CHAPTER 5

CHARACTERIZATION OF LIPOSOMES

5.1 INTRODUCTION

Both physical and chemical characteristics of liposomes influence their behaviour *in vivo* and *in vitro*. There are several examples demonstrating the importance of proper selection of liposome structures to obtain optimum and reproducible therapeutic effects (Section 2.2.9, Literature review). Physical and chemical characterizations are very important for a meaningful comparison of different liposome preparations or different batches prepared according to the same protocols. Biological considerations help to ensure safety of use in humans. As a rule, combinations of various characterization methods are used, as none of the existing techniques alone is able to describe liposomes adequately. The various techniques used in characterization have been extensively discussed previously (Section 2.2.9, Literature review). Liposome characterization should be performed immediately after preparation. One should also ensure that no major changes occur on storage so that a well-characterized product is injected and the liposome dispersion warrants optimal reproducibility of clinical effects.

5.2 EXPERIMENTAL

5.2.1 REAGENTS

Disodium hydrogen phosphate and potassium dihydrogen phosphate both of ExcelR grade (Qualigens Fine Chemicals, Mumbai); sodium chloride and ortho phosphoric acid, both of analytical reagent (A.R.) grade, iodine sublimed (S.D. Fine Chemicals Ltd., Boisar, Thane).

5.2.2 APPARATUS

Olympus microscope BX 40 (Olympus Optical Co. Lts., Japan); Malvern particle size analyser (Malvern Master sizer 2000 SM, U.K.); Scanning electron microscope (Jeol, JSM-840 SEM, Japan); Mettler DSC 20 Differential Scanning Calorimeter (Mettler Toledo, Switzerland); Karl fisher Autotitrator (Toshiwal Instruments (Bombay) Pvt. Ltd., Nasik; Remi Cooling centrifuge Model C-24 (Remi Equipments, Mumbai);

5.2.3 SOLUTIONS

Phosphate buffered saline, pH 7.4 (PBS) was prepared as per the method detailed in the Indian Pharmacopoeia (1985).

5.2.4 CHARACTERIZATION

The prepared liposomes containing cyclosporine (CsA), Leuprolide acetate and DNA both conventional and sterically stabilized, were characterized for the following attributes.

5.2.4.1 Morphology and lamellarity

Morphology and lamellarity of the conventional and sterically stabilized liposomes was ascertained from photomicrographs taken using an Olympus BX40 microscope at a magnification of 2,500 X. Figures 5.1 to 5.10 shows the photomicrographs of the various liposomes before extrusion using polycarbonate filter.

5.2.4.2 Size

The mean particle size of the prepared liposomes was obtained by using Malvern particle size analyser model SM 2000, which follows Mieś theory of light scattering. Diluted liposome suspension was added to the sample dispersion unit-containing stirrer and stirred at 2000 rpm in order to reduce the interparticle aggregation, and laser obscuration range was maintained between 10-20%. The average particle size was measured after performing the experiment in triplicate. Table 5.1 shows the mean particle size of the various liposomal formulations before extrusion and after extrusion through 0.2μm Nucleopore polycarbonate track-etch membrane filters (Whatman Inc. New Jersey, USA). The particle size distribution pattern of the liposomal formulations after extrusion was shown in the particle size analysis data.

5.2.4.3 Scanning electron microscopy (SEM)

The lyophilized liposome powder was coated with gold and then kept in the sampling unit as a thin film and then the photograph was taken at 11,000X magnifications using Jeol Scanning Electron Microscope (Jeol, JSM-840 SEM, Japan). Figures 5.11 to 5.16 show the scanning electron micrographs of various liposomal formulations.

5.2.4.4 Differential Scanning Calorimetry (DSC)

The DSC thermograms of representative batches of some of the prepared liposomes i.e. CL, CPL, LL, SLL5000-CC-PE, DL and SDL5000-CC-PE were taken on a Mettler DSC 20 Differential Scanning Calorimeter between 30 – 100°C at a heating rate of 10°C/min and nitrogen atmosphere. The thermograms are shown as figures 5.17 -5.22.

5.2.4.5 Entrapped volume

The prepared liposomal formulations were centrifuged for an appropriate period of time at 10,000 –15, 000 rpm (≈5000 - 7500g) at 0°C to form tight pellets. The water content of these pellets was determined by Karl fisher Autotitrator. The entrapped volume of the liposomal formulations was then calculated from these determinations and shown in table 5.1.

5.2.4.6 Zeta potential

Zeta potential of the liposomal dispersions in phosphate buffer pH 7.4 was measured in Malvern Zetasizer 3000 HS_A (Malvern Instruments, UK) and the values are shown in table 5.1.

5.2.4.7 Electrolyte induced flocculation test

This test has already been discussed in Chapter 4, Preparation of liposomes.

5.2.4.8 Drug entrapment

The entrapment of CsA, Leuprolide acetate and DNA in the prepared liposomes was studied using the methods described in Chapter 3, Analytical methods, for estimation of the drugs in liposomes. Table 5.1 shows the drug entrapment of the various liposomal formulations. Tables 5.2, 5.3 and 5.4 are analysis of variance (ANOVA) tables constructed for comparing the results obtained for the different liposomal formulations.

Table 5.1 Attributes of plain and sterically stabilized liposomes containing CsA, Leuprolide acetate and DNA

Liposome type	Mean Particle Size ± S.E.M (nm)		Zeta potential (meV)	Entrapped volume (µl/µmol of lipid)	Mean Drug entrapped* ± S.E.
	Before extrusion	After extrusion			
CPL	700.23 ±135.6	198.2 ± 54.6	+ 20	0.526	96.61 ± 2.45
CNL	689.6 ±144.8	185.3 ±32.6	-40.2	0.518	93.52 ± 3.22
CL	810.23 ±165.9	177.4 ±27.4	-7.02	0.520	92.13 ± 1.67
LL	600.7 ±170.2	188 ±27.4	-40.4	0.436	47.12 ±2.64
SLL5000	820.8 ±162.6	175 ±22.3	-20.4	0.420	37.8 ±3.46
SLL2000	842.3 ±170.6	179 ±30.2	-15.3	0.408	33.03 ±4.02
DL	756.3 ±159.9	182 ±33.8	-33.8	0.446	49.56 ±0.96
SDL5000	820.3 ±120.3	190.6 ±24.5	-23.2	0.432	45.34 ±1.46
SDL2000	900.6 ±130.6	236.2 ±25.3	-17.6	0.425	46.23 ±0.89
CDL	850.6 ±152.3	202.6 ±25.6	35.8	0.432	78.02 ±1.02
CSDL2000	820.3 ±160.5	205.6 ±22.9	+26.8	0.416	75.62 ±1.45

*n=3

Table 5.2 Analysis of variance (ANOVA) table for drug entrapment in conventional and charged liposomes containing cyclosporine

Source of Variation	Sum of Squares (SS)	DF	Mean Square (MS)	$F_{2,6}$	P-value	Tabulated F value
Between Groups	31.55	2	15.78	2.47	0.165	5.143
Within Groups	38.32	6	6.39			
Total	69.87	8				

Table 5.3 Analysis of variance (ANOVA) table for drug entrapment in conventional and sterically stabilized liposomes containing leuprolide acetate

Source of Variation	Sum of Squares (SS)	DF	Mean Square (MS)	$F_{2,6}$	P-value	Tabulated F value
Between Groups	291.026	2	145.51	19.83	0.0023	5.14
Within Groups	44.028	6	7.34			
Total	335.05	8				

Table 5.4 Analysis of variance (ANOVA) table for drug entrapment in conventional and sterically stabilized liposomes containing DNA

Source of Variation	Sum of Squares (SS)	DF	Mean Square (MS)	$F_{4,10}$	P-value	Tabulated F value
Between Groups	3179.61	4	794.90	596.96	7.54*10-12	3.48
Within Groups	13.32	10	1.33			
Total	3192.93	14				

