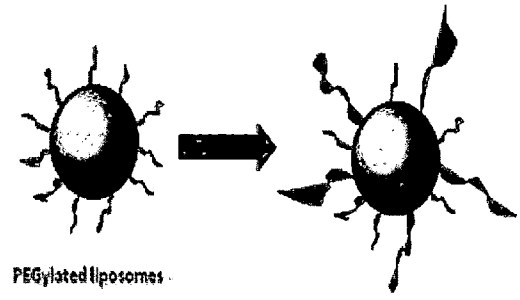
Chapter - 5

Preparation of PEGylated Liposomes



5.1. Introduction

The delivery of liposomes to the appropriate site, however, is still being investigated. For this purpose, both active targeting and passive targeting are considered. Conventional liposomes are tend to be trapped by the reticuloendothelial system (RES) such as liver and spleen before encountering the target. On the contrary, passive targeting, especially targeting to tumor tissues, could be achieved by reducing the RES trapping, since the vasculature in the tumor tissues is leaky enough to extravasate liposomes and circulating liposomes may accumulate passively in tumor tissues (Oku 1999). The development of liposomes containing lipid derivatives of PEG or saturated phospholipids such as DSPC with cholesterol has made targeted liposomal therapy more feasible by reducing the uptake by the RES system and there by prolonging the circulation time (Lundberg et al, 2000).

Particularly, PEG is useful because of its ease of preparation, relatively low cost, controllability of molecular weight and linkability to lipids or proteins including the antibody by a variety of methods. The presence of PEG reduces binding of serum proteins, i.e. opsonins marking the liposome for clearance by macrophages.

As a polymer for *in vivo* use, it should exhibit certain minimum properties, such as biocompatibility, biodegradability, non-immunogenicity and non-toxicity. Besides these advantages, it can be obtained under GMP conditions and it is FDA approved. The major role to play for PEG in bioconjugation for pharmaceutical and biotechnological use are giving stealth effect to biomolecule or carrier systems by shielding of antigenic and immunogenic epitopes, shielding receptor-mediated uptake by the RES, and preventing recognition and degradation by proteolytic enzymes, increased body residence time, modification of organ disposition, drug penetration by endocytosis and new possibilities of drug targeting. In addition to these properties, PEG facilitates conjugation by providing the functional groups required for conjugation. Now PEG derivatives are becoming available in a variety of activated and highly reactive end functional groups which need a minimum number of steps for conjugation. In a recent scenario more and more peptide and other macromolecules are delivered as a PEGylated form to overcome pharmacokinetic associated problems. Successful protein biopharmaceuticals include PEGylated interferons (PEGasys® and Intron®), PEGylated growth hormone receptor antagonist (Somavert®), PEGasparaginase (Oncospar®), adenosine deaminase (ADAGEN®), and granulocyte colony stimulating factor (Neulasta®) (Manjappa et al., 2011).

In our research we used lipid derivative of PEG (DSPE-mPEG$_{2000}$) to provide stealth effect to the liposomes which in turn avoid macrophages uptake and increase the blood circulation time leading to passive accumulation of drug loaded liposomes in solid tumors.

5.2. Materials and Methods

Sepharose CL-4B and Ellman's reagent were purchased from Sigma Aldrich, Mumbai, India. Foetal bovine serum was purchased from Himedia Lab, Mumbai, India. BCA kit was purchased from Bangalore Geni, Bangalore. DSPE-mPEG$_{2000}$-Maleimide was purchased from Laysan Bio In., (Arab, Alabama USA). All other chemicals used were of analytical reagent grade and were used without further purification.

5.3. Preparation of PEGylated liposomes

PEGylated liposomes were prepared by pre-insertion technique using thin film hydration method. 1mol%, 3mol% and 5mol% DSPE-mPEG$_{2000}$ were co-dissolved along with other phospholipids during film formation. The other steps followed are same as conventional liposomes. Prepared PEGylated liposomes were evaluated for pH, mean particle size and zeta potential, % DTX and impurity content. They were also tested for their steric stability using *in vitro* tests.

5.3.1. Sodium sulphate induced flocculation

1mL of PEGylated (1, 3, and 5 mol%) anionic liposomal suspensions were mixed with 4mL sodium sulphate solution of different concentrations (0.0M to 1.0M). The mixtures were then incubated in a shaker incubator at 37 °C with constant mild shaking for 2 hours to assess their electrolyte induced flocculation. The % transmittance of the resultant suspension was measured at 630nm using a UV-visible spectrophotometer. The measured % transmittance was converted to absorbance (Absorbance = 2- log %T). The resultant suspensions were also assessed for mean particle size and zeta potential (Lin et al, 1999).

5.3.2. *In vitro* serum protein adsorption (opsonisation study)

In vitro serum/liposome incubation:

To 300µL of non-PEGylated and PEGylated liposomal suspensions in a 2mL polypropylene micro test tubes (Eppendorf tubes), 1000µL of 100% foetal bovine serum was added and the liposome/serum mixture (76.9% final serum concentration) was incubated in a shaker incubator (Scigenic ORBITEK, Germany) for 1hr at 37 °C. The incubation mixture was immediately cooled using an ice/water bath for 5 min to stop the reaction.

