CHAPTER 5

EXPERIMENTAL BAPINEUZUMAB LOADED NANOPARTICLES

5.1 Introduction:

Alzheimer's disease most commonly leads to dementia. Increasing age is the greatest risk factor for Alzheimer's disease. Its prevalence approximately doubles every 5 years after the age of 60 – 1 in 10 individuals over 65 years and nearly half of those over 85 are affected by the disease (Mount & Downton, 2006).

One of the present dominant theory of Alzheimer's disease etiology and pathogenesis is related to the amyloid cascade hypothesis (Hardy & Selkoe, 2002; Selkoe, 1994), which states that overproduction of beta-amyloid peptide, or failure to clear this peptide, leads to Alzheimer's disease primarily through amyloid deposition, which is supposed to be involved in neurofibrillary tangles formation; these lesions are then associated with cell death which is reflected in memory impairment, the hallmarks of this dementia. In vitro amyloid formation is a complex kinetic and thermodynamic process and the reversibility of amyloid plaque growth in vitro suggests steady-state equilibrium between Aβ peptides in plaques and in solution (Maggio & Mantyh, 1996).

With the progression of Alzheimer's disease animal models, immunology is being applied in the treatment of conformational diseases to stimulate clearance of brain amyloid plaques, either as active or passive immunization. Active immunization approaches employ amyloid generating epitopes and/or immunogenic amyloid peptide conjugates, as well as various routes of administration and types of adjuvants.

Passive immunization techniques involve delivering antibodies or antibody fragments directed against specific amyloid epitopes, that is the immunity is provided and doesn't require the body to evoke any.

To further elaborate passive immunotherapy of Alzheimer's disease, it requires repeated administration of anti-AβP antibodies. For that reason, human anti-AβP antibodies should be preferably used to prevent an immune response to the currently available murine monoclonal immunoglobulins. Several methods are known to obtain human anti-AβP antibodies. One method is humanization of murine anti-AβP

antibodies by replacing framework portions of the murine anti-AβP antibodies with human framework sequences using recombinant DNA technology (Jones, Dear, Foote, Neuberger, & Winter, 1986). Alternative methods are the generation of human monoclonal anti-AβP antibodies in vitro by phage library display techniques (McCafferty, Griffiths, Winter, & Chiswell, 1990), or in vivo by immunization of animals whose immunoglobulin loci that have been replaced by human genes (Kuroiwa et al., 2002). In comparison to active immunization, passive immunization offers more control over both safety and efficacy.

Immunoglobulins G (IgG) have limited access to the brain. Only 0.1% of an intravenous dose was shown to pass via extracellular pathways through them blood–brain barrier (BBB) into the brain (Banks et al., 2002). Depending upon the region where the antibody would act on the aggregated peptide, the antibodies are classified as the one which target the C – terminal, N – terminal, the central region of the amyloid peptide or at a particular epitope at the oligomer.

Bapineuzumab is an IgG1, N – terminus anti amyloid humanized monoclonal antibody. It has been proven to reduce the amyloid plaques which are in excess in the brain. This occurs by binding to fibrillar and soluble Aβ and activates microglial phagocytosis and cytokine production. (http://www.alzforum.org/therapeutics/bapineuzumab).

Bapineuzumab entered a phase III trial and released preliminary analysis of the phase II results (Wyeth, 2008). The phase II trial was a randomised, double-blind, placebo controlled trial testing three doses of a humanized Aβ antibody in 240 participants. In each of the escalating doses of the antibody, about 32 patients received active agent and 28 received placebo. Although the study did not attain statistical significance on the primary efficacy endpoints in the whole study population over the 18-month trial period, in the subgroup of participants who did not have the *APOE* ε4 allele clinically significant benefits were recorded on several scales, including the mini-mental state examination and the Alzheimer's disease assessment scale battery. Furthermore, in the same subgroup, MRI showed less loss of brain volume in treated patients than in control patients. These findings suggest that this form of therapy might be effective.

However, some patients in the treatment group, but not in the control group, had vasogenic oedema, a serious adverse event.

Such an adverse reaction can be avoided by targeting the agent at the proper site of action. This could be achieved by using a nanocarrier. The advantage associated with nanosystems is that the absorption, biodistribution and elimination of drug in the body are dependent on the inherent properties of nanosystems like size, surface properties and charge.

"Bioconjugation", which refers to the covalent derivatization of biomolecules, provides a means to various goals (Jeet Kalia & Raines, 2010). One of them is attaching an antibody to a carrier substrate for its delivery to the target site.

Therefore, the objective of the investigation was to formulate a novel nanoparticulate formulation of Bapineuzumab by conjugation, for targeting it to brain and to open new avenues in passive immunization for Alzheimer's disease.

5.2 Materials

Bapineuzumab was purchased from Arihant Traders (India), Bovine serum albumin, 2 - iminothiolane (Traut's reagent), hydrochloride,5,5'-dithio-bis(2-nitro-benzoic acid)(Ellman's reagent), maliemide-ω-ester were purchased from Sigma Aldrich, India. Hydroxylamine was obtained from Himedia, India. L – cysteine was procured from Qualigens, India. The polypropylene column for gravity size exclusion chromatography and affinity chromatography was purchased from BioRad, India. Sephacryl S200 HR and MabSelect were procured from GE Healthcare, USA. Tris buffer, sodium chloride, glycine, sodium hydroxide were purchased from Sigma Aldrich, India. Other reagents and solvents were locally procured. The chemicals in kits were used as received. Distilled water was used for all experiments.

