PREPARATION AND CHARACTERIZATION OF IMMUNOLIPOSOMES DIRECTED AGAINST METASTATIC CANCER

M.Sc. Thesis

By

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DISCIPLINE OF BIOSCIENCES AND BIOMEDICAL ENGINEERING

INDIAN INSTITUTE OF TECHNOLOGY INDORE

JUNE 2019

PREPARATION AND CHARACTERIZATION OF IMMUNOLIPOSOMES DIRECTED AGAINST METASTATIC CANCER

A THESIS

Submitted in partial fulfillment of the

requirements for the award of the degree

of

Master of Science

by

NEETU RAJAK

DISCIPLINE OF BIOSCIENCES AND BIOMEDICAL ENGINEERING

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **PREPARATION AND CHARACTERIZATION OF IMMUNOLIPOSOMES DIRECTED AGAINST METASTATIC CANCER** in the partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE** and submitted in the **DISCIPLINE OF BIOSCIENCES AND BIOMEDICAL ENGINEERING, Indian Institute of Technology Indore**, is an authentic record of my own work carried out during the time period from July 2018 to June 2019 under the supervision of Dr. Sharad Gupta, Assistant Professor, IIT Indore and Dr. Tathagata Mukherjee, Deputy General Manager- R&D, Gennova Vaccine Formulation Centre and Research Laboratory, Pune.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

Neetu 26-06-19

Signature of the student with date
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This is to certify that the above statement made by the candidate is correct to the best of my/our knowledge.

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Convener, DPGC
Date:

Signature of Dr. Avinash Sonawane
Date 26.06.2019

Signature of Dr.Hem C. Jha
Date

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Dr. Sanjay Singh, CEO, Gennova Biopharmaceuticals Limited (GBL), for giving me this opportunity to experience research venture in an industrial setup. I am also grateful to Prof. Pradeep Mathur, Director, Indian Institute of Technology Indore for providing us this opportunity to pursue a research experience.

I am incredibly grateful to my research guide, Dr. Sharad Gupta, for supporting me throughout the project and always encouraging me to give my best. He had shown unmatched faith in me. In spite of his busy schedule, he was still there to help me with my project as well as my academic problems. I am extremely thankful to Dr. Prashant Kodgire for recommending me to GBL. He always understood the career venture I wanted to choose. I am also grateful for all the suggestions and advice he gave me during my project.

I express my profound gratitude to my co-guide, Dr. Tathagata Mukherjee, for his crucial suggestions and guidance during the project. Despite all my mistakes, he constantly trusted me and encouraged me for the best. His dynamic and positive temperament inspires me. I am indebted for all the valuable guidance he showered me with, in my critical career decisions. In spite of his busy schedule, he made himself available to clear even my small doubts. He had molded me into a better person for life!

I would like to express my sincere gratitude to Dr. Praveen Agarwal for providing me with scientific insights during my project. I am inspired by his scientific attribute. I have learned so much from him. I am indebted for his valuable career advice. I would like to especially thank Ms. Veeni Awatramani for her guidance throughout the project. This project would not have come this far without her mentorship. She has been very patient in teaching me. She has always encouraged me to pursue my heart. She has helped me throughout my journey and always made sure that I was doing fine. I am grateful to have a mentor like her.

I am also grateful to Dr. Jaideep Moitra, Mr. Swarnendu Kavira, and Mr. Arjun Singh for giving me full freedom to work in their labs. I would like to extend my heartiest thanks to Mrs. Aditi Laghate and Mr. Ankit Shrivastava for teaching me various downstream techniques.

I sincerely thank Mr. Pavan Kardile, Dr. Durgesh Kumar, and Dr. Navdeep Raghuwanshi for their help and support. I am thankful to Mr. Tushar Bedre, Mr. Madhva, and Mr. Bhagwat for all the assistance they provided to me.

I would like to thank my colleague and friend Ms. Nidhi S. Kumar, Ms. Janhavi Gopal, Mr. Gaurav Adawade, and Ms. Akanksha Rawade, for their constant support. They have helped me with experiments and also in reviving the environment around me.

This acknowledgement cannot be complete without thanking my beloved senior, Ms. Saumya Jaiswal, who had helped me at my each difficult moment. I express my sincerest gratitude to Ms. Anshu Kumar for all the help and support. I would also like to thank all my Lab member for their support.

I would like to extend my gratitude to Dr. Debasis Nayak for his support and encouragement. I thank Dr. Parimal Kar, coordinator at IIT Indore, Dr. Kiran Bala, DPGC convenor, IIT Indore and Dr. Hem C Jha, HOD, BSBE, IIT Indore for all the support. I would also like to thank my project PSPC committee members Dr. Hem C Jha and Dr. Avinash Sonawane, for their valuable comments.

I especially thank my beloved friends Kalpana Kumari, Priyanka Yadav, Suchi Agrawal, Rahul Lamba, Shubham Chaudhary Mohini Phopse, Leesha Alva, Kakoli Aditya and Shubham Reddy for their help, support and for making this journey more beautiful.

Lastly, I express my deepest gratitude to my parents and my elder brother for their unparalleled support. They have always trusted me with all my decisions and stood by me at all times. I am grateful for their unconditional love.

Neetu Rajak

This thesis

Is dedicated to

My family, friends,

And

My mentor

Dr. Tathagata Mukherjee

Abstract

The clinical efficacy of conventional chemotherapeutics is limited due to poor pharmacokinetics of the drugs, limited systemic circulation lifetime, undesirable bio-distribution, and non-specific cellular uptake. The inability to deliver the therapeutic drug specifically to the target tissues results in severe and harmful toxic effects on normal organs and tissues. Thus, site specific delivery of anti-cancer therapeutics is an area actively being explored. One of the most promising platforms to achieve this is through the use of liposomal technology. Liposomes are lipid bilayer vesicles which can be used for encapsulation of a diverse array of biomolecules as well as synthetic drug molecules. Various liposomal drugs have even been commercialized. In addition to drug encapsulation, liposomes offer the advantage of being amenable for site-specific delivery by modification of their surface with various types of ligands such as peptides, carbohydrates and antibody fragments against antigens that are over-expressed on the surface of cancer cells. Due to their high specificity, antibodies have proven to be the most potential ligands for site specific delivery of liposomal drugs. Such liposomes conjugated with antibodies or their fragments are referred to as immunoliposomes. This study aims to explore the possibility of creating immunoliposomes with a therapeutic antibody targeted against metastatic cancers. The approach used includes digesting the antibody to generate different fragments and conjugating the appropriate fragment with functionalized liposomes followed by characterization of immunoliposomes for their structural and functional properties.

Keywords: Therapeutic, liposome, antibody, specific uptake, immunoliposomes.

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LIST OF ABBREVIATIONS

DLS	-	Dynamic light scattering
DSPE	-	1,2-Distearoyl-sn-glycerol-3-phosphoethanolamine
DTT	-	D,L-Dithiothreitol
EDTA	-	Ethylenediaminetetracaetic acid
ELISA	-	Enzyme linked immunosorbent assay
EPR-		Enhanced permeability and retention
HRP	-	Horseradish peroxidase
HSPC	-	Hydrogenated soy phosphatidylcholine
kDa	-	Kilo Dalton
LUV	-	Large unilamellar vesicle
M.W.	-	Molecular weight
MAL	-	Maleimide
MEA	-	Merceptoethylamine
MUV	-	Multilamellar vesicle
NFDM	-	Non-fat dry milk
PAGE	-	Polyacrylamide gel electrophoresis
PBS	-	Phosphate buffered saline
PDI -		Polydispersity index
PEG	-	Polyethylene glycol
SDS	-	Sodium dodecyl sulphate
SEC-		Size exclusion chromatography
SUV	-	Small unilamellar vesicle
TBS-		Tris buffered saline
TCEP	-	Tris(2-carboxyethyl)phosphine hydrochloride

Chapter 1

Introduction

Conventional chemotherapeutics used for the treatment of cancer show decadence over a period of time due to their various limiting aspects. Most anticancer drugs fail to differentiate between normal and cancerous cells, resulting in systemic toxicity as well as a non-specific distribution [1,2]. These factors intercept the adequate drug concentration at tumorous tissues and hence increase the chances of multi-drug resistance [3]. To counteract these problems, nanoengineered drug delivery systems (nDDS) are ideal. nDDS are nano-carriers designed for site-specific delivery of the encapsulated drug. The various types of nDDS studied to date are dendrimers, nanorods, micelles, nanoparticles, and liposomes. Out of all these systems, liposomes have been the most successful drug delivery carriers [4]. This is due to their biocompatibility, biodegradability, controlled release of drug, and ability to encapsulate diverse molecules [5,6].

1.1 Liposomes

Liposomes are spherical vessels composed of amphipathic lipid molecules with one hydrophilic head and two non-polar hydrophobic tails on the end [7]. When dissolved in an aqueous phase, the apolar aliphatic chains interact with each other through hydrophobic interactions while the polar head groups interact with the hydrophilic environment, forming a lipophilic layer encircling an aqueous core in the center as shown in Figure 1.1 [8,9].Liposomes can be prepared using artificial or natural phospholipid. The permeability and charge of these lipid bilayers can be controlled with the choice of phospholipids [10].Liposomes can be classified based on a number of lipid bilayers present. They are generally classified into two categories as unilamellar vesicles (ULV) and multilamellar vesicles (MLV).

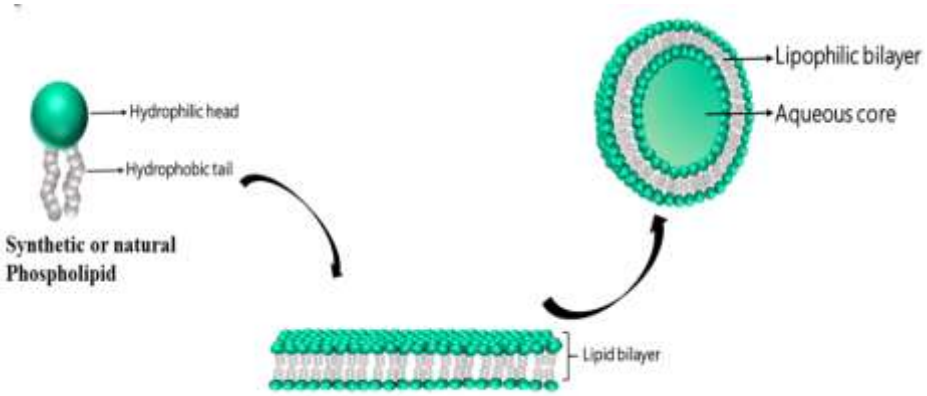

Figure 1.1 : Formation of liposomes- *Lipid molecules in aqueous solution form lipid bilayer followed by formation of liposomes.*

ULV have a single lipid bilayer and are further divided into small unilamellar vesicles (SUV), large unilamellar vesicles (LUV) and giant unilamellar vesicles (GUV) as shown in Figure 1.2 [11,12]. MLV contain two or more concentric lipid bilayers with sizes ranging between 1-5 µm [13]

1.1.1 Methods for preparation of liposomes

Various techniques are available for the preparation of liposomes. A method can be selected based on factors such as the concentration of drug to be loaded, desired half-life, and size of liposomes required, cost, and reproducibility for manufacturing [14].

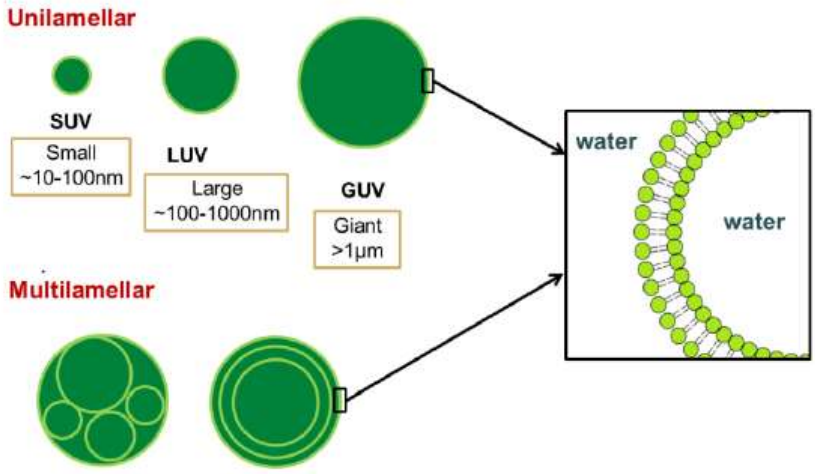

Figure 1.2: Classification of liposomes- *Structure of unilamellar liposomes and multilamellar liposomes [5].*

1.1.1.1 Reverse Phase Evaporation Method

This method includes the formation of inverted micelles followed by sonication in the organic phase and buffered aqueous phase. The organic phase is then slowly evaporated, which increases the viscosity of the solution [15]. Few micelle structures disintegrate into individual phospholipid, and these free-floating phospholipids complete lipid layer of residual micelle structures, converting them into a liposome [16]. Liposomes produced using this method have higher aqueous space and are suitable for drug delivery [10].

1.1.1.2 Thin film hydration

In this technique, lipid components are dissolved in an organic solvent followed by the evaporation of the solvent, to give a lipid film which is later dispersed in an aqueous solution [17]. Although very commonly used for encapsulating drugs within the liposomes, this technique has low encapsulation efficiency and gives liposomes with a high polydispersity index [18].

1.1.1.3 Detergent depletion method

In this technique, lipid film is hydrated with detergent. The detergent attaches to the hydrophobic sites of lipid chain and protects it from aqueous phase forming a micelle structure [19]. Later the detergent molecules are removed through dilution. This method usually results in low liposomal production. It has high encapsulation efficiency, but it usually produces large MLVs [20].

1.1.1.4 Ethanol injection method

In this method, the lipids are dissolved in ethanol, and this ethanolic solution is injected into saline or other aqueous media and mixed immediately so that the lipid molecules are dispersed homogenously in the solution [21].

After the preparation of liposomes, post-formation processing is required to obtain liposomes of the desired size and lamellarity. Few methods generally used for obtaining a homogenous liposome solution are sonication, extrusion, and high-pressure homogenization [18].

1.1.2 Sizing of liposomes

1.1.2.1 Sonication

The heterogeneous mixture of liposomes containing MLV and ULV are subjected to ultrasonic radiations. The high energy applied results in the formation of ULV. ULVs are then separated from MLV by ultracentrifugation [22].

1.1.2.2 High pressure homogenization

In this technique, the liposomal mixture is passed through a homogenizer. The homogenizer has a peristaltic pump. The solution is passed through a pump into a two piston pump. The piston is maintained at high pressure and maintained at a specific temperature [23]

1.1.2.3 Membrane extrusion method

In this method, the liposomal mixture is passed through a membrane filter using an extruder. The membrane filter of different pore size can be used. The extruder is maintained at lipid phase transition temperature, and the sample is passed under defined pressure [24-26]. The extruder contains different rings and membrane support. The extruder is arranged as shown in Figure 1.3 [27]

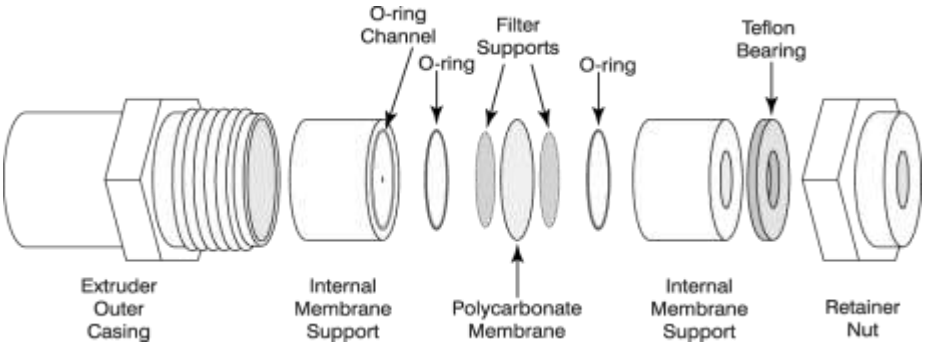

Figure 1.3 : Extrusion apparatus- *Assembly of extruder* [27]

1.2 Second generation liposomes: Stealth liposomes

To increase the therapeutic efficiency of liposomes, changes in the lipid composition, size, and charge of the vesicles are done. Interaction of high density lipoprotein (HDL) and low density lipoprotein (LDL) (present in blood plasma) with conventional liposomes results in the early release of the encapsulated drugs [8]. Incorporation of cholesterol in the liposomes is known to induce dense packing as well as to increase the stability of liposomes preventing their fusion with HDL or LDL [18].

