5. Preparation and Conjugation of antigens to Nanoparticles

5.1 Nanoparticles for nasal delivery of TB-antigen

Vaccine-delivery systems are generally particulate (e.g., emulsions, microparticles and liposomes) and have comparable dimensions to the pathogens, which the immune system evolved to combat. Increasingly more sophisticated delivery systems are being developed in which immunostimulatory adjuvants may be incorporated with the antigen. The rationale for this approach is to ensure that both the antigen and adjuvant are delivered into the same population of antigen-presenting cells. Enhancement of adjuvant activity through the use of micro- and nano-particulate delivery systems is particularly exciting, as synergistic effects are often seen resulting in immune responses stronger than those elicited by the adjuvant or delivery system alone. Micro- and nanoparticles also offer the possibility of enhancement of their uptake by appropriate cells through manipulation of their surface properties (Singh 2007). In last decade, there is significant development in vaccine and polymer science, and with that it is possible to design formulation with antigen which target immune system efficiently when delivered through Nasal route (Giri 2008). Most promising vaccine carrier candidates includes Poly Lactide (PLA), Poly Glycolide (PGA) and their derivatives Poly-Lactide-co-Glycolide (PLGA). Nano-particles (in the range of 200-500 nm) fabricated from biodegradable polymers (like PLA, PLGA), are found more suitable than micro-particles for surface presentation at APCs as well as encapsulation of antigens for localized or targeted delivery of antigens (Vajdy 2001, Singh 2002 and Alpar 2005).

5.1.1 Adjuvant in the preparation of Nanoparticles for TB mucosal vaccine with sub-unit proteins

Dimethyl dioctadecyl ammonium bromide (DDAB) is a synthetic lipophilic quaternary amine. DDAB is a well-known adjuvant used for the development of TB vaccine. DDAB has also been reported to stimulate both serum antibody response and cell-mediated immunity against the co-administered antigen. DDAB has already been used intramuscularly widely in the human population with no toxic effects reported. Recently, it has been demonstrated that DDAB induces both mucosal and systemic

immune responses when intranasally co-administered with protein antigens. It's been reported that intranasal administration of DDAB with Ag85 complex proteins induced significantly higher protection than antigen alone-treated mice (Giri 2008, Unpublished Observations). One could postulate that the mucosal adjuvant property of DDAB comes partially from its ability to increase the amount of antigens crossing the epithelium that covers the nasal cavity. DDAB is a depot-forming agent and acts as a carrier of antigen by direct binding of antigen or modification at the oil/water interface. Another nonexclusive explanation could be that DDAB generates inflammatory stimuli, thereby promoting the uptake of antigen. Indeed, the systemic adjuvant properties of DDAB have been ascribed to its capacity to stimulate an inflammatory response (synthesis of IL-1 and interferons) and the recruitment of activated mononuclear cells (Olsen 2001).

5.2 Incorporation vis a vis surface presentation of antigens to nanoparticles

(1) The antigens can be entrapped to the nanoparticles (NPs) by double emulsion technique, where antigen will remain as a core of NPs. The antigens are water soluble and w/o/w emulsions are generally prepared for such cases. There are several advantages of this technique which include (Sminia 1999, Singh 2002):

i) When antigens are entrapped in the polymeric material, polymer coat provides a definite protection to the antigens against biological environment and prevents premature enzymatic degradation. This prevents premature degradations of antigens before their reaching to the desired site for their action. This is very much essential when we aim for mucosal route for antigen delivery where antigens interact with enzymes present at various mucosal sites.

ii) Selection and surface modification of coat material is the key to control release pattern of core material from the NPs and hence, pre-programmed targeted release can be achieved easily.

iii) Most of the coat materials being used now days are biodegradable polymers and their metabolites do not harm the body.

iv) Possible easy set up at research scale, cost effective and re-producibility, making this method most adaptable for the research

v) It is reported that NPs prepared from biodegradable polymers act as adjuvants and potentiate the immune response (Singh 2007).

When we aim for the immune response, it is very much essential that antigens must interact well and for sufficiently longer time with the Antigen Presenting Cells (APCs) to elicit the immune response. When antigens are coated with polymer, the coat, not the core material (antigens) comes in contact with the APCs until they degrade completely and release the antigens for their action. The type of interaction of polymeric nano- or micro particles is non-specific and hence, initially, disease non- specific immune response will be obtained (Davis 2001, Koping-Hoggard 2005, Giri 2008).

(2) Surface presentation of antigens on NPs is an alternative method for the formulation, when it is aimed to target APCs for eliciting disease specific immune response. This method offers several advantages which include (Kasturi 2005, Wong 2007, Xu 2009):

i) Surface presentation of antigen facilitates direct interaction of antigens with antigen presenting cells to elicit disease specific immune response.

ii) The covalent conjugation chemistry between antigen and polymer is used for this purpose which also helps to release antigen at the predetermined rate, helps for longer stay of antigen with APCS for stronger immune response.

For surface presentation of the antigen, an interactive polymer surface is required and pre-prepared polymer like PLGA, PLA and PLG lacks such interactive groups. Generalized methodology is not possible for one group of antigens and it also varies with end group chemistry of antigens. Again, this can be possible only with protein based antigens where amino and sulpho links are used for covalent conjugation (Ratzinger 2010).

The basis for the selection of Polylcatide polymer is very sensible for our work as we were in need of conjugation of amino acid for the surface presentation of protein based antigens. Biotin (M.W. 244 Da and belongs to B

group of vitamin) conjugates with protein readily without altering protein folding. Moreover, incorporation of biotin and poly Ethylene Glycol during polymerization of lactide is easy, cost-effective, reproducible and practicable and as a final product we get Biotin-PEG-PLA block polymer. Therefore, we chose synthesizing polylactide polymer with biotin ligand where biotin reacted with amino group of protein for the surface presentation of antigens. The intention of incorporation of PEG to the block polymer was to help NPs to overcome mucosal barrier as we aimed for the Nasal mucosal delivery of the formulations.

5.3 Preparation of Antigen-loaded conventional PLGA-nanoparticles

5.3.1 Materials and Reagents

PLGA (50:50) was a kind gift from Purac Biomaterials, Netherland. Proteins were prepared at our laboratories. Polyvinyl vinyl alcohol (PVA) 88% hydrolyzed MW 25000, from was purchased from PolyScience, USA, Dimethyl dioctadecyl Ammonium Bromide (DDAB) was purchased from MP Biomedicals, USA. Probe sonicator (Vibra-Cell VCX 750 from Sonics & Materials, Inc., USA) and magnetic stirrer (Remi Equipments, India) were used.

5.3.2 Preparation of nanoparticles by double emulsion method

Poly lactide-co-GLycolode (50:50) (PLGA) and Dioctyl Decyl Ammonium Bromide (DDAB) were dissolved in 1 mL of dichloromethane (DCM) and antigens (Ag-85B and ESAT-6) 2 mg/mL were solubilised separately in PBS (pH 7.4). Two separate formulations for antigens (ESAT-6 and Ag-85B) were prepared by double emulsion technique, where inner core of NPs containing antigens. Various proportion of protein: polymer ratios and inner aqueous phase to organic phase were tried for the initial set of experiments. Antigen solutions were sonicated with PLGA solution in DCM at 0.5 sec on and off cycle for 3 minutes at 36 Amplitude. The system was kept in ice bath throughout sonication. After sonication, the emulsion was transferred to 10 mL of PBS and homogenized at 2000 RPM for 1 hour. Again the system was kept in ice bath. Following homogenization, the emulsion was transferred to 100 mL of PBS

and stirred for 6 hours at speed of 5 or 6 on a 4-position magnetic stirrer. About 500 uL of particle suspension was taken for size and ZP measurement. Particles were collected by centrifugation at 15000 rpm for 20 min (Beckman, Avanti J-25) and supernatant was decanted. Particles were washed with ultra-pure water two times and collected by centrifugation. Finally, particles were suspended in 1 mL of 1 M PBS.