5.3 Equipments and Instruments

1. High speed magnetic stirrer (Remi, MS500, Remi equipment, India)

2. Digital pH meter (Lab India Ltd, India)

3. High speed centrifuge (Sigma 3K30, Germany)

4. Ultracentrifuge (Optima L – 100 XP, Beckmann, USA)

5. Particle size Analyser (Zeta sizer Nano series, Malvern Instruments, UK)

5. UV – VIS spectrophotometer (UV 1800, Shimadzu, Japan)

6. Lyophilizer (Heto, Drywinner, Germany)

7. Differential Scanning Calorimeter (Shimadzu, Japan)

8. X- ray Diffractometer (Panalytical's Xpert Pro, Netherlands)

9. Transmission Electron Microscope (Tecnai 20, Philips, Holland)

10. Fourier Tranform Infrared spectrophotometer (Bruker, Germany)

11. Atomic Force Microscopy (Bruker Dimension Icon, USA)

5.4 Formulation of Bapineuzumab loaded nanoparticles by surface modification of Bovine Serum Albumin nanoparticles.

The optimization for blank nanoparticles was done in the similar manner as that described in Chapter 4.

5.4.1 Modification of Bapineuzumab:(S. Wagner et al., 2010)

Bapineuzumab was modified by introduction of thiol group using 2 – iminothiolane. The reagent (6.9 mg of 2-iminothiolane) was dissolved in 50 ml phosphate buffer pH 8.0. Bapineuzumab was diluted to a concentration of 1 mg/ml in phosphate buffer (pH 8.0). In order to introduce thiol groups, 250.0 µl (which represents 50-fold molar excess) and 500.0 µl (which represents 100-fold molar excess) of 2-iminothiolane were added to 500.0 µl Bapineuzumab solution and the volume of the samples was

adjusted with phosphate buffer (pH 8.0). These samples were incubated at 20 °C under constant shaking at 700 rpm for 2 and 5 hours respectively. The reaction was stopped by addition of 500.0 µl hydroxylamine solution. This solution was prepared by adding 2.8 mg in 10ml of phosphate buffer, pH 8.0). This mixture was incubated for another 20 min. The concentration and time was optimized by thiol quantification and checking for dimer formation.

5.4.2 Purification of modified Bapineuzumab: (S. Wagner et al., 2010)

Purification necessary to separate the modified antibody from unmodified antibody was undertaken through size exclusion chromatography by gravity column.

The procedure involved packing the 20 cm X 1 cm Bio Rad polypropylene column with Sephacryl HR S200 HR(GE Healthcare, USA), washing the packed column with distilled water, followed by column equilibration and sample loading. After sample was loaded, elution was carried out with 50 mM Tris + 150 mM NaCl buffer (pH 7.4). Twenty such eluting fractions were collected and analyzed by UV (UV 1800, Shimadzu, Japan) at 280 nm.

Additionally, a check was made to detect formation of dimer, if any, by analyzing the elute at 320 nm in UV (UV 1800, Shimadzu, Japan).

5.4.3 Thiol quantification of modified Bapineuzumab:(S. Wagner et al., 2010)

The pooled fractions of purified and modified antibody were taken and subjected to reduction using Ellman's reagent. Afterwards the samples were measured photometrically (UV 1800, Shimadzu, Japan) at 412 nm. In order to calculate the number of introduced thiol groups, L- cysteine standard solutions (1.2 mg/mL, 2.4 mg/mL, 3.6 mg/mL, 4.8 mg/mL) that were treated in the same way like the antibody solution was used.

5.4.4 Binding of antibody to nanoparticles:(S. Wagner et al., 2010)

First blank albumin nanoparticles were prepared by the technique mentioned in section *5.4.1*. The nanoparticle dispersion was concentrated by centrifugation as described earlier. To this concentrated dispersion, maliemide-ω-ester (20 mg/mL in phosphate buffer, pH 8.0) was added as the surface modifier. This was incubated for 1 hour at 20 ° C under constant stirring.

This produced the surface modified albumin nanoparticles.

The dispersion of blank nanoparticles prepared using the method described in Chapter 4 was taken and 500 µL of the surface modifier was added to it under magnetic stirring (Remi, MS500, Remi equipment, India). Thereafter, 2mL of thiolated antibody was added to surface modified nanoparticles. This was allowed to incubate for 12 hours at 20 ° C under constant stirring.

After 12 hours, the supernatant was collected by centrifugation at 35,000 RPM, at 4º C for 30 minute (Optima L-100XP, Beckman, USA). This was analyzed through affinity chromatography by gravity column in a 5 cm X 1 cm Bio Rad polypropylene column packed with MabSelect (GE Healthcare, USA).

Briefly, the procedure involved washing the packed column with distilled water. Equilibration of the column was carried out with 50 mM Tris + 150 mM NaCl (pH 7.4), followed by elution with 150 mM Glycine buffer (pH= 3.5). After elution, the column was washed with distilled water, 50 mM Tris + 1 M NaCl (pH 7.4) to reconcile the amount of Bovine Serum Albumin ((Healthcare, 2008)

Twenty elutes of 500 µL each were collected which were analyzed by UV at 280 nm (UV 1800, Shimadzu, Japan) for calculating the unbound antibody.

The amount of antibody bound to the nanoparticle surface was calculated as difference between the amount of antibody obtained after thiolation and purification and the amount of antibody determined in the supernatant obtained after the conjugation step.

