Development and use of polyhydroxybutyrate biopolyester as particulate vaccine beads.

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Abstract

Poly(3-hydroxybutyric acid) (PHB) is the most commonly produced polyhydroxyalkanoate formed naturally inside many genera of bacteria and archaea when nutrients are limited and a carbon-source is available in excess. These water-insoluble biopolyester spherical beads in the size range of 20-800 nm can be recombinantly produced by insertion of the required PHB biosynthesis genes into alternative bacterial hosts and then culturing the organisms under suitable conditions. A gene fusion can also be made to enable production of PHB beads which display the selected proteins abundantly at the surface of the bead.

Vaccines are needed which stimulate cell-mediated immunity and are effective at reducing intracellular infections such as tuberculosis, neosporosis and many viral infections. These diseases are responsible for a huge burden to human and animal health. Particulate vaccines target antigen presenting cells and cellular immune responses to protein antigens are enhanced when particulate vaccines are used.

This thesis describes the development of a novel vaccine delivery system in which PHB beads were engineered to display vaccine antigen on the surface of the beads.

Investigations were made into the process of vaccine bead design, production and validation to enable their use in vaccine trials. PHB synthesis genes from *Cupriavidus necator* were inserted into production strains to enable production of PHB. *Escherichia coli* was initially used as a bacterial production host and then *Lactococcus lactis* was introduced as an alternative, due to its lack of lipopolysaccharide, previous use as a production host for recombinant proteins and history of safe use for a range of human foods and products. To expand the repertoire of PHB vaccine beads, different vaccine antigens were used: hepatitis C core antigen and mycobacterial antigens (antigen-85A and 6 kDA early secretory antigenic target). Antigen specific cellular immune responses were produced in mice vaccinated with PHB vaccine beads and protection against tuberculosis was observed in mice immunized with these vaccines.

Preliminary studies into the mechanism of uptake of PHB beads by dendritic cells (DCs) showed PHB beads were taken up readily by DCs, with maturation of DCs and subsequent secretion of interleukin-12.

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Preface:

This thesis is written according to the regulations of the latest version of the Handbook for Doctoral Study, Version 7 – January 2011, published by the Doctoral Research Committee. This thesis complies with the format of a thesis based on publication as described in the handbook.

The list below presents the publication status of all chapters in this thesis. Published papers do not appear in chronological order.

Chapter 1A

Biopolyester particles: preparation and applications. I. A. Rasiah, N. Parlane, K. Grage, R. Palanisamy, A.C. Jahns, J.A. Atwood and B. H. A. Rehm.

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This book chapter review was jointly written by all authors. Natalie Parlane made particular contributions to the section describing poly lactic-co-glycolic acid.

Chapter 1B

Bacterial polyhydroxyalkanoate granules: Biogenesis, structure and potential use as micro-/ nano-beads in biomedical applications. Katrin Grage, Anika C. Jahns, Natalie Parlane, Rajasekaran Palanisamy, Indira A. Rasiah, Jane A. Atwood and Bernd H. A. Rehm.

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This review was jointly written by all authors. Natalie Parlane made a specific focus on the section describing structure of PHA granules.

Chapter 1 C

Introduction to Immunity and Vaccines.

This chapter has been written by Natalie Parlane as an introductory chapter for this thesis only and is not intended for publication

Chapter 2

Bacterial polyester inclusions engineered to display vaccine candidate antigens for use as a novel class of safe and efficient vaccine delivery agents. Natalie A. Parlane, D. Neil Wedlock, Bryce M. Buddle and Bernd H. A. Rehm.

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All experiments were carried out by Natalie Parlane except for construction of the pCWE *Spe*I-Ag85A-ESAT-6 and pHAS-Ag85A_ESAT-6 plasmid, which were done by Jessica Koach and Gina Pedersen.

Chapter 3

Vaccines displaying mycobacterial proteins on biopolyester beads stimulate cellular immunity and induce protection against tuberculosis. Natalie A. Parlane, Katrin Grage, Jun Mifune, Randall J. Basaraba, D. Neil Wedlock, Bernd H. A. Rehm, Bryce M. Buddle

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All immunological experiments were devised and carried out by Natalie Parlane. Construction of Ag85A-ESAT-6 plasmids and cloning into *L. lactis* was done by Katrin Grage and Jun Mifune. Randall Basaraba analysed all histopathology.

Chapter 4

Production of a particulate Hepatitis C vaccine candidate by engineered *Lactococcus lactis.* Natalie A. Parlane, Katrin Grage, Jason W. Lee, Bryce M. Buddle, Michel Denis and Bernd H. A. Rehm

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All immunological experiments were devised and completed by Natalie Parlane. Construction of plasmids pET-HCc-phaC and pNZ-HCcCAB and cloning was done by Katrin Grage and Jason Lee.

Chapter 5

Uptake by dendritic cells of polyhydroxybutryate vaccine beads produced in *Lactococcus lactis* and *Escherichia coli.* Natalie A. Parlane, Bryce M. Buddle, D. Neil Wedlock and Bernd H. A. Rehm

Manuscript in preparation: For submission to Immunology and Cell Biology with additional experimental results.

All experiments were devisd and carried out by Natalie Parlane.

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Abbreviations

AFB	acid-fast bacilli
Ag85A	antigen 85A
APC	antigen presenting cell
BCG	bacille Calmette-Guérin
BSA	bovine serum albumin
CBA	Cytometric bead array
CFU	colony forming units
CTL	Cytotoxic t cell
DC	dendritic cell
BMDC	bone-marrow derived dendritic cell
MoDC	monocyte derived dendritic cell
DMEM	Dulbeco's Modified Eagle media
ELISA	enzyme linked immunosorbent assay
ESAT-6	early secreted antigenic target- 6kDa
FCS	foetal calf serum
GC-MS	gas chromatography-mass spectroscopy
H&E	haematoxylin and eosin
IL	interleukin
IFN	interferon
LPS	lipopolysaccharide
LB	Luria-Bertani

MALDI-TOF MS	matrix-assisted laser desorption-ionization time-of-flight mass spectroscopy
MFI	median fluorescence intensity
MHC	major histocompatibility complex
NLR	NOD-like receptor
OD	optical density
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PHA	polyhydroxyalkanoate
PHB	polyhydroxybutyrate
PLA	Polylactic acid
PLGA	poly(lactic-co-glycolic acid)
PRR	pattern recognition receptor
s.c	subcutaneous
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SEM	standard error of the mean
TB	tuberculosis
TCR	T cell receptor
TEM	transmission electron microscopy
Th	T helper
TLR	Toll-like receptor
TNF	tumour necrosis factor

WT	wild-type
-/-	deficient or knock-out
2-ME	2 mercaptoethanol

Chapter 1: General Introduction

Microorganisms can form a number of intracellular inclusions including polyhydroxyalkanoate (PHA) polymers. Poly(3-hydroxybutyric acid) (PHB) was the first PHA discovered by Lemoigne in 1925 and this is the most commonly produced polyester in bacteria. These water-insoluble biopolyester granules are formed naturally inside many genera of bacteria and archaea when nutrients are limited and a carbon-source is available in excess. Spherical granules in the size range of 20-800 nm accumulate and may fill most of the intracellular space. PHAs can be recombinantly produced by insertion of the required PHA biosynthesis genes into alternative bacterial hosts and then culturing the organisms under suitable conditions. Key enzymes in PHA synthesis are PHA synthases, which catalyze the stereo-selective polymerization of (*R*)-3-hydroxyacyl-CoA to PHA with release of CoA. A fusion can also be made of PHA synthase genes and specific proteins to enable production of PHA granules/beads which display the selected proteins abundantly at the surface of the bead. Chapter 1A describes biopolyesters more broadly and details PHA background, preparation, *in vivo* and *in vitro* production and applications. The biogenesis, structure and uses of PHA granules are reviewed in Chapter 1B. This also describes recent applications of PHA for biotechnological and biomedical applications and introduces PHA tracking using *in vivo* mouse experiments. Chapter 1C outlines the general immune response, then vaccines and adaptive immunity followed by more detail of particulate vaccines.

Chapter 1A: Biopolyester particles: preparation and applications

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1.1 Introduction

Biopolyester particles are micro- and nanometer sized particles made from natural and renewable resources. In the past few decades, great advances have been made in the use of such particles throughout various fields of science and medicine, mainly due to their properties such as biodegradability and biocompatibility (4, 148). In particular, the area of drug delivery has been highly impacted with the range of polymeric biomaterials which have been processed into micro- and nano-spheres as well as capsules with the specific purpose of encapsulating or attaching pharmacologically active substances (120). The main goal in the design of such particles has been the controlled release of a bioactive agent to a specific site at a therapeutically optimal rate (198).

Several categories of biodegradable polymer particles have been conceived for various applications. These include (i) naturally occurring particles such as the polyhydroxyalkanoate (PHA) granules produced intracellularly by many bacteria (11, 183), (ii) particles which are chemically synthesized from extracted PHA (94), (iii) micro- and nano- particles based on polylactide (PLA) (120), (iv) co-polymers of PLA with glycolic acid, such as PLGA (151) and particles produced from polymalate (poly(β-malic acid), PMLA (173) (Figure 1). While these categories are by no means exclusive, they represent the major types of biotechnologically relevant as well as long-circulating, particulate carriers which have now taken a leading position in drug formulation and delivery.

The use of particles as carriers has several advantages over traditional oral and intravenous methods of drug administration, including high stability, ability to carry and administer several drugs at the same time at a specific site and the possibility of several routes of administration, such as oral or topical administration as well as inhalation (71). As vaccine delivery systems, the advantages include the administration of multiple antigens in a one-off immunization allowing the simultaneous uptake of various antigens by the respective immune cells, protection of the antigen *in vivo* and control over the rate of release (106, 168).

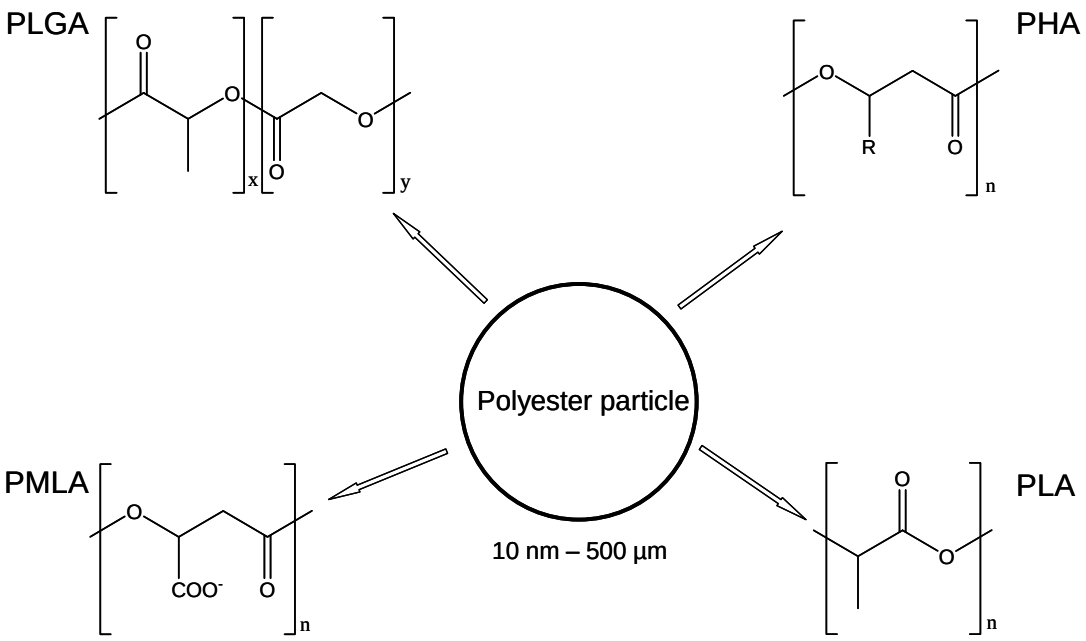

Figure 1: Overview of different biopolyester constituents

One of the fundamental requirements of particles used in therapeutic applications is biodegradation of the polymer within the human body. PLA and PLGA degrade *in vivo* by hydrolysis into lactic acid and lactic acid plus glycolic acids, respectively, which get incorporated into the cellular tricarboxylic acid cycle and hence oxidized (33). Similarly, PMLA is degradable by simple hydrolysis of the ester bond of the main chain, yielding malic acid which is in turn completely biodegraded to carbon dioxide and water (121). The degradation behaviour of these polymer materials has been the subject of much research (4, 23, 54), especially with regard to the mechanisms of degradation (121) and factors affecting the degradation processes *in vivo* (2, 192).

In addition to the growing number of medical applications, particles derived from biological polymers are well known for their use in biotechnological settings. In particular, particles made of the PHA polyhydroxybutyrate (PHB) have been widely investigated. These include (i) particles which have been synthesized from extracted PHA (14, 188) and (ii) granules which are intracellularly formed in recombinant bacteria and subsequently isolated (183). PHA particles have been successfully engineered to functionally display specific biotechnologically relevant fusion proteins on their surface such as streptavidin (167), binding peptides for inorganic materials (97) an antibody (74)

and to immobilize a thermostable α-amylase (181). These novel, functionalized beads are considered to be valuable tools for various applications in biomedicine and industry.

This review provides an overview of the main categories of biodegradable polyester particles used in biotechnological and therapeutic applications. Each type of particle, the composition of the respective biopolymer and the preparative methods for particle formation will be discussed. Finally, attention will be drawn to the wide variety of biomedical applications of each group, thus highlighting the level of progress achieved in this area in the past few decades.

1.2 Polyhydroxyalkanoates

Introduction

PHAs are bacterial storage compounds which are formed in times of imbalanced nutrient availability when a carbon source is available in excess but other nutrients have been depleted. They are deposited as water-insoluble spherical inclusions or PHA granules inside the cell and can be mobilized when conditions are suitable. PHA accumulation is quite common among both Gram-negative and Gram-positive bacteria and has even been shown to occur in some members of the *Archaea* (3, 59, 83, 89, 140, 208). Depending on the synthesizing organism (and to some extent on the substrate) PHAs are composed of monomers of different chain lengths and have been divided into short chain length (PHA_{SCL}) and medium chain length PHAs (PHA_{MCL}). PHA_{SCL} is mainly found as poly (3-hydroxybutyric acid) (PHB). While physical and thermal properties of the different PHAs vary, they are all biocompatible and biodegradable (27, 210). There is considerable interest in applications of PHA, but due to the relatively high production costs compared to oil-based plastics, PHAs are currently mainly attractive for use in the medical field, e.g. for sutures or implants like heart valves (65, 86).

PHB biosynthesis has been extensively investigated in the Gram-negative bacterium *Cupriavidus necator* (formerly *Ralstonia eutropha*) where it involves three steps starting from acetyl-coenzyme A (CoA) (164, 165, 199). Two molecules of acetyl-CoA are condensed to acetoacetyl-CoA by the enzyme β-ketothiolase (PhaA). Acetoacetyl-CoA reductase (PhaB) reduces acetoacetyl-CoA to (*R*)-3-hydroxybutyryl-CoA and these hydroxyacyl-CoA monomers are subsequently polymerized by PHB synthase (PhaC). The PHA synthase is the key enzyme of PHA biosynthesis in general; for PHAs other

than PHB different hydroxyacyl-CoA percursors derived from different metabolic pathways are used. Nascent polyester chains assemble to form the PHA granule, with the PHA synthase remaining covalently bound to the polyester molecule and thus immobilized at the granule surface (75, 134). Other proteins that associate with the granule surface in a non-covalent manner are PHA depolymerising enzymes (PhaZ in *C. necator*), regulatory proteins (PhaR in *C. necator*) and structural phasins (PhaP in *C. necator*) (67, 84, 85, 100, 141, 169, 175-177, 224, 232).

PHAs can be recombinantly produced e.g. in *Escherichia coli* by heterologously expressing the required PHB biosynthesis genes while providing appropriate cultivation conditions (125-128). PHA production in plants has also been considered (170, 201). Recently, native PHA granules and *in vitro* synthesized PHA beads have been increasingly considered for applications as functionalized micro- or nanobeads in biotechnology and biomedicine (73).

Preparation of PHA particles

PHA micro- and nanoparticles can be prepared chemically/*in vitro* from extracted PHA or be produced *in vivo* inside the (mainly bacterial) cell and subsequently isolated. In both cases a protein of interest can be immobilized at the bead surface through fusion to a granule associated protein. However, in case of *in vitro* synthesized beads proteins are also bound *in vitro* to pre-fabricated beads, whereas protein binding to *in vivo* produced beads occurs during PHA granule formation inside the cell. *In vitro* bead preparation is usually the method of choice for drug loading/encapsulation.

In vitro production

As PHA has long been considered as an alternative plastic, a range of methods have been developed for the extraction of PHA from cells, the aim being the isolation of the material and not the preservation of its spherical nature / granule shape (Lee (1996) and references therein; also reviewed in Kessler *et al*, (2001), Zinn *et al*, (2001)) (107, 124, 236). Extraction is usually preceded by biomass harvest and, depending on the method, cell disruption. A common method to isolate PHA from the biomass is solvent-based extraction. Cells (generally lyophilized) are resuspended in a solvent such as chloroform or methylene chloride in which PHA is soluble (25, 117). After all other cell debris has been removed, for example by filtration; the PHA can be precipitated with methanol or ethanol. Non-chlorinated solvents such as acetone have also been used, however, acetone only solubilises PHA_{MCL}, not PHB (229). While solvent-based

extraction yields PHA of high purity without affecting molecular weight, it is necessary to use large volumes of solvent, which is expensive and might also be undesirable for other reasons. Water-based extraction methods comprise digestion and/or solubilization of non-PHA cellular material. Hypochlorites, in general sodium hypochlorite, have been used in this method and were found to enable relatively cheap extraction of very pure PHA, but do significantly degrade PHA in the process (18, 179). The combined use of sodium hypochlorite and chloroform was reported to reduce degradation while still enabling isolation of high purity PHA (81, 82). Similarly, hypochlorite has also been used in combination with surfactants (180). Several other protocols have been developed which use different combinations of detergents and/or enzymes to solubilise cell components (31, 47, 48). Extraction of PHA with supercritical fluids, mainly supercritical carbon dioxide, has been reported to be particularly effective for removing lipid contaminants, resulting in extremely pure PHA containing only low levels of endotoxin (225).

Starting from purified PHA, micro- or nanoparticles for subsequent functionalization can be prepared by variants of the oil-in-water emulsion method (94). Lee and coworkers (123) and Wang and coworkers (221) used slightly varied versions of the oil-in-water emulsion method to generate microbeads from PHB and nanobeads from poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHx), respectively. In both cases, the polymer was dissolved in chloroform and an aqueous solution with or without surfactant added. The separate phases were emulsified with a homogenizer or by sonication, and PHB or PHBHHx beads harvested by evaporation of the chloroform and/or centrifugation, followed by repeated washing. According to Horowitz *et al,* the addition of surfactant results in the formation of beads containing amorphous PHA, which crystallizes upon removal of the surfactant, i.e. by washing with water (94). Omission of the surfactant leads to crystallization of the PHA as soon as the chloroform evaporates.