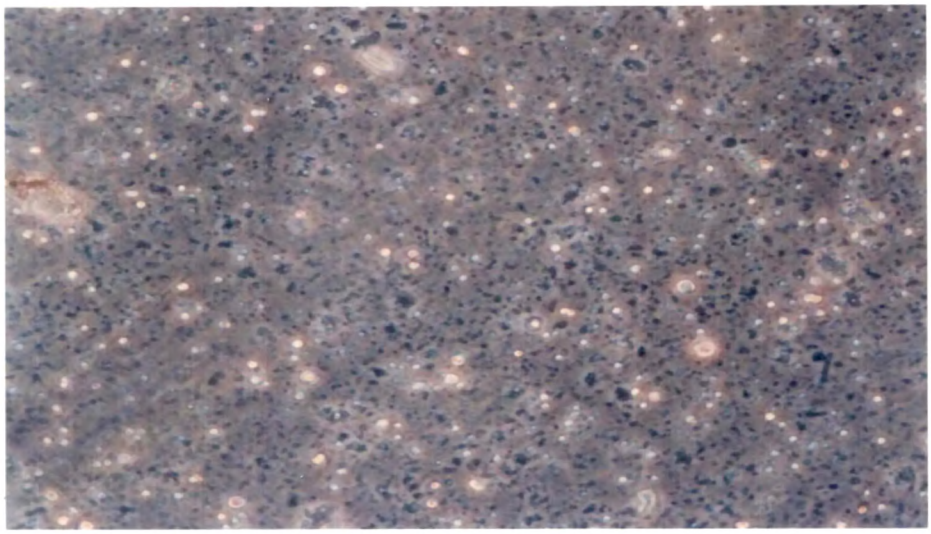

Figure 5.1 Photomicrograph of conventional liposomes containing cyclosporine

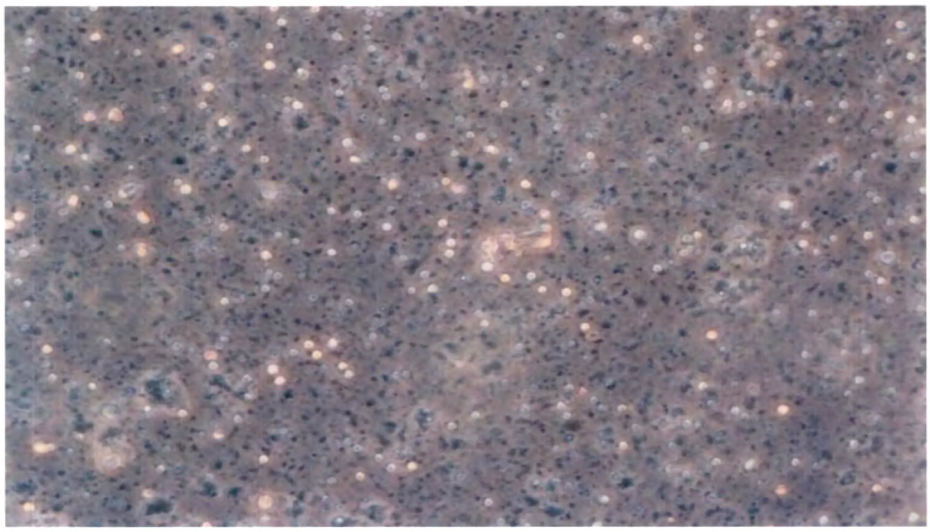

Figure 5.2 Photomicrograph of positive charged liposomes containing cyclosporine

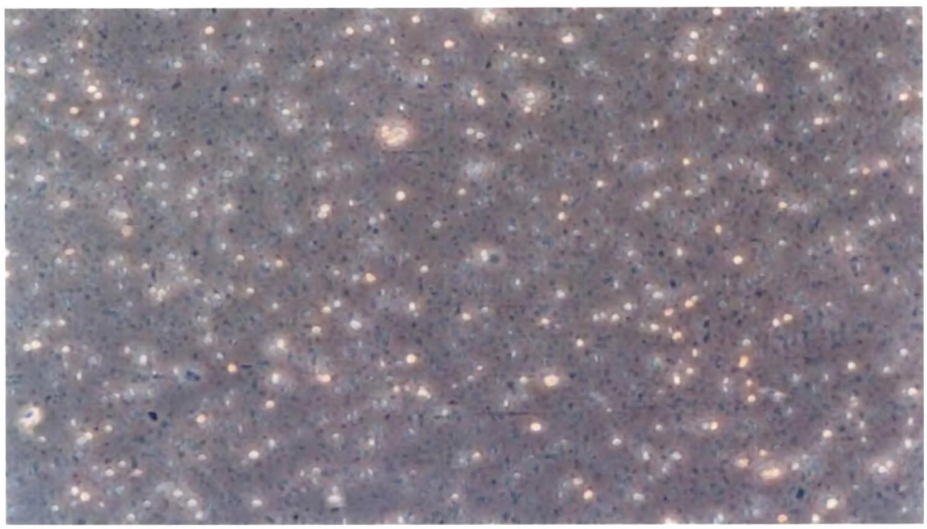

Figure 5.3 Photomicrograph of negative charged liposomes containing cyclosporine

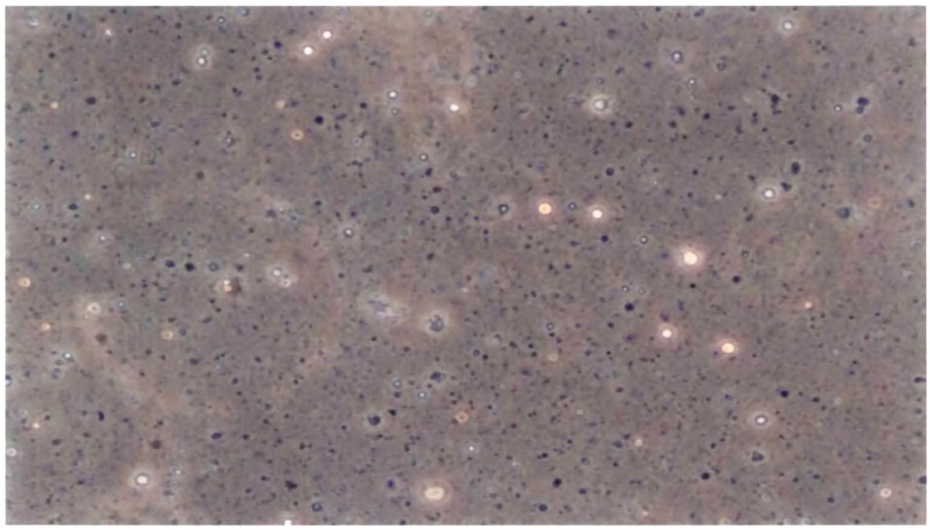

Figure 5.4 Photomicrograph of conventional liposomes containing leuprolide acetate

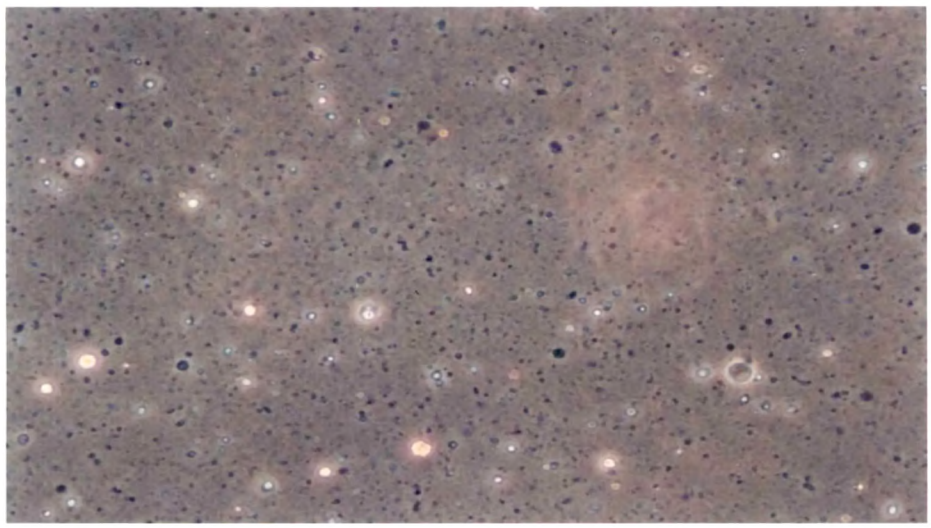

Figure 5.5 Photomicrograph of liposomes containing leuprolide acetate sterically stabilized using methoxy polyethylene glycol 5000 – activated with cyanuric chloride - phosphatidylethanolamine conjugate

