Aerosol Delivery of Recombinant Antigen 85B in Microparticle Vaccine Systems for Protection Against Tuberculosis

By

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ABSTRACT

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(Under the direction of Dr Anthony J. Hickey, PhD, D.Sc)

The only available tuberculosis vaccine, bacille Calmette Guérin (BCG) has highly variable efficacy. Antigen 85B (Ag85B) is a protein secreted by *Mycobacterium tuberculosis* (*MTB*) in the early culture and a promising subunit antigen. Pulmonary vaccination is an attractive immunization route. It was proposed that Ag85B/adjuvants in microparticle formulations intended for pulmonary delivery would target macrophages, elicit related cell-mediated immune response sufficient to protect guinea pigs from tuberculosis. Three specific aims were adopted to test the hypotheses. (1) To express recombinant Ag85 (rAg85B) from *E. coli* suitable for inclusion in polymeric microparticles for (2) *in vitro* screening of particle formulations with respect to related cell-mediated immunity and for (3) assessment of protection from infection in a guinea pig model of tuberculosis.

rAg85B was expressed from two *E. coli* strains and encapsulated by spray-drying in poly(lactide-co-glycolide) (PLGA) microparticles with adjuvants muramyl dipeptide (MDP) or trehalose dibehenate (TDB) to be delivered as dry powder aerosols. The antigenicity of the recombinant forms was evaluated by monitoring IL-2 secretion from T hybridoma cells DB-1 after recognition of $Ag85B_{97\text{-}112}$ epitope presented by THP-1, antigen presenting cells. The

powders were administered as single or multiple doses to guinea pigs to evaluate protection from a low dose aerosol infection with *MTB* strain *H37Rv*.

Spray-dried microparticles exhibited mass median aerodynamic diameters (MMAD) of 2.5-3.0μm, suitable for inhalation aerosols. The amount of IL-2, an indicator of antigenicity, released from T hybridoma cells following PLGA-Ag85B microparticle administration to THP-1 phagocytic cells, was 92-360 fold that of rAg85B solutions. Surface-associated rAg85B played a significant role in the observed IL-2 release. This formulation extended epitope presentation as demonstrated by duration and extent of IL-2 production.

BCG prime: PLGA-Ag85B aerosol boosts (x2) appeared to enhance protection from infection with virulent *MTB* strain *H37Rv* compared to BCG alone. However, further work is required to demonstrate this effect conclusively. This is a clinically meaningful observation since many susceptible individuals in the developing world have been immunized with BCG.

The results indicate that microparticles encapsulating rAg85B were immunologically effective, with preliminary evidence for protection and, potential for pulmonary vaccination to prevent *MTB* infection.

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LIST OF ABBREVIATIONS AND SYMBOLS

ACI	Andersen Cascade Impactor
Ag85B	antigen 85B
AIDS	acquired immune deficiency syndrome
APC	antigen presenting cell
BCG	bacillus Calmette-Guerin
BAL	bronchoalveolar lavage
BALT	Bronchus-associated lymphoid tissue
BET	Brunauer-Emmett-Teller
CCI	Carr's compressibility index
CFC	chlorofluorocarbon
CFU	colony forming unit
CH_2Cl_2	methylene chloride
CFP	culture filtrate protein
CMD	count median diameter
CMI	cell-mediated immunity
CMIS	common mucosal immune system
COPD	chronicle obstructive pulmonary disease
CR	complement receptor
CT	cholera toxoid
CTL	cytotoxic T-lymphocytes
D_{10}	diameter which 10% of particles are smaller than

D_{50}	diameter which 50% of particles are smaller than
D_{90}	diameter which 90% of particles are smaller than
DMEM	Dulbecco's modified Eagle's medium
d_p	projected area diameter
DPI	dry powder inhaler
DSC	differential scanning calorimetry
DTH	delayed-type hypersensitivity
ELPI	Electrical Low Pressure Impactor
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FPF	fine particle fraction
gor	glutathione reductase
GSD	geometric standard deviation
IPTG	isopropyl-β-thiogalactopyranoside
ISCOM	immunostimulant complexes
LB	Luria-Bertani
LPS	lipopolysaccharide
mAGP	mycolyl-arabinogalactan-peptidoglycan complex
MALT	mucosal-associated lymphoid tissues
MDP	muramyl dipeptide
MDR-TB	multidrug-resistant tuberculosis
MMAD	mass median aerodynamic diameter
MMD	mass median diameter

MMR	macrophage mannose receptor
MPL	monophosphoryl lipid A
MTB	mycobacterium tuberculosis
NOS	nitric oxide synthase
PLGA	poly (lactide-co-glycolide)
PMA	phorbol myristate acetate
pMDI	pressurized metered-dose inhaler
PTFE	polytetrafluoroethylene
RD	regional domain
RH	relative humidity
RNI	nitrogen intermediates
SD	spray-drying
SE	solvent evaporation
SEM	scanning electron microscopy
SOD	superoxide dismutase
TB	tuberculosis
TCR	T cell receptor
TDB	trehalose dibehenate
TDM	trehalose dimycolate
T_g	glass transition temperature
Th1	T helper type 1
TMM	trehalose monomycolate
trxB	thioredoxin reductase

VMD	volume median diameter
WHO	World Health Organization
XDR TB	extensively drug-resistant tuberculosis

1. Introduction

1.1 Tuberculosis and its current vaccination status

Tuberculosis (TB) has emerged as a global public health epidemic. The most recent data from the World Health Organization (WHO) indicated that there were approximately 9 million individuals who developed active TB disease and more than 2 million died due to TB in 2003 (Denrell, 2003). The global epidemic of TB has affected the United States where there were 14,511 cases reported in 2004 (4.9 cases per 100000 individuals) (Xia and Lee, 2004). Despite decreasing numbers of cases since 1992 in the United States, TB remains a serious public health problem among certain patient populations and is highly prevalent in many urban areas (Blumberg, Leonard and Jasmer, 2005). The incidence of TB has increased dramatically in areas where resources are inadequate to identify and treat all cases. Roughly one third of the world's population is infected with TB. It remains latent in all but 5-10% of those infected as long as the immune system is healthy (Friedrich, 2005).

Rising rates of multidrug-resistant tuberculosis (MDR-TB) have contributed to worsening treatment outcomes in some regions. This is a major public health problem because treatment is complicated, cure rates are well below those for drug-susceptible TB, and patients may remain infectious for months or years, despite receiving the best available therapy (Goble, Iseman, Madsen *et al.*, 1993). Chronic MDR-TB is caused by a single strain of *Mycobacterium tuberculosis* (*MTB*): W-Beijing family. Acquisition of drug resistance by *MTB* results from mutations (caused by nucleotide substitutions, insertions or deletions) in specific resistance-determining regions of the genetic targets or activating enzymes of anti-

TB chemotherapeutic agents (Post, Willcox, Mathema *et al.*, Ramaswarmy and Musser, 1998).

The worldwide emergence of extensively drug-resistant tuberculosis (XDR TB) and a provisional definition for this form of TB were first reported in November 2005. The U.S. data indicated that 74 TB cases reported during 1993-2004 met the case definition for XDR TB. In October 2006, the World Health Organization convened an Emergency Global Task Force on XDR TB, which revised the case definition to specify resistance to at least isoniazid and rifampin among first-line anti-TB drugs, resistance to any fluoroquinolone, and resistance to at least one second-line injectable drug (amikacin, capreomycin, or kanamycin). National TB Surveillance System data were analyzed for reported XDR-TB cases during 1993-2006; a total of 49 cases (3% of multidrug-resistant (MDR) TB cases) met the revised case definition for XDR TB. Of these, 17 (35%) were reported during 2000-2006. XDR TB presents a global threat and a challenge to TB-control activities in the world (Migliori, Besozzi, Girardi *et al.*, 2007).

In addition, tuberculosis is associated with increased mortality in HIV infection and is considered to be the leading cause of death among HIV-infected persons worldwide (Reyn and Vuola, 2002). Approximately one-third of the 40 million HIV-infected persons are co-infected with TB, which is implicated in up to one-half of all AIDS death (Worley, 2006). In 2006, TB still remains a major opportunistic infection in HIV-infected patients, often the first indication of immunodeficiency (Johnon and Decker, 2006). TB may be readily transmitted to individuals via the respiratory route at any stage of HIV infection. Currently, the incidence of TB in HIV-infected persons is 100 times greater than that of the general population. HIV-infected persons have increased risk for MDR-TB (Gordin, Nelson, Matts *et al.*, 1996).

1.1.1 Tuberculosis pathogen: *Mycobacterium tuberculosis (MTB)*

Mycobacterium tuberculosis (*MTB)* is transmitted by aerosol infecting the lungs. It is the etiological agent of TB in humans. Humans are a reservoir for this bacterium. *MTB* is a nonmotile, rod-shaped bacterium that is 2-5 μm in length and 0.2-0.3 μm in width (Grosset, 1993). In the classic case of tuberculosis, the *MTB* complexes appear in the well-aerated upper lobes of the lungs because *MTB* is an obligate aerobe. The bacterium is a facultative intracellular parasite, usually occupying the compartment vesicles of macrophages.

The envelope of *MTB* consists of two distinct parts: the plasma membrane and the cell wall. The cell wall structure of *MTB* is unique among prokaryotes and is a major determinant of virulence for the bacterium. The cell wall complex contains peptidoglycan attached to lipids. Over 60% of the mycobacterial cell wall is lipid. The lipid fraction of *MTB* cell wall consists of three major components (Fig 1.1): (1) Mycolic acids are 1-alkyl branched 2-hydroxy fatty acids, typically with 70-90 carbon atoms. Mycolic acids make up 50% of the dry weight of the cell envelope. Mycolic acids are strong hydrophobic molecules that form a lipid shell around the organism and affect permeability properties at the cell surface. They are thought to be a significant determinant of virulence in *MTB*. (2) Cord Factor (trehalose dimycolate) is responsible for the serpentine cording appearing in culture. Cord factor is toxic to mammalian cells and is also an inhibitor of polymonocyte migration. Cord factor is most abundantly produced by virulent strains of *MTB*. (3) Wax-D (autolytic product of cell wall mycolyl-arabinogalactan-peptidoglycan complex (mAGP)) in the cell envelope is the major component of Freund's complete adjuvant (FCA) (Besra and Chatterjee, 1994). Generally, high concentration of lipids in the cell wall has been associated

with *MTB* resistance to antibiotics, to killing by acid and alkaline compound, to lethal oxidation and its survival inside macrophages.

Pulmonary TB begins when droplet nuclei containing the microorganism reach the alveoli. The development of pulmonary tuberculosis from its onset to its various clinical manifestations may be pictured as a series of interactions and confrontations between host and invader. (1) The inhaled bacillus may multiply, or may be eliminated by alveolar macrophages before any lesion is produced. (2) Small caseous lesions may progress, or may heal or stabilize before they are detectable. (3) Larger caseous lesions may grow locally and shed bacilli into the blood and lymph to infect the lungs again to form the secondary lesions, or they may heal or stabilize. (4) Alternatively, caseous lesions may liquefy and introduce bacilli and their products into the bronchial tree, making arrest of the disease more difficult (Dannenberg and Rook, 1994). In general, each successive stage of the disease meets with weaker host responses. Furthermore, in a given lung, each tuberculous lesion is independent, i.e., each lesion may be at a different stage, independent of other lesions.

1.1.2 Macrophage activation and immune responses

Once the first small caseous tuberculous lesion is established, all subsequent stages of disease are capable of causing both tissue-damage and macrophage-activation. The tissue-damaging response is often produced during delayed-type hypersensitivity (DTH) reactions to high concentration of bacillary products inhibiting the numbers of bacilli appearing extracellularly. This damage results in granuloma formation. Macrophage-activation causes an accumulation of activated microbicidal macrophages around the caseous center of the lesions. The activated macrophages can kill and digest the bacilli they ingest intracellularly.

The mechanism of macrophage activation and initial immune response may be more important for vaccination (Fig 1.2). *MTB* is phagocytosed by resident alveolar macrophages after deposition in the lungs. The receptors involved in the specific uptake of pathogens are the macrophage mannose receptor (MMR) and complement receptor types 1 (CR1), 3 (CR3), and 4 (CR4) (Zimmerli, Edwards and Ernst, 1996). Toll-like receptors can stimulate pathogen uptake when interact with ligands (Thoma-Uszynski, Stenger and Takeuchi, 2001). IL-12 is induced following phagocytosis of *MTB* by macrophages, driving development of cell-mediated immunity (CMI), which is important in protection from *MTB* infection (Ladel, Szalay, Reidel *et al.*, 1997). CD4 Th1 cells are of primary importance in this protection from initial infection. *MTB* resides primarily in a vacuole within the macrophage, resulting in MHC class II presentation of mycobacterial antigen to CD4 T cells. The primary effector function of CD4 T cells is believed to be the production of IFN-γ, and possibly other cytokines, sufficient to activate macrophages. The *MTB* bacilli within the vacuoles may have access to the cytoplasm (Teitelbaum, Cammer and Maitland, 1999). The antigen epitope – MHC class I complex can be recognized by CD8 T cells, which then produce cytokines IFN-γ and TNF-α and act as cytotoxic cells. IFN-γ is the key activating agent that triggers antimycobacterial effects of macrophages (Flesch and Kaufmann, 1987). It improves or augments the antigen presentation in macrophages, leading to a recruitment of more CD4+ T-lymphocytes and cytotoxic T-lymphocytes (CTL) (Flesch, 1993)]. TNF-α, which can be secreted by macrophages, dendritic cells and T cells, induces chemokine production from macrophages and endothelial cells and also up-regulates adhesion molecules on vascular endothelial cells, resulting in cellular influx of further inflammatory cells (Hartmann and Plum, 1999). TNF-α, in synergy with IFN-γ, induces the production of nitric oxide and

related nitrogen intermediates (RNIs) by macrophages via the action of the inducible form of nitric oxide synthase (NOS).

MTB can persist inside host phagocytic cells, the macrophages. These pathogens can survive the antimicrobial mechanisms of macrophages which are designed to destroy any incoming microbe. Pathogenic mycobacteria have evolved strategies to survive within macrophages by preventing phagosome–lysosome fusion, thereby creating a niche that allows them to persist within an otherwise hostile environment (Muellera and Pieters, 2006). Firstly, *MTB* arrests the normal maturation process of phagosomes to phago-lysosome (Gatfield, Albrecht, Zanolari *et al.*, 2005) by changing the endosomal pH and inhibit the acidification of the early phagosome. Secondly, *MTB* produces chemicals such as superoxide, by the action of the enzyme superoxide dismutase (SOD), which might inhibit host defenses by interfering with host apoptosis (Skeiky and Sadoff, 2006). *MTB* avoids the development of a localized, productive immune response that could activate the macrophages leading to the destruction of the intracellular pathogens (Van Heyningen, Collins and Russell, 1997).

1.1.3 Recent status in TB vaccine development

There are treatment strategies available for drug-susceptible TB, MDR-TB and HIV-co-infected TB. However, the most desirable approach is to prevent TB through vaccination.

The currently available TB vaccine is attenuated strain of *Mycobacterium bovis* bacillus Calmette-Guerin (BCG). BCG is unable to stimulate an adequate cell-mediated immunity. It only protects against disseminated and meningeal TB in young children but is not effective against pulmonary TB, the most prevalent form of the disease in adults (Friedrich, 2005). The protective effect of BCG varies from 0%, overall efficacy of two different BCG vaccines compared with placebo in India (Tuberculosis Research Center

(ICMR), 1999), to as high as 80% effective in children, adolescents and young adults, with effects lasting up to 60 years in a study of native Americans (Aronson, Santosham, Comstock *et al.*, 2004; Comstock, 1994; Fine, 1989). The potential reasons for variable efficacy of BCG include: 1) interference with the immune response to BCG caused by previous exposure to environmental mycobacteria; 2) differences among BCG vaccine sub-strains; and phenotypic changes in the vaccine during passage from the original cultures and during the manufacturing process; 3) the deletion of protective antigens from BCG; 4) variability in dose, route of administration, age of administration and genetic differences among vaccinees; and lyophilization of the vaccine (Brewer and Colditz, 1995; Fine, Carneiro, Milstien *et al.*, 1999).

The concerns of safety, loss of sensitivity to tuberculin skin test, and particularly the variable efficacy of BCG increase the need for new and effective vaccines for TB. Several strategies have been adopted to address this need. They are listed as followed.

A. Improved efficacy of BCG (Table 1.1)

1) BCG overexpressing antigens. Horwitz's group had produced a TB vaccine candidate, rBCG30, which overexpresses Ag85B and induces increased protection compared with BCG strain in animal challenge studies (Horwitz and Harth, 2003);

2) BCG with the ability to escape endosome. Listeria monocytogenes Hly (listeriolysin) is a sulphydryl-activated cytolysin that forms pores in the membrane of the early phagosome. Kaufmann's group had produced rBCG vaccine that excretes Hly to punch holes in the endosome and allows leakage of BCG to cytosol. Therefore $CD8^+$ T cells responses are enhanced (Hess, Miko, Catic *et al.*, 1998).

3) Reducing BCG interference with immunity. Host superoxides produced by macrophages kill *MTB* and can amplify host T-cell recruiting signals. Kernodle's group has shown that secretion of superoxide dismutase (SOD), by MTB, to inactivate superoxides promotes granuloma formation and, therefore, contributes to inhibition of host defenses and persistence of the organism. rBCG with reduced secretion of functional SOD (Kernodle, 2004) is more potent than the parent strain. It has also been proposed, by Jacob *et al.*, that the deletion of genes of nlaA, which inhibit apoptosis of infected cells, might lead to a superior vaccine (Jacobs, 2005).

4) Reintroduction of deleted genes. This strategy reintroduces regional domain (RD) 1 locus which encodes the immunodominant and protective ESAT-6 and CFP-10 proteins (Pym, Brodin, Majlessi *et al.*, 2003) which were deleted during BCG attenuation.

B. New attenuated live TB vaccines (Table 1.2)

1) Inactivation of Phop, which regulates expression of many *MTB* proteins, attenuated *MTB* virulence while maintaining the pattern of immune responses associated with the parental *MTB* strain (Perez, Samper, Bordas *et al.*, 2001).

2) Auxotrophic mutant vaccine candidates (requiring the addition of a nutrient(s) for survival) have been produced. These mutants maintain their infective capability and have a limited duration of replication in the host (Guleria, Teitelbaum, McAdam *et al.*, 1996; Jackson, Phalen, Lagranderie *et al.*, 1999). The deletion of proteins involved in *MTB* lipid metabolism by disrupting both the panC, panD genes and lysA gene produced vaccine mc^2 6020 (panC panD lysA), which does not replicate *in vivo*. Deletion of the RD1 region from the panC, panD mutant produced vaccine $mc^2$6030 (panC panD ΔRD1) (Sambandamurthy, Derrick, Jalapathy *et al.*, 2005; Sambandamurthy and Jacobs, 2005; Sambandamurthy, Wang, Chen *et*

al., 2002). Both of these highly attenuated mutants were safer than BCG in immunocompromised animals and provided similar levels of protection in mouse challenge studies.

C. Subunit vaccines (Table 1.2)

1) Biochemical approaches initially identified several of the most abundant proteins secreted by *MTB*, including Ag85 complex, MPT32, PhoS, DnaK, GroES, MPT46, MPT53, MPT63 and the 19kDa lipoprotein (Andersen and Hansen, 1989; Nagai, Wiker, Harboe *et al.*, 1991; Young and Garbe, 1991). There are also antigens in low molecular weight range, ESAT-6 (6-10kDa) and Ag85B, MPT59 (26-34kDa) (Skjot, Oettinger, Rosenkrands *et al.*, 2000). *MTB*32 (a secreted serine protease)(Skeiky, Lodes, Guderian *et al.*, 1999) and *MTB*39 (a member proline-proline-glumatic acid family of protein on the cell wall)(Dillon, Alderson, Day *et al.*, 1999) have also been identified. Antigen vaccine candidates, which have advanced towards clinical development using animal protection models, include Ag85A, Ag85B, ESAT-6, TB10.4, *MTB*9.9, *MTB*39 and *MTB*41.

2) Recombinant fusion proteins are genetically generated. An example employs Ag85B and ESAT-6 (Hybrid-1)(Olsen, van Pinxtern, Rasmussen *et al.*, 2001), *MTB*39 and *MTB*32 (*MTB*72F)(Skeiky, Alderson, Ovendale *et al.*, 2004). These fusion proteins can be more immunogenic than the individual components.

3) Prime-boost vaccine strategies. The attraction of the heterologous prime-boost approach is that it preferentially expands TB-specific pre-existing memory cells against antigenic epitopes shared by both the prime and booster vaccines. This approach also minimizes the potential for generating antibody and T-cell neutralizing effects. An early study with a BCG prime-boost approach with adjuvanted proteins of Ag85A (Brooks, Frank, Keen *et al.*, 2001)

resulted in enhanced protection compared to non-BCG-primed in animals. This suggested that subunit antigens as boosters to BCG-primed animals might generally induce superior protection.

4) DNA vaccines (Table 1.2)

Poxviruses and adenoviruses are potent DNA vaccine delivery vehicles. Intranasal mucosal delivery of a recombinant adenoviral (Ad5 based) construct expressing *MTB* antigen Ag85A (AdAg85A) conferred protection that was superior to that seen with BCG vaccination against a pulmonary *MTB* challenge (Wang, Thorson, Stokes *et al.*, 2004). DNA vaccines showed great potential for use in heterologous prime-boost immunization strategies. Priming with BCG and boosting with a recombinant Modified Vaccinia Ankara virus (MVA) that expresses Ag85A induced increased levels of CD4 and CD8 T cells and enhanced protection in animals compared with a vaccination regimen using either BCG or MVA85A alone (Goonetilleke, McShane, Hannan *et al.*, 2003).

1.1.4 *MTB* secreted protein Antigen 85B (Ag85B) as the vaccination subunit antigen

Many current vaccine candidates have been selected from extensive screening of *MTB* antigens in animal models. Because secreted and surface-exposed antigens seem to be the first antigen encountered by the immune system, these antigens have been widely screened for immunogenicity and protection. *MTB* proteins ESAT-6, Ag85 complex, Hsp60 and hybrid protein: a fusion protein of ESAT-6 and Ag85B, a 72kDa fusion protein based upon the *MTB*32 and *MTB*39 antigens of *MTB* have shown protective capacity (Haile and Kallenius, 2005).

The increasing number of proteins and enzymes recently identified as being involved in cell envelope biogenesis includes the three predominant secreted proteins of *MTB*:

antigens 85A, 85B and 85C. The three antigens form Ag85 complex, which is up to 41% of the total culture filtrate protein. All three purified enzymes (68-80% identical at the amino acid level) have recently been shown to catalyze the transfer of a mycoloyl residue from one molecule of α,α' trehalose monomycolate (TMM) to another, leading to the formation of α,α' trehalose dimycolate (TDM or cord factor). Ag85 complex is involved in the final stage of mycobacterial cell wall assembly (Belisle, Vissa, Sievert *et al.*, 1997). The fibronectin-binding activity exhibited by all three components suggests that they might also be involved in pathogenicity (Abou-Zeid, Ratliff and Wiker, 1988). By conferring on mycobacteria the ability to bind fibronectin, these antigens could facilitate their entry into host cells and promote their adherence to mucosal surfaces (Ehlers and Daffe, 1998). Ag85 complex interacts with the immune system at an early stage of the infectious process and induces both humoral and cell-mediated immune responses in *MTB*-infected patients (Daffe, 2000; Wiker and Harboe, 1992) and animal models. The early-stage antigens would be most useful in a preventive vaccine. In addition, successful naked DNA vaccines against TB, employing the Ag85A (Baldwin, D'Souza and Orme, 1999) (Denis, Tanghe and Palfliet, 1998) and Ag85B genes (Lozes, Huygen, Content *et al.*, 1997; Ulmer, Liu and Montgomery, 1997), have been reported to elicit protection against *MTB*. DNA-Ag85B provided better protection than DNA-ESAT6 and DNA-MPT64 (Kamath, Feng, Macdonald *et al.*, 1999). Moreover, co-immunization with DNA-Ag85B, DNA-ESAT6 and DNA-MPT65 provided better protection than that induced by any single DNA vaccine (Kamath, Groat, Bean *et al.*, 2000). A recombinant BCG strain overexpressing *MTB* Ag85B elicited a higher level of protection than traditional BCG (Dhar, Rao and Tyagi, 2004). Recently, the fusion protein of antigen 85B with *Listeria monocytogenes* (Miki, Nagata, Tanaka *et al.*, 2004) or ESAT-6 (Olsen, van

Pinxtern, Rasmussen *et al.*, 2001) was reported to elicit strong immune responses and protective effects against *MTB*. The immune response to low molecular weight secretory proteins of *MTB* in bronchoalveolar lavage (BAL) from minimal pulmonary TB and non-TB patients has been reported. Among polypeptides predominantly recognized by BAL lymphocytes, only Ag85A and Ag85B induced both NO and interleukin-12 in alveolar macrophages (Sable, Goyal, Verma *et al.*, 2007). According to these reports, Ag85 complex or Ag85A and Ag85B molecules seem to be the ones of the most promising candidates for future subunit TB vaccines. Since antigen 85B elicits the strongest immune responses and also the most abundant subunit among 85A, B and C, antigen 85B will be the focus protein subunit for vaccination delivery in this research.

1.1.5 Antigen 85B protein expression

Usually investigators have separated the *MTB* Ag85B and multiple sub-unit protein complex from cell culture filtrate. *MTB* is exceptionally slow growing and biohazardous. Therefore, the growing, separation and purification procedures make it difficult to obtain large quantities of Ag85B from the culture filtrate. For example, growth of *MTB* for 2-3 weeks in 150 liters of broth culture was required to produce 100mg of Ag85B (Horwitz, 1995). The evaluation of Ag85 proteins for vaccine development and clinical trials imposes a practical requirement that they are produced in recombinant form in a rapidly growing, nonpathogenic host.

Several reports on expression of Ag85A and Ag85B indicated that intracellular proteins or secreted antigens have been achieved (Harth, Lee, Wang *et al.*, 1996; Kremer, Baulard, Estaquier *et al.*, 1995), but the high-level expression and secretion of these proteins in native form has proven difficult. Horwitz *et al.* reported sufficiently high-levels of

expression and secretion of the native form of recombinant Ag85A and B proteins using *Mycobacterium smegmatis* and *Mycobacterium vaccae*, two rapidly growing nonpathogenic mycobacteria, as hosts (Harth, Lee and Horwitz, 1997). Other investigators indicated that the recombinant Ag85 subunits can be successfully expressed in *Corynebacterium glutamicum* (Salim, Haedens, Content *et al.*, 1997), *E. coli* (Lakey, Voladri, Edwards *et al.*, 2000) and plant leaves (Dorokhov, Sheveleva, Frolova *et al.*, 2007). Lakey *et al.* employed *Escherichia coli* systems and concluded that the poor expression of some mycobacterial genes was due, in part, to the presence of low-usage *E. coli* codons. They used site-directed mutagenesis to convert low-usage codons to high-usage codons for the same amino acid in *MTB* genes for Ag85A and Ag85B. Replacement of five codons in the wild-type gene for Ag85B increased recombinant protein production in *E. coli* 54-fold, yielded 27mg/litre of Ag85B (Lakey, Voladri, Edwards *et al.*, 2000). The recombinant Ag85B (rAg85) elicited proliferation and interferon-gamma (IFN-γ) production by lymphocytes from healthy tuberculin reactors and was recognized by monoclonal antibodies to native Ag85B, indicating that the recombinant antigen contained T cell and B cell epitopes (Lakey, Voladri, Edwards *et al.*, 2000). The Ag85B used in this project was obtained by overexpression in same *E. coli* systems as Lakey's.

E. coli is a frequently used host since it facilitates protein expression by its relative simplicity, its inexpensive and fast high-density cultivation, the well-known genetics and the availability of a large number of compatible molecular tools. However, expression of recombinant proteins with *E. coli* as the host often results in insoluble and/or nonfunctional proteins. In Lakey's Ag85B *E. coli* expression system, inclusion bodies and aggregates were

found in the proteins produced (Lakey, Voladri, Edwards *et al.*, 2000). There are several strategies to increase ratio of soluble Ag85B in the *E. coli* cytoplasm.

- Protein expression at reduced temperatures

The aggregation reaction is, in general, favored at higher temperatures due to strong temperature dependence of hydrophobic interactions that determine the aggregation reaction (Kiefhaber, Rudolph, Kohler *et al.*, 1991). A direct consequence of temperature reduction is the partial elimination of heat shock proteins that are induced under overexpression conditions (Chesshyre and Hipkiss, 1989). Furthermore, the activity and expression of a number of *E. coli* chaperones are increased at lower temperature (Ferrer, Chernikova, Yakimov *et al.*, 2003; Mogk, Mayer and Deuerling, 2002). A sudden decrease in cultivation temperature inhibits replication, transcription and translation, which favor protein folding. However, bacterial growth is decreased resulting in a decreased amount of biomass (Sorensen and Mortensen, 2005).

- Special *E. coli* strains used to improve soluble expression

Cysteines in the *E. coli* cytoplasm are actively maintained in a reduced state by pathways involving thioredoxin reductase (*trxB*) and glutaredoxin (*gor*). The disulfide bond-dependent folding of heterologous proteins is improved in the *origami* strains (Novagen) which have these two mutated reductases. Disruption of the *trxB* and *gor* genes encoding the two reductases, allow the formation of disulfide bonds in the *E. coli* cytoplasm (Bessette, Åslund, Beckwith *et al.*, 1999).

- Molecular chaperones drive folding of recombinant proteins

A possible strategy for the prevention of inclusion body formation is the co-overexpression of molecular chaperones. GroEL (Hsp60 chaperone family) operates the

protein transit between soluble and insoluble protein fractions and participates positively in deaggregation and reduction of inclusion bodies (Mogk, Mayer and Deuerling, 2002).

Since the molecular chaperones in the cytosol can help to prevent protein misfolding and aggregation (Hartl and Hayer-Hartl, 2002) and site-mutated reductases are favorable for disulfide formation, *E. coli,* strain o*rigami B,* with chaperone vector was used in this project.

One drawback in using an *E. coli* system to overexpress protein is endotoxin production. Lipopolysaccharide (LPS) is the major component of Gram-negative bacterial outer membranes (Reeves, Hoobs, Valvano *et al.*, 1996). This endotoxin is continuously shed during bacterial growth. It activates the pattern recognition receptor Toll-like receptor 4 (TLR 4), which is expressed on a subset of epithelial cells (Backhed, Soderha, Ekman *et al.*, 2001). Activation of the TLR 4 intracellular signaling pathway stimulates a proinflammatory response in epithelial cells, leading to epithelial production of various proinflammatory mediators that instructs cells in the underlying tissue and cells belonging to the immune system (Ba¨ckhed, So¨derha¨, Ekman *et al.*, 2001). The injection of LPS, into experimental animals causes a wide spectrum of nonspecific pathophysiological reactions such as: fever, changes in white blood cell counts, disseminated intravascular coagulation, hypotension, shock and death (Bacteriology, 2002). Although LPS could be used as an adjuvant, the presence of large amounts as a contaminant in the antigens would interfere with the research into antigenicity and immunogenicity induced by the subunit antigen. It may also interfere with the normal functions of the host following immunization. Polymixin B can remove most of the endotoxin from solutions. Polymixins are a family of antibiotics that contain a cationic cyclopeptide with a fatty acid chain. Polymixin B neutralizes the biological activity of endotoxins by binding to the lipid A portion of LPS (Pierce Biotechnology).

1.2 Pulmonary aerosol vaccination is an alternative immunization approach

Vaccination is one of the most powerful strategies to prevent infectious diseases in underserved region. The most common pathogens enter the body via mucous membranes in the nose, lungs and gastrointestinal tract. Respiratory viral and bacterial infections are a major cause of morbidity and mortality throughout the world (Woodland and Randall, 2004). Many pathogens involve or utilize the respiratory tract as a portal of entry into the body (Brandtzaeg, 1992; Breiman, Butler and McInnes, 1999). Therefore, pulmonary aerosol vaccination could be a potentially powerful way to rapidly immunize the population, inducing protection by exposing airways to vaccines. This route of vaccine administration, which follows the natural route of infection, may best mimic the induction of immunity in the respiratory tract by pathogens and may lead to more general systemic immunity. Aerosol vaccination is a noninvasive, nontraumatic method of antigen delivery that avoids the risk of transmitting hepatitis B, HIV and other blood borne agents through improper injection practices. Therefore, pulmonary immunization is a potential alternative to conventional parenteral delivery approaches.

1.2.1 Pulmonary vaccination belongs to mucosal immunization

Mucosal immunity is the first line of defense against pathogens entering the body via mucosal surfaces. Pulmonary, nasal and oral immune systems contribute almost 80% of all immunocytes. These cells are accumulated in, or transit between, various mucosal-associated lymphoid tissues (MALT), which together form the largest mammalian lymphoid organ system (Mestecky, 2005).

Mucosal immunization can induce a systemic immunity: generation of secretory IgA antibodies, which are able to cross epithelial membranes and prevent future entry of

pathogens through the mucosal site. Furthermore, lymphocytes that were stimulated by antigens in the mucosal inductive site migrate via regional lymph nodes and thoracic duct to the blood stream and other mucosal effector sites. This migration leads to secretory IgA production at other mucosal sites of MALT (gut, nasal and genitourinary-associated lymphoid tissues) and has been termed the common mucosal immune system (CMIS) (Kiyono, Kweon, Hiroi *et al.*, 2001; Lugton, 1999; MacDonald, 2003; van Ginkel, Nguyen and McGhee, 2000). Meanwhile, circumstantial evidence indicates the existence of cell-mediated immunity (CMI) in CMIS (Gallichan and Rosenthal, 1996). The CMIS appears to have organ selectivity as enhanced memory is seen at the site of mucosal priming compared to that of distant mucosal sites (Mestecky, 1987). Maintenance of the memory T cells depends on the tissues in which exposure to antigen first occurred (Mackay, 1993). Mucosal vaccination, rather than systemic immunization, gave long-lived cytotoxic T memory lymphocytes (Gallichan and Rosenthal, 1996).

Pulmonary immunization appears very promising since the lungs contain a highly responsive immune system. The pulmonary epithelium has a crucial role in host defense against inhaled pathogens as it presents physical barriers which include the mucociliary escalator, secretion of antimicrobial agents (Larrick, Hirata, Balint *et al.*, 1995; McCray and Bentley, 1997; Wright, 1997), chemokines (Bivas-Benita, Ottenhoff, Junginger *et al.*, 2005) and cytokines in the mucus layer covering the airway epithelium to prevent colonization of microorganisms. Pulmonary macrophages and dendritic cells play an important role in both innate and adapted immunity. Alveolar macrophages are abundant, numbering over a billion in the periphery and interstitium of the lungs (Bezdicek and Crystal, 1997). Dendritic cells are present in the epithelial linings of the conducting airways, submucosa below the airway

epithelium, within alveolar septal walls (Holt, Stumbles and McWilliam, 1999). These two cell populations are professional antigen presenting cells (APC). They are able to phagocytose, process and present antigens to stimulate T cells. Primary stimulation of T cell clones within the pulmonary lymphoid tissue is induced when macrophages and dendritic cells migrate to bronchial lymph nodes and home to T cell paracortical area (Banchereau, Briere, Caux *et al.*, 2000; Poulter, 1997). Bronchus-associated lymphoid tissue (BALT), the respiratory part of MALT, is located mostly at bifurcations of the bronchus in animals and humans. Local T-cell responses have been noted. BALT can be thought of as a functional analogue to mucosal lymphoid aggregates in the intestine and, thus, deemed as a component of CMIS (Bienenstock and McDermott, 2005). There is evidence that mice genetically lacking spleen, lymph nodes and Peyer's patches can generate strong primary B- and T-cell responses to inhaled influenza. These responses appear to be initiated at sites of the induced BALT (iBALT) which function as an inducible secondary lymphoid tissue for respiratory immune responses (Moyron-Quiroz, J., Kusser *et al.*, 2004). Exposure of the lungs to various aerosol formulations designed to protect against influenza virus was more effective at inducing a local immune response than either intranasal administration or parenteral injections indicating that this route of administration is relevant for immunization (Smith, Bot, Dellamary *et al.*, 2003).

The physiological features of lymphoid tissues in the respiratory tract suggest there is potential for pulmonary immunization. Consequently, pulmonary vaccination is a reasonable strategy for protecting the population from infectious diseases where pathogens enter the body through the lungs via inhalation.

In tuberculosis pathology, *MTB* persists in macrophages in the lungs of the host. Therefore, pulmonary delivery of vaccines to the macrophages to initiate a cell mediated immune response is a mechanism of achieving pulmonary TB immunization.

1.2.2. Current status of pulmonary vaccine delivery

The potential for immunization of humans by aerogenic vaccination was recognized in the Soviet Union (Aleksandrov and Gefen, 1958) and the United States (Eigelsbach, Tigertt, Saslaw *et al.*, 1962) forty years ago. The Russian investigators employed dry vaccine preparations containing living vegetative cells or spores of attenuated strains for vaccination to humans and indicated that aerogenic vaccination was as effective as subcutaneous vaccination against plague, tularemia, brucellosis and anthrax (Aleksandrov, Gefen, Garin *et al.*, 1959; Aleksandrov and Gefen, 1958). Airborne BCG vaccination was conducted at University of Illinois in 1968. BCGs were nebulized to different age groups of people (Rosenthal, McEnery and Raisys, 1968). Rosenthal *et al.* indicated that tuberculosis pathology in human beings was similar to that in guinea pigs rather than mice or rabbits, providing important information on BCG aerosol immunization as well as animal models for tuberculosis. Rubella immunizations via the pulmonary, nasal and subcutaneous administrations were compared in 46 volunteers early in 1973. The humoral immunity via pulmonary delivery was comparable to that of subcutaneous vaccination (Ganguly, Ogra, Regas *et al.*, 1973).

Pulmonary immunization is an appealing means of protecting populations in biodefense strategies. *Bacillus anthracis* infection in humans occurs as cutaneous, gastrointestinal, or inhalational anthrax depending upon the route of exposure. Inhalational anthrax, the form most likely to occur during a bioterrorist attack, is difficult to diagnose

early, and despite antibiotic therapy, has a high fatality rate. In 1957, 32 volunteers were exposed to an aerosolized dry spore of live anthrax vaccine, composed of a mixture of strains STI-1 and No. 3, for 15 min in the Soviet Union. Few general adverse reactions were recorded (Aleksandrov, Gefen, Garin *et al.*, 1959). Later a skin test indicated that the aerosol method induced higher immunity than subcutaneous and scarification methods previously used for immunization in the Soviet Union (Shlyakhov, 1968) (Shlyakhov and Rubinstein, 1994). The pattern of CMI after aerosol anthrax immunization was investigated in human subjects. There were five phases in the kinetic pattern. In phase II (starting at 7 days post-vaccination) an exponential rise to a maximum at day 15 occurred. In later phases, up to a year of reduction in skin reaction occurred. The loss of the skin reaction on day 30 is a characteristic feature of post-vaccination anthrax CMI. This may be explained by a blockade of macrophages by lethal anthrax toxin released by the multiplying vaccine strain (Shlyakhov, Rubinstein and Novikov, 1997).

Francisella tularensis is a facultative intracellular bacterial pathogen. Infection may be caused by inhalation of contaminated air. Therefore, *Francisella tularensis* is considered a serious biohazard (Tarnvik, 1989). A live attenuated vaccine strain *Francisella tularensis* strain LVS was nebulized and administered to humans (Hornick and Eigelsbach, 1966). Immunity to aerogenic virulent LVS challenge appeared to be greater than the conventional parenteral vaccine administration.

The only successful clinical case of pulmonary vaccination on a large scale, with characterization of aerosol device technology, is a pulmonary measles immunization study. Measles vaccination by pulmonary aerosol delivery has considerable appeal. Approximately 4 million children in Mexico were exposed to measles vaccine aerosols and a high level of

successful prevention was achieved (Cutts, Clements and Bennett, 1997). The custom made system, Classical Mexican Device, used an International Product Inc. (IPI) jet nebulizer driven by an Evans industrial air compressor to deliver aerosols of the reconstituted Edmonston Zagreb strain of attenuated measles vaccine virus. The pulmonary delivery system produced 52-64% seroconversion, which compared favorably with the expected seroresponse to subcutaneous administration (4-23% seroconversion) in school children (Bennett, Fernadez de Castro, Valdespino-Gomez *et al.*, 2002). IFN-γ production in cellular immunity was more robust in infants who received aerosol vaccines (Wong-Chew, Islas-Romero, Garcia-Garcia *et al.*, 2005). Many children received a much larger dose than that necessary for immunization without any side effects. The necessary immunization dose for different age groups of children has been estimated (Coates, Tipples, Leung *et al.*, 2006). The WHO has identified three nebulization devices manufactured by Omron, Trudell and Aerogen that meet the desired performance criteria to replace the classical Mexican device (Laube, 2005). In order to overcome the cold-chain requirement and maintain the biological stability of measles vaccine, a pulmonary dry powder aerosol formulation is under development. The attenuated EZ strain of measles has been micronized by jet-milling after lyophilization. The measles vaccine was dispersible after blending with carrier lactose (LiCalsi, Maniaci, Christensen *et al.*, 2001). The shift from liquid to powder formulation coupled with appropriate dry powder inhalation technology potentially strengthens the measles vaccine. The current Measles Aerosol Project by WHO aims at licensing, by 2007, at least one method for respiratory delivery of current measles vaccines (Henao-Restrepo and Auguado, 2006).