Conventional Liposomes in the blood are easily recognized by Mononuclear phagocyte system (MPS) which could be escaped by coating the liposome with molecules to produce spatial barrier [13,28]. Poly-(ethylene glycol) is widely used to coat the liposomes. PEG contains a flexible chain when attached to liposome, the chains block the periliposomal space involved in recognition by immune cells and reduces the MPS uptake [28]. These long-circulating, PEGylated liposomes are known as stealth liposomes. PEG molecules are incorporated into liposomes using various approaches. The most widely used method is to use PEG-conjugated lipid molecule for liposomal preparation, e.g., DSPE-PEG as shown in Figure 1.4 [29]

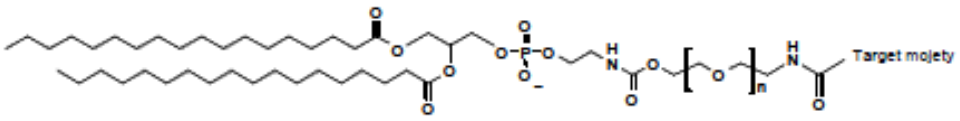

Figure 1.4 : Structure of PEG conjugated lipid molecule- *DSPE-PEG moiety* [29]

1.3 Targeting of liposomes-Passive and active targeting

Majority of liposomal carriers use passive targeting for delivery of drugs. Passive targeting of liposomes involves improved retention time of liposomes in the circulation by means of using PEGylation [30]. High retention time allows for exploitation of the EPR effect seen in tumor tissues. Generally, solid tumors lack a full-fledged vascular system, and

usually, their vasculature is highly fenestrated, which allows extravasation of nano-size molecules passively. This phenomenon is exploited for tumor drug delivery [31-34].

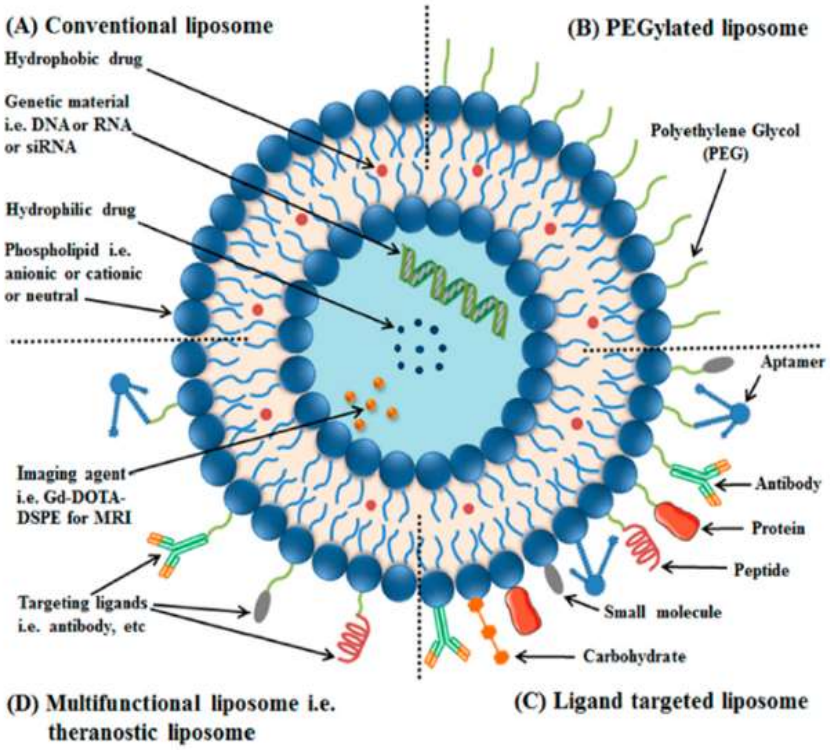

Figure 1.5 : Surface modified liposomes- *Liposomes are functionalized by conjugating functional moieties onto the surface of liposome* [34]

However, the sensitivity of target delivery is enhanced by coupling the overexpressed receptor ligands to the liposomal surface, also known as active targeting or ligand-based targeting [35]. This approach is carried out by conjugating the liposomes with ligands such as antibodies, peptides, or carbohydrate that can specifically attach to cell receptors as shown in Figure 1.5 [36]. These ligands can directly be attached to PEG chains or can be inserted into liposomes [37].

Active targeting of drugs emphasizes on targeting the overexpressed receptors or selectively expressed receptors on cancer cells for example

EGFR, FGFR, VEGF, folate receptors and transferrin receptors as shown in Figure 1.5 [38,39].

1.4 Immunoliposomes

Liposomes are conjugated to various types of ligand for active targeting. Immunoliposomes are liposomes conjugated with antibody or antibody fragment [40]. Liposomes can be conjugated to either the whole antibody or antibody fragment. Various coupling techniques are available for ligation of ligand to liposomes.

1.4.1 Antibody Fragmentation

Structurally, antibodies can be divided into multiple fragments using appropriate methods. Enzymatic digestions are the most commonly used methods used for generating these antibody fragments as shown in [41]. The 2 main fragments generated by enzymatic digestion are as follows:

1.4.1.1 Fab fragments

Fab fragments consist of a complete light chain (variable V_L + constant C_L) joined to a variable (V_H) and a portion of the constant region (C_{H1}) of the heavy chain by di-sulphide bonds as shown in Figure 1.6 [41].

The enzyme papain digests immunoglobulin into two Fab fragments, and an Fc fragment composed of two heavy chains joined to each other by di-sulphide bonds at the hinge region. Fab fragments recognize and bind to antigens, whereas the Fc portion helps in eliciting effector immune responses [42].

1.4.1.2 F(ab')$_2$ fragments

The enzyme pepsin digests an antibody into one F(ab')$_2$ fragment and degraded Fc fragments [43]. Since F(ab')$_2$ fragments are held together by disulphide bonds, mild reduction of F(ab')$_2$ fragments result in the production of Fab' fragment which contains a free thiol group at the hinge

region along with an antigen binding site [44]. The structures of the F(ab')$_2$, Fab' and Fab fragments are shown in Figure 1.6.

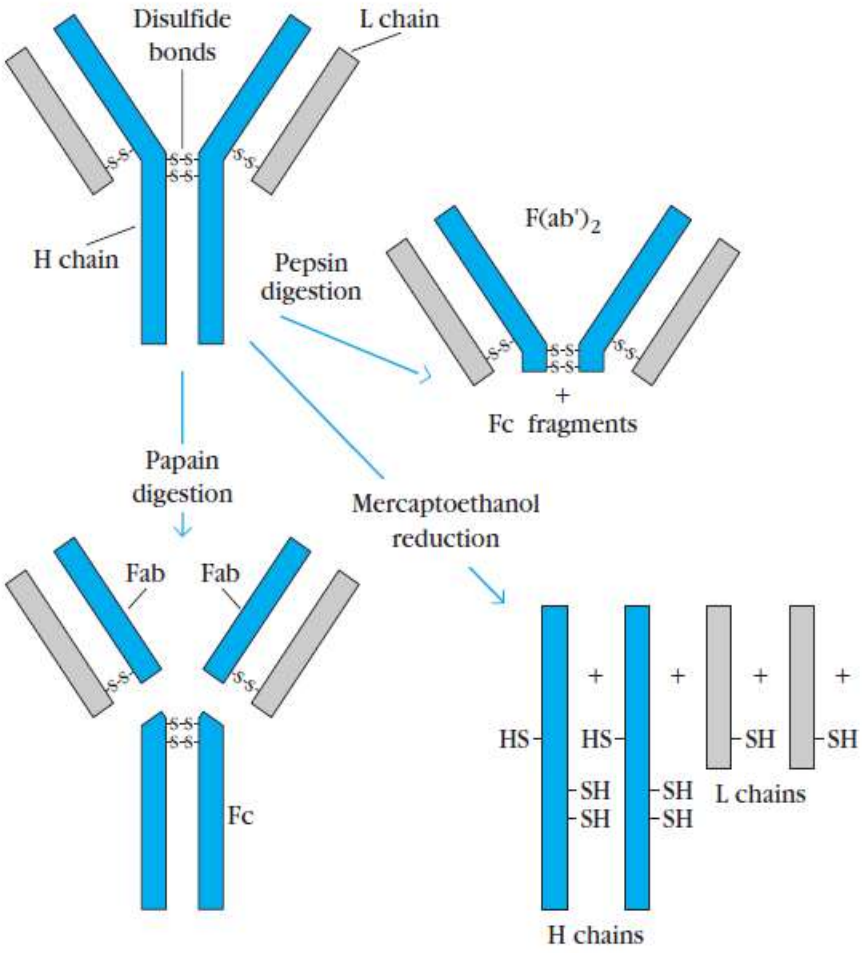

Figure 1.6 : Structure of antibody fragments- *Enzymatic digestion of IgG into F(ab)$_2$, Fab', light and heavy chain* [41]

1.5 Conjugation of antibody fragments with liposomes

Antibody fragments are coupled to liposomes using various methods that are simple, reproducible, stable, non-toxic, and does not alter the efficiency of therapeutic effect [44]. Generally, stealth liposomes (PEGylated liposomes) are used for preparing immunoliposomes due to their high circulation time. Different end-functionalized-PEG molecules are used for the preparation of stealth liposomes. These functionalized PEG molecules are linked to lipid moiety and contain a reactive functional group.

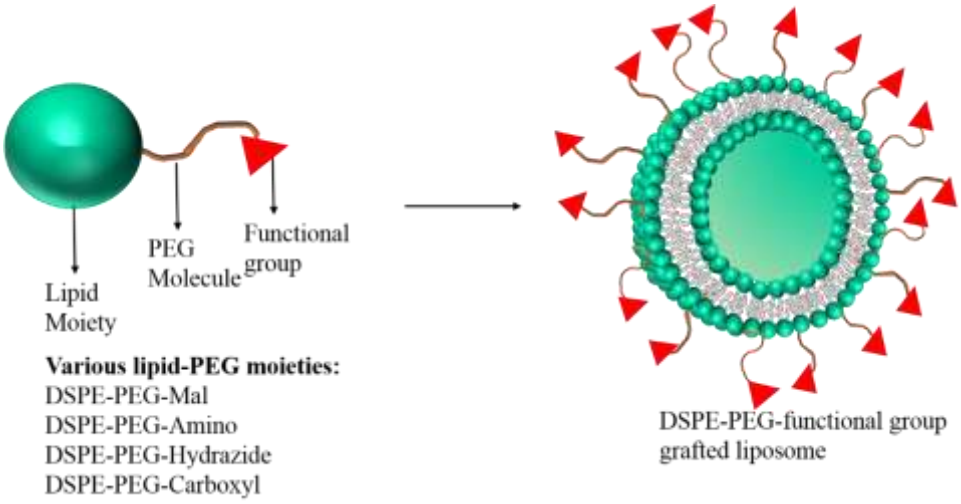

Figure 1.7: Surface functionalized liposome: *Liposomes prepared using PEG-function group conjugated lipids*

Several PEG molecules popularly used are DSPE-PEG-Maleimide, DSPE-PEG-NHS, DSPE-PEG-Amine, and Azide-PEG-NHS, etc. Liposomes prepared using these derivatized lipid-PEG contain the reactive functional group onto the surface, as shown in Figure 1.7, which is used for conjugation to antibody fragment through bond formation [44].

Different conjugation chemistry has been employed based on the chemical groups present on protein and liposome.

1.5.1 NHS-ester conjugation chemistry

N-Hydroxysuccinimide-ester (NHS-ester) reacts with primary amines under the slight alkaline condition to produce amide group as shown in

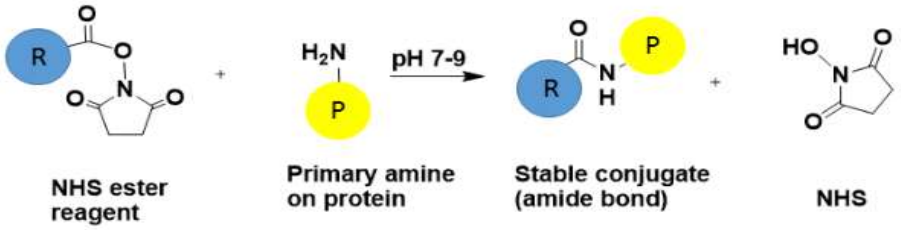

Figure 1.8: NHS-ester conjugation chemistry: *R-molecule with NHS group (antibody, lipid or any molecule), P-molecule with NH_2 group*

Figure 1.8, amines are present at the N-termini of each protein (α-amine) and in the side chain of lysine residues (ε-amine). NHS-ester conjugation chemistry is not appropriate for antibody-liposome coupling. Since it involves N-terminal amine where the antigen binding site of an antibody is present, it can affect the antigen binding efficiency of antibody [45].

1.5.2 EDC conjugation chemistry

1-Ethyl-3-(3-dimethylaminopropyl) carbodimide reacts with the carboxylic acid group. The COOH-group is present at the C-termini of protein and also

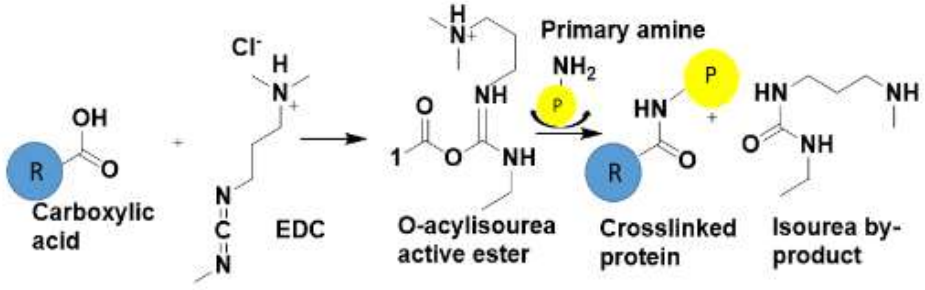

Figure 1.9: EDC conjugation chemistry- *R is molecule with carboxylic group (antibody, lipid or any molecule); P is molecule with NH_2 group (antibody, lipid or any molecule)* [23]

in the side chain of acidic amino acid residue (aspartic acid and glutamic acid). EDC reacts with COOH-groups to produce O-acylisourea intermediate. The O-acylisourea is immediately substituted by nucleophilic attack of primary amino groups. This primary amine group forms an amide bond with the COOH-group [45]. The reaction proceeds as shown in Figure 1.9

1.5.3 Maleimide conjugation chemistry

Maleimide reacts with SH-group irreversibly, forming a thio-ether bond. The reaction is favorable at near neutral pH ~6.5 to 7.5. The SH group is found in the side chain of cysteine residue present in the protein. Maleimide conjugation chemistry is most appropriate for conjugation of antibody fragments because antibody fragment (Fab') contains free thiol group at the

hinge region [45]. The maleimide reacts with thiol as shown in the Figure 1.10

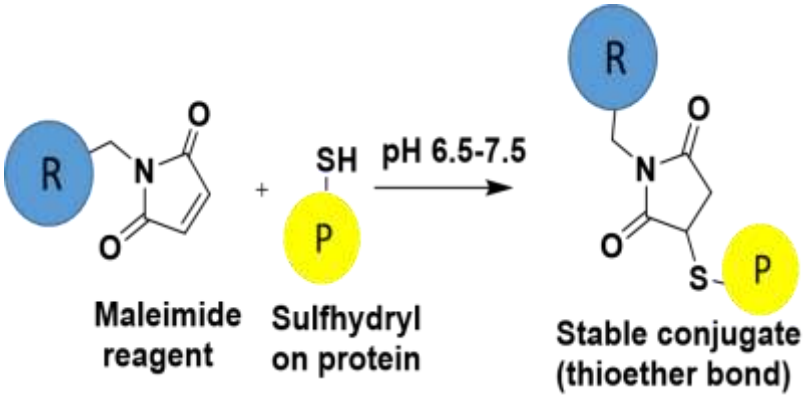

Figure 1.10: Thiol-maleimide conjugation chemistry-*R is any molecules thiol maleimide group and P is any molecule with sulfhydryl group*

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Chapter 2

Review of literature

Cancer is a disease caused due to the abnormal growth of cells. It is one of the leading cause of death worldwide with an ever increasing number of occurrences [46]. It is caused due to accumulated abnormalities in cell cycle regulation, which results in uncontrolled cell growth referred as a tumor. The tumor can be benign or metastatic in nature [47]. A tumor that remains restrained to its original location and lacks the ability to invade neighboring tissue is referred to as benign tumors. However, in benign tumor can be treated by surgery.

