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); Cellulose acetate strips for electrophoresis (Himedia Laboratories Ltd., Mumbai); Paper electrophoresis set-up (Bangalore Genei, Bangalore); Glass Iodine chamber (Durga Glass Works, Baroda).

5.2.3 SOLUTIONS

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

0.01M sodium phosphate buffer pH 6.8 containing 0.15M sodium chloride was prepared by dissolving the appropriate quantity of water and adjusting the pH with ortho phosphoric acid.

5.2.4 CHARACTERIZATION

The prepared liposomes containing methotrexate (MTX), tamoxifen citrate (TMX) and cytarabine (CYT) 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 5.12 show 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 Mie's 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 Nuclepore 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 5.13 to 5.17 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. ML, SML5000-CC-PE, TL, STL2000-CC-PE, CL, SCL5000-CC-PE, SCL2000-CC-PE and SCL5000-IC-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 5.18 –5.25.

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 Paper electrophoresis

The charge of the liposomes was determined using paper electrophoresis. The method referred by Betagiri *et al* (1993) was modified for this study. In brief, electrophoresis was conducted on cellulose acetate strips in pH 7.4 phosphate buffered saline for 1h at 20V/cm. The liposomes were visualized by treatment of the strips with iodine vapor. Figures 5.26 –5.28 show the photographs of the results of the electrophoresis study carried out for the various liposomal formulations.

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 MTX, TMX and CYT 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 MTX, TMX and CYT

Liposome type	Mean Particle Size ± S.E. (nm)		Entrapped volume (μl/μmol of lipid)	Mean Drug entrapped* ± S.E.
	Before extrusion	After extrusion		
ML	782.6 ± 148.2	182.3 ± 34.6	0.546	95.8 ± 1.42
SML5000-CC-PE	812.3 ± 164.9	165.4 ± 24.5	0.523	89.5 ± 2.66
SML2000-CC-PE	875.5 ± 140.4	173.3 ± 27.6	0.537	88.6 ± 1.79
SML5000-IC-PE	780.1 ± 152.3	172.1 ± 22.5	0.516	92.7 ± 2.01
TL	1031.6 ± 174.3	197.2 ± 41.0	0.562	89.0 ± 0.66
STL5000-CC-PE	945.2 ± 152.3	176.5 ± 22.6	0.554	84.4 ± 1.03
STL2000-CC-PE	927.9 ± 175.6	183.2 ± 26.7	0.519	85.3 ± 1.65
STL5000-IC-PE	910.6 ± 132.2	190.6 ± 24.5	0.513	83.5 ± 2.41
CL	1165.2 ± 196.2	236.2 ± 25.3	0.415	83.5 ± 1.60
SCL5000-CC-PE	1024.6 ± 177.6	205.6 ± 22.9	0.472	84.2 ± 1.26
SCL2000-CC-PE	987.1 ± 153.8	185.1 ± 29.7	0.448	83.8 ± 1.48
SCL5000-IC-PE	1074.8 ± 171.4	195.0 ± 31.1	0.433	82.2 ± 2.11

*n=3

Table 5.2 Analysis of variance (ANOVA) table for drug entrapment in conventional and sterically stabilized liposomes containing methotrexate

Source of Variation	Sum of Squares (SS)	DF	Mean Square (MS)	$F_{3,8}$	P-value	Tabulated F value
Between Groups	96.24	3	32.08	6.43	0.0158	4.066
Within Groups	39.89	8	4.99			
Total	136.12	11				

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

Source of Variation	Sum of Squares (SS)	DF	Mean Square (MS)	$F_{3,8}$	P-value	Tabulated F value
Between Groups	51.74	3	17.25	6.60	0.0148	4.066
Within Groups	20.89	8	2.61			
Total	72.63	11				

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

Source of Variation	Sum of Squares (SS)	DF	Mean Square (MS)	$F_{3,8}$	P-value	Tabulated F value
Between Groups	8.19	3	2.73	1.43	0.303	4.066
Within Groups	15.22	8	1.90			
Total	23.41	11				

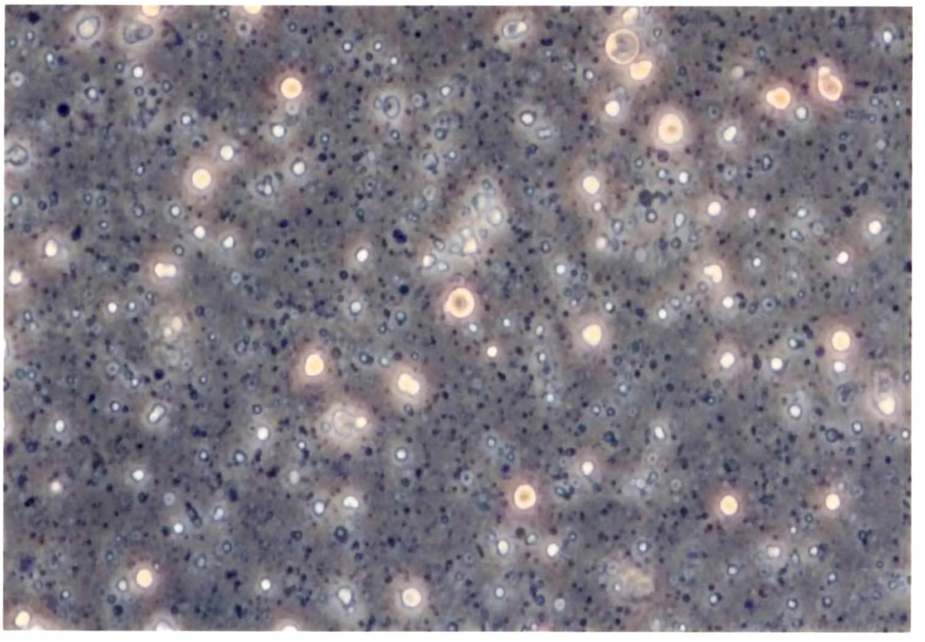

Figure 5.1 Photomicrograph of conventional liposomes containing methotrexate

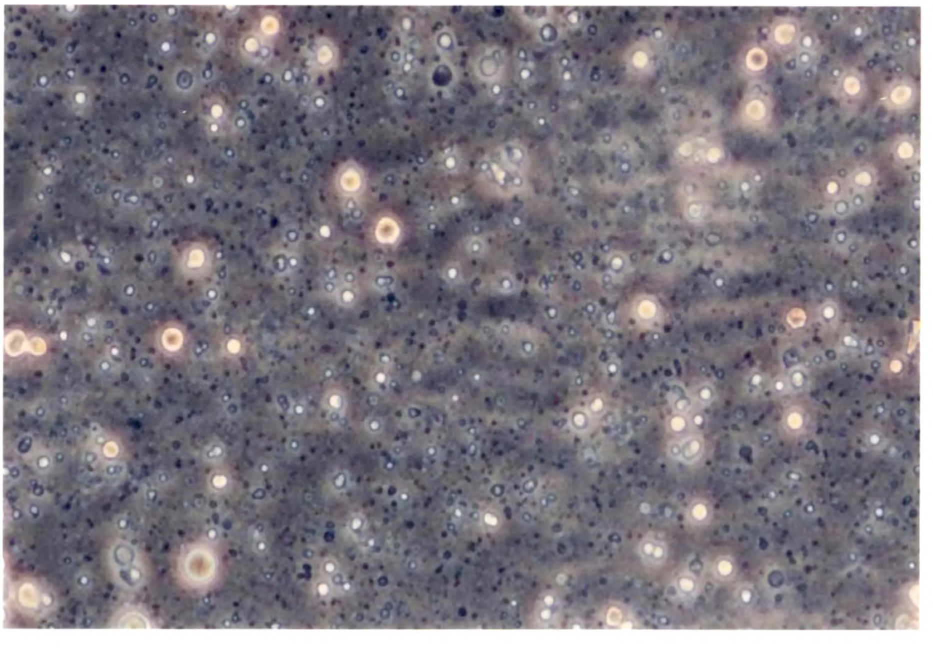

Figure 5.2 Photomicrograph of liposomes containing methotrexate sterically stabilized using methoxy polyethylene glycol 5000 – activated with cyanuric chloride-phosphatidylethanolamine conjugate

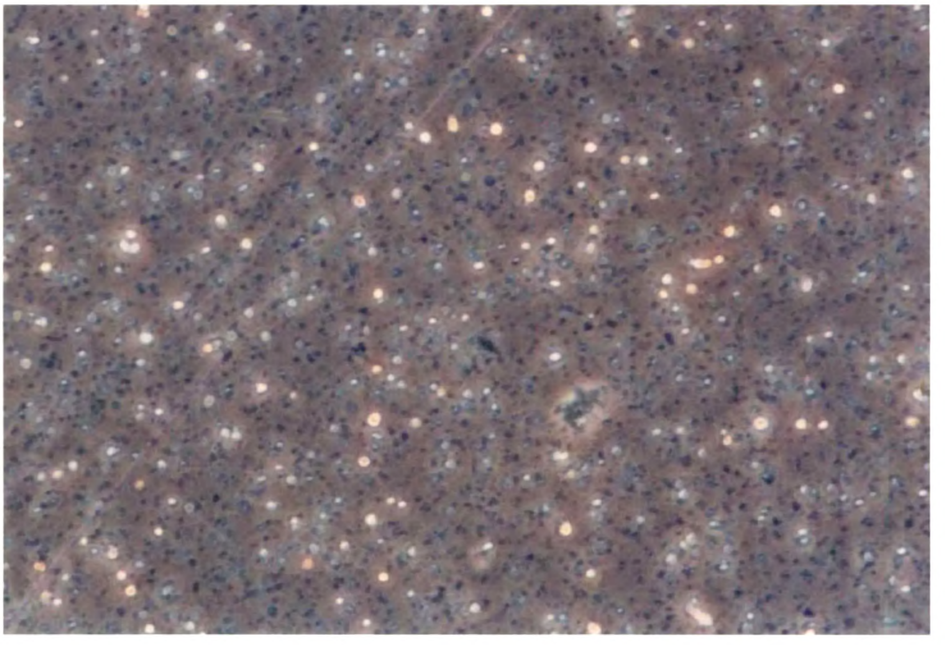

Figure 5.3 **Photomicrograph of liposomes containing methotrexate sterically stabilized using methoxy polyethylene glycol 2000 – activated with cyanuric chloride-phosphatidylethanolamine conjugate**