Clinical trials have demonstrated equivalence, or superiority in immune responses for measles and rubella delivered as inhaled aerosols with respect to alternative routes of administration (Sepulveda-Amor, Valdespino-Gomez, Garcia-Garcia *et al.*, 2002).

Pulmonary DNA immunization is a new and promising vaccination approach. DNA vaccines have the advantage of inducing a strong cellular immunity with a preference to cytotoxic T lymphocyte (CTL) and T helper type 1 (Th1) responses. The use of pulmonary delivery of DNA vaccines was recently described where plasmids encoding ovalbumin, hepatitis B surface antigen (Lombry, Marteleur, Arras *et al.*, 2004) and HLA-A*0201-restricted T cell epitopes of *Mycobacterium tuberculosis* (Bivas-Benita, Ottenhoff, Junginger *et al.*, 2005) were delivered to a mouse model. Increased immunity as measured by antibody and cytokine production was achieved.

1.2.3. Aerosol technology available for pulmonary delivery of vaccine

Many aerosol exposure methods have been used to vaccinate animal models. Intratracheal instillation and insufflation allow direct delivery of liquids and powders, respectively, to the lungs. Animal exposure to aerosols has been achieved through a range of exposure chambers (whole body and nose-only chambers). For clinical trials, aerosol vaccine delivery requires delivery devices and formulations in which the antigens are incorporated.

The site and efficiency of deposition of aerosolized particles in the respiratory tract is critically influenced by their particle size (defined as aerodynamic diameter), size distribution, particle shape and density (Gupta and Hickey, 1991). If the aerodynamic particle size is > 5 μm, inertial impaction is the primary mechanism for deposition of the particles in the upper and central airways. The particles ≤ 3 μm, which have not deposited by impaction, deposit in the lower airways by sedimentation. Relatively low velocities, along with longer residence

times, in the lower airways favor the deposition of submicron size particles (< 1μm) by the diffusion process. Diffusion and sedimentation are the major mechanism of deposition in the lower airways of the lungs. Since particles that are subject to diffusion have little mass, this mechanism is not considered important for therapeutic or vaccine aerosol purpose. Aerosol vaccination usually depends on the target pathogen and the sites of the inductive immunity. Larger particles (> 5μm) are needed for the vaccination to prevent upper respiratory infections by respiratory viruses or bacteria (for example, *Bordetella pertussis* and *Chlamydia pneumoniae* (Reynolds, 2002)) and smaller particles (≤ 3 μm) for lower respiratory tract infections (*Streptococcus pneumoniae, Bacillus anthracis* as the examples (Reynolds, 2002)).

Inhalers can be classified into three major categories: nebulizer, pressurized metered-dose inhaler (pMDI) and dry powder inhaler (DPI).

1.2.3.1 Nebulizers

Two types of nebulizers are commercially available: air jet and ultrasonic nebulizers. Generally air jet nebulizers can generate smaller particle sizes (mass median aerodynamic diameter 2-5 μm), which more easily penetrate to the small airways. Nebulizers have some advantages: constant output can deliver aerosols of most solutions and provide large doses with very little patient co-ordination or skill; larger total doses can be delivered than with MDI and DPI devices; disposable nebulizers are inexpensive. However, treatments using these nebulizers are time-consuming and inefficient, resulting in the waste of active ingredient.

Advances in technology have led to novel nebulizers that reduce waste and improve delivery efficiency. An enhanced delivery design, Pari LC Star (Pari, Germany), increases

aerosol output by directing auxiliary air and causing more generated aerosol to be swept out of the nebulizer for inhalation (Leung, Louca and Coates, 2004). Breath-actuated nebulizers, e.g. AeroEclipse (Trudell Medical International, London, ON, Canada) and Halolite (Medic-Aid Limited, West Sussex, UK) have recently been developed. The AeroEclipse controls an actuator piston to produce aerosol in inspiration and at rest position in patient's expiration (Leung, Louca and Coates, 2004). The Halolite monitors a patient's breathing pattern in the first three breaths and then targets the aerosol delivery into the first 50% of each inhalation. This ensures the aerosols are delivered to the patient during inspiration only, thereby eliminating drug loss during expiration (Denyer, Dyche, Nikander *et al.*, 1997). A number of metered-dose liquid inhalers, including AERx (Aradigm, Hayward, CA, USA), AeroDose (Nektar-AeroGen, San Carlos, CA, USA) and Respimat (Boehringer Ingelheim, Ingelheim, Germany), have been developed to produce fine aerosols in the respirable range by forcing the drug solution through an array of nozzles with 30–75% of the emitted dose being deposited in the lungs (Dolovich, 1999; Ganderton, 1999).

Pulmonary vaccination studies have been performed by nebulization of live attenuated pathogens, such as *Francisella tularensis* (Colan, Shen, Kuolee *et al.*, 2005; Hornick and Eigelsbach, 1966), measles morbillivirus (Wong-Chew, Islas-Romero, Garcia-Garcia *et al.*, 2005), BCG (Cohn and Davis, 1958; Lefford, 1977), and Rubella rubivirus (Ganguly, Ogra, Regas *et al.*, 1973). Nebulization delivers vaccine aerosols to the lower respiratory tract. However, there is a potency loss problem. Complex-molecules have been reported to frequently degrade in response to the shear force of jet nebulization (Schwarz, Johnson, Black *et al.*, 1996). The stability of measles vaccine was determined during nebulization by the "Classical Mexican Device". There was a 71% loss of vaccine potency after the nebulizer

was run continuously for 20min. The loss in viral potency was of the order of one third when the nebulizer was run in cycles of 30s on, 10s off (Coates, Tipples, Leung *et al.*, 2006). Immunity may be elicited in response to small numbers of viable pathogens in the lungs. However, the issue of vaccine dose reproducibility in the mass vaccine campaign should not be neglected.

1.2.3.2 Pressurized metered-dose inhalers (pMDI)

pMDIs represent the majority of pharmaceutical aerosol products. They are comprised of drug formulation filled or packaged under pressure along with the energy source, a liquefied propellant, in a canister equipped with a valve, to meter accurate and precise doses, and an actuator. A pre-determined volume of non-aqueous liquid is discharged per actuation to offer the precise dose delivery on demand. pMDIs deliver only a small fraction of the drug dose to the lungs (10-20% of emitted dose). There is a cold propellant effect due to the evaporation of propellant when the aerosols impact on the back of the throat, which can be ameliorated by the use of a spacer. Poor hand–mouth coordination is another obstacle in the optimal use of a pMDI. Recently, the breath-actuated pMDIs have been developed to eliminate co-ordination difficulties by firing in response to the patient's inspiratory flow. The Autohaler (3M Pharmaceuticals, Minnesota, USA), increased lung deposition from 7.2% with a conventional MDI to 20.8% of the dose using the breath-activated pMDI (Newman, Weisz, Talaee *et al.*, 1991). Lately (May 2006) Accentia Biopharmaceuticals (Tampa, Florida) launched a new breath-activated, dose-counting inhaler (MD Turbo) (http://www.mdturbo.com/h_whatismdt.html). This device helps to coordinate the press-and-breathe action needed for proper use of an inhaler apart from counting the remaining doses in the inhaler.

Few vaccines have been delivered by propellant-driven metered dose aerosols. The hydrophobic propellant is not a "friendly" environment for most of the vaccine strains or aqueous soluble antigen proteins. Surfactants or cosolvents may be needed for pMDI vaccine formulation. Brown *et al.* delivered *Streptococcus suis* bacteria into the respiratory tract of swine in the presence of surfactants using liquefied dimethylether as propellant (Brown, George and Matteson, 1997). About 6-12% of bacteria were delivered to the deep lungs. After aerosolization, only 17-38% of the cell-wall proteins were associated with the bacteria and 30-50% of antigenicity in the respirable bacteria was retained after actuation. This report demonstrated that small particle aerosols of the bacteria vaccine from pMDI can be generated, but with significant loss of antigenicity (Brown, George and Matteson, 1997).

1.2.3.3 Dry powder inhalers (DPI)

The emergence of novel dry powder inhalers (DPIs) was driven largely by the Montreal protocol which addressed, in part, eliminating chlorofluorocarbons (CFCs) from traditional pMDIs. There are a wide range of commercially available DPI devices, from single-dose devices (Aerolizer (Novartis, Basel, Switzerland) and Handihaler (Boehringer Ingelheim, Ingelheim, Germany)) to multiunit dose devices provided in a blister pack (Diskhaler and Diskus (GlaxoSmithKine, Middlesex, UK)) or reservoir-type system (Turbuhaler (AstraZeneca, London, UK)) (Hickey, 2004b). Generally, inert carriers, most commonly lactose, are needed for dispersion of small particles of the active ingredients. Aerosols are created by directing the air through loosely-packed powders. Lung deposition varies from 12-40% of the emitted dose among the different DPIs (Hickey, 2004b). Insufficient deaggregation of the active ingredient from coarse carrier particles contributes to the low active ingredient deposition. Active DPIs are being investigated to reduce patients'

inspiratory effort to disperse the fine particles. Aspirair (Vectura, Wiltshire, UK) is triggered by the patient's inhalation. This inhaler generates an aerosol plume significantly slower than most inhalers currently available. Therefore, the use of Aspirair reduces the amount of drug that is unintentionally deposited in the mouth and throat, and subsequently swallowed rather than reaching the lungs (Tobyn, Staniforth, Morton *et al.*, 2004). Spiros (Dura pharmaceuticals, San Diego, CA) uses a battery-driven propeller to aid the dispersion of powders. Inhance Pulmonary Delivery System (Nektar, San Carlos, CA) uses compressed air to aerosolize the powder and then converts it into a standing cloud in a holding chamber. This makes the generation of aerosol independent of patients' inspiratory effort.

In addition to the general advantages of dry powder vaccination, a unique feature of the route of administration is that, the alveolar APCs (especially macrophages and/or dendritic cells) are phagocytic and respond to small-size particulates by eliciting cell-mediated and humoral immunity. These particulates could be whole vaccine strains, subunit proteins or DNAs formulated in particulates.

Dry powder aerosol vaccination has been employed to immunize humans and animals. In the early 1960s, Russian investigators used dry vaccines of attenuated bacterial strains to immunize experimental animals against a number of diseases including, plague, tularemia, brucellosis and anthrax (Middlebrook, 1961). Large particle aerosols of a live, temperature-sensitive recombinant influenza virus, were generated by spinning-top aerosol generator to immunize the mice (Jemski and Walker, 1976). These dry powder particles of influenza virus provided 89% survival after challenge.

A dry powder measles formulation was suggested for delivery from Spiros inhalers (LiCalsi, Christensen, Bennett *et al.*, 1999). These are durable, handheld and could be either

single or multidose devices. The delivery efficiency of Spiros technology was demonstrated by pulmonary imaging with radiolabelled albuterol sulfate. Scintigraphy results showed uniform deposition of radiolabelled drug throughout the tracheobronchial region and significant and uniform deposition in the alveolar region (Hill, Vaughan and Dolovich, 1996). The studies performed with nebulized measles vaccine provide evidence that the aerosol vaccine to the lungs need not be greater than the currently accepted minimum subcutaneous dose of 1000$TCID_{50}$ (tissue culture ID_{50}). The estimated measles vaccine concentrations could vary from 3 to 40% in lactose depending on $TCID_{50}$ from different manufacturers. This blend is suitable for dispersion from inert lactose (LiCalsi, Christensen, Bennett *et al.*, 1999).

1.2.4. Components of pulmonary vaccine formulations

Formulation is an important factor that affects the stability of subunit antigens or live attenuated/inactivated vaccine strains. The formulations also dictate the aerosol delivery device for vaccine administration. Delivery devices need various formulation strategies to meet the physico-chemical and aerodynamic criteria to generate the respirable aerosols.

1.2.4.1. Adjuvants

Adjuvants help to elicit early, high and long-lasting immune responses with limited quantities of antigen. They are the focus of vaccine research because the purified, synthetic subunits and DNA vaccines are frequently poor immunogens and require adjuvants to evoke immune responses (Degen, Jansen and Schijns, 2003; Seong and Matzinger, 2004). With the use of adjuvants an immune response can be selectively modulated to Th1 or Th2 type (Marciani, 2003), which is important for protection against diseases caused by intracellular viruses, parasites and bacteria.

There are several mechanisms of action for adjuvants: depot generation (aluminum compounds, immunostimulant complexes (ISCOM), emulsions, oil adjuvants (Freund's complete/incomplete adjuvant)); immunomodulation (modification of cytokine networks, includes lipopolysaccharide (LPS), monophosphoryl lipid A (MPL), lipopeptides, CpG motif, muramyl dipeptide (MDP), cholera toxoid (CT)); and delivery vehicles (liposomes, biodegradable polymer microparticles) for antigens in targeting to APCs. The delivery vehicle function of adjuvants will be discussed in detail in the section describing particulate delivery systems.

The only approved adjuvant for humans is alum (potassium aluminum sulfate). Alhydrogel (aluminum hydroxide) and adju-phos (aluminum phosphate) are also used in research and preclinical trials. Aluminum compounds have a good safety record in vaccines. Usually they elicit a strong humoral response but poor Th1 immunity. MF59 and MPL have also been used in human trials (Giannini, Hanon, Moris *et al.*, 2006; Kahn, Sinangil, Baenziger *et al.*, 1994; Martin, De Donato, Minutello *et al.*, 1995) but there are no experimental reports on pulmonary vaccine delivery.

In TB vaccine development, *MTB* cell wall components exhibit adjuvancy (Fidler, 1992) and may readily be incorporated into particles due to their balance of hydrophobic and hydrophilic moieties. They may be useful in promoting the antigenicity of *MTB* derived molecules, such as Ag85, in a similar manner to the behavior of an intact microorganism (Lindblad, Elhay and Silva, 1997).

In TB vaccination investigations, several adjuvants have been suggested for coincorporation with the subunit antigens. Muramyl peptides are the smallest components in *MTB* cell wall. They can induce production of a cascade of cytokines, including IL-1α, 1β

and IL-6. Lymphocytes recruited in response to cytokines and chemokines release IL-2 and IFN-γ. The use of the cell wall components to stimulate immune response is well suited to the restrictions on dose that can be delivered by aerosol. They can be delivered to macrophages by incorporation in liposomes applied to cancer therapies (Philipis, Chedid and Bernard, 1987; Phillips, Moras and Chedid, 1985) and TB vaccination (Pettis, Hall, Costa *et al.*, 2000). Trehalose dimycolate (TDM) is a component in *MTB* cell wall. Due to TDM's toxicity profile, synthetic analogue trehalose dibehenate (TDB) has been made to maintain its adjuvancy while reducing side effects. Anderson *et al.* reported that TDB could enhance CMI and antibody responses of TB subunit antigens Ag85B-ESAT6 when they were encapsulated in cationic liposomes. Immunization of mice with this formulation induced a strong, specific Th1 type immune response characterized by substantial production of the interferon-gamma and high levels of IgG2a isotype antibodies. The lymphocyte subset releasing the interferon-gamma was identified as CD4 T cells (Davidsen, Rosenkrands, Christensen *et al.*, 2005; Holten-Andersen, Doherty, Korsholm *et al.*, 2004). Lipopolysaccharide (LPS) is composed of a hydrophilic polysaccharide and a lipophilic phospholipid (lipid A). LPS-induced cell activation requires a tripartite receptor complex in which one protein bind tightly to Toll-like receptor TLR 4 and TLR2. Binding of LPS by TLRs stimulates innate immunity, which in turn, activates adaptive immunity to activate NF-κB (Shizuo, 2003), induce nitric oxide-independent killing of intracellular tubercle bacilli in human alveolar macrophages (Thoma-Uszynski, Stenger and Takeuchi, 2001). Analogue monophosphoryl lipid A (MPL) retains adjuvancy at a lower toxicity (Ismaili, 2002). Anderson *et al.* have demonstrated that MPL could be used as one of the adjuvants in TB vaccine (Olsen, van Pinxtern, Rasmussen *et al.*,

2001) (Brandt, 2000). Other adjuvants have been validated in animal studies, such as saponin. MDP and TDB are the major adjuvants used in this project.

1.2.4.2 Microparticle systems

Microparticle systems have been a common theme in the vaccine delivery field. They enhance the immune response greatly in comparison to soluble antigens. They have adjuvant functions and stimulate the immune response to help Th1 induction (Raychaudhuri and Rock, 1998). Microparticle systems include liposomes, lipid-based and biodegradable polymer-based microparticles. Some subunit antigens and DNA vaccines use viral vectors for pulmonary delivery. Recently, the biodegradable microparticles have drawn a lot of attention in the vaccine delivery.

Biodegradable polymeric microparticles showed good adjuvant activity. Antigen uptake by APCs was enhanced by the association of antigen with polymeric microparticles. The biodegradable and biocompatible polyesters, poly(lactide-co-glycolides) (PLGA) are the primary candidates for the development of microparticles because they have been used in humans for many years as suture material and controlled-release delivery systems. Microparticle particle size (1-10μm) can be manipulated to target macrophages. Macrophages can present the antigens 100- to 1000- fold more efficiently to MHC class I and II pathways than soluble antigens alone when antigens are attached to small particles (Raychaudhuri and Rock, 1998). There is a greater potential for the microparticles residing in the lungs for extended period of time to be taken up by APCs. These microparticles can protect live vaccine strains or subunit antigens against rapid degradation by extracellular enzymes. They may also offer controlled-release dissolution profiles for antigen, which allows the development of single-dose vaccines. In contrast to alum, PLGA microparticles

have been shown to be effective for induction of CTL response (Raychaudhuri and Rock, 1998). *Yersinia pestis* subunit and anthrax rPA have been delivered intranasally or intratracheally in PLGA formulations leading to an improved immune response with respect to other formulations (Eyles, Sharp, Williamson *et al.*, 1998; Eyles, Williamson, Spiers *et al.*, 2000; Flick-Smith, Eyles, Hebdon *et al.*, 2002) (Table 2).

1.2.4.3 Dispersion of vaccines and microparticles

Dry powder aerosol delivery is an attractive delivery method for pulmonary immunization since it has many advantages over nebulizer and MDI delivery of aqueous and nonaqueous droplets. However, dry powder vaccines are not currently commercially available. Dispersion of these particles must be demonstrated for dry powder vaccine delivery to become a reality. The interaction energy between micron-sized particles needs to be overcome to efficiently generate an aerosol.

Sugar carriers (Steckel and Bolzen, 2004), sodium chloride salt (Chan, Clark, Gondal *et al.*, 1997) and redistribution of drug particles from coarse particles to the fine particle component can improve drug aerosol dispersion (Lucas, Anderson and Staniforth, 1998). It has been reported that some fine particle addition to the carrier can improve the dispersion of the active pharmaceutical ingredient (Shah and Misra, 2004).

Self-dispersion is another approach for aerosol particles. Large porous particles have mean geometric diameter up to 30μm, with good flow and dispersion properties (Edwards, Hanes, Caponetti *et al.*, 1997). However, large porous particles evade phagocytosis by macrophages. Recently van der Walle *et al.* reported that PLGA microparticles could be manufactured with novel dimpled surfaces for pulmonary delivery of DNA (Mohamed and van der Walle, 2006). The hollow microparticles, with a low density (0.24g/cm^3) and dimples,

were produced by addition of high molecular weight hydrophobic blocks of pluronic. The calculated aerodynamic diameter of microparticles was 3.8μm and the dimpled microparticles showed good aerosol dispersion properties. Porous particles larger than 10 μm are not suitable formulations for vaccine delivery in which the targeting APC is alveolar macrophages. There is an exception to this observation, which is a nanoparticle aggregate which disassembles upon deposition into its smaller component particles.

1.2.5. PLGA microparticles as the pulmonary aerosol delivery carriers

Microparticles of poly (lactic-co-glycolic acid) (PLGA) copolymers have been a major focus as antigen carriers (Alpar, Somavarapu, Atuah *et al.*, 2005; Eldridge, Staas, Meulbroek *et al.*, 1991; Jiang, Gupta, Deshpande *et al.*, 2005; Tamber, Johansen, Merkle *et al.*, 2005). PLGA microparticles have been used to deliver vaccines against TB in parenteral formulations. Ag85A DNA vaccine was adsorbed onto cationic PLGA microparticles (i.m) and similar protection levels against aerosol challenge was observed in mice using doses of PLGA-DNA two orders of magnitude lower than with naked DNA itself (Mollenkopf, Dietrich, Fensterle *et al.*, 2004). A single-shot prime-boost vaccine formulation based on a mixture of two different PLGA microparticles containing DNAhsp65 and the recombinant hsp65 protein was developed. This formulation clearly presented good efficacy and diminished lung pathology in both mice and guinea pigs (de Paula, Silva, Carlos *et al.*, 2007). Mice immunized with 71-kDa cell wall associated protein of *MTB H37Rv* in PLGAs exhibited significantly higher T-cell stimulation and cytokine release in comparison to the same protein emulsified in Freund's incomplete adjuvant (FIA) as well as a BCG vaccinated group throughout the post-immunization (p.im.) period. The protective effect of 71-kDa-PLGA was sustained (85%) while that of 71-kDa-FIA began to wane (70%) when increasing

the interval of challenge to 16 weeks post immunization. Further, the 71-kDa-PLGA immunized group exhibited a significantly higher ($p < 0.001$) clearance of bacterial load from the lungs and livers in comparison to the 71-kDa-FIA immunized group (Dhiman and Khuller, 1998). The early secreted antigenic target 6-kDa protein ESAT-6 was encapsulated in poly(lactide) (PLA) microparticles and intranasally instilled into mice. Microencapsulated ESAT-6 induced greatest numbers of ESAT-6 specific IFN-γ and IL-4 secreting cells in the lung and mediastinal lymph nodes (MLN). Similarly, ESAT-6 specific recall responses were strongest following intranasal immunization of mice with microparticle-encapsulated antigen (Carpenter, Williamson and Eyles, 2005). The research reports indicated that, PLGA carrier systems have great potential as adjuvant to enhance immunity. Their depot effect, sustained release manipulation and long-term protective properties of PLGAs brought them attention in antigen delivery for tuberculosis.

There are no reports on vaccines delivered in PLGA carriers as aerosol into the lungs, the primary site of *MTB* infection in the host, to elicit an immune response. PLGA microparticles have been employed to deliver therapeutic drugs into the lungs. These microparticles encapsulating rifampicin (Garcia-Contreras, Sethuraman, Kazantseva *et al.*, 2006) (Suarez, 2001) were delivered as aerosol to tuberculosis-sensitive guinea pig model. Aerosolized particles reduced most measures of tuberculosis (TB) infection. PLGA nanoparticle-based inhalable sustained drug delivery system containing antitubercular drugs enhanced relative bioavailability (compared to oral administration) for encapsulated drugs (12.7-, 32.8- and 14.7-fold for rifampicin, isoniazid and pyrazinamide, respectively) (Pandey, Sharma, Zahoor *et al.*, 2003). These studies are evidence for the potential of inhaled aerosol therapy for the treatment of TB. Respirable and sustained-released PLA particles containing

ipratropium bromide, a drug used to treat asthma and chronic obstructive pulmonary disease (COPD) (Taylor, Hickey and VanOort, 2006), provided an increased duration of effect in a guinea pig model of bronchoconstriction. Surface-modified PLGA nanospheres with chitosan improved pulmonary delivery of calcitonin due to the retention of nanospheres adhered to the bronchial mucus and lung tissue and the consequential sustained release at the adherence site (Yamamoto, Kuno, Sugimoto *et al.*, 2005). Some peptide drugs were also administered in respirable PLGA carriers (Niwa, Takeuchi, Hino *et al.*, 1995). These therapeutic aerosols while intended mechanistically to achieve different objectives, indicated potential for vaccines to be administered to the lungs. A number of new technologies are available to prepare respirable PLGA microparticles (Dailey, Kleemann, Wittmar *et al.*, 2003; Mohamed and van der Walle, 2006; Ozeki, Beppu, Mizoe *et al.*, 2006) that would facilitate pulmonary delivery of vaccines.

1.2.6 Particle size characterization and physico-chemical characterization of dry powders

1.2.6.1 Particle size characterization

Quantitative descriptors of particle size are one of the specific properties of powders. The projected area diameter (d_p) is obtained from two-dimensional images, generated by microscopy, of a particle. It represents the diameter of a circular disc with the same projected area as the particle being examined (Martin and Bustamante, 1993). The equivalent volume diameter (d_v) is the diameter of a sphere of the same volume to the particle. These two particle size characterizations have limited application to particles deposited in the respiratory tract. The aerodynamic diameter (d_a) is the dimension used to represent airborne

particle size. The d_a is the diameter of a unit density sphere that has the same terminal setting velocity in air as the actual particle (Hinds, 1999).

$$V_{TS} = \rho_0 d_a^2 g/18\eta = \rho_p d_e^2 g/18\eta\chi$$

V_{TS}: terminal settling velocity of the unit density sphere

ρ_0: unit density;

ρ_p: particle density;

g: the acceleration of gravity

η: air viscosity:

χ: shape factor

This equation states that the diameter of particles having uniform density, increases proportionately to the terminal settling velocity. A large particle is more likely to deposit by inertial impaction than a small one. However, if density varies, a larger, low-density particle can have the same terminal settling velocity as a smaller high-density particle. This is important since some manufacturing methods produce low-density particles, which may result in a significant difference between the directly observable, geometric diameter and the aerodynamic diameter.

Powders consisting of particles of similar size (monodisperse) can be described by a single diameter. However, most powders consist of a range of particle sizes (polydisperse) that affect their flow and dispersion. The entire distribution of particle sizes must be characterized. The median diameter is used to represent the 50th percentile of the distribution and geometric standard deviation is used to represent the breadth of the particle sizes for log-

normal distributions. The median diameter of a size distribution can be based on the count, mass, or volume and would be termed count median diameter (CMD), mass median diameter (MMD) and volume median diameter (VMD). The geometric standard deviation (GSD) for a normal distribution can be calculated from the following equation (Hinds, 1999).

$$GSD = D_{84\%} / D_{50\%} = D_{50\%} / D_{16\%} = [D_{84\%} / D_{16\%}]^{1/2}$$

where $D_{84\%}$ and $D_{16\%}$ represent the diameters at the cumulative percentile of 84% and 16%. For an aerodynamic size distribution, the median is identified as the mass median aerodynamic diameter (MMAD).

The span is an alternative measure of the breath and uniformity of a distribution, which is commonly used to describe formulations where laser diffraction technique has been used to collect equivalent volume diameter data.

$$Span = (D_{90\%}-D_{10\%}) / D_{50\%}$$

Where, $D_{90\%}$ and $D_{10\%}$ represent the diameters at the cumulative percentiles of 90 and 10%.

The cascade impactor is used to separate airborne particles into aerodynamic size classes (Lodge and Chan, 1986). A cascade impactor operates on the basis of inertial impaction (Hinds, 1999). These devices consist of a series of stages with orifices that decrease in size with each succeeding stage. Particles are drawn into the impactor on the conveying air stream produced by a vacuum pump. Collection surfaces serve to obstruct the path of the airflow. Since the orifice size decreases with succeeding stages, the linear velocity

of air increases at each successive stage. Therefore, the inertia and stopping distance of specific size fractions of particles increase at each stage increasing their probability of deposition (Baron and Willeke, 2001). This allows particles with a range of aerodynamic diameters to be deposited on each collection surface with particles having a higher aerodynamic diameter depositing on the upper stages. Additionally, the amount of powder dispersed from the delivery device can be quantified and an emitted dose can be calculated.

1.2.6.2 Physico-chemical property characterization

The surface area of the particle influences particle interactions in a powder due to the number of contact points. Surface area for a single spherical particle is equivalent to πd^2 and, therefore, increases proportionally to the square of the diameter, d. However, in a defined mass of powder there are many more small particles than large ones. On a population basis, the sum of the surface area of small particles is greater than that of large particles in any specific powder. The surface area is most commonly measured by gas adsorption at the surface of a known mass of powder (Crowder, 2003).

Particles consist of organized structures of molecules the dimensions of which dictate the crystal system. Pharmaceutically the underlying molecular structure contributes to the stability of the powder system. Thermal analysis is one means of studying the structure. The principle underlying thermal analysis is to apply energy, in the form of heat that can be absorbed by the solid and may contribute to a re-ordering of the system into another form or result in dissociation of important elements of the structure. Differential scanning calorimetry (DSC) is the method most frequently employed to study these phenomena. DSC measures differences in the amount of heat required to increase the temperature of a sample with respect to a reference. DSC can sense the phase transition, denaturation and unfolding events

in peptides and proteins. DSC may also be used to measure kinetic phase transitions, such as glass transitions.

The hygroscopicity, capacity to take on moisture, of a dry powder formulation can influence flow and dispersion properties. Powders with higher moisture contents are more likely to aggregate due to surface tension effects and capillary forces, and hygroscopicity can increase the probability of these effects occurring (Hickey and Ganderton, 2001). Moisture content can be determined by difference, measuring the changes in weight of a powder before and after heating assuming there is no degradation in the measured contents.

The zeta potential is defined as the difference in potential between the surface of the tightly bound layer and the electroneutral region of the solution. The zeta potential has practical application in the stability of systems containing dispersed particles because this potential governs the degree of repulsion between adjacent, similarly charged, dispersed particles. Tabata *et al.* pointed out that the size and surface charge can affect their phagocytosis by macrophages (Tabata and Ikada, 1988). Phagocytosis is enhanced as the absolute value of zeta potential increases for both the negatively and positively charged surface. Therefore, measurement of zeta potential of particles may provide certain prediction on their phagocytosis by macrophages.

Aerosol particles can acquire charge by flame charging, static electrification, diffusion charging, and field charging (Hinds, 1999). Of these mechanisms, static electrification plays the predominant role during DPI actuation. Static electrification occurs when particles interact with surfaces or detached from the bulk phase resulting in a separation of charges. In DPIs, this takes the form of surface charging, or triboelectrification. When particles are separated from each other or the surfaces of the dosage form or surfaces

of the inhalers, charge can be transferred resulting in a net charge on the particles. Particles experience significant friction during DPI discharge which tends to amplify this effect. The Electrical Low Pressure Impactor (ELPI) enables real time particle size distribution and concentration measurement. The ELPI is composed of a unipolar corona aerosol charger and a low-pressure cascade impactor (Fig 1.3). Deposition inside the impactor is dependent on particle aerodynamic diameter. Unlike conventional cascade impactors which are disassembled and the stages assayed chemically or gravimetrically after operation, ELPIs detect deposited particles by measuring the current resulting from dissipation of the particles' electrical charge. The collected particle fractions can be measured simultaneously on all stages using a multichannel electrometer. Thus, when the ELPI is operated with charger turned on, it functions as a near real-time particle sizer. However, when operated with the charger off, the native particle charge is measured. The ELPI is a low-pressure impactor; operation at reduced absolute pressures allows collection of particles down to a size of 30 nm

The static and kinetic surface charge properties, of PLGA microparticles, will be evaluated by zeta potential and real-time particle sizing, respectively.

1.3 Statement of problem

Currently, the only marketed TB vaccine is BCG. However, BCG has variable efficacy ranging from 0 to 80% in the worldwide clinical trials. Due to BCG's variable protection as a vaccine in the prevention of *MTB* infection, a major scientific effort over the past several years has produced hundreds of potential TB vaccine candidates. Vaccine candidates are usually delivered via parenteral routes. There has been much less research into alternative delivery systems and administration routes for vaccination to elicit effective immunity to *MTB*. The site of antigen exposure will determine the immune response and

circulating antigen-specific T cells. The pulmonary route of exposure, by which most TB patients acquire their primary infection, might be a promising route for TB vaccination. Different delivery systems, especially particulate systems, are easily recognized by antigen presenting cells, may help to target macrophages in the lungs to elicit improved immunity. Aerosol immunization with a promising vaccine candidate mycobacterial subunit antigen 85B (Ag85B), a subunit of antigen 85 complex, with immunostimulatory adjuvants as components in microparticle systems is proposed.

1.4 Hypothesis and Specific aims

1.4.1 Hypothesis

Ag85B/adjuvants in microparticle formulations intended for pulmonary delivery

- target macrophages.
- elicit related cell-mediated immune response *in vitro*.
- protect guinea pigs against challenge with virulent *MTB* (*H37Rv*).

1.4.2 Specific aims:

I. To evaluate the ability of Ag85B subunit together with different adjuvants loaded into microparticles.

(a) Ag85B were obtained as recombinant protein expressed in *E. coli*. The antigenicity of rAg85B will be evaluated by T hybridoma cell recognition assay.

(b) Poly (lactide-co-glycolide) (PLGA) microparticles were loaded with rAg85B with/without adjuvants by spray-drying method. The formulation parameters were optimized using factorial experimental design with multivariate statistical analysis. Different batches of formulations were manufactured with various loadings of rAg85B and adjuvants.

II. To elucidate the T hybridoma cells responses to different formulations; characterize the physico-chemical properties of microparticle systems as aerosols.

(a) The phagocytosis of microparticle systems by macrophage-like cells was evaluated.

(b) The proliferative nature of effector T cells after recognition of macrophage activation was determined. IL-2, which is released from hybridoma T cells and stimulates proliferation of T cells, was measured by T hybridoma cell recognition assay. The ability of different microparticle formulations to affect epitope presentation was measured by this bioassay upon macrophage inoculated by microparticle formulations.

(c) Microparticle formulations were characterized to establish their suitability as aerosols.

III. To evaluate the effect of vaccination with selected aerosol microparticle adjuvant/ antigen combinations. Protection will be measured using the guinea pig model of tuberculosis involving a low-dose virulent challenge.

(a) Optimized adjuvant/antigen microparticle combinations were selected based on results obtained in specific aims 1-2 and delivered as aerosols to guinea pigs prior to challenge with low-dose virulent *MTB H37Rv*. The bacterial burden in lungs and spleens, weight changes after challenge of infected guinea pigs was compared with appropriate positive (BCG) and negative controls.

1.5 Significance

The *MTB* secreted subunit protein, Ag85B, is a promising antigen for inclusion in a vaccine against tuberculosis. Microparticle formulation systems for pulmonary delivery, a new route of delivery of TB vaccine, may protect the protein antigens from extracellular degradation. Furthermore, local delivery may result in reduced potential side effects and dosage amounts. Guinea pig protection studies with optimized formulations illustrated the

protective effects of this recombinant protein in the microparticle systems delivered as aerosols. These studies may offer the first evidence for the effect of a protein subunit vaccine delivered to the lungs for protection against tuberculosis, which has significant implication for worldwide disease control.

Fig 1.1 Cell wall structure of *Mycobacterium tuberculosis.*

(Picture was modified from that obtained from http://student.ccbcmd.edu/courses/bio141/lecguide/unit1/prostruct/u1fig11.html with permission of Dr. Gary Kaiser in Community College of Baltimore County, Catonsville Campus).

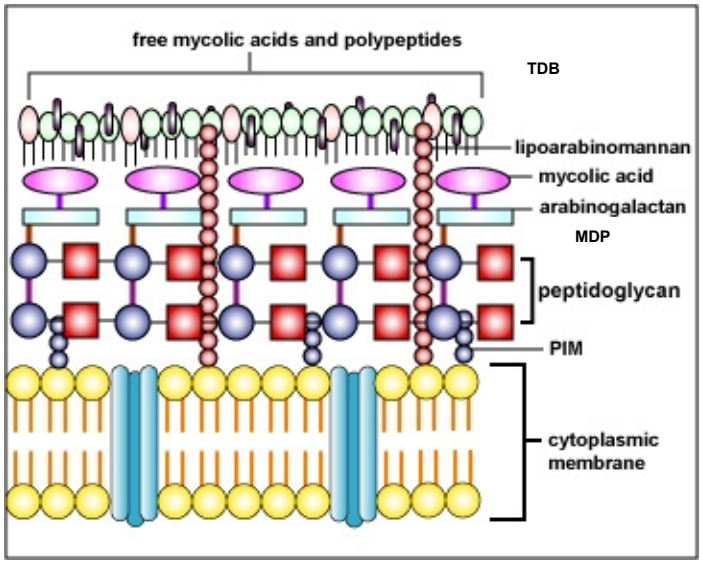


Fig 1.2 Alveolar macrophage activation and immune responses.

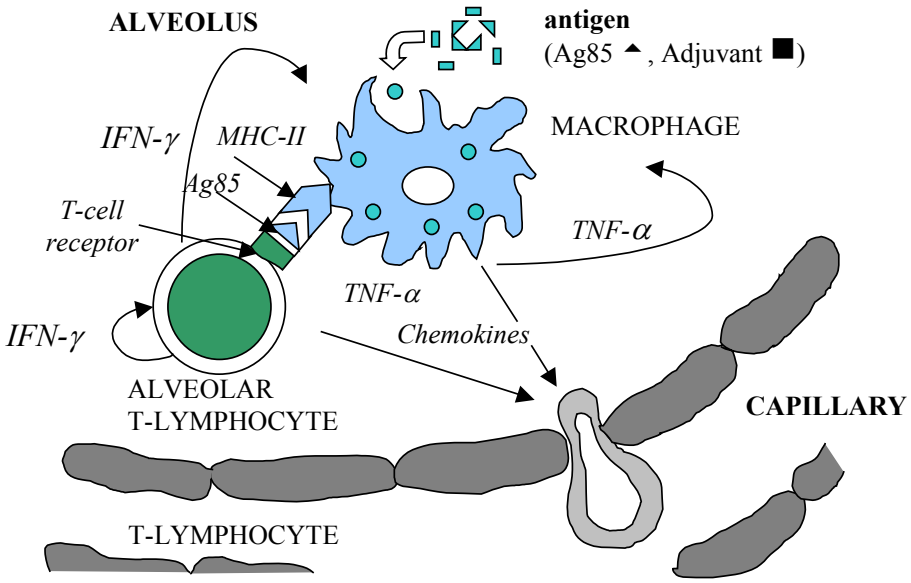


Fig 1.3. Electrical Low Pressure Impactor (ELPI) operating principle.

(http://www.dekati.com/cms/elpi/operating_principle)

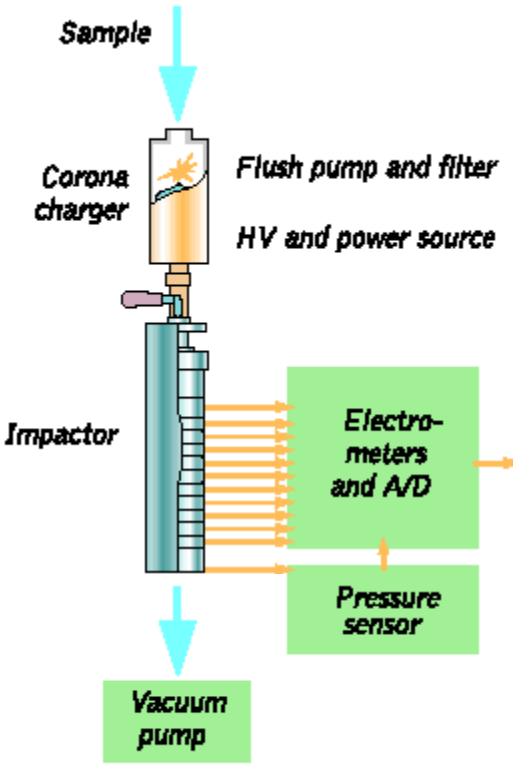


Table 1.1 Summary of new-generation tuberculosis vaccine to improve BCG

Vaccine	Description	Development stage	Researchers/sponsors
Live mycobacterial vaccines: modified BCG			
rBCG30	BCG Tice engineered to overexpress Ag85B	Phase I trial in US, completed in 2004; no serious adverse events	M. Horwitz, UCLA; D. Hoft, St Louis University, US; T. Littlejohn, Winston, Salem, North Carolina, US Sponsor: Sequella, Aeras*
rBCG-Aeras 403	BCG Danish with endosome escape and overexpression of several proteins including Ag85A, Ag85B and TB10.4	Ongoing pre-clinical studies and GMP production; Phase I clinical trial planned for 2006	R. Sun, D. Hone, M. Stone, J. Sadoff, Aeras*
rBCGΔ Ure:CHly$^+$	BCG Pasteur with endosome escape	Ongoing pre-clinical studies and GMP production; Phase I clinical trial planned for 2006	S. Kaufmann, Max Planck Institute for Infection Biology, Berlin, Germany
BCG:RDI	BCG Pasteur with reintroduction of RD-1 locus which contains protective Ags	Ongoing pre-clinical studies	S. Cole, Institute Pasteur, Paris, France
Pro-apoptotic BCG	BCG Tice with diminished superoxide dismutase activity	Ongoing pre-clinical studies	D. Kernodle, Vanderbilt, US Sponsor: NIH, VA, Aeras*

*Supported by the Bill and Melinda Gates Foundation. Phase I clinical trial: trial that assesses the safety and immunogenicity of a drug or a vaccine in healthy volunteers. BCG, bacille Calmette–Guérin; GLP, good laboratory practice; GMP, good manufacturing practice; NIH, National Institutes of Health; SSI, Statens Serum Institute; TB, tuberculosis; VA, Medical Research Service, Department of Veterans Affairs.

