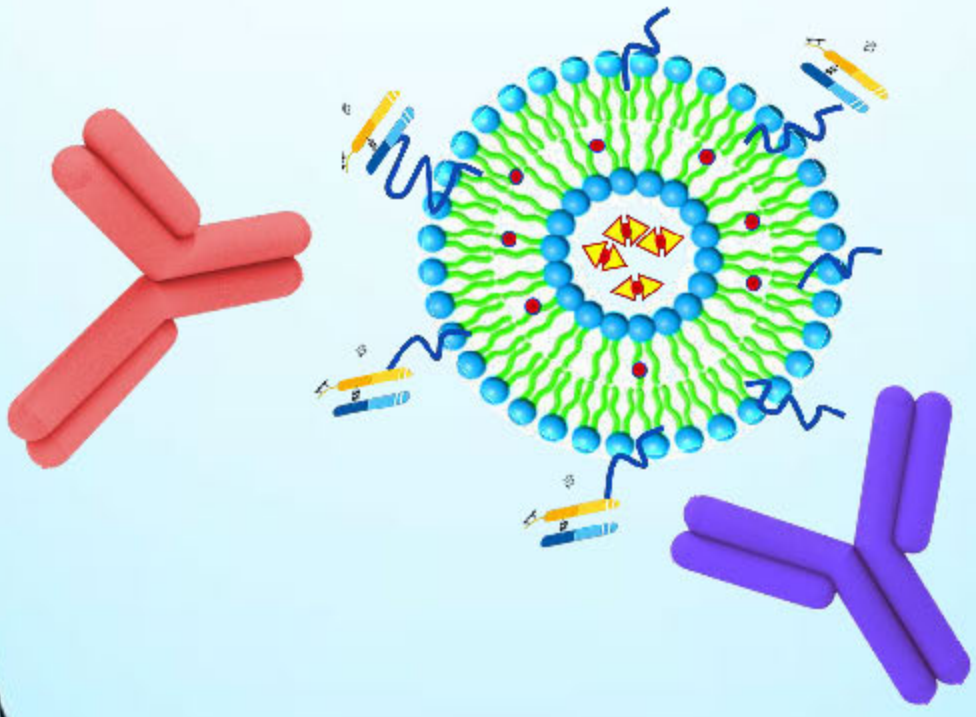
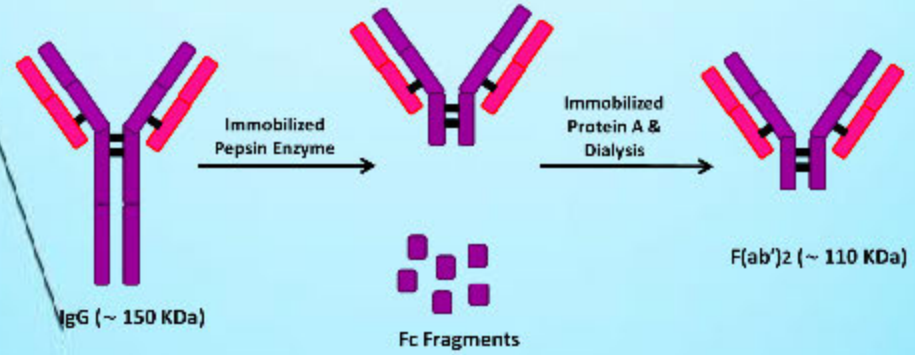
Chapter-6
Preparation and Characterization of Immunoliposomes





6.1 Introduction

Functionalization of Liposomes and Preparation of Immunoliposomes

Liposome surface functionalization facilitates enormous potential applications of liposomes, such as enhanced stability, bioactive liposome conjugates, and targeted delivery (1), (2). Various biomolecules have been conjugated onto liposome surface for variety of biomedical applications. For this purpose, Maleimide-functionalized DSPE is generally used. The maleimide is reactive towards thiol-containing ligands (3). The maleimide-thiol reaction gained great popularity in the field of surface modification of drug delivery systems (DDS) and bio-conjugation of drugs to antibodies. Maleimide react with thiols resulting in the formation of stable thioether bonds. The specificity to thiols, fast aqueous reaction kinetics and mild reaction conditions explain their broad pharmaceutical application in DDS functionalization. Numerous examples exist in the literature for the use of maleimide-thiol-reactions in the area of functionalized nanoparticles and liposomes. Although the hydrolysis tendency of maleimide is well-known, qualitative and quantitative information on the stability and reactivity of maleimide groups during preparation and in final formulations are not focused generally. This is surprising, since hydrolysis of maleimide prevents nanoparticle functionalization and results in an increase of negative surface charge due to the hydrolysis product maleic acid. Oswald et al. investigated the stability of 1,2-distearoyl-sn-glycero-3-phosphoethanol- amine-N-[maleimide-2000] (DSPE-PEG2000-Mal) during the preparation of liposomes via two common preparation methods, which can be distinguished by the insertion of DSPE-PEG2000-Mal during or after the liposome formation process (pre-insertion and post-insertion process). The liposomes prepared by the pre-insertion method had 63% active maleimide groups remaining on their surface. The activity decreased dramatically during the purification process down to 32%. The preparation by post-insertion method showed minimal effects on maleimide activity. 76% of maleimide groups were active and therefore available for coupling reaction after post-insertion (4). Figure 6.1 is the graphical representation of surface modification of liposomes loaded with PTX and PTX-DMβCD ICs. The liposomes were first made functionalized by addition of DSPE-PEG2000-Mal to the PEGylated surface of liposomes. The maleimide group of DSPE-PEG2000-Mal is responsible to form

thioether linkage with thiol group of antibody or antibody fragment to form antibody decorated targeted immunoliposomes.

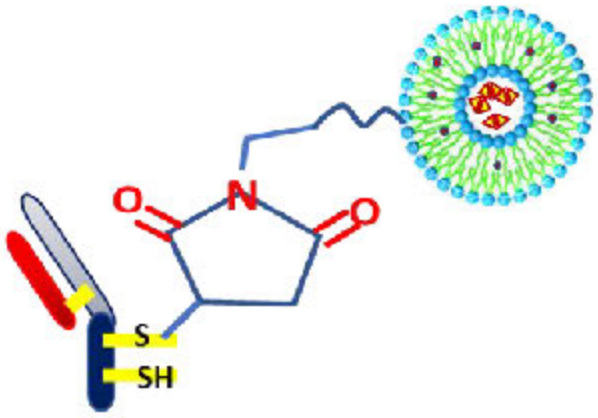


Figure 6.1 Graphical representation of Thio-ether linkage and conjugation of antibody to functionalized liposome

Fragmentation of Monoclonal Antibody

A proteolytic enzymatic digestion is the widely used approach for the antibody fragmentation. The Thermo Scientific™ Pierce™ $F(ab')_2$ Preparation Kit assists effective generation of $F(ab')_2$ from IgG (mouse anti-FSHR monoclonal antibody). A nonspecific endopeptidase, immobilized Pepsin has been used in this kit which is active at acid pH only and at neutral or alkaline pH it gets irreversibly denatured. Pepsin digestion mainly produces a $F(ab')_2$ fragment having molecular weight around 110 kDa that can be confirmed by SDS-PAGE under non-reducing conditions and numerous small peptides of the Fc portion (Figure 6.2). The resulting $F(ab')_2$ fragment is composed of a pair of Fab′ units connected by two disulfide bonds. The Fc fragment is extensively degraded and can be separated from $F(ab')_2$ by dialysis, gel filtration or ion exchange chromatography. This kit contains the necessary components for $F(ab')_2$ generation and subsequent purification. Immobilized Pepsin is advantageous because the digestion can be immediately stopped by simply removing the resin from the antibody digest solution. The included Spin Columns allow easy manipulation of the resin and maximum $F(ab')_2$ recovery. The prepacked, immobilized Thermo Scientific™ NAb™ Protein A Plus Spin Column binds the large Fc fragments and undigested IgG, allowing the $F(ab')_2$ fragments to pass through the

column for efficient purification. This complete kit makes $F(ab')_2$ generation and purification simple, fast and effective (Figure 6.3).