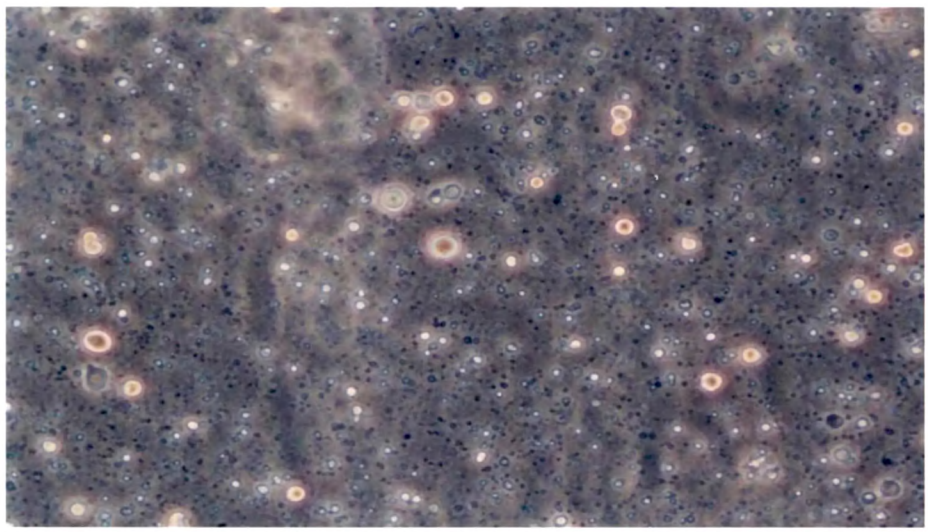

Figure 5.6 Photomicrograph of liposomes containing leuprolide acetate sterically stabilized using methoxy polyethylene glycol 2000 - activated with cyanuric chloride - phosphatidyl ethanolamine conjugate

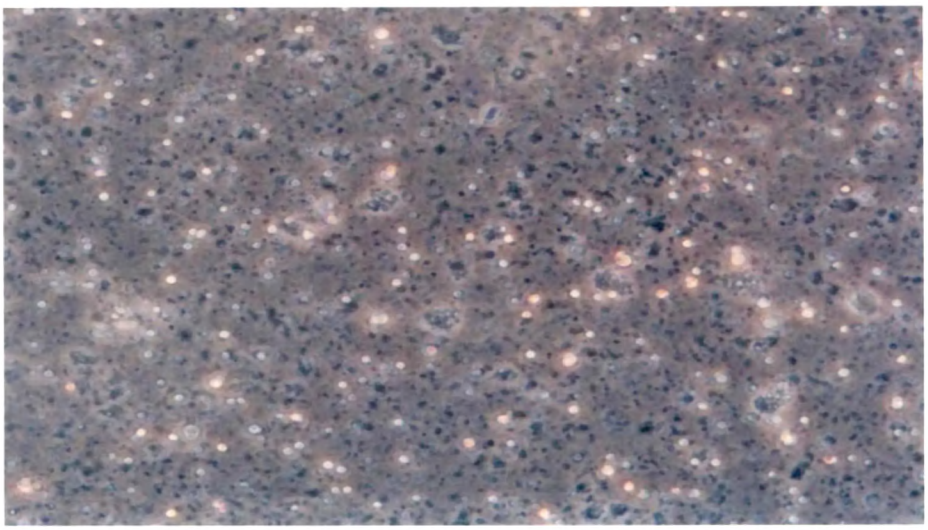

Figure 5.7 Photomicrograph of conventional liposomes containing DNA

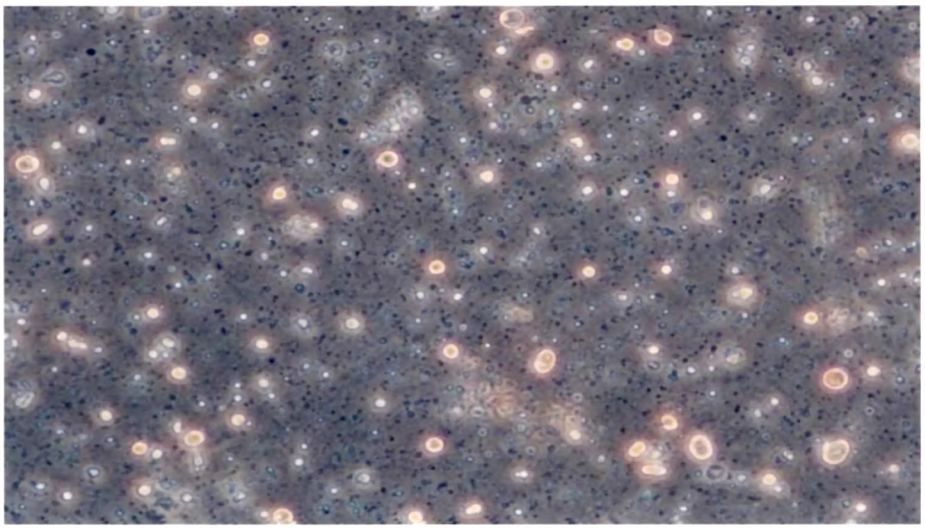

Figure 5.8 Photomicrograph of liposomes containing DNA sterically stabilized using methoxy polyethylene glycol 5000 – activated with cyanuric chloride -phosphatidyl ethanolamine conjugate

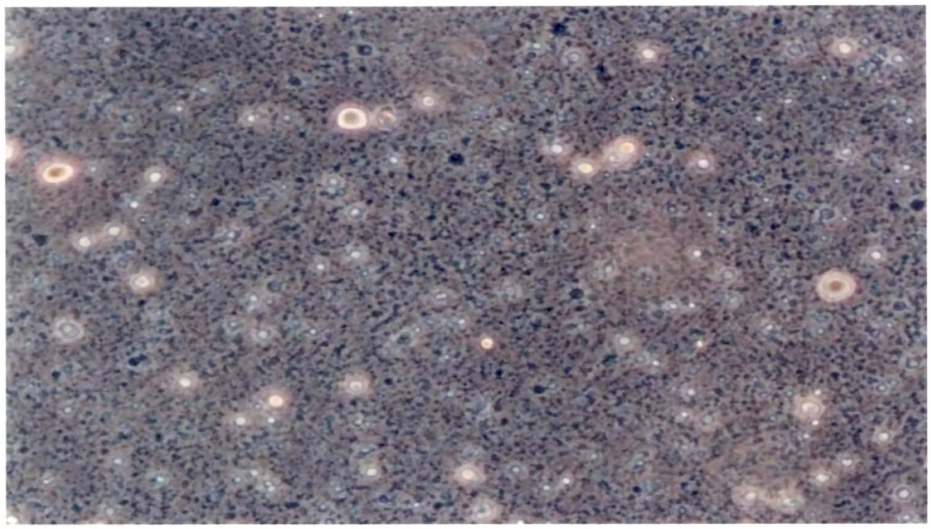

Figure 5.9 Photomicrograph of liposomes containing DNA sterically stabilized using methoxy polyethylene glycol 2000–activated with cyanuric chloride -phosphatidyl ethanolamine conjugate