Yao and coworkers generated PHBHHx nanoparticles loaded with the lipid dye rhodamine B isothiocyanate (RBITC) by a modified oil-in-water solvent diffusion technique (230). RBITC and PHBHHx were dissolved in dichloromethane and the dichloromethane mixture slowly added to a sonicated poly(vinyl alcohol) solution. After further sonication and extended gentle stirring, the particles were collected by centrifugation and subsequently washed in water.

For drug delivery, microcapsules and microspheres of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) containing different chemicals/drugs have been prepared by slightly varied solvent emulsion/evaporation techniques, all using dichloromethane as solvent and poly(vinyl alcohol) as emulsifier (66, 108). In addition, double emulsification methods (water-in-oil-in-water and oil-in-water-in-oil) were developed (37), and different copolymer compositions and molecular weights were investigated (56, 80). The primary aim of this research was to understand how the polymer properties and the preparation technique (41) influence the physical properties of the resulting microspheres as well as drug loading and release (see below).

In vivo production

In order to exploit the advantage of having proteins naturally immobilized at the bead surface, it is possible to use *in vivo* produced PHA granules as functionalized micro-/nanobeads. In this case, isolation conditions have to be gentler to preserve granule shape/properties and protein functionality. Production generally happens in recombinant strains, not necessarily natural PHA producers, but this does not impact on the (common) isolation methods. These methods usually involve a form of density gradient centrifugation, using either a glycerol or a sucrose gradient.

In earlier publications, native PHA granules were prepared by repeated centrifugation of cell lysates layered onto glycerol, using swing-out rotors (145, 184). Much purer granule preparations were obtained when glycerol gradients, consisting of four layers with glycerol concentrations between 10.5 and 9.0 M, centrifuged at 90,000*g* were used for further purification (75). Alternatively, glycerol gradient centrifugation can be performed with only two layers of 88% and 44% (v/v) glycerol at 100,000*g* (166). Both linear and discontinuous sucrose gradients have been used for the isolation of native PHA granules (64, 113, 133). Preparation of a linear gradient can be a complex procedure (142), but it has also been generated without the need for a special apparatus by freezing a discontinuous gradient (consisting of 2 M, 1.66 M, 1.33 M and 1M sucrose layers) in liquid nitrogen and then thawing it at 4°C (133). Alternatively, PHA granules have been isolated on a discontinuous sucrose gradient consisting of two layers of 20% and 15% (w/w) sucrose (113). In both cases centrifugation was done at 110,000*g*.

For some applications of functionalized PHA beads in protein purification, no prior isolation of the beads is required (see below) (13, 15)

Applications of PHA particles

PHA beads, naturally or artificially derived, are very versatile and can be used for a broad range of applications, mostly in biotechnology and biomedicine. Over the years, different strategies of how the PHA system can be used for protein purification have been evolved. All these different approaches have focused on efficiency of purification and low costs compared to commercially available chromatography systems. In the recently published applications the granule-associated phasins were employed as the basis for the purification of the desired proteins. In a very simple approach, the N terminal granule binding domain of PhaF, a phasin from *Pseudomonas putida* GPo1 (177), was used as a bio-tag for specific binding of the protein of interest to the purification matrix, e.g. *in vivo* produced PHA granules. Mild detergent treatment enabled the elution of the tagged protein (149). Further developed methods have used inteins for facilitated release of the target protein (14). Proteins of interest are fused via an intein linker to the phasin protein PhaP and therefore attached to the natively formed granules inside the cell. For better attachment, multiple copies of PhaP were employed. The protein of interest is released using the intein cleaving site. This system was successfully established in the natural PHA producer *C. necator* as well as in engineered *E. coli.* This does not necessarily require isolation of the engineered particles (13, 15), because the crude cell extracts containing the particles can be directly used for protein purification. Recently, a slightly modified purification method was published. It is still based on the PhaP-intein-X fusion, with X being the protein of interest, but it separates the protein production from the actual purification process, resembling common chromatography techniques. The PHA particles which serve as purification matrix are artificially (*in vitro*) made out of chemically purified PHA. The crude extract of the recombinantly produced PhaP-tagged protein is then incubated with the artificially prepared matrix. PhaP mediated binding and subsequent intein cleavage leads to purification of the protein (222). A completely different approach was employed by Brockelbank *et al* (2006), in which the ZZ domain of protein A from *Staphylococcus aureus* was fused to the PHA synthase PhaC, enabling affinity purification of antibodies of the IgG class (28). These engineered beads performed equally when compared to commercially available protein A beads. In this last example the protein to be purified did not need a special purification tag, whereas in all other methods the protein of interest was tagged with a granule-binding protein to enable attachment to the purification matrix.

Micro- and nano-PHA beads displaying certain functionalities are very valuable and beneficial, especially for biotechnological applications. In most cases these beads are of non-biological origin and the production involves chemical cross-linking (188). Several studies have described the one-step production of functionalized PHA beads in engineered *E. coli*. In all examples the N terminus of the PHA synthase was fused to the respective protein that should be displayed at the bead surface, for example streptavidin (167) or *β*-galactosidase (166, 167) and *α*-amylase (181) as examples of immobilized enzymes. These beads are functional tools for various applications, ranging from bio-labeling using the strong binding between streptavidin and biotin to supplementing washing detergents with a starch hydrolyzing enzyme that shows high temperature stability.

Because of its biodegradable and biocompatible properties, PHA is being investigated for its applicability in biomedicine. Immunoassays are an especially widely used tool. For these assays, the proteins must be in a functional state, but immobilized, all criteria which can be fulfilled using PHA beads. *E. coli* has been engineered to display the PhaP-bound mouse interleukin-2 (IL2) or the myelin oligodendrocyte glycoprotein (MOG) on PHA granules (11). These eukaryotic proteins were functional albeit their production in a prokaryotic host and performed successfully in fluorescence activated cell sorting (FACS). The PHA beads were produced in one step. An enterokinase site between the PhaP protein and the respective fusion partner allowed cleavage of the fusion partner and could hence be used for protein purification. In a related study it was shown that two independent functionalities can be displayed at the PHA bead surface either in the form of two functionalized proteins or one bifunctional protein (9). Beads displaying the separate GFP-PhaC and PhaP-MOG fusion proteins, as well as the GFP-PhaP-MOG bifunctional fusion protein showed discrete signals in FACS analysis and no overlapping or interference could be observed. Lee *et al* (2005) immobilized proteins on artificially derived PHA particles, using the substrate binding domain (SBD) of the PHA depolymerase (123). The SBD tagged proteins were produced in *E. coli* and then bound to the PHA particles in a way described previously for some of the protein purification methods. This method also resulted in functionally displayed proteins and FACS analysis could be successfully performed.

Biopolyester particles are already being used for applications in the delivery of drugs or similar compounds. PHA particles are just emerging in this area. PHA

copolymers, especially poly(hydroxybutyrate-hydroxyvalerate) (PHBV), have been investigated with regard to encapsulation and release of different types of drugs (8, 41, 55, 57, 66, 80, 108). These studies have mainly focused on the formation of drug loaded particles, distinguishing between monolithic microspheres and reservoir-type microcapsules. Interestingly, stabilizers, e.g. gelatine, had a big influence on the drug release properties of the particles. The dispersion of the drug in gelatine, followed by the encapsulation of the drug-gelatine mixture in PHBV proved to be disadvantageous for medical applications. These particles showed a triphasic release pattern, typically for microspheres, with alternating periods of slow and more rapid drug release (37, 57). The release properties could be changed to a zero order kinetics profile, when the drug was encapsulated in PHBV and the whole particle coated with gelatine. These particles showed a linear release profile over a period of 30 days, which would be more favourable for biomedical applications (37). Recently, Yao *et al* (2008) (230) described the use of prefabricated PHA beads for targeted drug delivery to macrophages. The mannosylated human α1-acid glycoprotein and the human epidermal growth factor were attached to PHA particles by using PhaP as binding domain as described above. To mimic the drug load the PHA particles were stained with a lipid soluble dye. Macrophages were reported to recognize the attached proteins with their specific receptors and the particles were ingested by endocytosis suggesting potential applications in targeted drug delivery.

Another biomedical application of PHA particles in the diagnostic area was recently conceived. Multifunctional PHA beads, displaying an IgG binding domain and a special peptide sequence for binding inorganics (gold or silica) were proposed as useful tools for bioimaging and diagnostics. It could be shown that both functionalities reacted independently from each other and no inhibition or interference could be detected. These beads allow the antibody based targeted delivery of a contrast agent to selected tissues (97). Furthermore, beads displaying the single-chain variable fragment (scFv) of an anti-*β*-galactosidase antibody were engineered, using the PHA synthase as anchoring motif (74). These functionalized beads were successfully used for the purification of *β*-galactosidase as example for a custom-made high affinity purification matrix. Once again, formation of these beads resulted cost-effectively from a microbial one-step production process. The displayed antibody fragment was correctly folded and instantly active and did not require any additional cross-linking. These examples demonstrate the

versatility of functionalized PHA beads either derived from extracted PHA or directly formed inside engineered microbial cells for applications in biotechnology and medicine.

1.3 Polylactides

Background

PLA, a biopolymer derived from lactic acid (2-hydroxypropionic acid), has been widely studied for its applications in the medical field and also for use in the food packaging industry. PLA is biodegradable (4), bioresorbable (120) and exhibits properties such as low immunogenicity and toxicity, which have led to FDA approval for its use in human injections (131). In addition, lactic acid based polymers have tensile strength and rheological properties suitable for applications as packaging materials and food containers (10) as well as various films, fibres and homeware (186).

Lactic acid, the basic unit of PLA, is produced by microorganisms, animals and plants in nature (197). Lactic acid exists as two optically active forms, namely the L(+) and D(-) isomers. While both forms are produced by fermentation in bacteria, only the L(+) isomer is found in mammalian systems. Since lactic acid is present in the biochemical pathways of most living organisms, polymers based on this organic material will degrade to natural metabolic compounds (205, 215).

PLA is usually produced as a mixture between the isomeric forms and therefore the nature of the polymeric material formed varies depending on the ratio between these. The process of PLA formation is a stereocomplex crystallization, or racemic crystallization and has been described by Tsuji (207). PLA made purely from L(+) lactide is called poly (L-lactide), or PLLA, while PDLA is the corresponding lactide made from D(-) lactide. PLLA is a semicrystalline powder with a melting temperature of about 180°C, whereas introduction of the D(-) isomer reduces the melting temperature and the degree and rate of crystallization (186). Thus, the degree of crystallinity determines the physical and mechanical properties of the polymer (19) as well as its degradation rate (21).

High molecular weight PLA (about 100,000 Daltons) can be produced by different routes (for schemes see Sodergard and Bhardwaj as well as Mohanty (19, 197)), namely (a) direct condensation polymerization which initially produces a low molecular

weight pre-polymer that is then polymerized by chain coupling agents (19, 95, 235), (b) azeotrophic dehydrative condensation (10, 19) and (c) ring opening polymerization (ROP). The ROP route involves generation of the cyclic dimer lactide (3,6-dimethyl-1,4-dioxane-2,5-dione) which is ring-opening polymerized to high molar mass polymers (197). ROP is the most commonly used method for PLA production, as this chemical process can be controlled and the properties of the polymers varied for different applications (87, 207). ROP is also used to incorporate other ring formed monomers such as glycolide into the lactide polymer. These copolyesters are described later in this review.

Due to the diverse properties of PLA polymers caused by its constituent lactide and the various polymerization methods, PLA has been processed for a variety of different applications in the biomedical field (190). These include suture lines (137), orthopedic implants (130) and microspheres (120, 202). Among these applications, the microspheres have been reported as being of most interest, because of their unique structure and tailorable properties (93). The remainder of this section will therefore focus on particles made of PLA, their preparation and applications.

PLA particles

PLA based particles are now widely used in medicine as long-circulating carriers for the controlled release of pharmacological substances (152) such as drugs (44, 159), hormones (45) and for the delivery of proteins and antigens (106, 217, 219). One of the most important features of particles used for this purpose is the amount of control over the release rate of the desired substance in the recipient tissues. To this end, structural properties of the particles are often tailored and much research has been conducted in this area (44, 131, 150). PLA can be formulated into particles of a wide size range, from nanoparticles and nanocapsules to microspheres and microcapsules. The term microsphere is generally used to describe small, porous spheres from 1-500 μm in diameter, whereas nanoparticles are generally 10-1000 nm in size (148). In drug delivery, particle size is a fundamental parameter which determines the route of administration of the drug (120). Microspheres can be administered intramuscularly or subcutaneously (101) but for intravenous administration and transport via blood circulation or across the mucosal membrane, and in the case of oral administration, particles in the nm range (and particularly less than 500 nm) are required (101, 120, 148). The significance of particle size is basically related to the rate and extent to which the particles are removed

from the circulation and their distribution in tissues (129). The size of the particles also affects loading of the drug and its release from the particles into the tissues. Katare *et al* (106) investigated the size of PLA microparticles in a study of the delivery of the tetanus toxoid (TT) by a single point intramuscular injection in rats. Parameters such as antigen load and the use of extra adjuvant were also considered for eliciting the optimal antibody response. Size ranges for the PLA particles with entrapped antigen 50-150 μm, 10-70 μm, 2-8 μm and less than 2 μm. They reported that the greatest encapsulation efficiency (60.4%) and TT load (53.2 μg /mg) were obtained with the particle size range of 2-8 μm. Serum antibody titres varied extensively depending on the size of the particles. Once again, particles of 2-8 μm elicited the highest titres whereas in the largest size particle range the titre was considerably lower. Interestingly, particles of less than 2 μm exhibited a low titre. The authors suggested that the differences were related to the uptake of the particles by macrophages, and that since the upper limit for this was considered as 5 μm, the 2-8 μm range was optimal. The lower response of the smallest group of less than 2 μm was thought to be due to less efficient antigen processing.

Apart from size, factors which influence the performance of microparticles include porosity (and the related % solid content) (44, 93), surface charge (6) and hydrophobicity (20). As with size, the optimum degree for porosity will depend on the situation for which they are prepared. For example, microspheres with a highly porous interior structure are considered as more advantageous than non-porous particles for the administration of inhaled drugs. The large size to density ratio of porous microspheres has been reported to generate a more easily aerosolized particle, resulting in a higher uptake of the inhaled drug (93). On the other hand, for the treatment of arthritic conditions, a less porous particle type has been reported as more suitable by Cui *et al* (44). This study compared the entrapment and release of the drug melittin, by particles of PLA and the polylactide-glycolide copolymer PLGA. The level of porosity of the two types of polymer particles was different, with the pure PLA particles being more porous and sponge-like, whereas increasing the molar fraction of the glycolide in the PLGA particles resulted in a harder, smoother surface. Although the results showed a significant "initial burst" release in both particle types, it was particularly high for the pure PLA, suggesting a diffusion of the substance through the pores and indicating the lesser efficiency of pure PLA particles in this instance.

The ability to control the rate and extent of the release of the substance is of paramount importance for PLA and other particles used in drug/antigen delivery systems. For this reason, PLA particles are frequently coated with carbohydrates (139, 158), electrolytes (90), polyethylene glycol (68) and other substances to increase stability (231), period of release (90) and blood-circulation time, and to reduce initial burst (156).

Preparation of PLA particles

Several methods are available for the preparation of PLA micro- and nano-particles. It is important to note that the method of preparation and the experimental parameters involved will directly influence the particles produced and their ability to interact with the drugs/antigens to be delivered (39, 93, 120). The three main preparation methods are the (a) emulsion based solvent evaporation methods, (b) the spray-drying method and (c) nanoprecipitation. Usually, adaptations of the first two methods are used for the microencapsulation of proteins and drugs in PLA particles. Emulsion based methods consist of four steps: (i) dissolution of the bioactive compound in an organic solvent (ii) formation of an emulsion of the organic solvent phase with an aqueous phase (iii) extraction of the solvent by evaporation which hardens the droplets into solid microparticles and (iv) drying of the microsphere particles (63). The solvent extraction/evaporation method has been used extensively, for example to prepare microspheres coated with collagen (93), enclose pesticides (202), adsorb vaccine antigens (214), encapsulate drugs (50, 143, 233) among other medical applications of PLA particles. In the study of Takei *et al* (202), this method was used to encapsulate a pesticide acetamiprid into PLA microspheres. The entrapment efficiency of the pesticide decreased with increasing concentration of this chemical in the oil phase. An important part of the process in the solvent emulsion method is the addition of stabilizing agents to the initial droplets and to the final particles. Examples of such surfactants are sodium dodecyl sulfate and cetyltrimethyl ammonium chloride (152), alginate, polyvinylpyrrolidone and gelatin (39, 101). In terms of particle size, PLA nanoparticles and nanospheres are produced by the same methods as microparticles and microspheres, with the manufacturing parameters adjusted to generate nanometer sized droplets, essentially by using a smaller ratio of the droplet phase to the suspension and a much higher stirring rate (7).

The spray-drying method for preparation of PLA particles involves dispersion of the drug in an organic solution of the PLA polymer, followed by immediate

nebulization in a hot air flow (120). This method was used by Blanco *et al* (2006) (23) to prepare microspheres of PLA and other co-polymeric (various ratios of PLA:PLGA) particles. The PLA was dissolved in 2% dichloromethane with constant stirring, and then sprayed through the nozzle of a spray drying device. The microspheres were collected in the separator of the spray-dryer and dried in a vacuum oven. PLA particles produced by this method were small (1.3 μm), slightly porous and with a smooth surface. The particles were analyzed for their degradation rates, and it was apparent that the pure PLA particles showed the slowest rate of degradation. Bishara *et al* (22) used a similar method from D,L-PLA, producing PLA particles of 2-4 μm. It has been suggested that while this method may be suitable for industrial-scale production of microspheres, the non-uniform particle sizes would be a likely limitation (120).

The nanoprecipitation method for the preparation of PLA particles is a relatively easy method, in which an organic solution of the polymer is simply added to water. PLA particles will immediately form by precipitation, following which the organic solvent can be evaporated off (21, 129). Mainly hydrophobic compounds are entrapped by this method (120). Peltonen *et al* (163) used nanoprecipitation to investigate improved entrapment efficiency of the bronchoconstriction-reducing drug sodium chromoglycate, within PLA nanoparticles. They reported that the loading of the hydrophilic drug into the hydrophobic nanoparticles was difficult but that it was improved by lowering of the pH of the outer medium. Hyvonen *et al.* in their nanoprecipitation study of PLA nanoparticles reported that the crystallinity of the particles decreased upon nanoprecipitation (96).