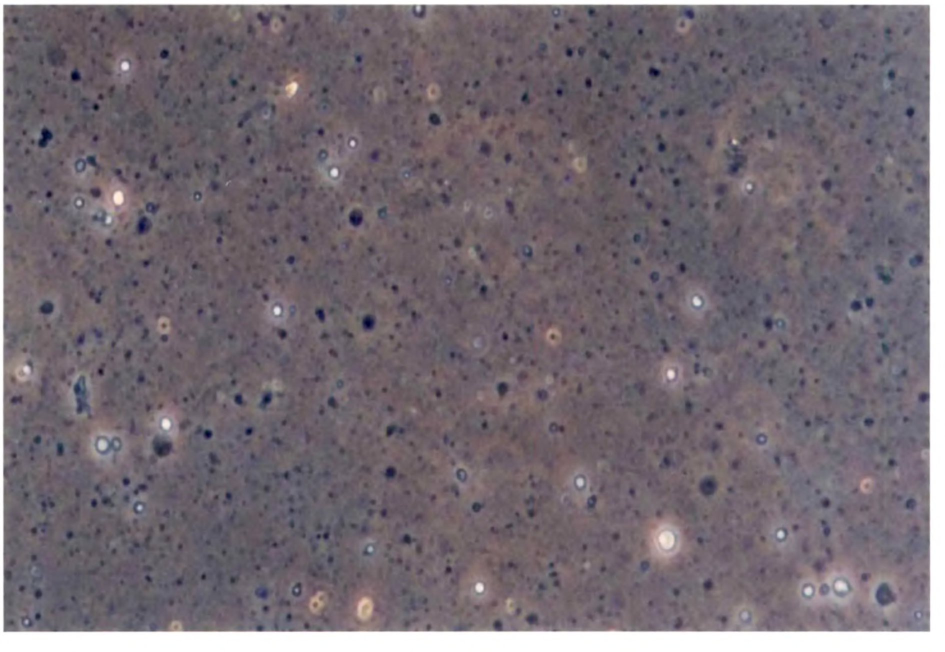

Figure 5.4 **Photomicrograph of liposomes containing methotrexate sterically stabilized using methoxy polyethylene glycol 5000 – activated with imidazoline carbonyl phosphatidyl ethanolamine conjugate**

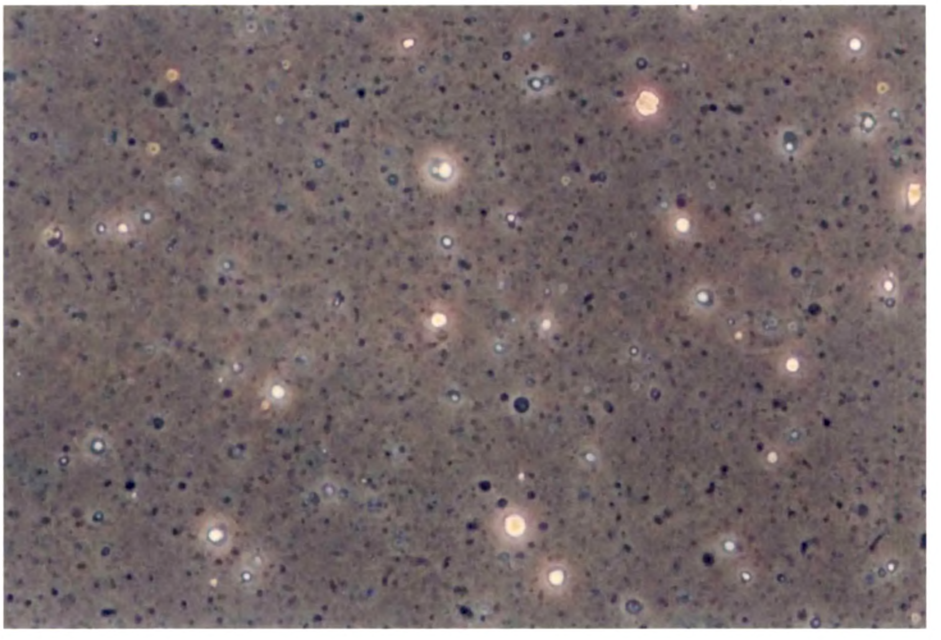

Figure 5.5 Photomicrograph of conventional liposomes containing tamoxifen citrate

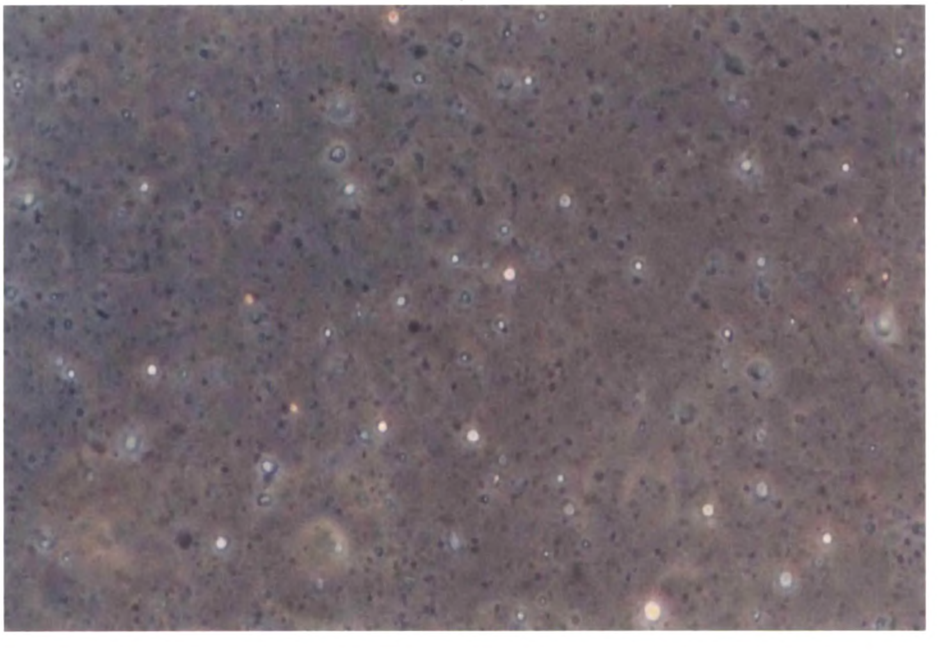

Figure 5.6 Photomicrograph of liposomes containing tamoxifen citrate sterically stabilized using methoxy polyethylene glycol 5000 – activated with cyanuric chloride-phosphatidylethanolamine conjugate

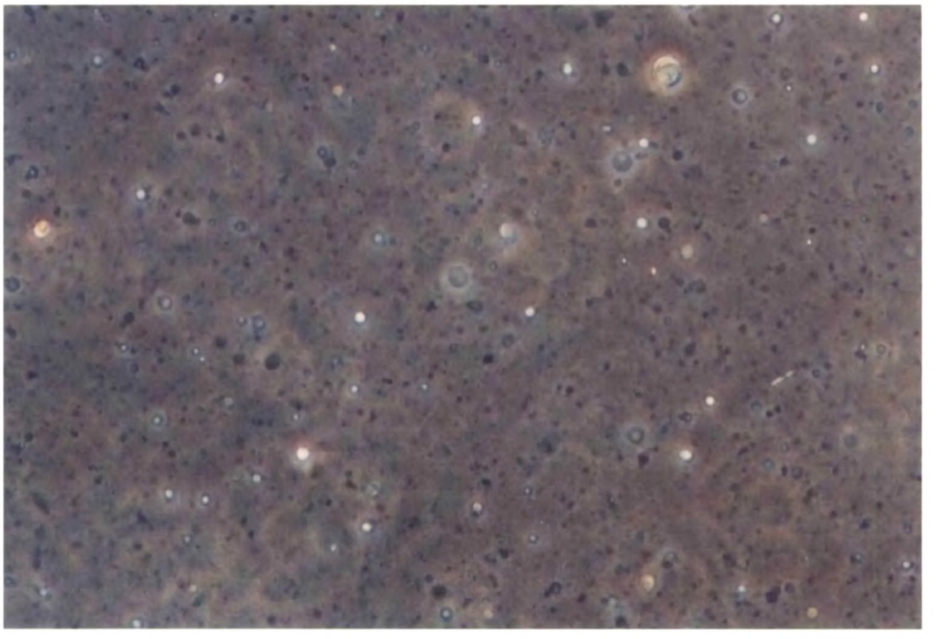

Figure 5.7 Photomicrograph of liposomes containing tamoxifen citrate sterically stabilized using methoxy polyethylene glycol 2000 – activated with cyanuric chloride-phosphatidylethanolamine conjugate