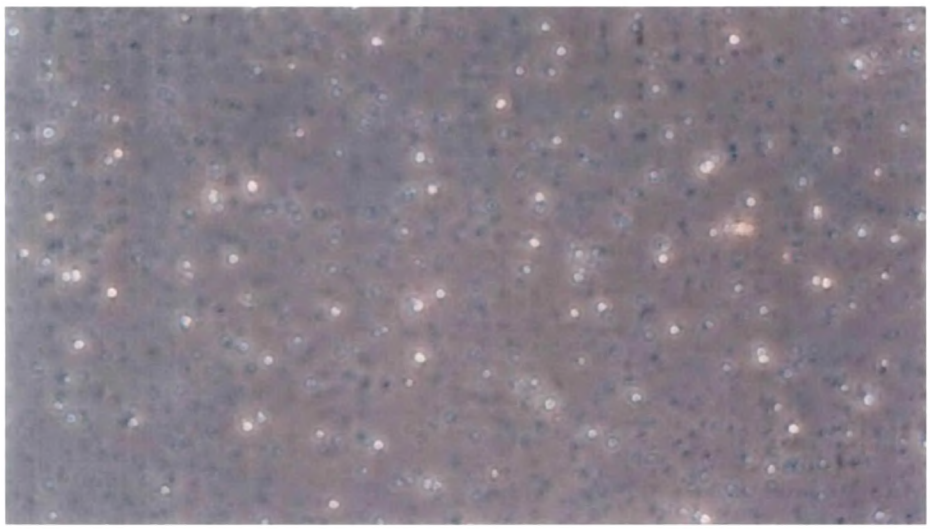

Figure 5.10 Photomicrograph of cationic liposomes containing DNA sterically stabilized using methoxy polyethylene glycol 2000–activated with cyanuric chloride-phosphatidyl ethanolamine conjugate

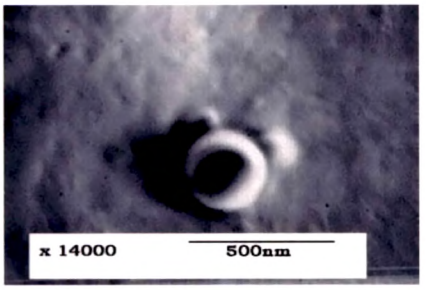

Figure 5.11 Scanning electron micrograph of conventional liposomes containing cyclosporine

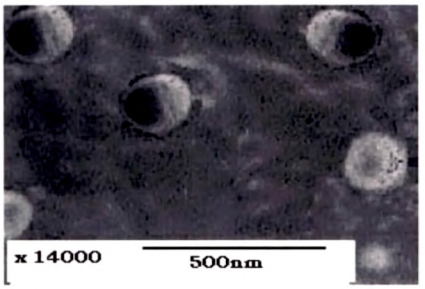

Figure 5.12 Scanning electron micrograph of conventional liposomes containing leuprolide acetate

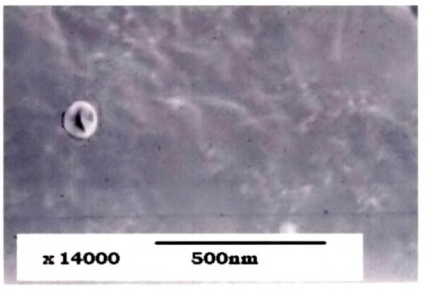

Figure 5.13 Scanning electron micrograph of liposomes containing leuprolide acetate sterically stabilized using mPEG5000-CC-PE

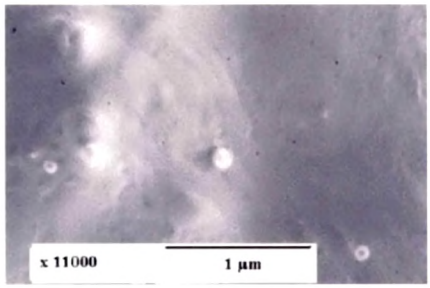

Figure 5.14 Scanning electron micrograph of conventional liposomes containing DNA

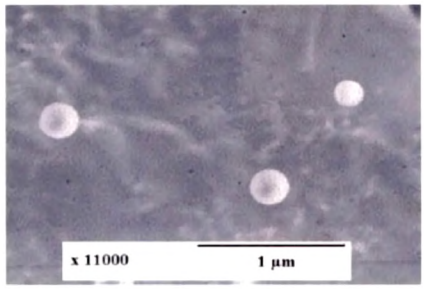

Figure 5.15 Scanning electron micrograph of conventional liposomes containing DNA sterically stabilized using mPEG2000-CC-PE