Table 1.2. New generation of TB vaccines in live *MTB*, subunit antigens and DNA vaccines

Live mycobacterial vaccines: modified Mycobacterium tuberculosis			
MTB PhoP	Deletion of virulence associated gene phoP from *MTB* MT103 strain	Ongoing pre-clinical studies and GLP production	B. Martin, University of Zaragoza, Spain; B. Gicquel, Institute Pasteur, Paris, France
MTB $mc^2$6030	*MTB H37Rv* with deletion of panCD and RD-1 locus	Ongoing pre-clinical studies and GMP production; Phase I clinical testing planned for 2006	W. Jacobs, Albert Einstein College of Medicine, New York Sponsor: NIH
MTB $mc^2$6020	*MTB H37Rv* with deletion of the lysA and the panCD locus	Ongoing pre-clinical studies and GMP production; Phase I clinical testing planned for 2006	W. Jacobs, Albert Einstein College of Medicine Sponsor: NIH
Subunit vaccines			
*MTB*72F	Recombinant fusion protein (*MTB*39 and *MTB*32) in AS02A and AS01B adjuvants	Phase I trials completed in US and Europe; no serious adverse events; additional trials ongoing in Europe	Y. Skeiky, S. Reed, Corixa Corp. Seattle, US Sponsor: Glaxo Smith Kline, Aeras*
Hybrid-1 (85B–ESAT6)	Recombinant fusion of Ag85B–ESAT-6 in IC31 adjuvant	Phase I clinical trials commenced in 2005	P. Andersen, SSI, Denmark Sponsor: SSI, TBVAC
HyVac-4 (Ag85B–TB10.4)	Recombinant fusion of Ag85B–TB10.4 in IC31 adjuvant	Ongoing pre-clinical studies and GLP production; Phase I clinical trial planned for 2007	P. Andersen, SSI, Denmark Sponsor: SSI, Aeras*
Heatshock protein	Nascent BCG proteins associated with purified heat-shock proteins	Ongoing pre-clinical studies	C. Colaco, ImmunoBiology, UK Sponsor: ImmunoBiology, Aeras*
Naked DNA and viral-vectored vaccines			
Hsp65 (GroEL) DNA	Conserved antigen from Mycobacterium leprae for immunotherapy	Ongoing pre-clinical studies	D. Lowrie, National Institute for Medical Research, London UK. Sponsor: Sequella Inc.
MVA85A	Attenuated strain of vaccinia (modified vaccinia virus) expressing Ag85A	Completed and ongoing Phase I clinical trials; immunogenic, no serious adverse events reported	A. Hill, H. McShane, Oxford University, UK Sponsor: Wellcome Trust
Aeras 402 (Ad35.TB-S)	Non-replicating Ad35 expressing multiple TB proteins including Ag85A, Ag85B and TB10.4	Ongoing pre-clinical studies; GMP production for Phase I clinical trials planned for 2006	Aeras*, Crucell Sponsor: Bill and Melinda Gates Foundation

2. Manufacture and screening of PLGA microparticle formulations containing rAg85B

2.1 Introduction

Manufacture of PLGA microparticles containing rAg85B, and screening formulations with regards to eliciting immunity, is required to develop particles suitable for aerosol vaccine deliveries. It is also necessary to validate the immunological advantages of these PLGA antigen formulations compared to simple solution formulations.

Supplies of Ag85B were once quite limited since it was purified from *MTB* culture. *MTB* is both exceptionally slow growing and biohazardous. Therefore, the growing, separation and purification procedures make it difficult to obtain sufficient quantities of Ag85B antigens from the culture filtrate. *E. coli* systems have been reported to overexpress this antigen (Lakey, Voladri, Edwards *et al.*, 2000). However, the inclusion bodies and aggregation influenced the antigen's overall immunogenicity. In order to increase the ratio of soluble form of Ag85B, a couple of strategies were used in the *E. coli* expression system or procedure in this project. Firstly, the *E. coli* culture temperature was reduced to 22°C after isopropyl-β-thiogalactopyranoside (IPTG) induction; Secondly, *E. coli* stain *Origami* B was used. This strain has a chaperone vector and mutated reductases that are favorable for disulfide bond formation and appropriate conformation in expressed proteins. There is one disulfide bond in Ag85B.

A number of microencapsulation techniques have been developed to date to make the microparticles of polymers. The choice of the technique depends on the nature of the polymer, the active ingredient and the intended use (Arshaday, 1991; Jalil and Nixon, 1990;

Wu, 1995). The microencapsulation method employed must include the following requirements: 1) Stability and biological activity of the active ingredients should not be adversely affected, during the encapsulation procedure, or in the final microparticle product. 2) The yield of the microparticles having the required size range and encapsulation efficiency should be high. 3) The microparticles should be produced as a free flowing powder and should not exhibit aggregation or adherence.

Three common methods are used to manufacture microparticles: solvent evaporation, phase separation and spray-drying.

Solvent evaporation methods include single emulsion and double emulsion processes. Single emulsion process involves oil-in-water (o/w) and oil-in-oil (o/o). The polymer is first dissolved in a water immiscible, volatile organic solvent. The active ingredient is then added to the polymer solution and dissolves or disperses as particles (Wu, 1995). This polymer-solvent-active ingredient solution/dispersion is then emulsified in a larger volume of water or oil (water for o/w, oil for o/o) in the presence of an emulsifier to yield an emulsion. The emulsion is then subjected to solvent removal by either evaporation or an extraction process to harden the oil droplets (Arshaday, 1991). In the former casem the emulsion is maintained at reduced or atmospheric pressure (where solvent vapor pressure is low) to enable the volatile solvent to evaporate. In the latter case, the emulsion is transferred to a large quantity of water into which the solvent associated with the oil droplets diffuses and evaporates. The solid microparticles are washed and collected by filtration, sieving or centrifugation. These are then dried under appropriate conditions or are lyophilized to give final product. The disadvantage of the single emulsion process is poor encapsulation efficiency of water-soluble active ingredient. The water-soluble ingredient will diffuse, or partition, from the dispersed

oil phase into the aqueous continuous phase and the hydrophilic ingredient is deposited on the microparticle surface (Wada, Hyon, Ike *et al.*, 1988).

The double emulsion process water-in-oil-in-water (w/o/w) is well suited to encapsulate water-soluble ingredients. An aqueous solution of the water-soluble ingredient is added to an organic phase containing PLGA with vigorous stirring to form the first microfine w/o emulsion. This emulsion is added gently with stirring into a large-volume of water containing an emulsifier to form w/o/w emulsion. The emulsion is then subjected to solvent removal by either evaporation or extraction process (Lewis, 1990). A number of hydrophilic drugs e.g. leuprolide acetate (Okada, Doken, Ogawa *et al.*, 1994), a luteinizing hormone-releasing hormone (LH-RH) agonist (Kamijo, Kamei, Saikawa *et al.*, 1996) have been encapsulated into microparticles by this method.

The phase separation process consists of decreasing the solubility of the encapsulating polymer by addition of a third component to the polymer solution in an organic solution. At a particular point, the process yields two liquid phases: the polymer containing coacervate phase and the supernatant phase depleted in polymer. The active ingredient, which is dispersed/dissolved in the polymer solution, is coated by the coacervate. Three steps are included: i) phase separation of the coating polymer solution, ii) adsorption of the coacervate around the drug particles, and iii) solidification of the microparticles. The polymer is first dissolved in an organic solution. Water-soluble drugs like peptides and proteins are dissolved in water and dispersed in the polymer solution (w/o emulsion). An organic nonsolvent is then added to the polymer–drug–solvent system, with stirring, which gradually extracts the solvent carrying the polymer. As a result the polymer is subjected to phase separation and it forms flexible “soft” coacervate droplets that entrap the drug. This

system is transferred to a large quantity of another organic nonsolvent to harden the microdroplets and form the final microparticles, which are collected by washing, sieving, filtration, or centrifugation, and are finally dried (Jain, 2000). In the phase separation method the phase equilibrium is not reached and hence the process involves a non-equilibrium system (Wu, 1995). Therefore, the formulation and process variables significantly affect the kinetics of the entire process and ultimately the characteristics of the final microparticles. Due to absence of any emulsion stabilizer in the coacervation process, agglomeration is a frequent problem. The coacervate droplets are extremely sticky and adhere to each other before the complete phase separation or the hardening stages of this method. Adjusting the stirring rate and temperature is known to rectify this problem (Wu, 1995).

Biodegradable PLGA microparticles have been successfully prepared by double-emulsion and phase-separation methods. The coacervation method tends to produce particles which are agglomerated, there is difficulty in production scale manufacturing, the method requires large quantities of organic solvent, and it is difficult to remove residual solvents from the final microparticle product (Takada, Uda, Toguchi *et al.*, 1995). The double-emulsion method on the other hand requires many steps, rigid control of the temperature and viscosity of the inner w/o emulsion, and difficulty in encapsulating higher concentrations of hydrophilic drugs (Jalil and Nixon, 1990; Takada, Uda, Toguchi *et al.*, 1995). Contrary to these methods, the spray drying (SD) method is very rapid, convenient, easy to scale-up, involves mild conditions, and is less dependent on the solubility parameter of the drug and the polymer (Wagenaar and Muller, 1994). The spray-drying method can produce microparticles by dissolving the hydrophobic ingredient into organic solvent together with PLGA or from the w/o emulsion in which the hydrophilic ingredient in buffer solution

dispersed into PLGA organic solutions. Spray-drying can generate particles less than 5μm, which is the most promising alternative method to produce aerosol particles (Chan, 2006).

PLGA microparticle manufacture by a spray-drying technique to produce respirable aerosols is the method of choice for the present studies. Compared with the solvent evaporation (SE) method of preparing microparticles, spray-drying (SD) has some advantages. Respirable particles can be separated in the manufacturing procedure by cyclone. Dispersion properties of particles from SD are better than those from SE since particles with irregular surface morphology and low bulk density, desirable physical properties, are usually obtained by SD; the manufacturing procedure is continuous and batch sizes are bigger; the loading efficiency is very good since losses into the continuous phase do not occur as in SE. However, the yield may be low, especially in small batch preparation. In SD, the feed rate, atomization pressure, inlet temperature, nitrogen flow rate, aspirator speed and polymer concentration are important factors controlling the product yield and particle sizes. Some other factors, such as nozzle size and water: organic solvent ratio in the emulsion systems, also play a role. Conditions can be optimized using a statistical method. Factorial experimental design may be used to determine important factors influencing particle properties.

The ability of PLGA formulations to elicit an antigen specific immune response is an important property to evaluate. An *in vitro* assay was developed for screening antigenicity of different formulation. This T hybridoma cell recognition assay is specific for human immune response. THP-1 cells (human monocytes) are exposed to Ag85B or particles containing Ag85B. These cells then present the Ag85B epitopes on their surface to T hybridoma cells DB-1. The CD4 T hybridoma DB1 cells (kindly provided by Dr W. Henry Boom, Case

Western Reserve University) were derived from transgenic mice with human MHC genes for HLA-DR1. They are restricted to human HLA alleles and respond to human MHC molecules, which present $Ag85B_{97-112}$ epitope (Canaday, Gehring, Leonard *et al.*, 2003). After T cell recognition of Ag85B epitope 97-112, IL-2 will be secreted. IL-2 was monitored since it stimulates proliferation of T cells and plays a very important role in the consequential CMI. This assay provides the *in vitro* antigenicity screening which also has implications for use in humans. Different loadings of rAg85B, with or without the adjuvants MDP or TDB, of different concentrations in the PLGA formulations were screened by this assay. The advantages of microparticle delivery systems compared to solution formulations of antigen can be assessed by this *in vitro* assay. These studies provide important evidence of immune response required to justify *in vivo* animal challenge studies using optimized particle formulations to assess the protective effect of the vaccine.

2.2. Materials and methods

Recombinant Antigen 85B: rAg85B protein was produced from two *E. coli* strains. The first *E. coli* strain was *JM109DE3*, containing Ag85B gene with His tag (courtesy of Dr Douglas Kernodle, Vanderbilt University). 1L Luria-Bertani (LB) broth with 50μg/ml carbenicillin was inoculated with 20 ml of an overnight culture and grown at 37°C until the OD_{600} reading reached 0.4-0.5. The culture was induced by isopropyl-β-thiogalactopyranoside (IPTG) and grown at 22°C overnight. The *E. coli* cells were pelleted and then probe-sonicated. The supernatant was passed through a nickel-affinity column (Ni Sepharose™ 6 Fast Flow, Amersham Biosciences, Piscataway, NJ). The eluted fractions with His-tag proteins were further purified by Superdex 75 peptide column (Amersham Biosciences, Piscataway, NJ) with 20mM Tris, 1M sodium chloride, pH 7.5 as the eluting buffer. The rAg85B was

quantified at UV 280nm with extinction coefficient of UV_{280} of 1.0 for a 1.0mg/mL protein solution (Lakey, Voladri, Edwards *et al.*, 2000). The rAg85B purified from *JM109DE3* is referred to as rAg85B JM.

The second *E. coli* strain was o*rigami B* strain carrying mutated thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes and a chaperone GroEL /GroES vector pGro7. The plasmid vector *pRSETB* was introduced into o*rigami B* cells by a standard transformation protocol. Transformants were selected on agar plates with four antibiotics: carbenicillin, tetracycline, chloramphenicol and kanamycin. 1L LB broth and 50μg/mL carbenicillin, 12.5μg/mL tetracycline, 15 μg/mL kanamycin and 20μg/ml chloramphenicol were inoculated with 20mL of an overnight o*rigami B* strain and grown at 37°C to reach OD_{600} reading 0.4-0.5. The culture was induced by 1mg/mL L-arabinose and 0.2mM IPTG and shaken at 22°C for 20 hours. The remaining steps of the protein purification procedure were performed as described above. rAg85B purified from o*rigami B* is referred to as rAg85B Chap.

Endotoxin in the protein preparations was removed with Detoxin-Gel^{TM} Endotoxin Removing Gel (Pierce, Rockford, IL). The endotoxin level was detected by QCL-Chromogenic LAL (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) and was < 0.025ng/mg after purification. The proteins were dialyzed in 0.1M ammonium bicarbonate overnight and then lyophilized for 48h.

SDS-PAGE: A precast 12% SDS-PAGE gel (Biorad, Hercules, CA) was used to determine the purity of the recombinant proteins. The gel was stained with Coomassie blue.

Cells and media: THP-1 cells (American Type Culture Collection) were maintained in RPMI 1640 (Invitrogen Corp., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS)

(Hyclone, Logan, UT), 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 10 mM HEPES buffer, nonessential amino acids, 1% of antibiotics/ antimycotics (Invitrogen Corp., Grand Island, NY) which contain 100U/ml of penicillin, 100μg/ml of streptomycin and 0.25 μg/mL of amphotericin B. The CD4 T- hybridoma DB1 cells (kindly provided by Dr W. Henry Boom, Case Western Reserve University) were derived from transgenic mice with human MHC genes for HLA-DR1. They are restricted to human HLA alleles and respond to human MHC molecules, which present $Ag85B_{97-112}$ epitope (Canaday, Gehring, Leonard *et al.*, 2003). DB-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp., Grand Island, NY) supplemented as indicated above (complete DMEM). Infection medium was DMEM supplemented with 10% non-heat-inactivated FBS with antibiotics/antimycotics.

T-hybridoma cell recognition assay: The anitigenicity of rAg85B was evaluated by a modified CD4 T cell hybridoma recognition assay (Gehring, Rojas, Canaday *et al.*, 2003). THP-1 cells were incubated in 96-well flat-bottom plates (1.5×10^5 cells/well) with 10ng/ml of phorbol myristate acetate (PMA, Sigma, St Louis, MO) in infection medium for 24 h to promote adherence to plates. Cells were washed once with infection medium and incubated with 100U/ml of recombinant human IFN-γ (Endogen, Woburn, MA) for 24 h. The cells were washed twice with infection medium prior to Ag exposure and were exposed to 100μl of rAg85B at various concentrations or 100μL of microparticle suspension in infection medium at 250μg/ml. DB1 T-hybridoma cells (Gehring, Rojas, Canaday *et al.*, 2003) (10^5 cells/well, 100μL, specific for recognition of $Ag85B_{97-112}$) were added into wells at the same time as antigens. The cells were co-incubated at 37°C for 24h and supernatants were

harvested. ELISA was used to measure the amount of IL-2 produced by T-hybridoma cells. (Biosource, Camarillo, CA) (Fig 2.1).

Microparticle manufacture: PLGA polymer (700mg)(MW 84.7kd, L:G 75:25, intrinsic viscosity 0.68 dL/g in chloroform, Durect Corp., Pelham, AL) was dissolved in 200ml methylene chloride. Either 1 or 2mg rAg85B were dissolved in 2.4ml of 20mM sodium phosphate buffer, pH 7.4 with or without the adjuvants muramyl dipeptide (MDP) or trehalose dibehenate (TDB, Sigma, St Louis, MO). The molecular structure and adjuvancy of these molecules are well documented (Powell, Foster, Becker *et al.*, 1988; Ritzinger, Meredith, Takayama *et al.*, 1981). The aqueous and organic phases were probe-sonicated for three 10s periods on an ice bath immediately prior to spray-drying. The microparticles were manufactured using a spray-dryer (Buchi Mini Spray-drier B-191, Buchi, Flawil, Switzerland) under an optimized condition.

Encapsulation efficiency: The rAg85B amount was measured by DMSO-NaOH-SDS method. 2mL DMSO were added to a vial containing 10-20mg of PLGA microparticles containing rAg85B and occasionally shaken for one hour. Then 10ml of 0.05N NaOH with 0.5% SDS were added and the mixture was gently mixed and allowed to stand for one hour. After centrifugation, the supernatant was subjected to Lowry's method for protein quantification of rAg85B. A HPLC method was used for MDP quantification. The HPLC instrument employed was an Agilent 1100 series. HPLC conditions for MDP were: C18 reversed phase column (4.6 × 25mm), mobile phase 97% 25mM ammonium phosphate buffer, pH 7.0 and 3% methanol at a flow rate 1ml/min and UV wavelength of 200nm.

PLGA microparticle release profiles: Microparticles of 90mg PLGA-rAg85B (0.14% w/w)-MDP (0.5% w/w) were put into 2ml of 67mM potassium phosphate buffer (pH 7.4)

containing 0.1% Tween 80 solution, and shaken in 37°C water bath. A volume of 1.5ml of suspension was centrifuged for 10min (Beckman GS-15R centrifuge, rotor F2402H at 12000rpm, 4°C) and 1ml of supernatant was removed at intervals for measurement of rAg85B and MDP content. The rest of the sample and 1ml of fresh medium were added to the dissolution vial. Lowry's method was used for protein analysis of rAg85B and HPLC method for MDP quantification.

Phagocytosis of PLGA microparticles: PLGA-sodium fluorescein microparticles were prepared for phagocytosis assay. Following PMA treatment, the THP-1 cells were incubated, with 5 particles/cell, for 24 hours. Particles were visualized using a Zeiss LSM5 Pascal Confocal Laser Scanning Microscope at excitation and emission wavelengths 488 and 524nm, respectively.

Determination of the role of surface-associated-rAg85B on PLGA microparticles: PLGA-rAg85B JM (0.28% w/w)-MDP (0.5% w/w) microparticles were placed in 67mM potassium phosphate buffer, pH 7.4, and vortexed for 3 hours to remove the surface-associated antigen and then centrifuged. The supernatant solution was separated into two aliquots. One aliquot were mixed with PLGA control microparticles and the other aliquot were used directly in the assay. The residual PLGA-rAg85B-MDP microparticles, after the initial burst, were dried and reconstituted in the fresh medium into suspension at the microparticle concentration of 250μg/ml. THP-1 cells were exposed to 100 μl of each sample, which were then subjected to the T cell hybridoma recognition assay.

The surface-adsorbed-rAg85B PLGA microparticles were prepared. A certain amount of rAg85B JM in 20mM sodium phosphate buffer was placed into PLGA control microparticles. The mass ratio of rAg85B to microparticles was the same as that of spray-

dried PLGA microparticles containing rAg85B (0.56% w/w). This formulation was used when the liquid completely evaporated. These dried microparticles were reconstituted in infection medium at 250μg/ml. 100μl of the suspension was used in T hybridoma recognition assay.

Six-day antigen presentation assay: THP-1 cells were incubated in 96-well flat-bottom plates (1.5×10^5 cells/well) with 10ng/ml of PMA in infection medium for 24 h. Cells were washed once with infection medium and incubated with 100U/ml of recombinant human IFN-γ for 24 h. The cells were: rinsed twice with infection medium; pulsed with 200μl rAg85B Chap (25μg/ml) or 200μl 83μg/ml of microparticles encapsulating rAg85B Chap (0.14% w/w) and MDP (0.5% w/w) and further incubated for 6h; then cells were washed extensively with infection medium to remove the antigen solution and microparticles. 200μl DB-1 cells at density of 1×10^6 cells/ml were added at each time point (from day 1 to 6) to the wells. After 24h co-incubation, the supernatant was harvested for IL-2 ELISA assay.

Statistics: A one-way ANOVA was used to compare the multiple groups. The student's t-test was used for analysis between any two individual groups. The statistical significance was set at $p< 0.05$.

2.3 Results and discussions

2.3.1 rAg85B expression in *E. coli* systems.

To reduce the potential for protein misfolding and aggregation, IPTG induction and further fermentation were performed at 22°C. The preparation of rAg85B JM yielded 16mg/L after purification and was the sole protein band visible in SDS-PAGE (Fig 2.2A). A second preparation of antigen, rAg85B Chap, was purified from the *E. coli* strain o*rigami B*. This o*rigami B* strain of *E. coli* carries mutated thioredoxin reductase (*trxB*) and glutathione

reducates (*gor*) genes and a chaperone GroEL /GroES vector which facilitate disulfide bond formation in the less reducing environment of the cytosol and could improve folding of Ag85B. The yield of rAg85B Chap was 4.5mg/L after purification. The mass of the purified rAg85B JM as determined by mass spectrometry (Nanospray-ESI-MS on the Applied Biosystems Q-star Pulsar mass spectrometer, Foster City, CA) was 34kD (Fig 2.2B).

The antigenicity of soluble rAg85B was tested in the T-hybridoma cell recognition assay involving the human monocytic cell line THP-1 and DB-1 T-hybridoma cells which specifically recognize $Ag85B_{97\text{-}112}$ epitope presented by MHC class II. This assay system and DB-1 T hybridoma cells were developed by Gehring *et al.* (Gehring, Rojas, Canaday *et al.*, 2003). Upon recognition of complex of MHC-II epitope, DB-1 cells produce IL-2, which can be measured by ELISA. Using this T-hybridoma cell recognition assay, presentation of the specific Ag85B peptide-MHC Class II complex by APC to T-hybridoma cells was measured. Both rAg85B preparations were taken up by THP-1 cells. Antigens were processed, and presented to DB-1 hybridoma cells as indicated by IL-2 production (Fig 2.2C). IL-2 production reached a plateau at a rAg85B concentration of 25-50μg/ml. rAg85B Chap induced higher IL-2 secretion from T-hybridoma cells DB-1 than the rAg85B JM, in the concentration range of 12.5-150μg/ml tested. The endotoxin level in the recombinant Ag85B was apparently lower than 0.025ng/mg after purification.

Two soluble rAg85B protein preparations used in this study differed in antigenicity based on T-hybridoma cell recognition assay. The rAg85B Chap preparation, purified from an *E. coli* strain carrying the mutated thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes and the chaperone GroEL /GroES vector elicited larger IL-2 production than rAg85B JM. Under physiological conditions, the *E. coli* cytoplasm is maintained in a reduced

state that does not favor the formation of stable disulfide bonds in proteins. The o*rigami B* mutations impair the reduction potential of both thioredoxins and glutathione (Bessette, Åslund, Beckwith *et al.*, 1999). Furthermore, the chaperones provide a central compartment for a single protein chain to fold unimpaired by aggregation (Hartl and Hayer-Hartl, 2002). Ag85B has one disulfide bond and one free –SH group. The purified rAg85B Chap may be structurally different from the rAg85B JM preparation, explaining the difference in antigen processing. Further investigation is needed to define the difference between the different rAg85B proteins.

2.3.2 Manufacture of PLGA microparticle

2.3.2.1 Spray-drying condition optimization

The Buchi B191 spray-dryer was used for microparticle manufacture. There are six parameters (nitrogen flow rate, aspiration pressure, atomization pressure, solution feed rate, inlet temperature and polymer concentration) affecting the outcome of the particle production. The preparation of particles in the size range 1-5 μm is most important since these particles can potentially deposit in the lungs. Yield is a secondary outcome that is not as important as particle size in the present study. The experiments were complicated by a feed tubing material incompatibility with the organic solvent methylene chloride. The tubing materials influenced the particle manufacture. Polytetrafluoroethylene (PTFE) was used in most of the feed tube part. However, the tubing portions under the pump and connections to the nozzles need to be resilient. Norprene was used in these connections but it was soluble to some extent in methylene chloride. In order to minimize the amount of oily material, extracted from tubing, and collected in the cyclone and jar, conditions were optimized by experimental design to nitrogen flow 600L/hr, atomization pressure 3.0 bar and aspiration rate 50%.

The other three parameters (polymer concentration, inlet temperature and feed rate) were optimized for PLGA microparticle manufacture. The particle size was the most important criteria for optimization. A factorial experimental design was used. Two-level, three factors, full fraction of experiment numbers were utilized (Table 2.1). Main effects of inlet temperature, feed rate and their interaction significantly affect the particle sizes. Polymer concentration at this range (0.35 to 0.5% w/w) did not play any role to change the particle size (Appendix 1A). The interaction between feed rate and inlet temperature is shown in Appendix 1B. It predicted that higher feed rate and lower inlet temperature in the tested range would produce the smallest positive particle size. The cuboidal plot indicated that the predicted smallest D_{v50} 3.62 μm likely be manufactured under condition of B- (lower inlet temperature) and C+ (higher feeding rate). The polymer concentration in the tested range did not affect the particle sizes (Appendix 1C). In order to obtain higher desirability in prediction, the optimized inlet temperature needed to be higher than 63°C at the feed rate of 50% of the pump (Appendix 1D).

From the results of factorial experimental design, the optimized condition for PLGA microparticles manufacture was: PLGA polymer concentration in methylene chloride (CH_2Cl_2) 3.5mg/ml, inlet temperature 65°C, atomization pressure 3.0 bar, aspirator rate 50%, feeding pump rate 50% (4.5ml/min), N_2 flow control 600L/hr.

Under this optimized condition, 24 batches of PLGA microparticle formulations with different source and loadings of rAg85B, different loadings of MDP, TDB, were generated (Table 2.2). The yield was in the range of 24-33%. The particle sizes and distributions are summarized in Chapter 3.

The spray-dried microparticles were observed as raisin like structures showing irregular surface under the scanning electron microscopy (SEM). (Fig 2.3A) The projected area equivalent diameter was in the range of 3-4μm. The particles morphology, shrunk and wrinkled, was due to the spray-drying conditions used. Rapid drying and evaporation leave a soft flexible shell that collapses at the end of spray-drying. The interior structure of the microparticles was hollow (Fig 2.3B). The polymers formed the shells of the microparticles.

2.3.2.2 Release profile of rAg85B and MDP from PLGA microparticles

PLGA microparticles exhibited a pulsatile release profile of rAg85B. This release profile corresponded with the microparticle structure. The PLGA-rAg85B (0.14% w/w)-MDP (0.5% w/w) preparations provided a pulsatile release profile of rAg85B with high initial burst as demonstrated by *in vitro* dissolution (Fig 2.4). In the first day 58% of the rAg85B was released from the microparticles. The cumulative quantity of rAg85B released reached a plateau of 66% on Day 3. Subsequently, a second period of release delivered an additional 14% (80% total release) by Day 20 and by Day 31, ~100% antigen was released. MDP was released from the microparticles in a large initial burst of 85% on day 1. The remaining 15% of MDP was continuously released over a period of 46 days. The initial burst most likely reflects release of surface associated protein and adjuvant.

The release profile difference between rAg85B and MDP may be explained by their molecular size. rAg85B is a 34kD protein. The large molecular size of rAg85B (34kD) inhibits diffusion from the polymer shell, until erosion of the polymers. MDP is a small molecule with molecular weight of 492 Da. The small size of MDP made it easy to penetrate through the surface pores and dissoluted.

2.3.3 PLGA microparticle formulations can enhance the antigenicity of rAg85B.

2.3.3.1 PLGA microparticle uptake by activated THP-1 cells.

THP-1 cells are human monocytes from peripheral blood. They can be activated by some reagents, such as phorbol myristate acetate (PMA), and become macrophage-like cells. There are complement and Fc-mediated receptors expressed on THP-1 cell surfaces (Tsuchiya, Yamabe, Yamaguchi *et al.*, 1980). HLA haplotypes of THP-1 were HLA-A2, -A9, -B5, -DRW1 and -DRW2 (Tsuchiya, Yamabe, Yamaguchi *et al.*, 1980). The monocytic nature of the cell line demonstrates lysozyme production, the ability to phagocytose particles and restore T-lymphocyte response to Con A. During culture, THP-1 maintained these monocytic characteristics for over 14 months (Tsuchiya, Yamabe, Yamaguchi *et al.*, 1980). Due to the macrophage-like and easy culturing properties, and as a human origin cell line required to interfere with humanized DB-1 cells, THP-1 cells are used as antigen presenting cells in the T cell recognition assay to help screening the PLGA microparticle formulations.

In order to visually detect the phagocytosis of PLGA microparticles, sodium fluorescein (5% w/w) was encapsulated in PLGA under the same optimized spray-drying condition. Confocal microscopy was used to detect the location of PLGA-sodium fluorescein. Following PMA treatment, THP-1 cells were incubated with 5 particles/cell for 24 hours. The similar sized PLGA-fluorescein microparticles were taken up by THP-1 cells after 24 hours incubation (Fig 2.5). This assay illustrated that these PLGA microparticles can be taken up by THP-1 cells. This phagocytosis can include these microparticles into the cells, and makes the antigens encapsulated in the particulate accessible to the APCs.

2.3.3.2 Presentation of PLGA microparticles containing rAg85 to DB-1 T hybridoma cells.

In this project, muramyl dipeptide (MDP) and trehalose dibehenate (TDB) were used as adjuvants for encapsulation into the microparticles. MDP and trehalose dimycolate (TDM) are components of *MTB* cell wall. Trehalose dibehenate (TDB) is the synthetic analogue of TDM. The use of the cell wall components and their analogues as adjuvants to stimulate the immune response is well suited to vaccine optimization.

PLGA microparticles containing adjuvants only, PLGA- MDP, PLGA- TDB and PLGA-TDB-MDP formulations at different loading of adjuvants (0.1, 0.5 and 1% w/w) were added to THP-1 cells and coincubated with DB-1 cells. Similar IL-2 production was elicited by each of the above PLGA formulations in the T-hybridoma cell recognition assay and all responses were of the same order of magnitude (50-90 pg/ml) (Fig 2.6)**.** The addition of adjuvants alone to the PLGA microparticles did not produce large differences in IL-2 secretion with respect to the PLGA control particles. The PLGA particles themselves induced only a modest IL-2 response compared to the media only control, showing 80pg/ml versus 50pg/ml respectively.

PLGA particles encapsulating rAg85B (0.14% w/w) and MDP (0.5% w/w) (PLGA-rAg85B-MDP) elicited significantly greater IL-2 secretion than soluble rAg85B or PLGA particle only controls. Soluble rAg85B solution alone (36ng dose/well equivalent to microparticle dose) elicited a very weak IL-2 response of 79 pg/ml (± 2.1 SD). In comparison, the IL-2 released after PLGA-rAg85B-MDP exposure was 2194pg/ml (± 211 SD), which represented a 92-fold greater IL-2 response to PLGA-rAg85B-MDP microparticles than to the same amount of rAg85B in solution (after subtracting the medium

control). Notably, the effect of PLGA-rAg85B-MDP on IL-2 production was dependent on PLGA association or encapsulation. Mixing PLGA – MDP (0.5% w/w) microparticles with 12.5 μg/ml soluble rAg85B (100μl mixture for assay), which represented a larger amount of rAg85B than present in the PLGA-rAg85B-MDP microparticles used above, was also compared in the T cell hybridoma recognition assay to the encapsulated Ag85B particles. IL-2 secretion was approximately 170 pg/ml (± 14 SD), significantly lower than that observed with the PLGA-rAg85B-MDP (Fig 2.8A)**.** This demonstrated that mixing soluble rAg85B with PLGA-MDP elicited a significantly lower response (p=0.003). In conclusion**,** Fig 2.7A shows that encapsulation of rAg85B into PLGA-MDP microparticles significantly magnified the antigenicity of rAg85B.

We also compared PLGA-MDP microparticle formulations encapsulating rAg85B JM and Chap in the hybridoma cell assay. IL-2 concentrations of 2194 pg/ml (± 211 SD) and 2790 pg/ml (± 735 SD) were produced in response to PLGA-rAg85B JM-MDP and PLGA-rAg85B Chap-MDP, respectively (Fig 2.7B). PLGA–microparticles containing rAg85B Chap gave a larger IL-2 response (360-fold after subtracting medium control) than the same amount of soluble rAg85B Chap that is consistent with observation of encapsulated rAg85B JM. There was no significant difference in IL-2 response between PLGA microparticle formulations encapsulating rAg85B JM or rAg85B Chap (p = 0.249), even though soluble rAg85B JM and Chap elicited statistically different IL-2 responses in the concentration range of 12.5-150 μg/ml (p <0.001). Therefore rAg85B JM was used for further study due to its high yield in *E. coli* expression.

The loading of rAg85B was increased two-fold in PLGA-MDP (0.5% w/w) microparticles to 0.28% (w/w). The IL-2 response to rAg85B presented in these particles

increased in comparison to those loaded with 0.14% reported above (Fig 2.8A). PLGA-rAg85B JM (0.28% w/w)-MDP microparticles resulted in secretion of 4532 pg/ml (± 216 SD) of IL-2, about a 1.24 fold increase in response to doubling rAg85B content. Testing an alternative adjuvant, PLGA-TDB (0.5%) microparticles containing rAg85B also elicited strong IL-2 secretion. PLGA- rAg85B (0.28% w/w)-MDP, PLGA-rAg85B (0.28% w/w)-TDB and PLGA-rAg85B (0.28% w/w) induced IL-2 responses of 4532 (± 216 SD), 4211 (± 146 SD) and 4282 (± 165 SD) pg/ml, respectively (Fig 2.8A). We also tested PLGA-rAg85B-TDB (0.5%) microparticles with added MDP (0.5%), and these particles did not appear to increase the IL-2 responses in the T-hybridoma cell assay. However, the presence of rAg85B dramatically changed the response range of IL-2 secretion (Fig 2.8B).

Interestingly, these results demonstrate that the presence of the adjuvants MDP or TDB at loading of 0.5% (w/w) in the microparticles in the concentration ranges tested did not influence the magnitude of response to rAg85B.

PLGA microparticles exhibit a strong adjuvant effect as shown above. At 0.5% loading of MDP or TDB there was no significant improvement in rAg85B (0.14%) antigenicity. However, adjuvants at other concentration range or combination may have significant enhancement in immunity elicitation. Different loadings of MDP or TDB were included with rAg85B into PLGA microparticles. In the case of 0.1 and 1% loading of MDP, with rAg85B (0.14%), the existence of MDP could not enhance antigenicity of rAg85B, neither in the rAg85B loading of 0.28%. There were no significant difference between the PLGA-rAg85B and PLGA-MDP (0.1%)-rAg85B (0.28%)(Fig 2.9). For TDB, 0.1% loading in PLGA-rAg85B (0.28%) elicited significantly more IL-2 secretion in T cell recognition assay than that of PLGA-rAg85B (0.28%). While higher TDB content was not as good as

lower loading (0.1%). Therefore, TDB 0.1% enhanced antigenicity of rAg85B in PLGA formulations (Fig 2.10).

The microparticle formulation of rAg85B has been shown to initiate higher levels of T cell recognition as indicated by IL-2 production than soluble rAg85B, which is important for Th1 CMI. There are two explanations for the PLGA-rAg85B formulation giving a greater response better than soluble rAg85B. The first possibility is that exogenous soluble proteins can be taken up by endocytosis or fluid-phase pinocytosis. However, the uptake of rAg85B from solution is not as efficient as that from microparticle formulations. Macrophages are particularly sensitive to particles ranging from 1-10μm. The phagocytosis of one microparticle with large quantities of rAg85B attached to the surface and within the polymer matrix, will result in higher levels of the protein in the phagosomal compartment than endocytosis of rAg85B soluble molecules. Secondly, it is also possible that particulate antigens are processed differently from soluble antigens. Rock *et al.* reported that distinct epitopes from Ovalbumin were generated with differing efficiencies, by macrophages and B cells, from particles compared to soluble antigens. This may be due to changes in the structure of the bound antigen, conformation or its accessibility to proteases (Vidard, Kovacsovics-Bankowski, Kraeft *et al.*, 1996). The PLGA used in this study was a polymer of 75:25 lactide to glycolide. The generally hydrophobic surface exhibits hydrophilic regions which favor hydrophobic as well as electrostatic interaction with antigen proteins. Further studies are required to elucidate the effect of particle-antigen interaction on the epitope presentation.

Subunit vaccines have the advantage of reducing side effects of possible infection that whole organisms may generate from vaccination. However, they frequently have weak

antigenicity and immunogenicity and adjuvants are often needed for subunit antigen vaccination (Marciani, 2003). PLGA microparticles have the depot effect associated with encapsulation of the antigens; therefore, microparticles themselves are adjuvants (Raychaudhuri and Rock, 1998). Our data confirm this adjuvant effect compared to soluble antigen. Addition of other adjuvants may further improve the antigenicity. However, the incorporation of adjuvants may also limit their use in humans. Furthermore, the use of adjuvants may be complicated by cross interaction of other naturally occurring adjuvants. For example, lipopolysaccharides can sensitize an animal's response to MDP (Takada and Galanos, 1987; Takada, Yokoyama and Yang, 2002). There was no statistically significant difference in IL-2 secretion between PLGA-rAg85B and PLGA-rAg85B-MDP at the concentrations (0.5% w/w). However, TDB 0.1% loading enhanced the rAg85B antigenicity in PLGA microparticles. Therefore, PLGA-rAg85B and PLGA-TDB (0.1%)-rAg85B are good formulations in terms of immunity elicitation.

2.3.3.3 The influence of surface-associated-rAg85B on PLGA-microparticles.

Surface-associated antigens and adjuvants were removed by washing and the residual microparticles were tested in the T-hybridoma cell recognition assay to determine whether the existence of surface-associated antigen was necessary for the antigen presentation activity of the formulation. The IL-2 response showed that neither initial-burst solution, initial-burst solution plus PLGA control microparticles, nor residual dried-microparticles after initial burst induced a similar level response as the PLGA-rAg85B-MDP microparticles (Fig 2.11A). Therefore, surface-association of rAg85B appeared to be important for the T-hybridoma cell response to PLGA-rAg85B-MDP.

The existence of surfactants in microparticles may reduce the nature and extent of phagocytosis by alveolar macrophages (Evora, Sorino, Rogers *et al.*, 1998; Jones, 2002; Jones, Dickinson, Gumbleton *et al.*, 2002). In our microparticle preparation, no surfactants or emulsifiers were used in the single emulsion before spray-drying. The large initial release of rAg85B indicated that the single emulsion was not sufficiently stable to hold the internal water droplets in water-in-oil emulsion. However, PLGA-subunit microparticle with this high initial burst of rAg85B was effective as vaccine formulations. The subunit proteins are most likely associated to the microparticle surfaces by means of physical chain entanglement via electrostatic and hydrophobic interaction (Sugiyama, Mitsuno and Shiraishi, 1996). Proteins / peptides may be embedded in the microparticle matrix but some portion of peptide chains extend from the particulate surface. The initial release may be explained by the extensive mixing, removing almost all of the rAg85B from the microparticle surface where diffusion from the microparticles would be the rate limiting phenomenon. A small quantity of protein would be left at the surface (Basinska, 2001) given the low concentrations (< 1μg/ml) and the high solubility of rAg85B. Following the initial release of rAg85B, the microparticles would behave as PLGA alone with respect to interaction with macrophages, until the remaining rAg85B was released from the microparticles by a mixture of diffusion and erosion mechanisms (Witt and Kissel, 2001).