Applications of PLA particles

While the physicochemical, chemical and mechanical properties of PLA polymers make them more suitable than other non renewable source based polymers for manufacturing products for industrial packaging, their use in the medical device market as drug delivery and therapeutic devices has greatly appreciated over the past few decades (24, 46, 69, 209). In particular, micro and nanospheres, particles and capsules made of PLA on its own or combined with other polymers have received tremendous attention as a means of delivering drugs (4, 35, 198). The advantages of using biodegradable polymers lie in controlling the release of drugs or proteins and their stability. Moreover, their degradation by simple hydrolysis of the ester bond and does not require the presence of enzymes to catalyze this hydrolysis.

Previously, micellar oil-dispersions, liposomes and hydrophilic polymer-photosensitizer conjugates have been used as potential drug carriers with varying degrees of success. PLA and their copolymers have now become the most important polymers for drug delivery systems and were approved by the Food and Drug Administration (42). This is because their biodegradation leads to pharmacologically inactive substances, which are absorbed by the body or removed by the metabolism (43).

Biodegradable PLA makes it an appropriate carrier to deliver drugs which are lipophilic in nature. In ovarian cancer diagnosis, the photodetection (PD) method using photosensitizers (PS) is used to accurately pinpoint the location of malignant tissue and scattered micrometastases in advanced cancer stages (200). Due to the lipophilic nature of the exogenous PS such as Hypercin (Hy) systemic administration is problematic. This can be mitigated by entrapping PS in biodegradable polymers as carrier materials. Zeisser-Labouebe (234) used PLA nanoparticles loaded with Hy to study the fluorescence PD of ovarian metastases in Fischer 322 rats bearing ovarian tumours. They found that using PLA nanoparticles resulted in higher accumulation and bioavailability of the drug within tumour tissues, thereby improving PS availability within tumours. In addition, lower concentrations of Hy were found in healthy surrounding tissues.

The selectivity of PS loaded PLA nanoparticles is also favourable for Photo Dynamic Therapy (PDT). PDT is a method in cancer treatment in which the cancer cells and tissues are destroyed by the uptake of PS, followed by photoirradiation (35). PLA nanoparticles would allow targeted delivery because of the possibility to chemically cross-link specific proteins to their surface (157, 159).

In parenteral administration, the PLA nanoparticles are opsonised by mononuclear phagocytes, removed from the blood stream and sequestered in organs such as liver and spleen, avoiding the effective delivery of the nanoparticles to organs other than those of the reticuloendothelial system (116). In a study by Bazile (16) a radiolabelled [^{14}C]-PLA_{50} (PLA coated with human serum albumin) was synthesized to follow the fate of this new drug carrier after intravenous administration to rats. From whole-body autoradiography and quantitative distribution experiments they observed that the ^{14}C-labelled polymer is rapidly accumulated in liver, bone marrow, lymph nodes, spleen and peritoneal macrophages. To control the opsonization process and to

improve the surface properties of the system, the surface of the nanoparticles was modified by either attaching or coating with a hydrophilic polymer such as poly ethylene glycol (PEG) (162, 198).

Despite its advantages, the hydrophobic nature of PLA tends to result in adsorption of non specific proteins, which reduces the effectiveness of the drug delivery. This uncontrolled protein adsorption to the lipophilic PLA was combated by synthesizing a diblock copolymer with hydrophilic PEG (204). Also, in oral administration of drugs, there is evidence that PLA nanoparticles are degraded by enzymes in the body fluids such as intestinal fluids and that this degradation is affected by the surface composition of the particles (16). In a study by Landry *et al* (119), degradation of albumin coated PLA_{50} was performed in simulated gastric and intestinal fluids (USP XXII) in order to model the degradation process. These authors observed that once the readily digestible albumin coat was digested by gastric and intestinal fluids, the exposed PLA_{50} was degraded, mainly due to an enzymatic cleavage process. They concluded that coating PLA with slowly digestible or non-digestible agents would be beneficial in a drug delivery system.

Quantum dots (QD) or semiconductor nanocrystals are nanoparticles with a typical diameter of 2-8 nm that possess unique luminescent properties (34, 146). QDs have been used as novel fluorescent markers in biological labeling and diagnostics (99, 160). Since QD molecules are neither water soluble, biocompatible or have functional groups linked with biomolecules, many of these applications require a combination of the QDs with polymers (34, 206). The polymers are versatile surface modifiers because of their processability and multiple chemical functionalities (220). In a study by Guo *et al* (78), CdSe (Cadmium and Selenium) QDs were modified to make them water soluble and emit strong fluorescence, by incorporating the CdSe QDs with PLA. The fluorescence of the modified CdSe QDs was stable for more than 30 days *in vitro*. They suggested that the use of PLA could reduce the possibility of QDs loss from the particles. The results demonstrated that PLA particles encapsulated with CdSe QDs had high potential for biological labeling especially in aqueous biological conditions. QDs are known for their cytotoxicity, due to desorption of free Cd (QD core degradation), free radical formation, and interaction of QDs with intracellular components (99). Polyethylene glycosylated (PEG) QDs have been shown to have reduced cytotoxicity, but modification of these to produce PEG-amine for biological activity renders them

cytotoxic once again (187). To overcome the problem of cytotoxicity, Guo *et al* (77) modified CdSe QDs by incorporating these into poly(D, L)-lactide (PDLA) nanoparticles. The resulting luminescent nanoparticles were coated with a layer of different molecules including F-68, cetyl trimethyl ammonium bromide (CTAB) and sodium dodecyl sulphate (SDS). The results suggested that CdSe QDs surface modified with F-68 have low cytotoxicity.

1.4 Poly(lactic-co-glycolic acid)

Background

PLGA is a co-polymer which is used in a host of Food and Drug Administration (FDA) approved therapeutic devices, owing to its biodegradability and biocompatibility. Unlike the homopolymers of lactic acid (polylactide) and glycolic acid (polyglycolide) which display poor solubilities, PLGA can be dissolved by a wide range of common solvents, including chlorinated solvents, tetrahydrofuran, acetone or ethyl acetate.

PLGA nanoparticles are biodegradable, biocompatible and approved for human use by the FDA. PLGA is hydrolysed to lactic and glycolic acids, both of which are by-products of various metabolic pathways in the body. By altering the ratio of the monomers used during synthesis, the degradation rate can be controlled (4), making PLGA a common choice in the production of a variety of biomedical devices such as: grafts, sutures, implants, prosthetic devices, micro and nanoparticles.

Preparation of PLGA nanoparticles

While there have been differing techniques employed to produce nanoparticles, the emulsification-diffusion technique was developed in 1996 to overcome toxicity issues with solvents and low yields obtained with gelatine stabilizers. There are single (oil-in-water) and double (water-in-oil-in-water) emulsification techniques (224). Each methodology results in a different release profile of the drug. Propylene carbonate (PC) was added as an alternative low toxicity stable solvent for the polymers (178). In this method, the polymer is dissolved in PC and then emulsified via homogenization with an aqueous surfactant added. The addition of water while stirring allows the PC to diffuse out of the polymer emulsion into the water causing precipitation of the polymer into nanoparticles. The size of the nanoparticles reportedly decreased as the stabilizer concentration increased up to 5% w/v but ultimately the size was determined by the

globule size throughout the process of emulsification. The nanoparticle size is therefore influenced by the concentration of the polymer, the rate the emulsification solution is stirred and the concentration of the stabilizer (178).

Various techniques are used to form drug-polymer micro- or nano-particles. A weakness with the use of implants is that the preparative process involves high or elevated temperatures, non uniform content and large variations in individual drug release patterns and of course the surgery required to implant them (114). Biodegradable microparticles developed to overcome this are usually formed by solvent evaporation or organic phase separation, spray drying or supercritical fluid technology, all of which involve several steps within each process and many formulation factors to be controlled (114). Again, rapid release, difficulty with injecting the viscous solution and concerns about the toxicity of the solvents used has led to alternative production methods involving a polymer phase emulsified into an external phase. The partial water miscibility of the organic solvents allowed the properties of the microparticles to be manipulated resulting in porous microparticles due to the polymer solidifying and trapping organic solvents within (114). It was found that by having an external oil phase, precipitation of the polymer was significantly lower than in implants resulting in reduced initial drug release (114).

The size also affects the distribution of targeted and non-targeted nanoparticles. A study by Cheng *et al* (38) demonstrated that there were at least four parameters to consider: the concentration of polymer, the drug loading, the water miscibility of the solvent used and the ratio of water to solvent. Likewise, aggregation can be triggered by temperature using pluronics as solvents and particle size can be predicted from the concentration of PLGA used (62).

Differences in formulation can affect the *in vivo* uptake of drugs (151) and similarly polymer degradation is influenced by a number of factors: co-polymer composition, molecular weight and size of particles (4). These are all optimized for the drug used and the therapeutic requirements. In addition to the above applications, modifications and polymer combinations expands the realm of PLGA uses. PLGA can be combined with chitosan to decrease the drug release rate while enhancing particle uptake by cells (76, 109). Cationic drug delivery has been investigated using PLGA based particles incorporating polyvinyl sulphonate-covinyl alcohol-graft-PLGA [P(VS-VA)-g-PLGA] (221). In this study degradation rate was determined by sulphonation.

Surfactants may be used in the formulation of PLGA variants. Polysorbate or poloxamer 188 coated PLGA allows some drugs to be transported across the blood-brain barrier (115). Sodium dodecyl sulphate was used by Xu and Czernuszka (228) while Feczkó *et al* (58) compared the effect of three different surfactants on the preparation of PLGA. In addition, pluronics, non-ionic macromolecular surface active agents, are used for the solubilisation and controlled release of drugs and have been incorporated into PLGA to produce an amphiphilic co-polymer which enhances protein loading and stability (228).

Different formulations can be used to provide multi-functional delivery agents. For use in the early detection and treatment of cancer, PLGA has been loaded with both anticancer agent and quantum dots to allow *in vivo* imaging and, and then coated onto carbon nanotubes prior to intra-venous injection of laboratory animals (76).

Applications of PLGA in Drug delivery

PLGA is a good candidate for therapeutic agent delivery. Drug delivery is often limited by the ability to target drugs to a specific site or tissue. In addition, efficient cytosolic delivery is difficult because the receptors for the drugs are in the cytosol or an organelle must transport the drug to the site. The surface of the nanoparticle and the way it interacts with the cell affects the uptake and subsequent intracellular trafficking of the nanoparticle containing or carrying the therapeutic agent (212). Nanoparticles from PLGA can be formulated to encapsulate low molecular weight drugs or macromolecules such as proteins or plasmid DNA (212). The polymer matrix helps to prevent premature degradation of the drug allowing a slower release. Usually around 100nm in diameter, PLGA nanoparticles are taken up by cells via endocytosis and the material inside the nanoparticle released slowly as the particle degrades. As a 'nanocarrier', PLGA has been shown to undergo almost 85% exocytosis yet the nanoparticles that escape from the cellular endosomes into the cytosol are able to remain there and release their content slowly (211). Adequate uptake by cells is also important if the nanoparticles are to deliver therapeutic agents intracellularly. In the study by Vasir and Labhasetwar (211), PLGA nanoparticles were functionalised with poly-L-lysine (PLL) on the surface and showed a three-fold increase in cellular uptake compared to unmodified PLGA nanoparticles.

PLGA is already used for human drug delivery for a range of medications with FDA approval. A review by Wischke and Schwendeman (227) notes 21 products which

have been/are marketed for slow release of compounds. For example, Risperdal Consta® which is used to treat schizophrenia is given 2 weekly while Lupron Depot® may be administered 1-3 monthly for endometriosis or fibroids and every 4 months for prostate cancer treatment. These are all administered by the intra-muscular route while Arestin® microspheres which incorporate an antibiotic (mimocycline) in PLGA beads and are used to treat periodontal disease in adults, are administered into the periodontal socket.

Parenteral administration of drugs involves safety concerns, is inconvenient and tiresome especially for the non-hospitalized person who requires frequent medication. Therefore oral delivery is sought and investigated by many research groups and companies. However, many drugs cannot presently be orally delivered due to difficulties related to degradation in the acidic and protease-rich environment of the gastrointestinal tract. Formulation strategies involving PLGA are being used to investigate oral delivery of many drugs (49), although none have yet been licensed for human use. Some examples showing the range of drugs being investigated are: Doxorubicin, a potent anticancer drug was loaded into orally delivered nanoparticles and results in rats showed enhanced bioavailability and lower toxicity (103); oral delivery of Eudragit® microparticles of PLGA entrapping insulin were evaluated in diabetes induced rats and successfully mediated stability of blood sugar levels (154). The anti-cancer drug 5-fluorouracil was incorporated into PLGA nanoparticles and showed enhanced bioavailability following oral delivery (132).

Topical use of PLGA loaded with flurbiprofen as a treatment for post-ophthalmic surgery pain and inflammation has been investigated *in vitro* and *ex vivo* with promising results (213).

Vaccine delivery has utilised PLGA in different formulations (196). Although effective tuberculosis vaccines remain elusive, studies have used PLGA based vaccines with some success (111). However, alternative delivery systems were still found to be more effective. Moreover PLGA is also being investigated for oral delivery of vaccines for oral immunotherapy (185), hepatitis B (79) and rotavirus vaccines (155).

Also studied for gene delivery, nanoparticles as a carrier have the gene of interest encapsulated inside a polymer matrix, which could then be used for gene therapy (138). Gene silencing using si(RNA) which incorporates polyethylenimine (PEI) as a cationic polymer into the PLGA matrix has recently undergone evaluation (161).

PLGA coated plasmid DNA has applications in the fields of vaccine delivery and disease treatment. With regard to vaccine delivery, PLGA vaccines using antigens relevant to human infections has also been carried out in mice using different routes of administration (223).

Specific targeting utilising PLGA particles has recently attracted interest by scientists. Magnetic PLGA nanoparticles have been developed and could be directed by externally applied magnetic fields to enable tissue-specific drug delivery (98). Similarly magnetic targeting has also been applied for cochlear treatment (53).

Delivery of chemotherapy drugs for treatment for retinoblastoma using PLGA nanoparticles has been studied and results indicated that PLGA loaded nanoparticles can provide a degree of sustained delivery (110).

1.5 Polymalate

Background

Polymalate, the anion of poly(β-malic acid), is a biopolyester produced by slime moulds and some fungi (182). The polyester backbone is formed by condensation of the hydroxyl groups and the β-carboxyl groups of the linearly arranged malate monomers, while the α-carboxyl groups represent side groups of the polymer backbone (60). This polyester can also be chemically produced from the corresponding lactones (216). PMLA is water-soluble, biodegradable and biocompatible as well as it can be chemically modified to alter its properties. Recently, poly(β-malic acid) and its derivatives have been investigated with regard to their potential for biomedical applications (122). PMLA is non toxic *in vitro* and *in vivo*, non-immunogenic, stable in the bloodstream and cells can easily take up the polymer (26, 32, 52, 70, 122). Polymalate is especially suitable for temporary therapeutic applications (122) and because of its properties more beneficial for repetitive treatment than for example viral delivery vectors (102, 189, 218).

Natural production of poly(malic acid) was first reported for *Penicillium cyclopium* (193-195) and later also for *Aureobasidium pullulans* (135, 136, 153). The best studied producer is the slime mould *Physarum polycephalum* (60, 112, 122). Phylogenetically, *P. polycephalum* belongs to the multicellular eukaryotes (12). During their life cycle slime moulds (myxomycetes) develop a plasmodium that differentiates into spores and later into single-celled amoebae, which might again form a plasmodium after mating (29). The plasmodium is a vegetative multinuclear giant cell with synchronously dividing

nuclei. Only in the plasmodium state myxomecetes produce PMLA, it accumulates to high concentrations in the nuclei (104, 105, 191).

Although the biochemical pathways are not fully revealed yet, it seems that PMLA functions as a storage and carrier molecule for proteins required to maintain the synchronous division of the nuclei in the giant plasmodium cell (5, 51, 104). Polymalate mimics the distance of the phosphate groups in the DNA backbone (92) and therefore competes with DNA for the binding of histones, DNA polymerases and other nucleic proteins (1, 5, 51, 60, 92). Binding to polymalate causes inactivation of the DNA polymerase (60). To maintain the synchronicity of the dividing nuclei a tight regulation between DNA polymerase, histones and other nucleic acid binding proteins is necessary. The level of polymalate remains constantly high in the nuclei and excess polymer is exported into the culture medium and cleaved to L-malate by polymalatase (112).

So far, no PMLA synthase activity has been found in plasmodial lysates but *in vivo* studies indicated the existence of a PMLA synthetase with β-L-malyl-AMP ligase and PMLA polymerase activity. The results suggested that the polymerase activity is regulated by a GTPase-dependent signal pathway which leads to inactivation of the enzyme when the plasmodium is injured (226).

Polymalate particle preparation

Polymalate is a water-soluble polymer which can be easily modified at the α-carboxyl groups. Although it can be produced chemically, most applications favour to use the naturally produced optically pure material. In most cases, polymalate is isolated from the natural producer *Physarum polycephalum*. The polymalate producing plasmodia of this slime mould are grown in shaking Erlenmeyer flasks for 2 to 3 days at 21°C to 27°C, depending on the production strain. The actual polymer is isolated from the liquid culture medium using adsorption to DEAE-cellulose as first purification step. Several batches can be combined and then further purified. Pure polymalate is obtained by repeated chromatography on DEAE-cellulose, alcohol precipitation, size exclusion chromatography and lyophilisation (91). It is advantageous to use relatively young cultures for the polymer purification as hydrolytic decomposition to L-malate starts from the onset of the growth. Early stage cultures also assure a polymer with a high-average molecular mass, ranging from 50 kDa to several hundred kDa (91, 182).

Different methods are used to form water insoluble polymalate particles; most of them involve at least one chemical modification step. In the emulsion solvent evaporation method, naturally produced polymalate is modified to a methyl ester which can then be dissolved in chloroform. Depending on the application the appropriate partner is mixed into the polymer solution and stirred until the solvent evaporated completely. Emulsifiers can be added to facilitate mixing. During the solvent evaporation, water-insoluble microspheres are formed which are rinsed in the end with distilled water and recovered by freeze-drying (171). The same method can be used to mould distinct shapes. Polymalic acid can be mixed with surfactants to form stable ionic complexes which are mixed with the respective application partner and dissolved in chloroform. The mixture can then be poured into moulding forms, e.g. discs, the solvent evaporates and the remaining spheres are available for the designed application (172).

For chromatographic applications, sepharose can be used to display polymalate, but the polymer needs to be highly pure. Minimization of covalent binding of malyl residues to the sepharose is necessary and can be achieved using different amines and amides as supplements (72).