Isolation of liposomes from the incubation mixture:

To separate liposomes from serum proteins, the incubation mixture (1300µl) prepared above was loaded onto a Sepharose CL-4B column (10mL pipette loaded with Sepharose CL-4B gel) and eluted with double distilled water. Fraction of 600µl was collected and replaced with distilled water every time. To determine the liposome concentration in the fractions, each fraction (600µl/fraction) was analyzed for phospholipid content using Stewart method (discussed in analytical methods) with slight modifications (Stewart, 1980). The fractions with the highest lipid content, typically fractions 7, 8, 9 and 10 from each column, were pooled and analysed for mean particle size, zeta

potential, lipid content, and adsorbed amount of serum proteins. The samples were stored at -70 °C until use (Ishidha et al., 2006).

Determination of % recovery of liposomes:

The % recovery of the liposomal suspension after separation from the column was determined by measuring the phospholipid content of the liposome before incubation with foetal serum and after separation through the column using Stewart method (Stewart, 1980) with slight modifications. *Before Incubation with serum*: To 0.1mL liposomal dispersions in 2mL polypropylene micro test tubes, 1 ml of distilled water was added (1.1mL). To 0.2mL of above liposomal dispersions in test tubes, added 3.0mL ammonium ferrothiocyanate solution and 3.0mL chloroform. The biphasic system was then vigorously vortexed for 3 minutes. *After Recovery from serum:* To 0.2mL of the recovered liposomal suspension (from pooled 2.4mL) in test tube, added 3.0mL ammonium ferrothiocyanate solution and 3.0mL of chloroform. The biphasic system was then vigorously vortexed for 3minutes.

On separating the lower chloroform phase from both the test tubes with a syringe the optical density of the chloroform phase was read at λ_{max} 472nm against chloroform as a blank and the amount of lipid present was estimated by calibration curve generated (described in analytical methods).

The percentage of liposomes recovered after separation of liposome serum mixture through Sepharose CL-4B column was calculated using below mentioned equation.

% Liposome Recovered =

$$\frac{\text{Total amount of lipid in recovered liposome}}{\text{Total amount of lipid in liposomal dispersion incubated}} \times 100$$

Determination of mean particle size and zeta potential:

The adsorption of serum proteins on to the liposomal surface was investigated by comparing the hydrodynamic diameter and zeta-potential of the liposomes before and after incubation with foetal bovine serum.

Estimation of total serum proteins associated with recovered liposomes:

Quantification of the serum proteins in the pooled liposomal fractions was performed using the BCA Protein Assay Kit (Bangalore Genei, Bangalore). The lipid in the sample did not interfere with the protein assay under our experimental conditions. The known amounts of bovine serum albumin were used to generate a standard curve (see analytical method). The protein concentrations in test samples and standards were measured at a wavelength of 562nm using spectrophotometer. The protein binding index (P_B: grams of total protein/mol of total lipid), was calculated as described earlier (Chonn et al. 1992). To 0.1 ml of recovered liposomal dispersions in 2mL polypropylene micro test tubes, 1mL of methanol was added in order to extract the lipids. The methanol was evaporated on a water bath maintained at 50 °C. To the residue, added 1

ml of distilled water and centrifuged at 6000 rpm for 15 min in order to settle down the lipids. To 40µL of supernatants in test tubes, added 160µL sodium azide solution (0.05 % w/v) to make up the volume to 200µL. To these test tubes 2mL of BCA working reagent was added and analysis was carried out at enhanced protocol (60 ^{0}C for 30 minutes). After incubation, cooled all tubes to room temperature and measured the absorbance at 562nm (A-562) of each tube against water as reference.

5.4. Preparation of functionalised pegylated liposomes

5.4.1. Introduction

In bioconjugation modification, PEG has been used repeatedly as a linker. PEG remains as a spacer arm between liposome and conjugated ligand molecules (antibodies and other proteins or peptides). Various homo and hetero-bifunctional PEG derivatives, which can be employed successfully in the preparation of immunoliposomes using proper conjugation strategy, are well explained (Manjappa et al., 2010). Use of functionalized phospholipids enables researchers to do protein lipidation, attach various peptides or other biologics to the surfaces of lipid emulsions or liposomes that allows some desired target features to be obtained, and increase of the plasma half-life of the modified drugs with the introduction of PEG chains into the target drugs. In the present study we used homo-functional phospholipid, DSPE-mPEG$_{2000}$-Maleimide, in which the maleimide group easily react with –SH groups of antibody *via* covalent thioether linkage.

5.4.2. Identification of maleimide functional group of DSPE-mPEG$_{2000}$-maleimide

The presence of maleimide group in the functional phospholipid, DSPE-mPEG2000-Maleimide, purchased from Laysan Bio In., (Arab, Alabama USA) was tested using Fourier transform infrared spectrophotometer (Bruker, Germany).

