Chapter – 5

Preparation and Optimization of ligand (Hyaluronic acid-HA) grafted liposomes

Targeting agent or a ligand can be grafted onto the liposomal surface to render them tumour specific. Amongst the availability of plethora of targeting strategies, one of the most commonly used approach is to attach the targeting agent to the distal end of a PEG molecule on the outside of the liposome. This has proved to increase the targeting ability compared to if the targeting agent is attached to the lipid head group. There have been several conjugation-chemical approaches to achieve the targetability in case of colloidal and vesicular drug delivery systems. Several studies, both *in vitro* and *in vivo*, have been performed using targeted liposomes. Among the most studied tumor targets with liposomes are the folate receptor, Human EGF receptor 2 (HER-2), CD-19 and the transferring receptor. A number of studies with folate-targeted liposomes loaded with anthracyclines have been performed showing that the folate receptor is suitable for liposome delivery with high specificity and internalization abilities. Several other therapies using liposomes targeting the folate receptor have been suggested, such as antisense delivery (Wang, 1995) and photodynamic therapy (Qualls, 2001). Liposomes targeting the folate receptor loaded with boronated compounds have also been studied with promising results (Pan, 2002a, Pan, 2002b and Sudimack, 2002.). The HER-2 has been studied as a target since early 1990's. Suzuki et al studied doxorubicin loaded liposomes with antibodies targeting either the p185 residue or the p125 residue, and it was shown that targeting p185 was superior.

Immunoliposomes loaded with doxorubicin have been shown to be better in animal studies than free doxorubicin, non-targeted liposomal doxorubicin. Lopez de Menezes et al, 1998 have targeted the tumor antigen CD-19 successfully on B-lymphoma cells both *in vitro* and *in vivo*. They have also performed *ex-vivo* experiments targeting CD-19 positive B-cells of multiple myeloma patient blood. The *in vivo* studies in mice showed that doxorubicin in immunoliposomes targeting CD-19 gave much better results than free doxorubicin or doxorubicin in non-targeted liposomes. Sarti, 1996 showed that transferrin liposomes interacted specifically with cultured cells and that they were internalized via receptor mediated endocytosis. Iinuma, 2002 developed cisplatin loaded transferrin liposomes that proved to increase the cisplatin levels of disseminated tumor cells in ascites significantly.

Immunogenic response elicited by antibodies and lability of proteins like transferrin are major constraints in popularizing these ligands for liposomal targeting. However, there also exists an alternative approach where aforementioned demerits can be easily overcome. This newer approach is based on the phenomenon of over expression of lectins (specific carbohydrate-binding proteins) by mammalian malignant cells as compared to normal ones (Gabius, 1988 and Monsigny, 1994). Studies have shown that in vitro targeting of cytotoxic liposomes loaded with melphalan lipid derivative by means of specific carbohydrate determinants (vectors) to HL-60 and human lung adenocarcinoma cells revealed higher cytotoxic activity than unvectored liposomes (Blume, 1993]. Earlier, Segawa, 1993 reported higher uptake of liposomes equipped with the lactose derivatized polysaccharide by the liver cancer cells *in vitro* than lactose-free liposomes. Lactose grafted Adriamycin loaded liposomes restrained tumour growth of AH66 hepatoma in nude mice more effectively than liposomes without lactose. (Ichinose, 1998)

Hyaluronic acid (Hyaluronan, HA) is a high molecular weight glycosaminoglycan and is a component of extracellular matrix essential for cell growth, structural organ stability and tissue organization. It is a polymer of repeating disaccharide units of D-glucoronic acid and N-acetyl-D-glucosamine.

HA has a binding affinity to specific cell surface receptors such as CD44, a receptor for HA-mediated motility (RHAMM) and lymphatic vessel endothelial hyaluronan receptor (LVYE-1). The principal receptor of HA is known as CD44 that can regulate cell proliferation and movement. After internalization into cells, HA is fragmented into endosomes and released into the cytoplasm. Several downstream pathways after HA activation are deregulated in cancer and some of the pathways lead to tumour growth, progression and metastasis. Fluctuations in CD44 and HA expressions have been widely observed in tumours from cancer patients specifically with upregulation of CD44 in certain cancers.(Narang, 2010)

Most malignant tumours exhibit high levels of HA (Lokeshwar, 2000) including lung carcinomas. (Penno, 1994). Though CD44 is expressed on various normal host cells, it has been found that these cell types are either not in contact with main bloodstream or require activation before binding to HA. Approaches to manipulate this CD44-HA interaction usually include of delivery of high molecular weight HA (Zeng, 1998), anti CD44 antibody (Zawadzki, 1998),

CD44 receptor globulin (Zawadzki, 1998, Sy et al., 1992) etc. Due to EPR phenomenon and highly leaky vasculature of tumours, ligand grafted liposomes can easily extravasate and can gain access to the innermost layers of tumour. (Netti et al, 1999). CD44 may be a potential targeting moiety for treatment of cancers that over express these receptors. High molecular weight HA-drug conjugates have been studied to achieve the same. (Luo, 1999) The concept is that an appropriately designed ligand with a modest affinity for the receptor, incorporated into liposomes at a suitable surface density, will interact with a greater avidity with cells that have a high number of CD44 receptors on their surface than with cells with a low number of receptors. The liposome provides an ideal surface to test such a hypothesis because the surface density of the ligand in the lipid bilayer can be controlled; furthermore, the ligands are mobile in a fluid bilayer and can rearrange to minimize steric constraints in the interaction with multiple receptors in an adjacent surface. (Abney, 1987)

In the current investigation we have attempted to target the ligand (HA) grafted liposomes to facilitate site specific delivery of cytotoxic agents (Etoposide and Docetaxel) within tumour cells. Grafting of HA as a ligand to liposomal surface is believed to augment the affinity of grafted liposomes for lung cancer cells that over express CD44 receptors, thus directing the liposome encapsulated cytotoxic drug straightway to lung cancer cells.

It has been hypothesized that HA can be grafted to liposomal surface by formation of covalent linkage between carboxylic acid groups present on HA and amine group of phosphatidylethanolamine present in lipid component of liposomes via reductive amination in presence of a suitable coupling agent such as sodium cyanoborhydride or coupling facilitated by EDC. (Szoka, 2001) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) induced coupling method was adopted in this investigation to graft HA to liposomal surface as per procedure reported in literature. (Margalit, 2004) Surface modification of liposomes by carbodiimide chemistry yields stable amide bonds. As a prerequisite, the polymer should be having free carboxyl groups at the surface that can be activated by 2-(diethyl amino methyl carbodiimide)-EDC. (Margalit et al, 2004). This method avoids any harsh treatment that may be detrimental to drug or lipid constituents and also avoids use of any reagent that may lead to formation of toxic intermediates.

