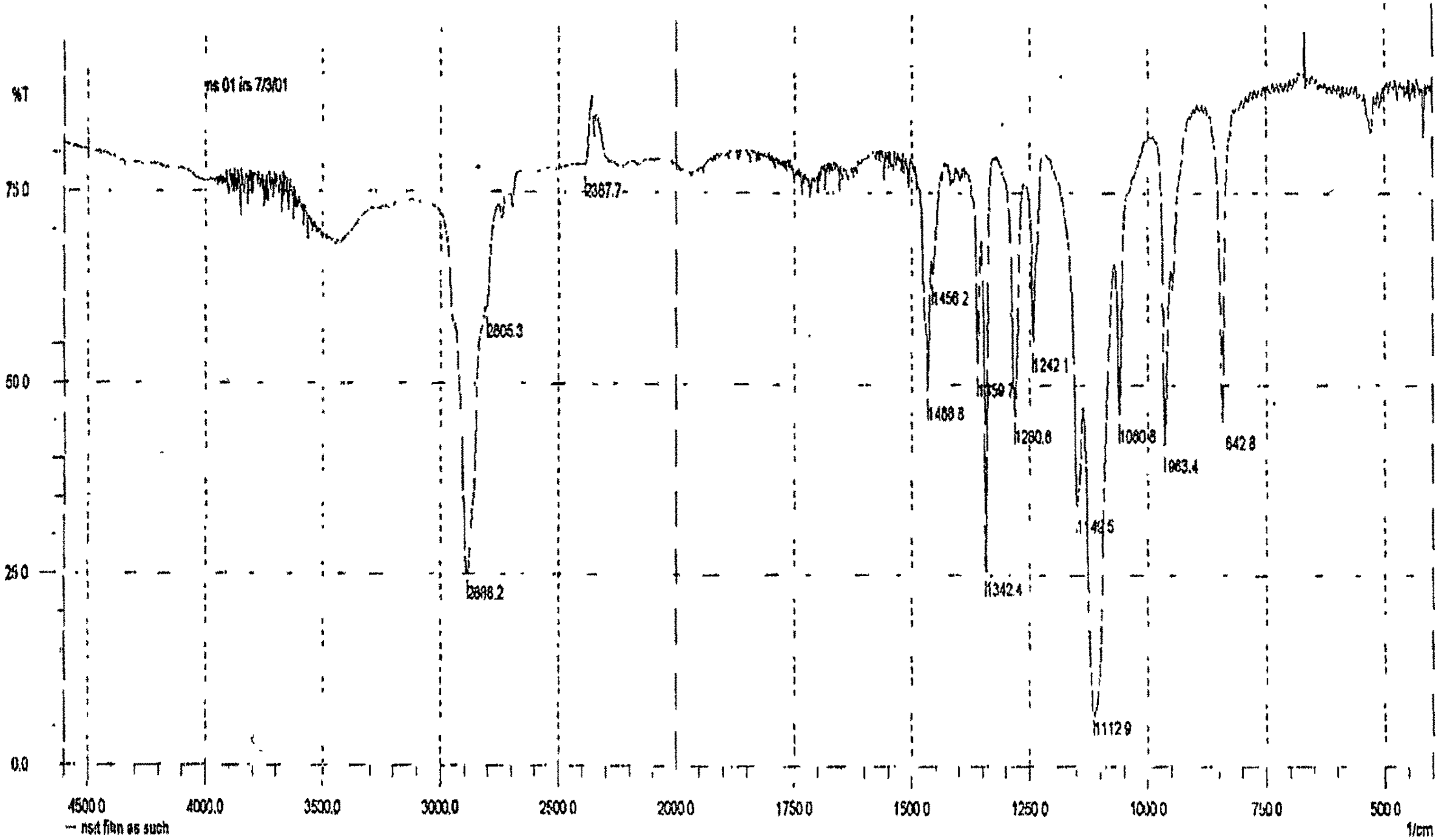


Figure 4.10 **Mid infra-red spectrum of methoxy polyethylene glycol 5000 activated with cyanuric chloride (mPEG5000-CC)**

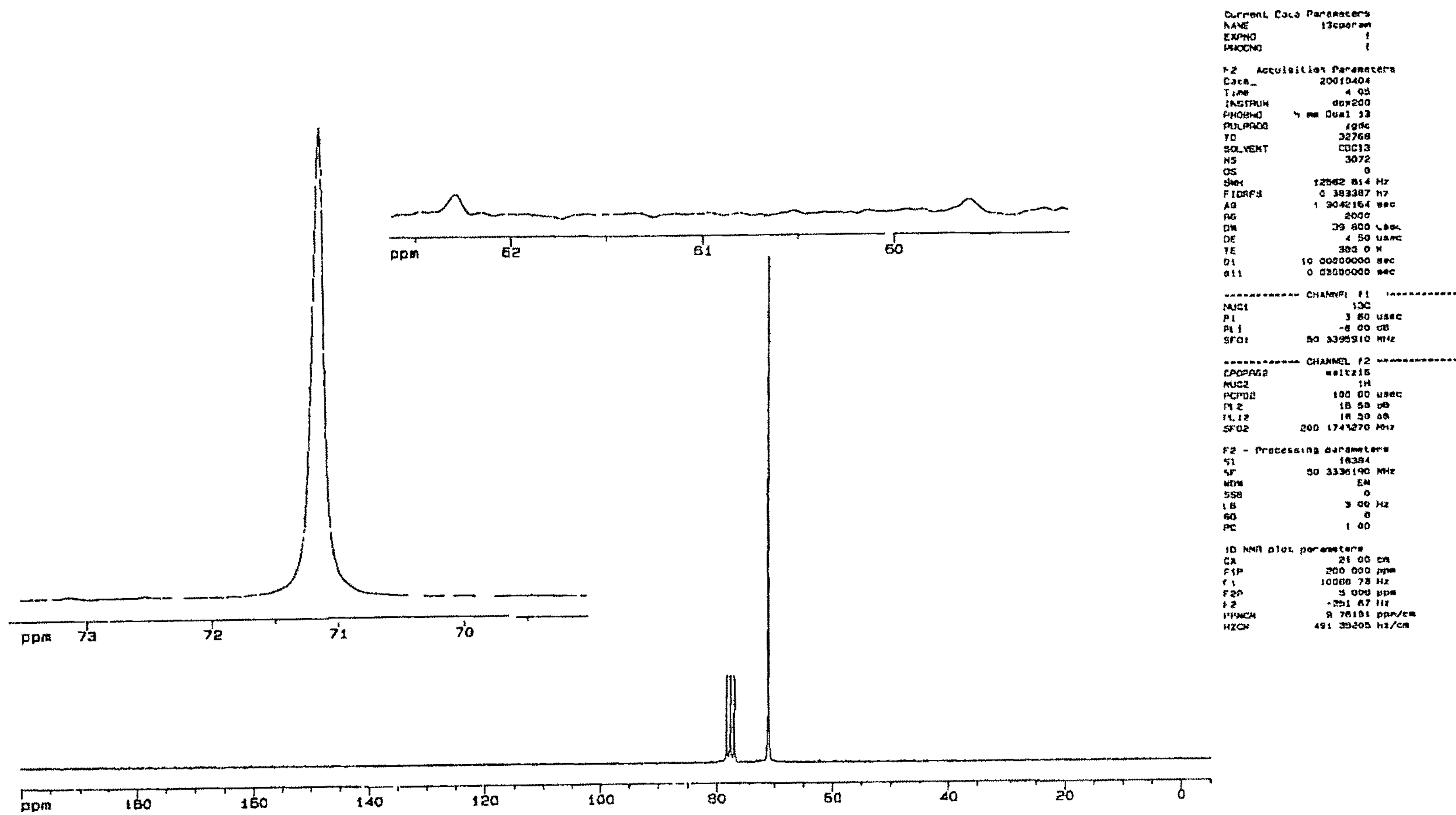
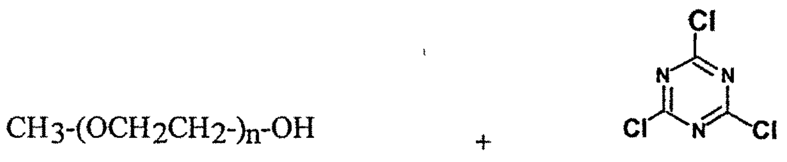


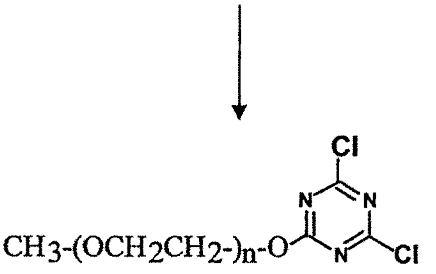
Figure 4.11 ^{13}C-NMR spectrum of methoxy polyethylene glycol 5000 activated with cyanuric chloride (mPEG5000-CC)

Figure 4.12 Reaction scheme for the synthesis of methoxy polyethylene glycol 5000 activated with cyanuric chloride -phosphatidylethanolamine conjugate (mPEG5000-CC-PE)

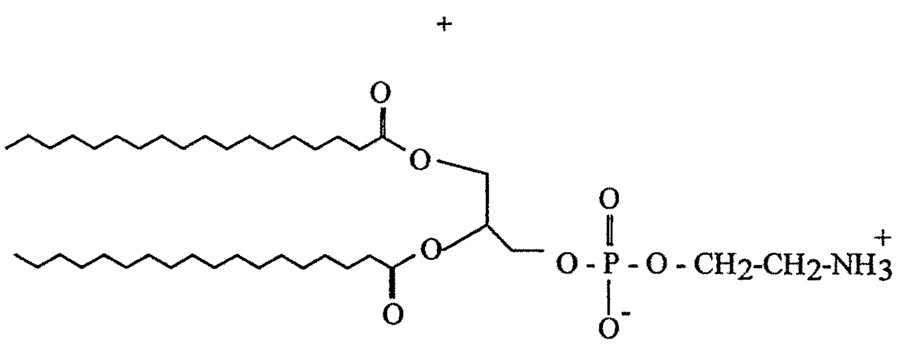


Methoxy Polyethylene glycol (Average mol. wt. 5000, mPEG5000)

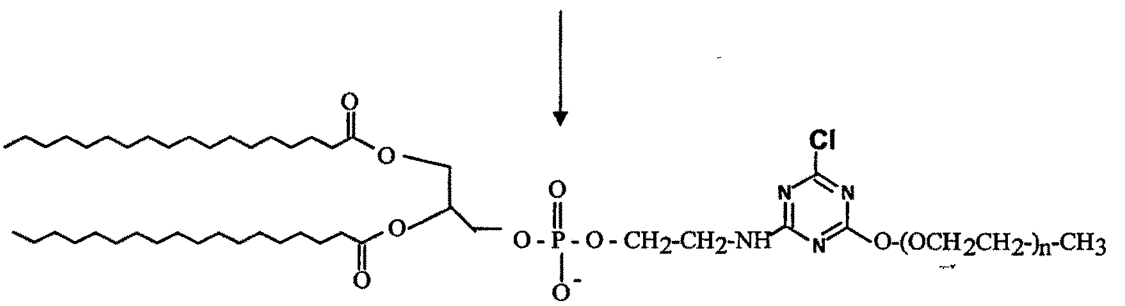
2,4,6-trichloro-s-traizine / cyanuric chloride (CC)



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



Phosphatidylethanolamine



mPEG5000-CC-PE

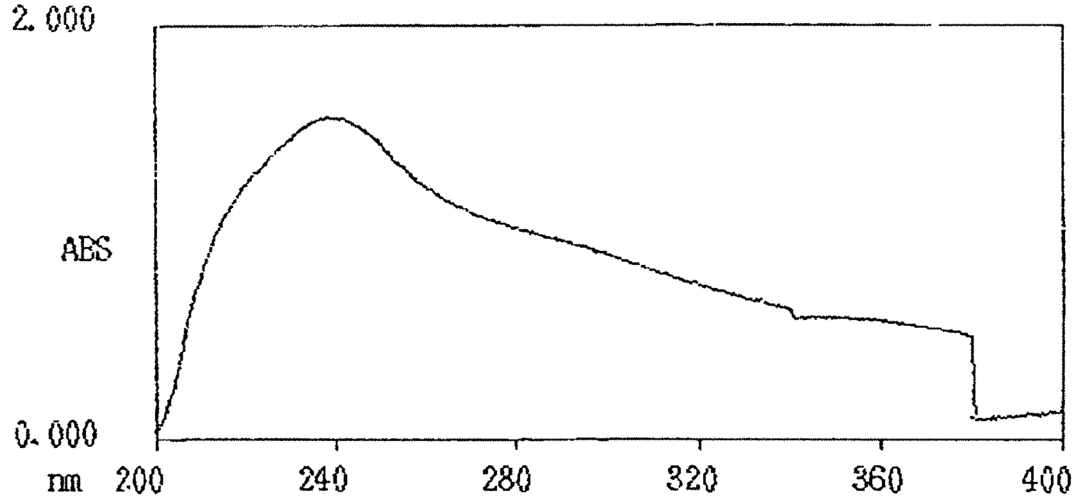


Figure 4.13 Ultraviolet absorptivity scan of Phosphatidyl ethanolamine (PE) in methanol (1 mg/ml)

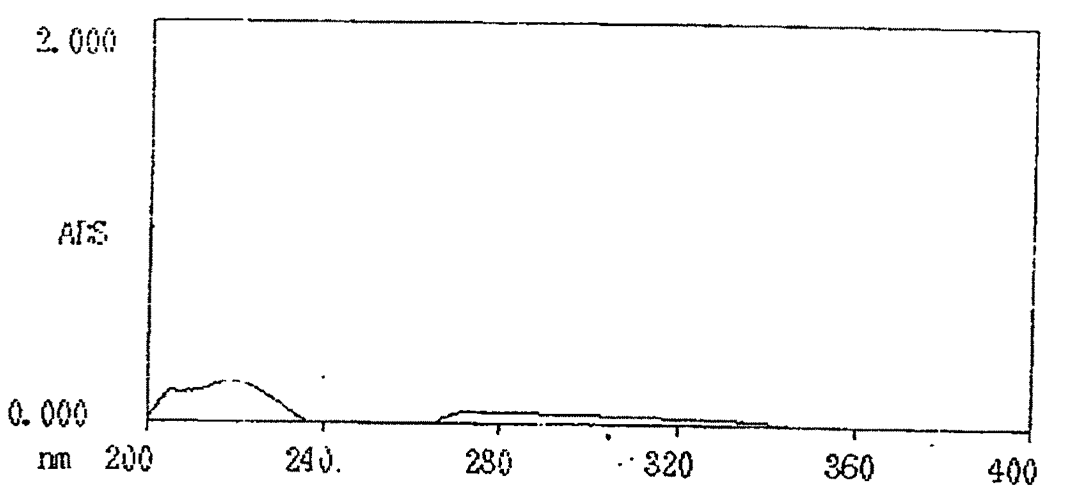


Figure 4. 14 Ultraviolet absorptivity scan of methoxy polyethylene glycol 5000 activated with cyanuric chloride – phosphatidyl ethanolamine conjugate (mPEG5000-CC-PE) in methanol (5mg/ml)

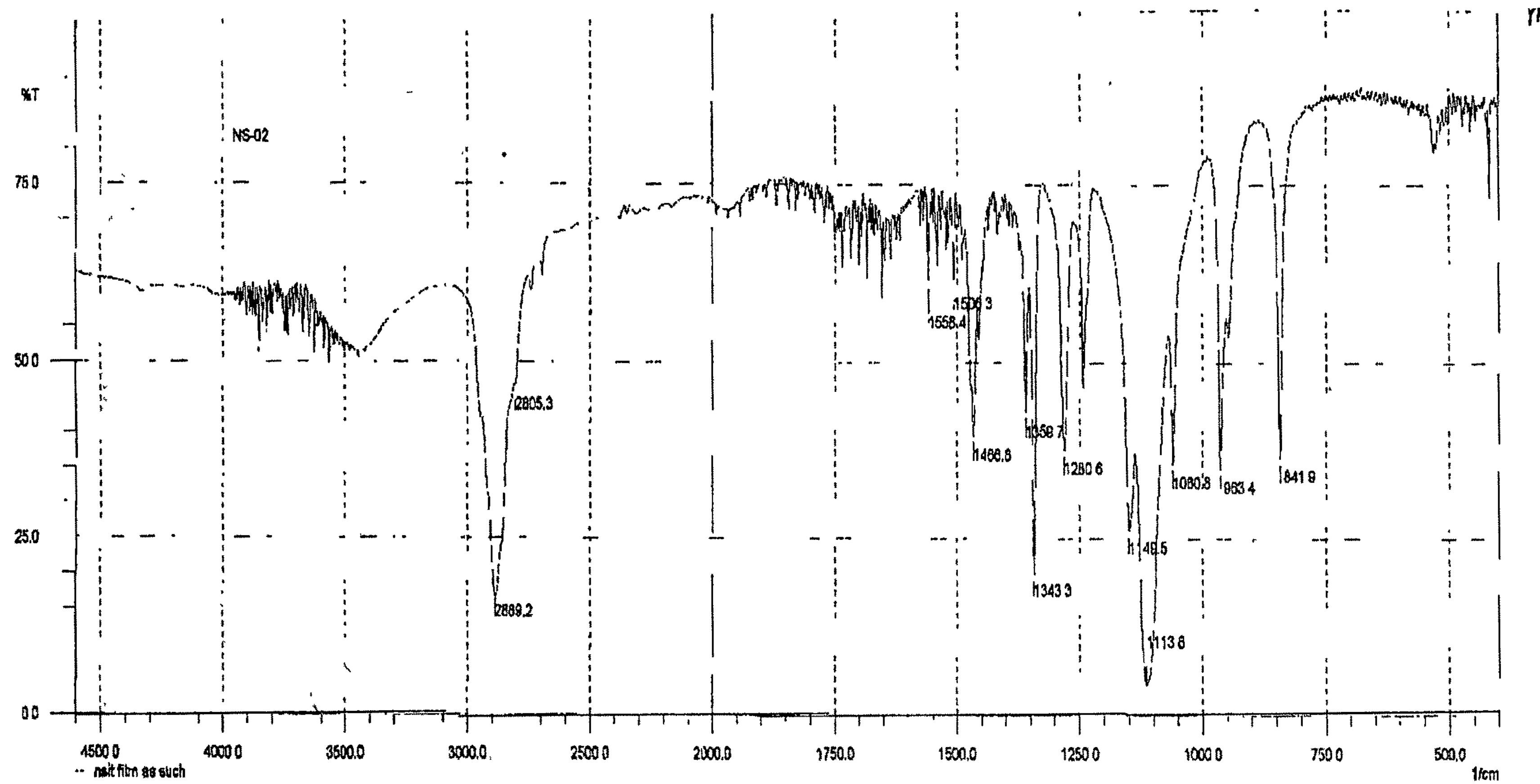


Figure 4.15 Mid infra-red spectrum of methoxy polyethylene glycol 5000 activated with cyanuric chloride -phosphatidyl ethanolamine conjugate (mPEG5000-CC-PE)