5.5 Lyophilization of Bapineuzumab Loaded Nanoparticles and Optimization of Cryoprotectant (Anhorn et al., 2008)

The optimized nanoparticle formulation was lyophilized using lyophilizer (Heto Drywinner). Different cryoprotectants (Trehalose dihydrate, Mannitol and Sucrose) at different ratio (1:1w/w, 1:2w/w, 1:3w/w) were tried to select the cryoprotectant which showed minimum increment in particle size. Nanoparticulate suspension (2 ml) was dispensed in 10 ml semi-stoppered vials with rubber closures and frozen for 24 h at -80 °C. Thereafter, the vials are lyophilized (Heto Drywinner, Allerod, Denmark) using different cryoprotectants like trehalose, sucrose and mannitol in different concentrations. Finally, glass vials were sealed under anhydrous conditions and stored

until being re-hydrated. Lyophilized NPs were re-dispersed in exactly the same volume of distilled water as before lyophilization. NP suspension was subjected to particle size measurement as described earlier.

5.6 Characterization of Bapineuzumab loaded nanoparticles.

5.6.1 Particle Size

The size and polydispersity index of the nanoparticles were determined using Malvern Zetasizer NanoSeries nano-ZS (Malvern Instruments Limited, Worcestershire, UK). Each sample was diluted ten times with filtered distilled water to avoid multi scattering phenomena and placed in a disposable sizing cuvette. Polydispersity index was studied to determine the narrowness of the particle size distribution. The size analysis of a sample consisted of 3 measurements, and the results are expressed as mean size ±SD.

5.6.2 Zeta Potential

Zeta potential was also measured using a Zetasizer (NanoSeries nano-ZS Malvern Instruments Limited, Worcestershire, UK). Each sample was suitably diluted with filtered distilled water and placed in a disposable zeta cell. Zeta limits ranged from -200 to +200 mV. The electrophoretic mobility (µm/sec) was converted to zeta potential by in - built software using Helmholtz-Smoluchowski equation. Average of 3 measurements of each sample was used to derive zeta potential.

5.6.3 Entrapment efficiency

To quantify the unbound antibodies, the formed nanoparticles were first centrifuged at 35,000 RPM, at 4º C for 30 minute (Optima L-100XP, Beckmann, USA) to separate the supernatant. The supernatant was then analyzed by Affinity Column Chromatography for unbound antibody. Affinity chromatography by gravity column in a 5 cm X 1 cm Bio Rad polypropylene column packed with MabSelect (GE Healthcare, USA). Twenty elutes of 750 µL each was collected. The fractions

collected were analyzed by UV- Visible Spectrophotometer (UV 1800, Shimadzu, Japan) at 280 nm.

The amount antibody bound to the nanoparticle surface was calculated as difference between the amount of thiolated antibody added for conjugation and the amount of antibody determined in the supernatant obtained after the conjugation step.

5.6.4 Transmission Electron Microscopy(Qi et al., 2014)

A sample of 25 mg/mL was utilized for the study. 2 µL of this suspension was placed over a copper TEM grid (150 mesh), negatively stained with 2 µL phosphotungstic acid (2% w/v) for 10 min and allowed to dry. The NPs were visualized at 200 kV under Transmission Electron Microscope (Tecnai 20, Phillips, Holland) and images were captured using Gatan Digital Micrograph software.

5.6.5 Atomic Force Microscopy (Moribe et al., 2012)

To visually ensure the proper linking of antibody onto the surface of nanoparticles, Atomic Force Microscope (AFM) (Bruker Dimension Icon, USA) equipped with ScanAsyst Software was used. Sample was loaded on a Mica plate (MUSCOVITE MICA, V- 1 quality,USA) and spread uniformly on its surface. The mica plate was stored in a dessicator without dessicant for 15 min to immobilize them onto the surface. This mica plate was then placed under the microscope. After setting the desired field of vision, the silicon tip cantilever was allowed to oscillate in the tapping mode with a spring constant of approximately 0.05 N/m and a resonance frequency of approximately 37 kHz. The AFM topography image which reflects the topographic features of the surface was obtained from the amplitude change of the cantilever oscillation. AFM was done on blank nanoparticles and Bapineuzumab loaded nanoparticles.

AFM measurement was performed on a vibration isolated table within an insulation cover.

5.6.6 Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted for actively loaded Bapineuzumab nanoparticles to check for the integrity of the antibody after its attachment to the surface modified nanoparticles. The procedure involved the use of 12% Resolving gel, 5 % Stacking gel and a reducing dye for loading the sample. Lane 1 was loaded with low molecular weight protein marker and Lane 2 was loaded with actively loaded Bapineuzumab nanoparticles.

5.6.7 Indirect Enzyme Linked Immunosorbent Assay (ELISA) (Moribe et al., 2012)

Indirect Enzyme Linked Immunosorbent Assay (ELISA) was also performed to check for the activity of the antibody attached to the surface of nanoparticles. The procedure involved coating a 96 well microtitre plate with the antigen i.e.Human β - amyloid peptide (1 – 42) using carbonate bicarbonate buffer. The antigen was coated in triplicate. The plate was then stored at 2°C overnight. Then blocking buffer was added after appropriate washings. After this standard dilutions of Bapineuzumab solutions (30 – 480 ng/mL) and nanoparticle suspension were added in triplicate in each well respectively. After incubation, HRP- conjugated secondary antibody was added to each well. After incubation, 200µL TMB was added and the mix was allowed to react for 10 minute. Finally, 0.1 M H_2SO_4 was added as the stop solution. The microtitre plate was read photometrically at 450 nm in a microplate reader (BioRad, India).