The two-step precipitation-dialysis method is used to form nanoparticles of different organic esters of polymalic acid. The polymer is dissolved in a water- miscible solvent; the solution obtained is then slowly stirred into water. The nanoparticles will precipitate and can be filtered and concentrated using rotary evaporation (144, 203) or dialysis against distilled water using a cellulose membrane (173). Depending on the chemical properties of the product and the desired purity, additional alcohol extraction and washing steps might be necessary, as well as adding stabilizers to the original solution (17).

Applications

To date, all studies looking at possible applications of poly(malic acid) (PMLA) particles have been aiming at drug delivery, trying to benefit from the excellent biocompatibility and biodegradability of PMLA (which is metabolized via the tricarboxylic acid cycle). As pure PMLA is highly water soluble and rapidly hydrolyzed in an aqueous environment, micro- or nano-particles were in all cases generated from modified PMLA, i.e. from copolymers.

Stochiometric ionic complexes of PMLA and alkyltrimethylammonium surfactants (*n*ATMA·PMLA) were considered to have interesting properties for controlled drug release. These copolymers were found to adopt a well defined supramolecular structure with alternating layers of both components (paraffinic and polyester phase) (174). Discs of 5 mm diameter, either pure or loaded with erythromycin, were used to investigate hydrolytic degradation and antibiotic release (172). Apart from a relatively high degradability of *n*ATMA·PMLA complexes in general (compared to similar complexes made with polyglutamic acid), the length of the surfactant alkyl side chain was shown to influence hydrolysis (by determining hydrophobicity and crystallinity). This should enable precise adjustment of the hydrolytic degradability. The only slight reduction of molecular weight during degradation and the detection of malic acid but not medium size polymer chains as degradation products led to the conclusion that *n*ATMA·PMLA complexes are depleted primarily by surface erosion (instead of bulk degradation) which was considered to be favourable for even drug release. Up to 30 wt% erythromycin could be dispersed in the *n*ATMA·PMLA complex where it was found to localize in the paraffinic subphase. The experimental data further indicated that erythromycin release was due to degradation, not diffusion.

In a similar study, microspheres were generated from methylated PMLA (PMLA-Me) and degradation and drug release compared for different methylation degrees (Portilla-Arias *et al.*, 2008a). The particle diameter could be adjusted in the range of 1-20 μm. Interestingly, PMLA-Me microspheres appeared to degrade more slowly than films of the same polymer. Both surface and bulk erosion seemed to contribute to the degradation mechanism. As for *n*ATMA·PMLA complexes (172), erythromycin release from PMLA-Me microspheres was found to be determined by polymer degradation, the role of diffusion being negligible (171).

PMLA-Me nanoparticles prepared from 75% methylated PMLA were investigated as protein delivery carriers (173). This copolyester was insoluble in water, but still quite hydrophilic and readily degradable. Nanoparticles prepared in DMSO displayed the most narrow size distribution of all solvents tested (diameter approx. 100 nm) and a high negative value of zeta-potential which was considered to be advantageous as it allows modification of surface-exposed carboxyl groups and prevents nanoparticle aggregation by electrostatic repulsion. The hydrolytic degradation

behaviour was similar to that reported for PMLA-Me microspheres (171, 173). Six proteins with different molecular weights and/or isoelectric points were used to load the nanoparticles by encapsulation (coprecipitation of protein and polymer), physical absorption (incubation of freeze-dried nanoparticles in protein solution) or chemical immobilization (activation of surface carboxyl groups of nanoparticles, followed by covalent binding of protein) (173). The loading efficiency was found to depend on both the protein and the entrapment method, and loading affected nanoparticle size and zeta-potential. Similar to the antibiotics in previous studies (171, 172), protein release took place in a time frame corresponding to the hydrolytic degradation of the nanoparticles (173). Conclusions drawn from a comparison of all protein release profiles included: Independent of the loading method, the rate of protein release decreased with increasing isoelectric point. Release profiles for encapsulated and chemically immobilized proteins were similar and suggested that a certain amount of particle degradation was required for protein release. Release profiles of physically absorbed proteins could be divided into two groups according to their isoelectric point: Basic proteins behaved similar to those loaded by the other two methods, while acidic ones were released faster and more evenly. In addition, the effect of loading and release on protein activity was studied and was also found to depend on the loading method.

Martinez Barbosa and coworkers investigated degradation mechanism and *in vitro* cytotoxicity of nanoparticles prepared from another group of PMLA hydrophobic derivatives, poly(benzyl malate) (PMLABe), poly(hexyl malate) (PMLAHe) and poly(malic acid-*co*-benzyl malate) (PMLAH/Be) (144). Nanoparticles were between 100 and 200 nm in diameter. While particles seemed more prone to aggregation at acidic pH, the pH was not found to have a major influence on degradation rates. The observed decrease in molecular weight was fastest for PMLAH/Be and slowest for PMLABe particles. NMR studies indicated that degradation of all polymers occurred by random hydrolysis. For the *in vitro* cytotoxicity studies, the different nanoparticles and their degradation products, respectively, were incubated with J774 A1 murine macrophage-like cells and the IC_{50} (concentration required to kill 50% of cells) determined. For all polymers, the IC_{50} decreased with increasing degradation time (and thus increasing amount of degradation products) and with increasing incubation time with cells. PMLAH/Be nanoparticles were reported to display the highest and PMLABe particles the lowest toxicity. The authors concluded that the observed cytotoxicity of PMLABe, PMLAHe and PMLAH/Be nanoparticles is caused by degradation products and not by

simple contact of nanoparticles with the cell. They further stated that the overall *in vitro* cytotoxicity was relatively low for all tested nanoparticles, but a direct comparison e.g. to pure PMLA was said not to be feasible due to its high water solubility.

With the aim of developing a sustained release formulation, microspheres of the biodegradable polyester poly-vephyllinemalate (Veph-malate) were generated by cross-linking the bronchial dilator Vephylline with malic acid (17). Particle diameters ranged from 1 to 10 μm (88). In comparison to Vephylline itself Veph-malate was found to be essentially non-toxic (17). In addition, release of the drug from Veph-malate microspheres was slower and more even than from Vephylline hydrogels. Further investigation of the release mechanism revealed that a two-step degradation process initially only led to the formation of oligomers and only later to the release of active Vephylline monomer, resulting in a delayed and prolonged effect of the drug compared to pure Vephylline (118). This two-step release profile was found to be pH dependent (88). Overall, the Veph-malate microspheres were considered to be useful as a prodrug ensuring sustained release of Vephylline and to be suitable for parenteral application (17, 118).

1.6 Conclusion

Biodegradable micro- and nano-particles have become vital tools for a wide variety of applications in research, biotechnology and medicine (Figure 2). This review has addressed the main groups of biopolyester particles in current use, with respect to their composition, preparation and applications. It is very evident from the ever-increasing range of patents and publications, that this is an area of exceptional growth and development.

One promising approach is the engineering of microbial cells for the manufacture of custom-made beads by hijacking the natural storage granule biogenesis system (Rehm 2007). This in particular allows the one-step production of polymer beads with a protein-based function at high density and functional orientation already attached to its surface. This intracellular production does also provide the unique advantage of utilizing a natural environment for the functional attachment of the relevant protein, hence enabling the functional display of difficult to express proteins.

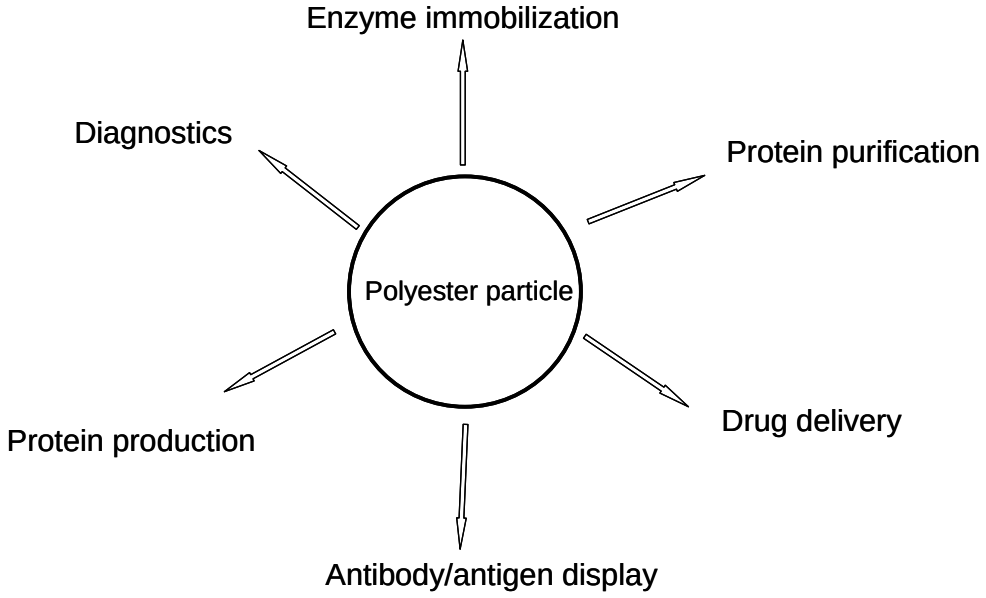

Figure 2: Schematic of biopolyester applications

Recently, the use of supercritical fluids (SCF) has gained attention as an advanced method for many biotechnological processes, including the formation of drug encapsulated PLA and PGLA particles (30, 40, 147). In addition, SCF has been used to incorporate food materials into biopolyester particles, such as the incorporation of β-carotenoid into particles of the PHB co-polymer with 3-hydroxyvalerate (PHBV) (61). In these methods, carbon dioxide and/or water in the supercritical state are used to co-precipitate a bioactive substance and the biopolymer, such as in the microencapsulation of the corticosteroid budesonide into PLA particles (36, 143). The use of carbon dioxide in the supercritical state is reported as useful for producing particles in a more controlled fashion than the conventional methods (30) and to enhance the solubility of drugs which are poorly soluble in aqueous and organic media (147).

While the use of biopolyester particles continues to grow in many areas, there are several challenges that remain to be overcome. For example, it seems that the size and uniformity of the particles have been identified as particularly important parameters in many contexts. In addition the reduction of initial burst and solubility improvement are critical issues in drug delivery. In industrial settings, product quality is often dependent on the level of existing control over the physical characteristics of the

particulate vehicles. Similarly, medical applications require particle characteristics that promote consistency and reproducibility. Therefore, much effort is now being invested in improving and tailoring particle properties, for bacterial as well as synthetically produced biopolymer particles. There is no doubt that the development and fine-tuning of these particles, and their potential applications, will continue to expand well into the future.

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Chapter 1B: Bacterial polyhydroxyalkanoate granules: Biogenesis, structure and potential use as micro-/nanobeads in biotechnological and biomedical applications

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1.8 Abstract

Polyhydroxyalkanoates (PHAs) are naturally occurring organic polyesters that are of interest for industrial and biomedical applications. These polymers are synthesized by most bacteria in times of unbalanced nutrient availability from a variety of substrates and they are deposited intracellularly as insoluble spherical inclusions or PHA granules. The granules consist of a polyester core, surrounded by a boundary layer with embedded or attached proteins which include the PHA synthase, phasins, depolymerising enzymes and regulatory proteins. Apart from ongoing industrial interest in the material PHA, more recently there has also been increasing interest in applications of the PHA granules as bionano-/microbeads after it was conceived that fusions to the granule associated proteins (GAPs) provide a way to immobilize target proteins at the granule surface. This review gives an overview of PHA granules in general, including biogenesis and GAPs, and focuses on their potential use as nano-/microbeads in biotechnological and biomedical applications.

1.9 Introduction

Bacterial polyhydroxyalkanoate (PHA) granules which are found as naturally occurring spherical inclusions are becoming increasingly recognised as potential functionalised beads for use in biotechnological and biomedical applications.

PHAs are polyesters which serve as carbon and energy storage for bacteria and become deposited as insoluble spherical inclusions in the cytoplasm. Most bacterial genera and even members of the family *Halobacteriaceae* of the *Archaea* are known to synthesize PHA (1-6) which is produced in conditions of nutrient limitation but where carbon is available in excess (7-10). Bacteria are able to accumulate as much as 80% of their dry weight in PHA (11, 12), with reversal of the PHA polymerization process in conditions of carbon starvation (13, 14). One of the most common PHAs is poly(3-hydroxybutyrate) (PHB) which is synthesized from 3-hydroxybutyrate (3HB), but different bacteria use hydroxy fatty acids of varying chain length, generating a range of PHAs.

Due to properties such as biocompatibility, biodegradability and production from renewable resources, there is considerable interest in the potential applications of PHAs. With chemical modification or through the creation of co-polymers, a range of material properties can be achieved, e.g. PHAs which are less brittle and more flexible

while retaining tensile strength. These polymers have been developed for use in industrial or medical applications and have been shown to be well tolerated by mammalian systems (15). Due to the comparatively high production costs, PHAs are currently mainly attractive for use in the medical field, e.g. for sutures or implants like heart valves, stents and bone scaffolding (15, 16).

The key enzyme for PHA biosynthesis is the PHA synthase. This enzyme polymerizes (*R*)-3-hydroxyacyl-CoA thioester monomers into polyester with the release of coenzyme A. Depending on the organism, there are several classes of PHA synthases using different *(R*)-3-hydroxyacyl-CoA precursors which can be provided by different pathways (17). In *Cupriavidus necator*, the most investigated PHB producer (18), *(R)*-3-hydroxybutyryl-CoA monomers are generated from acetyl-CoA by the action of two other enzymes (16, 19, 20). The three PHB biosynthesis genes are organized in one operon, the *phaCAB* operon. β-ketothiolase (encoded by *phaA*), condenses two molecules of acetyl-CoA to acetoacetyl-CoA and this is subsequently reduced to *(R)*-3-hydroxybutyryl-CoA by the NADPH-dependent acetoacetyl-CoA reductase (encoded by *phaB*). The PHB synthase (encoded by *phaC* in *C. necator*) then converts the thioester monomers into the polyoxoester PHB. The polymer aggregates to form a spherical inclusion or granule of usually 50 - 500 nm in diameter with the amorphous hydrophobic PHA polyester at the core and attached or embedded proteins at the surface, including the PHA synthase, PHA depolymerases, structural and regulatory proteins (21, 22).

In this review, we summarize the current literature on PHA granules, their biogenesis and structure, and on protein engineering approaches of associated proteins aiming at the design of PHA granules as bio-beads for use in various biomedical applications.

1.10 Structure of PHA granules

The structure of PHA granules has not been fully determined but the major constituent of granules is PHA, often PHB, with small amounts of protein and lipid (23). *In vivo* the hydrophobic polyester core is largely amorphous (24) with water as a component that prevents crystallization by acting as a plasticizer (25). This is the mobile state of PHA, i.e. the form that is subject to the action of synthesizing and degrading enzymes. After isolation, PHA is often crystalline (see below).

Initial studies, including electron microscopy in the 1960s (26), have shown the polyester core to be surrounded by a 4 nm boundary layer, which most likely comprises a phospholipid monolayer (27) with embedded and attached proteins (22, 28). While most data seem to be consistent with a monolayer, alternative membrane models, e.g. comprising inner and outer protein layers sandwiching phospholipids, have been suggested (29). More recent electron microscopy data indicated that the thickness of the surface layer surrounding the PHA granules to be 14 nm which the authors took as an indication of the size of the associated proteins (30). However, it cannot entirely be ruled out that the boundary layer primarily consists of proteins and that attachment of membrane material is only an isolation artifact.

In addition to EM, a variety of techniques have been used to investigate PHA, including wide-angle X-ray scattering (31), nuclear magnetic resonance spectroscopy (32) and confocal microscopy (30) (Figure 1). Using wide-angle X-ray scattering, Kawaguchi and Doi confirmed that PHA in native granules is amorphous, even after isolation, and that certain treatments seemed to initiate crystallization, presumably by removing a lipid component (31). Recently, contrast-variation small-angle neutron scattering was used to probe granule organization and results were consistent with the phospholipid monolayer model (33, 34). Atomic force microscopy (AFM) allows imaging at nano scale while being rapid and less damaging to preparations than EM (35, 36). Recently, analysis of PHA granules by AFM has shown an additional network layer with globular areas, most likely also incorporating structural phasin proteins (37). AFM was also used to show porin-like structures in the surrounding membrane which were suggested to provide a portal to the amorphous polymer core and be the site of PHA metabolism and depolymerisation (38).

Investigation of PHA granules is strongly influenced by the preparation technique because denaturation and crystallisation of PHA often occurs through physical stress such as excessive Sonication (37), freeze-thaw cycles or exposure to solvents, detergents or alkalis (39). To avoid denaturation during the purification process, PHA granules can be purified using mechanic (e.g. French Press) or enzymatic cell lysis followed by density gradient centrifugation. These techniques should allow accurate analysis and consistent end-use of PHA granules.

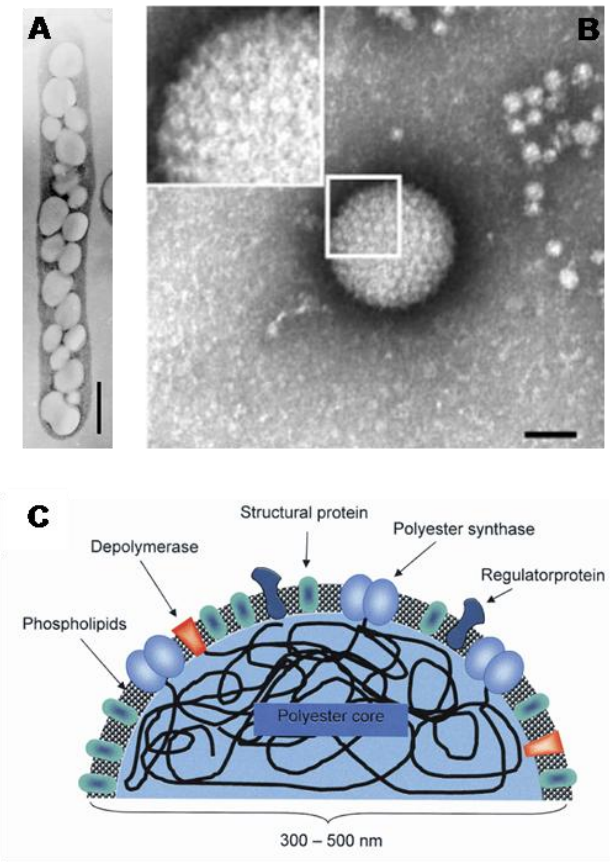

Figure 1: Different representations of PHA granules
A: TEM image of C. necator cell filled with PHA granules. Bar, 500 nm. (Journal of Bacteriology, 2005, 187, 3814-3824, doi:10.1128/JB.187.11.3814-3824.2005 (54), reproduced with permission from American Society for Microbiology.) **B**: TEM image of single PHA granule isolated from C. latum zooming in on the paracrystalline-like layer of particles covering the granule. Bar, 50 nm. (Applied and Environmental Microbiology, 2007, 73, 586-593, doi:10.1128./AEM.01839-06 (30), reproduced with permission from American Society for Microbiology.) **C**: Schematic depiction of a PHA granule with granule associated proteins. (Reproduced with permission, from Rehm, B. H. A., 2003, Biochemical Journal, 376, 15-33, DOI 10.1042/BJ20031254 (58). © the Biochemical Society.)