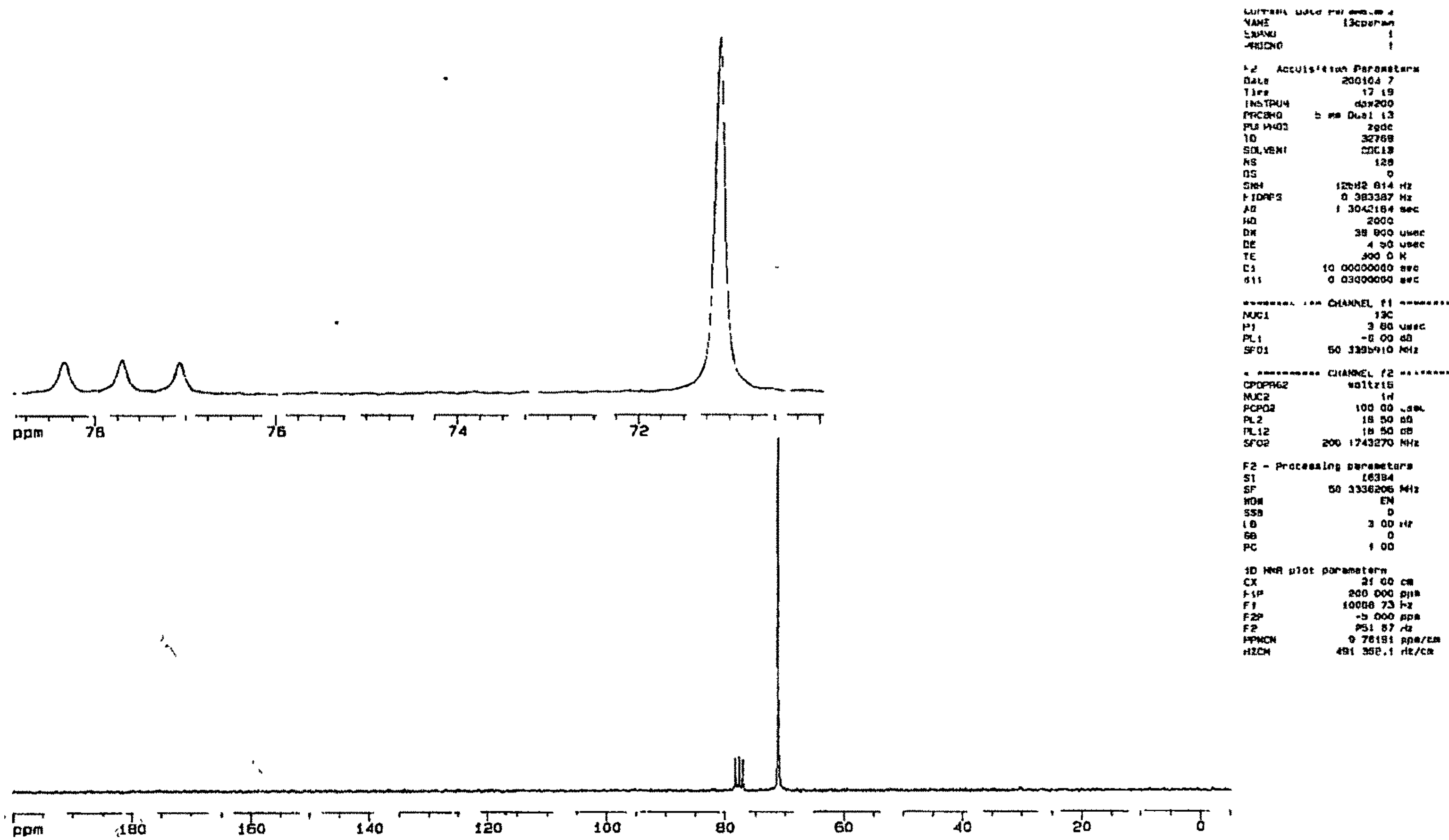


Figure 4.16 ^{13}C-NMR spectrum of methoxy polyethylene glycol 5000 activated with cyanuric chloride -phosphatidyl ethanolamine conjugate (mPEG5000-CC-PE)

Figure 4.17 Flowchart for the preparation of sterically stabilized liposomes containing methotrexate using methoxy polyethylene glycol 5000 activated with cyanuric chloride – phosphatidyl ethanolamine conjugate (mPEG5000-CC-PE)

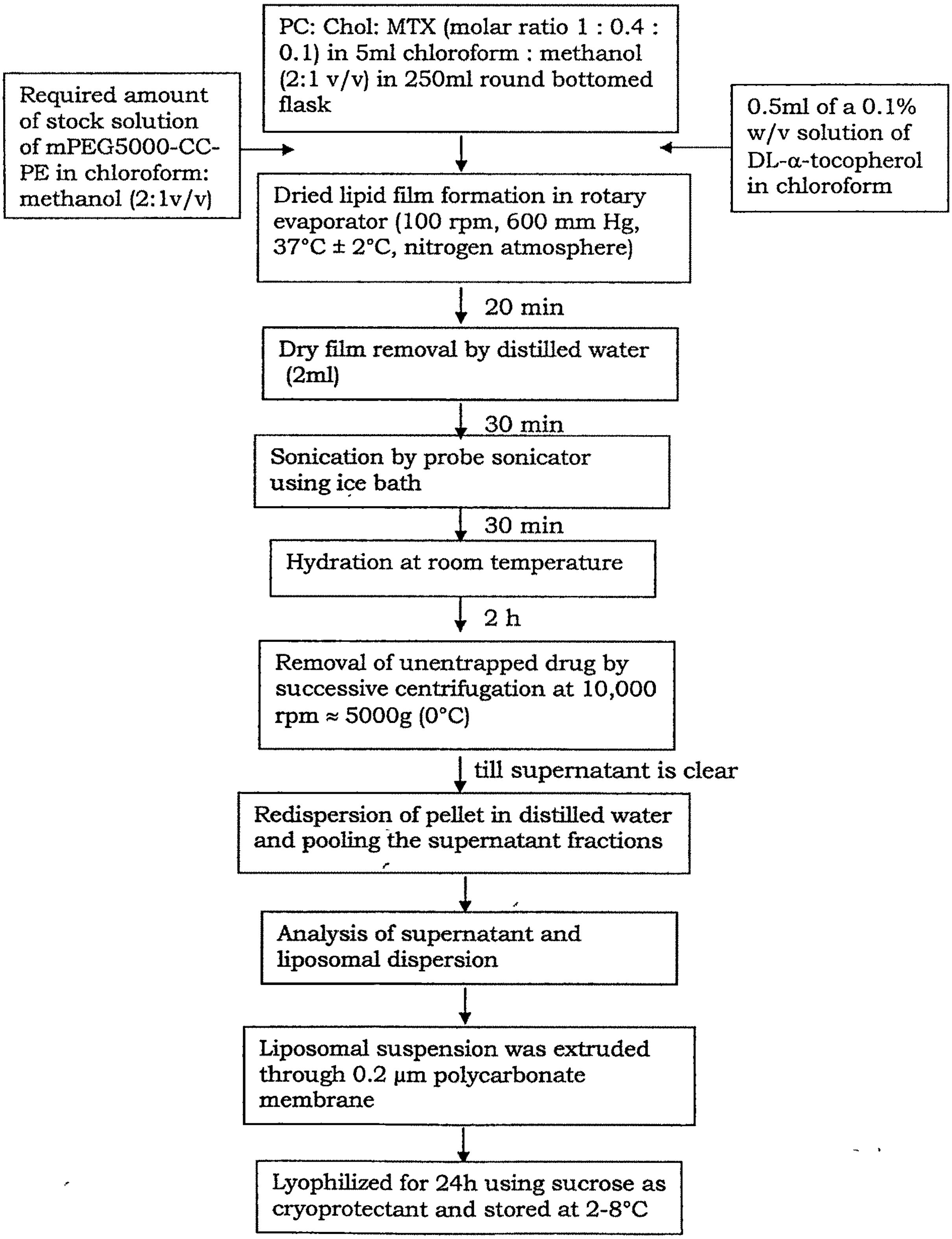


Table 4.7 Optimization of mPEG5000-CC-PE concentration, required for steric stabilization of methotrexate containing liposomes, using electrolyte induced flocculation test

Concentration of sodium sulphate (in M)	Mean absorbance ± S.E. at 400nm of methotrexate liposomes containing mPEG5000-CC-PE			
	3 mol%	5 mol%	7mol%	9mol%
0.0	0.551±0.016	0.563±0.029	0.594±0.026	0.531±0.017
0.4	0.585±0.031	0.574±0.031	0.587±0.034	0.546±0.034
0.8	0.602±0.022	0.588±0.012	0.612±0.041	0.544±0.046
1.2	0.625±0.016	0.601±0.038	0.627±0.025	0.568±0.029
1.6	0.654±0.036	0.592±0.011	0.651±0.019	0.579±0.016
2.0	0.677±0.010	0.611±0.046	0.644±0.026	0.571±0.031

Figure 4.18 Optimization of mPEG5000-CC-PE concentration, required for steric stabilization of methotrexate containing liposomes, using electrolyte induced flocculation test

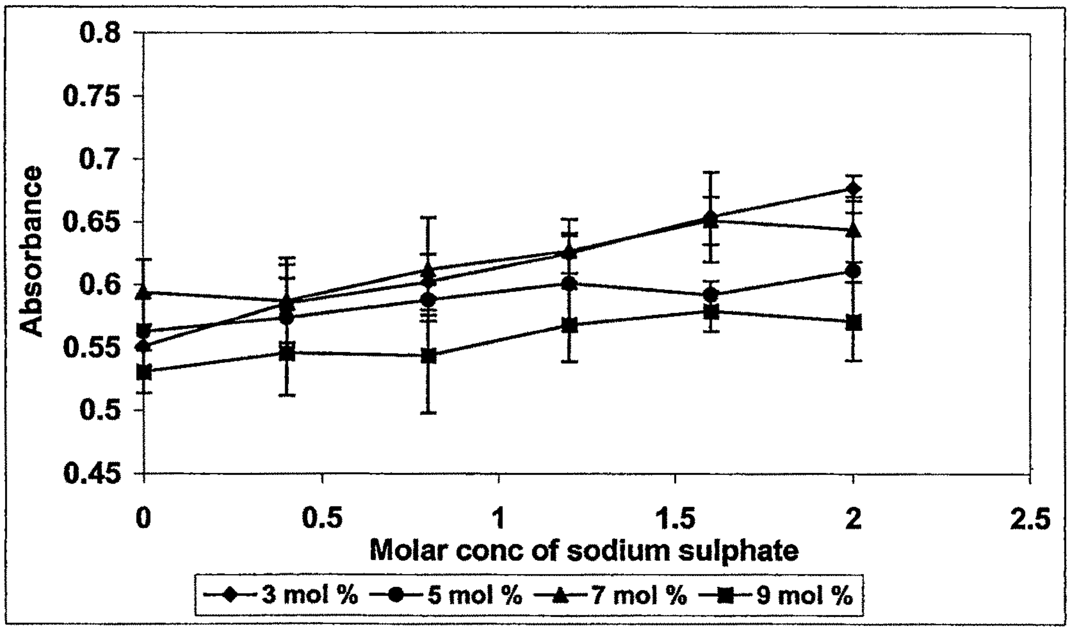


Figure 4.19 Flowchart for the preparation of sterically stabilized liposomes containing tamoxifen citrate using methoxy polyethylene glycol 5000 activated with cyanuric chloride-phosphatidyl ethanolamine conjugate (mPEG5000-CC-PE)

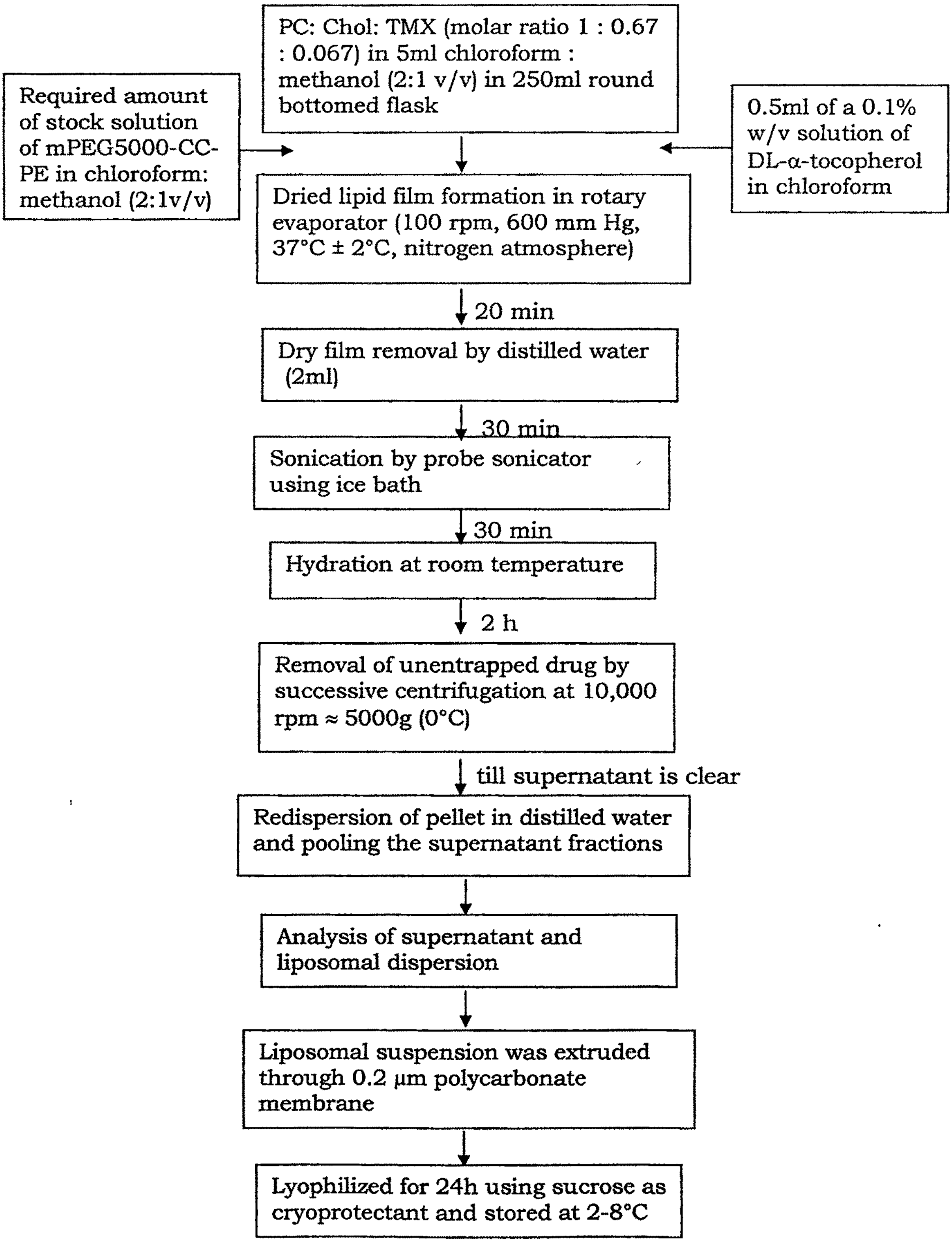


Table 4.8 Optimization of mPEG5000-CC-PE concentration, required for steric stabilization of tamoxifen citrate containing liposomes, using electrolyte induced flocculation test