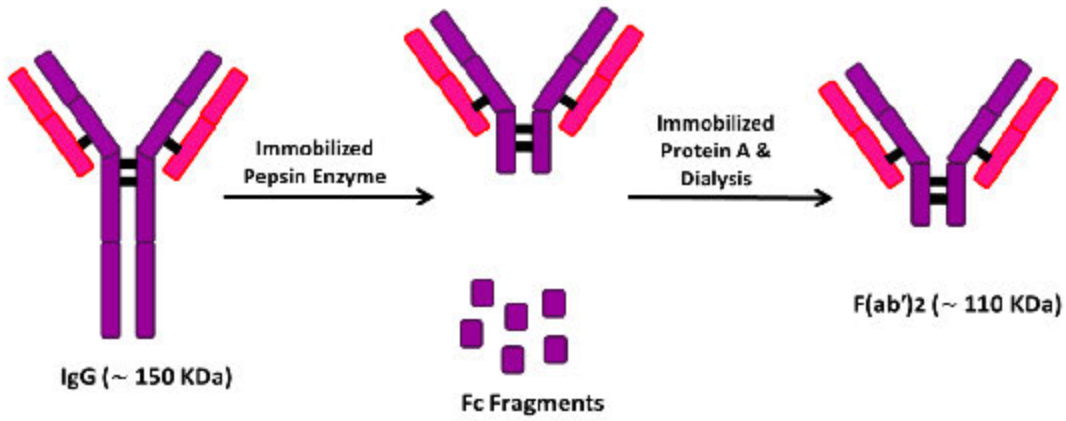


Figure 6.2 Schematic diagram of antibody digestion by Pepsin.

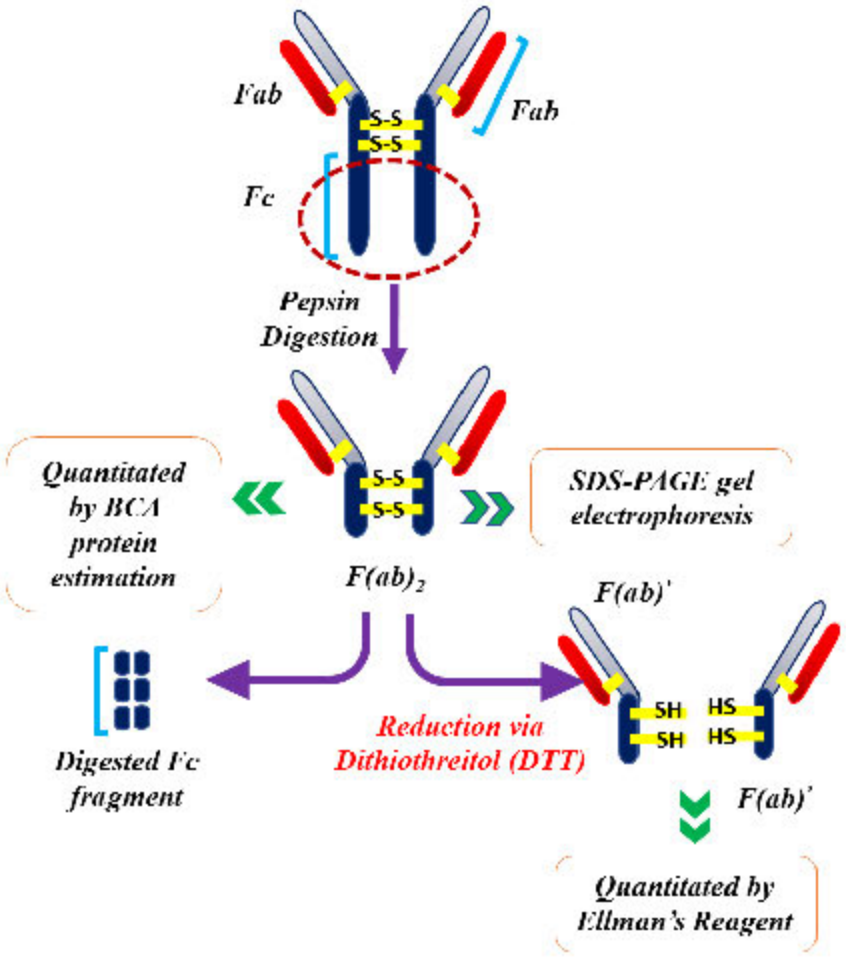


Figure 6.3 Antibody digestion and confirmation.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis technique is mainly used for qualitative protein characterization, for identification of protein homogeneity and for the estimation of molecular masses of protein subunits. The bifunctional bisacrylamide is able to cross-link the adjacent polyacrylamide chains to form a three-dimensional sieve like network of fibres and pores. Ammonium persulfate and N,N,N',N'tetramethylethylenediamine; (TEMED) are responsible components those by generating free radicals catalyse the polymerization reaction. The pore size of a gel depends on the concentration of acylamide which can be adjusted or can be optimised for various protein samples based on molecular weight of samples and the resistance imparted by gel to migration of proteins to achieve better resolution of method.

Majorly, the analytical polyacrylamide gels electrophoresis of proteins is performed under conditions that confirm dissociation of the proteins into their distinct polypeptide subunits and that reduce aggregation. Sodium dodecyl sulfate (SDS), a strong anionic detergent is usually used in combination with reducing agent or heat to allow dissociation of proteins before loading on the gel. The SDS binds to denatured polypeptides and thus polypeptides become negatively charge and display a constant charge-to-mass ratio irrespective of type of protein sample used. As the amount of SDS that binds to polypeptide is always proportional to its molecular mass and is not dependent on its sequence, the migration mobility of SDS-polypeptide complexes through polyacrylamide gels majorly depends on the size of the polypeptide. Being anionic, SDS complexes migrate toward the anode in an expected way, with complexes of low molecular mass migrating faster and the larger ones migrating slower comparatively. Thus, molecular mass of a protein sample can be determined from its relative mobility and the purity can be evaluated depending on intensity of its band as compared to known molecular mass standards.

The SDS-PAGE can be carried out either in reducing conditions or non-reducing conditions. In reducing conditions the reducing agents such as dithiothreitol (DTT) or 2-mercaptoethanol (2-ME) are added to reduce disulfide bonds of protein that allow complete denaturation and dissociation of sample proteins and results in polypeptide backbone unfolding and successive complexation with SDS. On the other hand, non-reducing conditions, the reducing agents are not added and thus oligomeric

form of the protein is preserved by presence of intact disulfide bonds in protein samples.

The most common method of electrophoresis for characterization of complex protein mixture uses a discontinuous buffer system which involves two connecting, but different gels. They are separating or resolving lower gel and the stacking upper gel. Both the gels vary in porosities, ionic strength and pH. The stacking gel is a macroporous as it contains with low concentration of acrylamide and the Tris-HCl, pH 6.8 buffer is used for formulation this layer. While the resolving gel less porous with high concentrations of acylamide as compared to stacking gel and, Tris-HCl, pH8.9 buffer is used for this one.

Additionally, the pH and ionic strength of buffer in reservoir differs from the buffer used to cast the gel. Tris- glycine, pH 8.3 is generally used as electrode buffer in the electrophoresis tank. The discontinuity of buffer plays a role to concentrate sample in the stacking gel to improve its resolution. As soon as the power is applied, voltage drop develops throughout the sample solution that pushes proteins in stacking gel for better resolution. All the components of system contain 0.1 % SDS. A moving boundary region is promptly made by highly mobile chloride ions forming a leading edge in the front and the comparatively slow glycinate ions in the rear trailing edge. A localised high-voltage gradient forms between the leading and trailing ion fronts forces SDS-polypeptide complexes to form a thin stack zone or band and migrate between the chloride and glycinate phases. Between the trailing and the leading edge there is a zone of steeper voltage and lower conductivity that sweeps the polypeptide from samples to deposit at surface of resolving gel. The macroporous stacking gel does not hinder the movement of proteins while at the interface both the gels, due to restrictive less porous gel and buffer discontinuity, the polypeptides experience an increase in retardation. Thus, in the resolving gel, proteins generally migrates slowly in towards the anode. Molecular sieving is required for the SDS- polypeptide complexes to separate on the basis of their respective molecular masses. Figure 6.4 shows SDS-PAGE gel electrophoresis assembly which was used in this work.

