9. Summary and conclusions

Mycobacterium tuberculosis (*M.Tb.*) continues to cause widespread morbidity and mortality in children and adults worldwide, despite the availability of relatively simple diagnostic tools, inexpensive and effective drugs, and public health infrastructures in most countries for control and treatment of tuberculosis (TB). In adolescents and adults, TB is primarily caused by reactivation of latent/persistent *M.Tb* bacilli and progression to active pulmonary disease. *M. bovis* bacille Calmette-Guérin (BCG) vaccine, widely used as TB vaccine for newborns and effective in preventing disseminated *M.Tb* disease in young children, is unable to prevent pulmonary (reactivation) TB in adolescents and adults. Different vaccine strategies will probably be required for the various needs that exist within a population in which some individuals have been previously immunized with BCG, co-infected with HIV and/or latently infected with *M. Tb.* In the last 15 years, new strategies to improve or replace BCG in the laboratory have led to several promising vaccine candidates that are actively being evaluated in human clinical trials include recombinant BCG strains, attenuated *M.Tb* strains, DNA, protein and other sub-unit vaccines. The focus of all these researches remains on boosting the BCG protection by adopting prime-boost strategy with new developed vaccine and extending protection against disease to the adult. Subunit protein vaccines, based on antigens of *M.Tb.* which are both important for bacterial growth and pathogenicity, and can also activate cell mediated immune response in infected individuals; have emerged as potential vaccine strategy.

Early Secretory Antigen Target-6 (ESAT-6) and Antigen 85B are such two potential vaccine candidates for the development of protein based sub-unit vaccine. Both have proven role in eliciting immune response against *M.Tb* infection. The bright side of such vaccine candidates includes their sure safe administration in the immuno-compromised individuals. The next important aspect with these candidates will remain on the development of suitable delivery vehicles which should be able to deliver these proteins at Antigen Presenting cells (APCs) without compromising their

antigenic activity. Many researchers have reported encapsulation of these proteins in biodegradable polymeric nano or micro particles.

Mucosal vaccine is an attractive alternative to conventional parenteral vaccine which offers several significant advantages which include easier administration, systemic and local immune response and eradication of needle- associated limitations. Nasal route is the entry for causative organism *M.Tb.* Therefore; Nasal vaccine for TB is more realistic immunization approach. The main objective of Nasal vaccine for TB is to elicit immune response in the respiratory tract and prevent development of *M.Tb* infection in the lungs, which is primary site of infection. To achieve this objective, the important aspect is the interaction of vaccine candidates with Antigen Presenting Cells (APCs). To get an access to APCs which are located in epithelia, the formulation carrying the vaccine candidate must cross the mucosal barrier.

Proteins by nature diffuse in the mucus spontaneously, although they will have very limited bioavailability due to premature degradation. To elicit strong immune response, they must reach epithelia and interact with APCs for sufficient time for the proper recognition by the immune system components. This requires a suitable delivery system.

Mucus Penetration Technology (MPT), an interesting finding from our collaborator group, Prof Justin Hanes at Johns Hopkins University, Baltimore, MD- USA showed that dense coating of low molecular weight Poly Ethylene Glycol (PEG) can avoid adhesion to mucus, thereby enabling nanoparticles to rapidly penetrate mucus secretions. In particular, nanoparticles as large as 500 nm either covalently modify with a dense coating of low molecular weight (PEG) or non-covalently coated with poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (also known as Pluronic) are capable of penetrating normal human mucus nearly as rapidly as they would in pure water.

With this reference, we have extended application of MPT in the protein based vaccine delivery system. We also believed that surface presentation of antigens would

be better recognized and interacted with APCs than the antigens encapsulated in the biodegradable particulate system. For surface presentation of antigens, the polymer must have biological ligand which can interact with antigens.

The protein expression from PMR LB 7/RV3875/Esat-6 and PMR LB 47/RV1886c/Ag 85B were optimized and 1 L of bacterial culture was able to give about 20 mg of ESAT-6 protein and 15 mg of Ag 85B proteins. These proteins were evaluated for their molecular weight, concentration and antigenic activity by SDS-PAGE, BCA assay and In-direct ELISA respectively and compared with standard proteins provided by the Colorado State University (CSU), USA. And we found, the lab synthesised proteins had the equivalent molecular weight and antigen activity as those given by the CSU, USA.