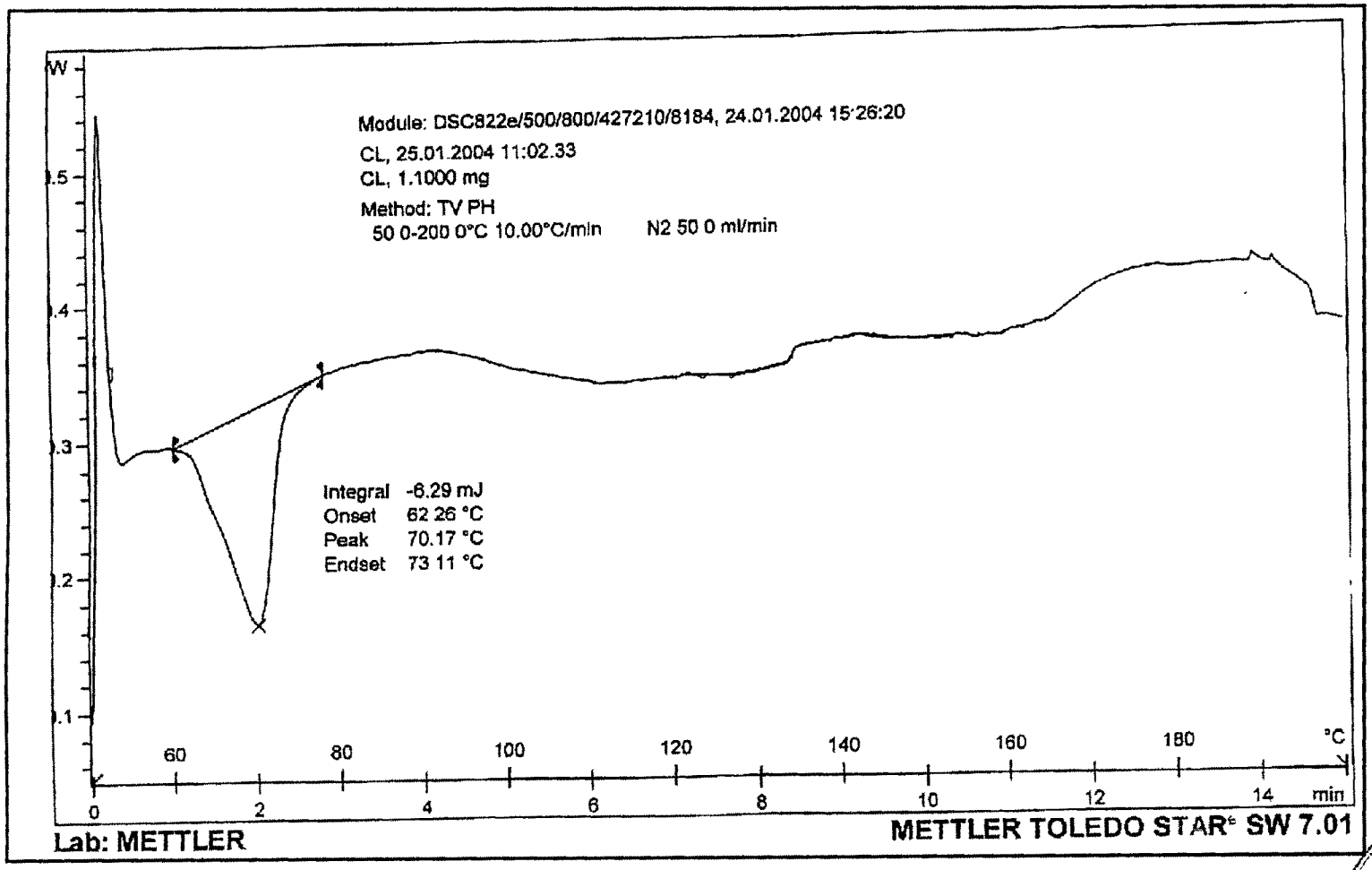

Figure 5.16 DSC thermogram of conventional liposomes containing cyclosporine

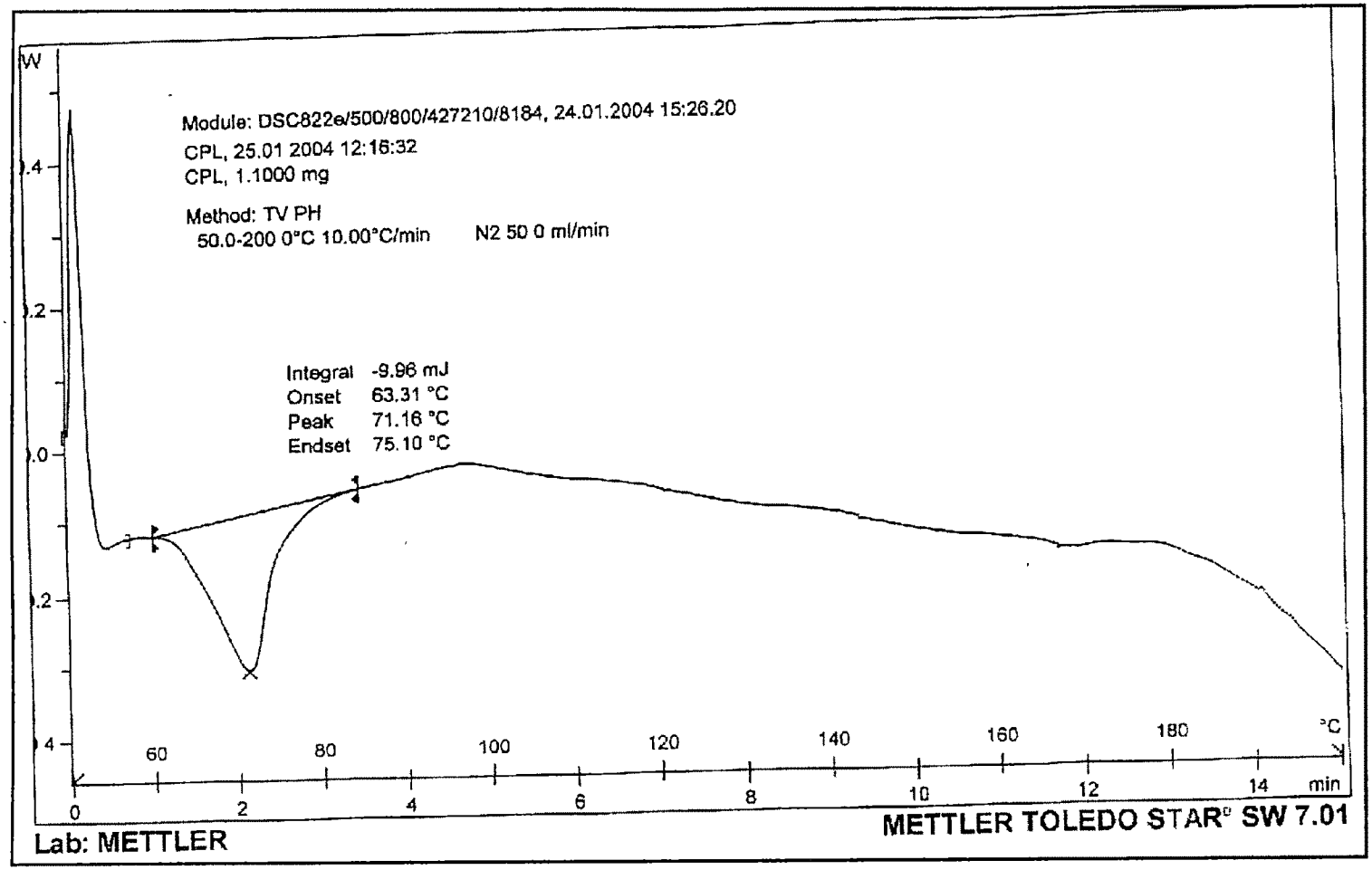

Figure 5.17 DSC thermogram of positive charged liposomes containing cyclosporine

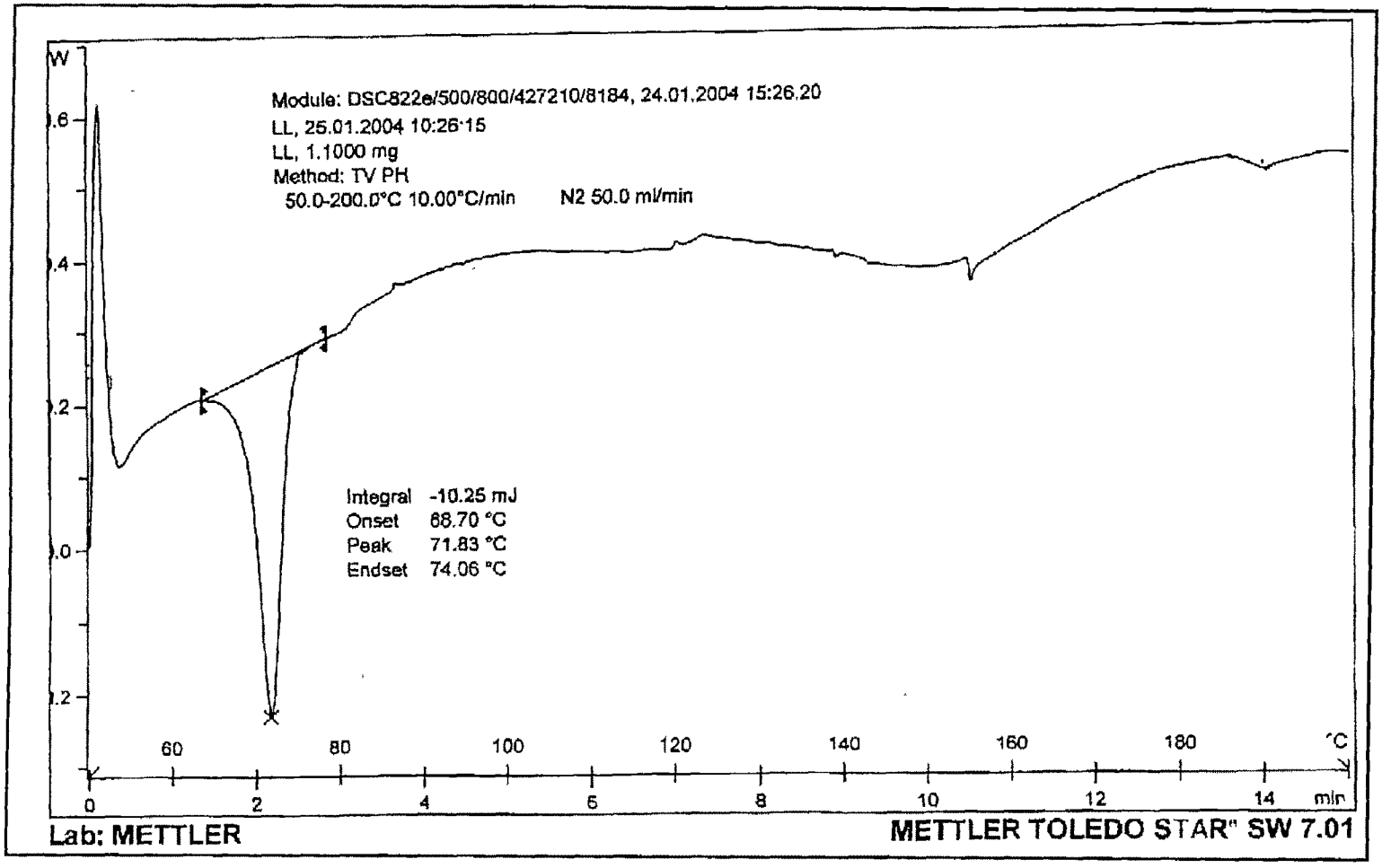

Figure 5.18 DSC thermogram of conventional liposomes containing leuprolide acetate