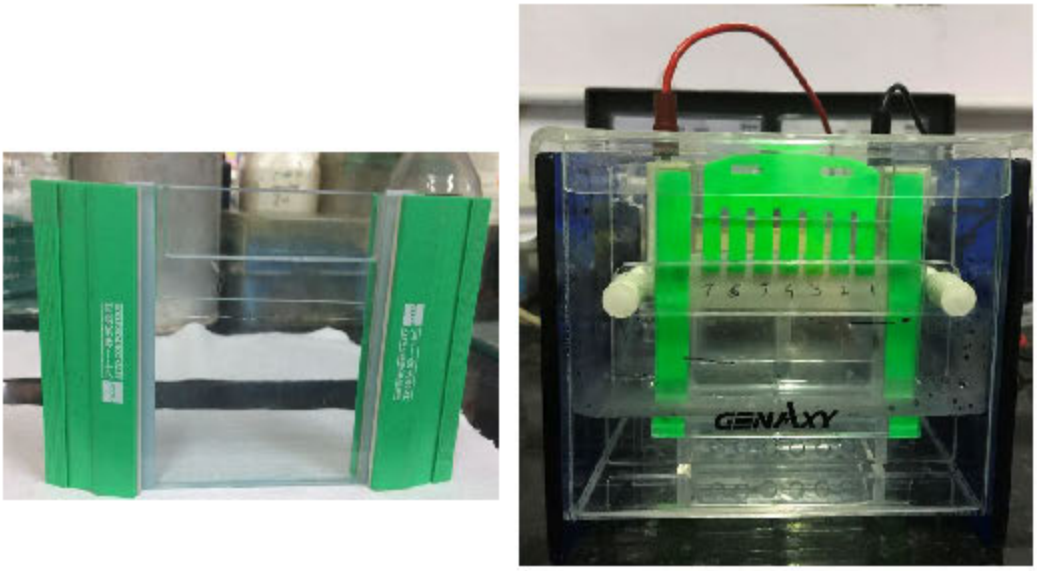


Figure 6.4 SDS-PAGE gel electrophoresis assembly.

Silver staining method

Silver staining is an efficient and very sensitive (detects in nanograms) technique for detection of proteins by staining them after SDS-PAGE electrophoretic separation. The protein bands stained after silver staining technique can be easily visualized without using any other specific advanced equipment.

The basic principle of silver staining is that the silver ions are bound by the protein, which can be further reduced under suitable conditions to generate metallic silver which can be visible as bands on gel. The polyacrylamide gels are first impregnated with soluble silver ions (Ag+) and developed by treatment of a reductant. The proteins in the gel can bind the soluble silver and promote the reduction of silver ions to metallic silver ions (Ag^0). Those metallic silver ions are insoluble and visible as bands on gel. Those bands can confirm presence of protein at the specified area of gel. The deposition of metallic silver stimulates further deposition in an autocatalytic manner and thus it is a very sensitive method to detect and stain proteins in nanogram levels.

Following are the sequential steps of silver staining;

Fixation:

The first step of fixation concentrates the macromolecules in the gel and prevents them from diffusing out of the gel during successive steps of staining. Fixation also eradicates the interfering constituents (Tris buffer, SDS, amino acids, ampholytes) responsible to give a high background and poor contrast.

Sensitization:

The gel is sensitised by treatment with reagents those chemically modify the macromolecule or proteins which will ultimately increase the sensitivity of protein molecules towards reaction with silver ions and promotes reduction of silver ions to metallic silver ions. The excess of sensitizing reagents can give high background staining and thus after this step the gel needs to be properly washed using distilled or deionized water to remove excess sensitizing reagents.

Silver impregnation:

In this step, the gel is impregnated with silver agents. There are two main methods. One is use of silver nitrate as a silver agent which is also called acid method and another is use of basic silver-ammonia also called as basic method. After this step the gel needs to be properly washed using distilled or deionized water to remove excess silver agents.

Image development:

The image is generally developed using formaldehyde as a developing agent that reduces silver to metallic silver. The reaction only takes place at high pH and thus sodium bicarbonate is added here in this step.

Stopping and Preservation:

The stopping step avoids further reduction of silver ions and preservation can be done to prevent cracking of gel during drying.

6.2 Materials and Instruments

Materials:

Sr No	Chemicals/Materials	Source/Manufacturer
1	DSPE-PEG(2000) Maleimide	Avanti Polar Lipids, USA.
2	Amicon Ultra-15 Centrifuge filter unit with Ultracel-50 membranes (50KDa)	Millipore, USA.
3	Pierce™ F(ab')2 Micro Preparation Kit	Thermo Scientific, USA.
4	Mouse anti-FSHR monoclonal antibody	R&D Systems Inc., USA
5	Goat anti-mouse (whole IgG) FITC tagged secondary antibody	Sigma Aldrich, Mumbai, India
6	Dithiothreitol (DTT)	Sigma Aldrich, Mumbai, India
7	Silver Nitrate	Sigma Aldrich, Mumbai, India
8	Ellman's reagent	Sigma Aldrich, Mumbai, India
9	A protein marker (Precision Plus Protein™ Unstained Standards)	Bio-Rad, USA
10	SDS (Sodium Dodecyl Sulphate)	Himedia, Mumbai, India
11	Acrylamide	Himedia, Mumbai, India
12	Bis-Acrylamide	Himedia, Mumbai, India
13	Tris Base	Himedia, Mumbai, India
14	Glycine	Himedia, Mumbai, India
15	TEMED (N,N,N',N'-Tetramethylethane-1,2-diamine)	Himedia, Mumbai, India
16	Bromo Phenol Blue	Himedia, Mumbai, India
17	Glycerol	Himedia, Mumbai, India
18	Ammonium Persulphate	Himedia, Mumbai, India

All other chemicals used were of analytical reagent grade and were used without any further purification.

Instruments:

Sr. No.	Instruments	Company
1	Rotary evaporator	IKA Rotavapor RV-10, IKA® India Private Limited
2	Centrifuge (CPR-30)	Remi Elektrotechnik Ltd., Vasai, India
3	Shaker Incubator	Scigenics Orbirek, Mumbai, India
4	UV-visible spectrophotometer	Shimadzu 1800, Japan
5	RP-HPLC	Shimadzu LC-20AT, Japan

6	FT-IR Spectrometer	Bruker, Germany
7	Mini gel dual assembly	Genaxy scientific, Delhi, India.

6.3 Methods

6.3.1 Preparation of functionalized PEGylated double loaded liposomes

6.3.1.1 Identification of maleimide functional group of DSPE-mPEG 2000 maleimide

For identification of maleimide functionality in the pegylated lipid, Fourier transform infrared spectrophotometry was used. Fourier transform infrared spectrophotometer/FTIR (Bruker, Germany) range between 4000 and 400 cm−1, with a resolution of 2 cm^{-1} was used. All powder samples were compressed into KBr disks for the FTIR measurement.

6.3.1.2 Preparation Method

Functionalization of PEGylated double loaded liposomes were carried out according to the method previously reported with slight modification (5), (6). The quantity of DSPE mPEG 2000-Maleimie linker that was employed for functionalization was 1 mol%. A micellar solution of 1 mol% of DSPE mPEG 2000-maleimide was prepared and pre-equilibrated at temperature of 55°C in a round bottom flask. Optimized batch of PEGylated double loaded liposomes containing 5 mol% of DSPE mPEG 2000 was taken in round bottom flask, and the flask was maintained at a temperature above the Tg of the highest melting lipid i.e. at 55°C± 2°C in a rotary evaporator for 10 minutes. The flask was removed from the rotary evaporator and the above prepared pre-equilibrated micellar solution of linker lipid was slowly added drop wise to the DLPLs. This mixture was again allowed to incubate in rotary flask evaporator at 50 rpm in water bath at a temperature of 55°C for 2 hours. After incubation period, the functionalized DLPLs were passed through Sepharose CL-4B column to separate the micelles from the liposome fraction and collected for analysis of lipids. From the pooled samples, analysis for liposomes was carried out to determine particle size, zeta potential and amount of PTX.