Prepared HA grafted liposomes were subjected to characterization in terms of vesicle size, zeta potential, percentage drug entrapment. As reported earlier optimization of coupling efficiency and incubation time were carried out to achieve modest surface density of HA on liposomal surface. This was achieved by correlating changes in zeta potential with HA concentration. (Jain et al, 2008)

5.1 MATERIALS AND METHODS

Materials	Source
Hyaluronic acid (1.5 Mda)	Sigma Aldrich Corporation, Mumbai
EDC	HiMedia, Mumbai, India
Potassium dihydrogen phosphate, Disodium hydrogen phosphate, borax, boric acid, sodium tetraborate, sulphuric acid	S.D.Fine Chemicals Ltd., Mumbai, India.
Dialysis membrane (Molecular weight cut off: 12000 Dalton; thickness: 200µm and porosity 0.45 µm)	Sigma Aldrich Corporation(India) Ltd., Mumbai, India,
Equipments	**Source**
Calibrated pipettes of 1.o ml, 5.0 ml and 10.0 ml, volumetric flasks of 10 ml, 25 ml, 50 ml and 100 ml capacity, beakers (250 ml) and other necessary glassware	Schott and Corning Ltd., Mumbai.
Analytical Balance	Precisa 205A SCS, Switzerland
pH meter	Systronics 335, India
Rotary Flash Evaporator	Superfit Equipments,

	Mumbai,India.
Vortex Mixer	Sphinx, Mumbai
Magnetic stirrers and heating mantle	Remi, Mumbai
Cyclomixer, three blade stirrer	Remi, Mumbai
Sigma cooling centrifuge	3k 30, Sigma Laboratory Centrifuge, Osterode, GmbH
Lyophilizer, DW1, 0-60E	Heto Drywinner, Denmark
UV Visible Spectrophotometer	Shimadzu Uv-1601, Japan.

5.1.1 Preparation and Optimization of HA grafted Etoposide liposomes (HAETPLIP) and HA grafted Docetaxel liposomes (HADOCLIP)

Covalent attachment of carboxyl group of HA with free amino group of Dipalmitoyl Phosphatidyl ethanolamine (DPPE) present in liposomes using EDC as a coupling agent was performed according to earlier procedures with suitable modifications. (Margalit, 2000). Briefly, HA was dissolved in deionized water with constant stirring followed by addition of EDC for pre-activation at pH=4 at 37^0C for 2 h by controlled titration with HCl. This activated HA was then added separately to ETP and DOC loaded liposomes buffered at pH=8.6 using borate buffer and incubated for 24 hours at 37^0C. Non grafted HA was removed using mini column centrifugation (Sephadex G-75 packed column) followed by washing thrice with distilled water. The liposomes were collected after ultracentrifugation using Sigma Cooling centrifuge, Osterode, Germany) at 30,000 rpm for 30 minutes. The resultant pellet was suspended in distilled water, lyophilized (HetoDryWinner, Denmark) and preserved 4^0C until for further use.

Change in zeta potential was used as a criterion to assess the amount of HA grafted to liposomal surface. Two process variables viz. total liposomal formulation to HA weight ratio for ETP and

DOC liposomes [Fig. 5.1 (a) and Fig. 5.1 (b)] respectively and incubation time for optimum HA grafting to liposomal surface of ETP and DOC liposomes [shown in Fig. 5.2 (a) and Fig. 5.2 (b)] and were by correlating them with change in zeta potential of the liposomal dispersion (Jain et al, 2008, 40). Particle size, zeta potential were measured using Zetasizer 3000 HS and percentage drug entrapment was computed for newly formed HA grafted ETP and DOC liposomes (HAETPLIP and HADOCLIP) respectively.

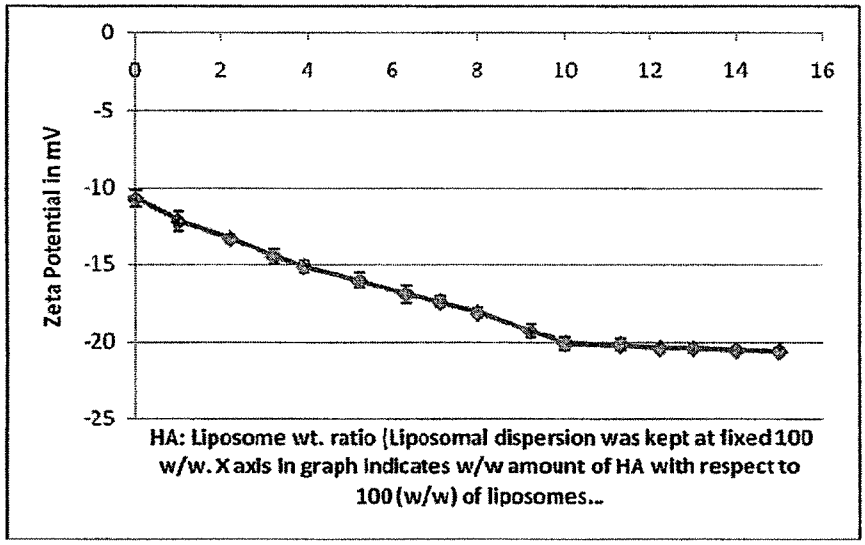

Fig 5.1 (a)-Preparation of formulations containing different weight ratios of ETP liposomes and HA followed by optimization of total HA:liposome weight ratio by correlating HA:liposome ratio with change in zeta potential at 25 C.(X axis indicates weight ratio of HA considering liposomal weight fixed at 100 w/w). All results are expressed as mean± S.D. (n=3)

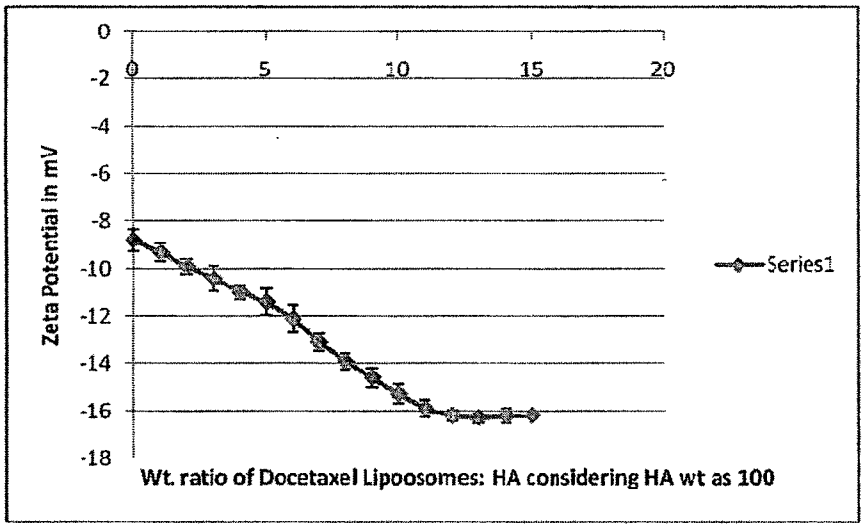

Fig 5.1(b)-Optimization of HA: Total DOC liposomes weight ratio by zeta potential determination method (Values expressed as mean ± S.D., n=3)