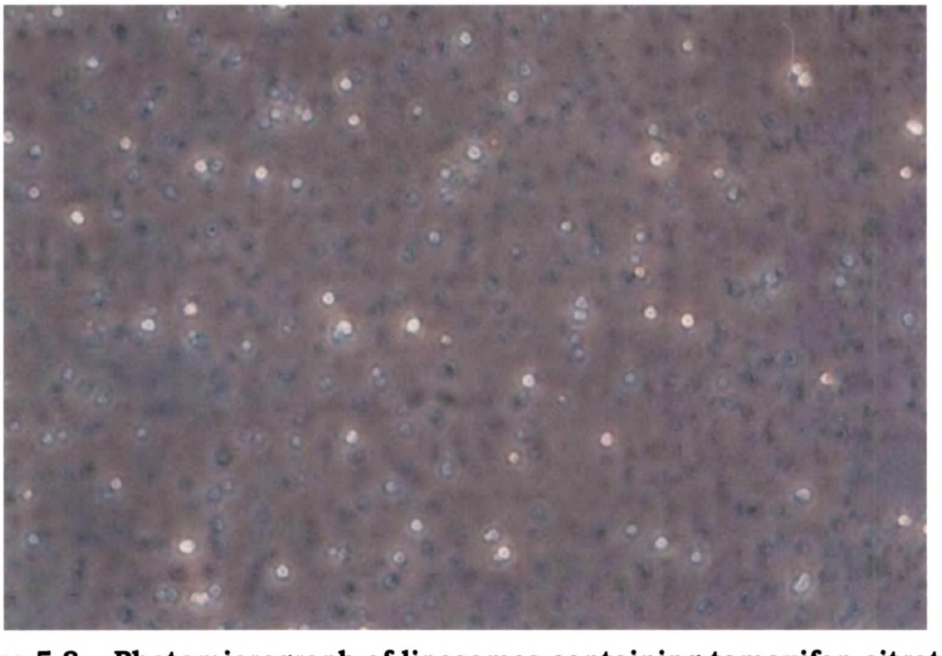

Figure 5.8 Photomicrograph of liposomes containing tamoxifen citrate sterically stabilized using methoxy polyethylene glycol 5000 – activated with imidazoline carbonyl-phosphatidyl ethanolamine conjugate

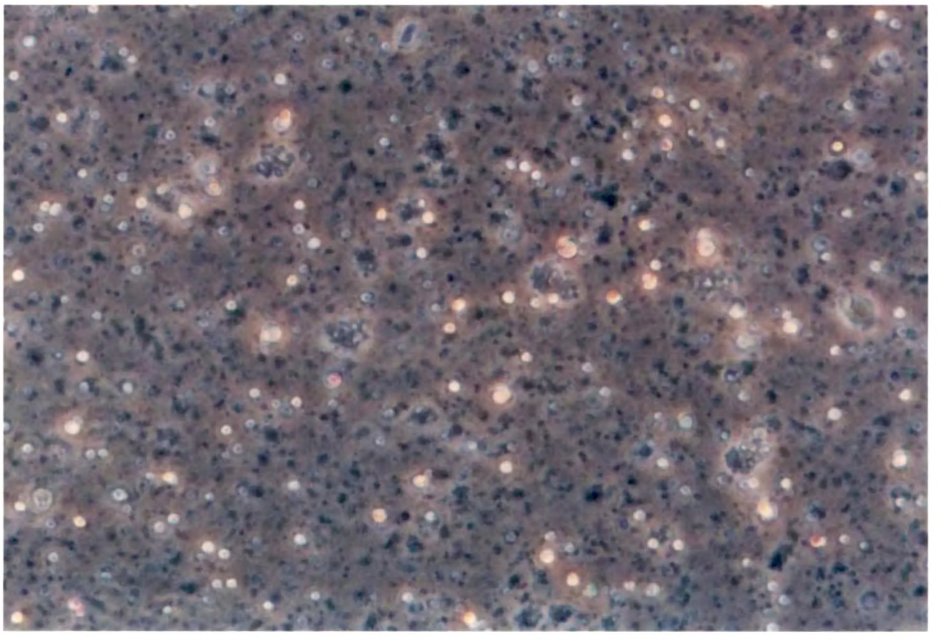

Figure 5.9 Photomicrograph of conventional liposomes containing cytarabine

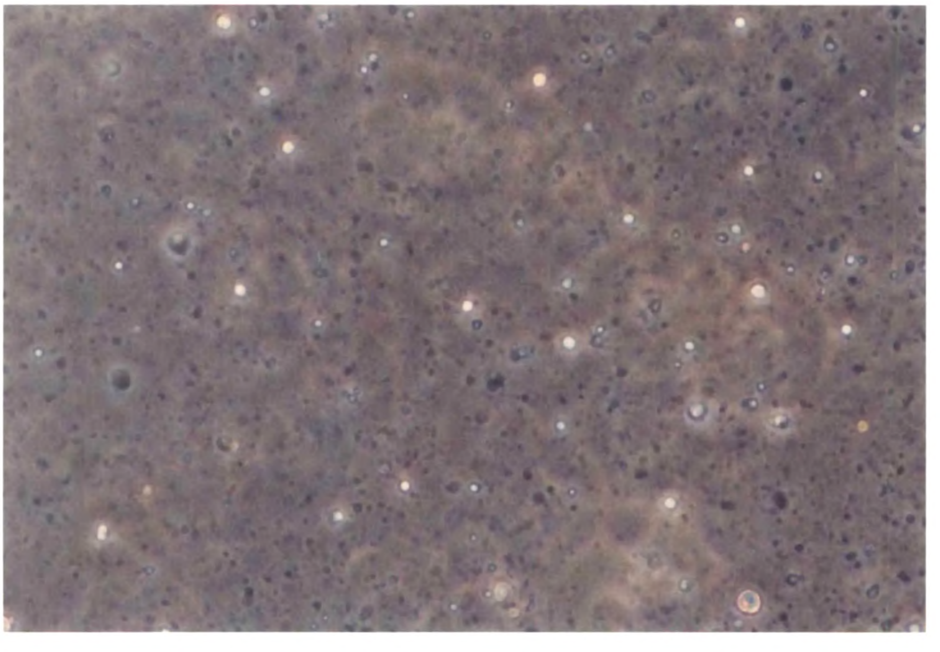

Figure 5.10 Photomicrograph of liposomes containing cytarabine sterically stabilized using methoxy polyethylene glycol 5000 – activated with cyanuric chloride-phosphatidylethanolamine conjugate.

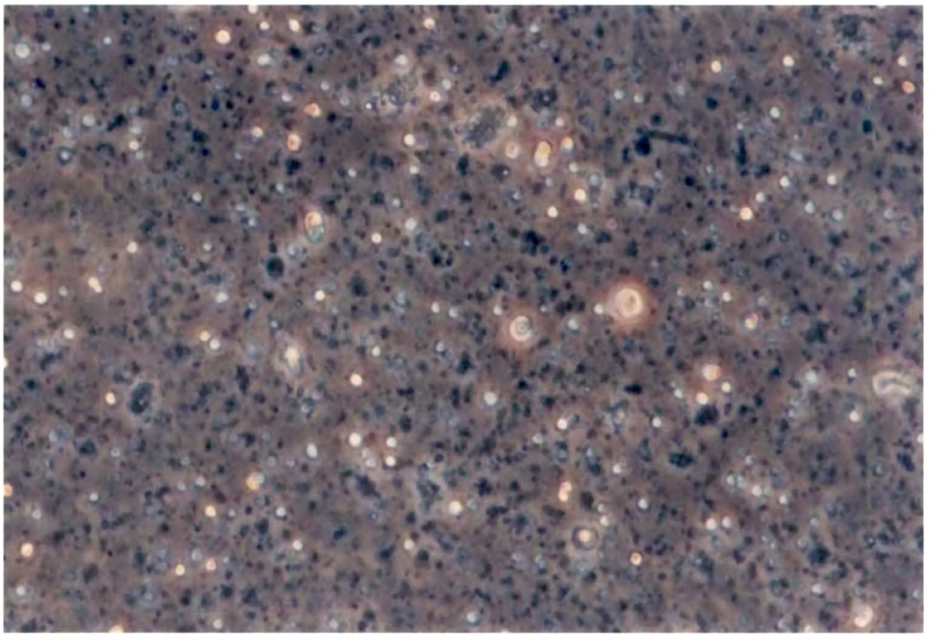

Figure 5.11 Photomicrograph of liposomes containing cytarabine sterically stabilized using methoxy polyethylene glycol 2000 – activated with cyanuric chloride-phosphatidylethanolamine conjugate

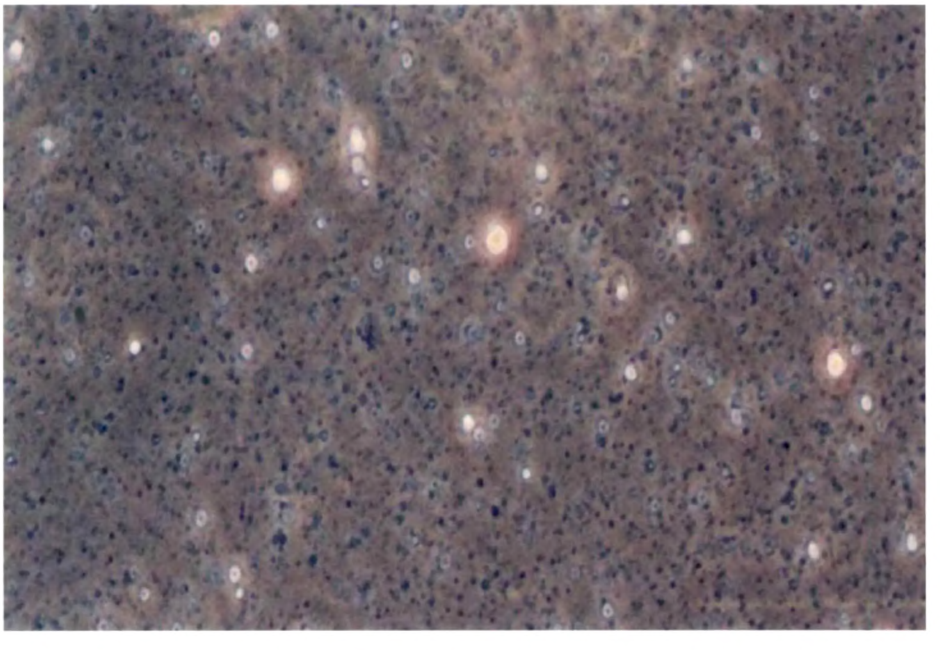

Figure 5.12 Photomicrograph of liposomes containing cytarabine sterically stabilized using methoxy polyethylene glycol 5000 – activated with imidazoline carbonyl-phosphatidyl ethanolamine conjugate