Only tumors that have the property to spread to other body parts are classified as cancer such as metastatic cancer. In the case of metastatic cancer, the cells of the primary tumor are able to invade distant body parts. Thus the treatment of metastatic cancer is more complex due to its metastasizing property [48,49]. Metastasis is the major cause of all cancer related deaths [50]. The cancer keeps spreading to different parts and cannot be treated with localized therapies such as surgery and radiation. Majorly chemotherapy is employed for the treatment of cancer [51].

Chemotherapy involves the application of drugs or chemicals to destroy cancer cells. Mostly, cancer cells take up the drug more rapidly because of their high metabolic rate, but few other normal cells such as small intestine cells, bone marrow cells, and hair follicles also take up the drugs non-specifically. It results in non-specific toxicity to cells. Conventionally, anticancer drugs are administrated via orally or intravenously. Oral administration of drugs exposes it to various metabolic pathways resulting in disordered pharmacokinetics and decreased effective dose [52]. It will then be required to administer larger doses, which will further increase the toxicity [53]. In many cases, anticancer drugs synthesized from a natural

plant source or synthetic are hydrophobic, making it insoluble [54]. Thus free drug administration invites various problems of cytotoxicity, insolubility, and bioavailability.

Liposomal encapsulation of drug limits the cytotoxicity increases bioavailability, and prevents biodegradation of drugs. Liposomes passively accumulate in tumor tissues via EPR effect, as shown in Figure 2.1 [55,56]. Tumor tissue vasculature shows wide fenestration. The gap between capillary endothelium of tumor blood vessels is wider (100 to 780 nm) as compared to the narrower (5 to 10 nm) gaps in normal tissue vessels [57]. The liposomes translocate through these gaps selectively into tumor tissues, a process termed as extravasation. Liposomes of size smaller than 400 nm have been shown to extravasate into tumor tissue. However, extravasation of liposomes of size <200 nm is much more efficient, as shown by Hobbs et al. (1998) [58]. Also, tumor tissue lacks a proper lymphatic drainage system, which results in retention of liposomes in the vicinity [59]. The circulation of these liposomes in blood vessels is increased by coating with PEG molecules. The PEG molecule produce steric hindrance. Thus preventing the liposomal-blood interaction and reducing the RES uptake [57].

FDA had approved 6 nano-size medicine: DaunoXome, Marqibo, Abraxanerentuximab, Trastuzumab emtansine, and Doxil, Majority of them are liposomal formulations [60]. Doxil is a liposomal formulation, in which doxorubicin is encapsulated inside the aqueous core of liposomes. Encapsulating doxorubicin increases its in-vivo circulation half-life and toxicity as compared to the drug itself. Doxil is used for the treatment of Kaposi's sarcoma, ovarian cancer, and multiple myeloma. [61]. However, Doxil has also been reported to show skin toxicity in feet and hands [61]. This is because of the non-specific delivery. This shows that even liposomal drugs are not very specific.

The specificity of liposomes is further increased by conjugation with ligands such as antibodies or peptides, which bind to overexpressed target cell receptors and hence increase the overall efficiency of treatment.

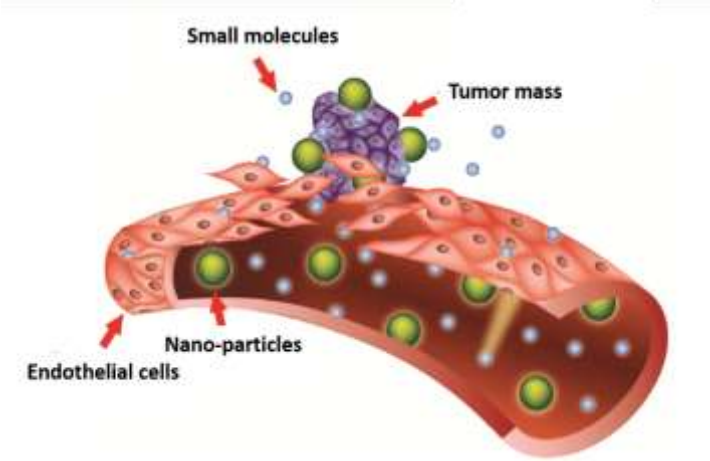


Figure 2.1 EPR effect: Tumor vessels: *Translocation of liposomes into the tumor mass through wide fenestrations* [18]

As shown in Figure 2.1, ligand bound liposomes become more specific to their target site. Antibody conjugated liposomes are referred to as Immunoliposomes. In this project, we hypothesized to prepare immunoliposomes by ligating the liposomes to a novel antibody-antibody X. To prepare immunoliposomes, firstly, stealth liposomes were prepared. Liposomes coated with PEG molecules are referred to as stealth liposome. The liposomes were prepared using derivatized lipid molecule. These lipid molecules are linked with a functional group at the end. For this project, maleimide attached lipid moieties were taken. Liposomes prepared had maleimide group on the surface. Antibody X was the ligand of choice. Antibody X Antibody X -Fab' fragment is conjugated to liposomes. Since Fab' contains a free thiol group in the hinge region, thiol-maleimide chemistry is used for conjugation. The immunoliposomes were characterized for size and specificity.

2.1 Objectives

a. To optimize the fragmentation of antibody to obtain Fab' fragment

b. To optimize purification of antibody fragment
c. To prepare liposomes of size 90-120 nm
d. To conjugate antibody fragments to liposome
e. To characterize immunoliposomes for its size, PDI and specificity

Chapter 3

Materials and Methods

3.1 Materials

Antibody X and Antigen Y were provided by the Downstream Processing Department at Gennova Biopharmaceuticals Ltd. Pepsin, Ethylenediaminetetracaetic acid (EDTA), Merceptoethylamine (MEA), Monosodium phosphate monohydrate, Disodium phosphate, 2-Iminothiolane hydrochloride, NaCl, Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and Bovine Serum Albumin were bought from Sigma-Aldrich; Acetic acid, HCl, Coomassie brilliant blue G-250, Coomassie brilliant blue R-250, Methanol, Tween 20 and Sodium Carbonate were obtained from Merck; Tris, Glycine were obtained from Affymetrix; Citric Acid Monohydrate, Cholesterol, Trisodium citrate were bought from Emplura; Bromophenol Blue, Glycerol and Carbonate-bis-Carbonate buffer capsules were purchased from Sigma. Non-fat dry milk (Blotting grade blocker) from procured from Bio-Rad. mPEG-MAL 2000, DSPE-PEG-MAL 2000were bought from Laysan Bio, Inc., HSPC was bought from COATSOME®, Sodium thiosulfate was obtained from Fisher Scientific, Silver nitrate was bought from Emparta®; Micro BCA™ Protein Assay Kit was purchased from Thermo Scientific™.

3.2 Instrument and Software

Various instruments used during the project are specified as followed.

COMMERCIAL NAME	SOFTWARE	USED FOR
Epoch™ Microplate Spectrophotometer	Gen5™ (BioTek®)	Absorbance

ChemiDoc™ XRS+ System	Image Lab (Bio-Rad)	Chemiluminescence
Agilent, Waters	Open Lab (Agilent), Empower (Waters)	SE-HPLC
Zetasizer Nano ZS	Zeta sizer , (Malvern Instruments)	DLS for size and PDI

3.3 Preparation of Buffers and Working Solutions

3.3.1 Preparation of 5X non-reducing sample buffer for SDS-PAGE

1.21 g of Tris (MW: 121.14 g/mol) was mixed in 10mL Milli Q water to prepare 1M Tris solution. The pH of this solution was adjusted to 6.8, with concentrated hydrochloric acid. 50 mL of 5X sample buffer required for loading of SDS PAGE samples was prepared as mentioned in Table 3.1

Table 3.1: Composition of 5X Sample Buffer

Component	Quantity added
1M Tris, pH = 6.8	6.25 mL
Bromophenol blue	50.00 mg
Glycerol	25.00 mL
SDS	5.00 g
Water	18.75 mL

The bromophenol blue was dissolved with gentle movement of the tube to avoid frothing due to SDS. Aliquot of 450 µL of 50 mL 5X sample buffer were prepared and stored at -20°C.

For the preparation of 5X reducing sample buffer, 50 µL of 1M DTT was added to the 450 µL aliquot of the 5X non-reducing sample buffer. This reducing sample buffer was prepared freshly each time prior to use.

3.3.2 Preparation of 2X non-reducing sample buffer for SDS PAGE

1.21 g of Tris (MW: 121.14 g/mol) was dissolved in 10 mL Milli Q water to prepare 1M Tris solution. The pH of this solution was adjusted to 6.8, with concentrated hydrochloric acid. 50 mL of 2X sample buffer required for the loading of SDS PAGE samples was prepared as mentioned in Table 3.2.

Table 3.2: Composition of 2X Sample Buffer

Component	Quantity added
1M Tris, pH 6.8	5 mL
Water	22.5 mL
Glycerol	10 mL
10% SDS	12.5 mL
Bromophenol blue	100 mg

The bromophenol blue was dissolved with gentle movement of the tube to avoid frothing due to SDS. 450 µL aliquots of this 50 mL 5X sample buffer were prepared and stored at -20°C.

For the preparation of 2X reducing sample buffer, 50 µL of 1M DTT was added to the 450 µL aliquot of the 2X non-reducing sample buffer. This reducing sample buffer was prepared freshly each time prior to use.

3.3.3 Preparation of 10X running buffer for SDS PAGE

For the preparation of 1 L of 10X SDS PAGE running buffer, the components mentioned in Table 3.3 were dissolved in 800 mL of Milli Q water and stirred on magnetic stirrer to dissolve.

The final volume of the solution was made up to 1000 mL.1L of the 1X working solution of the running buffer was prepared by adding100 mL of the 10X buffer to 900 mL of Milli Q water.

Table 3.3 Composition of 10X Running Buffer for SDS PAGE

Component	Quantity added (g)
Glycine	144.0
Tris	30.30
SDS	10.00

3.3.4 Preparation of 10X blotting buffer for Western blot

For 1L blotting buffer, all the components mentioned in the Table 3.4 : Composition of 10X blotting buffer for Western blot were weighed and dissolved in 800 mL of Milli Q with constant stirring and the final volume was made up to 1000 mL.

For preparing a 1L 1X working solution, 100 mL of 10X blotting buffer was mixed with 700 ml of Milli Q followed by 200 ml methanol

Table 3.4 : Composition of 10X blotting buffer for Western blot

Component	Quantity added (g)
Glycine	144.0
Tris	30.30

3.3.5 Preparation of 10X phosphate buffered saline (PBS)

For preparing 1L, 10X phosphate buffered saline, components mentioned in the Table 3.5were weighed and dissolved in 800 mL of Milli Q with constant stirring on a magnetic stirrer, and the final volume was made up to

Table 3.5: Composition of 10X

Component	Quantity added (g)
$NaH_2PO_4.H_2O$	3.2
Na_2HPO_4	10.9
NaCl	90

1000 mL. 1L of the 1X PBS working solutions were prepared by adding 100 mL of 10X PBS to 900 mL of Milli Q water.

3.3.6 Preparation of 1X PBST

1L of 1X PBST was prepared by adding 500 µL of 0.05% Tween 20 to 1L of 1X PBS followed by mixing on a magnetic stirrer till frothing was observed.

3.3.7 Preparation of 10X Tris buffered saline (TBS)

For preparing 1L 10X Tris buffered saline, components mentioned in the Table 3.6 were weighed and dissolved in 800 mL of Milli Q with constant

Table 3.6 : Composition of 10X TBS

Component	Quantity added (g)
Tris	24.2
NaCl	87.7
pH	7.4

stirring on a magnetic stirrer. The final volume was made up to 1000 mL. 1L of the 1X TBS working solutions were prepared by adding 100 mL of 10X TBS to 900 mL of Milli Q water.

3.3.8 Preparation of 1X TBST

1L of 1X TBST was prepared by adding 500 µL of 0.05% Tween 20 to 1L of 1X TBS followed by mixing on a magnetic stirrer till frothing was observed.

3.3.9 Preparation of Bradford reagent

For the preparation of 1L of Bradford reagent, 102.4 mg of Coomassie Brilliant Blue (CBB) G-250 was first dissolved in 50 mL of absolute ethanol as mentioned in Table 3.7. Following this, 100 mL of orthophosphoric acid was added to it. The solution was stirred for 15 min and the volume was made up to 1L with Milli Q water. This solution was filtered with Whatman filter paper No. 1 and stored in an amber colored bottle at 4-8°C.

Table 3.7 : Composition of Bradford reagent

Component	Quantity added
CBB G-250	102.4 mg
Absolute ethanol	50 mL
Orthophosphoric acid	100 mL

3.3.10 CBB R-250 staining solution

For the preparation of 1L of CBB-R250 staining solution, CBB R-250, 1.25 g was dissolved in 450 mL of methanol. Then 100 mL of glacial acetic acid was added to this and the volume was made up to 1L with Milli Q water as mentioned in Table 3.8

Table 3.8 : Composition of CBB R-250 Staining solution

Component	Quantity added
CBB R-250	1.25 g
Methanol	450 mL
Glacial acetic acid	100 mL

3.3.11 Destaining solution

1L of destaining solution was prepared as per the composition mentioned in Table 3.9

Table 3.9 : Composition of distaining solution

Component	Quantity
Methanol	450 mL
Glacial acetic acid	100 mL
Water	450 mL

3.3.12 Preparation of 0.1M sodium citrate buffer

0.1M solution of citric acid was prepared by dissolving 21.01 g in 1L of Milli Q water and 0.1 M solution of sodium citrate, $C_6H_5O_7Na_3.2H_2O$ (M.W: 141.96 g/mol) was prepared by dissolving 29.41 g in 1L of Milli Q water. 40 mL of 0.1 M citric acid solution was mixed with 10 mL of sodium citrate to produce 0.1 M sodium citrate buffer of pH 3.5.

500 mL of 20 mM Sodium citrate buffer was prepared by adding 100 mL 0.1 M citrate buffer to 400 mL Milli Q.