5.4.3. Preparation of functionalized PEGylated liposomes

The functionalised PEGylated liposomes were prepared as per the early reported methods with slight modifications (Allen et al., 1991; Iden and Allen, 2001; Moreira et al., 2002). 1 mol% of DSPE-mPEG$_{2000}$-Maleimide was used to prepare functionalised liposomes. Prior to experiments, PEGylated liposomes (containing 4mol% DSPE-mPEG$_{2000}$) to be functionalised were taken in a round bottomed flask, attached to Rota evaporator and allowed to rotate in water bath maintained at 65±2 ^{0}C. Also, the concentrated micellar DSPE-mPEG$_{2000}$-Maleimide (1 mol%; 7.5mg dissolved in 0.2mL of distilled water) solution was first equilibrated to 65±2 ^{0}C and added slowly drop by drop with constant mixing to round bottomed flask containing PEGylated liposomes equilibrated to 65±2 ^{0}C. The flask was then re-attached and allowed to rotate at 50rpm in water bath maintained at 65±2 ^{0}C for period of 1.5 to 2hr. After incubation of the mixtures the micelles were separated from the liposomes by Sepharose CL-4B column chromatography (as discussed before). Liposome fraction in the void volume was

collected for lipid analysis (Stewart, 1980). The collected liposomes were pooled and analysed for mean particle, zeta potential and % DTX and impurity content.

5.4.4. Confirmation of functional lipid insertion over PEGylated liposomes

The insertion of functional phospholipid, DSPE-mPEG$_{2000}$-Maleimide, on PEGylated liposomes was confirmed by Ellman's sulphydryl group estimation assay (thermo scientific protocol with slight modifications) and as per the reported method with slight modifications (Rajeeva singh, 1994). Indirectly, maleimide groups can be assayed by first reacting them (prepared functionalised PEGylated liposomes) with a known amount of thiol, present in excess, and then assaying the remaining unreacted thiol using Ellman's reagent (Yoshitake et al., 1979; Roberta and Rouser 1958; Alexander 1958). The amount of maleimide is calculated as the difference between the initial amount of thiol and the amount of unreacted thiol after complete reaction of all maleimide groups. The assay involves the reaction of maleimide (prepared functionalised PEGylated liposomes) with an excess of cysteine and quantitation of the remaining cysteine using Ellman's reagent. The amount of maleimide is calculated as the difference between the amounts of initial cysteine and assayed remaining cysteine.

Prepared a set of test tubes (Blank, positive control and sample tubes) in triplicate and 1.5mM cysteine hydrochloride solution in 0.1M sodium phosphate (pH 8) containing 1mM EDTA. For positive control tubes we have added 200µL non-functional PEGylated liposomes and 250µL of 1.5mM cysteine solution. In blank test tubes added 200µL non-functional PEGylated liposomes, 250µL 0.1M sodium phosphate solution (pH 8) and no cysteine solution. For sample test tubes we have added 200µL of prepared functional PEGylated liposomes and 250µL of 1.5mM cysteine. To all prepared blank, positive control and sample test tubes added 2.3ml of 0.1M sodium phosphate solution (pH 8) containing 1mM EDTA, mixed well, purged with nitrogen gas, covered the mouth completely and kept overnight in a cold room with constant mixing. 50µL of Ellman's reagent (4mg in 1mL 0.1M sodium phosphate solution, pH 8) was added to all test tubes, mixed well and kept at room temperature for 15 minutes. The unreacted cysteine was then determined by measuring the absorbance of the above solutions (yellow coloured solution) at 412nm using UV-visible spectrophotometer. The amount of cysteine remain unreacted was calculated as per the thermo scientific protocol.

5.5. Results and Discussion

5.5.1. Preparation of PEGylated liposomes

The PEGylated liposomes were prepared using DSPE-mPEG$_{2000}$ at different mol% by pre-insertion technique. The mean particle size, % DTX and impurity content and pH of formulation were analysed and represented in Table 5.1. Upon increasing the DSPE-mPEG$_{2000}$ concentration the DTX loading increases and we observed significant increase at 5mol%. This might be due to increase in total lipid content after inclusion of DSPE-mPEG$_{2000}$. Although the mean particle size remains unchanged at 1 and 3 mol%, we observed increased in mean particle size at 5mol%. The formation of impurity (7-

epidocetaxel) increases with increase in DSPE-mPEG$_{2000}$ concentration. This increase in impurity generation might be better correlate to increased formulation pH (observed pH of 5±0.11 at 5mol%) after inclusion of 5mol% DSPE-mPEG$_{2000}$. The zeta potential of prepared PEGylated liposomes was found to be slightly increased (-56±2mV) as compared to conventional liposomes (-42.5±1.7mV). The DSPE-mPEG$_{2000}$ concentration on the liposome surface was optimized based on *in vitro* electrolyte induced flocculation and serum protein adsorption study.

Table 5.1. Effect of DSPE-mPEG$_{2000}$ concentration on % drug content and mean particle size

DSPE-mPEG$_{2000}$ concentration	% DTX Content	% 7-EDTX Content	MPS(nm) with PDI	pH
CLs	45.72±4.59	0.144±0.128	107±6(0.275±0.024)	4.4±0.02
PLs-1 mol %	50±4.43	0.18±0.045	108±2 (0.262±0.0070)	4.5±0.043
PLs-3 mol %	60±2.43	0.32±0.121	113±1 (0.249±0.0240)	4.7±0.022
PLs-5 mol %	78.358±2.863	0.462±0.062	119±6 (0.228±0.0403)	5.0±0.11

Values are Mean±SD, n=3. DTX: Docetaxel; EDTX: epidocetaxel; MPS: Mean particle size; CLs: conventional liposomes; PLs: PEGylated liposomes. Significant increase in the DTX loading was observed at 3 mol% (*$p<0.05$) and 5mol% (***$p<0.0001$) as compared to CLs. The 5 mol% PEGylated liposomes showed significantly increased DTX loading than 3 mol% PEGylated liposomes (*$p<0.05$, $p=0.0017$).