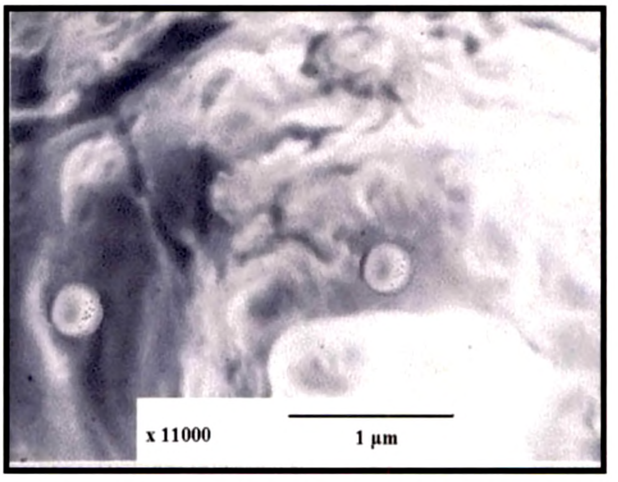

Figure 5.13 Scanning electron micrograph of conventional liposomes containing methotrexate

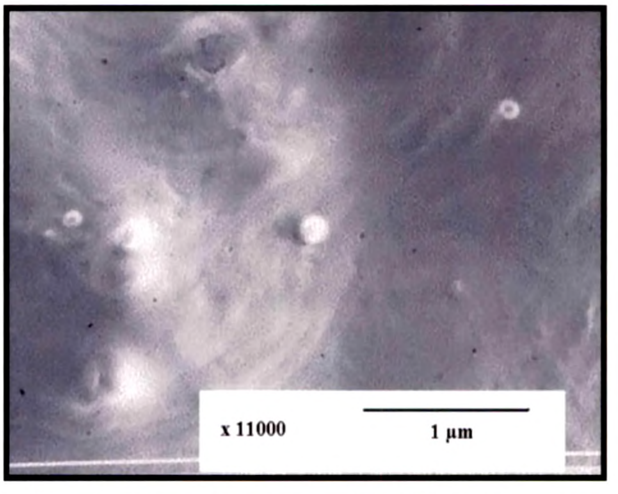

Figure 5.14 Scanning electron micrograph of liposomes containing methotrexate sterically stabilized using mPEG5000-CC-PE

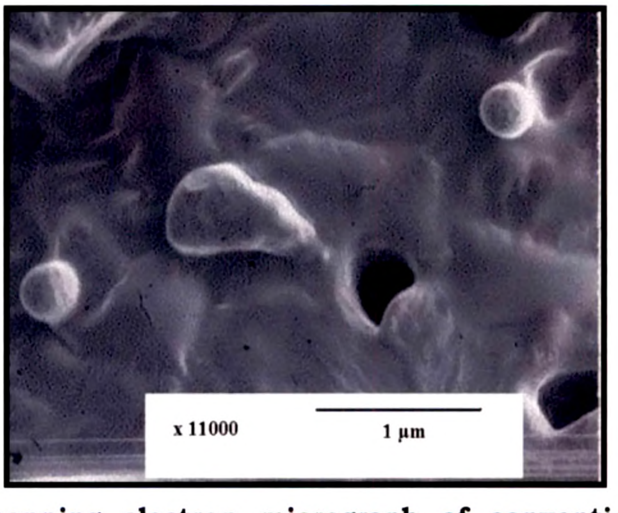

Figure 5.15 Scanning electron micrograph of conventional liposomes containing tamoxifen citrate sterically stabilized using mPEG2000-CC-PE

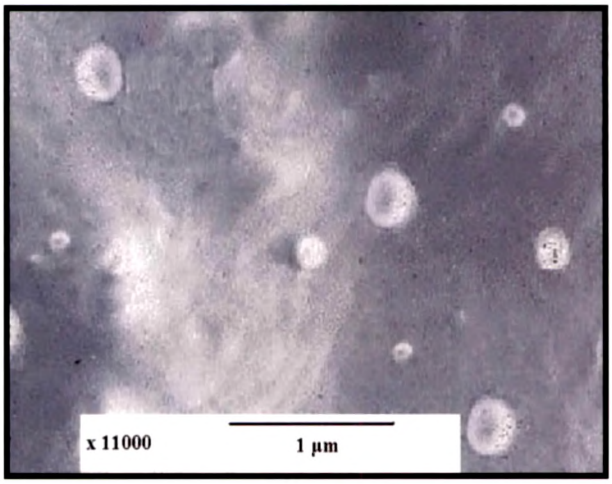

Figure 5.16 Scanning electron micrograph of conventional liposomes containing cytarabine

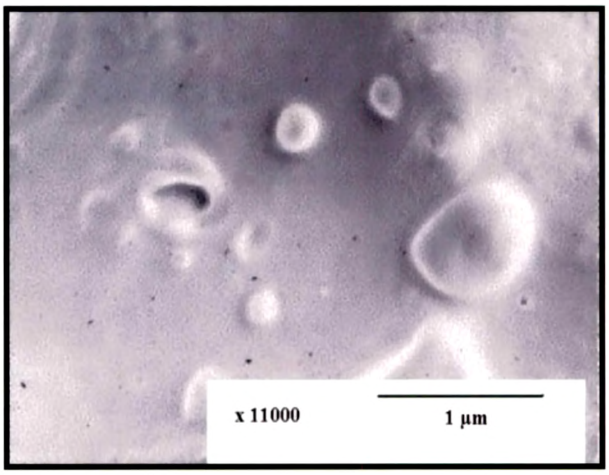

Figure 5.17 Scanning electron micrograph of conventional liposomes containing cytarabine sterically stabilized using mPEG5000-IC-PE