PLGA microparticles with surface-adsorbed rAg85B showed an adjuvant effect, as shown by IL-2 secretion of 721 ± 158 pg/ml, that was not as strong as that of spray-dried particles containing rAg85B, where IL-2 production of 3245 ± 77 pg/ml occurred (Fig 2.11B). However, the surface-adsorbed-antigen PLGA formulations provided stronger antigenicity than the solution formulation, which stimulated IL-2 secretion at the level of 80

pg/ml. These data illustrated that the surface-associated antigen is important to enhance the antigenicity. However, its adjuvancy is much weaker than the spray-dried formulations containing rAg85B. The extent of the antigen detachment from the microparticles might be the reason for this difference. In spray-drying procedure, the antigens may be entangled and embedded in the microparticle shells which is a strong attachment to the particles. The physical adsorption force of antigens on particle surface may not be strong enough to hold the antigens to the particle surface during the reconstitution procedure. Large quantities of antigens may go into solution instantaneously.

2.3.3.4 Epitope presentation by THP-1 cells exposed to PLGA microparticles encapsulating rAg85B.

Prolonged epitope presentation by macrophages may be an advantageous property for a vaccine. The ability of PLGA microparticles containing rAg85B to present epitope over time was studied. Epitope presentation was compared over six days by THP-1 cells pulsed with soluble rAg85B Chap solution alone or the PLGA-rAg85B Chap (0.14% w/w) -MDP (0.5% w/w) (Fig 2.12). The absolute amount of IL-2 response differed for soluble antigen versus microparticles encapsulating antigen. By assaying T cell recognition daily we were able to study the epitope presentation of a given formulation on the initial Day 1 (Set at 100%) compared to subsequent Days 2 and 3, and the IL-2 response was unchanged. However, after Day three the level of IL-2 response fell. A more dramatic decrease in IL-2 response was observed with soluble antigen than with the PLGA-rAg85B-MDP microparticles. On Day six, 16% of the IL-2 response that was elicited on Day one was observed for soluble rAg85B, while 54% of the response observed with the microparticle formulation of rAg85B remained. The THP-1 cells also showed different morphology after

exposure of rAg85B solution and microparticle formulations. Photomicrographs were taken on day 3 (Fig 2.13). rAg85B solution did not show significant difference in cell morphology from the control. Few cells exhibited pseudopodia (Fig 2.13B). While the THP-1 cells exposed to rAg85B microparticles (Fig 2.13C) seemed morphologically more activated and extended more pseudopodia compared to the control and rAg85B solutions. Thus, the microparticle formulation of rAg85B not only amplified the response, but also provided longer-lasting epitope presentation. This prolonged presentation favors T cell recognition and the subsequent cytokine secretion.

In the first three days of the assay, the epitope presentation did not change compared to Day one. Antigen processing, forming complexes with MHC class II and sorting onto APC surface take some time, so there is concern over kinetic stability with respect to the complex of MHC class II: peptide epitopes. The kinetic stability of a MHC class II: peptide complex physiologically influences the recognition and expansion of specific T cells. Peptide epitopes must be loaded onto MHC class II in endocytic compartments and exported to the surface of APCs; this complex must be stable during the transit to the draining lymph node sustaining TCR signaling once contact between $CD4^+$ T cells and the antigen-bearing APCs (Sant, Chaves, Jenks *et al.*, 2005). If $t_{1/2}$ of the complex is less than 5 hours, T cell immunity will not be generated *in vivo*. Immunodominant peptide complexes with MHC class II could possess extremely long half-lives of more than 150 hours (Sant, Chaves, Jenks *et al.*, 2005). Ag85B peptide 91-108 is a dominant CD4 T cell recognition-epitope and has been recognized by 85% of healthy donors (Valle, Megiovanni, Merlo *et al.*, 2001). It is inferred that the epitope Ag85B $_{97\text{-}112}$: MHC II complex must be sufficiently stable, although kinetic

stability data is not available. On day six, the PLGA microparticle formulation of rAg85B still retained 54% of epitope presentation on APC surface, while soluble rAg85B had 16%.

Another explanation for the extended production of IL-2 is that the replication of macrophages might prolong epitope presentation. The doubling time for THP-1 cells before differentiation is ~ 26 hours (ATCC, Information). However, macrophages which lose migration ability exhibit much slower replication, usually granulocyte-macrophage colony-stimulating factor (GM-CSF) is needed to stimulate the growth (Nakata, Akagawa, Fukayama *et al.*, 1991). The macrophage is one type of the cells can release GM-CSF (Bezdicek and Crystal, 1997) with the secretion level of 29-260 pg/ml / 5 $\times 10^5$ cells / 24 hours (Dakhama, Israël-Assayag and Cormier, 1996). It may be inferred that the estimated GM-CSF secretion would be 0.1-1 U/ml in the bioassay used in this study (10^8 Units/mg of GM-CSF (Komuro, Keicho, Iwamoto *et al.*, 2001)). There was a 15-34% increase in macrophage numbers in the initial 7 days under the GM-CSF concentration of 0.2-2U/ml (Nakata, Akagawa, Fukayama *et al.*, 1991). Therefore, the estimated increase in macrophage numbers is 7-17% at GM-CSF concentration of 0.1-1 U/ml. The newer cells may phagocytose the microparticles debris remaining and start to present the epitopes. Since the half life of epitope:MHC class II on THP-1 cell surface is the same to both of the formulation assay, if the macrophage replication is the major reason for the epitope presentation difference on day 6, then 7-17% of epitope presentation difference would be expected. About 38% epitope presentation difference was observed (Fig 2.13). The higher level of epitope presentation stimulated by microparticle formulation was most likely due to the encapsulated antigen in microparticles internalized into the THP-1 cells have depot effect and made the epitope presentation on cell surface longer. The release of antigens from

microparticles increases the density of epitopes in the phagosome (Torres, Ramachandra, Rojas *et al.*, 2006) which favors the T cell recognition. However, the possibility that replicated macrophages phagocytose the microparticle debris after washing step could not be completely excluded.

2.4 Summary

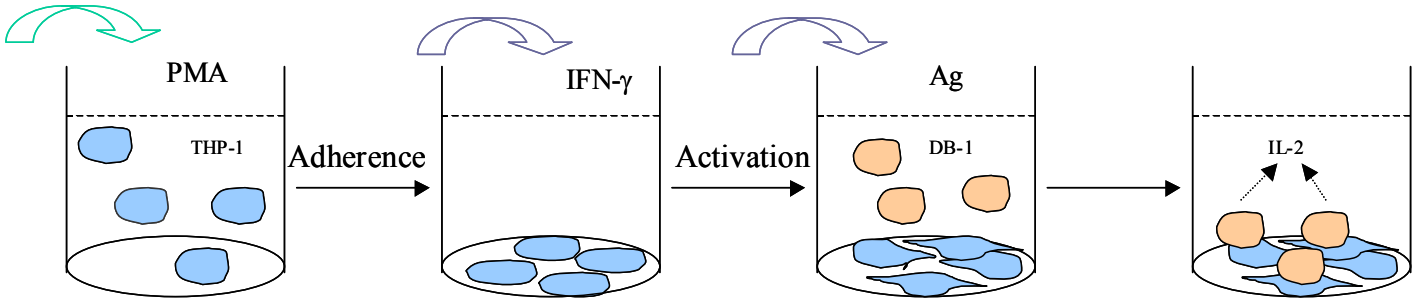
PLGA microparticles encapsulating rAg85B were delivered to THP-1 cells for processing and presentation in the context of MHC class II to a CD4 T-hybridoma cell line DB-1. Microparticle formulations containing rAg85B were more effective in stimulating THP-1 cells to present antigen to the DB-1 T-hybridoma cells than rAg85B protein in solution. Submicrogram amounts of rAg85B in microparticles induced 92-360 fold larger IL-2 production than the same amount of soluble rAg85B. This effect was enhanced when the quantity of rAg85B incorporated into the microparticles was doubled.

rAg85B is one of the most promising vaccine candidates with both class I and II epitopes. The ability of rAg85B in the microparticle systems to elicit CD4 T-hybridoma cell activation has been demonstrated in an *in vitro* T cell recognition assay. In conclusion, two expression systems were used to produce rAg85B. These antigens were incorporated at different concentrations into PLGA microparticles by a spray drying process. Microparticles delivered rAg85B more efficiently to macrophages than soluble antigen delivery. In turn, rAg85B can be processed and presented well at the macrophage surface in the context of MHC class II. PLGA-rAg85B microparticles stimulate an antigen specific CD4 T cell hybridoma response that is two orders of magnitude greater than that observed for soluble rAg85B. The PLGA-rAg85B particle effect can't be recapitulated by mixing of soluble or initial burst solution with control PLGA particles. This suggests that the strong adjuvant

effect of the particles requires surface absorption or encapsulation. These formulations provided extended epitope presentation on the APC surface for up to 6 days. This may mimic the boosting effects of independently administered vaccines but further studies are required to establish this proposition. Finally, the PLGA microparticles can be prepared in a particle size range suitable for pulmonary delivery as aerosols.

Sufficient evidence was obtained from the *in vitro* assessment of microparticles containing rAg85B to justify their evaluation in protecting animals from infection with small aerosol inocula of *MTB*.

Fig 2.1 Principle of T hybridoma cell recognition assay.

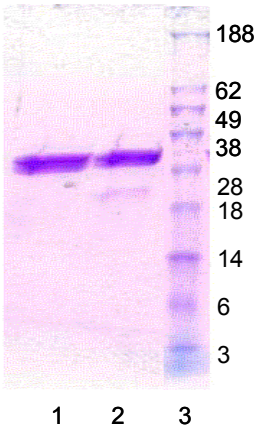


PMA: phorbol myristate acetate

DB-1: T hybridoma cells

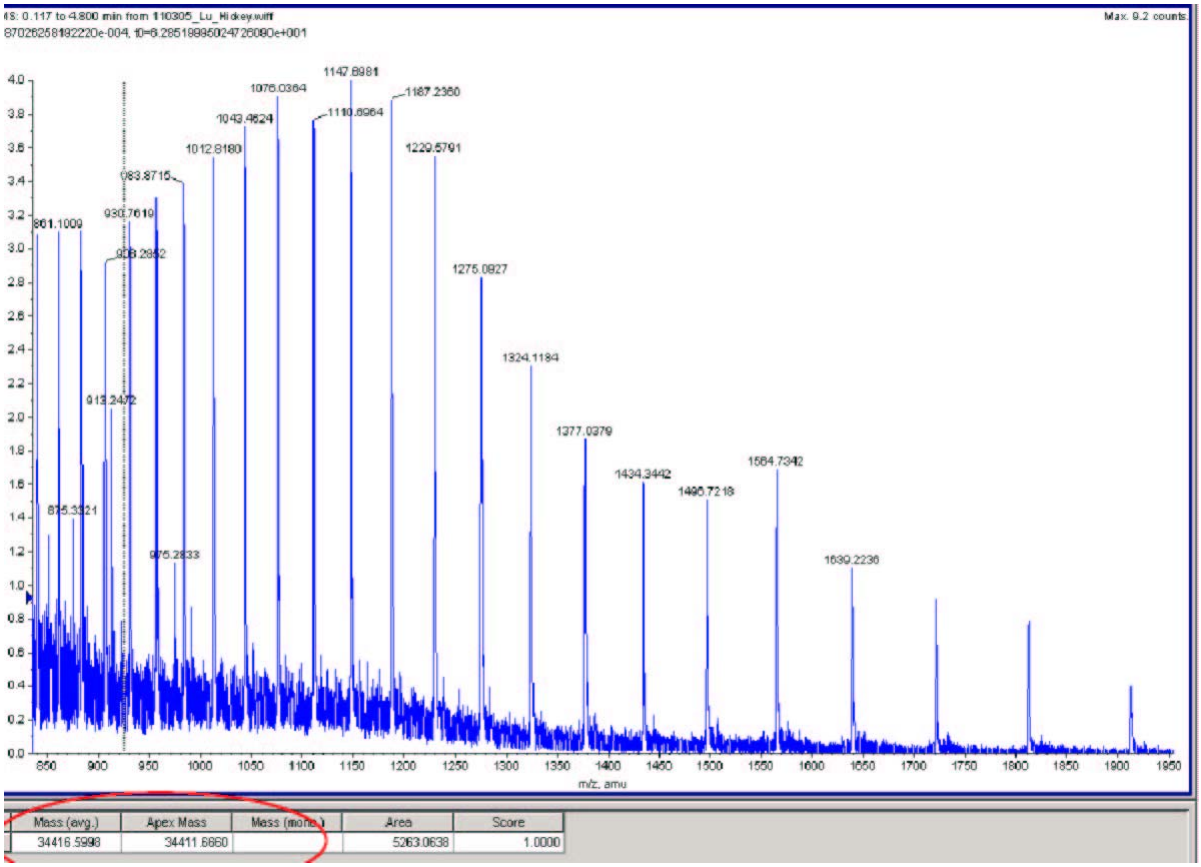
Fig 2.2 The characterization of recombinant Ag85B.

A) SDS-PAGE of two recombinant antigen 85B. Lane 1, rAg85B JM; Lane 2, rAg85B Chap; Lane 3, molecular weight marker.



B) Mass spectrometry of rAg85B JM. MW is 34412 Da

x-axis is m/z, y-axis is relative abundance.

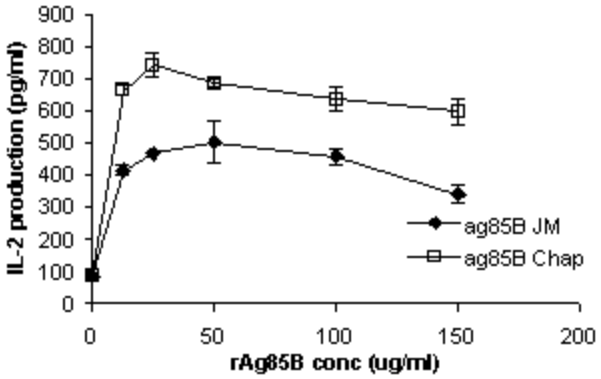


B

Calculations
1564.7342 = (MW+n)/n (1)
1639.2236 = (MW+n+1)/ (n+1) (2)
where n is the number of positive charges on one molecule,
MW is the molecular weight of rAg85B

MW = 34416
n = 22

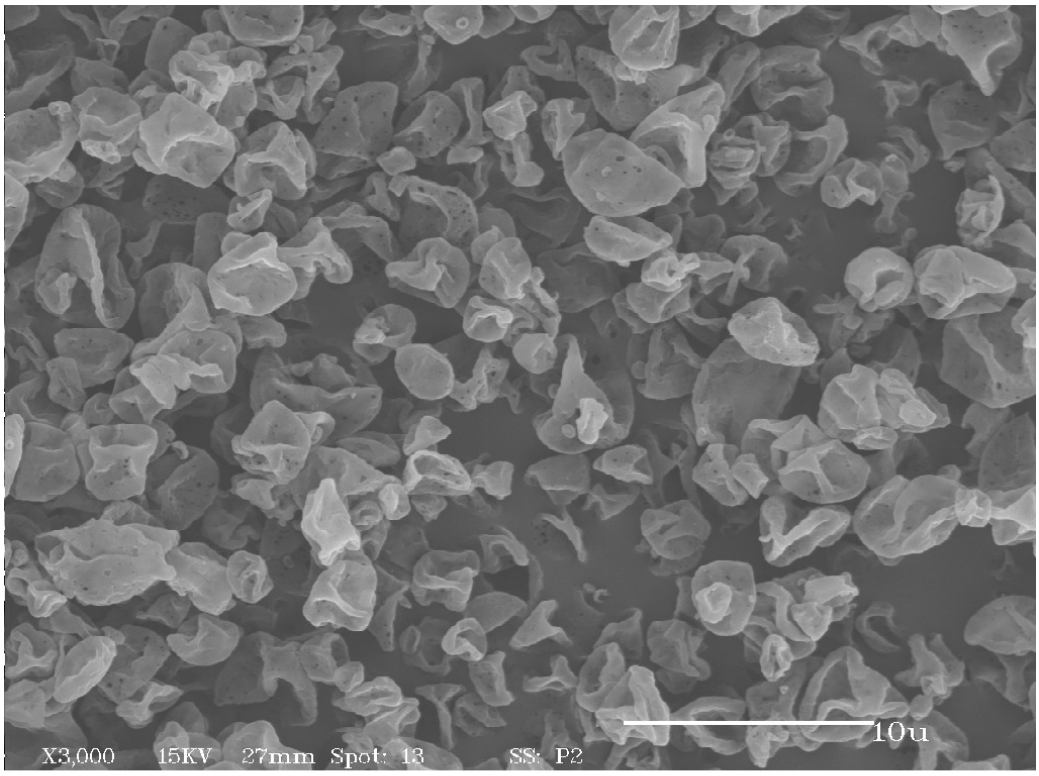
C) Antigenicity of two recombinant Ag85Bs in solution evaluated by T cell recognition assay. Blank square is rAg85B Chap, solid diamond is rAg85B JM.



C

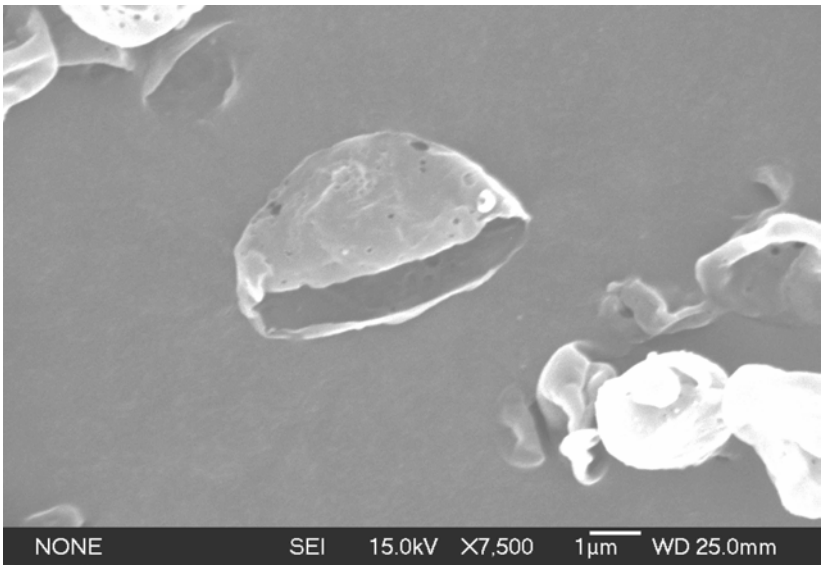
Fig 2.3 Morphology of PLGA microspheres.

A) SEM of PLGA-MDP (0.5%)-rAg85B (0.14%) microspheres. The standard bar in the picture was 10μm.



A

B) Interior morphology of PLGA microparticles.



B

Fig 2.4 Release profile of rAg85B JM and MDP from PLGA- rAg85B JM-MDP. The dissolution was performed in 67mM sodium phosphate buffer, pH 7.4 at 37°C (shaken in waterbath). n=2

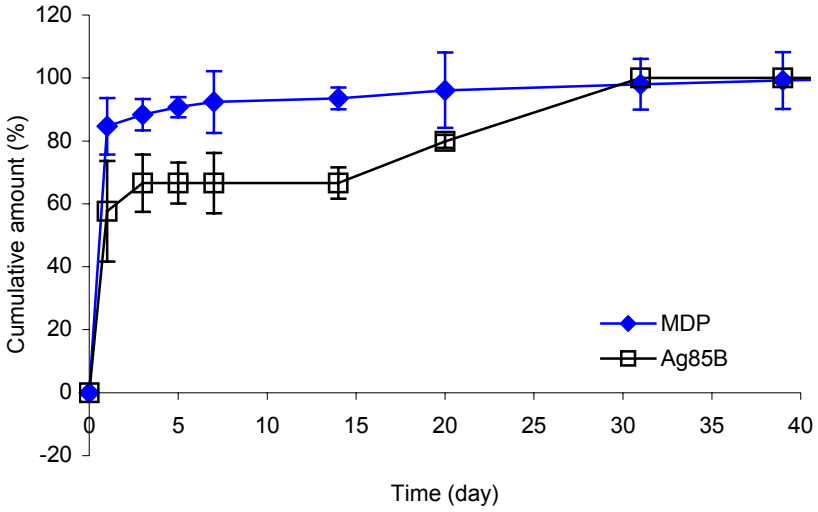


Fig 2.5 Confocal microscopy of phagocytosis of PLGA-sodium fluorescein particles by activated THP-1 cells. (1) Confocal image; (2) Normal image; (3) Overlap of the confocal and normal images.

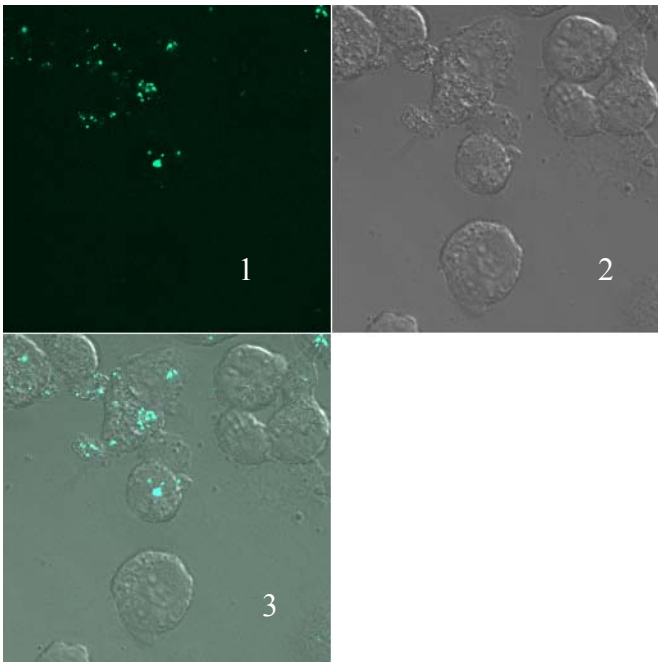


Fig 2.6 T hybridoma cell responses to different loadings of PLGA-adjuvants microparticles, as measured by IL-2 ELISA. (n=3, bar is standard deviation.)

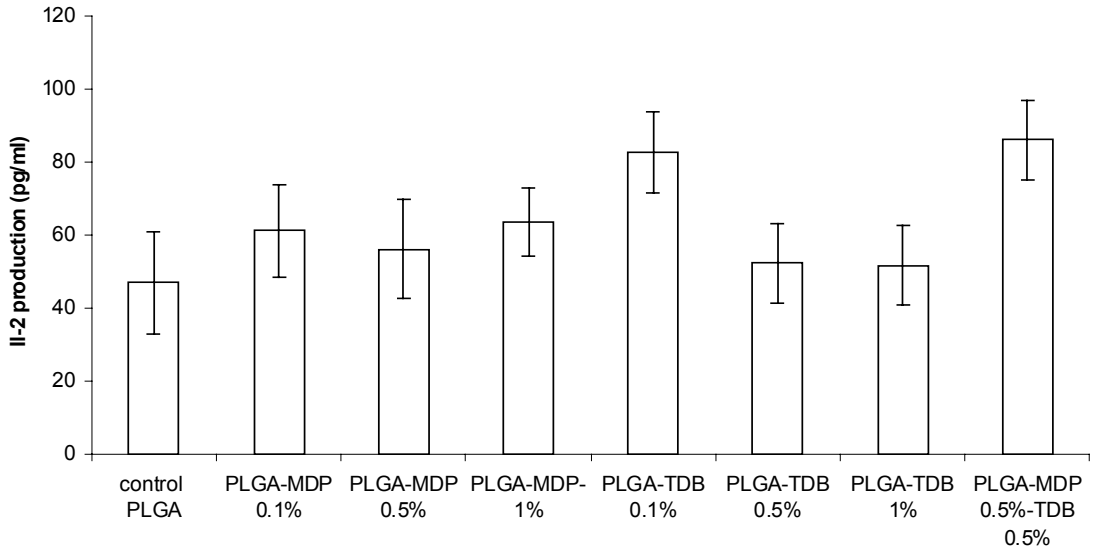
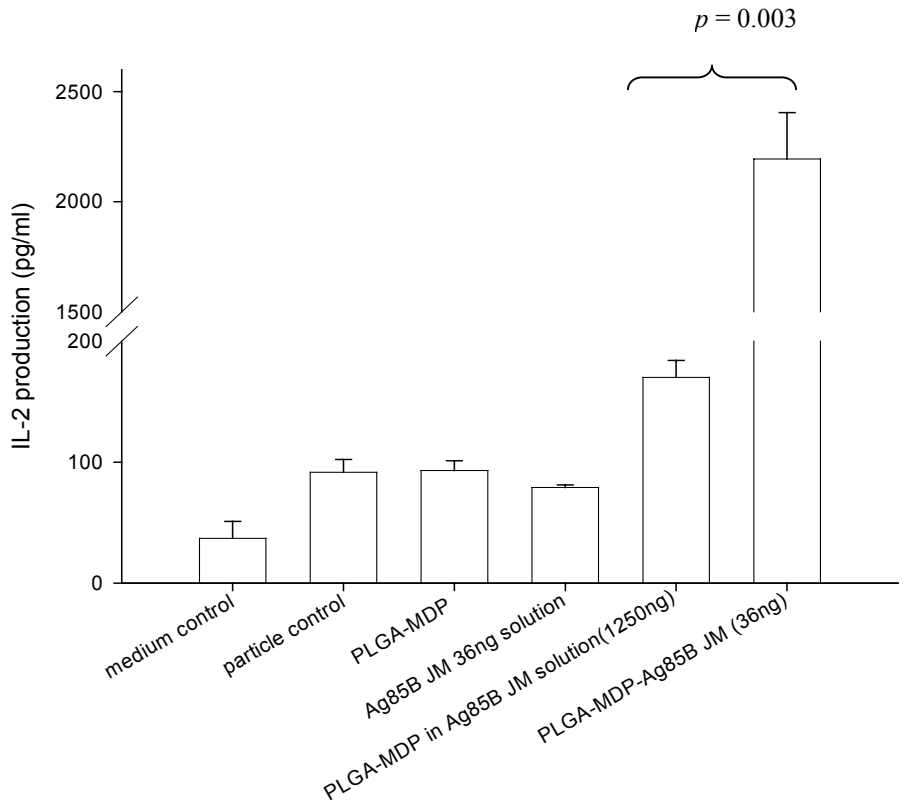


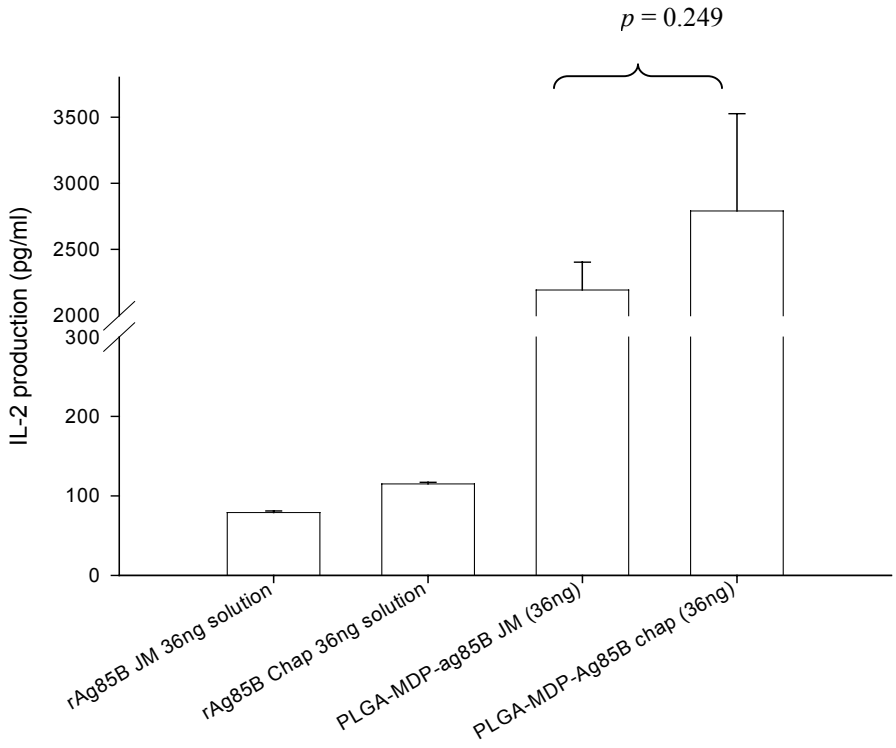
Fig 2.7 T hybridoma cell responses to different formulations, as measured by IL-2 ELISA. (n=3, bar is standard deviation in normal scale). All PLGA formulations contained MDP 0.5% loading and Ag85B as of 0.14% loading.

A) The adjuvant effect of PLGA microspheres;



A

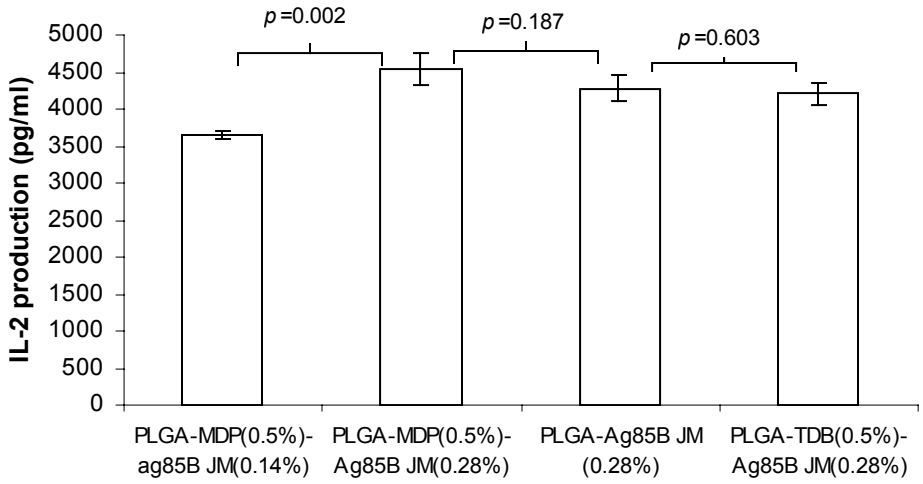
B) The T hybridoma cell responses to Ag85B Chap and JM strains in different formulations. The *p* values were labeled in the figures.



B

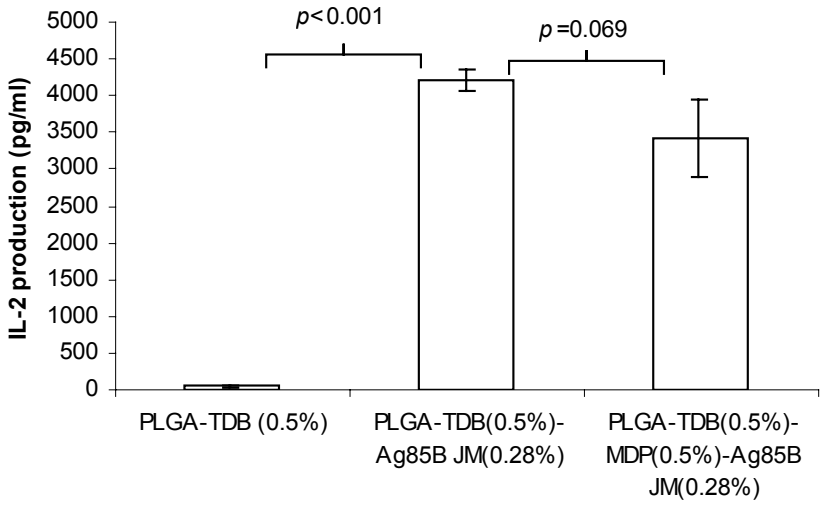
Fig 2.8 T hybridoma cell responses elicited by different doses and formulations of rAg85B in PLGA microspheres, as measured by IL-2 ELISA. (n=3, bar is standard deviation).

A) Different doses of Ag85B JM in PLGA microspheres at loading of 0.14 and 0.28% and with/without the adjuvant MDP or TDB;



A

B) Addition of MDP in the PLGA-TDB-Ag85B formulation did not improve the T hybridoma cell response.



B

Fig 2.9 MDP adjuvant effect on rAg85B-PLGA formulations.

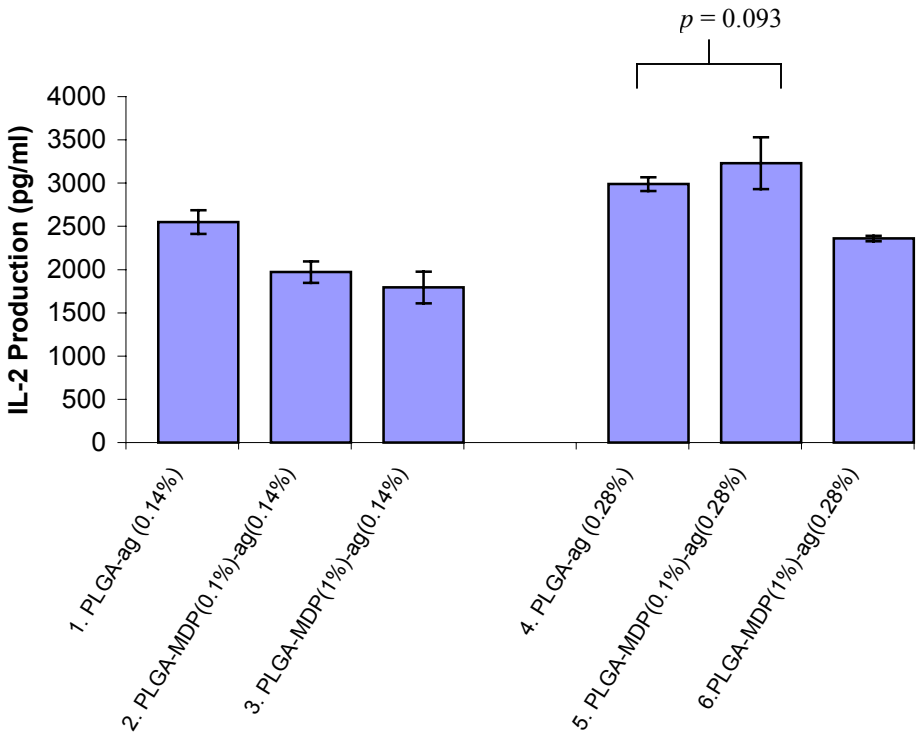


Fig 2.10 TDB adjuvant effect on rAg85B-PLGA formulations.

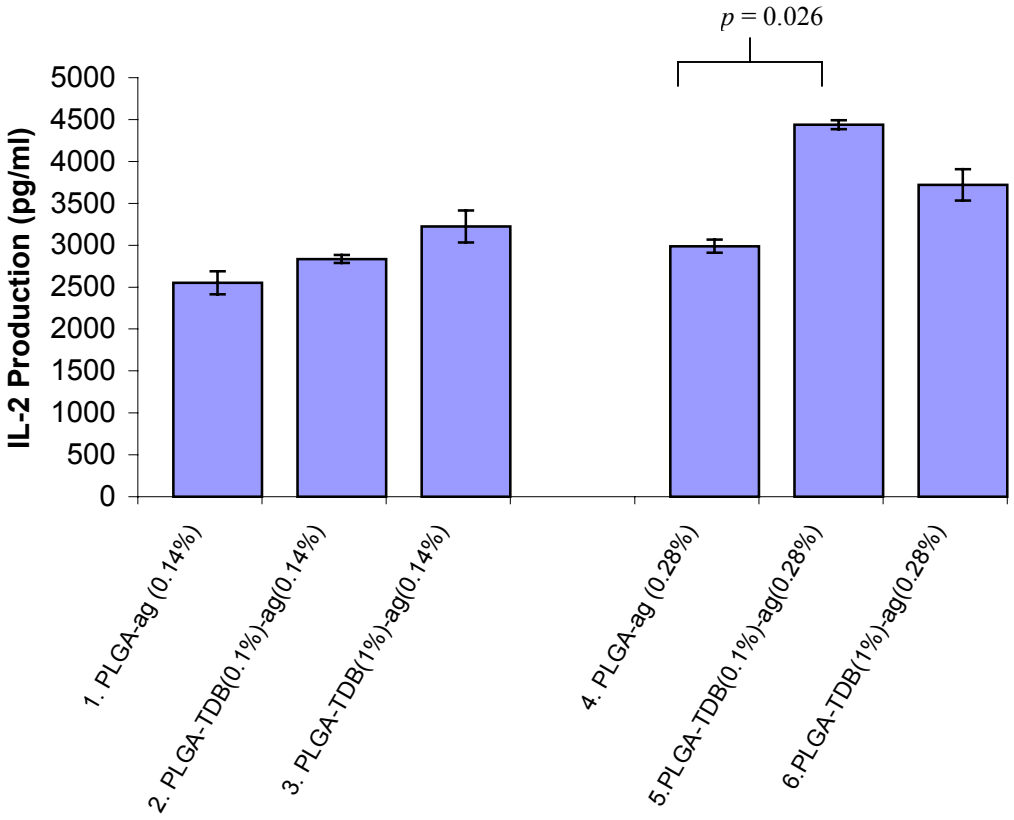
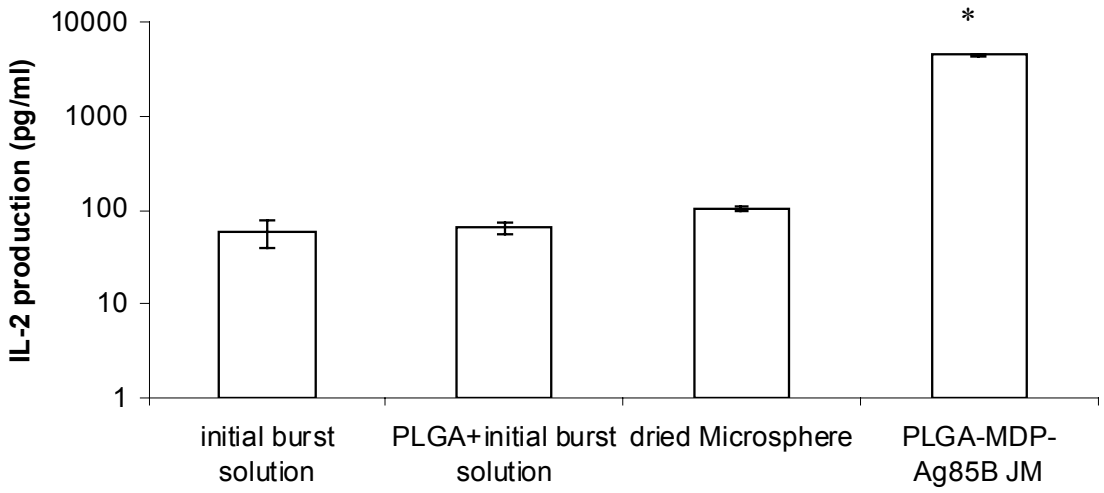


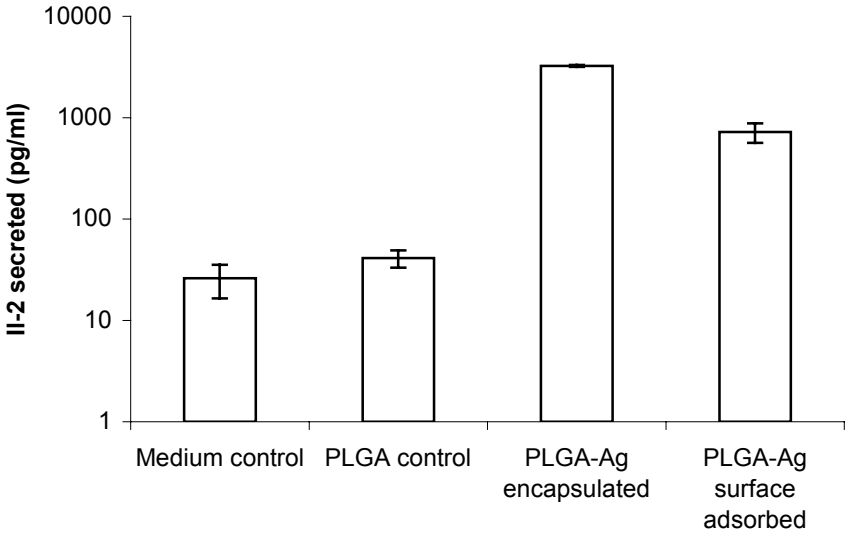
Fig 2.11 The influence of surface-associated PLGA-rAg85B-MDP formulation on T hybridoma cell activation, measured by IL-2 ELISA. (n=3, bar is standard deviation). (*p< 0.001)

A) Spray-dried formulation with/without initial burst;



A

B) Particles with only surface-adsorbed Ag85B JM.