We determined the role combination of phospholipids (HSPC:DPPC), instead of individual phospholipids, in DTX loading by preparing PEGylated liposomes (5mol%) containing HSPC and DPPC both and individually. We observed increased DTX loading with PEGylated liposomes composed of HSPC and DPPC both as compared to PEGylated liposomes composed of individual phospholipids. About 11% and 20% increase in DTX loading was observed in PEGylated liposomes composed of HSPC and DPPC both as compared to HSPC and DPPC alone, respectively (Table 5.2). Therefore it concludes that the use of combination of phospholipids (rather than single phospholipid) will enhance the hydrophobic drug loading in the bilayer membrane. The increased % impurity formation was observed with PEGylated liposomes prepared with HSPC alone (about 0.6% increase) and DPPC alone (about 0.5% increase) as compared to liposomes composed of both. This might be due to altered rigidity of membrane composed of both phospholipids as compared membrane composed of individual phospholipids. No significant change in and mean particle size was observed but still slight increase in mean particle size was observed with HSPC liposomes.

Table 5.2. Comparison of PEGylated liposomes (5mol%) composed of HSPC and DPPC both and HSPC and DPPC individually.

PEGylated liposomes prepared with	% DTX content	% impurity (7-epidocetaxel)	Mean particle size (nm) with PDI
HSPC and DPPC	78.358±2.863	0.462±0.062	119±6 (0.228±0.0403)
HSPC alone	66.73±4.23	1.067±0.123	124±4 (0.248±0.067)
DPPC alone	57.92±3.82	0.9314±0.081	116±3(0.241±0.055)

Values are Mean±SD, n=3. DTX: Docetaxel; PDI: Polydispersity index. Significant increase in DTX loading with PEGylated liposomes composed of HSPC and DPPC combination as compared to PEGylated liposomes composed of HSPC (*$p<0.05$, $p=0.024$) or DPPC (*$p<0.05$, $p=0.0014$) individual phospholipids.

5.5.2. Sodium sulphate induced flocculation

In the present study, the existence of a hydrated mPEG barrier on liposome surface was investigated by electrolyte flocculation study. This is based on the fact that the physical stability of a colloidal system is mainly dependent upon the competitive processes of attraction (vander Waals forces) and repulsion (either electrostatic repulsive force or steric stabilizing barrier or both) (Florence and Attwood, 1988). If particles are mainly stabilized electrostatically, destruction of the electrostatic double layer surrounding the particles will result in aggregation of the particles into clusters with a corresponding increase in optical turbidity. However, if the particles are mainly stabilized by a hydrated steric stabilizing barrier, the colloidal system should be stable even if the electrostatic double layers have been destroyed.

The results of Na_2SO_4 induced flocculation of non-PEGylated and PEGylated liposomes are shown in Figure 5.1 and Table 5.3. The non-PEGylated liposomes remain unstable in Na_2SO_4 solution and they start flocculate even at 0.2M solution and flocculation still increased with increase in Na_2SO_4 concentration to 1.0M. This unstability can be attributed to the interaction of Na+ ions with negatively charged liposomes and thereby destruction of electrostatic barrier surrounding the liposomes leading to flocculation. When the liposomes were modified with 1mol%, 3mol%, and 5mol% of DSPE-$mPEG_{2000}$, the critical flocculation concentration for liposomes was found to be 0.6M and below this concentration the liposomes remained stable. All PEGylated liposomes showed less flocculation and more stability than conventional liposomes. The decrease in flocculation was observed with increase in mPEG concentration on liposome surface at 0.2M. At above 0.2M concentration, we surprisingly found increased flocculation with increase in DSPE-$mPEG_{2000}$ concentration. The liposomes surface modified with 1mol% DSPE-mPEG2000 showed better stability even at higher sodium sulphate concentration (up to 0.8M) then 3 mol% and 5 mol%. The relatively small increase in the stability of PEGylated liposomes in Na_2SO_4 solution was thought to be due to the fact that Na_2SO_4 can not only compress the electrostatic double layer but also dehydrate the mPEG layer

on the liposome surface. The similar result was reported by Stolnik et al. (Stolnik et al., 1994) and they found that when PLGA and polystyrene nanoparticles were coated with poloxamine 908 or PLA-PEG copolymer, which creates a steric PEO barrier on the particle surface, the stability of the resulting colloidal systems in Na_2SO_4 solutions was increased but they still flocculated at a critical concentration around 0.5M.

The mean particle size and zeta potential of non-PEGylated and PEGylated liposomes after incubation with different concentrations of Na_2SO_4 solution were measured (Table 5.4). The PEGylated liposomes retained their original particle size at 0.2M and 0.4M Na_2SO_4 but at above 0.4M the mean particle size was increased to micrometer (above detection level of Zeta seizer). The zeta potential of all formulations was drastically decreased to neutral.

Table 5.3. Influence of sodium sulphate concentration on absorbance of the liposomal preparation.

