Design and engineering of self-assembling antigens towards particulate vaccines

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Abstract

Natural and synthetic self-assembling polymers and proteins could be bioengineered to display and/or encapsulate antigens to serve as innovative antigen carrier systems for the induction of desirable immunities. Polyhydroxyalkanoates (PHAs) are naturally occurring polyesters synthesized as cytoplasmic polyester inclusions (polyester particles) by various bacteria. The particles have been used as an antigen delivery platform by translationally fusing antigens to the particle surface-associated protein, PHA synthase. Furthermore, it has been found that protein inclusion bodies contain a large amount of correctly folded and biologically active proteins and could be engineered to perform as an antigen carrier system. Tuberculosis (TB) is a global health issue for both humans and animals. Inaccurate diagnosis and inefficacious vaccination make TB control problematic. The Mantoux tuberculin skin test gives false positive results if humans or animals are vaccinated with the Bacille Calmette-Guérin (BCG) strain or exposed to environmental mycobacteria. BCG cannot provide effective protection against TB. Subunit vaccines have great promise to protect against infectious diseases, but they are often weak immunogenically. A strategy to circumvent this problem is the use of self-assembly particulate vaccines, which could present multiple copies of antigens and serve as a depot for prolonged multivalent antigen display to induce enhanced immunogenicity. In this thesis, four specific TB diagnostic antigens — CFP10, Rv3615c, ESAT6, and Rv3020c — were displayed on polyester particles. The results showed that polyester particles displaying TB antigens specifically distinguished TB-infected from non-infected cattle. Antigen immunogenicity was dramatically enhanced after the display on polyester particles, which lowered the antigen concentration (0.1 to 3 μg dose/inoculum) required for skin tests. Mycobacterial vaccines H4 (Ag85B-TB10.4) or H28 (Ag85B-TB10.4-Rv2660c) were bioengineered to display H4/H28 on polyester particles and/or self-assemble H4/H28 into protein inclusion bodies. The results demonstrated that polyester particle-/protein inclusion body-based particulate TB vaccines increased overall immunogenicity by enhancing humoral (for example, IgG1 and IgG2c) and cellular (for example, IFNγ and IL17A) immune responses when compared to respective soluble antigens.

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Chapter 5.
General discussion, conclusion, and future work.
This chapter was written by **Shuxiong Chen** for this thesis and not for publication purposes.

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List of Abbreviations

A full list of abbreviations used

^{o}C	Degree Celsius
Ap	Ampicillin
Ag85B	Mycobacterial antigen Ag85B
APC	Antigen-presenting cells
BL21	*E. coli* production strain
ClearColi BL21	An endotoxin–free host derived from wild-type BL21 (DE3) *E. coli* cells.
BSA	Bovine serum albumin
BCG vaccine strain	Live-attenuated *Mycobacterium bovis* Bacile Calmette-Guerin vaccine strain
CFP10	The 10-kDa culture filtrate protein
CFP10-PhaC	The 10-kDa culture filtrate protein-PHA synthase
CFP10-Rv3615c-PhaC-ESAT6	The 10-kDa culture filtrate protein-Rv3615c-PHA synthase-the 6-kDa early secretory antigenic target
CFP10-Rv3615c-PhaC-ESAT6-Rv 3020c	The 10-kDa culture filtrate protein-Rv3615c-PHA synthase-the 6-kDa early secretory antigenic target-Rv3020c
CI	Confidence interval
Cm	Chloramphenicol
DDA	Dimethyl dioctadecyl ammonium bromide
DIVA	Differentiate infected from vaccinated animals
DNA	Deoxyribonucleic acid
DTH	Delayed-type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
ESAT6	The 6-kDa early secretory antigenic target
EC50	The half maximal effective concentration
FDA	Food and Drug Administration
g	Gravity/gram
HIV	Human immunodeficiency virus

H4	Mycobacterial antigen Ag85B- mycobacterial antigen TB10.4
H28	Mycobacterial antigen Ag85B- mycobacterial antigen TB10.4- latency-associated antigen Rv2660c
IFNγ	Interferon-γ
IgG	Immunoglobulin G
IPTG	Isopropyl â-D-1-thiogalactopyranoside
kDa	Kilo Daltons
LB	Luria-Bertani (broth)
MALDI-TOF/MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MW	Molecular weight
OIE	Office International des Epizooties
PCR	Polymerase chain reaction
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline containing 0.05% (v/v) Tween 20
PhaA	β-ketothiolase
PHAs	Polyhydroxyalkanoates
PHA_{SCL}	Short chain length PHAs
PHA_{MCL}	Medium chain length PHAs
PhaB	Acetoacetyl-CoA reductase
PhaC	PHA synthase
PHB	Poly(3-hydroxybutyric acid)
PPDs	Purified protein derivatives
Rv3615c	Mycobacterial antigen Rv3615c
Rv3020c	Mycobacterial antigen Rv3020c
Rv2660c	Latency-associated antigen
PRRs	Pattern recognition receptors
SEM	Standard error of the mean
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SICCT	Single intradermal comparative cervical test
TB10.4	Mycobacterial antigen TB10.4
TB	Tuberculosis

TBE	Tris-Borate-EDTA buffer
TEM	Transmission and Electron Microscopy
Tet	Tetracycline
Tris	Trishydroxymethylaminomethane
Th	T helper
v/v	Volume per volume
w/v	Weight per volume

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Chapter 1. Introduction

Tuberculosis (TB) is an infectious disease caused by various tuberculous strains of mycobacteria and is a major health problem worldwide for both humans and animals (1, 2). The 2018 global TB report has shown that some cases of TB infection are not detected by health systems, and inaccurate diagnosis is one of the important contributing factors. Moreover, the lack of efficacious TB vaccines makes control of TB transmission problematic (1-4). Bovine TB causes great economic hardship for agriculture (5). The Mantoux tuberculin skin test is a primary diagnostic tool for both human and bovine TB, but this test cannot accurately distinguish TB-infected from Bacille Calmette-Guérin (BCG)-vaccinated and non-pathogenic mycobacteria-sensitized humans or cattle (2, 6, 7). In addition, the only available licensed TB vaccine, live attenuated BCG, cannot provide adequate protection against this disease (1, 2). Live vaccines are also associated with safety concerns as they may revert to disease-causing pathogens, especially in immunocompromised patients (8). Encouragingly, subunit vaccines generally have no infective risk to patients, but they have low immunogenicity and are unable to elicit desirable immune responses (8-11). Thus, innovative antigen carrier systems are required to produce low-cost skin test reagents with high specificity and sensitivity, and to augment the immunogenicity of subunit vaccines (12, 13).

Recently, particulate antigen carrier systems have drawn significant attention as they possess inherent immunogenicity due to augmented uptake by antigen-presenting cells (APCs) (14-17). Particulate carriers could be densely coated with immunogenic antigens and immobilization also enhances antigens stability (18, 19). In particular, polyester particles have been successfully bioengineered to display various desired proteins and this could be achieved by fusing them to particle surface-associated proteins, such as polyhydroxyalkanoates (PHA) synthase PhaC, as they remain covalently embedded on the surface after particle formation (7, 20). In addition, it has recently been found that protein inclusion bodies/protein particles are comprised of not only misfolded proteins but also a significant amount of correctly folded and biologically active proteins (12, 21-24). Protein inclusion bodies comprised of antigenic

peptides are able to act as antigen carrier systems for efficient antigen delivery and elicit desirable immune responses (12, 21).

In this chapter, the disease, diagnostic tools, and vaccines of TB (Section 1.1) will be reviewed, and adjuvants' function (Section 1.2) will be discussed. This chapter will continue with a description of the formation and application of versatile antigen carrier systems, including polyester particles (Section 1.3) and protein inclusion bodies (Section 1.4). The endotoxin free expression system (Section 1.5) used in this study will also be discussed. Finally, the primary aim of this study, which was to assess the immunogenicity of TB antigens displayed on the surface of different carrier systems to facilitate the generation of highly specific and sensitive TB skin test reagents and efficacious TB vaccines (Section 1.6), will be introduced.

1.1 Tuberculosis (TB)

TB is an airborne infectious disease caused by pathogens of the *Mycobacterium tuberculosis* complex (1, 2, 25). Pathogens typically infect the lungs but could also affect other organs of the body (3, 26). TB kills more people than other lethal infectious diseases, such as human immunodeficiency virus (HIV) and malaria (2, 3, 26). Furthermore, some TB-infected cases are being missed by health systems, and inaccurate diagnosis is thought to be one of the major factors causing this issue (2, 27). Vaccination has been considered to be one of the most effective approaches to circumvent the threat of challenging infectious diseases worldwide (12, 26, 28). However, although the only available licensed TB vaccine — live, attenuated *Mycobacterium bovis* (*M. bovis*), known as BCG — is used globally, it mainly prevents TB in children and cannot effectively control the global TB epidemic (1, 2). Thus, it is a global health priority to deveop a highly specific and sensitive diagnostic reagent for accurate and efficent TB diagnosis as well as an efficacious vaccine for TB prevention (2).

1.1.2 *Mycobacterium tuberculosis* infection

The lungs are the most common point of entry and site of TB infection for tuberculous mycobacteria species (26, 29). These bacteria are acid-fast and rod-shaped bacilli, which are shielded by a lipid-rich cell wall composed of long-chain fatty acids and other components (30). TB in humans is mainly caused by *M. tuberculosis* and *Mycobacterium africanum*; a related species, *M. bovis*, infects a variety of hosts, including cattle and humans (31, 32).

M. tuberculosis behaves as an intracellular pathogen after invading hosts. It evades the immune system by hiding within macrophages that cannot kill this intracellular pathogen as mycobacteria are capable of arresting the phagosome maturation (33). After initial infection, but prior to the disease, the pathogen is mainly restricted within tuberculous granulomas, which are organized host immune structures composed of macrophages and T lymphocytes. The granulomas are critical for the inhibition of tuberculous mycobacterial infections but are not able to completely eradicate infections; therefore, the granulomas force *M. tuberculosis* into a dormant state (34).

Most people are healed after infection. The granulomas become caseous necrosis, which can resolve by fibrin deposition, sclerosis, and calcification with disappearance of the mycobacteria (35). However, up to 10% of infected individuals develop active TB during their lifetime (26, 30). Late onset of the disease is due to the endogenous reactivation of dormant tuberculous mycobacteria (36). The caseous necrosis liquefies in the active disease. *M. tuberculosis* in these lesions then becomes extracellular and starts to replicate. It is capable of disseminating to other organs causing extra-pulmonary TB as, for example, meningeal TB, the most frequent form (35). Patients with active pulmonary TB are extremely contagious and are able to transmit the bacilli easily within or between populations through minute droplets by coughing, expectorating, and even during conversation (26).

Both humoral and cell-mediated immune responses play an important role against mycobacterial pathogens; however, it is generally accepted that the cell-mediated immune response generated by polyfunctional CD4+ T cells is more critical for control of *M. tuberculosis* as it correlates with the prevention of infection (37, 38).

Nevertheless, the mechanism(s) of polyfunctional CD4+ T cells against *M. tuberculosis*, induced by vaccines or natural infection, have not yet been defined (39). It is certainly conceivable that polyfunctional CD4+ T cells-derived multiple proinflammatory cytokines, including interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), and interleukin 2 (IL2), are crucial for protection against intracellular *M. tuberculosis* (37, 39-41). For example, IFNγ, one of the main markers of cell-mediated immunity, and TNFα act synergistically to inhibit *M. tuberculosis* replication in murine macrophage cells. Hence, IFNγ and TNFα synergistically augment the ability of macrophages to contain *M. tuberculosis* infection (39, 42, 43). IL2 induces the proliferation and enhances the survival of activated T cells and promotes memory T cell development during primary infection (39, 44, 45). The production of IFNγ, TNFα, and IL2 cytokines by polyfuctional CD4+ T cells activated by different vaccines, correlates with various degrees of protection (39).

1.1.3 Diagnostic tools

TB remains a serious public health problem worldwide, caused by different tuberculous mycobacteria (29, 46, 47). The pathogen may affect any organ systems, but pulmonary TB (80%) is the most common form (3, 26). One fifth of TB-positive individuals have extra-pulmonary disease, 5–17% of which is gastrointestinal (GI) TB, the sixth most common form of non-pulmonary locations (48, 49). The diagnosis of GI TB is troublesome as it could mimic other GI disorders (50-52).

The 2018 Global TB Report stated that approximately 10 million new cases occurred in 2017 (2). However, only 6.4 million cases, representing 64% of the estimated new cases, were reported (2). Inaccurate diagnosis is one of the main factors contributing to the difference between the estimated number of new cases and the number of reported cases (2, 27). TB is also a critical animal health issue, with more than 50 million cattle infected with *M. bovis* globally (5). This mycobacterial pathogen can be detected in the tissue and milk of TB-infected cattle, which have been considered as the potential sources for TB transmission (53, 54). Several diagnostic tools, including the tuberculin skin test, QuantiFERON-TB Gold In-Tube (an *in vitro* blood test), and other techniques, such as imaging methods or smear microscopy, have been used for TB diagnosis (6, 32, 55). However, the tuberculin skin test is the only available skin test to diagnose both

human and bovine TB throughout the world (55-57). The tuberculin skin test discriminates TB-infected from non-infected individuals according to the delayed-type hypersensitivity (DTH) immune responses (32).

As an example, in cattle that develop cell-mediated immunity to antigens in the tuberculin skin test reagent, a DTH reaction will occur in 48–72 hours (1, 32). In particular, after intradermal injection of the reagent, TB-infected cattle exhibit raised skin at the injection site due to a DTH reaction whereas no skin changes occur at the injection site in non-infected cattle (32). The bovine tuberculin skin test reagent is the purified protein derivatives (PPDs), obtained from cell lysates of *M. bovis* (58, 59). The skin test reagent has shown low specificity due to the cross-reactivity of non-specific antigens in PPDs with the antigens in the BCG strain and/or non-tuberculous environmental mycobacteria. Therefore, due to the low specificity of PPDs, the tuberculin skin test could not accurately distinguish TB-infected from BCG-vaccinated and environmental mycobacteria-sensitized cattle (2, 6, 7, 58, 60). Hence, in order to augment the specificity of the TB skin test, the development of a new differential diagnostic reagent comprising specific TB antigens is required (2, 7, 27, 61).

Three specific diagnostic antigens, CFP10, ESAT6, and Rv3615c, have been found in *M. tuberculosis* (62-64). The corresponding ortholog antigens have also been identified in other pathogenic mycobacterial strains, including *M. bovis*, but are absent in the majority of non-pathogenic mycobacteria or the BCG vaccine strain (31, 62-64). Peptide cocktails containing CFP10, ESAT6, and/or Rv3615c have shown increased skin test specificity while retaining a high sensitivity in regards to bovine TB diagnosis (60, 65, 66). Recently, a study has demonstrated that the addition of an immunogenic mycobacterial antigen, Rv3020c, to the peptide cocktail containing CFP10, ESAT6, and Rv3615c could further enhance skin test sensitivity without affecting specificity (66).

1.1.4 Current vaccines

BCG has been in use for almost 100 years and is the only available licensed vaccine to protect against TB (46). This vaccine provides extensive protection against severe forms of TB, especially meningeal and miliary TB, in childhood; however, BCG is not able to prevent pulmonary TB in adults (46, 67). BCG efficacy is highly variable in all age

groups (46, 67). This may indicate that immunological memory does not continue lifelong after vaccination. BCG is also not considered for use in HIV-infected infants/children as it carries the risk of disseminating BCG disease in these young ones (46, 68, 69). Moreover, BCG may not be able to provide protection in the region where environmental mycobacteria are abundant. The existence of environmental mycobacteria could impart some level of anti-TB immunity on a population, which might, in turn, limit the effect of BCG or inhibit essential vaccine replication (67).

There is a significant demand for efficacious new vaccines that can provide adequate protection for the prevention of TB (4, 70). Currently, immense efforts have been made to either improve or develop new TB vaccines, such as recombinant BCG and subunit vaccines, as shown in **TABLE 1** (4, 70-72). Recombinant BCG, an updated version of the current BCG vaccine, restores protective genes absent in the current BCG strain and knocks out unwanted virulent genes (73). These BCG strains are live-attenuated vaccines and are highly immunogenic but have inherent safety issues, given the potential of these attenuated strains to revert to disease-causing pathogens (8). Conversely, non-viable subunit vaccines, comprised of antigenic parts of the pathogen, cannot replicate nor revert to virulent forms (8). Hence, subunit vaccines are generally considered to be safe but tend to induce a weak immune response and, thus, the administration of multiple dosages may be required (8-10).

TABLE 1. *Development stages of current TB vaccines*

Vaccine candidate name	Type	Phase	References
TB/Flu-04L	Attenuated influenza producing Ag85A and ESAT6	I	(74)
DAR-901	Heat-killed non-tuberculous-mycobacteria	I	(75)
ChAdOx1.85A/MVA85A	Chimpanzee adenovirus producing Ag85A/modified vaccinia virus Ankara producing Ag85A	I	(76)
MTBVAC	*M. tuberculosis* MT103 with deletion of *phoP* and *fadD26* gene	I	(77)
AdHu5Ag85A	Human adenovirus type 5 producing Ag85A		(78)
AERAS-402	Human adenovirus type 5 producing Ag85A, Ag85B, and TB10.4	IIa	(79)
AERAS-402 and MVA85A	AERAS-402 prime followed by MVA85A boost	I	(80)
H1 + CAF01	Fusion protein Ag85B-ESAT6 + CAF01 adjuvant	I	(81)
H1 + IC31	Fusion protein Ag85B-ESAT6 + IC31 adjuvant	IIa	(82, 83)
ID93 + GLA-SE	fusion protein containing Rv1813, Rv2608, Rv3619, and Rv3620 + GLA-SE adjuvant	IIa	(70, 84)
VPM 1002	Modified recombinant BCG	IIa	(85)
RUTI	Lysate of *M. tuberculosis*	IIa	(86, 87)
H4/Aeras-404 + IC31	Fusion protein Ag85B-TB10.4 + IC31 adjuvant	IIa	(88)
H56/Aeras-456 + IC31	Fusion protein Ag85B-ESAT6-Rv2660c + IC31 adjuvant	IIa	(89)
M72F + AS01	Fusion protein Mtb32a-Mtb39a in AS01 adjuvant	IIb	(90)
MVA85A/AERAS-485	Modified vaccinia virus Ankara producing Ag85A	IIb	(91, 92)
Mycobacterium Vaccae	Lysate of *Mycobacterium vaccae*	III	(93, 94)
Mycobacterium indicuspranii	Lysate of *Mycobacterium indicuspranii*	III	(89)

Effective TB control requires two vaccination regimens, pre-exposure and post-exposure vaccines (26, 59, 95). The pre-exposure vaccine is applied prior to infection with *M. tuberculosis* and prevents disease in the naive population (26, 59). For example, pre-exposure vaccine H4 contains two early stage antigens, Ag85B and TB10.4, which are normally secreted during the acute phase of infection (96). These two antigens have been found in both *M. tuberculosis* and BCG (96-98). Vaccine H4 is currently in phase IIa clinical trials (4). Mathematical modeling shows that the most efficient method to accelerate global TB control is the combination of pre-exposure and post-exposure vaccines (95).

Post-exposure vaccination targets adolescents and/or adults with latent TB infection to prevent disease outbreaks in *M. tuberculosis*-infected individuals (26). Post-exposure vaccines are also called multistage vaccines as they integrate latency antigens of *M. tuberculosis* (26, 59). The development of post-exposure vaccines involves classical preventive vaccine target antigens and major latency-associated antigens (98-100). For instance, the post-exposure vaccine H28 combines the pre-exposure vaccine H4 (Ag85B-TB10.4) backbone and the latency-associated antigen Rv2660c (46, 96, 97, 100). In non-replicating and nutrient-starved conditions, Rv2660c is strongly up-regulated, and its production level is increased 100–300 fold (101). It has been demonstrated that H28 has the potential to perform as a prophylactic vaccine as it prevents re-activation of TB in the mouse model of long-term persistent TB and also provides essential protection in the naive population (102).

1.2 Adjuvants

As described in Section 1.1.4, subunit vaccines are generally safe but tend to show low immunogenicity as they have limited antigen diversity and cannot replicate, unlike live-attenuated vaccines, such as BCG (8-11). Thus, the addition of an adjuvant is often required to induce a desirable protective immune response (8, 11, 14, 15). Adjuvants are compounds formulated with low immunogenic subunit vaccines to produce stronger immune responses when compared to vaccines alone (14). Adjuvants are currently in widespread use, but there is no clear understanding of their exact cellular and molecular mechanisms (14, 15).

Adjuvants have been divided into two groups, immunostimulatory molecules and delivery vehicles, based on their mechanisms of action (14, 15). Encouragingly, most adjuvants, such as aluminium salts and emulsions, possess both immunostimulatory and delivery functions (14, 15, 103). The majority of immunostimulatory adjuvants are ligands for pattern recognition receptors (PRRs), such as ligands for Toll-like receptors, C-type lectin receptors, and NOD-like receptors (15). The innate immune responses heavily rely on PRRs. In the past decade, many PRRs and signaling pathways involved in the innate immunity have been defined (14). Recent data suggests that adjuvants

stimulate T and B cell immune responses by interacting with components of the innate immune system, rather than by direct engaging the lymphocytes (104, 105).

Adjuvants that function as vaccine delivery systems are generally particulates (such as aluminium salts, lipid vesicles, microparticles, or emulsions) (15, 16). In particular, particulates could serve as a depot for slow antigen release and enhancing the recruitment and subsequent uptake by APCs (14-17). Furthermore, particulates have a comparable size to the pathogens and can potentially mimic the pathogen-host surface interaction (67, 103). Recently, studies have shown that polyester particles may possess adjuvant properties as they could perform as vaccine carrier systems to induce augmented immunogenicity when compared to vaccines alone (9, 106). They have been widely used in the development of a variety of efficacious vaccines against different disease-causing pathogens, including *M. tuberculosis*, Hepatitis C, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, and *Neisseria meningitides* (9, 67, 106-108). Polyester particle-based particulate vaccines are able to display multiple copies of antigens and, thus, enhance trapping and retention of antigens in local lymph nodes. The covalent display of antigens on polyester particles prompts antigen uptake by APCs, hence leading to the induction of a desired protective immune response (9, 13, 16, 106).

1.3 Polyester particles

Polyester particles are comprised of polyhydroxyalkanoates (PHAs), natural polyesters produced by a variety of microorganisms (7, 20). PHAs are deposited as water-insoluble cytoplasmic polyester inclusion bodies (particles), which could serve as carbon and energy sources (7, 20). PHA synthase, PhaC, is the key enzyme required for PHA biosynthesis. Synthesized PHA chains could self-assemble into an amorphous hydrophobic polyester core (109, 110). PhaC remains covalently embedded on the particle surface after the self-assembly process (7, 20). Polyester particles could perform as a versatile platform for the display of immunogenic antigens of interest towards the development of particulate vaccines and diagnostic reagents via bioengineering of PhaC as this synthase remains covalently attached to the surface after particle formation (7, 9, 61, 108).

1.3.1 Polyhydroxyalkanoates

PHAs are naturally occurring polyesters comprised of (*R*)-3-hydroxy fatty acids synthesized by a range of bacteria and some archaea in unbalanced nutrient conditions when an excess carbon source is available (7, 20). PHAs are deposited as intracellular polyester inclusion bodies in the cytoplasm and function as a carbon and energy source (7, 20). Polyester particles are composed of an amorphous hydrophobic polyester core coated with PHA synthase, PhaC (7, 9, 108). The size of the polyester particles varies and typically ranges between approximately 100 and 500 nm in diameter (7, 19). Generally, on average, bacteria are able to produce 5 to 10 polyester particles per cell (111, 112). The mass of polyester could be accumulated greater than 80% of the cellular dry weight (111, 112).

The length and composition of PHA constituents — hydroxyalkanoic acids — contribute to the diversity and properties of PHAs (110, 113). Over 150 different hydroxyalkanoic acids have been found to associate in the formation of PHAs (110, 113, 114). These polyesters have been classified into two major classes based on the length of carbon chain: short chain length PHAs (PHA_{SCL}), which have 3–5 carbon atoms and medium chain length PHAs (PHA_{MCL}), which have 6–14 carbon atoms (110, 115-117). Generally, the most common form of polyesters isolated from microorganisms is poly(3-hydroxybutyric acids) (PHB) (118, 119). In addition, PHA_{SCL} are hard and have a high level of crystallinity and melting temperature (110, 116, 117). However, PHA_{MCL} are more elastomeric and have a low level of crystallinity and melting temperature (110, 115, 117, 120).

Polyesters have been widely exploited in medical fields due to their attractive properties, such as being biocompatible, elastomeric, and modifiable (7, 108, 121). Their biocompatibility allows polyesters to be used in medical applications without eliciting unwanted immune responses (7, 9). Moreover, polyesters could be tolerated by mammalian systems and have been used in medical implants, such as bone scaffolding, due to their biocompatibility and elastomeric properties (121). In addition, recombinant DNA technologies have been extensively used to study and modify biopolyesters (9, 27, 61). Indeed, many desired proteins have been successfully displayed on the surface of polyester particles by bioengineering of the embedded PHA synthase, PhaC (7, 9, 20,

108). These polyester particles displaying desired proteins have successfully been produced by recombinant *Escherichia coli* cells (9, 27, 61).

1.3.2 PHA synthases

PHA synthases have been divided into four major classes based on their primary structure, subunit composition, and substrate specificity (110, 114, 122). Class I and II PHA synthases have a single subunit, PhaC, with a molecular weight (MW) between 60 kDa and 73 kDa (123). Class I PHA synthases prefer short-chain length 3-hydroxy fatty acids for polyester production and are mostly found in *Cupriavidus nector* (*C. nector*) (114, 124). However, Class II synthases are mainly found in *Pseudomonas aeruginosa* (*P. aeruginosa*) and, preferentially, use medium-chain length 3-hydroxy fatty acids for polyester biosynthesis (114, 124).

In spite of PhaC, Class III and IV PHA synthases each contain an additional subunit, PhaE and PhaR, respectively (110, 114, 123). Class III synthases are mostly found in *Allochromatium vinosum*; contain two subunits, PhaC and PhaE, which have a similar molecular weight (WM) of 40 kDa; and use both short- and medium-chain length 3-hydroxy fatty acids as substrates for polyester production (110, 123). The PhaC subunit of Class III synthases shows a 21%–28% protein sequence similarity to Class I and II PhaC, but the subunit PhaE does not exhibit any protein sequence similarity to PhaC (110, 123). The composition of Class IV synthases is similar to Class III synthases, in which PhaE is replaced with PhaR, a relatively small protein with a MW of 22 kDa (110, 114, 123). Class IV synthases are mainly found in *Bacillus megaterium* and preferentially use short-chain length 3-hydroxy fatty acids for polyester synthesis (110, 114).

PHA synthases exist as monomers and dimers, which are present in equilibrium and the dimerized synthases are the active form required for polyester biosynthesis (110, 125). The presence of suitable substrates induces dimerization of PHA synthases, and the subsequent dimerized forms polymerize available substrates to self-assemble into polyester particles (126). A mutagenic study demonstrates that the highly conserved tryptophan-425 of the Class I PHA synthases is needed to form a hydrophobic surface for dimerization of PhaC by mediating protein–protein interactions (127, 128).

Moreover, Class I and II synthases form homodimers, but Class III and IV synthases could form multimeric heterodimers (110).

1.3.3 Self-assembly of polyester particles

PHA biosynthesis requires three main enzymes, PHA synthase (PhaC), β-ketothiolase (PhaA), and acetoacetyl-CoA reductase (PhaB), encoded by *phaC*, *phaA*, and *phaB*, respectively (7, 109, 110). Class I and II PHA synthases require the expression of gene *phaC*. However, Class III and IV synthases are encoded by both *phaC* and *phaE*/*phaR* (110). Generally, PhaA and PhaB enzymes produce the substrate (*R*)-3-hydroxyacyl-CoA, which is polymerized to form PHAs by PhaC dimer (or PhaC and PhaE/PhaR for Class III/IV PHA synthases) (129, 130).

As mentioned in Sections 1.3.1 and 1.3.2, the most common form of polyesters is PHB, found in *C. nector*, which produces Class I PHA synthase (114, 118, 119, 124). Particularly, two acetyl CoAs were condensed by enzyme PhaA to form acetoacetyl-CoA, which is reduced to (*R*)-3-hydroxybutyryl-CoA by enzyme PhaB. Ultimately, the polymerization of the monomeric substrate, (*R*)-3-hydroxybutyryl-CoA, is catalyzed by the PhaC dimer to form PHB with the release of CoA (129, 130).

The formation of polyester particles is a self-assembly process (7). The exact mechanism of polyester particle assembly is unknown; however, two models of particle formation, the micelle model and the budding model (**Fig. 1**), are proposed. The micelle model is based on the amphipathic property of growing PHA chains (110, 131, 132). In particular, the dimerized PHA synthase remains covalently attached to the growing PHA chain, the increased hydrophobicity of which converts the soluble synthase into an amphipathic molecule (110, 131-133). The elongating PHA chains aggregate to form a hydrophobic core of polyester particles surrounded by a protein layer containing PHA synthases (110, 133). The micelle model is supported by polyester particle formation *in vitro* using purified PHA synthases and relevant substrates (110).

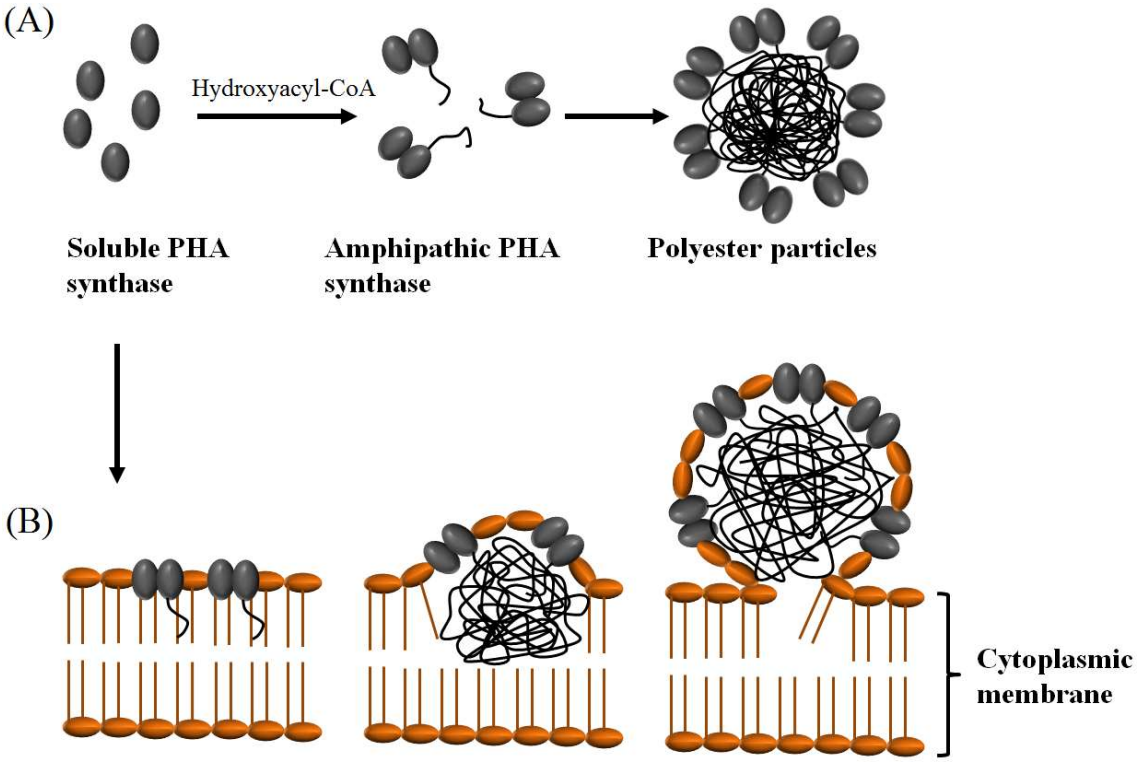

Figure 1. Models of polyester particle self-assembly. (A) Particle self-assembly by micelle model. (B) Particle self-assembly by budding model from cytoplasmic membrane.

The budding model proposes that the cytoplasmic membrane is involved in polyester particle formation (134). In particular, dimerized PHA synthases localize to the inner cytoplasmic membrane where the elongating PHA chains are synthesized and self-assembled into spherical particles between the phospholipid bilayer (110). Eventually, polyester particles are budded into the cytoplasm and surrounded by a phospholipid monolayer and PHA synthases (110, 133). However, there is no evidence that polyester particles have a phospholipid layer *in vivo* (135). A study has shown that several different natural polyester producers, such as *C. nector* and *Pseudomonas putida*, contain polyester particles coated with particle-associated proteins only, such as PHA synthases. Moreover, a phospholipid layer is not found on the particle surface (136).