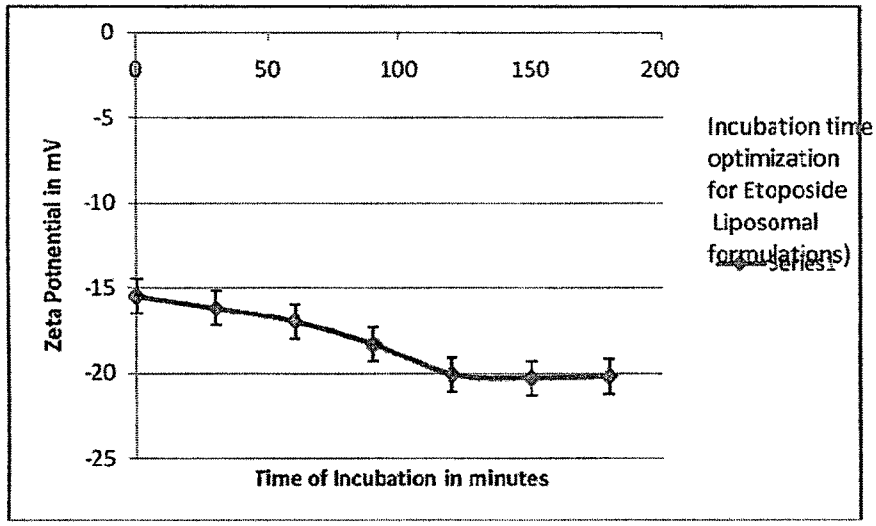

Fig 5.2 (a) - Incubation time optimization for HA: ETP liposomes (weight ratio 10:100) grafting at 25 C. (Values expressed as mean ± S.D., n=3)

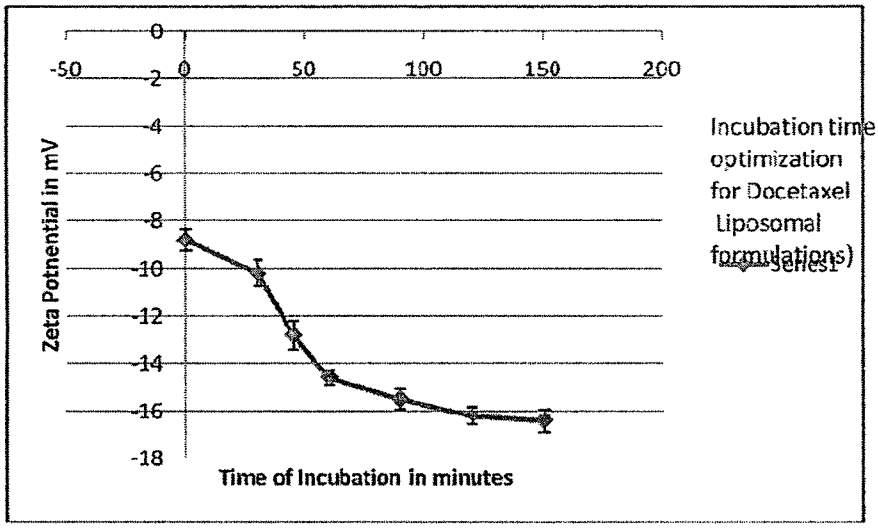

Fig 5.2 (b) - Formulations containing different ratios of DOC liposomes and HA at incubation time optimization for HA grafting at 25 C. (Values expressed as mean ± S.D.)

5.1.2 Estimation and optimization of concentration of coupling agent (EDC)

EDC is extensively used as a coupling agent to facilitate grafting of HA to surface of drug loaded liposomes and is an efficient coupling agent ensuring higher coupling efficiency of ligand and is relatively free from formation of toxic intermediates unlike other coupling agents. Hence, EDC was preferred as a coupling agent to facilitate HA grafting on liposomal surface of ETP and DOC.

It is essential to find out the optimum concentration of EDC necessary to facilitate successful HA grafting on drug loaded liposomal surface. Liposomal dispersion equivalent to 5 mg was taken and HA coupling was carried out (as described in above section 5.1.1) in presence of varied concentrations of EDC. EDC concentration was optimized in terms of HA density obtained on liposomal surface, particle size and zeta potential of HAETPLIP (Table 5.1, [Fig. 5.3 (a), 5.3 (b)] and HADOCLIP [Table 5.2, Fig 5.4 (a) and (b)]

Table 5.1: Summary report of effect of concentration of coupling agent (EDC) on HA grafting efficiency, particle size and zeta potential for HA grafted Etoposide loaded liposomes. (HAETPLIP)

Sr. No.	EDC concentration in µg*	Surface density of HA (µg/5 mg of liposomal dispersion)*	Zeta potential in mV*	Particle size in nm*
1	1	25 ± 5.11	-12.2 ± 0.62	101 ± 5
2	2	40 ± 7.4	-13.3 ± 0.24	132 ± 13
3	3	78.5 ± 9.6	-14.5 ± 0.51	147 ± 18
4	4	112.9 ± 13.2	-15.1 ± 0.37	166 ± 6.0
5	5	145.8 ± 8.7	-16.0 ± 0.48	188 ± 22
6	6	197.6 ± 11.5	-16.9 ± 0.59	196 ± 10
7	7	263.4 ± 24.6	-17.4 ± 0.34	203.5 ± 13
8	8	322.7 ± 12.1	-18.1 ± 0.32	211 ± 12
9	9	401.2 ± 10.9	-19.3 ± 0.45	215 ± 21
10	**10**	**500** ± 6.62	**-20.1 ± 0.41**	**217** ± 5
11	11	497.1 ± 27.7	-20.2 ± 0.37	370 ± 19

***-Mean ± S.D. (n=3). All results are expressed as mean± S.D. (n=3)**

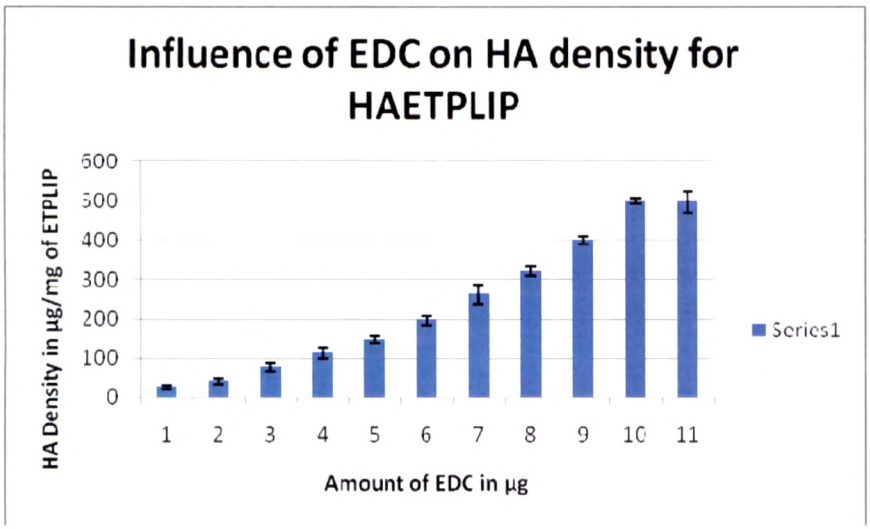

Fig 5.3 (a)- Plot of HA density Vs. Amount of EDC depicting the influence of EDC amount on Etoposide liposomal surface density of HA. All results are expressed as mean± S.D. (n=3)