Concentration of sodium sulphate (in M)	Mean absorbance ± S.E. at 400nm of tamoxifen citrate liposomes containing mPEG5000-CC-PE			
	3 mol%	5 mol%	7mol%	9mol%
0.0	0.587±0.021	0.545±0.011	0.524±0.022	0.530±0.016
0.4	0.592±0.026	0.565±0.032	0.523±0.031	0.533±0.018
0.8	0.621±0.011	0.559±0.018	0.532±0.035	0.542±0.037
1.2	0.654±0.026	0.574±0.016	0.545±0.019	0.538±0.034
1.6	0.662±0.014	0.578±0.013	0.552±0.034	0.545±0.016
2.0	0.667±0.015	0.582±0.010	0.560±0.011	0.550±0.026

Figure 4.20 Optimization of mPEG5000-CC-PE concentration, required for steric stabilization of tamoxifen citrate containing liposomes, using electrolyte induced flocculation test

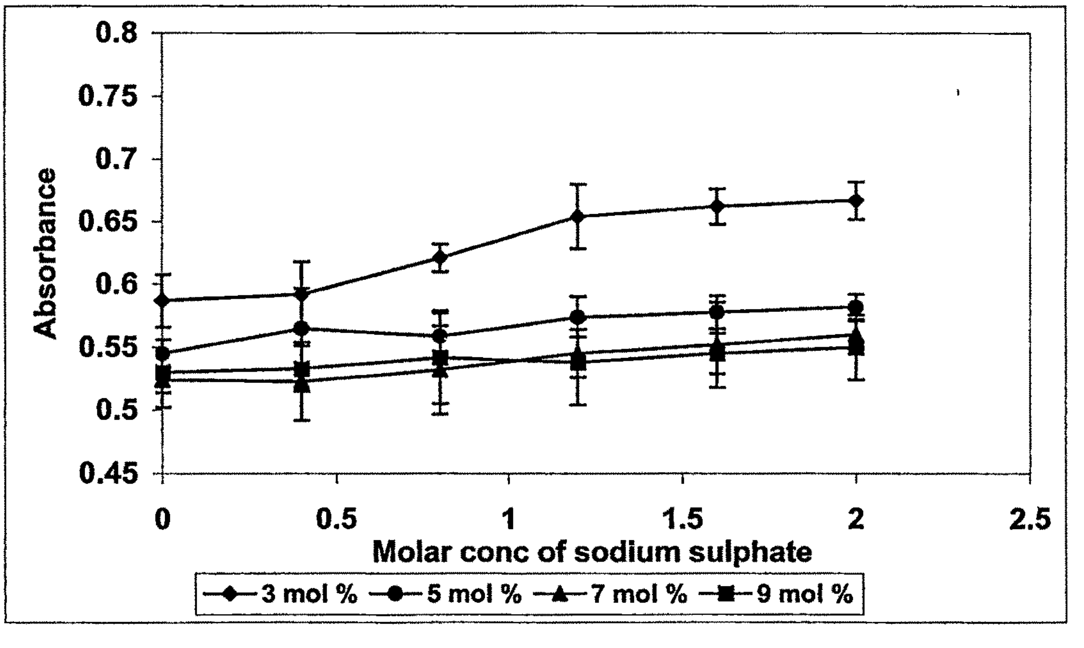


Figure 4.21 Flowchart for the preparation of sterically stabilized liposomes containing cytarabine using methoxy polyethylene glycol 5000 activated with cyanuric chloride – phosphatidyl ethanolamine conjugate (mPEG5000-CC-PE)

PC : Chol in 5ml chloroform : methanol (2:1 v/v) in 250ml round bottomed flask

↓

Required amount of stock solution of mPEG5000-CC-PE in chloroform: methanol (2: 1 v/v) →

← 0.5ml of a 0.1% w/v solution of DL-α-tocopherol in chloroform

Dried lipid film formation in rotary evaporator (100 rpm, 600 mm Hg, 37°C ± 2°C, nitrogen atmosphere)

↓ 20 min

Dry film removal using 2ml of cytarabine solution (2ml)

← Cytarabine dissolved in distilled water (5mg/2ml) Molar ratio PC: Chol: CYT: 1: 0.625: 0.125

↓ 30 min

Sonication by probe sonicator using ice bath

↓ 30 min

Hydration at room temperature

↓ 2 h

Removal of unentrapped drug by successive centrifugation at 10,000 rpm ≈ 5000g (0°C)

↓ till supernatant is clear

Redispersion of pellet in distilled water and pooling the supernatant fractions

↓

Analysis of supernatant and liposomal dispersion

↓

Liposomal suspension was extruded through 0.2 μm polycarbonate membrane

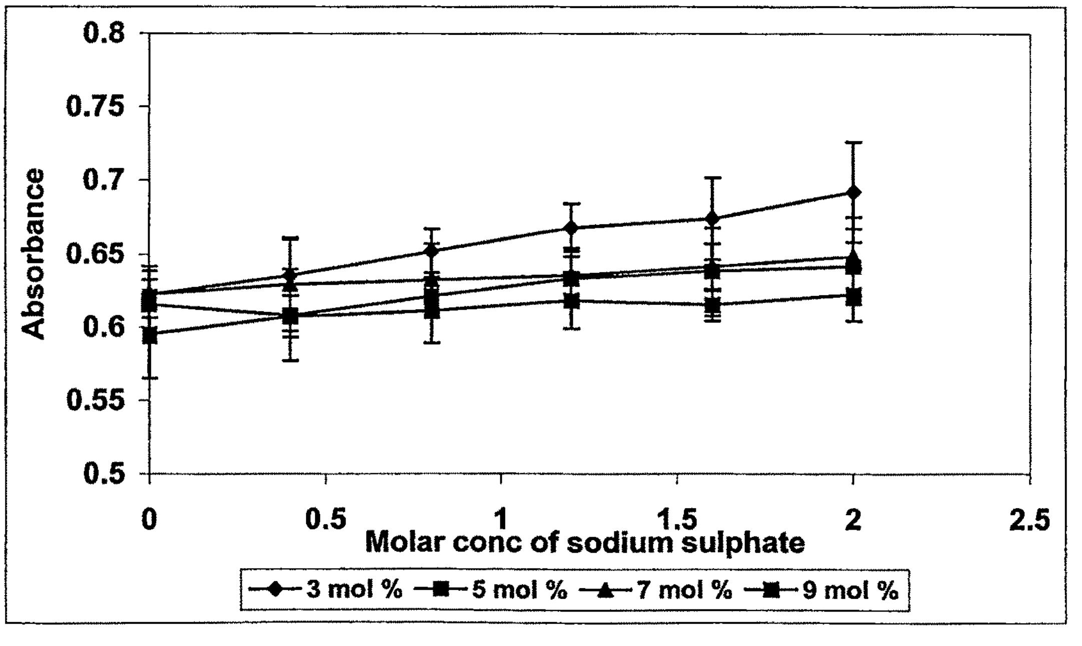
↓

Lyophilized for 24h using sucrose as cryoprotectant and stored at 2-8°C

Table 4.9 Optimization of mPEG5000-CC-PE concentration, required for steric stabilization of cytarabine containing liposomes, using electrolyte induced flocculation test

Concentration of sodium sulphate (in M)	Mean absorbance ± S.E. at 400nm of cytarabine liposomes containing mPEG5000-CC-PE			
	3 mol%	5 mol%	7mol%	9mol%
0.0	0.622±0.016	0.615±0.026	0.622±0.010	0.595±0.030
0.4	0.635±0.025	0.608±0.031	0.629±0.032	0.607±0.014
0.8	0.652±0.015	0.621±0.013	0.632±0.025	0.611±0.022
1.2	0.668±0.016	0.633±0.015	0.635±0.019	0.618±0.019
1.6	0.674±0.028	0.638±0.030	0.641±0.016	0.615±0.011
2.0	0.692±0.034	0.641±0.026	0.648±0.027	0.622±0.018

Figure 4.22 Optimization of mPEG5000-CC-PE concentration, required for steric stabilization of cytarabine containing liposomes, using electrolyte induced flocculation test



4.2.9.5 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 were 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 stirred magnetically 24 h at room temperature. 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 methoxypolyethylene glycol 2000 and mPEG2000-CC in methanol taken on a Shimadzu 1601 UV-Visible spectrophotometer are shown in Figures 4.23 and 4.24 respectively. The identity of mPEG2000 activated with cyanuric chloride was ascertained by taking its mid infrared spectrum on a Shimadzu FTIR-8300 spectrophotometer (Figure 4.25).

4.2.9.6 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 4.26. 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 4.27, 4.28 and 4.29 respectively.

4.2.9.7 Preparation of liposomes using mPEG2000-CC-PE

Tables 4.10, 4.11 and 4.12 as well as Figures 4.31, 4.33 and 4.35 present the results of the optimization of the amount of mPEG2000-CC-PE required for steric stabilization of liposomes containing methotrexate, tamoxifen citrate and cytarabine 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 mPEG2000-CC-PE was added to the lipid solution before it was subjected to evaporation for lipid film formation. The amount of mPEG2000-CC-PE added was compensated by removal of an equimolar amount of PC from the system. The amount of mPEG2000-CC-PE required for steric stabilization was optimised by subjecting triplicate batches of liposomes, formed with different amounts of the mPEG2000-CC-PE, to the electrolyte induced flocculation test which is described earlier in this chapter. The preparation methods are shown as flowcharts (Figures 4.30, 4.32 and 4.34 respectively for liposomes containing methotrexate, tamoxifen citrate and cytarabine).

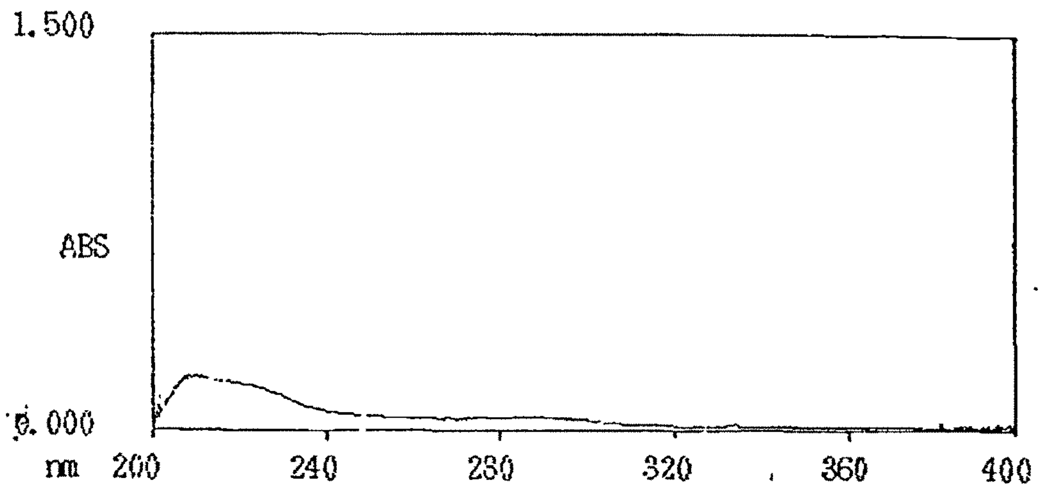


Figure 4.23 Ultraviolet absorptivity scan of methoxy polyethylene glycol 2000 in methanol (1mg/ml)

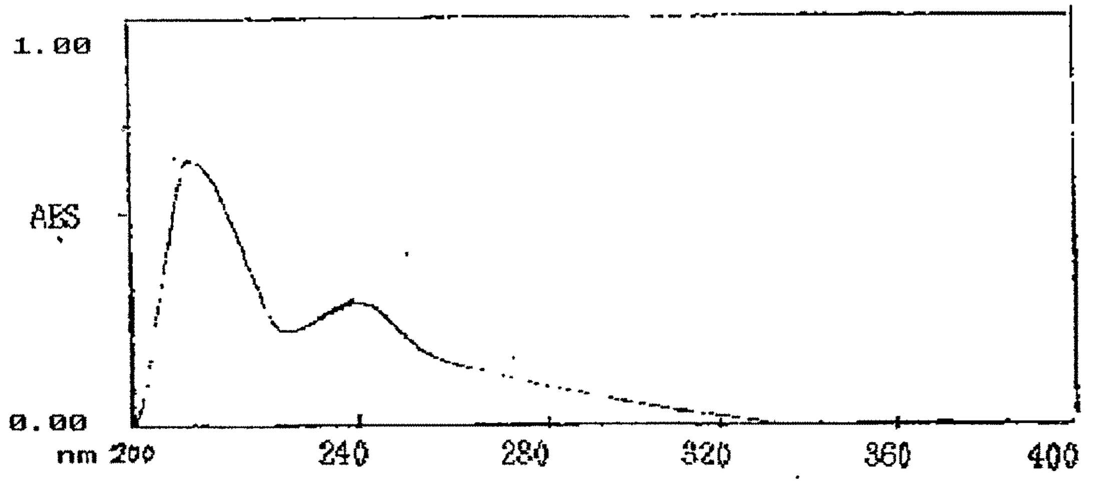


Figure 4.24 Ultraviolet absorptivity scan of methoxy polyethylene glycol 2000 activated with cyanuric chloride (mPEG2000-CC) in methanol (1mg/ml)

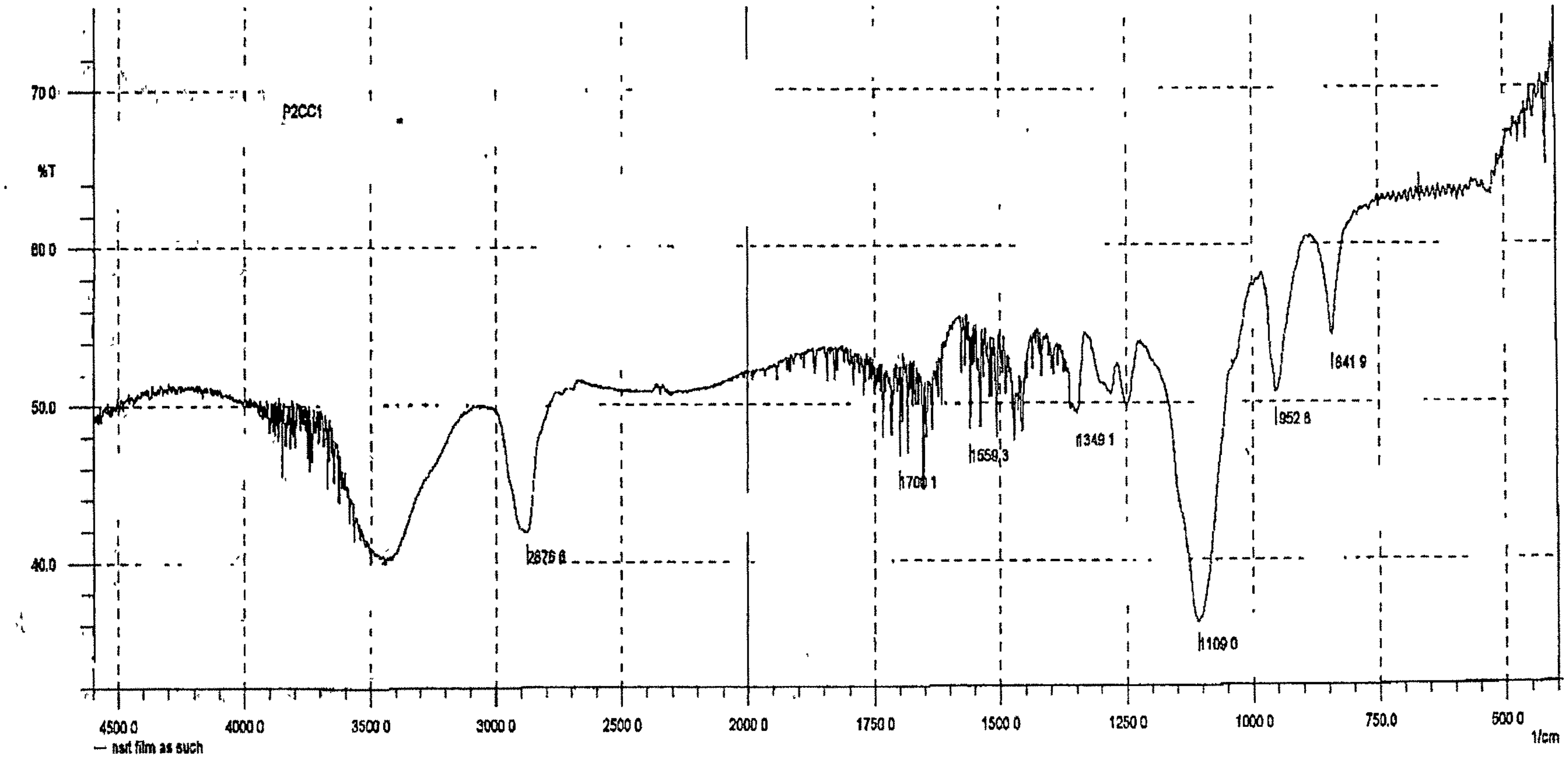
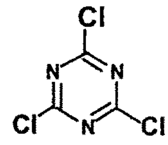


Figure 4.25 **Mid infra-red spectrum of methoxy polyethylene glycol 2000 activated with cyanuric chloride (mPEG2000-CC)**