6.3.1.3 Confirmation of functionalization

Ellman's assay was used for confirmation of post-insertion of DSPE-mPEG2000 Maleimide functional moiety to the DLPLs by determination of sulphydryl group available for reaction with the reagent. A known amount of thiols are reacted in excess with maleimide, after which the unreacted thiol present in the reaction is determined using Ellman's reagent. Thus, this method is an indirect method of estimation of maleimide. The quantitation of maleimide is then done using the following formula:

$$\text{Amount of maleimide} = \frac{\textit{Initial amount of thiol}}{\textit{amount of unreacted thiol after reaction}}$$

Herein, the thiol group for initial reaction with maleimide is furnished by using cysteine as standard, wherein cysteine is reacted in excess to conjugate each maleimide functional moiety. The excess cysteine remaining after the reaction is complete is quantitated using Ellman's assay.

The procedure for estimation is briefly described below:
Solution of Cysteine hydrochloride (1.5 mM) and Sodium phosphate (pH 8, 0.1 M) solution containing 1 mM EDTA were prepared. A set of three test tubes in triplicate was prepared as follows in table 6.1:

Table 6.1 Estimation of functionalization.

Sr no.	Test tube	Content
1	Reaction blank	Non-functional PEGylated liposomes (200µl) + 2.3 ml Sodium phosphate (pH 8, 0.1 M) containing 1 mM EDTA
2	Positive control	Non-functional PEGylated liposomes (200µl) + 250 µl Cysteine hydrochloride solution (1.5 mM) + 2.3 ml Sodium phosphate (pH 8, 0.1 M) containing 1 mM EDTA
3	Samples	Functionalized DLPLs (200µl) + 250 µl Cysteine hydrochloride solution (1.5 mM) + 2.3 ml Sodium phosphate (pH 8, 0.1 M) containing 1 mM EDTA

All the above tubes were mixed well and stirring was done in cold room overnight after purging the tubes with nitrogen gas and covering the mouth of tubes with plugs. After overnight stirring, 50µl of Ellman's reagent (prepared by adding 4 mg of reagent in 1 ml of 0.1 M sodium phosphate (pH 8.0) was mixed and stirred for 15 min at room temperature. The absorbance of the above solution was determine using UV visible spectrophotometer at 412 nm to determine the unreacted cysteine.

6.3.2 Fragmentation of Monoclonal Antibody

6.3.2.1 Preparation and purification of F(ab')$_2$ fragments of anti-FSHR antibody

For preparation and purification of F(ab')$_2$, Pierce™ F(ab')$_2$ Micro Preparation Kit from up to ten 0.125 ml samples containing 25-250 µg of IgG was used. Fragmentation of whole antibody to F(ab')$_2$ and its purification was carried out as described in the provided protocol along with the kit (7),(8).

1. Equilibration of immobilized pepsin

The immobilized pepsin vial was gently swirled to attain uniform suspension. From that 65 µl of slurry was taken using a cut pipette tip and placed in 0.8 ml spin column. The column was caped and placed into 1.5 ml microcentrifuge tube to centrifuge at 5000 x g for 1 min. After centrifugation, the collected buffer was discarded and the resin was washed using 130 µl of digestion buffer (20 mM sodium acetate pH 4.4, 0.05 % sodium azide) centrifuged again at 5000 x g for 1 min. The buffer collected was discarded and the washing step was repeated for 2 more times that allowed complete equilibration of immobilized pepsin using digestion buffer provided. At the end, bottom of the spin column containing equilibrated immobilized pepsin was plugged with rubber cover.

2. Preparation of IgG sample

The bottom closure of Zeba Desalt spin column was twisted off and its red cap was loosened. The Zeba Desalt spin column was then placed in collection tube and the column was centrifuged at 1500 x g for 1 min to take out storage solution. The mark was made on the outer surface of column where the compacted resin slanted ascending and this column was kept in centrifuge tube in such a way that the mark was facing external for all successive centrifugation steps. 300 µl of digestion buffer

was added into the Zeba Desalt spin column and it was centrifuged at1500 x g for 1 min. The buffer collected at the bottom after centrifugation was removed. This step was repeated for another 3 times and in each run the collected buffer was discarded. After this, the Zeba Desalt spin column was kept in a new collection tube and its cap was removed. To this Zeba Desalt spin column, the 250 µl of intact or whole monoclonal antibody (50 µg) was added slowly in the centre of the compacted resin bed. Then, the cap was placed again and it was centrifuged at at1500 x g for 2 min. The antibody sample was collected in collection tube after centrifugation.

3. Generation of Fragments

The 250 µl of prepared IgG sample (section 2) was added in the spin column comprising the equilibrated immobilized resin (section 1). The top cap of spin column was placed and the bottom was plugged using a rubber cover. It was briefly vortexed to ensure even mixing of antibody sample and immobilized resin contacting digesting peptide pepsin. Then, the digestion reaction was allowed to take place by keeping the column for incubation at 37 ^{0}C for 2.5 hr with constant end-over-end mixing. After specified period of time the bottom rubber cover of spin column was removed and it was placed in microcentrifuge tube to centrifuge at 5000 x g for 1 min to allow separation of digest from immobilized pepsin. The column was placed in a new microcentrifuge tube and further washed with phosphate buffer saline/ PBS (130 µl) by centrifuging at 5000 x g for 1 min. Both the digested fraction 250 µl and 130 µl were mixed to obtain 380 µl of total digested fraction. The digested fraction was evaluated by 10% SDS-PAGE under non-reducing conditions to confirm complete digestion. The bands of antibody fractions were visualized by silver staining method.

4. Purification of $F(ab')_2$ fragments

The Nab Protein A column, PBS and IgG elution buffer were allowed to equilibrate at room temperature. The bottom closure of Nab Protein A column was twisted off and the yellow cap was loosened. The column was then placed in a collection tube and centrifuged for 1 min at 1000 x g to take of the storage solution of the column. The column was equilibrated by adding 400 µl of PBS, briefly mixed and centrifuged at 1000 x g for 1 min. The flow-through was discarded. The step was repeated for 1 more time. The bottom of the equilibrated Nab Protein A column was plugged using a rubber cover and the obtained 380 µl of digest (section 3) was added

to this column. The column was capped tightly, mixed gently and incubated at room temperature for 10 min with end-over-end mixing. The bottom rubber cap was removed then and the cap was loosened. The column was kept in a new collection tube and centrifuged at 1000 x g for 5 min. The flow-through samples collected in the collection tube containing $F(ab')_2$ and Fc fragments was kept. For best recovery, the column was washed again using 200 µl of PBS and the step was repeated. The collected flow-through was mixed with the first fraction of $F(ab')_2$ and Fc fragments. The $F(ab')_2$ fragments were purified using ulrtacel membrane filter unit (50 KDa) from Fc fragments. The recovery and yield of $F(ab')_2$ fragments was estimated by Bradford method of protein estimation.

6.3.2.2 Reduction of F(ab')$_2$ to Fab'

Reduction of $F(ab')_2$ to Fab′ was carried out using DTT. DTT equivalent to 10 mM (approx. 15 µg) was added to $F(ab')_2$ solution previously comprising of 1 mM EDTA. This reaction mixture was kept for incubation for 1 hr at room temperature with continuous end-over-end mixing (Xu et al., 2002). The DTT was removed from Fab′-SH fragments using Zeba desalt spin column. After incubation, the samples were instantly transferred to the Zeba desalt spin column and allowed to incubate for 10 min at room temperature. The column was then placed into a new collection tube containing EDTA equivalent to 1 mM and was centrifuged at 1000 x g for 2 min to purify and separate Fab′-SH fragments from DTT. The Fab′-SH fragments were analysed by 10% SDS-PAGE under non-reducing condition to confirm complete reduction of $F(ab')_2$ to Fab′-SH. Buffers containing 1 mM EDTA was used throughout the procedure to avoid metal ions catalysed oxidation of reduced sulfhydryl groups to disulfide. Presence of –SH group in F(ab′)-SH was also estimated by Ellman's assay method. The prepared F(ab′)-SH fragments were then used for immunoliposomal formulation.

6.3.3 Preparation of Immunoliposomes

The Fab′-SH fragments were mixed and co-incubated with priorly functionalized PEGylated PTX loaded liposomes. The functionalized PEGylated liposome contains maleimide at the terminal end of PEG chain. The anti-FSHR Fab′-SH fragment and DSPE-mPEG2000 were mixed at molar ratio of 1:100 µM/ µM (weight ratio was 1:50 µg/µg). The incubation was kept overnight in cool conditions under N_2 atmosphere. After that, the ILs was incubated overnight in cool conditions/

cold room with excess quantity of cysteine so that the unconjugated leftover maleimide groups were occupied by cysteine. The extra unreacted cysteine was then removed from ILs using ultracel membrane (50 KDa). The prepared ILs were evaluated using 10% SDS-PAGE and were analysed for particle size, zeta potential, entrapment efficiency and % drug content. The total Fab′ conjugated on ILs was estimated by Bradford protein estimation method. The immunoreactivity of prepared Fab′ and ILs were also evaluated by Bradford's protein assay method.