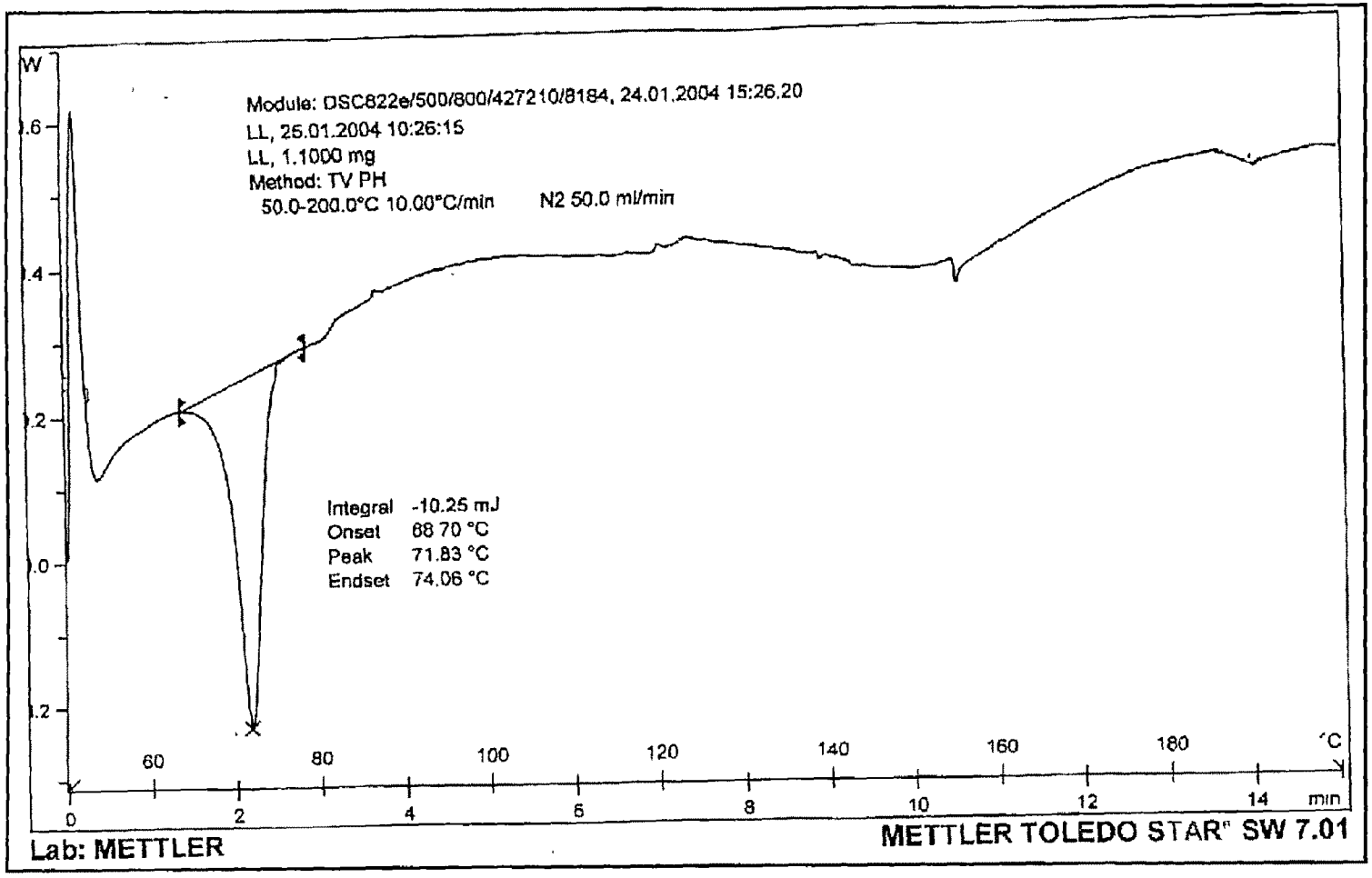

Figure 5.1 DSC thermogram of liposomes containing leuprolide sterically stabilized using methoxy polyethylene glycol 2000 -activated with cyanuric chloridephosphatidyl ethanolamine conjugate (mPEG2000-CC-PE)