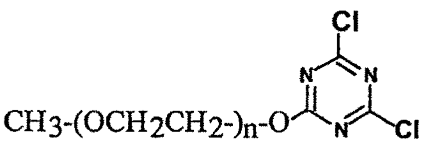
Figure 4.26 Reaction scheme of synthesis of methoxy polyethylene glycol 2000 activated with cyanuric chloride-phosphatidyl ethanolamine conjugate (mPEG2000-CC-PE)

$CH_3-(OCH_2CH_2-)_n-OH$ +



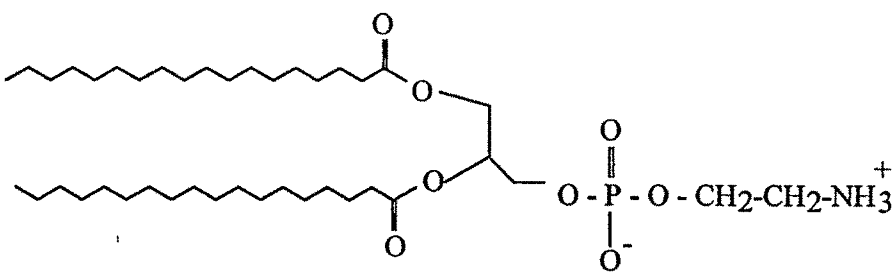
Methoxy Polyethylene glycol (Average mol. wt. 2000, mPEG2000)

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

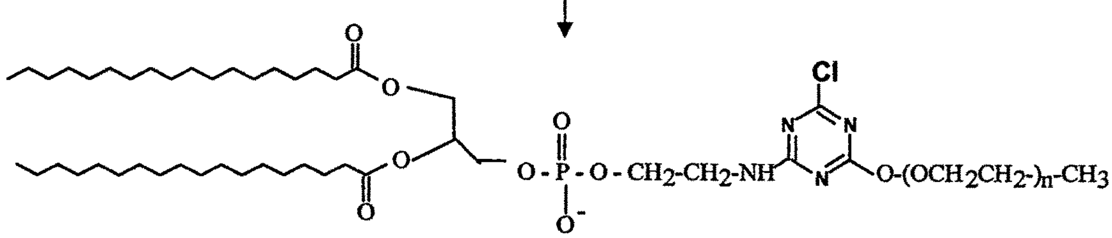


methoxy polyethylene glycol 2000 activated with cyanuric chloride (mPEG2000-CC)

+



Phosphatidylethanolamine



mPEG2000-CC-PE

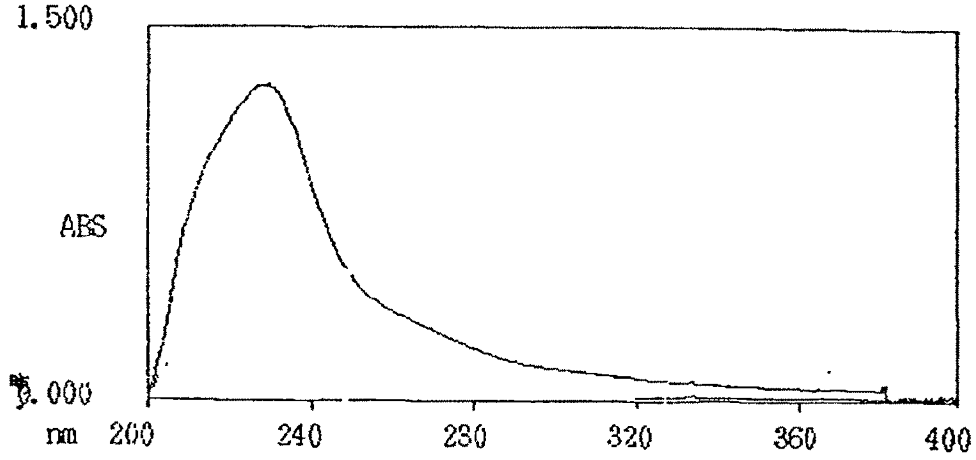


Figure 4.27 Ultraviolet absorptivity scan of methoxy polyethylene glycol 2000 coupled with cyanuric chloride -phosphatidyl ethanolamine conjugate (mPEG2000-CC-PE) in methanol (1mg/ml)

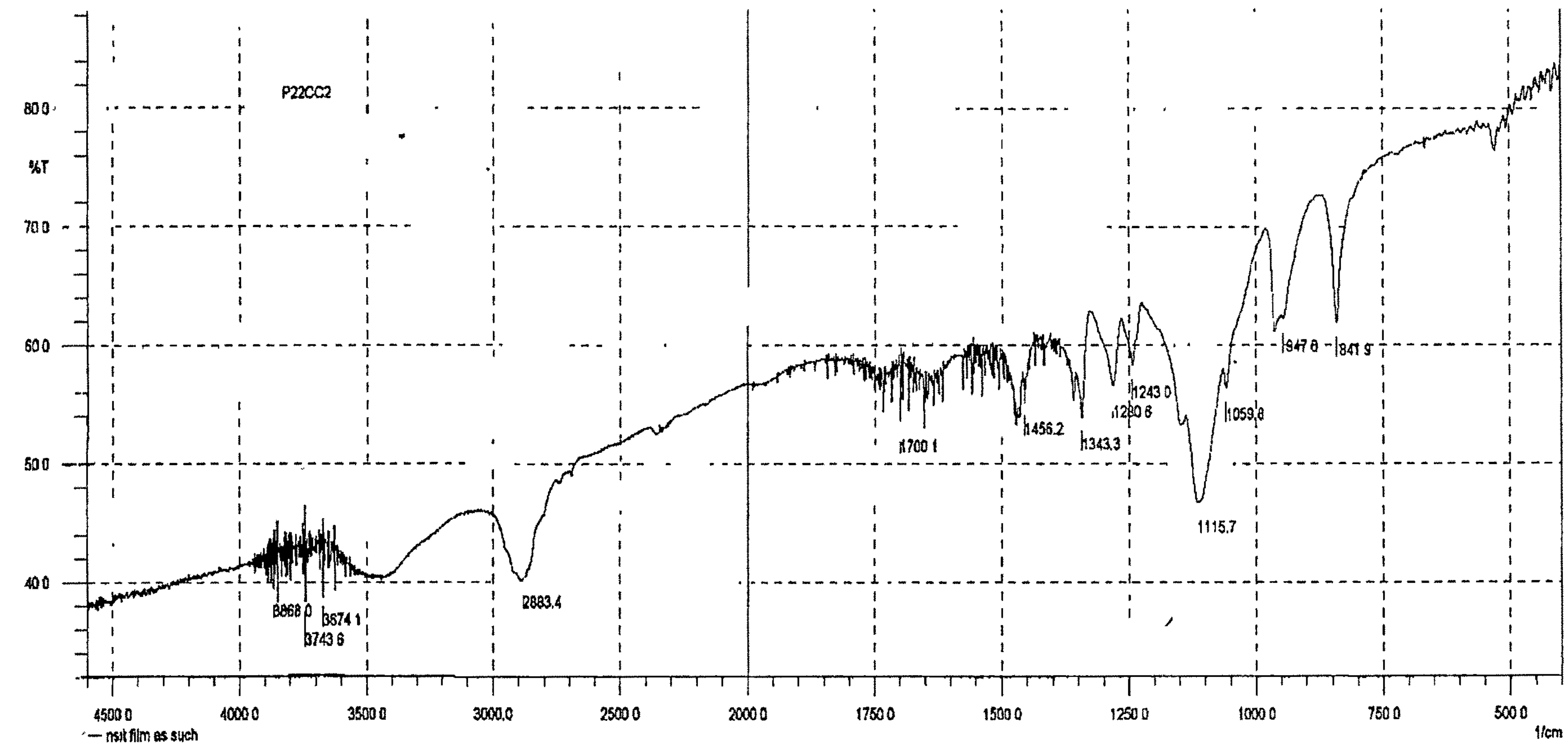


Figure 4.28 **Mid infra-red spectrum of methoxy polyethylene glycol 2000 activated with cyanuric chloride – phosphatidyl ethanolamine conjugate (mPEG2000-CC-PE)**

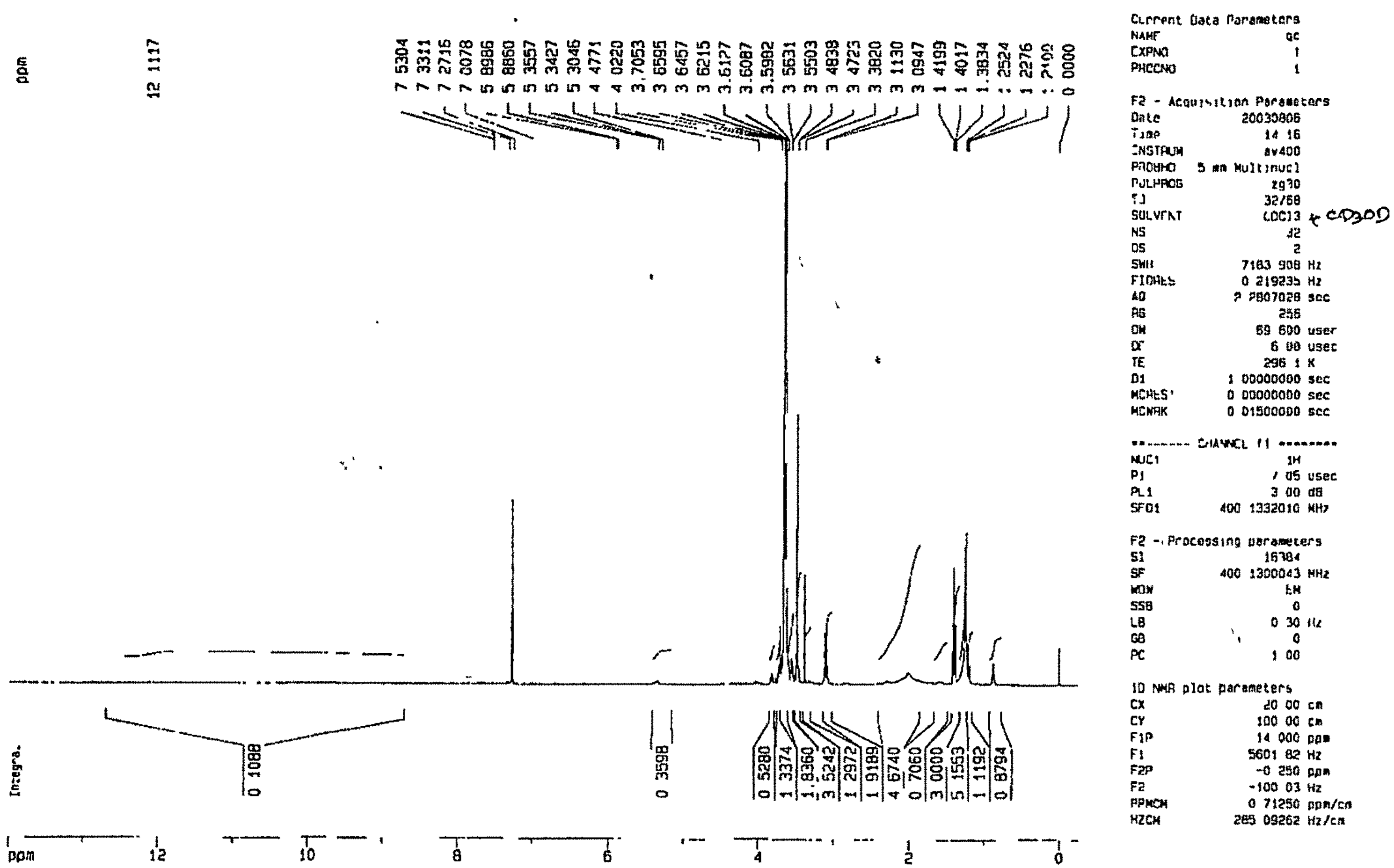


Figure 4.29 ^{13}C-NMR spectrum of methoxy polyethylene glycol 2000 activated with cyanuric chloride – phosphatidyl ethanolamine conjugate (mPEG2000-CC-PE)

Figure 4.30 Flowchart for the preparation of sterically stabilized liposomes containing methotrexate using methoxy polyethylene glycol 2000 activated with cyanuric chloride-phosphatidylethanolamine conjugate (mPEG2000-CC-PE)

PC: Chol: MTX (molar ratio 1 : 0.4 : 0.1) in 5ml chloroform : methanol (2:1 v/v) in 250ml round bottomed flask

Required amount of stock solution of mPEG2000-CC-PE in chloroform: methanol (2:1 v/v) →

← 0.5ml of a 0.1% w/v solution of DL-α-tocopherol in chloroform

↓

Dried lipid film formation in rotary evaporator (100 rpm, 600 mm Hg, 37°C ± 2°C, nitrogen atmosphere)

↓ 20 min

Dry film removal by distilled water (2ml)

↓ 30 min

Sonication by probe sonicator using ice bath

↓ 30 min

Hydration at room temperature

↓ 2 h

Removal of unentrapped drug by successive centrifugation at 10,000 rpm ≈ 5000g (0°C)

↓ till supernatant is clear

Redispersion of pellet in distilled water and pooling the supernatant fractions

↓

Analysis of supernatant and liposomal dispersion

↓

Liposomal suspension was extruded through 0.2 μm polycarbonate membrane

↓

Lyophilized for 24h using sucrose as cryoprotectant and stored at 2-8°C

Table 4.10 Optimization of mPEG2000-CC-PE concentration, required for steric stabilization of methotrexate containing liposomes, using electrolyte induced flocculation test

Concentration of sodium sulphate (in M)	Mean absorbance ± S.E. at 400nm of methotrexate liposomes containing mPEG2000-CC-PE			
	3 mol%	5 mol%	7mol%	9mol%
0.0	0.613±0.026	0.623±0.010	0.602±0.041	0.612±0.017
0.4	0.624±0.035	0.631±0.031	0.607±0.026	0.609±0.016
0.8	0.632±0.021	0.644±0.021	0.613±0.035	0.619±0.013
1.2	0.659±0.011	0.649±0.025	0.620±0.015	0.628±0.025
1.6	0.674±0.015	0.661±0.016	0.619±0.019	0.625±0.019
2.0	0.680±0.028	0.668±0.019	0.624±0.020	0.631±0.034

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

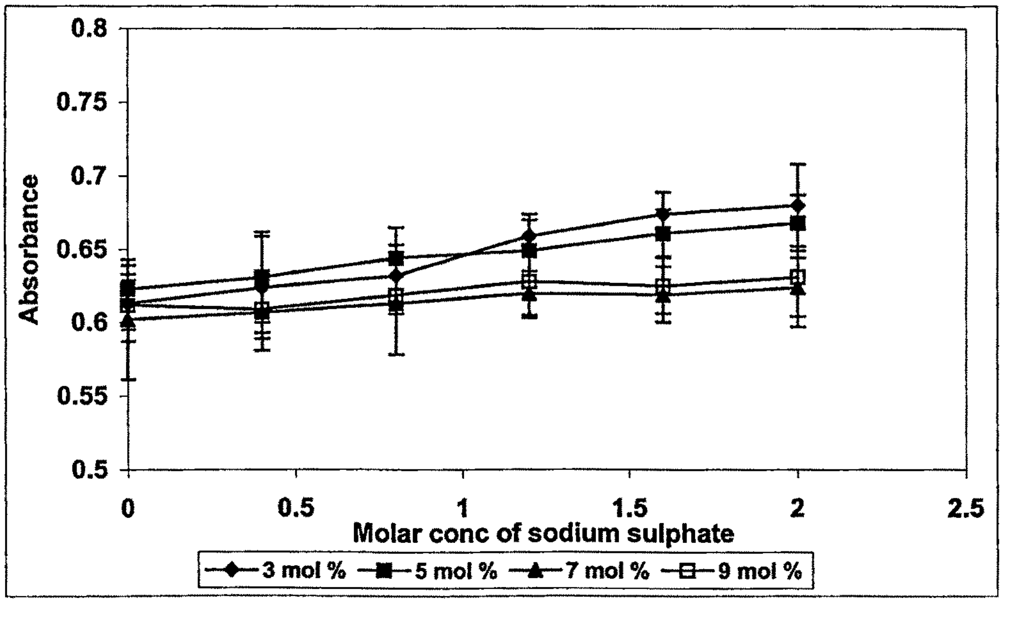


Figure 4.32 Flowchart for the preparation of sterically stabilized liposomes containing tamoxifen citrate using methoxy polyethylene glycol 2000 activated with cyanuric chloride-phosphatidylethanolamine conjugate (mPEG2000-CC-PE)