B

Fig 2.12 Kinetics of epitope presentation, as indicated by IL-2 release from T-Hybridoma cells exposed to macrophages pulsed with rAg85B Chap solution or PLGA-rAg85B (0.14% w/w)-MDP (0.5% w/w). (n=3, bar is standard deviation)

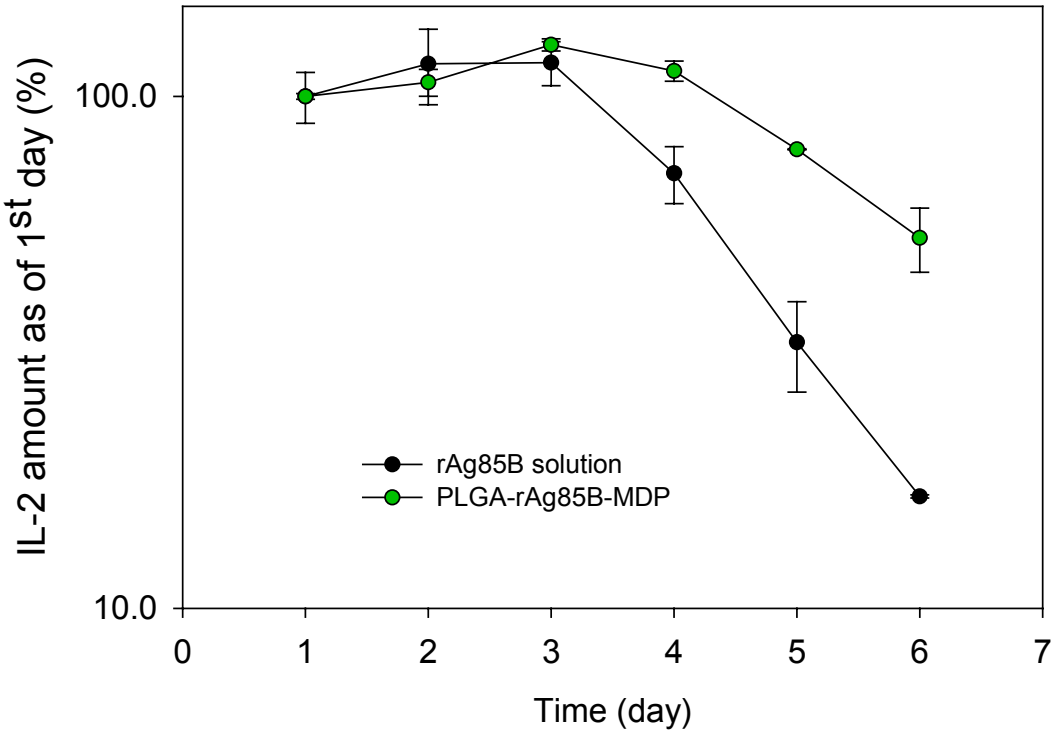
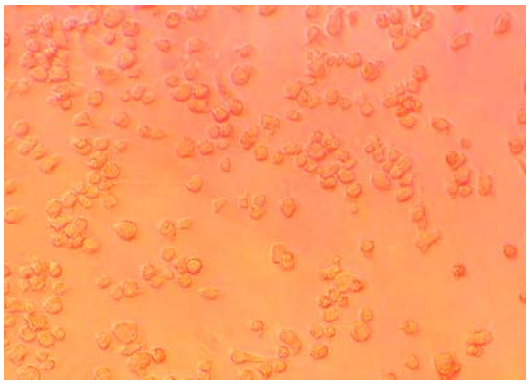


Fig 2.13 Morphology of THP-1 cells on day 3 (after 6 hour rAg85B exposure on day 0).

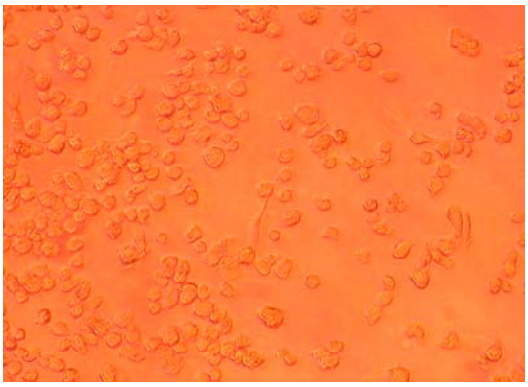
(A) THP-1 control;

(B) THP-1 after exposure of rAg85B Chap solution;

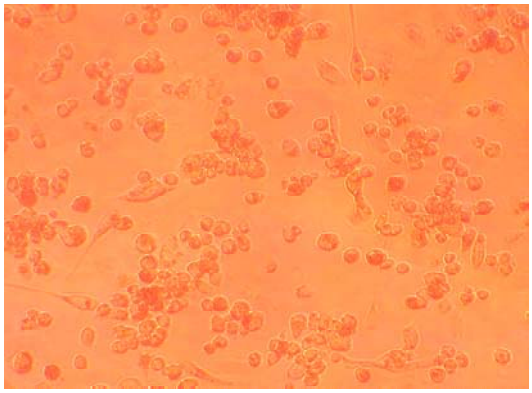
(C) THP-1 after exposure of rAg85B-MDP-PLGA microparticles.



A



B



C

Table 2.1 Two-level, 3 factors experimental design for PLGA microparticle manufacture condition optimization.

Std	Run	Block	Polymer conc (% w/v)	Inlet T (°C)	Feed rate (%)*	Yield (%)	D_{g50} (μm)	D_{v50} (μm)
3	1	Block 1	0.35	80	30	23.22	3.0	77.68
4	2	Block 1	0.50	80	30	16.99	2.1	79.37
6	3	Block 1	0.50	65	50	38.32	4.2	10.70
5	4	Block 1	0.35	65	50	29.00	2.6	11.6
2	5	Block 1	0.50	65	30	18.06	2.8	29.24
8	6	Block 1	0.50	80	50	12.61	2.8	28.72
1	7	Block 1	0.35	65	30	49.22	2.7	13.30
7	8	Block 1	0.35	80	50	20.68	2.4	16.17

* 100% feed rate with the current tubing is 9ml/min; 50% of the pump is 4.5ml/min and 30% of the pump is 2.7ml/min.

Table 2.2 The PLGA microparticle formulations manufactured under the optimized conditions.

Batch of PLGA microsphere	MDP (w/w loading %)	TDB (w/w loading %)	rAg85B JM (w/w loading %)	rAg85B Chap (w/w loading %)
1	-	-	-	-
2	0.1	-	-	-
3	0.5	-	-	-
4	1.0	-	-	-
5	-	0.1	-	-
6	-	0.5	-	-
7	-	1.0	-	-
8	-	-	0.14	-
9	0.1	-	0.14	-
10	0.5	-	0.14	-
11	1	-	0.14	-
12	-	0.1	0.14	-
13	-	1	0.14	-
14	0.1	-	0.28	-
15	0.5	-	0.28	-
16	1	-	0.28	-
17	-	0.1	0.28	-
18	-	0.5	0.28	-
19	-	1	0.28	-
20	-	-	0.28	-
21	0.5	0.5	0.28	-
22	0.5	-	-	0.14
23	-	-	0.56	-
24	-	0.1	0.56	

3 Physico-chemical property characterization of dry powder PLGA formulations as aerosols

3.1 Introduction

Respirable particles containing antigen were manufactured and they elicited an immune response in a complex cell culture model of marker immunity. However, their suitability for pulmonary delivery depends on their physico-chemical properties. In order to deliver PLGA-microparticles containing antigens to the lungs, aerodynamic particle size, distribution and general physico-chemical properties must be characterized to meet the criteria suitable for respirable aerosols.

The target particle morphology, size and distribution for dry powders are important factors for aerosols. Scanning electric microscopy (SEM) is a technique capable of producing highly-resolution images of a sample (Martin and Bustamante, 1993). SEM images have a characteristic 3D appearance and are useful for judging the surface structure of particles. The equivalent diameter of a circular disc of the same projected area as the particle being studied, can be obtained through SEM 2D images (Martin and Bustamante, 1993). Laser diffraction is one of the most commonly used light scattering methods of particle sizing. Particles are passed through a laser (frequently Helium-Neon, λ= 682.3nm) creating a diffraction pattern on a detector array. An algorithm is used to calculate the particle size distribution from this diffraction pattern (Ranucci, 1992). Algorithms vary and are proprietary to specific instrument manufacturers. Diffraction patterns are correlated with the volume diameters of equivalent volume spheres to the particles being studied. The mass median aerodynamic

diameter (MMAD) and size distribution representing a population of particles, can be obtained by measurement of mass in different particle size ranges after aerosols are dispersed and deposited on different stages of an inertial sampler, e.g. the Andersen Cascade Impactor (ACI). Assuming a log-normal distribution, the median diameter is used to represent the fiftieth percentile of the distribution and geometric standard deviation (GSD) is used to represent the breadth of the particle size distribution. The aerodynamic diameter is that of an equivalent volume of sphere of unit density with the same terminal settling velocity as the particle being studied (Hickey, 2004a). The emitted dose from the device upon aerosolization can be measured using the USP sampling Apparatus B for delivered-dose uniformity. The typical operating conditions for sampling dry powder aerosols are under 60L/min flow rate. The emitted doses range from 75-125% of the nominal dose to meet the USP criteria (Pharmacopeia., 2005). The fine particle fraction (FPF) is the portion of particles with sizes below than 5 μm. The fine particles are designated as those having the potential to be deposited in the lungs.

Besides the particle size and distribution, a number of other physical properties of the particles may be characterized which influence the performance of dry powder aerosols. They includes: flow properties, specific surface area, powder moisture content; surface charge properties and crystallinity that relate to storage stability and aerosol dispersion efficiency and reproducibility.

Bulk and tapped density are combined measures to assess powder flow properties. The bulk density is the mass of a powder divided by the volume prior to packing. Packing is usually achieved by tapping a volume of powder. The tapped density is the packed mass divided by the minimum volume of occupancy of the powder obtained after it has been

tapped until no further decrease in volume occurs (Carr, 1965). The Carr's compressibility index (CCI) may be used to quantify the powder flow using the two density terms and is defined as:

CCI = (Tapped density – bulk density) x 100% / Tapped density (Carr, 1965)

As powder flow improves, the bulk density approaches the tapped density and any further tapping will not cause a decrease in volume. Therefore, as the CCI approaches 0, powder flow improves as particle interactions decrease (Carstensen, 1993). A free-flowing powder has a CCI less than ~20-21% (Carr, 1965). Hausner ratio is another term to indicate the flow properties of powders.

Hausner ratio = tapped density / bulk density (Hausner, 1967)

According to Wells (Wells, 1988), a Hausner ratio of <1.20 is indicative of good flowability of the material, whereas a value of >1.50 suggests poor flow. The Hausner ratio and the Carr index, which are measures of particle interaction, have been widely used to estimate the flow properties of powders. These terms were developed to assess compressibility for tabletting and have since been adopted for assessment of aerosol powders.

When a powder is poured onto a surface from a fixed height, it forms a cone. The angle formed by the cone to the horizontal surface can be calculated and is known as the static angle of repose (Crowder, 2003). This angle is related to the potential flow properties of a powder. As the static angle of repose increases, powder flow decreases. A large static

angle of repose is indicative of large cohesive forces within the powder. Powders with static angle of repose < 40° are considered to be free-flowing powders; while angles >50°, indicate that the powders flow poorly or not at all (Carr, 1965). The static angle of repose has been defined as an indirect measure of particle size, shape, porosity, cohesion, fluidity, surface area and bulk (Carr, 1965).

The surface area of a particle can be computed from measurement of the amount of a gas adsorbed onto a sample of powder to form a monolayer (Martin and Bustamante, 1993). This technique is accurate and relevant to further applications. When a mixture of nitrogen and helium are passed through a cell containing powder, adsorption and desorption of nitrogen gas can be measured with a thermal conductivity detector. Over a range of gas pressures, adsorption occurs non-linearly, in the simplest case asymptotically to monolayer surface coverage, but can be described by the Brunauer-Emmett-Teller (BET) equation. This equation allows the calculation of volume of gas adsorbed without specific assumptions regarding the nature of adsorption. The specific surface area is the surface area per unit mass of the sample (Martin and Bustamante, 1993).

Moisture content measurement, by moisture balance determination, involves monitoring the weight change of a material after heating to vaporize associated water. It is useful in the measurement since any water present in the material may result in loss of activity of proteins or affect the physical stabilities (Chang, Reeder and Carpenter, 1996) (Dong, Korber, Lopez Esguerra *et al.*, 2006) and influence dispersibility of dry powders. Water weight will be lost during heating allowing percent weight loss of the material to be calculated. Moisture content analysis and DSC allow determination of the stability of the materials in different temperature ranges (Brittain, 1995).

Differential scanning calorimetry (DSC) is a method of monitoring phase transitions that occur when the temperature of a material is increased. The glass transition temperature (T_g) of a material is the temperature at which a supercooled noncrystalline liquid existing below its crystallization temperature (amorphous state) converts to a glass. The amorphous state and its glass are metastable and kinetically-dependent transitions. Above T_g, the material becomes rubbery (amorphous), lacking long-range order and capable of elastic or plastic deformation without fracture. To assure long-term stability of the dry solid formulation, the solid-state material should exist in the glassy state rather than in the amorphous state in which it has a higher molecular mobility (Carpenter, Pikal, Chang *et al.*, 1997).

Zeta potential is an important and useful indicator of electrical charge on particle surfaces. It can be used to predict and control the stability of suspensions (Martin and Bustamante, 1993). Zeta potential is the difference in electrical potential between a tightly bound layer of ions on particle surfaces and the bulk liquid in which the particles are suspended. It can be quantified by tracking the charged particles when they migrate in a voltage field, as performed by a zeta potential analyzer. The greater the zeta potential, the more likely the suspension is to be stable because the charged particles repel one another and, thus, overcome the natural tendency to aggregate. As a result, higher values of zeta potential imply more stable suspension, and lower values indicate colloid instability, which could lead to aggregation.

Electrostatic charge can accumulate on aerosol powders. These surface charge properties can affect dry powder performance during mixing and device filling; passage of the drug through the device upon actuation and inhalation; solid-state adhesive and cohesive

interfacial interactions; and ultimately, lung deposition and therapeutic effect. Studies of medicinal aerosol electrostatics have been attempted previously. Electrostatic charge carried by a DPI aerosol cloud has been measured by Faraday cage (Adhiwidjaja, Matsusaka, Yabe *et al.*, 2000), which precluded analysis of different size fractions and charge distribution. Use of the electrical low pressure impactor (ELPI™) for studying surface charge properties of dry powder particles allows a more comprehensive investigation of the phenomenon, with accurate determination of magnitude and polarity of charges on particles in the respirable size range over time. Furthermore, since each impactor stage is electrically isolated from the next, charges can be measured across individual stages, allowing size specificity of charging to be evaluated. ELPI was used to examine the surface electrostatic properties of PLGA microparticles that evolved as dry powders were generated as aerosols.

The PLGA microparticles containing Ag85B intended for aerosol delivery were characterized in terms of physical stability and for their dispersion properties both for administration to animals and from inhaler to humans.

3.2 Materials and methods

Projected area equivalent diameter: Scanning Electron Microscopy (SEM, Model 6300, JEOL, Peabody, NY, USA) was used for projected area equivalent diameter measurement. The stubs were coated with gold-palladium alloy (150-250Å) using a sputter coater (Polaron 5200, Structure Probe Incorporated Supplies, West Chester, PA, USA). The coater was operated at 2.2kV, 20mV, 0.1torr (argon) for 90 seconds. An accelerating voltage of 15kV was used.

Volume median diameter (VMD): A small amount of each batch of PLGA formulations was suspended in 0.1% Tween 80 and sonicated for 1 min. The suspensions were placed in a

sample cell which had an integral stir bar and a volume of ~15ml for detection. The ideal suspension concentration was obtained at a laser obscuration of ~ 20-25%. D_{v50} and span were measured using a laser diffraction particle sizer (Malvern 2600, Worcestershire, UK).

Mass median aerodynamic diameter (MMAD): PLGA-disodium fluorecein (5% w/w) microparticle powders were dispersed from dry powder inhalation/dispersion devices (Inhalator® (Boehringer Ingelheim, Ingelheim, Germany) suitable for human use and DP-3 insufflator (PennCentury, PA) for aerosol insufflation to animals). An eight-stage Andersen MKII nonviable cascade impactor (Graseby Andersen, Smyrna, GA) was used to collect the powders for characterization with respect to mass deposited on different stages. Dry powder aerosolization was performed under 60 and 28.3L/min flow rates (n=3). The plates of the impactors were coated with 1% silicon oil in hexane. glass-fiber filters (Pall Corp., Ann Arbor, MI) with a pore size of 0.22μm were used below the last stage of the impactor. 10mL of 20mM sodium phosphate buffer, pH 7.4 were placed in the preseparator. ~ 3mg of PLGA microparticle powders were filled in a gelatin capsule (#3) and inserted into Inhalator®. Following piercing of the capsule, sampling was continued for 10s. For the insufflator, ~3mg of powders were filled into the powder chamber. A 10mL syringe was used to push air through the chamber and puff the powder out. 10mL chloroform and 10mL 20mM sodium phosphate buffer, pH 7.4 were used to wash the plates, preseparator and USP induction port. After strong vortexing (Vortex-Genie 2, G-560, Scientific Industries, Inc., Bohemia, NY) and 30min exposure to dissolution medium, the supernatant was decanted and assayed by UV spectrophotometry at 490nm. The supernatant in the filter stage was filtered through 0.2μm pore size PVDF filter (Whatman Inc., Clifton, NJ) to remove the debris of glass filters.

Bulk and Tapped density: Approximately 1g PLGA microparticle powders were placed in a graduated cylinder. The mass/volume before tapping was calculated as bulk density. The tapped density was obtained following about 100 taps which allowed the powder volume to plateau.

Static angle of repose: PLGA microparticle powders were poured through a glass funnel onto a flat collection surface until the angle of repose did not change with addition of powder. The angle to the horizontal surface was measured. At least 1g of PLGA microparticle powders was needed for this purpose.

Specific surface area: Single point surface area analysis was performed on PLGA microparticle powders using Quantasorb Jr (Quantachrome Instruments, Boynton Beach, FL). Accurately weighed PLGA microparticle powders were degassed at 25°C for 22hr, 40°C for 3hr with nitrogen or no heating. The samples were subjected to repeated adsorption and desorption of the adsorbate, 30% nitrogen in helium gas.

Moisture content: Each sample was loaded onto an aluminum tray to achieve a coating with a thin layer of powder. The initial weight of the powder was recorded. The powder was heated to 105°C and held for 30min. The final weight of the powder was measured accurately using a moisture balance (Mettler LJ16 Moisture Analyzer, Mettler-Toledo, Columbus, OH). The difference between final and initial powder weight was calculated and attributed to the water content in the sample, recorded as a percent of the total weight.

Thermal analysis: Differential scanning calorimetry (Perkin Elmer DSC 6, Wellesley, MA) was performed on powder samples. Known quantities of the powders were sealed in an aluminum pan and differences in heat flow were measured against an empty reference pan. Analysis was performed at a ramp rate of 10°C/min, unless otherwise mentioned.

Zeta potential: Zeta potential was measured for 0.2-0.3mg/mL PLGA microparticles suspended in 5mM sodium phosphate buffer, pH 7.4 (Zeta meter 3.0 Zeta meter, Inc, Staunton, VA and Zeta Plus, Brookhaven Instrument, Holtsville, NY).

Kinetic surface charge properties: Upon aerosolization, real-time particle size measurements and surface electrostatics of dry powder aerosols dispersed from gelatin capsules were examined using an electrical low-pressure impactor (ELPI™, Dekati Ltd, Tampere, Finland). The DPI was connected to the mouthpiece and standard USP induction port, which was connected to the ACI pre-separator (loaded with 15 mL of 20mM sodium phosphate buffer, pH 7.4). A custom-manufactured aluminum connection to the pre-separator split the 60 L/min stream in two; 40 cm Tygon™ tubing (R3603) was used to connect the two outlets with ELPI™ and ACI, each connected to a vacuum pump operating at 30 L/min. Microparticle powders ~ 3mg were aerosolized at 60 L/min using Inhalator® (Boehringer Ingelheim, Ingelheim, Germany) under ambient conditions of 23 -25°C and 34-35% relative humidity). The apparatus is shown in Fig 3.1.

3.3 Results and discussions

3.3.1 PLGA microparticles particle size and distribution

In the 24 batches of PLGA microparticle formulations, most of the D_{v50} were in the range of 3.6-6.0μm (Table 3.1). There was a tendency for formulations containing TDB to exhibit larger particle sizes, in some cases above 7μm (7.7μm, batch 14). However, the equivalent projected area diameter D_{p50} was lower, in this example 3.6 μm. There was some aggregation of PLGA microparticles containing TDB in 0.1% Tween 80. PLGA microparticle formulations with TDB were more aggregated than other formulations,

probably because of the poor solubility of TDB in methylene chloride leading to bridges between the particles. Most of the particle distributions, expressed as Spans, were less than 2.

The mass median aerodynamic diameter (MMAD) is known to influence lung deposition. PLGA-sodium fluorecein (5% w/w) particles were sampled by Andersen non-viable Cascade Impactor and chemical analyses were performed to generate stage mass data suitable for interpretation utilizing log probability mathematical fits. After the gelatin capsule was pierced, the microparticle formulation was dispersed from Inhalator® at flow rate of 60L/min. The powders deposited predominantly on stage 3 (cutoff 2.3μm), ~ 27% of the emitted dose. Approximately 17% and 8% of the emitted dose deposited on mouth-throat piece and preseparator, respectively. They were aggregates or particles larger than 8.6μm (Figure 3.2A). The MMAD was 2.5μm, which was much smaller than the projected area diameter 3.2μm and $_{Dv50}$ of 4.6 μm, respectively (Fig 3.2B).

The DP-3 dry powder insufflator is an easy-to-use, hand-operated pulmonary drug delivery device designed to produce an aerosol cloud of fine particles from the end of a small-diameter delivery tube (web). About 3mg of microparticle powders were loaded into the chamber of DP-3 insufflator. At a flow rate of 60L/min, the powders were dispersed in air from a syringe. Twenty-seven percent of the microparticles deposited on stage 3, the largest quantity among the stages. The preseparator and mouth-throat piece collected 17 and 7%, respectively. The size distribution was bimodal with MMAD 2.8 μm (Fig 3.3). The dispersion studies from DP-3 insufflator were also performed at 28.3L/min to assess whether a flow rate dependence of shear would influence particle size. The microparticles deposited predominantly (25%) on stage 4 (cutoff size 2.1 μm) (Fig 3.4). In addition, 22% was collected on stage 3 (cutoff size 3.3μm). This bimodal distribution gave a MMAD of 3.0μm.

The MMAD, FPF and emitted dose in PLGA aerosol the dispersions under these two flow rates were similar. However, a difference was observed in the proportion collected in preseparator. A greater proportion was collected in the preseparator at 60 than 28.3L/min (17 vs 10% of emitted dose). This demonstrated that, under relatively higher flow rate (60L/min), the powders might have more inertia and impact easily in the preseparator.

Since the particles in the size range of micrometers are very sensitive to the humidity, the dispersion properties of the PLGA microparticle formulations were investigated after 28 days storage under 85% RH environment (saturated KCl solution). The deposition pattern was similar to the dry powder from the desiccators with MMAD of 3.2μm (Fig 3.5). However, a difference in emitted dose between the dry and moisture-treated powders was observed at flow rate of 28.3 L/min (Table 3.2).

The fine particle fractions (FPF, size < 4.7 μm) of dry powder and moisture-treated powders were similar, 71 (± 7.8) vs 78 (± 6.4)%. This indicated that the powder emitted from the insufflator behaved similarly at both humidities. The emitted dose of dry powder was 92 (± 2.1)%. The humidified powder gave variable emitted dose from 16 to 92%. This variability was also observed when some powders were retained in the insufflator, which was rarely the case in the dry powders without moisture-treatment. The exposure to high humidity appeared to increase the moisture content of the microparticles.

The DP-3 insufflator is suitable for the pulmonary delivery of PLGA aerosols to the animals. Even at slow flow rate, the deposition pattern and MMAD of this formulation were satisfactory.

3.3.2 Flow properties of PLGA microparticle powders

Large angles of repose correlate with poor the powder flow. The results, of 39.1 ± 1.9° were close to 40° (a transition angle of powder flow property, free flow powder < 40°). The Carr's compressibility index (CCI) was 25.0 ± 1.0%, close to free flow powder of 20-21°. The Hausner ratio was 1.32 ± 0.23. Therefore, the PLGA microparticle powders have intermediate flow properties (between the extremes of easily or poorly flowing, Table 3.3). The irregular surfaces of microparticles made the air drag force possibly bigger (Chan, 2006) and reduced the contact area or points between particles, which can help to improve the aerosol dispersion (Telko and Hickey, 2005). The low powder density also influenced the powder performance. From the dispersion point of view, even without carriers the microparticles alone would be predicted to aerosolize well from devices.

From the aerosol dispersion performance data under two humidity conditions, moisture treatment induced poor dispersion properties of PLGA powders which were observed as aggregated clumps. The interparticulate forces between small particles must be strong enough to withstand the external powder handling. The forces can be van der Waals, electrostatic, capillary and mechanical interlocking or solid bridging (Hickey, 2004a). Electrostatic interaction will be reduced under high humidity condition. However the presence of moisture leads to large capillary forces. Once the moisture content exceeds a certain limit, capillary forces will have a significant influence, as the presence of moisture in the interparticulate spaces would lead to the microparticle aggregation. Also the wetting of the particles may deform the morphology of the particles and increase the contact area between particles (Zeng, Martin and Marriott, 2000). Solid bridging may be induced by partial melting of the powders; solid diffusion; wetting agents may dry and harden; and

recrystallization of dissolved materials (Zeng, Martin and Marriott, 2000). Since the powders were all stored in a freezer, melting was excluded. Solid diffusion and recrystallization are the most likely explanation for solid bridging. In the presence of moisture, some components in microparticle powders may dissolve, diffuse to the surface or may form into new crystals during the treatment procedure. Solid bridges would be formed upon the material precipitation and crystallization, and further induce particle aggregations. Therefore, capillary forces and solid bridging may be two important factors in reduced flowability of the powders after moisture treatments.

3.3.3 Specific surface area and moisture content

Particle size is a primary determinant of surface area. As the number of irregularities increases, and the particle size decreases, the surface area increases (Lowell, 1979). The specific surface area was measured under 3 degassing conditions: room temperature degassed, no degassing and degassed at 40°C for 3 hr. The specific surface area values of PLGA microparticles were: 15.7, 14.7 and 6.7m^2/g. The degassing temperature, of 40°C, was close to the PLGA glass transition temperature. Freshly prepared microparticles were raisin like, collapsed hollow spheres. The heating procedure, which was held at 40°C, would increase the polymer mobility and potentially change the morphology of the microparticles.

The larger surface area (Table 3.4) of microparticles may correlate with high surface energy that would be a factor affecting their stability, especially in the presence of adsorbed moisture. The moisture content in the PLGA powders at different time points under ambient conditions was analyzed. At time 0, there was 0.7% moisture content. At 4hr and 48 hr, the moisture content increased to 2.18 and 2.96%, respectively (Table 3.5). The moisture content plateaued after 4hr. This low moisture adsorption was consistent with the high

hydrophobicity of this PLGA polymer (75:25 L:G). However, the microparticle formulations with moisture contents above 2% were observed as clumps. The components in the PLGA microparticles were 0.8% of sodium phosphate (at pH 7.4 there are monobasic and dibasic sodium phosphate) and 0.14-0.56% of rAg85B. Monobasic and dibasic sodium phosphates are slightly and very hygroscopic, respectively. 1 mole of sodium phosphate dibasic can adsorb 2-7 moles of water depends on humidity and temperature (Windholz, Budavari, blumetti *et al.*, 1983). Therefore, the PLGA microparticles with the sodium phosphate buffer components did not appear to resist the elevated humidity and became aggregated, which subsequently influenced dispersion. Whether Ag85B has strong hygroscopicity is not known, but as a protein it is likely to exhibit heterogeneous hydrophobic and hydrophilic regions. The moisture occupied on the PLGA surface and interparticulate spaces likely induced the capillary force and solid bridging between particles (see section 3.3.2). Consequently the powder dispersion was interfered.

3.3.4 Thermal behavior of PLGA microparticles

The DSC technique provides qualitative and quantitative information about the physicochemical status of formulations which was reported to be involved in the endothermic or exothermic process (Dubernet, 1995). DSC is useful for monitoring different samples of the same material to assess structural similarities or differences or the effects of additives on the thermal properties of a material. Using the DSC to evaluate the thermal properties of polymeric materials and microparticles allows the nature of the drug inside the polymer matrix to be assessed, which may emerge in solid solution, metastable molecular dispersion or crystallization (Dubernet, 1995)

In the PLGA microparticle formulations, the natural states of adjuvants MDP/ TDB, Ag85B and their interaction with PLGA polymers might be detected by analysis of the DSC thermagrams. The spray-dried PLGA (84.7kD, L:G=75:25) microparticles were scanned at 10°C/min from 10°C to 300°C. The PLGA degraded at about 271°C. Therefore, the scanning range was set below 250°C. There was a glass transition with onset of 51.4°C in the initial scan of the PLGA control microparticles and there was no crystal transition peak in the tested temperature range. After cooling to 10°C, the 2^{nd} and 3^{rd} scans were performed. The T_g onsets for 2^{nd} and 3^{rd} scans were 46.1 and 46.4°C (Fig 3.6, Table 3.6). Thus, a maximum of two scans were conducted in each sample. Comparing the T_g of the PLGA microparticles in different scans, the initial scan gave much higher T_g onset. This may be explained by the rapid solidification of the polymer during the spray-drying process. The PLGA microparticles in all the scans were in the amorphous state.

The existence of Ag85B decreased the T_g onset of PLGA control microparticles after encapsulation Ag85B. The reduction of T_g onset, with respect to PLGA control, in the initial scans was 1.3 and 3.2°C for 0.28 and 0.56% w/w loading of Ag85B in PLGA microparticles, respectively. The T_g onset in the 2^{nd} repeated scan was reduced 0.5 and 0.4°C for these two Ag85B loadings, respectively (Fig 3.7, Table 3.7). The changes in the T_g onset temperatures demonstrated that there was interaction between Ag85B and PLGA microparticles. There was no melting peak for Ag85B at 180°C. This may indicate that, Ag85B is in the amorphous state and the Ag85B might disperse in the PLGA as the solid solute. The very low loading of Ag85B made it difficult to detect further in the solid-state.

However, MDP or TDB alone in the PLGA microparticles significantly depressed the T_g. 1% w/w of loading, MDP and TDB reduced the T_g temperature by 4.08 and 4.21°C in

the initial scans, and 1.14, 4.00°C in the 2nd scan, respectively. TDB depressed the T_g to a greater extent than MDP. But this depression in T_g was minimized when co-encapsulated with Ag85B (Fig 3.8, Table 3.7). The typical transition peaks of TDB were absent in all the scans of polymeric particles containing TDB. This also indicated that most likely TDB dispersed in the polymer matrix. In order to investigate whether TDB is truly dispersed in polymers stably or in the metastable state, possibly due to the relative weak interaction between polymer and the adjuvants, an annealing step was employed at 40°C for overnight. The thermagram was the same as that of without annealing. This indicated that TDB was truly dispersed in the polymer microparticle matrix.

The T_g of PLGA microparticles changed significantly upon the addition of the small molecules of adjuvants. The addition of small molecules into the polymer matrix increased the size of free volume cavities of the polymer. Increments in free volume induced by mixing plasticizers with amorphous polymers, represents one of the mechanisms that can explain the depression of polymer T_g (Sperling, 2001). MDP and TDB both have hydrophilic groups which interact with the polar chain groups, disrupt polymer chain–chain hydrogen bonding, removing further barriers to bond rotation and chain mobility. TDB has long fatty acid chains which would interact with high hydrophobic PLGA (75:25 L:G) to interfere with the polymer chain interactions. The small molecules and the polymer might develop strong interactions between each other, leading to plasticization of the polymer. The consequences in DSC are a lower T_g, and the absence of the fusion event of the small molecules (Dubernet, 1995).

Ag85B, MDP and TDB all behave as plasticizers to PLGA microparticles. The addition of Ag85B into PLGA-Adjuvants actually reduced the T_g depression of PLGA. This

might be due to that, the interactions between Ag85B and adjuvants minimize the interactions between adjuvants and PLGA polar chains.

Knowing T_g of polymer formulations has significant application in pharmaceutical field. It is known that the diffusion coefficients of small molecules through the polymer matrix increase several orders of magnitude when in the glass transition region (Karlsson, Stubbs, Karlsson *et al.*, 2001; Sperling, 2001). Stability of the pharmaceutical formulations will be achieved by storing the products at a temperature of at least 40°C lower than T_g.

3.3.5 Surface charge properties

The PLGA formulations containing different adjuvants and varied amounts of Ag85B showed negative zeta potential, implying negative charges on the microparticle surfaces (Table 3.8). This may be attributed to the presence of ionized carboxyl groups on the microparticles surface (Stolnik, Garnett, Davies *et al.*, 1995). The zeta potential values were in the range of –38mV to –51mV. The colloid particles or suspensions with more positive or negative zeta potentials (instead of close to zero) are more stable in the suspension system by reducing the particle aggregations (Martin and Bustamante, 1993). Polymer microparticles with the large absolute charge values (either positive or negative) on their surfaces would be easily phagocytosed by macrophages (Tabata and Ikada, 1988). Therefore, macrophage-like, THP-1 cells would phagocytose PLGA microparticles easily.

ELPI was used for evaluation of the surface charge properties after PLGA microparticles were aerosolized from the Inhalator®. With the "charger off" condition, the powders deposited on different stages were bipolar (i.e. both positive and negatively charged) after PLGA control dry powder was aerosolized from Inhalator®. Particles from 0.1 to 1.6μm in size had the most negative charges of –4000-5000fA. The particles from 1.6-10μm bore

positive charges with the maximum positive current of +13000fA. This made the overall charge of the deposited powder positive. The ELPI charge property was different from the zeta potential measurement, which had negative values. Zeta potential measures the particles suspended in the buffer solution while the ELPI evaluates the true dry-state powder property which included all factors involved in the induction of charge in the DPI-PLGA microparticle system. It has been noted that a particular triboelectrification effect occurs between PLGA microparticles and DPI capsule material, inhaler materials (Peart, Orban, McGlynn *et al.*, 2002). This contact can induce bipolarity of charge on the PLGA control microparticles. When the charger was on, all the particles were given positive charges and stage 9 (cutoff size 1.6μm) and 10 (cutoff size 2.5μm) collected most of the powder (+42000 fA) (Figure 3.9).

PLGA-Ag85B microparticles showed different surface properties upon aerosolization when charger was off. The powders were almost unipolar except the particle sizing from 4.0-10.0μm with slight positive charge (+2000-3000fA). The particles on stage 9 deposited as negative charged particles with the value of –17000fA. The magnitude of negative charges made the overall surface charge negative after aerosolization. The only difference between PLGA control and PLGA-Ag85B formulations was the presence of Ag85B. The existence of Ag85B resisted the bipolar contact charge induction. There are positively and negatively charged amino acid residues in Ag85B protein. Ag85B may behave like an electron buffer when electrons move by the triboelectrification between particles and dry powder inhaler materials. It was estimated that ~53% of the microparticle surface area was covered by rAg85B (assuming that the particle size is 5μm, polymer density is 0.68g/ml, Ag85B loading is 0.56% and Ag85B exists as the nanosize particle (4.5nm diameter) on the microparticle

surface). The presence of Ag85B has the potential to change the surface properties of the microparticles.

When the powder is bipolar, the collision between particles occurs more rapidly than uncharged particles. When the particles are unipolar, the collision rate is smaller than observed for uncharged particles. Thus, highly unipolar particles are less aggregated than particles that are not (Philip, Mehta, Mazumder *et al.*, 1997). This implies that PLGA – Ag85B microparticles may be dispersed easily and that the potential for lung deposition may be improved. When the charger was on, all the particles were coated with positive particles and the largest quantity of positively charged particles were deposited on stage 9.

3.4 Summary

The PLGA microparticle formulations exhibited aerodynamic sizes (MMAD0 from 2.5 to 3.0μm, which was suitable for pulmonary delivery to humans (Inhalator®) and animals (insufflator). The thermal behavior of PLGA formulations containing Ag85B, and/or adjuvants, indicated an interaction between PLGA polymer and MDP, TDB and Ag85B as seen in the depression of the PLGA glass transition temperature. The T_g also indicated the storage temperatures for these PLGA formulations should be less than 10°C to reduce the possible mobility of the components in PLGA. The surface charge properties of PLGA control and PLGA-Ag85B were different after aerosolization from Inhalator®. PLGA-Ag85B retained the unipolar property and this may be good for aerosol dispersion and lung deposition. Overall, the PLGA microparticle formulations were suitable for delivery as aerosols. For the purposes of the present work this observation supports the decision to continue into animal studies of protection from tuberculosis. However, in the event that protection is afforded to the animals, the Inhalator® data demonstrates that the particles are

suitable for delivery to humans, which would be required in the clinical safety, tolerability and protection studies that are beyond the scope of the present work.

Fig 3.1 Experimental apparatus for ELPI measurement of kinetic surface charge properties of PLGA microparticles upon aerosolization. (Courtesy of M. Telko)

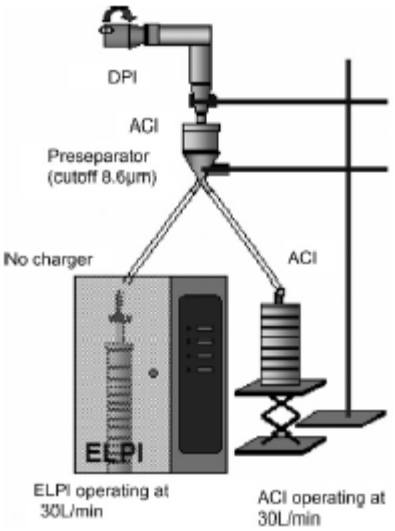
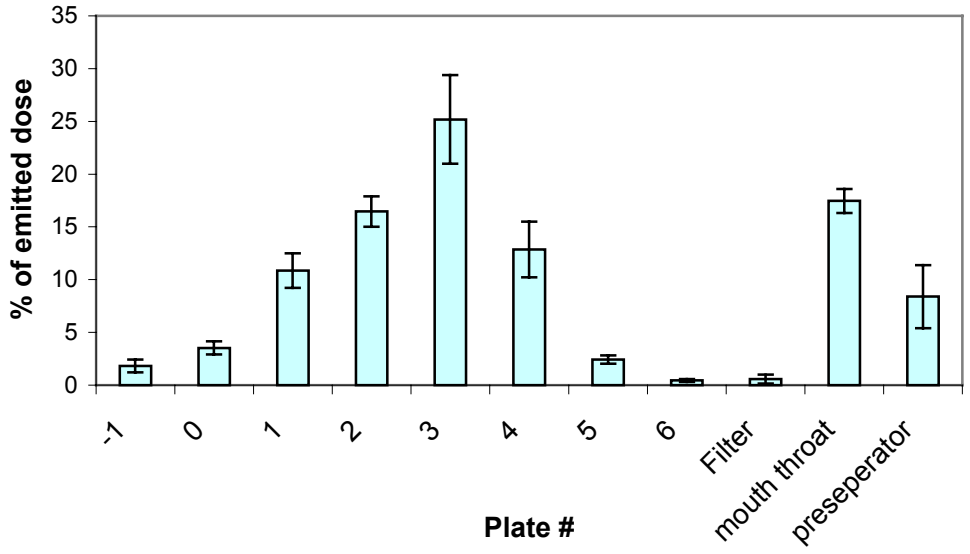


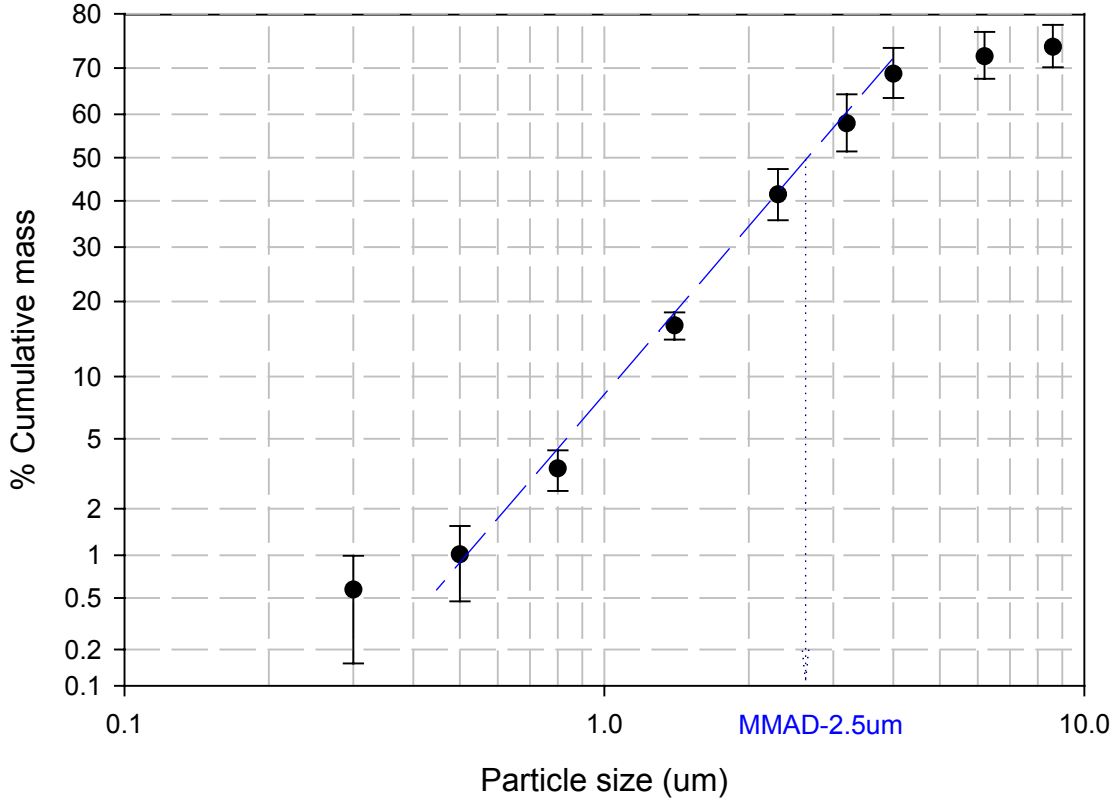
Fig 3.2 Deposition of PLGA-sodium fluorescein microspheres after dispersion from Inhalator® at 60L/min for 10s. Powders were stored in desiccators at room temperature.

