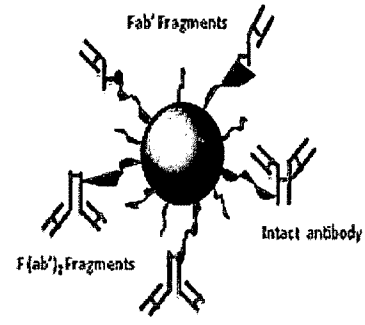
Chapter - 6

Preparation of Immunoliposomes



Conjugation of antibodies to functional PEG derivatives (Immunoliposome)

6.1. Introduction

Active targeting of liposomes to tumor cells is generally attempted by conjugating ligands to the liposomal surface which allow a specific interaction with the tumor cells. Several type of ligands have been used for this purpose, including antibodies or antibody fragments, vitamins, glycoproteins, peptides (RGD-sequences), and oligonucleotide aptamers. Among the different approaches of active targeting, immunoliposomes using antibody or antibody fragment as a targeting ligand and a lipid vesicle as a carrier for both hydrophilic and hydrophobic drugs, is a fascinating prospect in cancer therapy (Mastrobattista et al., 1999). The use of an antibody molecule as a homing device has been especially facilitated by the development of the hybridoma technology, which makes it possible to produce a large quantity of a monoclonal antibody to a wide variety of cell determinants (Kohler and Milstein, 1975). However, only a limited number of preclinical studies reported successful targeting of immunoliposomes *in vivo* (Peeters et al., 1987). As systemic administration is the most practical route for the treatment, immunoliposomes must be developed so that physiological barriers can be overcome. Therefore, the development of liposomes with RES impeding potential is a necessary step before attempting the use of immunoliposomes. Given a suitable antibody with high specificity and affinity for the target antigen, the critical factor is the accessibility of target cells to immunoliposomes. Efficient target binding of the injected immunoliposomes occurs only when the target cell is in the intravascular compartment or can be accessible through leaky vascular structures. Thus, in terms of targeting drug delivery by immunoliposomes, two anatomical compartments are of considerable interest for targeting sites. One is located at a readily accessible site in intravascular, such as the vascular endothelial surface, T cells, B cells or thrombus. Another is a much less accessible target site located in the extravascular. This site involves a solid tumor, an infection site, or an inflammation site, where vascular structure is leaky (Maruyama et al., 1999). The process of targeted drug delivery with immunoliposomes can be roughly divided into two phases: the transport phase, in which the immunoliposomes travel from the site of administration (often i.v. administration) to the target cells, and the effector phase that includes the specific binding of immunoliposomes to the target cells and the subsequent delivery of entrapped drugs (Mastrobattista et al., 1999). Immunoliposomes for the treatment of tumor should satisfy a number of requirements aimed at maximum targeting effect of immunoliposome administered systemically in the bloodstream. Antigen binding site of the liposome-conjugated antibody must be accessible for unperturbed interaction with antigen on the surface of target cells. The blood clearance of immunoliposomes must be minimized in comparison with rate of extravasation in the tumor. Immunoliposome must allow efficient loading and retention of a selected anticancer drug. And finally, the drug and antibody incorporation must be stable enough to permit liposomal entry into the tumor tissue without the loss of either of these agents (Maruyama et al., 1999).

The ability to conjugate an antibody to another protein or to drug delivery system is critically important for lots of applications in life science research, diagnostics, and therapeutics. Antibody conjugates have become one of the most important classes of biological agents associated with targeted therapy for cancer and other diseases. There are literally many markers that have been identified on tumor cells to which monoclonal antibodies have been developed for targeted therapy (Carter et al., 2004). The preparation of antibody conjugates to find and destroy cancer cells *in vivo* has become one of the leading strategies of research into investigational new drugs (McCarron et al., 2005). In most cases, the site-specific delivery of drugs involves the successful development of defined monoclonal antibody conjugates that can target diseased cells without affecting normal cells. The unique structural characteristics of antibody molecules supply a number of choices for modification and conjugation schemes (Goding, 1986a; Harlow and Lane, 1988a). The chemistry used to effect conjugate formation should be chosen to yield the best possible retention of antigen binding activity. A detailed illustration of antibody structure is shown in Figure 6.1.

Antibody molecules possess a number of functional groups suitable for modification or conjugation purposes. Crosslinking reagents may be used to target lysine ε-amine and N-terminal α-amine groups. Carboxylate groups also may be coupled to another molecule using the C-terminal end as well as aspartic acid and glutamic acid residues. Although, both amine and carboxylate groups are as abundant in antibodies as they are in most proteins, the distribution of them within the three dimensional structure of an immunoglobulin is nearly uniform throughout the surface topology. For this reason, conjugation procedures that utilize these groups will crosslink somewhat randomly to nearly all parts of the antibody molecule. This in turn leads to a random orientation of the antibody within the conjugate structure, often blocking the antigen binding sites against the surface of another coupled protein or molecule. Obscuring the binding sites in this manner results in decreased antigen binding activity in the conjugate compared to that observed for the unconjugated antibody (Hermanson, 2008). Conjugation chemistry finished with antibody molecules generally is more successful at preserving activity if the functional groups utilized are present in limiting quantities and only at discrete sites on the molecule. By proper selection of the conjugation chemistry and knowledge of antibody structure, the immunoglobulin molecule can be oriented so that its bivalent binding potential for antigen remains available. Two site-directed chemical reactions are especially useful in this regard. The disulfides in the hinge region that hold the heavy chains together can be selectively cleaved with a reducing agent [such as 2-mercaptothylamine (MEA), dithiothreitol (DTT), or tris (2-carboxyethyl)phosphine (TCEP)] to create two half-antibody molecules, each containing an antigen binding site (Palmer and Nissonoff, 1963; Sun et al., 2005).

The second method of site-directed conjugation of antibody molecules takes advantage of the carbohydrate chains typically attached to the C_H^2 domain within the Fc region. Mild oxidation of the polysaccharide sugar residues with sodium periodate will generate

aldehyde groups. A crosslinking or modification reagent containing a hydrazide functional group then can be used to target specifically these aldehydes for coupling to another molecule. Directed conjugation through antibody carbohydrate chains thus avoids the antigen binding regions while allowing the use of intact antibody molecules. This method often results in the highest retention of antigen binding activity within the ensuing conjugate. However, care should be taken in using this method, because some antibody molecules can be glycosylated near the antigen binding area, thus potentially interfering with activity upon conjugate formation (Béduneau et al., 2007; Simard and Leroux, 2009). Another limitation to the use of this strategy is the necessity for the antibody molecule to be glycosylated. Antibodies of polyclonal origin (from antisera) are usually glycosylated and work well in this procedure, but other antibody preparations may not possess polysaccharide. In particular, some monoclonals may not be post-translationally modified with carbohydrate after hybridoma synthesis. Recombinant antibodies grown in bacteria also may be devoid of carbohydrate. Before attempting to use a conjugation method that couples through polysaccharide regions, it is best to test the antibody to see whether it contains carbohydrate-especially if the immunoglobulin is of hybridoma or recombinant origin.