1.11 PHA granule assembly

The process of PHA biosynthesis, the polymerization of (*R*)-3-hydroxyacyl-CoA to PHA, leads to the formation of spherical inclusions which start to assemble as the PHA synthase converts soluble substrate monomers into insoluble high molecular weight polymer. Two different models have been discussed to explain this process which will be described below. During the polymerization process, the synthase remains covalently attached to the growing polyester chain and continues to incorporate more substrate until metabolic or spatial constraints terminate the polymerization procedure, i.e. the substrate has been depleted or all available space in the cell has been used. The

size of PHA granules and the number of inclusions per cell seem to vary between organisms (with the diameter usually ranging between 100 and 500 nm diameter and 5-10 granules per cell), and it has been debated if fusions between granules occur or if they are successfully prevented by the granule associated proteins (GAPs), in particular the phasin PhaP (22, 40). PhaP also impacts on the granule surface to volume ratio and thus the number of granules per cell (40, 41) and on PHA synthase activity (42, 43) (see below).

Apart from the metabolic background (which determines the provision of suitable substrate), factors which could potentially influence the molecular weight of the polymer include the PHA synthase concentration and presence of PHA depolymerising enzymes. The latter are only present in the native host and there are indeed indications that recombinant production yields higher molecular weight polymer than the native system (44). An inverse correlation between PHA synthase concentration and molecular weight has so far only been shown for *in vitro* as well as recombinant production in *E. coli* and not for production in the native host (45-47).

The exact mechanism by which the nascent polyester chains with attached synthesizing enzymes (enzyme-nascent polyester units) congregate to form the boundary layer-covered PHA granule has not been elucidated. Two models for granule biogenesis have mainly been discussed (48). The first one is the 'micelle' model which is based on the assumption that the PHA synthase is present in the cell as a soluble enzyme, more or less randomly distributed in the cytoplasm. Once polymerization starts, the nascent polyester chain converts the initially soluble enzyme into an amphipathic molecule and the increasingly hydrophobic PHA chains aggregate into a micelle-like structure. In this model, the constituents of the boundary layer, i.e. phospholipids and other GAPs apart from the synthase, would gradually become incorporated as the self-assembled PHA inclusion increases in size. The second model is the more recent 'budding' model which suggests that the PHA synthase localizes to the inner face of the cytoplasmic membrane, either inherently or as soon as a PHA chain emerges from the enzyme. In this case, biosynthesis of the polyester would be directed into the intermembrane space where the extending chains would accumulate until eventually PHA inclusions surrounded by a phospholipid monolayer would bud off the membrane. While the 'micelle' model is supported by the fact that PHA granules can be

produced *in vitro* in the absence of membranes (45, 49, 50), most of the recently emerging evidence is in favour of the 'budding' model.

Jendrossek *et al.* analyzed early stages of PHB accumulation *in vivo* in *Rhodospirillum rubrum*, *C. necator* and in recombinant *E. coli* by confocal laser scanning fluorescence microscopy (CLSM), visualizing PHB granules by Nile red staining and fusion of EYFP (enhanced yellow fluorescent protein) to a phasing (51). In young cultures in the initial stages of PHB production, they observed PHB granules predominantly at or near the cell poles and near the cell wall. Jendrossek similarly analyzed *Caryophanon latum* by CLSM in combination with Nile red staining and by TEM in combination with immunogold staining and found that PHB granules in early stages of formation were localized close to the cytoplasmic membrane (30). Also in 2005, Peters and Rehm reported their fluorescence microscopy studies of emerging PHA granules in *Pseudomonas aeruginosa* PAO1 and recombinant *E. coli* employing either the PHA synthase from *P. aeruginosa* or *C. necator* which were both fused to GFP (green fluorescent protein) at their N terminus (52). In both organisms, nascent PHA granules were observed to localize to the cell poles and occasionally to the poles and to the centre of the cell, i.e. to the future cell poles. This localization occurred independently of septum formation but seemed to require proper nucleoid structure and segregation as was demonstrated by analysing PHA granule formation in a *mukB* mutant which is affected in nucleoid structure and segregation. This study suggested that nucleoid occlusion, i.e. spatial competition between nucleoid and PHA granules might play a role in intracellular localisation of granule formation. This was the first evidence that the cytoskeleton might be involved in PHA granule formation (52). Further investigations revealed no evidence that the nascent polyester chain is responsible for subcellular localization of the synthase (e.g. by anchoring it to the membrane), as even an inactive mutant of the *C. necator* PHB synthase still localized to the cell poles (53). The deletion of either the N or the C terminus of the synthase, respectively, did also not affect proper positioning. These results indicate that the core region of the *C. necator* PHB synthase might be responsible for polar localization. The observations of Jendrossek *et al.* and Peters *et al.* provide support for the budding model as early stage PHA granules were localized at the cell poles and thus (a) close to the membrane and (b) not randomly distributed. However, these findings might also indicate that the situation is more complex as according to the model one would expect PHA granules to emerge along the circumference of the entire cell, not only at the poles.

Tian *et al.* have suggested a third model based on their kinetic studies of PHB granule biogenesis in *C. necator* by TEM (54). They observed dark-stained elongated structures or 'mediation elements' in the centre of the cell with small granules attached. In older cultures these elements were no longer visible, which could either mean that they had been degraded or that they were covered by granules. The authors proposed these mediation elements to serve as scaffolds for the initiation of granule formation, which would be analogous to the cellulosome. Although the observations of Tian *et al.* are currently not compatible with the polar localization of nascent PHA granules described by other researchers as described above, they also support a non-random distribution.

Another question is how synthesis of the PHA chain is terminated. In particular, does it happen in a way which enables the PHA synthase to reinitiate synthesis and generate more than one polymer chain? Calculations addressing this question have mainly been based on data derived from *in vitro* PHA biosynthesis. Based on the amount and molecular weight of the PHA produced *in vitro* by different organisms, it was calculated that both the PHA synthase from *C. necator* (class I) and from *P. aeruginosa* (class II) synthesized not more than one polyester chain per molecule of enzyme, while the *Allochromatium vinosum* synthase (class III) produced multiple chains (42, 45, 49). Tian *et al.* made an attempt to determine this ratio for the *in vivo* situation in *C. necator* and obtained a ratio of PHB molecules to PHB synthase molecules of 60 to 1 (47). Thus, some indications for chain transfer have been obtained in single cases but no definite conclusions could be drawn so far. Comparison of the molecular weight of PHA produced from different carbon sources led to the suggestion that some of them might act as chain transfer agents in chain termination and that the actual chain transfer agent *in vivo* might be 3-hydroxybutyric acid which is not enzyme bound (55).

1.12 Granule-associated proteins

Proteins associated with the phospholipid granule surface play a major role in PHA synthesis and degradation, and in granule formation (56). These proteins have been designated to four classes (names in brackets for *C. necator*), namely the polyester or PHA synthases (PhaC), the depolymerases (PhaZ), regulatory proteins (PhaR) and phasins (PhaP) (Figure 1).

PHA synthase

The PHA synthase, which is the key enzyme of PHA biosynthesis, catalyses the stereo-selective conversion of *(R)*-3-hydroxyacyl CoA thioester substrates to PHA, with the concomitant release of coenzyme A (57, 58). The ongoing increase in the number of published bacterial genomes has resulted in a corresponding increase in the number of putative PHA synthases. Currently, the nucleotide sequences of at least 88 PHA synthases have been obtained, including two potential PHA synthase genes from the halobacterial species *Haloarcula marismortui* and *Haloferax mediterranei* (2, 6). Based on their primary structures, as well as the number of subunits and substrate specificity, PHA synthases have been assigned to four major classes (58).

Class I and class II PHA synthases consist of only one type of subunit (PhaC) with molecular weights between 61 and 73 kDa (59). The PHA synthases belonging to class I (e.g. *C. necator*) utilize *(R)*-3-hydroxy fatty acid substrates consisting of 3-5 carbon atoms and produce PHA composed of short length monomers (PHA_{SCL}) (58, 60) whereas those of class II (e.g. *P. aeruginosa*) utilize *(R)*-3-hydroxy fatty acids with 6-14 carbon atoms and synthesize medium chain length PHA (PHA_{MCL}) (61, 62). PHA synthases of class III (e.g. *A. vinosum*) consist of two subunits, namely PhaC of 40 kDa with similarity to classes I and II polyester synthases and PhaE with no similarity to these, also of 40 kDa (63, 64). Class IV PHA synthases, found in the genus *Bacillus,* also consist of two subunits, one being the 40kDa PhaC subunit and the other a 20kDa PhaR subunit (65). The PHA molecules synthesized by the enzymes in classes III and IV are made of PHA_{SCL}. A small number of bacterial PHA synthases do not fit into the above classification (66, 67). The archaeal PHA synthases investigated so far seem to be similar to class III enzymes (2, 6).

Among the proteins associated with the granule surface, only the PHA synthase is required for PHA granule formation, in the presence of a suitable substrate. This not only allows *in vitro* synthesis (45), it also makes recombinant production of PHA e.g. in *E. coli* relatively straightforward (68). In addition, the PHA synthase stays covalently attached to the granule surface and tolerates N-terminal fusions with other proteins. Therefore, it is possible to engineer PhaC fusions for the immobilization and functional display of these proteins on the granule surface (see below) (69-72).

Comparison of the primary sequences of the PHA synthases has shown six conserved blocks and eight identical amino acids (58). While the N-terminal region has

no conserved sequences, this region may have a role in the level of PHA synthase expressed and in the yield of PHA (73). The C-terminal region of approximately 40 amino acids is more conserved in class I and II PHA synthases, consisting mainly of hydrophobic amino acids which suggests a role for this region in binding of the synthase to the hydrophobic granule core (58). With regard to secondary structure, predictions from multiple sequence alignments have indicated that the PHA synthases mainly contain variable-loop and α-helical secondary structures (74). The α/β hydrolase region, which has been shown to be essential for enzymatic activity (75), has been strongly suggested to exist in the C-terminal portion of the protein, based on a conserved domain homology search (76). In addition, the presence of a conserved lipase-like box in the primary structure where the catalytic site serine of the lipase is replaced by a cysteine in the PHA synthase (G-X-[S/C]-X-G), further indicates homology to lipases (77). Three conserved amino acid residues (cysteine, aspartic acid and histidine) are thought to be critical for the catalytic mechanism by forming a catalytic triad. PHA synthases exist in an equilibrium of monomeric and dimeric forms *in vitro*, however when the *(R)*-3-hydroxyacyl-CoA substrate is provided, significant dimerization is suggested to occur, with one subunit of the active dimer attaching to the growing polyester chain while the other subunit binds a new *(R)*-3-hydroxyacyl CoA substrate molecule (78). Evidence has also been presented by mutational studies of the PHA synthase of *A. vinosum*, that the conserved aspartic acid residue plays an important role in chain elongation while digestion of the polyester chain-enzyme complex and HPLC analysis have shown that the polyester chain stays covalently attached to the conserved cysteine of the enzyme (79, 80).

PHA depolymerases

PHA depolymerases, enzymes which degrade PHA, consist of two groups, namely the intracellular depolymerases that degrade the amorphous PHA within granules of the accumulating bacteria, and the extracellular depolymerases which are secreted by most bacteria to utilize denatured PHA present in the environment from e.g. other non-living cells (81). PhaZ refers to the intracellular depolymerases found on the PHA granule surface. These are necessary for the mobilization of the PHA granules as a source of energy (14).

Intracellular depolymerases have been investigated much less than the extracellular depolymerases and the mechanism by which intracellular native PHA

granules can be re-utilized is not well understood. There are some studies which have addressed the mobilization of intracellular PHA and the PhaZ encoding genes of *C. necator* (14, 82-84). While the first described PhaZ of *C. necator* was designated PhaZ1 (83), subsequent (putative) depolymerases have been identified in *C. necator* and designated PhaZ2 to PhaZ5 (84-86). The genome sequence of *C. necator* revealed seven genes for PHA depolymerase isoenzymes and two for PHA oligomer hydrolases (87) but for few there is actual direct evidence for their *in vivo* function. Following an alternative nomenclature, these putative depolymerases have also been designated PhaZa1 to PhaZa5, PhaZb, PhaZc and PhaZd1/2 (88).

Saegusa *et al* reported the cloning and sequencing of the intracellular *phaZ* of *C. necator* and the demonstration of PHA degrading activity when amorphous PHA granules were provided as the substrate (83). Although PHB metabolism has been reported to be cyclic in nature with PHB synthesis and degradation occurring at the same time (82, 89), it has been a matter of discussion that simultaneous synthesis of PHB from acetyl-CoA and degradation of PHB to 3HB would be a waste of energy (83). Very recently, Uchino *et al.* presented evidence that the PHA depolymerase PhaZa1 from *C. necator* is responsible for the degradation of PHB granules, albeit not exclusively, and that the enzyme degrades the polymer by thiolysis into 3HB-CoA instead of 3HB which would help to explain the previously apparently futile cycle of simultaneous PHB biosynthesis and degradation (88, 90).

PHA depolymerases investigated in bacteria other than *C. necator* include the recently described PhaZ of *Pseudomonas putida* and *Azotobacter chroococcum* (91, 92).

Phasins

Phasins are the most abundant protein found at the PHA granule surface and are synthesized in very large quantities under storage conditions, representing as much as 5 % of the total cellular protein (40, 93-96). Phasins are noncatalytic proteins, consisting of a hydrophobic domain which associates with the PHA granule surface and a predominantly hydrophilic domain exposed to the cytoplasm of the cell. There is evidence that this amphiphilic layer of phasins stabilizes PHA granules and prevents coalescence of separated granules (40, 94, 95).

Phasins are low-molecular-weight proteins (mostly between 11 and 25 kDa) and have been identified and isolated from many PHA_{SCL}-producing bacteria due to their

association with PHA granules (40, 93-97). Phylogenetically, phasins are non related and share no sequence homology (93). The phasin protein of *Rhodococcus ruber* binds to the PHA granule surface via two hydrophobic domains at the C terminus of the protein (95, 97). In contrast, no distinct region in the PhaP1 protein from *C. necator* could be identified to be responsible for the binding of this protein to the granule surface (93, 98). Therefore, binding capacity due to secondary or maybe also tertiary and quaternary structure of the protein has been suggested (98). PhaP1 of *C. necator* is the most investigated member of this class of proteins. Genome analysis of *C. necator* identified three PhaP homologues which need to be further investigated (85). An initial study confirmed that PhaP1 is the major phasin protein and was recently characterized as a planar triangular protein that occurs as trimer (98). Mutants defective in one of the *phaP1* homologues did not show any differences in phenotype compared to the wild type (21). Phasins are not essential for PHA accumulation, but strains unable to produce any phasin protein accumulate only one single large PHA granule, taking up all available space in the cell (40). Overproduction of PhaP leads to the formation of many small granules (41). The influence on granule size has been demonstrated both *in vivo* and *in vitro* (49, 95, 96, 99, 100). Although phasins are not necessarily required for PHA production their synthesis and abundance is closely correlated to PHA accumulation.[10] Phasins are only produced under accumulating conditions (40, 41, 101) and the amount of protein produced parallels the level of PHA in the cell (43, 46, 101, 102). Additionally, phasins are thought to positively influence synthase activity although no evidence for direct interaction was reported so far (42, 49, 103, 104). The occurrence of phasins on the granule surface obviously prevents other proteins not related to PHA metabolism from binding in an unspecific manner to the PHA granule surface which could be disadvantageous for the overall metabolism of the cell.[40] In the absence of PhaP this protective function can be partially resumed by other phasin like proteins such as BSA (42, 49) or HspA (105).

Recombinantly produced PhaP1 protein from *C. necator* was shown to be able to bind to triacylglycerol (TAG) inclusions in *Rhodococcus opacus* and *Mycobacterium smegmatis*, indicating the capability of PhaP to bind to any type of hydrophobic inclusion, irrespective of the compound stored in the core of the inclusion (106).

First crystals of the phasin protein PhaP from *Aeromonas hydrophila* were obtained in 2006 (107).

Regulatory proteins

PHA granule synthesis and phasin production are tightly regulated by the effectiveness of the transcriptional regulator PhaR. Genes encoding proteins homologous to PhaR are widely distributed among PHA_{SCL}-producing bacteria, indicating an important role in the regulation of PHA_{SCL} biosynthesis (94, 95). So far, the PhaR proteins from *C. necator* and *Paracoccus denitrificans* have been further investigated and binding of the regulatory protein to DNA sequences upstream of the respective *phaP* and *pha*R genes could be shown for both organisms (41, 108, 109). Additional evidence was derived from mutagenesis studies performed in *C. necator*, where no PhaP protein could be detected in a *phaC* deletion strain, whereas a *phaC*/*pha*R deletion strain as well as a *pha*R deletion strain synthesized large amounts of PhaP protein (102). Deletion of *pha*R completely disconnected PhaP accumulation from PHB production in *C. necator* (102). Based on these findings the following regulatory model has been suggested for *C. necator* (41, 56, 102). Under conditions non-permissive for PHA biosynthesis, PhaR binds to the *phaP* promoter region and inhibits transcription. Under PHA- accumulating conditions, the PHA synthase starts synthesizing polyester chains, and PHA granules are formed. PhaR, with high binding capacity to hydrophobic surfaces, binds to the PHA granule surface, hence lowering the cytoplasmic concentration to a point too low to sufficiently repress the transcription of *phaP*. This leads to synthesis of PhaP, which immediately binds to PHA granules; no soluble PhaP is detectable in the cytoplasm. In later stages of the accumulation, when PHA granules reached the maximum size, most of the granule surface will be covered with PhaP protein, leaving no space for efficient PhaR binding. Increasing cytoplasmic concentration of PhaR again allows binding to the respective DNA sequences and repressing transcription of *phaP* and its own gene, indicating an efficient autoregulation of *pha*R expression to prevent synthesis of more PhaR than is required for sufficient repression of *phaP* expression (41, 56, 102). The same type of PhaP/PhaR regulation was found for *P. Denitrificans* (109). Additionally, simultaneous binding of PhaR to DNA and PHA granule surface *in vitro* as well as *in vivo* could be shown for this organism (109, 110). These results indicated a bi-functional character for PhaR and implied that the protein has two separate domains for binding to the two molecules. Binding of PhaR to the PHA granule surface seems to be irreversible and mainly driven by nonspecific hydrophobic interactions, implying high affinity but low specificity. PhaR of *P. denitrificans* was the first regulatory protein reported to interact directly with PHA

granules. In contrast to PHA binding, DNA binding is reversible and highly specific, presumably involving the N terminal region of the PhaR protein, which shows high sequence homology among PhaR homologues.[110] Recent studies suggested PhaR to be a more global PHA-responsive repressor, involved not only in the expression of *phaP* but also in the expression of genes involved in other metabolic pathways (102, 109, 111).