5.7 Results and discussion

5.7.1 Modification of Bapineuzumab and its purification:

Various methods can be used for bioconjugation of antibodies to nanoparticles. Some of them include linkages by amide bonding, linkages generated by cycloaddition, linkages containing carbon nitrogen double bonds, linkages containing thioethers. Out of all these methods, the technique involving introduction of thioether linking was utilized to modify the antibody. (Jeet Kalia & Raines, 2010)

To optimize the amount of 2 – iminothiolane and the reaction time for modification of antibody, studies were carried out with 50 – fold molar excess of 2 - iminothiolane and

100 – fold of 2 - iminothiolane molar excess for 2 and 5 hour respectively for each. The elutes collected after the experiments were analyzed by UV (1800, Shimadzu) at 280 nm for modified and unmodified antibody. The absorbances were noted down, the concentration of each modified and unmodified antibody was then calculated. Additionally the elution pattern was also observed.

The elution obtained after treating the antibody with 50 – fold molar excess of 2-imminothiolane for 2 hours is shown in Figure 5.1

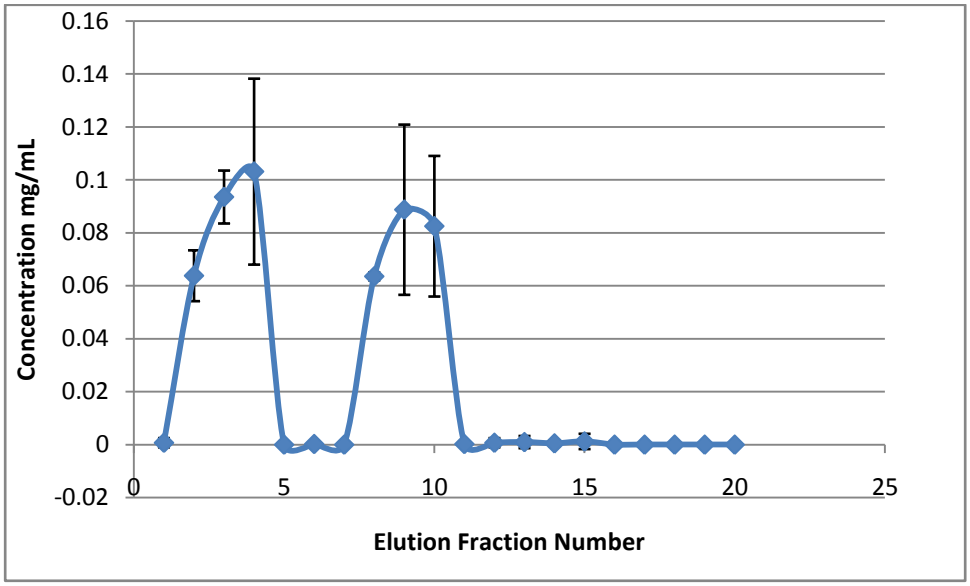


Figure 5.1: Elution of antibody after thiolation with 50 – fold molar excess for 2 hour.

From Figure 5.1, it can be seen that during elution, two peaks were formed. The first peak represents the modified antibody and the second peak is the unmodified antibody. Since the molecular weight of unmodified antibody was higher than unmodified antibody, it failed to get into the pores of the resin, and washed out as elute along with the equilibration buffer. (Healthcare, 2008)

The elution obtained after treating the antibody with 50 – fold molar excess of 2-imminothiolane is shown in Figure 5.2

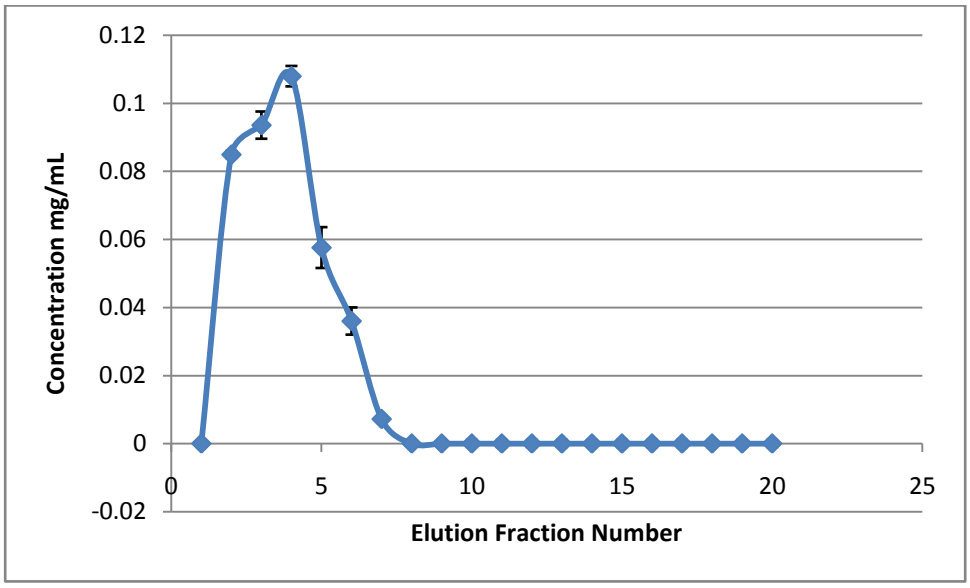


Figure 5.2: Elution of antibody after thiolation with 50 – fold molar excess of 2-imminothiolane for 5 hour.