Useful enzymatic derivatives of antibody molecules may be prepared that still retain the antigen binding sites. Two principal digested forms of IgG antibodies are useful for creating immunological reagents. Enzymatic digestion with papain produces two small fragments of the immunoglobulin molecule, each containing an antigen binding site (called Fab' fragments), and one larger fragment containing only the lower portions of the two heavy chains (called Fc, for "fragment crystallizable") (Figure 6. 2a) (Coulter and Harris, 1983). Alternatively, pepsin cleavage produces one large fragment containing two antigen binding sites [called $F(ab')_2$] and many smaller fragments formed from extensive degradation of the Fc region (Figure 6.2b) (Rousseaux et al., 1983). The $F(ab')_2$ fragment is held together by retention of the disulfide bonds in the hinge region. Specific reduction of these disulfides using 2-mercaptoethylamine (MEA) or other reducing agents produces two Fab' fragments each of which has one antigen binding site. Papain and pepsin, and similar enzymes including bromelain, ficin, and trypsin, cleave immunoglobulin molecules in the hinge region of the heavy chain pairs. Depending on the location of cleavage, the disulfide groups holding the heavy chains together may or may not remain attached to the antigen binding fragments that result. Elimination of immunogenic effect of Fc portion and of the increased RES clearance through specific recognition by the phagocytic cells carrying Fc receptor was achieved by using antibody fragments instead of the whole antibody. Antibody fragments also allow better way of conjugation to the liposomes containing functionalised PEG derivatives through unique thiol groups in the hinge region (Maruyama et al., 1999).

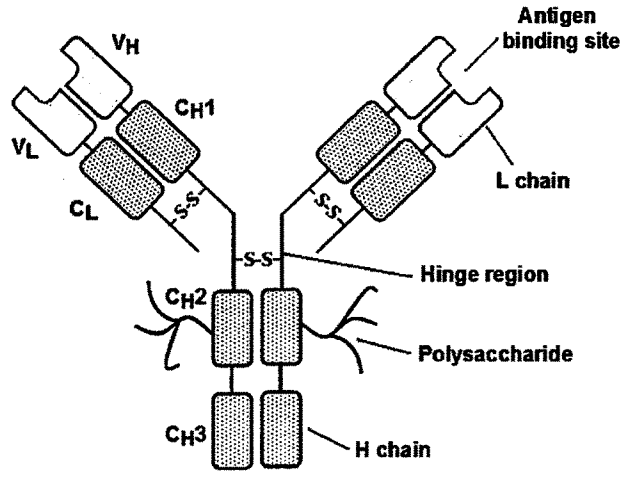


Figure 6.1. Modified structure of an IgG antibody molecule

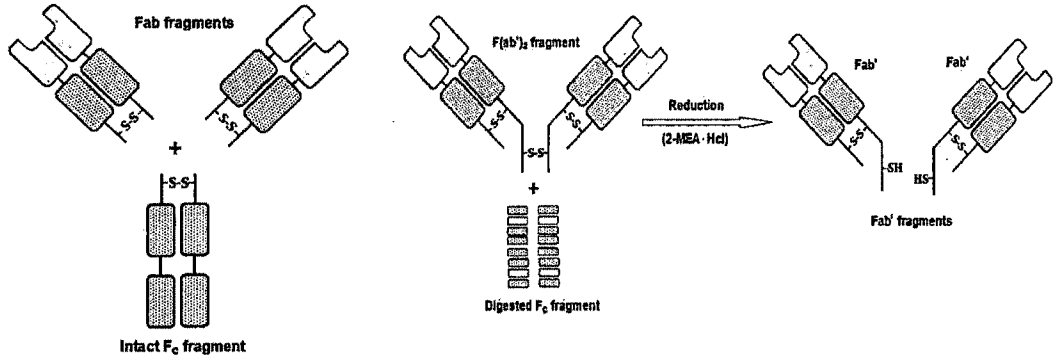


Figure 2a. **Figure 2b.**

Figure 6.2. Enzymatic digestion of IgG antibodies. **(a).** Papain digestion of IgG antibodies primarily results in cleavage in the hinge region above the interchain disulfides. This produces two heavy-light chain pairs, called Fab′ fragments, each containing one antigen binding site. The Fc region normally can be recovered intact. **(b).** Pepsin digestion of IgG class antibodies results in heavy chain cleavage below the disulfide groups in the hinge region. The bivalent fragments that are formed are called $F(ab')_2$. The remaining Fc region normally is severely degraded into smaller peptide fragments. $F(ab')_2$ fragments are then reduced with 2-mercaptoethylamine hydrochloride (MEA·HCl) which yield Fab′ fragments.

Angiogenesis is defined as the formation of new blood vessels from existing ones. For solid tumors (1-2mm^3), oxygen and nutrients can reach the center of the tumor by simple diffusion. Because of their non-functional or non-existent vasculature, non-angiogenic tumors are highly dependent on their microenvironment for oxygen and the supply of nutrients. When tumors reach 2 mm^3, a state of cellular hypoxia begins, initiating angiogenesis (Fabienne et al., 2010). Neuropilins (NRP 1 and NRP 2) are membranous receptors capable of binding two disparate ligands, class 3 semaphorins (SEMA 3A) and vascular endothelial growth factors (VEGF-A165), and regulating two diverse systems, neuronal guidance and angiogenesis. NRP-1 is expressed by a wide variety of human tumour cell lines and diverse human neoplasms, and are implicated in mediating effects of VEGF and semaphorins on the proliferation, survival and migration of cancer cells. NRP-1 is expressed in patient specimens from lung, breast, prostate, pancreatic and colon carcinomas. NRP1 has also been found in several other tumours including melanoma, astrocytoma and neuroblastoma. These findings taken together with the expression of NRPs in diverse neoplasms, suggests a possible role for this molecule in tumour invasion and metastasis in addition to its involvement in tumour vascularisation (Bielenberg et al., 2006).

Thus here we hypothesize that the liposomes, as a carrier of anticancer drug, conjugated with anti-neuropilin-1 antibodies (Intact/Fab' fragment), as a targeting ligand, have 1. kill angiogenic blood vessels and, indirectly, the tumor cells that these vessels support and 2. penetrate into the tumor interstitial space and function as a sustained release system, resulting in direct cancer cell kill, including cytotoxicity against cells that are at the tumor periphery and are independent of the tumor vasculature. 3. Also they invade into the tumor cells by endocytosis through neuropilin-1 receptors express on cancer cells. Thus, this combined strategy has the potential to overcome some major limitations of conventional chemotherapy.

6.2. Materials and Methods

Rabbit anti-neuropilin-1 polyclonal antibody was purchased from Santa Cruz Biotechnology, US. Goat anti-rabbit (whole IgG) FITC tagged secondary antibody, Dithiothreitol (DTT) and Ellman's reagent were purchased from Sigma Aldrich, Mumbai, India. Antibody modification kit was purchased from Thermo Scientific, US. Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-50 membranes (50KDa) were purchased from Millipore, US. Protein markers were purchased from Fermentas Molecular Biology Tools, Thermo Scientific, US. All other reagents used were of analytical grade and were used without further purification.