PhaF and PhaI have been reported to be granule-associated proteins with regulatory function for *Pseudomonas oleovorans* and a model similar to the *C. necator* PhaP/PhaR system has been suggested (112). However, PhaR does not show sequence homology to PhaF or PhaI and, unlike PhaR, PhaF is also involved in the regulation of PHA synthase production in *P. oleovorans* (112, 113).

1.13 Applications of PHA granules

As mentioned briefly in the introduction, PHAs have been considered as bio-based and biodegradable alternatives to conventional petroleum-based plastics for over 20 years and have more recently, over approximately the last 10 years, attracted increasing interest for medical applications (114-117). PHAs are used because of their biocompatibility, their modifiable physical and thermal properties and also because of their biodegradability, but they are generally used as a chemically extracted bulk material. Only very recently, researchers have started to exploit the particular spherical structure or 'bead' nature of PHA granules. The general properties of PHA as a material in combination with the size and shell-core composition of PHA granules open up a broad range of applications in biotechnology and medicine, from protein purification to drug delivery (Figure 2).

Protein purification

Protein purification methods typically aim to recover a high yield of protein, free of contaminants, and without denaturing the biological activity. Consequently, separation methods must be sufficiently mild so as not to irreversibly alter the protein's structure. Techniques that meet these requirements include affinity-based methods which take advantage of bonding interactions between a protein analyte and an immobilizing matrix. These methods must be individually optimized for each protein which can be expensive and time consuming (118). Producing the protein of interest fused to an affinity tag generally simplifies the purification procedure. Following purification of the protein the tag can be easily removed enzymatically. Although this

approach is widely used and considered reliable for purifying the native target protein, the cost and number of separation steps involved can make the method cumbersome (119). Recently, self-cleaving affinity tags based on inteins have been introduced to eliminate the need for expensive proteolytic enzymes (121, 121). Despite the success of the self-cleaving affinity tagged purification process, the cost of these methods, especially the cost of affinity resins, and the relatively low binding capacity for the tagged protein prohibit large scale industrial protein purification (122).

The large-molecular-weight spherical structure of PHA granules with the surface-associated proteins (PHA synthase, phasins, etc.) and the low-cost production make the granules a useful tool for protein immobilization and purification (119, 123, 124). Banki *et al.* developed a protein purification system which combines two technologies, namely PHA production in recombinant *E. coli* and intein-mediated self splicing, implementing the specific affinity of the *C. necator* phasin PhaP to the PHA granules (122). In this system, the protein of interest is produced fused to the C terminus of PhaP which acts as an affinity tag. Both the tagged protein and the PHA granules are co-produced in *E. coli* and the protein binds to the granules - which act as an affinity matrix - via the phasin tag. After cell disruption, granules with bound protein can be separated from other cellular components by simple centrifugation. Following appropriate washing, the protein of interest is released by intein self-cleavage. Banki *et al.* used multiple phasins (2-3 repeats) and reported the successful purification of several test proteins (maltose binding protein (MBP), β-galactosidase (LacZ), chloramphenicol acetyltransferase (CAT) and NusA) with yields of 30-40 mg of protein per litre of culture (122). The authors suggested that 'fine-tuning', e.g. of the granule size might further improve results.

Barnard *et al.* developed an analogous system for *C. necator* with the aim of overcoming the general disadvantages of using *E. coli* as a protein production host (e.g. inclusion body formation) (125). In addition, using a natural PHA producer has the advantage of having to recombinantly produce only one protein, the PhaP-tagged target protein. Barnard *et al.* demonstrated purification of GFP and LacZ from *C. necator* and further reported that PhaP is functional both as an N- and a C-terminal tag.

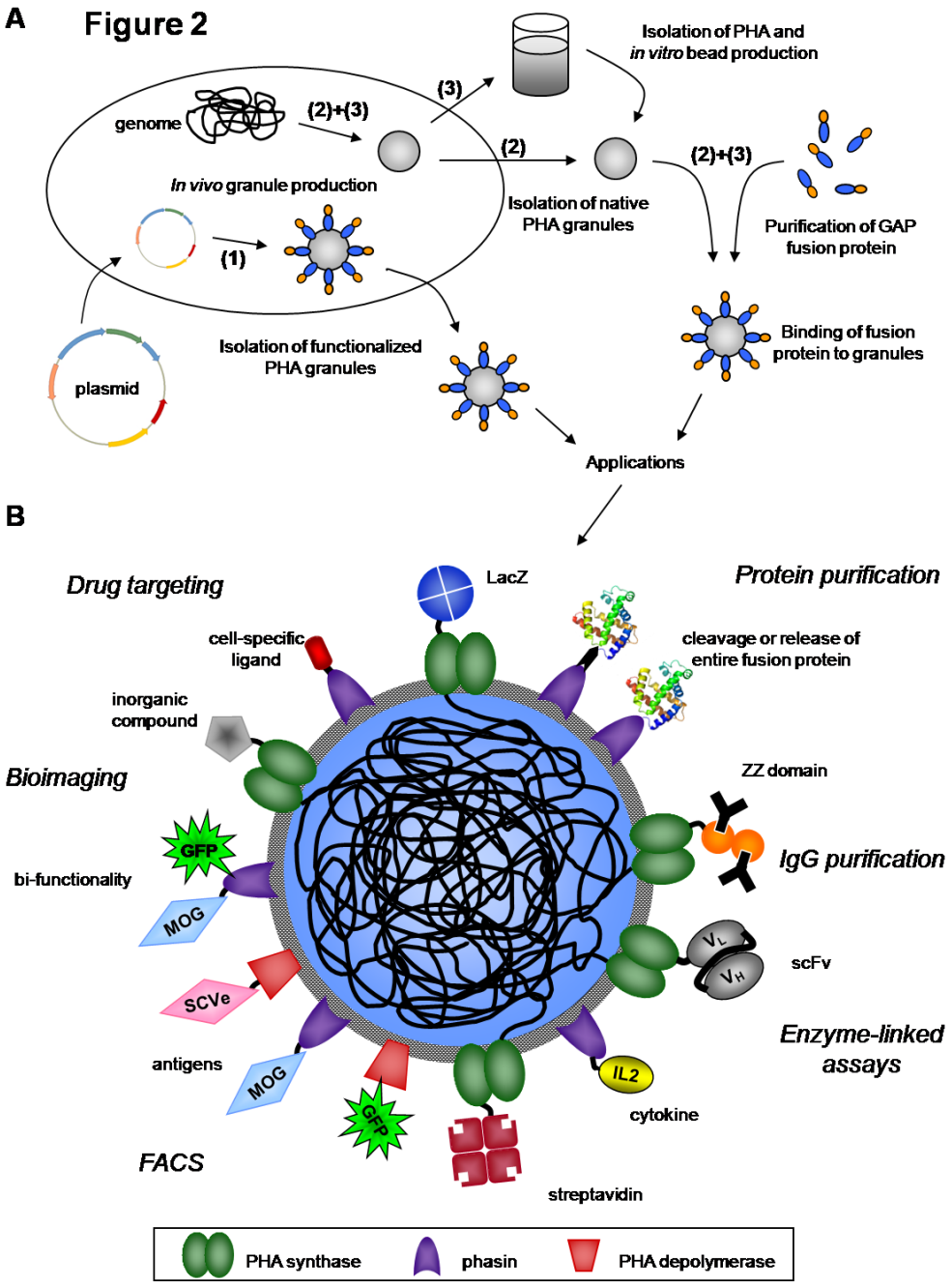

Figure 2: Potential applications for PHA granules
A: Different approaches for the generation of functionalized PHA beads. (1) A plasmid encoded fusion of the target protein and a GAP is recombinantly produced in a PHA synthesizing host strain. (PHA synthesis can be natural or recombinant.) The fusion protein associates with PHA granules as they form and functionalized PHA granules are isolated from the cell (2, 3). Native PHA granules are formed by a natural PHA producing organism. This is either followed by isolation of these native granules (2) or by chemical extraction of the PHA and subsequent *in vitro* bead production (3). In a last step, the separately produced (and purified) GAP fusion protein is allowed to bind to the PHA granules/beads *in vitro* (2 and 3). **B:** Schematic overview of the different proteins and other compounds which have so far been immobilized and functionally displayed at the PHA granule surface, pointing out potential applications.

Recently, Wang *et al.* chose a slightly different approach to the same goal of PHA-based protein purification (126). The protein production step was separated from the PHA production step and the protein purification procedure. While the target proteins (EGFP (enhanced GFP), MBP and LacZ) were tagged with *Aeromonas hydrophila* PhaP and recombinantly produced in *E. coli*, the PHA beads were produced *in vitro* from chemically extracted PHA bulk material. Following incubation of the PHA beads with protein crude extract, the target proteins were also retrieved by intein-mediated cleavage. This suggested that phasins do not only bind to emerging native granules but also to crystalline PHA. Moreover, the authors suggested one main advantage of their system, namely suitability for a wider range of target proteins, including eukaryotic proteins, as the tagged protein can be produced in any host organism independent of PHA granule formation. One might also expect stronger target protein production if this process does not have to compete with PHA biosynthesis in the cell. On the other hand, compared to the methods of Banki *et al.* and Barnard *et al.* the method of Wang *et al.* requires more steps and also additional processing of the PHA. For all the PHA-based protein purification methods described here, one has to keep in mind that they are not suitable for the purification of proteins which themselves have a high affinity for PHA granules. In this case one would expect problems with separation and poor yield as the target proteins would compete with the phasins in binding to the granules (127).

Banki *et al.* suggested that the self-contained system consisting of phasin tag and PHA affinity matrix is particularly suitable for large-scale purification with moderate purity requirements. Moldes *et al.* took an even simpler approach (128). They used the N-terminal region of the *P. putida* phasin PhaF as a tag for protein purification and simply released the purified tagged protein (fusion of PhaF and target protein) by detergent treatment.

Biological Nano-/Micro-Beads

The use of nanoparticles in drug delivery, target specific therapy, molecular imaging, and as biomarkers or biosensors, in diagnosis and many other biomedical fields is increasing rapidly (129, 130). Recently, it has been conceived that PHA granules have great potential for development towards these applications.

In 2005 it was demonstrated that the fusion of GFP to the N terminus of the PHA synthase did not affect PHA granule formation (52) which encouraged further studies to engineer the PHA synthase to enable immobilization of the enzyme β-galactosidase (131). Immobilized β-galactosidase was stable for several months under various storage conditions. This proof-of-principle work showed that protein engineering of the PHA synthase to produce functionalized PHA granules could be a useful tool for developing bionano- or microbeads for various applications. The PHA synthase has the advantages of providing a covalent interaction with the granule as well as a simpler recombinant production system in which no other genes apart from the PHA biosynthesis genes have to be heterologously expressed.

In order to develop a system for purification of immunoglobulin G (IgG), Brockelbank *et al.* engineered the PHA synthase by fusing the IgG binding ZZ domain of protein A from *Staphylococcus aureus* to the PhaC N terminus (69). The IgG binding capacity of the ZZ domain-displaying granules (ZZ-PHA granules) was confirmed by enzyme–linked immunosorbent assay (ELISA). ZZ-PHA granules enabled efficient purification of IgG from human serum and performed equally well compared to commercial protein A-Sepharose beads with regard to both purity and yield (69). In another recent work an anti-β-galactosidase scFv (single-chain variable fragment of an antibody) was immobilized at the surface of PHA granules following the same principle of using PhaC as a self-assembly-promoting fusion partner (70). The scFv-displaying beads were successfully used for specific binding and elution of their antigen β-galactosidase. The functional display of the scFv was further assessed by a quantitative enzyme linked assay measuring β-galactosidase activity. Both approaches indicated the functional display of the antibody fragment at the bead surface which makes these scFv-displaying beads a potential tool for diagnostic or therapeutic applications (70). The main advantage of this system is the simple one-step production as opposed to laborious multiple steps required for immobilization of antibodies using conventional methods.

The strong streptavidin-biotin bond can be used to attach various biomolecules to one another or onto a solid support. This is a powerful tool for purification or detection of these molecules. Protein engineering of streptavidin for *in vivo* assembly of streptavidin beads was recently published by Peters and Rehm (72). Different variants of streptavidin (mature full length, core and monomeric) were tested as C-terminal fusions

to the PHA synthase, and the performance of the enzyme and the resulting streptavidin beads was analyzed. The PHA synthase retained its activity in all fusions, but the mature full length streptavidin performed best with regard to biotin binding. It was demonstrated that the *in vivo* generated streptavidin beads are applicable for ELISA, DNA purification, enzyme immobilization and flow cytometry (72). In another study, Jahns *et al.* employed PHA granules as biological template structures for molecular biomimetics (71). The PHA synthase was fused to genetically engineered proteins for inorganics (GEPIs) and additionally to the ZZ domain of *S. aureus*. This approach resulted in the production of PHA granules with a multifunctional surface displaying both specific binding sites for certain inorganic substances (gold or silica) and for IgG. These bio-beads could serve as suitable tools for medical bioimaging procedures where an antibody-mediated targeted delivery of an inorganic contrast agent is desired (71).

The examples of functionalized bio-beads described so far are based on fusions to the PHA synthase and recombinant production mainly in *E. coli*. In order to expand the range of possible applications, the feasibility of displaying immunologically relevant eukaryotic proteins on the surface of the PHA granules was explored by Bäckström *et al* (132). In this study, mouse myelin oligodendrocyte glycoprotein (MOG) and interleukin-2 (IL2) were individually immobilized at the granule surface *in vivo* in *E. coli* by generating fusions to the C terminus of PhaP (132). Isolated beads displaying either MOG or IL2 were analyzed by fluorescence activated cell sorting (FACS) using monoclonal antibodies that recognize correctly folded MOG or IL2, respectively. Although both proteins are secreted proteins which normally form inclusion bodies when produced in the *E. coli* cytoplasm, they could be successfully produced in a properly folded state at the surface of PHA granules in this host. When an enterokinase recognition site was incorporated between PhaP and IL2, the latter could be cleaved off, demonstrating that the system enables purification of eukaryotic proteins. Moreover, the excellent long term storage performance further supports the potential of these beads for diagnostic applications (132).

In a follow-up proof-of-concept study, bi-functional PHA granules were generated which simultaneously displayed two protein-based functions suitable for FACS analysis (133). GFP was either displayed fused to the N terminus of PhaC and MOG to the C terminus of PhaP or both proteins were fused to the N and C terminus of the phasin, respectively. This showed that bi-functional PHA nanobeads displaying

e.g. a fluorescent protein and a protein with a specific interaction partner (antigen, receptor) could be used in diagnostics.

While most of the published reports on functionalised PHA bionano- and microbeads have involved protein engineering of the phasins or the PHA synthase, Lee *et al.* targeted the substrate binding domain (SBD) of the PHA depolymerase from *Alcaligenes faecalis* (124). They reported the *in vitro* production of microbeads from extracted PHA to which separately synthesized GAP-tagged target proteins could subsequently be bound. Beads of 2μm diameter were generated and the model proteins EGFP, RFP (red fluorescent protein) and SARS-CoV (severe acute respiratory syndrome corona virus) envelope protein were immobilized at the surface via a fusion to the N terminus of the SBD. All model proteins, including the SARS-CoV envelope protein were successfully detected by FACS, which demonstrates the suitability also of this method for generating functionalized PHA beads for e.g. immunoassays or - as the authors suggested - the study of protein-protein interactions.

Targeted drug delivery

There is now general agreement based on a large amount of data that PHA-based medical devices are indeed well tolerated by the human body (134, 135). Thus, functionalized PHA granules seem to be excellent candidates for targeted drug delivery as they combine the properties of a biocompatible polymer with the properties of a bio-bead. Though the *in vivo* PHA production system has the advantage of being relatively cost effective compared to other drug carrier systems on the market, medical applications naturally require materials of extreme purity. Therefore, suitable methods would be needed for endotoxin removal from bacterially produced PHA bionanobeads (16, 136). To date, the biocompatibility of PHA granules has not been specifically studied, but when considering the major constituent of the granules, PHA, it is expected to be similar to the biocompatibility of PHA alone, pending the biological activity (toxicity, immunogenicity) of the surface proteins. Various drug delivery and drug targeting systems based on biodegradable polymers are currently under development (137, 138). Several of the functionalized PHA bio-beads described above would be suitable for targeted drug delivery, but so far there has only been one report of *in vivo* animal tests (139).

Recently, Yao *et al.* exploited one of the *C. necator* phasins as a tag to develop a receptor-mediated drug delivery system (139). PhaP was fused to the cell-specific ligands

mannosylated human α1-acid glycoprotein (hAGP) and human epidermal growth factor (hEGF). hAGP is recognized by receptors on macrophages, hEGF by receptors on hepatocellular carcinoma cells. The fusion proteins were produced in *Pichia pastoris* and *E. coli*, respectively, purified and immobilized on *in vitro* generated and rhodamine B isothiocyanate (RBITC)-loaded PHA beads. Fluorescence microscopic examination showed that both ligand-PhaP-nanobeads were taken up by the correct type of cell *in vitro* and directed to the correct tissue in *in vivo* mouse experiments, demonstrating targeted delivery of the model drug RBITC.

1.14 Outlook

To date, a range of proteins and other molecules have been successfully immobilized at the surface of PHA granules, indicating that these bacterial storage compounds have potential to be developed into powerful tools for diagnostic and therapeutic biomedical applications. A particular advantage of PHA granules as functionalized bionano- and/or microbeads - apart from the simple and cost-effective production - is the oriented immobilization of e.g. proteins via the GAP-tag and thus high binding capacity of the resulting beads. Future work should include improved strategies for size control of *in vivo* produced beads as well as improved methods for pyrogen removal.

1.15 References: Chapter 1B

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Chapter 1C –Introduction to immunity and vaccines

1.16 The immune response and vaccines

The purpose of the immune system is to protect the host from infectious agents and foreign substances. Physical barriers are the first line of defense and include the skin, mucosal secretions, flushing of surfaces by tears or urine, the presence of commensal bacteria and production of anti-microbial products. If these physical barriers are breached then immune responses are activated to detect, control and eliminate invading pathogens. The immune response is usually categorized into two functional systems, innate and adaptive immunity. Both require the host to recognize the invading agent as foreign, but by different processes, and there is interaction between both systems (5).