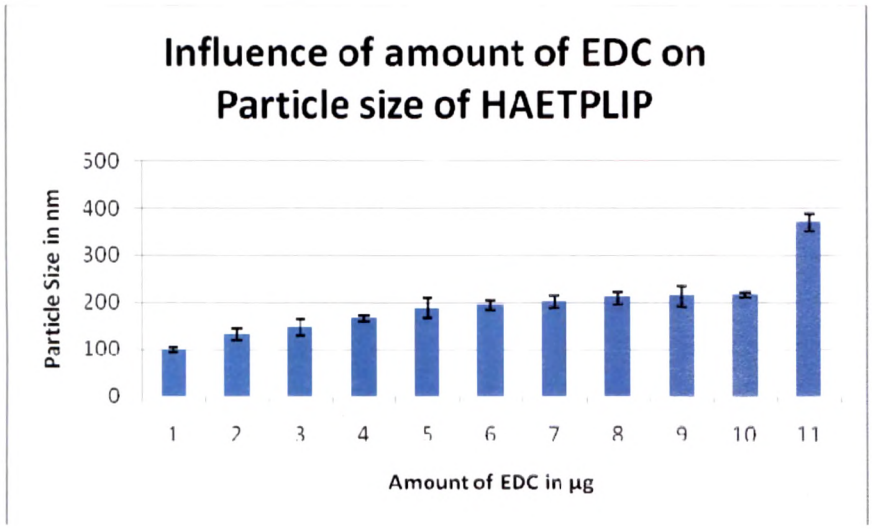

Fig. 5.3 (b) - Plot of Particle size of HAETPLIP Vs. Amount of EDC depicting influence of amount of EDC added on particle size of HA grafted Etoposide liposomes. All results are expressed as mean± S.D. (n=3)

Table 5.2: Summary report of effect of concentration of coupling agent (EDC) on HA grafting efficiency, particle size and zeta potential for HA grafted Docetaxel loaded liposomes. (HADOCLIP)

Sr. No.	EDC concentration in µg*	Surface density of HA (µg/5 mg of liposomal dispersion)*	Zeta potential in mV*	Particle size in nm*
1	1	20.34 ± 2.98	-9.3 ± 0.37	118 ± 9
2	2	43.89 ± 3.75	-9.9 ± 0.32	134 ± 10
3	3	61.23 ± 5.12	-10.4 ± 0.54	162 ± 12
4	4	100.3 ±10.1	-11.0 ± 0.28	189 ± 4
5	5	143.7 ± 12.6	-11.4 ± 0.56	207 ± 15
6	6	201.1 ± 25.8	-12.1 ± 0.59	219 ± 20
7	7	258.54 ± 23.9	-13.1 ± 0.35	224 ± 18
8	8	301.9 ± 12.9	-13.9 ± 0.32	227 ± 12
9	9	355.1 ± 10.0	-14.6 ± 0.41	228 ± 14
10	10	418.2 ± 34.8	-15.3 ± 0.41	231 ± 20
11	11	502.7 ± 41.9	-15.9 ± 0.37	235 ± 10
12	12	598.3 ± 9.6	-16.2 ± 0.23	235 ± 8
13	13	598 ± 31.3	-16.3 ± 0.19	326 ± 16
14	14	597.8 ± 40.9	-16.2 ± 0.32	404 ± 23

***-Mean ± S.D. (n=3)**

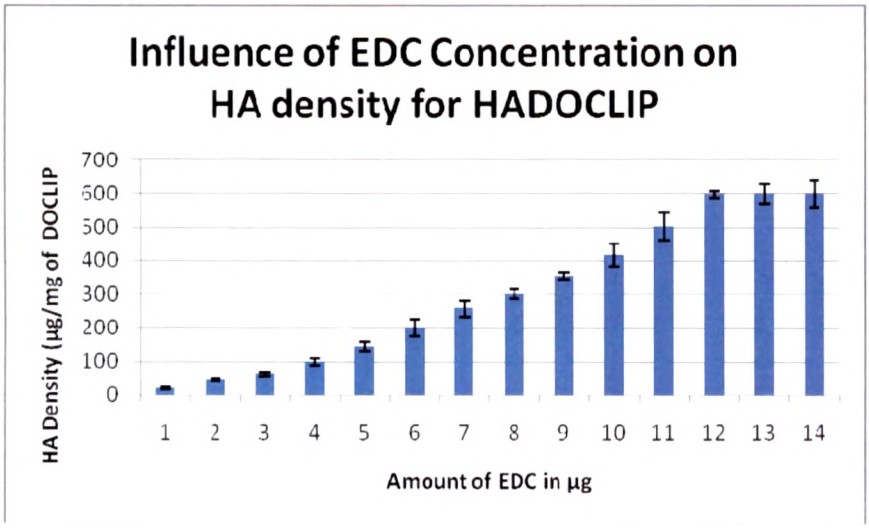

Fig. 5.4 (a)-Plot of HA density Vs. Amount of EDC depicting the influence of EDC amount on Docetaxel liposomal surface density of HA.

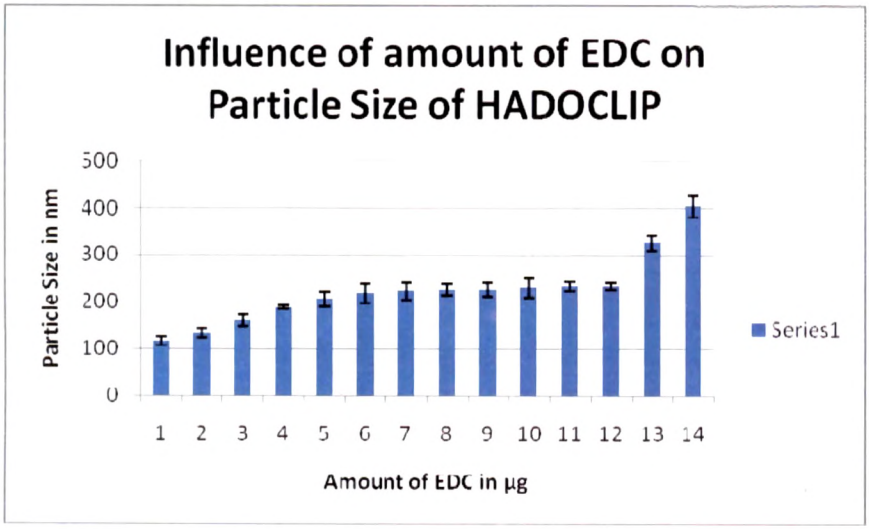

Fig. 5.4 (b) - Plot of Particle size of HADOCLIP Vs. Amount of EDC depicting influence of amount of EDC added on particle size of HA grafted Docetaxel liposomes. All results are expressed as mean± S.D. (n=3)

HA grafted liposomes of ETP and DOC were characterized for their particle size, zeta potential and percentage drug entrapment. The comparative overview of optimized batches of non grafted ETP liposomes (ETPLIP), DOC liposomes (DOCLIP), HA grafted liposomes of ETP (HAETPLIP) and DOC (HADOCLIP) is given in Table 5.3.

Table 5.3: Summary report of characterization of Non grafted and HA grafted liposomal Etoposide and Docetaxel.

Formulation Code	Particle Size (nm) *	Zeta Potential (mV)*	Percentage Entrapment *
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)

5.1.3 Spectrophotometric determination of HA

This method was based on modified Uronic acid Carbazole reaction of Bitter and Muir (Bitter, 1962) based on DISCHE's carbazole reaction.(Dische, 1947) Serial dilutions of HA ranging from standard concentrations of 0.5, 1,10, 20, 30, 40 and 50 µg/ml were prepared in double distilled water.

First, 3 ml of 0.025 M sodium tetraborate in sulphuric acid was added to tubes and cooled at 4 C. 0.25 ml of different standard concentrations were added slowly to tubes followed by initial vigorous agitation of the tubes to ensure complete reaction followed by constant cooling. Later on, the tubes were heated for 10 minutes in water bath and cooled to ambient temperature followed by addition of 0.1 ml of 0.125% carbazole in ethanol, tubes were agitated moderately and heated for 20 minutes in a heated water bath followed by cooling at ambient room temperature and absorbance was measured spectrophotometrically at 530 nm using UV-Visible Spectrophotometer-UV1601 (Shimadzu, Japan) using Double Distilled water as a blank.

