CHAPTER 4
FORMULATION AND CHARACTERIZATION

4. FORMULATION AND CHARACTERIZATION

4.1 Formulation

4.1.1 Introduction

There are many potential barriers to the effective delivery of a drug in its active form to solid tumors. Most small-molecule chemotherapeutic agents have a large volume of distribution on i.v. administration (Speth et al., 1988; Chabner and Longo, 1996). The result of this is often a narrow therapeutic index due to a high level of toxicity in healthy tissues. Through encapsulation of drugs in a macromolecular carrier, such as a liposome the volume of distribution is significantly reduced and the concentration of drug in the tumor is increased.

Liposomes are spherical lipid bilayers from 50 nm to 1000 nm in diameter that serve as convenient delivery vehicles for biologically active compounds. The field of liposome research has expanded considerably over the last 35 years. It is now possible to engineer a wide range of liposomes varying in size, phospholipid composition and surface characteristics to suit the specific application for which they are intended. In comparison with other drug carriers, liposomes have some advantages like biological degradability and relative toxicological and immunological safety. It is impossible to describe the details of such a vast field in a single review and do justice to all the relevant studies.

In order to improve the drug concentration in the cells, gemcitabine entrapment in a liposome capsule was next performed, using a combination of various liposome preparation procedures. In particular, a pH gradient method was used (Marilena et al., 2004). The presence of ammonium sulphate in the internal compartments of liposomes provide an acidic environment that elicit the protonation of gemcitabine in order to drastically reduce the drug back-diffusion from liposomes thereby reduce the drug leakage from the liposomes (figure 4.1). Liposomal preparation by pH gradient method provided an encapsulation efficiency of ~80%.

4.1.2 Active loading of gemcitabine into liposomes via a transmembrane ammonium sulphate gradient

Active loading through a pH gradient is a technique based on the membrane permeability of the free base of a hydrophilic drug, whereas its charged protonated form is membrane impermeable. The drop in pH is caused by an ammonium sulphate transmembrane gradient having liposomes with internal ammonium sulphate surrounded by an ammonium sulphate free medium. Encapsulated ammonium ions are in equilibrium with uncharged ammonia and protons. The capability to permeate the liposomal membrane is dependent on size and charge of the species according to the following relation: NH_3>>> H^+>> NH_4^+> SO_4^{2-}> $(NH_4)2SO_4$. A shift in equilibrium to the right (Equation 1), a reduction in the pH within the liposomes, when uncharged ammonia diffuses out of the vesicles, leaving the protons behind.

Equation 1: $NH_4^+ \rightleftharpoons H^+ + NH_3$

Simultaneously, the neutral form of the drug, in this case the free base of gemcitabine, is expected to diffuse into the vesicle where it becomes protonated, due to the low pH, and thus trapped. This decreases the proton concentration within the liposomes, however more ammonia will subsequently be produced and diffuses out of the vesicle increasing the proton supply facilitating the drug uptake (Haran et al., 1993; Fenske et al., 2003).

In order to create pH gradient, liposome formation is carried out using ammonium sulphate solution as hydrating agent followed by removal of external ammonium sulphate. Removal of ammonium sulphate in the outer aqueous phase, build up pH gradient. This was executed by size exclusion chromatography (SEC) using Sephadex G50 gel (Haran et al., 1993). The incorporation of a drug into liposomes by such a remote loading method is conducted under heat at or above the phase transition point of phospholipids that make up membranes of the liposomes. It is a common practice to heat a mixture of a suspension of liposomes, into which the incorporation of a drug (GEM), and a solution of the drug at approx. 56°C for 30 minutes (Keisuke et al., US Patent).

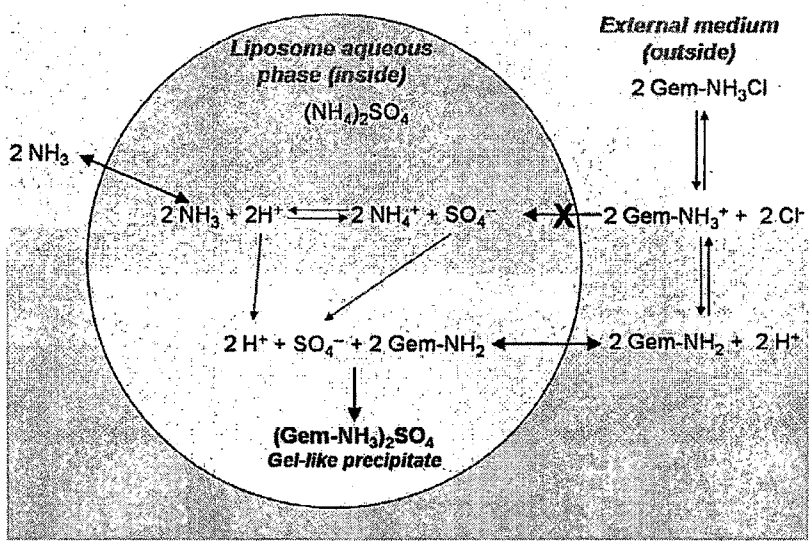

Figure 4.1 Schematic representation of the gemcitabine encapsulation process within liposomes by means of the presence of a pH gradient elicited by the co-encapsulation of a 200 mM ammonium sulphate solution

4.2 Materials and Methods

4.2.1 Materials

Gemcitabine was obtained as gift sample from Eli Lilly, indianapollis, Folic acid, *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide(DCC), triethylamine, polyoxy ethylene bis-amine(MW, 3350, NH2-PEG-NH2), cholesterol (CHOL), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazoliumbromide (MTT), succinic anhydride, pyridine, methanol, chloroform and Sephadex G-25 were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). Hydrogenated soybean phosphatidylcholine (HSPC), Monomethoxy polyethylene glycol 2000-distearoyl phosphatidyl ethanolamine (mPEG-DSPE) was obtained as gift sample from Lipoid GmbH, Germany. All reagents and solvents were of analytical or HPLC grade and were used without further purification.

4.2.2 Synthesis of F-PEG-DSPE

The synthesis was carried out as shown in Fig. 4.2. First, NHS ester of FA, folate-PEG-amine and N-Succinyl DSPE were synthesized by methods described previously by Stephenson and Kempen. This was followed by the synthesis of folate-PEG-DSPE by reacting folate-PEG-amine with N-Succinyl DSPE. Briefly, for synthesis of one, NHS ester of FA, 5g FA is dissolve in 100mLof DMSO, 2.5mL of triethylamine. A 1.1 molar excess of NHS (2.6g) and DCC (4.7g) is added the mixture is stirred overnight at room temperature in dark. Folate-PEG-bis-amine, PEG-bis-amine (500 mg) were dissolved in 2mL DMSO with 1.1M excess of NHS folate (88.3mg) and the reaction was allowed to proceed overnight at room temperature. The product folate-PEG-amine was then purified by Sephadex G-25 gel-filtration chromatography. For synthesis of Second, to synthesize an N-Succinyl, 100mg DSPE dissolved in anhydrous 5 mL chloroform ($CHCl_3$), 10μL pyridine was reacted with 1.1M excess of 14.7mg of succinic anhydride the mixture was incubated overnight at room temperature. Finally, to synthesize folate- PEG-DSPE, the carboxyl group of N-succinyl DSPE is activated by reacting with 1M equivalent of DCC for 4 hrs at RT. An equimolar quantity of folate-PEG-amine dissolved in $CHCl_3$ is added and the mixture is allowed to react overnight at RT. The solvent was then removed on a rotary evaporator and the product is washed twice with cold acetone. The identity of the product was confirmed by thin-layer chromatography (TLC) and FTIR Studies (Kempen, 1988; Stephenson et al., 2004).

