Chapter – 6

Characterization of drug loaded (HA grafted and non grafted) liposomesliposomes

Chapter 6 Characterization of Drug Loaded (HA grafted and non grafted) liposomes

INTRODUCTION

Characterization of newly developed formulations is undertaken to obtain insight regarding the physico chemical properties before moving ahead to variegated pharmaceutical applications, bulk scale production followed by commercialization. Characterization of a formulation can be conducted at two different levels: physico-chemical characterization in terms of particle size, zeta potential, and percentage drug entrapment whereas biopharmaceutical characterization mainly comprises of determining drug release and pharmacokinetic profile in animals by in vivo studies.

Many sophisticated and sensitive techniques such as laser light scattering (LLS), Dynamic light scattering (DLS), Photon correlation spectroscopy for particle size and size distribution, Scanning electron microscopy for morphology, X ray photo electron spectroscopy (XPS), Nuclear Magnetic Resonance (NMR), Fourier Transform Infra Red (FTIR) for surface chemistry, Differential scanning calorimetry (DSC) for thermal properties and drug excipients interaction etc. are used for overall characterization of newly developed formulations.

Scrutiny of physico chemical properties by thorough characterization facilitates optimization of various process and formulation variables that critically determine the performance and stability of a new formulation. Parameters such as density, molecular weight, crystallinity affect drug properties while surface charge, hydrophilicity, hydrophobicity affect the formulation interaction with microenvironment.

Chapter 6 Characterization of Drug Loaded (HA grafted and non grafted) liposomes

6.1 EQUIPMENTS

Equipment	Source
pH meter	Systronics 335, India
Balance	Precisa 205 A, SCS, Switzerland
Cooling centrifuge	3K30, Lab centrifuge, Osterode, Gmbh.
Cyclomixer	Remi, Mumbai,India
Particle size Analyzer	Malvern Instruments, Worcestershire, U.K.
Zeta Sizer	NanoZS, Malvern Equipments Ltd., U.K.
UV-Visible Spectrophotometer	UV-1601, Shimadzu, Japan.
DSC	Mettler Toledo DSC 20, Switzerland
Probe Sonicator	Ralsonics, Mumbai,India.
Glassware	Schott and Corning, Mumbai. Merck, Mumbai,India.
Polycarbonate membranes	Whatmann, USA.
Dialysis bag (1000 Mol.wt. cut off)	Sigma Aldrich Corporation, Mumbai,India.

Chapter 6 Characterization of Drug Loaded (HA grafted and non grafted) liposomes

Chemicals	Source
6-coumarin	Neelikon Dyes, Mumbai,India.
Triton X	S.D.Fine Chemicals, Mumbai,India.
Centrifuge tubes, microtips,micropipettes and miscellaneous plasticware	Tarsons Ltd., Kolkatta, India.

6.2 PHYSICAL CHARACTERIZATION

Particle size determination was based on principle of laser diffractometry (Malvern Mastersizer 2000 series, Malvern, Worcestershire, U.K.) using Hydro 2000 SM sampling unit. Apparatus consisted of a He-Ne laser (5 mW) and sample holding cell of 50 ml capacity. Each sample in sufficient quantity was dispersed in distilled water to achieve obscuration range between 5-10 %. Samples were stirred using a blade stirrer at 1000 rpm to keep particles in suspended form and measurements were recorded for volume mean diameter (VMD) which is related to mass median diameter divided by density of particles.

Polydispersity Index (PDI) of powder was defined from span.

$$\text{Span} = \frac{[D(V,_{90}) - D(V,_{10})]}{[D(V,50)]} \qquad \text{--------(1)}$$

D(V,90, V,50 and V,10) are equivalent volume diameters at 90, 50 and 10 % cumulative volume respectively.

6.2.1 Particle Size Measurement

The size of non grafted and Hyaluronic acid (HA) grafted liposomes was measured by dynamic light scattering with a Malvern Zetasizer 3000 HS. Diluted liposome suspension was added to the sample cuvette and then cuvette was placed in zetasizer. Sample was stabilized for two minutes and reading was measured. The average particle size was measured after performing the experiment in triplicate and results were recorded in Table 6.1.

6.2.2 Zeta Potential Determination

Zeta potential of developed non grafted and HA grafted liposomes was determined using Malvern Zetasizer 3000 HS (Malvern Instruments, Malvern, UK). The zeta potential was calculated by Smoluchowski's equation from the electrophoretic mobility of liposomes at 25 °C (Mu and Feng 2001) and results were recorded in Table 6.1.