A) Deposition pattern (n=3, mean ± SD)



A

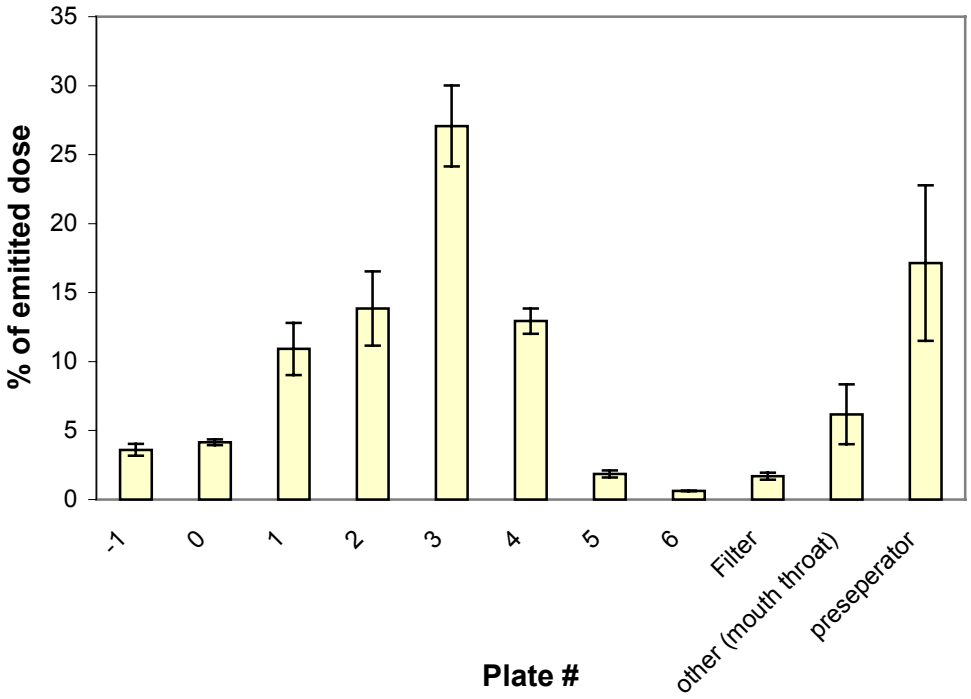
B) MMAD of this powder under the same conditions. (n=3, mean ± SD)



B

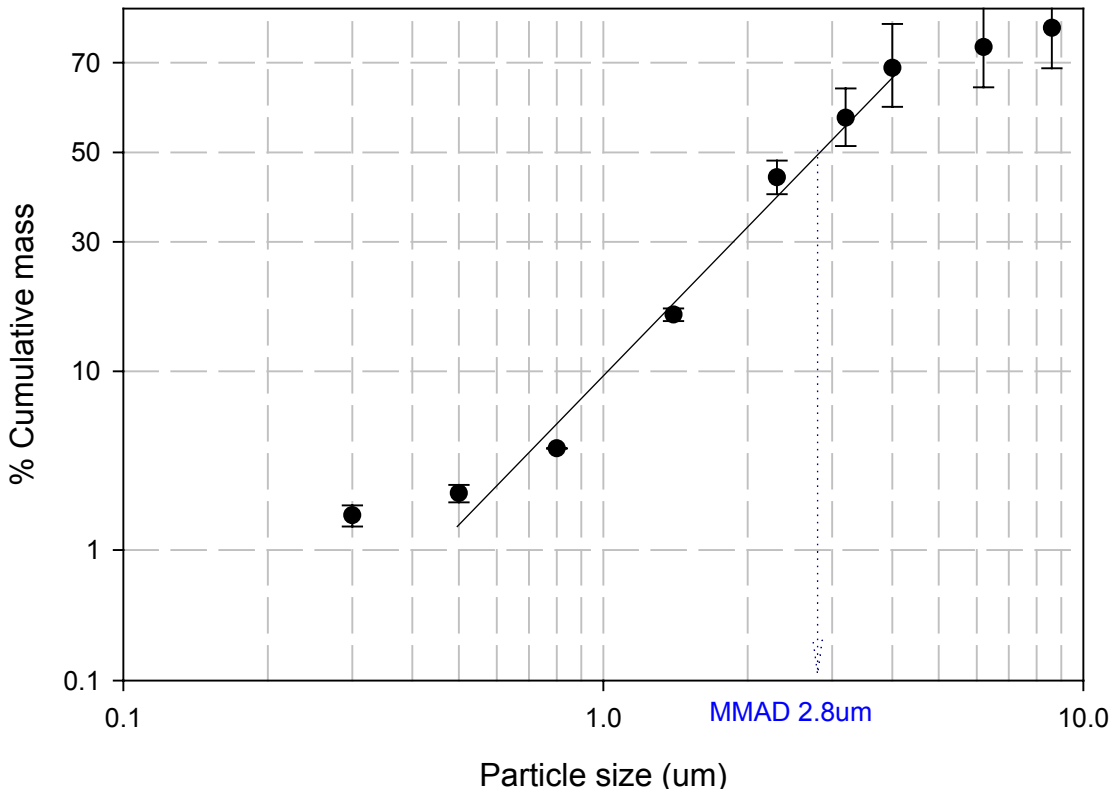
Fig 3.3 Deposition of PLGA microspheres after dispersion from Insufflator DP-3 sampled at 60L/min airflow rate for 10s. Powder stored in desiccators at room temperature.

A) Deposition pattern. (n=3, mean ± SD)



A

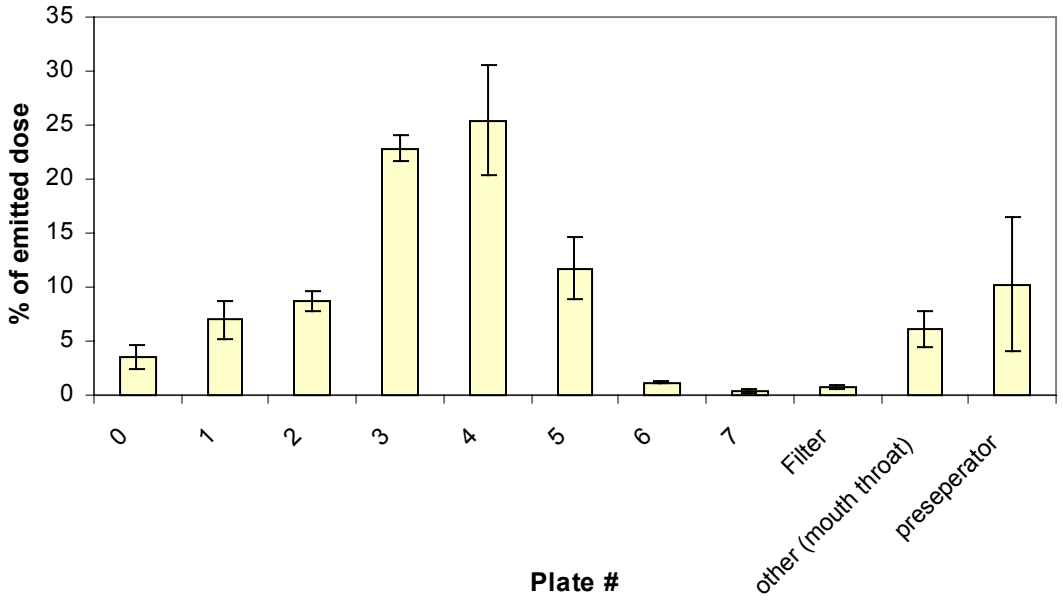
B) Aerodynamic size and distribution (n=3, mean ± SD)



B

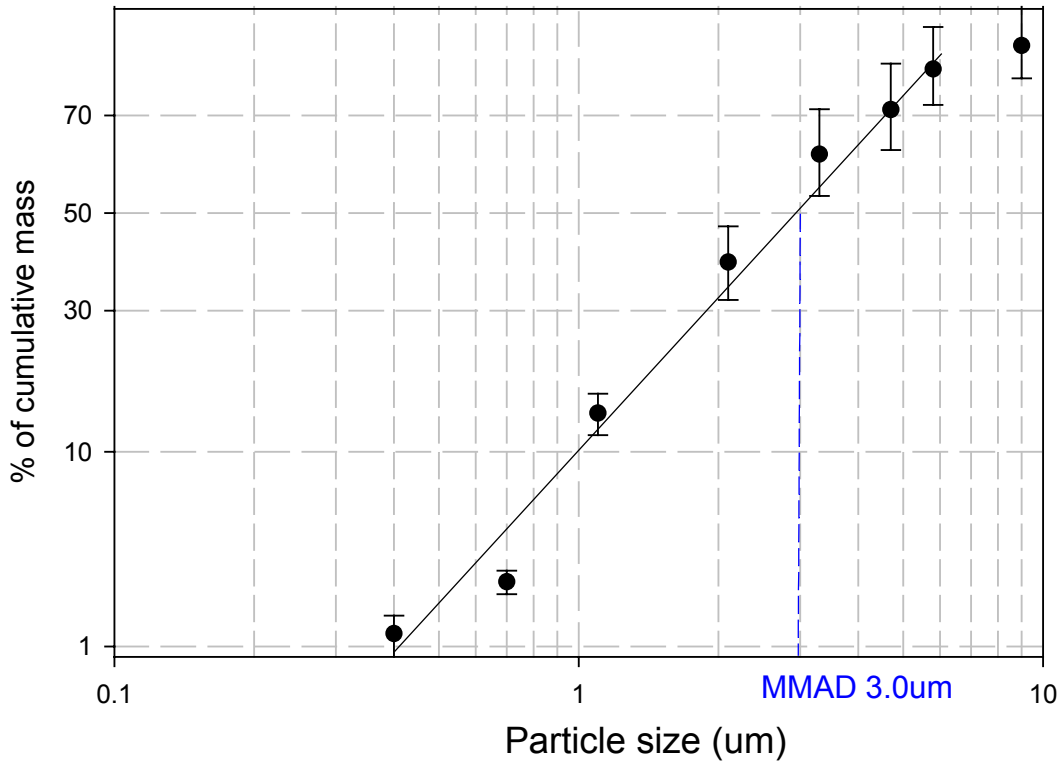
Fig 3.4 Deposition of PLGA microspheres after dispersion from Insufflator DP-3 at 28.3L/min for 10s. Powder stored in desiccators at room temperature.

A) Powder deposition on different stages (n=3, mean ± SD)



A

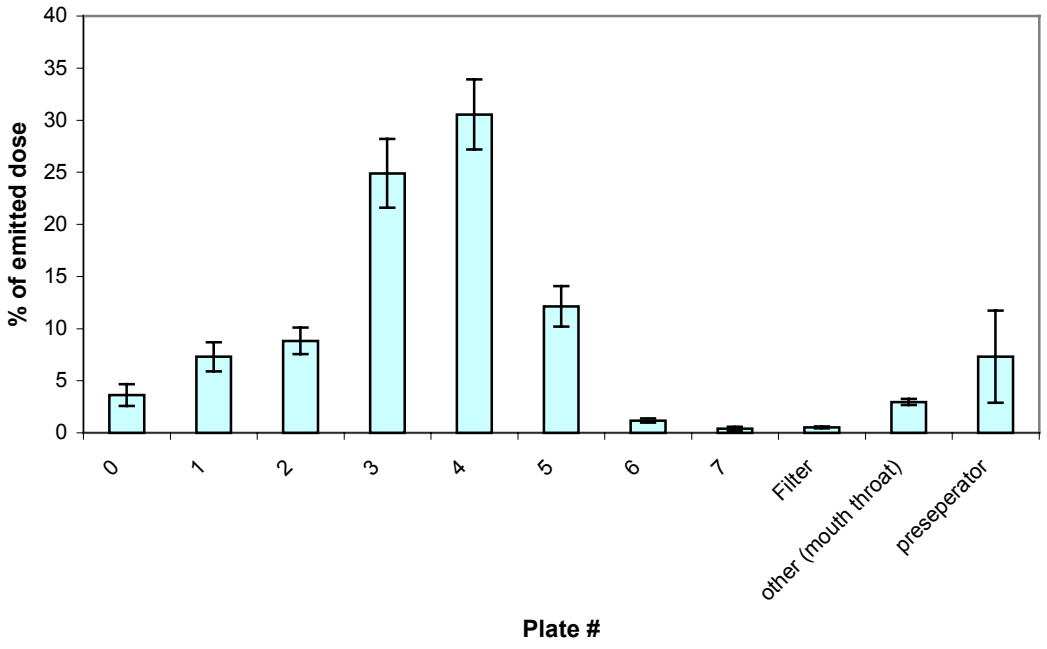
B) Aerodynamic size and distribution (n=3, mean ± SD)



B

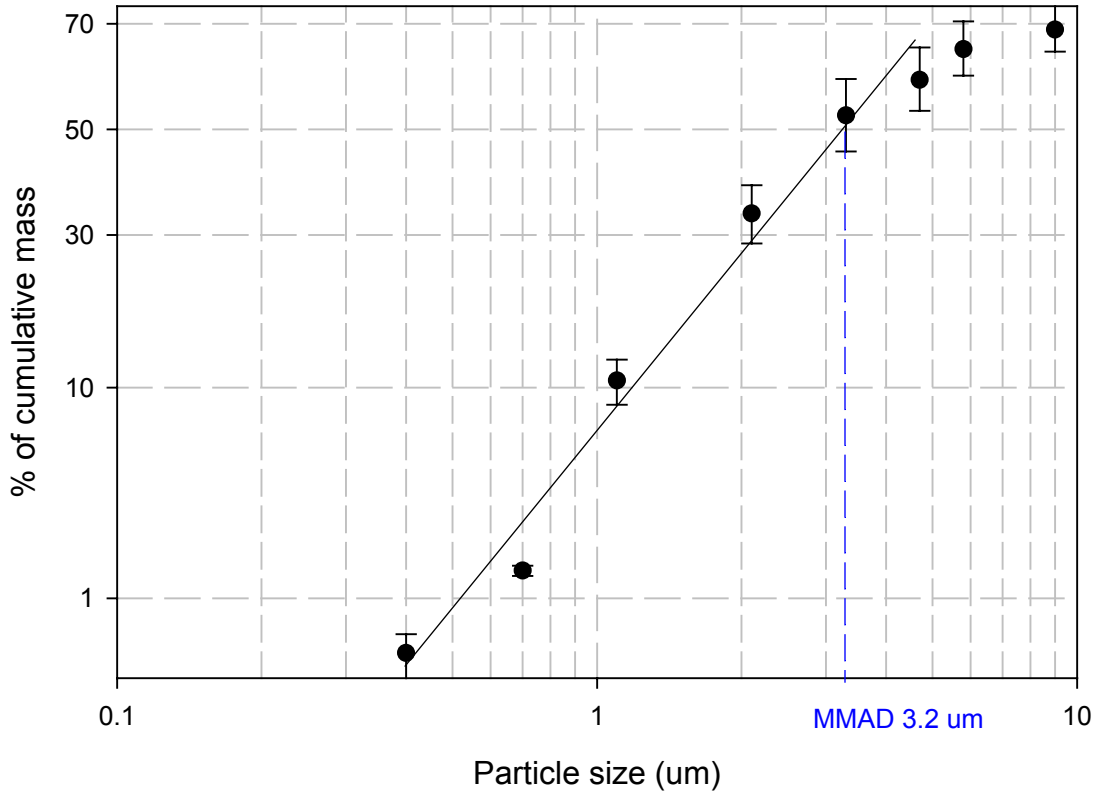
Fig 3.5 Deposition of PLGA microspheres after dispersion from Insufflator DP-3 at 28.3L/min for 10s. Powders were stored over saturated KCl solution for 28days at room temperature.

A) Powder deposition on different stages (n=3, mean ± SD)



A

B) Aerodynamic size and distribution (n=3, mean ± SD)



B

Fig 3.6 DSC thermagrams of spray-dried microparticle at repeated heat scanning at 10°C /min and speed of 20 and 30°C/min.

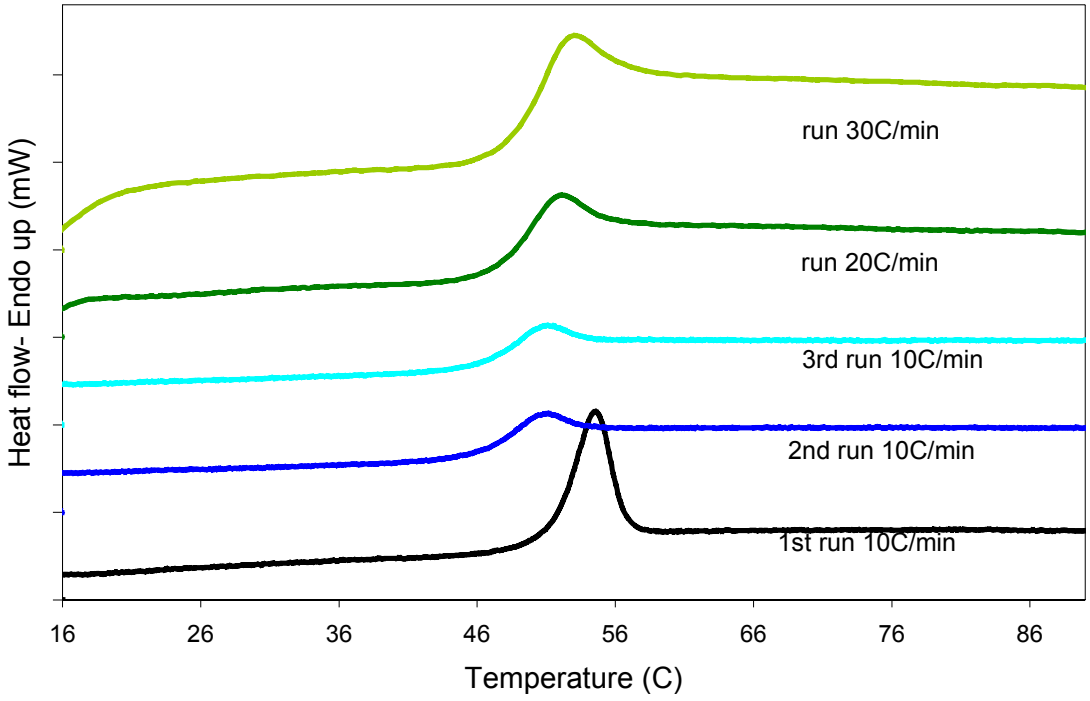


Fig 3.7 DSC thermagrams of spray-dried microparticle PLGA-Ag85B of different loadings (0.28 and 0.56% w/w) at scan rate of 10°C/min.

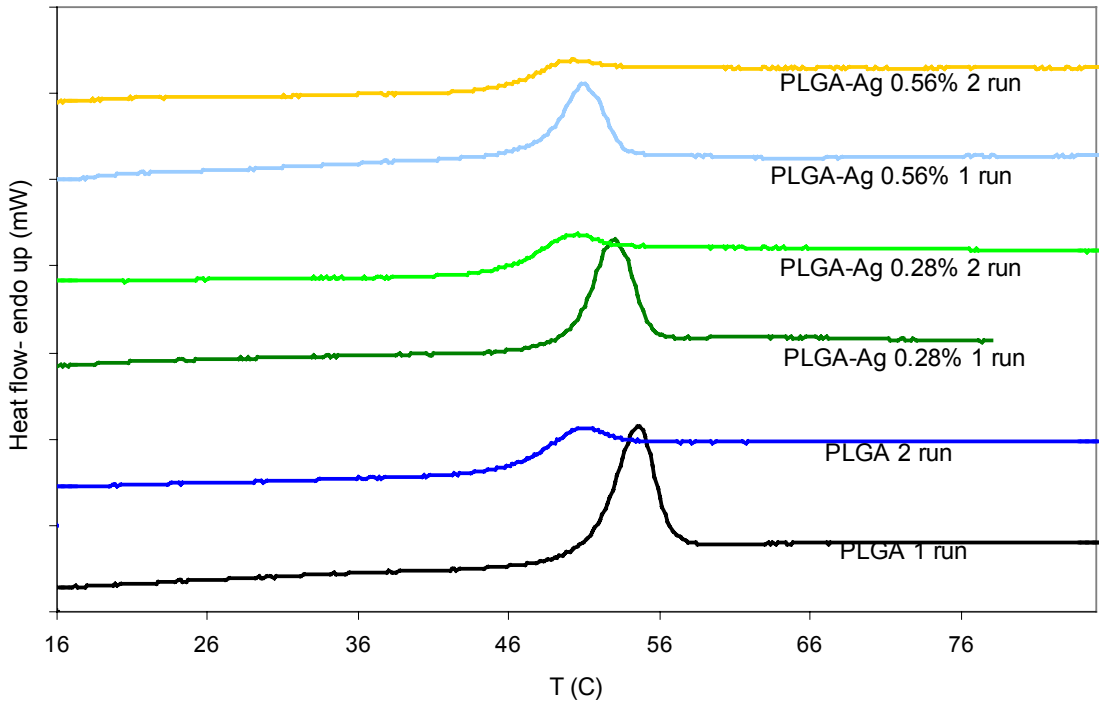
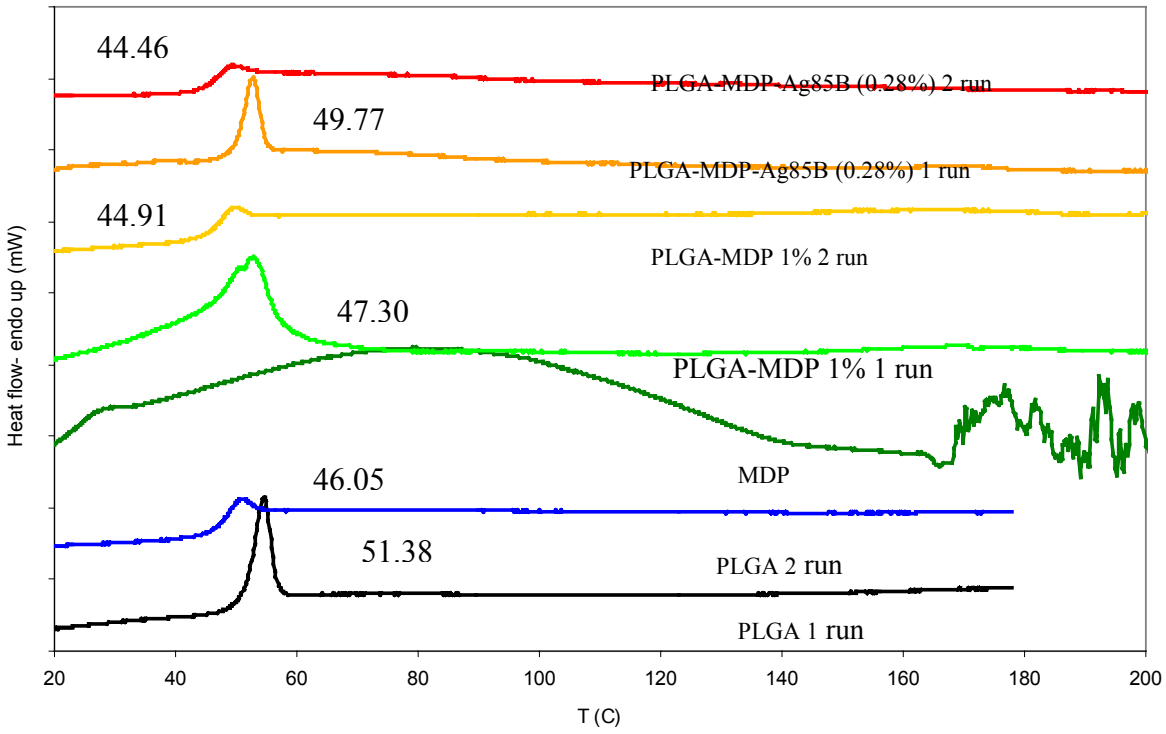


Fig 3.8 DSC thermagrams of PLGA microparticle containing adjuvants with/without Ag85B. Scan rate was 10°C/min.

A) MDP as the adjuvant;



B) TDB as the adjuvant

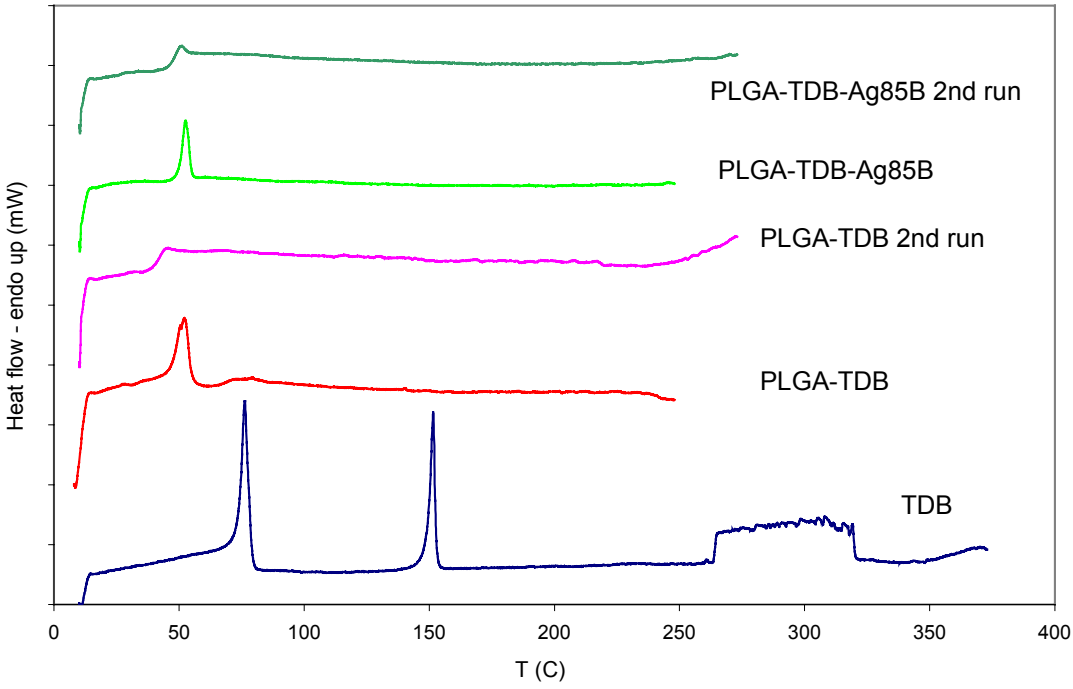
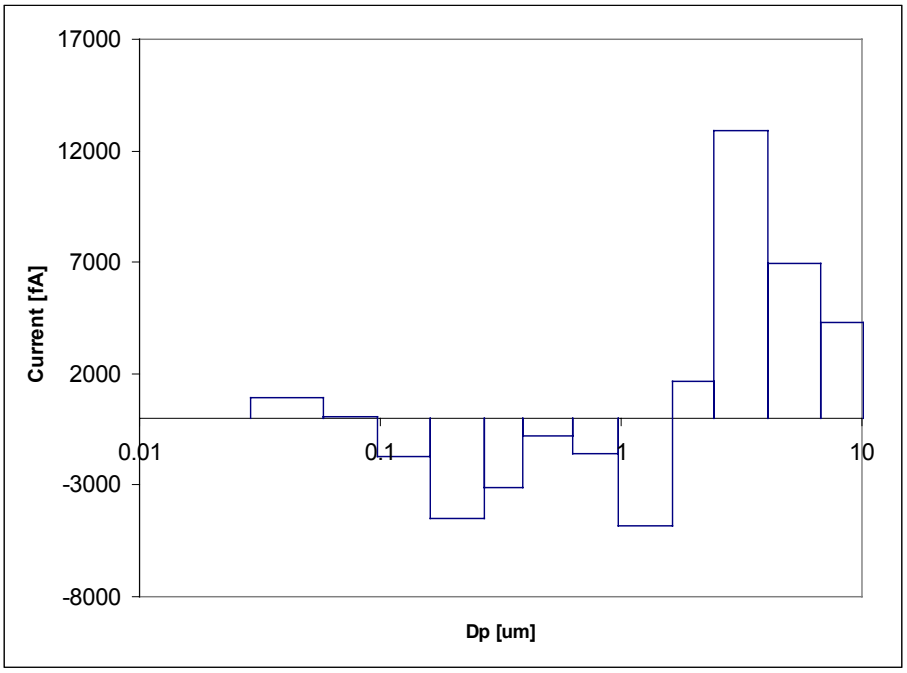


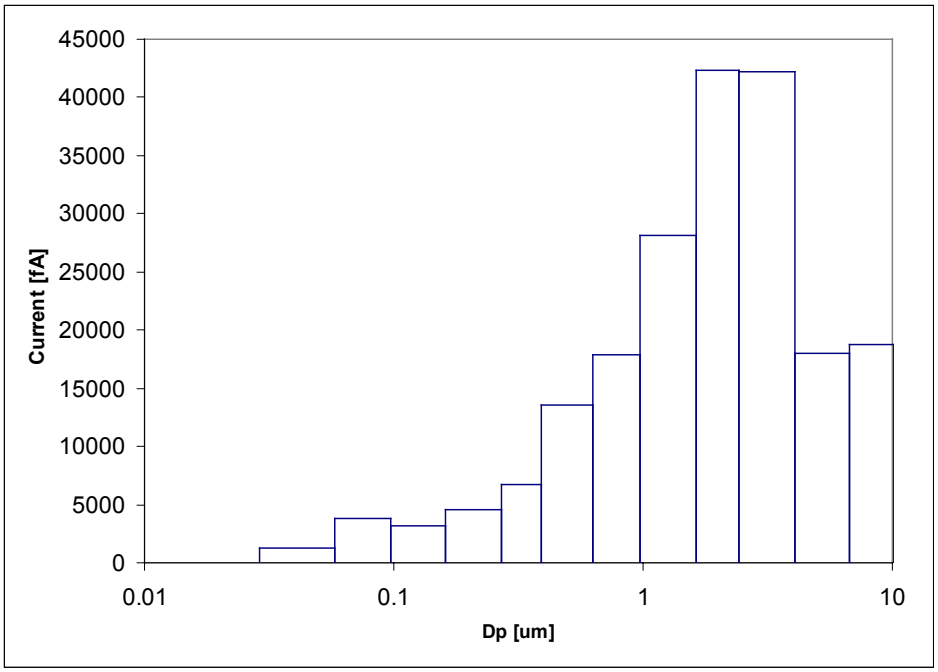
Fig 3.9 The surface charge properties of PLGA microparticle formulations detected by ELPI upon aerosolization.

A) Charger off;



A

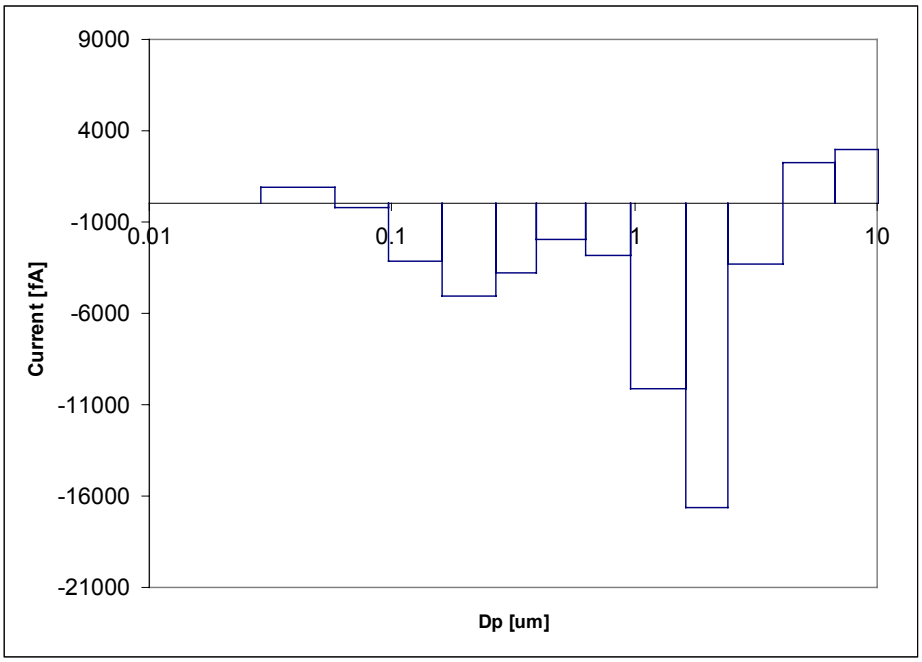
B) Charger on



B

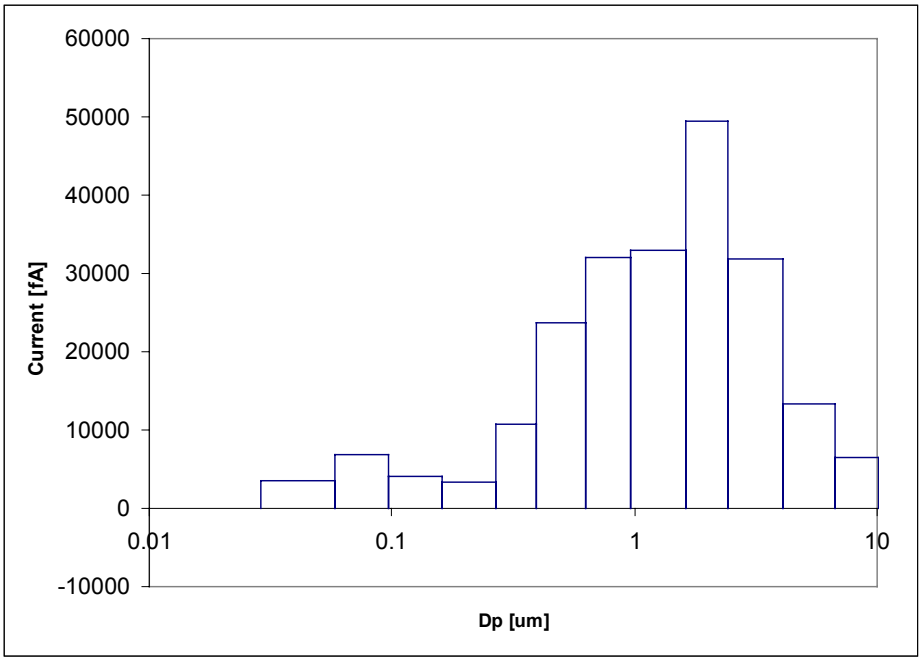
Fig 3.10 The surface charge properties of PLGA-Ag85B microparticle formulations detected by ELPI upon aerosolization.

A) Charger off;



A

B) Charger on



B

Table 3.1 Median volume particle size and distribution of PLGA microparticle formulations.

Batch of PLGA microparticle	MDP (w/w loading %)	TDB (w/w loading %)	rAg85B JM (w/w loading %)	rAg85B Chap (w/w loading %)	D_{v50} (μm)	Span
1	-	-	-	-	4.61±1.00 Dp50: 3.17	1.16±0.16 1.32(GSD)
2	0.1	-	-	-	5.93±0.07	1.42±0.04
3	0.5	-	-	-	7.18	2.09
4	1.0	-	-	-	5.59	1.40
5	-	0.1	-	-	5.84±0.13	2.14±0.30
6	-	0.5	-	-	6.36±1.19	1.98±0.54
7	-	1.0	-	-	8.98	2.35
8	-	-	0.14	-	4.30±0.13	1.38±0.34
9	0.1	-	0.14	-	5.03±0.07	2.54±0.19
10	0.5	-	0.14	-	5.78±0.04 Dp50: 3.74	0.29±0.04 1.33(GSD)
11	1	-	0.14	-	3.61±0.05	0.87±0.04
12	-	0.1	0.14	-	3.73±0.24	1.47±0.55
13	-	1	0.14	-	3.90±0.19	1.05±0.24
14	0.1	-	0.28	-	3.97±0.48	1.14±0.51
15	0.5	-	0.28	-	6.66±0.02 Dp50: 4.40	1.88±0.05 1.30(GSD)
16	1	-	0.28	-	4.28±0.36	1.19±0.36
17	-	0.1	0.28	-	3.55±0.01	0.78±0.01
18	-	0.5	0.28	-	7.40±0.05 Dp50:4.30	1.68±0.06 1.28(GSD)
19	-	1	0.28	-	3.93±0.51	1.24±0.54
20	-	-	0.28	-	3.76±0.14 Dp 4.25	1.88±1.05 1.25(GSD)
21	0.5	0.5	0.28	-	7.71±0.05 Dp50:3.60	2.07±0.01 1.26(GSD)
22	0.5	-	-	0.14	5.99±0.09 Dp50:3.49	0.27±0.03 1.32(GSD)
23	-	-	0.56	-	5.14±0.11	1.34±0.14
24	-	0.1	0.56		4.23±0.13	1.18±0.24

D_{v50}: median volume diameter
D_{p50}: median projected area diameter
GSD: geometric standard deviation

Table 3.2 Mass median aerodynamic size (MMAD), fine particle fraction (FPF) and emitted dose of microparticle formulations under different conditions.

	Inhalator	**DP-3**	**DP-3**	**DP-3**
Operating condition	60 lpm	60 lpm	28.3 lpm	28.3 lpm
Powder condition	desiccated	desiccated	desiccated	85% RH
MMAD (μm)	2.5 ± 0.4	2.8 ± 0.3	3.0 ± 0.1	3.2 ± 0.2
FPF of emitted dose (%)	68.8 ± 5.2 [a]	68.9 ± 8.4 [a]	71.1 ± 7.8 [b]	78.5 ± 6.4 [b]
Emitted dose (%)	90.1 ± 2.7	92.9 ± 8.1	92.2 ± 2.1	57.8 ± 32.9
FPF of total dose (%)	61.2 ± 3.7 [a]	64.0 ± 8.2 [a]	65.5 ± 4.0 [b]	45.4 ± 14.5 [b]

a: aerodynamic size < 4μm
b: aerodynamic size < 4.7μm

Table 3.3 Flow properties of PLGA microparticle formulations (n=3, mean ± SD)

Bulk density (g/ml)	0.083 ± 0.02
Tapped density (g/ml)	0.11 ± 0.09
Static angle of repose (°)	39.10 ± 1.90
Carr's compressibility index (%)	25.00 ± 1.00
Hausner ratio	1.32 ± 0.23

Table 3.4 Specific surface areas of PLGA microparticles under different degassing conditions.