Sodium sulphate	Absorbance			
	CLs	PLs-1 mol%	PLs-3 mol%	PLs-5 mol%
DW	0.053±0.003	0.049±0.006	0.054±0.004	0.064±0.007
0.2 M	0.373±0.054	0.113±0.012	0.090±0.005	0.085±0.010
0.4 M	0.919±0.083	0.175±0.043	0.164±0.009	0.112±0.004
0.6 M	0.961±0.093	0.240±0.021	0.834±±0.044	1.508±0.123
0.8 M	1.110±0.056	0.351±0.044	0.923±0.072	1.445±0.11
1.0 M	1.143±0.077	0.554±0.034	0.972±0.088	1.445±0.15

Values are Mean±SD, n = 3. CLs: Conventional liposomes; PLs: PEGylated liposomes

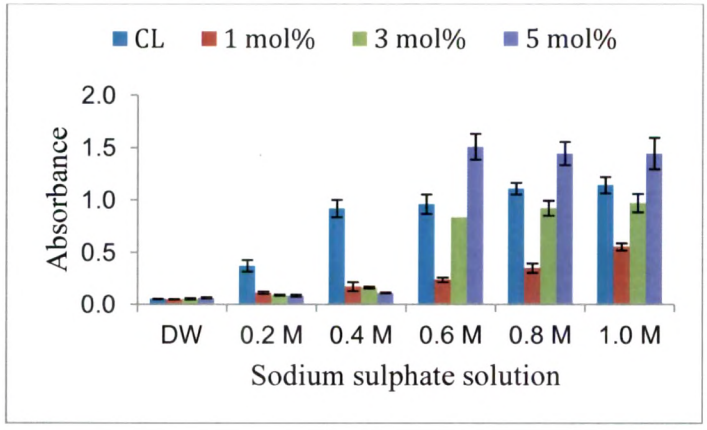


Figure 5.1. Influence of sodium sulphate concentration on absorbance of the liposomal preparation. Values are Mean±SD, n = 3. All PLs showed significantly less flocculation as compared to CLs ($p<0.001$) at 0.2M and 0.4M. At 0.6M, 0.8M and 1M the PLs with 1mol% PEG showed significantly less flocculation as compared to CLs ($p<0.0001$), whereas 3mol% and 5mol% PLs showed significantly more flocculation as compared to 1mol% PLs ($p<0.0001$).

Table 5.4. Influence of sodium sulphate concentration on mean particle size of liposomes

Sodium Sulphate Concentration	Mean particle size (nm)			
	CLs	PLs-1 mol%	PLs-3 mol%	PLs-5 mol%
DW	109±8	109±3	100±2	101±4
0.2 M	336±25	157±7	169±6	138±2
0.4 M	1315±56	182±5	253±11	200±7
0.6 M	-*	287±6	630±18	-*
0.8 M	-*	355±12	-*	-*
1.0 M	-*	-*	-*	-*

Values are Mean±SD, n=3. -* indicates values above 1micrometer. CLs: Conventional liposomes; PLs: PEGylated liposomes. The mean particle size of CLs significantly increased ($p<0.0001$) as compared to all PEGylated liposomes at both 0.2M and 0.4M sodium sulphate concentrations.

5.5.3. *In vitro* serum protein adsorption (opsonisation study)

It has previously been demonstrated that liposomes rapidly bind a complex profile of plasma proteins *in vitro* upon exposure to human plasma or serum (Bonte and Juliano, 1986; Juliano and Lin, 1980). It is hypothesized that the presence of PEG on the liposomal surface attracts a water shell, resulting in reduced adsorption of plasma proteins such as opsonins and, as a consequence, impaired recognition of the liposomes by the cells of the mononuclear phagocyte system (MPS) following intravenous administration. This results in increased blood circulation times of these liposomes (Ishida et al., 2006). It is widely believed that serum proteins associating with the liposomes are a determinant factor in the clearance of liposome. Chonn et al. (Chonn et al., 1992) concluded that the ability of liposomes to interact with serum proteins, expressed as the P_B value, is indeed related to their clearance rate: the higher the amount of protein bound, the faster the liposomes were cleared. Ishida et al. also concluded that the accelerated clearance of PEGylated liposomes involves selectively bound rather than non-specifically bound proteins.

The results to this stage clearly indicate that the amount of blood protein associated with liposomes in the circulation dramatically affects liposome clearance behaviour *in vivo*. It should be noted, however, that the *in vivo* analysis is limited by the amount of liposomes recovered, especially for rapidly cleared liposomes. In order to further characterize the surface properties of liposomal systems in relation to protein binding, and known clearance properties, it would be useful to develop an *in vitro* assay. In this regard, it is first important to show that the amount of protein bound to liposomal surface *in vitro*. The *in vitro* determinations, being simpler and allowing for greater

recoveries of liposomes should therefore be a useful assay for predicting the clearance behaviour of liposomes of novel liposome compositions (Chonn et al., 1992).

Furthermore, liposomes having similar membrane surface charge imparted by different anionic phospholipids can exhibit significantly different protein binding abilities. This was quantified by measuring the protein binding ability (P_B; grams of total protein/mol of total lipid) which, in turn, was related to the clearance behaviour of the liposomes in the circulation.