5.3.3 Experiment Design for Preparation of PLGA nanoparticles by double emulsion techniques

Considering effect of various parameters (include amount of polymer, amount of protein and ratio of protein and polymer, volume of aqueous phase, organic phase and their ratio, time for the stirring to evaporate organic phase, time for the sonication for the preparation of primary emulsion and time for the homogenization for the preparation of secondary primary emulsion) preliminary experiments were conducted. From our studies and observation, we concluded that, protein: polymer ratio and inner aqueous phase: outer organic phase ratio played influential role on particle size and % entrapment efficiency (%EE). Hence, 3^2 full factorial design was applied to find the optimized condition for particle size and %EE. Two variables were taken at its three levels: low, medium and high, which were represented by transform values of -1, 0 and +1, respectively. Values of these selected variables are shown in Table 5.1.

Factors	**Levels used, Actual (coded)**		
	Low (-1)	Medium (0)	High (+1)
protein: polymer ratio	1:10	1:25	1:50
inner aqueous phase: outer organic phase ratio	1:8	1:4	1:2

Table 5.1 Factorial design parameters and experimental conditions.

The number of experiments required for factorial design is dependent on the number of independent variables selected. The response was measured for each trial and then simple linear equation (1), interactive equation (2) and quadratic model equation (3) were fitted by carrying out multiple regression analysis and F-statistic to identify statistically significant terms.

$$Y = b0 + b1X1 + b2X2 \quad (1)$$

$$Y = b0 + b1X1 + b2X2 + b12X1X2 \quad (2)$$

$$Y = b0 + b1X1 + b2X2 + + b12X11 + b22X22 + b12X1X2 \quad (3)$$

In developing the regression equation, the test factors were coded according to equation 4.

$$x_i = (X_i - X_i^X) / \Delta X_i \quad (4)$$

Where xi is the coded value of the i^{th} independent variable, X_i is the natural value of the i^{th} independent variable, X_i^X is the natural value of the i^{th} independent variable at the center point and ΔX_i is the step change value. Multiple regression according to quadratic model was carried out using equation 5.

$$Y = b_0 + \sum_i b_i X_i + \sum_i \sum_y b_{ij} X_i X_j + \sum b_{ii} X_i^2 \quad (5)$$

Where Y is the measured response, b0 is the intercept term, bi, bij and bii are, respectively the measures of the variables Xi, XiXj and Xi 2. The variable XiXj represents the first order interactions between Xi and Xj (i < j) (Adinarayana 2002). The multiple regression was applied using Microsoft excel in order to deduce the factors having significant effect on the formulation properties. The best fitting mathematical model was selected based on the comparisons of several statistical parameters including the coefficient of variation (CV), multiple correlation coefficient (R2), adjusted multiple correlation coefficient (adjusted R2) and the predicted residual sum of square (PRESS). Among them, PRESS indicates how well the model fits the data and for the chosen model it should be small relative to the other models under consideration (Akhnazarova 1982). Three dimensional response surface plots and two dimensional contour plots resulting from equations were obtained by the NCSS software.

5.3.4 Size and Zeta Potential Measurement

For size measurement, 500 uL of particle suspension was transferred into disposable type micro cuvette (ref. 67.758, Sarstedt AG & Co.) and particle size were measured by dynamic light scattering (DLS) using a Zetasizer (Nano ZS90, Malvern). For zeta potential (ZP) measurement, 1 mL of particle suspension (diluted in 10 mM NaCl to make suspension 1 mL) was transferred into a disposable capillary cell (DTS1060, Malvern, USA) and ZP by Laser Doppler electrophoresis (LDE) using a Zetasizer (Nano ZS90, Malvern, USA).

5.3.5 Determination of % Entrapment Efficiency

For determining % Entrapment Efficiency (%EE), MicroBCA® assay was used (Thomas 2011). SpectraMax® Plus^{384}Absorbance Microplate Reader from Molecular Device, USA was used to read the microplate and method followed for the protein estimation is described in the section (3.9.1).

The antigens (Ag-85B and EAST-6) were extracted from nanoparticles. For extraction of antigen from nanoparticles, particles were suspended in 1 mL of PBS and vortexed for 30 seconds, which was followed by centrifugation at 15000 rpm for 10 minutes. Supernatant were decanted. Extraction was done thrice with 1 ml of 100 mM PBS. From each of these 1 mL supernatant, 150 µL, in triplicate, was added to microplate well and 150 µL of BCA agent was added to each well. Blank reading was carried with 150 µL of PBS instead of supernatant. Then the plate was shaken for 30 seconds and incubated at 37^{0}C for 60 minutes. Plate was read with microplate reader spectrophotometer at 540 nm. This process was followed for both the antigens separately and estimation of protein was calculated. Antigen loading efficiency was calculated on the basis of cumulative amount of antigen found in three supernatant, which was then subtracted from actual amount of antigen used initially (1mg). This value was considered as actual loading.

The percentage encapsulation efficiency was calculated using following equation (6):

$$\% \text{ Encapsulation efficiency} = \frac{\text{Actual loading}}{\text{Theoretical loading}} \times 100 \qquad (6)$$

5.3.6 Results and Discussions

There is considerable excitement within the nanotechnology field with regard to the potential use of nanosystems as carriers for mucosal vaccine delivery. Indeed, many of the vaccines available, including protein antigens and DNA vaccines, are very unstable and need to be protected from degradation in the biologic environment. In addition, their efficacy is limited by their poor capacity to cross biologic barriers and reach the target sites. As a consequence, the design of appropriate antigen carriers that could help overcome these problems has become a significant challenge. The information accumulated regarding the *in vivo* behaviour of these nanocarriers indicates that they are able to facilitate the transport of the associated antigen across the nasal epithelium, thus leading to efficient antigen presentation to the immune system (Giri 2008). Furthermore, the results suggest that not only the size and surface properties but also the polymer composition and the structural architecture of the nanosystems are critical for the optimization of these antigen carriers (Salem 2001).

We selected two polymers (PLGA and B-PEG-PLA) from Polylactide group of polymers, which have different composition and surface properties. There are two ways of carrying antigens with the polymer: either they are encapsulated in the polymer or they are surface presented on the polymers. We opted for both the ways of antigen presentations with the polymers.

Several techniques have been reported to prepare biodegradable NPs from polymers. Some of the commonly used preparation methods are emulsion-evaporation, double solvent evaporation, salting out, emulsification/diffusion, solvent displacement and nanoprecipitation. Emulsion-evaporation is one of the most frequently used methods yielding spherical NPs with smooth surfaces (Bandi, 2004) and hence was used to prepare NPs of this investigation.