There was no resolution of modified antibody and unmodified antibody achieved with 50 – fold molar excess and 5 hour incubation (Figure 5.2). Also, the elution occurred in the initial fraction collected. This could be due to dimerization of antibody. This is a risk associated with the introduction of thiol groups into antibodies, wherein oxidative bridges are formed. Due to the overall increase in molecular weight of the antibodies, it eluted out all at once, leaving no scope for resolution (S. Wagner et al., 2010)

Similar observation was noted for reaction involving 100 - molar excess at 2 and 5 hours. The elution pattern is shown in Figure 5.3 and Figure 5.4 respectively.

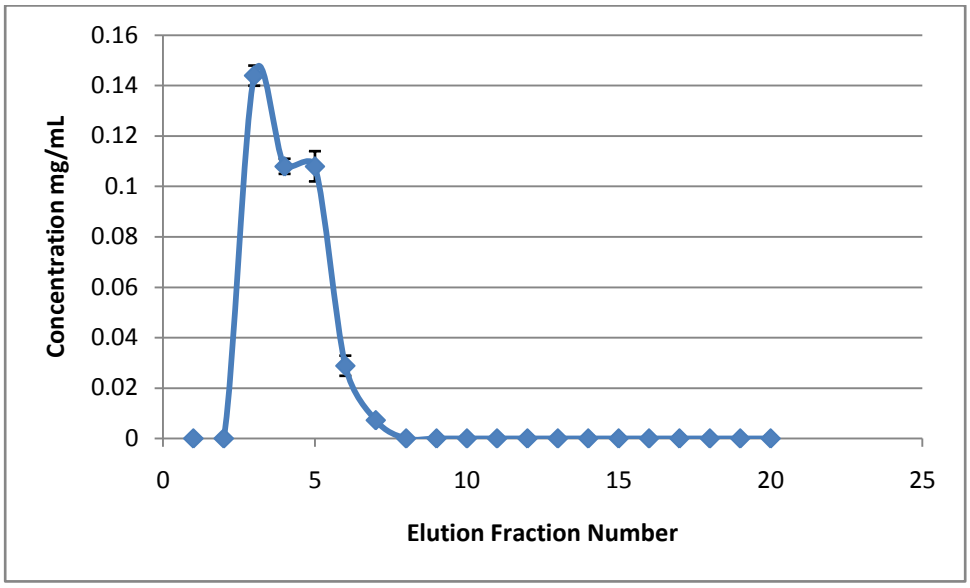


Figure 5.3: Elution of antibody after thiolation with 100 – fold molar excess of 2-imminothiolane for 2 hour.

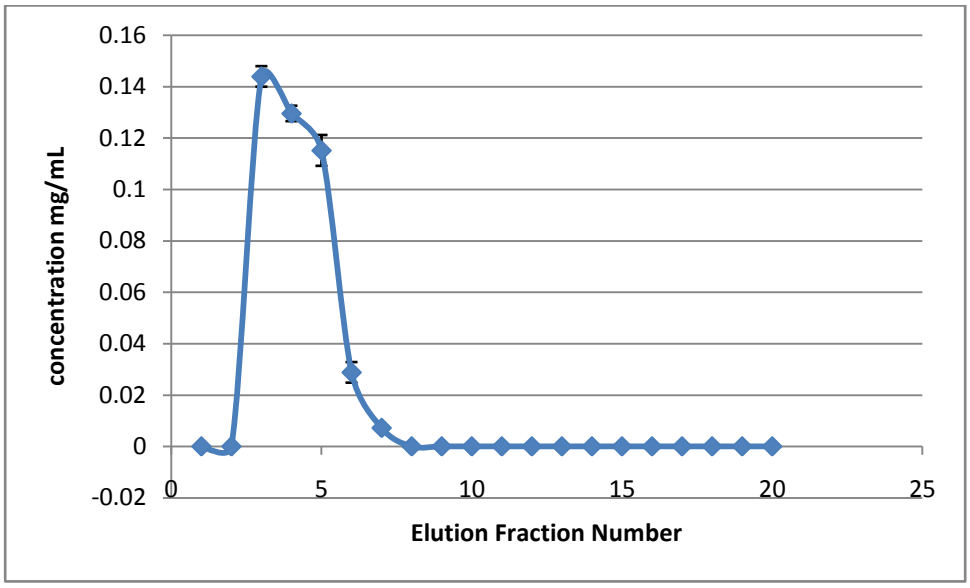


Figure 5.4: Elution of antibody after thiolation with 100– fold molar excess 2-imminothiolane for 5 hour.

Therefore, a 50-fold molar excess of 2 – iminothiolane with an incubation period of 2 hours was set for modification of antibody.

5.7.3 Thiol quantification of modified Bapineuzumab:

In 1959, Ellman introduced a reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), which has found extensive use in the estimation of free thiol groups in native and denatured proteins . The procedure is based on the reaction of the thiol with DTNB to give the mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB) which is quantified by the absorbance of the dianion at 412 nm (Riddles, Blakeley, & Zerner, 1979).

For the purpose of quantification of thiols introduced on Bapieuzumab, the comparison of modified Bapineuzumab was done against cysteine, as explained in method *5.4.3*. Cysteine is a unique among the amino acids found in proteins. It contains one thiol group per molecule, hence making it easy to utilize it as a standard for thiol content. The thiol of cysteine is a powerful nucleophile, and cysteine is the most easily oxidized of the amino acids, forming covalent crosslinks between peptide chains. (Nobs, Buchegger, Gurny, & Allemann, 2003)

After thiolation of antibody with 50 – fold molar excess or 100 – fold excess for 2 and 5 hours respectively, the modified antibody was subjected to treatment with Ellman's reagent to calculate the thiol increase for modified antibody

The results of the same can be seen in Table 5.1

Table 5.1: Thiol concentration in modified antibody after thiolation

Molar excess of 2-imminothiolane	**Time of incubation (hr)**	**Thiol concentration in modified antibody (µg/mL)***
100	2	0.39
100	5	0.48
50	2	0.26
50	5	0.34

*: Studies done in triplicate.