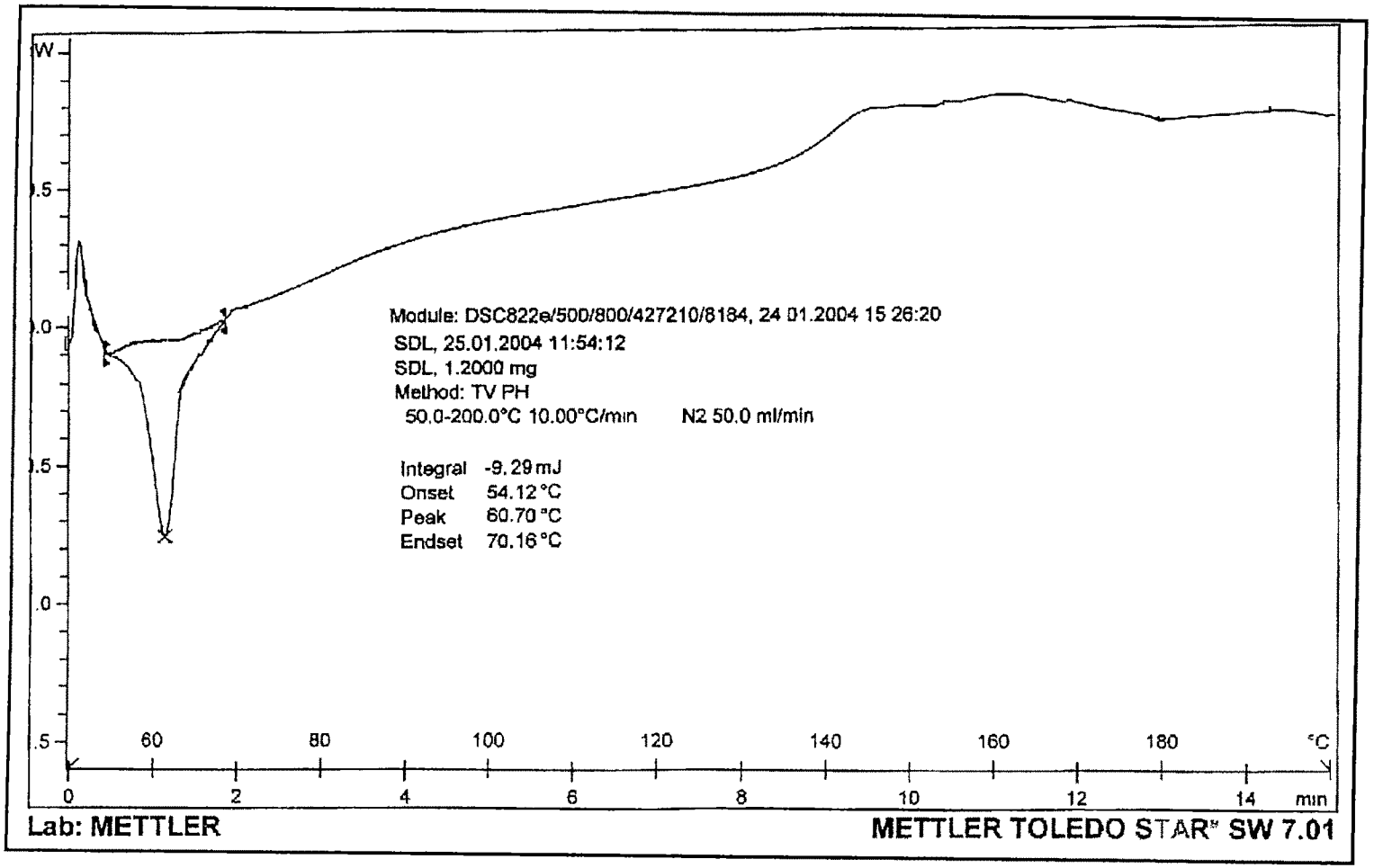

Figure 5.20 DSC thermogram of conventional liposomes containing DNA

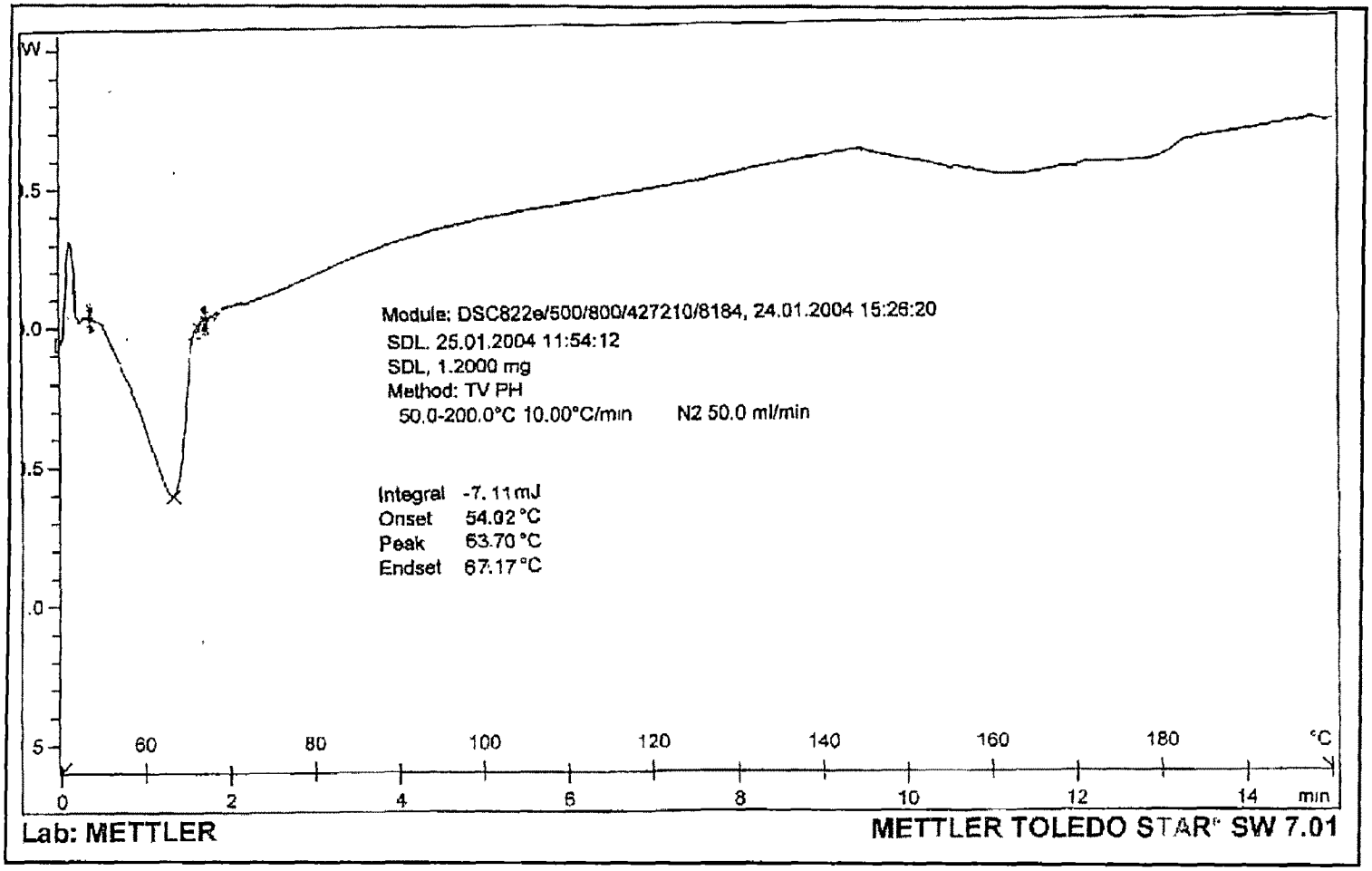

Figure 5.21 DSC thermogram of liposomes containing DNA sterically stabilized using methoxy polyethylene glycol 5000 -activated with cyanuric chloride -phosphatidyl ethanolamine conjugate (mPEG5000-CC-PE)

5.3 RESULTS AND DISCUSSION

An attempt was made to characterize the conventional and sterically stabilized liposomes containing CsA, leuprolide acetate and DNA. It was envisaged that such characterization would help in gaining a deeper insight into the factors that affect the performance of these liposomes both *in vitro* and *in vivo*.

5.3.1 MORPHOLOGY AND LAMELLARITY

The close examination of the photomicrographs of the prepared liposomes (figures 5.1 –5.10) indicates that the majority of the prepared liposomes were spherical and multilamellar before extrusion. Some unilamellar structures are also observed, the presence of which can be attributed to the sonication process used for size reduction of the prepared liposomes.

The SEM photograph (figures 5.11 –5.15) of the liposomes after extrusion through 0.2μm track-etch polycarbonate filters showed that the liposomes are spherical in shape. The surface of the conventional and sterically stabilized liposomes does not show any visual difference.

5.3.2 PARTICLE SIZE

Table 5.1 shows the mean particle size of the prepared liposomes before and after extrusion through 0.2μm Nuclepore polycarbonate filters. The sonication time of 15 minutes was found to be sufficient to reduce the particle size of less than 900nm. The sonication time of 15min was found to be adequate to reduce the size of the conventional liposomes of CsA, leuprolide acetate and DNA to around 900nm. Increase in sonication time to 30min does not significantly reduce the particle size further. Hence the sonication time was fixed at 15min during the preparation of the liposomes.

The liposomes were sequentially extruded through 2-stacked Nucleopore 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 resulting liposomes were of uniform particle size range of around 200nm, which is very important for *in vivo* biodistribution and tumour targeting.

The particle size of the sterically stabilized liposomes of CsA, leuprolide acetate and DNA (Table 5.1) indicates that, in general, there is no significant change in particle size of these liposomes compared to that of the corresponding conventional liposomes containing CsA, leuprolide acetate and DNA. Hence it is evident that under similar preparative conditions, such as lipid composition, charge and presence of protecting polymer only minimally influence vesicle size.

5.3.3 DIFFERENTIAL SCANNING CALORIMETRY (DSC)

DSC of some of the prepared liposomal formulations viz. CL, CPL, LL, SLL5000-CC-PE, DL, and SDL2000-CC-PE, was done with a view to understand the influence of insertion of charge and steric stabilizing agents, in the bilayer, on the phase transition temperature of the system. Also, DSC thermograms would be able to identify whether the agents are completely miscible with the components of the bilayer or not. The thermograms are shown as figures 5.16-5.21.

Examination of the thermograms of the charged liposomes and sterically stabilized liposomes prepared using poly ethylene glycol derivatives (mPEG5000-CC-PE and mPEG2000-CC-PE) showed that the agents introduced are completely miscible with the other components of the bilayer. This is because all the thermograms of the sterically stabilized liposomes and the charged liposomes show a single-phase transition peak and no shoulders on these peaks are visible (Bedu-Addo and Huang, 1995).