For the preparation of PLGA NPs, two parameters (particle size and %EE) were optimized using 3^2 factorial designs. 3^2 factorial designs allow determination of the influence of the factors investigated and their interactions requiring a minimum of experiments. Most of the experiments involve study of effects of two or more factors; in such cases factorial designs are most efficient in studying the joint effect of the

factors on a response. In a factorial design, all combinations of the levels of the factors are investigated. Moreover, the design gives explanation of the responses as a function of the parameters investigated (Joshi 2010).

Nine batches of (each of ESAT-6 and Ag-85B) double emulsion was prepared using 3^2 factorial designs, varying two independent variables, protein: polymer ratio (X1) and inner aqueous phase: outer organic phase (X2). The particle size (PS) and % EE were taken as taken as dependent variables and the results are recorded in Table 5.2.

Batch no.	X1	X2	PS	%EE ESAT-6	PS	%EE Ag-85B
1	-1	-1	105 ± 08	6.45 ± 0.09	118 ± 10	5.45 ± 0.11
2	-1	0	344 ± 12	8.07 ± 0.87	360 ± 13	7.83 ± 0.08
3	-1	1	320 ± 15	10.39 ± 0.11	381 ± 17	8.39 ± 0.09
4	0	-1	130 ± 07	7.23 ± 0.087	138 ± 07	6.35 ± 0.13
5	0	0	229 ±12	10.45 ± 0.15	289 ±15	8.14 ± 0.11
6	0	1	347 ±14	11.2 ± 0.22	361 ±18	8.45 ± 0.13
7	1	-1	269 ±14	6.75 ± 0.08	281 ±11	5.75 ± 0.08
8	1	0	306 ±16	10.38 ± 0.17	301 ±18	6.39 ± 0.09
9	1	1	421 ± 22	13.8 ± 0.13	432 ± 25	7.32 ± 0.11

Table 5.2 Particle size (PS) and % Entrapment efficiency (%EE) with 3^2 factorial designs

As shown in table 5.2, the maximum %EE (13.8) for ESAT-6 was observed at 1 level of X1 and X2 while PS was also increased to 421 nm., while in the case of Ag-85B, the maximum %EE (8.54%) was found at 0 level of X1 and 1 level of X2. However, particle size increased to 361 nm.

The two-dimensional contour plots and three dimensional response surface plots were generated by NCSS software and presented as figure 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8. The contour plots for PS for ESAT-6 and Ag-85 B (figure 5.1 and 5.5 respectively) were found to be linear which indicate linear relationship between X1 and X2 variables. From the contour, it was observed that higher amount of protein: polymer ratio and higher the aqueous phase to organic phase gave larger size of the

NPs. The response surface plots for PS for ESAT-6 and Ag 85B (figure 5.2 and 5.6 respectively) showed increase in PS with increased in protein: polymer ratio and aqueous phase to organic phase ratio.

As shown in figure 5.3, in the case of ESAT-6, at -1 level of X2, (inner aqueous phase: outer organic phase ratio), %EE first increased from 6.45% to 7.23% and then slightly decreased to 6.75% at -1, 0 and 1 levels of X1 respectively. Similar pattern was also observed at level 0 of X2, where %EE increased from 8.07% to 10.45% and then slightly decreased to 10.38%. However, at level 1 of X2, we observed increase in %EE from 10.39% to 11.2% and then to 13.8% at -1, 0 and 1 levels of X1 respectively. However, the increase from -1 to 0 level of X2 was marginal (10.39% to 11.2% EE). Similar patterns were also observed in the case of Ag85B (as shown in the figure 5.7).

Thus, relationship was nonlinear but the increase in %EE with increase in level of X2 was higher at 1 level of X1 in comparison to 0 and -1 levels of X1.

We aimed for 200 nm PS for our work, which was observed at level 0, 0 of X1 and X2 variable and also we observed maximum %EE at that point, we decided 0,0 is the best fit value for our experiments.