5.1.4 Fourier Transform Infra red Spectroscopy

Liposomal samples of ETPLIP, HAETPLIP, DOCLIP and HADOCLIP were mixed with potassium bromide and compressed on a hydraulic press to make pellets. Spectra were obtained by scanning the samples between 400 and 4000 cm^{-1} on a FTIR (Perkin Elmer). Spectra were recorded for ETPLIP, HAETPLIP, DOCLIP and HADOCLIP and are shown in [Fig. 5.5(a)], [Fig. 5.5(b)], [Fig. 5.6(a)] and [Fig. 5.6(b)] respectively.

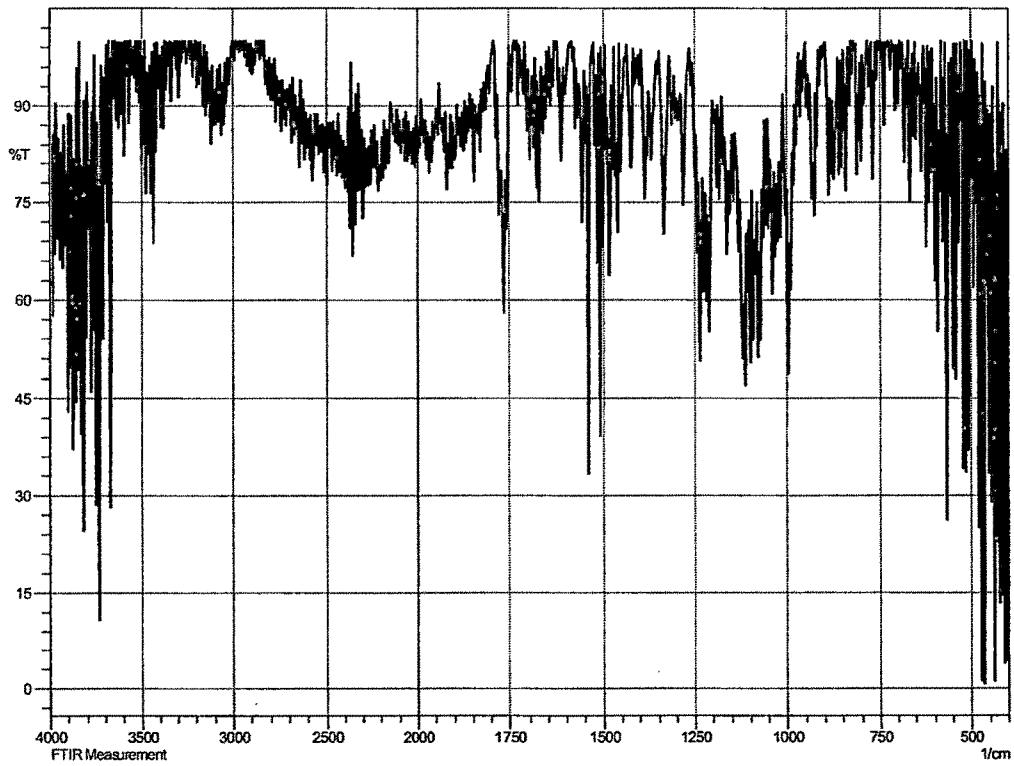

Fig 5.5(a)-FTIR Spectrum of Etoposide Liposomes (ETPLIP)

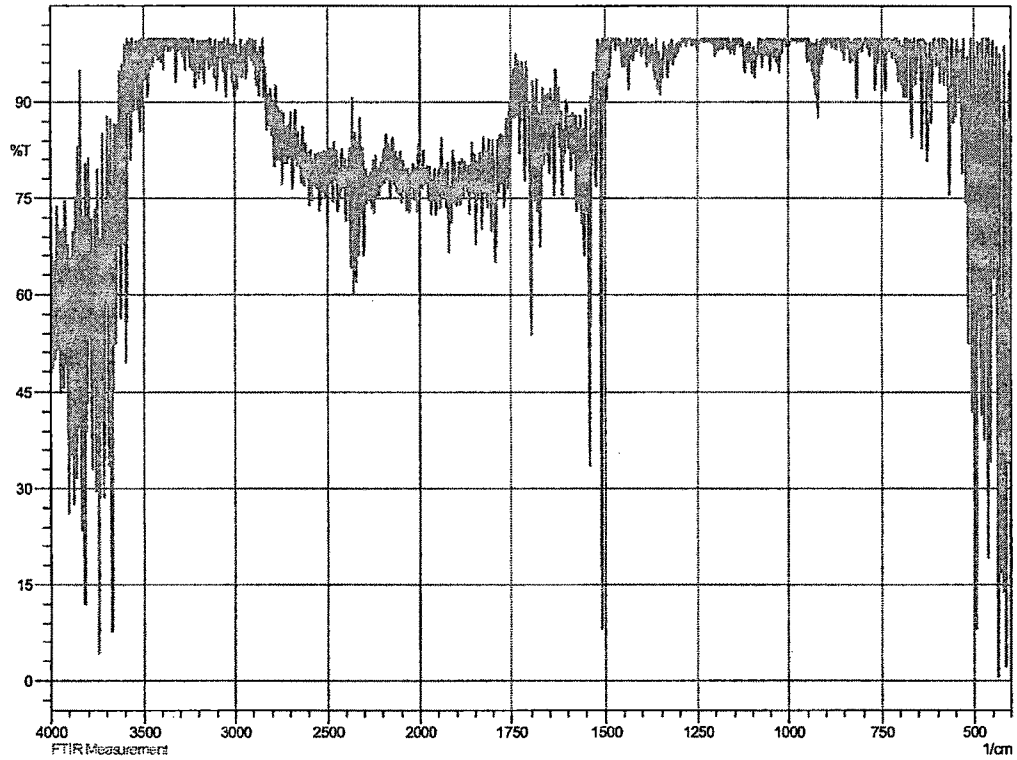

Fig. 5.5(b)-FTIR Spectrum of HA grafted Etoposide liposomes (HAETPLIP)