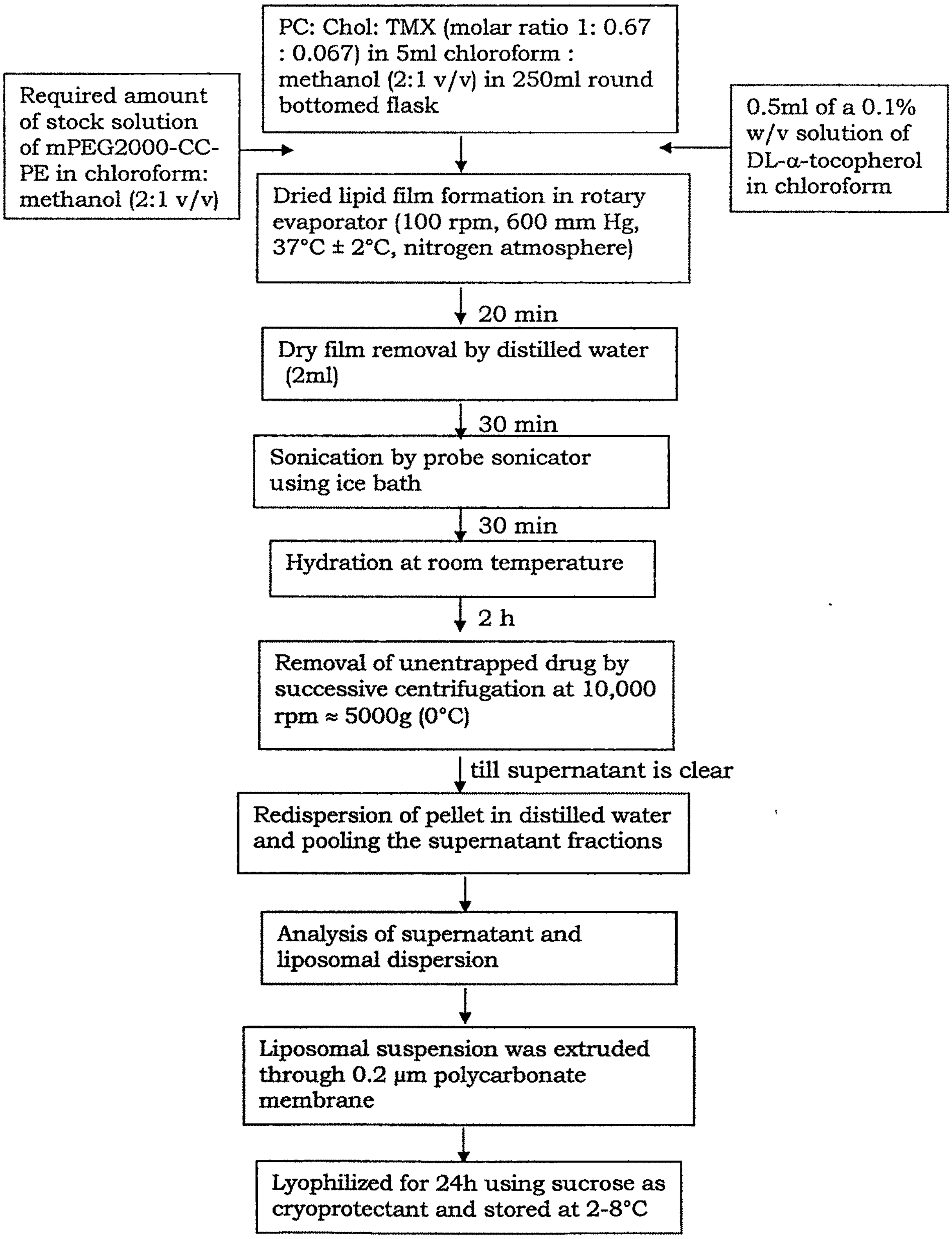


Table 4.11 Optimization of mPEG2000-CC-PE concentration, required for steric stabilization of tamoxifen citrate containing liposomes, using electrolyte induced flocculation test

Concentration of sodium sulphate (in M)	Mean absorbance ± S.E. at 400nm of tamoxifen citrate liposomes containing mPEG2000-CC-PE			
	3 mol%	5 mol%	7mol%	9mol%
0.0	0.598±0.016	0.615±0.017	0.601±0.021	0.615±0.032
0.4	0.612±0.013	0.624±0.026	0.598±0.016	0.611±0.012
0.8	0.623±0.025	0.635±0.024	0.603±0.015	0.617±0.018
1.2	0.633±0.035	0.645±0.016	0.610±0.031	0.621±0.014
1.6	0.639±0.016	0.646±0.018	0.614±0.011	0.624±0.016
2.0	0.645±0.019	0.652±0.034	0.619±0.024	0.630±0.031

Figure 4.33 Optimization of mPEG2000-CC-PE concentration, required for steric stabilization of tamoxifen citrate containing liposomes, using electrolyte induced flocculation test

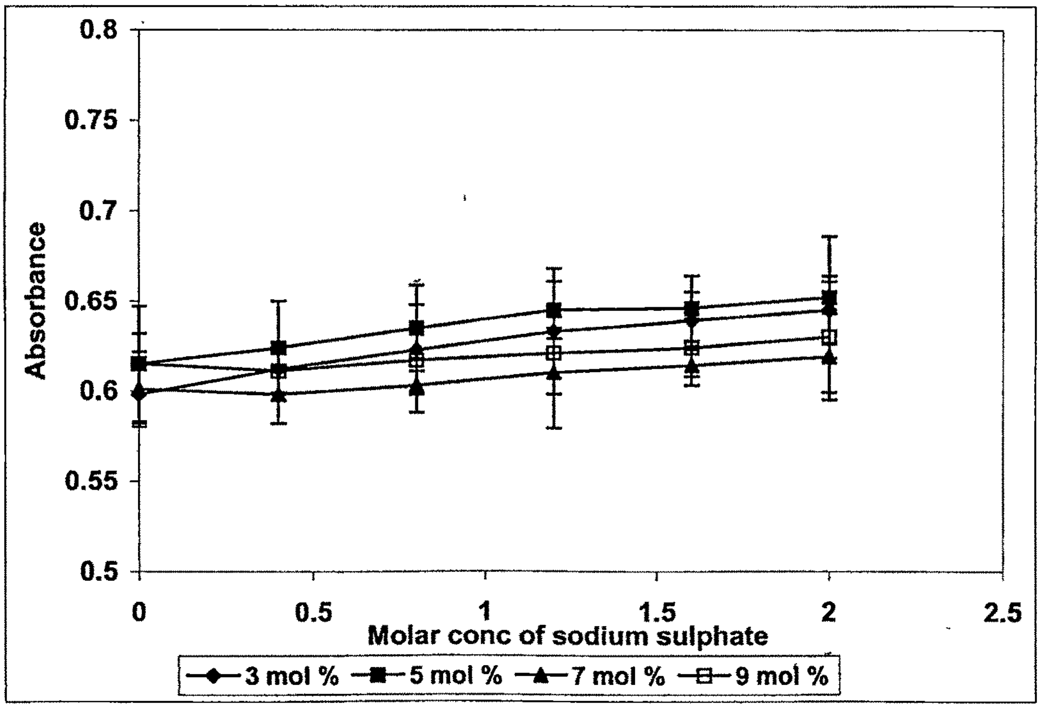


Figure 4.34 Flowchart for the preparation of sterically stabilized liposomes containing cytarabine using methoxy polyethylene glycol 2000 activated with cyanuric chloride-phosphatidyl ethanolamine conjugate (mPEG2000-CC-PE)

PC : Chol in 5ml chloroform : methanol (2:1 v/v) in 250ml round bottomed flask

Required amount of stock solution of mPEG2000-CC-PE in chloroform: methanol (2: 1 v/v)

0.5ml of a 0.1% w/v solution of DL-α-tocopherol in chloroform

Dried lipid film formation in rotary evaporator (100 rpm, 600 mm Hg, 37°C ± 2°C, nitrogen atmosphere)

↓ 20 min

Cytarabine dissolved in distilled water (5mg/2ml)
Molar ratio PC: Chol: CYT: 1: 0.625: 0.125

Dry film removal using 2ml of cytarabine solution (2ml)

↓ 30 min

Sonication by probe sonicator using ice bath

↓ 30 min

Hydration at room temperature

↓ 2 h

Removal of unentrapped drug by successive centrifugation at 10,000 rpm ≈ 5000g (0°C)

↓ till supernatant is clear

Redispersion of pellet in distilled water and pooling the supernatant fractions

↓

Analysis of supernatant and liposomal dispersion

↓

Liposomal suspension was extruded through 0.2 μm polycarbonate membrane

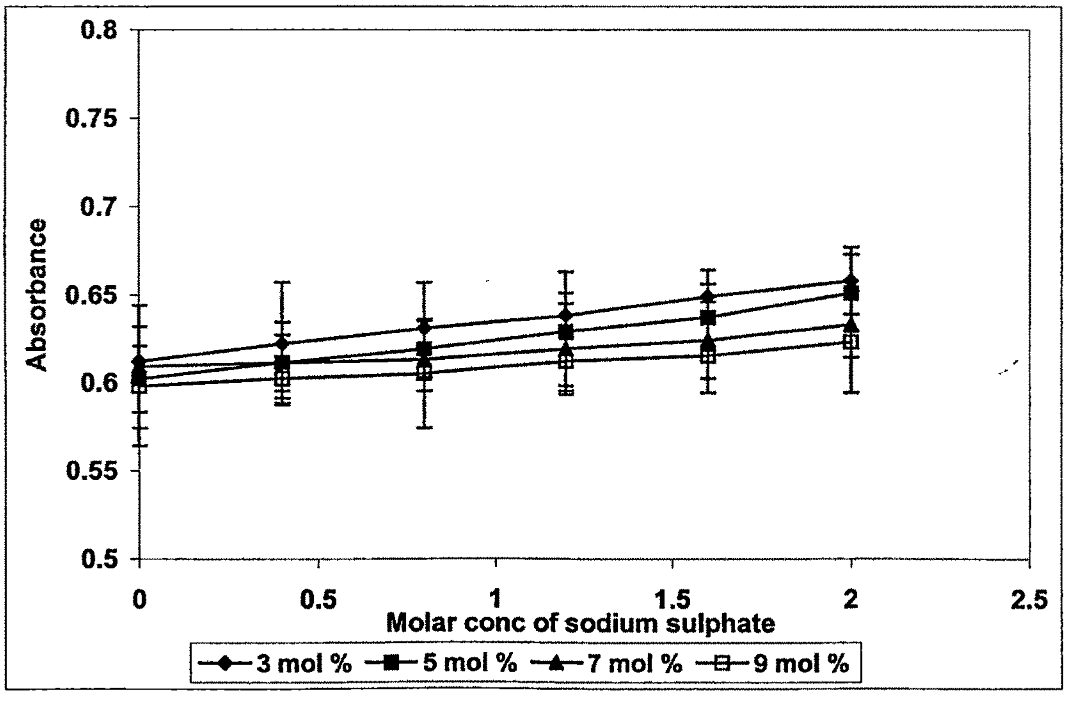
↓

Lyophilized for 24h using sucrose as cryoprotectant and stored at 2-8°C

Table 4.12 Optimization of mPEG2000-CC-PE concentration, required for steric stabilization of cytarabine containing liposomes, using electrolyte induced flocculation test

Concentration of sodium sulphate (in M)	Mean absorbance ± S.E. at 400nm of cytarabine liposomes containing mPEG2000-CC-PE			
	3 mol%	5 mol%	7mol%	9mol%
0.0	0.612±0.009	0.602±0.019	0.609±0.035	0.598±0.034
0.4	0.622±0.035	0.611±0.023	0.611±0.016	0.602±0.011
0.8	0.631±0.026	0.619±0.016	0.613±0.018	0.605±0.031
1.2	0.638±0.013	0.629±0.034	0.619±0.026	0.612±0.014
1.6	0.649±0.015	0.637±0.019	0.624±0.022	0.615±0.021
2.0	0.658±0.019	0.651±0.022	0.633±0.019	0.623±0.029

Figure 4.35 Optimization of mPEG2000-CC-PE concentration, required for steric stabilization of cytarabine containing liposomes, using electrolyte induced flocculation test



4.2.9.8 Synthesis of methoxy polyethylene glycol 5000 activated with imidazoline carbonyl and coupled with phosphatidyl ethanolamine conjugate (mPEG5000-IC-PE) (Allen et.al., 1991)

Methoxy polyethylene glycol activated with imidazoline carbonyl was obtained from Sigma and was used without further purification. 117mg of Phosphatidylethanolamine (PE) was taken in a three-necked round-bottomed flask and was dissolved in 10ml of chloroform. 1.02g of methoxy polyethylene glycol activated with imidazoline carbonyl dissolved using 5ml of chloroform and was added to the phosphatidylethanolamine in the molar ratio of 1:2. Chloroform was evaporated under vacuum. Triethylamine (10.12mg) was dissolved in benzene and added to the methoxy polyethylene glycol activated with imidazoline carbonyl mixture in a molar ratio of 1: 1. The flask containing the mixture was heated to 95°C for 6 h under calcium chloride guard tube and a condenser system with water circulation to condense the benzene vapours. The final product was isolated by precipitating with diethyl ether and dried in a rotary flash evaporator. The product was then collected and stored at 0°C until further use. The scans of methoxy polyethylene glycol activated with imidazoline carbonyl over the ultraviolet wavelength range and mid infrared is shown in Figures 4.36 and 4.38 respectively. 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 4.37, 4.39 and 4.40 respectively. The entire reaction scheme is shown in Figure 4.41.

4.2.9.9 Preparation of liposomes using mPEG5000-IC-PE

Tables 4.13, 4.14 and 4.15 as well as Figures 4.43, 4.45 and 4.47 present the results of the optimization of the amount of mPEG5000-IC-PE required for steric stabilization of liposomes containing methotrexate, tamoxifen citrate and cytarabine 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-IC-PE was added to the lipid solution before it was subjected to evaporation for lipid film formation. The amount of mPEG5000-IC-PE added was compensated by removal of an equimolar amount of PC from the system. The amount of mPEG5000-IC-PE required for steric stabilization was optimised by subjecting triplicate batches of liposomes, formed with different amounts of the mPEG-IC-PE, to the electrolyte induced flocculation test which is described earlier in this chapter. The preparation methods are shown as flowcharts (Figures 4.42, 4.44 and 4.46 respectively for liposomes containing methotrexate, tamoxifen citrate and cytarabine).

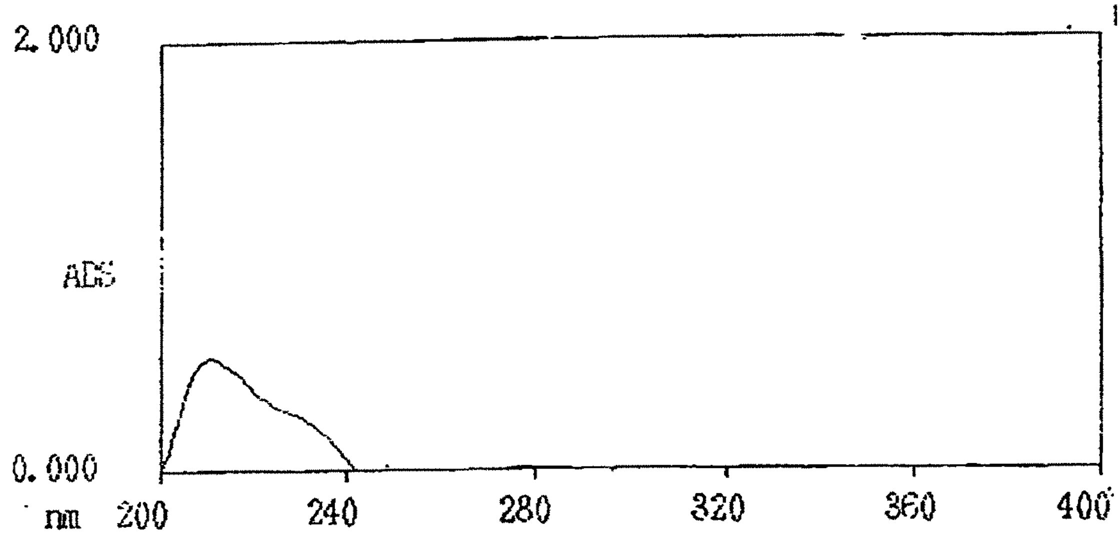


Figure 4.36 Ultraviolet absorptivity scan of methoxy polyethylene glycol 5000 activated with imidazoline carbonyl (mPEG5000-IC) in methanol (1mg/ml)

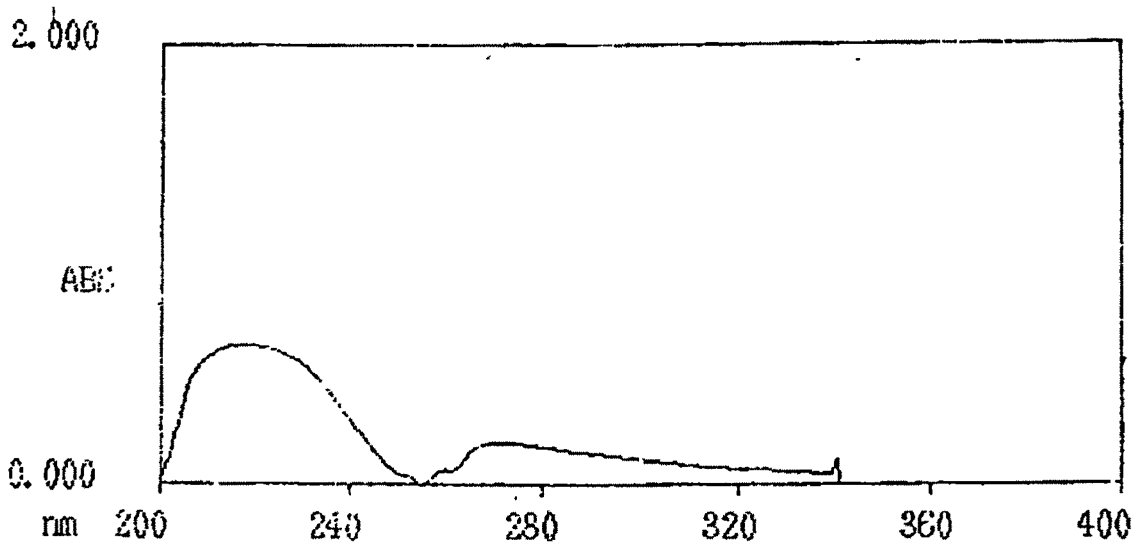


Figure 4.37 Ultraviolet absorptivity scan of methoxy polyethylene glycol 5000 activated with imidazoline carbonyl – phosphatidyl ethanolamine conjugate (mPEG5000-IC-PE) in methanol (1mg/ml)