Biotin-PEG-PLA was synthesized in two easy steps: biotin attachment to bi-functional PEG followed by PLA attachment to PEG- biotin by ring opening polymerization of the lactide monomer. Gel Permeation Chromatography was performed to evaluate molecular weight of the polymer and average molecular weight of the polymer was around 20K. This was also indicating polymerization of the monomer lactide.

Another parameter which is very necessary to evaluate for polymer characterization is the attachment of ligand. Here biotin is the ligand. The attachment of biotin to NH2-PEG-OH was achieved through N-hydroxy- succinimide chemistry. The graft polymerization of Lactide to the HO-PEG-biotin proceeds through a ring opening polymerization mediated by Sn(Oct)2 complex. Analysis of PEG-biotin by H^1NMR spectroscopy showed the appearance of a triplet at 2.05 ppm that can be assigned to the methylene from the biotin chain α to the amide and appearance of a broad singlet belonging to the free amino proton at 7.85 ppm. These signals were not present on the NMR spectra of NHS-biotin. The biotin group was identified through the two methane protons from the cyclic biotin structure at 4.3 and 4.2 ppm and two urea protons from cyclic biotin structure at 6.45 and 6.35 ppm. In NMR spectra's of PLA-PEG-biotin, biotin signals could not be seen as the signal from the PLA overwhelmed

the biotin signal. (CH; 1.53 ppm and CH_3, 5.22 ppm). The data of GPC and NMR clearly indicated that polymerization has occurred with the graft polymerization reaction and there was proper attachment of PEG and Biotin to the backbone of the polymer PLA.

Two different types of nanoparticles were prepared: one from B-PEG-PLA polymer, where a single emulsion technique was used while conventional nanoparticles were prepared from Poly Lactide-co-Glycolide (PLGA) (50:50) by double emulsion techniques. Two separate types of batches were prepared in the case of B-PEG-PLA nanoparticles, one was containing Dioctyldecyl ammonium bromide (DDAB) and one was prepared without it. Here, DDAB was used as an adjuvant.

The 3^2 factorial designs were used to optimize particle size and %EE for antigens encapsulated in the PLGA nanoparticles: 2 parameters at three levels were taken into consideration. These two parameters included protein: polymer ratio and inner phase aqueous phase: outer organic phase. Protein: polymer ratio was kept at three different levels 1:10, 1:25 and 1:50. The inner aqueous phase: outer organic phase ratio was kept at three levels: 1:8, 1:4. 1:2. NCSS software was used to analyse 3^2 factorial design models. The %encapsulation efficiency of PLGA nanoparticles were measured by determining amount of protein present in washings by BCA assay and we found 11.8 ± 1.78% for ESAT and 8.38 ± 1.06 for Ag-85B. Size of nanoparticles was measured by dynamic light scattering (DLS) using a Zetasizer (Nano ZS90, Malvern). For the measurement of the size, 500 µL of particle suspension was transferred into disposable type micro cuvette (ref. 67.758, Sarstedt AG & Co.). For zeta potential measurement, 1 mL of particle suspension was transferred into a disposable capillary cell (DTS1060, Malvern) and was measured by laser Doppler electrophoresis (LDE) using a Zetasizer (Nano ZS90, Malvern). The size of PLGA nanoparticles were found 349.8 ± 38.84 nm and zeta potential was -31.00 ± 3.07 mV.

The single emulsion method was used for the preparation of B-PEG-PLA nanoparticles and similarly as described in previous section, size and zeta potential was determined. The results for B-PEG-PLA nanoparticles size and zeta potential for

batches containing cationic surfactant DDAB were 215.35 ± 36.83nm and 2.97 ± 1.70 mV respectively. When DDAB was added in the concentration of 10% w/w of polymer the nanoparticles size and zeta potential was found 205.54 ± 19.80 nm and -4.58 ± 1.56 mV. Near neutral zeta potential was the indication of PEGylation of PLA. Various quantity of DDAB was added while preparation of nanoparticles and we optimized the concentration of DDAB to 10% w/w of polymer weight, where zeta potential was found close to zero. This 10% addition of DDAB did not affect the size of nanoparticles prepared from B-PEG-PLA.