	Degassing condition	Specific surface area (m^2/g)
1	No degassing, r.t	14.71 ± 0.46
2	r.t 22hr	15.76 ± 0.12
3	40°C 3hr	6.65 ± 0.14

Table 3.5 Moisture content of PLGA microparticles at room temperature and ambient humidity (34-35% RH)

Time (hr)	Moisture content (%)
0	0.70
4	2.18
48	2.96

Table 3.6 Glass transition onset temperature and heat capacity of spray-dried PLGA microparticles.

Scanning rate (°C/min)	No of run	T_g onset (°C)	ΔCp (J/g*°C)
10	1	51.38	0.272
	2	46.05	0.482
	3	46.48	0.472
20	2	47.42	0.413
30	2	48.18	0.476

Table 3.7 Glass transition onset temperatures of different PLGA formulations.

Sample	initial run T_g onset (°C)	2nd run T_g onset (°C)
PLGA	51.38	46.05
PLGA-Ag 0.28%	50.06	45.52
PLGA-Ag 0.56%	48.17	45.67
PLGA-MDP 1%	47.30	44.91
PLGA-MDP 1%-Ag 0.28%	49.77	44.46
PLGA-TDB 1%	47.17	42.05
PLGA-TDB 1%-Ag85B 0.28%	49.98	46.10

Table 3.8 Zeta potentials of different PLGA formulations.

Zeta potential	**Zeta meter 3.0**		
	counts	Mean (mV)	SD
PLGA control	20	-50.9	6.5
PLGA-MDP(0.5%)-Ag85B(0.28%)	20	-45.0	8.6
PLGA-TDB(0.5%)-Ag85B (0.28%)	21	-56.6	4.6
PLGA-MDP(0.5%)-TDB(0.5%)-Ag85B(0.28%)	25	-49.5	7.3
9a PLGA-MDP(0.5%)-Ag JM(0.14%)	21	-41.6	6.2
10a PLGA-MDP(0.5%)-Ag Chap(0.14%)	20	-42.0	9.1
PLGA-MDP-(0.5%)	20	-49.9	7.4
PLGA-TDB(0.5%)	21	-46.6	6.0
PLGA-MDP(0.5%)-TDB(0.5%)	22	-45.7	8.6
	ZetaPlus		
	Zeta potential (mV)		SE
PLGA control	-49.7		0.9
PLGA-Ag85B (0.14%)	-45.3		1.1
PLGA-Ag85B (0.28%)	-38.4		2.3
Ag85B	-37.6		1.2

4 Study of Ag85B in microparticle systems delivered into the lungs for protection against tuberculosis in guinea pig model.

4.1 Introduction

The effectiveness of a vaccine is demonstrated by protection from microorganisms that induce diseases. Ultimately, the protective effect must be afforded to a human population. However, in the first instance, animal models of disease have proven helpful in assessing protection from virulent challenge. There is a substantial history of vaccine development for prevention of tuberculosis, in which protection has been evaluated in guinea pigs.

In 1995, Horwitz *et al.* demonstrated that intradermal immunization 3-4 times with 100μg Ag85B /time or a combination of Ag85B with four other culture filtrate proteins protected guinea pigs from weight loss (a weight gain of 15% was observed), death, lung destruction and bacterial burden in the lungs (reduced 0.8 log cfu (colony forming unit)) and spleens (reduced 0.8 log cfu) compared with control animals (Horwitz, 1995). Quantities of 300-400μg of the subunit antigen Ag85B, and the same amount of four other antigens protected animals from infection to some extent. Delivery of a small amount of a vaccine composed of PLGA and Ag85B might be suitable for homologous immunization (using the same antigen for prime and boost vaccination) due to the efficiency of particle uptake by macrophages.

Currently, BCG is the standard against which new vaccines are compared. rBCG30, a recombinant BCG expressing Ag85B (Horwitz, Harth, Dillon *et al.*, 2000), and a BCG overexpressing ESAT-6 (Pym, Brodin, Majlessi *et al.*, 2003) have been reported to afford

superior protection compared to BCG. Multiple BCG vaccination is not a good strategy as indicated by decreased survival in guinea pigs infected with *MTB* (Basaraba, Izzo, Brandt *et al.*, 2006), the reduced protection level in neonatal calves after revaccination with BCG (Buddle, Wedlock, Palane *et al.*, 2003) and little, or no, efficacy in clinical trials (Kubit, Czajka, Olakowski *et al.*, 1983; Leung, Tam, Chan *et al.*, 2001; Tala-Heikkila, Tuominen and Tala, 1998).

For a subunit antigen against tuberculosis, another largely neglected approach to improve the immune resistance of the human population to tuberculosis is to enhance the immunity against *MTB* of persons previously vaccinated with BCG by heterologous boosting with subunit antigens.

Only a few animal models have proven relevant to the pathogenesis of tuberculosis in humans (McMurray, 1994; Orme and McMurray, 1997). The guinea pig is sensitive to *MTB* infection, manifesting a pulmonary infection at low bacterial doses, while the alternative model, the mouse, is resistant to such exposure. The guinea pig reflects the natural history of the progressive infection of *MTB* and lung pathology seen in humans with active tuberculosis. Pulmonary infection induces "classical" granulomas replete with necrosis and cell breakdown, which has many similarities to human tuberculosis. The progress of the early infection is of importance because there is a silent tuberculosis bacillemia between initial implantation of the organism within the alveolus and the subsequent development of specific cell-mediated immunity. This results in seeding of other parts of the lungs and other organs (Smith, McMurray and Wiegeshaus, 1970). In humans, infection is nearly always contained and most organisms are killed once the cell-mediated response is mature. However, viable organisms persist in sites that were inoculated during the early bacillemic phase. The factors

responsible for the continued containment of *MTB* are poorly understood, but clearly include maintenance of an intact cell-mediated response. As a result, the guinea pig model is currently considered the "gold standard" for tuberculosis candidate vaccine testing (Orme, 2005b). This model will be employed in the present studies for vaccination followed by a virulent low dose *MTB* (*H37Rv*) aerosol challenge. It is important to note that guinea pigs exhibit an excellent antigen specific DTH response in skin, which allows evaluation of systemic immunity following vaccination. Successful aerosol vaccination of guinea pigs with BCG has been demonstrated (Lagranderie, Frehel and de Chastellier, 1991). However, this observation was not pursued to a formulation strategy and the use of subunit proteins has not been evaluated.

After the APCs phagocytose the pathogens or vaccines, the epitopes presented with MHC class I/II are recognized by naïve T cells. Naïve CD4 T cells can differentiate upon activation into either Th1 or Th2 cells, which differ in the type of cytokines they produce. The role of effector T helper cells is to control whole immune responses by secreting a multitude of cytokines and by expressing surface molecules. Th1 cells are associated with IFN-γ, IL-12 and TNF-α whereas Th2 cells typically produce IL-4, IL-5, IL-10 and IL-13. CD4 T cells polarization depends on the cytokine environment to which the cells are exposed (Langenkamp, Messi, Lanzavecchia *et al.*, 2000). Naïve CD8 T cells are predestined to become cytotoxic. Proliferating CD8 T cells acquire effector function and migrate to the peripheral site of inflamed tissue to eliminate infected cells and release cytokines capable to slow down pathogen replication. Cytotoxic T-lymphocytes (CTL) responses against certain pathogens seem to require the presence of CD4 T cells during priming of naïve CD8 T cells.

Hence, a potent CTL vaccine should contain both CD4 and CD8 epitopes (Ridge, Di Rosa and Matzinger, 1998).

Homologous and heterologous vaccination regimens will be used in this protection study. Single dose and multiple doses of PLGA-Ag85B and PLGA-TDB (0.1%) - Ag85B were used as the immunization formulations. In multiple dose studies, one group of animals were primed with BCG and then boosted by PLGA-Ag85B aerosol formulations. Guinea pigs challenged with low dose inocula of bacteria were used for anti-*MTB* immunization study.

4.2 Materials and methods

Animals: Dunkin-Hartley guinea pigs ~350g were obtained from established breeders (Charles River Breeding Laboratories, Portage, MI). The animals were housed in the university core facility and provided food, water and husbandry.

Aerosol Vaccination: The vaccination regimen is shown in Fig 4.1. Five groups were employed in the single dose regimen. Six guinea pigs were assigned to each group. PLGA placebo as microparticle control, BCG as positive control and untreated animals as negative controls were included in this regimen. BCG (1×10^3 cfu, Tice strain, Organon USA Inc, Roseland, NJ) was subcutaneously administered to guinea pigs. PLGA-Ag85B (0.56% w/w), PLGA-TDB (0.1% w/w)-Ag85B (0.56% w/w) were the vaccination formulations containing subunit antigen. Animals were anesthetized with a mixture of ketamine (50mg/kg), xylazine (5mg/kg) prior to insufflation. ~10mg of PLGA microparticle formulations were insufflated (Model DP-3, Penncentury, PA) into the airways of guinea pigs. These two treatment groups were intradermally boosted 6 weeks later with Ag85B 100μg, TDB 200μg in 0.2% Pluronic L62 and 0.1% Tween 80 in 100μl PBS due to the absence of skin response to purified protein

derivative (PPD) and rAg85B. Half of the negative controls were administered with the same soluble antigen formulation mentioned above as the boosting control. In the multiple dose regimen, the animals were primed with PLGA-Ag85B (0.56% w/w, 10mg insufflation), PLGA-TDB (0.1% w/w)-Ag85B (0.56% w/w, 10mg insufflation) and BCG (s.c. 1×10^3 cfu). The animals were boosted 4 weeks later with ~10mg of PLGA-Ag85B (0.56% w/w)(for PLGA-Ag85B and BCG groups) and PLGA-TDB (0.1% w/w)-Ag85B (0.56% w/w)(for PLGA-TDB-Ag85B group) and 10 weeks after initial vaccination by insufflation of approximately ~3mg of PLGA-Ag85B (2.24% w/w)(for PLGA-Ag85B and BCG groups) and PLGA-TDB (0.1% w/w)-Ag85B (2.24% w/w)(for PLGA-TDB-Ag85B group). Animal weights were recorded twice a week. Six weeks after the last boost, the vaccinated animals were transferred to BL-3 lab.

Cutaneous delayed type hypersensitivity (DTH): The skin test was performed one day before virulent strain challenge. Purified protein derivative (PPD) (100TU, Mycos Research, Loveland, CO) from *MTB H37Rv* and Ag85B (50μg in 100μl PBS) were injected intradermally (i.d.) on the two flanks of the animals. The injection sites were measured 24 hours after i.d. injection for mean diameter of induration or erythema.

Aerosol Exposure to MTB virulent strain *H37Rv*: A whole body exposure chamber was employed for quantitative experimental infection of animals by inhalation. A Collison nebulizer-Venturi unit (Waltham, MA) was employed to atomize the suspension of *MTB,* strain *H37Rv* (ATCC, Manassas, VA), at a concentration of 5×10^4 cfu in phosphate buffered saline (PBS), and mixed it with secondary air before it was drawn into the exposure chamber (University of Wisconsin, Mechanical Engineering Workshop, Madison, WI) by a vacuum pump. The droplet cloud was delivered to the exposure chamber, thereby exposing the

confined guinea pigs (Grover, Kim, Wiegeshaus *et al.*, 1967; Wiegeshaus, McMurray and Grover, 1970). Five minutes after nebulization, the primary air supply was stopped and airflow through the chamber continued for 10 minutes. A series of HEPA filters placed in the exhaust airline between the exposure chamber and vacuum pump removed microorganism before air discharge. Nebulization of this amount of *MTB*, *H37Rv,* has been shown to result in the inhalation and retention of 3-5 cfu in the periphery of the lungs (Smith, 1991).

After exposure to the virulent bacilli, guinea pigs developed primary pulmonary lesions that resemble typical human tubercles (McMurray, 1994). There is an exponential increase in viable mycobacteria recovered from lungs of guinea pigs between days 3 and 21 post-infection. Between days 14 and 18, the first organisms appear in the trachebronchial lymph nodes draining the lung fields. At approximately 18 to 21 days, mycobacteria are detected in the spleen (McMurray, 1994).

Necropsy and Bacteriology: The necropsy and bacteriology studies were performed 4 weeks after virulent *MTB* challenge. Animals were anesthetized with pentobarbital (175mg/kg) and exsanguinated prior to necropsy. The peritoneal and chest cavities were exposed and the right lung lobe was inspected for grossly visible primary lesions prior to resection and homogenization. Lobes taken for histological evaluation were kept in 10% formalin until used. Guinea pig spleens were removed aseptically and weighed. The organ weights were recorded. Tissue samples were homogenized using Teflon-glass homogenizers, the homogenate was diluted with sterile saline and aliquots were inoculated onto duplicate Middlebrook 7H10 plates of oleic acid albumin agar. Plates were sealed with oxygen permeable tape and incubated at 37°C. Colonies were counted after 21 days.

4.3 Results and discussions

4.3.1 Weight of animals after virulent strain challenge

Sudden weight loss is one of the syndromes of active tuberculosis. Guinea pig models also demonstrate this symptom of infection (Baldwin, 1998). The weights of animals were measured twice a week until necropsy studies. The weight changes after virulent *MTB* challenge were recorded for single and multiple doses groups. In single dose groups, the mean values of total weight gain for groups of PLGA-Ag85B, PLGA-TDB-Ag85B formulations, BCG and untreated controls were 7.6, 2, 116.2 and 95.3g, respectively. BCG positive control groups showed consistent positive weight gain until 21 days post challenge and started to lose weight on day 26 before necropsy. Untreated controls and PLGA placebo groups gained weight slightly less than BCG and the weight loss began 18 days after challenge. The weight gains of PLGA-Ag85B and PLGA-TDB-Ag85B were much less than the untreated controls at almost each time point (Fig 4.2A). PLGA-TDB-Ag85B groups lost weight from day 14, much earlier than any other groups in single dose regimen. It should be noted that these groups were boosted i.d. with Ag85B (see methods section). This additional step was taken when there was no apparent immune response to the prime vaccine with this antigen as indicated by the absence of response to PPD and rAg85B. The weight change observed for the solution Ag85B boosted group requires further investigation.

In the multiple-dose groups, there was a trend that all the animals gained weight until day 22 post-challenge. Untreated controls and PLGA-TDB-Ag85B treated groups began losing weight on day 26. While BCG prime: PLGA-Ag85B boosts and multiple doses of PLGA-Ag85B consistently gained weight to the conclusion of the study. The total weight gains for multiple doses of PLGA-Ag85B, PLGA-TDB-Ag85B, BCG prime:PLGA-Ag85B

boosts and untreated controls were 266 ± 6, 66 ± 19, 264 ± 15 and 122 ± 4g, respectively (Fig 4.2B). Treatments of homologous multiple doses of PLGA-Ag85B and BCG prime: PLGA-Ag85B aerosol boosts resulted in almost the same weight gain and the weight gains in these two groups were significantly higher than those of the untreated control group.

4.3.2. DTH response in treated animals

Six weeks after single dose vaccination, BCG groups had DTH induration responses to purified protein derivative (PPD) but no apparent responses to Ag85B except some erythema in the tested area. The animals vaccinated with PLGA formulations containing Ag85B showed erythema at the injection site in response to Ag85B but no response to PPD. Therefore, 100μg Ag85B, 200μg TDB in 0.2% Pluronic L62 and 0.1% Tween 80 in 100μl PBS was intradermally applied to boost the PLGA-Ag85B and PLGA-TDB-Ag85B groups. Six weeks following the boost, the control animals boosted with Ag85B solution showed responses to Ag85B. Both treatments of aerosol PLGA-Ag85B: i.d. solution Ag85B and aerosol PLGA-TDB-Ag85B: i.d. solution Ag85B showed strong DTH responses to Ag85B but only slight or no reactions to PPD. The diameters of induration for Ag85B responses were 13.8 mm ± 2.9 (SD), 14.2 mm ± 4.1 (SD) and 11.0 mm ± 1.4 (SD) for groups of PLGA-Ag85B: solution Ag85B, PLGA-TDB-Ag85B: solution Ag85B and untreated control with Ag85B solution, respectively. There was no significant difference among these three groups in terms of induration diameters. BCG groups responded strongly to the PPD test, but Ag85B skin test only induced redness in this group.

In the multiple-dose groups, BCG prime: PLGA-Ag85B boost groups had responses to PPD but negligible DTH responses to Ag85B. PLGA-Ag85B and PLGA-TDB-Ag85B did not respond to PPD, only slight redness to Ag85B skin test.

Currently, PPD is the only reagent for diagnosis of infection with tuberculosis. Upon inoculation into the skin of sensitized individuals, a delayed-type hypersensitivity (DTH) response is evoked (Koch, 1897). This reagent allows the detection of individuals sensitized by mycobacteria (Hart, 1967). It is also used to assess the degree of immunity conferred by the only tuberculosis vaccine BCG (WHO, 1963). However DTH reaction does not correlate well with vaccine-derived protection from infection (Fine, Stern, Ponnighaus *et al.*, 1994). The specific antigen can also be used in the skin test to detect the systemic cell-mediated immunity. In this study, PPD and Ag85B were used for detection of DTH responses prior to challenge. After i.d solution Ag85B boosting of the single dose groups, there were significant DTH responses to Ag85B rather than redness before id solution antigen boost. However, in these groups consistent protection was not observed in the lungs and spleens. However, there was slight protection effect in spleen for PLGA-TDB-Ag85B formulations. PPD did not elicit a DTH response in the groups of PLGA formulations containing Ag85B. In the multiple dose groups, BCG prime:PLGA-Ag85B boost responded to PPD to some extent. All the animals in this regimen did not respond to Ag85B. These results concurred with the lack of DTH induced by *MTB* culture filtrate protein (CFP) in guinea pigs despite protective effects in the lungs (Baldwin, 1998).

The skin test results may help guide the development of a TB vaccine. The existence of strong adjuvant may help antigens to induce strong DTH reaction (Baldwin, 1998) but it cannot be inferred that this will result in protection. The solution Ag85B formulation consisted of 100μg Ag85B, 200μg TDB and 0.2% Pluronic L62. This formulation, containing a large quantity of TDB, is a very strong adjuvant. However, it's relatively large induration readings did not correlate with a protective effect in guinea pigs (see section of

4.3.3). Pais *et al.* reported that the specificity of T cells involved in the classical tuberculin reaction to PPD were different from those involved in protective immunity (Pais, Silva, Smedegaard *et al.*, 1998). Whether this is applicable to the skin response to Ag85B is unknown. However, the immunological specificity required to respond to a single antigen would suggest that this might be the case.

4.3.3. Mycobacterial burden in organs

The lungs and spleens were resected for determination of number of colony forming units of *MTB*, at necropsy. In single dose regimens, there was no difference between untreated control and control boosted with Ag85B solution. The formulations of PLGA placebo, PLGA-Ag85B and PLGA-TDB-Ag85B did not significantly reduce the *MTB* burden in the lungs compared with the untreated controls (Fig 4.3A). PLGA-TDB-Ag85B showed a drop (0.78 log cfu/g) in bacterial burden compared with the untreated control ($p< 0.05$) in spleen (Fig 4.3B). PLGA-TDB-Ag85B most likely reduced systemic *MTB* disseminated from the lungs at some time during the development of infection in guinea pigs. However, this adjuvant antigen combination was not as good as BCG in reducing the bacterial burden in lungs and spleens.

There were three vaccination strategies in the multiple-dose regimen. Neither of homologous multiple doses of PLGA formulations containing Ag85B effectively reduced the *MTB* burden in the lungs or spleens compared with untreated controls. The only effective vaccination regimen was the priming with BCG and then boosting twice with PLGA-Ag85B aerosols. This regimen significantly reduced the *MTB* burden in lungs and spleens compared with untreated controls ($p<0.05$) (Fig 4.4). Although in the multiple dose groups the *MTB* burden in the lungs of untreated controls seemed greater than that in single dose groups, BCG

prime: PLGA-Ag85B boost groups had lower *MTB* burden (0.3 Log cfu/g) compared with the BCG groups and the untreated controls (1.5 log cfu/g) in the raw data. This *MTB* reduction in the lungs by PLGA-Ag85B after BCG priming was substantial, considering the Ag85B mass of ~83μg in PLGA formulation. The data variation among the BCG prime:PLGA-Ag85B boost group was small (SD = 0.16) which indicated that the guinea pigs responded similarly among individuals within the group. Therefore, the boosted PLGA-Ag85B enhanced the immunization of BCG. A logical control experiment for these studies would involve boosting a BCG vaccine with BCG alone. However, this is known to result in a serious life threatening immune repsonse that would be unacceptable (Basaraba, Izzo, Brandt *et al.*, 2006).

The raw data (Table 4.1) were analyzed by comparing the protective effect (log cfu/g untreated group – log cfu/g treatment group) to evaluate the multiple doses versus single dose regimens. Positive values indicate fewer bacteria in the organs and greater protection (Fig 4.5). The lungs showed no significant difference between multiple dose and single dose regimens for PLGA-Ag85B and PLGA-TDB-Ag85B. However, statistically there was a significant difference between BCG and BCG prime:PLGA-Ag85B boost groups. Animals boosted twice with PLGA-Ag85B, by insufflation, exhibited enhanced lung protection (p = 0.014).

Comparison of the spleen data did not show any significant difference in the effect resulting from single dose and multiple doses of PLGA-Ag85B. However, PLGA-TDB-Ag85B treatments showed different spleen protection pattern between single and multiple dose regimens ($p < 0.05$). The single dose reduced the bacterial burden (0.78 log cfu/g)

compared with untreated control but additional boosts obviously depleted the protective effect of the first dose.

Neither single dose nor homologous multiple doses of PLGA formulations containing Ag85B appeared to protect the lungs from *MTB* infection as indicated by the similar bacterial burden in lungs compared with untreated controls. The memory CD4 T cells require restimulation before acting on target cells (Janeway, Travers, Walport *et al.*, 2001). Single dose immunization with PLGA formulations is insufficient for this purpose. Multiple doses repeatedly stimulate CD4 T memory cells specific for Ag85B epitopes. However, in the present studies, no protection was observed in the lungs or spleens. The ability of the immune system – T cells to recognize antigens in *in vitro* bioassay, and the ability to induce protection following immunization *in vivo* do not necessarily correlate with each other. CD4 Th1 immunity is an important factor in protection from tuberculosis and IFN-γ is a relevant cytokine marker to evaluate this response. However, high level of IFN-γ in the lung parenchyma does not imply protection. There is experimental evidence that despite large IFN-γ and IL-2 cytokine responses with respect to IL-4 and IL-5, no protective effects were seen in mice after virulent *MTB* challenge (Majlessi, Simsova, Jarvis *et al.*, 2006).

Although protection was not evident in animals treated with single dose and homologous multiple dose of PLGA formulations containing Ag85B, there were some differences between these two treatments. Firstly, the single dose of PLGA-TDB-Ag85B resulted in a statistical reduction of spleen *MTB* burden ($p \leq 0.05$) while the multiple doses of PLGA-TDB-Ag85B did not decrease bacterial burden in the spleen. Dendritic cells may be involved in the response to aerosol immunization. In the single dose, due to particle distribution, some of the aerosol powders deposited in the central airway where it is believed

that dendritic cells are located (Gong, McCarthy, Telford *et al.*, 1992). After dendritic cells take up particles, they become mature and migrate to lymph nodes to present epitope to T cells. The T cells either come back to the infected area or permeate through the lymph nodes and serve in the systemic cell-mediated immunity. Repeated doses of aerosol particles possibly gained greater access to alveolar macrophages. Under these circumstances, the number of activated macrophages might exceed that of the dendritic cells. This could depress the function of dendritic cells. Macrophages can profoundly inhibit T cell responses to antigen presented by respiratory dendritic cells possibly by soluble inhibitors secreted by macrophages (Gong, McCarthy, Telford *et al.*, 1992; von Garnier, Filgueira, Wilstrom *et al.*, 2005). Therefore, the immunity invoked by a large involvement of dendritic cells with respect to macrophages could diminish as more macrophages are activated.

Secondly, a difference between single and multiple doses was observed with respect to the weight gain. In single dose, PLGA formulations containing Ag85B both lost weight significantly compared with untreated controls. In multiple doses, groups of heterologous BCG prime: PLGA-Ag85B boost and homologous PLGA-Ag85B both gained weight with respect to untreated controls. BCG prime: PLGA-Ag85B boost reduced *MTB* burden in lungs and spleens, which correlated with the weight-gain data. Histopathology data may explain the discrepancy of weight gain and non-protection in lungs and spleens for group of PLGA-Ag85B. Lesion severity is, undoubtedly, a critical component of survival of infected animals. The degree of lymphocytic infiltration may have a better correlation with weight gain, survival and protection in guinea pig model (Baldwin, 1998).

TB vaccine development should not ignore the identification of vaccines that can effectively boost preexisting immunity (McShane and Hill, 2005). There are several reasons

for this strategy. One is simplicity. A large proportion of the worldwide population has been immunized with BCG or exposed to infection with mycobacteria. Therefore, a more realistic use of subunit antigen vaccines would be to boost individuals previously vaccinated or those who may be at risk of reactivation disease due to latent tuberculosis. Another consideration is the ethical dilemma related to a first study in man. In order to conduct a Phase III trial on any TB vaccine in an endemic region, BCG would be given beforehand in all of the study groups, including the control group. These constraints would require any vaccine tested to build on, and be evaluated with respect to, BCG-initiated immunity (Xing, Santosuosso, McCormick *et al.*, 2005). A heterologous regimen was used in the guinea pig study. The vaccination regimen of BCG prime: PLGA-Ag85B aerosol boosts significantly enhanced the protection efficacy of BCG alone in terms of *MTB* burden in lungs and spleens. In consideration of the amount of Ag85B used, only 83μg subunit antigen was used in these two boostings. The aerosol fine particle fraction (<5μm) of this formulation was 65%, and in theory the fine particle dose would only be 54μg. The protective effect of this pulmonary boosting immunization in lungs was comparable to that performed by Horwitz *et al.* who intradermally boosted Ag85B 100μg after BCG priming (Horwitz, Harth, Dillon *et al.*, 2005). Our data confirmed that Ag85B is capable of enhancing protective immunity upon boosting (Horwitz, Harth, Dillon *et al.*, 2005) although different administration routes were used. It is critical that the primary immunization establishes a favorable immune response (balanced Th1 / Th2 immunity) (Sable, Goyal, Verma *et al.*, 2007). Priming with BCG appears to allow a favorable immune response to Ag85B protein, which is also expressed and secreted by BCG. However, further studies are required to confirm the observed results before the evidence is considered conclusive.

4.4 Summary

The pulmonary boost of PLGA-Ag85B appeared to enhance the protection afforded by BCG alone. This heterologous prime:boost immunization strategy was observed to reduce *MTB* burden in the lungs and spleens in guinea pigs compared with repeated single subunit antigen immunization strategy, which did not demonstrate protection within the limits of the experimental design. Although there was no influence in terms of *MTB* burden in lungs and spleens, the weight gain of guinea pigs in homologous multiple-dose group of PLGA-Ag85B was comparable to BCG prime:PLGA-Ag85B boost groups. This weight gain indicates that the guinea pig while apparently demonstrating the same degree of infection as some of the other groups (based on bacterial burden) may not exhibit the same severity of disease and degree of metabolic involvement. Histopathology and immunological (cytokine and chemokine profiles) data are needed for further interpretation of the data. There is the necessarity to repeat the protection study to confirm these results.

The PLGA microparticle systems delivered as aerosols appear to be a good method of boosting to enhance the BCG-induced immunity. PLGA microparticles encapsulating Ag85B may be an effective delivery system for pulmonary administration of vaccine to prevent *MTB* infection.

Fig 4.1 Vaccination regimens for single dose and multiple dose groups.

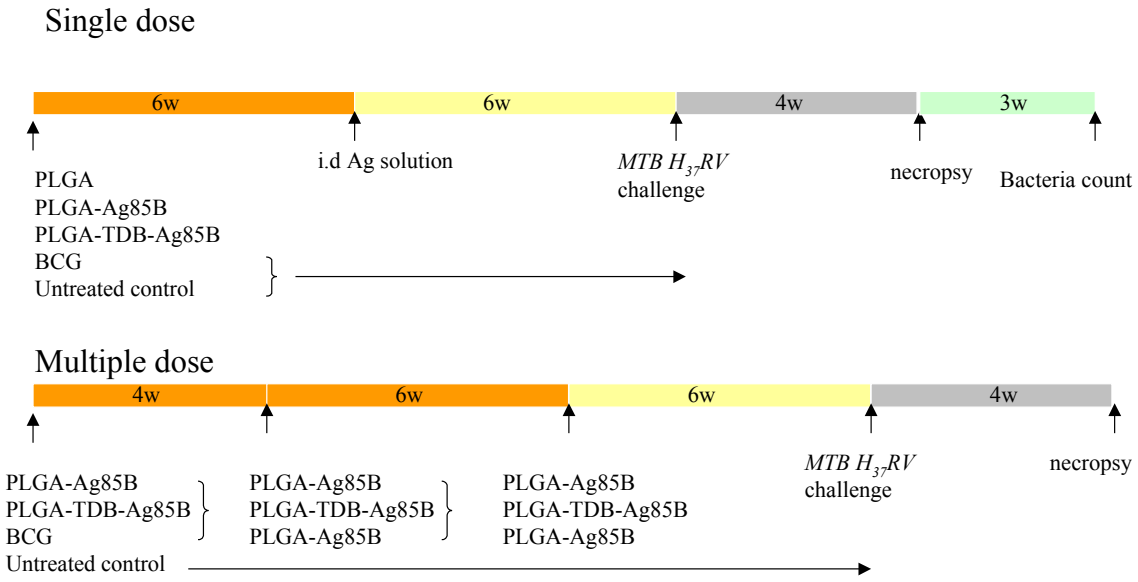
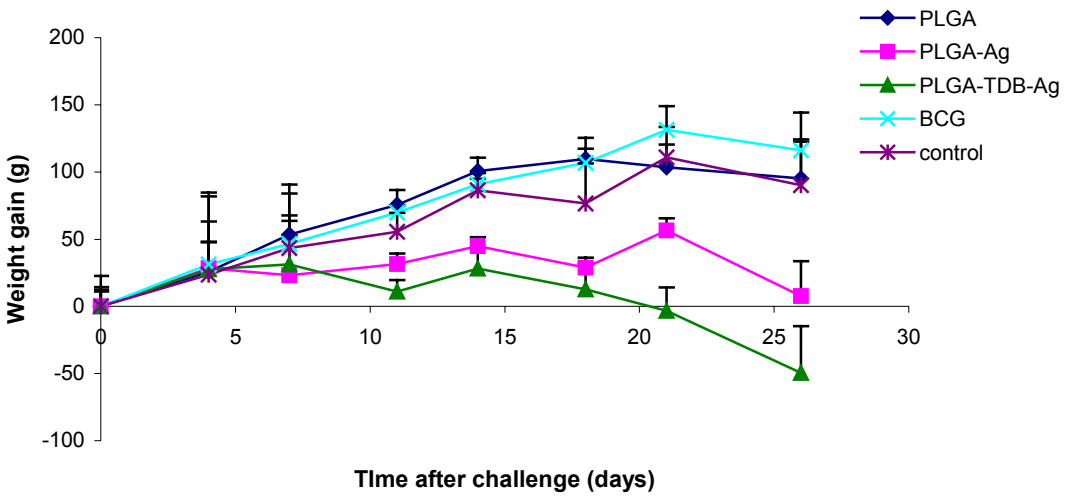


Fig 4.2 Weight profiles of guinea pigs with respect to time following infection in different dose regimens after virulent *MTB* challenge.

A) Single dose groups;



B) Multiple dose groups.

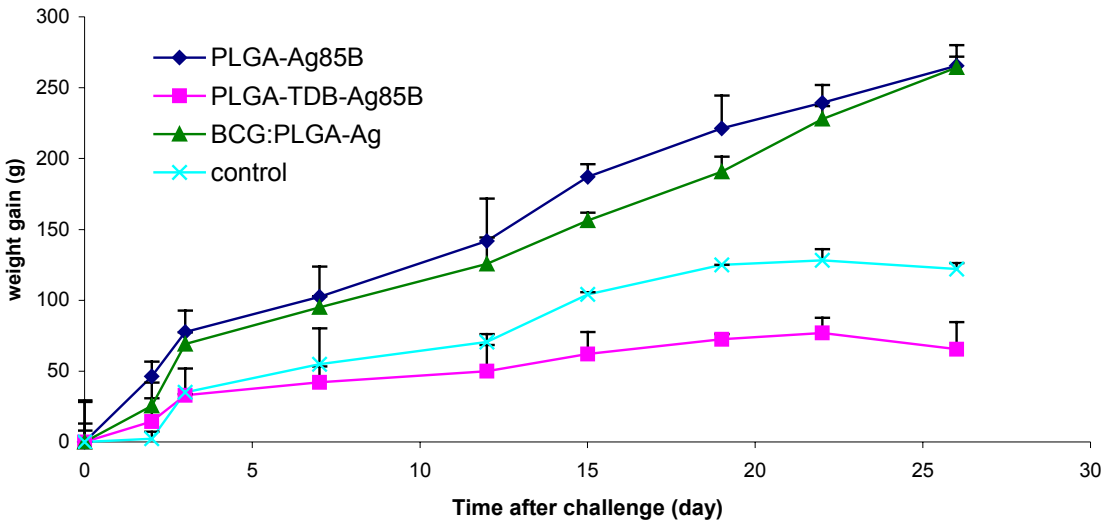
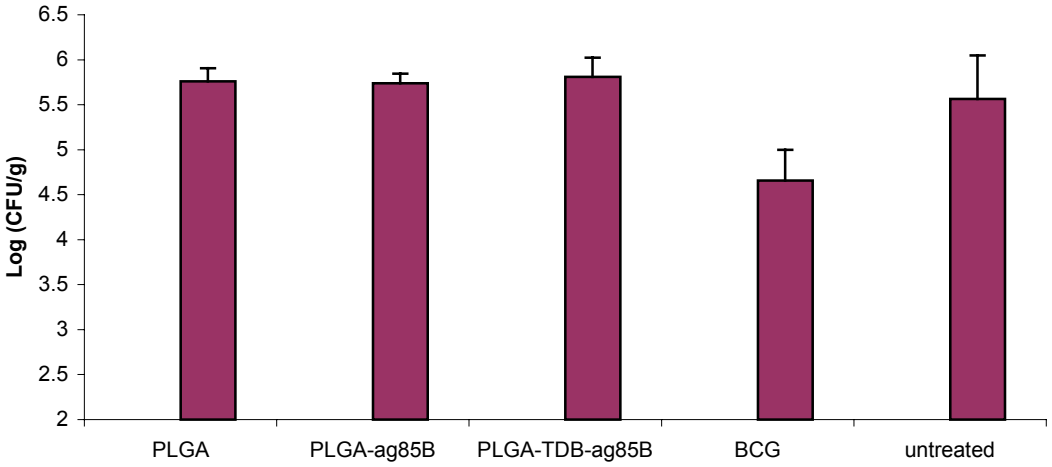


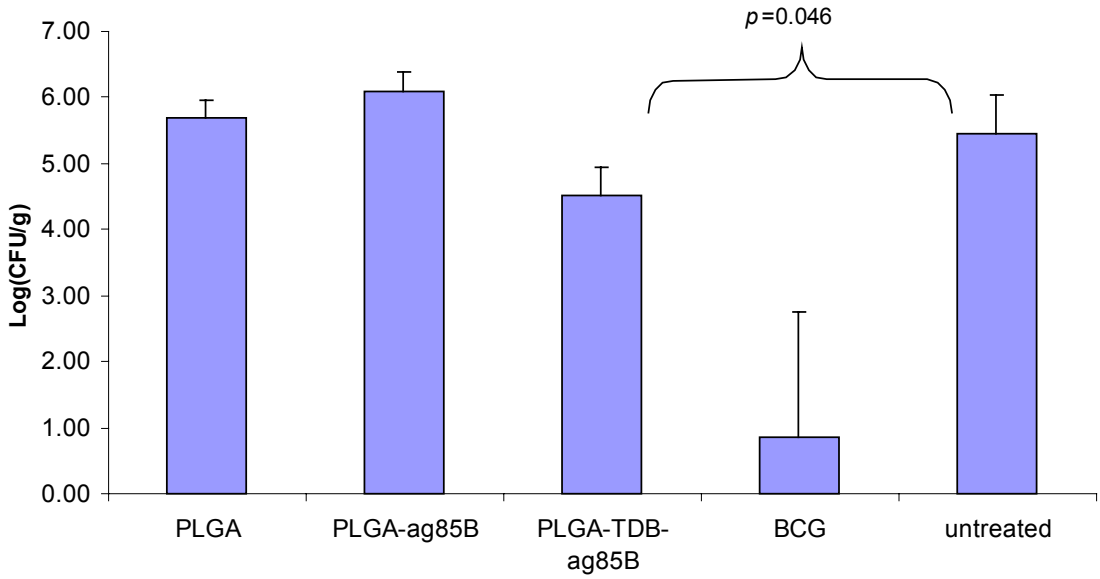
Fig 4.3 Bacterial burdens in different organs after single dose vaccination.

A) In lungs; (n=6, mean ± SD)



A

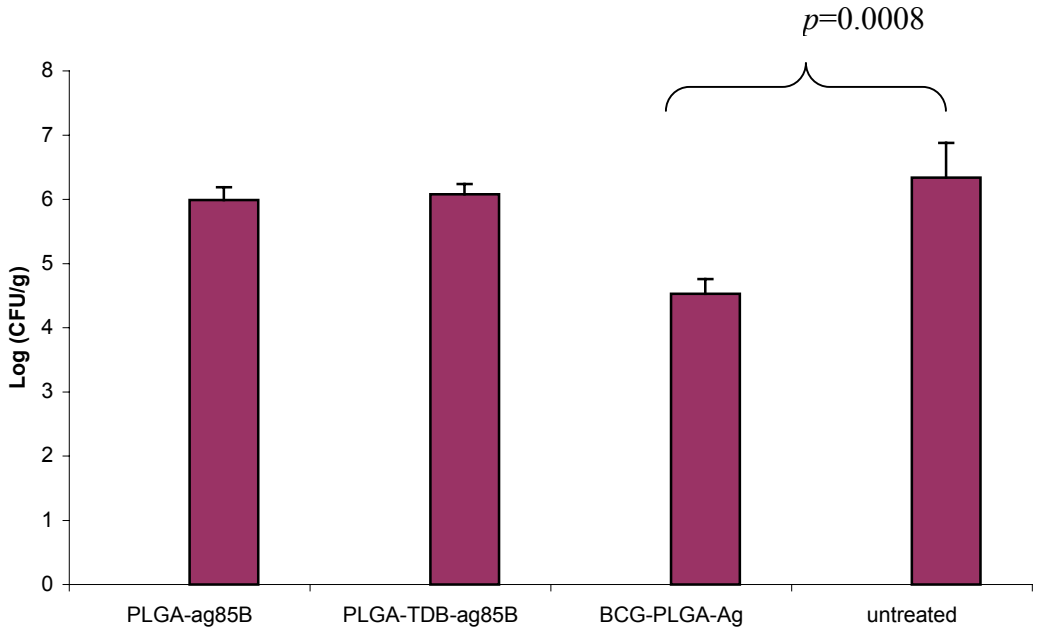
B) In spleens (n=6, mean ± SD)



B

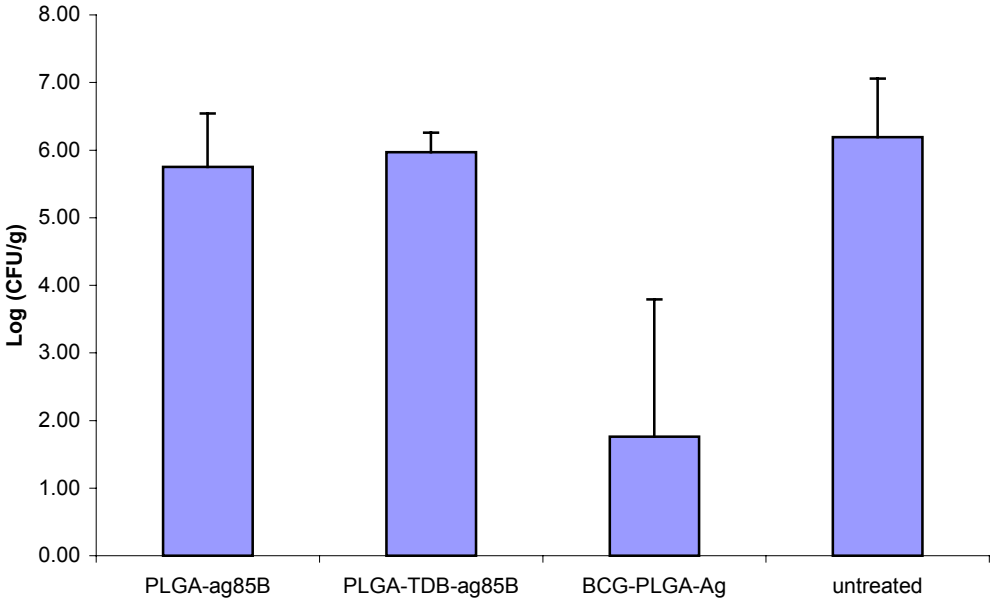
Fig 4.4 Bacterial burdens in different organs after multiple dose vaccination.