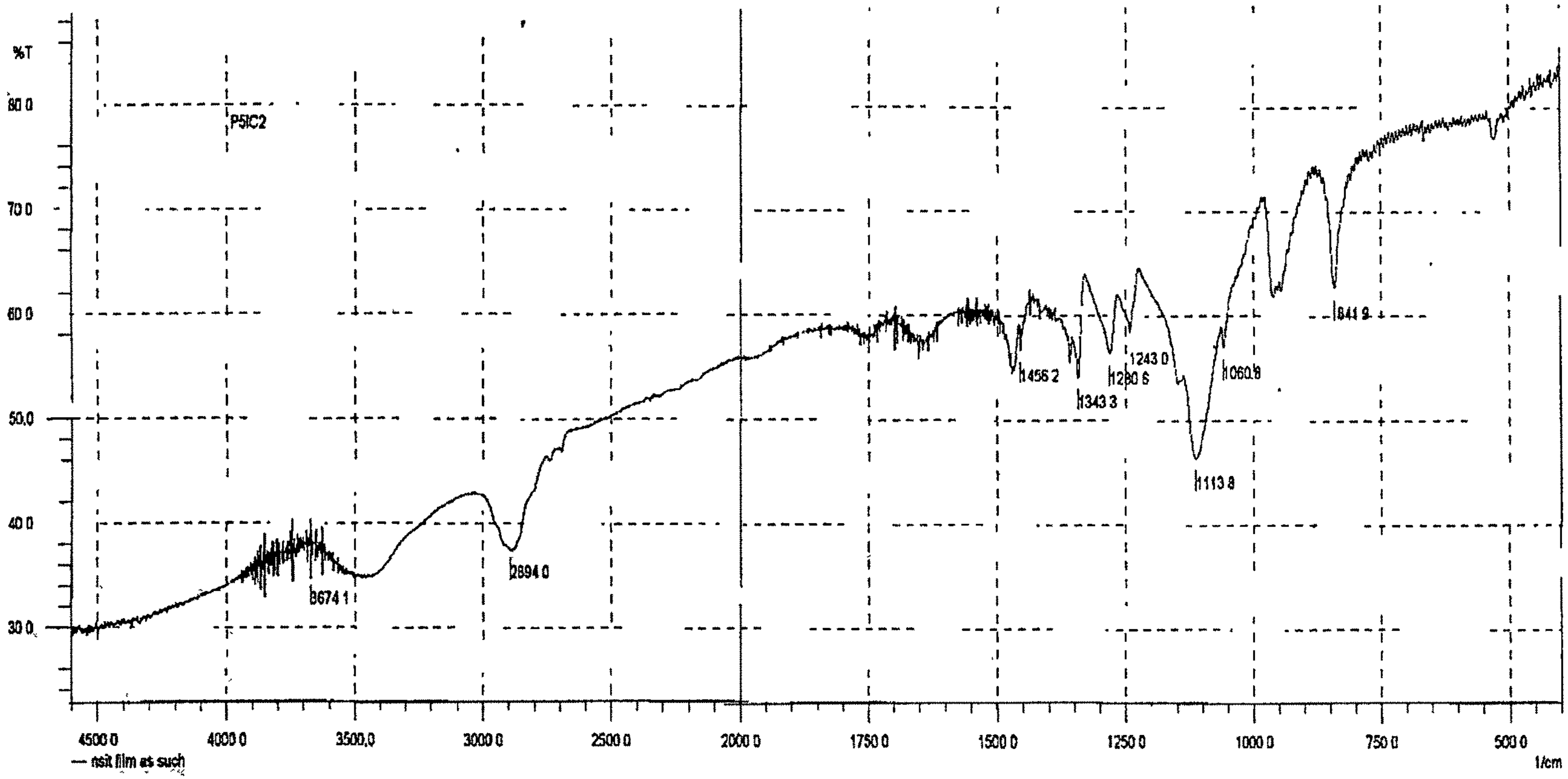


Figure 4.39 **Mid infra-red spectrum of methoxy polyethylene glycol 2000 activated with imidazoline carbonyl -phosphatidyl ethanolamine conjugate (mPEG5000-IC-PE)**

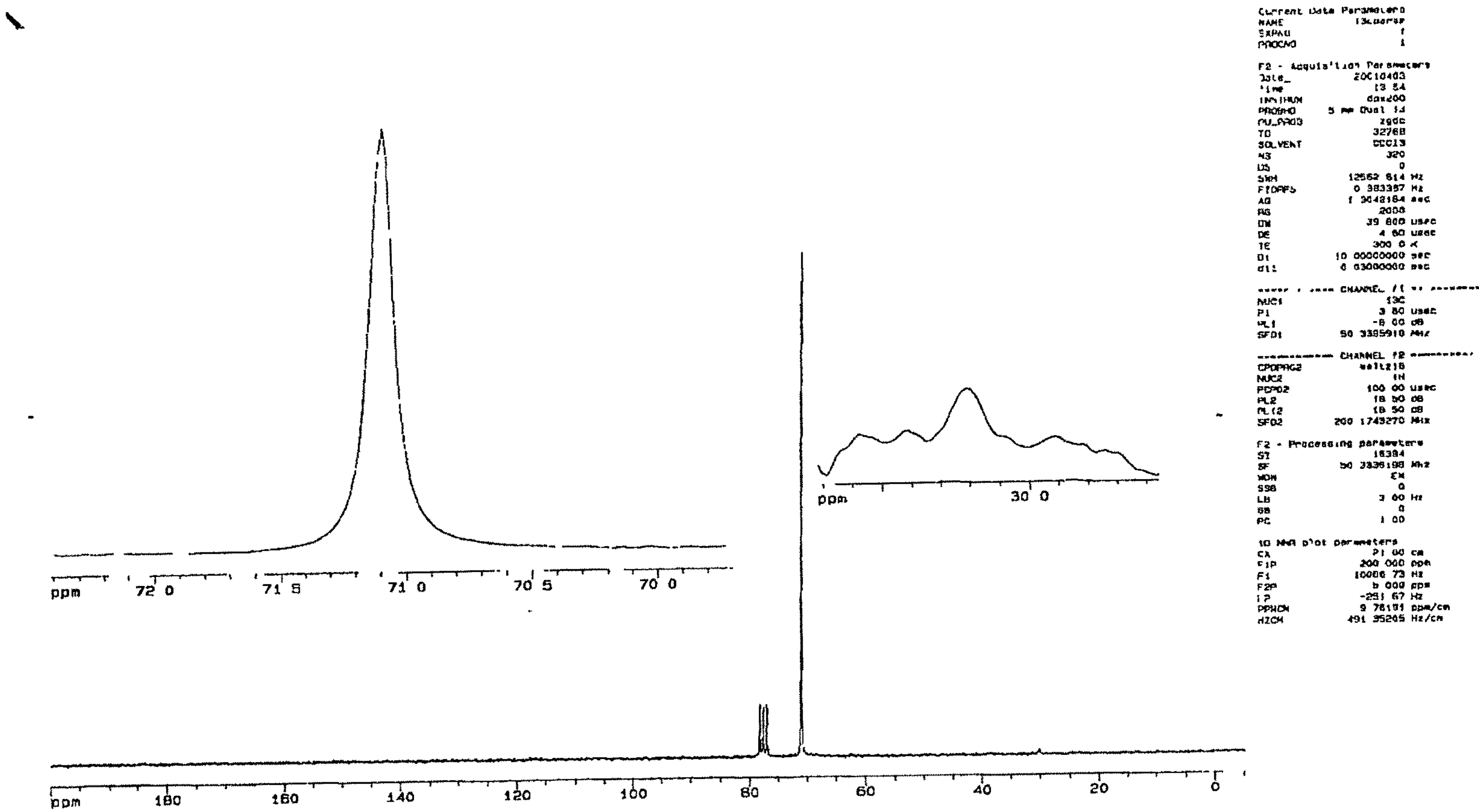
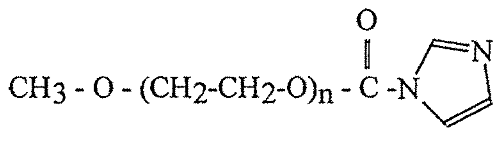
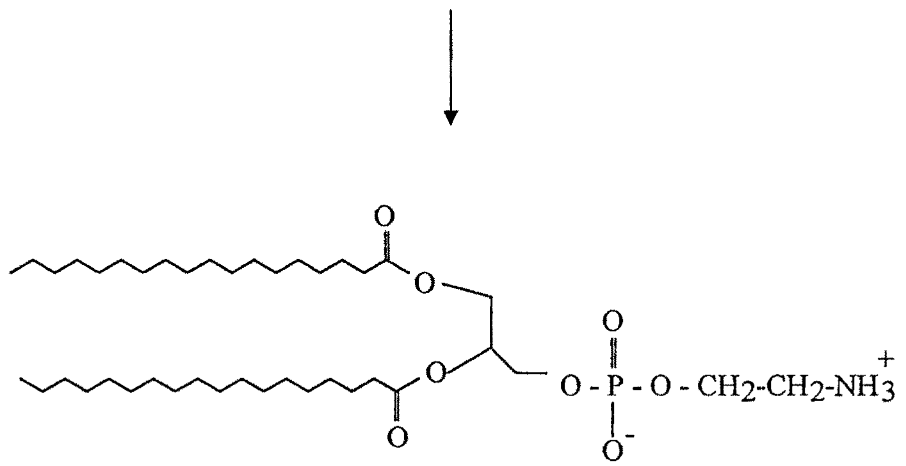


Figure 4.40 ^{13}C-NMR spectrum of methoxy polyethylene glycol 5000 activated with imidazoline carbonyl – phosphatidyl ethanolamine conjugate (mPEG5000-IC-PE)

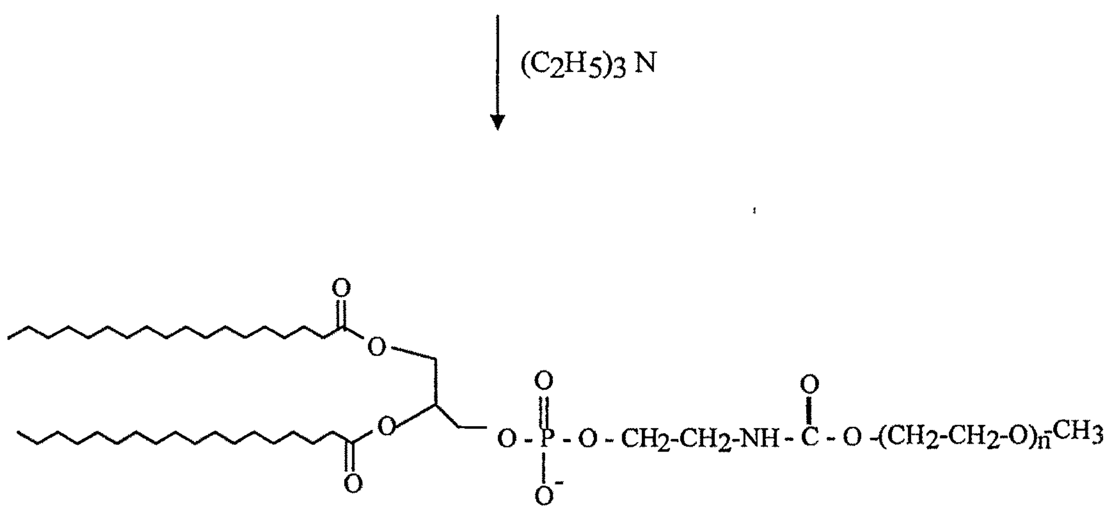
Figure 4.41 Reaction scheme for synthesis of methoxy polyethylene glycol 5000 activated with imidazoline carbonyl and coupled with phosphatidylethanolamine conjugate (mPEG5000-IC-PE)



Activated mPEG5000 with imidazoline carbonyl



Phosphatidylethanolamine



mPEG5000-IC-PE

Figure 4.42 Flowchart for the preparation of sterically stabilized liposomes containing methotrexate using methoxy polyethylene glycol 5000 activated with imidazoline carbonyl - phosphatidylethanolamine conjugate (mPEG5000-IC-PE)

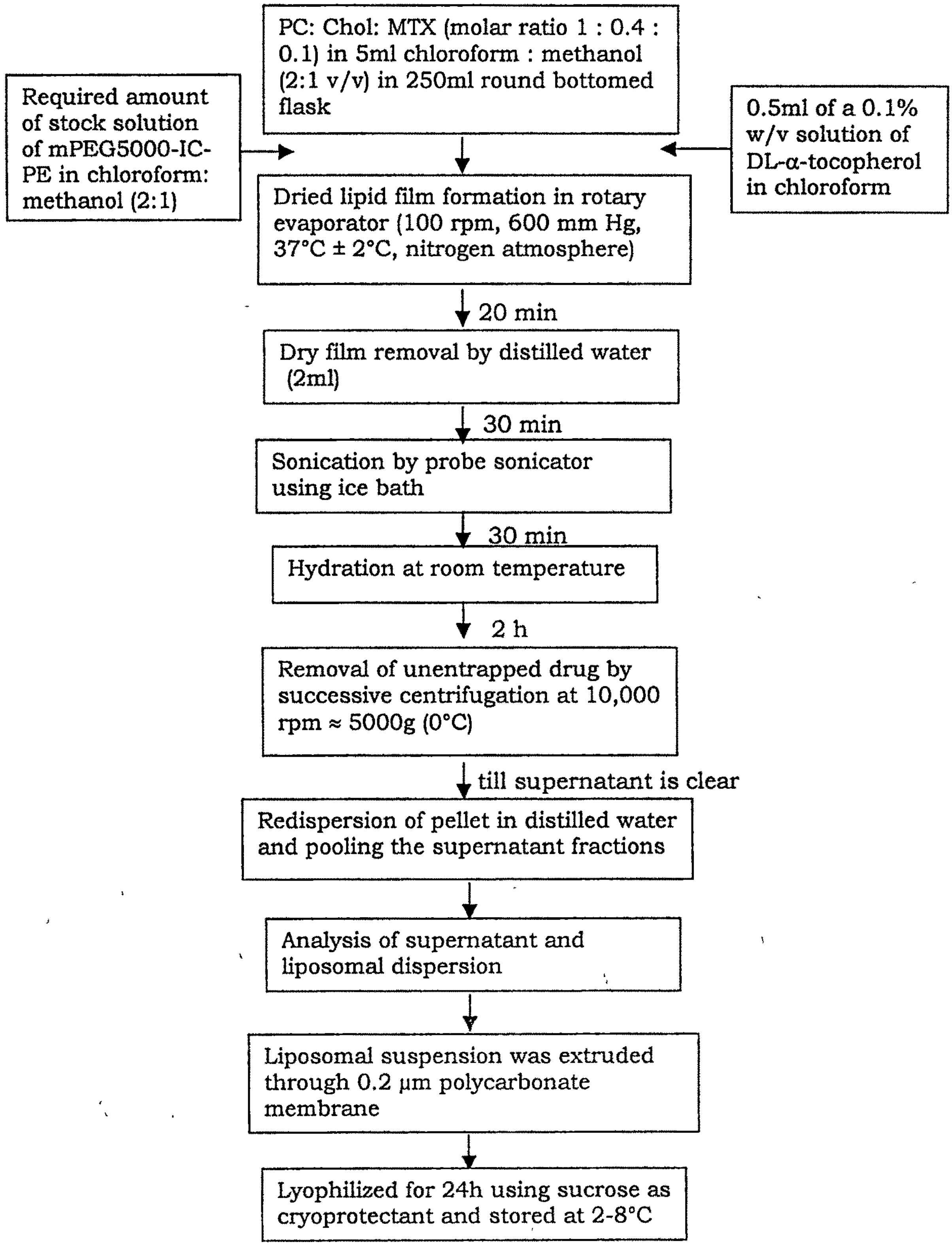


Table 4.13 Optimization of mPEG5000-IC-PE concentration, required for steric stabilization of methotrexate containing liposomes, using electrolyte induced flocculation test