6.3. Modification of antibody

6.3.1. Preparation and purification of $F(ab')_2$ fragments of anti-neuropilin-1 antibody

Pierce® $F(ab')_2$ Micro Preparation Kit to generate and purify $F(ab')_2$ fragments from up to ten 0.125 ml samples containing 25-250μg of IgG was used in our project. $F(ab')_2$ fragments were generated and purified as per the protocol provided along with kit.

A. Immobilized pepsin equilibration:

Gently swirled the immobilized pepsin vial to obtain an even suspension and using a cut pipette tip, placed 65μl of the 50% slurry (i.e., 32.5μl of settled resin) into a 0.8 ml spin column. Caped the column and placed into a microcentrifuge tube (1.5mL). Centrifuged column at 5,000 × *g* for 1 minute and discarded the buffer. Washed the resin with 130μl digestion buffer (20 mM sodium acetate, pH 4.4; 0.05% sodium azide) and centrifuged the column at 5,000 × *g* for 1 minute and discarded the buffer. This step was repeated for two more times to completely equilibrate the immobilized pepsin with digestion buffer. Caped the bottom of the spin column with rubber cap.

B. IgG sample preparation:

Twisted off the bottom closure of a Zeba Desalt Spin Column and loosened the red cap and placed the column in a collection tube. The column was centrifuged at 1,500 × *g* for 1 minute to remove storage solution. Placed a mark on the side of the column where the compacted resin is slanted upward and the column was placed in centrifuge tube with the mark facing outward in all subsequent centrifugation steps. Added 300μl of digestion buffer to column and centrifuged at 1,500 × *g* for 1 minute to remove buffer. Repeated this step three additional times, discarding buffer from the collection tube. Placed the column in a new collection tube, removed the cap and slowly applied 250μl sample (containing 50μg of intact antibody) to the center of the compacted resin bed. Replaced the cap and centrifuged at 1,500 × *g* for 2 minutes to collect the sample.

C. Fragment generation:

250μl of the prepared IgG sample was added to the spin column containing the equilibrated immobilized pepsin (Section A). Top cap and bottom plug on the spin column was placed and briefly vortexed to mix. Incubated the digestion reaction for 2.5 hours, for rabbit IgG (rabbit neuropilin-1 polyclonal antibody), at 37°C with a constant end-over-end mixing during the incubation. Removed the bottom cap, placed the column into a microcentrifuge tube and centrifuged at 5,000 × *g* for 1 minute to separate digest from the Immobilized Pepsin. Washed the resin with 130μl of PBS and placed the column into a new tube and centrifuged at 5,000 × *g* for 1 minute. Added the wash fraction to the digested antibody and the total sample volume becomes 380μl. The digest was evaluated *via* 10% SDS-PAGE under non-reducing condition (sample loading buffer containing no reducing agent) to assess digestion completion. The bands were visualized using silver staining method.

D. *F(ab')$_2$ purification:*

The SDS-PEGE analysis followed by silver staining of separated antibody digest confirms the complete digestion of neuropilin-1 antibody. However, the purification of F(ab')$_2$ from undigested antibody present in undetectable range was done as per the protocol given.

Equilibrated the NAb Protein A Column, PBS and IgG Elution Buffer to room temperature. Snapped off bottom closure and loosen top yellow cap on the Protein A Column. Placed the column in a collection tube and centrifuge for 1 minute at 1,000 × *g* to remove storage solution (contains 0.02% sodium azide) and discarded the flow-through. To equilibrate column, added 400µl of PBS and briefly mixed, centrifuged for 1 minute, discarded the flow-through, and repeated this step for one more time. Capped the bottom of column with the rubber cap and applied 380µl sample to column and capped the top tightly. Resuspended the resin and sample by inversion and incubated at room temperature with end-over-end mixing for 10 minutes. Loosened the top cap, removed the bottom cap, placed column in a new collection tube and centrifuged for 5 minute. The flow-through fraction containing F(ab')$_2$ and Fc fragments was saved. For optimal recovery, we washed the column with 200µl of PBS, centrifuged for 5 minute, collected the flow-through and mixed F(ab')$_2$ fraction.

The F(ab')$_2$ fragments were purified from Fc fragments by ultracel membranes (50KDa). The concentration of purified F(ab')$_2$ was determined by Bradford protein estimation method.

6.3.2. Reduction of F(ab')$_2$ into Fab'-SH

The amount of DTT equivalent to 10mM was added to F(ab')$_2$ solution (~15µg) containing 1mM EDTA and incubated at room temperature for 1hr with end-over-end mixing (Xu et al., 2002). The Fab'-SH fragments were purified from DTT by using Zeba Desalt Spin Columns. After incubation, the sample was immediately transferred to Zeba Desalting column and incubated for 10minutes at room temperature. Placed the column in a collection tube containing EDTA equivalent to 1mM and centrifuged at 1000 × *g* to separate the Fab'-SH fragments from DTT. The fragments were analysed *via* 10% SDS-PAGE under non-reducing condition for assessing complete reduction of F(ab')$_2$. We have used chromatographic buffers containing 1mM EDTA to prevent metal ions catalysed oxidation of reduced sulfhydryls into disulfides. The Fab' fragments were also analysed for presence of –SH groups using Ellman's assay. The prepared Fab'-SH fragments were immediately used for immunoliposomes preparation.

6.4. Preparation of immunoliposomes

The thiolated anti-neuropilin-1 Fab' fragments were mixed with the prepared functionalised PEGylated liposomes containing maleimide-terminated linker at the, anti-NRP-1 Fab'/Mal-mPEG$_{2000}$-DSPE, molar ratio of 1/100, µM/µM (weight ratio of 1:50, µg/µg) and the mixture was incubated overnight in cold room under N_2. Subsequently,

the unconjugated maleimide groups were reacted by incubating immunoliposomes with excess amount of cysteine for overnight in cold room. The excess unreacted cysteine was then removed by ultracel membranes (50KDa). The prepared immunoliposomes were confirmed by 8% SDS-PAGE gel permeation chromatography and were analysed for mean particle size, % drug content, total protein content by bradford protein estimation method, and for immunoreactivity.

6.5. Characterization

6.5.1. SDS-PAGE analysis of $F(ab')_2$, Fab'-SH and immunoliposomes

The SDS-PAGE gel permeation chromatography was performed to determine the digestion of intact antibody into $F(ab')_2$, reduction of $F(ab')_2$ into Fab'-SH, and conjugation of Fab'-SH to functionalised PEGylated liposomes (immunoliposomes) (Béduneau et al., 2007). The SDS-PEGE was performed as described detail in western blotting analysis technique (chapter: determination of neuropilin-1 protein expression in A549 and B16F10 cell line) under non reducing condition (sample loading buffer containing no reducing agents). The chromatographic buffers containing 1mM EDTA were used for separation of loaded $F(ab')_2$, Fab'-SH and immunoliposomes. The 10%, to identify $F(ab')_2$ fragment, and 8%, to identify Fab'-SH and immunoliposomes, SDS-PAGE gel permeation chromatography was performed.