3.3.13 Preparation of 1M sodium phosphate buffers

1M solution of sodium dihydrogen phosphate monohydrate, $NaH_2PO_4.H_2O$ (M.W= 137.99 g/mol) was prepared by dissolving 20.7 g in 150mL of Milli Q water and 1M solution of disodium hydrogen phosphate, Na_2HPO_4 (M.W: 141.96 g/mol) was prepared by dissolving 21.3 g in 150 mL of Milli Q water. The two solutions were mixed in the quantities mentioned in Table 3.10 to obtain 50 mL of 1M sodium phosphate buffers of pH 6.0, 6.5, 7.0, 7.5 and 8.0.

Table 3.10 : Composition of 1M Sodium Phosphate Buffers

pH	Volume of 1M $NaH_2PO_4.H_2O$ (mL)	Volume of 1M Na_2HPO_4 (mL)
7.0	19.50	30.50
8.0	3.40	46.60

500 mL of 50 mM sodium phosphate buffer (pH 7) was prepared by adding 25 mL of 1M sodium phosphate buffer (pH 7) to 475 mL of Milli -Q water.

3.3.14 Preparation of developing solution for silver staining

To prepare 100 mL developing solution, 3% sodium carbonate solution was prepared by mixing 3 g of sodium carbonate in 100 ml Milli-Q. 2 mL 0.02%

sodium thiosulphate and 50 µL of 37% formaldehyde was mixed with 100 mL of 3% sodium carbonate solution.

3.4 Analytical Techniques

3.4.1 SDS PAGE

Polyacrylamide gels were placed in the Invitrogen™ Mini Gel Tank apparatus. 1X running buffer was added to the gel compartment of the apparatus. Protein samples mixed with respective sample buffer were heated for 5 min. at 85°C. Samples were loaded along with protein marker Bio-Rad Precision Plus Protein® Standard-All Blue. Gels were run at 150V for 60min. After the gels were run, they were further subjected to CBB-R250 staining or silver staining or Western blotting.

3.4.2 CBB-R250 Staining

After SDS PAGE was performed, the gels were removed from the cassette and placed in the gel staining box. 200 mL of Coomassie staining solution was added to the gel staining box, and the gel was kept in this solution for 60 min. Following this, the gels were destained with 2 washes (of 20 min. each) of the destaining solution until the protein bands were clearly visible.

3.4.3 Silver staining

After the SDS PAGE was performed, the gels were removed from the cassette and placed in the gel staining box. Fixing solution (50% methanol and 10% glacial acetic acid in water) was then added to the gel box (approx. 200 mL). Gels were kept for 30 min. in this and later transferred to 5% methanol solution for 15min followed by washing with Milli Q water for 10 min. This was followed by the sensitization step with 0.02% sodium thiosulphate in Milli Q for 2min. After this, the gels were washed with Milli Q water for 2 min. Following this, the gels were kept in 0.2% silver nitrate solution for 30 min. The gels were then treated with the developing solution till the bands were clearly visible. Citric acid monohydrate was used to

terminate the developing reaction. Gels were later given 2 washes with Milli Q and scanned with the ChemiDoc™ XRS+ instrument (Bio-Rad).

3.4.4 Western blot

After the SDS PAGE was performed, the gels were removed from the cassette and were subjected to Western blotting. Invitrogen nitrocellulose paper sandwich was used for wet blotting. The apparatus used for wet blotting was Bio-Rad Mini Trans Blot®. The transfer was carried out at 100V for 90 min. The obtained blots were then placed in 10 mL of blocking solution for 1 h. Blocking solution composed of 3% NFDM and 2% BSA prepared in 1X TBST. This was followed by 3 washes of 5 minutes each with 1X TBST. After the washes, the blots were treated with 10mL of 5000 times diluted (in Blocking) Anti-kappa antibody for 1h. 3 washes of 5 minutes each with 1X TBST were performed followed by visualization.

The blots were developed using Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare), and the bands were viewed using the ChemiDoc™ XRS+ instrument (Bio-Rad).

3.4.5 Dot blot

Dot blot apparatus Bio-Rad BioDot™ was assembled as shown in Figure 3.1 and washed with 1X PBS thrice. The samples were added into the well

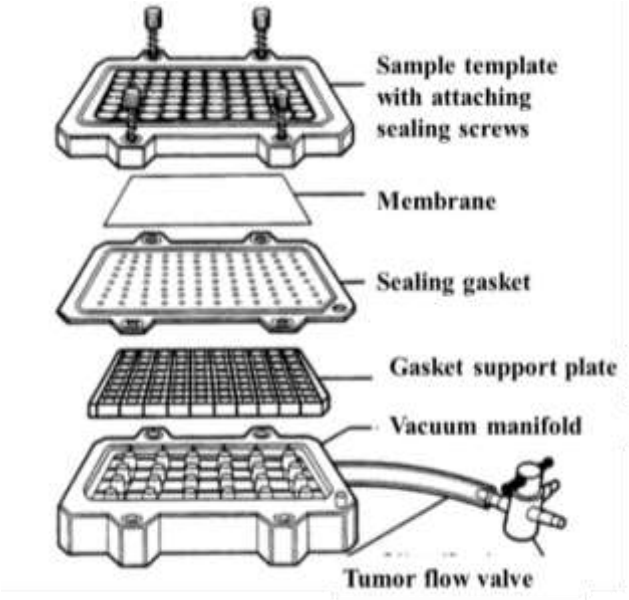


Figure 3.1 : Dot blot apparatus assembly (Bio-Dot Microfiltration apparatus)

followed by addition of 10%SDS. The sample was left under gravity for 30 min. The sample was then sucked through using gentle vacuum and the blot was again washed with 1X PBS twice followed by vacuum drying.

The blot was transferred to 20 mL blocking solution for 1 hr. The blocking was composed of 5% NFDM in 1X TBST. It was followed by washing with 1X TBST thrice with an interval of 5 min. After the washes, the blots were treated with 10mL of 20,000 times diluted (in Blocking) Primary antibody for 3h followed by three washing with 1X TBST. After the washes, the blots were treated with 10 mL of 20,000 times diluted (in 5% NFDM in 1X TBST) Anti-mouse antibody tagged with the enzyme Horse Radish Peroxides (HRP) for 1h. 3 washes of 5 minutes each with 1X PBST were performed subsequent to antibody treatment.

The blots were developed using Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare) and the bands were viewed using the ChemiDoc™ XRS+ instrument (Bio-Rad).

3.5 Methods

3.5.1 Preparation of antibody fragment

3.5.1.1 Generation of antibody fragment F(ab')$_2$

Antibody X (MW: 150 kDa) was digested with enzyme pepsin (MW: 35 kDa) to generate F(ab')$_2$ fragments. Digestion was setup at pH 3.5 (20 mM citrate buffer) with antibody and pepsin at concentration of 1 mg/mL each using molar ratio 1:10 and 1:20 as reported by Jones et

Table 3.11 : Reaction mixture for optimization of pepsin

Components (µL)	1:10	1:20	Control
Volume of Antibody	195	195	97.5
Volume of Pepsin	5	2.5	-
Volume of Citrate Buffer	-	2.5	2.5
Total Volume	**200**	**200**	**100**

al.2003 [62]. Reaction mixtures were set up as mentioned in Table 3.11 kept at 37°C in an incubator and quenched by adding 0.2 M Tris, pH 8.8 at each time point. Time points taken: 0 min, 15 min., 30 min, 45 min, 60 min, 75 min, 90 min, 105 min, and 120 min. SDS PAGE was performed using 10% acrylamide gels followed by CBB-R250 staining for analysis.

3.5.1.2 Scale-up of the digestion reaction and purification of F(ab')$_2$

A scale-up digestion of antibody with pepsin was performed using the optimized conditions to optimize the purification process of the fragments. Antibody concentration taken was 7 mg/mL.

Various Chromatographic techniques were tested to separate the desirable F(ab)$_2$ fragments from unwanted Fc and other fragments. ÄKTA Avant 25 (GE Healthcare Lifesciences) was used for chromatographic separation.

Size exclusion chromatography was performed using a column of fractionation range 3 to 70 kDa. The column was equilibrated with 20mM PBS pH 7.2 and the sample containing F(ab')$_2$; unreacted pepsin was loaded into the column. The column had a loading volume of 0.5 ml. The sample was run at a flow rate of 0.5 mL/min. and several fractions were collected. All the fractions were analyzed through SDS PAGE using 10% acrylamide gels followed by CBB-R250 staining.

3.5.1.3 Generation of Fab'

To attain the most efficient reduction of F(ab')$_2$ to Fab' using mild reducing agent Merceptoethylamine (MEA)2 stoichiometric ratios with 2 different temperatures were tested. Antibody X (MW : 150 kDa) in a concentration 0.64 mg/mL was reacted with MEA (MW : 77.15 g/mol) of stock concentration 10 mg/mL in the presence of 5 mM EDTA using molar ratios F(ab')2 : MEA – 1:2000 and 1:5000 as reported in Zhou et al [63,64]. The reaction was performed at RT and at 37°C as mentioned in Table 3.12. The reactions were quenched with Iodoacetamide at different durations (0.5 h, 1 h and 2 h) to optimize the reaction time.

Table 3.12 : Reaction mixture for optimization of reduction of $F(ab')_2$

Components (µL)	1:2000	1:5000	Control
Volume of $F(ab)_2$	79	79	79
Volume of MEA	7.7	17.3	-
Volume of 0.5 M EDTA	1	1	1
Volume of Milli Q	12.3	2.7	20
Total Volume	**100**	**100**	**100**

SDS PAGE was performed using 10% acrylamide gels followed by CBB-R250 staining for analysis.

3.5.2 Preparation of Liposomes

Liposomes were prepared by the ethanol injection method followed by high pressure extrusion to obtain unilamellar liposomes of appropriate size. In the ethanol injection method, all liposomal components i.e., HSPC, Cholesterol, DSPE-PEG 2000 or DSPE-PEG 2000-MAL were weighed and dissolved in 500 µL Absolute ethanol at 65°C. The dissolved lipids were then added to 9.5 mL of pre-warmed (at 65°C) sodium phosphate buffer, pH 7 to give a 10 mL liposomal solution consisting of a non-homogenous population of liposomes. To obtain unilamellar liposomes of a size ≈ 90 nm,

Table 3.13 : Composition of Non-functionalized and functionalized liposome

Non functionalized liposome	Functionalized liposome
HSPC	HSPC
Cholesterol	Cholesterol
DSPE-mPEG	DSPE-PEG-MAL

the liposomal solution was extruded through Polycarbonate membranes of appropriate sizes to obtain the required homogenous solution of liposomes

Similarly, functionalized liposomes were prepared by adding DSPE-PEG-MAL in place of DSPE-mPEG. Different composition of functionalized and non-functionalized liposomes are as mentioned in Table 3.13.

The characterization of liposomes for their size and poly dispersity index (PDI) was done using the Zetasizer Nano ZS (Malvern Instruments).

3.5.3 Conjugation of Fab' with Liposomes

3.5.3.1 Conjugation of Fab' with mPEG, DSPE-PEG-MAL and functional liposome

Fab' contains a free thiol group on the cysteine residue situated at the hinge region of the heavy chain. Thus, the thiol-maleimide conjugation chemistry was chosen for conjugation. Conjugation reactions of Fab' (in PBS with 5 mM EDTA, pH 7.2) with 2 kDa mPEG-MAL, DSPE-PEG-MAL and functionalized liposomes were set up as mentioned in Table 3.14 . Maximum Fab': PEG ratio of 1:20 was taken. Fab' (MW: 50 kDa) at a concentration of 1.2 mg/mL was reacted with mPEG-MAL, DSPE-PEG-MAL in presence of 50 mM sodium phosphate buffer. pH 7.4 in 2 mL micro centrifuge tubes with constant mixing on LabRoller™ (Labnet International Inc.). The reaction mixtures were kept at RT overnight and analyzed using SDS PAGE followed by western blot.

Table 3.14: Reaction Mixture for conjugation of Fab' with mPEG, DSPE-PEG-MAL

Component s(µL)	Reaction 1	Reaction 2	Control
Volume of Fab'	15	15	15
Volume of mPEG MAL	20	-	-
Volume of DSPE-PEG MAL	-	20	
Volume of Phosphate buffer	215	215	215
Volume of PBS	-	-	20
Total Volume	**250**	**250**	**250**

Similarly, Fab' fragments were also conjugated with functionalized and non-functionalized liposomes as mentioned Table 3.15. Fab' at a concentration of 1.2 mg/mL was reacted with a maximum possible liposomal solution such that the final strength of Fab'in the reaction mixture is 0.1 mg/mL.

Table 3.15 : Conjugation of Fab' with functionalized liposome and Non-functionalized liposomes

Components (µL)	Functionalized Liposome
Volume of Fab'	15
Volume of functionalized Liposome	235
Volume of Non-Functionalized Liposome	-
Total Volume	**250**

The reaction mixture was set up in a 2 mL silicon centrifuge with constant mixing on LabRoller™ (Labnet International Inc.) at RT for overnight and analyzed using SDS PAGE followed by western blot.

3.5.3.2 Conjugation of Fab' to mPEG, DSPE-PEG-MAL and functionalized liposome in the presence of reducing agent TCEP

Conjugation reactions of Fab' (in PBS with 5 mM EDTA, pH 7.2) with mPEG-MAL, DSPE-PEG-MAL (both has molecular weight 2 kDa) and functionalized liposomes were set up as mentioned in Table 3.16. Maximum Fab': PEG ratio of 1:20 was taken. TCEP was added to the Fab' in a ratio of 1:3 (Fab': TCEP). TCEP-Fab' (MW: 50 kDa) at a concentration of 1.2 mg/mL was then reacted with mPEG-MAL, DSPE-PEG-MAL in presence of 50 mM sodium phosphate buffer. pH 7.4 in 2 mL

microcentrifuge tubes with constant mixing on LabRoller™ (Labnet International Inc.).

The reaction mixtures were kept at RT overnight and analyzed by SDS PAGE followed by Western blotting.

Table 3.16 : Reaction Mixture for conjugation of TCEP-Fab' with mPEG, DSPE-PEG-MAL

Components (µL)	Reaction 1	Reaction 2	Control
Volume of TCEP-Fab'	20	20	10
Volume of mPEG MAL	20	-	-
Volume of DSPE-PEG MAL	-	20	
Volume of Phosphate buffer	210	210	210
Volume of PBS	-	-	20
Total Volume	**250**	**250**	**250**

Table 3.17 : Conjugation of TCEP-Fab' with functionalized liposome and Non-functionalized liposomes

Components (µL)	Functionalized Liposome
Volume of TCEP-Fab'	20
Volume of functionalized Liposome	230
Volume of Non-Functionalized Liposome	-
Total Volume	**250**

Similarly, TCEP-Fab' fragments were also conjugated with functionalized and non-functionalized liposomes as given in Table 3.17. Fab' at a concentration of 1.2 mg/mL was reacted with maximum possible liposomal

solution such that the final strength of Fab'in the reaction mixture is 0.1 mg/mL.