Concentration of sodium sulphate (in M)	Mean absorbance ± S.E. at 400nm of methotrexate liposomes containing mPEG5000-IC-PE			
	3 mol%	5 mol%	7mol%	9mol%
0.0	0.565±0.014	0.542±0.032	0.554±0.026	0.545±0.027
0.4	0.588±0.022	0.548±0.016	0.560±0.018	0.552±0.016
0.8	0.601±0.016	0.545±0.023	0.564±0.013	0.561±0.030
1.2	0.611±0.011	0.552±0.016	0.571±0.035	0.560±0.043
1.6	0.615±0.026	0.558±0.011	0.576±0.021	0.568±0.011
2.0	0.623±0.028	0.567±0.024	0.580±0.012	0.572±0.026

Figure 4.43 Optimization of mPEG5000-IC-PE concentration, required for steric stabilization of methotrexate containing liposomes, using electrolyte induced flocculation test

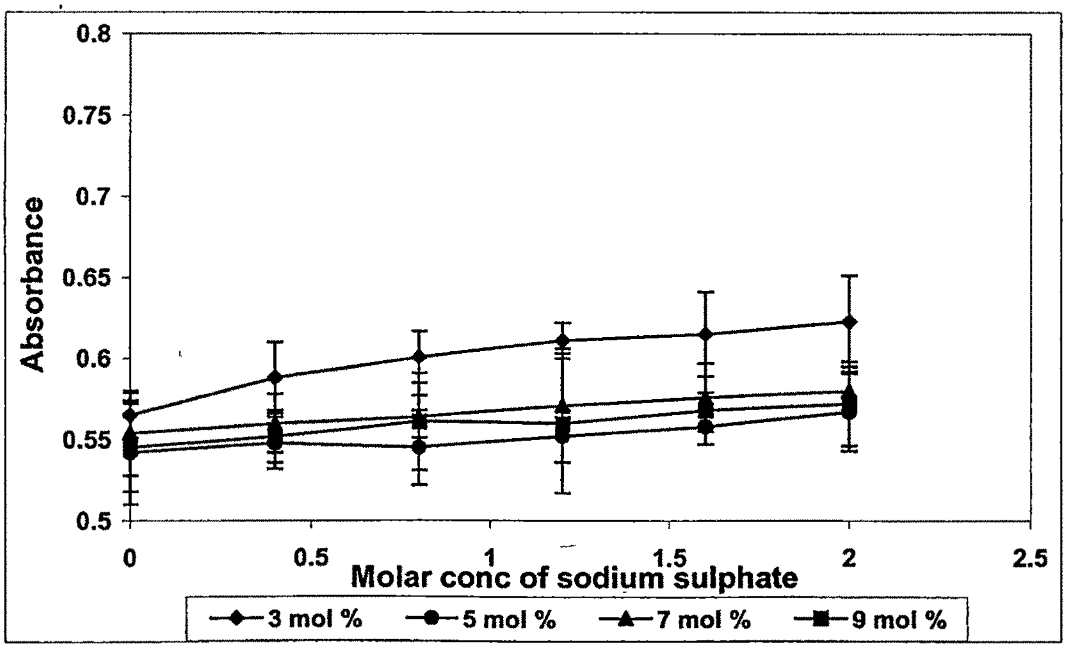


Figure 4.44 Flowchart for the preparation of sterically stabilized liposomes containing tamoxifen citrate using methoxy polyethylene glycol 5000 activated with imidazoline carbonyl - phosphatidylethanolamine conjugate (mPEG5000-IC-PE)

PC: Chol: TMX (molar ratio 1 : 0.67 : 0.067) in 5ml chloroform : methanol (2:1 v/v) in 250ml round bottomed flask

Required amount of stock solution of mPEG5000-IC-PE in chloroform: methanol (2:1 v/v) →

← 0.5ml of a 0.1% w/v solution of DL-α-tocopherol in chloroform

↓

Dried lipid film formation in rotary evaporator (100 rpm, 600 mm Hg, 37°C ± 2°C, nitrogen atmosphere)

↓ 20 min

Dry film removal by distilled water (2ml)

↓ 30 min

Sonication by probe sonicator using ice bath

↓ 30 min

Hydration at room temperature

↓ 2 h

Removal of unentrapped drug by successive centrifugation at 10,000 rpm ≈ 5000g (0°C)

↓ till supernatant is clear

Redispersion of pellet in distilled water and pooling the supernatant fractions

↓

Analysis of supernatant and liposomal dispersion

↓

Liposomal suspension was extruded through 0.2 μm polycarbonate membrane

↓

Lyophilized for 24h using sucrose as cryoprotectant and stored at 2-8°C

Table 4.14 Optimization of mPEG5000-IC-PE concentration, required for steric stabilization of tamoxifen citrate containing liposomes, using electrolyte induced flocculation test

Concentration of sodium sulphate (in M)	Mean absorbance ± S.E. at 400nm of tamoxifen citrate liposomes containing mPEG5000-IC-PE			
	3 mol%	5 mol%	7mol%	9mol%
0.0	0.640±0.010	0.638±0.024	0.628±0.023	0.624±0.021
0.4	0.654±0.026	0.634±0.016	0.632±0.022	0.629±0.011
0.8	0.662±0.012	0.641±0.035	0.635±0.017	0.632±0.020
1.2	0.671±0.024	0.644±0.016	0.638±0.016	0.636±0.029
1.6	0.675±0.032	0.652±0.015	0.635±0.035	0.640±0.013
2.0	0.688±0.028	0.655±0.021	0.642±0.021	0.638±0.018

Figure 4.45 Optimization of mPEG5000-IC-PE concentration, required for steric stabilization of tamoxifen citrate containing liposomes, using electrolyte induced flocculation test

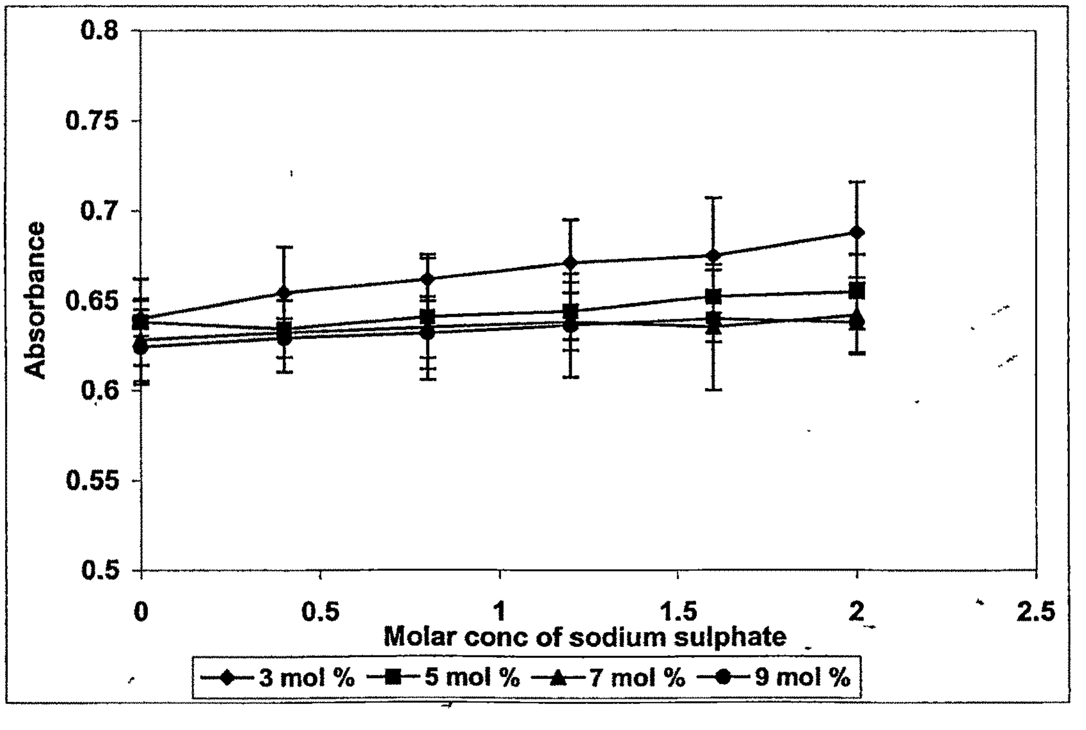


Figure 4.46 Flowchart for the preparation of sterically stabilized liposomes containing cytarabine using methoxy polyethylene glycol 5000 activated with imidazoline carbonyl-phosphatidyl ethanolamine conjugate (mPEG5000-IC-PE)

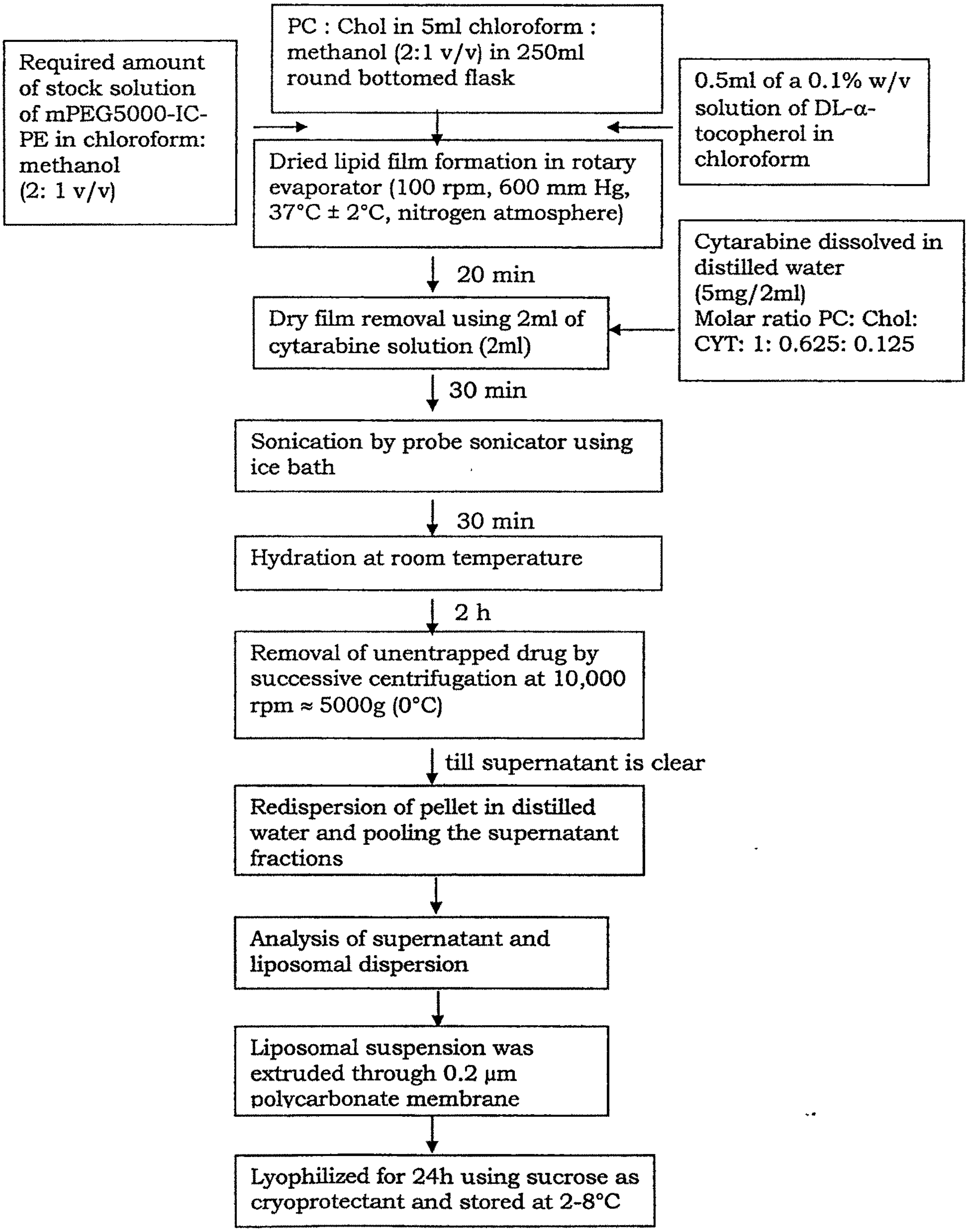
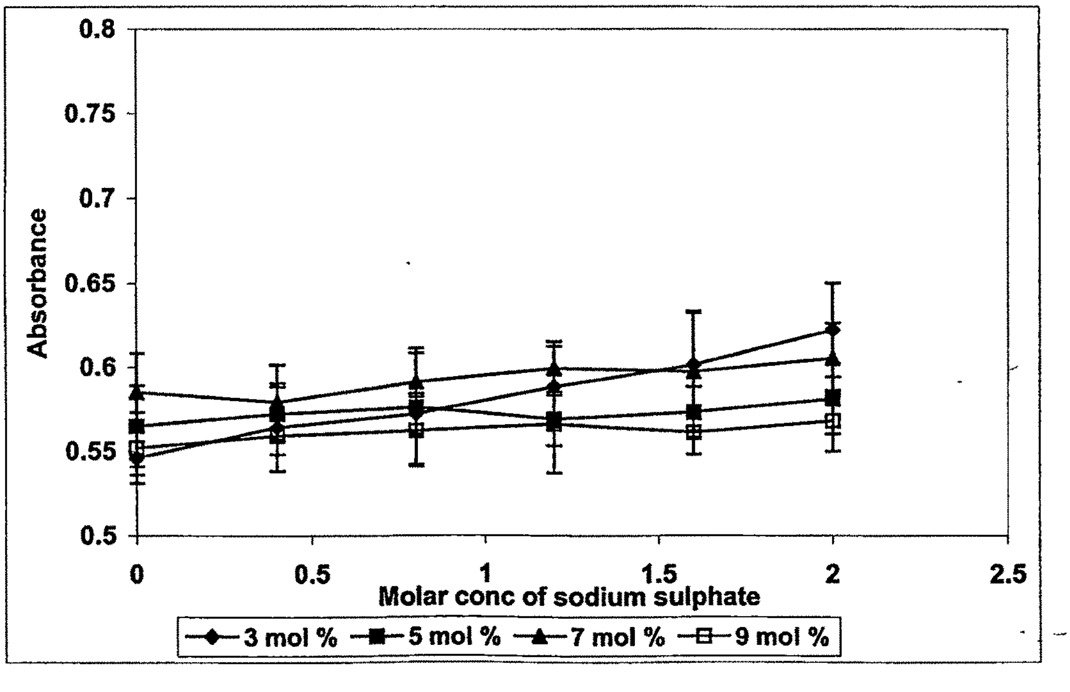


Table 4.15 Optimization of mPEG5000-IC-PE concentration, required for steric stabilization of cytarabine containing liposomes, using electrolyte induced flocculation test

Concentration of sodium sulphate (in M)	Mean absorbance ± S.E. at 400nm of cytarabine liposomes containing mPEG5000-IC-PE			
	3 mol%	5 mol%	7mol%	9mol%
0.0	0.546±0.10	0.565±0.024	0.585±0.023	0.552±0.021
0.4	0.564±0.026	0.572±0.016	0.579±0.022	0.559±0.011
0.8	0.572±0.012	0.576±0.035	0.591±0.017	0.562±0.020
1.2	0.588±0.024	0.569±0.016	0.599±0.016	0.566±0.029
1.6	0.601±0.032	0.573±0.015	0.597±0.035	0.561±0.013
2.0	0.622±0.028	0.581±0.021	0.605±0.021	0.568±0.018

Figure 4.47 Optimization of mPEG5000-IC-PE concentration, required for steric stabilization of cytarabine containing liposomes, using electrolyte induced flocculation test



4.3 RESULTS AND DISCUSSION

An attempt was made to prepare sterically stabilized liposomes containing anticancer drugs with the objective of increasing circulation time, thereby increasing the tumour uptake of drugs and reducing the side effects associated with these drugs. Methotrexate (MTX) and Tamoxifen citrate (TMX), predominantly lipophilic anticancer drugs and Cytarabine (CYT), a predominantly hydrophilic anticancer drug were chosen for the study due to their frequency of use and the range of side effects associated with their use.

4.3.1 OPTIMIZATION OF THE PREPARATION OF CONVENTIONAL LIPOSOMES OF MTX, TMX AND CYT

To start with, liposome formulation was optimised before proceeding for steric stabilization. Accordingly, conventional liposomes containing MTX, TMX and CYT were prepared initially followed by introduction of the agents, responsible for steric stabilization, into these formulations.

Multilamellar vesicles (MLVs) were chosen for the entrapment of MTX and TMX since the internal space of these vesicles contains concentric lipid layers making them good carriers for lipophilic compounds such as MTX and TMX. Such compounds can be included in the lipid mixture at the time of drying. Additional advantages of MLVs include ease of preparation, mechanical stability (an important requisite for sterically stabilized liposomes which have long blood circulation times) and ease of scale up (using a microfluidizer) (New, 1990). Being a water-soluble drug, large unilamellar vesicles (LUVs) of CYT would have been more suitable as these have a high proportion of aqueous compartment in them, which can hold hydrophilic drugs. However, MLVs provided better entrapment of CYT compared to other methods like reverse phase evaporation. The lipid film hydration technique was selected for the preparation of MLVs of MTX, TMX and CYT, as it is simple and reproducible (New, 1990). Though high melting lipids are recommended as membrane compounds for sterically stabilized liposomes, egg PC was taken as the chief compound of the liposomal membrane due to its comparative ease of availability and low cost over other high melting lipids such as distearoyl phosphatidyl choline and dipalmitoyl phosphatidyl choline. MTX and TMX 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).