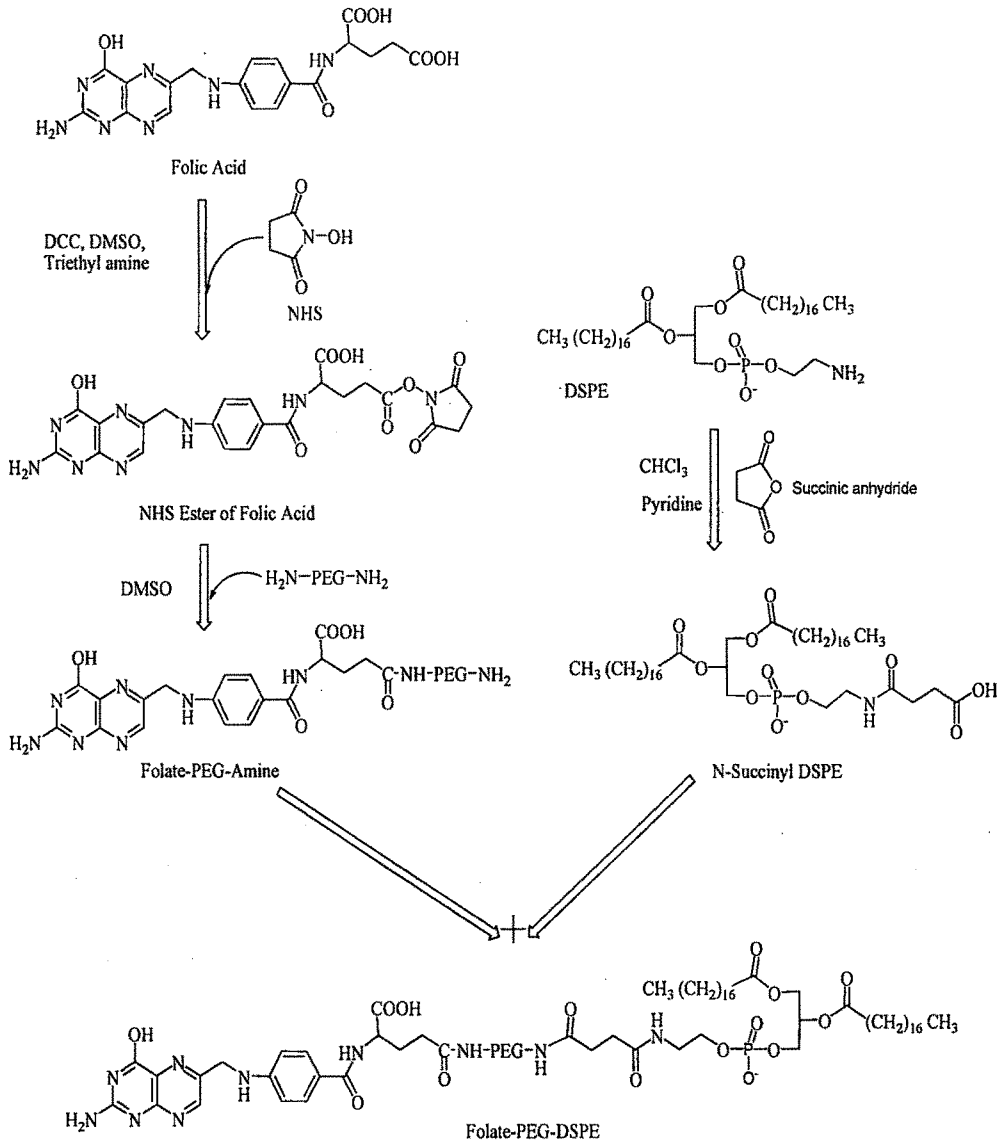

Figure 4.2 Synthesis of Folate-PEG-DSPE

4.2.3 Method

4.2.3.1 Preparation of liposomes by pH gradient method

Liposomes were prepared by thin film hydration method. GEM was remote-loaded into the liposomes by a transmembrane pH gradient method. The lipid compositions of the conventional (CL), Stealth (SL) and the Folatetargeted (FT) liposomes were HSPC/CHOL at molar ratio of 8:2, HSPC/CHOL/mPEG-DSPE at molar ratio of 7.5:2:0.5 and HSPC/CHOL/ /F-PEG-DEPE at molar ratio of 7.75:2:0.25, respectively. Briefly, the lipids (45 mg total) were dissolved in 3 mL $CHCl_3$: methanol and dried into a thin film by rotary evaporation and then further dried under vacuum. The lipid film was hydrated with 2mL of 200mM ammonium sulfate ($(NH_4)_2SO_4$) for 60 min at 57±3°C with vortex mixing. The liposomal suspension was then probesonicated (2×2min×0.6cycle×80amplitude) using a serotorius probsonicator to produce small unilamellar vesicles (SUVs). The un-entrapped $(NH_4)_2SO_4$ outside of the liposomes was removed by centrifugation method by using HEPES buffer at 25000RPM, 4°C at 30 min, 3 cycles. The mean diameter of the liposomes was determined by dynamic light scattering using malvern mastersizer. GEMHCl (6 mg/mL) was dissolved in deionized H_2O and added to the liposomes at a GEM-to-lipid ratio of 0.25 M, followed by 30 min incubation at 57±3°C. Residual free GEM un-entrapped in the liposomal preparation was removed by size exclusion chromatography on a Sephodex G25 column. GEM concentration in the liposomes was determined by measuring absorption at 268 nm on a Shimadzu UV–spectrophotometer following liposome lysis in methanol.

4.2.3.2 Thin film formation

Lipid mix with cholesterol at different molar ratio were taken in a 100 mL of round bottom flask (RBF) and dissolved in 4 mL chloroform: methanol [$CHCl_3$: MeOH] (2:1) mixture. The organic solvent was evaporated using rotary flask evaporator under vacuum on a thermostatic water bath at 57±3°C at a speed of 100 rotations per minutes (RPM) of the rotor. This was continued until the evaporation of organic solvent and leaving behind a dry thin lipid film deposited on the walls of the flask.

4.2.3.3 Hydration of thin film

Thin film was hydrated with 2 mL of ammonium sulphate solution rotary flask evaporator under thermostatic water bath at 57±3°C at a speed of 100 rotations per minutes (RPM) of the rotor. The multilamellar vesicles (MLVs) formed after hydration was kept at room temperature for 2 hrs for annealing. The liposomes were characterized for microscopic observation was performed by using Olympus microscope (BX40F4, Tokyo, Japan) at 40X magnification and photographed using digital camera.

4.2.3.4 Production of Small Unilamellar Vesicles (SUVs)

Size reduction of the MLVs were carried out using Probe sonicator (Labsonic, Sartoris, Germany) at 80% amplitude 0.6 cycles for 2 min×2 to produce a clear translucent solution.

4.2.3.5 Drugs Incubation

The suspension of SUVs obtained were mixed with GEMHCl solution (different quantities of GEMHCl dissolved in deionized H_2O), followed by a 30-min incubation at 57±3°C. Residual free GEM HCl in the liposomal preparation was removed by size exclusion chromatography on a Sephodex G25 column/ centrifugation method by using HEPES buffer centrifugation at 25,000 RPM for 30 min at 4°C for 3 cycle (Sigma 3K30).

4.2.4 Determination of Entrapment Efficiency

The percent entrapment efficiency of liposome was calculated by estimating the free drug in the supernatant after centrifugation at 25,000 RPM for 30 min at 4°C (Sigma 3K30), the supernatant was removed for estimating free drug and estimating entrapped drug in the liposome after lysis with methanol. Briefly, 0.2 mL of liposome was diluted upto 1.0 mL with methanol and centrifuged at 3,000 RPM for 15 min at 4°C (Sigma 3K30). Both supernatant and sediment was estimated for GEM after suitable dilution using UV spectrophotometer at 268 nm.

$$\%\text{Entrapment efficiency} = \frac{\text{Total drug added - Free drug}}{\text{Total drug added}} \times 100$$

$$\%\text{Entrapment efficiency} = \frac{\text{Entrapped drug}}{\text{Total drug added}} \times 100$$

4.2.5 Optimization of Process parameters

4.2.5.1 Vacuum, Speed or rotation (Rotation per minute) and Film Formation Time

Liposomal batches were prepared by varying the process parameters like vacuum, rotation per minute (rpm) and time, the film was observed by keeping a constant lipid to cholesterol ratio. The film formation time was dependent on vacuum and speed of rotation. Vacuum was increased progressively from 400 to 600 mmHg and speed of rotation was varied between 80 to 120 rpm. The above procedure was repeated three times. The effect of vacuum, rpm and film formation time on Liposomal formulation shown in table 4.1.