6.2.3 Percent Drug Entrapment

To determine percent drug entrapment (PDE), free and entrapped drug was measured. The free ETP and DOC (un-entrapped) in the liposomal dispersion were separated by controlled centrifugation. Liposomal dispersion was centrifuged at a speed of 30,000 rpm for 20 minutes at 4^0C using by ultracentrifugation and the liposomal dispersion was removed without disturbing the drug pellet. Etoposide drug pellet was dissolved in methanol: chloroform (9:1) mixture and estimated for un-entrapped drug content. Fixed volume of liposomal suspension was withdrawn and dissolved in methanol: chloroform (9:1) mixture and estimated for entrapped drug content. Similarly, Docetaxel drug pellet was dissolved in ethanol and analyzed spectrophotometrically at 228 nm for drug content and results were recorded in Table 6.1.

PDE is expressed as:

$$PDE = \frac{\text{drug entrapped}}{\text{drug entrapped + free drug}} \times 100$$

Table 6.1 Characterization of Etoposide loaded liposomes (ETPLIP), Hyaluronic acid (HA) grafted Etoposide Liposomes (HAETPLIP), Docetaxel loaded liposomes (DOCLIP) and HA grafted Docetaxel liposomes (HADOCLIP) in terms of particle size, zeta potential and PDE.

Formulation Code	Particle Size (nm)*	Zeta Potential (mV)*	Percentage Drug Entrapment (PDE) *
ETPLIP	190 ± 3.7	-10.7 ± 0.57	80.2 ± 3.4
HAETPLIP	217 ± 2.1	-20.2 ± 0.37	73.1± 4.08
DOCLIP	195 ± 3.0	-8.8 ± 1.2	70.1 ± 2.8
HADOCLIP	235 ± 1.8	-16.2 ± 2.9	64.2± 3.04

***-Mean ± S.D. (n=3)**

6.3 CHARACTERIZATION OF 6-COUMARIN LOADED LIPOSOMES

6-coumarin loaded liposomes prepared and grafted with HA (discussed in methods, Ch 4) were characterized for particle size, zeta potential and results were recorded in Table 6.2.

Table 6.2 Characterization of 6-coumarin loaded non grafted and HA grafted liposomes in terms of particle size, zeta potential and surface density of HA.

Formulation Code	Particle Size (nm)*	Zeta Potential (mV)*	Surface density of HA*
6-Coumarin loaded liposomes	192±4	-9.9±0.5	---
HA grafted 6-coumarin loaded liposomes	232 ±5	-18.5 ±1.5	500 µg

***-Mean ±S.D. (n=3)**

Chapter 6 Characterization of Drug Loaded (HA grafted and non grafted) liposomes

6.4 DRUG-EXCIPIENT INTERACTION BY DSC

DSC study was performed in order to assess the physical state of drugs (Etoposide-ETP and Docetaxel-DOC) in liposomes as well as to study drug excipient interaction that can precipitate out incompatibility, if any. DSC of samples was carried out by scanning the samples using Differential Scanning Calorimeter. Thermograms were analyzed using Mettler Toledo star SW 20. An empty aluminium pan was used as the reference for all determinations. During each scan, 2 to 3 mg of sample was heated in a hermetically sealed aluminium pan at a heating rate of 10 C/min from 25 to 300^{0}C under a nitrogen atmosphere. Results were recorded in Fig 6.1(a), 6.1 (b) and 6.1 (c) for Plain Etoposide drug, blank liposomes(without drug) and Etoposide loaded liposomes (ETPLIP) and those for Docetaxel were shown in Fig 6.2 (a), 6.2 (b) and 6.2 (c) for plain Docetaxel drug, blank liposomes (without Docetaxel) and Docetaxel loaded liposomes (DOCLIP).