After preparation of B-PEG-PLA nanoparticles, 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 was 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 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. When 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 there is not much compromise in antigenic power of protein with biotinylation. This was done by Indirect ELISA. When evaluated for antiginicity 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.

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

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

This portion of our work is partly an extension of Mucus Penetration Techniques developed by Dr. Justin Hanes, Professor, Johns Hopkins Medical Institutions, Baltimore, USA. All various types of particles including two controls PS-PEG, a representative of diffusive particles and PS-COOH, a representative of Immobile particles, two proteins ESAT-6, a 9.8 kDa protein and Ag 85B, a 32 kDa protein conjugated to B-PEG-PLA NPs fabricated with and without DDAB were studied for mucus penetration. It was observed that ESAT-6 conjugated particles were diffusing faster (almost as similar to PS-PEG nanoparticles) in mucus than Ag-85B conjugated

NPs. This observation could be justified with difference in molecular weight of ESAT-6 (MW 9.8 kDa) to that of Ag-85B (MW 32-34 kDa).

Another noticeable finding of this experiment was a change in % diffusive particles. In the case of Ag85B, 51.17 ± 5.40% was found when DDAB was not added and 73.30 ± 5.71% when DDAB was added. Several reports on study of Ag-85B indicated that Ag85B make a complex with DDAB (Brandt 2000, Doherty 2002). This complex might be one reason for the improved diffusion of Ag85B conjugated NPs.
Hanes group reported in their earlier work on transport of conventional PLGA nanoparticles v/s mucus penetrating nanoparticles and showed that conventional nanoparticles got trapped in the mucus

The stability of lyophilized protein conjugated nano-particles were conducted at three different temperature -20^{0}C, 2-8^{0}C and RT (~+20^{0}C) over 45 days. The nanoparticles conjugated with antigens were found stable at -20^{0}C and 2-8^{0}C over 45 days. However they were not found stable when stored at RT.

For animal studies, vaccination with *M. bovis* BCG remains the gold standard against which all other vaccines need to be compared. The ability of the vaccination protocols to generate immune response was assessed by protection assay. To determine the ability of nasal vaccination to generate immune response, groups of BALB/6 mice were lightly anaesthetized (with Isoflurane, USP 2% in oxygen) and given one dose of 30 µg of antigen formulation in 50 µL of PBS through nostril with the help of a thin tip attached to micropipette. The antigen conjugated to adjuvanted NPs is a stable suspension formulation containing both the antigens ESAT-6 and Ag85B in 30 µg each of antigens and 10% w/w of DDAB as an adjuvant. As a control group of mice, they were received 50 µL of PBS (negative control), 50 µL of solution of both the antigens (ESAT-6 and Ag85B in 30 µg concentration and 200 µg of DDAB) (antigens in pure form) and plain NPs 20 mg/ 50 µL (adjuvant effect of NPS). All these groups of mice received respective dose through nasal route. 4 weeks resting time was given all groups mice to develop immunity.

After immunization, mice were challenged with low dose (10^2 CFU) *M.Tb* (Pasteur strain) by inhalation route. One group of mice was sacrificed on the following day of infection to verify microbial infection and rest of the groups were sacrificed at 4^{th} week and 8^{th} week of the infection. The lungs and the spleen were collected in 2.5 mL of ice cold sterile PBS aseptically and homogenized with cup and pestle type of homogenizer manually. From this, 10^{-2} and 10^{-4} serial dilutions were made and 0.5 mL was placed on 7H11 agar plates. Numbers of colonies were counted after 3 week incubation at 37^0C, 5% CO_2 incubator.

The protection assay of the Intra Nasal administration of equal amount of ESAT-6 and Ag-85B conjugated- DDAB-adjuvanted Nano-particles was compared with the protection ability of combination of proteins in pure form, proteins encapsulated in PLGA nanoparticles when given by intra nasal route and BCG vaccines by subcutaneous route. The bacterial load in lungs homogenates was also determined in the case of un-vaccinated mice and when plain NPs were given through intra nasal route.