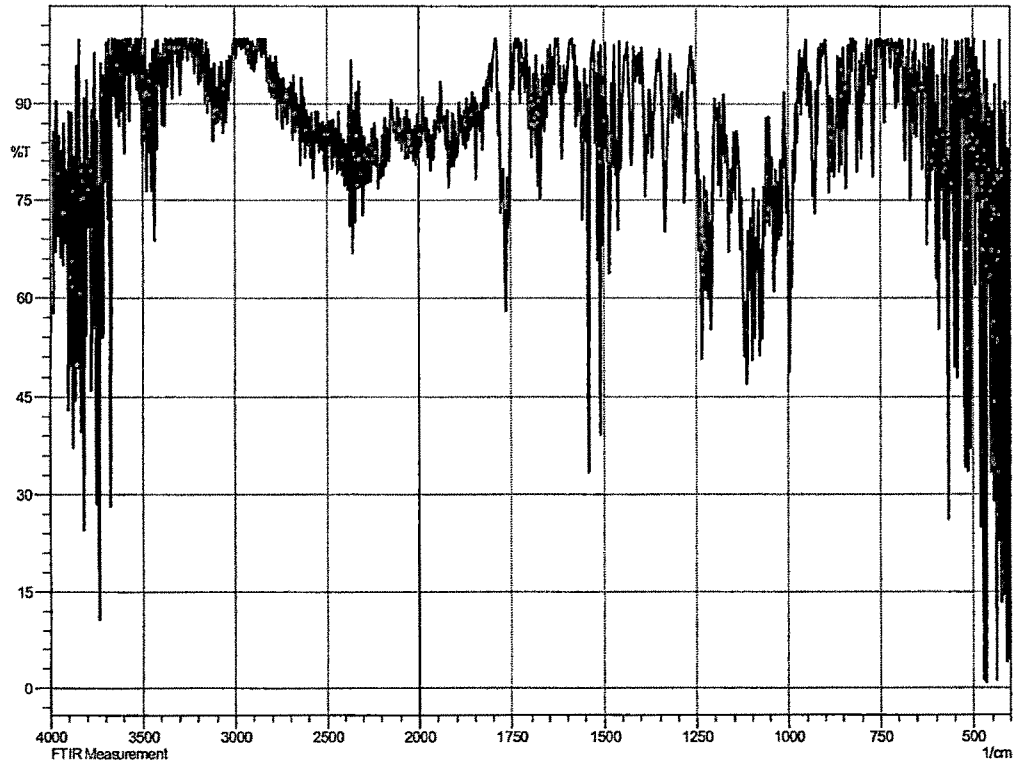

Fig 5.5 (a) - FTIR Spectrum of Etoposide Liposomes (ETPLIP)

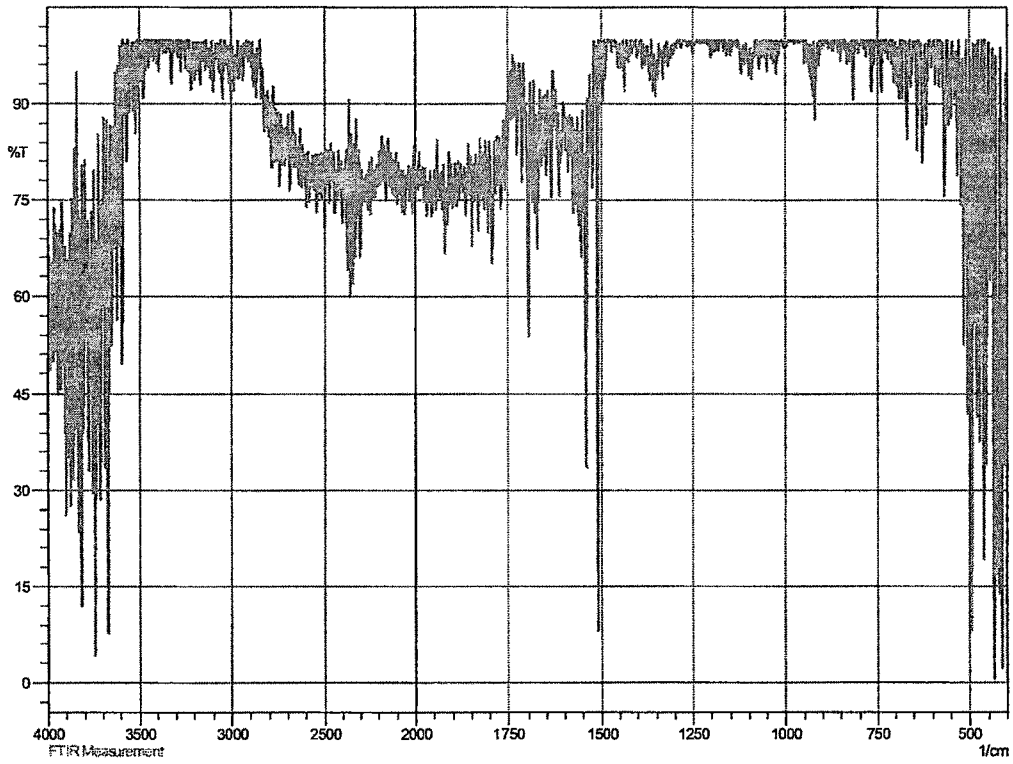

Fig. 5.5(b)-FTIR Spectrum of HA grafted Etoposide liposomes (HAETPLIP).

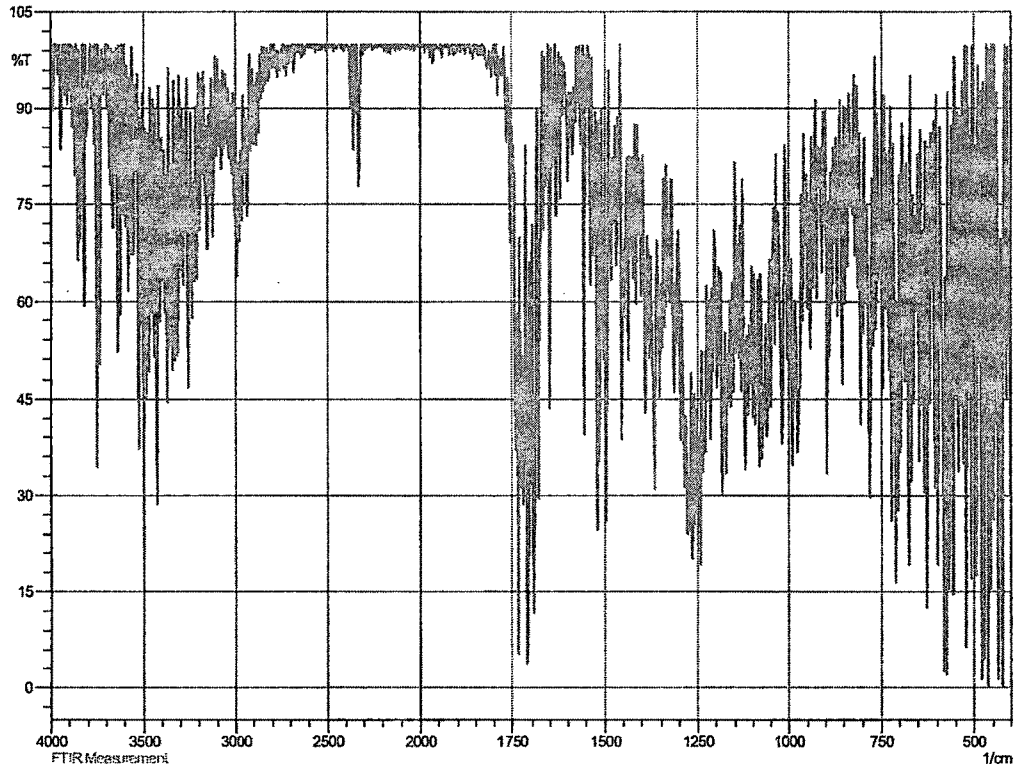

Fig 5.6 (a)-FTIR Spectrum of Docetaxel Liposomes (DOCLIP)