1.3.4 Polyester particles as diagnostic reagents and particulate vaccines

Polyester particles have been engineered to display a variety of desired proteins, and this could be achieved by fusing them to PHA synthase PhaC as it remains covalently attached to the surface after the particle self-assembly process (7, 9, 61, 108). It has

been demonstrated that multiple regions of the N- and C-terminus of PhaC are dispensable and could be replaced by proteins of interest without affecting polyester particle formation (137). Moreover, various foreign protein functions, such as antigens, binding domains, and enzymes, have been successfully displayed on the surface of polyester particles (7, 9, 108). Production and display of foreign proteins on the surface of polyester particles are easy, efficient, and cost-effective as particle formation is implemented intracellularly in bacterial cells by one-step production, and additional cross-linking *in vitro* is not needed after particle isolation and purification (7, 11, 27, 61). Overall, the successful functionalization of the particle surface provides engineered polyester particles' broad applications, including diagnostic reagents and subunit vaccines development (7, 9, 27, 61, 138).

1.3.4.1 Polyester particle-based diagnostic reagents

As described in Section 1.1.3, the Mantoux tuberculin skin test is the only available skin test to diagnose TB. However, false positive results could be obtained if humans or cattle are exposed to non-tuberculous environmental mycobacteria or vaccinated with the BCG strain (6, 58, 60, 139, 140). Thus, a new, highly accurate skin test reagent composed of specific TB antigens is required to circumvent the low specificity of the current tuberculin skin test (60).

Recently, a highly specific and sensitive TB skin test reagent has been developed by displaying particular TB diagnostic antigens, CFP10, ESAT6, and Rv3615c, on the surface of polyester particles (27, 141). Particularly, three skin test diagnostic reagents — avian PPD, bovine PPD, and polyester particle-displaying CFP10-Rv3615c-ESAT6 — are all very sensitive and capable of identifying *M. bovis*-infected cattle (27, 141). However, only the triple antigen polyester particle is able to accurately identify BCG-vaccinated and environmental mycobacteria-sensitized cattle, but avian PPD and bovine PPD show dramatically reduced specificities (27, 141).

1.3.4.2 Polyester particle-based particulate vaccines

A number of efficacious polyester particle-based particulate vaccines have been successfully developed through the bioengineering of the surface-embedded PHA

synthase, PhaC (9, 106, 108). For example, the food-grade bacterium *Lactococcus lactis* has been engineered to produce polyester particles that abundantly display the hepatitis C virus core antigen. Immunization with the particulate hepatitis C vaccine in mice has elicited antigen-specific Th1 and Th17 immunity (106). Moreover, antigens from *P. aeruginosa* have been displayed on polyester particles. Mice immunization with the resulting particulate vaccine has triggered strong and specific Th1 and Th2 immune responses (108). In addition, polyester particles have been covalently coated with proteins and carbohydrate antigens from *Neisseria meningitidis* (*N. meningitidis*). The particulate vaccine containing proteins and carbohydrates has induced protective immunity in mice against *N. meningitidis* infection (9).

Studies have shown that mice appear healthy following immunization (9, 106). There are no abnormal behaviors and adverse effects, such as suppuration at the injection site, suggesting polyester particle-based particulate vaccines are safe and have no toxic effects (7, 9, 106, 108). In addition, there is an indication that polyester particles may possess adjuvant properties as the surface antigenic proteins could induce antigen specific CD4+ T cell effector responses and lead to the development of improved immunity (9, 106, 142, 143).

1.4 Protein inclusion bodies

Protein inclusion bodies (protein particles) are large insoluble protein aggregates, which have been commonly observed in bacteria used for overproduction of recombinant proteins (144, 145). Initially, it was thought that protein inclusion bodies are comprised of misfolded and inactive proteins (145). However, studies have demonstrated that protein inclusion bodies produced in bacteria cells contain properly folded as well as biologically active proteins (12, 21, 144-146). Thus, protein inclusion bodies contain not only misfolded proteins but also a large amount of properly folded and biologically active proteins (12, 21-24). Promisingly, recent studies have shown that protein inclusion bodies could perform as antigen carrier systems for efficient antigen delivery and the ultimate induction of desirable immune responses (12, 21).

1.4.1 Protein inclusion body formation

The formation of protein inclusion bodies *in vivo* is a self-assembling process (22). In particular, the biosynthesis of protein inclusion bodies is illustrated in **Fig. 2**. Recombinant proteins are often overproduced and overwhelm bacterial cell repair machinery (22, 147). Unlike Eukaryotic cells, bacteria lack compartmentalization and thus protein synthesis in the cytoplasm occurs simultaneously at multiple locations (22). Synthesized polypeptides are at different translational folding states, including misfolded and properly folded proteins (22). Hydrophobic interactions between misfolded proteins lead to the formation of small proto-aggregates, during the process of which properly folded and biologically active proteins are also incorporated into proto-aggregates (22). The growing proto-aggregates become larger and eventually fuse together to form protein inclusion bodies (22). It has been observed that protein inclusion bodies often have a high level of purities. This is probably because protein intermediates only aggregate with themselves but do not aggregate with non-homologous cellular proteins (148, 149).

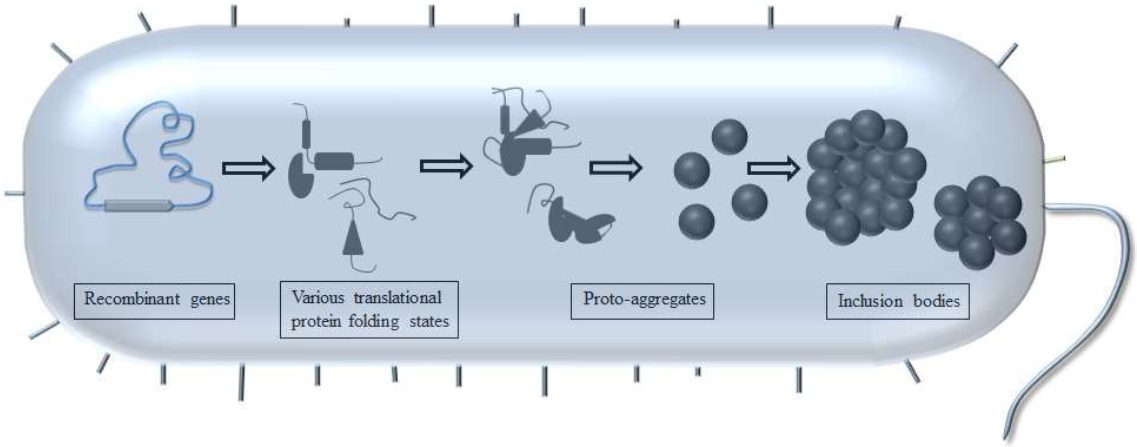

Figure 2. Schematic overview of protein inclusion body formation in bacterial cells.

The size and shape of protein inclusion bodies are diverse and dependent on various factors, including the cultivation time and host cells (22, 150). Protein inclusion bodies are spherical in shape at the early cultivation time point. However, the spherical shape of protein inclusion bodies will be changed when they reach the host strain cell wall. In particular, after long-term cultivation, protein inclusion bodies are usually elongated

into a cylinder-like structure, which could potentially occupy the whole cytoplasmic space (22, 148). In addition, as the proto-aggregates are fused together, an amorphous matrix fills the spaces between the proto-aggregates and imparts protein inclusion bodies to give the appearance of a porous surface (22). Usually, only one protein inclusion body is formed in bacterial cells, and two are rare. When cell division occurs, the protein inclusion body remains in one cell and continues to grow. However, protein synthesis and inclusion body formation will start *de novo* in a new cell (22).

1.4.2 Potential of protein inclusion bodies as particulate vaccines

Similar to polyester particle-based particulate vaccines, protein inclusion body-associated particulate vaccines, which are comprised of immunogenic antigens, could also serve as a depot for a tight and prolonged antigen binding to elicit increased immunogenicity (8, 11). Interestingly, protein inclusion bodies can tolerate ultra-sonication, high pressure, and other mechanical disruptions during the isolation and purification process (151). Moreover, protein inclusion bodies are able to retain their functionalities after lyophilization and freeze-thawing, and remain stable under long-term storage conditions (23, 150).

Recently, protein inclusion bodies have been used to develop various vaccines for protection against different diseases (21, 152, 153). For example, the first application of protein inclusion bodies was the development of a vaccine to protect against malaria, a disease transmitted through the bite of an infected *Anopheles* mosquito (154). Briefly, the circumsporozoite protein antigens from rodent malaria species *Plasmodium berghei* has been bioengineered for the self-assembly of immunogenic protein inclusion body vaccines (154). Encouragingly, the vaccine without formulation with an adjuvant could provide a long-lasting protection in different mouse strains (154). Protein inclusion bodies have also been used to develop a universal influenza A vaccine, which contains the external domains of the viral membrane proton channel M2 (155). High titers of antibodies with neutralizing activity have been observed in vaccinated animals (155). Particularly in mouse models, the animals have been completely protected from the influenza challenge (156). In addition, the protein inclusion body platform has been used to develop a vaccine to protect against coronavirus that can cause severe acute respiratory syndrome. This protein inclusion body-based vaccine contains multiple

copies of the B cell epitope from the C-terminus of the virus spike protein and has resulted in an immense production of conformation–specific antibodies with the ability of neutralizing coronavirus *in vitro* (157). Thus, the protein inclusion body platform shows promise and also has the potential to be an efficient and stable antigen carrier system for the development of various vaccines against different pathogens.

1.5 Endotoxin free expression system

Recombinant Gram-negative bacteria, such as *E. coli*, are often used as a versatile host for DNA manipulation and the production of heterologous proteins (158-160). However, Gram-negative bacteria are known to contain lipopolysaccharide (LPS) endotoxins, which constitute a major component of their outer membrane and have been demonstrated to induce strong immune responses in both humans and animals (161, 162).

LPS endotoxins could be co-purified with functionalized biological products derived from Gram-negative bacteria, which is undesirable in certain applications (163). Vaccine delivery agents derived from Gram-negative bacteria have to meet Food and Drug Administration (FDA) guidelines for approval, and these only allow a maximum of 20 endotoxin unit per medical device (164). Multiple purification steps are required to remove endotoxins to an acceptable level approved by the FDA. Commonly used methods are ion-exchange chromatography, ultrafiltration, and oxidizing agents, which may be ineffective or too harsh (161, 162, 165). Therefore, alternative production organisms that make endotoxin-free vaccines are advantageous.

ClearColi BL21 (DE3) is an endotoxin–free host derived from wild-type BL21 (DE3) *E. coli* cells. As aforementioned, the endotoxin LPS could induce a strong immune response in both human and animals (161, 162, 166). LPS is composed of a distal hydrophilic polysaccharide and a hydrophobic domain (Lipid A) (161, 162). In *E. coli*, Lipid A contains a disaccharide backbone and 6 acyl chains (the number of acyl chains varies in different Gram-negative bacteria), a determinant of endotoxicity (166). Thus, these acyl chains are responsible for the major biological activity of endotoxins, which

are known to elicit a wide variety of pathophysiological effects, such as systemic inflammation, fever, coagulopathy, shock, and even death (161, 162, 165).

The endotoxin free *ClearColi* BL21 (DE3) cells contain a modified LPS, Lipid IV_A. The *ClearColi* cells are comprised of seven separate gene deletions, including *gutQ*, *kdsD*, *lpxL*, *lpxM*, *pagP*, *lpxP*, and *eptA*. These mutations cause the removal of the oligosaccharide chain and two of the six acyl chains from LPS (167). *ClearColi* cells also contain an additional compensating mutation (*msbA148*), which enables the cells to maintain viability in the presence of Lipid IV_A (167). When compared to the six acyl chains of LPS, the four acyl chains of Lipid IV_A do not trigger the endotoxic response in mammalian cells (168). Moreover, the growth rate of the *ClearColi* may be slower than the wild type BL21 (DE3) cells, but their final protein production levels are very similar (167).

1.6 General hypothesis

Particulate platforms (polyester particles and protein inclusion bodies) are able to perform as antigen carrier systems for the development of specific and cost–effective TB skin test reagents and of efficacious particulate subunit vaccines to protect against TB.

1.7 Aim and scope of this study

TB is a global health problem for both humans and animals. Efficacious TB vaccines are important to control this disease worldwide. Nevertheless, specific and cheap diagnostic reagents to accurately distinguish TB-infected from non-infected humans and cattle are also critical to restrict TB transmission globally. Due to the non-specific antigens in PPDs, the current Mantoux tuberculin skin test has low specificity and is unable to accurately identify TB-infected humans and cattle if they are vaccinated by BCG and/or sensitized by environmental mycobacterial strains. The only licensed vaccine, BCG, fails to provide adequate protection in humans. Subunit TB vaccines often exhibit low immunogenicity and tend to provide inadequate levels of protection as they have limited antigen diversity and cannot replicate, unlike live-attenuated vaccines.

The primary aim of this study was to use innovative polyester particles and protein inclusion bodies as the platforms to develop highly specific and cost-effective TB skin test reagents as well as efficacious particulate TB vaccines. Briefly, this study is divided into three parts shown below:

- Specific mycobacterial antigens, CFP10, Rv3615c, ESAT6, and Rv3020c, were displayed on polyester particles to develop specific and cheap bovine TB skin test reagents.
- Polyester particles were used as the antigen carrier system to deliver subunit TB vaccines H4 (Ag85B-TB10.4) or H28 (Ag85B-TB10.4-Rv2660c) to develop polyester particle-based particulate TB vaccines.
- TB vaccines H4 or H28 were bioengineered towards the self-assembly of protein inclusion bodies (protein particles) containing only mycobacterial antigens.

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Preface to Chapter 2

TB is a global health issue for humans and cattle and tuberculous strains of mycobacteria are the major cause of the disease. The Mantoux tuberculin skin test is a primary tool for TB diagnosis, but this test often shows low specificity as the non-specific antigens in the reagent (PPDs) could cross-react with the anigens in the BCG vaccine strain and environmental mycobacteria. Thus, the Mantoux tuberculin skin test tends to give false positive results when humans and cattle are vaccinated with the BCG strain or sensitized with environmental mycobacteria.

Specific TB diagnostic antigens, CFP10, ESAT6, Rv3615c, and Rv3020c have been found in tuberculous mycobacteria, such as *M. tuberculosis* and *M. bovis*, but are absent in the majority of environmental mycobacteria and the BCG vaccine strain. In Chapter 2, the polyester particle was used as the antigen carrier system to display the four TB diagnostic antigens for the development of specific as well as cost-effective bovine TB skin test reagents.

Chapter 2. Display of antigens on polyester inclusions lowers the antigen concentration required for a bovine tuberculosis skin test

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2.1 Abstract

The tuberculin skin test is the primary screening test for the diagnosis of bovine tuberculosis (TB), and use of this test has been very valuable in the control of this disease in many countries. However, the test lacks specificity when cattle have been exposed to environmental mycobacteria or vaccinated with *Mycobacterium bovis* bacille Calmette-Guérin (BCG). Recent studies showed that the use of three or four recombinant mycobacterial proteins, including 6-kDa early secretory antigenic target (ESAT6), 10-kDa culture filtrate protein (CFP10), Rv3615c, and Rv3020c, or a peptide cocktail derived from those proteins, in the skin test greatly enhanced test specificity, with minimal loss of test sensitivity. The proteins are present in members of the pathogenic *Mycobacterium tuberculosis* complex but are absent in or not expressed by the majority of environmental mycobacteria and the BCG vaccine strain. To produce a low-cost skin test reagent, the proteins were displayed at high density on polyester particles through translational fusion to a polyhydroxyalkanoate synthase that mediates the formation of antigen-displaying inclusions in recombinant *Escherichia coli*. Display of the proteins on the polyester particles greatly increased their immunogenicity, allowing for the use of very low concentrations of proteins (0.1 to 3 μg of mycobacterial protein/inoculum) in the skin test. Polyester particles simultaneously displaying all four proteins were produced in a single fermentation process. The polyester particles displaying three or four mycobacterial proteins were shown to have high sensitivity for detection of *M. bovis*-infected cattle and induced minimal responses in animals exposed to environmental mycobacteria or vaccinated with BCG.

2.2 Introduction

Control of bovine tuberculosis (TB), which is caused by infection with *Mycobacterium bovis*, is critical, as this disease is of great economic and zoonotic importance. Programs for eradication in cattle are primarily based on use of the tuberculin skin test for diagnosis of the disease, with slaughter of reactor animals. This approach has been instrumental in eradication of this disease from a number of countries (1). The tuberculin skin test is a delayed type hypersensitivity (DTH) test that was developed more than 100 years ago for the diagnosis of TB, and it has proved to be a simple,

inexpensive, robust, and widely accepted test. However, the test lacks specificity when animals have been sensitized to environmental mycobacteria or have been vaccinated with the human TB vaccine, *M. bovis* bacille Calmette-Guérin (BCG) (2). Estimates of sensitivity for a single intradermal test in cattle using purified protein derivative (PPD) prepared from *M. bovis* (bovine PPD) have ranged from 63.2% to 100% (median, 83.9%), with specificity between 75.5% and 99.0% (median, 96.8%) (2). Development of a more specific skin test reagent would be highly desirable. The use of BCG vaccine alone or as part of a heterologous prime-boost combination is currently being considered by a number of countries for control of bovine TB (3). BCG cannot be used currently, as it compromises interpretation of the tuberculin skin test, and development of a test to differentiate infected from vaccinated animals (DIVA) would be essential.

The tuberculin skin test utilizes bovine PPD, which is a poorly defined mixture of proteins, lipids, and carbohydrates, including components that are present in nonpathogenic environmental mycobacteria. The two major formats for the tuberculin skin test are the caudal fold test, in which bovine PPD is injected intradermally in the caudal fold of the tail, and the single intradermal comparative cervical test (SICCT), in which PPDs are injected in the neck. The SICCT compares reactions induced following intradermal injection of bovine PPD and *Mycobacterium avium*-derived PPD (avian PPD), to control for environmental sensitization.

Three highly immunogenic specific TB antigens, namely, 6-kDa early secretory antigenic target (ESAT6), 10-kDa culture filtrate protein (CFP10), and Rv3615c, are expressed by members of the pathogenic *M. tuberculosis* complex, which includes *M. bovis*, but are not expressed by the majority of nonpathogenic environmental mycobacteria and the BCG vaccine strain (4-7). Those proteins, or peptides derived from those proteins, have been shown to enhance skin test specificity in cattle, while maintaining relatively high sensitivity for the diagnosis of bovine TB (8, 9). More recently, the addition of peptides from a fourth specific mycobacterial protein, Rv3020c, further enhanced test sensitivity (10). The tuberculin skin test is used as the primary screening test for bovine TB, and three major criteria must be satisfied before bovine PPD can be replaced with specific antigens. Use of specific antigens must provide improved test specificity with minimal loss of test sensitivity, and the cost of the reagents must be similar to that of PPD. Recent research has indicated that the first two

criteria could be met, while use of recombinant proteins or a peptide cocktail would increase the cost of the reagents.

The cost of reagents could be reduced by using low concentrations of the proteins, by displaying them on nanoparticles, potentially increasing their immunogenicity, and producing them as a recombinant fusion protein. This has been achieved by displaying ESAT-6, CFP10, and Rv3615c on polyester inclusions (bioparticles) produced by *Escherichia coli*, and preliminary results have indicated their utility in skin testing for the diagnosis of bovine TB (11). Polyester inclusions are naturally produced by various bacteria during imbalanced nutrient availability in which excess carbon is available and is deposited as spherical water-insoluble cytoplasmic inclusions (12, 13). The polyesters are composed of (*R*)-3-hydroxy-fatty acids with different carbon chain lengths (14). Foreign proteins have been displayed on the polyester particles by translationally fusing them to a polyester synthase (PhaC), which has mediated formation of protein-displaying particles in recombinant *E. coli* (15, 16). The particles are 100 to 500 nm in diameter, and particles contain an amorphous hydrophobic polyester core surrounded by proteins, including the fusion protein composed of PhaC and foreign proteins (12, 17). Interestingly, immunological studies using antigen-displaying particles revealed that the particles showed adjuvant properties by enhancing the immune response to the displayed antigen, compared to its soluble counterpart (18).

This paper extends findings from the earlier study (11) by demonstrating that bioparticles displaying ESAT-6, CFP10, and Rv3615c (3-protein bioparticles) were effective in identifying experimentally and naturally *M. bovis*-infected cattle, as well as distinguishing them from BCG-vaccinated noninfected animals. In addition, bioparticles were designed and produced to display four mycobacterial proteins, i.e., ESAT-6, CFP10, Rv3615c, and Rv3020c (4-protein bioparticles) simultaneously, potentially increasing test sensitivity (10). Such bioparticles were successfully produced and analyzed, and skin test performance experiments showed that they were effective in identifying *M. bovis*-infected animals in skin tests, using very low concentrations of the mycobacterial proteins.

2.3 Materials and methods

2.3.1 Animals

The groups of cattle used to assess the skin test performance of the bioparticle reagents are shown in **Table 1**. The noninfected animals were from TB-free herds located in TB-free regions of New Zealand, some of which had been naturally exposed to environmental mycobacteria, as indicated by strong skin test or interferon gamma (IFNγ) responses to avian PPD. The BCG-vaccinated cattle were cattle that had been vaccinated subcutaneously with BCG (2×10^5 to 8×10^5 CFU; Statens Serum Institute, Denmark) at 2 to 4 weeks of age and revaccinated with the same dose of vaccine at 2 years of age (19). The animals were skin tested 11 weeks after revaccination. The three groups of cattle had been experimentally infected endobronchially with approximately 6,000 CFU of *M. bovis*, as described previously (20). The naturally *M. bovis*-infected cattle were from infected herds from the west coast of the South Island and Waikato, New Zealand, and the three groups of animals were identified as infected with *M. bovis* on the basis of positive results for initial caudal fold skin tests with bovine PPD. All of the experimentally *M. bovis*-infected cattle and all except one of the naturally *M. bovis*-infected cattle were confirmed as infected through culture of *M. bovis* from tissues obtained at slaughter, with confirmation for the remaining animal on the basis of typical gross and histopathological lesions. All animal manipulations were approved by the Grasslands Animal Ethics Committee, New Zealand.

TABLE 1. *Cattle tested with comparative cervical skin tests with biobead skin test reagents*

Group	No. of animals	Age when tested (mo)	Bioparticles tested	Time after vaccination, challenge, or skin test
Control	12	27	3-protein[a]	NA[b]
	24	9	4-protein[c]	NA
BCG-vaccinated	12	27	3-protein	11wk after vaccination
Experimentally *M. bovis*-infected	10	12	3-protein	27 wk after challenge
	12	33	3-protein	11 wk after challenge
	10	12	4-protein	10 wk after challenge
Naturally *M. bovis*-infected	11	Mixed ages	3-protein	11 wk after initial skin test
	9	Mixed ages[d]	3-protein and 4-protein	15 wk after initial skin test
	7	Mixed ages[d]	4-protein	10 wk after initial skin test

[a] The 3-protein bioparticles displayed three mycobacterial proteins (ESAT-6, CFP10, and Rv3615c) on their surfaces.

[b] NA, not applicable.

[c] The 4-protein bioparticles displayed four mycobacterial proteins (ESAT-6, CFP10, Rv3615c, and Rv3020c) on their surfaces.

[d] Cattle were also tested in the caudal fold test with the 4-protein bioparticles.

2.3.2 Antigens

Bovine and avian PPDs were supplied by AsureQuality (Upper Hutt, New Zealand) and Prionics (Lelystad, The Netherlands). Purified recombinant proteins, i.e., ESAT-6, CFP10, and Rv3615c, were supplied by Lionex Diagnostics and Therapeutics GmbH (Germany).

2.3.3 Production of polyester particles displaying mycobacterial proteins

Polyester particles displaying three mycobacterial proteins, i.e., ESAT-6, CFP10, and Rv3615c (3-protein bioparticles), were produced in *E. coli* BL21(DE3) as described previously (11). In order to produce polyester particles that simultaneously displayed four mycobacterial antigens, i.e., ESAT-6, CFP10, Rv3615c, and Rv3020c (4-protein bioparticles), we designed and constructed a hybrid gene encoding all four antigens fused to a polyester synthase as a single polypeptide. Briefly, the DNA fragment encoding the antigens ESAT-6 and Rv3020c was synthesized by Genscript (USA), with codon optimization for expression in *E. coli*. This DNA fragment was subcloned directly into the 3' end of the polyester synthase gene from the plasmid construct pET-14b cfp10-linker-rv3615c-phaC-linker-malE, resulting in a hybrid gene encoding the single fusion protein CFP10-Rv3615c-PhaC-ESAT6-Rv3020c. The cloning strategy is outlined in **Fig. 1**. The resulting plasmid, pET-14b cfp10-linker-rv3615c-phaC-linker-esat6-linker-rv3020c, was transferred into *E. coli* BL21(DE3) (pMCS69) to assess production of polyester particles. The plasmid pMCS69 contains the genes *phaA* and *phaB* from *Ralstonia eutropha*, which both mediate synthesis of the polyester precursor (*R*)-3-hydroxybutyryl-CoA.

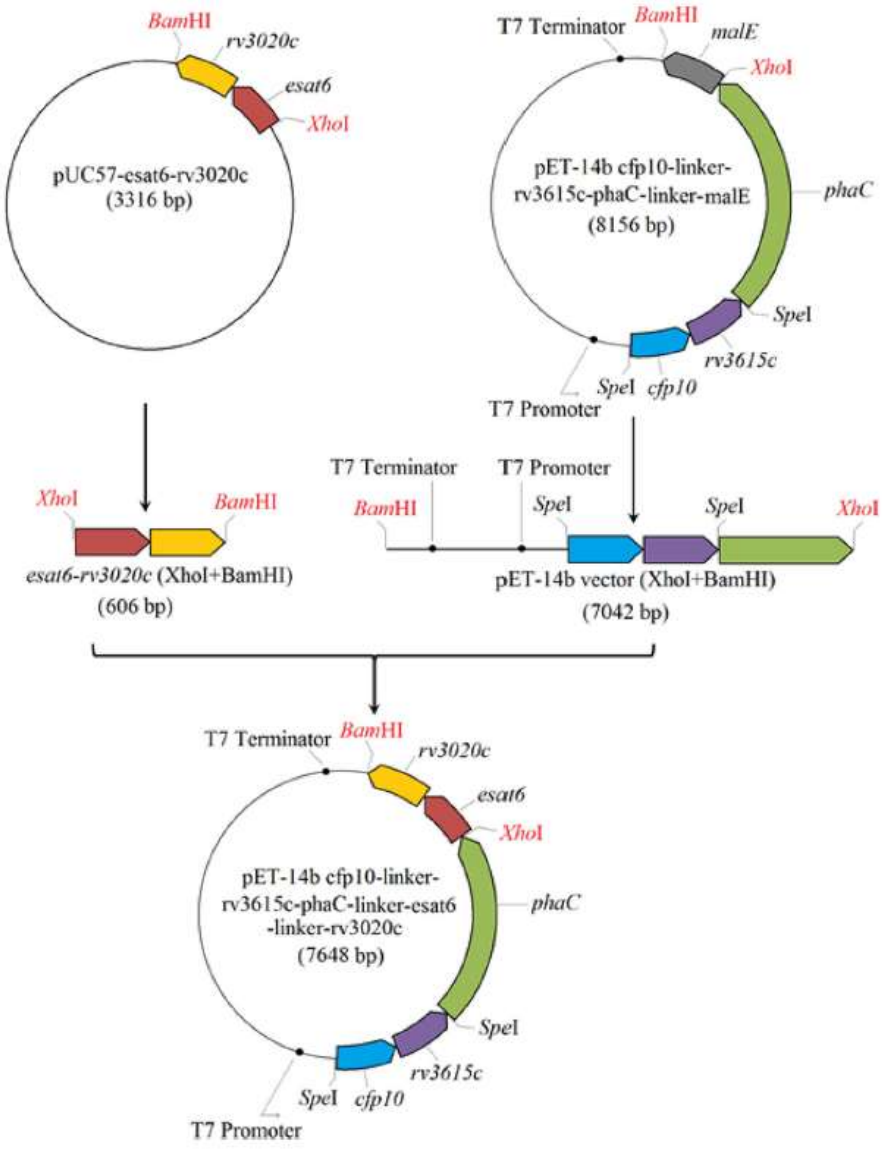

Figure 1. Cloning strategy for production of the CFP10-Rv3615c-PhaC-ESAT6-Rv3020c fusion protein.

To confirm that the particle preparations were sterile, samples of the preparations were spread on LB agar. In the later experiments that compared the use of the 4-protein and 3-protein bioparticle preparations, the particles were γ-irradiated (12.5 kGy; MSD Ltd., Upper Hutt, New Zealand) as an additional step to ensure sterility. Dextran at a final concentration of 15% (USP grade; Pharmacosmos A/S, Holbaek, Denmark) was added to the bioparticle preparations to keep the particles in suspension.

2.3.4 Analysis of proteins attached to polyester particles

For analysis of the proteins, the fusion protein consisting of mycobacterial proteins and PhaC protein was separated from the polyester particles by SDS-PAGE on a 8% polyacrylamide gel and was stained with Coomassie blue. Proteins of interest were excised from the gels and subjected to tryptic peptide fingerprinting using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (21). The mycobacterial protein concentration contained in the particles was estimated by first measuring the total amount of protein contained in the particles. Then the proportion of protein attributed to the fusion protein was determined by densitometry, and the amounts of mycobacterial proteins contained in the fusion protein were calculated from the molecular weights (MWs) of the mycobacterial proteins in comparison with that of the PhaC protein.

2.3.5 Testing for sensitizing effects

The method used to determine whether the bioparticles had sensitizing effects was based on the method used to test batches of bovine tuberculin (22). Briefly, a group of three guinea pigs that had not been treated previously with any material that could interfere with the test were injected intradermally on the abdominal flank with 0.1 ml of the 4-protein bioparticle skin test reagent, containing 0.3 μg of mycobacterial proteins, on three occasions, with intervals of 5 days. The concentration of bioparticles was one-tenth of the highest cattle dose and was equivalent to the one-tenth of the cattle dose of bovine tuberculin (500 IU) used to assess sensitization in guinea pigs. Each guinea pig, together with each of three control guinea pigs that had not been injected previously, was injected intradermally in the abdominal flank 15 days after the third injection with the same dose of the bioparticle reagent. The guinea pigs were examined 24 to 28 h later, to determine whether there was any difference in the reactions between the two groups of animals.

2.3.6 Skin testing of cattle

The comparative cervical skin test in cattle was undertaken by injecting 0.1-ml volumes of the reagents intradermally in the mid-neck region. Up to six inoculation sites were used on each side of the neck, with at least 50-mm spaces between the sites. The hair at each site was clipped, and the skin thickness at the injection site was measured with calipers immediately prior to injection and 72 h later, with results being expressed as the change in skin thickness (in millimeters). Changes in skin thickness of < 1 mm could not be measured accurately, and the positive cutoff value was set at a ≥ 1-mm increase in skin thickness between 0 and 72 h postinoculation. Different concentrations of the bioparticles displaying mycobacterial proteins were prepared by dilution in phosphate-buffered saline (PBS). Bovine PPD (5,000 IU/0.1 ml for PPD from AsureQuality [Upper Hutt, New Zealand] or 3,000 IU/0.1 ml for PPD from Prionics [Lelystad, The Netherlands]) was included in each test. Manufacture and supply of the AsureQuality PPDs were discontinued midway through the study. The comparative caudal fold skin test was performed by intradermally injecting a 0.1-ml volume containing the 4-protein bioparticle reagent into the caudal fold on one side of the tail and a 0.1-ml volume containing bovine PPD (3,000 IU; Prionics) into the caudal fold on the other side. The caudal folds were palpated 72 h after injection, and any detectable lumps were measured with calipers. For one group of seven cows, two different doses of the 4-protein bioparticle reagent were injected intradermally approximately 50 mm apart in one caudal fold, with bovine PPD being injected in the other caudal fold.

2.3.7 Statistical analyses

A mixed-effects model was used for comparisons of different test reagents or concentrations of reagents (treatment), with treatment serving as the fixed effect and animal as the random effect. For analysis of data for experimentally infected cattle tested with the 3-protein bioparticles, the results from two studies were combined, requiring a meta-analysis. For this analysis, a mixed-effects model was used, with treatment serving as the fixed effect and animal plus treatment nested within experiment as the random effects. A mixed-effects model was used for comparison of injection sites, reagents, and times of test reading (hours), with site, reagent, and time as the fixed effects and animal as the random effect. The results from these models provided

multiple comparisons of the predicted means, with *p* values adjusted by the Benjamini-Hochberg method (23). The analyses were performed with the R packages nlme, lme4', and predictmeans in R 3.2.0 (24, 25)**.** Statistical significance was denoted by *p* values of < 0.05.