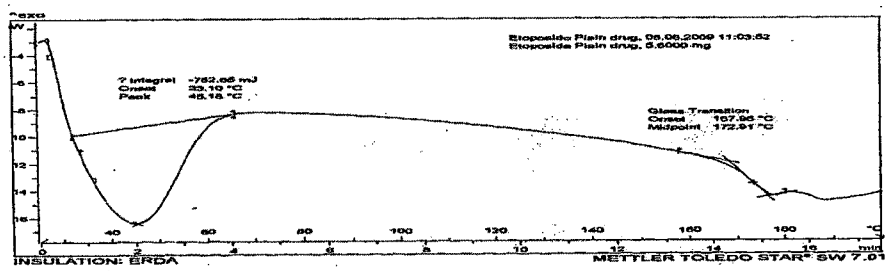

Fig 6.1(a)-DSC Thermogram of pure Etoposide plain drug

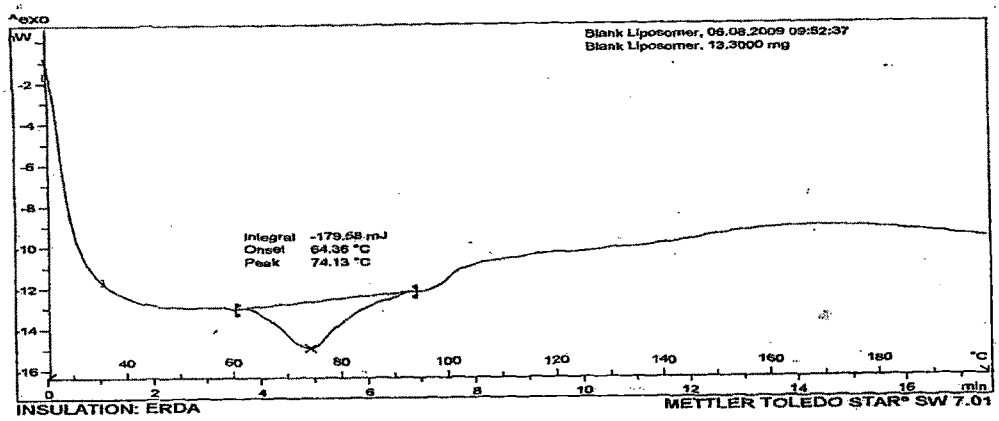

Fig 6.1 (b)-DSC Thermogram of Blank Liposomes (without drug)

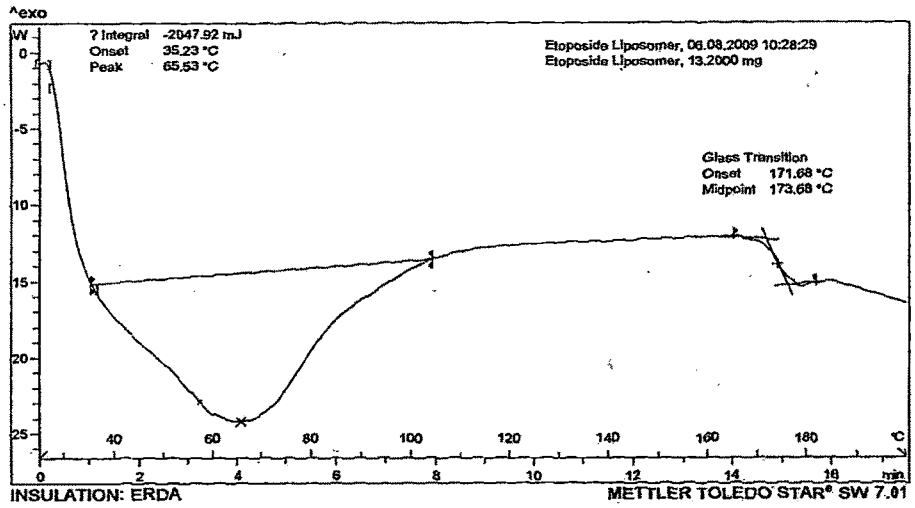

Fig 6.1 (c))-DSC Thermogram of Etoposide liposomes.

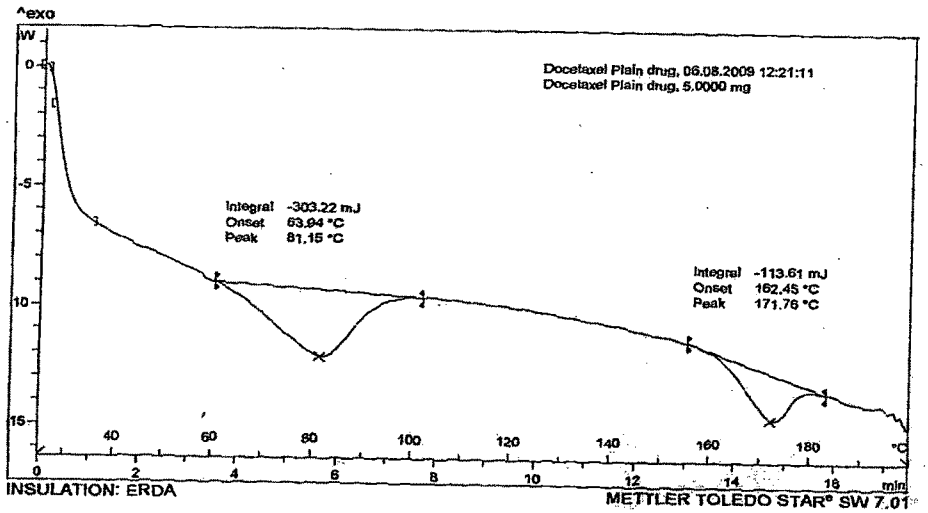

Fig 6.2 (a)-DSC Thermogram of Plain Docetaxel.