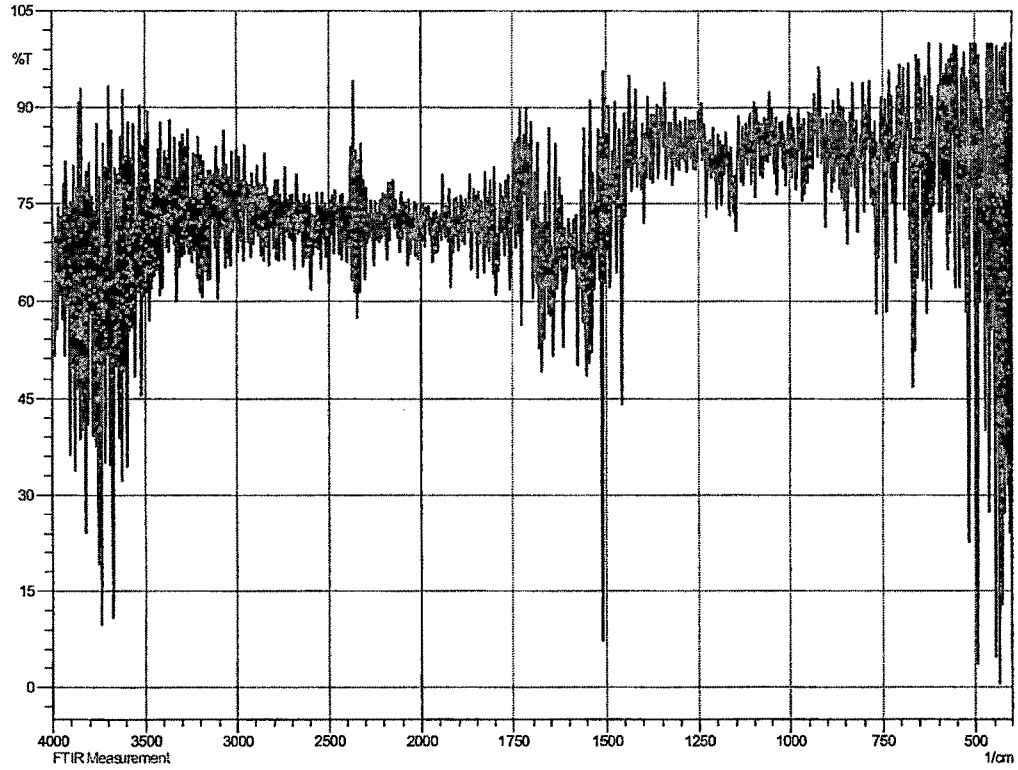

Fig 5.6(b)-FTIR Spectrum of HA grafted Docetaxel Liposomes (HADOCLIP)

5.1.5 Preparation and Optimization of HA grafted coumarin loaded liposomes

HA was dissolved in deionized water with constant stirring followed by addition of EDC for pre-activation at pH=4 at 37 C for 2 hours by controlled titration with HCl. This activated HA was then added to coumarin loaded liposomes buffered at pH=8.6 and incubated for 24 hours at 37 C. Non grafted HA was removed using mini column centrifugation (Sephadex G-75 packed column) followed by washing thrice with distilled water. The liposomes were collected after ultracentifugation using Sigma Cooling centrifuge, Osterode, Germany) at 30,000 rpm for 30 minutes. The resultant pellet was suspended in distilled water, lyophilized at 4^0C (HetoDryWinner, Denmark) and preserved until for further use.

Change in zeta potential was used as an indicator to determine the amount of HA grafted to liposomal surface. Two process variables viz. incubation time for optimum HA grafting to

liposomal surface and total liposomal formulation to HA weight ratio were by correlating them with change in zeta potential of the liposomal dispersion (Zetasizer 3000 HS, Malvern Instruments Co. U.K.) (Jain et al, 2008). Particle size and zeta potential were measured using Zetasizer 3000 HS, (Malvern Instrument, U.K.).

5.2 DISCUSSION

Etoposide (ETP) and Docetaxel (DOC) loaded liposomes were prepared by Thin Film Hydration (TFH) technique and surface modification using HA was done by carbodiimide coupling technique. HA grafted liposomes of Etoposide (HAETPLIP) and Docetaxel (HADOCLIP) were prepared by incubating the drug loaded optimized liposomes with Hyaluronic acid (HA) in the presence of coupling agent –EDC. HA gets grafted to liposomal surface by virtue of reaction between carboxylic acid (-COOH) of HA and amine ($-NH_2$) functional group of DPPE in liposomes, in presence of coupling agent like EDC, resulting in formation of an amide linkage.

The grafting efficiency of HA was determined as a function of two major formulation variables: incubation time for HA:liposome grafting and HA: total liposome weight ratio. The two major formulation variables (incubation time and total liposomal formulation to HA weight ratio) were optimized by measuring change in zeta potential of the dispersion (Jain et al, 2008)

The rationale underlying correlating zeta potential change with HA grafting is based on the fact that increase in surface density of HA on liposomal surface would be accompanied with corresponding reduction in zeta potential owing to negative charge imparted by HA to liposomal surface. This reduction in zeta potential was utilized to optimize the incubation period and total HA uptake. The zeta potential of HA grafted liposomes would become constant at a concentration of HA when all HA binding sites of liposomes would be saturated with HA and further HA uptake would not be possible. For optimization of HA to liposomal ratio, formulations with different HA:total liposome weight ratios were prepared and incubated for 24 h at 25^0C. The HA to liposomal ratio at which there was no significant change in zeta potential was identified as optimum weight ratio. [Fig 5.1(a) and 5.1(b)]

The surface density of HA grafted on liposomal surface can be determined by modified uronic acid carbazole reaction of Bitter and Muir based on DISCHE's carbazole reaction (Dische, 1947). Briefly, treatment of HA based carriers with 0.025 M sodium tetraborate in sulphuric acid and 0.125 % carbazole in absolute ethanol, resulted in disintegration of HA into glucoronic acid. This glucoronic acid yielded stable violet color with carbazole which can be quantified at 530 nm. It was observed that at HA:liposome weight ratios $\geq$ 10:100 there was no significant reduction in zeta potential and hence, 10:100 was considered to be optimum HA:liposome ratio. This may be due to the saturation of all HA binding sites on liposomes above 10:100 HA:liposome weight ratios for ETP liposomes and 12:100 HA:liposome weight ratio for DOC liposomes.

In order to optimize the second major formulation variable- incubation time for successful grafting of HA to liposomal surface, liposomal formulations with optimum HA: total liposome weight ratio (determined and optimized in previous step, shown in [Fig 5.2(a), 5.2(b)] were prepared and incubated for different time intervals followed by recording their zeta potential. The time period of HA incubation with liposomes, at which no further significant reduction in zeta potential was considered as the optimum incubation period which was found to be of 120 minutes. [Fig 5.2(a) and Fig.5.2 (b)]

EDC was added in varying amounts in range of 1 to 14 µg. Influence of EDC on HAETPLIP and HADOCLIP in terms of change in size, zeta potential and surface density of HA was studied and reported in Table 5.1, Fig. 5.3(a), Fig. 5.3(b), Table 5.2, Fig. 5.4 (a) and Fig. 5.4 (b) respectively. Optimization of EDC amount required for successful HA grafting was also done by correlating EDC concentration with changes in particle size, zeta potential and surface density since these are the major variables that determine the efficiency of HA grafting process.