2.4 Result

2.4.1 Engineering of *E. coli* for production of 4-protein bioparticles

A plasmid encoding PhaC and the mycobacterial genes *esat6*, *cfp10*, *Rv3615c*, and *Rv3020c* was constructed as described in Materials and Methods. Briefly, antigen Rv3615c was inserted between CFP10 and the N terminus of PhaC, and antigens ESAT6 and Rv3020c were fused to the C terminus of PhaC, resulting in a single fusion protein containing the four mycobacterial proteins. Recombinant production of this fusion protein facilitated the formation of intracellular polyester particles in the *E. coli* cells. Formation of intracellular polyester particles in the *E. coli* cells was indicated by isolation of a white suspension from disrupted cells (data not shown). Gas chromatography-mass spectrometry analysis confirmed that the cells were accumulating the polyester polyhydroxybutyrate, which constitutes the core of the polyester particles, contributing about 34% of the cellular dry weight. SDS-PAGE analysis demonstrated that antigen-displaying particles showed a prominent protein with an apparent MW of 107 kDa for the PhaC-4 mycobacterial fusion protein, compared to a MW of 98 kDa for the PhaC-3 mycobacterial fusion protein (data not shown). The MW of the PhaC protein alone was 64 kDa. The components of the fusion protein were identified by tryptic peptide fingerprinting using MALDI-TOF MS (see **Table S1** in the supplemental material). Densitometric analysis of the SDS-PAGE results showed that the fusion proteins CFP10-Rv3615c-PhaC-ESAT6-Rv3020c and CFP10-Rv3615c-PhaC-ESAT6 accounted for approximately 70.5% of the total protein in their corresponding particle fractions (data not shown).

Sedimentation of the particles in the injection syringe was overcome by the addition of dextran to a final concentration of 15% (wt/vol). All animal studies with the 4-protein bioparticles, and with the 3-protein bioparticles used for comparison with the 4-protein

bioparticles, utilized preparations that contained 15% dextran and had been γ-irradiated as an added safeguard to ensure sterility. Testing of the 4-protein bioparticles in experimentally infected cattle demonstrated that the addition of 15% dextran and γ-irradiation did not affect the magnitude of the skin test responses (data not shown).

2.4.2 Testing for sensitizing effects in guinea pigs

The 4-protein bioparticles were tested for induction of sensitization at a dose of 0.3 μg mycobacterial protein (one-tenth of the cattle dose). There was no difference in the reactions at the skin test sites of the guinea pigs that had received multiple doses of the skin test reagent at 5-day intervals versus those that had received a single dose of the reagent, when examined 25 h postinoculation. Each of the three vaccinated guinea pigs had a small zone of erythema at the site of inoculation (3, 4, and 5 mm in diameter), with identical readings for the three nonvaccinated animals. In addition, two of the three vaccinated guinea pigs had a small area of induration (2 mm in diameter; 0 mm for the other guinea pig), while all three nonvaccinated guinea pigs had a 2-mm-diameter area of induration at the site of inoculation.

2.4.3 Reactivity of 3-protein bioparticles in cattle

All of the 22 experimentally infected cattle produced positive responses in the comparative cervical skin test (≥1-mm increase in skin thickness) 72 h following injection of the 3-protein bioparticles (3 μg of mycobacterial protein), three recombinant proteins (30 μg of total mycobacterial protein; 10 μg of each protein), and bovine PPD (5,000 IU; AsureQuality). Twenty-one of the 22 cattle tested positive with the 3-protein bioparticles (1 μg of mycobacterial protein/dose) (**Fig. 2*A***). The only significant difference between the reagents was that the mean response with bovine PPD was greater than that with the 3-protein bioparticles (1 μg/dose; $p < 0.05$). In naturally infected cattle, the two concentrations of the 3-protein bioparticles (1 and 3 μg/dose) produced responses in 10 of the 11 infected animals, while all tested positive with bovine PPD (5,000 IU; AsureQuality) (**Fig. 2*B***). The mean response for the 3-protein bioparticles (3 μg/dose) was significantly greater than that for bovine PPD ($p < 0.01$). One animal tested negative with the two concentrations of the 3-protein bioparticles

(Fig. 2*B*) and also with the three recombinant proteins used at a dose of 10 μg of total mycobacterial protein (data not shown). There was insufficient recombinant protein for testing at the recommended dose of 30 μg of total recombinant protein. Twelve cattle that had been vaccinated with BCG vaccine at 2 to 4 weeks of age and revaccinated 2 years later were skin tested 11 weeks after revaccination. Eleven of the 12 cattle tested positive with bovine PPD (5,000 IU; AsureQuality), 2 of 12 tested positive with the 3-protein bioparticles (3 μg/dose), and none tested positive with the 3-protein bioparticles (1 μg/dose) or the recombinant proteins (30 μg/dose) (**Fig. 2*C***). The noninfected cattle included 12 animals, some of which had been naturally exposed to environmental mycobacteria; six of those animals responded positively to avian PPD (2,500 IU; AsureQuality) in the skin test (data not shown). All tested negative with the 3-protein bioparticles (1 and 3 μg/dose) and the recombinant proteins (30 μg/dose), while two animals showed reactivity with bovine PPD (5,000 IU) (**Fig. 2*D***).

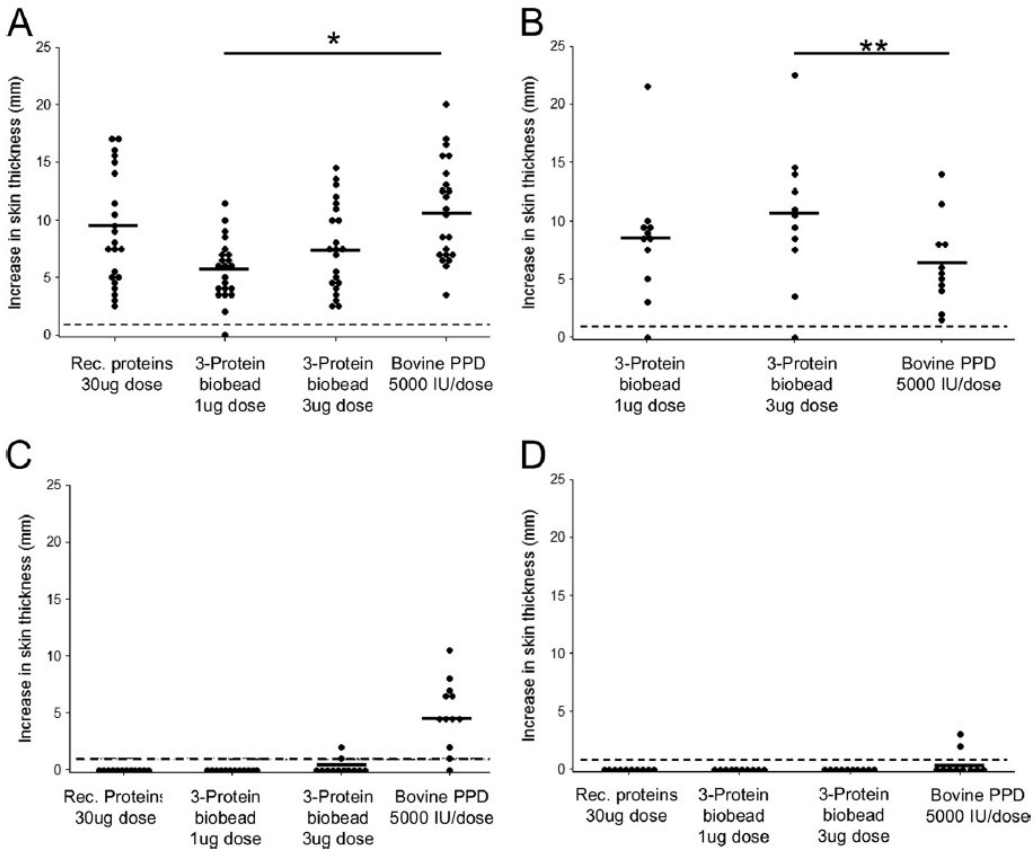

Figure 2. Skin test responses to the 3-protein bioparticles in cattle. Comparative cervical skin test responses for recombinant (Rec.) ESAT-6, CFP10, and Rv3615c proteins (total protein dose of 30 μg), 3-protein bioparticles (ESAT-6, CFP10, and Rv3615c) at 1 μg mycobacterial protein and 3 μg mycobacterial protein, and bovine PPD (5,000 IU/dose; AsureQuality) are shown. Results are presented as increases in skin thickness between 0 and 72 h postinoculation. (A) Cattle experimentally infected with *M. bovis* (n = 22). (B) Cattle naturally infected with *M. bovis* (n = 11); the recombinant proteins (30 μg dose) were not available for testing for this group. (C) BCG-vaccinated cattle (n = 12). (D) Naive (noninfected and nonvaccinated) cattle (n = 12). Dashed horizontal lines, 1-mm positive cutoff value. *, $p < 0.05$; **, $p < 0.01$. In panel A, there were no significant differences between the recombinant proteins and the two doses of the 3-protein bioparticles ($p > 0.05$). Horizontal bars, means.

2.4.4 Reactivity of 4-protein bioparticles in cattle

Concentrations of the 4-protein bioparticles ranging from 3 to 0.01 μg of mycobacterial protein were tested in the comparative cervical skin test in five cattle that had been experimentally challenged with *M. bovis* 10 weeks previously. There were no significant differences between the mean responses for the 4-protein bioparticle preparations containing 3, 1, 0.33, or 0.11 μg mycobacterial protein. In contrast, the mean responses for the 4-protein bioparticles containing 0.04 or 0.01 μg mycobacterial protein were significantly lower than those for the four higher doses of the bioparticles (p <0.05) (**Fig. 3**). A total of 24 noninfected animals (9 months of age) were tested with the 4-protein bioparticles (3 μg/dose) and bovine PPD (3,000 IU; Prionics) in the comparative cervical skin test, and no detectable increases in skin thickness of ≥ 1 mm were detected in any of those animals (data not shown).

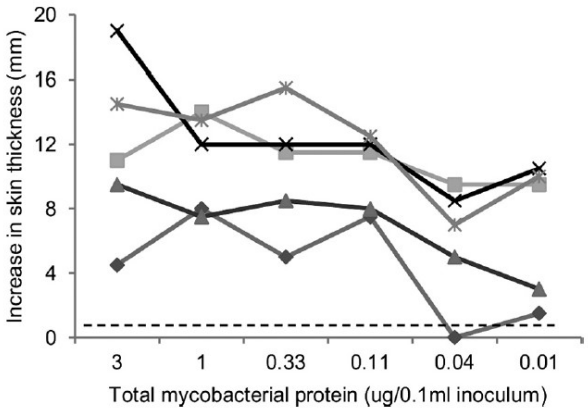

Figure 3. Skin test results for 4-protein bioparticle dose titration. Comparative cervical skin test responses in experimentally infected cattle (n = 5) for dilutions of the 4-protein bioparticles (ESAT-6, CFP10, Rv3615c, and Rv3020c) containing 3, 1, 0.33, 0.11, 0.04, or 0.01 μg of total mycobacterial protein/dose are shown. Responses for different animals are shown with different symbols. Results are presented as increases in skin thickness between 0 and 72 h postinoculation. Dashed horizontal lines, 1-mm positive cutoff value. Mean responses for dilutions of 4-protein bioparticles containing 3, 1, 0.33, and 0.11 μg mycobacterial protein were significantly greater than those for dilutions containing 0.04 and 0.01 μg protein (p < 0.05).

In a comparative cervical skin test, all nine naturally infected animals produced positive responses to the 3- and 4-protein bioparticle preparations (3 μg mycobacterial protein) and bovine PPD (3,000 IU, Prionics) (**Table 2**). The mean response for bovine PPD was significantly greater than those for the 3-protein and 4-protein bioparticles ($p < 0.01$), while responses for the two bioparticle preparations were very similar. In a comparative caudal fold test that was performed on the same day as the cervical skin test, there were no significant differences between the mean responses for the 4-protein bioparticles (3 μg mycobacterial protein) and bovine PPD (3,000 IU; Prionics) (Table 2). Similar results were observed in a second group of 7 naturally infected animals in which two doses of the 4-protein bioparticles were compared with bovine PPD in the cervical and caudal fold skin tests conducted on the same day (**Fig. 4**). The skin responses were significantly greater for bovine PPD than for the two doses of the 4-protein bioparticles in the comparative cervical test ($p < 0.001$) but not in the caudal fold test. For the latter group of animals, the overall responses in the cervical test were significantly greater than those in the caudal fold test ($p < 0.001$). No significant differences were detected when the tests were read at 72 and 96 h postinjection.

TABLE 2. *Skin test responses for 3- and 4-protein bioparticles in naturally M. bovis-infected cattle in comparative cervical and caudal fold tests performed on the same day (n = 9)*[a]

	Increase in skin fold thickness (mm)[b]				
	Comparative cervical skin test			Comparative caudal fold skin test	
Animal no.	3-protein bioparticles	4-protein bioparticles	Bovine PPD	4-protein bioparticles	Bovine PPD
1	1.5	2	5.5	2	1.5
2	2.5	4.5	9	5	4
3	3	4	8	2	6
4	4.5	4.5	9.5	1.5	3
5	10.5	10	25	5	6
6	11	11.5	14.5	7	6
7	12.5	12	12	9	3
8	12.5	14.5	11	9	4
9	13.5	15.5	18	9	5
Mean ± SEM	7.9 ± 1.6	8.7 ± 1.7	12.5 ± 2.0[c]	5.5 ± 1.0	4.3 ± 0.5

[a] Comparative cervical skin test responses to 3-protein bioparticles (ESAT-6, CFP10, and Rv3615c) (3 μg total mycobacterial protein), 4-protein bioparticles (ESAT-6, CFP10, Rv3615c, and Rv3020c) (3 μg mycobacterial protein), and bovine PPD (3,000 IU/dose; Prionics) and comparative caudal fold skin test responses to 4-protein bioparticles (ESAT-6, CFP10, Rv3615c, and Rv3020c) (3 μg total mycobacterial protein) and bovine PPD (3,000 IU/dose; Prionics) are shown. Animals are listed from lowest to highest for responses to 3-protein bioparticles in the comparative cervical skin test. SEM, standard error of the mean.

[b] Increase in skin fold thickness between 0 and 72 h after inoculation.

[c] The mean for bovine PPD was significantly greater than the means for the 3-protein and 4-protein biobeads in the comparative cervical skin test ($p < 0.01$).

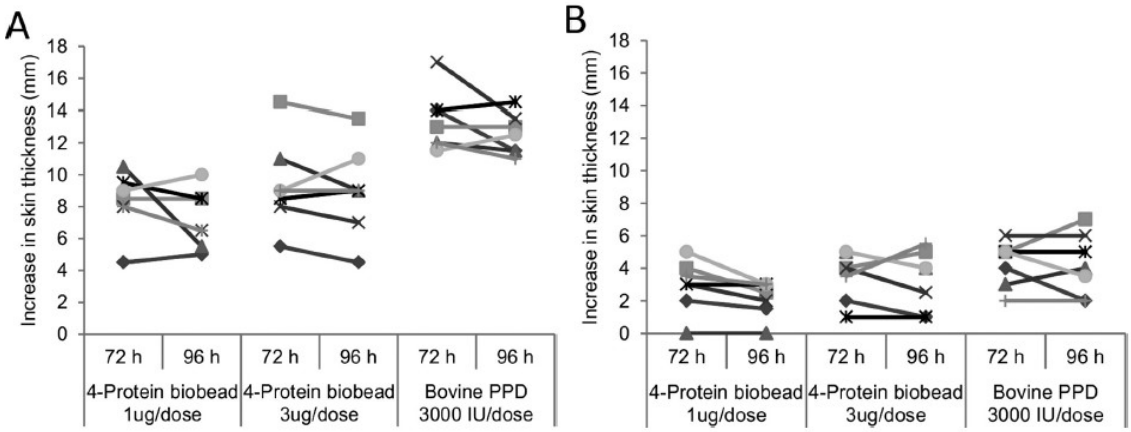

Figure 4. Skin test responses to two doses of the 4-protein bioparticles in naturally *M. bovis*-infected cattle ($n = 7$) in comparative cervical and caudal fold tests performed on the same day. Responses for different animals are shown with different symbols. (A) Comparative cervical skin test responses for the 4-protein bioparticles (ESAT-6, CFP10, Rv3615c, and Rv3020c) at doses of 1 and 3 μg mycobacterial protein and bovine PPD (3,000 IU/dose, Prionics). (B) Comparative caudal fold skin test responses for the 4-protein bioparticles (ESAT-6, CFP10, Rv3615c, and Rv3020c) at doses of 1 and 3 μg mycobacterial protein and bovine PPD (3,000 IU/dose, Prionics). The mean response for bovine PPD was significantly greater ($p < 0.05$) than those for the two doses of the 4-protein bioparticles in the comparative cervical skin test but not in the comparative caudal fold skin test. Overall responses in the comparative cervical skin test were significantly greater ($p < 0.001$) than those in the caudal fold skin test, while there were no significant differences between readings at 72 and 96 h postinoculation.

2.5 Discussion

Recent studies have demonstrated that skin testing with three or four specific mycobacterial proteins, or peptides derived from those proteins, could be used to diagnose bovine TB in cattle (8, 10). The reagents, at a recommended dose of 10 μg for each protein or peptide pool, were shown to be more specific than bovine PPD (tuberculin) and could also be used as a DIVA reagent to differentiate *M. bovis*-infected animals from animals vaccinated with BCG vaccine. As the skin test is the primary screening test for TB diagnosis in cattle, the cost of the skin test reagents is an important consideration. It was reported recently that display of three specific mycobacterial proteins (ESAT-6, CFP10, and Rv3615c) on bacteria-produced polyester inclusions (bioparticles) could greatly increase their immunogenicity for the skin test (11). This

markedly reduces the cost of the reagents, as lower concentrations of the proteins can be used and only a single fermentation is required with the proteins displayed as a fusion protein on single bioparticles.

In the current study, no significant differences in mean increases in skin thicknesses were observed between the 3-protein bioparticles and the three recombinant proteins in the comparative cervical skin test with experimentally infected cattle, despite concentrations of the mycobacterial proteins in the bioparticles being 10- and 30-fold lower than those of the recombinant proteins. Comparisons between the 3-protein bioparticles and bovine PPD revealed contrasting results for different groups of infected animals. Significantly smaller mean increases in skin thickness were observed with the low dose (1 μg of mycobacterial protein) than with bovine PPD in experimentally infected animals, while significantly greater mean skin thicknesses were observed with the high dose (3 μg of mycobacterial protein) than with bovine PPD in a group of naturally infected animals.

The comparative sizes of responses to recombinant proteins versus bovine PPD also varied in studies undertaken in Spain (9) and the United Kingdom (8) with naturally infected cattle; no differences were noted for the pool of the three recombinant proteins versus bovine PPD in one study (9), while responses were smaller for the proteins in the other study (8). Comparisons of test sensitivity in naturally infected animals need to be interpreted with caution, as the animals often are selected for retesting based on initial positive responses to bovine PPD. The sizes of the responses to specific proteins or to the complex mixture of components in PPD could depend on the stage of infection and the infecting strain of *M. bovis*. Test sensitivities for specific proteins would be expected to be lower than those for bovine PPD, due to the large number of immunogenic proteins present in PPD. However, a recent study in guinea pigs showed that some protein-protein interactions in PPD may abrogate the DTH response for TB, possibly through induction of an anti-inflammatory T cell type immune response (26). In the current study, the low dose of the 3-protein bioparticles (1 μg mycobacterial protein) and the three recombinant proteins had high specificity, with no positive responses in naive or BCG-vaccinated animals. It is possible that low concentrations of *E. coli* products in the bioparticle preparations could produce weak skin test responses,

although minimal responses have been observed following testing of control bioparticles (11).

Studies in the United Kingdom showed that the addition of peptides derived from the mycobacterial protein Rv3020c to a peptide cocktail derived from ESAT-6, CFP10, and Rv3615c proteins increased test sensitivity without compromising specificity (10). Based on these findings, bioparticles were constructed that contained the fusion protein CFP10-Rv3615c-PhaC-ESAT6-Rv3020c, which included the enzyme PhaC required for bioparticle formation. Analyses by tryptic peptide fingerprinting using MALDI-TOF MS and SDS-PAGE confirmed the identity of the fusion proteins and functionality. A critical requirement for a skin test reagent is that it must not induce sensitization. Using the Office International des Epizooties (OIE) World Organisation for Animal Health protocol for testing batches of tuberculin for sensitizing effects (22), one-tenth of the cattle dose of the 4-protein bioparticles did not induce sensitization in guinea pigs.

Dilutions of the 4-protein bioparticles were tested in the comparative cervical skin test with experimentally infected cattle, and dilutions containing 3 to 0.11 μg of mycobacterial protein/0.1-ml dose induced skin test responses of similar sizes. In contrast to those findings, Whelan et al. (8) noted marked decreases in the sizes of skin test responses for naturally TB-infected animals when the dose of recombinant proteins was decreased from 5 or 10 μg to 1 μg for each individual protein. The more rapid decline in the dose-response relationship may be related to the testing of naturally infected animals, for which responses often are weaker and more variable.

The 4-protein bioparticle preparation (3 μg mycobacterial protein/0.1 ml dose) was shown to be effective in both the comparative cervical and caudal fold skin tests for identifying naturally infected cattle. Although the 4-protein bioparticles (3 μg/dose) and bovine PPD positively identified the same numbers of animals, the sizes of the responses were greater for bovine PPD in two groups of naturally infected animals with the cervical test but not the caudal fold test. In addition, the sizes of the responses overall were greater in the cervical test than in the caudal fold test. Francis et al. (27) considered that tuberculin skin testing in the cervical region was more sensitive than that in the caudal fold of the tail. In the comparative cervical test, responses to the 3-

and 4-protein bioparticle preparations were very similar for individual animals (Table 2), and there were no differences in the responses for 1- and 3-μg/dose 4-protein bioparticle preparations (Fig. 4). Overall, there were no differences in the responses to the 4-protein bioparticles or bovine PPD when the cervical and caudal fold tests were read at 72 versus 96 h postinoculation. Pollock et al. (28) considered that skin test reactions to ESAT-6 were often greatest at 96 h postinoculation, while Whelan et al. (8) considered that there were no differences between the reactions to ESAT-6, CFP10, and Rv3615c proteins at 72 versus 96 h. Measuring responses to both bovine PPD and specific mycobacterial proteins at 72 h postinoculation would be the most practical option.

In recent caudal fold field testing of the 4-protein bioparticles in noninfected cattle, a small hard lump, < 1 mm in size, was observed at the inoculation site for the 3-μg protein dose in a small proportion of the animals, while such reactions were less frequent with the 1-μg protein dose (B. Buddle, unpublished observations). This suggested that the 1-μg protein dose may be the preferable dose for skin testing.

Overall, this study has demonstrated that bacterial polyester particles displaying three or four TB-specific antigens have high sensitivity and specificity in the skin test when used at very low concentrations. A large field trial involving up to 50,000 cattle is currently in progress in New Zealand to determine test sensitivity and specificity for the 4-protein bioparticle reagent, in comparison with those for bovine PPD, using the comparative caudal fold test. The display of mycobacterial proteins on polyester particles should allow the development of a highly specific, cost-effective, skin test reagent for the diagnosis of *M. bovis* infection in cattle.

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We thank Gary Yates, Maree Joyce, and Melissa Surrey for the culture of *M. bovis*, staff from Polybatics Ltd. for producing the 3- and 4-protein bioparticles, Peter Oden for the caudal fold testing, and Dongwen Luo for the statistical analyses.

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2.9 Supplementary material

TABLE S1. *MALDI-TOF/MS analysis of CFP10-Rv3615c-PhaC-ESAT6-Rv3020c proteins*

Protein/Protein sequence	Peptide fragments assigned to the various protein regions
CFP10-Rv3615c-PhaC-ESAT6-Rv3020c (MW: 109.4 kDa)	
MAEMKTDAATLAQEAGNFERISGDLKTQIDQVESTA GSLQGQWRGAAGTAAQAAVVRFQEAANKQKQELDE ISTNIRQAGVQYSRADEEQQQALSSQMGFGPGGGGG	CFP10: T6-R20, T27-R57, A86-P102
PMTENLTVQPERLGVLASHHDNAAVDASSGVEAAAG LGESVAITHGPYCSQFNDTLNVYLTAHNALGSSLHTA GVDLAKSLRIAAKIYSEADEAWRKAIDGLFTTS	Rv3615c: P108-R119, I194-K204
ATGKGAAASTQEGKSQPFKVTPGPFDPATWLEWSRQ WQGTEGNGHAAASGIPGLDALAGVKIAPAQLGDIQQ RYMKDFSALWQAMAEGKAEATGPLHDRRFAGDAWR TNLPYRFAAAFYLLNARALTELADAVEADAKTRQRIR FAISQWVDAMSPANFLATNPEAQRLLIESGGESLRAG VRNMMEDLTRGKISQTDESAFEVGRNVAVTEGAVVF ENEYFQLLQYKPLTDKVHARPLLMVPPCINKYYILDL QPESSLVRHVVEQGHTVFLVSWRNPDASMAGSTWD DYIEHAAIRAIEVARDISGQDKINVLGFCVGGTIVSTAL AVLAARGEHPAASVTLLTTLLDFADTGILDVFVDEGH VQLREATLGGGAGAPCALLRGLELANTFSFLRPNDLV WNYVVDNYLKGNTPVPFDLLFWNGDATNLPGPWYC WYLRHTYLQNELKVPGKLTVCGVPVDLASIDVPTYI YGSREDHIVPWTAAYASTALLANKLRFVLGASGHIAG VINPPAKNKRSHWTNDALPESPQQWLAGAIEHHGSW WPDWTAWLAGQAGAKRAAPANYGNARYRAIEPAPG RYVKAKAHMVLAVAIDKRGGGGG	PhaC: V233-R248, I275-R286, D290-K302, R313-R320, F327-R337, F358-R392, I407-R419, Y462-R511, D518-K625, L668-K710, A776-R785, A801-R812
LEMTEQQWNFAGIEAAASAIQGNVTSIHSLLDEGKQS LTKLAAAWGGSGSEAYQGVQQKWDATATELNNALQ NLARTISEAGQAMASTEGNVTGMFAGGGGG	ESAT6: W877-R893

MSLLDAHIPQLIASHTAFAAKAGLMRHTIGQAEQQA MSAQAFHQGESAAAFQGAHARFVAAAAKVNTLLDI AQANLGEAAGTYVAADAAAASSYTGF	Rv3020c: H946-R976

Preface to Chapter 3

Specific and cost-effective skin test reagent is important for TB diagnosis. In addition, an efficacious TB vaccine is critical to control TB transmission. BCG is the only available licensed TB vaccine, but this vaccine is not able to provide adequate protection against this disease and has the risk to revert to disease-causing pathogen, especially in immunocompromised patients. However, in contrast to the live BCG, non-viable subunit vaccines are relataively safe but often show low immunogenicity as it has limited antigen diversity. It has been demonstrated that display of antigens on the surface of antigen carrier systems could enhance antigen immunogenicity towards the development of efficacious vaccines.

Previous studies have shown that polyester particle has been used as the innovative antigen delivery system for the development of various efficacious vaccines against different pathogens, such as hepatitis C virus, *P. aeruginosa*, and *N. meningitidis*. In Chapter 3, an endotoxin free *E. coli* strain was bioengineered to produce polyester particle displaying immunogenic mycobacterial recombinant peptides Ag85B-TB10.4 (H4) or Ag85B-TB10.4-Rv2660c (H28). The immunogenicity of polyester particle-based particulate mycobaterial vaccines was compared with the free soluble controls and evaluated using mice model.

Chapter 3. Innovative antigen carrier system for the development of tuberculosis vaccines

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3.1 Abstract

A major obstacle to tuberculosis (TB) subunit vaccine development has been the induction of inadequate levels of protective immunity due to the limited breadth of antigen in the vaccine preparations. In this study, immunogenic mycobacterial fusion peptides, Ag85B-TB10.4 (designated H4) and Ag85B-TB10.4-Rv2660c (designated H28), were covalently displayed on the surface of self-assembled polyester particles. This study investigated whether polyester particle displaying mycobacterial antigens could provide augmented immunogenicity, i.e. offer an innovative vaccine formulation, when compared to free soluble antigens. Herein, polyester particle-based particulate vaccines were produced in an endotoxin-free *Escherichia coli* strain, and emulsified with the adjuvant dimethyl dioctadecyl ammonium bromide (DDA). C57BL/6 mice were used to study the immunogenicity of formulated particulate vaccines. The result of humoral immunity showed the antibodies only interacted with target antigens and not with PhaC and the background proteins of the production host. The analysis of Th1 cellular immunity indicated that a relatively strong production of cellular immunity biomarkers, IFNγ and IL17A cytokines, was induced by particulate vaccines when compared to the respective soluble controls. This study demonstrated that polyester particles have the potential to perform as a mycobacterial antigen delivery agent to induce augmented antigen-specific immune responses in contrast to free soluble vaccines.

3.2 Introduction

TB remains a major global health problem as it causes approximately 2 million deaths annually, along with 10 million new cases (1, 2). The pathogenic mycobacterial strain, *Mycobacterium tuberculosis*, is the main causative agent (3). The only available and widely-used vaccine for TB, live-attenuated BCG, has been unable to control this disease (2, 4). In addition, live vaccines are associated with safety concerns as they may revert to disease-causing pathogens especially in immunocomprised patients (5). Subunit vaccines generally have no infective risk to patients (5, 6). Some promising antigen candidates have been selected for developing new TB vaccines (7-10). A recombinant vaccine H4 comprises two secreted early stage antigens, Ag85B and

TB10.4, present in both *M. tuberbulosis* and BCG (7, 8, 10, 11). Approximately one-third of the world's population is infected with latent *M. tuberculosis*, with a 10% lifetime risk of developing active disease due to resuscitation of latent *M. tuberculosis* (12, 13). Rv2660c is an *M. tuberculosis* antigen that is preferentially produced during latent infection (12, 13). Therefore, combining the H4 vaccine backbone with the latency-related antigen Rv2660c resulted in the multistage, multivalent antigen, H28 (12-14).

Polyester particles are composed of polyhydroxyalkanoates (PHAs), which are naturally occurring biopolyesters produced by various microorganisms in imbalanced nutrient environments when an excess carbon source is available (15-18). PHA synthesis requires three enzymes, PhaC (PHA synthase), PhaA (β-ketothiolase), and PhaB (acetoacetyl-CoA reductase). The key enzyme, PHA synthase PhaC, catalyses the polymerization of (*R*)-hydroxyacyl-CoA thioester monomers, synthesized by enzymes PhaA and PhaB, to PHAs (18, 19). The PHA chains self-assemble into the hydrophobic core of particles (16, 20). We have hijacked this natural polyester particle forming process and use bioengineering to display desired proteins on the particle surface. This is achieved by translational fusion of foreign proteins, such as antigens, to PhaC as this enzyme remains covalently embedded on the surface after the self-assembly process of the polyester particles (18, 21, 22). This concept of using biopolyester particles for vaccine delivery is further underpinned by their unique favourable properties such as biocompatibility with mammalian systems (23-28). Hepatitis C and TB antigens displayed on the particle surface have demonstrated increased immunogenicity when compared to their soluble forms, suggesting that polyester particles are capable of increasing the immunogenicity of vaccines when desired antigen candidates are displayed on their surfaces (29-31). In addition, polyester particles could serve as a depot for prolonged multivalent antigen display to provide enhanced immunogenicity. Therefore, these particles are able to function as adjuvants to enhance immune responses (25), and the surface antigens could elicit specific CD4+ T cell effector responses and ultimately lead to protective immunity (25-27, 31).

Subunit vaccines tend to exhibit low immunogenicity as they have restricted antigen diversity and cannot replicate, unlike live-attenuated vaccines (5, 6). This compromises the capability of subunit vaccine to elicit protective immunity and thus a versatile

vaccine delivery system is often required to combat this issue (5, 6). In this study, polyester particles were used to display the vaccines H4 and H28, respectively. This paper describes the bioengineering of *ClearColi* BL21 (DE3), an endotoxin free host, to produce H4- or H28-displaying polyester particles, the immunogenicity of which was evaluated in C57BL/6 mice. This study aims to assess whether particulate TB vaccines are better than soluble forms in regards to induction of immune responses associated with protective immunity.

3.3 Materials and methods

3.3.1 Bacterial strains and growth conditions

Bacterial strains applied in this study were illustrated in **Table 1**. XL1-Blue was used for molecular cloning and grown in Luria broth (LB) (Difco Laboratories Inc, Detroit, MI, USA) supplemented with ampicillin (100 μg/ml) at 37°C. *E. coli* BL21 (DE3) was used for polyester particle production and media for growth of this production strain contained 0.5% (wt/vol) NaCl, 1% (wt/vol) glucose, ampicillin (100 μg/ml), and chloramphenicol (50 μg/ml). Moreover, 2% of the overnight cell cultures were inoculated to a new growth media and firstly incubated at 37°C at 200 rpm for about 3 h. Cultures were induced by 1 mM IPTG when the OD600 reached approximately 0.5 and the incubation was continued at 25°C for 48 h.