Table 4.1 Optimization of RPM, vacuum, time

Vacuum (mmHg)	Rotation (rpm)	Time (min)	Quality of film
400	80	50	Non uniform and thick
500	100	60	Dry, uniform and thick
600	120	70	Non uniform
500	80	60	Thin but leaves gap in between & irregular
600	**100**	**60**	**Uniform, dry and thin film**
400	120	60	Non uniform

4.2.5.2 Hydration Time

The film was hydrated with the hydration media for different time intervals from 40 min to 80 min and evaluated to optimize hydration time, for complete hydration of lipid film. The above procedure was repeated three times. The effect of hydration time was shown in table 4.2.

Table 4.2 Optimization of Hydration Time

Hydration time	Effect on Hydration of Lipid
40 min	Not properly hydrated leaves film behind.
60 min	**Complete hydration**
80 min	Complete hydration but loss of liposomal suspension

4.2.5.3 Sonication Cycles

To convert MLVs into SUVs, the prepared MLVs are subjected to sonication by using probesonicator. Sonication of liposomal suspension was optimized by altering time, cycle and amplitude according to the size requirements. The procedure was repeated three times. The effect of sonication on liposomal formulation was shown in Table 4.3.

Table 4.3 Optimization of Sonication

Sl. No.	Time (Min)	Cycle	Size (nm) *
1	1	60% Amplitude, 0.4 cycles ×2	170 nm ± 6.3
2	2	60% Amplitude, 0.6 cycles ×2	145 nm ± 5.4
3	3	60% Amplitude, 0.8 cycles ×2	140 nm ± 5.1
4	1	80% Amplitude, 0.4 cycles ×2	130 nm ± 2.5
5	**2**	**80% Amplitude, 0.6 cycles ×2**	**95 nm ± 3.5**
6	3	80% Amplitude, 0.8 cycles ×2	90 nm ± 4.1
7	1	100% Amplitude, 0.4 cycles ×2	120 nm ± 3.6
8	2	100% Amplitude, 0.6 cycles ×2	92 nm ± 5.5
9	3	100% Amplitude, 0.8 cycles ×2	90 nm ± 3.3

*** Mean±SD (No. 3)**

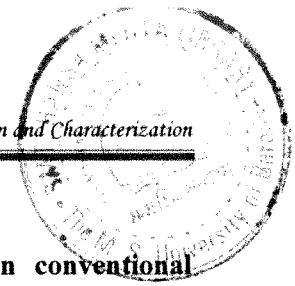

Table 4.4 Optimization of drug:lipid, lipid:cholestrol ratio in conventional liposomes(CL)

Sl. No.	Drug: Lipid(mole/mole)	Lipid (HSPC): Cholestrol	Size* (nm)	%EE*
1	0.15	7:03	126.24± 5.69	67.53 ± 3.24
2	**0.25**	**8:02**	**103.60± 4.58**	**74.83 ± 2.65**
3	0.35	9:01	120.21± 6.71	63.45 ± 4.96
4	0.25	7:03	136.24± 3.46	68.04 ± 3.27
5	0.35	8:02	148.24± 4.56	71.87 ± 4.41
6	0.15	9:01	121.33± 6.31	66.71 ± 3.86
7	0.35	7:03	156.41± 5.41	63.18 ± 5.12
8	0.15	8:02	116.24± 7.31	69.33 ± 4.56
9	0.25	9:01	110.24± 4.02	66.71 ± 3.91

*** Mean±SD (No. 3)**

Table 4.5 Optimization of drug:lipid and lipid mixture ratio in stealth liposomes(SL)

Sl. No.	Drug: Lipid(mole/mole)	HSPC:mPEG-DSPE: Cholestrol	Size* (nm)	%EE*
1	0.25	7.75:0.25:2	146.78± 8.12	67.53 ± 3.24
2	**0.25**	**7.50:0.50:2**	**120.30±6.46**	**73.36± 2.34**
3	0.25	7.25:0.75:2	115.91± 8.31	63.45 ± 4.96
4	0.25	7.0:1.0:2	141.24± 3.51	68.04 ± 3.27

*** Mean±SD (No. 3)**

Table 4.6 Optimization of drug:lipid and lipid mixture ratio in folate targeted liposomes(FT)

Sl. No.	Drug: Lipid(mole/mole)	HSPC:DSPE-PEG-Folate: Cholestrol	Size (nm)	%EE
1	0.25	7.75:0.10:2	136.31± 5.36	63.48 ± 4.61
2	0.25	7.50:0.20:2	129.65±6.16	68.51± 3.82
3	**0.25**	**7.25:0.25:2**	**98.43±4.18**	**75.16± 2.92**
4	0.25	7.0:0.30:2	118.36± 5.42	68.14 ± 4.46

*** Mean±SD (No. 3)**

Table 4.7 Comparison between CL, SL and FT formulations

Formulation	Size* (nm)	Zeta* (mV)	PDI*	%EE*
CL	103.60± 4.58	4.71 ± 0.97	0.074 ± 0.010	74.83 ± 2.65
SL	120.30±6.46	-7.33 ± 2.13	0.097 ± 0.017	73.36± 2.34
FT	98.43±4.18	3.61 ± 1.31	0.085 ± 0.013	75.16± 2.92

*** Mean±SD (No. 3)**

4.3 Results and Discussion

- **Rotation Speed and vacuum**

  The effect of rpm and vacuum on the quality of film formed was evaluated by determining quality of film formed at different rpm and vacuum conditions. These parameters were optimized for formation of smooth film with complete removal of the solvent residue. The presence of residual solvent may lead to physical destabilization of liposomes by interfering with the co-operative hydrophobic interactions among the phospholipids methylene groups, which hold the structure together (Martin et al., 1990). The optimization chart is shown in table 4.1.

- **Hydration Time**

  The film was hydrated for different time intervals between 40 to 80 min, and was evaluated for complete hydration of lipid film with maximum entrapment and uniform size. The results reveal that after 40 minutes the film was not properly hydrated, some portion of lipid was still remained unhydrated on the surface of the RBF; at 60 minutes the film was completely hydrated and gives homogenous suspension of liposome with optimized entrapment efficiency. Optimized hydration time is shown in table 4.2.

- **Sonication cycles**

  Sonication is important to convert MLVs to SUVs for drug delivery system, by using probesonicator the size will be optimized by using different parameters like amplitude, time cycle etc. the optimization chart was shown in table 4.3.

- **Optimization of Drug : Lipid ratio, lipid: cholesterol and selection of lipids**

  Different concentrations of Drug: Lipid ratio was optimized on the basis of size and EE. As Drug: Lipid ratio was increased from the optimized ratio, entrapment efficiency was not increased. The effect of lipid (Phospholipid) to cholesterol ratio was evaluated with respect to entrapment efficiency, size, PDI and stability of CL, SL and FT Liposomes. With the Lipid: Cholesterol ratio (8:2) the size, EE was found to be good and stable. The optimization chart is shown in table 4.4.

This method is a simple, efficient, safe, economical and fast transmembrane loading procedure for efficient active loading of gemcitabine into liposomes using the transmembrane gradient. The resulting liposomes loaded with the GEM drug are stable and safe. A storageable form of loadable liposomes has stability over 3 months. This procedure is applicable for sustained release of liposome encapsulated drugs from ammonium liposomes. Liposomal suspension having a greater concentration of ammonium ions inside the liposomes than outside, by removing the external ammonium ions by centrifugation method by using HEPES buffer at 25000RPM× 4°C × 30 min× 3 cycles, here in each cycle remove the supernatant and add equal quantity of 50mM HEPES buffer, thereby it establish a pH gradient from inside to outside the liposomes. By this increase the loading and decrease the drug leakage by decreasing back diffusion drug from liposomes (Barenolz et al., 1993; Guoqin et al., 2010; Marilena et al., 2004).

4.4 Characterization of liposomes

4.4.1 Particle Size Analysis

The particle size (z-average) and poly dispersity index (PDI) of the liposomes was analyzed by photon correlation spectroscopy (PCS) using a Malvern Zetasizer Nano (Malvern Instruments; UK). 0.2 mL of liposomes suspension was diluted to 1.0 mL with distilled water (DW) and measured after an equilibration time of 1 minute. The Zetasizer Nano is operating with a 4 mW He-Ne-Laser at 633 nm and non invasive back-scatter technique (NIBS) at a constant temperature of 25°C. The measurements were conducted in the manual mode. The size distribution by intensity and volume was calculated from the correlation function using the multiple narrow mode of the Dispersion Technology Software version 4.00 (Malvern, Herrenberg, Germany). Thereby, the resulting size distributions show the hydrodynamic diameter. The average particle size and PDI was calculated after performing the experiment in triplicate. PDI of 0.0 represents a homogenous particle population while 1.0 indicates a heterogeneous size distribution in the liposome. The particle size analysis results of liposomal formulation were shown in figure 4.3-4.5 and table 4.7.