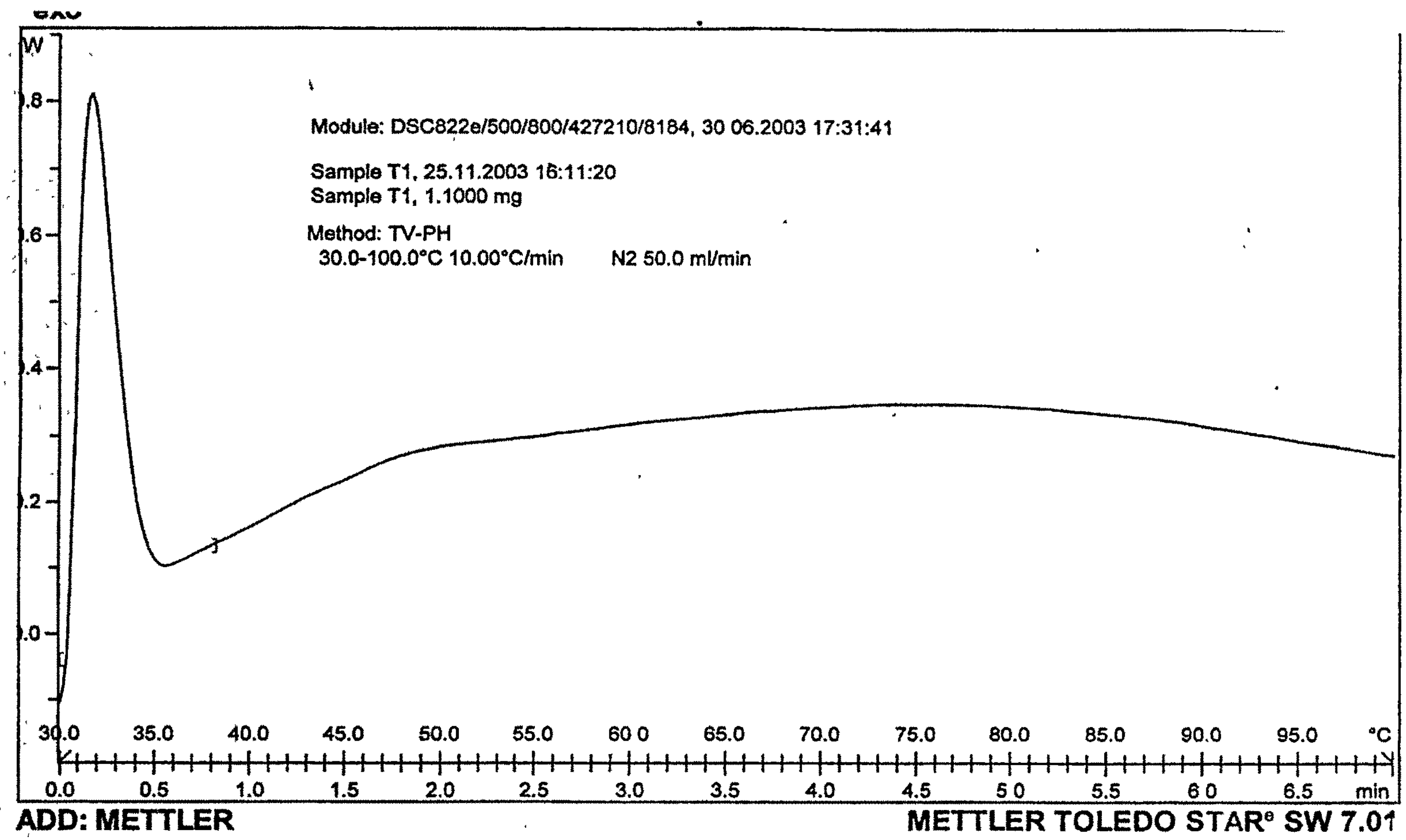

Figure 5.18 DSC thermogram of conventional liposomes containing methotrexate

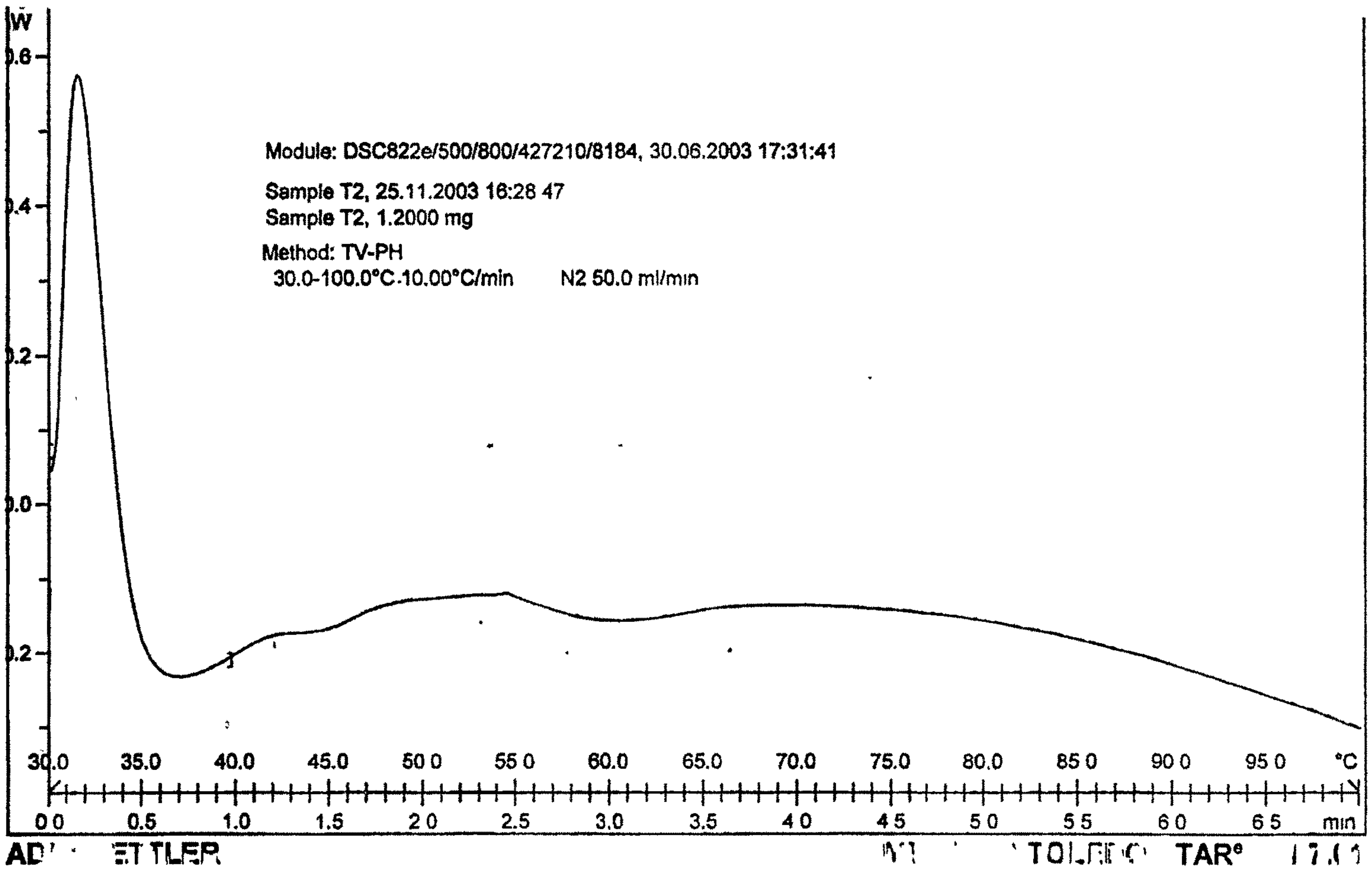

Figure 5.19 DSC thermogram of liposomes containing methotrexate sterically stabilized using methoxy polyethylene glycol 5000 –activated with cyanuric chloride –phosphatidyl ethanolamine conjugate (mPEG5000-CC-PE)

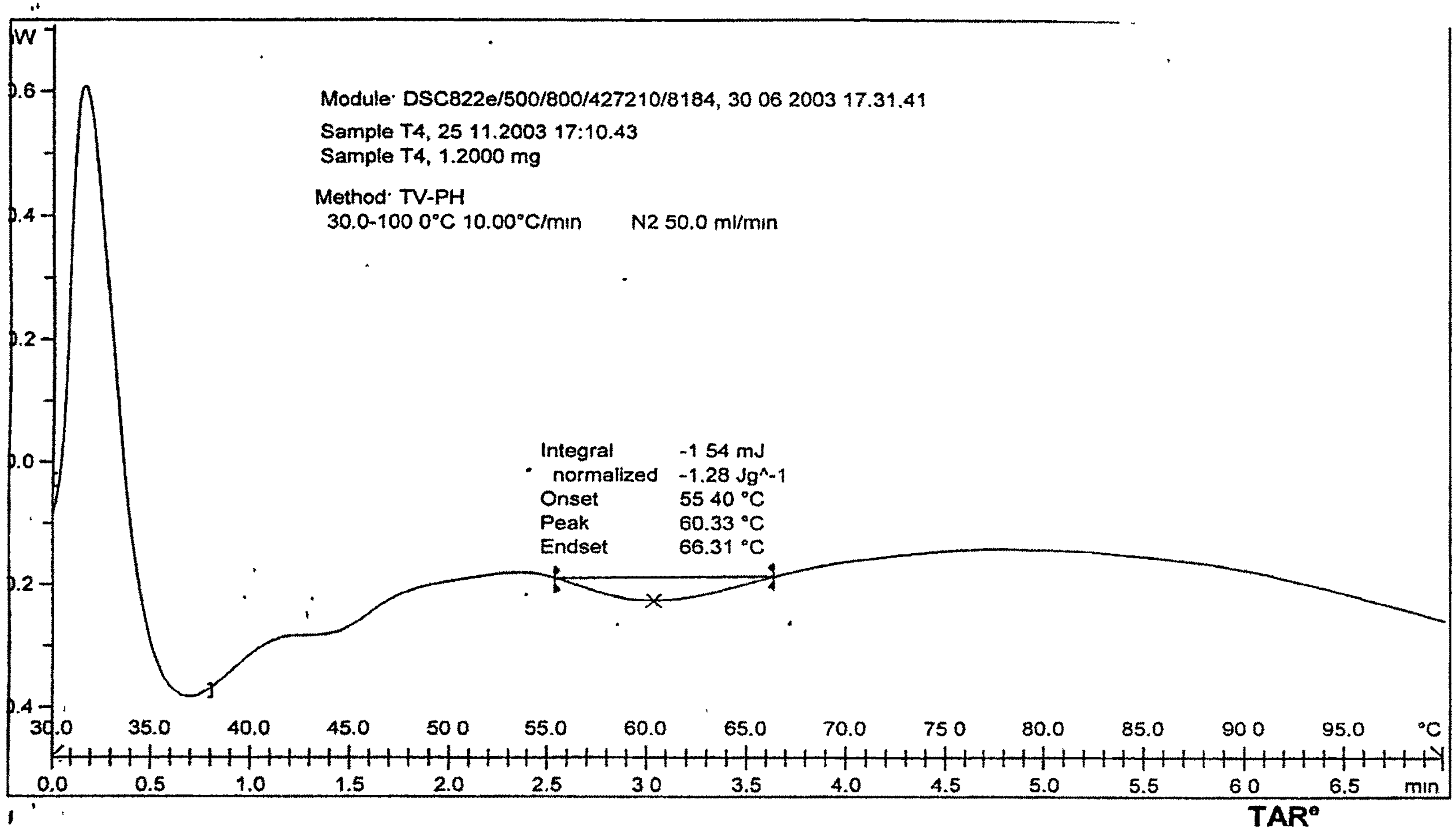

Figure 5.20 DSC thermogram of conventional liposomes containing tamoxifen citrate