As indicated in Table 5.1 and Fig 5.3 (a) and 5.3 (b), varying amounts of EDC as a coupling agent were used for successful grafting of HA to ETPLIP and DOCLIP. Successful HA grafting results in increased surface density of HA on ETP and DOC loaded liposomes. HA grafting results in reduction in zeta potential owing to negative charge imparted by HA to drug loaded liposomes. As indicated in Table 5.1, Fig 5.3(a) and 5.3(b), progressive reduction in zeta

potential with corresponding increase in HA surface density on ETPLIP can be used as an indicator to assess HA grafting to ETPLIP. However, in case of ETP liposomes at concentrations of EDC beyond 10 μg, there was no further reduction in zeta potential indicating that all HA binding sites on Etoposide loaded liposomal surface were saturated and there was no further grafting of HA to ETPLIP and hence, zeta potential nearly became constant. Similarly, progressive reduction in zeta potential with corresponding increase in HA surface density on DOCLIP can be used as an indicator to assess HA grafting to DOCLIP. However, at concentrations of EDC beyond 12 μg (Table 5.2, Fig. 5.4(a) and Fig. 5.4(b), there was no further reduction in zeta potential indicating that all HA binding sites on DOCLIP surface were saturated and there was no further grafting of HA to DOCLIP and hence, zeta potential nearly became constant. Increase in particle size at concentration of EDC beyond 10 μg in case of HAETPLIP and 12 μg in case of HADOCLIP may be attributed to aggregation of HA.

The particle size, zeta potential and percentage drug entrapment of ETPLIP, HAETPLIP, DOCLIP and HADOCLIP were recorded in Table 5.3. 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. Reduction in zeta potential of HA grafted drug loaded liposomes may be attributed to negative charge imparted to liposomal surface by HA. Zeta potential of ETPLIP and HAETPLIP was observed to be -10.7 $\pm$ 0.57 mV and -20.2 $\pm$ 0.37 mV whereas the same for DOCLIP and HADOCLIP was observed to be -8.8 ± 1.2 mV and -16.2 ± 2.9 mV respectively. Percentage drug entrapment was found to be 80.2 $\pm$ 3.4 % for ETPLIP, 73.1 $\pm$ 4.08 % for HAETPLIP, 70.1 $\pm$ 2.8 % for DOCLIP and 64.2 $\pm$ 3.04 % for HADOCLIP.

The reduced percentage drug entrapment observed in HAETPLIP and HADOCLIP may be attributed to leaching out of some drug during HA attachment on liposomal surface.

The reason for subjecting the drug delivery system to FTIR was to confirm the grafting of HA to Etoposide Liposomes (ETPLIP) and Docetaxel liposomes (DOCLIP). FTIR spectrum of Etoposide liposmes (ETPLIP) is shown in Fig 5.5(a). Sharp peak at 1540 cm^{-1} and 1760 cm^{-1} reveals the carbonyl stretch of triglycerides or carboxylic esters present in lipoidal excipients of

lipids. A band at 3420 cm^{-1} can be attributed to $-NH_2$ and –OH group stretching vibration of phosphatidylethanolamine component lipid or phosphatidylcholine component of liposomes (ETPLIP).

On comparing the IR spectrum of ETPLIP [Fig. 5.5(a)] and HAETPLIP [Fig 5.5(b)] formed following grafting of ETPLIP by HA, it can be observed that spectrum of HAETPLIP shows shifting of carbonyl peak (1760 cm^{-1}) to 1690 cm^{-1} indicating formation of amide band. Primary amides display one or more than one bands in region of 1510 cm^{-1} to 1650 cm^{-1} caused by primary amide or secondary amide II bending. Similarly, low concentrations of amides exhibit band at 1690 cm^{-1}. These bands in region of 1510 to 1700 cm^{-1} reveal the formation of amide bond confirming the grafting reaction.

From the IR data it is clear that HA grafted Etoposide liposomes (HAETPLIP) had characteristic peaks of amide bond which could be a good testimonial for successful grafting reaction between HA and lipid excipients like phosphatidylethanolamine.

FTIR spectrum of Docetaxel liposomes (DOCLIP) is shown in [Fig 5.6(a)]. Sharp peaks at 1740 cm^{-1} and 1710 cm^{-1} reveal the carbonyl stretch of triglycerides or carboxylic esters present in lipoidal excipients of lipids. A band at 3450 cm^{-1} can be attributed to $-NH_2$ and –OH group stretching vibration of phosphatidylethanolamine component lipid or phosphatidylcholine component of liposomes (DOCLIP).

On comparing the IR spectra of DOCLIP [Fig 5.6 (a)] and HADOCLIP [Fig 5.6(b)] formed following grafting of DOCLIP by HA, it can be observed that spectrum of HAETPLIP shows shifting of carbonyl peaks (1740 cm^{-1}) to moderately sharp intense peak at 1660 cm^{-1} indicating formation of amide band. Primary amides display one or more than one bands in region of 1510 cm^{-1} to 1650 cm^{-1} caused by primary amide or secondary amide II bending. In case of HADOCLIP, a sharp intense peak at 1510 cm^{-1} and those of milder intensity are observed in region of 1550 to 1650 cm^{-1} reveal formation of primary amide bonds formed following grafting reaction. The amide bands observed in HADOCLIP spectra are suggestive of successful HA grafting to Docetaxel liposomes containing DPPE.

Hence, FTIR analysis could serve as a valuable tool to confirm grafting reaction apart from other tests performed for determining efficiency of grafting reaction such as determination of surface density determination for ligands by modified uronic acid detection and other photometric methods.

5.3 CONCLUSION

Hyaluronic acid (HA) was selected as a ligand to target CD44 receptors over expressed in lung cancers and hence, targeted drug loaded liposomes were prepared by grafting of HA to surface of Etoposide and Docetaxel loaded liposomes. HA was grafted to drug(s) loaded liposomes in different HA:drug loaded liposome weight ratios by carbodiimide coupling technique using EDC as a coupling agent in different amounts for different incubation periods. Carboxylic acid functional groups in HA were covalently bonded to free amine group of phosphatidylethanolamine of lipid component of liposomes resulting in formation of a stable amide bond. Success of HA grafting to liposomal surface was assessed and confirmed by correlating surface density of HA to zeta potential. HA grafting to drug loaded liposomal surface was optimized for different weight ratios of HA:liposomes and incubation time to determine the optimum surface density of HA to render drug(s) loaded liposomes site specific. 10:100 and 12: 100 HA:liposome weight ratios were found to be optimum for Etoposide loaded and Docetaxel loaded liposomes. There was progressive reduction in zeta potential of HA grafted liposomes with increase in HA surface density owing to negative charge imparted to liposomal surface by HA. Zeta potential became constant beyond optimum weigh ratio of HA:liposomes indicating that all HA binding sites on liposomal surface were completely saturated with HA. Optimum incubation period was found to be of 2 hours for HA grafting. The amount of EDC required to achieve optimum surface HA density was found to be 10 µg in case of HAETPLIP and HADOCLIP. It was found that increase in HA amount beyond optimized HA:liposomes weight ratio resulted in significant rise in particle size of HAETPLIP and HADOCLIP as all HA binding sites on liposomal surface would be saturated with HA resulting in aggregation of excess HA.

FTIR analysis of HA non grafted and grafted drug loaded liposomes indicated characteristic amide peaks at 1690 cm^{-1} in case of HAETPLIP and 1660 cm^{-1} in case of HADOCLIP indicating

formation of stable, primary amide bond indicating successful grafting of HA to surface of Etoposide and Docetaxel loaded liposomes. Optimized HA grafted liposomes of Etoposide and Docetaxel were subsequently converted to dry powder inhalers (DPIs) for local and site specific delivery to lung cancer.

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