Figure 5.5 represents the increase in thiol groups in modified antibody in comparison to unmodified antibody.

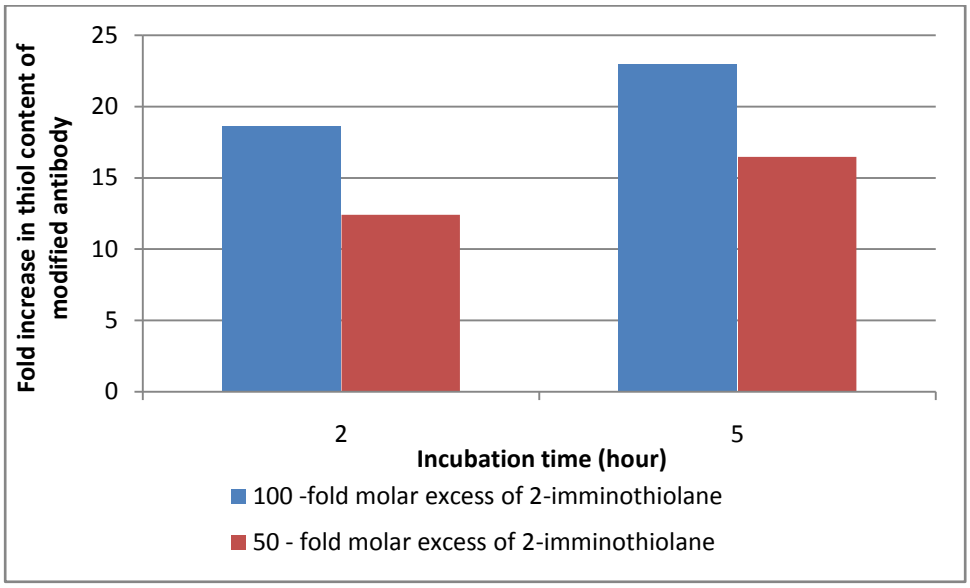


Figure 5.5: Thiol quantification of modified antibody.

It was observed in Figure 5.5 that the increase in thiol groups on antibody was time and concentration dependent. Higher the molar excess for longer time, more was the thiol content. However, with the increase in molar excess and longer incubation time, the problem of dimerization was observed. Hence to get best of thiolation efficiency without affecting the integrity of antibody, 50 - fold molar excess with incubation for 2 hours was fixed for antibody modification.

Thiol quantification showed thiolated antibodies had 12 – 15 times more thiol group in comparison to unmodified antibody. Therefore these additional thiol groups can get involved in bond formation with the surface modified Bovine Serum Albumin through the cross linker.

5.7.4 Surface modification of BSA nanoparticles

The crosslinker used for surface modification of BSA nanoparticles was maliemide-ω-ester

The schematic reaction for typical thiol-reactive functional groups like maleimides is shown in the figure 5.6.

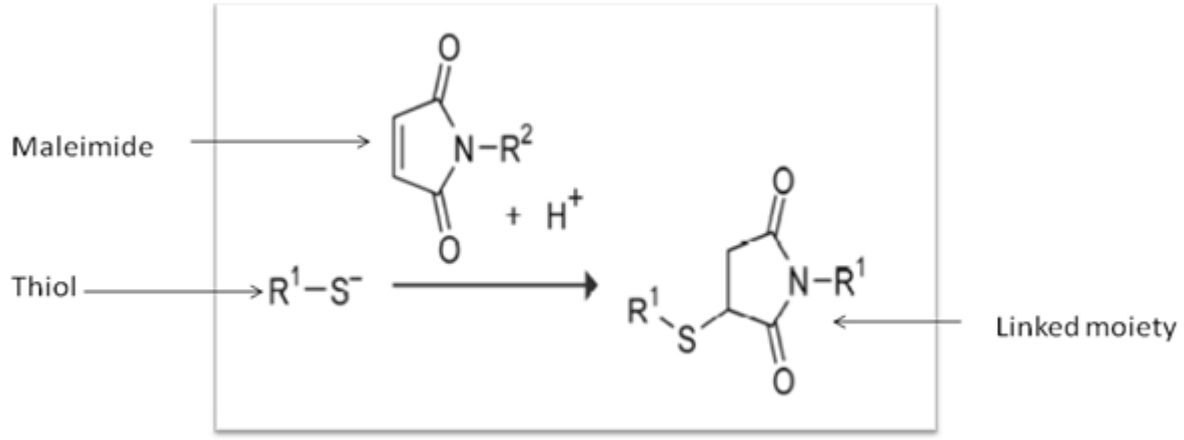


Figure 5.6: Reaction scheme involving thiol and maleimide for formation of covalent bond.(wikibooks)

Herein the thiol group of modified antibody reacted with the maleimide group of the linker to result in surface modification of blank bovine serum albumin nanoparticles with antibodies.

The surface modified nanoparticles, when administered through nose, would carry the antibody to the site of action by surpassing the Blood Brain Barrier, and thus help in targeting it to the amyloid plaques in the brain.