6.5.2. Silver staining method

Principle:

Silver staining is the most sensitive method for permanent staining of proteins or nucleic acids in polyacrylamide gels. It creates a record of the electrophoresis result that can be viewed without any special equipment. It is, however, a complex, multi-step process, and many variables can influence the result. High purity reagents and precise timing are necessary for reproducible, high-quality results.

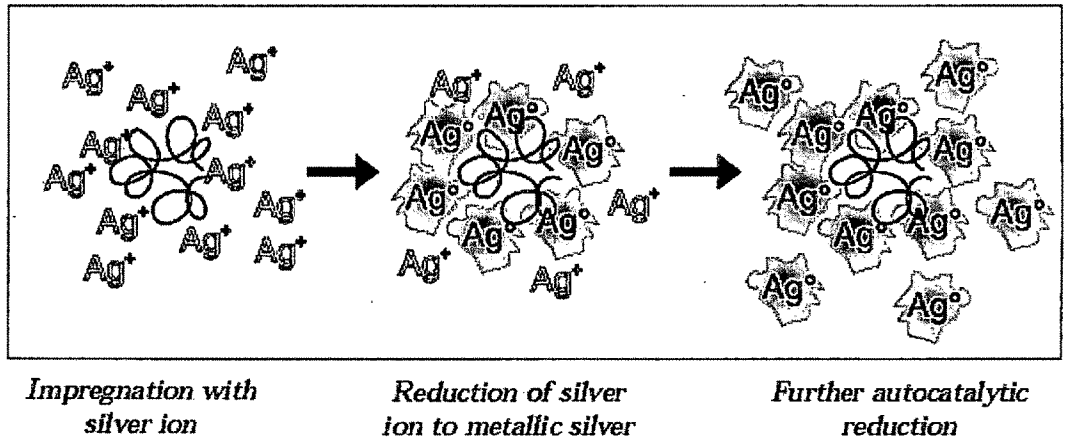


Figure 6.3. Mechanism of silver staining of separated proteins on SDS-PAGE gel

In silver staining, polyacrylamide gels are impregnated with soluble silver ion (Ag^+) and developed by treatment with a reductant. Macromolecules in the gel promote the reduction of silver ion to metallic silver (Ag^o), which is insoluble and visible, allowing protein or nucleic acid containing bands to be seen. The initial deposition of metallic silver promotes further deposition in an autocatalytic process, resulting in exceptionally high sensitivity.

The silver staining process consists of the following steps: Fixing, sensitization, silver impregnation, development, stopping, and gel preservation. Water washes are also included between some of the steps.

Fixing: In the fixing step, the gel is treated with acid. This renders the macromolecules in the gel insoluble and prevents them from diffusing out of the gel during subsequent staining steps. Substances in the gel that interfere with silver staining such as buffers, ions, denaturants, detergents or carrier ampholytes, are washed out of the gel during this step.

Sensitization: The gel is treated with reagents that chemically modify proteins, rendering them more reactive toward silver, and reagents that accelerate the subsequent reduction of silver ion. This step greatly enhances the sensitivity of silver staining for protein but is not necessary when silver staining DNA. Excess sensitization reagent results in a high level of background staining, so the gel is washed thoroughly with distilled or de-ionized water following the sensitization step.

Silver impregnation: In this step, the gel is treated with silver nitrate. Mildly acidic conditions prevent silver ion from being reduced to metallic silver. The gel is briefly washed following this step to remove excess silver from the gel surface.

Development: The development solution contains formaldehyde, which reduces silver ion to metallic silver. This reaction only proceeds at high pH, so sodium carbonate is included to render the development solution alkaline.

Stopping and preservation: The stopping solution prevents further reduction of silver ion. The preservation solution contains glycerol, which prevents the gel from cracking during drying.

Protocol:

The separated proteins on 8% and 10% SDS-PPAGE gel were identified using highly sensitive silver staining method. The optimized laboratory protocol was used for staining the proteins.

a. The gel was kept overnight in fixing solution composed of methanol: water: glacial acetic acid (50:40:10, v/v).
b. After overnight incubation with fixing solution the gel was washed with 5% methanol for 10 minutes.
c. Gel was washed twice with distilled water, 5 minutes each.
d. Gel was incubated with 0.02% sodium thiosulphate for 2 minutes.
e. Washed the gel with distilled water 3 times, 3 minutes each.

f. Incubated the gel in 0.2% silver nitrate solution for 30 minutes.
g. Gel was washed with distilled water twice, one minute each.
h. Gel was kept in developing solution (2.19% sodium carbonate, 2mL of 0.02% sodium thiosulphate solution and 125µl formaldehyde solution) until the bands are visualised.
i. When the bands are of desired intensity, gel was removed and placed in a stopping solution (1.4% EDTA). The gels were photographed, and preserved.

6.5.3. Ellman's assay to confirm the presence of –SH groups on Fab' fragments

The presence of sulfhydryl groups on the prepared Fab' fragments was tested before incubation with functionalised liposomes to prepare immunoliposomes using Ellman's reagent. The concentration of thiols present was calculated as per the thermo scientific protocol (as explained in the chapter 5; preparation of functionalised liposomes). The 10µl (~0.706µg of Fab' fragment) of the prepared Fab' fragment solution was diluted to 250µl with reaction buffer (0.1M sodium phosphate pH 8, containing 1mM EDTA) in a test tube. The blank test tube containing 250µl reaction buffer was prepared. To both the test tubes added 2.5mL reaction buffer and 50µl Ellman's reagent (4mg dissolved in 1mL reaction buffer). Mixed well and incubated at room temperature for 15 minutes. The absorbance of the blank ant test samples were read at 412nm using UV-visible spectrophotometer. The absorbance of test sample after subtracting the blank absorbance was used for sulfhydryl estimation using molar absorptivity of TNB (2-nitro-5-thiobenzoic acid) as described in chapter 5; preparation of functionalised liposomes.

6.5.4. Determination of concentration Fab' fragments present in unknown solution and over liposomal surface

The 0.5mL of prepared immunoliposomes was dissolved in methanol to extract the lipids. The methanol in eppendorf tube was then evaporated at 50 ^{0}C to get residue of lipids and Fab' fragments at the bottom of tube. The residue was then dissolved in 0.1mL distilled water, centrifuged, and the supernatant was used to determine the amount of Fab' fragments conjugated over liposomes.

Standard 0.5mg/ml bovine serum albumin (BSA) solution in milliQ water was prepared. 1.25, 2.5, 3.75 and 5µg of BSA from standard solution (2.5µl, 5µl, 7.5µl and 10µl, respectively) were transferred to 96 well plate in triplicate. Volume was made up to 25µl with milliQ water. 25µl water was kept as blank. 25µl of prepared Fab' fragment solution and extracted Fab' fragments from immunoliposomes were transferred 96 well plate in triplicate. 100µl of Bradford's reagent was added to all sample and blank wells and absorbance was taken at 595nm using ELISA reader with SoftMax Pro software. Protein concentration was automatically determined by the software, using the BSA standard.