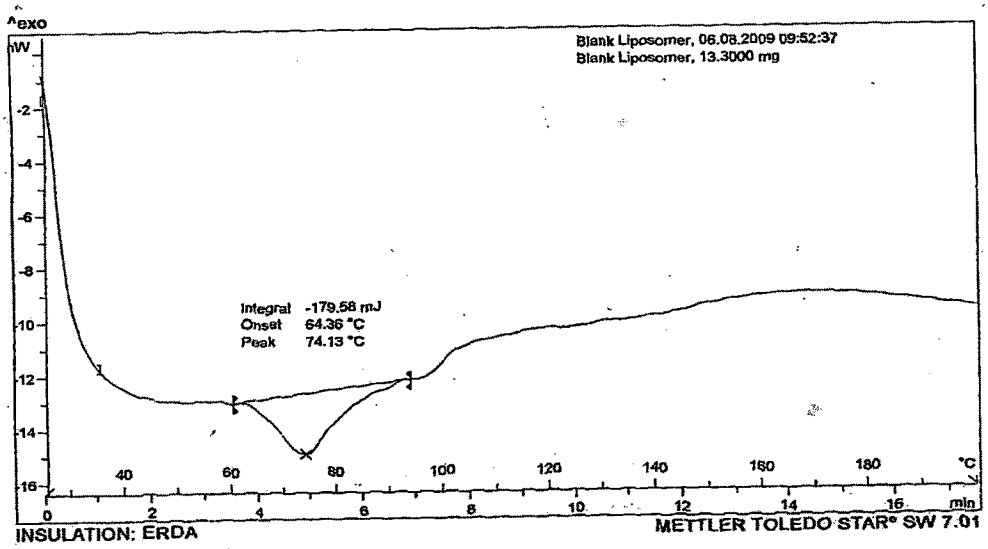

Fig. 6.2 (b)-DSC Thermogram of Blank liposomes (without drug)

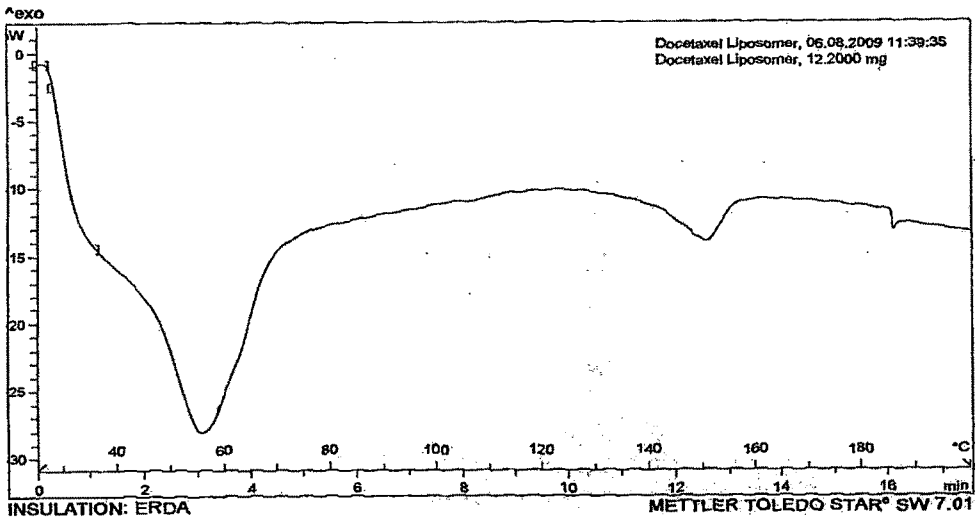

Fig. 6.2 (c)-DSC Thermogram of Docetaxel liposomes

6.5 In vitro Drug Release Studies for non grafted and HA grafted liposomes

In vitro drug release studies were conducted in Phosphate buffer saline (PBS) pH=7.4 to determine the drug release profile from the HA grafted and non grafted liposomes. Drug loaded liposomes (non grafted and grafted) were placed into the dialysis membrane and placed in a beaker containing 100 ml of PBS at 37± 1^0C with continuous magnetic stirring to mimick the sink conditions. Aliquots (1ml) were withdrawn at regular intervals and replaced with the same volume of fresh dissolution medium and the amount of Etoposide and Docetaxel released at given time interval was quantified spectrophotometrically and results for ETP formulations were recorded in Table 6.3 and Fig 6.3 while those for DOC formulations were recorded in Table 6.4, Fig. 6.4.