TABLE 1. *Bacterial strains, plasmids and primers used in this study.*

Strains or plasmids	**Characteristics[a] or sequence***	**Sources or references**
E. coli		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *pro*AB *lacI*^{q} *lacZ*Δ*M15* Tn*10* (Tet^{R})]	Stratagene
ClearColi BL21 (DE3)	F^{-} *ompT hsdS$_{B}$ (r_{B}^{-} m_{B}^{-}) gal dcm lon* λ(DE3 [*lacI lac*UV5-T7 gene 1 *ind1 sam7 nin5*]) *msbA148* Δ*gutQ* Δ*kdsD* Δ*lpxL* Δ*lpxM* Δ*pagP* Δ*lpxP* Δ*eptA*	Lucigen
Plasmids		
pET-14b	Ap^{R}; T7 promoter	Novagen
pET -14b PhaC	pET-14b containing *phaC* fragment gene	(32)
pUC57-H4	Cloning vector, ColE1 origin, Apr; *Bam*HI fragment gene *h4*	GenScript
pUC57-H28	Cloning vector, ColE1 origin, Apr; *Bam*HI fragment gene *h28*	GenScript
pET-14b His6-H4	pET-14b derivative containing *Nde*I/*Bam*HI fragment gene *his6-h4*	(33)
pET-14b His6-H28	pET-14b derivative containing *Nde*I/*Bam*HI fragment gene *his6-h28*	(33)
pET -14b PhaC-H4	pET-14b containing *Bam*HI fragment gene *h4* fused to the 3' end of *phaC*	This study
pET -14b PhaC-H28	pET-14b containing *Bam*HI fragment gene *h28* fused to the 3' end of *phaC*	This study
pMCS69	Cm^{R} ; T7 promoter, pBBR1MCS derivative containing *phaA* and *phaB*, from *C. necator*, colinear to *lac* promoter	(34)
Oligonucleotides		
H4_Fwd	ttcGGATCCttcagccgtccgggtctgccgg	This study
His6_H4_Fwd	ccCATATGcaccaccaccaccaccacttcagccgtccgggtctgccgg	This study
H4_Rev	agccGGATCCttaaccgccccatttagctgcttcggcggtgtcg	This study

[a] Tet^{R}, tetracycline resistance; Cm^{R}, chloramphenicol resistance; Ap^{R}, ampicillin resistance; *h4*, *ag85b-tb10.4*; *h28*, *Ag85B-TB10.4-Rv2660c*.

***** Uppercase represents the gene sequence of restriction enzymes

3.3.2 Plasmids construction for production of polyester particle and soluble vaccines

Plasmids and oligonucleotides used in this study are shown in Table 1. The molecular cloning techniques were carried out as outlined elsewhere (35). DNA sequences of all constructed plasmids were confirmed by the Massey Genome Service (Massey University, Palmerston North, Manawatu-Wanganui, New Zealand). The biosynthesis of polyester particle displaying mycobacterial antigens is illustrated in **Fig. 1**. Particularly, the polyester synthase PhaC is required for the biosynthesis of polyester particles and is encoded by the gene *phaC* on a pET-14b plasmid. Plasmid pMCS69 contains the genes-encoding enzymes PhaA and PhaB that produce precursor molecules for polyester synthesis. pET-14b and pMCS69 were transformed into *E. coli* BL21 (DE3).

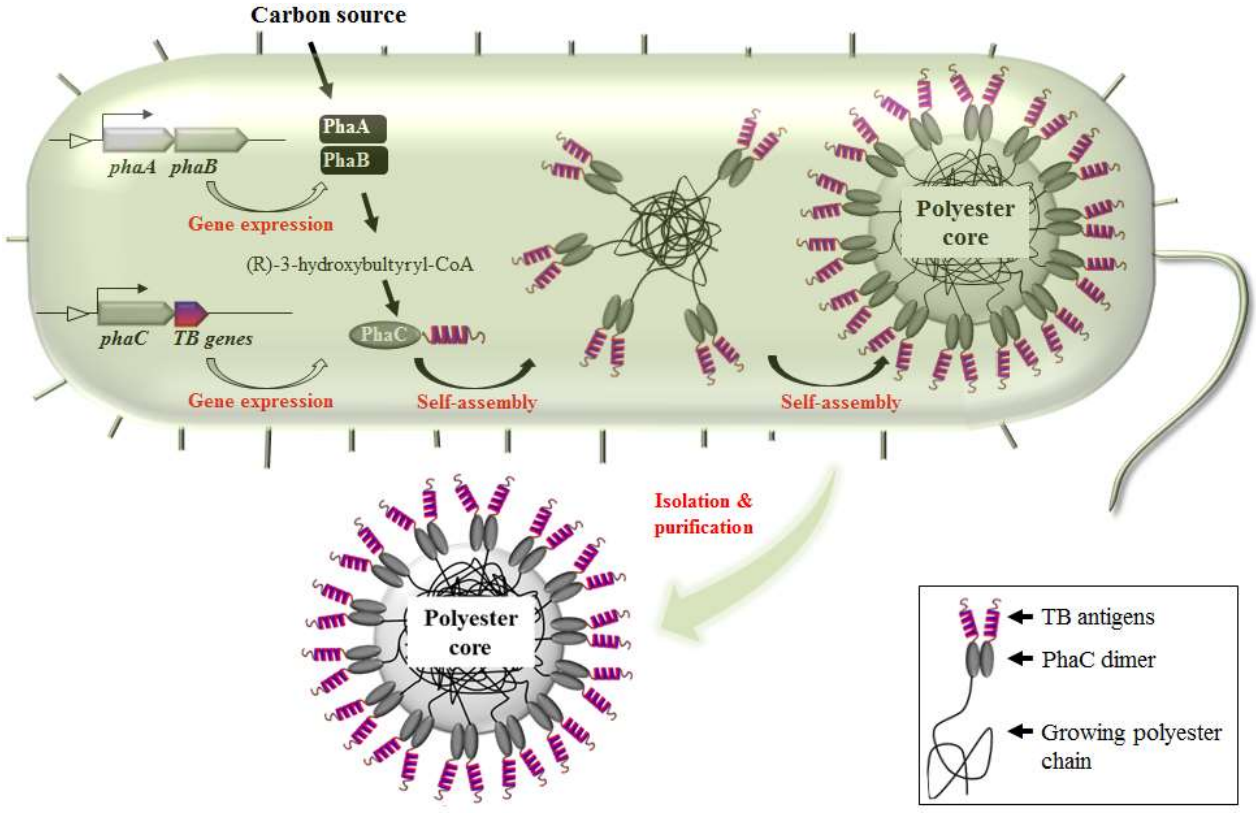

Figure 1. Schematic overview of polyester particle formation *in vivo*. Polyester synthase PhaC is the key enzyme required for polyester chain biosynthesis and polyester particle self-assembly in the presence of (*R*)-hydroxybutyryl-CoA, the precursor molecules synthesized by PhaA and PhaB enzymes.

The gene fragments encoding the recombinant antigens H4 with amino acid sequence and H28 with amino acid sequence were synthesized by GenScript (Piscataway, NJ, USA) and codon-optimized for *E. coli* strains. A *Bam*HI restriction site was inserted at both the 5' and 3' end of each gene fragment.

The plasmid constructs for production of polyester particle vaccines were made as follows. The gene fragments *h4* and *h28* were excised from a pUC57 vector and then subcloned into linearized pET-14b PhaC, generated by *Bam*HI digestion, using T4 DNA ligase to formulate the final plasmids, pET-14b PhaC-H4 and pET-14b PhaC-H28. The plasmid construction for production of soluble TB vaccine controls, pET-14b His6-H4 and pET-14b His6-H28, was described previously (33). Target gene sequences in all resulting pET-14b plasmids were confirmed by Massey Genome Service (Massey University, Palmerston North, New Zealand). Plasmids pET-14b encoding PhaC-TB antigens were respectively transformed into *ClearColi* BL21 (DE3) harbouring pMCS69 for polyester particle production. Plasmids pET-14b encoding TB antigens alone were transformed into *ClearColi* BL21 (DE3) for free soluble vaccine preparation.

3.3.3 Isolation and purification of vaccine samples

Particle isolation and purification procedures were described elsewhere (33). Briefly, cells were subjected to mechanical disruption using a microfuidiser M-110P (Microfluidics, Westwood, MA, USA). Particles were sedimented and sequentially washed three times as previously described and stored at 4°C for further analysis (31, 36). In addition, soluble recombinant vaccines, His6-H4 and His6-H28, were also prepared as described previously (33).

3.3.4 Characterization of polyester particles

The fusion protein percentage of total protein in the particle fraction was determined by densitometry using Image Lab Software (Bio-Rad Laboratories, Irvine, CA, USA). A BSA standard curve, ranging between 50 ng and 500 ng, was used to determine the protein concentrations. Morphology and size of polyester particles were analyzed by transmission electron microscopy (TEM). A Mastersizer 3000 (Malvern Panalytical Ltd,

Malvern, UK) was used to measure the size distribution of polyester particles before and after formulation in DDA. Zeta potential of all vaccine samples was analyzed by the Zetasizer Nano ZS (Malvern Panalytical Ltd, Malvern, UK). Target protein bands were excised from Bis-Tris gel and subjected to protein identification using matrix-assisted laser desorption ionisation–time-of-flight mass spectrometry (MALDI-TOF/MS), performed by The Centre for Protein Research (Otago University, Dunedin, New Zealand).

3.3.5 Vaccine formulation and immunization

Dimethyl dioctadecyl ammonium bromide (DDA; Sigma-Aldrich, St. Louis, MO, USA) was prepared at a concentration of 10 mg/ml using sterile Tris-HCl buffer (10 mM, pH 7.5). Each dose of formulated vaccines contained 2 μg of TB antigens and 200 μg of DDA in a volume of 200 μl Tris-HCl buffer. Mycobacterial vaccines were H4 and/or H28 displayed on polyester particles and their free soluble forms, produced by *ClearColi* BL21 (DE3), an endotoxin free host. All vaccines were freshly formulated with DDA solution before use.

The Otago University Animal Ethics Committee (Otago University, Dunedin, New Zealand) approved all animal experiments, which were performed using 6- to 8-week-old female C57BL/6 mice. Mice were group-housed in individually ventilated cages in a specific pathogen free facility and were provided with standard mouse chow and water ad libitum. The sample size of 6 mice per group was calculated with the assistance of a biostatistician from the University of Otago, using estimated standard deviations from data that we have previously collected from similar studies and were based on the smallest detectable differences with 80% power when using a two-sided test at the 5% level of significance (37, 38). The Otago University animal unit bred all mice, originally purchased from Jackson Laboratories (Bar Harbor, ME, USA). Formulated vaccines were injected into mice subcutaneously on the flank 3 times at 9 days intervals. The animal experiments were not repeated.

3.3.6 Sample preparation and immunological assays

Three weeks after the final vaccination, mice were sacrificed by anaesthetic overdose. Sera were collected and single spleen cell suspensions were prepared as described previously (33). Splenocytes were stimulated with 100 µl of different stimuli, cRPMI medium and 40 µg/ml of soluble vaccines (His6-H4 and/or His6-H28), and incubated at 37°C in 5% CO_2 for 24 and 60 h, respectively. The BD CBA Mouse Th1/Th2/Th17 cytokine kit (BD Biosciences, Franklin Lakes, NJ, USA) was used to measure cytokine release, adapted to Falcon V bottom plates (Corning, Tewksbury, MA, USA). Data was acquired and analyzed using a FACS Canto with BD FACSDiva software (BD Biosciences, Franklin Lakes, NJ, USA).

Mice sera were also collected and analyzed by ELISA and immunoblot assay as described elsewhere (33). Briefly, serum antibody responses were analyzed by ELISA. One hundred microliters of soluble His6-H4 and/or His6-H28 at a concentration of 5 µg/ml were coated on high-binding plates (Greiner Bio-One GmbH, Frickenhausen, Germany) overnight at 4°C. In order to test the immunogenicity of polyester synthase, PhaC, plain polyester particles were also coated on high-binding plates. Sera from individual mice, primary antibodies, were serially diluted and added to the plates. Goat anti-mouse IgG1- or IgG2c-HRP (Abcam, Cambridge, UK) were used as secondary antibodies to detect the IgG1 and IgG2c antibodies, respectively. IgG titres were presented as the half maximum effective concentration (EC50) which gave the half maximum response/absorbance (mean value ± the standard error of the mean, $n = 6$) and estimated by a sigmoid curve fitted with a straight line ($y = mx + c$) using linear interpolation. An example of EC50 value calculation was shown in **Fig. S1**.

Immunoblot assay was implemented to investigate the specificity of the IgG response. Briefly, pooled sera from mice vaccinated with soluble His6-H4 and/or soluble His6-H28 were used to interact with soluble proteins, purified polyester particle vaccines, and whole cell lysate containing various particle vaccines. An anti-mouse IgG HRP-conjugate (Abcam, Cambridge, UK) was used to detect the bound IgG antibodies.

3.3.7 Statistical analysis

Minitab 17 was used to analyze all the statistical data. Statistical differences between groups were determined by one-way ANOVA with non-parametric Kruskal-Wallis test. Statistical analysis was implemented using Mann-Witney Wilcoxon test when the comparison was between two groups. Each data point stands for 6 mice ± the standard error of the mean. Statistical significance was identified when $p < 0.05$.

3.4 Results

3.4.1 Engineering and production of mycobacterial antigens-displaying polyester particles

Hybrid genes encoding antigens fused with PhaC mediated production of polyester particles displaying antigens at high density, while genes encoding His6-tagged antigens enable production and purification of soluble controls as illustrated in **Fig. 2*A***. The respective pET-14b plasmids were introduced into *E. coli* BL21 (DE3) cells harbouring pMCS69 plasmid, containing the gene-encoding enzymes (PhaA and PhaB) which synthesize the precursor molecules for polyester synthesis. *E. coli* cells containing pET-14b and pMCS69 plasmids were cultivated to produce polyester particles displaying H4 or H28. The formation of intracellular polyester particle vaccines mediated by the respective recombinant proteins in the *E. coli* cells was observed by TEM (**Fig. 2*B***). The size of purified polyester particles was diverse ranging between 100 nm and 700 nm in diameter; moreover, all particles showed a similar oval-shaped morphology (Fig. 2*B*).

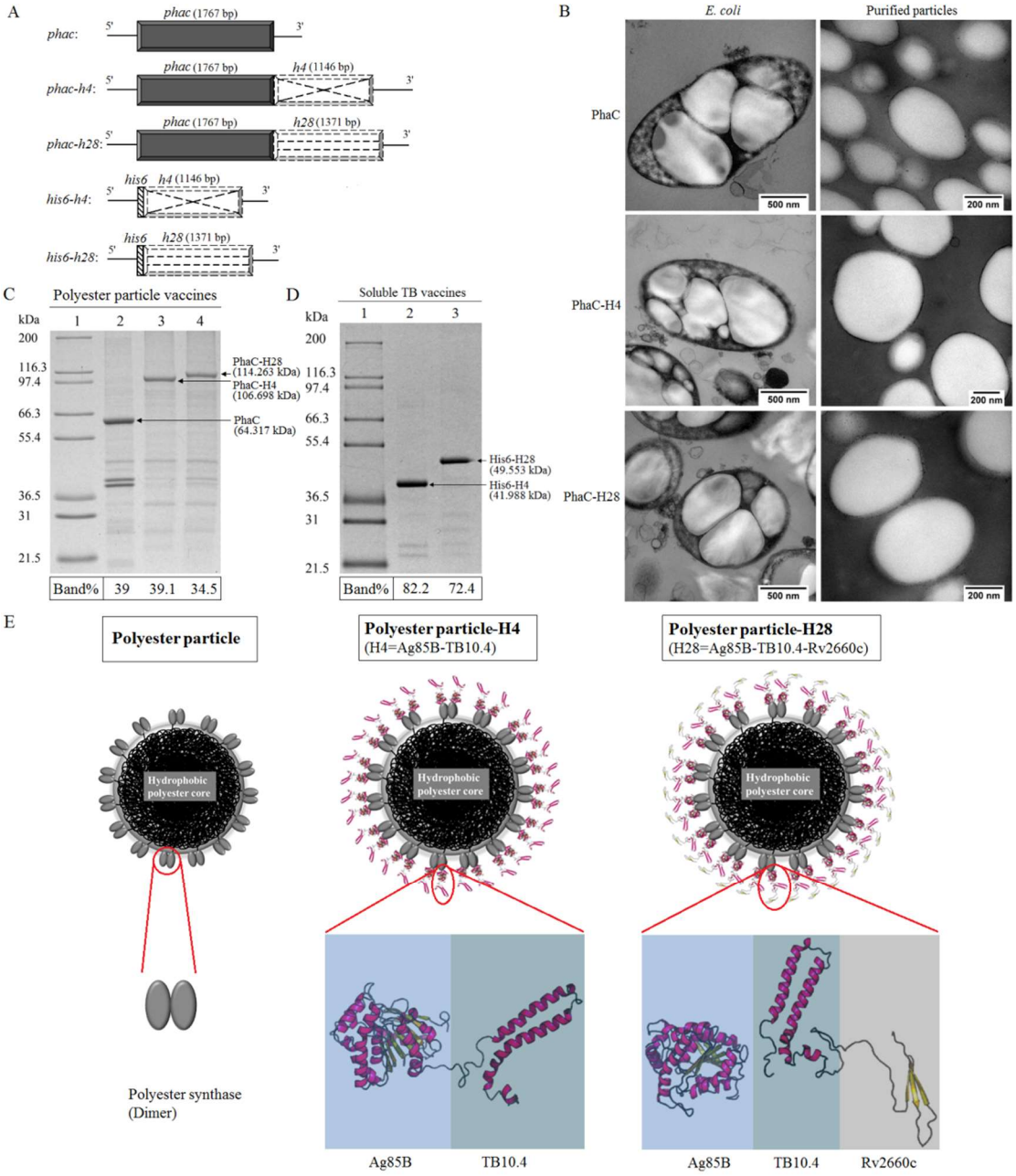

Figure 2. Design and engineering of polyester particles displaying mycobacterial antigens. *A*) Schematic diagram of recombinant genes for the production of polyester particles displaying H4 and H28 and of the free soluble His6-tagged H4 and H28. *B*) TEM images of *E. coli* harbouring different recombinant pET-14b plasmids. (Scale bar: *E. coli* cells, 500 nm; purified particles, 200 nm). *C*) Analysis of isolated polyester particle displaying H4/H28 antigens. Lane 1, molecular weight marker (GangNam-Stain prestained protein ladder; iNtRon); lane 2, PhaC (64.317 kDa); lane 3, PhaC-H4 (106.698 kDa); lane 4, PhaC-H28 (114.263 kDa). *D*) Protein profile of soluble His6-H4 and His6-H28 vaccines. Lane 1, molecular weight marker (GangNam-Stain prestained protein ladder; iNtRon); lane 2, His6-H4 (41.988 kDa); lane 3, His6-H28 (49.553 kDa). *E*) Schematic overview of recombinant mycobacterial vaccines-displaying polyester particles produced by *E. coli* cells. Depicted structure models of

mycobacterial proteins are deduced from the protein data bank using Raptor X (http://raptorx.uchicago.edu/StructurePrediction/predict/). Ag85B, protein structure was deduced from protein data bank (1F0N, p = 1.57e-06); TB10.4, protein structure was deduced from protein data bank (2KG7, p = 2.12e-02); Rv2660c, protein structure was deduced from protein data bank (4UFC, p = 1.31e-02).

To investigate whether mycobacterial antigens were displayed on the surface of polyester particles, SDS-PAGE was used to analyze the protein profile of polyester particles. The gel showed that molecular weights (MW) of dominant protein bands correspond with the theoretical MW of PhaC (64.317 kDa), PhaC-H4 (106.698 kDa), PhaC-H28 (114.263 kDa), His6-H4 (41.988 kDa), and His6-H28 (49.553 kDa) (**Fig. 2*C*** and **2*D***). Densitometry analysis showed that immobilized antigens (PhaC-H4 and PhaC-H28) and their free soluble forms (His6-H4 and His6-H28) respectively accounted for approximately 34.5-39.1% and 72.4-82.2% of the total protein in their corresponding sample fraction (Fig. 2*C* and 2*D*). The target protein sequences of all vaccine samples were identified by MALDI-TOF MS (**Table S1**). A schematic diagram of polyester particles displaying mycobacterial vaccines, H4 and H28, is illustrated in **Fig. 2*E***.

3.4.2 Characterization of formulated polyester particle vaccines

To analyze the effect of DDA adjuvant on polyester particle vaccines after emulsification, size distribution and Zeta potential of all formulated vaccine samples were characterized. Size distribution of DDA micelles was firstly measured and > 90% of the micelles were smaller than 200 nm (**Fig. 3*A*** and **Table S2**). Prior to emulsification with DDA, 50% of the plain polyester particles were distributed below 623 nm in diameter (Fig. 3*A* and Table S2). Moreover, polyester particles displaying different mycobacterial vaccines, H4 and H28, showed a similar size distribution pattern and allocated mainly in two size ranges, 0.15 μm–1 μm and 1 μm–100 μm (Fig. 3*A*).

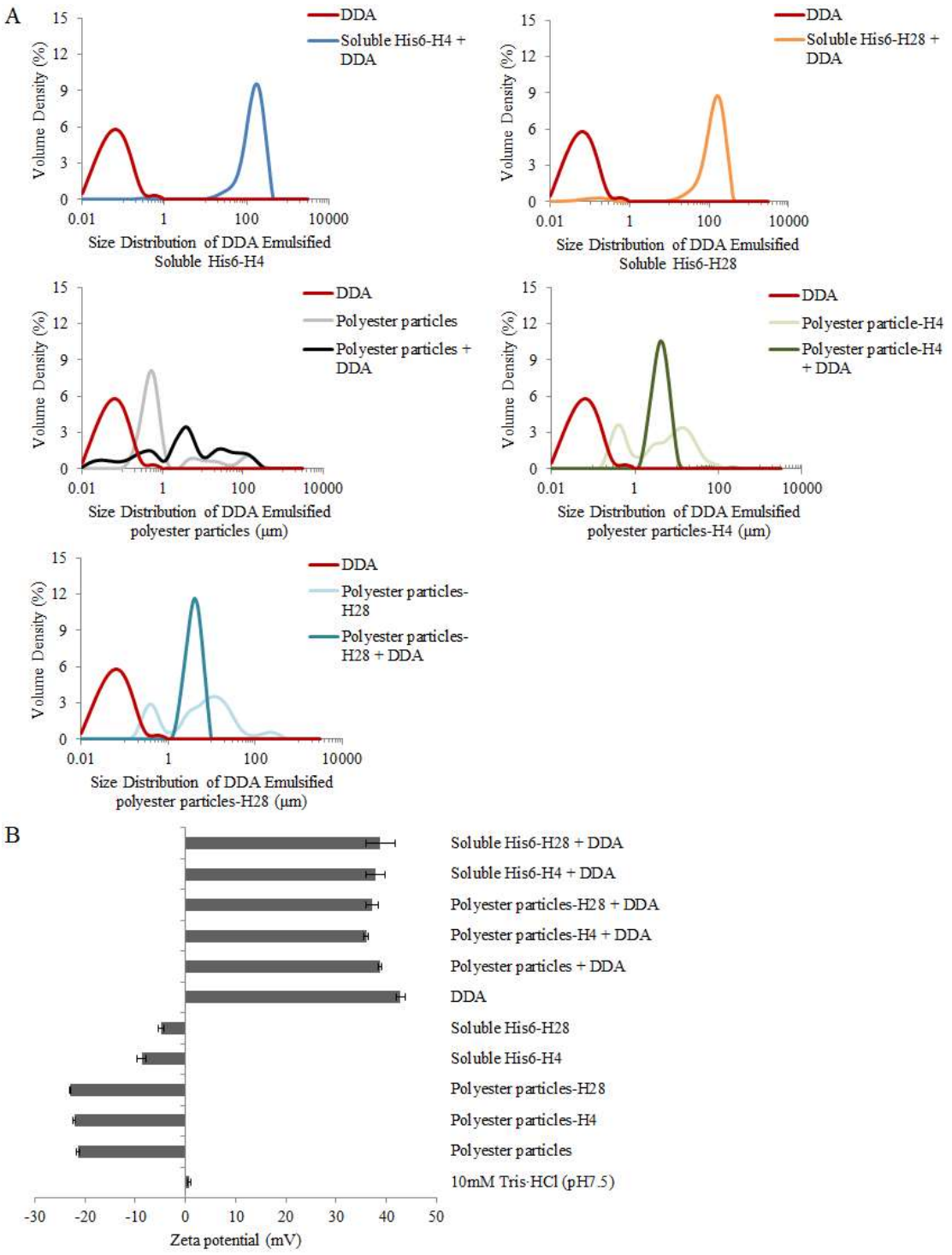

Figure 3. Size distribution and Zeta potential of various vaccine samples. *A*) Size distribution of polyester particle vaccines before and after emulsification in DDA micelles. Size of vaccine particle was measured 3 times consecutively by Mastersizer 3000 and the standard deviation is less than 0.01. *B*) Zeta potential of polyester particle vaccines before and after formulation with DDA adjuvant. The Zeta potential of all vaccine particles was measured 3 times by Zetasizer Nano ZS. Each data point of measurement stands for the mean ± 1 standard error of the mean.

After vaccine formulation, the changes in size distribution of polyester particles as well as DDA micelles were analyzed (Fig. 3*A*). The size distribution of DDA micelles was shifted from approximately 0.01 µm–0.3 µm to 20 µm–300 µm when soluble mycobacterial antigens were emulsified with DDA (Fig. 3*A*). Surprisingly, after emulsification of polyester particle vaccines in DDA, particles displaying H4 or H28 became monodispersed and size ranged between 1 µm–10 µm (Fig. 3*A*). Interestingly, the plain polyester particles were not monodispersed after DDA emulsification and size range was similar before and after formulation with DDA (Fig. 3*A*).

The Zeta potentials of polyester particle vaccines as well as the free soluble forms were also characterized before and after emulsification in DDA solution as shown in **Fig. 3*B***. Prior to DDA emulsification, the Zeta potentials of particle-H4 and particle-H28 were -22.1 ± 0.2 mV and -23.0 ± 0.1 mV (mean ± 1 standard error, n = 3), respectively. The negative charges of particles displaying H4/H28 were similar to the Zeta potential of the plain polyester particles (-21.4 ± 0.3 mV, n = 3). The soluble vaccines, His6-H4 (-8.7 ± 0.9 mV, n = 3) and His6-H28 (-4.9 ± 0.6 mV, n = 3), were less negatively charged when compared to the immobilized versions. DDA micelles possessed a strongly positively charged surface (42.9 ± 0.8 mV, n = 3). In addition, Fig. 3*B* showed that DDA imparted all vaccine samples a strongly positively charged surface after emulsification. Particularly, the Zeta potentials of particles + DDA, particle-H4 + DDA, particle-H28 + DDA, soluble His6-H4 + DDA, and soluble His6-H28 + DDA were 38.8 ± 0.3 mV, 36.0 ± 0.4 mV, 37.1 ± 1.2 mV, 37.9 ± 1.9 mV, and 38.8 ± 2.9 mV (mean ± 1 standard error, n = 3), respectively (Fig. 3*B*).

3.4.3 Experimental design

This experiment was implemented with the aim of investigating whether the polyester particles would perform as a safe antigen carrier system to enhance the immunogenicity induced by the H4/H28. In this experiment, all vaccine samples were produced in an endotoxin free *E. coli* strain. The amount of mycobacterial vaccines immobilized on particles and the corresponding free mobile forms were determined by densitometry using a BSA standard curve (**Fig. S2**) and shown in **Table S3**. These data were used to deduce the amount of polyester particle vaccines to be injected per dose.

Plain polyester particles, polyester particle-H4/H28, and soluble H4/H28 vaccines were prepared and freshly formulated with DDA for mice immunization (**Fig. 4**). Mice were immunized 3 times subcutaneously at 9 day intervals. All mice were healthy during the immunization period and did not show abnormal behavior (data not shown). Three weeks after the final vaccination, mice were sacrificed. The blood and spleen tissue were processed and prepared for antigen-specific immune response analysis.

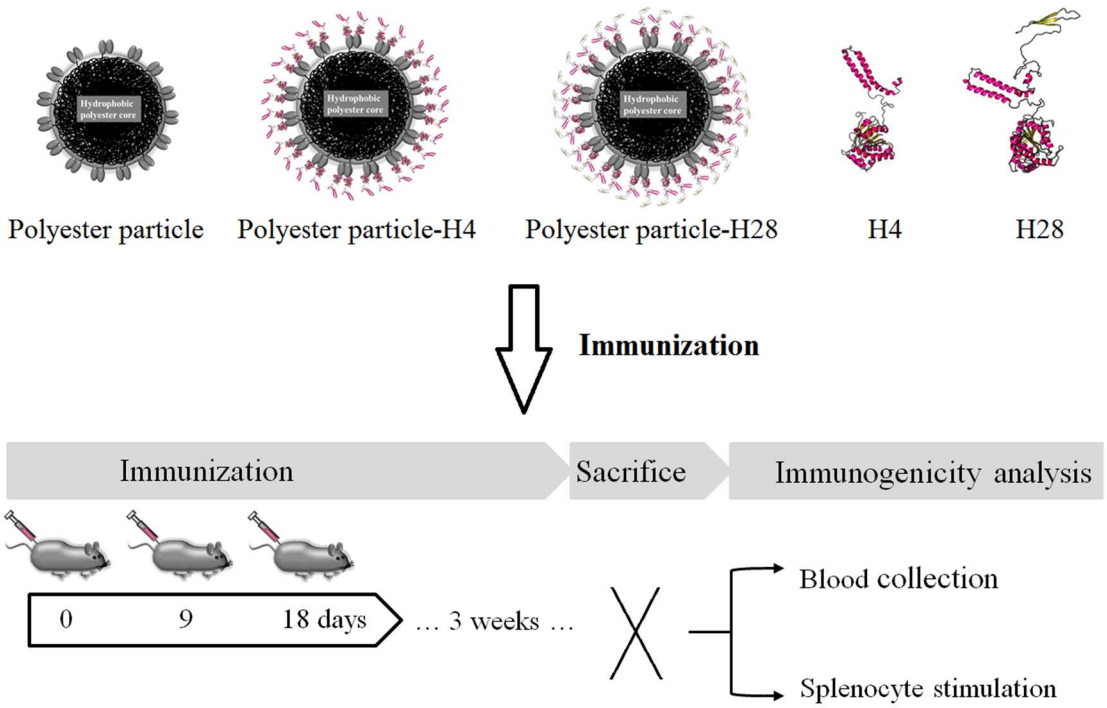

Figure 4. Schematic representation of mice immunization plan. Mice were immunized 3 times subcutaneously with polyester particles vaccine and soluble H4/H28 vaccines at 9 day intervals on the flank. Formulated TB vaccines contained 2 μg of antigens/dose, emulsified in dimethyl dioctadecyl ammonium bromide (DDA, 250 μg/dose) in a volume of 200 μl/dose. Three weeks after the final immunization, mice were lethally anaesthetised by pentobarbital sodium (100 mg/kg administered intraperitoneally). Blood sample and spleen tissue were obtained and prepared for immunological analyses.

3.4.4 Antibody responses

Antibody responses induced by different vaccines were analyzed by western blot and ELISA (**Fig. 5**). In order to measure the immunogenicity of the plain polyester particle (PhaC), the pooled sera from the mice immunized with the plain polyester particles alone (anti-PhaC antibody) were used to test the reactivity against different polyester particle-based vaccines. The western blot results showed that no protein bands were recognized by pooled sera from mice vaccinated with the plain polyester particles, indicating that the plain particle was not immunogenic and did not elicit antibody responses (Fig. 5). In addition, the plain particles were coated on a high-binding ELISA plate to interact with sera from mice immunized with various vaccines. The IgG1 and IgG2c responses were then measured. The results showed that weak IgG responses to PhaC were generated by mice immunized with plain polyester particle and particle displaying H4/H28 when compared to the placebo (DDA only). However, weak IgG1 responses to PhaC produced by the plain polyester particle was similar to the responses elicited by the particle vaccines displaying H4/H28 and the soluble controls, His6-H4 and His6-H28 (CI = 0.95%, $p = 0.273$, $n = 6$) (Fig. 5). Weak IgG2c responses to PhaC were also produced by the plain polyester particles and other vaccine groups (the particle vaccines displaying H4/H28 and soluble H4/H28) and there were no significant differences (CI = 95%, $p = 0.323$, $n = 6$) (Fig. 5).