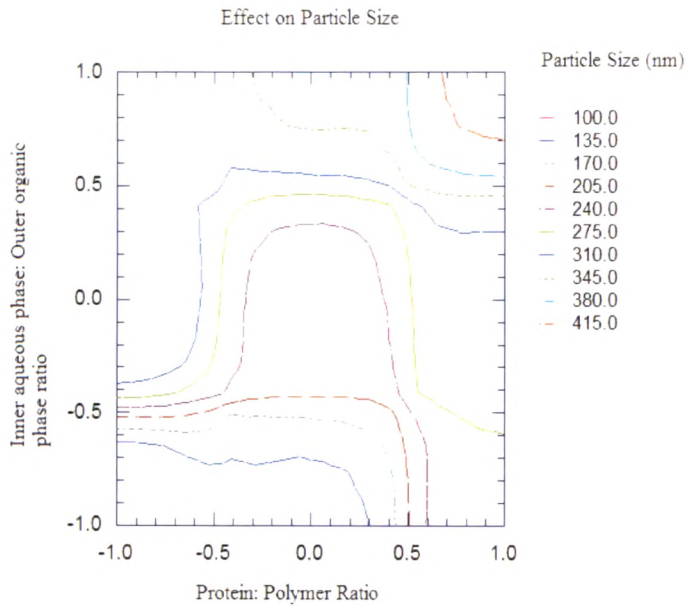

Figure 5.1 Contour plot of Particle size for ESAT-6

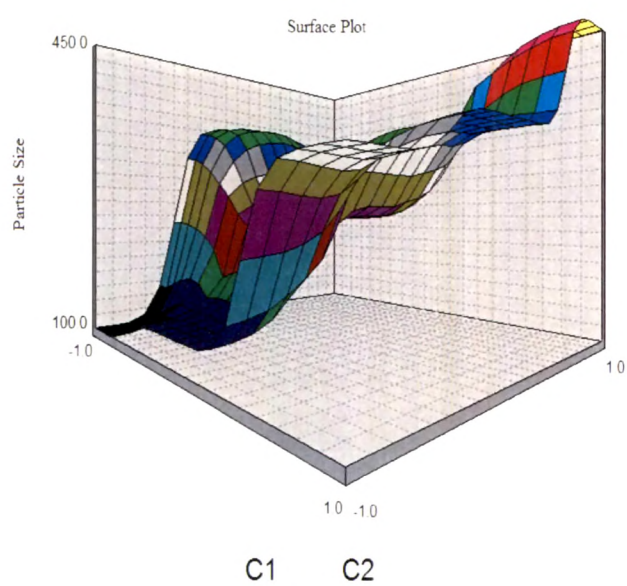

Figure 5.2 Surface plot of particle size for ESAT-6 encapsulated PLGA NPs

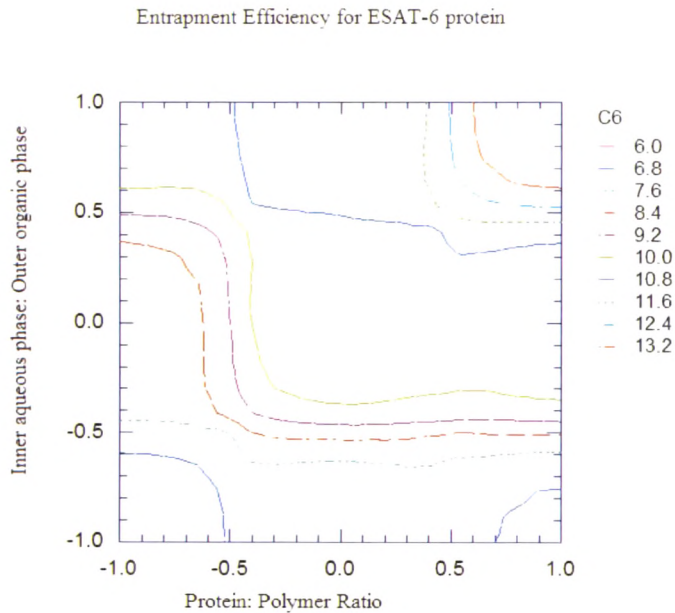

Figure5.3 Contour plot of %EE for ESAT-6 protein

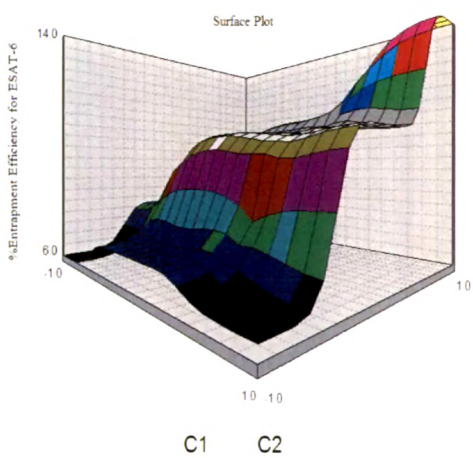

Figure 5.4 Surface plot of %EE for ESAT-6 protein

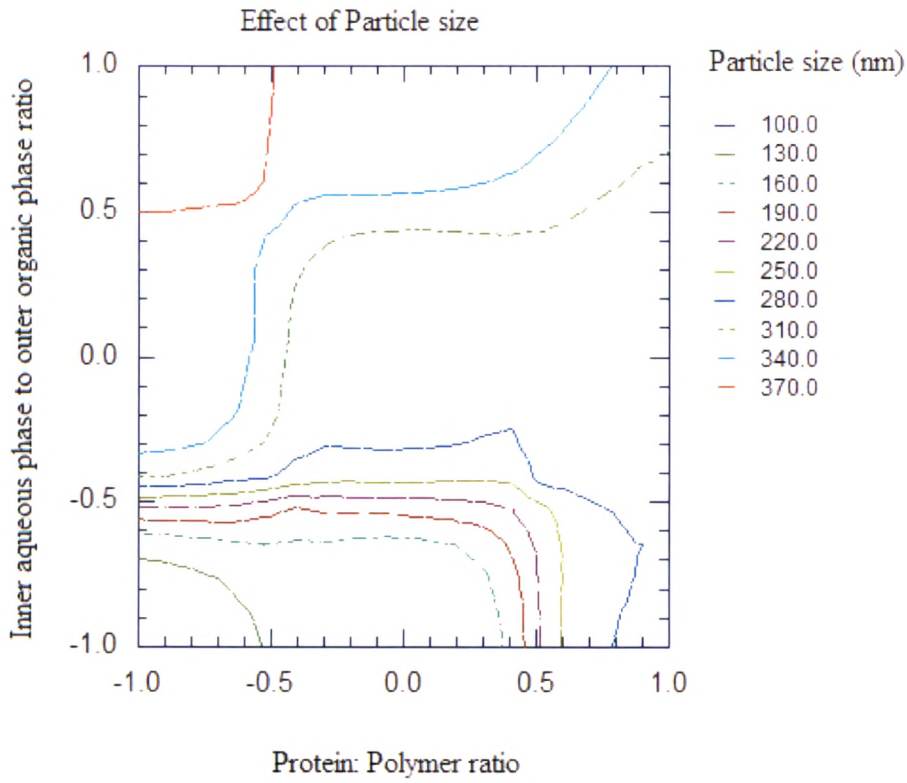

Figure 5.5 Contour plot of Particle size for Ag 85-B

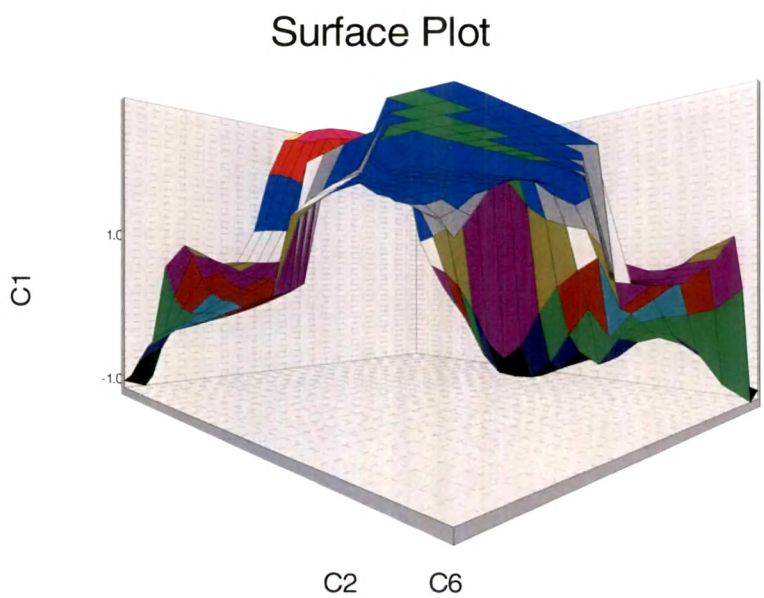

Figure 5.6 Surface plot of Particle size for Ag-85B

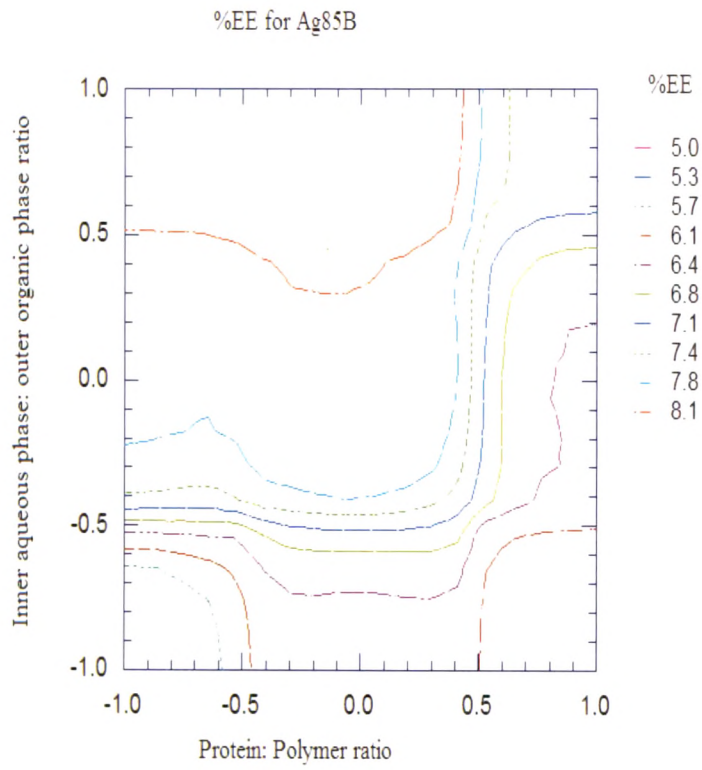

Figure 5.7 Contour plot of %EE for Ag85B

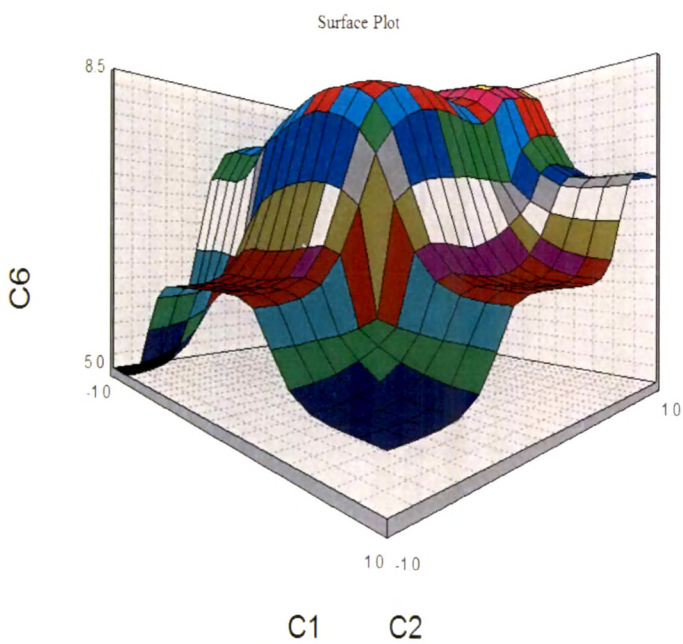

Figure 5.8 Surface plot of %EE for Ag85B

The size of NPs was also determined on the basis of our plan of study where we aimed to compare two different formulations: encapsulated antigens with surface presented antigens, where PLGA and B-PEG-PLA were used to prepare carrier particles respectively. In the case of B-PEG-PLA NPs, where uniform and dense PEGylation is the key to facilitate mucus penetration. Wang et al (2008) reported that 200 nm and 500 nm particle size gives dense and uniform PEG coverage when compared to 100 nm size. Therefore, we had choice of preparation of 200 nm or 500 nm NPs. Mice as the animal model and nasal route as route for administration were chosen for the studies. Mouse nostril restricts entry of 500 nm particle size (Davis 2001), therefore, we aimed PS as close to 200 nm. Best fit values obtained from 3^2 factorial design for %EE and PS was 1:4 ratio of inner to outer phase of emulsion and 1:25 ratio of protein: polymer ratio.

The formulation was prepared by using 1 mg of protein and 25 mg of polymer and 250 µg of DDAB. Proteins were solubilized in PBS (2 mg/mL) and 25 mg of PLGA and 250 µg of DDAB were dissolved in 2 mL of DCM. 500 µL of protein solution was sonicated with polymer solution (1:4 ratio of inner to outer phase volume ratio) and rest of the procedure was followed as described in section 5.3.2. Size was checked for its reproducibility and we found 238.8 ± 38.84 nm for ESAT-6 and 258.1 ± 15.54 nm for Ag-85B. % EE for ESAT-6 was 11.8 ± 1.78, while for Ag-85 B, % EE 8.38 ± 1.06 were observed.

With the double emulsion technique we could achieve about 8 and 11 %EE for Ag-85 B and ESAT-6 respectively. This indicated loss of great amount of antigens. We also believed that presence of organic solvent (DCM) and heat of sonication lead to degeneration of antigen. Therefore, to get maximum results from the formulation, we believed need for the alternative approach for the preparation of NPs as well as for the antigen presentation.

5.4 Single Emulsion Technique for Preparation of B-PEG-PLA nanoparticles

5.4.1 Materials and Reagents

B-PEG-PLA was synthesized at the Hanes Lab, Johns Hopkins University, Baltimore. Polyvinyl vinyl alcohol (PVA) 88% hydrolyzed MW 25000, was purchased from PolyScience, USA, Dimethyl dioctadecyl Ammonium Bromide (DDAB) was purchased from MP Biomedicals, USA. Probe sonicator (Vibra-Cell VCX 750 from Sonics & Materials, Inc., USA), magnetic stirrer (Bellco Glass, Inc., USA), and Centrifuge (Avanti J-25, Beckman, USA) were used.