Table 6.3 *In vitro* drug release profile of Etoposide Drug Solution (ETPSOL),non grafted Etoposide liposomes (ETPLIP) and HA grafted Etoposide liposomes (HAETPLIP) in Phosphate Buffer Saline (pH=7.4) Results are expressed as mean ± S.D.(n=3)

Time in hours	% drug release*		
	Etoposide Plain Drug (ETPSOL)	Etoposide Liposomes (ETPLIP)	HA grafted Etoposide liposomes (HAETPLIP)
1	34.8 ±1.13	6.12±2.11	3.12±1.98
2	44.8±2.04	9.84±2.08	5.78±0.67
3	53.1±1.03	14.87±0.97	9.86±1.02
4	63.5±2.08	21.22±1.12	12.65±0.95
5	71.2±0.98	30.04±0.76	19.11±0.87
6	80.4±1.1	39.71±1.01	26.59±1.13
7	88.6±2.05	47.84±0.88	35.27±1.65
8	97.4±3.12	55.43±0.65	42.75±2.87
19		61.29±0.21	51.11±2.3
18		72.6±0.45	60.01±1.97
24		84.12±2.25	72.35±0.23
36		91.72±3.09	79.68±1.88
48		97.34±1.1	88.41±2.4

***-Mean ±S.D.(n=3)**

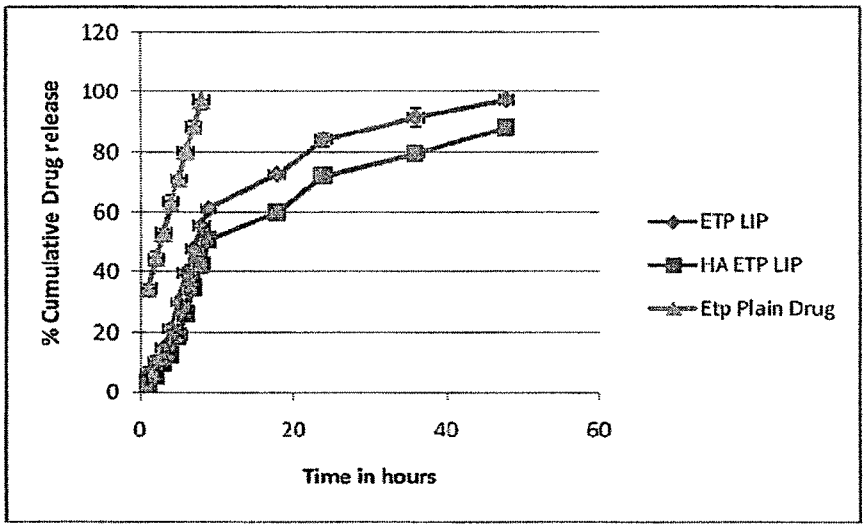

Fig 6.3 *In vitro* drug release profile of Etoposide Drug Solution (ETPSOL),non grafted Etoposide liposomes (ETPLIP) and HA grafted (HAETPLIP) in Phosphate Buffer Saline (pH=7.4) Results are expressed as mean ± S.D.(n=3)

Table 6.4 *In vitro* drug release profile of Docetaxel Drug Solution (DOCSOL),non grafted Docetaxel liposomes (DOCLIP) and HA grafted Docetaxel liposomes (HADOCLIP) in Phosphate Buffer Saline (pH=7.4) Results are expressed as mean ± S.D.(n=3)

Time in hours	% drug release*		
	Docetaxel Plain Drug (DOCSOL)	Docetaxel Liposomes (DOCLIP)	HA grafted Docetaxel liposomes (HADOCLIP)
1	32.3±0.18	4.13±2.11	2.13±1.12
2	42.6±2.21	10.78±2.08	6.44±1.98
3	54.9±1.09	14.4±1.35	9.86±2.16
4	64.4±0.98	22.68±1.72	13.57±2.06
5	72.2±1.65	32.55±1.03	19.98±1.89
6	81.3±0.87	41.23±1.01	26.57±1.78
7	89.4±1.31	50.56±1.86	31.78±2.66
8	98.1±1.12	60.78±1.44	39.3±1.84
9		68.12±2.03	44.82±1.76
18		74.22±1.54	52.89±2.09
24		81.52±1.19	59.46±1.56
36		88.61±1.66	64.56±1.97
48		92.57±1.71	70.23±1.68