6.5.5. Immunoreactivity of prepared Fab′ fragments and immunoliposomes

A549 cells were grown up to 90% confluency in 35mm tissue culture plates, harvested, and 0.6 million cells were transferred to different eppendorf tubes. Cells in different tubes were washed twice with PBS and then were fixed using 1% paraformaldehyde (PFA). The cells of 1st tube were considered as control for background fluorescence of the cells. The cells in the 2nd tube were considered as secondary control in which the cells were incubated with FITC-labelled secondary antibodies only, for one hour. The 3rd tube was considered as positive control in which the cells were first incubated with intact anti-neuropilin-1 antibody for one hour (1μg/10^6 cells), washed with FACS buffer twice followed by incubation with FITC labelled secondary antibody (1:200) for 1hr. The 4th, 5th, and 6th tube cells were first incubated with 1μg, 4μg, and 8μg Fab′ fragments, respectively for 2hrs. Similarly, the 7th, 8th, and 9th tube cells were incubated first with immunoliposomes equivalent to 1μg, 4μg, and 8μg of conjugated Fab′ fragment, respectively for 2hrs. After incubation, the cells (from tube 4th to 9th) were washed twice with FACS buffer and then incubated with intact anti-neuropilin-1 antibody (1μg/10^6 cells), for one hour. After one hour incubation, the cells were again washed twice with FACS buffer and incubated with FITC labelled secondary antibody (1:200) for one hour. During incubation periods the cells were re-dispersed at every 15minutes. After incubation the cells from all tubes (from 2nd to 9th) were washed twice with FACS buffer, resuspended in FACS buffer and acquired using FACS Calibur.

6.6. Results and Discussion

6.6.1. SDS-PAGE analysis of modified antibody and immunoliposomes

In the present research we have conjugated Fab′ fragments of anti-neuropilin-1 antibody over liposomes instead of whole antibody as it eliminates the immunogenic effect of Fc portion and the increased RES clearance through specific recognition by the phagocytic cells carrying Fc receptors. Fab′ fragments also allow better way of conjugation to the liposomes containing terminal functional maleimide group through unique thiol groups in the hinge region (Maruyama et al., 1997; Juweid et al., 1997). In contrast to whole antibody, Fab′ has the small size (MW: ~40kDa) and guarantees its tumor tissue/cell penetration ability. Also, its functional end groups for chemical conjugation are distanced from its binding site (Alexis et al., 2008).

The pepsin cleavage produces one large fragment containing two antigen binding sites [called $F(ab')_2$] and many smaller fragments formed from extensive degradation of the Fc region (Figure 6.2b) (Coulter and Harris, 1983). The $F(ab')_2$ fragment is held together by retention of the disulfide bonds in the hinge region. Specific reduction of these disulfides using MEA or other reducing agents produces two Fab′ fragments each of which has one antigen binding site.

Hence, in the present study we have generated and purified $F(ab')_2$ fragments of anti-neuropilin-1 antibody using Pierce® $F(ab')_2$ micro preparation kit (digestion of antibody using immobilised pepsin followed by purification of $F(ab')_2$ from undigested antibody

by protein A column). The prepared $F(ab')_2$ fragments were then reduced to Fab' fragments using dithiothreitol (equivalent to 10mM) (Xu et al., 2002; Sun et al., 2005).
The prepared $F(ab')_2$, Fab' fragments and immunoliposomes were identified by SDS-PAGE gel permeation chromatography (Béduneau et al., 2007). The 10%, to identify $F(ab')_2$ fragment, and 8%, to identify Fab'-SH and immunoliposomes, SDS-PAGE gel permeation chromatography was performed under non reducing condition (loading buffer containing no reducing agent). Under non reducing condition we can prevent the reduction of $F(ab')_2$ into Fab'-SH that happens when $F(ab')_2$ sample was heating in the presence of reducing agent in loading buffer during sample preparation. Also, after preparation of Fab'-SH by reduction with DTT, if we run the gel under reducing condition (to identify formation of Fab'-SH) that helps to reduce unreduced $F(ab')_2$ into Fab'-SH during sample preparation resulting in wrong interpretation of our reduction experimental results. This would help us to distinguish whether the reduction reaction completed during/after reduction experiment or during/after sample preparation for PAGE analysis. Therefore, to prevent these wrong interpretations of the results we performed SDPS-PAGE experiments under non-reducing condition. The chromatographic buffers containing 1mM EDTA were used as EDTA prevents the ion catalysed oxidation of Fab' fragments containing sulfhydryl groups into disulfides resulting in formation of $F(ab')_2$, during experiment, in the chromatographic gel in the presence of buffer.
Silver staining is the most sensitive method for permanent staining of proteins or nucleic acids in polyacrylamide gels as compared to other available methods. We used silver staining method as it is very sensitive method and we can easily detect small amount of separated undigested antibody, $F(ab')_2$, Fab' and Fc fragments. Also, using this method we can easily handle small amount of samples without wasting and when we have less amount of antibody for these types of projects.
The results of pepsin digestion of anti-neuropilin-1 antibody are shown in Figure 6.4a. The molecular weight of separated proteins are determined using software Alpha ease (Alpha Innotech). Lane 1, lane 2 and lane 3 are pepsin digest, protein marker (25, 34, 45, 68 and 120kDa), and BSA, respectively. In lane 1, one band is observed at molecular weight of ~78kDa corresponding to $F(ab')_2$ fragment. The second and third bands showed molecular weight of 42kDa and 23kDa corresponding to digested large and small Fc fragments, respectively. We observed no band at 120kDa corresponding to undigested antibody indicating the complete digestion of neuropilin-1 antibody. The gel was incubated in the developing solution as long as possible to obtain all possible bands with minimum background color.
In Figure 6.4b, the lane 1, lane 2, lane 3, lane 4, lane 5 and lane 6 represents protein marker (11, 25, 36, 55, 72, 95, 130 and 250kDa), Fab' fragment, $F(ab')_2$ digest, intact neuropilin-1 antibody, immunoliposomes, and BSA, respectively. We observed a single band at ~35kDa in lane 2 that confirms the presence of Fab' fragment formed by complete reduction of $F(ab')_2$ (lane 3 at ~78kDa) at 10mM DTT. In lane 4 we observed a

band at 120kDa corresponding to intact neuropilin-1 antibody. The immunoliposomes equivalent to double the amount of Fab′ fragments were loaded in the well. The conjugation of Fab′ fragments over liposomes can be confirmed by both the disappearance of Fag′ fragment band and appearance of new band corresponding to immunoliposomes in the lane 5. We observed disappearance of band at ~35kDa corresponding to Fab′ fragment and observed very big and well spreaded new band at ~12kDa indicating the formation of immunoliposomes. The immunoliposomes (composed of many lipid molecules and Fab′ fragments) are of high molecular weight, as compared to other proteins used in the study, diffuse very slowly in the gel (8%) and must show band near the well, or above protein marker with highest molecular weight (250kDa in our study). Instead, a new band at ~12kda corresponding to immunoliposomes was observed in our study. This indicated that the immunoliposomes diffused much faster than other proteins used in our study. The principle of gel permeation chromatography includes diffusion of negatively charged proteins (negative charge induced by heating proteins with SDS during sample preparation) towards positive electrode. The prepared immunoliposomes have zeta potential of -55±3.1mV and they gain still more negative potential during sample preparation for loading. Therefore, we can correlate this highest zeta potential of immunoliposomes, as compared to other proteins used in the study, for faster diffusion towards positive electrode in the gel. The shape of the immunoliposome band in the gel (lane 5) is might be due to slight heterogeneity of liposomal dispersion.