5.4.2 Preparation of plain NPs by Single emulsion method

Polymer 20 mg was dissolved in 1 mL DCM and sonicated with 10 mL of 1% PVA for 2 minutes at 37% amplitude for 0.5 second on and off cycle. Immediately, it was transferred to 250 mL of 0.1% of PVA solution drop wise and stirred at speed of 5 or 6 on a 4-position magnetic stirrer (Bellco Glass, Inc., USA) for 3 hours. About 1 mL of particle suspension was collected from the beaker for size and ZP measurement. Particles were collected by centrifugation at 15000 rpm for 20 min (Beckman, Avanti J-25) and supernatant was discarded. Particles were washed with ultra-pure water two times and collected by centrifugation.

5.4.3 Preparation of DDAB adjuvanted NPs by Single emulsion method

Three different concentrations of DDAB (5% w/w, 10%w/w and 20% w/w of the weight of polymer) were studied. 20 mg of polymer and DDAB were added in DCM and above described procedure for single emulsion method was followed to prepare NPs. Finally particles were suspended in 1 mL of 100 mM PBS.

5.4.4 Particles characterization

5.4.4.1 Size and Zeta Potential Measurement

100 µL particles suspension was diluted to 1 mL with 10 mM NaCl to 1 mL. From this, 500 uL of particle suspension was transferred into disposable type micro cuvette (ref. 67.758, Sarstedt AG & Co.) and particle size were measured by dynamic light scattering (DLS) using a Zetasizer (Nano ZS90, Malvern). For ZP measurement, 1mL of particle suspension was transferred into a disposable capillary cell (DTS1060,

Malvern, USA) and ZP was measured by Laser Doppler electrophoresis (LDE) using a Zetasizer (Nano ZS90, Malvern, USA).

5.4.4.2 Scanning Electron Microscopy of Nano Particles

The morphology of the NPs was analyzed using scanning electron microscopy (SEM). NPs suspensions were layered on the SEM studs, and they were allowed to dry at room temperature. Platinum coating was done prior to microscopy. Samples were then observed with JEOL JSM- 6700F field emission microscope set at 10 kV.

5.4.5 Labelling of Protein and Determination of Degree of Labelling

Preparation of B-PEG-PLA NPs was followed by covalent conjugation of proteins using biotin- avidin chemistry.

For covalent coupling, proteins were first labelled with biotin. Biotin is a naturally occurring vitamin that binds with high affinity with avidin and other streptavidin proteins. Because of its small size (244 Da); biotin can be conjugated to many proteins without altering its biological activity. N- Hydroxysuccinimide (NHS) ester activated biotins are selected where primary amine of protein react with sulpho group of biotin. Selection of the kit is highly dependent on which group from the protein will interact. For the given protein where we have decided upon biotinylation of protein by using EZ-Link NHS- Chromogenic Biotinylation Kit (cat#21625) where primary amine group will react with Sulfonated-NHS esters.

Sulfo-NHS-Biotin is moisture sensitive and hydrolyzes very fast. Hence, to avoid moisture adsorption precautions were taken while storing, opening and handling vials containing this reagent.

The conjugation chemistry of the biotin and protein can be explained with schematic diagram as shown in figure 5.9.