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.

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). 5ml 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 100rpm 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 was found to be a critical factor for uniform transparent film formation. This is evident from the fact that the film obtained when water bath was not used for heating 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, lower temperatures (≅ 30°C ± 2°C) even though gave uniform films, showed opaque deposits at certain places. Temperatures in the vicinity of 37°C ± 2°C gave even films which were transparent in all regions. Higher temperatures (≅ 45°C) were investigated in the preparation of liposomes with high PC: Chol ratios which failed to give satisfactory films probably due to higher cholesterol content which overwhelmed the bilayer structure preventing proper film formation. Vacuum was maintained at about 600mm of mercury by means of nitrogen introduction via a bleed valve. Lower values of vacuum (≅300mm 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. Drying for longer period of time rendered flaky film, which could not be 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 distilled water, which, apart from acting as hydration medium, also assisted in film removal and dispersion. Initially phosphate buffer saline (pH 7.4) (PBS) was tried as hydration medium in case of MTX liposomes. But it was found that the entrapment of drug got reduced due to the solubility of the drug in PBS. Hence, distilled water was selected as hydration medium for the study. A period of 30min was found to be adequate for complete film removal and dispersion in most cases. In cases where some film remained adhering to the walls of the flask, a few glass beads (0.3cm diameter) were 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). An ice bath was used to dissipate heat generated in the process (New, 1990). 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. Room temperature (≅30°C) was found to be adequate for the annealing process signifying that the phase transition temperature of the lipids used in the liposomes was lower than these temperatures. The time of hydration for swelling was determined for all the three types of liposomes and is discussed in greater detail later in this chapter.

The next step in the preparation of liposomes was the separation of unentrapped drug from the liposomes. Initial trials with minicolumn centrifugation method using Sephadex G-50 (New, 1990a) failed to give satisfactory results. Centrifugation at 10,000rpm for 30 min at 0°C was found to be suitable for separation of the unentrapped drug from the liposomes provided the process was repeated till the supernatant above the pellet was clear. In order to confirm the results, a few batches were also subjected to protamine aggregation studies (New, 1990a). Results indicated that centrifugation was quite effective for separation of free MTX, TMX and CYT from their respective liposomes. The results in terms of the free drug obtained by protamine aggregation were marginally lower than those obtained by centrifugation suggesting that some of the very small liposomes formed by sonication (most probably SUVs) were not pellatized by centrifugation. The difference in drug entrapment obtained by both the methods was not significant.

The liposomal suspension was then freeze dried at −20°C overnight and lyophilised for 24h using sucrose as cryoprotectant. The lyophilisation process is discussed in detail in the stability of liposomes chapter.

The study of the above parameters in the preparation of liposomes indicated the vulnerability of liposomes containing MTX, TMX and CYT to the preparation conditions for drug entrapped. Therefore, it was felt necessary to optimise the following conditions of drug containing liposomes for reproducibility of drug entrapment.

4.3.1.1 Conditions for preparation of MTX containing liposomes

The following conditions were optimised for MTX containing liposomes.

A. Composition of the lipid mixture

Trials were initiated with a molar ratio of 1: 0.2: 0.1 PC: Chol: MTX (ML1, drug entrapment = 70.1% ± 1.49, Table 4.1). Increase in the proportion of cholesterol in the lipid mixture (ML7, drug entrapment = 84.2% ± 1.58, Table 4.1) significantly enhanced the percentage of drug entrapped (Calculated t = 8.78, tabulated t' = 2.78, at 4 degree of freedom, P= 0.95). 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 of 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). Methotrexate, by virtue of being able to bind to phosphatidyl choline strongly, utilizes this space to gain access to the lipophilic regions of the bilayer. Increase in the cholesterol proportion increases the space, which can be used by MTX. Therefore a great number of MTX molecules can bind to the phosphatidyl choline molecules than in the earlier case accounting for the increased drug entrapment observed when the cholesterol proportion is increased. However, further increase in the cholesterol proportion in the lipid mixture (ML13, drug entrapment = 84.6% ± 1.01, Table 4.1) did not lead to any significant improvement in the percentage of MTX entrapped (Calculated t = 0.176, tabulated t' = 2.78, at 4 degree of freedom, P= 0.95) probably due to saturation of the available space. The films formed in the last case were not smooth even when the temperature of the bath was increased.

B. Hydration medium and Hydration Volume

Initially phosphate buffer saline (pH7.4) (PBS) was used as the hydration medium for hydrating the dry lipid film. But it was found that the drug entrapment was reduced to greater extent (≅ 30%). The reduction in entrapment of MTX may be due to the solubility of the drug in PBS, which dissolve the drug from the lipid film and led to reduction in drug entrapment. Hence the hydration volume was changed to distilled water, which provided better entrapment.

The volume of distilled water was optimised at 2ml for MTX liposomes (ML11, drug entrapment = 95.8% ± 1.42, Table 4.1). Increase in the hydration volume to 3ml (ML12, drug entrapment = 95.2% ± 1.56, Table 4.1) did not show any significant increase in the volume of drug entrapment as evaluated by student 't' test (Calculated 't' = 0.428, tabulated 't' = 2.78, at 4 degree of freedom, P=0.95)

C. Hydration time

Increase in the hydration time in the rotary flash evaporator from 15 min (ML10, drug entrapment = 90.2% ± 0.23, Table 4.1) to 30 min (ML11, drug entrapment = 95.8 ± 1.42, Table 4.1) led to significant increase (Calculated 't' = 4.38, tabulated 't' = 2.78, at 4 degree of freedom, P=0.95) in drug entrapment. Further increase in hydration time does not led to increase in drug entrapment. This is not surprising considering that MTX; being incorporates itself in the lipid bilayer rather than the aqueous compartment. A decrease in hydration time (15min) also associated with the incompletely formed liposomes when observed microscopically. All the liposome formulations were kept 2 h at room temperature for annealing to be completed.

D. Sonication time

The sonication time was optimised using the above conditions. A sonication time of 30 min was found to be sufficient for reduction in the particle size to around 1 μm without any significant change in the percent drug entrapment.

4.3.1.2 Conditions for preparation of TMX containing liposomes

The following conditions were optimised for TMX containing liposomes.

A. Composition of the lipid mixture

Trials were initiated with a molar ratio of 1: 0.5: 0.1 PC: Chol: TMX (TL1, drug entrapment = 62.3% ± 1.48, Table 4.3). Increase in the PC content in the lipid mixture (TL9, drug entrapment = 86.5 ± 0.16, Table 4.3) significantly enhanced the drug entrapment (Calculated t'= 21.64, tabulated t' = 2.78, at 4 degree of freedom, P= 0.95). Increase in the proportion of cholesterol in the lipid mixture (TL11, drug entrapment = 89% ± 0.66 compared to TL7, drug entrapment = 77.2% ± 1.58, Table 4.3) significantly enhanced the drug entrapment (Calculated t'= 10.12, tabulated t'= 2.78, at 4 degree of freedom, P= 0.95) due to the same reason explained in case of MTX containing liposomes. Therefore a great number of TMX molecules can bind to the phosphatidyl choline molecules than in the earlier case accounting for the increased drug entrapment observed when the cholesterol proportion is increased.

B. Hydration Volume

The volume of distilled water was optimised at 2ml for TMX liposomes (TL11, drug entrapment = 89% ± 0.66, Table 4.3). Increase in the hydration volume to 3ml (TL12, drug entrapment = 88.8% ± 0.29, Table 4.3) did not show any significant increase in the volume of drug entrapment as evaluated by student t' test (Calculated t' = 0.32, tabulated t' = 2.78, at 4 degree of freedom, P=0.95)

C. Hydration time

The hydration time in the rotary flash evaporator was optimised at 30 min (TL11, drug entrapment = 89% ± 0.66, Table 4.3) Further increase in hydration time to 60min (TL12, drug entrapment = 88.8% ± 0.29, Table 4.3) does not led to increase in drug entrapment (Calculated t' = 0.32, tabulated t' = 2.78, at 4 degree of freedom, P=0.95). This is again, not surprising considering that TMX; being highly lipophilic, incorporates itself in the lipid bilayer rather than the aqueous compartment. All the liposome formulations were kept 2 h at room temperature for annealing to be completed.

D. Sonication time

The sonication time was optimised using the above conditions. A sonication time of 30 min was found to be sufficient for reduction in the particle size to around 1 μm without any significant change in the percent drug entrapment.

4.3.1.3 Conditions for preparation of CYT containing liposomes

The following conditions were optimised for CYT containing liposomes.

Cytarabine being water soluble, drug loading in the liposome is highly sensitive towards formulation parameters. The variables like temperature, vacuum applied and hydration time were kept constant as these variables does not contribute much towards the change in drug entrapment, while the formulation variables, Drug / Lipid [Phosphatidyl choline (PC) and Cholesterol (Chol)] molar ratio, PC / Chol in percent ratio of total lipids and the volume of hydration medium which have been predicted to play a significant role in enhancing the percent drug entrapment (PDE) are taken as variable parameters. 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 cytarabine liposomes were prepared. The liposome batches were evaluated for its drug entrapment within the liposomal vesicles. Mathematical modelling was carried out to obtain a second order polynomial equation (full model, equation 1) (Anthony Armstrong et al, 1996).

A substantial high drug entrapment achieved in liposomes prepared by lipid film hydration method was 83.5% at X_1 (1 : 13), X_2 (60 : 40) and X_3 (2 ml). 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).

$$Y = 69.141 + 5.366 X_1 + 2.323 X_2 - 0.356 X_3 + 7.745 X_1^2 - 2.322 X_2^2 - 7.59 X_3^2 - 0.253 X_1X_2 - 3.658 X_2X_3 - 4.008 X_1X_3 + 5.863 X_1X_2X_3 \quad (1)$$

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 43.5 to 83.5 % (Table 4.5). Small values of the coefficients of the terms X_3, X_2^2 and X_1X_2 in equation 1 are regarded as least contributing in the preparation of cytarabine 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.

$$Y = 67.556 + 5.394\ X_1 + 2.29\ X_2 + 7.71\ X_1^2 - 7.53\ X_3^2 - 3.658\ X_2X_3 - 4.008\ X_1X_3 + 5.863\ X_1X_2X_3 \qquad (2)$$

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 = 0.000005) and PC / Chol ratio (p value = 0.0098) are significant. The second order main effects of both Drug / Lipid ratio (p value = 0.000034) and volume of hydration (0.000048) are significant, as is evident from their p-values. The interaction between X_2X_3, X_1X_3 and $X_1X_2X_3$ are found to be very significant with the p values of 0.00156, 0.00072 and 0.00014 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 tabulated 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 =5.366) was found to be maximum and hence the variable, drug: lipid ratio (X_1) was considered to be a major contributing variable for PDE of cytarabine 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 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 = 0.9178 for full model and 0.9011 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 = 0.8664 for full model and 0.8646 for reduced model) are also very high which indicates a high significance of the model. A higher value of correlation coefficients (R = 0.958 for full model and 0.9493 for reduced model) signifies an excellent correlation between the independent variables (Box *et. al.,* 1978). 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 cytarabine (CYT) in liposomes primarily based on the combination of the lipids and the ratio between the drug and the lipids. It is really very interesting, even though CYT is water soluble, the study showed that the contribution of the hydration volume is not significant when compared to other two factors. The sonication time was optimised using the above conditions. A sonication time of 30min was found to be sufficient for reduction in the particle size to around 1μm without any significant change in the percent drug entrapment.

4.3.2 STERICALLY STABILIZED LIPOSOMES OF MTX, TMX AND CYT

Sterically stabilized liposomes of MTX, TMX and CYT were then, prepared with an aim of altering the pharmacokinetics of these drugs inside the body thereby increasing the tumor accumulation of the drugs. It was envisaged that such a change in pharmacokinetic properties particularly plasma residence time might lead to better efficacy of these drugs in the treatment of cancer due to increased accumulation in the cancer sites owing to the enhanced permeation and retention effect.

Torchilin and Trubetskoy have formulated some general requirements towards polymers for liposomes protection (Torchilin and Trubetskoy, 1995). These polymers should be soluble and hydrophilic and have a highly flexible main chain. Literature survey revealed that hydrophilic materials like PEG derivatives could be used for steric stabilization.

Polyethylene glycol (PEG) grafting has been widely used as a method for reduction of the rapid clearance from circulation by RES, of liposomes (Section 2.3.5, Literature Review). Therefore, PEG-lipid conjugates were incorporated in liposomes for steric stabilization. The approach investigated in the study, in which PEG-lipid conjugate was incorporated along with the lipids, which formed as a part of the bilayer. The methoxy polyethylene glycol 5000 activated with cyanuric chloride-phosphatidyl ethanolamine conjugate (mPEG5000-CC-PE), methoxy polyethylene glycol 2000 activated with cyanuric chloride-phosphatidyl ethanolamine conjugate (mPEG2000-CC-PE) and methoxy polyethylene glycol 5000 activated with imidazoline carbonyl-phosphatidyl ethanolamine conjugate (mPEG5000-IC-PE) were chosen for the study to achieve steric stabilization.

All the polymers for steric stabilization were chosen that no charge be imparted to the liposomes since particles with neutral surfaces were the most appropriate with regard to blood persistence (Section 2.3.4, Literature Review). The concentration of the polymer necessary for steric stabilization was optimised using electrolyte induced flocculation test. This is a standard test to investigate whether a 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 electrostatically 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 400nm 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 4th power of the wavelength of the incident light, hence a lower wavelength (400 nm) was used for measurements (Betagiri et al, 1993).

4.3.2.1 Conventional liposomes containing MTX, TMX and CYT

Figures 4.2, 4.4 and 4.6 show the plots of absorbance against the concentration of electrolyte added for conventional liposomes containing MTX, TMX and CYT respectively (data in Tables 4.2, 4.4 and 4.6). The conventional liposomes containing MTX, TMX and CYT showed a gradual increase in flocculation as the concentration of the sodium sulphate increases from 0M to 2M.

4.3.2.2 Liposomes of MTX, TMX and CYT sterically stabilized liposomes using PEG derivatives

Methoxy polyethylene glycol 5000 (mPEG5000) activated with cyanuric chloride-phosphatidyl ethanolamine conjugate (mPEG5000-CC-PE) has been investigated as a sterically stabilizing agent. Figure 4.12 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 4.9-4.11 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 4.14 - 4.16 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. In all the cases, 3% 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% on molar basis of this reagent was found to provide steric stability to the liposomes of MTX, TMX and CYT (Tables 4.7 to 4.9 and Figures 4.18, 4.20 and 4.22 respectively). Further increase in mPEG5000-CC-PE concentration (7% and 9% molar ratio) does not produce any significant increase in protection to that of the liposomes prepared using 5% of the reagent. The flow chart for the preparation of steric stabilized liposomes containing MTX, TMX and CYT are shown in Figures 4.17, 4.19 and 4.21 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 4.26 outlines the synthesis of this agent. 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, mPEG2000-CC) as evidenced by quantitative UV spectroscopy and IR spectra (Figures 4.24 and 4.25 respectively). Reaction of this product with phosphatidyl ethanolamine using the method suggested by Blume and Cevc (Blume and Cevc, 1990) gave the agent, mPEG2000-CC-PE. The UV, IR and ^{13}C-NMR spectra of this product are shown in Figures 4.27-4.29 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, 3% and 5% 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. 7% on molar basis of this reagent was found to provide steric stability to the liposomes of MTX, TMX and CYT (Tables 4.10 to 4.12 and Figures 4.31, 4.33 and 4.35 respectively). Further increase in mPEG2000-CC-PE concentration (9% molar ratio) does not produce any significant increase in protection to that of the liposomes prepared using 7% of the reagent. The flow chart for the preparation of steric stabilized liposomes containing MTX, TMX and CYT are shown in Figures 4.30, 4.32 and 4.34 respectively.

Methoxy polyethylene glycol 5000 activated with imidazoline carbonyl and coupled with phosphatidylethanolamine conjugate (mPEG5000-IC-PE) has been investigated as a sterically stabilizing agent. Figure 4.41 outlines the synthesis of this agent. The synthesis of the compound was performed as per the method described by Allen and coworkers (Allen *et. al.,* 1991). A molar ratio of 1:2 of methoxy polyethylene glycol (mPEG5000) activated with imidazoline carbonyl to the phosphatidylethanolamine was found to give the desired product (methoxy polyethylene glycol 5000 activated with imidazoline carbonyl-phosphatidyl ethanolamine conjugate, mPEG5000-IC-PE) as evidenced by the UV, IR and ^{13}C-NMR spectra shown in Figures 4.37, 4.39 and 4.40 respectively. In all the cases, 3% 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% on molar basis of this reagent was found to provide steric stability to the liposomes of MTX, TMX and CYT (Tables 4.13 to 4.15 and Figures 4.43, 4.45 and 4.47 respectively). Further increase in mPEG5000-IC-PE concentration (7% and 9% molar ratio) does not produce any significant increase in protection to that of the liposomes prepared using 5% of the reagent. The flow chart for the preparation of steric stabilized liposomes containing MTX, TMX and CYT are shown in Figures 4.42, 4.44 and 4.46 respectively. The mechanism of protection afforded by PEG has been already elucidated in the previous chapter (Section 2.3.6, Literature Review)

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