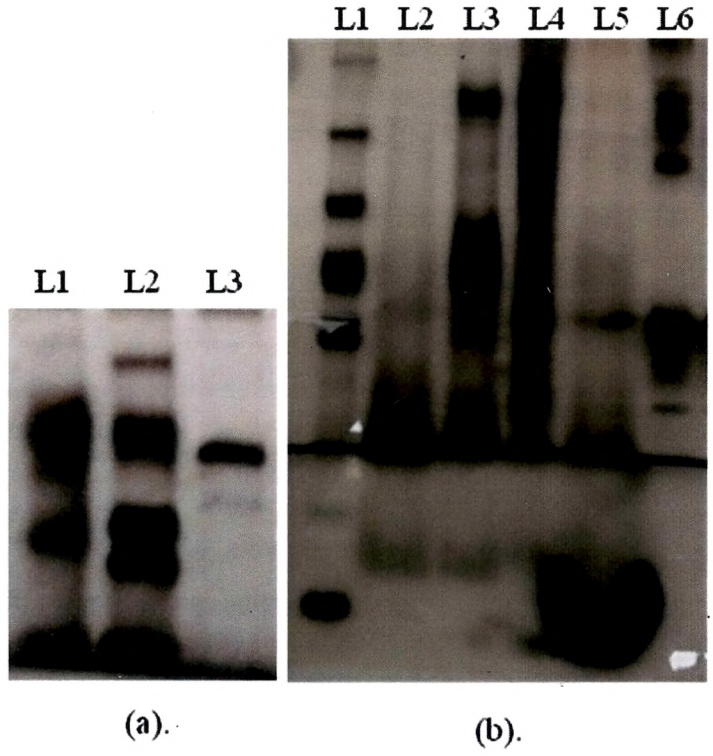


Figure 6.4. Modification of neuropilin-1 antibody and preparation of immunoliposomes. (a). Pepsin digestion of neuropilin-1 antibody (L1: antibody digest; L2: protein marker; L3: BSA). (b). Reduction of $F(ab')_2$ fragment and confirmation of Fab′ fragment and immunoliposomes (L1: protein marker; L2: Fab′ fragment; L3: $F(ab')_2$ fragment; L4: intact neuropilin-1 antibody; L5: immunoliposomes; L6: BSA).

6.6.2. Determination of presence of sulfhydryl groups on Fab′ fragments

After confirmation of reduced Fab′ fragments, the presence of sulphydryl group was identified using Ellman's reagent and quantified based on molar absorptivity. The amount of thiol groups present in 10μl of the sample containing ~0.706μg of Fab′ fragment was calculated below.

The reported molar absorptivity (molar extinction coefficient, which is expressed in units of $M^{-1}cm^{-1}$) of TNB (2-nitro-5-thiobenzoic acid) in this buffer system at 412nm is 14,150 (Riddles et al., 1983). Molar absorptivity, 'E' is defined as follows:

E= A/bc

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

Solving for concentration gives the following formula:

C=A/bE

A=0.156, b=1cm and E = 14,150 $M^{-1}cm^{-1}$.

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

= 1.1024×10^{-5} M/litre

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

The total volume of the solution being measured is

2.5 ml of Reaction Buffer

+ 0.25ml un-known sample

+ 0.05 ml of Ellman's Reagent Solution

2.80 ml of solution

The concentration of the unknown sample is 1.1024×10^{-5} M (1000ml contains 1.1024×10^{-5} M), then 2.80 ml of that solution contains =

$(2.8 \times 1.1024\times10^{-5})/1000 = 3.086\times10^{-8}$ moles

Therefore, the 10μl of the used sample (~0.706μg of Fab' fragment) contain 3.086×10^{-8} moles of thiols. This indicated the availability of sufficient numbers of sulphydryl groups on the prepared Fab' fragments for conjugation over liposomes through maleimide functional group *via* thioether linkage.

6.6.3. Preparation of immunoliposomes

The immunoliposomes were prepared by incubating prepared Fab' fragments with functionalised PEGylated liposomes overnight in cold room followed by incubation with excess amount of cysteine and purification of immunoliposomes from unconjugated Fab' fragments and cysteine by using ultracel-50 membranes (50kDa MWCO). The Table 6.1 shows the mean particle size, zeta potential, drug content, and total antibody content of prepared immunoliposomes. The lipid concentration was determined by Stewart method (Stewart, 1980) and found to be 0.0255mM/mL of final prepared immunoliposomes. The concentration of Fab' fragment in unknown solution was determined by Bradford protein assay (as described in analytical methods). To prepare immunoliposomes ~15μg (~0.4μM) of Fab' fragment was incubated with functionalised liposomes of 0.0255mM of total lipid/mL (40μM of functionalised lipid/mL). The weight ratio of Fab' fragment to functionalised lipid is 1:50, w/w (or 1:100, μM/μM). The concentration of Fab' fragments attached over liposomes was determined by Bradford assay and it was found to be ~14.42±0.12μg/mL (0.0255mM of total lipid). Therefore, the % Fab' fragment conjugated over liposomes was found to be 96.13%.

No change in the zeta potential was observed between Pegylated and immunoliposomes. The slight increase in mean particle size of the immunoliposomes is could be due to conjugation of Fab' fragments over PEGylated liposomes. The % drug content of

immunoliposomes was slightly decreased and is might be due to dilution that occur during the preparation and purification of immunoliposomes (Table 6.1).

Table 6.1. Comparison PEGylated liposomes and immunoliposomes

Formulation	Mean particle size (nm) with PDI	Zeta potential (mV)	% Drug content
PEGylated Liposomes	119±4 (0.228±0.040)	-56±2.1	78.35±2.86
Immunoliposomes	125±3 (0.182±0.038)	-55±3.1	73.23±3.54

Values are Mean±SD, n=3.

6.6.4. Determination of immunoreactivity of prepared Fab' fragment and immunoliposomes

The expression of neuropilin-1 receptors in A549 cells was determined by using western blotting, FACS and confocal microscopy techniques using polyclonal anti-neuropilin-1 antibody. The Fab' fragments of neuropilin-1 antibody were prepared for conjugation over liposomes (immunoliposomes). The intact antibody was first pepsin digested, followed by reduction with dithiothreitol. After this enzymatic and chemical modification and/or after conjugation to liposomes, the Fab' fragments may lose their immunoreactivity. Therefore, it is necessary to determine the immunoreactivity of Fab' fragments and immunoliposomes before determining its performance *in vitro*, against different tumor cell lines which over-express neuropilin-1 receptor, and *in vivo* in tumor induced animal models.