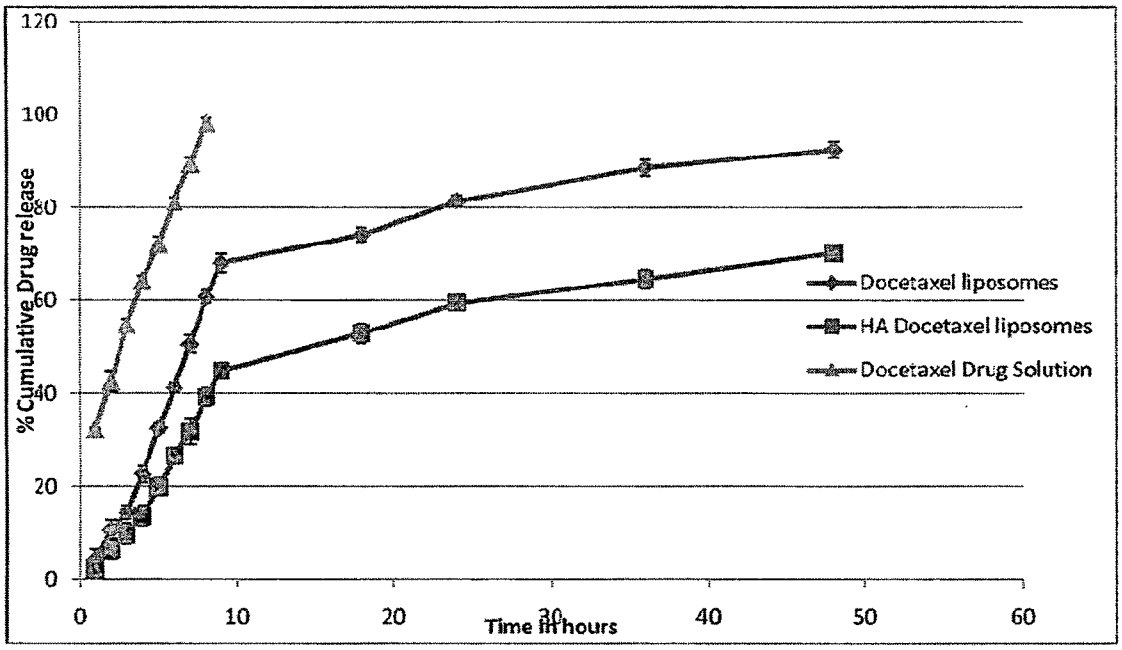

Fig 6.4 *In vitro* drug release profile of Docetaxel Drug Solution (DOCSOL),non grafted liposomes (DOCLIP) and HA grafted (HADOCLIP) in Phosphate Buffer Saline (pH=7.4) Results are expressed as mean ± S.D.(n=3)

6.6 DISCUSSION

The particle size of HAETPLIP and Etoposide loaded liposomes (non grafted ETPLIP) were found to be 217 ± 2.1 nm and 190 ± 3.7 nm respectively. A low polydispersity index of 0.187 and 0.109 was obtained for both the formulations indicating a narrow particle size distribution of the formed liposomes. The zeta potential of ETPLIP and HAETPLIP was observed to be -10.7 ± 0.57 mV and -20.2 ± 0.37 mV respectively. The results are shown in Table 6.1. The zeta potential was observed to decrease on grafting of HA to liposomes owing to negative charge imparted to liposomal surface by HA.

Percentage drug entrapment was found to be 80.2 ± 3.4 % for ETPLIP and 73.1 ± 4.08 % for HAETPLIP. The reduced entrapment observed in HA grafted Etoposide liposomes (HAETPLIP) may be attributed to leaching out of some drug during HA attachment on liposomal surface.

The prepared liposomes were falling in nanometric size range with HADOCLIP liposomes of size range 235 ± 1.8 nm and Docetaxel loaded liposomes (non grafted DOCLIP) were found to be 195 ± 3.0 nm. A low polydispersity index of 0.197 and 0.210 was obtained for both the formulations indicating a narrow particle size distribution of the formed liposomes. The zeta potential of DOCLIP and HADOCLIP was observed to be -8.8 ± 1.2 mV and -16.2 ± 2.9 mV respectively. The zeta potential was observed to decrease on grafting of HA to liposomes owing to negative charge imparted to liposomal surface by HA.