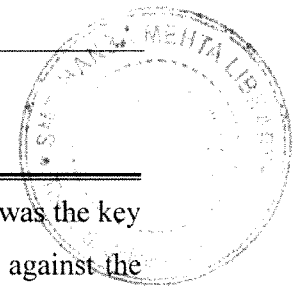
We found 2.5 x 10^2 CFU in the lungs of mice sacrified on the following day of the infection, which indicated successful transfer of *M.Tb* to the lungs of bacteria. When bacterial load were evaluated after 4 weeks of the bacterial infection, we found 9.1 x $10^7 \pm 5.53 \times 10^2$ and 1.25 x $10^4 \pm 1.3 \times 10^2$ in the case of ESAT-6 and Ag 85B encapsulated PLGA NPs and Ag85B and ESAT-6 conjugated DDAB adjuvated B-PEG-PLA NPs respectively while 2.8 x $10^4 \pm 2.19 \times 10^2$ CFU were found for the BCG vaccine. This indicates that antigen conjugate NPs and the BCG vaccine had almost same efficacy, however, PLGA NPs showed 10^3 more CFU. The comparative efficacy of conjugated NPs with the BCG was mainly attributed to the strong immnuno-dominant nature of these two proteins, presence of adjuvant DDAB and to a certain extent NPs (NPs also contribute in eliciting non-specific immune response to some extent). Actual TB infection produces these two proteins in copious amount while BCG is able to produce Ag-85 complex only. However, BCG fails to produce ESAT-6 antigen which plays vital role in the disease protection mechanism. Lesser efficacy in the case of PLGA encapsulated NPs can be explained with the fact that

encapsulated antigens were not recognized early by the APCs for the early development of specific immunity against the disease.

When protection assay performed after 8th week of the infection, the bacterial load reduction in lungs was found more in the case of antigen conjugated DDAB adjuvanted B-PEG-PLA NPs compared to the BCG vaccine. In 8 weeks of the time, the bacteria were grown in the lungs to 3.75 x 10^{12} in number of CFU which was reduced to 1.53 x 10^{8} number of CFU in BCG treated group and to 3.75 x 10^{4} number of CFU in the antigen conjugated DDAB adjuvanted B-PEG-PLA NPs. It indicated that at Day 56, the % log bacterial load reduction was about two times better in the case of antigens conjugated DDAB adjuvanted B-PEG-PLA NPs than the BCG vaccine. B-PEG-PLA, a PEGylated PLA degrades in body over 30 days, which may have allowed antigens to get free from the NPs for complete interaction with APCs which may have enabled them to provide longer and stronger immunity against the disease.

The key findings of the present studies from our research are:

- ✓ We performed experiments with pre-prepared PLGA and modified PLA polymer (Biotin- PEG-PLA) for the comparative studies of antigens encapsulated vis a vis conjugated NPs. We also performed protection ability of both the formulation in mice and compared with the standard BCG vaccine. We found that conjugated NPs provided better disease protection than the encapsulated NPs. Hence, we can conclude that the polymer composition and the structural architecture were critical for the formulation of the protein-based antigens.
- ✓ Protein labelling and covalent- conjugation of proteins with B-PEG-PLA polymer could be achieved without compromising protein antigenicity.
- ✓ We also found that PEGylation of PLA helped B-PEG-PLA NPs to penetrate well through the nasal mucus. Over 70% of Ag-85B and ESAT-6 conjugated adjuvanted B-PEG-PLA NPs penetrated through the mucus.



- ✓ The mucus penetration ability and surface presentation of antigens was the key combination for the given formulations to elicit immune response against the *MTb* when given intranasally.

Future scope of work:

However, detailed studies on immunoprofiling can provide supportive data on level of cytokins produced during the immune response. Some more animal studies work can also be conducted on this topic which may include:

- ✓ Role of protein based vaccine in "Long term memory of immune response" where studies may be continued for one year.
- ✓ Determination of long term immunity when given with BCG vaccine as prime-boost techniques
- ✓ Multiple administration of protein based vaccine at various time intervals and its effect on long term immune response
- ✓ In-vivo stability studies
- ✓ Preventive- curative combination therapy in the cases of latent infection, and reactivated infection for the control of active tubercle infection.

Reviewer's Comments