3.5.4 Thiolation of Fab' followed by conjugation with DSPE-PEG MAL

Fab' was thiolated using Traut's reagent or 2-iminothiolane hydrochloride. Fab' at concentration of 1.1 mg/mL was reacted with 2-iminothiolane hydrochloride at a concentration 0.035 mg/mL using Fab': iminothiolane molar ratios of 1:10 and 1:15. The reactions were performed in 0.1 M sodium phosphate buffer pH 8.0 in presence of 10 mM EDTA as per mentioned in Table 3.18 [65]. The reaction mixtures were kept at RT for 1 h and excess iminothiolane was removed by buffer exchanging with PBS, pH 7.2 containing 5 mM EDTA using 10K Amicon® Ultra 0.5mL centrifugal filter

Table 3.18 : Reaction mixture for thiolation of Fab'

Components (µL)	1:10	1:15	Control
Volume of Fab'	105	105	105
Volume of Traut's reagent	45	68	-
Volume of 0.5 M EDTA	5	5	5
Volume of Sodium phosphate buffer	25	25	25
Volume of Milli	70	47	115
Total Volume	**250**	**250**	**250**

Concentration of Thiolated Fab' was then checked using Bradford's reagent followed by PEGylation with DSPE-PEG MAL 2k to check the conjugation efficiency after thiolation. Thiolated Fab' at a concentration of 0.36 mg/mL was reacted with 1 mg/mL DSPE-PEG MAL(prepared in Milli Q) 2k in Fab': PEG molar ratios of 1:15, 1:20, 1:50 and 1:100 in sodium phosphate

buffer, pH 7 containing 5mM EDTA. The reaction was set-up as per mentioned in Table 3.19

The reaction mixtures were kept at RT overnight and analyzed using SDS PAGE.

Table 3.19 : PEGylation of Thiolated Fab'

Components (µL)	1:15	1:20	1:50	1:100	Control
Volume of Thiolated-Fab’	27	27	27	27	27
Volume of DSPE-PEG MAL	6	8	20	40	-
Volume of PBS + 5mM EDTA	57	55	43	23	58
Volume of Sodium phosphate buffer	10	10	10	10	10
Total Volume	**100**	**100**	**100**	**100**	**100**

3.5.5 Thiolated Fab’ affinity determination by enzyme-linked immunosorbent assay

Enzyme linked immunosorbent assay (ELISA) was employed to assess the effect of thiolation on the binding affinity of Fab’ to its antigen. For this, the binding affinities of thiolated Fab’ and non-thiolated Fab’ were compared.

Briefly, a PolySorp™, F 96 well plate was coated with 100 µL of 50 ng/mL Antigen Y to each well (prepared in 0.05M Carbonate-bis-carbonate buffer pH 9.6) and kept at 4°C on the shaker overnight. The plate was then kept at 37°C for 1 h followed by washing with 1X PBST thrice using an AquaMax® Plate Wash head (Molecular Device).300 µL of blocking solution (5%NFDM prepared in 1X PBS) was added to each well and the plate was kept at RT for 1h on a plate shaker, followed by washing with 1X PBST thrice.

Fab' and thiolated Fab' were diluted in 1%NFDM prepared in 1X PBS) and the concentrations used for ELISA were10,000 ng/ml, 5000 ng/ml, 1000ng/ml, 500 ng/ml, 100ng/ml, 50 ng/ml, 10 ng/ml, 5 ng/ml.100 µL of sample was added to each well and the plate was kept at RT for 1 h on shaker followed by washing with 1X PBST thrice. Following this, 100 µL of anti-kappa-HRP antibody (1:5000 diluted in 1% NFDM prepared in 1X PBS) solution was added to each well and kept for 1 h at RT on shaker followed by washing with 1X PBST thrice.100 µL of substrate (1:1 TMB:H_2O_2) was added to each well and kept at RT for 20 min in dark. 100 µL of 2N H_2SO_4 was added to each well and mixed properly. Plates were read at 450 nm using Epoch™ Microplate Spectrophotometer.

3.5.6 Conjugation of thiolation of Fab' to functionalized Liposome

Fab' was thiolated as mentioned in Section 3.5.7 and used for conjugation with Non- functionalized and DSPE-PEG MAL 100% functionalized liposomes.The details of the reaction mixtures are mentioned in Table 3.20.

Table 3.20 : Conjugation of Thiolated Fab' with Liposomes

Components (µL)	Reaction 1	Reaction 2	Control
Volume of Fab'	50	25	25
Volume of functionalized liposome	200	-	-
Volume of Non-functionalized liposome	-	100	-
Volume of 50mM Phosphate buffer pH 7.4	-	-	100
Total Volume	**250**	**125**	**125**

The reactionswere set up in 2 mLmicro-centrifuge tubes with constant mixing on LabRoller™ (Labnet International Inc.) at RT for overnight and analyzed using SDS PAGE followed by Western blot

3.5.7 Scale-up and purification of Immunoliposomes

A scale-up reaction of 1.2 mL was set up for purification of immunoliposomes was set up as mentioned in Table 3.21 thiolated Fab' at a concentration of 0.86 mg/mL was conjugated with 100% functionalized liposomes (final strength of Fab' in the reaction was 0.1 mg/mL)

The reaction was set up in a 15 mL tube with constant mixing on LabRoller™ (Labnet International Inc.) at RT for overnight and analyzed using SDS PAGE followed by western blot.

Table 3.21 : Scale-up of Liposomal conjugation reaction

Components (µL)	Reaction 1	Control
Volume of Fab'	130	70
Volume of functionalized liposome	988	-
Volume of 50mM Phosphate buffer pH 7.4	-	530
Total Volume	**1168**	**600**

To separate unreacted Fab' from the immunoliposomes (liposome conjugated Fab'), the reaction mixture was loaded onto Size exclusion chromatography column. The column was equilibrated with 20mM PBS pH 7.4 and the scaled-up sample was injected into the column. The Sample was run at a flow rate of 0.5 mL/min. and several fractions were collected and analyzed through SDS PAGE using 10% acrylamide gels followed by CBB-R250 staining

The size and PDI analysis of the sample were done using Zetasizer Nano ZS (Malvern Instruments).

3.5.8 Quantification of Fab' in Immunoliposomes

The amount of Fab' attached to the liposomes was determined using the Micro BCA™ Protein Assay Kit. Prior to the BCA assay, 0.3 mL Immunoliposomes were mixed with 0.4 mL methanol, 0.2 mL chloroform, and 0.1 mL water. The mixture was vortexed and centrifuged for 3 min. at 9000 g. The upper phase was discarded carefully followed by addition of 0.3 mL methanol. The sample was vortexed and centrifuged at 9000 g. The supernatant was discarded carefully and the remaining pellet was dried using a vacuum drier. The pellet was finally re-suspended in 20 mM PBS pH 7.4 containing 2% SDS.

The sample was diluted appropriately, and the protein concentration was determined using the Micro BCA™ Protein Assay Kit.

3.5.9 To determine the binding specificity of Immunoliposomes to Antigen Y using SEC-HPLC followed by Dot blot

The binding specificity of Immunoliposomes to Antigen Y was determined using SE-HPLC confirmed by Dot blot. A reaction mixture is containing Immunoliposomes and Antigen Y in Molar ratio 0.07: 1 was set-up along with a positive and two negative controls.

Only Antigen Y was taken as a positive control. Only Immunoliposomes and Functionalized-liposomes with Antigen Y were taken as a negative control.

All Four reaction mixtures were injected into the column and were run at a rate of 0.7 mL/min on SE-HPLC equilibrated with buffer: 20mM Tris, 300mM NaCl, and pH-7.2. The eluted samples were analyzed by Dot blot.

Chapter 4

Results and Discussion

In order to make the immunoliposome three components are required

a. The antibody X(or its fragment) for decorating the surface of the liposome
b. Liposome that is amenable to conjugation with the antibody X to form immunoliposome
c. Antigen Y that binds with the antibody X on the surface of the immunoliposome in order to prove that neutralization function of the immunoliposome.

4.1 Preparation of antibody fragment

The choice of an appropriate antibody fragment for conjugation with liposomes is critical for the safety, efficacy, and stability of the immunoliposome formed. Generally, only a portion of the antibody is conjugated to liposome instead of a whole antibody due to immunogenic effects of IgG-Fc portion [44], and stability issues. The Fc portion activates usual complement pathway and triggers Fc receptor mediated phagocytosis, which results in increased RES clearance [66]. Attachment of the whole antibody may result in a bulky structure which may result in the unstable formulation and ready clearance. Additionally, the mode of action of the immunoliposome formed will be 'neutralization'. Hence the Fc region of the whole antibody is not necessary for its function.

Various antibody fragments can be generated by enzymatic digestion of the full length antibody with or without chemical reduction depending on the desired fragment as detailed in section 1.4.1-Antibody fragmentation in the introduction chapter. Antibody fragment Fab' contains free thiol group at the hinge region (see Figure 1.6) which can be readily exploited for

conjugation to liposomes through a maleimide functionalized mPEG on the surface of the liposome [67].

Pepsin is an acidic protease, that digests the antibody below the inter-chain di-sulphide bonds to give an intact $F(ab')_2$ fragment (Figure 4.1)and

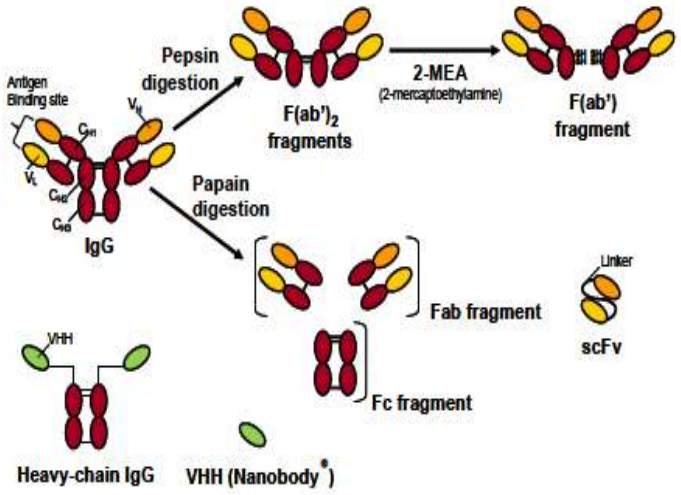


Figure 4.1 : Pepsin digestion of antibody fragmentation- *Digestion of IgG by pepsin and papain enzyme to generate $F(ab')_2$ and Fab fragment followed by reduction to generate Fab' fragment. various other ScFv fragments* [41].

degraded Fc fragments [41]. F(ab')2 can be further reduced chemically to yield Fab' with accessible thiol group for conjugation.

4.1.1 Generation of antibody fragment $F(ab)_2$

Antibody X was readily available at Gennova's R & D. It was fragmented to produce $F(ab')_2$ by pepsin digestion. Pepsin is active only at acidic conditions and works best at pH 2. However, the integrity of the antibody is highly compromised at such a low pH. Therefore, digestion condition needed to be determined where the activity of pepsin is the highest without hampering the stability of the antibody X. Jones *et al.*2003 on antibody fragmentation, revealed that digestion carried out at pH 3.5 does not compromise the stability of the antibody X. Hence, optimization of pepsin

digestion of antibody X was carried out at pH 3.5 with a varying stoichiometry of pepsin to the antibody (1:10 and 1:20).

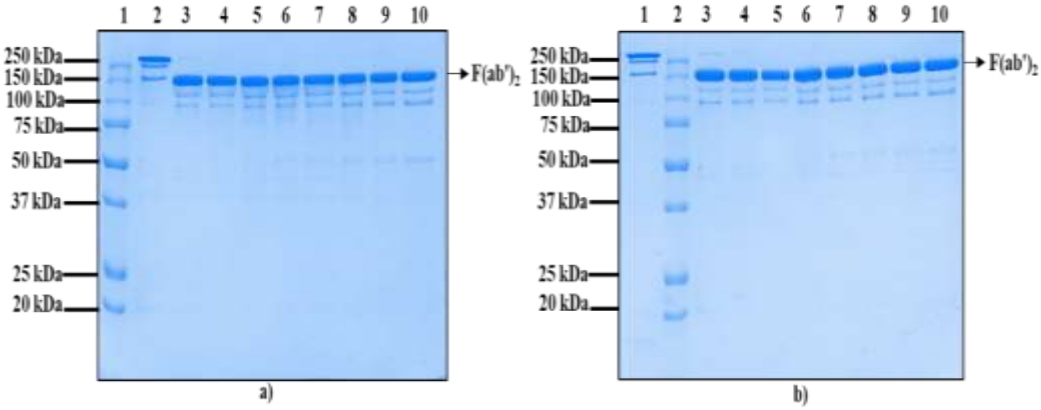


Figure 4.2: Optimization of antibody digestion by pepsin at pH 3.5 – Analysis by Coomassie brilliant blue R-250 staining: a) Enzyme/antibody ratio 1:10*; Lane 1- Precision Plus Protein™ All Blue Pre-stained Protein Standards (Bio-Rad); Lane 2- only antibody (control); Lane 3 – Reaction quenched after 15 min; Lane 4 - Reaction quenched at 30 min; Lane 5 - Reaction quenched at 45 min; Lane 6 - Reaction quenched at 60 min; Lane 7 - Reaction quenched at 75 min; Lane 8 – Reaction quenched at 90 min ;. Lane 9 – Reaction quenched at 105 min; Lane 10 – Reaction quenched at 120 min* **b) Enzyme/antibody ratio 1:20 ;** *Lane 1- only antibody (control); Lane 2- Precision Plus Protein™ All Blue Pre-stained Protein Standards (Bio-Rad); Lane 3 – Reaction quenched after 15 min; Lane 4 - Reaction quenched at 30 min; Lane 5 - Reaction quenched at 45 min; Lane 6 - Reaction quenched at 60 min; Lane 7 - Reaction quenched at 75 min; Lane 8 – Reaction quenched at 90 min;. Lane 9 – Reaction quenched at 105 min; Lane 10 – Reaction quenched at 120 min.*

For both the molar ratios, IgG was completely digested into $F(ab')_2$ (MW: 110 kDa) in about 30 min. As shown in Figure 4.2, Lane 2 shows the intact IgG which when digested shows a lower band of the antibody fragment $F(ab')_2$ as shown in Lane 3 to 10 for both the reaction conditions. Therefore, molar ratio 1:10 in 20mM Citrate buffer pH 3.5 was considered as the optimized reaction condition for pepsin digestion and was used for the scale-up of the reaction in order to produce more $F(ab')_2$ and purify it.

4.1.2 Scale-up of the digestion reaction and purification of F(ab')$_2$

To obtain large quantity of the digested F(ab')$_2$, digestion reaction was setup at higher scale. The digested sample was purified to separate degraded Fc

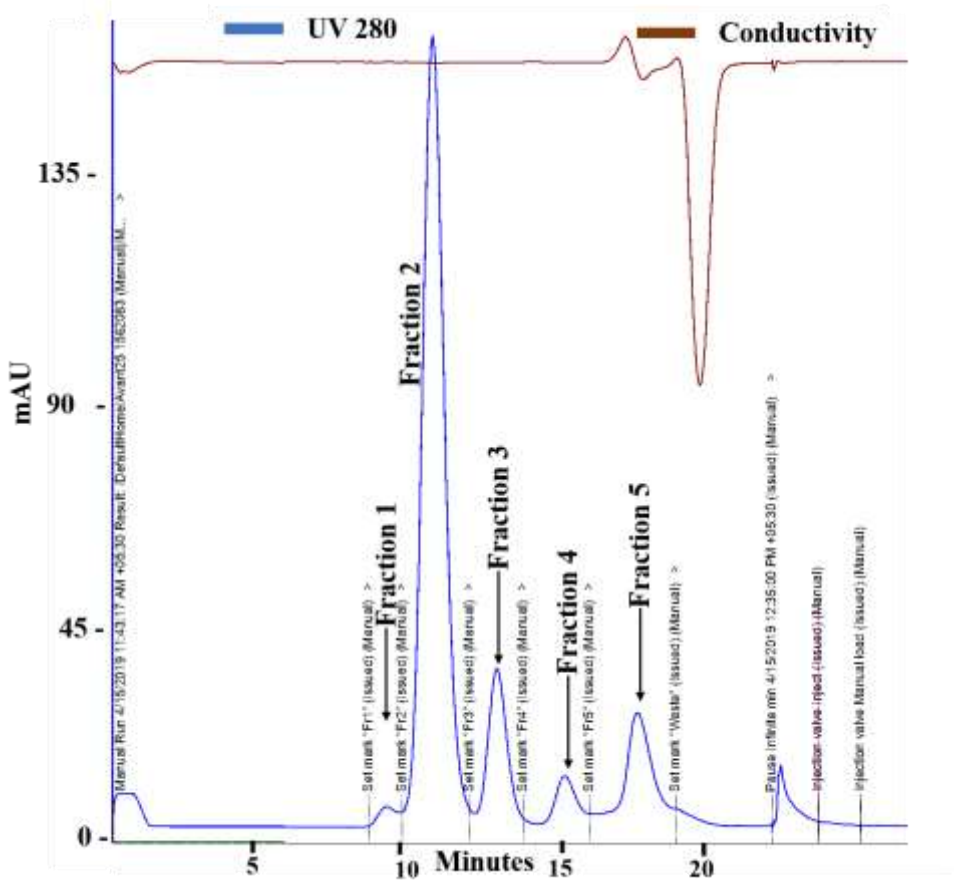


Figure 4.3 : Chromatogram showing purification of F(ab')$_2$- *Purification done using size exclusion chromatography using Size exclusion chromatography*

fragments and other lower molecular weight impurities from F(ab')$_2$ (MW: 110 kDa) using Size exclusion chromatography (SEC).