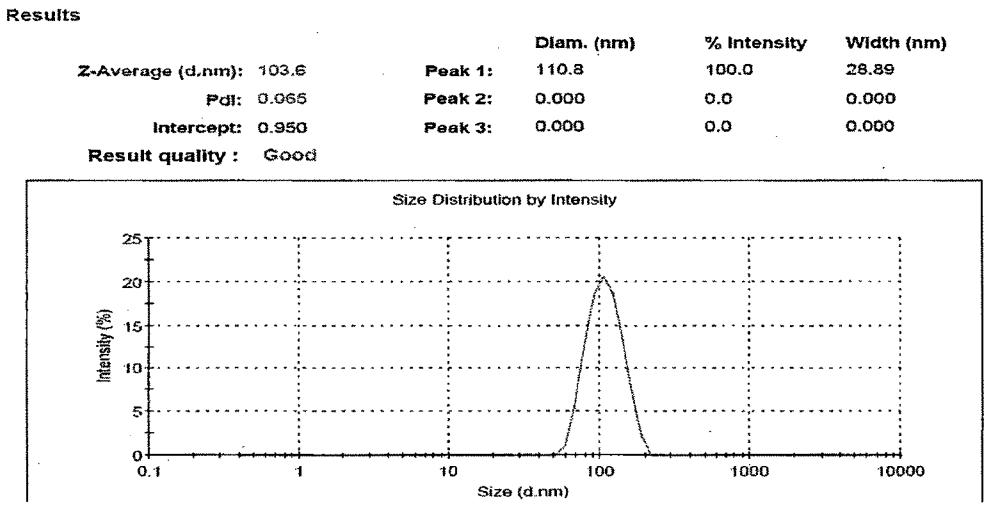

Figure 4.3 Particle Size analysis of the optimized batch of the Conventional liposomes (representative sample)

Results

			Diam. (nm)	% Intensity	Width (nm)
Z-Average (d.nm):	129.5	Peak 1:	142.6	100.0	46.12
PdI:	0.097	Peak 2:	0.000	0.0	0.000
Intercept:	0.932	Peak 3:	0.000	0.0	0.000
Result quality :	Good				

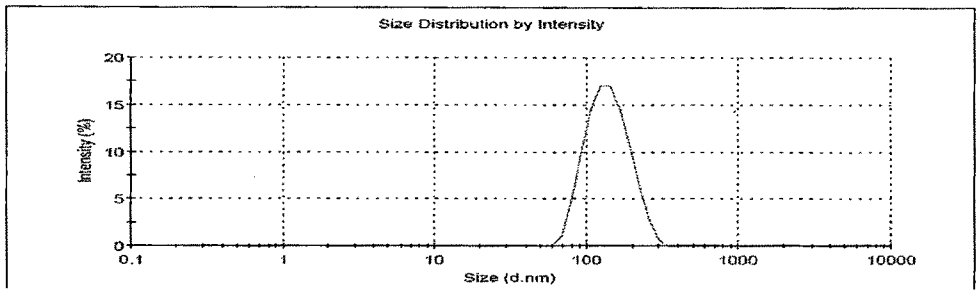

Figure 4.4 Particle Size analysis of the optimized batch of the Stealth liposomes (representative sample)

Results

			Diam. (nm)	% Intensity	Width (nm)
Z-Average (d.nm):	98.43	Peak 1:	105.2	100.0	26.88
PdI:	0.073	Peak 2:	0.000	0.0	0.000
Intercept:	0.967	Peak 3:	0.000	0.0	0.000
Result quality :	Good				

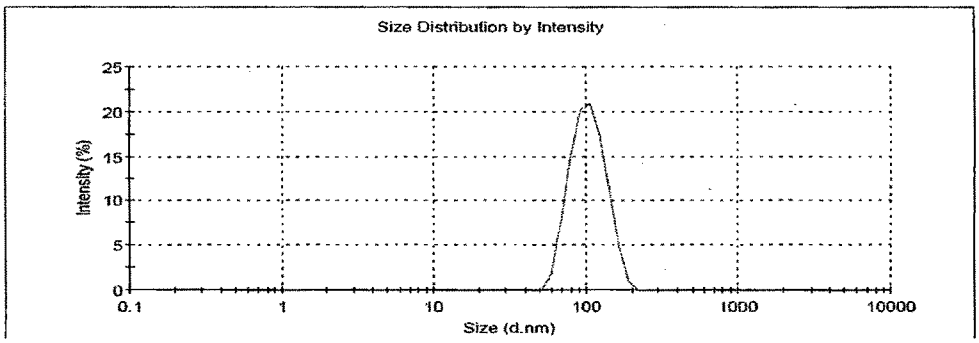

Figure 4.5 Particle Size analysis of the optimized batch of the Folate targeted liposomes (representative sample)

4.4.2 Zeta (ς) potential analysis

The zeta potential (ς potential) of the various liposome suspension prepared was measured by microelectrophoresis using Malvern Zetasizer Nano ZS (Malvern, Instrument, U.K.). Zeta potential of the liposome was measured after separation of the free drug from the liposome. 0.2 mL of liposome was diluted to 1 mL of DW. The determination of the zeta potential was realized at 25°C after injecting 1 mL of the

sample into a standard sample cell. The zeta potential data results of formulations are shown in figure 4.6-4.8 and table 4.7.

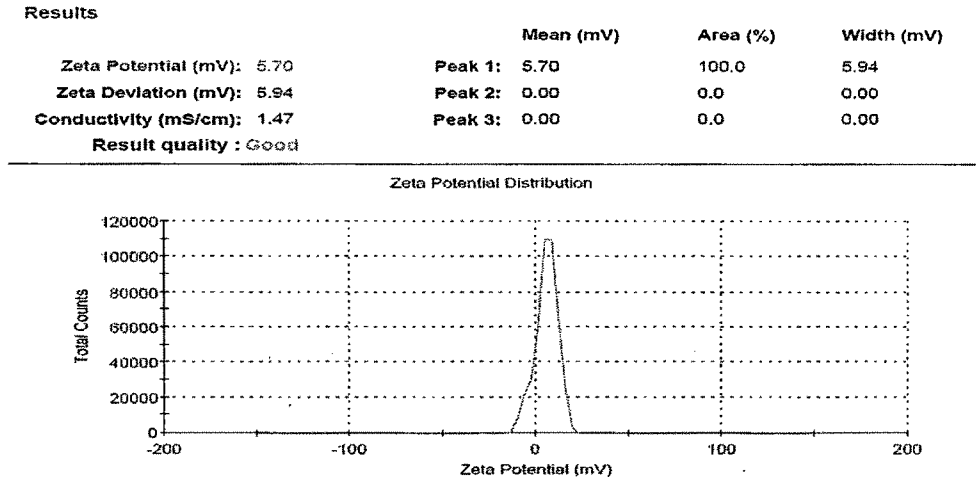

Figure 4.6 Zeta potential analysis data of the Conventional liposomes (representative sample)

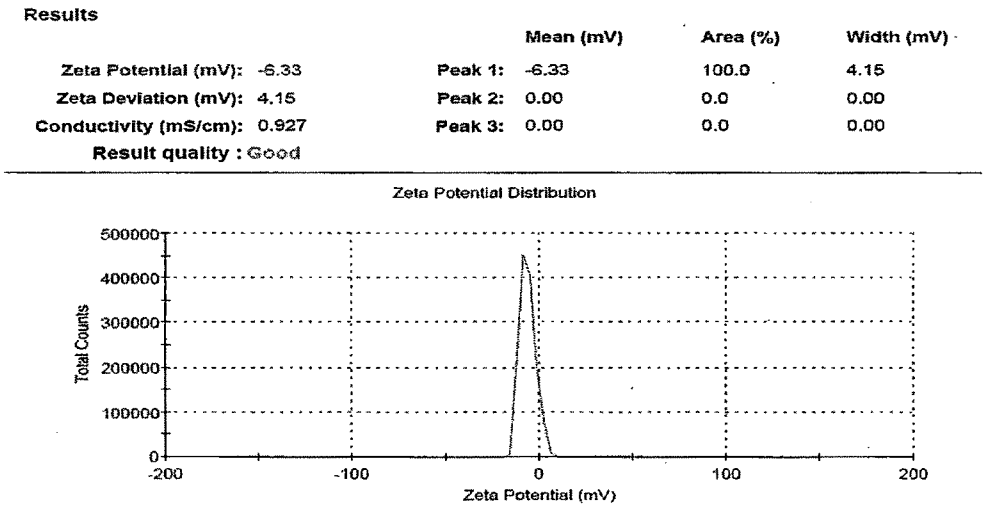

Figure 4.7 Zeta potential analysis data of the Stealth liposomes (representative sample)