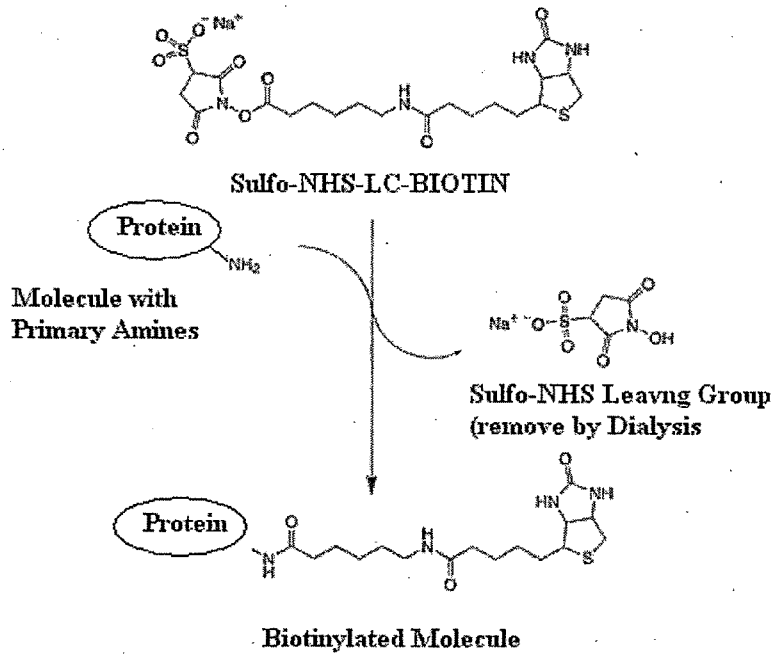

Figure 5.9 Schematic diagram of conjugation of protein with Sulfo-NHS- Biotin

The biotin labelling kit were purchased from Pierce, USA who also gave the direction for the optimization which was followed. The degree of protein labelling, protein concentration and protein activity were evaluated after biotin labelling.

5.4.5.1 Materials and Reagents

Biotinylation Kit (cat#21625), Dialysis Cassette of suitable MWCO (3.5 kDa and 20 kDa) were purchased from Pierce, USA.

5.4.5.2 Procedure for Biotinylation

The degree of labelling largely is dependent on molar concentration of biotin reagent, molar concentration of protein and ratio of these two. Theoretically we can explain: the extent of labelling is governed by the distribution of amine group on the protein and molar concentration of reactive group on biotin reagent. As indicated in manufacturer's instruction for Protein Labelling, 20 fold molar concentrations of protein were calculated as shown in formula 7:

$$\text{mL of Protein} \times \frac{\text{mg protein}}{\text{mL protein}} \times \frac{\text{mmol protein}}{\text{mg protein}} \times \frac{\text{20 mmol Biotin}}{\text{mmol protein}} = \text{mmol Biotin} \quad (7)$$

20 = 20 folds mmol concentration

The volume of biotin solution was calculated by using following formula (8):

$$\text{mmol biotin X } \frac{443\text{ mg}}{\text{mmol Biotin}} \text{ X } \frac{500\ \mu\text{L}}{2.2\text{ mg}} = \mu\text{L Biotin solution} \quad (8)$$

443 = molecular weight of Sulfo-NHS- Biotin

500 = µL volume of water in which 2.2. mg Sulfo-NHS- Biotin was added to make 10 mmol solutions.

Biotinylation of Protein

The 2 mg/mL protein solution was prepared for both the protein in PBS. The concentration of protein was again quantified by MicroBCA® assay just before labelling. The vial containing Sulfo-NHS- Biotin stored at -20^{0}C was equilibrated to room temperature before opening it to avoid moisture absorption. 2.2 mg was weighed accurately using scientific balance and dissolved in 500 µL of ultra-pure water. The calculated volume of Sulfo-NHS- Biotin solution was transferred to vial containing protein solution. The reaction was allowed to incubate for 90 minutes at 4^{0}C. Then, the contents from the eppendorff containing proteins and biotin reaction mixture was carefully transferred to the dialysis cassette (MWCO 3500 for both the proteins, as our purpose was to remove hydrolysed as well as unbound biotin. Dialysis cassettes were then placed in 1 L 100 mM PBS solution and buffer were changed twice over 8 hours of dialysis process. The entire dialysis process was conducted at 4^{0}C. The biotin- labelled protein was collected in a tube and stored at 4^{0}C until used for next determination and conjugation. The Labelled protein was again quantified for protein content by MicroBCA® assay.

The degree of biotin labelling of proteins were then evaluated by HABA-avidin assay as explained in the following section. Protein activity was determined by ELISA as discussed in 3.10.3.

5.4.5.3 Extent of biotinylation

Degree of Labelling (DoL) can be defined as the extent of biotin attachment to the protein molecule. There are three methods to find out DoL:

(1) Fluorescent method

Biotinylated proteins can be detected by fluorometric method which based on the displacement of a ligand tagged with a quencher dye from the biotin binding sites. Read-to-use kits are available from In-vitrogen for determination of DoL by fluorescence. The assay can detect from 4 to 80 pmol of biotin in a sample and provide a 50-fold higher sensitivity than the HABA biotin binding assay (Green 1970, Rao 1997).

(2) UV-spectroscopy method

If the mol number of tryptophan, tyrosin and cysteine is known, the degree of biotin can be measured by UV spectroscopy method. The protein typically shows absorbance at 280 nm. Covalently conjugated biotin shows absorption at 354 nm. The absorption of labelled biotin protein, if it is taken at 354 nm and 280 nm, it is possible to determine accurately molar concentration of biotin to molar concentration of protein. This method does not harm either to biotin or protein and Biotinylated Protein can be recovered after measurement for future use. The main precaution one should take care as non- reacted, hydrolyzed biotin also shows absorbance at 280 nm, which interferes in the result of protein absorbance (Green 1965).

(3) Colorimetric method

HABA (4′-hydroxyazobenzene-2-carboxylic acid) is a reagent that enables a quick estimation of the mole-to-mole ratio of biotin to protein. To quantify biotin label incorporation, a solution containing the Biotinylated protein is added to a mixture of HABA-avidin. Because of its higher affinity for avidin, biotin displaces the HABA from its interaction with avidin and the absorption at 500 nm decreases proportionately. By this method, an unknown amount of biotin present in a solution can be evaluated (Gan 1999, Livnah 1993).

From above three methods, we used colorimetric method for evaluation of degree of labelling.

Materials and Reagents

HABA- avidin reagent (available as lyophilized powder, ready for the reconstitution) was purchased from Sigma Aldrich, USA and manufacturer guidelines was followed for reconstitutions and storage.

Procedure

To the HABA-avidin lyophilized powder, 10 mL of PBS was added and mixed well. From this, 180 µL of the HABA/Avidin solution was added to each micro plate well. In one well, 20 µL of PBS was added. This was considered as blank reading. In remaining well, 20 µL of biotin labelled Protein solution was added. The microplate containing mixture was placed on shaker incubator (Benchtop GYROMAX 727 Incubator Shaker from Amerex Instruments, Inc., USA) for 30 seconds for complete mixing. Absorbance of the solution in the well was measured at 500 nm. The values of blank and test solution were recorded.

The online calculator for degree of biotin labelling from Pierce, USA was used. (http://www.piercenet.com/HABA/habacalcmp.cfm).

5.4.5.4 Determination of protein concentration before and after biotinylation

The concentration of protein was determined before and after biotinylation by Micro BCA assays kit, purchased from Pierce, USA to evaluate the difference in protein concentration due to biotinylation. The procedure discussed in 3.9.1 section was followed and protein concentration was calculated.