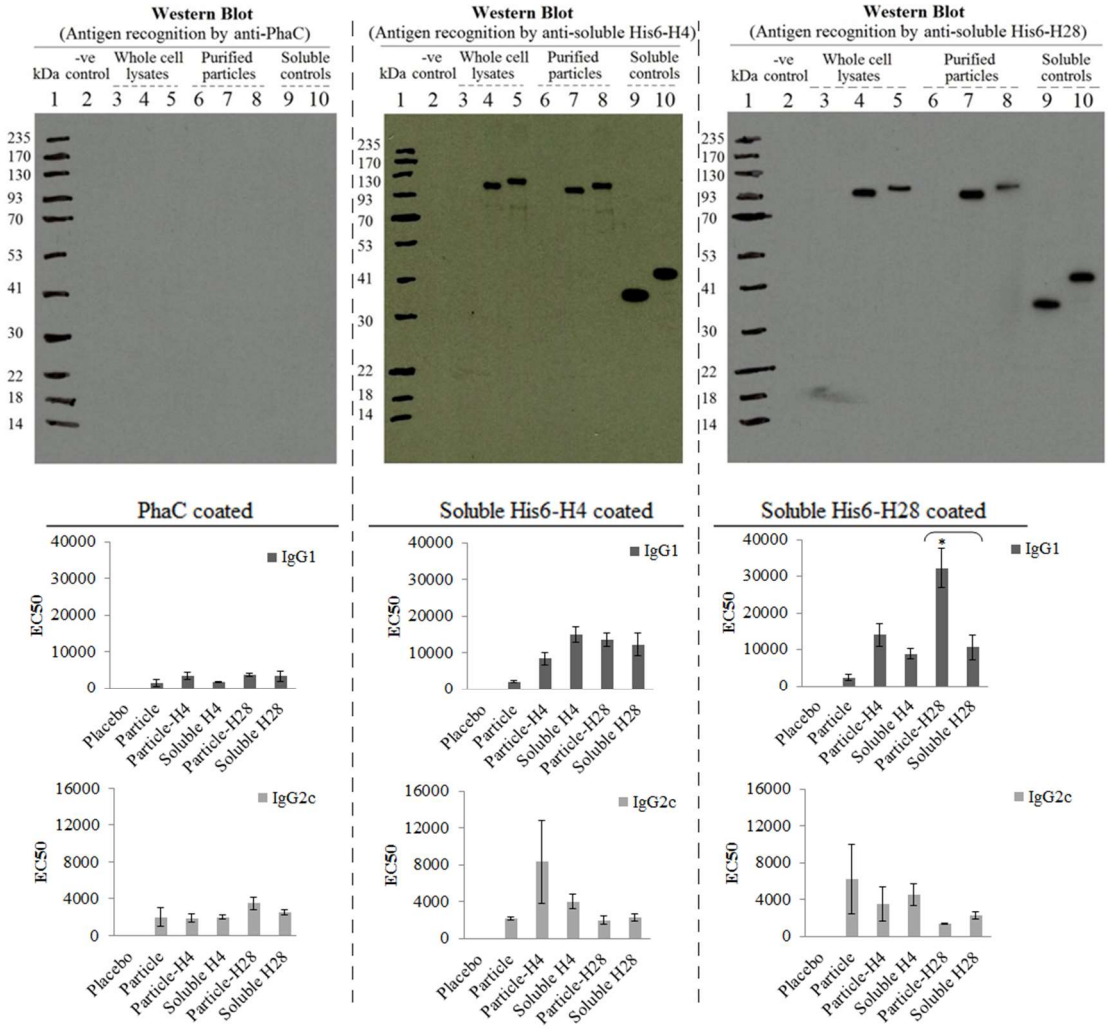

Figure 5. Analysis of antibody responses using western blot and ELISA. Specific antibody responses were analyzed by western blot using pooled sera from mice immunized with plain particle vaccine (PhaC) and soluble His6-H4/His6-H28. Lane 1, molecular weight marker (GangNam-Stain prestained protein ladder; iNtRon); lane 2, *E. coli*/ pET-14b; lane 3, *E. coli*/ pET-14b PhaC (64.317 kDa); lane 4; *E. coli*/ pET-14b PhaC-H4 (106.698 kDa); lane 5, *E. coli*/ pET-14b PhaC-H28 (114.263 kDa); lane 6, PhaC (64.317 kDa); lane 7, PhaC-H4 (106.698 kDa); lane 8, PhaC-H28 (114.263 kDa); lane 9, soluble His6-H4 (41.988 kDa); lane 10, soluble His6-H28 (49.553 kDa). IgG1 and IgG2c in response to different vaccines were analyzed by ELISA and presented as the EC50. Each data point stands for the results from 6 mice ± the standard error of the mean. Statistical significance [Confidence interval (CI) = 95%, $p < 0.05$, $n = 6$] was calculated by one-way ANOVA with Mann-Whitney test (Minitab 17). *, significantly greater than the group vaccinated with the soluble His6-H28 (CI = 95%, $p < 0.05$, $n = 6$).

Specificity of anti-His6-H4 and anti-His6-H28 antibodies' responses was measured using western blot (Fig. 5). In particular, all the polyester particle and the soluble vaccines applied in immunization were tested for reactivity against sera from mice vaccinated with soluble His6-H4 and His6-H28 vaccines. In addition, whole cell lysate with and without particle vaccines were also applied in this experiment. Fig. 5 showed that no protein bands were observed in whole cell lysate without particle vaccines and with the plain particle, indicating pooled sera from mice immunized with soluble His6-H4 and His6-H28 did not non-specifically interact with PhaC and background proteins from cell lysate. Indeed, the pooled anti-His6 H4 and anti-His6 H28 could specifically recognize the protein bands corresponding to PhaC-H4 (106.698 kDa), PhaC-H28 (114.263 kDa), soluble His6-H4 (41.988 kDa), and soluble His6-H28 (49.553 kDa), suggesting the induction of specific anti-His6-H4 and anti-His6-H28 antibodies, which only specifically recognize its target protein containing H4 or H28.

Interestingly, on soluble His6-H4 coated ELISA plate, mice vaccinated with free soluble His6-H4 showed a relatively high IgG1 response when compared to its immobilized version (polyester particle-H4) (CI = 95%, $p = 0.059$, $n = 6$) and the IgG1 responses were similar to mice immunized with polyester particle-H28 and its soluble form (CI = 95%, $p = 0.677$, $n = 6$) (Fig. 5). Nevertheless, on soluble His6-H28 coated plate, IgG1 response was higher in mice immunized with polyester-H4 than in mice vaccinated with soluble His6-H4 (CI = 95%, $p = 0.2521$, $n = 6$) (Fig. 5). IgG1 response to His6-H28 was significantly higher in mice immunized with polyester particle-H28 than in mice vaccinated with its free soluble antigen (CI = 95%, $p = 0.0301$, $n = 6$) (Fig. 5).

Furthermore, IgG2c response to His6-H4 was stronger in mice immunized with particle-H4 than in mice vaccinated with soluble His6-H4 (CI = 95%, $p = 0.418$, $n = 6$) (Fig. 5). However, a similar IgG2c response to soluble His6-H4 was observed in mice immunized with particle-H28, soluble His6-H28, as well as the plain particle (CI = 95%, $p = 0.854$, $n = 6$) (Fig. 5). On soluble His6-H28 coated plate, there was no significant difference of IgG2c response in mice vaccinated with particle-H4 and soluble His6-H4 (CI = 95%, $p = 0.226$, $n = 6$) and in mice immunized with particle-H28 and soluble His6-H28 (CI = 95%, $p = 0.059$, $n = 6$) (Fig. 5).

3.4.5 Cytokine responses

To measure the Th1 cell-mediated immune response induced by the vaccines, splenocytes from immunized mice were re-stimulated *in vitro* with soluble His6-H4/His6-H28 and cRPMI medium alone. Cytokines in supernatant released from splenocytes were measured at 24 h and 60 h, to analyze both the early and late release of cytokines.

cRPMI medium was a negative control and did not contain His6-H4/His6-H28 antigen. No detectable cytokines were released from splenocytes incubated with cRPMI medium suggesting that there was no cytokine leakage when splenocytes were unstimulated (data not shown). Upon the stimulation with soluble His6-H28, TNFα, IL10, and IL6 production was higher in polyester particle-H28 vaccinated group compared to the soluble His6-H28-immunized group ($p = 0.199$ for TNFα, $p = 0.126$ for IL10, $p = 0.174$ for IL6; CI =95%, $n = 6$) (**Fig. 6**). In response to soluble His6-H4 stimulation, there was not much difference of TNFα, IL10, orIL6 release between particle-H4 and soluble His6-H4 vaccinated groups ($p = 0.575$ for TNFα, $p = 0.066$ for IL10, $p = 0.63$ for IL6; CI =95%, $n = 6$) (Fig. 6); however, the particle-H28 immunized group still showed relatively high TNFα, IL10, and IL6 production when compared to soluble His6-H28 vaccinated group ($p = 0.411$ for TNFα, $p = 0.121$ for IL10, $p = 0.121$ for IL6; CI =95%, $n = 6$) (Fig. 6). The releases of IL2 were low in all vaccinated groups and below 100 pg/ml at the both earlier and later culture time point (Fig. 6 and **Fig. S3**).

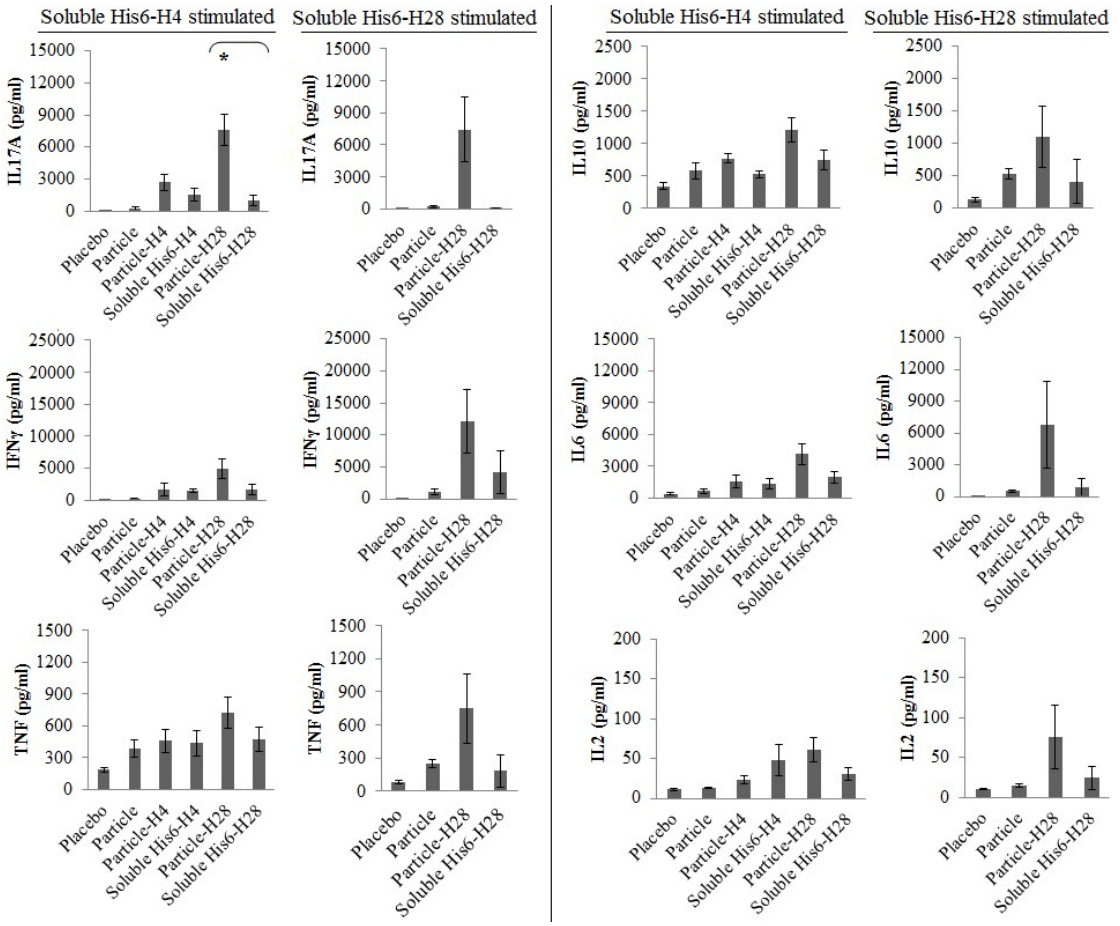

Figure 6. Cytokine responses of murine splenocytes after 60-hour stimulation with soluble His6-H4 and His6-H28 and analyzed by cytometric bead array. Each data point stands for the mean for 6 mice ± the standard error of the mean. Statistical significance [Confidence interval (CI) = 95%, $p < 0.05$, $n = 6$] was calculated by one way ANOVA with Mann-Whitney or Kruskal-Wallis Test (Minitab 17). *, significantly higher than the splenocytes from mice immunized with soluble His6-H28 (CI = 95%, $p < 0.05$, $n = 6$).

At the earlier culture time point with soluble His6-H4 stimulation, IL17A release by splenocyte from mice immunized with polyester particle-H28 was significantly higher compared to its soluble form vaccinated groups (CI = 95%, $p = 0.0131$, $n = 6$) (Fig. S3). Encouragingly, this significance remained at 60 h (CI = 95%, $p = 0.0081$, $n = 6$) (Fig. 6). Moreover, upon soluble His6-H28 stimulation at the earlier culture time point, the IL17A production from groups vaccinated with particle-H28 significantly higher when compared to other vaccinated groups (CI = 95%, $p = 0.001$, $n = 6$) (Fig. S3). However, this significance disappeared at the later culture time point (CI = 95%, $p = 0.124$, $n = 6$) (Fig. 6). Upon soluble His6-H4 stimulation for 24 h, the groups vaccinated with soluble vaccines showed relatively high IFNγ release when compared to the groups vaccinated

with particulate vaccines (p = 0.784 for particle-H4 and soluble His6-H4, p = 0.471 for particle-H28 and soluble His6-H28; CI =95%, n = 6) (Fig. S3). It was also observed that the IFNγ release in the soluble His6-H28 immunized group was higher than the particle-H28 vaccinated group at the early culture time point in response to soluble His6-H28 stimulation (CI = 95%, p = 0.8102, n = 6) (Fig. S3). However, at the later culture time point, there was a trend suggesting increased IFNγ production in groups immunized with particulate vaccines when compared to groups vaccinated with the corresponding soluble controls (p = 0.411 for particle-H4 and soluble His6-H4 in response to soluble His6-H4 stimulation, p = 0.121 for particle-H28 and soluble His6-H28 in response to soluble His6-H4 stimulation, p = 0.422 for particle-H28 and soluble His6-H28 in response to soluble His6-H28 stimulation; CI =95%, n = 6) (Fig. 6).

3.5 Discussion

This study evaluated the potential application of self-assembled polyester particles produced in *E.coli* cells as a carrier system for the delivery of mycobacterial antigens, H4 and H28. A number of studies have shown that polyester particles could be genetically engineered to display antigen of interests (29, 30, 39). This particle based vaccine delivery system had been shown to enhance antigen specific immune responses (25, 40). In contrast to live-attenuated vaccines, subunit vaccines are safe but often have low immunogenicity, thereby compromising their protective efficacy (5, 6). Herein, *E. coli* cells were bioengineered to overproduce a fusion protein, PhaC-H4 or PhaC-H28, which mediated intracellular formation of polyester particles displaying H4 or H28 antigens, respectively. Analysis of TEM images confirmed the intracellular formation of the TB antigens-displaying polyester particles (Fig. 2*B*). The polyester synthase PhaC remained anchored on the surface after particle formation and isolation (29, 41). Protein profile analysis by SDS-PAGE showed that the full length PhaC-TB antigen fusions were overproduced as MWs of fusion proteins corresponded to their theoretical MWs (Fig. 2*C*), suggesting the mycobacterial antigens H4/H28 were successfully displayed on the particle surface. This was further confirmed by identification of tryptic peptides derived from the fusion proteins using MALDI-TOF/MS analysis. The SDS-PAGE analysis also showed the impurities of purified polyester particle vaccines (Fig. 2*C* and

2*D*), indicating that some *E. coli* background proteins may be co-purified with polyester particles. However, these host cell background proteins were not immunogenic and did not induce detectable immune responses in mice (40).

As the particle size and its surface charges may affect cellular uptake and antigen processing (42-45), the size and Zeta potential of polyester particle displaying TB antigens were characterized before and after emulsification in DDA. The size distribution of the DDA micelle monodispersed peak was increased from 0.01 μm–0.3 μm to 20 μm–300 μm when the soluble mycobacterial vaccines, His6-H4/His6-H28, were emulsified in the adjuvant (Fig. 3*A*), suggesting that interactions between amphipathic DDA adjuvant and soluble vaccines occurred and might influence electrostatic interactions between DDA micelles, causing further micelle aggregation. Moreover, all polyester particle vaccines before DDA emulsification were not monodispersed and widely distributed from approximately 0.1 μm to 100 μm. However, after DDA emulsification, particles displaying H4/H28 were monodispersed and its size ranged between 1 μm and 10 μm (Fig. 3*A*), indicating DDA affected the physicochemical properties of polyester particle vaccines. Since particles ranging in size between 1 μm and 10 μm can be efficiently be taken up by antigen presenting cells (APCs) via phagocytosis, the formulated polyester particles will likely be efficiently taken up APCs (5, 6, 46). However, the correlation between cellular uptake and immune responses is controversial (43).

Surface charges of particles could influence cellular uptake (43, 47, 48). All the vaccine samples were negatively charged; however, they possessed a positively charged surface after they were emulsified in DDA (Fig. 3*B*). A study showed that uptake of particles, the size of which was larger than 0.5 μm in diameter, by dendritic cells was greatly enhanced when particles showed a positive surface charge (43). Some studies showed that particles with a negatively charged surface can promote cellular uptake (49, 50). However, some authors demonstrated that the surface charge was not critical for uptake of small size particles (<0.5 μm) (51). Therefore, the effect of surface charges on the cellular uptake remains to be established (43).

E. coli is a Gram-negative bacterium and contains the endotoxin lipopolysaccharide (LPS), which is pathogenic and induces strong endotoxic responses in human and

animals (52, 53). However, *ClearColi* BL21 (DE3) is an endotoxin free production host and only contains a modified LPS, which is not capable of triggering immune responses (54). All the polyester particle vaccines were produced by *ClearColi* cells. After vaccine formulation, the injectability of formulated particle vaccines was tested before they were applied into mice. After DDA emulsification, it was difficult to inject vaccines through a 20 gauge needle when the particle slurry was more than 10% (data not shown), at which percentage the TB antigens per dose was approximate 2.3-2.7 μg. In order to make the formulated solution injectable, particle percentages need to be less than 10%. Therefore, formulated particulate vaccines contained 2 μg of TB antigens/dose were determined and the slurry of particulate vaccines at this dosage was between 5–8%.

M. tuberculosis is an intracellular pathogen and its life cycle has a transient extracellular phase, where the target pathogen is very susceptible to the antimicrobial effects of antibodies (55). Therefore, not only cell-mediated immunity but also humoral immunity plays an important role to prevent and control this pathogen (55, 56). The humoral immunity was firstly analyzed by measuring the levels of IgG1 and IgG2c by ELISA.

To analyze the immunogenicity of the plain polyester particles, sera from mice immunized with different vaccine samples were used to interact with plain particles coated on ELISA plate. Very low IgG1 and IgG2 immune responses to plain particles were observed (Fig. 5). Moreover, the western blot result showed that no PhaC related protein bands were detected by the pooled sera from mice immunized with the plain polyester particle (PhaC). These results suggested that polyester particles itself are not immunogenic i.e. no carrier suppression. Some studies also confirmed that polyester particles are non-toxic and unlikely to elicit immune responses, and are well tolerated by mammalian systems due to their biocompatibility (23, 24, 40).

In addition, to investigate whether *E. coli* host cell proteins, co-purified with vaccine samples, are immunogenic and could induce humoral immunity, the whole cell lysates containing various vaccine samples were used to interact with pooled anti-His6-H4 and anti-His6-H28, respectively. The western blot showed that the pooled anti-His6-H4 and anti-His6-H28 did not interact with the background proteins from *E. coli* cells and only specifically recognized the free soluble His6-H4/ His6-H28 and their immobilized forms (Fig. 5). This indicated induction of only antigen-specific antibodies when mice

were vaccinated with particles displaying H4/H28 while no antibodies against potential host cell protein impurities were induced. A study also confirmed that production host cell proteins co-purified with particulate vaccines did not elicit a detectable humoral immune response in mice (40). Furthermore, overlapping peptides representing the PhaC anchoring protein did not restimulate splenocytes isolated from mice vaccinated with polyester particles either or not displaying antigens, which further suggested that the carrier system is not immunogenic and that there is no carrier suppression (57).

Cell-mediated immunity, contributed by T cells, plays an important role for protection against intracellular pathogens (58). Polyfunctional CD4+ T cells could simultaneously produce multiple Th1 cytokines, IFNγ, IL2, and TNFα, after *M. tuberculosis* infection or vaccination (58-60). The cell-mediated immune response was analyzed by measuring the various cytokine releases from splenocytes restimulated with soluble His6-H4/His6-H28. IFNγ, a Th1 cytokine strongly associated with cell-mediated immune response, has a significant role in protection from TB (58, 60). The particulate vaccines-immunized group showed a higher IFNγ production when compared to the soluble vaccines-immunized group in response to either soluble His6-H4 or His6-H28 stimulation at the later culture time point (Fig. 6). This may suggest mycobacterial vaccines displayed on polyester particle could enhance immune response in regard to IFNγ production. Moreover, antigen specific IFNγ production was dramatically increased in particle vaccines-immunized mice. In particular, splenocytes from mice vaccinated with polyester particles displaying H28 when restimulated with soluble His6-H28 at the later culture time point showed an IFNγ production of up to 20,000 pg/ml, which indicated a strong cell-mediated immune response (Fig. 6).

In addition, IL17A is also a biomarker of cell-mediated immunity development (61). At the earlier culture time point, the polyester particle-H4 vaccinated group produced more IL17A cytokine than the group-immunized with its free soluble version in response to soluble His6-H4 stimulation, but this IL17A production did not reach significance (CI = 95%, $p = 0.0828$, $n = 6$) (Fig. S3). However, the group-vaccinated with particle-H28 produced significantly more IL17A cytokine than their soluble form-immunized mice (CI = 95%, $p = 0.0131$, $n = 6$) (Fig. S3). In contrast, IL17A production at the later culture time point was greatly increased. Moreover, after 60 h incubation, all particulate vaccines (particle-H4 and particle-H28) immunized mice showed more IL17A

production than mice-vaccinated with soluble vaccines (Fig. 6), suggesting a strong activation of cell-mediated immunity. Analysis of antibody and cytokine responses suggested that particulate vaccines generally induced relatively strong immune responses when compared to free soluble vaccines. This could be because particulate vaccines may serve as an antigen depot for prolonged antigen display (5) and augment overall immunogenicity by enhancing B cell receptor cross-linking to cause high levels of B cell activation and promoting antigen cross-presentation for induction of both humoral and cell-mediated immune responses (5, 6, 46, 62). Although antigen specific Th1 cytokines play important role for protection against TB, reliable correlates between biomarkers and immune protection are still lacking, as high T cell responses do not always confer enhanced protection in immunized subjects (63). Therefore, future challenge experiments in mice will be necessary to evaluate vaccine efficacy.

It is not clear whether the augmented antigen-specific immune responses in this study were induced by the polyester particle delivery system or both the particle and the adjuvant DDA. This will be addressed in future experiments by using the polyester particle vaccine without the adjuvant DDA. Previous studies demonstrated that these polyester particle might have adjuvant property as vaccine efficacy had been improved regardless the addition of adjuvant (25, 31). Adjuvants are often classified as immunostimulatory molecules (such as TLR or CLR ligands) or delivery agents (such as aluminium salts, lipid vesicles, or emulsions) (64-66). Most adjuvants, such as aluminium salts and emulsions, possess both the functional properties (65). Polyester materials are biocompatible as they could be developed into various medical implants (67). This study also confirmed that polyester particle carrier is not immunogenic. Therefore, polyester particles may enhance antigen-specific immune responses via its particulate delivery effect.

In summary, this study demonstrated that mycobacterial antigens were successfully displayed on polyester particles *in vivo* by one-step production using an endotoxin free *E. coli* strain. Particles itself were not immunogenic and did not elicit detectable immune responses. Particles displaying mycobacterial antigens mediated antigen-specific humoral and cell-mediated immune responses. Polyester particle vaccines possessed positively charged surface and became monodispersed after formulation with DDA. These monodispersed particulate vaccines exhibited ascendant

immunological properties when compared to the soluble antigens. Hence, the polyester particle-based particulate vaccine approach offer a one-step and cost-effective vaccine manufacturing process and show the promise to become a safe, efficient and stable antigen carrier system for vaccination against intercellular pathogens, such as *M. tuberculosis*.

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3.7 Author contributions

Shuxiong Chen and Bernd H. A. Rehm designed the research; Shuxiong Chen analyzed the data; Shuxiong Chen, Sarah Sandford, Joanna R. Kirman, and Bernd H. A. Rehm performed the research; Bernd H. A. Rehm supervised the project; Shuxiong Chen wrote the original draft; Bernd H. A. Rehm revised the draft; and all authors reviewed and contributed to the final manuscript.

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3.9 Supplementary material

TABLE S1. *MALDI-TOF/MS analysis of PhaC-TB antigen fusion proteins*

Protein/Protein sequence	**Peptide fragments assigned to the various protein regions**
PhaC-Ag85B-TB10.4 (MW: 106.698 kDa)	
MATGKGAAASTQEGKSQPFKVTPGPFDPATWLEWS RQWQGTEGNGHAAASGIPGLDALAGVKIAPAQLGDI QQRYMKDFSALWQAMAEGKAEATGPLHDRRFAGD AWRTNLPYRFAAAFYLLNARALTELADAVEADAKT RQRIRFAISQWVDAMSPANFLATNPEAQRLLIESGGE SLRAGVRNMMEDLTRGKISQTDESAFEVGRNVAVTE GAVVFENEYFQLLQYKPLTDKVHARPLLMVPPCINK YYILDLQPESSLVRHVVEQGHTVFLVSWRNPDASMA GSTWDDYIEHAAIRAIEVARDISGQDKINVLGFCVGG TIVSTALAVLAARGEHPAASVTLLTTLLDFADTGILD VFVDEGHVQLREATLGGGAGAPCALLRGLELANTFS FLRPNDLVWNYVVDNYLKGNTPVPFDLLFWNGDAT NLPGPWYCWYLRHTYLQNELKVPGKLTVCGVPVDL ASIDVPTYIYGSREDHIVPWTAAYASTALLANKLRFV LGASGHIAGVINPPAKNKRSHWTNDALPESPQQWLA GAIEHHGSWWPDWTAWLAGQAGAKRAAPANYGN ARYRAIEPAPGRYVKAKAVLAVAIDKRGGGGGLEG GGGIEGRAPGSGGSFSRPGLPVEYLQVPSPSMGRDIK VQFQSGGNNSPAVYLLDGLRAQDDYNGWDINTPAF EWYYQSGLSIVMPVGGQSSFYSDWYSPACGKAGCQ TYKWETFLTSELPQWLSANRAVKPTGSAAIGLSMAG SSAMILAAYHPQQFIYAGSLSALLDPSQGMGPSLIGL AMGDAGGYKAADMWGPSSDPAWERNDPTQQIPKL VANNTRLWVYCGNGTPNELGGANIPAEFLENFVRSS NLKFQDAYNAAGGHNAVFNFPPNGTHSWEYWGAQ LNAMKGDLQSSLGAGMSQIMYNYPAMLGHAGDMA GYAGTLQSLGAEIAVEQAALQSAWQGDTGITYQAW QAQWNQAMEDLVRAYHAMSSTHEANTMAMMARD TAEAAKWGG	PhaC: V21-R36, I63-R74, D78-R100, F102-R108, F115-K139, F146-R180, I195-K234, Y250-R299, E371-R386, H443-K451, L456-K498, F501-K518, A564-R573 Ag85B: F621-R640, V644-R663, W717-R733, A796-R810, L827-R854 TB10.4: A973-R991

PhaC-Ag85B-TB10.4-Rv2660c (114.263 kDa)	
MATGKGAAASTQEGKSQPFKVTPGPFDPATWLEWS RQWQGTEGNGHAAASGIPGLDALAGVKIAPAQLGDI QQRYMKDFSALWQAMAEGKAEATGPLHDRRFAGD AWRTNLPYRFAAAFYLLNARALTELADAVEADAKT RQRIRFAISQWVDAMSPANFLATNPEAQRLLIESGGE SLRAGVRNMMEDLTRGKISQTDESAFEVGRNVAVTE GAVVFENEYFQLLQYKPLTDKVHARPLLMVPPCINK YYILDLQPESSLVRHVVEQGHTVFLVSWRNPDASMA GSTWDDYIEHAAIRAIEVARDISGQDKINVLGFCVGG TIVSTALAVLAARGEHPAASVTLLTTLLDFADTGILD VFVDEGHVQLREATLGGGAGAPCALLRGLELANTFS FLRPNDLVWNYVVDNYLKGNTPVPFDLLFWNGDAT NLPGPWYCWYLRHTYLQNELKVPGKLTVCGVPVDL ASIDVPTYIYGSREDHIVPWTAAYASTALLANKLRFV LGASGHIAGVINPPAKNKRSHWTNDALPESPQQWLA GAIEHHGSWWPDWTAWLAGQAGAKRAAPANYGN ARYRAIEPAPGRYVKAKAVLAVAIDKRGGGGGLEG GGGIEGRAPGSGGSFSRPGLPVEYLQVPSPSMGRDIK VQFQSGGNNSPAVYLLDGLRAQDDYNGWDINTPAF EWYYQSGLSIVMPVGGQSSFYSDWYSPACGKAGCQ TYKWETFLTSELPQWLSANRAVKPTGSAAIGLSMAG SSAMILAAYHPQQFIYAGSLSALLDPSQGMGPSLIGL AMGDAGGYKAADMWGPSSDPAWERNDPTQQIPKL VANNTRLWVYCGNGTPNELGGANIPAEFLENFVRSS NLKFQDAYNAAGGHNAVFNFPPNGTHSWEYWGAQ LNAMKGDLQSSLGAGMSQIMYNYPAMLGHAGDMA GYAGTLQSLGAEIAVEQAALQSAWQGDTGITYQAW QAQWNQAMEDLVRAYHAMSSTHEANTMAMMARD TAEAAKWGGMIAGVDQALAATGQASQRAAGASGG VTVGVGVGTEQRNLSVVAPSQFTFSSRSPDFVDETA GQSWCAILGLNQFH	PhaC: V21-R36, I63-R74, D78-R100, F102-R108, F115-K139, F146-R169, I195-K234, Y250-R299, E371-R386, H443-K451, L456-K498, F501-K518, A564-R573 Ag85B: F621-R640, V644-R663, W717-R733, A796-R810, L827-R854 TB10.4: A973-R991 Rv2660c: N1039-H1076
His6-Ag85B-TB10.4 (MW: 106.698 kDa)	
MHHHHHHFSRPGLPVEYLQVPSPSMGRDIKVQFQSG GNNSPAVYLLDGLRAQDDYNGWDINTPAFEWYYQS GLSIVMPVGGQSSFYSDWYSPACGKAGCQTYKWET	Ag85B: F8-F33, L45-Y68, S74-Y86, S91-Y102, S117-L132, A143-F150,

FLTSELPQWLSANRAVKPTGSAAIGLSMAGSSAMILA AYHPQQFIYAGSLSALLDPSQGMGPSLIGLAMGDAG GYKAADMWGPSSDPAWERNDPTQQIPKLVANNTRL WVYCGNGTPNELGGANIPAEFLENFVRSSNLKFQDA YNAAGGHNAVFNFPPNGTHSWEYWGAQLNAMKGD LQSSLGAGMSQIMYNYPAMLGHAGDMAGYAGTLQ SLGAEIAVEQAALQSAWQGDTGITYQAWQAQWNQ AMEDLVRAYHAMSSTHEANTMAMMARDTAEAAK WGG	K182-W215, K246-Y251, N262-W271 TB10.4: N299-Y313, N351-Y361, A374-G388
His6-Ag85B-TB10.4-Rv2660c (114.263 kDa)	
MHHHHHHFSRPGLPVEYLQVPSPSMGRDIKVQFQSG GNNSPAVYLLDGLRAQDDYNGWDINTPAFEWYYQS GLSIVMPVGGQSSFYSDWYSPACGKAGCQTYKWET FLTSELPQWLSANRAVKPTGSAAIGLSMAGSSAMILA AYHPQQFIYAGSLSALLDPSQGMGPSLIGLAMGDAG GYKAADMWGPSSDPAWERNDPTQQIPKLVANNTRL WVYCGNGTPNELGGANIPAEFLENFVRSSNLKFQDA YNAAGGHNAVFNFPPNGTHSWEYWGAQLNAMKGD LQSSLGAGMSQIMYNYPAMLGHAGDMAGYAGTLQ SLGAEIAVEQAALQSAWQGDTGITYQAWQAQWNQ AMEDLVRAYHAMSSTHEANTMAMMARDTAEAAK WGGMIAGVDQALAATGQASQRAAGASGGVTVGVG VGTEQRNLSVVAPSQFTFSSRSPDFVDETAGQSWCAI LGLNQFH	Ag85B: F8-R27, V31-R50, A183-R241 TB10.4: A360-R378 Rv2660c: M389-R440

TABLE S2. *Summary of polyester particle size distribution before and after emulsification in DDA. Dx (10, 50, 90) stands for x% of particle diameters are distributed below the value. Span is the width of the size distribution. Uniformity stands for the absolute deviation of the median. The specific surface area (m2/kg) is the total exposed particle surface area (m2) divided by the total wet weight of the particle (kg), analyzed by Mastersizer3000*

Samples	**Dx (10) (µm)**	**Dx (50) (µm)**	**Dx (90) (µm)**	**Span**	**Uniformity**	**Specific surface area (m^2/kg)**
Polyester particle	0.285	0.623	50.5	80.512	29.244	9986
Polyester particle-H4	0.352	4.88	28.5	5.766	1.995	5442
Polyester particle-H28	0.374	6.38	30.8	4.77	1.787	4624
DDA	0.0215	0.0632	0.179	2.493	0.867	123100
Polyester particle + DDA	0.101	3.9	84.3	21.59	6.146	23610
Polyester particle-H4 + DDA	2.34	4.35	7.85	1.268	0.693	1462
Polyester particle-H28 + DDA	2.32	4.17	6.97	1.116	0.34	1524
Soluble His6-H4 + DDA	67.4	164	300	1.415	0.433	120
Soluble His-H28 + DDA	42.2	145	276	1.616	0.491	2835

TABLE S3. *Amount of mycobacterial antigens displayed on the particle surface*

Vaccine samples	**Rec protein in 10% particle slurry (µg/µl)**	**µg of rec protein per mg of wet particle**	**MW ratio (Antigen: rec protein)**	**µg of antigen per mg of wet particle**
PhaC (Particle)	0.1547	1.547	N/A	N/A
PhaC-H4 (Particle-H4)	0.05112	0.5112	1:2.59	0.1974
PhaC-H28 (Particle-H28)	0.02961	0.2961	1:2.34	0.1265

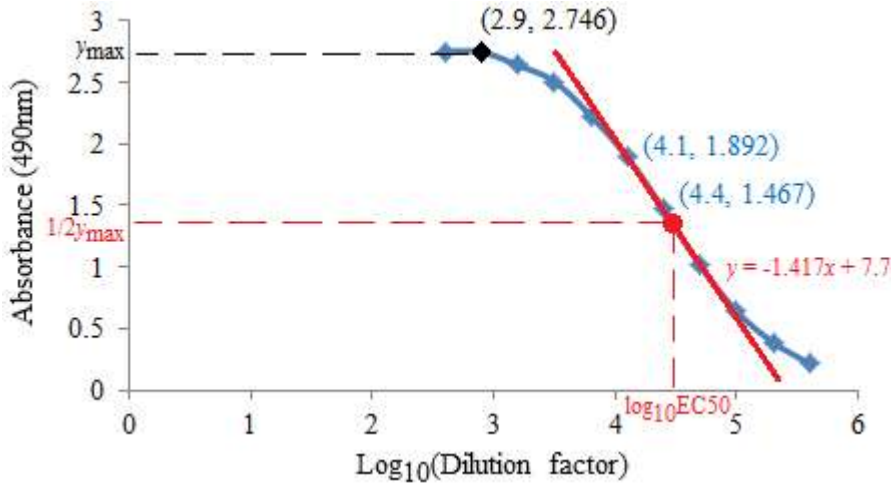

Figure S1. Methods of EC50 value calculation. EC50 was estimated using a sigmoid curve and a straight line (y = mx + c). The sigmoid curve was developed using serially diluted sera concentrations ranging from 1/400 to 1/409,600 and their corresponding absorbance. The straight line was determined using the linear interplotation between two known points, (4.1, 1.892) and (4.4, 1.467), on the sigmoid curve. The above methods demonstrated EC50 calculation of IgG1 response to soluble His6-H28 using a single serum obtained from a mouse immunized with polyester particle-H4. [Antigens Ag85B and TB10.4 are present in both H4 (Ag85B-TB10.4) and H28 (Ag85B-TB10.4-Rv2660c) vaccines. For the sera from mice immunized with polyester particle-H4, we look for the antibodies against Ag85B and TB10.4.]