The DSC thermograms of charged liposomes and sterically stabilized liposomal formulations of CsA, leuprolide acetate and DNA (figures 5.18-5.23) shows that phase transition temperature of the liposomes lies between 55° to 60°C. The incorporation of charge and polyethylene glycol derivatives (mPEG5000-CC-PE and mPEG2000-CC-PE) into the bilayer of the liposomes containing CsA, leuprolide acetate and DNA does not seem to alter the bilayer rigidity. It is evident from the similar phase transition temperatures obtained for the different liposomal formulations prepared using charged liposomes containing CsA and steric stabilizing agents as compared to that of the conventional liposomes containing leuprolide acetate and DNA.

5.3.4 ZETA POTENTIAL

The attachment of PEG neutralises the effect of any charged component. Liposome grafted with PEG prevents liposome clearance by neutralising the surface charge of liposomes and shielding various opsonins. While PEG-PE itself demonstrates slight electronegativity, with shorter PEG producing a more electronegative product, a larger PEG can effectively shield the charge of a phospholipid block as well as the charge of the whole liposome. This means that even if the surface potential of a PEG-liposome is negative, the net zeta potential of such a liposome is close to neutral.

The neutral liposomes containing cyclosporine in case of CL the zeta potential was around -7meV, the addition of positive charge lipid (Stearylamine) in case of CPL increased the zetapotential to approximately +20meV, while the addition of negative charge lipid (DSPG) in case of CNL sharply decreased the zeta potential to -40meV.

In the case of conventional liposomes containing DNA made of HSPC and DOTAP, the zeta potential was around 35.8 meV, the attachment of PEG to charged liposomes brought down their zeta potential to about 26.8meV. In the case of conventional liposomes containing leuprolide acetate and DNA made of HSPC and DSPG, the zeta potential was around -40.38 meV, the attachment of PEG to charged liposomes brought their zeta potential close to that of plain liposomes [-20.4meV , -15.3meV and -23.2meV, -17.6meV in case of SLL5000-CC-PE, SLL2000-CC-PE and SDL5000-CC-PE, SDL2000-CC-PE) repectively. Hence 5 mol% and 6 mol% PEG-PE in liposomes with a molecular weight (5000 and 2000) provide more efficient charge neutralisation.

5.3.5 ENTRAPPED VOLUME

Table 5.1 show the entrapped volume of the various liposomal systems obtained by centrifugation of the systems to form tight pellets and subsequent Karl Fisher titrimetry.

The entrapped volume of neutral liposomes containing CsA was found to be 0.520µl/µmol of lipid, which is near to the theoretical value of 0.5µl/µmol of lipid reported for MLV systems (Betagiri *et. al.*, 1993a). The entrapped volumes of charged liposomes containing CsA, shown in table 5.1 indicates

no major change from that of neutral liposomes, proved that the incorporation of charge does not have any significant effect on the entrapped volume. Proof of this conjecture comes in the form of no significant difference in drug entrapment (table 5.1) for liposomes containing cyclosporine compared to the charged liposomes containing cyclosporine, which can be explained only by such a phenomenon.

The entrapped volume of conventional liposomes containing leuprolide acetate determined as 0.436µl/µmol of lipid indicating once again that the formed liposomes are predominantly multilamellar. The entrapped volumes of sterically stabilized liposomes containing leuprolide acetate, shown in Table 5.1 indicates that there found to be a decrease in the entrapment volume from that of conventional liposomes, proved that the incorporation of steric stabilizing agents does have significant effect on the entrapped volume.

The entrapped volume of conventional liposomes containing DNA was found to be 0.415µl/µmol of lipid indicating once again that the formed liposomes are predominantly multilamellar. When poly ethylene glycol derivatives used as steric stabilizing agents into the liposomes containing DNA, a slight decrease in the entrapment volume was found. The entrapped volumes of sterically stabilized liposomes containing leuprolide acetate, shown in Table 5.1 indicates that there found to be a decrease in the entrapment volume from that of conventional liposomes, proved that the incorporation of steric stabilizing agents does have significant effect on the entrapped volume.

5.3.6 ELECTROLYTE INDUCED FLOCCULATION

The results of this test have already been discussed in chapter 4, Preparation of liposomes.

5.3.7 DRUG ENTRAPMENT

The entrapment of CsA, leuprolide acetate and DNA in conventional, charged and sterically stabilized liposomes was determined using the methods elaborated in Chapter 3, Analytical methods. Table 5.1 shows the results of these determinations.

Analysis of variance along with Tukey Multiple Kramer test was used to determine whether the results were significantly different from each other or

not. The analysis of variance (ANOVA) tables are shown as tables 5.2 –5.4 for liposomes containing CsA, leuprolide acetate and DNA respectively.

The entrapment of CsA in neutral liposomes was similar to that of positive and neutral charged liposomal formulations. ANOVA of drug entrapment values of charged liposomes containing CsA revealed no significant differences in drug entrapment was found when charges were used (calculated F = 2.47, tabulated F = 5.14 at 2 and 6 degrees of freedom in numerator and denominator respectively at the α = 0.05). This is expected since cyclosporine being highly hydrophobic, is associated with the bilayer forming unit hydrogenated phosphatidylcholine rather than with the charge incorporating agents like stearylamine and DSPG.

Significant differences are seen in the entrapment values of the liposomes when polyethylene derivatives are used as steric stabilizing agents in case of liposomes containing leuprolide acetate and DNA. ANOVA of drug entrapment values of liposomes containing leuprolide acetate and DNA sterically stabilized using different agents revealed significant differences in drug entrapment was found when these agents were used (calculated F = 19.83 and 596.96, tabulated F = 5.14 and 3.48 at 2 and 6, 4 and 10 degrees of freedom in numerator and denominator respectively at the α = 0.05). Tukey Multiple Kramer test was used to determine which of the batches were significantly different. But the incorporation of steric stabilizing agents in the bilayer cause a significant reduction in drug entrapment in case of liposomes containing leuprolide acetate and DNA. It is well documented that reduction in drug entrapment in the bilayer may be due to the complex formation between the PEG and drug (Zalipsky, 1995). The entrapment of leuprolide acetate and DNA in liposomes sterically stabilized by mPEG5000-CC-PE and mPEG2000-CC-PE is not significantly different from each other (difference of means = 4.98 and 1.123 and q = 3.188 and 1.686 for SLL5000-CC-PE and SLL2000-CC-PE, SDL5000- CC-PE and SDL2000-CC-PE compared to the tabulated q = 4.339 and 4.654). The reduction in drug entrapment may also be due to the fact that both the drug and the poly ethylene glycol were entrapped in the lipid bilayer. Further investigations into this aspect are required in order to satisfactorily explain the observed phenomena.

REFERENCES

Bedu-Addo, F.K., Huang, L. (1995). Interaction of PEG-phospholipid conjugates with phospholipid: implications in liposomal drug delivery. *Adv. Drug Deliv. Rev.,* 16, 235-247.

Betagiri, G.V., Jenkins, S.A., Parsons, D.L. (1993). "Stability of liposomes" in Liposome Drug Delivery Systems. Betagiri GV, Jenkins SA and Parsons DL.(eds.) Technomic Publishing Co. Inc., Lancaster, Pennsylvania, 27-46.

Betagiri, G.V., Jenkins, S.A., Parsons, D.L. (1993a). " Preparation of liposomes "in Liposome Drug Delivery Systems. Betagiri GV, Jenkins SA and Parsons DL.(eds.) Technomic Publishing Co. Inc., Lancaster, Pennsylvania, 1-26.

New, R.R.C. (1990). "Preparation of Liposomes "in Liposomes: A Practical Approach, New RRC (ed.) Oxford University Press, Oxford, 33-104.

Zalipsky, S. (1995). Chemistry of PEG conjugates with biologically active molecules. *Adv. Drug. Del. Rev.,* 1995, 16, 157-182.