SEC elutes molecules based on their molecular size. The column used had a fractionation range of 3 kDa – 70 kDa which was used to separate shown in Figure 4.3, fractions 1, 2, 3, 4 and 5 were collected and analyzed by SDS PAGE through CBB-R250 staining. As shown in the Figure 4.4, purified F(ab')$_2$ was separated from lower molecular impurities present in load and was obtained in fraction 2and 3 at a retention time of 10-12 mL.

The first largest peak, refer to fraction 1 of Figure 4.3, is confirmed to be of $F(ab')_2$in Figure 4.4, followed by smaller peak of lower molecular weight impurities.

From 3 mg of antibody X, 0.9 mg of $F(ab')_2$was obtained. The yield of the overall process is low and can be increased with further optimization efforts. Since, scope of the project was to make immunoliposomes and the yield of $F(ab')_2$ was considered sufficient to carry out its reduction to form Fab', it was decided to park the optimization of $F(ab')_2$ production for later

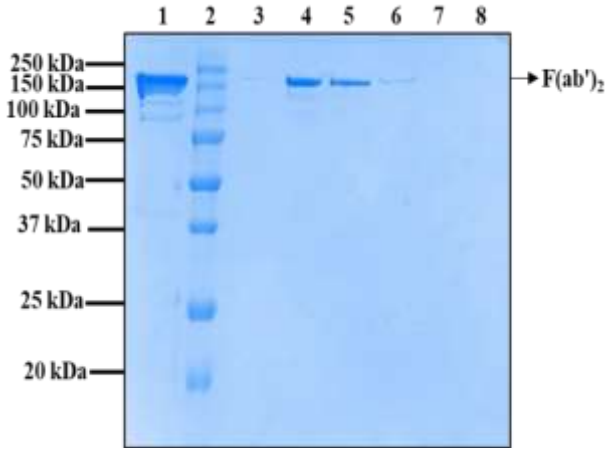


Figure 4.4: Purification of $F(ab')_2$ using Size exclusion chromatography- Analysis by CBB-R250 staining: *Fractions 1-6; Lane 1- Load; Lane 2- Precision Plus Protein™ All Blue Pre-stained Protein Standards (Bio-Rad); Lane 3-Fraction 1; Lane 4 – Fraction 2;Lane 5 – Fraction 3 ; Lane 6 - Fraction 4; Lane 7 - Fraction 5; Lane 8 – Blank.*

4.1.3 Generation of Fab'

To obtain the Fab', reduction of $F(ab')_2$was carried out using a mild reducing agent merceptoethylamine (MEA). Zhou *et al*.reported reduction of $F(ab')_2$using MEA in a molar ratio 1:2000, as mentioned in [64]. The same condition as tested at 25°C and 37°C at various time points. As shown in Figure 4.6(a), the reduction was minimal at 25°C while at 37°C, most of the $F(ab')_2$ had been reduced to Fab' with only a small amount of intact $F(ab')_2$ left after 2 hours.

To further optimize the reduction, the molar ratio was doubled (1:5000), keeping other parameters constant. As shown in Figure 4.5(b), at this higher ratio, the content of Fab' obtained at 25°C increased, while the time required

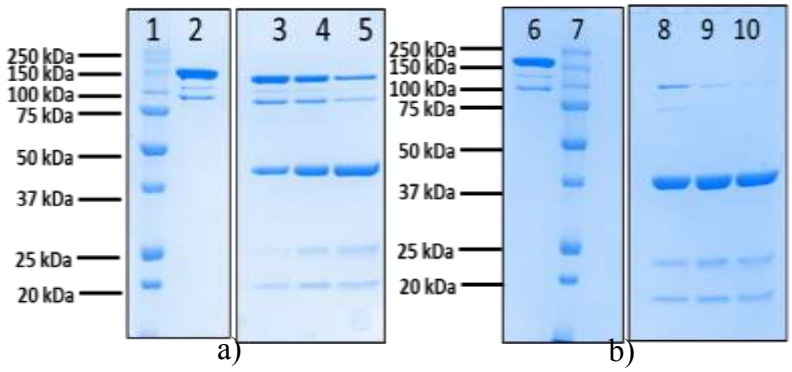


Figure 4.5:Optimization of reduction of $F(ab')_2$ to Fab Antibody/MEA ratio 1:5000– Analysis by CBB R-250 staining*: Lane 1- Precision Plus Protein™ All Blue Pre-stained Protein Standards (Bio-Rad); Lane 2- only antibody (control); Lane 3 – Reaction at RT quenched after 0.5 h; Lane 4 – Reaction at RT quenched at 1 h ; Lane 5 - Reaction at RT quenched at 2 h; Lane 6 - only antibody (control); Lane 7- Precision Plus Protein™ All Blue Pre-stained Protein Standards (Bio-Rad); Lane 8 – Reaction at* 37°C *quenched at 0.5 h; Lane 9 – Reaction at* 37°C *quenched at 1 h ; Lane 10 – Reaction at* 37°C *quenched at 2 h.*

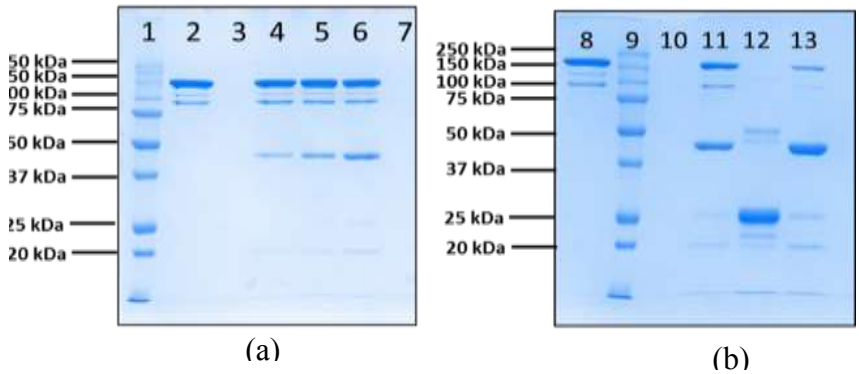


Figure 4.6:Optimization of reduction of $F(ab')_2$ to Fab Antibody/MEA ratio 1:2000'– Analysis by CBB R-250 staining: *Lane 1- Precision Plus Protein™ All Blue Pre-stained Protein Standards (Bio-Rad); Lane 2- only antibody (control); Lane 3- Blank; Lane 4 – Reaction at RT quenched after 0.5 h; Lane 5 – Reaction at RT quenched at 1 h ; Lane 6 - Reaction at RT quenched at 2 h; lane 7 - Blank; Lane 8- only antibody (control); Lane 9- Precision Plus Protein™ All Blue Pre-stained Protein Standards (Bio-Rad); lane 10 - Blank; lane 11 – Reaction at 37°C quenched at 0.5 h; Lane 12 – Reaction at 37°C quenched at 1 h ; Lane 13 – Reaction at 37°C quenched at 2 h.*

for reduction at 37°C decreased Complete reduction at 37°C was observed in just 1 h.

Thus, this ratio 1:5000 at 37°C for 1 h was selected as the optimum condition for reduction of $F(ab')_2$ to Fab'.

Scale up and purification of Fab' was not deemed to be necessary for establishing the proof of concept – i.e., preparation of a neutralizing immunoliposomes. Further stability of the Fab' (because of the presence of thiol group the Fab' readily dimerizes in absence of reducing agent needed to be determined, which is mini-project in itself [68]. Lastly, the reaction condition was so optimized that more than 95 % Fab' was obtained upon reduction of $F(ab')_2$. Hence it was decided that Fab' would be prepared fresh every time for conjugation reaction with functionalized liposome.

4.2 Preparation of liposomes

The ethanol injection method was used for the preparation of liposomes. The method involves the dissolution of lipid into ethanol followed by injection of lipid into the buffer, forming liposomes. The organic solvent (ethanol) and buffer were maintained at the transition temperature of lipid. The liposomes prepared to contain a heterogeneous mixture of ULV (unilamellar liposomal vesicle) and MLV (multilamellar liposomal vesicle).

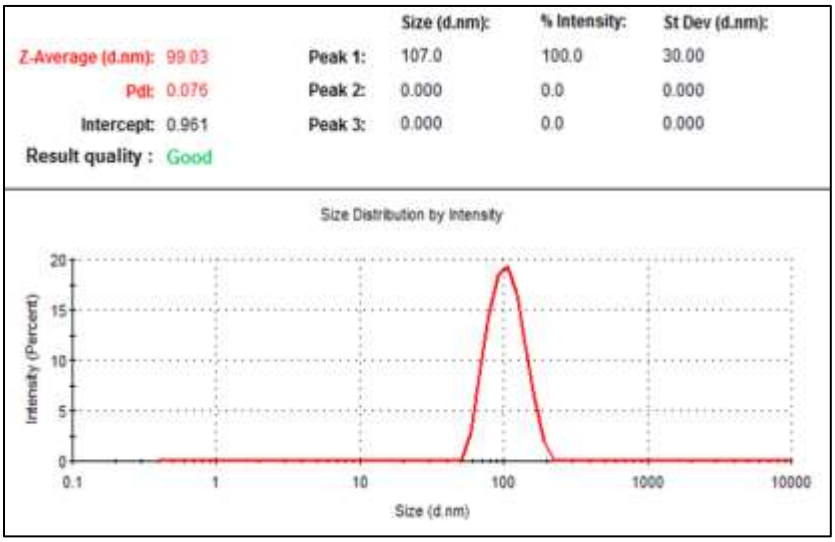


Figure 4.7 : Size and PDI of liposomes

Since ULV has higher circulation time as compared to MLV as reported by Senior J. et al.1982, ULVs were the choice of liposomes [69]. To remove MLV from mixture, liposomes were subjected to a set cycle of extrusion (optimized at Gennova-R & D). Extrusion is a method sizing the liposome and making the population homogeneous in term s of size

The size of liposome was checked using Zetasizer Nano ZS (Malvern Instruments), as shown in Figure 4.7, and was determined to be ~ 99 nm with a PDI (Poly dispersity Index)of0.07 which indicates homogenous liposomal population.

Two types of liposome were prepared (as shown in Figure 4.8):

a. The first type of liposome was decorated with 2 kDa mPEG without having any functional group. These were termed as 'non-functionalized liposomes'. These served as negative controls.
b. The second type of liposomes were decorated with 2 kDa mPEG having a maleimide functional group. These were termed as 'functionalized liposomes'. These served to form immunoliposomes.

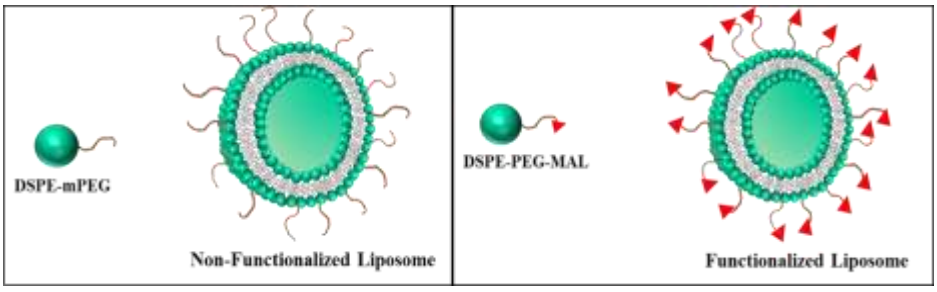


Figure 4.8: Structure of Non-functionalized and functionalized liposome

The properties of liposomes were not affected by the addition of the functionalized PEG. Since the composition of the liposome produced is proprietary information Gennova, hence it cannot be disclosed in the thesis.

4.3 Conjugation of Fab' with Liposomes

4.3.1 Conjugation of Fab' with mPEG, DSPE-PEG-MAL and functional liposome

Fab' contains a free thiol group which is exploited to conjugate the antibody fragment with PEG-MAL (mPEG modified with a maleimide functional group) or DSPE-PEG-MAL (mPEG modified with a maleimide functional group that is conjugated a lipid). All the mPEGs were of 2kDa molecular weight. An overnight conjugation reaction of Fab' (MW: 50 kDa) was set up at 25°C with PEG-MAL, DSPE-PEG-MAL as well as functionalized liposomes.

As shown in Figure 4.9, Fab'-2k PEG conjugated adduct could be seen in lane 3 around 55-60 kDa band, where the conjugation reaction was with PEG-MAL. A very faint band of conjugated adduct could also be seen with DSPE-PEG-MAL (lane 4). However no adduct was seen in the case of conjugation with functionalized liposomes (Lane 5). It indicated that either

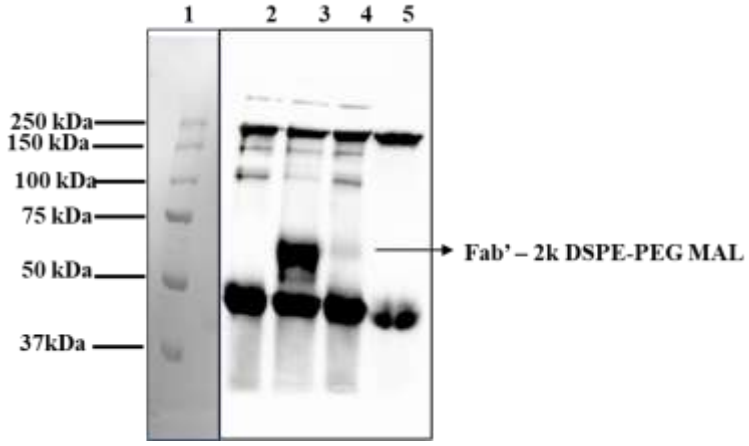


Figure 4.9 : Conjugation of Fab' with 2 kDa PEG, DSPE-PEG MAL AND functionalized liposome- Analysis by western blot: *Lane 1- Precision Plus Protein™ All Blue Pre-stained Protein Standards (Bio-Rad); Lane 2- only Fab' (control); Lane 3- Fab' conjugated with 2 kDa PEG; Lane 4- Fab' conjugated with 2 kDa DSPE-PEG MAL; Lane 5 - Fab' conjugated with 2 kDa functionalized liposomes*

thiol group present on Fab' is not accessible to bulkier groups for conjugation, since as the steric hindrance increased (like in case of bulkier

functionalized liposomes) the conjugation decreased. In case of conjugation with DSPE-PEG-MAL a very faint band of the adduct was seen. DSPE-PEG-MAL in solution could form micellar structures which could attribute to the steric hindrance and hence show less conjugated product formation. Clearly the thiol in the Fab' is not exposed enough to counter the steric hindrance and hence react to form an adduct with micelles or liposomes. Alternatively, the thiol group can also be made more reactive using reducing agents. However, reducing agents containing thiol groups cannot be used – like DTT, beta –merpactoethanol, etc. So non-thiol containing TCEP was used as a reducing agent.