5.4.5.5 Determination of protein activity measurement

The change in protein activity upon biotinylation was also evaluated by In-direct ELISA method. For the protein activity, the procedure described in 3.10.3 section was followed and protein activity was calculated.

5.4.5.6 Conjugation efficiency for each protein

The amount of protein conjugated to the NPs with Avidin-biotin covalent chemistry needs to be quantified. The protein concentration after biotinylation was determined by micro-BCA assay. This was considered as initial concentration. From this proteins

solution, 75 µL was added to 750 µL of Avidin Labelled-Nano particle suspension in PBS. Then incubation was done for half an hour at 4^0C and particles were recovered by centrifugation (15000 rpm for 15 minutes). Supernatant was also collected (Volume of supernatant was~ 600 µL). Particles were again suspended in 1 mL of 1 M PBS and washed twice to remove any un-conjugated proteins to it and recovered similarly by centrifugation and supernatant was also collected. Finally, the protein conjugated NPS were suspended in 100 µL of PBS. Later on, all three supernatant and particle suspension were analysed for protein content by BCA assay. For supernatants, no dilution was made, while for suspension, 10 times dilution was made. The total amount of protein was calculated in all three supernatant and in nano-particles suspension. Mass balance was checked and percentage of proteins conjugated were calculated using following formula (9):

$$\% \text{ Protein conjugation} = \frac{\text{Amount of protein in Nano-particle suspension}}{\text{Initial Protein Concentration}} \times 100 \quad (9)$$

5.5.6 Results and Discussions

The main advantages of double emulsion method is that antigens will be protected by polymeric materials which prevent premature degradation of antigens. However, we observed several limitations of this application in our work. With double emulsion method, mixture of aqueous phase containing antigens was sonicated in the presence of organic solvents. Presence of organic solvents and heat of sonication are sufficient for protein folding. Antigenicity of the antigen is very much dependent on protein structure. Moreover, the interactions of the antigens with APCS is the key to generate immune response, however, when antigens reside as core of the system, their chances of interacting with APCs are minimal until polymer coat degrades completely. Moreover, with approximately 200 nm size of NPS, the % encapsulation efficiency of water soluble antigen was also found very less, for Esat-6, it was 11.8% ± 1.78 and for Ag 85B, 8.38% ± 1.06. The estimation of antigen was done with micro BCA assay, where quantitation of protein was based on reactivity of amino group.

Upon fragmentation or de-folding, protein remains as amino acid group and correct estimation of unfolded protein was not possible.

We aimed at Nasal route for delivering vaccine where three areas are of major concern: penetration of antigen through nasal mucosa, presentation of antigens to APCs and long interaction of antigens with APCs. Proteins by nature diffuse in the mucus spontaneously; however, to generate strong immune response, they must reach to the epithelia and interact with APCs for sufficient time for the proper recognition by the immune system components. To achieve this aim, we believed that there was a need of customized design of a delivery system through which surface presentation of antigens and long interaction with APCs is possible. For surface presentation of antigens, it is essential to have interactive polymer surface, however, pre-prepared PLA and PLGA polymers lack such reactive groups (Desai 1991, Barrera 1993, Langer 1993, Langer 1998). Moreover, the long interaction of antigens with APCs, needs antigen presentation at epithelium. Therefore, we adopted the theory of mucus penetrating nanoparticles proposed by Dr. Justin Hanes and co-workers, Johns Hopkins University, Baltimore, USA. Hanes group has proved that dense coating of such polymers with low molecular weight PEG helps overcoming mucosal barrier. Therefore, we have also considered our work with Biotin-PEG-PLA (B-PEG-PLA) along with the PLGA. B-PEG-PLA, a PEGylated polylactide polymer, where PLA is the backbone of the polymer and provides structural integrity (Salem 2001), PEGlyation of PLA helps for mucus penetration (Lai 2009) and Biotin is the motif which facilitates protein conjugation for the surface presentation of the protein based antigens.

B-PEG-PLA is a PEGylated polylactide polymer. A low molecular weight PEG was used for the PEGylation, which is reported to reduce mucus adhesion and facilitate particles to penetrate through mucus (Wang 2008). Wang et al also observed that 200 nm and 500 nm particles have uniform and dense PEGylation than the 100 nm NPs and have comparative better mucus penetration and as mouse was considered animal model for the in-vivo studies, where 500 nm particles have limited passage through nostril of mouse, we decided to prepare 200 nm size of NPs for our studies.

In these studies, two formulations of nanoparticles (NPs) were prepared, one with adjuvant DDAB and one without it. NPs were prepared by single emulsion techniques. In this case, plain NPs were prepared from B-PEG-PLA polymer and later on, antigens were conjugated to NPs, therefore, %EE is not the parameter needed to optimize during the preparation of NPs. However, protein conjugation efficiency was important parameter in this case. For the conjugation, biotinylation kit was purchased from Pierce, USA and guidelines given by them were followed for the optimization of the conjugation reaction.

A series of experiments were conducted with different polymer amount (10 mg, 20 mg and 50 mg) to get ~200 nm NPs, we observed the size of NPs 105 ±12 nm, 205 ± 19 and 450 ± 35 nm for 10, 20 and 50 mg weight of polymer respectively. Therefore, we considered 20 mg B-PEG-PLA for the preparation of NPs. The ZP was observed to be -7.4 ± 2.5, -4.58 ± 1.56 and – 3.9 ± 1.1 mV for 10, 20 and 50 mg weight of polymer. The shift of ZP from -7.4 mV to (~) -4 mV can be explained as with increased amount of PEG, the amount deposited on the surface also increases, thereby increasing the coat thickness, which leads to progressive reduction in the ZP (Wang 2008).

Dioctyl Decyl Ammonium Bromide (DDAB), a cationic quaternary ammonium bromide, which has proven role as adjuvant in the TB vaccine preparation (Wedlock 2008), was incorporated in the formulation to increase antigenicity of the protein based antigens. Three different concentrations of DDAB (5% w/w, 10%w/w and 20% w/w of the weight of polymer) were incorporated and evaluated with the size and ZP parameter. When we added 5% of DDAB, the ZP was -2.15 ± 1.05 mV, 10% gave ZP +2 ± 0.75 mV while 20% showed + 7 ± 1.1 mV. The effect of addition of 5% and 10% DDAB did not alter much of the size of NPs (210 ±22 nm and 215.35 ± 36.83nm for 5% and 10% DDAB respectively) as they believed to make a thin layer over the NPs (Olsen 2001), however, 20% DDAB increased particle size to 280 ± 32 nm). Therefore, we decided to 10% w/w DDAB to include in our experiments, where ZP is close to zero and size of NPs remain ~200 nm.