Therefore, in the present study the serum protein binding ability of prepared anionic (DPPG) non-PEGylated and PEGylated liposomes were tested by incubating liposomes with 100% foetal bovine serum for 1hr at 37 ^{0}C with slight agitation in a shaker incubator. The liposomes were then recovered using Sepharose CL-4B column as described in method (Table 5.5).

The recovered liposomes were first analysed for mean particle size and zeta potential retention as serum protein adsorption can alter the liposomal size and zeta potential (Table 5.6). About 30nm increase in mean particle size of non pegylated liposomes was observed as compared to 1mol% (about 8nm), 3mol% (about 14nm) and 5mol% (negligible change) PEGylated liposomes. The 5 mol% DSPE-mPEG$_{2000}$ better retained its mean particle size and this might be due to decreased association of serum proteins as compared to 1mol%, 3mol% and non-PEGylated liposomes. The zeta potential of recovered non-PEGylated (-16.5±4.9mV) and PEGylated liposomes (-10.4±1.2 to -14.05±0.21mV) was found decreased (Table 5.6).

The total amount of serum proteins associated with recovered liposomes was estimated using BCA protein estimation kit. The liposome associated proteins were efficiently extracted and delipidated using procedure as described in method. This delipidation step was required because lipids interfere with most protein assays (Kessler and Fanestil, 1986). The amount of recovered liposomal surface associated serum proteins and P_B values are shown in Table 5.7 and Figure 5.2. The conventional liposomes (non-PEGylated) (P_B: 494.417±22μg/μM of total phospholipid recovered) showed more protein binding (P_B) as compared to PEGylated liposomes. The PEGylated liposomes with 5mol% DSPE-mPEG$_{2000}$ showed less (Statistically significant) amount of associated serum proteins than conventional liposomes and PEGylated liposomes (1mol% and 3mol%). Therefore, we can expect enhanced *in vivo* circulation time with 5mol% PEGylated liposomes as compared to conventional liposomes and other PEGylated liposomes (1mol% and 3mol%). Further, the 5 mol% PEGylated liposomes were used for *in vivo* therapeutic study in tumor induced animal model.

Table 5.5. The % liposomes recovered after separation through Sepharose CL-4B column

Formulation	Amount of lipid used in study (µg)	Amount lipid in recovered liposomes (2.4mL) (µg)	% Liposomes recovered
CLs	888.539	583.86±38.38	65.70±4.32
PLs-1 mol%	916.778	599.26±24.89	65.32±2.71
PLs-3 mol%	965.189	569.19±67.42	58.97±6.98
PLs-5 mol%	999.480	702.68±13.48	70.3±1.34

Values are Mean±SD, n=3. CLs: Conventional liposomes; PLs: PEGylated liposomes

Table 5.6. Mean particle size and zeta potential of anionic liposomes before and after incubation with foetal bovine serum

Formulation	Before incubation with serum		After separation through Sepharose CL-4B column	
	Size (nm) with PDI	Zeta potential (mV)	Size (nm) with PDI	Zeta Potential (mV)
CLs	107±6 (0.275±0.024)	-42.5±1.7	139±5 (0.2955±0.042)	-16.5±4.9
PLs (1 mol %)	108±2 (0.262±0.0070)	-55.5±2.4	116± 1(0.211±0.009)	-10.4±1.2
PLs (3 mol %)	113±1 (0.249±0.0240)	-55±1.4	127±5 (0.241±0.011)	-14.05±0.91
PLs (5 mol %)	119±6 (0.228±0.0403)	-54±2.1	122±3 (0.241±0.017)	-14.05±0.21

Values are Mean±SD, n=3. PDI: Polydispersity index; CLs: Conventional liposomes; PLs: PEGylated liposomes. The mean particle size of CLs was significantly increased ($p<0.0001$) after separation from the serum liposome mixture. The 3 mol% PLs showed significant increase in mean particle size ($p<0.05$, $p=0.021$) after separation from the serum liposome mixture. The zeta potential of all formulations was significantly decreased ($p<0.0001$) after separation from the serum liposome mixture.

Table 5.7. The amount of serum proteins associated with recovered anionic liposomes

Formulations	Amount of serum proteins (μg)	lipid in recovered liposomes (μM)	Protein binding (P_B; μg/μM of lipid)
CLs	355.31±18.56	0.718±0.046	494.417±22
PL-1 mol%	219.06±21.21	0.737±0.03	297.194±25
PL-3 mol%	228.31±11.49	0.7001±0.083	325.899±15
PL-5 mol%	209.12±11.93	0.863±0.015	242.085±15