4.3.2 Conjugation of Fab' to mPEG, DSPE-PEG-MAL and functionalized liposome in the presence of reducing agent TCEP

Presence of TCEP in the reaction would prevent the oxidation of free-thiol groups, thereby making the thiol-group more reactive for conjugation. The Fab' was conjugated to PEG-MAL, DSPE-PEG-MAL as well as the functionalized liposome in presence of reducing agent TCEP.

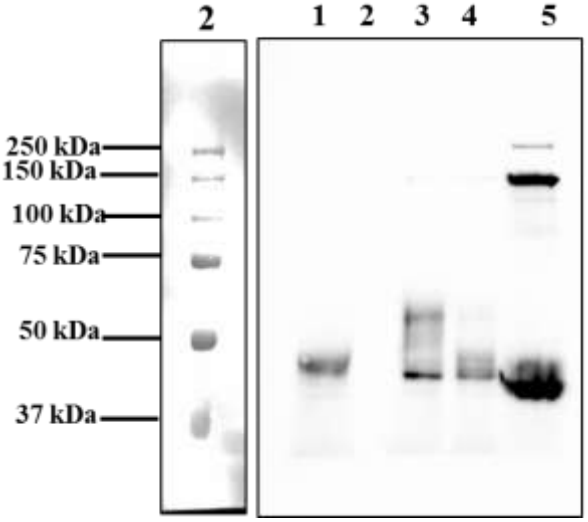


Figure 4.10 : Conjugation of TCEP Fab' with PEG, DSPE-PEG MAL and functionalized liposome- Analysis by western blot; *Lane 1- only Fab' (control)Lane 2- Precision Plus Protein™ All Blue Pre-stained Protein Standards (Bio-Rad);; Lane 3-TCEP Fab' conjugated with 2 kDa PEG; Lane 4- TCEP Fab' conjugated with 2 kDa DSPE-PEG MAL; Lane 5 – TCEP-Fab' conjugated with 2 kDa functionalized liposomes*

As shown in Figure 4.10, Fab'-PEG conjugated adduct could be seen in lane 3 around 55-60 kDa band. No band of conjugated adduct could be seen with DSPE-PEG-MAL (refer to Figure 4.10, lane 4) or functionalized liposomes (refer to Figure 4.10, lane 5). A band around 150 kDa was seen for the conjugation with functionalized liposomes. This could be Fab dimer but its absence in the remaining reactions was baffling. Clearly it is not a conjugate, hence deciphering it was shelved for a later date. There was no effect of reducing agent to the conjugation reaction.

The inaccessibility of thiol groups can be overcome by thiolation of a surface exposed amine side chain of lysine amino acid of the antibody using Traut's reagent.

4.4 Thiolation of Fab' followed by conjugation with DSPE-PEG MAL 2k

From the previous experiments, it was concluded that the thiol group present in the hinge region of Fab' is inaccessible for binding to maleimide group for bulky moieties like the functionalized liposomes.

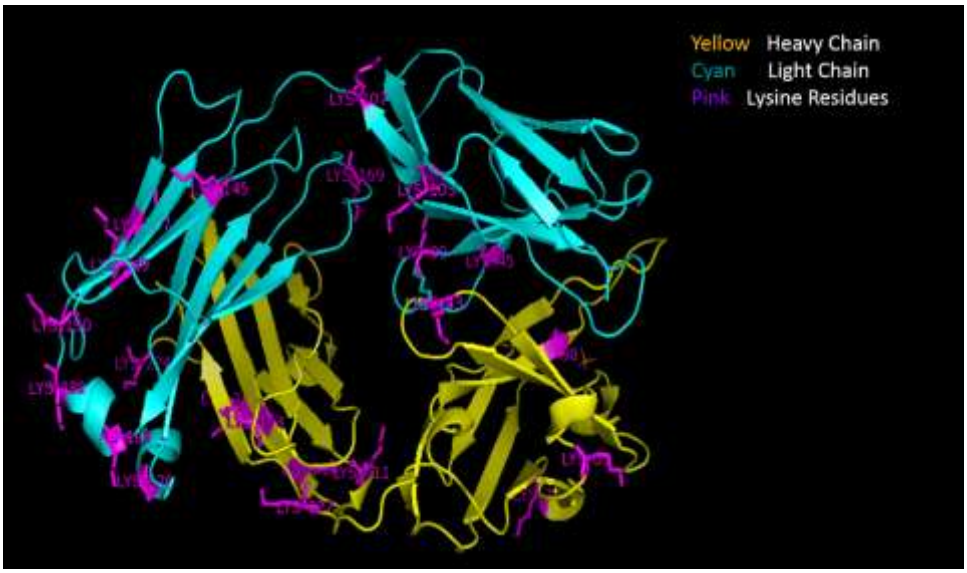


Figure 4.11: Structure of Fab' showing marked Lysine residues- *Structure made using PyMOL (TM) Evaluation Product – Copyright © 2008 DeLano Scientific LLC.*

During the literature study, methods that increase the binding affinity of protein to ligand were reviewed. Site specific modification is one of the successful method reported previously [63]. It includes the addition of free cysteine by modification of N-termini or C-termini by attaching a short linker peptide [70]. However, these methods are time consuming. Hence, an alternative approach was used.

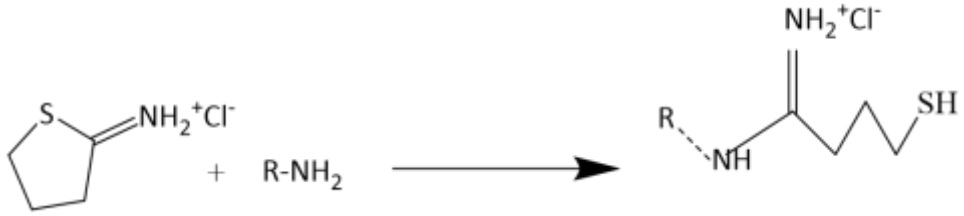


Figure 4.12: Traut's Reagent reacting with primary amines [40].

The Fab' was thiolated using Traut's reagent-2 iminothiolane. It is a cyclic thioimidate that reacts with primary amines as shown in Figure 4.12 [44]Primary amines are present at the N-terminal of protein as well as in the Lysine residues. There are 50 Lysine residues present in Fab' fragment of Antibody X as shown in Figure 4.11. The thiolation of Fab' fragment is required to be optimized to control the number of thiols attached because thiolation at the epitope binding site of the antibody X can affect the antigen binding efficiency of the fragment.

Fab' was thiolated using two ratios of Fab' to 2 iminothiolane-1:15 and 1:10 followed by conjugation with DSPE-PEG-MAL at varying stoichiometry.

As shown in Figure 4.13, conjugation was observed for all the reaction conditions explored. Furthermore, all the bands obtained had the same intensity. This is indicative that the thiolation ratio of 1:10 is enough to trigger conjugation and thiolating the Fab' more does not have an added advantage. Additionally, it was observed that there was no effect of increasing DSPE-PEG-MAL molar ratio.

Therefore, thiolation ratio 1:10 was considered as the optimum condition and was used for subsequent experiments.

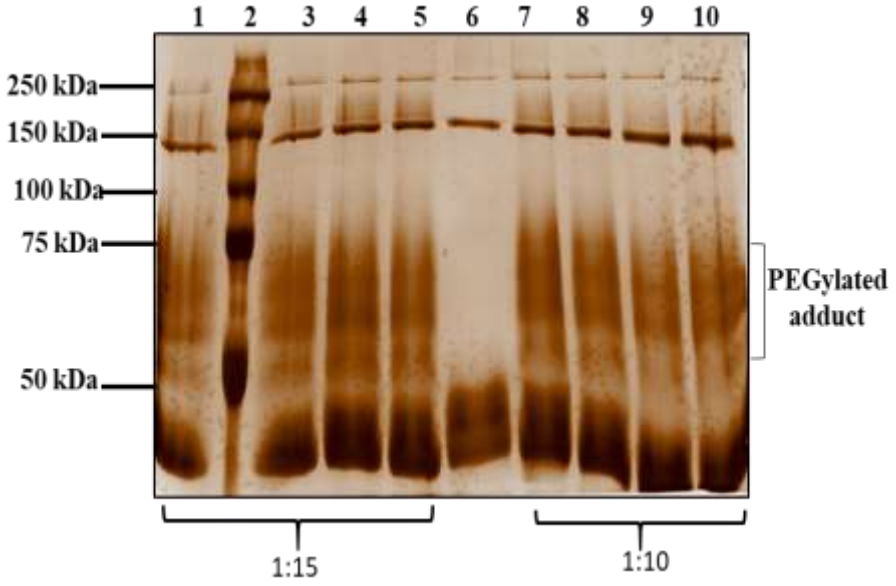


Figure 4.13 : Conjugation of 2 kDa DSPE-PEG MAL with thiolated Fab' - Analysis by silver staining: *Lane 1 - Thiol ratio 1:15 and PEGylation ratio 1:100; Lane 2 - Precision Plus Protein™ All Blue Pre-stained Protein Standards (Bio-Rad); Lane 3 - Thiol ratio 1:15 and PEGylation ratio 1:50; Lane 4 – Thiol ratio 1:15 and PEGylation ratio 1:20; Lane 5 - Thiol ratio 1:15 and PEGylation ratio 1:15; Lane 6 - Control; Lane Thiol ratio 1:10 and PEGylation ratio 1:100; Lane 8 - ; Lane Thiol ratio 1:10 and PEGylation ratio 1:50; Lane 9 – ; Lane Thiol ratio 1:10 and PEGylation ratio 1:20; Lane 10 – ; Lane Thiol ratio 1:10 and PEGylation ratio 1:15.*

4.5 Thiolated Fab' affinity determination by enzyme-linked immunosorbent assay

Fab' was thiolated using the Traut's reagent. Thiolation of amines of Lysine amino acid present in the epitope binding site of the Fab' can affect the binding affinity of Fab' to Antigen Y. The difference in the binding affinity ofFab' versus thiolated Fab' was determined by performing direct-ELISA. As shown in Figure 4.14, the binding affinity of thiolated Fab' was almost the same as that of Fab' which indicates that thiolation did not affect the binding affinity of Fab'.

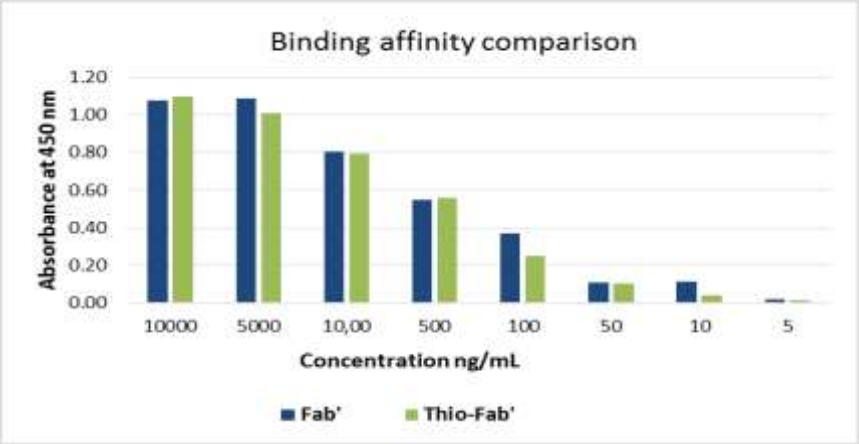
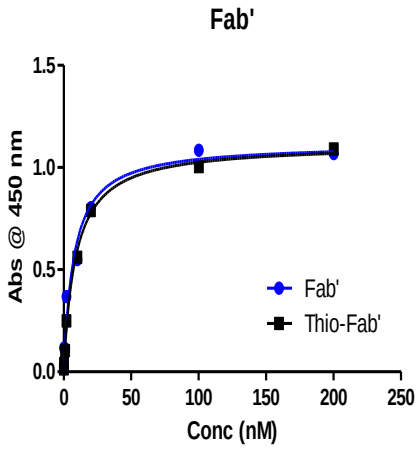


Figure 4.14 : Bar graph showing binding affinity of Fab' and thiolated-Fab' to Antigen Y

The results were confirmed by determining the dissociation constant for Fab' as well as thiolated Fab'. The dissociation constant Kd gives the measure of binding affinity between two molecules [71]. The dissociation constant is inversely proportional to the binding affinity of the molecule. As shown in Figure 4.15, the Kd value obtained for Fab' was 7.4 nM, whereas the Kd value for thiolated Fab' is 8.8 nM which suggests that difference in binding affinity of Fab' and thiolated Fab' is negligible.



	Fab'	Thio-Fab'
One site -- Specific binding		
Best-fit values		
Bmax	1.117	1.116
Kd	7.439	8.786
Std. Error		
Bmax	0.05075	0.02912
Kd	1.468	0.9629
95% Confidence Intervals		
Bmax	1.008 to 1.226	1.053 to 1.178
Kd	4.290 to 10.59	6.720 to 10.85
Goodness of Fit		
Degrees of Freedom	14	14
R^2	0.9602	0.9884
Absolute Sum of Squares	0.1048	0.03176
Sy.x	0.08651	0.04763
Number of points		
Analyzed	16	16

Figure 4.15 : Graph showing dissociation constant of Fab' and thiolated Fab'

4.6 Conjugation of thiolation of Fab' to functionalized Liposome

Thiolated Fab' was conjugated with functionalized liposomes to prepare immunoliposomes (ILs). Western blot was performed to confirm the presence of immunoliposomes. A band of conjugated adduct could be seen (Figure 4.16 lane 7), around 55 kDa along with a band of non-conjugated Fab' at 50 kDa, no adduct was seen for the conjugation reaction with non-functionalized liposomes (lase 5). This suggests successful attachment of Fab' to the maleimide functionalized liposome, which further indicate be separated from non-conjugated Fab' by SEC

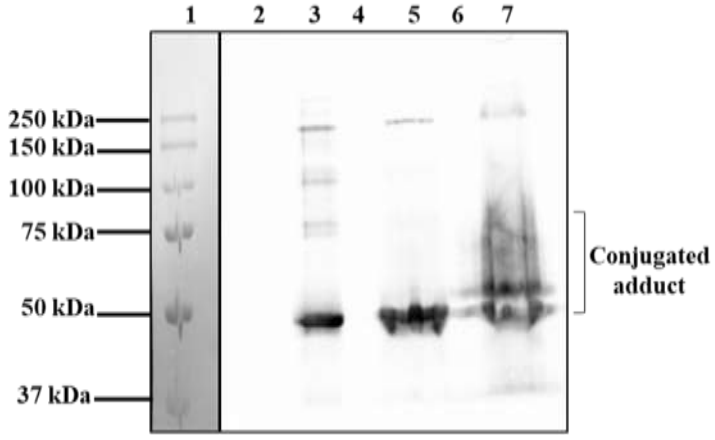


Figure 4.16: Conjugation of thiolated Fab' with functionalized liposome - Analysis by western blot: *Lane 1 - Precision Plus Protein™ All Blue Pre-stained Protein Standards (Bio-Rad); Lane 2 - Blank; Lane 3 -Control; Lane 4 - Blank; Lane 5 – thiolated Fab' conjugated with non-functionalized liposome; Lane 6 - Blank; Lane 7 – Conjugation of thiolated Fab' with functionalized liposome*

4.7 Scale-up and purification of Immunoliposomes

A 5 times scaled up the reaction of immunoliposomes was set up. The immunoliposomes were purified to separate unreacted Fab' using SEC. The column used had a fractionation range of 3 kDa – 70 kDa which separates small molecules such as thiolated Fab' (50kDa) and elute immunoliposomes in the void volume.