Radioimmunoassay (RIA) can be used to determine the immunoreactivity of these Fab' fragments and immunoliposomes. This technique need well skilled person and radio-active substance (Garg et al., 1991; Adamczyk et al., 1998). If a primary antibody is labeled with a fluorescent tag or an enzyme capable of producing a visible signal upon addition of the appropriate substrate, then probing with a primary antibody allows direct detection of the target antigen. Use of secondary antibodies provides several advantages over direct detection with labeled primary antibodies. Most importantly, primary antibodies are typically expensive to buy or produce, and researchers may not wish to risk poor recovery or complete inactivation by subjecting the antibody to a labeling procedure to make it detectable. In addition, using an appropriate secondary antibody can provide signal amplification by multiplying the number of label molecules that can be bound per target molecule.

For each target species, varieties of secondary antibody are available for particular immunoglobulin classes and fragment types. Some will bind all parts of whole IgG (heavy and light chains, H+L), or only the Fab or Fc region, or only the γ (gamma) chain. Still others are specific for IgM heavy chains (μ or Fc5μ), or the λ (lambda) or κ (kappa)

light chains common to all immunoglobulins (IgG, IgA, IgD, IgE, and IgM). In our study anti-rabbit IgG (whole molecule)-FITC antibody produced in goat (purchased from Sigma Aldrich, Mumbai, India) was used. Hence, it will bind all parts of rabbit anti-neuropilin-1 primary antibody.

The experiment was designed as described in the experimental part. Cells were first treated with increasing concentrations of Fab' fragments and immunoliposomes (equivalent to 1μg, 4μg and 8μg of Fab' fragments) for 2hr. Then, the cells were washed, and allowed to react with neuropilin-1 intact antibody (1μg/10^6 cells) for one hour. After 1hr the cells were washed and incubated with goat anti-rabbit IgG (whole molecule)-FITC antibody (1:200 dilution) for one hour. The cells were washed with FACS buffer, centrifuged, re-suspended in FACS buffer and acquired using FACS Calibur.

Under our experimental condition, there are two possible outcomes expected. Firstly, if the secondary antibody binds only to Fc portion of primary antibody then the decrease in shift (decrease in MFI; mean fluorescence intensity) will be observed with increase in the concentrations of Fab' fragments and immunoliposomes as compared to positive control cells indicating the immunoreactivity of Fab' fragments and immunoliposomes. This is because when the cells were first reacted with Fab' fragments and immunoliposomes, if they are immunoreactive, they occupy maximum receptors and might saturate the receptors at higher concentrations. This leads to decreased availability and/or un-availability of receptors when cells were treated with intact primary antibody. Therefore, less number and/or no primary antibodies will bind on cells, followed by less and/or no secondary antibody will bind on Fc portion of primary antibody resulting in decreased or no signal as compared to positive control. This decrease in signal indicates prepared Fab' fragments and immunoliposomes are immunoreactive.

Secondly, if the secondary antibody binds to all parts on primary antibody, then we can expect the increase in shift (MFI) and/or reach saturation with increase in Fab' fragments and immunoliposomes concentration as compared to positive control cells. This is because the secondary antibody is binding to both Fab' fragments/immunoliposomes and intact antibody. This leads to increase in shift (MFI) and/or reach saturation (additive shift at lower concentration) as compared to positive control. This again indicates that the prepared Fab' fragments and immunoliposomes are immunoreactive.

However, from the experimental results, we observed increased shift/MFI with increase in Fab' fragments and immunoliposomes concentration (Figure 6.5). This indicates both Fab' fragments and immunoliposomes retained their immunoreactivity after subjecting to enzymatic and chemical modification and after conjugation to liposomes, respectively. This also indicates that the secondary antibody tested is binding to all parts on primary antibody than Fc portion alone.

When compared the relative mean fluorescence intensity (RMFI) of Fab' fragment treated cell with RMFI of immunoliposomes treated cells, we observed a significant

difference between them (Table 6.2 and Figure 6.6). With immunoliposomes equivalent of 1μg we observed the RMFI (562.27±15.51%) that is more than RMFI of 8μg of Fab' fragments (430.65±16.73%). At immunoliposomes equivalent to 4μg and 8μg the RMFI was found remain same. This increased RMFI with immunoliposomes compared to similar concentrations of Fab' fragments used can be better explained. Each immunoliposome has more number of Fab' fragments over its surface and when immunoliposome bind to the cell through one Fab' fragment conjugated over it, there are still more Fab' fragments on liposomal surface available for binding to more number of secondary antibodies. The more number of Fab' fragments are retained on the cell surface, though they are not bind to cell receptors, *via* the one Fab' fragment of immunoliposomes that is binding to cell receptor. But the same was not happened in the case of Fab' fragments where they bind alone and provide site for one secondary antibody. Therefore, we observed more signal (RMFI) and saturation (might be due to saturation of receptors but it was not happened at the same concentrations of Fab' fragments used, so it might be due to insufficient secondary antibody quantity) with immunoliposomes than Fab' fragment.

There is no difference in the mean fluorescence of secondary control, treated with secondary anti-rabbit FITC, compared to only cells. It indicates that the secondary antibody is not reacting, non-specifically to cell surface proteins instead binding to primary antibody only that was observed in positive control cells.

When we considered the % RMFI of positive control cells (232.45±20.45%) and cells treated with 1μg Fab' fragments (345.63±12.34%), it is very clear that the modified Fab' fragments are equally immunoreactive to that of intact neuropilin-1 antibody. Therefore, the prepared immunoliposomes will efficiently interact with the cells which overexpress neuropilin-1 and they can be used in both *in vitro* and *in vivo* tests to determine their performance.