6.3.4 Characterization of Immunoliposomes

6.3.4.1 Size, Zeta and Entrapment efficiency for ILs

The prepared ILs were characterized for particle size, zeta potential and % drug retained by the same method as reported in chapter 5.

6.3.4.2 Drug release study from ILs

The method for determination of the above parameters was as reported in chapter 5.

6.3.4.3 SDS-PAGE analysis

The SDS-PAGE (Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis) technique was performed to evaluate and confirm digestion of intact whole anti-FSHR (Follicle Stimulating Hormone Receptor) monoclonal antibody (mAb) into $F(ab')_2$, reduction of F(ab′) to Fab′-SH and conjugation of Fab′-SH to functionalized PEGylated liposomes to form ILs. The SDS-PAGE was performed under non-reducing condition i.e. the sample loading buffer was not containing a reducing agent (9, 10). The detailed procedure of SDS-PAGE is given as follows:

Gel Casting and Electrophoresis:

Reagents:

- ***30 % w/v of Acrylamide solution***

  It was prepared by addition of 29 gm Acrylamide (29% w/v) and 1 gm of Bis-Acrylamide (1% w/v) in 100 ml of milliQ water. The pH of prepared solution

was confirmed to be 7 or less. The solution was stored at 2-8 ^{0}C in dark amber coloured bottle.

- ***1.5 M Tris solution (pH 8.8)***

  18.16 gm of Tris base was dissolved in 100 ml of milliQ water to get a 1.5 M Tris solution. pH was checked and adjusted to 8.8 using HCl.

- ***1 M Tris solution (pH 6.8)***

  121.11 gm of Tris base was dissolved in 100 ml of milliQ water to get a 1.0 M Tris solution. pH was checked and adjusted to 6.8 using HCl.

- ***10% w/v SDS solution***

  10 gm of SDS (Sodium Dodecyl Sulphate) was added in 100 ml of milliQ water and heated to 70 ^{0}C to dissolve SDS completely. The volume was adjusted to 100 ml after heating.

- ***10% w/v APS (Ammonium persulphate) solution***

  100 mg of APS was weighed and dissolved in 1 ml of milliQ water. In each SDS-PAGE experiments it was freshly prepared.

- ***TEMED*** (N,N,N',N'-Tetramethylethane-1,2-diamine) was purchased from Himedia, Mumbai, India and used as such.

- ***Tris-Glycine Electrophoresis Buffer (Tank Buffer)- 5X***

  It was prepared by addition of 250 mM Glycine, 25 mM Tris base and 0.1 % SDS in milliQ water. The 5X buffer was stored at 2-8^{0}C and the working 1X buffer was prepared by diluting the 5X buffer with milliQ water to 1X.

- ***Sample Loading Buffer***

  It was prepared by addition of 60.5 mg of Tris-cl, 200 mg of SDS, 10 mg of Bromo Phenol Blue and 1 ml of glycerol in milliQ water and the volume was made up to 10 ml.

- ***1X SDS loading buffer***

  50 mM tris-Cl (pH 6.8), 2% w/v SDS, 0.1% bromophrnol blue, 10% v/v glycerol.) – Composition of resolving gel and stacking gel are provided in Table 6.2 and Table 6.3 respectively.

Table 6.2 Composition of resolving gel.

Sr. No.	Components	10 % Resolving Gel*
1	H_2O	1.9 ml
2	30% w/v Acrylamide solution	1.7 ml
3	1.5 M Tris solution (pH 8.8)	1.3 ml
4	10% w/v SDS (Sodium Dodecyl Sulphate) solution	50 µl
5	10% w/v APS (Ammonium persulphate) solution	50 µl
6	TEMED	4 µl
	Total Volume	~ 5 ml

Note: APS and TEMED were added at the end.

* Depending on molecular weight of protein of interest the gel % was chosen.

Table 6.3 Composition of stacking gel.

Sr. No.	Components	10 % Resolving Gel*
1	H_2O	1.4 ml
2	30% w/v Acrylamide solution	333 µl
3	1.0 M Tris solution (pH 6.8)	250 µl
4	10% w/v SDS (Sodium Dodecyl Sulphate) solution	20 µl
5	10% w/v APS (Ammonium persulphate) solution	20 µl
6	TEMED	3 µl
	Total Volume	~2 ml

Note: APS and TEMED were added at the end.

6.3.4.2 Silver staining method

The optimized laboratory protocol was used for silver staining.

1. After complete run of SDS-PAGE electrophoresis, the gel was washed with distilled water for 10 min.
2. The gel was then kept in the fixing solution (Methanol: Glacial Acetic Acid: Distilled water; 30: 10:60 v/v) overnight with gentle shaking.

3. After specified time period the fixing solution was removed and gel was transferred to freshly prepared 30% v/v methanol in distilled water. The gel was kept in 30% methanol solution for 30 min with gentle shaking at room temperature
4. The gel was then washed with distilled water twice, each for 5 min with gentle shaking at room temperature.
5. The gel was then incubated in a freshly prepared Sodium thiosulphate pentahydrate solution (0.02% w/v $Na_2S_2O_3.5H_2O$) for 1-2 min.
6. The gel was then washed with distilled water for 5-10 secs.
7. Gel was then incubated in 0.2% w/v of silver nitrate ($AgNO_3$) solution at room temperature for 30 min with gentle shaking.
8. The gel was rinsed using distilled water.
9. Gel was then incubated in developing solution (2.25 w/v Na_2CO_3 solution, 0.05% v/v formaldehyde taken from its 40% standard solution and 2 ml of 0.02% w/v solution of $Na_2S_2O_3.5H_2O$) for around 10-15 min till the band develops.
10. Reaction was then stopped by transferring the gel in stopping solution (10% v/v of Glacial acetic acid or 1.4% w/v EDTA solution). The gels were photographed and preserved.

6.3.4.3 Ellman's assay

Before incubation of prepared Fab′-SH fragments with the functionalized liposomes, the presence of sulfhydryl groups on Fab′-SH was estimated by Ellman's assay method. The detailed procedure has been described in chapter 3 analytical methods. Briefly, the sample from prepared Fab′-SH fragment solution (10 µl of Fab′-SH or ~ 0.700 µg of Fab′-SH fragments) was diluted to 250 µl with reaction buffer (0.1 M sodium sulphate, pH 8 containing 1 mM EDTA) in one eppendorf tube. In another eppendorf tube 250 µl of only reaction buffer was taken. To both the tubes 2.5 ml of reaction buffer and 50 µl of ellman's reagent was added (4 mg of ellman's reagent was dissolved in 1 ml of reaction buffer). The solutions were mixed well and incubated for 15 min at room temperature. The absorbance if blank and the test samples were read at 412 nm wavelength using UV-visible spectrophotometer. The

absorbance of blank was deducted from the absorbance of the test samples. The sulfhydryl groups were estimated as described above in this chapter 3 (section 3.4.5)

6.3.4.4 Estimation of Fab' fragments

The 0.5 ml of prepared immunoliposomes was dissolved in methanol to extract the lipids. The methanol was then evaporated at 50 ^{0}C to get Fab′ and lipid residues. The leftover residues were then dissolved in 0.1 ml of distilled water. The undissolved lipids were separated by centrifugation. The supernatant water containing dissolved Fab′ fragments which were attached to immunoliposomes were estimated by Bradford's method of protein estimation. Thus, the amount of Fab′ fragments conjugated to immunoliposomes was determined.

Standard solution of Bovine serum albumin solution in milliQ was prepared at a concentration of 0.5mg/ml. Standard concentration of 0.25; 0.5; 0.75 and 1 µg of BSA was prepared as described in chapter 3. The volume in each well was made to 10 µl with milliQ water. As blank, 10µl of sample was added in a well. 10 µl of prepared Fab′ and priory extracted Fab′ from immunoliposomes were also transferred to 96 well plate. To each well 100µl of Bradford's reagent was added to each well and absorbance was taken at 595 nm using ELISA plate reader. The estimation was carried out in triplicate and the concentration of protein was determined using software automatically using BSA as standard.