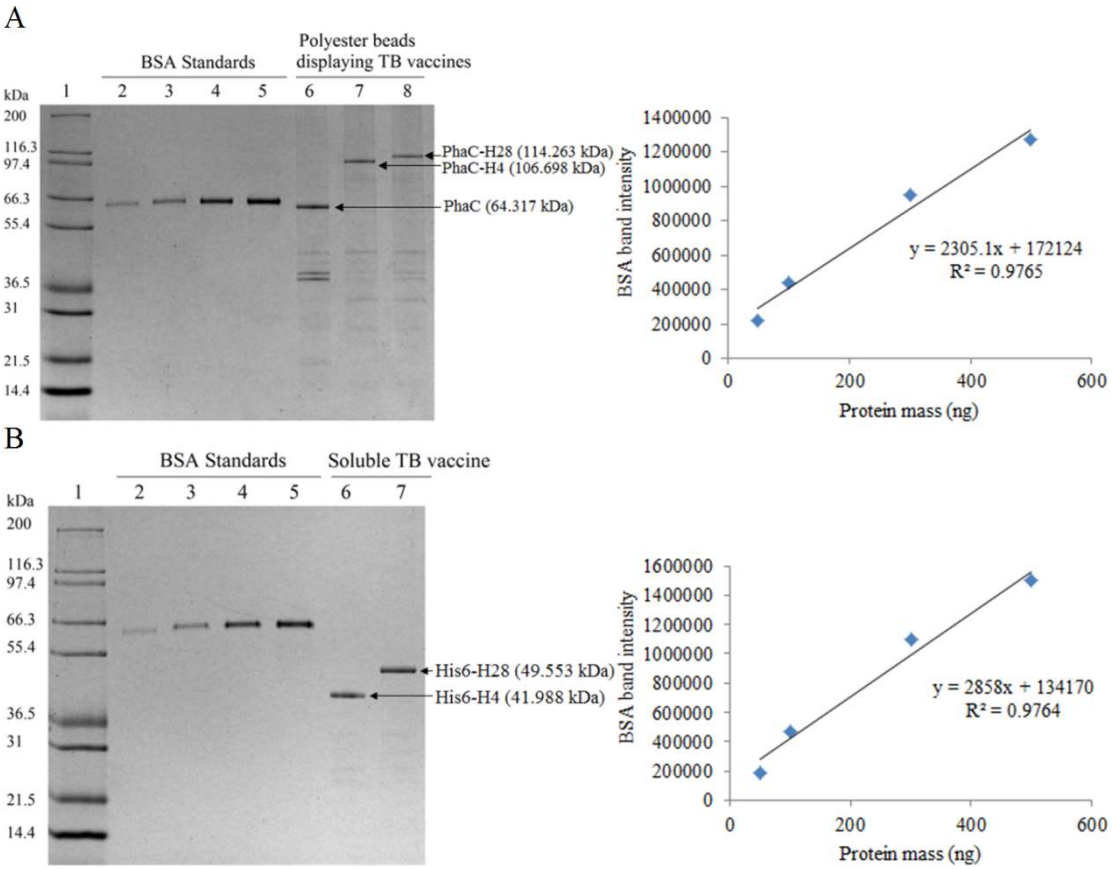

Figure S2. Vaccine concentration measurement. *A*) Measurement of recombinant mycobacterial antigens immobilized on the surface of polyester particles. *B*) Determination of free soluble recombinant mycobacterial antigens. BSA, ranging between 50 ng and 500 ng, were loaded on 10% Bis-Tris gel to develop a standard curve, used to determine the recombinant protein concentrations in 10% particle slurry. The images were taken and analyzed by the gel doc (BioRad Laboraties, Hercules, CA) and the Image Lab software (BioRad Laboratories, Hercules, CA), respectively.

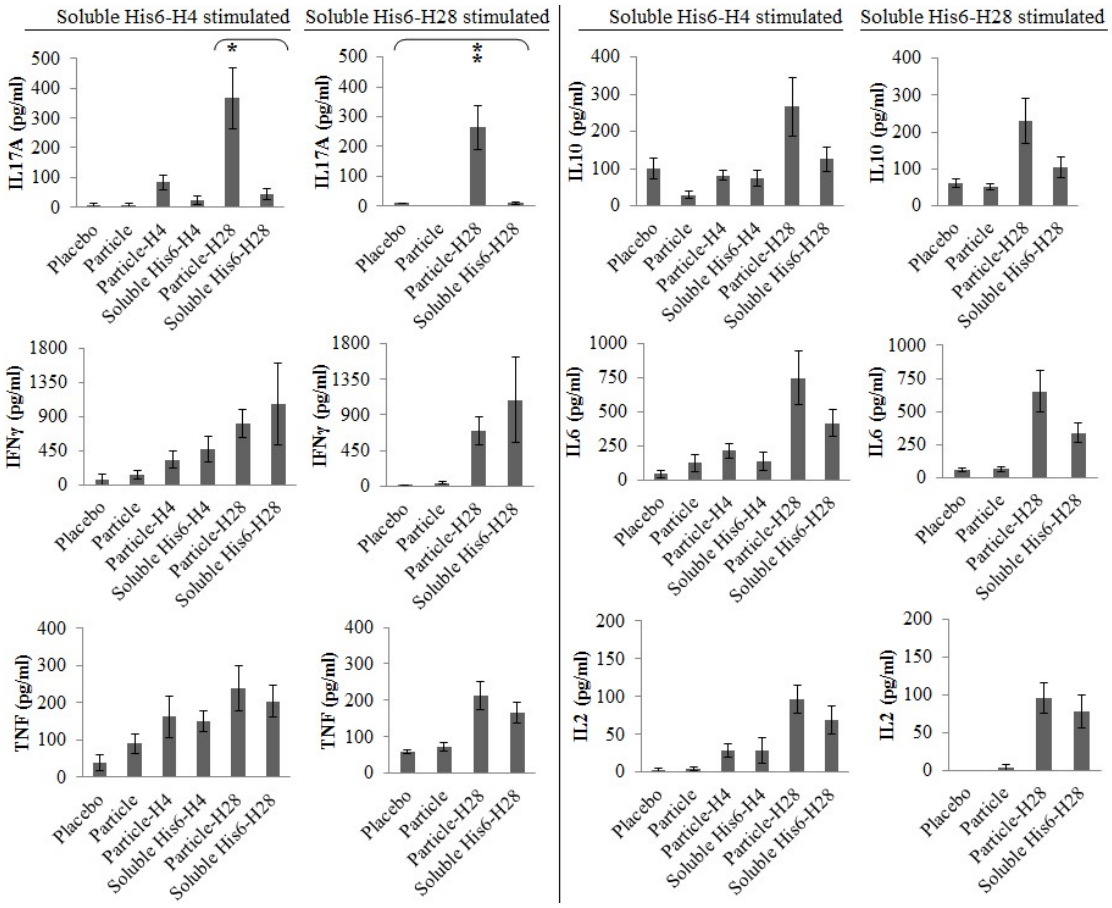

Figure S3. Cytokine responses of murine splenocytes after 24-hour stimulation with soluble His6-H4 and His6-H28 and measured by cytometric bead array. Each data point stand for the mean for 6 mice ± the standard error of the mean. Statistical significance (CI = 95%, $p < 0.05$, $n = 6$) is determined by one way ANOVA with Mann-Whitney or Kruskal-Wallis Test (Minitab 17). *, significantly higher than the splenocytes from mice immunized with soluble His6-H28 (CI = 95%, $p < 0.05$, $n = 6$). **, significantly higher than the splenocytes from mice immunized with DDA (placebo), particle, soluble His6-H28 (CI = 95%, $p < 0.05$, $n = 6$).

Preface to Chapter 4

A number of studies have shown that bioengineered antigenic peptides, such as antigens from *Plasmodium berghei* and coronavirus, are able to self-assemble into protein inclusion body which could perform as an immunogenic antigen carrier system for efficient antigen delivery and induction of desirable immune response. Protein inclusion body was generally thought to contain misfolded and inactive proteins. However, recent studies have demonstrated that protein inclusion bodies also contain a large amount of properly folded and biologically active proteins.

The previous chapter described the development of particulate mycobacterial vaccines using the polyester particle as the platform. The polyester particle-based mycobacterial vaccines (polyester particle-H4 and polyester particle-H28) showed increased immunogenicity when compared to the free soluble versions (H4 and H28). However, the platform, plain polyester particle, was not immunogenic. In Chapter 4, the immunogenic mycobacteria vaccine H4 or H28 was bioengineered towards the spontaneous formation of mycobacterial antigen inclusion body (antigen particle) in an endotoxin free *E. coli* strain. The immunogenicity of the mycobaterial antigen particle vaccines and the soluble forms was analysed. The experiment was performed using female C57BL/6 mice.

Chapter 4. Design of Bacterial Inclusion Bodies as Antigen Carrier Systems

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4.1 Abstract

Synthetic subunit vaccines hold great promise to prevent infectious diseases, but they often induce only weak immune responses. Here we exploited bioengineering techniques to develop immunogenic antigen particles comprised of self-assembling antigenic peptides. A bioparticle platform was developed to present repetitive copies of subunit antigens, which mimic the host-pathogen surface interaction to provide enhanced immunogenicity. Herein, mycobacterial fusion proteins H4 (consisting of Ag88B-TB10.4) and H28 (containing Ag88B-TB10.4-Rv2660c) were bioengineered to assemble H4 and/or H28 antigens into particulate vaccines inside an endotoxin-free *Escherichia coli* strain, to serve as a novel vaccine against tuberculosis (TB). A bioprocess for particle isolation was developed and their physico-chemical properties were investigated. Antigen particles formulated with the adjuvant dimethyl dioctadecyl ammonium bromide (DDA) were used to subcutaneously immunize C57BL/6 mice. Both soluble and particulate vaccines elicited functional and specific antibodies, which only interacted with target antigens and not production host cell proteins. Particulate vaccines induced a strong production of IFNγ and IL17A cytokines. When compared to soluble TB vaccines, the antigen particle vaccines demonstrated increased immunogenicity. Overall, this study provides proof of concept that selected antigens can be engineered to self-assemble into inclusion bodies to serve as particulate vaccines with improved immunological properties compared to the respective soluble antigens.

4.2 Introduction

Vaccination is one of the most effective ways to combat challenging infectious diseases globally.[1] It has emancipated us from the attack of a number of infectious diseases, such as polio, measles, and smallpox.[1c] However, the top three global infectious killers, TB, HIV and malaria remain without effective vaccines. Live-attenuated vaccines are highly immunogenic, but have an inherent safety issue, that the live, weakened vaccines may revert into disease-causing pathogens.[2] Subunit vaccines contain antigenic parts of the pathogen and so pose no infective risk to the patient.[3] However, subunit vaccines are often less immunogenic and fail to provide adequate protection.[2-3] To overcome this drawback, we explored a bioengineering strategy to manipulate antigenic peptides

towards the self-assembling of a new particulate TB vaccine platform.

TB is primarily an infectious disease of the lungs caused by *Mycobacterium tuberculosis* (*M. tuberculosis*).[4] TB kills more people annually than any other infectious diseases, and in 2016 there were estimated to be 10.4 million new cases.[5] There is a licensed TB vaccine, live, attenuated *Mycobacterium bovis*, known as Bacille Calmette-Guérin (BCG). The BCG vaccine, however, fails to provide effective protection in both humans and cattle.[5-6]

New TB vaccines, including highly purified antigenic protein subunit vaccines, are currently being tested in human clinical trials.[1a] However it is difficult to generate recombinant vaccines that can induce adequate levels of protective immunity, since recombinant subunit vaccines cannot replicate and therefore require the administration of multiple doses.[2] An approach to circumvent this issue is the use of self-assembling antigen particles, which are able to display multiple copies of subunit antigen in a well-organized structure with defined orientation. The particles can potentially mimic the shape, size and geometry of the pathogen-host surface interactions, thus enhancing their immunogenicity.[2, 7]

A few studies have shown that antigen particles can effectively trigger a protective immune response. Successful immunity induced by vaccine particles has been demonstrated against animal diseases, such as liver fluke, classical swine fever, and salmonid rikettsial septicaemia,[8] as well as against the human oral infections, gingivitis and periodontitis.[2, 7, 9] The proteins displayed on particles imparted increased antigen immunogenicity and stability compared to the soluble forms.[10] In this study, the design space of protein assemblies inside *E. coli* was explored toward developing a robust production process for synthetic multivalent particulate vaccines.

A number of immunogenic antigens have been considered for developing new effective TB vaccines.[11] Ag85B and TB10.4 are early stage antigens, and are normally secreted during the acute phase of infection. These two antigens are expressed by both *M. tuberculosis* and BCG.[11a, 12] The recombinant fusion protein Ag85B-TB10.4, designated vaccine H4, showed protective immunity in mice [11a, b, 11d] and was also safe and immunogenic in South African adults.[11e] Rv2660c is a latency-associated antigen,

which induced strong cellular and humoral immune responses in a Chinese latent TB infection population.[13] The vaccine H28, Ag85B-TB10.4-Rv2660c, combined the vaccine H4 backbone and the latency-associated antigen Rv2660c,[11a, 11d] and was able to protect mice against *M. tuberculosis* challenge.[11d]

This present study aimed to investigate the immunogenicity of self-assembling particles expressing mycobacterial antigens compared to the corresponding soluble antigen forms. This paper describes the bioengineering of the endotoxin free production host *ClearColi* BL21 (DE3) to produce antigen particle vaccines, containing the mycobacterial recombinant fusion proteins H4 or H28. Antigen particles and the corresponding soluble forms were prepared and used to immunize female C57BL/6 mice subcutaneously. The humoral and cellular immune responses were analyzed.

4.3 Results and discussion

4.3.1 Engineering of recombinant mycobacterial vaccines, H4 and H28, towards *in vivo* self-assembly of particles

Intracellular self-assembly of particles was observed when recombinant genes were over-expressed under a strong promoter.[14] Recombinant genes encoding TB vaccines H4 and/or H28 were genetically manipulated and cloned into the pET-14b expression vector containing the strong promoter, T7. The modular composition of the recombinant genes and encoded fusion proteins is outlined in **Figure 1A**. *E. coli* cells harboring the various plasmids containing *h4* and/or *h28* genes were cultivated to produce the recombinant proteins.

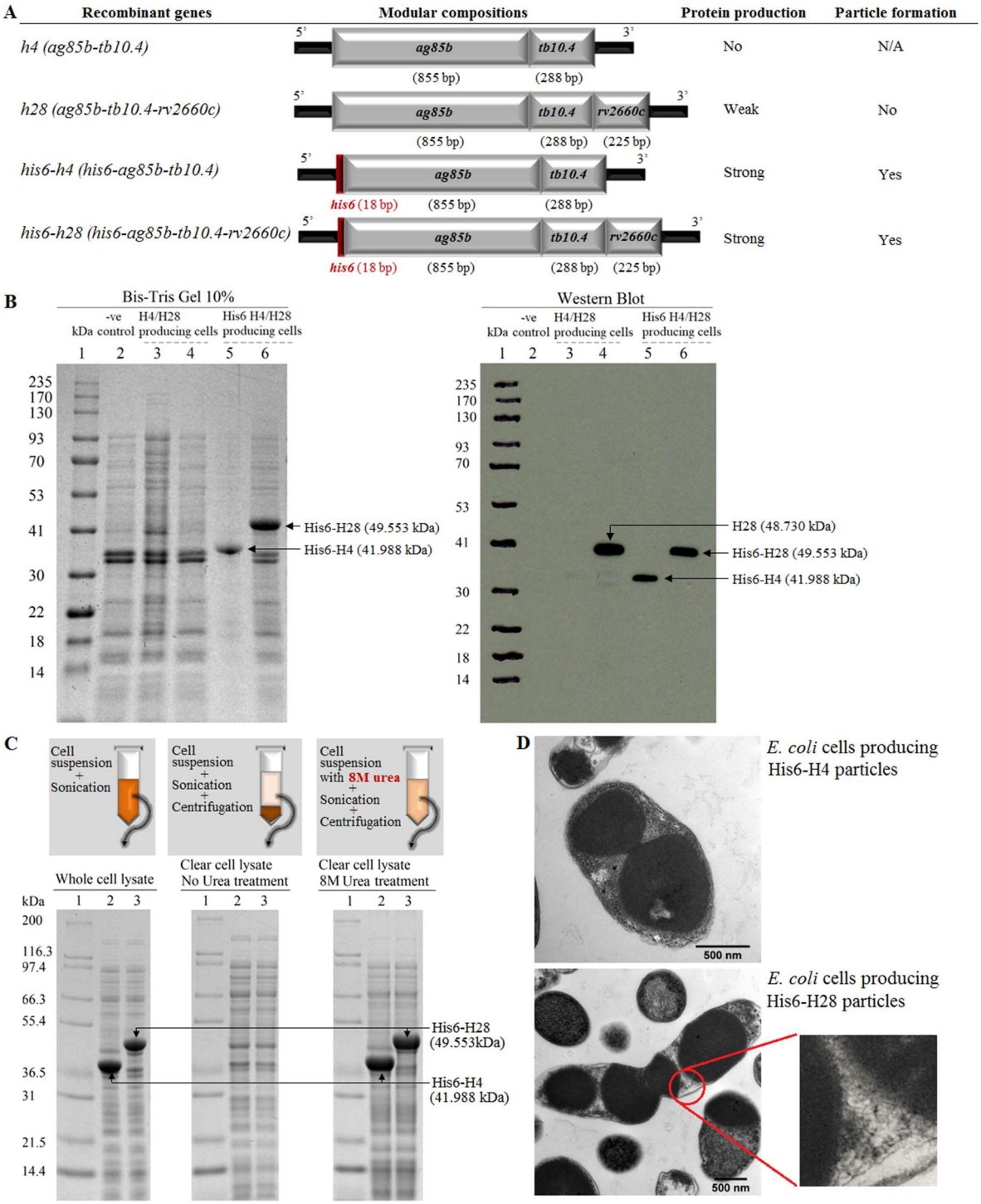

Figure 1. Bioengineering of mycobacterial antigen fusions for the intracellular formation of vaccine particles. A) Schematic overview of recombinant genes encoding fusion proteins for the production of insoluble H4 and/or H28 particles. B) Production of H4 and H28 recombinant proteins with or without His6 tag in *E. coli* cells. Protein profile of *E. coli* cells producing non-His6-tagged and His6-tagged H4 and H28 was analyzed with Bis-tris gel. Immunoblot of cells producing H4 and H28 with or without His6-tag was performed using pooled sera from mice immunized with soluble His6-H4. Lane 1, molecular weight marker (GangNam-Stain pre-stained protein ladder; iNtRon); lane 2, *E. coli*/ pET-14b; Lane 3, *E. coli*/ pET-14b H4,

41.166 kDa; lane 4, *E. coli*/ pET-14b H28, 48.730 kDa; lane 5, *E. coli*/ pET-14b His6-H4, 41.988 kDa; lane 6, *E. coli*/ pET-14b His6-H28, 49.553 kDa. C) Solubility analysis of His6-tagged H4 and H28 TB vaccines. Protein profile of His-tagged vaccines from whole *E. coli* cells was analyzed with Bis-tris gel. Supernatant fractions of cell suspension treated with or without 8 M urea were also investigated after sonication and centrifugation. Lane 1, molecular weight marker (Mark 12; Invitrogen); lane 2, His6-H4 (41.988 kDa); lane 3, His6-H28 (49.553 kDa). D) TEM images of *E. coli* cells producing insoluble H4 and H28 particles (scale bar, 500 nm).

The protein profile of cells producing H4 and H28 with and/or without His6 at the N-terminus was analyzed by Bis-Tris gel and immunoblot. The Bis-Tris gel showed dominant protein bands corresponding to proteins with theoretical molecular weights (MW) of His6-H4 (41.988 kDa) and His6-H28 (49.553 kDa) (Figure 1B), suggesting His6-H4 and His6-H28 proteins were heavily overproduced. However, no dominant protein bands corresponding to protein with MW of non-His6-tagged H4 (41.166 kDa) and H28 (48.730 kDa) were found.

The immunoblot demonstrated that His6-H4, His6-H28 as well as non-His6-tagged H28 were specifically detected, but non-His6-tagged H4 protein was not found (Figure 1B). Therefore, the *h4* gene fragment without *his6* at the 5' end was never expressed and H28 protein was produced weakly in *E. coli* host under the strong T7 promoter; however, attachment of the positively charged His6 tag at the N-terminus of vaccines H4 and H28 could successfully stimulate the protein overproduction in *E. coli* cells.

To investigate whether overproduced His6-H4 and His6-H28 were soluble, the supernatant fractions of crude cell lysate containing His6-tagged H4 or H28 were treated by 10 mM Tris buffer with or without 8 M urea and then analyzed on 10% Bis-Tris gel. The protein profile of whole cell lysate demonstrated that His6-H4 and His6-H28 protein bands were dominant (Figure 1C). However, after centrifugation, His6-H4 and His6-H28 protein bands were not found in the clear cell lysate without 8 M urea treatment. Interestingly, heavy His6-H4 and His6-H28 protein bands were observed in the supernatant fractions of crude cell lysate after 8 M urea treatment, suggesting that His6-H4 and His6-H28 recombinant antigens were insoluble.

The presence of these intracellular TB vaccine particles was observed by TEM. *His6-H4* and/or *His6-h28* recombinant genes encoded fusion proteins mediated intracellular formation of insoluble protein particles (Figure 1D). This is likely because the positively charged polyhistidine tag altered the intrinsic properties of H4 and H28, such as net charge, solubility, or the capability of folding correctly,[15] leading to overproduction of insoluble proteins and aggregation into protein particles. Nevertheless, in recombinant bacteria properly folded and biologically active proteins were found alongside misfolded proteins.[2, 7-8, 14b, 16]

As shown in Figure 1D, abundant cotton-like amorphous structures were observed and filled in the space between and inside the protein aggregate. This amorphous structure is likely a loose protein network, comprising unfolded proteins linked by hydrophobic interactions and properly folded protein precursors trapped inside the network.[17] Moreover, only one or two protein particles could be produced in *E. coli* cells. During cell division protein particles tend to remain in one daughter cell whereas protein production and particle formation will start anew in the other cell.[7]

4.3.2 Characterization of purified particulate TB vaccines

Antigen particles were released from cells using a Microfluidizer. The particles were oval in shape and their size ranged between 200 nm and 800 nm in diameter (**Figure 2A**). The shape and size of protein particles were diverse and depended on the shape of the host cells as well as the cultivation time.[7, 16] In the early stage of cultivation, protein particles tended to form spherical shapes; however, particles were not capable of retaining the spherical shape after they reached the cell wall and were usually elongated into large rod shapes after long-term cultivation.[7] The particle shape may affect the immune responses. A study showed that small spherical particles (193 nm) could generate stronger Th1 and Th2 immune responses against ovalbumin compared to the rod-shaped particles (1530 nm in length).[18] However, another study demonstrated that cellular uptake of rod-shaped particles was preferred compared to sphere- and disc-shaped particles.[19]

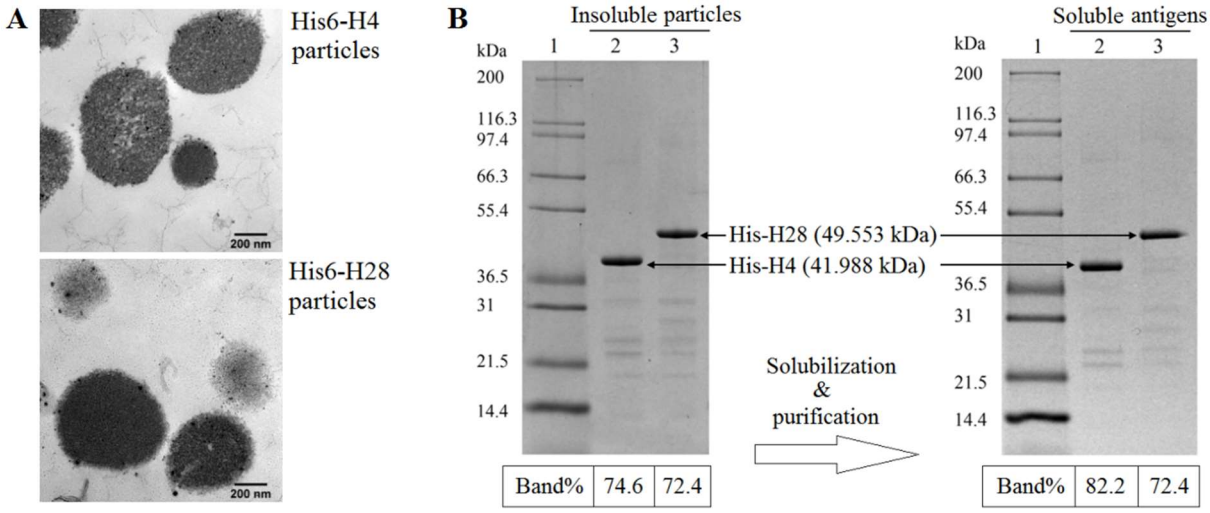

Figure 2. Analysis of purified His6-H4 and/or His6-H28 particles. A) Analysis of purified His6-H4 and/or His6-H28 particles by TEM (scale bar, 200 nm). B) Protein profile of purified TB vaccine particles and their soluble forms. The band % was analyzed by densitometry using Image Lab Software (Bio-Rad Laboratories, USA). Lane 1, molecular weight marker (GangNam-Stain prestained protein ladder; iNtRon); lane 2, purified His6-H4 particles/its soluble form (41.988 kDa); lane 3, purified His6-H28 particle/its soluble form (49.553 kDa).

The protein profile of purified particles was analyzed by Bis-Tris gel electrophoresis in Figure 2B. Densitometry analysis of the Bis-Tris gel showed that His6-H4 and His6-H28 vaccines accounted for 74.6% and 72.4% of the total protein in the His6-H4 and His6-H28 particle fractions, respectively (Figure 2B). Antigen particles were solubilized with 10 mM Tris buffer containing 8 M urea, followed by successive purification procedures. Bis-Tris gel electrophoresis was used to analyze the resulting solubilized His6-H4 and His6-H28 proteins, which accounted for 82.2% and 72.4% in their corresponding soluble protein fractions (Figure 2B). The dominant protein bands corresponding to the theoretical MW of His6-H4 and His6-H28, respectively, were excised and their protein sequences were confirmed by MALDI-TOF/MS (**Table S1**).

In order to investigate whether DDA could influence size distribution and surface charges of various vaccine particles, the size distribution (**Figure 3A** and **Table S2**) and Zeta potential (Figure 3B) of particles were analyzed before and after emulsification in DDA. Before emulsification in DDA, His6-H28 particles were monodispersed and their size was around 1 μm (Figure 3A). Large aggregates were observed in His6-H4 particles; however, the majority of these particles were distributed in a small size range,

similar to the size range of monodispersed His6-H28 particles (Figure 3A). In general, particles of a size range from 0.5 μm to 10 μm are taken up by antigen presenting cells (APCs) via phagocytosis.[3, 20] In contrast, soluble antigen or smaller particles are usually taken up by endocytosis. Uptake of particulate vaccines by phagocytosis into phagosomes has major consequences as this allows antigen cross-presentation ultimately inducing both humoral and cell-mediated immune responses.[21] When compared to protein particles, the size of DDA micelles was relatively small; indeed, 90% of the micelles were smaller than 179 nm (Table S2).

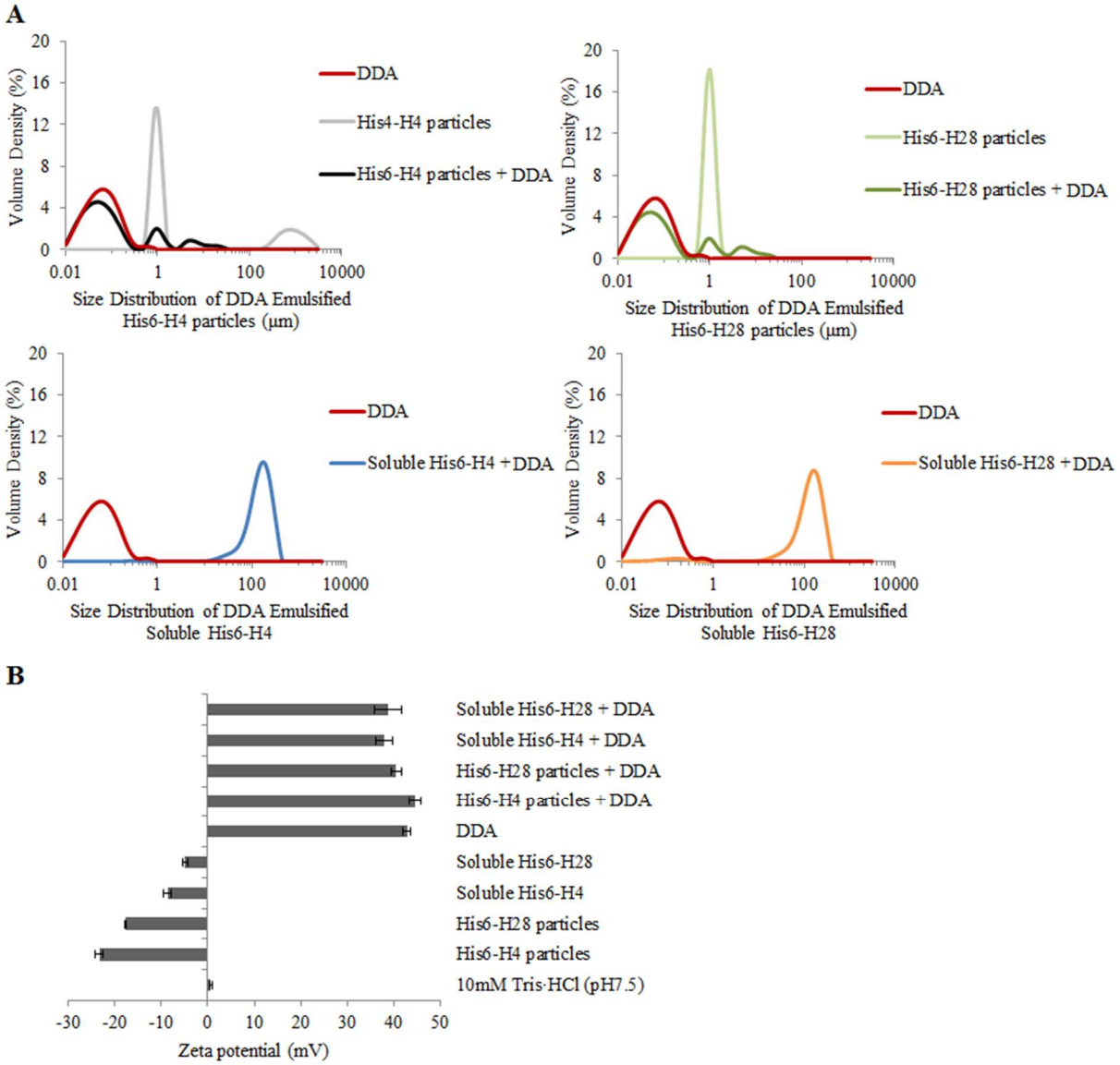

Figure 3. Particle size and Zeta potential of various formulated vaccine particle samples. A) Size distribution of vaccine samples emulsified in DDA micelles. Particle samples were solicited prior to the size distribution measurement. Size of particle samples was consecutively measured three times by Mastersizer 3000 and the standard deviation was less than 0.01. B) Zeta potential of various vaccine samples before and after emulsification in DDA. The Zeta potential of each vaccine sample was measured three times by Zetasizer Nano ZS. Each data point of measurement represents the mean ± the standard error of the mean.