To the NPs, proteins were conjugated by covalent-conjugation using biotin-avidin chemistry. There are two directional effect of protein conjugation to NPs: NPs morphology and protein characteristics. Before evaluating the two- way effect of conjugation, we determined the presence of proteins on the surface. The proteins have sulpho and amino as the end group, which contribute negative effect on the ZP. When we determined ZP with the protein conjugated NPs, we observed the ZP of conjugated NPs shifted to negative side. The ZP of ESAT-6 conjugated plain NPs was -13.23 mV while DDAB adjuvanted protein conjugated NPs showed -8.34 mV. Similarly, Ag-85B conjugated plain NPs showed -13.33 mV while presence of DDAB to the conjugated NPs showed -10.3 mV zeta potential. However, the size of NPs were not affected much with any of protein (ESAT-6 and Ag85B) conjugations to the NPS. In all four cases (two plains NPs and two adjuvanted NPs with both the proteins), particle size was in the range of 200 nm. 226.8 ± 5.71 nm, 237.4 ± 6.05 nm size were found for Ag 85B conjugated NPs and Ag 85B conjugated DDAB adjuvanted NPs respectively, while 220.5 ± 4.5 nm and 224.6 ± 5.9 size of particles were found for ESAT-6 conjugated NPs and ESAT-6 conjugated DDAB adjuvanted NPs.

The morphology of the NPs (before and after protein conjugation) was analyzed using scanning electron microscopy (SEM). Figure 5.10 show the SEM figures for the NPs prepared by single emulsion techniques from B-PEG-PLA and figure 5.11 and figure 5.12 show SEM images of NPs conjugated to the protein ESAT-6 and Ag85B respectively.

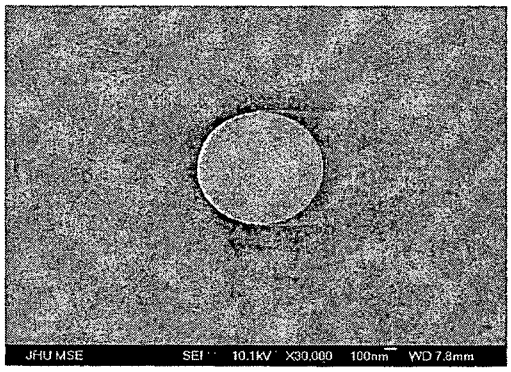

Figure 5.10 SEM figure: B-PEG-PLA NPs- 200 nm

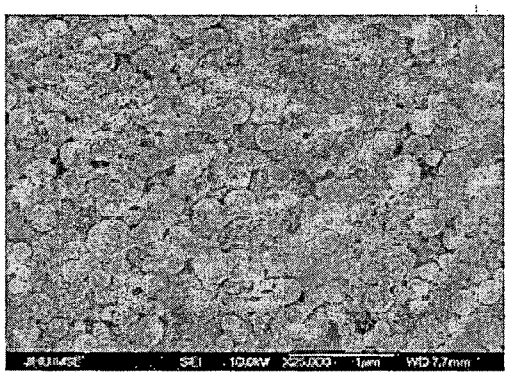

Figure 5.11 SEM figure: ESAT-6 conjugated Adjuvanted NPs

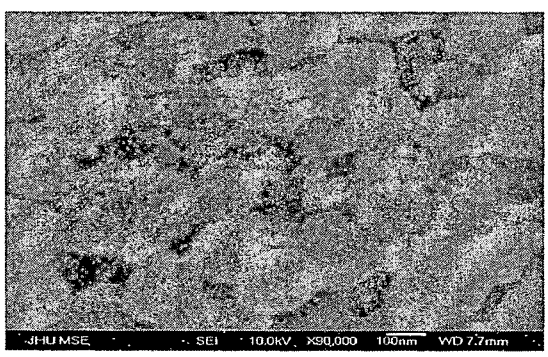

Figure 5.12 SEM figure: Ag 85B conjugated Adjuvanted NPs.

The SEM image 5.10 confirms that particles formed by single emulsion method have spherical shape. When antigens were conjugated to the NPs, spherical shape was maintained (figure 5.11 and figure 5.12), however, particles looks more aggregated after conjugations.

After preparation of NPs, the proteins were conjugated by covalent conjugation. For doing so, proteins were biotinylated first. Primary amine groups were reacted with the sulphoamine group of biotinylation reagents. 20 molar fold concentration of protein to biotin was recommended by manufacturer of biotinylation kit and that much quantity was added. To achieve optimum covalent-conjugation chemistry of avidin-biotin, 2-8 degree of biotin labelling was recommended for each protein. The DoL was determined by colorimetric analysis using HABA-avidin reagent. The original color of the reagent was orange which is due to HABA-Avidin complex.Biotin has more affinity for binding to avidin compared to HABA. Hence depending upon the quantity

of biotin present over the protein, HABA was replaced by biotin and color changed to yellow. Intensity of yellow color was the indication of amount of biotin present over protein. There were two ways to conduct this test, one with cuvette method and another with microwell plate. We have used microwell plate for the studies and calculation was done accordingly. For Ag-85B, with 20 fold molar concentration of biotin, DoL was ~ 4 while for ESAT-6 it was ~2.

The change in protein concentration after biotinylation was also determined by MicroBCA® reagent. The change in protein concentration before and after biotinylation was due to participation of primary amine groups in biotinylation. This primary amine groups may be responsible for antigenic activity of the proteins. Hence, we also decided the DoL should not cross 4 to avoid compromising in antigenic activity of the proteins.

Table 5.3 shows the consolidated results for the Dol and protein activity.

Protein	**Biotin recommended (µL)**	**Biotin solution Added (µL)**	**Concentration By BCA assay Biotinylation**		**Degree of Labelling By HABA-assay**	**Protein activity by ELISA**
			Before	After		
ESAT- 6	205.3	205	1.09 ± 0.009	0.98 ± 0.012	2.05	89.33 ± 2.33
Ag 85 B	94.23	100	1.52 ± 0.008	1.48 ± 0.098	3.57	87.27 ± 2.69

Table 5.3 Data for Protein labelling

We determined protein concentration for ESAT-6 before and after biotinylation by MicroBCA® assay, we found, 1.09 ± 0.009 mg/mL and 0.98± 0.012 mg/mL respectively. Similarly, Ag-85B concentration was determined and it was found 1.52 ± 0.008 mg/mL and 1.48 ± 0.098 mg/mL before and after biotinylation process.

We also determined the protein activity to evaluate antigenic power of protein with biotinylation. This was done by Indirect ELISA. When evaluated for protein activity after biotinylation and compared with antigenicity of protein in pure form, we found that 89.33 ± 2.33% and 87.27 ± 2.69% of antigenic power was retained by ESAT-6

and Ag-85B respectively. Closely 90% of the protein activity was retained after biotinylation in both proteins (ESAT-6 and Ag-85B). This can be explained with the fact that the biotinylation reagent has small spacer group of 6 carbon length which brought small change in the protein structure which didn't alter protein activity significantly.

Then the proteins were conjugated with B-PEG-PLA by using biotin-avidin chemistry. After conjugation to the NPs, ZP was evaluated for determining presence of proteins over the NPs. Shifting of ZP from neutral to negative side is one indication of presence of proteins on the NPs surface. When we evaluated for Ag-85B conjugation with NPs synthesized with and without adjuvant DDAB, we found ZP -10.3 ± 1.14 mV and -13.33 ± 0.68 mV respectively. Similarly, ZP was evaluated for ESAT-6 conjugation with NPs synthesized with and without DDAB; we found ZP values -8.34 ± 0.7 mV and -13.23 ± 1.11 mV respectively.

We also performed SDS-PAGE for the presence of proteins ESAT-6 and Ag-85B over NPs followed by coomassie staining and we found two clear visible bands at 10 kDa and 32 kDa for ESAT-6 and AG85-B respectively.

The results of ZP, SDS-PAGE and In-direct ELISA clearly indicated that protein conjugation had taken place without change in protein activity.

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Reviewer's comments