Values are Mean ± SD, n=3. CLs: Conventional liposomes; PLs: PEGylated liposomes

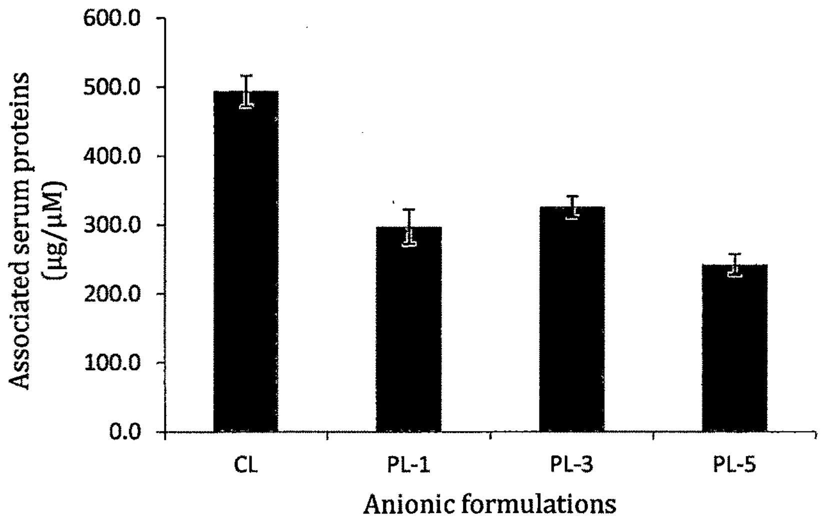


Figure 5.2. Effect of PEGylation on serum protein binding (P_B) of anionic liposomes. The CLs showed significantly high serum protein binding than all PEGylated liposomes (***$p<0.0001$). The PEGylated liposomes with 5 mol% DSPE-mPEG$_{2000}$ showed significantly less serum protein adsorption than 3 mol% DSPE-mPEG$_{2000}$ (*$p<0.05$).

5.5.4. Preparation of functionalised PEGylated liposomes

The presence of maleimide group in the functional phospholipid, DSPE-mPEG2000-Maleimide (Figure 5.3), purchased from Laysan Bio In., (Arab, Alabama USA) was tested using Fourier transform infrared spectrophotometer (Bruker, Germany). The FTIR spectrum (Figure 5.4) confirms the presence of maleimide group.

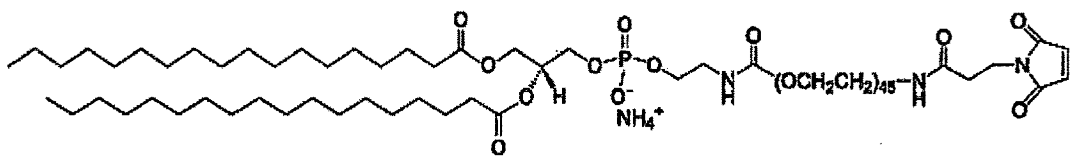


Figure 5.3. Chemical structure of DSPE-mPEG$_{2000}$-Maleimide

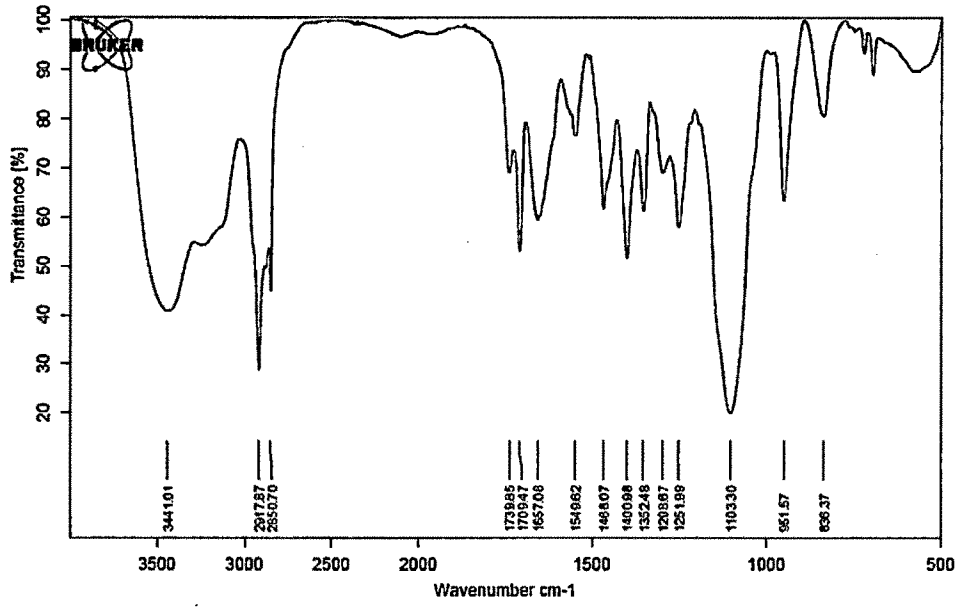


Figure 5.4. FTIR spectrum of functional phospholipid derivative, DSPE-mPEG$_{2000}$-Maleeimide. (3441cm^{-1}: N-H bending; 2917 cm^{-1}: C-H stretching; 1739 cm^{-1}: C=O stretching ester; 1709 cm^{-1}:C=O stretching imide; 1657 cm^{-1}: C=O stretching amide; 1409 cm^{-1}: C=O stretching imide ; 1103 cm^{-1}: C=O stretching imide; 836 cm^{-1}: C=O imide (Camelia and Maria, 2006).

When the functionalised liposomes were prepared by co-dissolving DSPE-mPEG$_{2000}$-Maleimide along with other lipids (pre-insertion method), the maleimide functional groups are distributed on both sides of the membrane, and a proportion of the functional groups, exposed on the outer surface of the liposome membrane, are available for coupling to ligand molecules. An alternative approach which overcomes these problems is post-insertion technique in which the functional phospholipid will be inserted on liposomal surface by incubating preformed liposome with micellar solution of functional phospholipids at 60 ^{0}C for a period of 1hr (Li et al., 2010). In this method, the all functional groups remain only on outer surface of membrane available for conjugation with ligands.