After emulsification in DDA, the changes in size distribution of DDA micelles and vaccine particles were also characterized (Figure 3A). Surprisingly, the distribution pattern and size range of DDA micelles and vaccine particles (His6-H4/His6-H28 particles) were similar before and after vaccine formulation. However, some aggregates ranged between 1.5 μm and 30 μm after emulsification of vaccine particles in DDA (Figure 3A). These aggregates were probably generated due to electrostatic interactions within and between particles and presented aggregated DDA micelles, vaccine particles, or their hybrids. In addition, emulsification of soluble antigens (His6-H4/His6-H28) with DDA interrupted the size distribution of DDA micelles (Figure 3A). The re-generated size range of particles was 20 μm – 300 μm (Figure 3A and Table S1).

Interestingly, there is very limited knowledge about the physicochemical interactions between antigen and adjuvant components in vaccine formulations.[22] In this study, the amphipathic adjuvant DDA in vaccine formulations might have influenced protein conformation via electrostatic interaction as the size and charge of particles changed after emulsification in DDA. This suggested the amphipathic property of DDA might affect protein folding within protein particles.

The Zeta potential of His6-H4/His6-H28 antigen particles in vaccine formulation buffer, 10 mM Tris-HCl buffer pH7.5, was negative (Figure 3B). Interestingly, after solubilization, the negative charges of their soluble forms were significantly decreased ($p < 0.05$) when compared to the particle forms (Figure 3B). DDA was strongly positively charged (Figure 3B). All the vaccine samples possessed a strongly positively charged surface after emulsification in DDA solution (Figure 3B). However, it is unknown whether the surface charge of vaccine particles will change *in vivo* after injection into mice.

Surface charge of particles might affect cellular uptake. Uptake of particles by dendritic cells is known to increase, when the particle surface is positively charged.[23] Moreover, cell membranes are dominated by negative surface charges, which could repel negatively charged particles.[24] However, a number of studies have shown that negatively charged particles were efficiently taken up by APCs presumably due to facilitated opsonization,[3, 25] or cationic sites at the cell membrane mediating adsorption of negatively charged particles.[24, 25b]

4.3.3 Vaccine formulation and mice vaccination

To determine the protein concentration of vaccine particles and their soluble forms, different amounts of BSA, ranging between 50 ng and 500 ng, were used as standards and analyzed by densitometry using Image Lab software (**Figure 4A**). Mice were immunized subcutaneously with 5 µg of TB antigens per dose, emulsified in DDA (250 µg/dose) in a volume of 200 µl. The immunization plan is illustrated in Figure 4B.

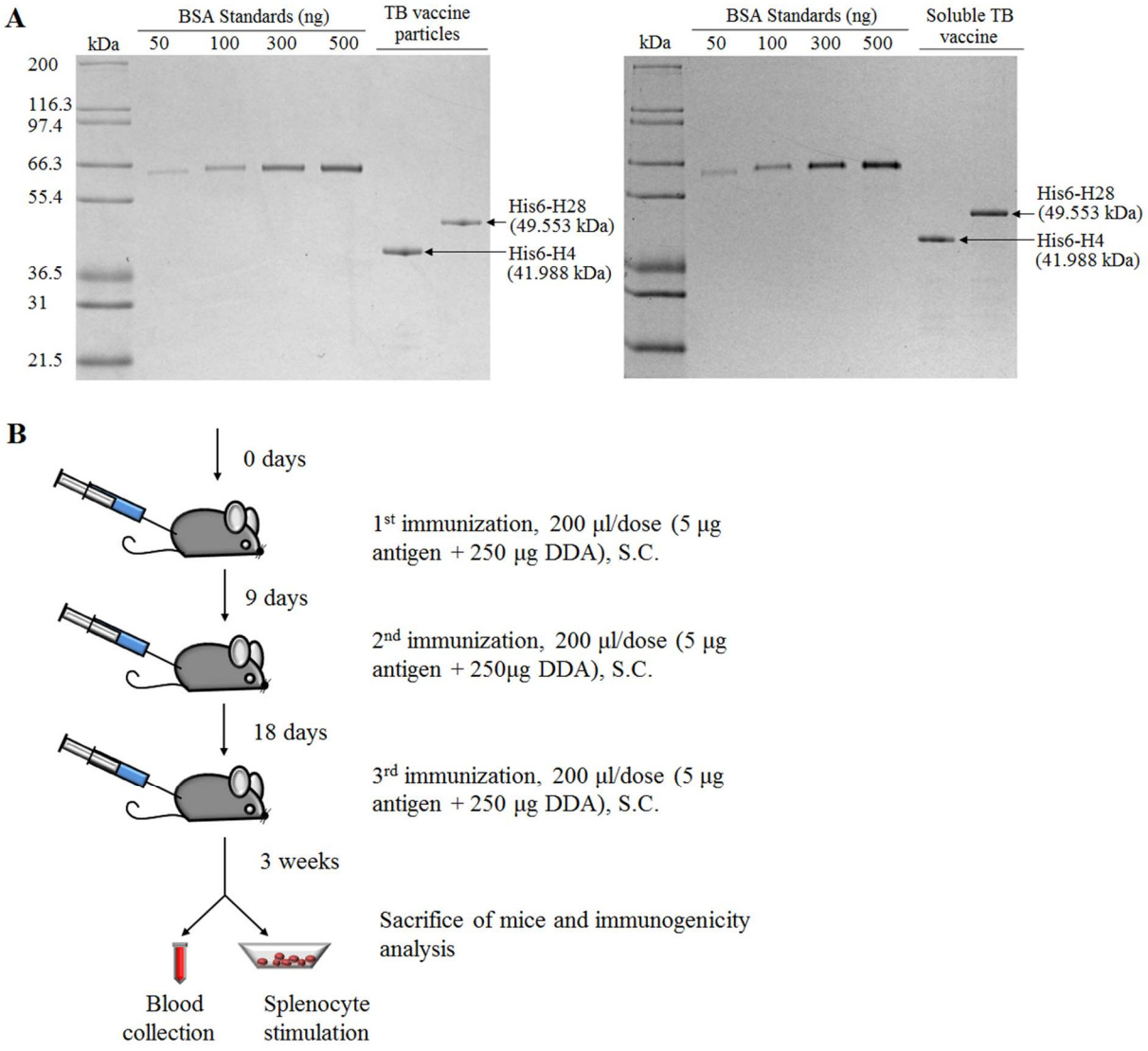

Figure 4. Vaccine formulation and mice immunization. A) Measurement of His6-tagged H4 and H28 antigen concentrations by densitometry. Different amounts of BSA standard ranging between 50 ng and 500 ng were loaded on Bis-Tris gel to generate a standard curve, used to determine the antigen concentrations. The image was taken by the gel doc (BioRad Laboraties, Hercules, CA), and analyzed with the Image Lab software (BioRad Laboratories, Hercules, CA). B) Mice immunization using formulated vaccines. There were 6 female C57BL/6 mice per group. Mice were vaccinated three times subcutaneously at 9 day intervals on the flank with experimental vaccines containing 5 µg of TB antigens/dose, emulsified in DDA (250 µg/dose) in a volume of 200 µl. Three weeks later after the third vaccination, pentobarbital sodium (150 µg/g body weight administered intraperitoneally) was used to lethally anaesthetize mice. Blood and spleen tissue were collected and processed for immunogenicity analysis.

E. coli is a common host for recombinant production of proteins for medical uses. However, the disadvantage of using *E. coli* as the production host is the presence of lipopolysaccharide (LPS) endotoxins, which could co-purify with various biological products.[26] LPS can induce a strong immune response in both human and animals,[26a, b] and can elicit a wide variety of pathophysiological effects such as systemic inflammation, fever, coagulopathy, shock and even death.[26a, b, 27] The process of removing LPS is costly and complicated.[26a, b] Hence, we chose *ClearColi* BL21 (DE3), an endotoxin free mutant derived from strain *E. coli* BL21 (DE3) that only produces a genetically modified non-toxic LPS, which is not able to trigger an endotoxic response in human cells.[28] All the vaccine particles prepared for vaccination were produced in *ClearColi* cells. Following immunization, all mice appeared healthy, gained weight and remained alive throughout the trial. No obvious abnormal behaviors and no adverse effects were observed (data not shown).

4.3.4 Vaccination of mice with His6-H4/His6-H28 particles and their soluble forms induces antigen specific immune responses

4.3.4.1 Antibody responses

Generally, both humoral and cellular immunity play a role against bacterial pathogens; however, the humoral immune response is considered less more important for control of intracellular pathogens where cellular immunity was correlated with prevention of infection.[29] In mice, Th1 immune responses predominantly stimulate an IgG2 response, whereas Th2 immunity drives an IgG1 response.[30] Since C57BL/6 mice have an IgG2c rather than an IgG2a gene,[31] in this study, IgG1 and IgG2c responses were measured by ELISA to characterize antigen associated humoral immune responses. The IgG1 response to soluble His6-H4 was significantly higher in mice immunized with soluble His6-H4 than in mice vaccinated with His6-H4 particles ($p < 0.05$) (**Figure 5A**). However, no significant difference was observed between mice immunized with soluble or insoluble His6-H28 ($p = 0.457$) (Figure 5A). The His6-H28-specific IgG1 response was significantly greater in mice vaccinated by His6-H4/His6-H28 particles than in mice immunized with soluble antigen ($p < 0.05$) (Figure 5A). Generally, the IgG1 responses to soluble His6-H4/His6-H28 were greater than the IgG2c responses (Figure 5A).

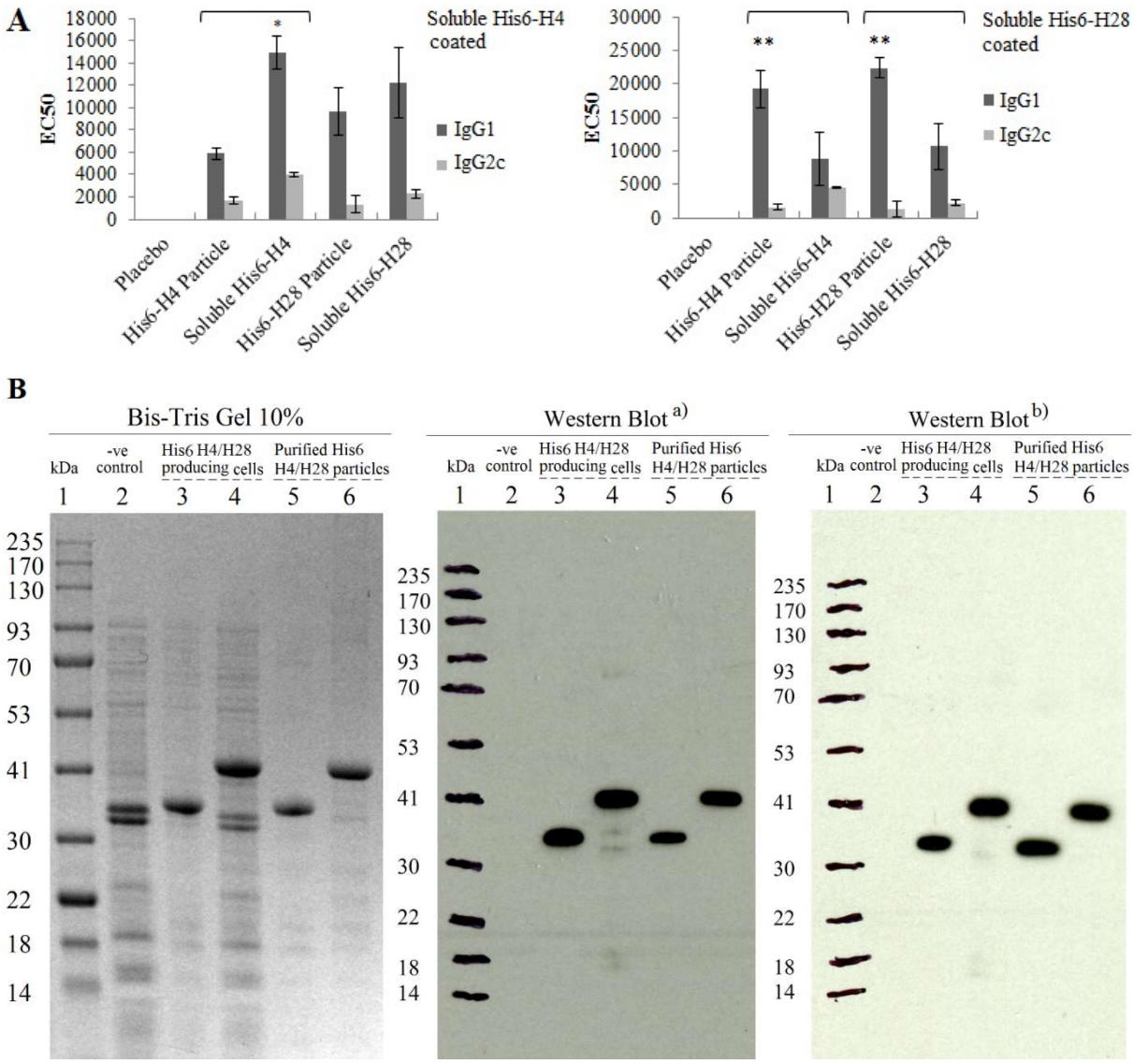

Figure 5. Antigen specific antibody response. A) Antibody responses in mice immunized with different vaccines presented as the EC50 in response to soluble His6-H4 and soluble His6-H28. Levels of specific antibodies of the IgG1 and the IgG2c isotype were measured by ELISA. Each data point represents results from six mice ± the standard error of the mean. Statistical significance is determined by one-way ANOVA (Minitab 17) and denoted when $p < 0.05$. *, significantly greater than the group vaccinated with His6-H4 particle ($p < 0.05$). **, significantly higher than the group vaccinated with the soluble vaccine versions ($p < 0.05$). B) Specific recognition of vaccine particles by pooled sera from mice immunized with soluble His6-H4 or soluble His6-H28. a) Antigen recognition by pooled anti-soluble His6-H4. b) Antigen recognition by pooled anti-soluble His6-H28. Lane 1, molecular weight marker (GangNam-Stain prestained protein ladder; iNtRon); lane 2, *E. coli*/ pET-14b; lane 3, *E. coli*/ pET-14b His6-H4, (41.988 kDa); lane 4; *E. coli*/ pET-14b His6-H28, (49.553 kDa); lane 5, insoluble His6-H4 particles (41.988 kDa); lane 6, insoluble His6-H28 particle (49.553 kDa).

The specificity of the IgG response was analyzed by immunoblot. Vaccine particles, whole cell lysate containing vaccine particles, and cell lysate alone without TB vaccines as the negative control were used to test the reactivity against pooled sera from mice immunized with soluble TB vaccines (Figure 5B). Pooled sera from mice immunized with soluble His6-H4/His6-H28 specifically recognized protein bands corresponding to the theoretical MW of His6-H4 for 41.988 kDa and His6-H28 for 49.553 kDa. Moreover, only the target protein band in whole cell lysate containing TB vaccine particles was recognized and no background proteins were detected in the negative control, the cell lysate harboring pET-14b empty vector; therefore, serum antibodies from mice immunized with soluble His6-H4/His6-H28 were very specific (Figure 5B).

4.3.4.2 Cytokine responses

Cell-mediated immune response by T cells is important for control of *M. tuberculosis* infection.[29b, 32] Ample studies have supported that polyfunctional CD4+ T cells producing various pro-inflammatory cytokines, including IFNγ,TNFα, and IL2, often correlated with protection against intracellular pathogens.[32-33] We evaluated the development of cell-mediated immunity by measuring the release of cytokines from splenocytes that were restimulated *in vitro* with soluble His6-H4 and soluble His6-H28 protein. In this study, cytokine release was measured at the early (24 h) and late (60 h) time points to allow for detection of cytokines that are released later, or are consumed, during culture.

No cytokines were detected, when splenocytes were incubated with cRPMI medium alone, suggesting there was no cytokine secretion in the absence of stimulus (data not shown). Upon the stimulation *in vitro* with soluble His6-H4 and/or His6-H28, mice vaccinated with TB vaccine particles and their soluble forms showed more cytokine release compared to mice vaccinated with the placebo (adjuvant alone) (**Figure 6** and **Figure S1**). There were no significant differences of the level of TNFα, IL10, and IL6 produced by splenocytes of mice from vaccinated groups ($p > 0.05$), although production of these cytokines was increased relative to the placebo control (Figure 6 and Figure S1).

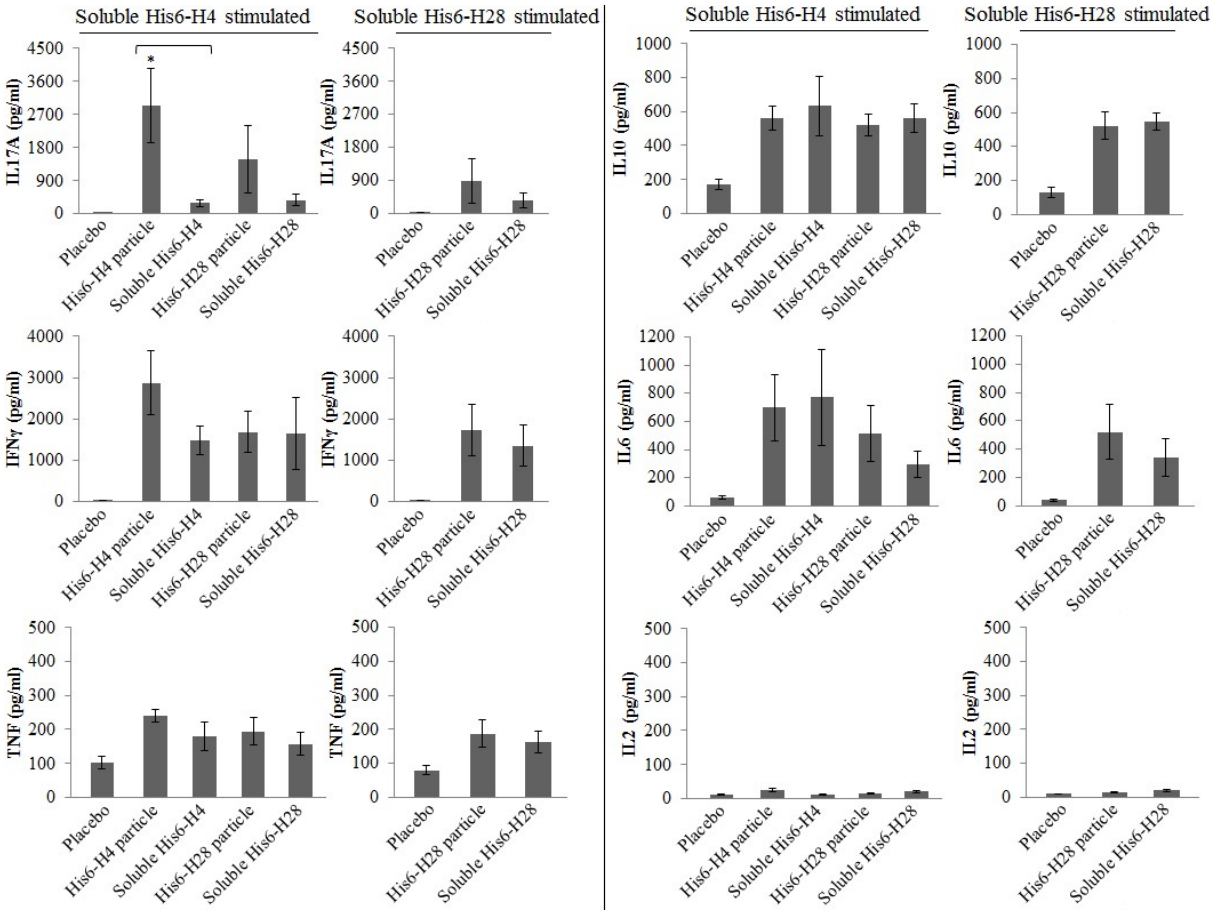

Figure 6. Cytokine release by murine splenocytes following 60 h stimulation with soluble His6-H4 and soluble His6-H28. Three weeks after final vaccinations, splenocytes were cultured for 60 h with soluble His6-H4 and soluble His6-H28. Release of cytokines was measured by cytometric bead array. Each data point represents the mean for 6 mice ± the standard error of the mean. Statistical significance is determined by one way ANOVA (Minitab 17) and denoted when $p < 0.05$. *, significantly greater than the splenocytes from mice vaccinated with soluble His6-H4 ($p < 0.05$).

IL17A and IFNγ are markers of development of cell-mediated immunity.[34] Upon stimulation with soluble His6-H4 for 60 h, IL17A production was significantly higher for the His6-H4 particle-vaccinated group than for the group immunized with the soluble protein ($p < 0.05$) (Figure 6). Although not significant ($p > 0.05$), IL17A production was also greater in the His6-H28 particle vaccinated group compared to the soluble His6-H28-immunized group in response to either soluble His6-H4 or soluble His6-H28 stimulation (Figure 6).

Following 60 h stimulation, the amount of IFNγ produced by splenocytes from mice vaccinated with His6-H4 particles was slightly increased compared to the soluble

protein-vaccinated group; however, this was not significant ($p = 0.166$) (Figure 6). At the earlier culture time point, when IFNγ levels were much lower, this did reach significance ($p < 0.05$) (Figure S1). Similarly, at the earlier culture time point IL17A production to soluble His6-H4 stimulation was significantly higher for the His6-H4 particle-immunized group than for the soluble form vaccinated group ($p < 0.05$) (Figure S1).

IL2 is a cytokine critical for T cell growth and differentiation; however, it is rapidly consumed during culture (Figure 6 and Figure S1). Therefore, IL2 levels were highest after 24 h of culture (Figure S1). Similar to IL17A and IFNγ, the levels of IL2 secreted by splenocytes at the earlier culture time point were significantly higher from mice vaccinated with His6-H4 particles compared to mice immunized with the soluble vaccine ($p <0.05$) (Figure S1). Here, immunogenicity results demonstrated that vaccine particles induced relatively strong immune responses as opposed to their soluble forms. The assembly of antigens into particles improves vaccine immunogenicity as it facilitates uptake by APCs when compared to the soluble antigen.[3, 35] The phagocytosis of antigen particles enables antigen cross-presentation towards induction of Th1- and Th2-type immune response, while an antigen depot might mediate prolonged epitope display and a stronger immune response.[3, 35-36] Moreover, IL17A, IFNγ, and IL2 are known to be important for protection against TB; however, their production post-vaccination does not always correlate tightly with enhanced protective immunity.[32b, 37] Future animal studies including challenge with the pathogen will investigate whether the induced immune responses prevent infection by *M. tuberculosis*.

4.4 Conclusion

This study demonstrated that selected antigens can be engineered to assemble into inclusion bodies inside an endotoxin-free *E. coli* strain and that these antigen particles can serve as particulate vaccine. These particles could be manufactured and formulated as novel particulate vaccine exhibiting superior immunological properties when compared to the soluble antigen. Antigen particles induced specific humoral and cell mediated immune responses. Antigen particles surfaces were negatively charged but became positively charged after emulsification in DDA. Moreover, formulation of

antigen particles with the amphipathic adjuvant DDA affected the particle size. The bioengineered antigen particle vaccines were safe and provided an efficient vaccine delivery system for immunization against TB. These antigen particles were immunogenic as they were able to generate strong and specific humoral and cellular immune responses that were significantly better than immunization with the soluble antigens.

4.5 Experimental section

Materials: All gene fragments were purchased from GenScript. Primers were synthesized and purchased from IDT. Cloning materials, including restriction enzymes, DNA polymerase, agarose, TBE buffer, SYBR safe DNA gel stain, plasmid isolation and purification kit, DNA cleaning kit etc., were purchase from Invitrogen, Roch, Merck, Bio-Lab, Zymo Research or Fisher. High-binding ELISA plate, Tween 20, and BSA from purchased from Greiner Bio-One, Invitrogen, and Gibco. Antibodies, including IgG1, IgG2c, and HRP-conjugated secondary antibodies, were purchase from Abcam. DDA was obtained from Sigma-Aldrich. CBA kit was purchased from BD Bioscience.

Plasmid Construction for Production of H4 and H28 Particulate Vaccines: Cloning techniques were performed as described elsewhere.[38] The cloning strategy for pET-14b His6-H4 and pET-14b His6-H28 was demonstrated in **Figure 7**. The gene fragments encoding the recombinant antigens H4 with amino acid sequence MFSRPGLPVEYLQVPSPSMGRDIKVQFQSGGNNSPAVYLLDGLRAQDDYNGWDINTPA FEWYYQSGLSIVMPVGGQSSFYSDWYSPACGKAGCQTYKWETFLTSELPQWLSANRA VKPTGSAAIGLSMAGSSAMILAAYHPQQFIYAGSLSALLDPSQGMGPSLIGLAMGDAG GYKAADMWGPSSDPAWERNDPTQQIPKLVANNTRLWVYCGNGTPNELGGANIPAEFL ENFVRSSNLKFQDAYNAAGGHNAVFNFPPNGTHSWEYWGAQLNAMKGDLQSSLGAG MSQIMYNYPAMLGHAGDMAGYAGTLQSLGAEIAVEQAALQSAWQGDTGITYQAWQ AQWNQAMEDLVRAYHAMSSTHEANTMAMMARDTAEAAKWGG and H28 with amino acid sequence MFSRPGLPVEYLQVPSPSMGRDIKVQFQSGGNNSPAVYLLDGLRAQDDYNGWDINTPA FEWYYQSGLSIVMPVGGQSSFYSDWYSPACGKAGCQTYKWETFLTSELPQWLSANRA VKPTGSAAIGLSMAGSSAMILAAYHPQQFIYAGSLSALLDPSQGMGPSLIGLAMGDAG

GYKAADMWGPSSDPAWERNDPTQQIPKLVANNTRLWVYCGNGTPNELGGANIPAEFL
ENFVRSSNLKFQDAYNAAGGHNAVFNFPPNGTHSWEYWGAQLNAMKGDLQSSLGAG
MSQIMYNYPAMLGHAGDMAGYAGTLQSLGAEIAVEQAALQSAWQGDTGITYQAWQ
AQWNQAMEDLVRAYHAMSSTHEANTMAMMARDTAEAAKWGGMIAGVDQALAATG
QASQRAAGASGGVTVGVGVGTEQRNLSVVAPSQFTFSSRSPDFVDETAGQSWCAILGL
NQFH were excised from pUC57 vector (GenScript, USA) by enzyme digestion with *Bam*HI (BioLabs, USA), followed by fragment separation using agarose gel electrophoresis with SYBR safe stain (Invitrogen, USA) and gel purification (Zymo Research, USA). Polymerase chain reaction (PCR) was used to introduce *his6* to the 5' end of gene fragments encoding H4 and H28. Meanwhile, the plasmid pET-14b CFP10-PhaC was digested with *Nde*I and *Bam*HI, and then the resulting linear pET-14b vector was ligated to *his6-h4* and/or *his6-h28*, generating the final plasmids, pET-14b His6-H4 and pET-14b His6-H28. These two plasmids are sequence confirmed by Massey Genome Service (Massey University, New Zealand) and then transformed into the endotoxin free production host, *ClearColi* BL21 (DE3) (Lucigen, USA).

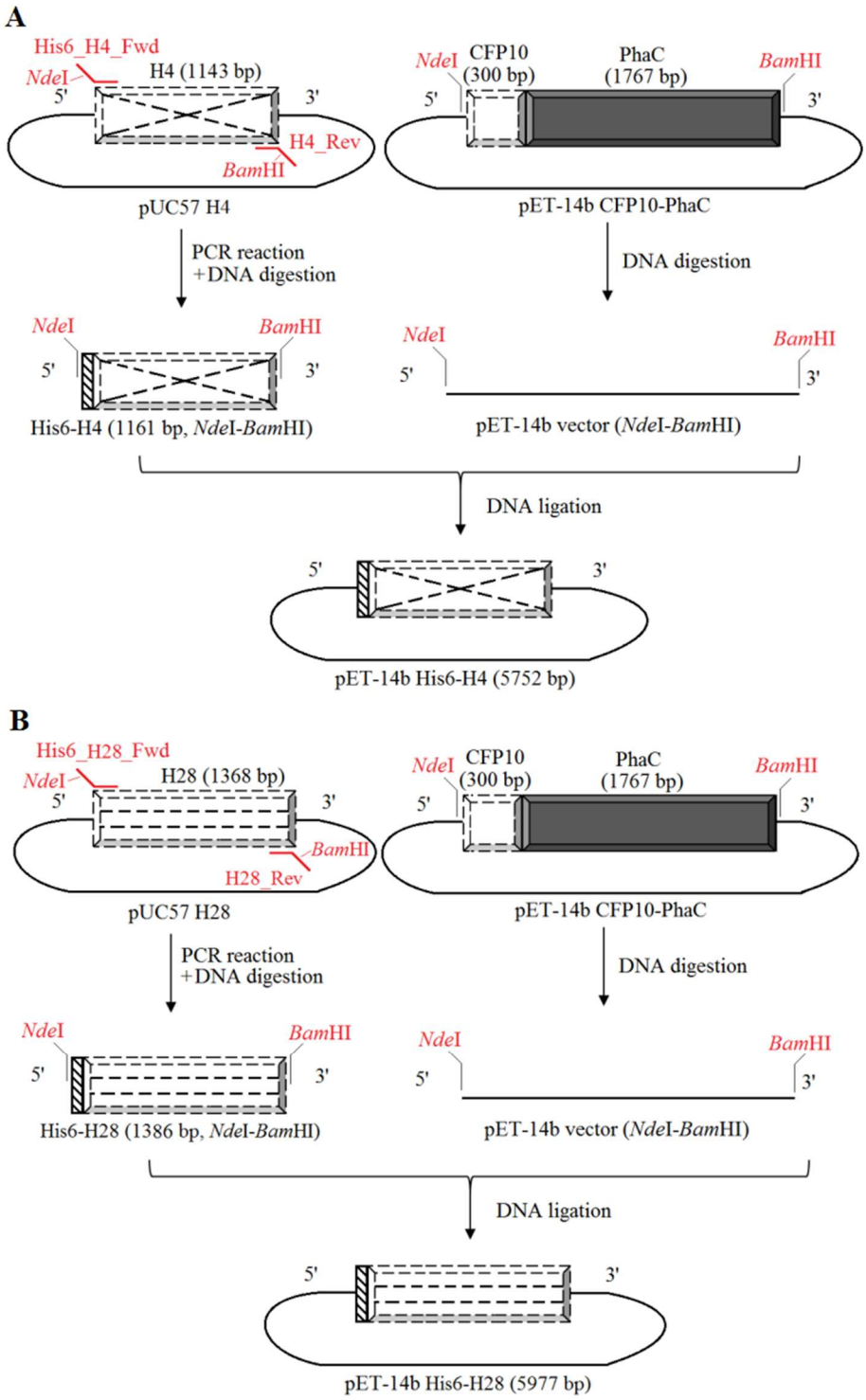

Figure 7. Construction of pET-14b His6-H4 (A) and pET-14b His6-H28 (B). The DNA fragment encoding the *h4/h28* gene was isolated from pUC57-H4/pUC57-H28 by DNA hydrolysis with *Bam*HI. The *his6* gene was introduced to the 5' end of purified *h4/h28* gene by PCR. The resulting *his6-h4/his6-h28* was ligated into the linearized vector pET-14b, generated by enzyme digestion with *Nde*I and *Bam*HI, using T4 DNA ligase to generate the final plasmid, pET-14b His6-H4/ pET-14b His6-H28.