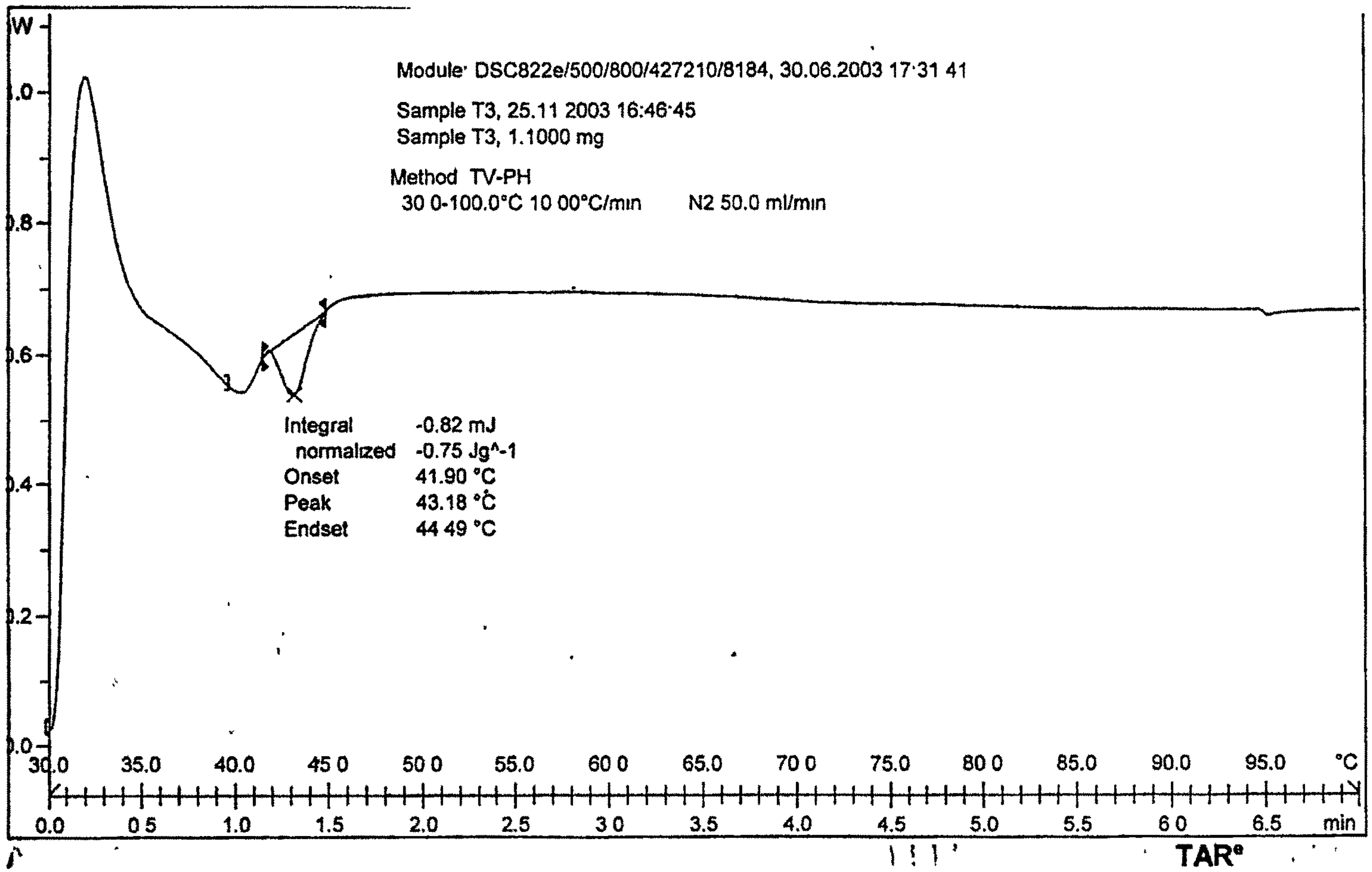

Figure 5.21 DSC thermogram of liposomes containing tamoxifen citrate sterically stabilized using methoxy polyethylene glycol 2000 –activated with cyanuric chloride –phosphatidyl ethanolamine conjugate (mPEG2000-CC-PE)

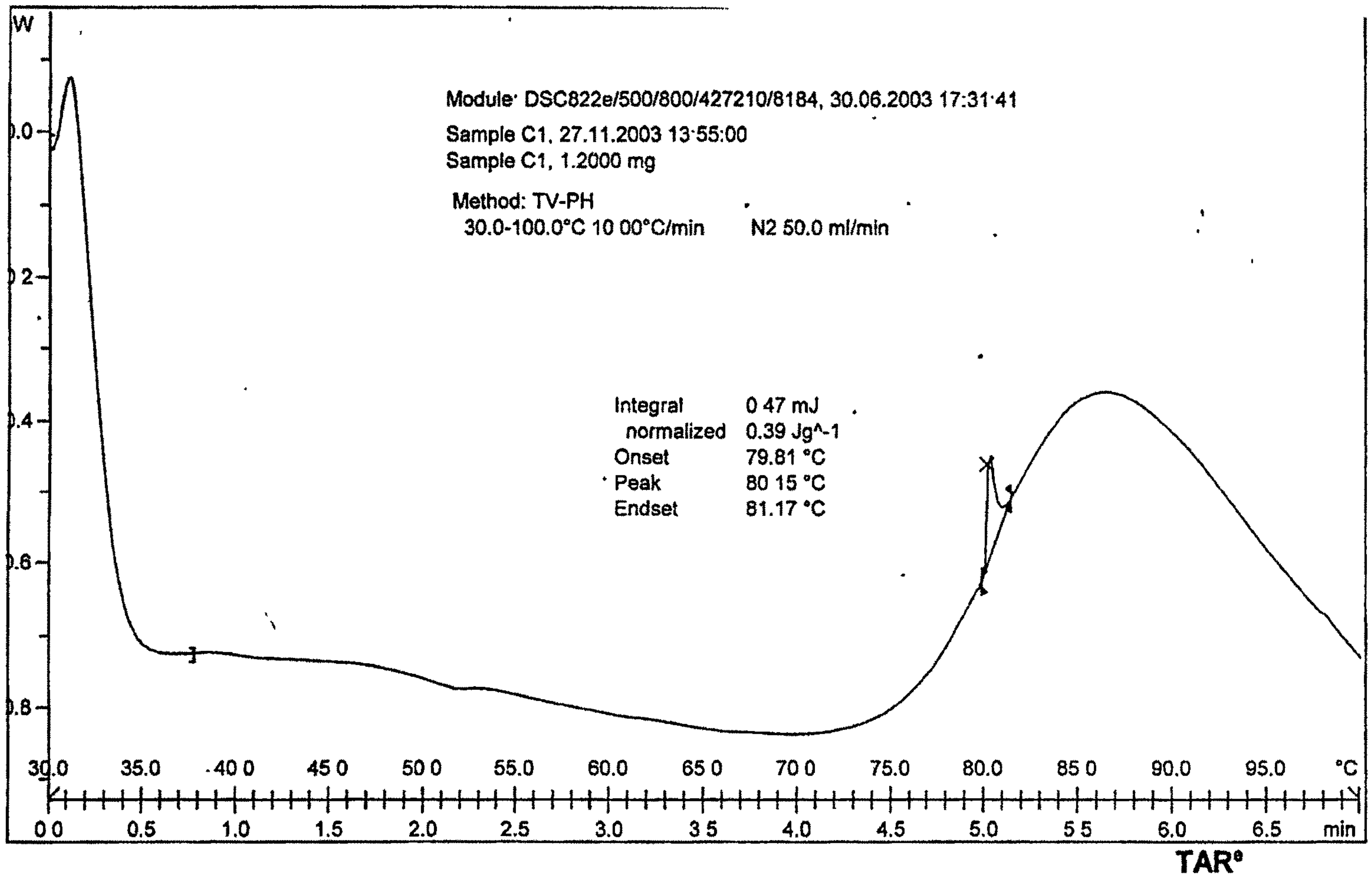

Figure 5.22 DSC thermogram of conventional liposomes containing cytarabine

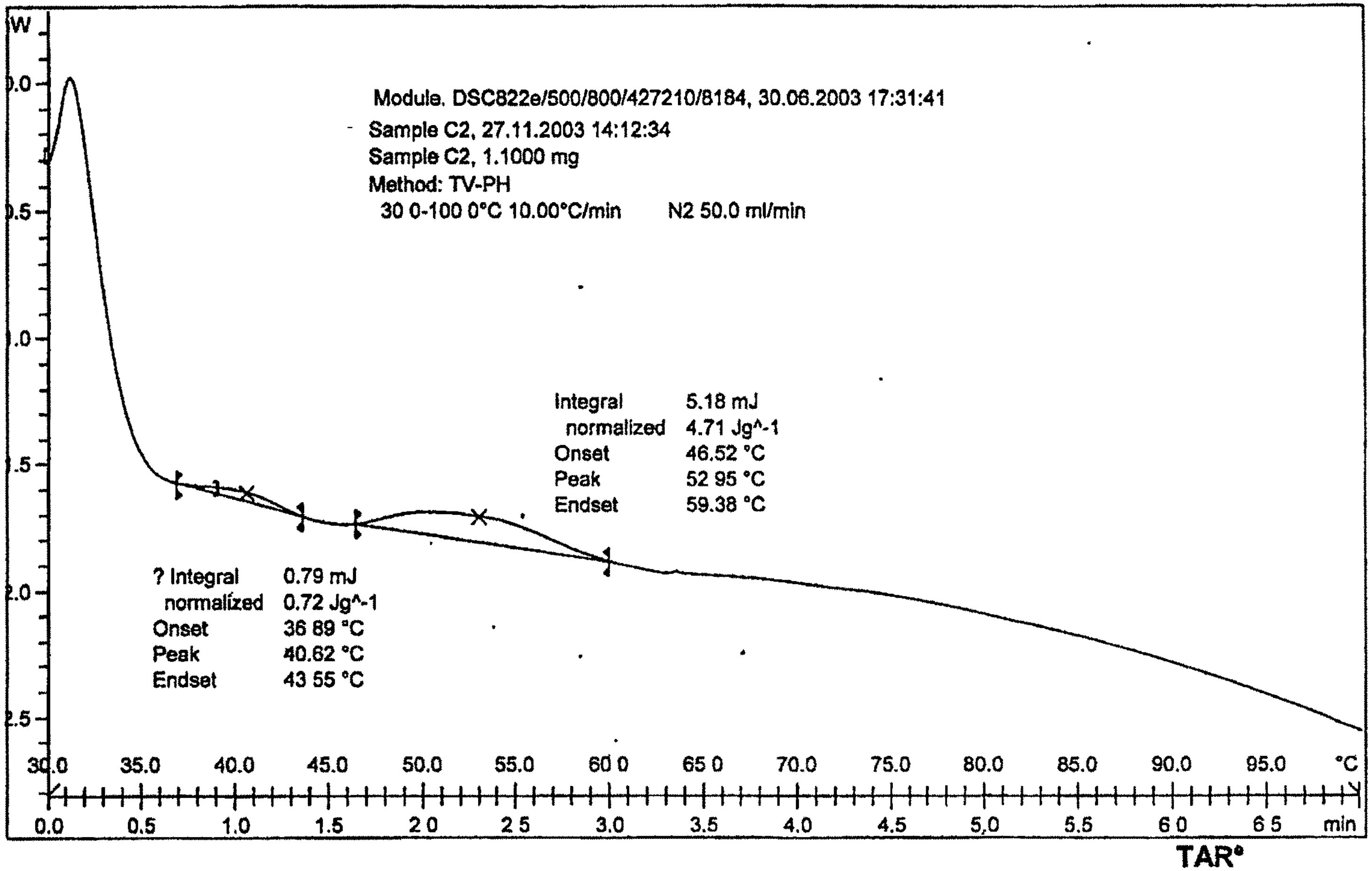

Figure 5.23 DSC thermogram of liposomes containing cytarabine sterically stabilized using methoxy polyethylene glycol 5000 –activated with cyanuric chloride –phosphatidyl ethanolamine conjugate (mPEG5000-CC-PE)