This post-insertion technique is a logical progression from the work of Uster et al. (Uster et al., 1996) and involves the coupling of ligands to the terminus of polyethylene glycol (PEG)-lipid derivatives in a micellar phase followed by the time and temperature dependent transfer of the ligand-coupled PEG-lipids into the bilayers of pre-formed, drug-loaded liposomes during a simple incubation step. As the antibodies (specially monoclonal and other proteins) are more heat sensitive (they may lose their biological activity) and this method involves time and temperature dependent transfer, we can make some slight modifications in the above method. We can first transfer the DSPE-mPEG$_{2000}$-Maleimide (functional lipid derivatives) in a micellar phase over PEGylated

liposomes and then we can incubate the functionalised liposomes with ligands at room temperature or in a cold room.

Hence, in our study the functionalised PEGylated liposomes were first prepared as per the early reported methods with slight modifications (Allen et al., 1991; Iden and Allen, 2001; Moreira et al., 2002) and then we used those liposomes to prepare ligand conjugated liposomes. Micellar solution of 1mol% of DSPE-mPEG$_{2000}$-Maleimide was then transferred over the surface of preformed docetaxel loaded PEGylated liposomes (containing 4mol% DSPE-mPEG2$_{000}$) as described in method. We observed no extra impurities generated after heating docetaxel loaded liposomes for additional 2hr at 65 ^{0}C during preparation of functionalised liposomes. The liposomes are slightly diluted after separation but no change in total drug content and mean particle size were observed.

5.5.5. Confirmation of presence of maleimide groups over PEGylated liposomes

The insertion of functional phospholipid, DSPE-mPEG$_{2000}$-Maleimide, on PEGylated liposomes was confirmed by Ellman's sulphydryl group estimation assay (thermo scientific protocol with slight modifications) and as early the reported method with slight modifications (Rajeeva singh 1994). The amount of maleimide is calculated as the difference between the initial amount of thiol (cysteine) and the amount of unreacted thiol (cysteine) after complete reaction of all maleimide groups (Table 5.8). Prepared a set of test tubes (Blank, positive control and sample tubes) (yellow product) analysed using UV-visible spectrophotometer at 412nm. The absorbance positive control tubes and sample tubes were subtracted from blank sample absorbance. Using the absorbance values we have calculated the amount thiols (cysteine) remained unreacted in samples as below mentioned.

Calculation of the Free Sulfhydryl Concentration

A 250µl aliquot of the 1.5mM standard cysteine solution mixed with 2.3 ml of reaction buffer (0.1M sodium phosphate of pH 8), 200µl non functionalised PEGylated liposomes and 50µl of Ellman's reagent solution gave an absorbance of 0.6703 (after subtracting the blank; 0.283) using a 1cm spectrophotometric cuvette. The reported molar absorptivity (molar extinction coefficient, which is expressed in units of $M^{-1}cm^{-1}$) of TNB in this buffer system at 412 nm is 14,150 (Riddles et al., 1983). Molar absorptivity, 'E' is defined as follows:

E= A/bc where A = absorbance, b = path length in centimeters, c = concentration in moles/liter (=M)

Solving for concentration gives the following formula: C=A/bE

A=0.6703, b=1cm and E = 14,150 $M^{-1}cm^{-1}$

Therefore, C= 0.6703/1(14,150) M

$= 4.737\times10^{-5}$ M/litre

This value represents the concentration of the solution in the spectrophotometric cuvette. To calculate the concentration of the unknown sample, it is necessary to account for dilution factors as follows:

The total volume of the solution being measured is

2.3 ml of Reaction Buffer

+ 0.2ml non-functionalised PEGylated liposomes

+ 0.25 ml of standard cysteine solution

+ 0.05 ml of Ellman's Reagent Solution

2.80 ml of solution

The concentration of the positive control solution is 4.737×10^{-5} M (1000ml contains 4.737×10^{-5} M), then 2.80 ml of that solution contains

$(2.8 \times 4.737\times10^{-5})/1000 = 1.326\times10^{-7}$ moles

These 1.326×10^{-7} moles of sulfhydryl are present in the positive control solution and in similar way we have calculated the sulfhydryl concentration of test samples. The amount of sulfhydryls reacted with functionalised liposomes ($0.6457\times10^{-7}\pm0.256\times10^{-7}$ moles) was calculated by subtracting test sample values from positive control sample values (Table 5.8). The obtained value of sulfhydryls reacted with functionalised liposome indicate the availability of sufficient maleimides for conjugation of ligands.

Table 5.8. Estimated cysteine (thiol) concentrations using Ellman's assay

Concentration of thiols (positive control samples)	$1.326\times10^{-7}\pm0.166\times10^{-7}$ moles
Concentration of thiols remain unreacted (functionalised liposomes)	$0.6807\times10^{-7}\pm0.151\times10^{-7}$ moles
Concentration of thiols reacted with functionalised liposomes (0.2ml)	$0.6457\times10^{-7}\pm0.256\times10^{-7}$ moles

Values are Mean±SD, n=3.

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