Percentage entrapment of Docetaxel was found to be 70.1 ± 2.8 % for DOCLIP and 64.2 ± 3.04 % for HADOCLIP. The reduced entrapment in HA grafted Docetaxel liposomes (HADOCLIP) may be attributed to leaching out of some drug during HA attachment on liposomal surface.

6-coumarin loaded liposomes were prepared to study the in vitro cell uptake of developed liposomal formulations by virtue of fluorescence produced by 6-coumarin using confocal microscopy. Hence, 6-coumarin liposomes were prepared by the identical technique as used for the preparation of drug loaded liposomes except the fact that in coumarin loaded liposomes, no

drug was loaded. It was hypothesized that 6-coumarin liposomes possessing the similar particle size and zeta potential as in case of drug loaded liposomes would exhibit similar behavior during cell uptake and can be used to visualize and characterize in vitro cell uptake.

6-coumarin loaded liposomes exhibited similar particle size and zeta potential as in case of ETPLIP and DOCLIP. The 6-coumarin liposomes optimized in terms of size and zeta potential were subjected to HA grafting (Ch 4) to prepared HA grafted coumarin loaded liposomes. The surface density of HA was found to be 500 µg similar to that found in case of HAETPLIP. Hence, HA grafted 6-coumarin liposomes were expected to exhibit same behavior as in case of HA grafted drug(s) loaded liposomes during cell uptake studies by confocal microscopy.

DSC studies were conducted to assess the physical state of drug in liposomes since the former plays a critical role in determining drug release profile *in vitro* as well as in intact biological systems. Drug entrapped in lipid matrix can exist in various physical states such as crystalline, amorphous and solid dispersion.

Fig. 6.1 shows the DSC thermograms of pure Etoposide, physical mixture of the HSPC/DPPE/cholesterol (Blank liposomes) and Etoposide-loaded liposomes. Pure Etoposide showed a sharp endothermic melting peak (Tm) at 45.18 °C [Fig. 6.1(a)]. Blank liposomes comprising of HSPC/DPPE/cholesterol showed distinct peak at 74.13 °C related to the melting peak of the lipids [Fig. 6.1(b)]. Etoposide melting peak was depleted in the calorimetric curve of Etoposide loaded liposomes [Fig. 6.1(c)] and also showed reduction in melting point of lipid mixtures from 74.13 °C to 65.63 ^{0} C indicating that drug may be present in the amorphous state in the liposomal samples. Depression of melting point in case of Etoposide loaded liposomes may be attributed to molecular dispersion formed by Etoposide in lipid matrix. Absence of characteristic drug peak suggested that the drug was molecularly dispersed in lipid matrix. The reduction in sharpness of endotherm is due to the presence of lipids in liposomes. It might be hypothesized that the liposome formation reduced the crystallization of Etoposide and resulted in molecular dispersion or solid state dispersion in lipid matrix.

As shown in [Fig 6.2 (a)], the drug Docetaxel exhibits quite sharp endotherms at 81.15^{0}C and 171.6^{0}C. [Fig. 6.2(b)] shows the endotherm at 75^{0}C which is mainly due to presence of lipid mixtures of HSPC, DPPE and Cholesterol. [Fig. 6.2(c)] shows broadened endotherm at 57 ^{0}C and similar broadened peak at 157^{0}C indicating entrapment of Docetaxel in lipid matrix of liposomes. Shifting of endotherm towards lower side indicated amorphous nature of liposomes. Broadened endotherms at 57^{0}C and 157^{0}C observed in thermograms of Docetaxel liposomes suggest that the drug has been molecularly dispersed in the lipoidal matrix and reduction in sharpness and height of endotherm indicates presence of lipids in liposomes with proper encapsulation of drug, thereby reducing sharpness of original drug peak and leading to shifting of endotherm. Depletion of Docetaxel endotherms [Fig 6.2(C)] is a testimony to the fact that Docetaxel was dispersed in amorphous form in the liposomal system. It might be hypothesized that formation of liposomes resulted in reduction in crystallinity of Docetaxel and one can conclude that Docetaxel was present in amorphous state of molecular dispersion. (Youseffi , 2009 and Dubernet, 1995)

The *in vitro* drug release profiles of optimized Etoposide Liposomes (ETPLIP) and HA grafted Etoposide Liposomes (HAETPLIP) formulations were studied using dialysis membrane. Percentage drug release versus time in Phosphate Buffer Saline –PBS (pH=7.4) is shown in Fig. 6.3. After 8 hours, the % drug release from non grafted and HA grafted liposomes (HAETPLIP) was found to be 55.43 $\pm$ 0.65% and 42.75 $\pm$ 2.87 %. After the initial burst release the release rate slowed down and the cumulative amount of drug released after 24 hours from non grafted and HA grafted liposomes (HAETPLIP) was found to be 84.12 $\pm$ 2.25 % and 72.35 $\pm$ 0.23 % respectively. Drug release from plain etoposide drug solution was found to be 100% in 8 hours.