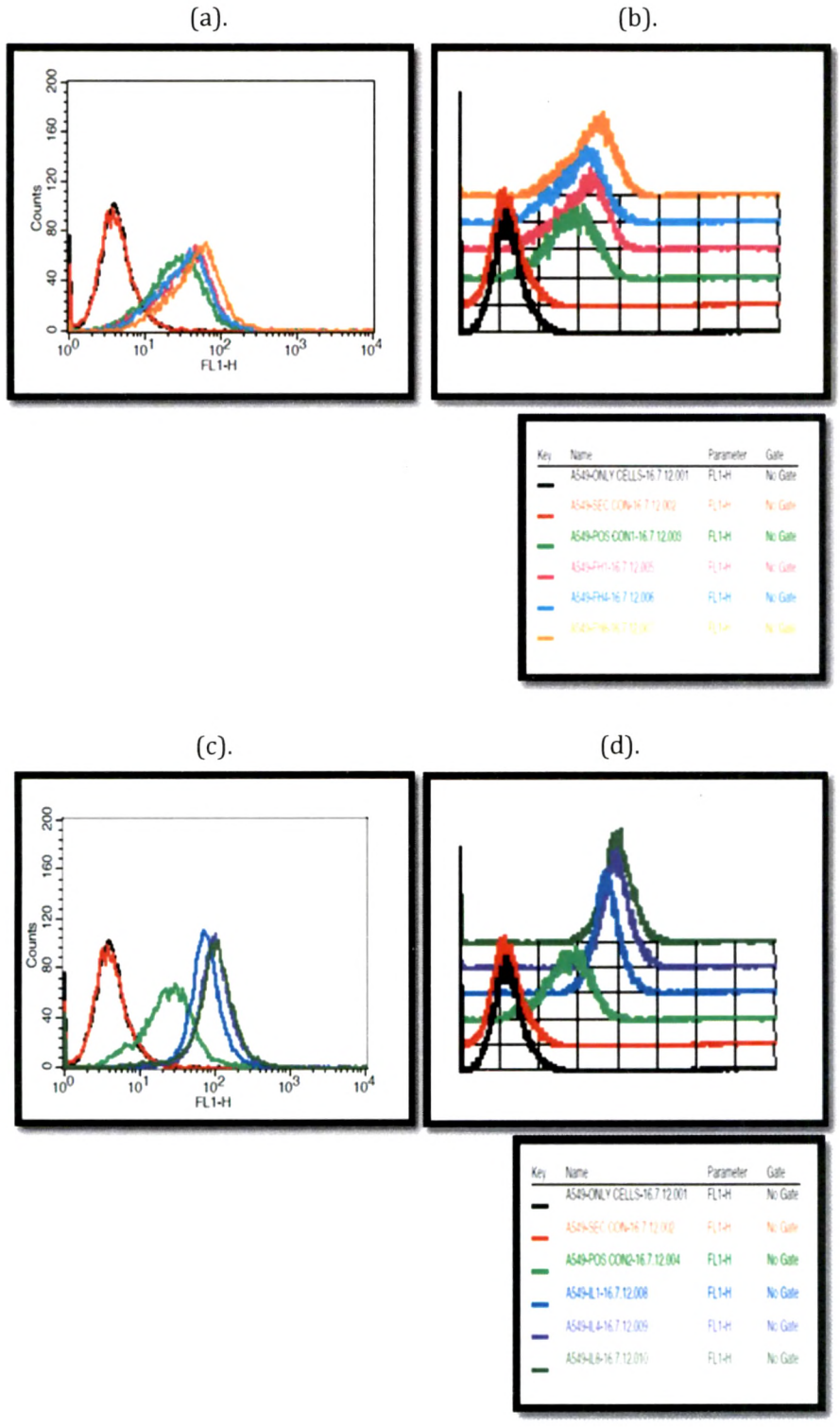
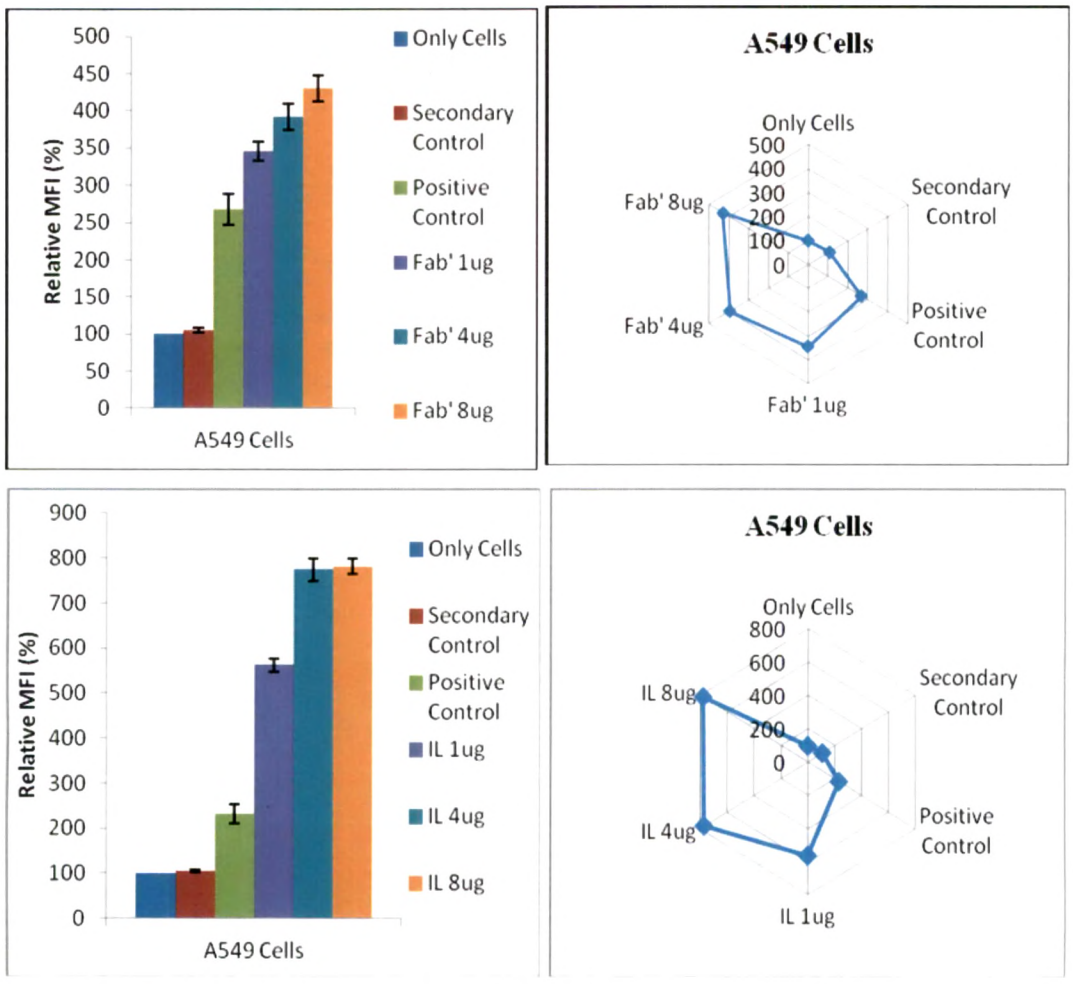


Figure 6.5. Immunoreactivity of prepared Fab′ fragments and immunoliposomes. (a). FACS analysis results of A549 cells trated with different concentrations of Fab′ fragments and its overlay graph (b). FACS analysis of A549 cells treated with different concentrations of immunoliposomes (c) and its overlay graph (d).

(a). (b).



(c). (d).

Figure 6.6. Graphs representing % RMFI of A549 cells treated with different concentrations of Fab′ fragments (a) and radar representation of the same values (b). %RMI values of A549 cells treated with different concentration of immunoliposomes (c) and radar representation of the same (d).

Table 6.2. Comparison of % realtive mean fluorescence intensity (RMFI) of A549 cells treated with different concentrations of Fab' fragments and immunoliposomes to determine their immunoreactivity towards neuropilin-1 receptor.

Experiment	% RMFI
Only Cells	100
Secondary Control	104.99±2.48
Positive Control	232.45±20.45
Fab' 1ug	345.63±12.34
Fab' 4ug	391.99±17.32
Fab' 8ug	430.65±16.73
IL 1ug	562.27±15.51
IL 4ug	774.06±25.39
IL 8ug	781.83±18.32

Values are mean±SD, n=3.

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