Results

		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV): 2.71	Peak 1:	2.71	100.0	10.2
Zeta Deviation (mV): 10.2	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 1.21	Peak 3:	0.00	0.0	0.00
Result quality : Good				

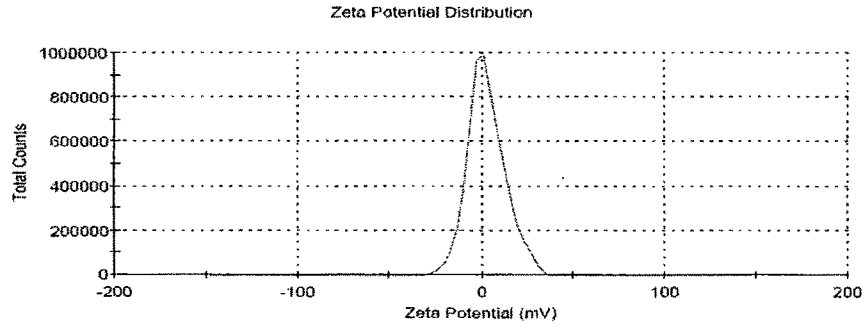

Figure 4.8 Zeta potential analysis data of the Folate targeted liposomes (representative sample)

4.4.3 Morphology

The prepared MLVs were characterized by optical microscopy and SUVs by transmission electron microscopy.

4.4.3.1 Olympus microscopy

Morphological evaluation was conducted using Optical microscope with polarizer BX 40, Olympus Optical Co. Ltd., at a magnification of 40X (shown as below), by this we can conclude that the prepared liposomes by TFH method were spherical in nature and MLVs before sonication. The photographs of MLVs are shown in figure 4.9.

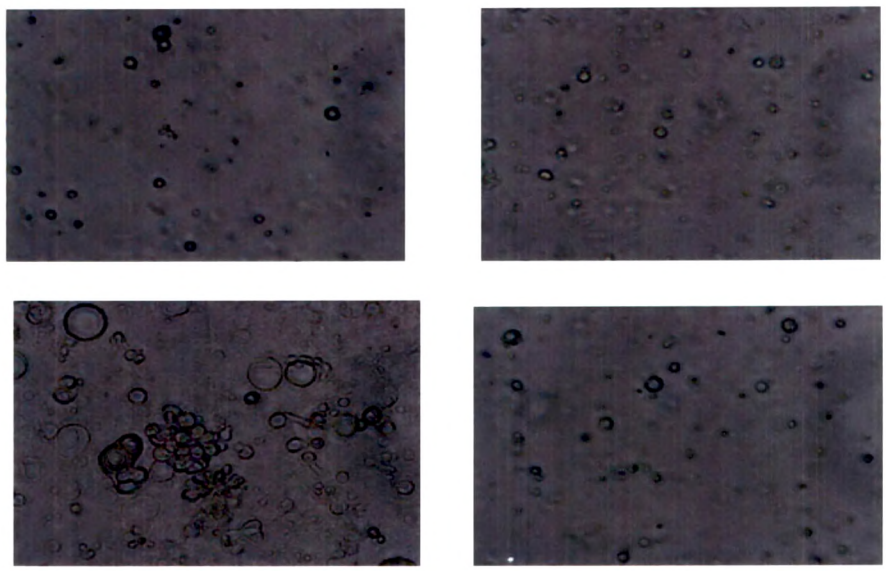

Figure 4.9 Photography of MLVs in Olympus microscopy

4.4.3.2 Transmission electron microscopy (TEM)

TEM is a microscopic technique whereby a beam of electrons is transmitted through an ultra thin specimen, interacting with the specimen as it passes through. An image is formed from the interaction of the electrons transmitted through the specimen, the image is magnified and focused onto an imaging device. To prevent charge build-up at the sample surface samples need to be coated with a thin layer of conducting material, such as carbon, where the coating thickness is several nanometers. For negative-staining 5 µL of dilute liposome dispersion was placed on a 200-mesh formvar copper grid (TAAB Laboratories Equipment, Berks, UK), allowed to absorb, and the surplus was removed by filter paper, stained with 5 µL of 2.5 % uranyl acetate for 30 seconds. Then the surplus was removed, and the sample was dried at room conditions before imaging the liposome with a transmission electron microscope operating at an acceleration voltage of 200 KV. The TEM images of the prepared SUVs are shown in figure 4.10.

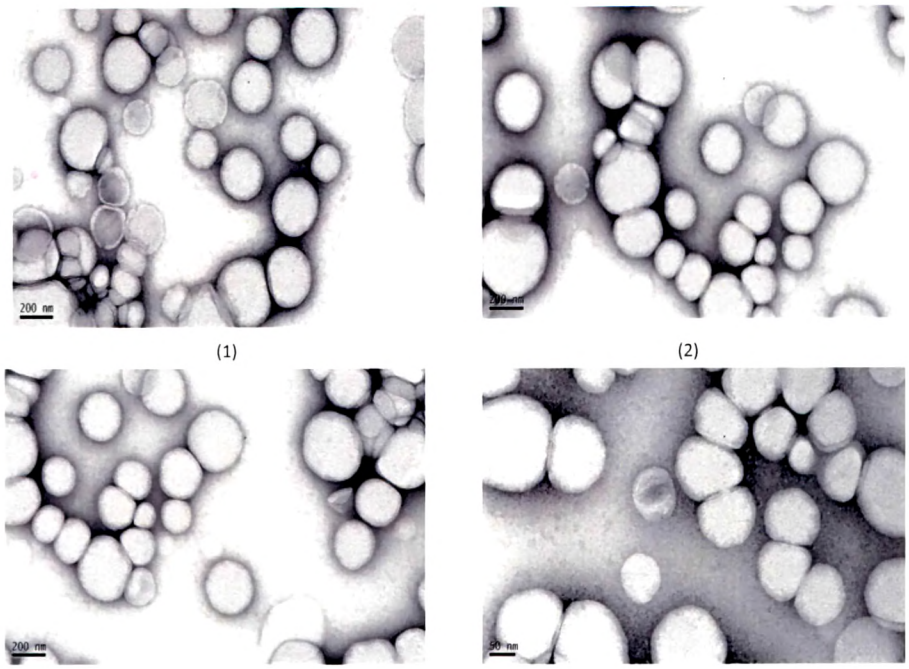

Figure 4.10 Photography of SUVs by TEM

4.4.4 Fourier transform infrared (FTIR) spectroscopy

The sample was ground with a specially purified salt (usually potassium bromide) finely (to remove scattering effects from large crystals). This powder mixture is then pressed in a mechanical die press to form a translucent pellet. These pellets were used to take FTIR spectra.

Folic acid and the synthesized lipid of DSPE-PEG-Folate were subjected to FTIR spectroscopic analysis to ascertain reaction. The obtained FTIR spectra are given in Figure 4.11-4.12. The characteristic peak of FA at 1694 cm^{-1} (C-O stretching) has been appeared in DSPE-PEG-Folate at 1637 cm^{-1}, indicating that the reaction has been takes place.