A) In lungs; (n=6, mean ± SD)



A

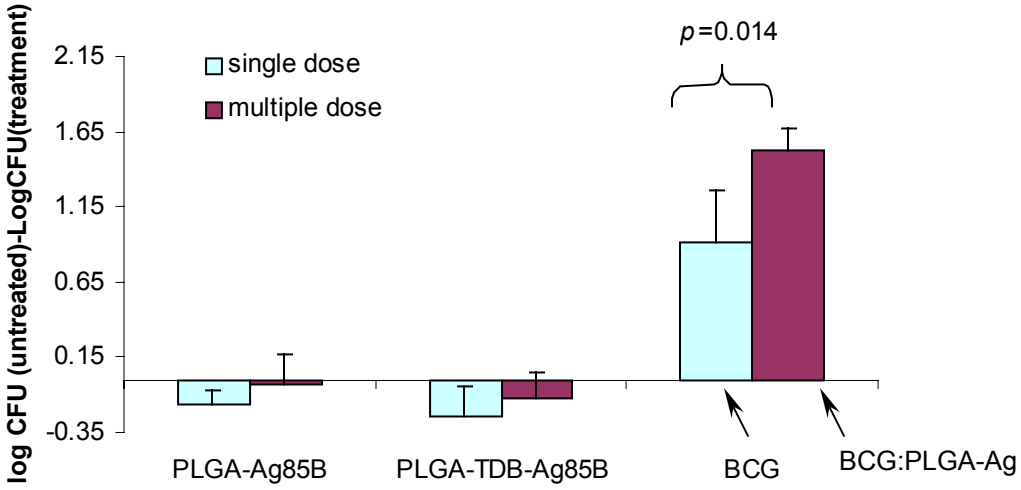
B) In spleens (n=6, mean ± SD)



B

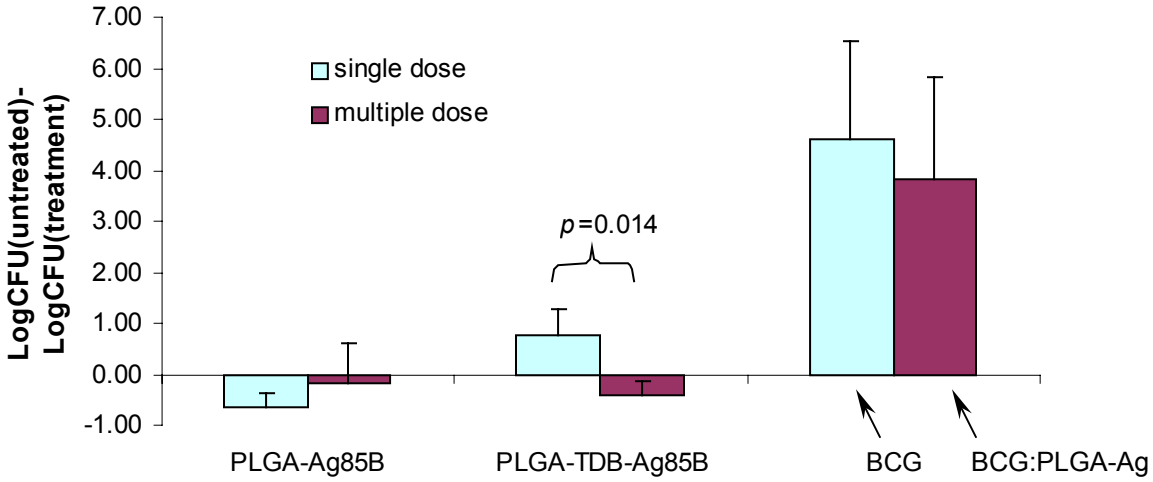
Fig 4.5 Protections in *MTB* burden in different organs between single and multiple doses vaccinations after standardization (Log (cfu of untreated)- Log (cfu of treatment)). In single dose BCG, in multiple doses group BCG prime and PLGA-Ag85B boost.

A) In lungs.



A

B) In spleens.



B

Table 4.1 Protections from TB in the lungs for different vaccination regimens.

Regimen	Dose	Organ	Protections (Log (cfu of untreated controls in each group) –log (cfu of treatment))
PLGA-Ag85B	single	lung	-0.17
PLGA-Ag85B	multiple	lung	-0.03
PLGA-TDB-Ag85B	single	lung	-0.24
PLGA-TDB-Ag85B	single	spleen	0.78
PLGA-TDB-Ag85B	multiple	lung	-0.12
BCG	single	lung	0.91
BCG:PLGA-Ag85B	multiple	lung	1.52

5. General Discussions

Tuberculosis remains one of the leading causes of death from a single infectious agent. Due to problems with current the TB vaccine, BCG's, including high variability in protection worldwide (Brewer, 2000; Fine, 1995) and diminishing immunity over 10-15 years (Brewer, 2000), more than 200 TB vaccine candidates have been tested in different animal and primate models (Ginsberg, 2000; Izzo, Brandt, Lasco *et al.*, 2005; Orme, 2005a) over the last ten years. However, research into alternative immunization routes has been rare. TB is primarily a respiratory mucosal infectious disease. Mucosal vaccination, especially pulmonary mucosal immunization, provides good protection against airborne infection (Lu and Hickey, 2007). Meanwhile, formulation of immunogens is one of the key factors influencing the final vaccination outcome. Microparticle formulation, which takes advantages of the macrophage sensitivity to particulates, is easily internalized by APCs to enhance immunity. Therefore, immunogens, formulations and immunization route are key factors that need to be considered in TB vaccine development.

The research described in this dissertation, addresses inclusion of TB secreted Ag85B in a microparticle formulation (PLGA microparticles) for aerosol delivery by the pulmonary route, demonstrating strong antigenicity in an *in vitro* cell assay and sufficient inmunogenicity in an *in vivo* guinea pig model. The heterologous vaccination regimen of BCG prime: PLGA-Ag85B aerosol boost appeared to enhance the immunity elicited by BCG alone. PLGA microparticle dry powders are suitable for delivery as inhalation aerosols. The powder state potentially increases the stability of the immunogens encapsulated in this

formulation thereby reducing the cold chain requirement compared with the traditional BCG parenteral formulations. This is meaningful in developing countries where the cold chain requirement for protein/whole organism solutions/suspensions may not be possible. In addition, the risk of transmission of diseases, in general, from inadvertent or deliberate re-use of needles is avoided. Pulmonary vaccination, using microparticle systems encapsulating subunit antigens, is a novel strategy in TB vaccine development. The heterologous boost strategy is an alternative to homologous regimens. The latter may not provide significantly greater or even equivalent protection compared with BCG in experimental models. This needle-free vaccination will not only ease patient compliance, but also enhance the local immunization effect. For campaigns of mass immunization, needle-free immunization would reduce the cost and needle-related contamination considerations.

There are three specific aims in this dissertation research. The achievements in each specific aim are outlined below (Table 5.1).

5.1 Recombinant Ag85B expression and PLGA microparticle formulation manufacture

The generation of recombinant Ag85B from *E. coli* systems with sufficient antigenicity and the manufacture of PLGA microparticle formulations encapsulating rAg85B/adjuvants were the main objectives in specific aim I. The *in vitro* T hybridoma cell recognition assay was utilized for antigenicity screening. Two rAg85B were expressed from two systems with sufficient antigenicity. The rAg85B from *Origami B* strain carrying mutated reductases and chaperone vector had higher antigenicity than that from JM strain in the tested concentration range. These antigens were incorporated at different concentrations together with different loadings of adjuvants MDP or TDB into PLGA microparticles by a spray drying process under the optimized conditions discovered by factorial experimental

design. The microparticles are in the suitable size range as aerosols. The morphology of PLGA microparticles was irregular and raisin-like with hollow interior structure as demonstrate by scanning electron microscopy. The dissolution profile of rAg85B was different from adjuvant MDP from PLGA microparticle formulations. rAg85B exhibited a pulsatile release which peaked at days 1 and 20. Following an initial burst during the first day, MDP showed a continuous release up to 30 days. The objectives of production of recombinant Ag85B with sufficient antigenicity to elicit a marker of immune response *in vitro* and the manufacture of PLGA microparticles encapsulating rAg85B with/without adjuvants in respirable sizes were fulfilled.

5.2 The immunological advantages of microparticle formulations over solution formulation of rAg85B and the physico-chemical suitability of PLGA microparticles as aerosols

A successful pulmonary vaccine formulation must meet the physical property requirements for aerosol delivery. Their immunological effects are important factors in their use for vaccine development.

The physico-chemical properties of microparticles provided more information on stability, flow property and hygroscopicity of formulations. The PLGA microparticle formulations exhibited an aerodynamic size (MMAD) range from 2.5 to 3.0μm, which was suitable for pulmonary delivery to humans (Inhalator®) and animals (insufflator). Moisture influenced the fine particle dose by decreasing the emitted dose from the insufflator. This microparticle formulation had moderately good flow properties. The thermal behavior of PLGA formulations containing Ag85B and/or adjuvants illustrated that there was an interaction between PLGA polymer and MDP, TDB and Ag85B as seen in the depression of

the PLGA glass transition temperature (T_g). The T_g also indicated that the storage temperatures for the PLGA formulations should be less than 10°C, to reduce the possible mobility of the components in formulation. The surface charge properties of PLGA control and PLGA-Ag85B were different after aerosolization from Inhalator®. PLGA-Ag85B retained the unipolar property and this may be good for aerosol dispersion and lung deposition. Overall, the PLGA microparticle formulations are suitable to be delivered as aerosols.

The PLGA microparticle formulations could be internalized into the macrophage-like cells THP-1. PLGA microparticles encapsulating rAg85B delivered antigen to THP-1 cells for processing and presentation in the context of MHC class II to a CD4 T-hybridoma cell line DB-1. Microparticle formulations containing rAg85B were more effective in stimulating THP-1 cells to present antigen epitopes to the DB-1 T-hybridoma cells than rAg85B protein in solution. This demonstrated the strong adjuvant effect of PLGA microparticles. Submicrogram amounts of rAg85B in microparticles induced 92-360 fold greater release of IL-2 than the same amount of soluble rAg85B. The PLGA-rAg85B particle effect cannot be duplicated by mixing dissolved antigen, or initial burst solution, with control PLGA particles, or by utilizing antigen associated with the surface of PLGA particles (prepared by suspending the particles in solution of antigen and drying). This suggests that the strong adjuvant effect of the particles requires surface association or encapsulation of antigens. These formulations provided extended epitope presentation on the APC surface for up to 6 days, which is favorable for T cell recognition. Therefore, these microparticle formulations offer the advantage of strong adjuvant effect and long epitope presentation on APC surfaces compared to the traditional solution formulations.

5.3 Protection effect of the aerosol vaccination in the guinea pig models after challenged with virulent strain *H37Rv*.

The effect of vaccination with selected aerosol microparticle adjuvant/ antigen combinations was evaluated in guinea pig models of tuberculosis involving a low-dose virulent challenge. The pulmonary boost of PLGA-Ag85B appeared to enhance the protection efficacy of BCG alone. This prime:boost immunization strategy was observed to reduce *MTB* burden in the lungs and spleens of guinea pigs compared with repeated single subunit antigen immunization strategy, which had no protection within the tested period of time. Although there was no influence in terms of *MTB* burden in lungs and spleens, the weight gain of guinea pigs in homologous multiple-dose group of PLGA-Ag85B was comparable to BCG prime:PLGA-Ag85B boost groups. The bacteriology data of heterologous regimen of subunit antigen boost via pulmonary route after BCG prime, confirmed that the effective boost vaccine candidates might be non-whole organism based or heterologous to BCG (Xing and Caters, 2007). The DTH skin test results indicated the discoordination between response to PPD / specific antigen and the protection outcome as of reduction the bacterial burdens in the lungs and spleens.

5.4 General conclusions

The key points in this dissertation research are antigen selection, formulation screening, vaccination route and strategies. Ag85B was selected as the vaccine subunit protein due to its known antigenicity and immunogenicity (Horwitz, 1995; Kamath, Feng, Macdonald *et al.*, 1999; Sable, Goyal, Verma *et al.*, 2007). Biotechnologies for generation of this subunit antigen are available. The *in vitro* immunonological assay T hybridoma cell recognition assay was specifically modified for recognition of Ag85B epitope 97-112

(Gehring, Rojas, Canaday *et al.*, 2003). This made the method of antigenicity screening accessible. Microparticle formulations of PLGA polymers encapsulating rAg85 with/without adjuvants MDP/TDB exhibited strong adjuvant effects compared with solution formulations of Ag85B. They enhanced rAg85B antigenicity by two orders of magnitude as measured by T hybridoma cell recognition assay. Only encapsulated Ag85B showed such strong antigenicity while soluble rAg85B with PLGA controls did not demonstrate enhanced antigenicity. Furthermore, the microparticle formulation stimulated the longer epitope presentation as observed in a 6-day bioassay. This may be explained by the depot effect provided by the microparticles containing rAg85B. Protein microparticles are effective delivery systems capable of eliciting strong cell-mediated response (Evans, Ward, Kern *et al.*, 2004), which is recognized as the essential element required for tuberculosis immunization. Pulmonary mucosal vaccination mimics the natural route of airborne infection with *MTB*. Homologous and heterologous vaccination regimens were used to assess the extent of protection from challenge with virulent MTB in the guinea pigs. Homologous immunization included administration of three aerosols (prime and two boosts), a regimen which is commonly followed for protein-based vaccination (Agger and Andersen, 2001). However, this strategy did not provide evidence of protection in the time frame of the study. Heterologous regimen with BCG prime: PLGA-rAg85B boosts appeared to depress the bacterial growth in the lungs and spleens. The lungs had significantly lower bacterial burden compared with that of BCG alone. Overall, the results illustrated that aerosols of PLGA microparticles were effective in boosting the BCG-induced immunity.

These results support the initial hypotheses. Ag85B/adjuvants in microparticle formulations intended for pulmonary delivery: (a) target macrophages; (b) elicit related cell-

mediated immune response *in vitro* and (c) gave preliminary indication of protection against challenge with virulent MTB *H37Rv* in guinea pig models. THP-1 phagocytic cells were shown to engulf microparticles. The *in vitro* T hybridoma cell recognition assay demonstrated the microparticle formulations containing rAg85B enhanced the antigenicity by two-order of magnitude as measured of IL-2 cytokine marker. Aerosol vaccination of the selected microparticle formulations encapsulating Ag85B demonstrated modest protection of guinea pigs against challenge with virulent *MTB H37Rv*. The BCG prime: PLGA-rAg85B aerosol boost appeared to protect the lungs and spleens from infection, as indicated by the estimates of bacterial burden in the organs. However, these initial studies require repetition before definitive conclusions can be drawn.

Due to safety concerns, in particular in immunocompromised persons, and to technical challenges regarding manufacture and reproducibility, live mycobacteria vaccines are not the product of choice of most vaccine manufacturers. The live attenuated microorganism vaccines elicit immunity based on their ability to replicate in the host. However, serious side effect may be observed in persons with severe immunosuppression and acute leukemia (Mitus, Holloway, Evans *et al.*, 1962). There was a risk of an increase in HIV viral load following measles-mumps-rubella (MMR) vaccination (Stanley, Ostrowski and Justement, 1996). One case of death was reported due to the measles pneumonitis following MMR vaccination of a patient with HIV infection (CDC, 1996). Subunit antigens have a record of relative safety since no microorganism replication is involved. An influenza vaccine: a trivalent influenza subunit vaccine provided clinically effective protection against influenza illness in HIV-1-infected patients (Yamanaka, Teruya, Tanaka *et al.*, 2005). Conventional influenza subunit vaccine Agrippal (Chiron, Siena, Italy) and adjuvanted

subunit vaccine Fluad (Chiron, Siena, Italy) both elicited seroconversion rate of 53-68% and 44-72% in HIV-1 patients (Gabutti, Guido, Durando *et al.*, 2005). Therefore, TB subunit antigen might offer a good safety profile to HIV patients. Since HIV patients are susceptible to co-infection with TB, there is urgency to elucidate the extent of protection offered by vaccines in these high-risk patients. Levels of serum antibodies to five *MTB* antigens increased before microbiological and clinical symptoms of active TB in HIV patients were identified (Gennaro, Affouf, Kanaujia *et al.*, 2007). Partial reconstitution of the CD4+-T-cell in CD4 gene knockout mice restored responses to tuberculosis DNA vaccines (D'Souza, Romano, Korf *et al.*, 2006). These reports indicated that TB non-bacterial vaccines possibly protect the host from *MTB* infection when HIV-infected patients have concurrent highly active antiretroviral therapy under which the $CD4^+$-T-cell counts are known to increase (Autran, 1999). Since the immunological responses to antigen vaccines may decrease (Arpadi, Markowitz and Baughman, 1996; CDC, 1993) as HIV disease progresses, vaccination early in the course of infection may be more likely to induce an immune response. Microparticles containing Ag85B might be used in HIV-positive individuals. Most HIV-positive people are immunized with BCG routinely in childhood, especially in developing countries; hence, most were "primed" with BCG well before being infected with HIV. If a booster vaccine administered to HIV-positive individuals before their immune system deteriorates is able to augment their immunoprotective capacity against tuberculosis, this may help protect these high-risk persons from one of the most common and devastating opportunistic infections in acquired immune deficiency syndrome (AIDS).

Microparticle formulations containing subunit antigen delivered via pulmonary route are a new development in vaccines for tuberculosis. These studies offer the first evidence for

the effect of a protein subunit vaccine delivered to the lungs, as a booster of BCG, for protection against tuberculosis, which has significant implication for worldwide disease control.

5.5. Future studies

Some questions emerge from the present studies that need to be addressed to fully interpret the outcome of the *in vitro* and *in vivo* studies.

A). Conformation of subunit antigen proteins.

The influence of the structural conformation of subunit antigens on epitope presentation and antigenicity in the cell-mediated immunity is not clear. The soluble forms of rAg85B from JM and *Origami B E. coli* strains appeared to result in different antigenicity in the T hybridoma cell recognition assay. The rAg85B from *Origami B* strain showed higher antigenicity in the concentration range tested than the JM strain (Fig. 1). The *Origami B* strain, rAg85B, is a mutant with respect to glutathione and thioredoxin reductases, which is favorable for the disulfide bond formation, and facilitates the antigen folding, assisted by concurrently co-expressed chaperones. Thus, a more appropriate conformation may be obtained in *Origami B* strain rAg85B. In order to answer this question, techniques of CD, FTIR, NMR may be used to access the 2D and 3D structure of antigens. This question is relevant to future vaccine development. If the conformation is unnecessary, then fusion of different epitopes may be a strategy to elicit stronger immunity. The literature contains some evidence that the conformational stability of the antigens may not be a concern and maintenance of the necessary primary and/or partial secondary structures might be sufficient to elicit effective antigenicity and immunogenicity. The fusion vaccine candidates, Ag85B-

ESAT-6 (Langermans, Doherty, Vervenne *et al.*, 2005), Mtb32F-Mtb39F (Mtb72F) (Skeiky, Alderson, Ovendale *et al.*, 2004) both showed stronger immunity than the single antigens.

B). The efficiency of epitope presentation for antigens associated with microparticles requires more detailed investigation.

PLGA microparticles appeared to act as adjuvants improving antigenicity. However, the explanation is not clear. There are three possibilities. Firstly, phagocytosis of microparticles by macrophages is much more efficient than pinocytosis of soluble antigens, in terms of the number of antigen molecules internalized. Secondly, differences in the mechanism of epitope presentation may occur between solution formulation and microparticle formulation. Vidard *et al.*'s research showed that the efficiency of presentation between solution and particle forms resulted in differences in response to ovalbumin. Macrophages generated distinct epitopes with different efficiencies from particles and solution formulations (Vidard, Kovacsovics-Bankowski, Kraeft *et al.*, 1996). Therefore, the clarification of the epitope presentation efficiency in two formulations for Ag85B may explain the adjuvant effect and provide meaningful base foundation for the future vaccine development.

A third explanation, that microparticles elicit immunity independently of the antigen was not supported by the T-cell recognition assay in which IL-2 production was not elevated for particles alone. However, in general particles are known to activate macrophages as part of the general housekeeping response (Bezdicek and Crystal, 1997).

C) Formulations

(1) The PLGA polymer was of L:G 75:25 and molecular weight of 84.7 Kd. The glass transition temperature of ~50°C suggested that the storage temperature may need to

be under 10°C. Since tuberculosis predominantly occurs in developing countries, where the cold-chain conditions are required, this may not be a suitable polymer. However, PLGAs with different molecular weight having higher glass transition temperatures are available and should be assessed for this application.

(2) Surfactants, such as Pluronic F68 and F127, which are considered safe (Kibbe, 2000) for human administration and do not reduce phagocytosis of particulates (Jones, 2002), may be added into emulsion in spray-drying to manipulate the release profile of Ag85B. The controlled continuous release or pulsatile release may be suitable for a CMI response, mimicking a boost. A single administration would result in the release of two or more distinct doses of drug at time intervals relevant to induction of the response.

D). Animal study

(1) In single dose vaccination regimens, it is not clear that the weight changes in groups of PLGA-Ag85B and PLGA-TDB-Ag85B were due to the soluble rAg85B boosting. Since only the groups boosted with soluble rAg85B reduced the weights significantly compared with untreated controls and PLGA controls. Therefore, the weight changes of the single dose groups PLGA-rAg85B and PLGA-TDB-rAg85B after virulent *MTB* challenge should be monitored independently, in the absence of a soluble rAg85B boost.

(2) Draining lymph nodes may be sampled for *MTB* burden and histopathology since the infection spreads initially to the lymph nodes. Lymph node lesions become severe and progress more rapidly than pulmonary lesions (Basaraba, Dailey, McFarland *et al.*, 2006). It was recognized that a rapid and progressive enlargement of intra-

thoracic hilar lymph nodes was due to rapidly progressing granulomatous lymphadenitis (Kraft, Dailey, Kovach *et al.*, 2004). The involvement of hilar lymph nodes following low-dose aerosol challenge in the guinea pig resembled the early, extra-pulmonary manifestation of tuberculosis. The granulomatous inflammation and necrosis of intra-thoracic hilar lymph nodes is an early and significant manifestation of low dose aerosol infection of guinea pigs with *MTB* (Basaraba, Dailey, McFarland *et al.*, 2006). The size, bacteriology and histopathology of lymph nodes may allow more detailed interpretation of the progress of the disease and host immunity after challenge.

(3) The IFN-γ, IL-10, IL-4 and antibodies to Ag85B may be evaluated. The subunit antigens occasionally exhibit weak *in vivo* immunogenicity compared to *in vitro* studies (Sable, Kalra, Verma *et al.*, 2007). This may, in part be a reflection of the narrow range of responses that are examined *in vitro*. For example, in the present studies only one cytokine, IL-2, was selected as the marker for immunogenicity (Lu, Garcia-Contreras, Xu *et al.*, 2007). Therefore, the immunogenicity of inducing CD4 Th1 cell-mediated immunity may be monitored. Recently, it has been postulated that the vaccines inducing balanced Th1 and Th2 response following immunization are most effective in protection from tuberculosis (Giri, Verma and Khuller, 2006; Sable, Verma and Khuller, 2005). The requirement of Th1 and Th2 cytokine balance is governed by IFN-γ, IL-10, IL-4 (Wigginton and Kirschner, 2001). Due to the limited availability of commercially available ELISA, or relevant reagents, for guinea pig proteins and the relatively short-half life of these cytokines, cytokine mRNA methods could be used for this purpose (Carding, Lu and Bottomly, 1992).

(4) Dendritic cells play an important and unique role in priming Th1 and Th2 CMI cytokine polarization, which has lead to the development of dendritic cell-based vaccine for TB (McShane, Behboudi, Goonetilleke *et al.*, 2002). Whether it also involves microparticle phagocytosis and stimulates the recognition of T cells is unknown. Identification of functions of lung dendritic cells and macrophages in processing, migration and presentation to T cells is important. This will improve the understanding of the reduction of *MTB* burden in lungs and other organs such as spleens.

In addition to the questions remaining from the completion of the dissertation research, new approaches may be taken to the future study of TB vaccines and their development. These approaches would be the subject of independent projects and would involve the elucidation of different mechanisms than those addressed in the present research.

(1) The heterologous regimen of subunit antigen Ag85B and subunit antigen DNA may be used as pulmonary prime and boost strategy. One of the advantages of DNA vaccines is that their translated antigen proteins have access to cytosol to elicit CD8 Th1 immunity which is recognized as an important component of immunity involved in the prevention of TB (Raychaudhuri and Rock, 1998). Therefore, a combined approach may provide better protection than the homologous vaccination.

(2) Pulmonary homologous of DNA vaccines, including Ag85B DNA, fusion antigen Ag85B-ESAT-6 DNA, Ag85B-MTB72F DNA may be a valuable alternative because of the multiple epitope presentation and the broader immunity that might be anticipated.

(3) A heterologous regimen of BCG parenteral prime: aerosol DNA boost; BCG parenteral prime: aerosol virus boost immunization regimen might have an application in protection from TB. Viral vectors have the ability to induce high levels of transgene product in a wide range of cell types, an advantage over plasmid DNA vectors or protein-based vaccines. Viral vectors have been used parenterally or mucosally to activate antigen-specific immune responses. These features make them attractive candidates boost vaccines for enhancing BCG prime immunization (Xing, Santosuosso, McCormick *et al.*, 2005). The limitation to viral-vector vaccines is that a strong antibody response may be engendered against the virus which would hinder repeated administration of the same virus to a previously exposed host.

(4) Regimens of BCG aerosol prime: aerosol subunit antigen/ aerosol DNA/ aerosol virus vector, have not been investigated. Both prime and boost via pulmonary mucosal immunization may provide better protections from TB.

(5) The morphology of BCG is a rod-like structure with length of 2-5μm and cross-section diameter of 0.2-0.3 μm. The BCG particles can deposit into the lungs easily due to their elongated shape and sensitively recognized by macrophages in the lungs. The generation of stable dry powder aerosols of BCG, which can maintain the individual shape of BCG while retaining sufficient immunogenicity, is a challenging task for TB vaccine formulations. This is approach is currently under investigation. (Wong, Sampson, Germishuizen *et al.*, 2007).

(6) Research into the influence of TB vaccines on HIV patients is rare. The BCG vaccine is not recommended for immunocompromised individuals, since it has been shown to be deleterious in some cases (D'Souza, Romano, Korf *et al.*, 2006). HIV patients are

immunocompromised of CD4 T cell, which is a very important immunocomponent to protect the hosts from TB. Whether the promising TB non-bacterial vaccines to normal populations are also functional to HIV-infected patients requires investigation.

(7) The use of pulmonary delivery of microparticles for vaccination to protect from diseases other than TB should also be investigated. It is anticipated that the lung would be an excellent site of vaccination to elicit mucosal humoral immunity that would be important in the treatment of a variety of diseases acquired by the pulmonary route, such as respiratory syncytial virus, influenza, *bacillus anthracis*, *streptococcus pneumoniae* and *Francisella tularensis* (Lu and Hickey, 2007).

5.6 Concluding marks

The current study of aerosol delivery of microparticle formulation of TB antigens illustrated that the pulmonary route is a promising route of delivery and means of vaccinating susceptible populations. Microparticle delivery systems may act as adjuvants and enhance the antigenicity. The observed protection from the BCG prime: aerosol PLGA-Ag85B boost is relevant to TB vaccine development and the approach to immunization in the field. The combination of suitable antigens, appropriate formulations, a novel delivery route, advanced technologies and appropriate vaccination regimens would facilitate a new vaccination strategy for protection against the insidious disease of tuberculosis.

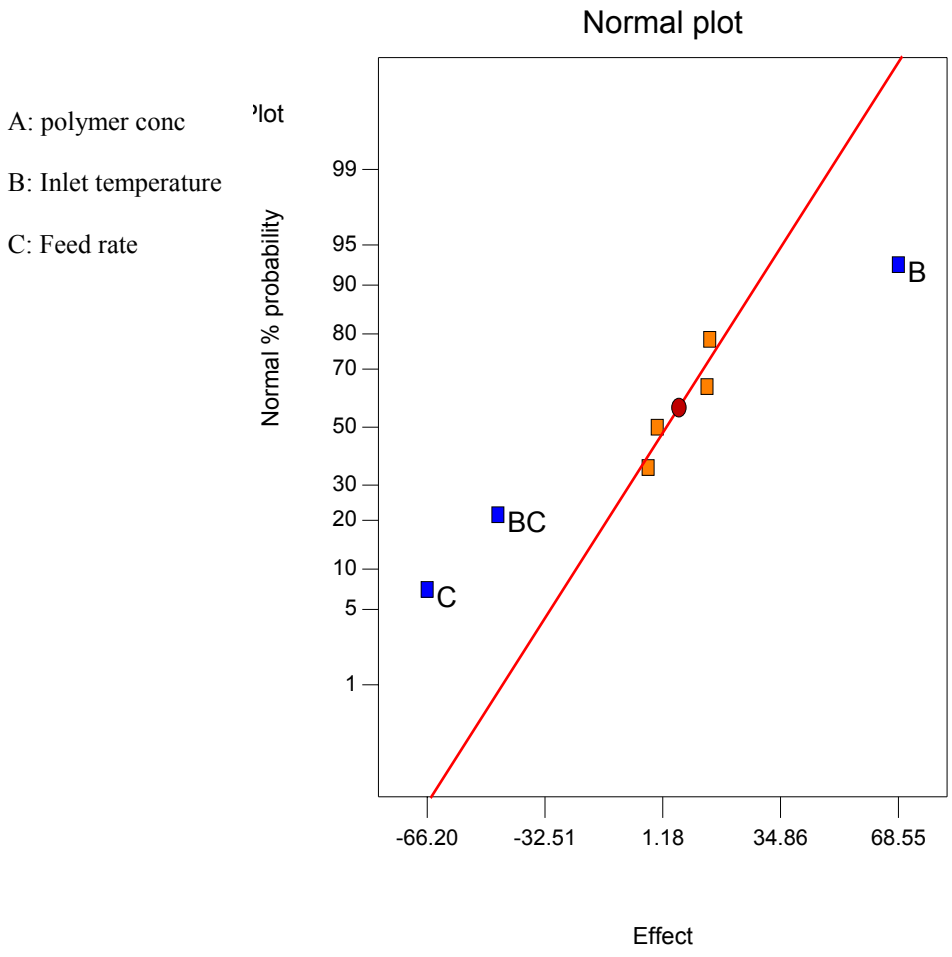
Table 5.1 Overview of the activity, procedure and brief results in each specific aim.

Specific aim	Activity	Procedure	Results
1 (rAg85B generation and PLGA microparticle manufacture)	Ag85B vector expression in two strains of *E.coli* Evaluation of antigenicity of rAG85B Optimization of spray-drying condition for PLGA microparticles Manufacture of PLGA microparticles	1. plasmid transformation 2. antigen expression 3. purification 4. MS of rAg85B 5. T hybridoma cell recognition assay to assess antigenicity 6. Fractional design to optimize spray-drying conditions 7. manufacture of formulations 8. morphology and release profile	rAg85B was obtained with sufficient antigenicity The optimized conditions were found and 24 batches of microparticle formulations were produced. Pulsatile release of rAg85B from formulation
2 (*In vitro* screening of microparticle formulations and physical property characterization of these formulations as aerosols)	Macrophage cell recognition of particles Immunological characterization of microparticles Physical property characterization of microparticles as aerosols	9. Phagocytosis of particles by THP-1 cells 10. microparticle adjuvant effect 11. epitope presentation elicited by microparticles 12. MMAD, emitted dose, FPF 13. flow property 14. Specific surface area, moisture adsorption 15. Thermal behavior	Microparticles can be taken up by macrophage-like cells. In vitro cell assay indicated the strong adjuvant effect of particles; prolonged epitope presentation was elicited by microparticle formulations. The physical properties showed that PLGA microparticle formulations are suitable to be delivered as aerosols
4 (Protection study after aerosol delivery of microparticle formulations)	Aerosol delivery of microparticles containing rAg85B Aerosol challenge with virulent *MTB*	16. pulmonary delivery of formulations to guinea pigs (single or multiple dose, homologous or heterologous regimen) 17. Challenge guinea pigs with virulent *MTB* 18. Necropsy and assessment of protection	Heterologous regimen BCG prime: PLGA-Ag85B aerosol boost appeared to improve the immunity elicited by BCG alone.

APPENDIX

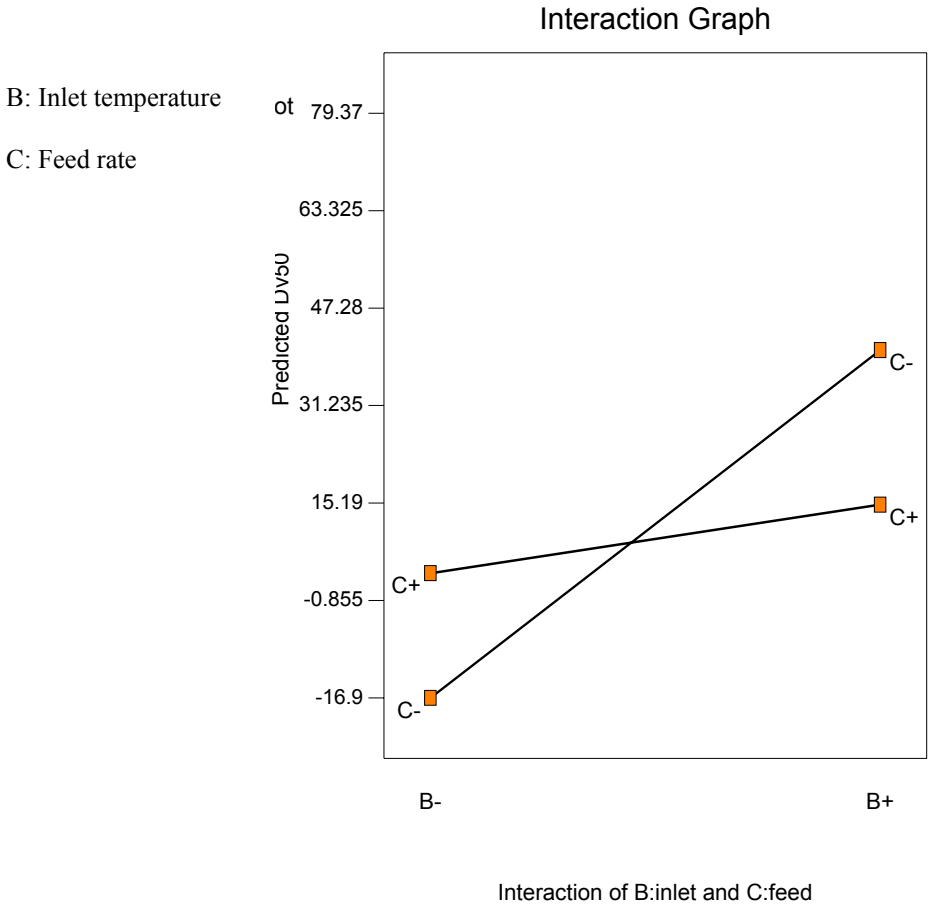
Appendix I. Factorial design experiment analysis to optimize the best spray-drying conditions.

A) Main effects of different factors affected the particle sizes in Design experiment;



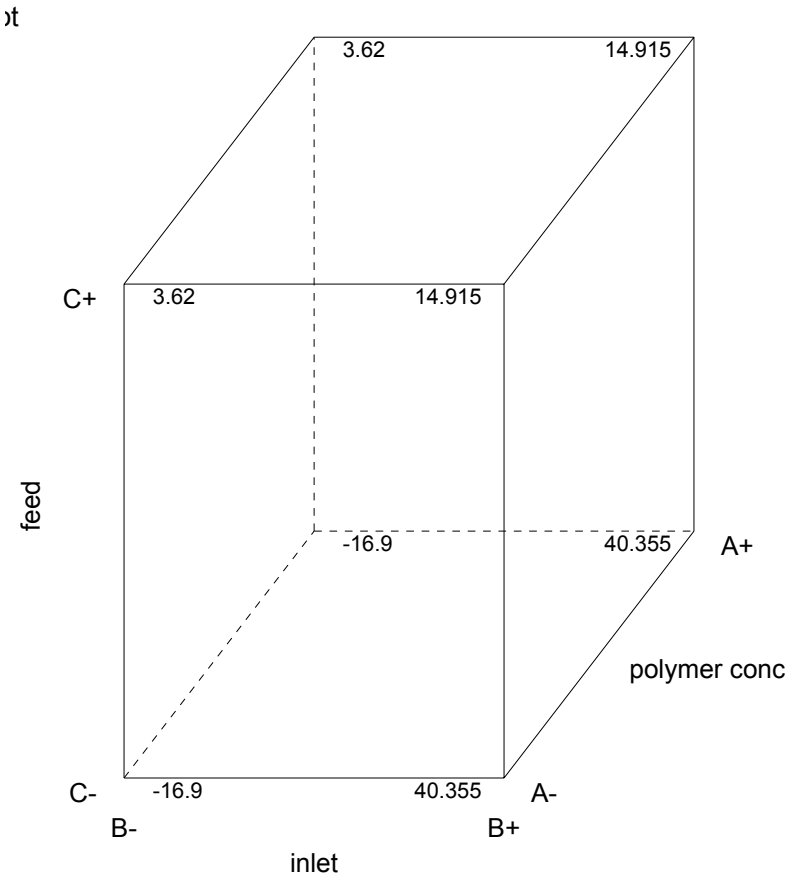
A

B) Interaction between inlet temperature and feed rate which affects particle sizes. [B]: inlet temperature; [C]: feed rate;



B

C) Prediction of particle sizes D_{v50} of PLGA microspheres influenced by three factors (polymer concentration, inlet temperature and feed rate) in spray-drying.

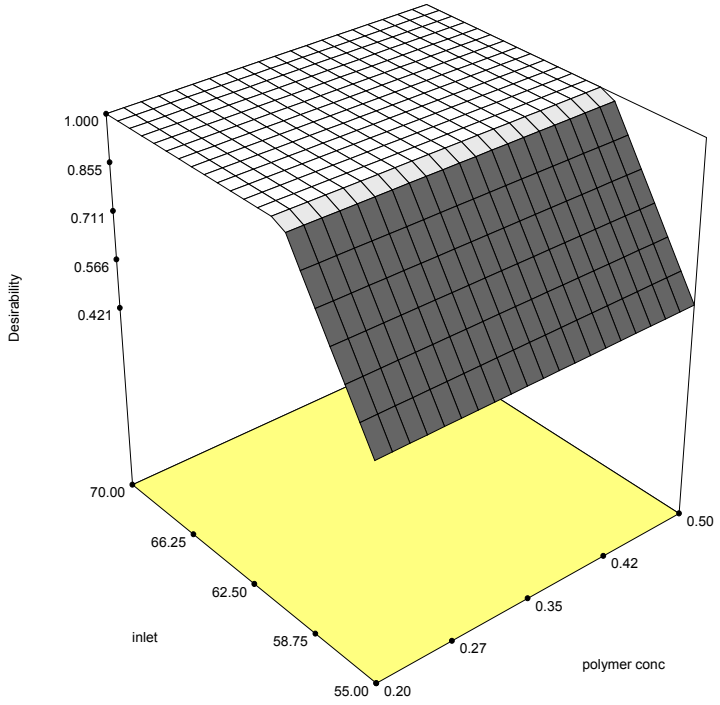


C

D) Prediction of the optimized conditions for spray-drying to produce PLGA microsphere formulations. Desirability reflects the most desirable value for each response variable (range from 0-1. The higher the value, the more ideal condition is to generate desirable value for response).

X=polymer conc
Y= Inlet

Actual constants:
Feed = 50.35



D

Appendix II. The calibrated cutoff aerodynamic diameters in Andersen Cascade Impactor at the flow rates of 60 and 28.3L/min.

Stage	MMAD (μm) at the flow rate of 28.3 LPM	60 LPM
-1	-	8.6
0	8.7	6.5
1	6.1	4.5
2	5.0	3.2
3	2.8	1.9
4	1.8	1.1
5	1.0	0.5
6	0.5	0.2
7	0.3	-

Appendix III. The cutoff aerodynamic diameters on each stage in electrical low pressure impactor (ELPI) at flow rate of 30L/min.

Stage	Cutoff diameter (μm)
1	0.0290
2	0.0575
3	0.0966
4	0.161
5	0.269
6	0.392
7	0.628
8	0.970
9	1.630
10	2.440
11	4.080
12	6.730
13	10.16

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