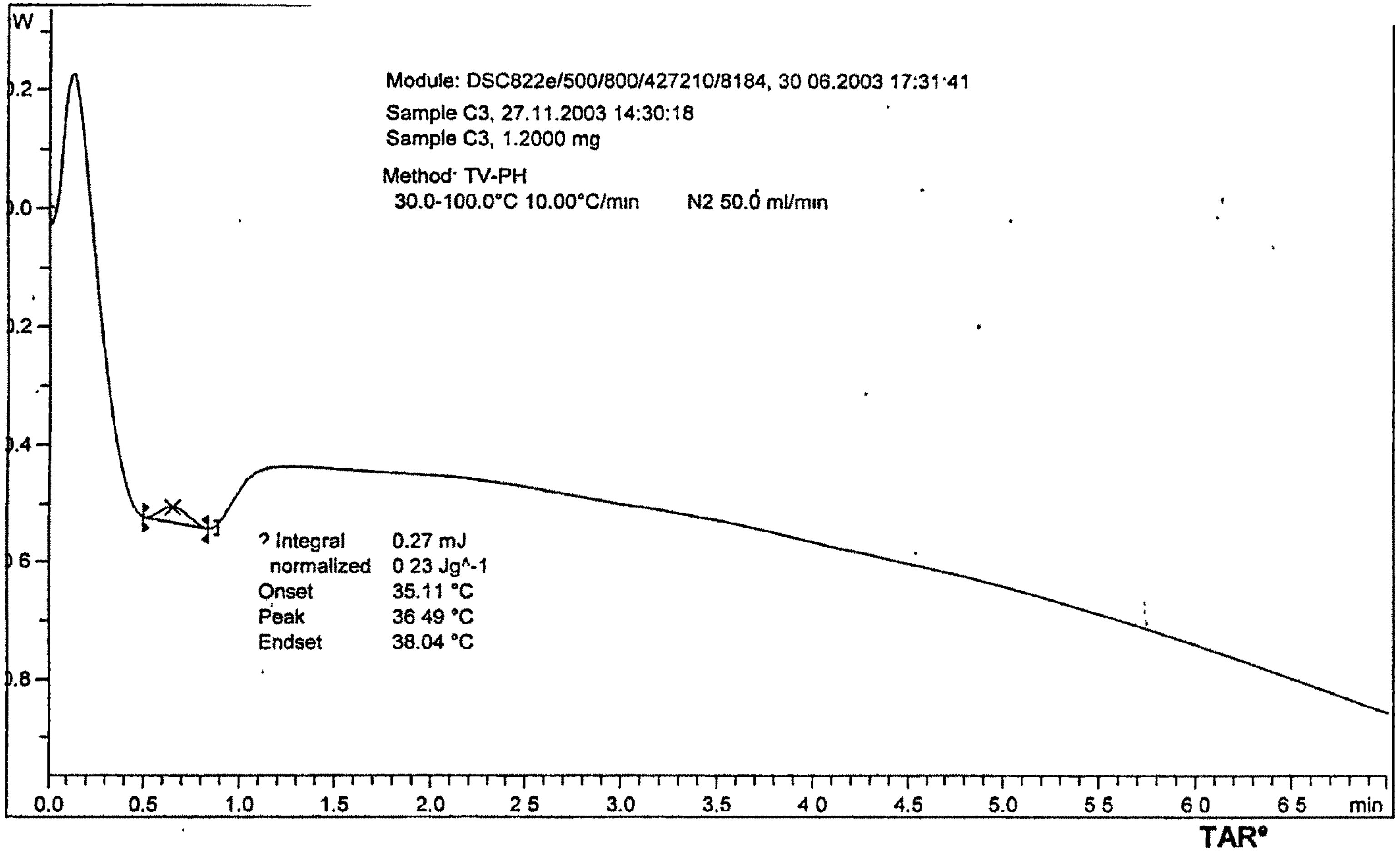

Figure 5.24 DSC thermogram of liposomes containing cytarabine sterically stabilized using methoxy polyethylene glycol 2000 –activated with cyanuric chloride –phosphatidyl ethanolamine conjugate (mPEG2000-CC-PE)

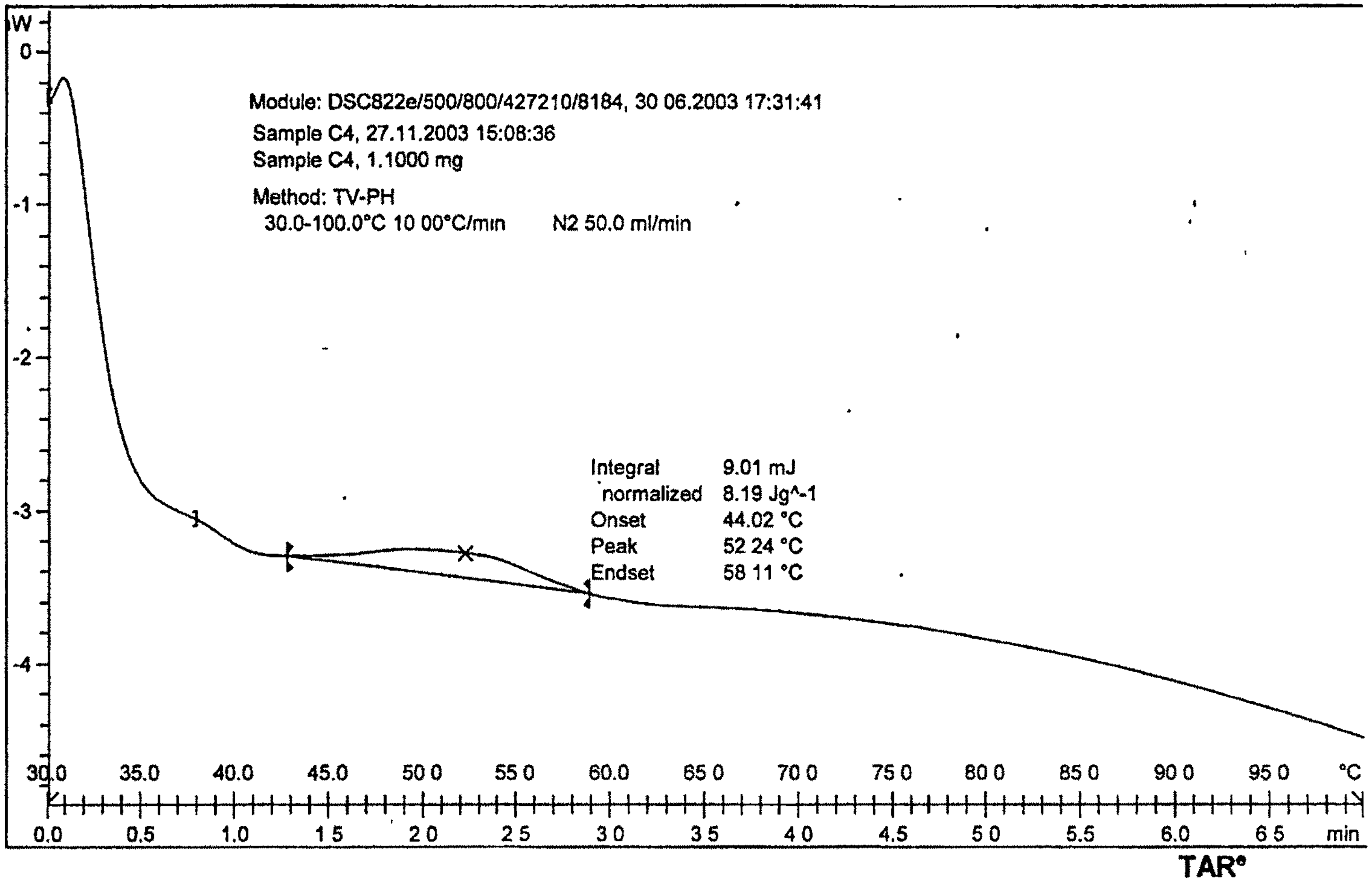

Figure 5.25 DSC thermogram of liposomes containing cytarabine sterically stabilized using methoxy polyethylene glycol 5000 –activated with imidazoline carbonyl –phosphatidyl ethanolamine conjugate (mPEG5000-IC-PE)

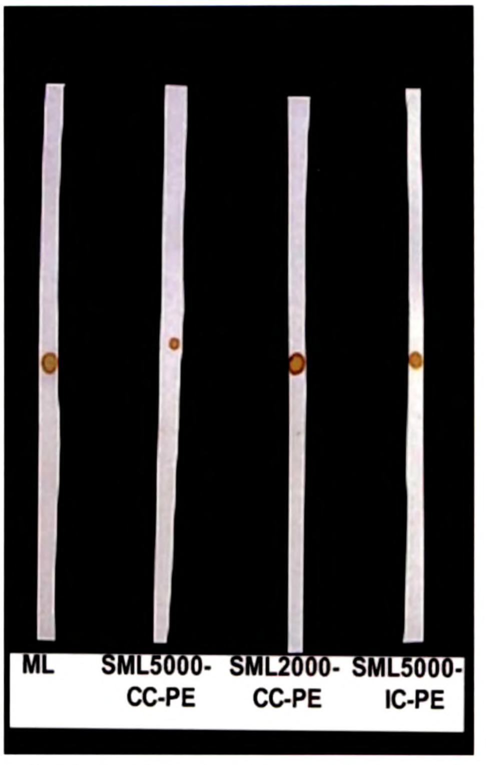

Figure 5.26 Photograph of electrophoresis studies carried out on liposomes containing methotrexate.