6.4 Result and Discussion

6.4.1 Preparation of functionalized PEGylated double loaded liposomes

6.4.1.1 Identification of maleimide functional group of DSPE mPEG 2000 maleimide

The chemical structure of DSPEmPEG2000 maleimide is presented in the figure 6.5 below. The presence of maleimide group was confirmed by FTIR spectroscopy (Figure 6.6) along with the characteristic peaks exhibited by functionalized lipid (Table 6.4).

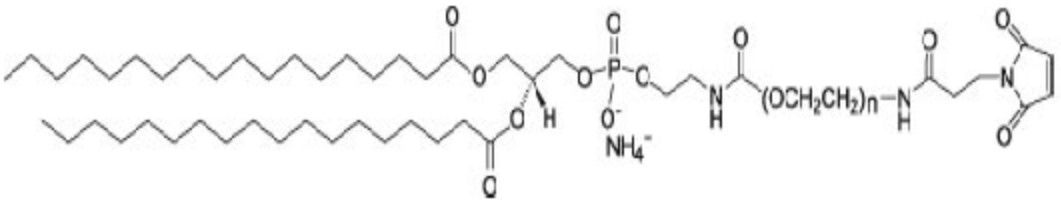


Figure 6.5 DSPE-mPEG2000-Maleimide structure

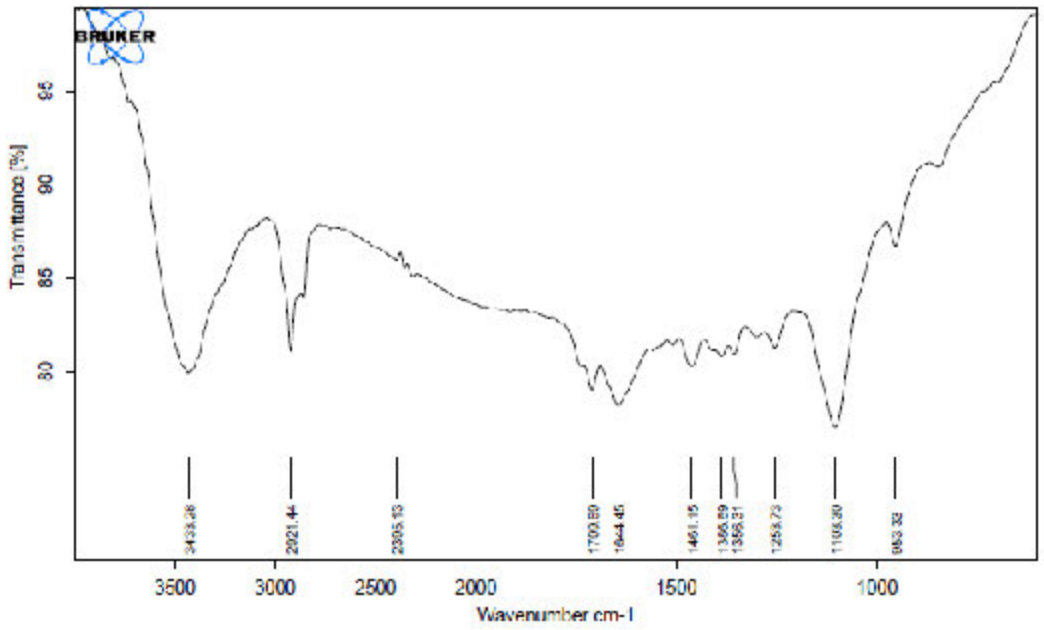


Figure 6.6 FTIR of DSPE mPEG2000 maleimide

Table 6.4 The characteristic peaks exhibited by the functionalizing lipid.

Type	Wave number (cm^{-1})
N-H bending	3433.28
C-H stretching	2921.44
C=O stretching imide	1709.69
C=O stretching amide	1644.45
C=O stretching imide	1461.15
	1103.30
C=O imide	836

6.4.1.2 Preparation Method

The selection of post insertion technique for conjugating the maleimide functional group was made as herein all the functional groups are exposed to the outer surface of the liposomal membrane and thus all will be available for conjugation with ligands. Wherein, in case of pre-insertion technique, the functionalizing lipid is co-dissolved in organic solvent with other lipids, in which case a part of the functional

group also get oriented to the inner membrane of the liposomes and is not available for conjugation reaction.

This post-insertion method is specifically useful in cases wherein antibodies, which are thermosensitive, are to be conjugated to the liposomes as only using mild reaction conditions are involved thus preserving the biological activity of the ligands. Herein, in a single incubation step carried out using post insertion technique, time and temperature dependent transfer of coupled ligand-lipid conjugate to the bilayer of drug loaded liposomes occurs that follows after coupling reaction between ligand and terminus of PEG-lipid derivative. We have used the above principle to conjugate functional lipid in micellar phase to the surface of PEGylated liposomes using only mild conditions of incubating at room or cold temperature.

Herein, micellar solution of functional lipid at 1mol% was prepared and transferred to the surface of PEGylated double loaded liposomes with a slight modification of the method as described. No impurity was formed after the conjugation reaction was carried out at 55°C for 2 hours and further after separation of functionalized liposomes after reaction, no change in drug content and particle size was observed.

6.4.1.3 Confirmation of functionalization

The confirmation of insertion of functional lipid was done by Ellman's assay with slight modification as reported in the previous section. The difference in amount of initial thiol content to the amount of thiol unreacted after completion of reaction of all maleimide groups gives the amount of maleimide conjugated to the DLPLs. Here, absorbance values for blank are subtracted from both the positive and samples test tubes (Table 6.5).

Table 6.5 Ellman's assay: Determination of reacted thiol concentration

Sample	*Concentration*
Positive control test tubes: Total thiol concentration	$1.322 \times 10^{-7} \pm 0.146 \times 10^{-7}$ moles
Sample test tube: Thiol unreacted with functionalized liposomes	$0.6639 \times 10^{-7} \pm 0.130 \times 10^{-7}$ moles
Thiol reacted with functionalized liposomes	$0.6581 \times 10^{-7} \pm 0.023 \times 10^{-7}$ moles

Values are mean±SD, n=3.

Calculation method for determination of free sulfhydryl content:

Molar absorptivity of Ellman's reagent (DTNB: 5,5-dithio-bis-(2-nitrobenzoic acid)) =

14,150 $M^{-1}cm^{-1}$(at 412 nm)

Molar absorptivity E = A/b*c

 c= A/E*b

Where, A = absorbance; b = path length (cm); c= concentration in moles/liter or molarity (M)

Absorbance reading for Blank Test tube = 0.256

Absorbance reading for positive control samples = 0.9241

Corrected absorbance reading for positive control samples = 0.9241-0.256 = 0.6681

 c = 0.6681/14,150*1

= 4.722 x 10^{-5}M/litre

Applying the correction factor for dilutions made:

Reaction buffer = 2.3 ml

Non-functionalized PEGylated liposomes = 200µl

Cysteine solution = 250 µl

Ellman's reagent solution = 50µl

Total volume = 2.8 ml.

Thus, the number of moles in the volume of 2.8 ml of above solution

= 2.8 x 4.722 x 10^{-5}/ 1000 = 1.322 x 10^{-7} moles present in positive control test tube.

Similarly, for estimation of sulfhydryl group in the sample test tubes, that determines the amount of maleimide group available for conjugation with the ligand (antibody), the values were obtained by subtracting the test samples thiol concentration from the positive control samples as provided in Table 6.5.

6.4.2 Fragmentation of Monoclonal Antibody

In present work, a Fab′ fragments of anti- FSHR monoclonal antibody have been armed over the liposomal surface loaded with paclitaxel for target based therapy of ovarian cancer. The use of Fab′ fragments as a targeting ligands instead of whole antibody could eliminate the Fc portion of whole antibody and its associated

immunogenic effects. The recognition of Fc portion was also avoided by phagocytes and thus RES (reticuloendothelial system) clearance of Fab′ was reduced providing a long circulation. In contrast to whole monoclonal antibody (MW: ~ 150 KDa), a small size Fab′ (MW: ~ 50 KDa) can have better tumor or cell penetration efficiency. Additionally, Fab′ fragments could be easily grafted over the liposomal surface comprising of terminal maleimide functional groups through which the thiol groups of the hinge region binds (Figure 6.1) (11).