5.7.5 Particle size, zeta potential and entrapment efficiency

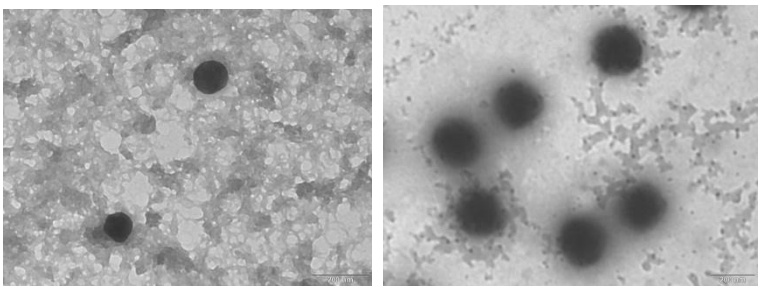
The particle size of actively loaded nanoparticles was found to be 110.45 ± 6.21 nm with a polydispersity index of 0.11 ± 0.02. The zeta potential was found to be -16 mV ± 0.18.

A negative zeta potential along with small particle size was thought to be desirable prerequisites for good brain uptake (Fornaguera, Feiner-Gracia et al. 2015).

69.89% ± 2.45 % of thiolated antibody got bound to the surface modified bovine serum albumin nanoparticles.

5.7.6 Transmission Electron Microscopy (TEM)

From the TEM images (Figure 5.7), it can be seen that both blank and loaded nanoparticles were spherical in shape and with a size less than 200 nm. Also, for loaded nanoparticles, a coat of antibody was clearly visible around the darkened core of BSA .



A B

Figure 5.7: TEM images of (A) blank BSA and (B) Bapineuzumab – BSA nanoparticles respectively.

5.7.7 Atomic Force Microscopy.

From AFM (Figure 5.8), the surface topography of blank nanoparticles was observed to be smooth with a particle size range of 98.12nm to 105.47 nm and a particle height of 4.6nm to 5.8 nm. Bapineuzumab loaded nanoparticles showed a rough surface, with undulation throughout indicating the presence of antibody on the surface of nanoparticles. The particle size was found to be in the range of 118.12 nm to 131.01 nm with a particle height of 12.76 nm to 15.42 nm.

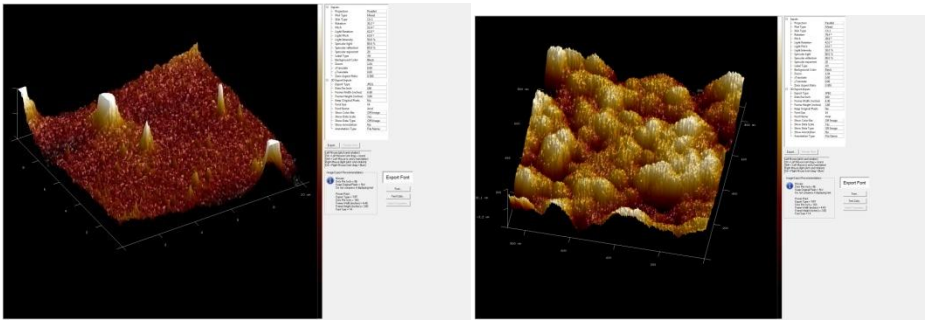


Figure 5.8: Detailed three dimensional AFM topography of Blank (BSA) and Bapineuzumab-BSA

5.7.8 Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS – PAGE gel as seen in Figure 5.9, was viewed utilizing Gel Doc XR+ Imager (Bio Rad, India) using the Image Lab Software. During the process of SDS – PAGE, the reducing gel causes the antibody to breakdown to two fragments of 25kD and the other of approximately 50kD respectively.

The protein marker was run on Lane 1 whereas the nanoparticulate formulation was run on Lane 2. The protein marker is a standard mix of proteins to encompass a range of molecular weight. The protein marker used in this case was in the range of 10 kD to 120 kD. After processing, the gel showed the presence of antibody in the nanoparticles with typical bands at 25 kD and approximately 50kD respectively at Lane 2. This indicated that during the process of formulation, the antibody did not undergo any change in its structure, thereby maintaining its integrity.