1.17 Innate immunity

Innate responses occur rapidly, are non-specific, do not require prior exposure to the pathogen and there is no long-lasting memory. Phases of innate immunity involve action of anti-microbial enzymes and peptides, recognition of foreign pathogen, complement activation, recruitment of inflammatory cells, phagocytosis and destruction of the pathogen. The innate response also initiates the adaptive immune response with activation and maturation of antigen presenting cells.

Cells with innate immune functions are neutrophils, macrophages, natural killer cells (NK), mast cells, basophils and dendritic cells.

Pattern recognition receptors (PRRs)

Pattern recognition receptors (PRRs) are found on innate immune cells and recognise evolutionarily conserved pathogen-associated molecular patterns (PAMPs) from microorganisms (42). Damaged host cells can also release molecules such as heparin and heat-shock proteins and these damage-associated-molecular-patterns (DAMPs) are also detected by PRRs. Different cells express different PRRs i.e complement receptor 1 is expressed on many follicular dendritic cells but not conventional dendritic cells.

Toll-like receptors (TLRs) are transmembrane PRRs, expressed as homo-dimers and hetero–dimers, which recognize specific PAMPs. Different cells express different PPRs i.e NK cells express TLR3, TLR7 and TLR8. Ten TLRs have been identified in humans and 12 in mice and they are found in distinct cellular compartments i.e. TLR1, TLR2, TLR4, TLR5 and TLR6 are expressed on the cell surface and recognise molecules derived mainly from bacterial membranes such as lipopolysaccharide (LPS), peptidoglycan and flagellin. Additionally TLR3, TLR7, TLR8, TLR9 and TLR11 are expressed in intracellular vesicles such as endosomes and endoplasmic reticulum and recognise nucleic acids which originate from intracellular viruses and pathogens (30, 51). Recognition of PAMPS by TLRs can activate nuclear factor κβ (NFκβ) and mitogen-activated protein kinase (MAPK) pathways which then induce the expression of pro-inflammatory cytokines, chemokines and co-stimulatory molecules. The interferon regulatory factor (IRF) pathway which induces type-1 interferon production is activated by TLR recognition of viruses (27, 53).

NOD-like receptors (NLRs) are PRRs located in the cytoplasm of cells and there are two major subfamilies: NLRCs (previously called NODs) recognize intracellular bacterial peptidoglycan products, then activate the NFκβ and MAPK pathways to induce production of inflammatory molecules. NLRPs (previously called NALPs) recognise and bind with peptidoglycan PAMPS and also DAMPs such as ATP, released due to hypotonic stress (40). NLRPs then assemble in the large multi-protein inflammasome with other proteins. This structural framework enables activation of caspase-1 and then interleukin-1 (IL-1) and interleukin-18 (IL-18) inflammatory cytokines are released which influences adaptive immunity (40).

RIG-I-like helicases (RLHs) are PPRs which detect cytoplasmic viral RNAs present in the cytoplasm of a virus-infected cell. Sensing of viral RNAs induces the production of interferon-α (IFN-α) and interferon-β (IFN-β). Activation of RLHs can also lead to production of pro-inflammatory cytokines via activation of NFκβ (66)

The mannose binding lectin (MBL) and ficollins are PRRs in blood and body fluids. They bind microorganisms resulting in activation of complement via the lectin pathway.

The mannose receptor found on dendritic cells and macrophages also recognises mannosylated microorganisms and binding of this receptor leads to phagocytosis.

Complement

Complement has a major role in immunity and is activated by several routes involving PRRs (23, 31). The lectin pathway involves serum MBL or ficolins binding pathogen surface carbohydrates. The classical pathway is activated when antigen/antibody complexes are bound by complement recognition factor C1q (41). In the alternative pathway, soluble complement factor C3 undergoes spontaneous hydrolysis and in the presence of factors B, D, and P can be deposited onto the pathogen surface (25, 67). All pathways generate a C3 convertase which cleaves C3 into C3a and C3b. C3b remains bound to the microbial surface and C3a is released. The resultant complement cascade has three major effects: 1) Production of C3a and C5a which act as chemoattractants for phagocytic cells to the site of infection and promote inflammation. C3a and C5a also bind to mast cells and basophils which results in degranulation and release of bioactive agents including histamine; 2) C3b is an opsonin enhancing phagocytosis; 3) The membrane-attack complex (MAC) disrupts cell membranes to cause cell lysis (36, 57).

1.18 Bridges between innate and adaptive immunity.

Innate responses such as TLR signalling, NLR signaling and complement activation can initiate and influence acquired immunity (5, 23). Dendritic cells (DC) are broadly classified as innate cells because they detect foreign proteins via PRR but DC have a major role in initiating the adaptive immune response to naïve T cells (20). Innate PRR and complement contribute to adaptive immunity. TLR ligation can activate both the Th1 and Th2 immune response (38, 68). Innate NFκβ activation resulting from TLR-PAMPs interaction, leads to maturation and up-regulation of co-stimulatory molecules B7.1 (CD80) and B7.2 (CD86) on antigen presenting cells (APC). Major histocompatibility complex (MHC) class I and MHC class II molecules are up-regulated and cytokines are produced which act on the adaptive system. Interleukin-12 (IL-12) release by DC activates NK cells and induces $CD4^+$ T cell differentiation into Th1 cells. The pro-inflammatory cytokine tumour necrosis factor-α (TNF-α) increases vascular permeability, acts as a chemoattractant for neutrophils and adaptive cells, and stimulates phagocytosis by macrophages which in turn enhances adaptive responses. Interleukin-1β (IL-1β) and interleukin-6 (IL-6) are pro-inflammatory cytokines with many similar

innate and adaptive actions to TNF-α. NLRP3 activation stimulates the release of IL-1 and IL-18. These can stimulate T cell differentiation and promote Th17 and Th1 responses (37). Complement has roles in innate immunity and promoting antigen-specific immune response for both antibody and T-cell immunity (31). With regard to vaccinology, complement activation is thought to aid in the clearance of vaccine antigens and is not usually considered or may be actively avoided in vaccine studies. In contrast Reddy *et al.* designed a biomaterial that activates complement so it could act as a molecular adjuvant and PAMP danger signal (55).

1.19 Adaptive immunity

In contrast to innate immunity, the adaptive immune response is slow but results in long-term cellular memory to protect from future assault by pathogens. Adaptive immunity may be the result of natural infection or as a consequence of vaccination (74).

During a primary immune response in the lymph node, DCs process antigen into peptides and present the peptide in association of MHC molecules to naïve T helper cells. MHC class I molecules are expressed by all nucleated cells and are able to process cytosolic antigens into short peptides for presentation to T cells which bear CD8 surface receptors. MHC class II molecules are expressed by APC and these cells process endocytosed extracellular proteins into longer peptides and then present these to $CD4^+$ T cells. In the presence of co-stimulatory molecules, binding of the T cell receptor (TCR) to the antigenic peptide bound to MHC class II on the DC leads to differentiation of $CD4^+$ T helper cells into effector T cells: Th1, Th2, Th17, Tregulatory (Treg) cells and the recently recognised T follicular helper cell (T_{FH}) (35). Differentiation is controlled by the cytokine milieu produced as a result of innate responses (38, 56). However, recent research suggests Th17 and Tregs can have unstable phenotypes (72). Effector T cells proliferate and release cytokines which direct the response towards humoral (predominantly antibody) or cell mediated immunity (CMI). Naïve B cells can be activated in a T-cell dependent or independent manner. The surface immunoglobulin of the B-cell receptor recognises and binds specific antigen in its native form and this binding provides the first signal towards activation. In T cell-dependant activation, the B cell internalizes and degrades the antigen into peptides which can then be displayed at the B cell surface bound to MHC class II molecules. This complex can be recognised by

T cells that have already differentiated in response to the same pathogen. The T cells secrete cytokines such as IL-4, IL-5 and IL-6 which causes the B cell to proliferate and differentiate into antibody-secreting plasma cells and memory cells. In addition, T cell-dependant activation stimulates affinity maturation of the antibody for the antigen and class switching from IgM, especially in the secondary immune response (59). In contrast, T cell-independent (TI) activation of B cells can be induced by two different classes of antigen and results in low affinity antibodies. TI-1 antigens such as lipopolysaccharide (LPS) or DNA activate TLRs expressed by B cells, and this causes polyclonal proliferation and differentiation (24). Alternatively TI-2 antigens such as bacterial capsular polysaccharide have highly repetitive epitopes which are able to cross-link B-cell receptors on the surface of antigen-specific mature B cells (44). DCs prime naïve T cells and in the secondary immune response upon reexposure to specific antigens other APC such as B cells and macrophages play a significant role in progressing the immune response by increasing presentation of antigen to T and B cells. Extracellular pathogens are generally eliminated by antibodies directed towards the surface molecules of the pathogen. Antibodies may also act to neutralize toxins, neutralize viral antigens, opsonise bacteria for subsequent phagocytosis and activate complement for bacterial cell lysis. In contrast, protection against intracellular pathogens, e.g. *Mycobacterium tuberculosis (M. tb)* requires CMI because the organisms are usually inaccessible to the extracellular action of complement and antibody (18). For intracellular pathogens, this CMI involves $CD4^+$ T cells supporting $CD8^+$ cytotoxic T cells (CTL) to lyse the infected target cell which bears processed antigen on its cells surface (65). CMI also involves the production of cytokines e.g. IFN-γ by helper T cells which can activate macrophages for enhanced killing of the pathogen (28). Protective immune responses to viruses usually require a robust CTL response, coupled with interferon cytokine release but antibody often plays a part in protection from re-infection. However, viruses have evolved many mechanisms to evade host defenses so persistence or reinfection is common (47, 48, 52).

The outcome of the primary immune response is to develop a small pool of memory T and B cells so that subsequent exposure to the pathogen will result in rapid differentiation of memory cells into effector cells which quickly proliferate resulting in an enhanced immune response.

1.20 Vaccines

Vaccination involves administration of antigen to induce adaptive immunity. To be effective, vaccines must be able to initiate an appropriate immune response towards the target antigen without causing unwanted effects (43). Traditionally, vaccines were composed of either whole organisms or inactivated toxin e.g. tetanus toxoid. The organisms could be either live attenuated microorganisms (e.g. morbillivirus, the cause of measles) or killed bacteria (e.g. *Bordetella pertussis,* the cause of whooping cough). Live vaccines often stimulate life-long immunity with a single vaccination but can potentially revert to virulence or cause disease, especially in immunocompromised hosts (21). For these safety reasons, modern licensing requirements prefer vaccines with defined components. Vaccines may need the addition of adjuvants and/or immunostimulatory molecules for successful activation of T cells and memory responses (4). TLR and NLR agonists can be incorporated into vaccines to ensure activation of innate responses. For example monophosphoryl lipid A, derived from LPS, signals via TLR4 and has been incorporated into a number of vaccines (1, 7, 69). Also Pam3Cys, a synthetic lipopeptide can be used in vaccines to induce signalling through TLR2/TLR1 (63, 69). TLR9 activation has been achieved by the use of unmethylated CpG motifs in a range of vaccines (26, 32). Activation of NLRP3 is important in respiratory disease and therefore vaccines which target NLRP3 is an area for vaccine development (37). Recently it has been shown that vaccine adjuvants such as alum target both NLRs and TLRs (12).

Earliest recordings of 'inoculation' to protect humans from small pox date back to India in the 8th century and China in the 10th century. Jenner is attributed with developing this practice in 1876 and vaccination has since been used to successfully prevent many diseases which affect humans and animals. However, there are many diseases which affect animals and humans for which there is no suitable vaccine. Effective prophylactic vaccines have been the focus of most research but therapeutic vaccines, predominantly for the treatment of cancer, are an emerging human-health field of research.

Most currently used vaccines elicit an antibody response. In contrast, vaccines to protect against disease caused by intracellular pathogens need vaccines which stimulate CMI and such sub-unit vaccines comprising known antigens and compounds are under development using a range of technologies. Some of these are discussed later in this section. Vaccines have been developed to protect from many viral infections but

a number of viruses still require effective vaccines. Influenza viruses are adept at antigenic drift and shift which makes vaccine production an annual challenge (9). Persistent viruses such as *Herpesviridae*, human immunodeficiency-virus and hepatitis viruses are able to subvert the immune system resulting in suppression of MHC class I molecules and impairment of DC maturation (3, 13). Therefore development of vaccines for these viruses requires further research.

Uptake of vaccine antigens

The entry process of microorganisms or their antigens to APC varies (10). Soluble protein or peptide vaccines have poor efficacy and often need to be combined with adjuvants to promote uptake by immune cells, usually through TLR activation (20). The immune response can be enhanced by co-administration of antigen and immunomodulators, often TLR ligands, on the same particle (15). Vaccine antigen delivery usually targets peripheral dendritic cells but a recent study found that targeting lymph-node resident DC was more likely to produce an immune response (54). Particulate vaccines are more readily taken up by dendritic cells and various studies indicate particle size affects immune responses. A Th1 response was initiated following vaccination with nanobeads ≤49nm and both Th1 and Th2 responses followed vaccination with larger 93-123nm beads (46). Similarly nano-vaccines with carrier size 40-50nm was shown to promote both Th1 response and IgG antibody, although sub-class was not tested (17). Some researchers (29) make the broader statement that micron-sized particles promote a Th2 response and nanoparticles promote a Th1 response. This view is quite widely held but in contrast a different study showed lipid vesicles with a size ≥225 nm induced a predominant Th1 response and vesicles ≤155nm induced a Th2 response (8). Intradermal vaccination experiments measuring interstitial flow of nanoparticles to lymph-node resident dendritic cells determined 50% of 25nm particles were found in the node compared to 6% of 100nm particles (54). It is often difficult to make direct comparisons between papers because of the different immunisation schemes, types of vaccine delivery system and antigens being used (49).

These papers show that immune responses can be tailored dependant on particle size but also highlight that manufacturing processes need to be controlled for particle size consistency. The adaptive immune response has developed in response to challenge by microorganisms of particular sizes and so it is logical to design vaccines similarly.

New technologies for particulate vaccines

There are a number of new technologies which are being used to deliver vaccine antigens, often with the goal of achieving a cell mediated immune response. Some of these are summarized below.

Virus like particles (VLP) have recently been developed and used as vaccine delivery systems for human diseases of cervical cancer caused by human papilloma virus (19) and hepatitis B (39). These VLP elicit an immune response from the derived human virus but recent research has aimed to develop versatile VLP from non-human viruses to avoid pre-existing neutralizing antibody responses (50).

Liposomes are vesicles of spherical shape that can be produced from non-toxic phospholipids and cholesterol. Liposomes are used for delivery of cancer drugs and antibiotics. Cationic liposomes have positively charged lipids and these interact with negatively charged cell membranes to enhance cellular uptake. They can be formulated with immunostimulatory molecules to enhance adjuvant properties (58). Virosomes are a type of liposomes that contain viral envelope proteins. They are similar to VLPs but are produced *in vitro* from native virus particles rather than the recombinant system of VLP. Virosomes are used as hepatitis A and influenza vaccines (16).

Chitosan is a polysaccharide derived form the exoskeleton of crustaceans but can be formulated in many ways to change solubility and degradation. Alginate-coated chitosan hepatitis B antigen nanoparticles were used for oral vaccination (6) or as an adjuvant (71) and conjugation to gold for DNA vaccination enhanced antibody responses (73). Chitosan has also been combined with poly D, L-lactide-co-glycolic acids (PLGA) to decrease cargo release rate while enhancing particle uptake by cells (22, 34). These papers suggest chitosan may be good for oral delivery but formulation must be carefully controlled.

Immunostimulatory compounds (ISCOMS) are formed when saponins such as QuilA are added to liposomes. CTL responses are primarily achieved (45) which is an ideal immune response to viral infections.

Biopolyesters, alone and in combination with other polyesters are commonly used in the formation of nanoparticles and microparticles for *in vivo* use. These are described in Chapter 1B but a brief summary is included here. Of the many

biopolyester, polylactic acids (PLA) and PLGA are highly biocompatible, biodegradable and have FDA approval for *in vivo* use. PLA and their co-polymers are used extensively for sutures and drug delivery (2, 62). Vaccine use is limited because micron rather than nanometre size particle size may not be optimum to elicit CMI (29). PLGA have been used in a variety of vaccine delivery systems (14, 60, 64). However some studies have shown immunisation with PLGA has given a varied response which may be due to degradation during production (11). PLGA vaccines have been shown to activate the NLRP-3 inflammasome leading to pro-inflammatory cytokine release and enhanced innate and adaptive immune responses (61). Polyhydroxybutyrate (PHB), the most common polyhydroxyalkanoate biopolyester has a range of uses as outlined in Chapter 1B and more recent *in-vivo* studies have been completed (33, 70). As an extension to *in-vivo* uses of PHB, this thesis incorporates investigations into the production and suitability of PHB as vaccine delivery beads.

1.21 References: Chapter 1C

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Aims and scope of this project

While many of particulate vaccine systems are effective and several are in current production, disadvantages arise in high production costs with expensive recombinant protein manufacture. Processes and production strategies to enable cost-effective vaccines would have benefits for both humans and animals. Animal vaccines need a low cost/unit to encourage wide-use. Higher costs are usually acceptable for human vaccines but if typical vaccine production costs were reduced then this would be more attractive to third-world countries or for use in animals.

Antibody responses obtained as a consequence of vaccination are most appropriate for diseases caused by extracellular microorganisms. Diseases such as tuberculosis, toxoplasmosis and neosporosis are caused by intracellular microorganisms and create a huge burden to human and animal health. Vaccines which stimulate CMI and are effective at reducing such intracellular infections remain elusive. In addition, protection against many viral infections require both an antibody and CTL responses and vaccines tailored for these responses are needed.

Polyesters produced by bacteria have previously been shown to have a wide-range of *in vitro* and *in vivo* uses. PHAs can be produced by *in vitro* production processes and are being used safely in medical devices such as sutures, stents and bone scaffolds but the high production and purification costs can be a limiting factor in their use as vaccine delivery vehicles. As an alternative, PHB polyester bead carriers with vaccine proteins displayed on the beads have a simpler manufacturing process than other biopolyesters. Investigation into production of PHB beads suitable for vaccination is an area which warranted further investigation.

The broad aim of the study was to use a polymer biosynthesis pathway to produce PHB beads for use as versatile vaccine delivery agents which could be engineered to display a choice of vaccine antigen on the surface of the beads.

Specific aims:

- **To determine if PHB beads could be produced which display vaccine antigens.** Initially, this entailed insertion of PHB synthesis genes from *Cupriavidus necator* and mycobacterial antigen genes into an *Escherichia coli* production strain. These genes encoded for PHB synthesis enzymes (PhaA, PhaB and PhaC) and the mycobacterial fusion protein, comprising antigen-85A and 6 kDA early secretory antigenic target (Ag85A-ESAT-6). Following bacterial growth, bead formation was observed using fluorescence microscopy and electron microscopy. Polyester in bacterial cells was determined by gas chromatography-mass spectroscopy (GC-MS). PHB beads were purified and assessed using SDS-PAGE, matrix-assisted laser desorption-ionization time-of-flight mass spectroscopy (MALDI-TOF), enzyme linked immunosorbent assay (ELISA) and flow cytometry. Electron microscopy was used to assess both intracellular beads and purified beads. In addition, wild-type PHB beads which did not display vaccine antigens were made for use as a control.