Enzymatic digestion of whole monoclonal antibody by pepsin results into fragmentation of whole intact antibody to a large fragment $F(ab')_2$ (MW: ~ 110 KDa) and small FC fragments. The $F(ab')_2$ contains two antibody binding sites held together by retention of the hinge region's disulphide bonds and the extensive digestion process yields degradation of Fc portion in small fragments (Figure 6.2 and 6.3) (12). $F(ab')_2$ by reducing agent such as DTT (dithiothreitol) results in two Fab′ fragments each of which contains one antibody binding site.

Thus, in this work we have generated a purified and immunoreactive $F(ab')_2$ fragments of anti-FSHR monoclonal antibody using Pierce™ $F(ab')_2$ Micro Preparation Kit from up to ten 0.125 ml samples containing 25-250 µg of IgG. Briefly, the procedure involves digestion of whole antibody first by immobilised pepsin enzyme followed by the purification of $F(ab')_2$ fragments from the undigested whole antibody using protein A column supplied with the kit. The prepared $F(ab')_2$ fragments were reduced to Fab' or Fab′-SH by reacting them with 10 mM DTT reducing agent. SDS-PAGE gel electrophoresis technique was used to confirm process completion of digestion of whole antibody to $F(ab')_2$, its purification and its reduction to Fab′-SH using 10% gel under non-reducing conditions. Reduction of $F(ab')_2$ to Fab′-SH may take place during the experiments when samples are heated with loading buffer containing a reducing agent giving a false interpretation and thus to avoid such situation, non-reducing (no reducing agent in sample loading buffer) was selected for the SDS-PAGE technique. Buffers containing 1 mM EDTA was used throughout the procedure in preparation of samples to avoid metal ions catalysed oxidation of reduced sulfhydryl groups to disulfide.

Silver staining was performed to permanently stain the proteins separated after SDS-PAGE gel electrophoresis as an efficient and the most sensitive staining method.

As the quantity of proteins present in this work antibody (mAb), $F(ab')_2$, Fab′ may be present in very less amount and thus silver staining method was chosen as the most sensitive one amongst all the staining methods available detecting proteins even in the nanogram levels. Thus, when the amount of antibody is less and we need to manage the experiments in small quantities of antibody, this method could help to stain small quantities of proteins or antibodies.

The results of SDS-PAGE gel electrophoresis after silver staining has been shown in Figure 6.7. Lane 2 is a standard protein marker showing bands with a known molecular weight. In lane 1, intact whole monoclonal antibody was loaded showing band ~ 150 KDa. Lane 3, was loaded with the prepared and purified $F(ab')_2$ fragments showing band ~ 120 KDa. There were no other bands seen in this lane confirming the purity of $F(ab')_2$ from undigested antibody and Fc fragments after digestion. Lane 4, confirms complete reduction of $F(ab')_2$ to Fab′ as it was loaded with the Fab′-SH showing band ~ 50 KDa after reduction. There was no band present for $F(ab')_2$ and complete reduction of $F(ab')_2$ was confirmed. The lane 5 was loaded with the standard BSA as a reference control showing band at ~ 65 KDa. The lane 6 was loaded with pepsin digest. In lane 6 one band was observed ~ 120 KDa corresponds to the presence of $F(ab')_2$ fragments. Other bands seen in lane 6, ~ 35 KDa and ~ 30 KDa were may be due to the presence of small Fc fractions of the digest. There was no band seen ~ 150 KDa corresponding to the undigested antibody, thus the results indicate complete digestion of whole anti-FSHR mAb.

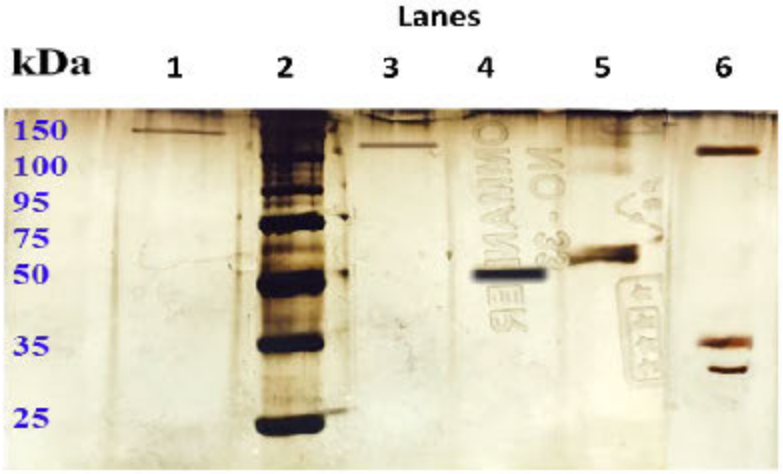


Figure 6.7 SDS PAGE gel after silver staining for antibody

6.4.3 Preparation of Immunoliposomes

The immunoliposomes were prepared by incubation of Fab'-SH fragments with functionalised PEGylated liposomes loaded with paclitaxel in cold room overnight. The incubation was followed by addition of excess amount of cysteine to neutralise the unreacted maleimide groups. The unconjugated Fab'-SH fragments and excess cysteine were separated to purify immunoliposomes using ultracel-50 membrane (50 KDa MDCO). The lipids concentration was estimated by Stewart method (Stewart, 1980 #10) and found to be 0.0116 mM/ml of the final immunoliposomes.

Table 6.6 Characterization of ILs

Formulation	Mean Particle size (nm) and PDI	Zeta potential (mV)	% Drug retained	% Antibody content on ILs
PEGylated	153.24±2.94	-3.27±0.09	96.45±1.65	-
ILs	165.79±3.81	-6.46±0.19	95.12±2.02	90.49±2.96