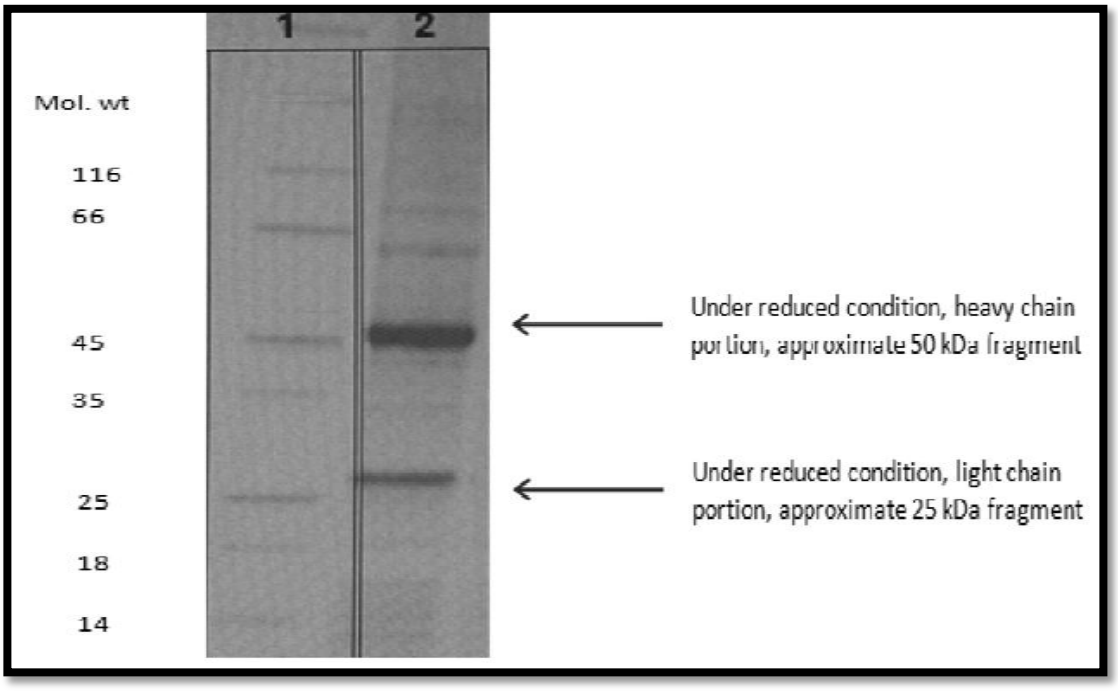


Figure 5.9: SDS – PAGE gel image for Actively loaded Bapineuzumab nanoparticles.

5.7.9 Indirect Enzyme Linked Immunosorbent Assay

The purpose of conducting ELISA was to ensure that the activity of the antibody was retained even after its covalent linking to BSA nanoparticles.

Figure 5.10 shows the comparative absorbances between standard Bapineuzumab solutions and Bapineuzumab nanoparticles . The higher the value of absorbance, higher is the concentration of antibody. The Bapineuzumab nanoparticles showed similar values of absorbance analogous to standard calibration curve, thus indicating that Bapineuzumab had retained its integrity and activity after conjugation with BSA nanoparticles. The trendline aids in having a comparing the nanoparticles to standard

solution of Bapineuzumab. For both, the points are scattered in the same area around the trendline.

Results of Indirect ELISA proved the efficacy of antibody loaded nanoparticles. The antibody attached to the surface of nanoparticles showed binding to the specific antigen coated on the 96 well plate. Therefore we concluded that the integrity of the antibody was maintained to bind on the N – terminal epitope of antigen (amyloid peptide)

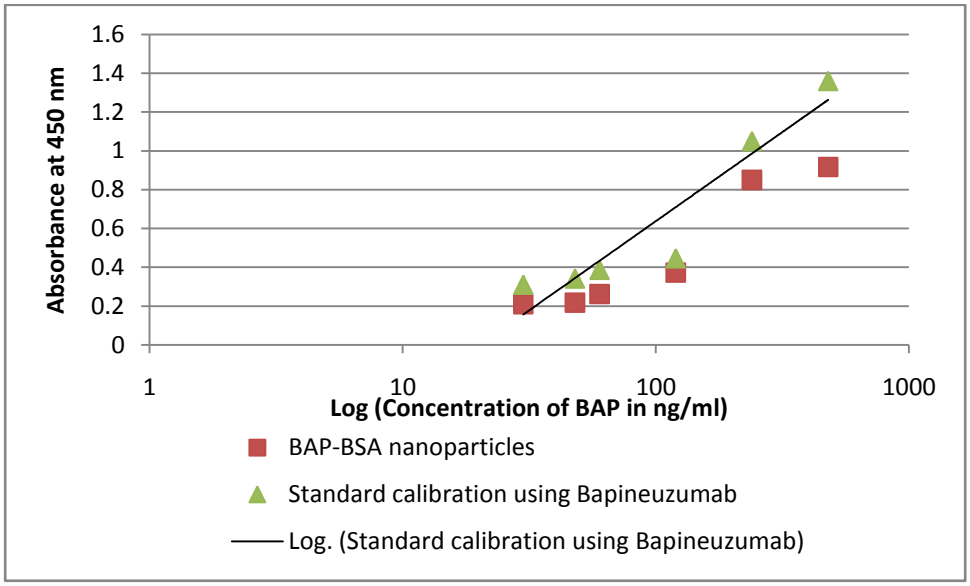


Figure 5.10: Absorbances corresponding to activity of Bapineuzumab in standard solution and in nanoparticles.

5.8 Lyophilization of Bapineuzumab loaded nanoparticles

Freeze drying causes increase in particle size of nanoparticles after lyophilization due to aggregation of particles during the process (Abdelwahed et al., 2006). If these aggregates are not separated during re-dispersion, it may cause instability to the system. The optimized Nanoparticle formulation was lyophilized using lyophilizer (Heto Drywinner, Vaccubrand, Denmark). Different cryoprotectants (Trehalose dehydrate, Mannitol and Sucrose) were used at different ratios to find out optimum concentration of cryoprotectant which showed minimum increment in particle size. As minimum increment of particle size was observed for 2% mannitol as cryoprotectant

in the ratio of 1:1 w/w, it was considered optimum for Lyophilization of Bapineuzumab loaded nanoparticles.

Table 5.2: Optimization of cryoprotectant for Bapineuzumab loaded nanoparticles

Cryoptrotectant	**Particle size after Lyophilization**	**Polydispersity Index**
Trehalose dehydrate (1:1)	351.3 ± 15.6	0.21
Trehalose dehydrate (1:2)	217.31 ± 12.44	0.34
Trehalose dehydrate (1:3)	380.36 ± 18.44	0.33
Sucrose (1:1)	294.14 ± 13.68	0.25
Sucrose (1:2)	177.39 ± 18.97	0.21
Sucrose (1:3)	300.91 ± 13.69	0.30
Mannitol (1:1)	141.25 ± 11.24	0.36
Mannitol (1:2)	220.34 ± 10.24	0.28
Mannitol (1:3)	266.71 ± 16.27	0.31

5.9 References

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