Gemcitabine HCl pure drug and the optimized formulation of CL were subjected for FTIR spectroscopic analysis for compatibility studies, and to ascertain whether there is any interaction between the drug and lipid used. The obtained FTIR spectra are

given in figure 4.13-4.16. The characteristic peaks of the pure drug were compared with the peaks obtained for formulation, are given in Table 4.8. From the data it is observed that a similar characteristic peak of gemcitabine was appeared formulation with minor differences. The characteristic peaks at 3392 (NH_2 stretching) and 1703 (C-O stretching) cm^{-1} appeared in both the pure GEM drug and its formulation, indicating no possible chemical interaction between drug and lipid. It has also been concluded that the characteristics bands of pure drugs were not affected after successful loading. Absence of any change in their peak position indicates no chemical interaction between drug and lipids used in the preparation of liposomes.

Table 4.8 FTIR spectral data of GEM pure drug and CL liposomal formulation

Group	Frequency of pure drug	Frequency of formulation
NH_2 stretching	3392.90 cm^{-1}	3392.30 cm^{-1}
C-O stretching	1703.30 cm^{-1}	1703.20 cm^{-1}

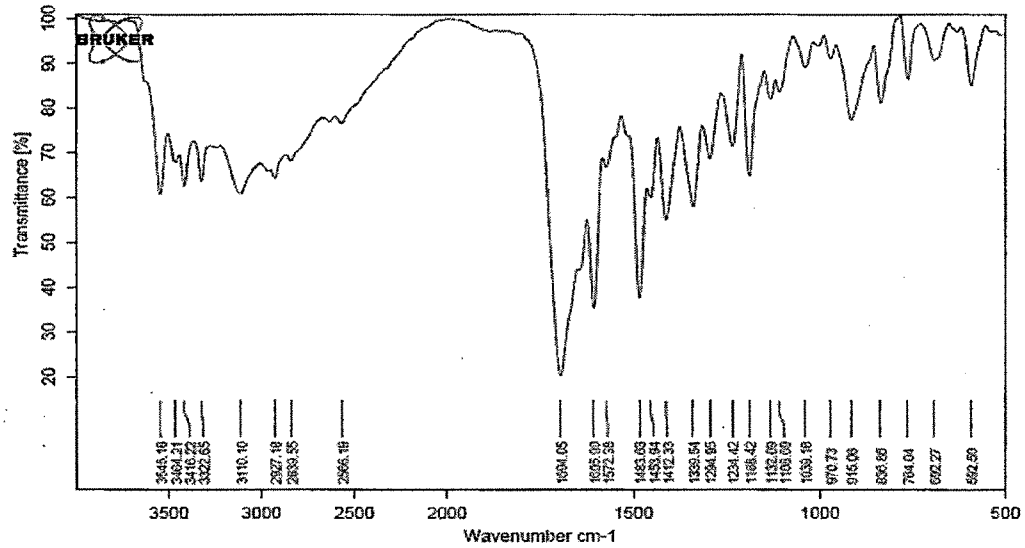

Figure 4.11 FTIR spectra of folic acid

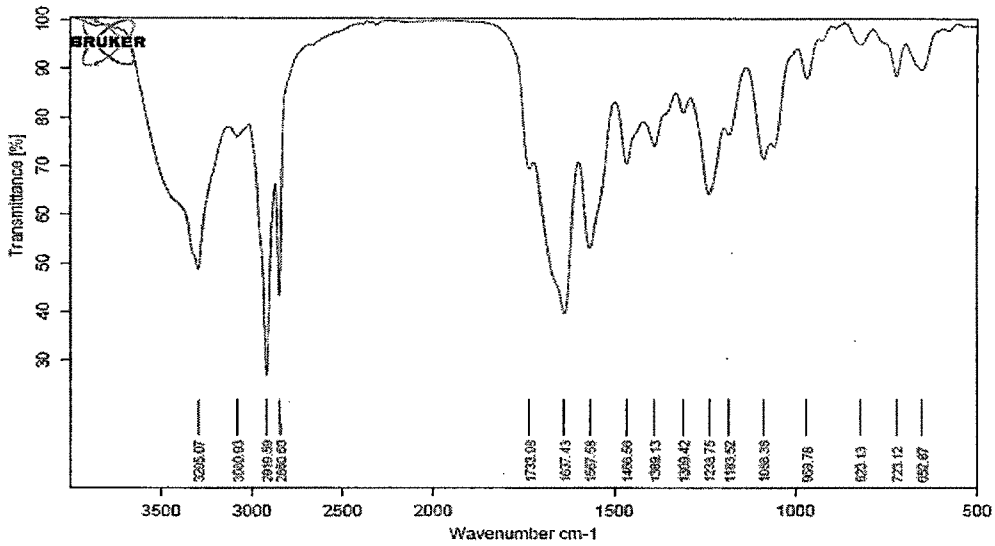

Figure 4.12 FTIR spectra of DSPE-PEG-Folate

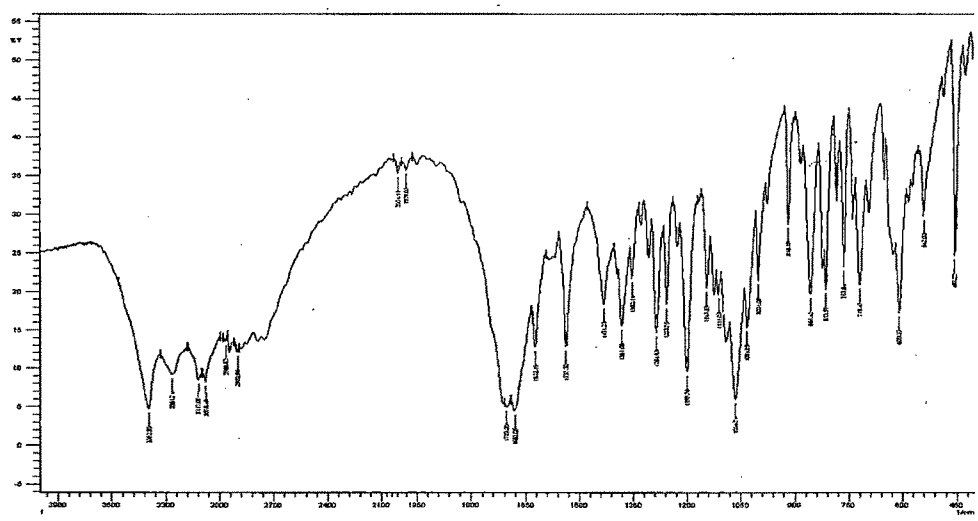

Figure 4.13 FTIR spectra of GEM Pure drug

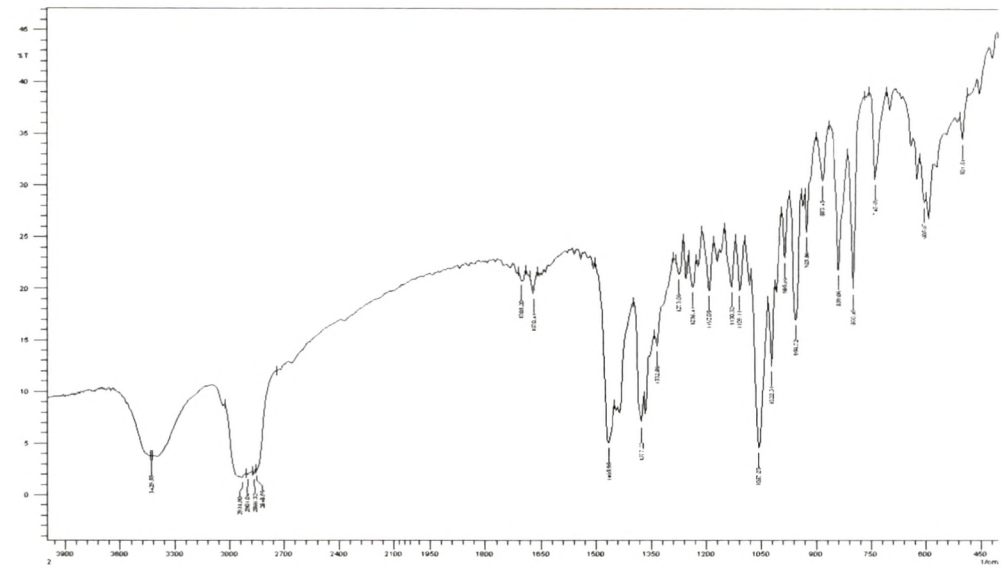

Figure 4.14 FTIR spectra of HSPC

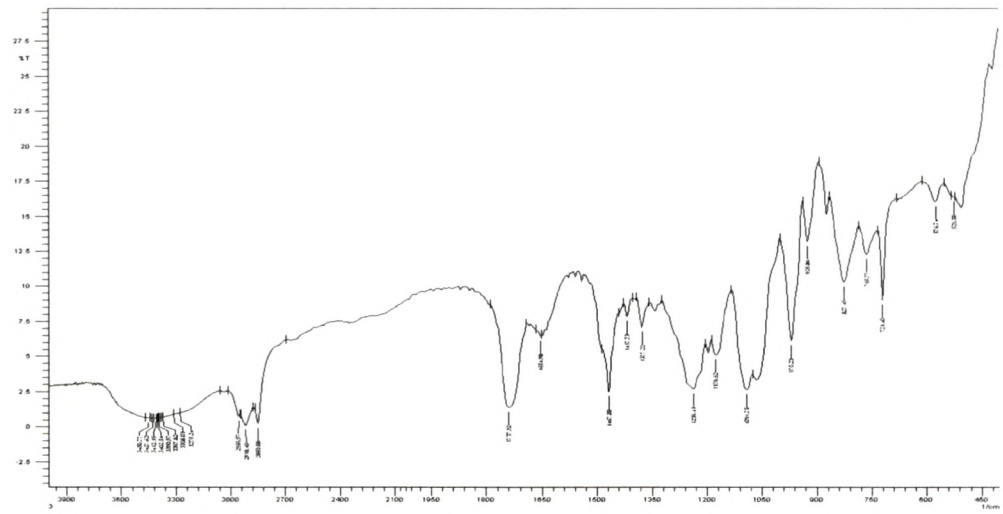

Figure 4.15 FTIR spectra of CL liposomal formulation

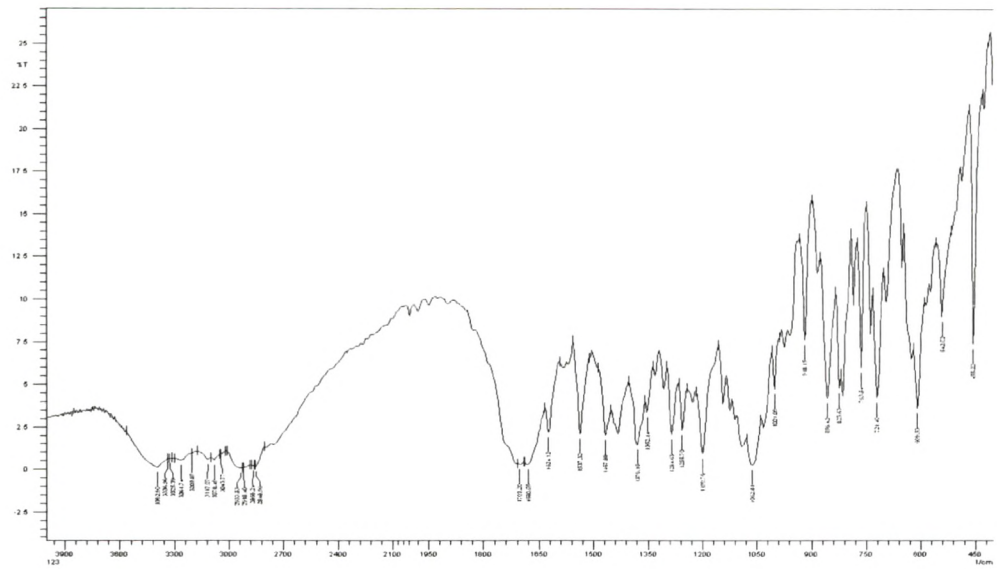

Figure 4.16 FTIR spectra of Cholesterol

4.4.5 Differential Scanning Calorimetric studies

In order to investigate the possible interaction between the drug and lipids, differential scanning calorimetry (DSC) studies were carried out. DSC thermogram of the formulation was compared with the DSC thermogram of pure drug sample. About 2-5 mg of sample was heated, in a hermetically sealed aluminum pan, at a heating rate of 10° C/min, from 10° C to 300° C under a nitrogen atmosphere. An empty aluminum pan was used as the reference for all measurements. Gemcitabine hydrochloride exhibits a sharp endothermic peak at 279.41 and 286.04°C. The obtained DSC thermograms are shown in figure 4.17-4.20.