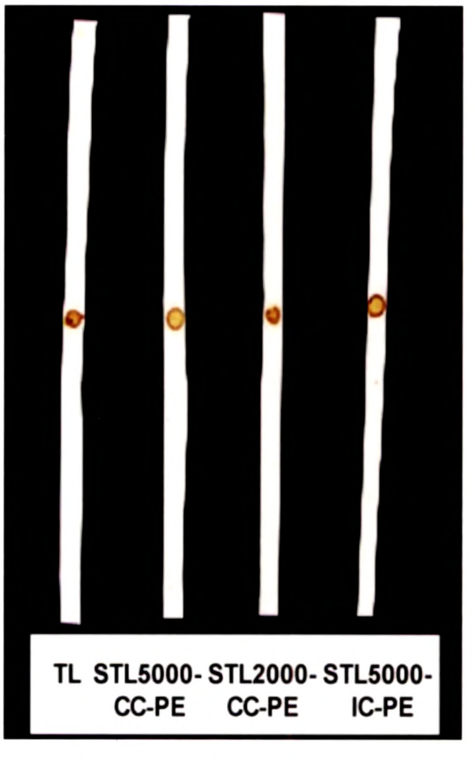

Figure 5.27 Photograph of electrophoresis studies carried out on liposomes containing tamoxifen citrate.

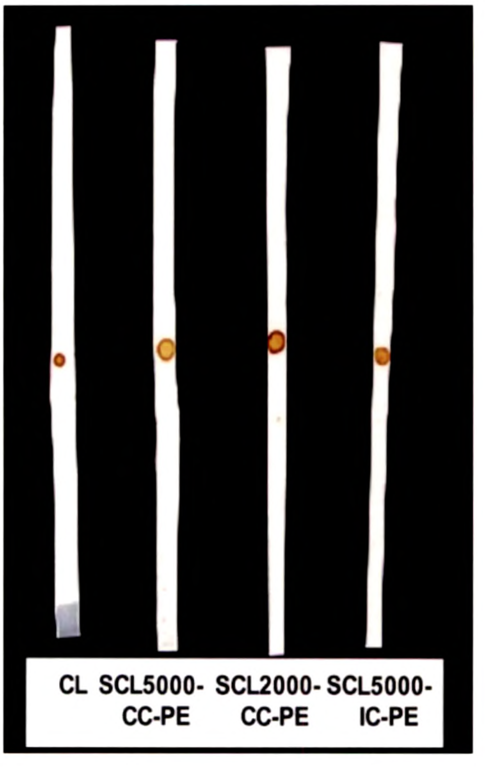

Figure 5.28 Photograph of electrophoresis studies carried out on liposomes containing cytarabine.

5.3 RESULTS AND DISCUSSION

An attempt was made to characterize the conventional and sterically stabilized liposomes containing MTX, TMX and CYT. 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 –5.12) 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 5.13 –5.17) 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 30min was found to be adequate to reduce the size of the conventional liposomes of MTX, TMX and CYT to around 1 μm. Further reduction in sonication time (15min) yielded very high particle size. Increase in sonication time to 60min does not significantly reduce the particle size further. Hence the sonication time was fixed at 30min during the preparation of the liposomes.

The liposomes 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 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 MTX, TMX and CYT (Table 5.1) indicates that, in general, the particle size of these liposomes is smaller as compared to that of the corresponding conventional liposomes

The entrapped volume of conventional liposomes containing methotrexate was found to be 0.546µl/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 sterically stabilized liposomes containing methotrexate, shown in Table 5.1 indicates no major change from that of conventional liposomes, proved that the incorporation of steric stabilizing agents does not have any significant effect on the entrapped volume.

The entrapped volume of conventional liposomes containing tamoxifen citrate determined as 0.562µl/µmol of lipid indicating once again that the formed liposomes are predominantly multilamellar. The entrapped volumes of sterically stabilized liposomes containing tamoxifen citrate, shown in Table 5.1 indicates no major change from that of conventional liposomes, proved that the incorporation of steric stabilizing agents does not have any significant effect on the entrapped volume.

The entrapped volume of conventional liposomes containing cytarabine 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 cytarabine, a slight increase in the entrapment volume was found. The most probable reason for this increase seems to be the association of water molecules with the polyethylene glycol molecules. Since, this phenomenon is not observed with corresponding liposomes containing methotrexate and tamoxifen citrate (hydrophobic drugs), the presence of hydrophilic cytarabine seems to be a major factor in this association. Cytarabine may be forming some kind of association with poly ethylene glycol molecules, which leads to a microenvironment of water around the system and a concomitant increase in entrapped volume. Proof of this assumption comes in the form of the slight increased values of drug entrapment (Table 5.1) for liposomes containing cytarabine sterically stabilized with poly ethylene glycol derivatives compared to that for conventional liposomes.

5.3.5 PAPER ELECTROPHORESIS

Figures 5.26 – 5.28 show the results of electrophoresis, carried out on cellulose acetate strips, of conventional and sterically stabilized liposomes containing methotrexate, tamoxifen citrate and cytarabine. The results indicate that none of the prepared liposomes carry any charge. This is to be expected since none of the materials used to prepare conventional as well as sterically stabilized liposomes containing methotrexate, tamoxifen citrate and cytarabine possess any charge at neutral pH.

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 methotrexate, tamoxifen citrate and cytarabine in conventional 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 methotrexate, tamoxifen citrate and cytarabine respectively.

The entrapment of methotrexate and tamoxifen citrate in conventional liposomes was greater than that of cytarabine. This is expected since methotrexate and tamoxifen citrate being hydrophobic, is associated with the bilayer whereas cytarabine is hydrophilic and therefore is entrapped in the aqueous compartment. It is well documented that entrapment in the bilayer results in high values of entrapment whereas entrapment in the aqueous compartment is low particularly for multilamellar vesicles (New, 1990).

ANOVA of drug entrapment values of liposomes containing methotrexate and tamoxifen citrate sterically stabilized using different agents revealed significant differences in drug entrapment was found when these agents were used (calculated F = 6.44 and 6.60, tabulated F = 4.07 at 3 and 8 degrees of freedom in numerator and denominator respectively at the 5% level). 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 does not cause any significant difference in drug entrapment in case of liposomes containing cytarabine (calculated F = 2.73, tabulated F = 4.07 at 3 and 8 degrees of freedom in numerator and denominator respectively at the 5% level).

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 methotrexate. When compared to conventional liposomes containing methotrexate, the entrapment is varied significantly to the liposomes sterically stabilized with mPEG5000-CC-PE and mPEG2000-CC-PE (difference of means = 6.33 and 7.03 and q = 4.913 and 5.456 for ML and SML5000-CC-PE and ML and SML2000-CC-PE respectively, compared to the tabulated q = 4.529). The entrapment of methotrexate in liposomes sterically stabilized by mPEG5000-CC-PE, mPEG2000-CC-PE and mPEG5000-IC-PE is not significantly different from each other (difference of means = 0.70, 3.53 and 4.23 and q = 0.543, 2.741 and 3.284 for SML5000-CC-PE and SML2000-CC-PE, SML5000-CC-PE and SML5000-IC-PE and SML2000-CC-PE and SML5000-IC-PE respectively, compared to the tabulated q = 4.529). The incorporation of the poly ethylene glycol into the bilayer decreases the drug entrapment significantly except in case of mPEG5000-IC-PE, difference of mean = 2.8 and q = 2.172. This may be due to the change in the hydrophobicity of the bilayer due to the addition of the polyethylene glycol into the lipid bilayer. 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.

Tamoxifen citrate loaded liposomes containing polyethylene glycol as steric stabilizing agent showed significant difference in the entrapment values of the liposomes. When compared to conventional liposomes containing tamoxifen citrate, the entrapment is varied significantly to the liposomes sterically stabilized with mPEG5000-CC-PE and mPEG5000-IC-PE (difference of means = 4.31 and 5.61 and q = 4.62 and 6.01 for TL and STL5000-CC-PE and TL and STL5000-IC-PE respectively, compared to the tabulated q = 4.529). The entrapment of tamoxifen citrate in liposomes sterically stabilized by mPEG5000-CC-PE, mPEG2000-CC-PE and mPEG5000-IC-PE is not significantly different from each other (difference of means = 0.83, 1.30 and 2.13 and q = 0.893, 1.393 and 2.287 for STL5000-CC-PE and STL2000-CC-PE, STL5000-CC-PE and STL5000-IC-PE and STL2000-CC-PE and STL5000-IC-PE respectively, compared to the tabulated q = 4.529). The incorporation of the poly ethylene glycol into the bilayer decreases the drug entrapment significantly except in case of mPEG2000-CC-PE, difference of mean = 3.473 and q = 3.723. The reason given above for methotrexate liposomes will also hold here.

Significant differences are not seen in the entrapment values of the liposomes when polyethylene derivatives are used as steric stabilizing agents in case of liposomes containing cytarabine. When compared to conventional liposomes containing cytarabine, the entrapment is not varied significantly to the liposomes sterically stabilized with mPEG5000-CC-PE mPEG2000-CC-PE and mPEG5000-IC-PE (calculated F = 2.73, tabulated F = 4.07 at 3 and 8 degrees of freedom in numerator and denominator respectively at the 5% level). The possible reason for non-significant change in the drug entrapment may due to position of the drug in the liposomes. The drug, cytarabine being hydrophilic in nature got entrapped in the aqueous compartment and the incorporation of the polyethylene glycols in the lipid bilayer does not cause any major change in the drug entrapment.

5.4 REFERENCES

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