The initial burst effect in case of liposomal formulations may be attributed to some amount of drug adsorbed on liposomal surface which might have been released at a faster rate. As compared to non grafted liposomes, the release rate was slower in HA grafted liposomes which may be due to augmented structural integrity of HA grafted liposomes. HA also acts as a hydrophilic carrier and provides an additional sustained release of drug entrapped inside the

liposomes. The results are in congruence with those obtained in case of ferritin coupled solid lipid nanoparticles. (Jain et al, 2008).

Similarly, *in vitro* drug release profiles of optimized Docetaxel Liposomes (DOCLIP) and HA grafted Docetaxel Liposomes (HADOCLIP) formulations were studied using dialysis membrane. Percentage drug release versus time in Phosphate Buffer Saline –PBS (pH=7.4) is shown in Fig. 6.4. In case of non grafted liposomes, percentage release at the end of 8 h was found to be 55.43 $\pm$ 0.65% and 84.12 $\pm$ 2.25 % at the end of 24 h. While, in case of HA grafted liposomal formulations (HADOCLIP) drug release was found to be 42.75 $\pm$ 2.87 % at the end of 8 h and 72.35 $\pm$ 0.23 % after 24 h. The initial fast release of drug may be attributed to burst effect as some amount of drug adsorbed on liposomal surface might have been released at a faster rate. However, the release rate got slowed down in HA grafted liposomes which may be attributed to augmented structural integrity of HA grafted liposomes. HA also acts as a hydrophilic carrier (similar to PEG) and provides an additional sustained release profile of drug entrapped inside the liposomes. The results are in congruence with those obtained in case of ferritin coupled solid lipid nanoparticles. (Jain et al, 2008).

Non grafted and HA grafted liposomes of Etoposide and Docetaxel characterized and optimized in terms of particle size, zeta potential, percentage drug entrapment, drug excipients interaction and in vitro drug release as well as in vitro biological studies where HAETPLIP and HADOCLIP exhibited superior performance in terms of cellular uptake, cytotoxicity and achievement and sustenance of intracellular drug levels and hence, HA grafted liposomal formulations of ETP and DOC were subsequently converted to Dry Powder Inhalers (DPIs) subsequently and were characterized for their stability aspects an in vitro lung deposition profile.

6.7 REFERENCES

- Betageri G.V., Jenkins S.A. and Parsons D.L., 1993. Liposome Drug Delivery Systems. PA, USA: Technomic Publishing company Inc; 16-17.
- Chien YW, 1992. In: Novel drug delivery system, Marcel Dekker Inc., New York and Basel.Fielding RM and Abra RM Pharm. Res, 9:220.
- Dubernet, C, 1995. Thermo analysis of microspheres. Thermochim.Acta 248, 259-269.
- Fry D.W., White C. and Goldman D.J., 1978. Rapid separation of low molecular weight solutes from liposomes without dilution. Anal. Biochem, 10: 809.
- Gemmell D.H. and Morrison J.C., 1957. The release of medicinal substances from topical applications and their passage through the skin. J Pharm Pharmacol; 9(10): 641-56.
- Higuchi, T., 1961. Rate of release of medicaments from ointment bases containing drugs in suspension. J. Pharm. Sci.; 50: 874-875.
- Jain A., Jain S., 2008. In vitro and cell uptake studies for targeting of ligand anchored nanoparticles for colon tumors. Eur.J.Pharmaceutics. 35: 404-416.
- New RRC. 1990. "Characterization of liposomes" in Liposomes: A Practical Approach, New RRC (ed.) Oxford University Press, Oxford; 105-161.
- New RRC., 1990. "Preparation of Liposomes" in Liposomes: A Practical Approach, New RRC (ed.) Oxford University Press, Oxford; 33-104.
- Reddy, L., H., Adhikari, J.,S, Dwarakanath, B.,S.,R., Sharma, R.,K., Murthy, R., S., R.,2006. Tumoricidal Effects of Etoposide incorporated into solid lipid nanoparticles After Intraperitoneal administration in Dalton's lymphoma bearing mice. *AAPS Journal.* 8(2):E254-E262.
- United States Pharmacopoeia, 2000. United States Pharmacopoeial Convention Inc., Rockville, USA; USP24: 2054.