As shown in Figure 4.17, Fraction 1, 2, 3, 4, 5, and 6were collected and analyzed by Western blot. As shown in A 5 times scaled up the reaction of immunoliposomes was set up. The immunoliposomes were purified to separate unreacted Fab' using SEC. The column used had a fractionation range of 3 kDa – 70 kDa which separates small molecules such as thiolated Fab' (50kDa) and elute immunoliposomes in the void volume., Lane 2- Fab', Lane 4 contains load (unpurified sample). Two bands can be observed in load, higher molecular weight band. of conjugated Fab' (faint) and lower molecular weight band of un-conjugated Fab' (thick). As it can be seen in lane 6 (concentrated conjugates), purified ILs were separated from un-conjugated Fab' present in the load.

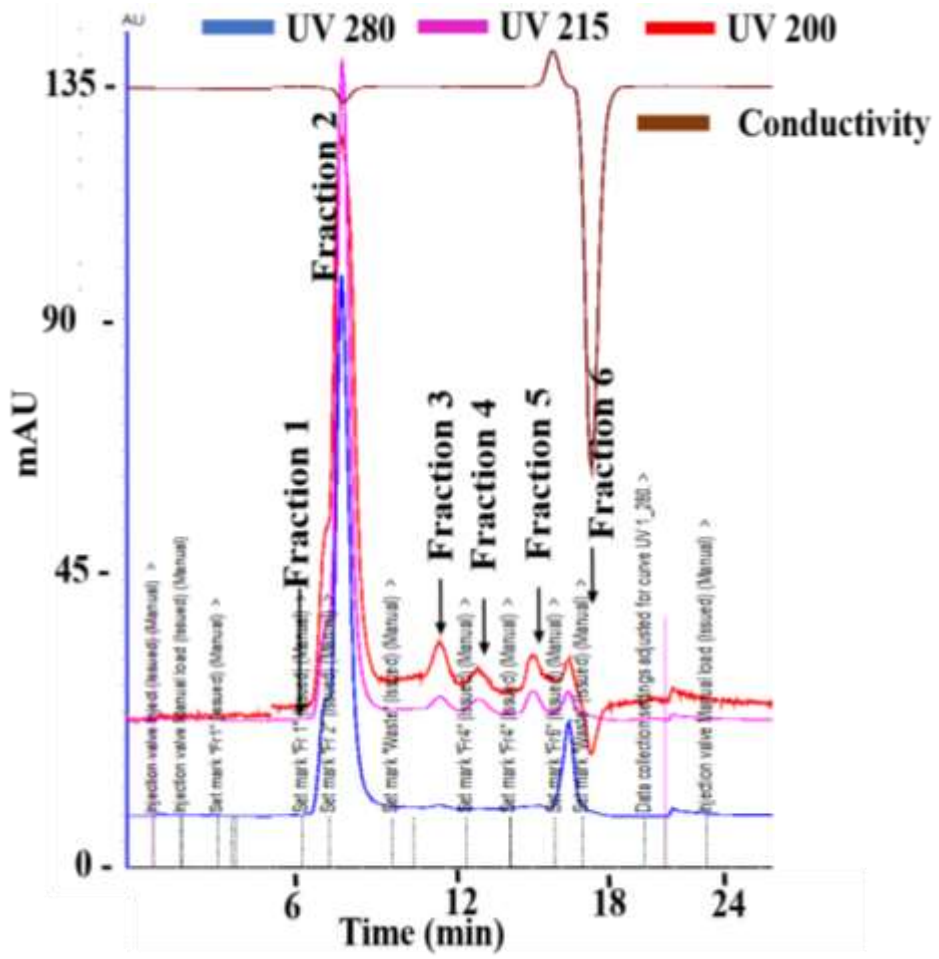


Figure 4.17: Chromatogram showing purification of ILs using Size exclusion chromatography

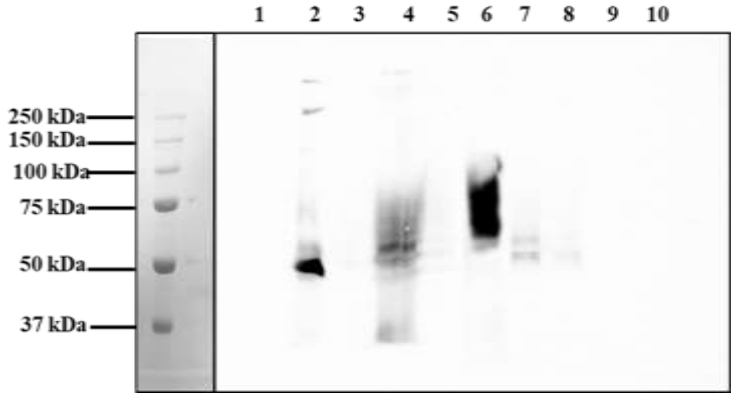


Figure 4.18: Purification of Immunoliposomes using SEC column - Analysis by western blot: *Lane 1 - Precision Plus Protein™ All Blue Pre-stained Protein Standards (Bio-Rad); Lane 2 – thiolated Fab'; Lane 3 – Blank; Lane 4 – Unpurified Immunoliposome; Lane 5 – Fraction 1; Lane 6 – Fraction 2; Lane 7 – Fraction 3; Lane 8 – Fraction 4; Lane 8 – Fraction 5; Lane 9 – Fraction 6, Lane 10 – Blank*

4.8 Size and PDI analysis of Immunoliposomes (ILs)

The size of Immunoliposomes was measured using Zetasizer Nano ZS (Malvern Instruments). The size of ILs was observed to be ~ 102 nm, which is similar to the size of un-conjugated liposomes (~ 99 nm) as observed in

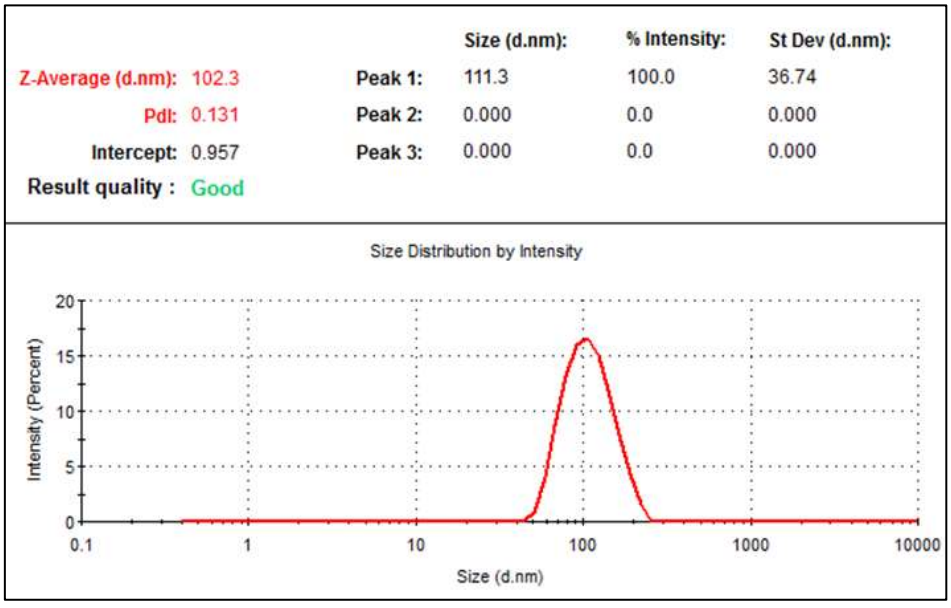


Figure 4.19: Size and PDI of immunoliposome

Figure 4.19. But the PDI was slightly higher ~ 0.1, which suggests that immunoliposomal population is slightly less homogenous in the mixture. An additional sizing step could be incorporated after purification to get a more homogeneous population.

Further to remove unconjugated liposomes from immunoliposomes (both of which come in the void volume of the SEC) an affinity chromatography can be performed with kappa select affinity chromatography where the immunoliposomes with a stick to the beads and the un-conjugated liposome will come out in the flow through. This purification step could not be performed due to time constraints.

4.9 Quantification of Fab' in Immunoliposomes

The amount of protein conjugated to immunoliposomes was checked using the Micro BCA™ Protein Assay Kit. The protein precipitation method was used to separate the lipid portion from immunoliposomes priorly [72]. The concentration of the protein conjugated onto immunoliposomes was determined to be 6 μg/mL. This is very low, and there is much scope for improving the yield of the process, but the quantity was sufficient enough to carry out the binding experiment with antigen Y.

4.10 To determine the binding specificity of Immunoliposomes to Antigen Y

The binding specificity of Immunoliposomes to antigen Y was determined by incubating the immunoliposomes with antigen Y for 20 min at 25°Cto allow the formation of antigen-antibody complex. The reaction samples were analyzed by SE-HPLC. If the antigen reacts with ILs it will co-elute and if not it will elute late in the column.

As shown in the Figure 4.20, the larger molecular weight Immunoliposomes were eluted first followed by the Antigen Y.

The peaks observed in the chromatogram, Figure 4.20, were collected as 200 µL aliquots in micro-titer plate and were analyzed by Dot blot (antigen detection by anti-antigen antibodies) as shown in Figure 4.21.

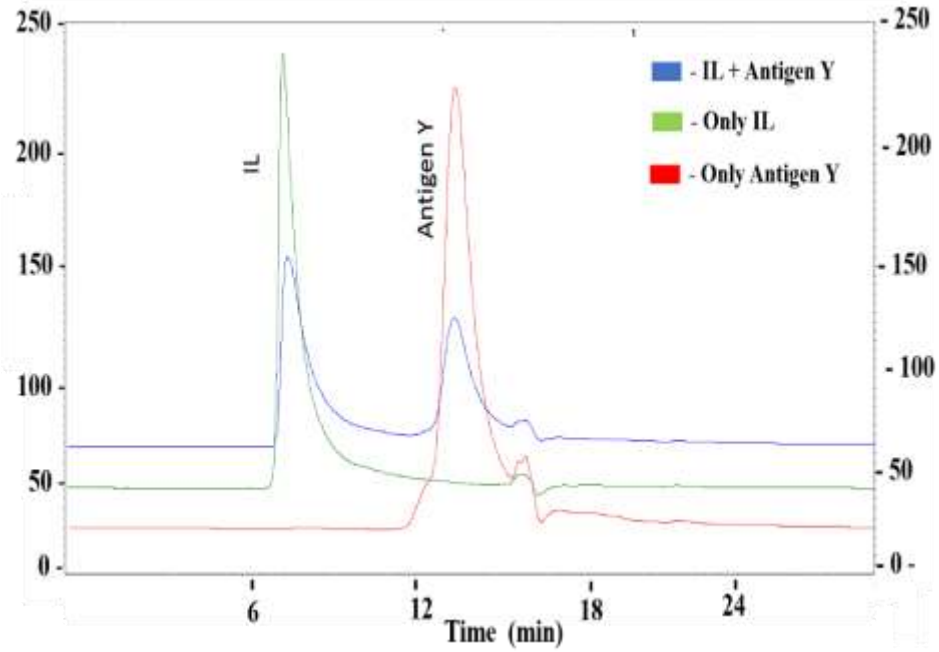


Figure 4.20: SE-HPLC chromatogram of all samples

For the reaction mixture (ILs + Antigen Y), peak was collected from 8 min to 18 min and were analyzed by Dot blot. The positive signal in the Dot blot obtained for initial aliquots collected from the peak representing IL sample (Figure 4.21 lane 1).It is indicative of the presence of Antigen Y in the particularly collected aliquote which would only be possible if ILs form complex with Antigen Y. The signal eventually fades away followed by a higher intensity signal. The higher intensity signal is of unbound Antigen Y.

For negative control sample containing only ILs, the peak was collected from 8 min to 11 min. In the Dot blot, (refer to Figure 4.21, lane 5 and 6) a negative signal was obtained for this sample as there were no antigen Y present in the sample.

For a sample containing only antigen Y, the peak was collected from 14 min to 18 min. In the Dot blot, (Figure 4.21, lane 7) a positive signal was obtained for this sample as it was a positive control.

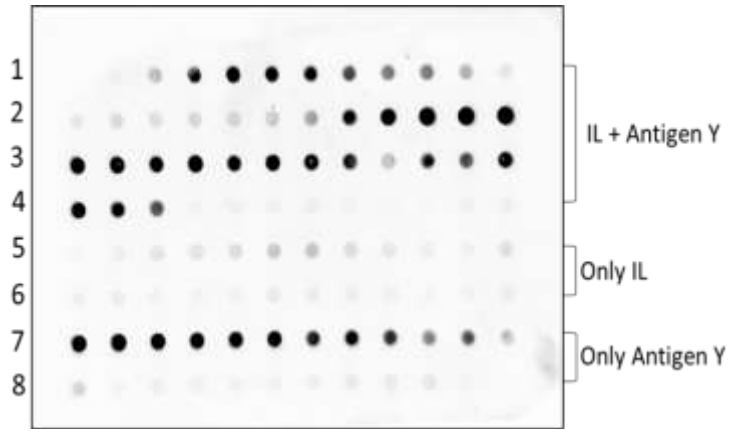


Figure 4.21: Dot blot of SE-HPLC fractions

The results obtained here conclude the binding of ILs to antigen Y but still are not enough to determine whether the binding was specific or non-specific. There may be a possibility of binding of Antigen Y to free maleimide groups present on the surface of immunoliposomes. If that is the case, then the binding specificity can be confirmed by blocking the maleimide group on the surface of liposome using cysteine and then incubating it with antigen Y. If a positive signal is seen, then it would mean non-specific binding to the immunoliposomes.

Chapter 5

Conclusion and future prospects

The project aimed to prepare and characterize immunoliposomes directed against a type of metastatic cancer. The first step for preparation of immunoliposomes is obtaining a suitable antibody fragment. The optimum conditions for obtaining the antibody fragment $F(ab)_2$ through pepsin digestion was successfully determined. Further, the Fab' fragment was obtained by reduction of $F(ab)_2$ using MEA.DSPE-PEG-MAL 2000 functionalized liposomes of about 90-100 nm were successfully prepared. During the course of the project it was found that Fab' produced by Fragmentation of IgG was unable to conjugate with PEG inserted in the functionalized liposome. Thus Fab' was thiolated using Traut's reagent without affecting its antigen binding property, and then the process of conjugation with liposomes was optimized. The conjugation of thiolated Fab' with functionalized liposomes was successful, resulting in the production of Immunoliposomes. The produced ILs were purified and characterized. The antigen binding specificity of Immunoliposomes was assessed by formation of an antigen-antibody complex, which was checked by antigen specific Dot blot.

This study establishes the 'proof of concept' for preparation of Immunoliposomes. The Immunoliposomes should be further characterized for morphology, specificity, stability, and toxicity. The process of conjugation should be further optimized to increase the yield of ILs. The specificity of ILs to Antigen Y can be determined by blocking the surface reactive groups of liposomes (Maleimide). Subsequently, the specificity and binding affinity of Immunoliposomes can be increased following requisite changes in the formulation of Immunoliposome. The immunoliposomes stability should be checked. It is also crucial to determine the surface morphology of the Immunoliposomes which can be achieved by

Transmission Electron Microscopy. Furthermore, anticancer drugs should be encapsulated in the ILs and studied for drug release profile. The *in-vitro* and *in-vivo* studies to check the therapeutic efficiency of Immunoliposomes should also be performed.

Chapter 6

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