Bacterial Strains and Growth Conditions: Bacterial strains used in this study are shown in **Table 1**. XL1-Blue, a molecular cloning host, was grown in Luria broth (LB) (Difco, Detroit, MI) containing ampicillin (100 μg/ml) at 37°C. *ClearColi* BL21 (DE3), an endotoxin free host, was used for protein production. An overnight *ClearColi* cell culture at a volume of 10 ~ 20ml was used to inoculate 1 litre of LB medium supplemented with 0.5% (wt/vol) NaCl, 1% (wt/vol) glucose, and ampicillin (100 μg/ml), and was incubated at 37°C at 200 rpm for approximately 3 h. The cell culture was induced by IPTG at a final concentration of 1 mM when the OD600 reached approximately 0.5 and the incubation was continued at 37ºC for 48 h.

Table 1. Bacterial strains, plasmids, and oligonucleotides used in this study.

Strains/plasmids/ oligonucleotides	Characteristics[a]	Sources or references
E. coli		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *pro*AB *lacI*^{q} *lacZ*Δ*M15* Tn*10* (Tet^R)]	Stratagene
ClearColi BL21 (DE3)	F^- *ompT hsdS*$_B$ (*r*$_B^-$ *m*$_B^-$) *gal dcm lon* λ(DE3 [*lacI lac*UV5-T7 gene 1 *ind1 sam7 nin5*]) *msbA148* Δ*gutQ* Δ*kdsD* Δ*lpxL* Δ*lpxM* Δ*pagP* Δ*lpxP* Δ*eptA*	Lucigen
Plasmids		
pET-14b	Ap^R; T7 promoter	Novagen
pET-14b cfp10-phaC	pET-14b derivative containing *Nde*I fragment gene *cfp10* fused to the 5' end of *phaC*	[39]
pUC57 H4	pUC57 containing *Bam*HI fragment gene *h4*	GenScript
pUC57 H28	pUC57 containing BamHI fragment gene *h28*	GenScript
pET-14b H4	pET-14b derivative containing *NdeI*/*Bam*HI fragment gene *h4*	This study
pET-14b H28	pET-14b derivative containing *NdeI*/*Bam*HI fragment gene *h28*	This study
pET-14b His6-H4	pET-14b derivative containing *NdeI*/*Bam*HI fragment gene *his6-h4*	This study
pET-14b His6-H28	pET-14b derivative containing *NdeI*/*Bam*HI fragment gene *his6-h28*	This study
Oligonucleotides		
His6-H4/28_*Nde*I	5'-CCCATATGCACCACCACCACCACCACTTCAGCCGTCCGGG TCTGCCGG-3'	This study
His-H4_*Bam*HI	5'-AGCCGGATCCTTAACCGCCCCATTTAGCTGCTTCGGCGGT GTCG-3'	This study
His-H28_*Bam*HI	5'-CCGGATCCTTAGTGGAATTGGTTCAGACCCAGAATGGCAC -3'	This study

[a] Tet^R, tetracycline resistance; Ap^R, ampicillin resistance; *h4*, *ag85b-tb10.4*; *h28, ag85b-tb10.4-rv2660c*

Protein Particles Isolation and Purification: After growth at 37°C for 48 h, cells were harvested by centrifugation at 6000 × g for 20 min. Cells were re-suspended in 0.5x lysis buffer and then mechanically disrupted using a microfluidizer M-110P (Microfluidics, USA). Cell lysate was centrifuged at 8000 × g for 20 min at 4°C to pellet protein particles, which were then sequentially washed three times by 0.5x lysis buffer (10 mM Tris, 5 mM EDTA, 0.04% w/v SDS, pH11), wash buffer (10 mM Tris, 5 mM EDTA, 2 M urea, 2% v/v Triton X-100, pH7.5), and Tris buffer (10 mM Tris.HCl, pH7.5). Purified protein particles were stored in 10 mM Tris buffer pH7.5, with 20% ethanol at 4°C for further analysis. The process for isolating and purifying protein particles from crude cell lysate is illustrated in **Figure S2**.

Soluble Recombinant Protein Preparation: Purified protein particles were solubilized by re-suspension in 10 mM Tris buffer (10 mM Tris.HCl, pH7.5) containing 8M urea, followed by sonication for 2 min at 4~12 W depending on the volume. After centrifugation at 8000 × g for 20 min at 4°C, solubilized protein solution was filtered by 0.2 μm filter and then dialyzed 3 times using 10 mM Tris buffer at 4°C. Solubilized protein was concentrated using 30,000 WMCO if the concentration was too low after ultracentrifugation at 100,000 rpm for 1 hour at 10°C. Soluble protein was stored in 10 mM Tris buffer at 4°C. The method for preparing soluble recombinant protein is shown in Figure S2.

Analysis of Protein Particles: Purified protein particles were separated on a 10% Bis-Tris gel, densitometry analysis of which was used to determine a fusion protein percentage/purity of the total protein in particle fractions using Image Lab Software (Bio-Rad Laboratories, USA). The amount of a fusion protein was calculated using different amounts of BSA as a standard curve ranging between 50 ng and 500 ng. The molecular morphology and size of protein particles were visualized by transmission electron microscopy (TEM). Aggregation of protein particles in the ultimate storage solution, 10 mM Tris buffer pH7.5 with 20% ethanol, was measured by Mastersizer 3000 (Malvern, UK). Zeta potential of protein particles and their soluble forms was also analyzed by Zetasizer Nano ZS (Malvern, UK). Target protein bands on Bis-Tris gel were excised and subjected to protein identification using matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF/MS). Protein sample preparation and identification using MALDI-TOF/MS were performed by The

Centre for Protein Research (Otago University, Dunedin, New Zealand).

Vaccine Formulation and Immunization: Formulated vaccines contained 5 μg of TB antigens/dose, emulsified in dimethyl dioctadecyl ammonium bromide (DDA; 250 μg/dose; Sigma-Aldrich, USA) in a volume of 200 μl Tris buffer (10 mM Tris.HCl, pH7.5). TB vaccines were His6-H4 particles, His6-H28 particles, and their soluble forms, produced in endotoxin free host *ClearColi* BL21 (DE3). DDA (250 μg/dose) alone was the negative control. The adjuvant, DDA, was prepared at a concentration of 10 mg/ml in sterile Tris buffer. DDA powder was added into the sterile Tris buffer and heated in an 80°C water bath with stirring until dissolved. The homogeneous white DDA solution was allowed to cool to room temperature (25°C). Vaccines were mixed with the DDA solution freshly before use.

All animal experiments were approved by Otago University Animal Ethics Committee (Dunedin, New Zealand). This animal study was performed using 6- to 8-week-old female C57BL/6 mice, originally purchased from Jackson Laboratories (Bar Harbor, Maine, USA) and bred within the Otago University animal unit. There were 6 mice per group. Formulated vaccines were injected into mice subcutaneously on the flank in a volume of 200 μl. Mice were immunized 3 times, 9 days apart.

ELISA: Serum antibody responses were analyzed by enzyme-linked immunosorbent assay (ELISA). High-binding plates (Greiner Bio-One, Germany) were coated overnight at 4°C with 100 μl of 5 μg/ml purified soluble TB vaccines, His6-H4 and/or His6-H28, diluted in phosphate-buffered saline containing 0.05% (v/v) Tween 20, pH 7.5 (PBST). As controls, plates were also coated overnight at 4°C with 100 μl of PBST. Plates were washed three times with PBST and blocked with 3% (wt/v) bovine serum albumin (BSA) for 1 hour at 25°C. Plates were washed with PBST and incubated with primary polyclonal antibodies, sera taken from individual mice diluted with PBST at concentrations ranging from 1/400 to 1/409,600, at 25°C for 1 hour. After three times wash with PBST, plates were incubated with secondary HRP-conjugated antibodies, anti-mouse IgG1- or IgG2c-HRP (Abcam, UK) diluted with PBST at a concentration of 1/20,000, for 1 hour at 25°C. After washing, o-phenylenediamine (OPD) substrate (Abbott Diagnostics, IL, USA) was added on plates and incubated for 15 min at 25°C. The reaction was stopped by adding 50 μl of 0.5 N H_2SO_4, and the results were

measured at 490 nm on an ELx808iu ultramicrotiter plate reader (Bio-Tek Instruments Inc., USA).

Immunoblot Assay: To investigate the specificity of the IgG response, pooled sera from mice immunized with soluble His6-H4 and/or soluble His6-H28 were diluted 2,000-fold and used for immunoblotting against whole cell lysate containing various vaccine particles and purified vaccine particles after they were transferred from Bis-Tris gel to nitrocellulose membranes (Life Technology, USA). An anti-mouse IgG HRP-conjugate (Abcam, United Kingdom) was diluted 20,000-fold and used for detection of bound IgG antibodies. Signal was developed by incubating the membrane with SuperSignal West Pico Stable Peroxide Solution, and SuperSignal West Pico Luminol/Enhancer Solution (Thermo Scientific, USA). Film was developed with X-ray film developer.

Sera Collection and Preparation of Single Spleen Cell Suspension: Three weeks after vaccination, mice were culled by anaesthetic overdose, with 150 μg pentobarbital sodium (National Veterinary Suppliers Ltd, Palmerston North, New Zealand) per gram of body weight, administered intraperitoneally. Blood was collected from the descending vena cava and allowed to clot at room temperature (25°C). Following centrifugation, serum supernatants were frozen at -80°C until analysis.

Single cell suspensions were prepared from spleens by teasing the tissue through a 70 μM cell strainer (Corning, USA). Cells were then washed twice with iRPMI medium (Life Technologies, USA) supplemented with penicillin (100 U/ml; Life Technologies, USA) and streptomycin (100 U/ml; Life Technologies, USA), then red blood cells were lysed using red blood cell lysis buffer (Sigma-Aldrich, USA). Cells were washed and resuspended in cRMPI (Life Technologies, USA) supplemented with penicillin (100 U/ml), streptomycin (100 U/ml), and 5% (wt/v) fetal calf serum (FCS; Life Technologies, USA). Cells were stained with Trypan blue (1:100) and counted using a haemocytometer.

Splenocyte Stimulation and Measurement of Cytokines in Supernatants: Single spleen cell suspensions were prepared in cRPMI medium at a cell concentration of 5×10^6/ml, 100 μl of which was added to U-bottomed 96-well plates (Life Technologies, USA). Cells were stimulated with 100 μl of cRPMI medium alone or 40 μg/ml of soluble

His6-H4 or soluble His6-H28 antigen. The culture was incubated at 37°C in 5% CO_2 for 24 or 60 h. Cytokine release in supernatant was measured using BD CBA Mouse Th1/Th2/Th17 cytokine kit (BD Biosciences, USA) with Falcon V bottom plates (Corning, USA) according to manufacturer's instructions. Data was acquired using a FACS Canto with BD FACSDiva software (BD Biosciences, USA).

Statistical Analysis: Analyses of the cytokine and antibody responses were performed by using the one-way ANOVA analysis. Each data point represents results from six mice ± the standard error of the mean. Statistical significance is determined when $p < 0.05$. Statistical analysis was carried out using Minitab 17.

4.6 Acknowledgements

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4.8 Supplementary material

Table S1. *MALDI-TOF/MS analysis of His6-H4 and His6-H28 proteins.*

Protein/Protein sequence	Peptide fragments assigned to the various protein regions
His6-H4 (His6-Ag85B-TB10.4) (MW: 106.698 kDa)	
MHHHHHHFSRPGLPVEYLQVPSPSMGRDIKVQFQSGGNNSPAVYLLDGLRAQDD YNGWDINTPAFEWYYQSGLSIVMPVGGQSSFYSDWYSPACGKAGCQTYKWETFL TSELPQWLSANRAVKPTGSAAIGLSMAGSSAMILAAYHPQQFIYAGSLSALLDPSQ GMGPSLIGLAMGDAGGYKAADMWGPSSDPAWERNDPTQQIPKLVANNTRLWVY CGNGTPNELGGANIPAEFLENFVRSSNLKFQDAYNAAGGHNAVFNFPPNGTHSWE YWGAQLNAMKGDLQSSLGAGMSQIMYNYPAMLGHAGDMAGYAGTLQSLGAEI AVEQAALQSAWQGDTGITYQAWQAQWNQAMEDLVRAYHAMSSTHEANTMAM MARDTAEAAKWGG	Ag85B: F8-F33, L45-Y68, S74-Y86, S91-Y102, S117-L132, A143-F150, K182-W215, K246-Y251, N262-W271 TB10.4: N299-Y313, N351-Y361, A374-G388
His6-H28 (His6-Ag85B-TB10.4-Rv2660c) (114.263 kDa)	
MHHHHHHFSRPGLPVEYLQVPSPSMGRDIKVQFQSGGNNSPAVYLLDGLRAQDD YNGWDINTPAFEWYYQSGLSIVMPVGGQSSFYSDWYSPACGKAGCQTYKWETFL TSELPQWLSANRAVKPTGSAAIGLSMAGSSAMILAAYHPQQFIYAGSLSALLDPSQ GMGPSLIGLAMGDAGGYKAADMWGPSSDPAWERNDPTQQIPKLVANNTRLWVY CGNGTPNELGGANIPAEFLENFVRSSNLKFQDAYNAAGGHNAVFNFPPNGTHSWE YWGAQLNAMKGDLQSSLGAGMSQIMYNYPAMLGHAGDMAGYAGTLQSLGAEI AVEQAALQSAWQGDTGITYQAWQAQWNQAMEDLVRAYHAMSSTHEANTMAM MARDTAEAAKWGGMIAGVDQALAATGQASQRAAGASGGVTVGVGVGTEQRNL SVVAPSQFTFSSRSPDFVDETAGQSWCAILGLNQFH	Ag85B: F8-R27, V31-R50, A183-R241 TB10.4: A360-R378 Rv2660c: M389-R440

Table S2. Summary of particle size distribution of various TB vaccine samples emulsified in DDA. Dx (10, 50, 90) represents x% of particle diameters are distributed below the value. Span is the width of the distribution. Uniformity represents the absolute deviation of the median. The specific surface area of particles (m^2/kg) is the total exposed surface area (m^2) divided by the total wet weight (kg) and is calculated by Mastersizer3000.

	Dx (10) (µm)	Dx (50) (µm)	Dx (90) (µm)	Span	Uniformity	Specific surface area (m^2/kg)
DDA	0.0215	0.0632	0.179	2.493	0.867	123100
His6-H4 particles	0.74	1.12	1090	971.36	250.575	4373
His6-H28 particles	0.73	1.03	1.46	0.707	0.217	5716
His6-H4 particles + DDA	0.0209	0.0715	1.86	25.782	15.622	112100
His6-H28 particles + DDA	0.0213	0.0752	3.97	52.584	15.561	108200
Soluble His6-H4 + DDA	67.4	164	300	1.415	0.433	120
Soluble His6-H28 + DDA	42.2	145	276	1.616	0.491	2835

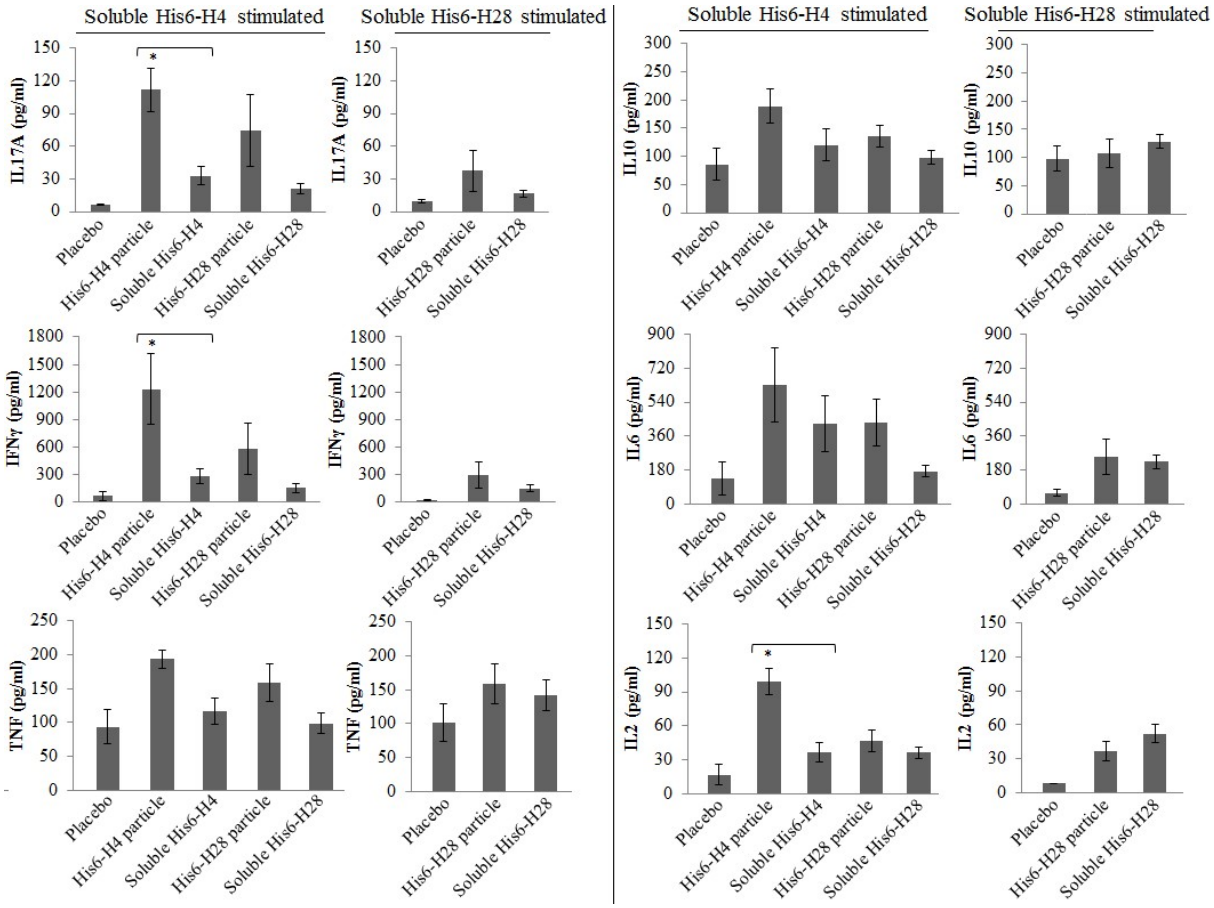

Figure S1. Cytokine release by murine splenocytes following 24 h the stimulation with soluble His6-H4 and soluble His6-H28. Three weeks after final vaccinations, splenocytes were cultured for 24 h with soluble His6-H4 and soluble His6-H28. Release of cytokines was measured by cytometric bead array. Each data point represents the mean for 6 mice ± the standard error of the mean. Statistical significance is determined by one way ANOVA (Minitab 17) and denoted when $p < 0.05$. *, significantly greater than the splenocytes from mice vaccinated with soluble His6-H4 ($p < 0.05$).

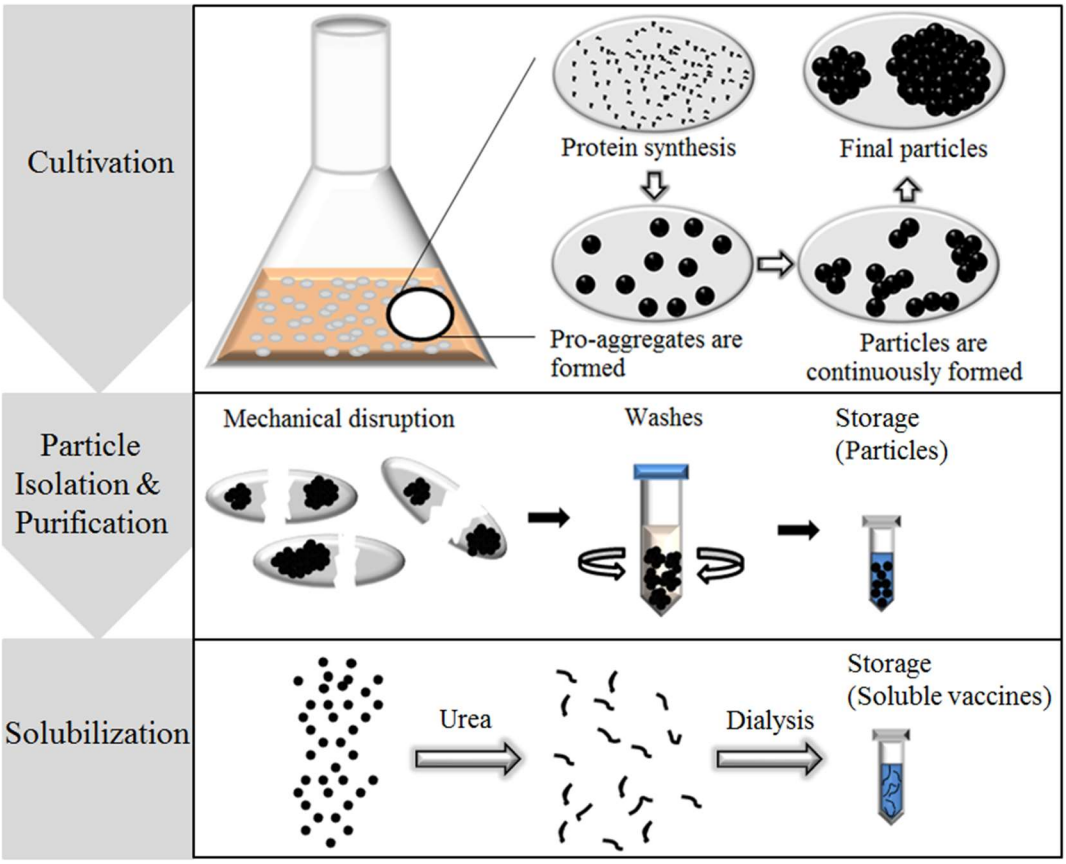

Figure S2. Schematic overview of protein particle isolation and purification and of soluble vaccine preparation.

Chapter 5. General discussion, conclusion, and future work

This study aimed to use various self-assembling particles as the antigen carrier systems to facilitate the development of a highly specific new TB skin test reagent and efficacious subunit TB vaccines. Indeed, polyester particles displaying four diagnostic TB antigens (CFP10, Rv3615c, ESAT6, and Rv3020c) were produced in *E. coli* BL21 (DE3) for the preparation of bovine TB skin test reagents (Chapter 2). Furthermore, vaccines H4 (Ag85B-TB10.4) or H28 (Ag85B-TB10.4-Rv2660c) were displayed on polyester particles and protein inclusion bodies (protein particles) to develop efficacious particulate TB vaccines. In particular, polyester particle-based TB vaccines (Chapter 3) and protein particle-associated TB vaccines (Chapter 4) were produced in a bioengineered endotoxin-free strain of *E. coli*. All the experiments conducted in this thesis resulted in three peer-reviewed publications as shown in Chapters 2, 3, and 4. Specific findings were interpreted and discussed in each chapter. In this section, a summary of general findings and future work will be discussed.

5.1 General discussion

Free soluble antigens used for the development of various vaccines and diagnostic reagents often have low immunogenicity and thus the addition of adjuvants and/or antigen carriers are needed to elicit desirable immunities (1-4). Natural and synthetic self-assembling polymers and proteins have been exploited to display and/or encapsulate antigens to perform as versatile antigen carriers capable of inducing desirable immune responses (5, 6). Although there are some antigen carrier systems available such as chitosan and liposomes, polyester particles and protein particles exhibit some advantages over the other carrier systems. For example, the formation of polyester particles and protein particles is a self-assembly process *in vivo* (2, 7). The display of immunogenic antigens on the surface of these carriers is time-saving and cost–effective as the formation of these particulates is performed in bacterial cells through a one-step production (2, 6, 8). Thus, additional *in vitro* cross-linking is not required after isolation and purification of these antigen carriers (2, 7).

Polyester particles consist of PHAs, naturally occurring biopolyester produced by various bacteria and Archaea (9, 10). A variety of desired antigens have been successfully immobilized on polyester particles by bioengineering of the particle surface protein, PhaC (6, 7, 11). Previous studies demonstrated that polyester particle technology was exploited to develop different particulate vaccines to efficaciously protect against various pathogens, such as hepatitis C virus, *M. tuberculosis*, and *N. meningitides*, in mice models with no observation of adverse effects (11-13). Recently, immunogenic antigens had been bioengineered towards the self-assembling of protein particle-based particulate vaccines, which could provide protective immunity against various diseases, such as malaria and severe acute respiratory syndrome (14-16).

Although efficacious TB vaccines are important to protect against TB, accurate and cheap diagnostic tools have also been considered as substantial factors to control TB transmission worldwide (17-20). The 2018 Global TB Report indicated that there were approximately 10 million estimated new cases in 2017, 36% of which was not reported. This omission may be mainly caused by inaccurate diagnosis (18). TB is also a global animal health issue (21), and TB–infected cattle is the potential source for human TB transmission (22, 23). Currently, the tuberculin skin test is the primary screening tool for the diagnosis of human and bovine TB. However, this test lacks specificity due to the cross-reactivity of antigens in the non-specific skin test reagent, PPDs, with the antigens in the BCG vaccine and non-tuberculous environmental mycobacteria (24-28). Thus, the development of a specific and cheap skin test reagent for accurate TB diagnosis is pivotal and was described in Chapter 2.

In order to develop a specific skin test reagent for accurate TB diagnosis, selected antigens must be absent in both BCG and the majority of environmental mycobacteria to avoid non-specific cross-reactivity (29-31). Particularly, immunogenic mycobacterial antigens, CFP10, Rv3615c, ESAT6, and Rv3020c antigens are only present in pathogenic mycobacterial strains, including *M. tuberculosis* and *M. bovis*, but are not observed in BCG and other non-tuberculous environmental strains (30-34). The skin test is the primary screening tool for TB diagnosis and hence cost-effective production of the reagent is needed. The cost of diagnostic reagent could be potentially decreased by increasing antigen immunogenicity, which in turn lowers the antigen concentrations required for skin test (11, 35, 36). This has been achieved by immobilizing

mycobacterial antigens, CFP10, Rv3615c, and ESAT6, on polyester particles (35). Furthermore, the biomanufacture of a polyester particle-based skin test reagent is cost–effective as the particle formation and antigen immobilization occurs simultaneously *in vivo* in one step and no further cross-linking between antigens and particles *in vitro* is required after particle isolation (2).

A previous study demonstrated that a peptide cocktail containing mycobacterial antigens, CFP10, Rv3615c, ESAT6, and Rv3020c, dramatically increased skin test specificity (34). To further enhance the test specificity and sensitivity as well as to reduce the cost of the skin test reagent, these four antigens (CFP10, Rv3615c, ESAT6, and Rv3020c) were covalently and densely displayed on polyester particles by translationally fusing the antigens to the particle surface protein, PhaC. This study that was described in Chapter 2 illustrated that polyester particles displaying three (CFP10, Rv3615c, and ESAT6) or four (CFP10, Rv3615c, ESAT6, and Rv3020c) TB antigens have high sensitivity and specificity for detection of TB-infected cattle. Very low concentrations of immobilized antigens (0.1 to 3 μg dose/inoculum) were required to induce skin thickness in a similar size. Furthermore, a study showed that there was a significant decrease in the size of the skin test reaction between the 10 and 1 μg dose of free recombinant antigens (4). These findings suggest that the immunogenicity of antigens was greatly enhanced after immobilization on polyester particles and lowered the antigen concentration required for the skin test. Accordingly, polyester particles displaying three and/or four TB antigens could act as specific, sensitive, and cost-effective diagnostic reagents. These two skin test reagents could also potentially be used to diagnose human TB as the ortholog antigens are present in *M. bovis* as well as in *M. tuberculosis*.

In addition to the development of skin test reagents, Chapters 3 and 4 focused on the design and preparation of efficacious TB vaccines by using polyester particles and protein particles as the antigen carriers to deliver recombinant mycobacterial fusions H4 or H28. The current licensed TB vaccine, live attenuated BCG, could not provide adequate protection against TB (37, 38). Synthetic subunit vaccines are promising alternatives to potentially prevent TB disease, but they often show low immunogenicity (1, 11, 39). In order to overcome the drawbacks of subunit vaccines, polyester particles and protein particles were used as antigen carrier systems to develop efficacious

particulate subunit TB vaccines.

Polyester particles are comprised of an amorphous hydrophobic polyester core surrounded by the synthase PhaC (7, 11). Polyester possesses various properties, including biocompatibility, which enables polyester to be tolerated by mammalian cells and is unlikely to elicit non-specific immune responses. Thus, polyester materials are not immunogenic (7, 11, 40). The humoral response results in Chapter 3 showed that antibodies induced by polyester particle-based particulate TB vaccines only specifically interact with TB antigens and not with PhaC. Therefore, the polyester particle delivery system is not immunogenic and unable to induce detectable immune responses. On the other hand, vaccines H4 or H28 were bioengineered towards the self-assembly of mycobacterial protein particles. Hence, the protein particles consist of immunogenic mycobacterial antigens, which impart the protein particles high immunogenicity.

It is generally agreed that a strong cell-mediated immune response plays a critical role in protecting against the intracellular pathogen, *M. tuberculosis* (41-43). However, humoral immunity may also play a role against this pathogen as it has a transient extracellular phase (44, 45). Thus, to develop efficient and protective vaccines against an intracellular pathogen, such as *M. tuberculosis*, it is pivotal to induce a strong cellular as well as humoral immunity. The IgG titer result showed that mice vaccinated with polyester or protein particles displaying H4/H28 elicited a high and specific titer, recognizing the H4 and H28 recombinant fusion proteins in the whole cell lysate (Chapters 3 and 4). Th1 cytokines, IL17A and INFγ, are the biomarkers for the development of cellular immunity (42, 43, 46). This thesis demonstrated particulate TB vaccines (polyester or protein particles displaying H4/H28) elicited strong production of IL17A and INFγ when compared to the soluble controls (Chapters 3 and 4). Generally, particulate vaccines showed increased immunogenicity. This may be because the display of antigens on particles facilitates the uptake by APCs (1, 2). Particles perform as an antigen depot, which might prolong epitope display and induce a stronger immune response (2, 47, 48). Nevertheless, although the Th1 cytokines IL17A and INFγ are crucial for protection against TB, they do not correlate with protective immunity (36, 42, 49, 50). A future challenge study needs to be performed to assess vaccine efficacy.

5.2 General conclusion

Overall, this thesis shows that polyester particles displaying specific TB diagnostic antigens, CFP10, Rv3615c, ESAT6, and Rv3020c, have high specificity and sensitivity in bovine TB skin test. The production and display of foreign antigens on the surface of polyester particles are simple, efficient, and cost-effective as the particle formation is implemented intracellularly in bacterial cells by one-step production and additional cross-linking *in vitro* is not needed after the particle isolation and purification. The display on polyester particles increases antigen immunogenicity, which in turn decreases the cost and antigen concentration needed for skin test. In addition, the mycobacterial vaccines (H4 and H28) displayed on the polyester particles showed enhanced immunogenicity when compared to the soluble vaccines. Our studies also demonstrated that the antigen particle is more immunogenic and could act as a better antigen delivery system when compared to its soluble form.

5.3 Future work

A number of studies indicated that polyester particles may have adjuvant properties as the immunogenicity of surface antigens was augmented when they were displayed on polyester particle, which could lead to the development of improved immunity (11-13, 51). However, the plain polyester particle itself is non-toxic, non-immunogenic, unlikely to induce immune responses, and can be well tolerated by mammalian systems due to its biocompatibility. It indicated that polyester particle may augment antigen-specific immune responses by the particulate delivery effect (49, 52, 53). Our studies also demonstrated that polyester particle-based particulate TB vaccines increased the overall immunogenicity when compared to the soluble vaccines. However, it is unknown whether the enhanced antigen-specific immune responses were elicited by polyester particle alone or both the particle and DDA. This issue needs to be addressed in the future experiment using the polyester particle-based vaccines alone without the addition of adjuvant DDA.

Both humoral and cellular immunities are important to protect against bacterial pathogens. However, the cell-mediated immune response is more important for control

of intracellular pathogens (44, 54, 55). The cytokines IL17A and IFNγ are the biomarkers for development of cell-mediated immunity and are important for the protection against mycobacterial infection (56-59). Our studies showed that particulate TB vaccines (polyester particle-TB vaccines and mycobacterial antigen particle vaccines) induced strong production of IL17A and IFNγ when compared to soluble TB vaccines. However, enhanced protective immunity is not always correlated with high IL17A and IFNγ production (36, 42, 49, 50). Therefore, future animal experiments using challenge with the pathogen need to be performed in order to investigate whether the particulate TB vaccines induced immune responses could prevent the infection by *M. tuberculosis*.

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Appendix

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Name/Title of Principal Supervisor: Prof. Bernd H. A. Rehm

Name of Published Research Output and full reference:

Natalie A. Parlane, Shuxiong Chen, Gareth J. Jones, H. Martin Vordermeier, D. Neil Wedlock, Bernd H. A. Rehm, Bryce M. Buddle. (2015). Display of antigens on polyester inclusions lowers the antigen concentration required for a bovine tuberculosis skin test. Clinical and Vaccine Immunology, 20(1).

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Shuxiong Chen, Sarah Sandford, Joanna Kirman, and Bernd H. A. Rehm. (2018). Design of bacterial inclusion bodies as antigen carrier systems. Advanced Biosystems, 2(11)

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