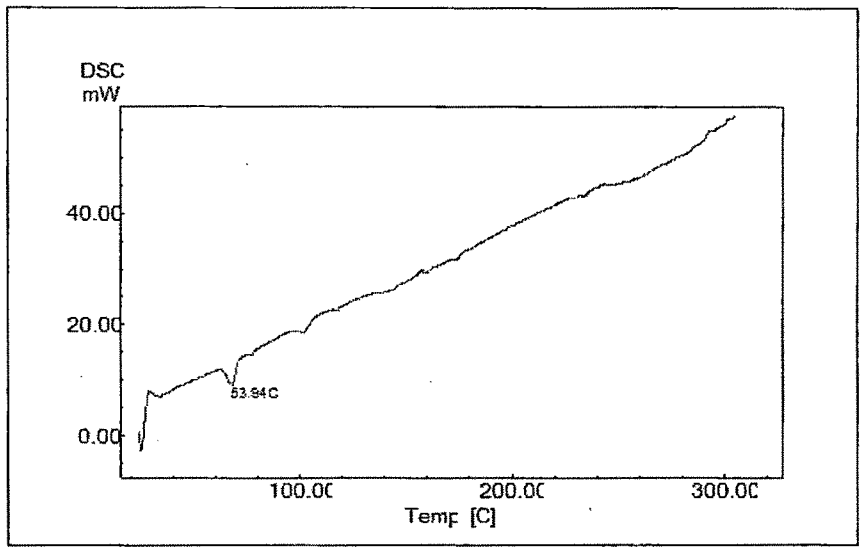

Figure 4.17 DSC of HSPC

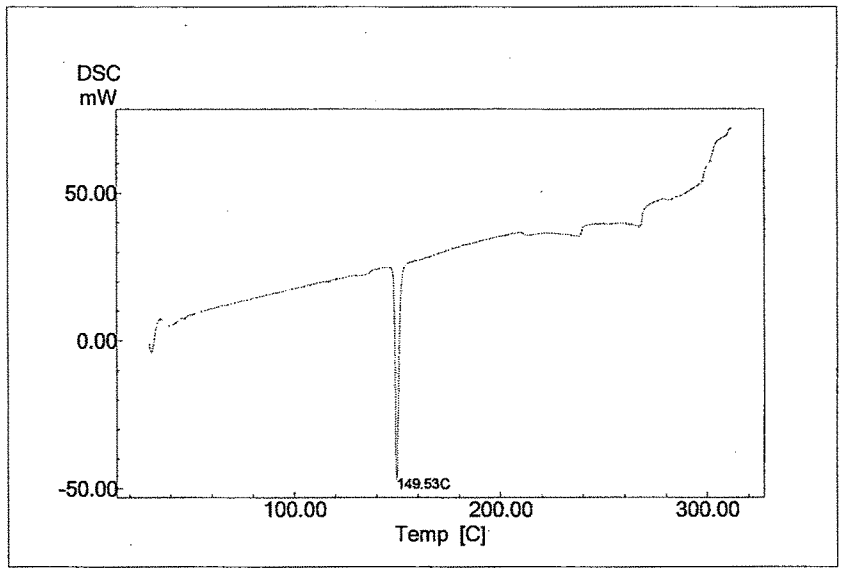

Figure 4.18 DSC of Cholesterol

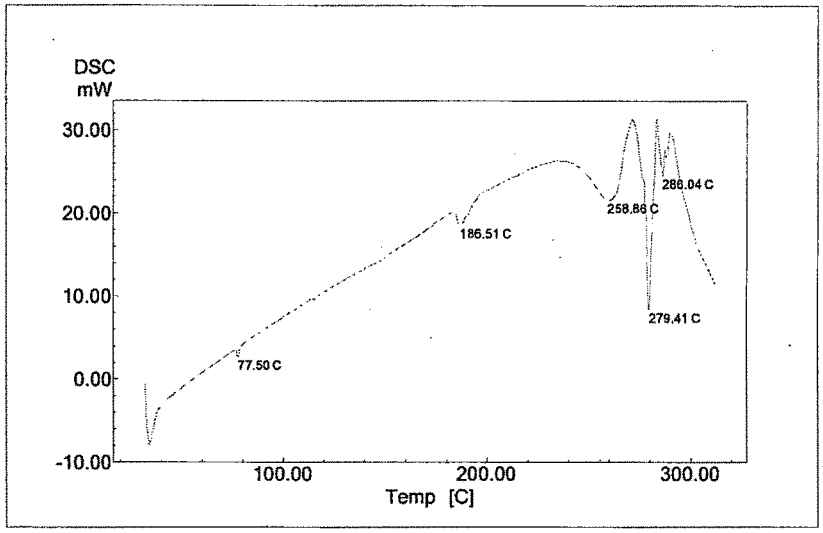

Figure 4.19 DSC of Pure drug

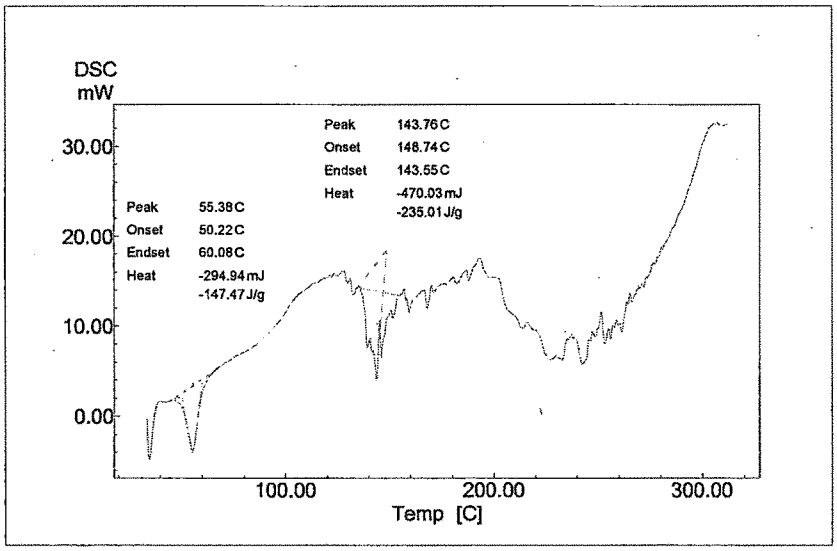

Figure 4.20 DSC of CL Liposomal formulation

4.4.6 *In vitro* diffusion Studies

4.4.6.1 Materials and method

4.4.6.2 *In vitro* Release of Gemcitabine

Phosphate buffer solution (PBS) at pH 7.4 was selected as the release medium. Liposomal suspension transferred into a dialysis membrane (MW cut-off: 15000 Da). The dialysis bag was placed in 50 mL PBS (pH 7.4). The release study was performed at 37°C in magnetic stirrer. At selected time intervals, 5 mL buffered solution from the receptor compartment was removed and replaced with 5 mL fresh buffer solution. Gemcitabine concentration was estimated in the sample by UV-visible spectrophotometry method based on the absorbance intensity at 268 nm.

Table 4.9 *In vitro* release of GEM from GEM liposomes at pH 7.4

Time (hrs)	% Cumulative Release at pH 7.4		
	CL	SL	FT
1	4.03± 0.312	3.41± 0.41	3.83± 0.514
2	6.44± 0.542	4.82± 0.368	4.54± 0.443
4	7.33± 0.432	6.11± 0.545	5.56± 0.367
6	8.32± 0.981	7.32± 0.785	7.11± 1.01
8	10.12± 1.31	8.73± 0.84	7.98± 0.73
12	14.98± 0.78	11.48± 1.21	10.52± 1.01
24	18.38± 2.13	15.71± 1.78	13.92± 1.41
48	22.64± 1.72	20.04± 2.13	18.33± 0.79
72	27.41± 1.45	23.12± 2.13	21.95± 1.14
96	32.14± 2.13	27.32± 1.98	25.65± 1.65

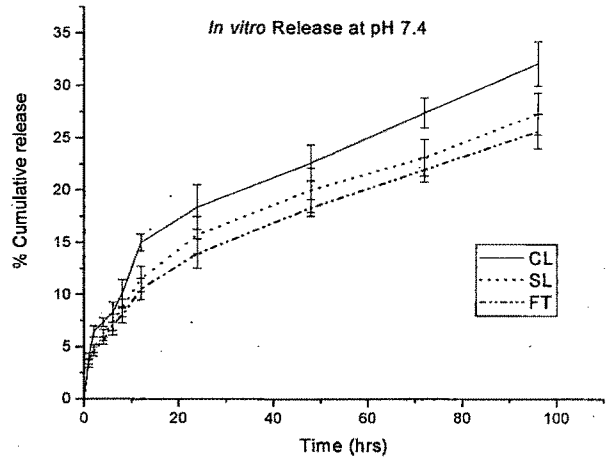

Figure 4.21 *In vitro* release studies of GEM at pH 7.4 buffer

4.4.6.3 Results and discussion

4.4.6.4 *In vitro* gemcitabine release study

In vitro diffusion of formulations is a valuable tool to predict the behaviour of a particular formulation with respect to drug transport across the membrane. Various parameters pertaining to formulations such as flux, partition coefficient and diffusion coefficient can be derived using *in vitro* evaluation techniques. The *in vitro* diffusion studies can also be used as a screening tool to screen the best formulation out of many. One of the disadvantages of *in vitro* evaluation techniques is that the method does not mimic the behavior of living organs/ tissues, for example degradation of drug compound in the presence of enzymes, capricious blood supply or metabolism etc.

The *in vitro* release of gemcitabine-loaded liposomes in buffered solution (pH 7.4) is shown in figure 4.21 and table 4.9. Reduction in the release of GEM from the FT and SL in comparison to conventional liposomes occurred because the linkers incorporated in previous formulations retained drug in the bilayer by making it more rigid. The slower and continuous release may be attributed to slow trans-layer permeation kinetics and diffusion from the interior. It has been shown that the aqueous medium slowly penetrates the internal structure of the liposomes and causes progressive degradation of the polymer chains (Lin Jia et al., 2010).

Reference

1. Barenolz, Yechezkel, Haran, Gilad Method of amphiphatic drug loading in liposomes by pH gradient. United States Patent 1993; 5192549.
2. Chabner BA, Longo DL. Cancer Chemotherapy and Biotherapy: Principles and Practice, Lippincott-Raven, Philadelphia. 2nd ed. 1996:409-434.
3. Fenske, Mauer, Cullis PR. Encapsulation of weakly-basic drugs, antisense oligonucleotides, and plasmid DNA within large unilamellar vesicles for drug delivery applications. In: Torchilin VP, Weissig V, editors. Liposomes. 2nd ed. New York: Oxford university press; 2003:167-191.
4. Guoqin Niu, Brian Cogburn, Jeffrey Hughes. Preparation and Characterization of Doxorubicin Liposomes. Methods in Molecular Biology 2010;624: 211-219.
5. Haran G, Cohen R, Bar LK, Barenholz Y. Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphiphatic weak bases. Biochim Biophys Acta 1993;1151:201-215.
6. Keisuke Yoshino, Keiko Yamashita, Yoshitaka Ogata. (http://www.faqs.org/patents/app/20100021531#ixzz0j20NsIBa,Inventors:)
7. Kempen H.J.M.. Synthetic glycolipids, a process for the preparation, thereof and several uses for these, synthetic glycolipids. 1988 U.S. Patents 4,751,219.
8. Lin Jia, Jian-Jun Zheng, Shu-Man Jiang, Kai-Hong Huang. Preparation, physicochemical characterization and cytotoxicity in vitro of gemcitabine-loaded PEG-PDLLA nanovesicles. World J Gastroenterol 2010;16(8):1008-1013.
9. Marilena Celano, Maria Grazia Calvagno1, Stefania Bulotta1 et al. Cytotoxic effects of Gemcitabine-loaded liposomes in human anaplastic thyroid carcinoma cells. BMC Cancer 2004;4:63.
10. Speth PAJ, van Hoesel QGCM, Haanen C. Clinical pharmacokinetics of doxorubicin. Clin Pharmacokinet. 1988:15;15–31.
11. Stephenson SM, Low PS, Lee RJ. Folate Receptor-Mediated Targeting of Liposomal Drugs to Cancer Cells. Methods in Enzymology 2004;387:33-50.