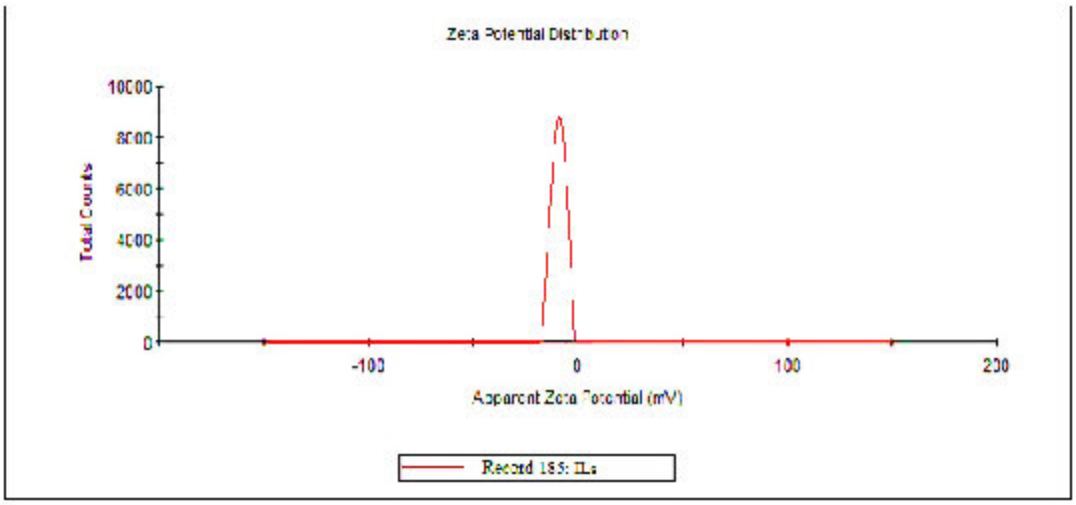


Figure 6.8 Zeta potential for ILs

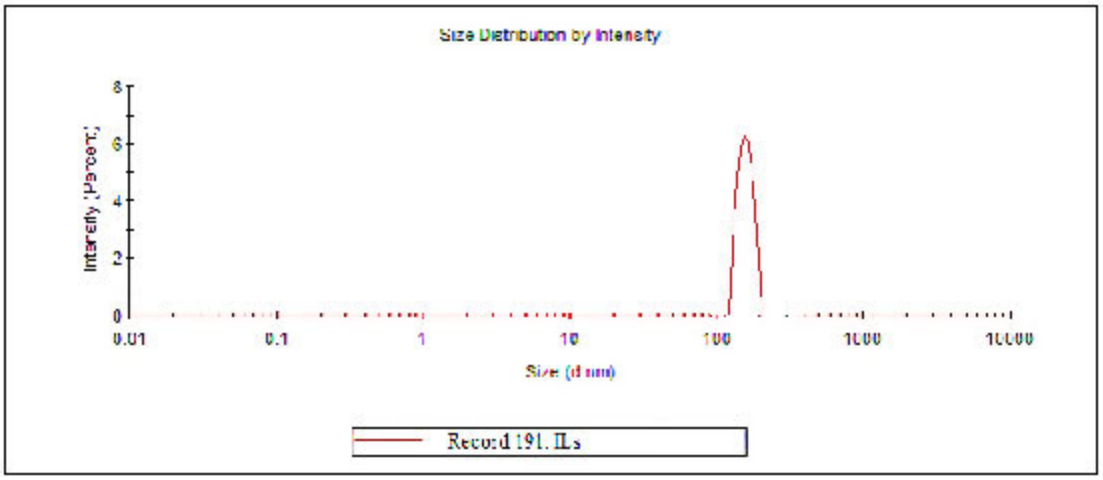


Figure 6.9 Size distribution for ILs

6.4.4 Characterization of Immunoliposomes

6.4.4.1 Size, Zeta and Entrapment efficiency for ILs

The Table 6.6 shows the mean particle size, zeta potential, percent drug retained and the antibody content of prepared immunoliposomes along with zeta potential and size distribution of ILs in figure 6.8 and figure 6.9. No significant change in size, zeta potential of % drug content was observed due to the conjugation of Fab′ fragments to form immunoliposomes as compared to PEGylated ones.

6.4.4.2 Drug release study:

The release of PTX from the ILs displayed similar release profile as that of DLPLs. This confirms that the functionalization of liposomes has not impacted the surface characteristic of liposomes. Figure 6.10 shows in vitro release profile of PTX from ILs which was found to be similar as compared to DLPLs. Thus, conjugation of antibody fragments on liposomes have not altered it drug releasing profile.

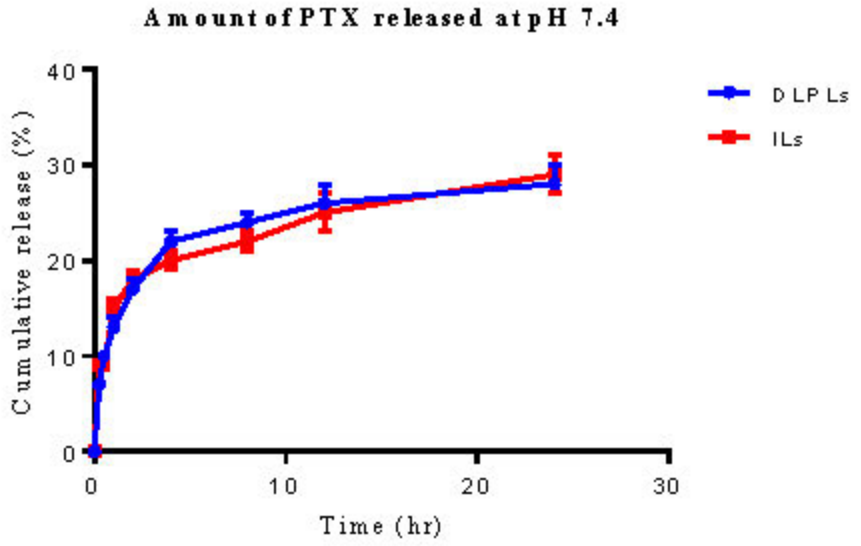


Figure 6.10 In vitro release of PTX from DLPLs and ILs up to 24 hr at 37 °C in phosphate buffer saline at pH 7.4

6.4.4.3 Ellman's assay

The presence of sulfhydryl groups on reduced Fab′ fragments were determined by Ellman's assay. The quantity of sulfhydryl/thiol group present in tested 10 µl or ~0.700 µg of Fab′-SH fragments sample was calculated as follows;

Molar absorptivity of Ellman's reagent (DTNB: 5,5-dithio-bis-(2-nitrobenzoic acid)) = 14,150 $M^{-1}cm^{-1}$(at 412 nm)

Molar absorptivity E = A/b*c

 c= A/E*b

Where, A = absorbance; b = path length (cm); c= concentration in moles/liter or molarity (M)

A= 0.171

Thus, C=0.156/1 (14,150) M

= 1.208 x 10^{-5} M/litre

This value represents the concentration of sulfhydryl groups of the solution filled in the cuvette. The real concentration would be after considering dilution factor.

So, the total volume of solution measured was;

Reaction Buffer-2.5 ml

Unknown sample-0.25 ml

Ellman's reagent solution- 0.05 ml

So the total volume was 2.8 ml.

Now if 1000 ml contains 1.208×10^{-5} M; 2.8 ml contains;

$= (2.8 \times 1.208 \times 10^{-5})/1000 = 3.036 \times 10^{-8}$ moles

Thus, the 10 µl or ~0.700 µg of Fab′-SH fragments contain 3.036×10^{-8} moles of thiol. The results indicated the presence of sulfhydryl groups on the prepared Fab′ fragments as it gets conjugated via maleimide thioether linkage with liposomes.

6.4.4.4 Estimation of Fab' fragments

The fab′ concentration in the unknown sample was estimated by Bradford's protein estimation method (as described in chapter 3; analytical methods). The immunoliposomes were prepared by incubating known amount of Fab′ fragments (~ 15 µg or ~ 0.4 µM) with functionalised DLPLs. The incubation was carried out at various molar ratios of Fab′-SH and functionalized lipids i.e. 1:50w/w.

The concentration of Fab′ fragments conjugated over liposomal surface was estimated by Bradford protein estimation method which was found to be ~ 13.5 ± 0.15 µg/ml (0.0116 mM/ml of total lipids). Thus, % Fab′ fragments conjugated over liposomal surface was found to be 90.49±2.96%.

6.5 References

1. Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. Nature reviews Drug discovery. 2005;4(2):145-60.
2. Janzen J, Song X, Brooks DE. Interfacial thickness of liposomes containing poly(ethylene glycol)-cholesterol from electrophoresis. Biophysical Journal. 1996;70(1):313-20.
3. Schelte P, Boeckler C, Frisch B, Schuber F. Differential reactivity of maleimide and bromoacetyl functions with thiols: application to the preparation of liposomal diepitope constructs. Bioconjugate chemistry. 2000;11(1):118-23.
4. Oswald M, Geissler S, Goepferich A. Determination of the activity of maleimide-functionalized phospholipids during preparation of liposomes. International journal of pharmaceutics. 2016;514(1):93-102.
5. Allen TM, Hansen C, Martin F, Redemann C, Yau-Young A. Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo. Biochimica et biophysica acta. 1991;1066(1):29-36.
6. Moreira JN, Ishida T, Gaspar R, Allen TM. Use of the post-insertion technique to insert peptide ligands into pre-formed stealth liposomes with retention of binding activity and cytotoxicity. Pharmaceutical research. 2002;19(3):265-9.
7. Lamoyi E, Nisonoff A. Preparation of F(ab')2 fragments from mouse IgG of various subclasses. Journal of immunological methods. 1983;56(2):235-43.
8. Fab: Thermofischer. Available from: https://tools.thermofisher.com/content/sfs/manuals/MAN0011655_Pierce_Fab2_Micro_Prep_UG.pdf
9. Laemmli UK. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature. 1970;227(5259):680-5.
10. SDS page protocol. Available from: http://www.molecularcloning.com/.
11. Maruyama K, Takizawa T, Takahashi N, Tagawa T, Nagaike K, Iwatsuru M. Targeting efficiency of PEG-immunoliposome-conjugated antibodies at PEG terminals. Advanced Drug Delivery Reviews. 1997;24(2):235-42.

12. Crivianu-Gaita V, Romaschin A, Thompson M. High efficiency reduction capability for the formation of Fab' antibody fragments from F(ab)2 units. Biochemistry and Biophysics Reports. 2015;2:23-8.