- **Could different bacterial production hosts can be used to display antigens on PHB beads?** Initially *E. coli* was used as a bacterial production host and then *Lactococcus lactis* was introduced as an alternative, due to its lack of LPS endotoxin, previous use as a production host for recombinant proteins and history of safe use for a range of human foods and products.

- **To ascertain if specific immune responses could be stimulated following vaccination with PHB beads and was an adjuvant required.** Firstly, recombinant proteins were made in *E. coli* for use as vaccination controls. These proteins were insoluble so extensive experimentation was required to ensure an appropriate product. Then vaccination dose-response experiments using PHB beads which displayed antigen, with and without an adjuvant were carried out in mice. Antibody isotype and IFN-γ were initially measured but this was expanded to include a range of other cytokines in later experiments. Statistical analysis was used to compare groups of vaccinated mice.

- **To determine if an effective immune response had developed.** Following vaccination of mice with PHB beads which displayed Ag85A-ESAT-6, tuberculosis challenge studies were undertaken to find out if there was a correlation between immune response and protection from disease. In addition to assessing immune responses, spleens and lungs were analysed by histopathology and cultured for mycobacteria and compared to the gold-standard *Mycobacterium bovis* Bacille Calmette-Guérin (BCG). Statistical analysis was used to compare groups of vaccinated and challenged mice.

- **Were there differences in immune responses when different production hosts or different antigens were used?** Initial experiments used mycobacterial antigens displayed on PHB beads. Subsequently, to expand the repertoire of PHB vaccine beads hepatitis C core antigen genes, instead of mycobacterial genes, were inserted into *E. coli and L. lactis* bacterial production hosts and vaccination trials in mice were completed and immune responses measured and assessed statistically.

- **To demonstrate the uptake of PHB beads by dendritic cells and mechanism of the initial immune response.** Dendritic cells are thought to initiate adaptive immune responses, so PHB beads were incubated with human and mouse dendritic cells. Transmission electron microscopy (TEM) was used to visualize the uptake, then activation markers of dendritic cells were analysed and cytokine release by cells was measured. Monocyte derived human DCs and mouse bone-marrow derived DCs were cultured with PHB beads. Antibodies to TLR2 and TLR4 were used to investigate the role of signaling via these TLRs. TLR4 knock-out mice (TLR4--/-) were used in an alternative experiment.

Chapter 2

Bacterial polyester inclusions engineered to display vaccine candidate antigens for use as a novel class of safe and efficient vaccine delivery agents

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2.1 Abstract

Bioengineered bacterial polyester inclusions have the potential to be used as a vaccine delivery system. The biopolyester beads were engineered to display a fusion protein of two key antigens involved in the immune response to the infectious agent that causes tuberculosis *Mycobacterium tuberculosis*, notably Antigen 85A (Ag85A) and the 6 kDa early secreted antigenic target (ESAT-6) from *Mycobacterium tuberculosis*. Polyester beads were successfully produced (henceforth called Ag85A-ESAT-6 beads) by recombinant *Escherichia coli* and these beads abundantly displayed the tripartite fusion protein comprising the two antigens and the bead forming enzyme, PhaC, at its surface. The ability of the Ag85A-ESAT-6 beads to enhance immunity in mice to the displayed antigens was investigated. The beads were not toxic for the animals, as determined by weight gain and absence of lesions at the inoculation site in immunized animals. *In vivo* injection of the Ag85A-ESAT-6 beads in mice induced significant humoral and cell-mediated immune responses to both Ag85A and ESAT-6. Vaccination with Ag85A-ESAT-6 beads was efficient at stimulating immunity on their own, and this ability was enhanced by administration of the beads in an oil-in-water emulsion. In addition, vaccination with the Ag85A-ESAT-6 beads induced significantly stronger humoral and cell-mediated immune responses than vaccination with an equivalent dose of the fusion protein Ag85A-ESAT-6 alone. The immune response induced by the beads was of a mixed Th1/Th2 nature, as assessed from the induction of the cytokine interferon-gamma (Th1 immune response) and increased levels of IgG1 (Th2 immune response). Hence engineered biopolyester beads displaying foreign antigens represent a new class of versatile safe and biocompatible vaccine.

2.2 Introduction

Bioengineered nano-/micro-structures manufactured by microorganisms are becoming increasingly attractive because of their functional properties suitable for applications in various fields, particularly the medical sciences (9, 25, 29). Biopolyester beads comprising polyhydroxyalkanoate (PHA) are produced as intracellular inclusions by a wide range of bacteria and Archaea when a carbon-source is available in excess (30). PHA synthesis requires the key enzyme, polyester synthase, to catalyze the stereo-selective polymerization of (*R*)-3-hydroxyacyl-CoA to PHA. Self-assembly of polyester chains results in the formation of polymer granules with a hydrophobic core and the

PHA synthase protein remains covalently attached at the surface (28). These spherical granules range in size from 50 to 300 nm and accumulate in the intracellular space (34).

Such biopolyester beads can be engineered to display the PHA synthase protein and its fusion partners on the surface at high density (23). There have been recent examples where biopolyester beads were specifically engineered, produced in bacteria and then harvested for their potential applications as life science tools. For example, biopolyester beads have been produced which display the IgG binding domain ZZ from protein A (6) for use as an alternative to protein A latex beads for a variety of diagnostic tests. Another study produced beads which displayed green fluorescent protein (GFP) to enable tracking following *in vivo* administration (22). Beads have been developed with covalently attached enzymes, suggesting an application in immobilization and stabilization of biocatalysts (24). Recently, biopolyester beads have been produced which display immobilized antibody single chain fragments (scFv) as well as multiple binding functions including the binding of inorganic compounds (4, 11, 14).

Our interest in these biopolyester beads is to explore their properties for use as vaccine delivery agents. Potential advantages associated with using these beads as vaccine delivery agents include their size, versatility and inherent biocompatibility with living tissues. Particles smaller than 2 μm in size are readily phagocytosed by macrophages and dendritic cells (20) suggesting the value of using nano-/micro-sized particles as vaccine delivery systems. The concept of using nano-/micro-particles for delivering vaccines has already been explored, for example, biodegradable biocompatible polyesters polylactide and poly-D, L-lactide-co-glycolic acid have been used as vaccine delivery systems (31) or carriers of adjuvant systems (15). Employing PHA beads for delivery of vaccines may present additional advantages, such as low cost, ease of production and mode of surface functionalization. Novel vaccines are required for a variety of infectious diseases, including tuberculosis, for which no truly efficacious vaccine has yet been designed (16). A number of antigens have been considered for developing new tuberculosis vaccines (3, 19, 33). Early secreted antigenic target 6-kDa protein (ESAT-6) is found in *Mycobacterium bovis* and *Mycobacterium tuberculosis* but not in the vaccine strain *M. bovis* BCG (12). This antigen is recognized immunologically in tuberculosis infected humans (27), cattle (26) and mice (5). The Ag85 complex is composed of three homologous proteins Ag85A, Ag85B and Ag85C (1). Antigen 85A

has been used in a number of immunisation studies and has been shown to elicit an immune response and in some cases, enhanced protection (10, 13).

This paper describes the development and microbial production of bio-engineered biopolyester beads displaying on their surface a functional antigen comprising a fusion protein of polyester synthase, Ag85A and ESAT-6 and subsequent evaluation of antigen-specific immune responses in immunized mice.

2.3 Materials and methods

Bacterial strains and growth conditions.

Escherichia coli DH5α (Invitrogen. CA, USA) was grown in Luria broth (Difco, Detroit, MI, USA), supplemented with 1% (w/v) glucose and ampicillin (75 μg/ml). Media for growth of *E. coli* BL21 (DE3) (Invitrogen) in addition contained chloramphenicol (30 μg/ml).

Plasmids, oligonucleotides and construction of plasmids for production of Ag85A-ESAT-6 displaying polyester beads.

All plasmids and oligonucleotides are listed in Table 1. DNA sequences of new plasmid constructs were verified by DNA sequencing. In addition to the polyester synthase gene (*phaC*), PHA biosynthesis requires the enzymes PhaA and PhaB for precursor synthesis, and these enzymes were encoded by plasmid pMCS69. The plasmid DK1.2-Ag85A-ESAT-6 containing a hybrid gene comprised of the coding region (without the secretory signal sequence) of Ag85A (N-terminal component) and the coding region of ESAT-6 (C-terminal component) was a kind gift from Lynne Slobbe, University of Otago, New Zealand (36). A DNA fragment, encoding the Ag85A-ESAT-6 fusion protein and including a translation initiation site and start codon was isolated from this plasmid by PCR using primers Ag85A-*Spe*I and ESAT-6-*Spe*I and ligated into the vector pCWE *Spe*I at the *Spe*I restriction site. The resultant construct, pCWE *Spe*I-Ag85A-ESAT-6 had the mycobacterial fusion gene at the N-terminus of PhaC and downstream of p*lac*. Bacteria transformed with this plasmid did not produce detectable levels of polyester and consequently a second construct was made using the pET-14b based plasmid pHAS which contains the T7 promoter. The DNA segment comprising the Ag85A-ESAT-6-phaC hybrid gene was obtained by hydrolysis of pCWE *Spe*I-Ag85A-ESAT-6 with *Xba*I and *Cla*I and subcloned into pHAS using the restriction sites *Xba*I and *Cla*I sites of pHAS to generate the plasmid pHAS-Ag85A-ESAT-6. This

construct was used to produce biopolyester beads displaying the vaccine candidate antigen Ag85A-ESAT-6 to be evaluated in this study.

Table 1: Bacterial strains and plasmids used in this study

Strains/Plasmids	Description	Source/reference
Strains		
E. coli BL21(DE3)	F⁻ *ompT hsdS*$_B$(*r*$_B$⁻ *m*$_B$⁻) *gal dcm* (DE3)	Invitrogen
DH5α	DH5α F⁻ *end A1 hsd R17* (r_k^-, m_k^-) *sup E44 thi-1* λ^- *rec A1 gyr A96 rel A1* Δ*(lac-arg)* F⁻	Invitrogen
Plasmids		
pET-14b	Ap^r,T7 promoter	Novagen
pHAS	pET14b derivative containing the *Nde*I/*Bam*HI inserted *phaC* gene from *C. necator*	(35)
pMCS69	pBBR1MCS derivative containing genes *phaA* and *phaB* from *C. necator*	(2)
pCWE *Spe*I	pBluescript SK(-) derivated containing the PHA synthase gene from *C. necator*	(23)
DK1.2-Ag85A-ESAT-6	pBluescript II SK (+) containing fusion between Ag85A and ESAT-6	(32)
pCWE *Spe*I-Ag85A-ESAT-6	pCWE derivative containing Ag85A-ESAT-6 hybrid gene inserted into *Spe*I site	This study
pHAS-Ag85A-ESAT-6	pHAS containing Ag85A-ESAT-6 hybrid gene upstream of *pha*C	This study
Oligonucleotides		
Ag85A-*Spe*I	5'-gctactagtaataaggagatatacatatgttttcccggccggg-cttgc-3'	This study
ESAT-6-*Spe*I	5'-tgcactagttgcgaacatcccagtgacgtt-3'	This study

***In vivo* functionality of the polymer synthesizing enzyme PhaC.**

To assess whether the PhaC fusion partner still catalyses polyester synthesis and mediates intracellular granule formation, the polyester content of bacterial cells harboring the various plasmids was assessed by gas chromatography-mass spectroscopy (GC-MS) analysis. The amount of accumulated polyester corresponds to the *in vivo* PhaC activity. Polyester content was quantitatively determined by GC-MS after conversion of the polyester into 3-hydroxymethyl ester by acid-catalyzed methanolysis.

Isolation of polyester granules.

Polyester granules were isolated as previously described (25). Briefly, bacteria were disrupted and the whole cell lysate was centrifuged at 4000 *g* for 15 min at 4°C to sediment the polyester beads. Beads were purified via glycerol gradient ultracentrifugation.

Analysis of proteins attached to the polyester beads.

The concentration of proteins attached to the beads was determined using the *Bio-Rad Protein* Assay, (Bio-Rad; CA, USA). Proteins were separated by SDS-PAGE using NuPAGE® gels (Invitrogen) and stained with SimplyBlue Safe Stain (Invitrogen). The amount of Ag85A-ESAT-6 PhaC fusion protein relative to the amount of total proteins attached to the beads was detected using a Gel Doc™ XR and analysed using Quantity One software (version 4.6.2) (Bio-Rad laboratories, Hercules, CA, USA). 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).

Enzyme-linked immunosorbent assay (ELISA).

Maxisorb plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with purified Ag85A-ESAT-6 beads or wild-type beads, diluted in carbonate-bicarbonate coating buffer, pH 9.6 (Sigma-Aldrich) ranging from 1 mg/ml to 0.015 mg/ml protein concentration over serial dilutions. Plates were washed with phosphate buffered saline (PBS) containing 0.05% (v/v) Tween 20 (PBST) and blocked with 3% (w/v) bovine serum albumin (BSA) in PBS for 2 h at 25°C. Plates were then washed in PBST and incubated with mouse antibody to ESAT-6 (Abcam, Cambridge, UK). Following washing with PBST, plates were incubated for 1 hour at room temperature with anti-mouse IgG:horse radish peroxidase conjugate (Sigma-Aldrich) diluted in 1% (w/v) BSA in PBS. After further washing, o-phenylenediamine (OPD) substrate (Sigma-Aldrich) was added and incubated for 30 minutes at room temperature. The reaction was stopped with 0.5 M H_2SO_4 and the absorbance was recorded at 495 nm on a VERSAmax microplate reader.

Flow cytometry.

Twenty-five micrograms of purified Ag85A-ESAT-6 beads or wild-type beads were washed twice in ice-cold flow cytometry buffer (PBS, 1% (w/v) foetal calf serum

(FCS), 0.1% (w/v) sodium azide) and detected with mouse anti-ESAT-6 antibodies (Abcam, Cambridge, UK). Following washing in flow cytometry buffer, beads were stained with rat anti-mouse Fluorescein isothiocyanate (FITC) labeled antibody (BD Pharmingen, CA, USA), incubated for 30 minutes on ice in the dark and washed again. A BD FACScalibur (BD Biosciences, CA, USA) was used to collect at least 10,000 events for each sample and analysed using CellQuest software.

Immunisation of mice.

All animal experiments were approved by the AgResearch Grasslands Animal Ethics Committee (Palmerston North, New Zealand). Groups of female C57BL/6 mice (purchased from the animal breeding facility of the Malaghan Institute of Medical Research, Wellington, New Zealand) aged 6-8 weeks old were immunized by the subcutaneous route, 3 times at 2 week intervals with wild-type beads, Ag85A-ESAT-6 beads or recombinant Ag85A-ESAT-6 protein either alone or mixed with 20% (v/v) Emulsigen™ (MVP Laboratories, Omaha, NE, USA) adjuvant (100 μL/injection). Non-immunized or PBS immunized control animals were included in each set of experiments.

Immunological assays.

Three weeks after the last immunisation all mice were anaesthetized intraperitoneally using 87 μg ketamine (Parnell laboratories, Alexandria, NSW, Australia) and 2.6 μg xylazine hydrochloride (Bayer, Leverkusen, Germany) per g of body weight. Blood was collected by cardiac puncture, allowed to clot and centrifuged prior to serum being collected and frozen at -20°C until assayed. Mice were euthanized, spleens removed and a single cell suspension was prepared by passage through an 80 gauge wire mesh sieve. Spleen red blood cells were lysed using a solution of 17 mM Tris-HCl and 140 mM NH_4Cl. After washing, the cells were cultured in DMEM (Dulbecco's Modified Eagle media, Invitrogen) supplemented with 2 mM glutamine (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 5 x 10^{-5} M 2-mercaptoethanol (Sigma) and 5% (w/v) FCS (Invitrogen) in triplicate wells of flat-bottomed 96 well plates at a concentration of 5 x 10^5 cells/well in a 200 μL volume. The cells were incubated with medium alone or in medium containing either Ag85A or ESAT-6 (5 μg/ml) or a combination of both antigens. Concanavalin A (ConA; Sigma, final concentration of 5 μg/ml) was used as a positive control. Cells were incubated at 37 °C and 10% CO_2 in air.

Measurement of IFN-γ.

Culture supernatants were removed after 4 d of incubation and frozen at -20°C until assayed. Levels of IFN-γ in culture supernatants were measured by ELISA according to manufacturer's recommendations (BDBiosciences) using a commercial pair of antibodies and standards (BD Pharmingen). Briefly, maxisorb plates (Nunc) were coated overnight with monoclonal antibody, washed and blocked with PBS containing 10% (w/v) FCS. After washing, culture supernatants or standards were added to the wells and plates incubated. After further washing, biotinylated detection antibody was added, incubated and washed prior to addition of an avidin horseradish peroxidase conjugate. The assay used o-phenylenediamine (OPD) substrate, and was read at 495nm on a VERSAmax microplate reader. Standard curves were constructed using SOFTmax PRO software and cytokine values were determined from the curve. Limit of detection for IFN-γ was 0.05 ng/ml.

Measurement of serum antibody.

Antibody in sera was measured by ELISA. Maxisorb (Nunc) plates were coated overnight with 5 μg/ml Ag85A or ESAT-6 and then blocked using 10% (w/v) FCS in PBS. After washing in PBST, dilutions of serum (from 1/10 - 1/10000) were added and incubated. Following washing, anti-mouse IgG1:HRP or IgG2c:HRP (ICL, Newberg, Oregon, USA) was added and plates incubated. Plates were washed and TMB used as a substrate prior to reading at 450 nm on a VERSAmax microplate reader. Monoclonal anti-ESAT-6 or anti-Ag85A antibodies (Abcam) were titrated and included as a positive control for the IgG1 plates. Results were expressed in titres representing the reciprocal of the serum dilution which gave half the maximal OD.

Statistical Analysis.

Analyses of the IFN-γ and antibody responses were performed by Kruskal-Wallis one-way analysis of variance.

2.4 Results

2.4.1 Microbial production and characterisation of biopolyester beads displaying Ag85A-ESAT-6 at their surface.

Plasmids pHAS-Ag85A-ESAT-6 and pHAS were introduced into *E. coli* BL21 (DE3) cells harbouring plasmid pMCS69 and the transformants cultured to produce

Ag85A-ESAT-6 and wild-type beads, respectively. GC/MS analysis of respective cells confirmed the presence of the polyester, polyhydroxybutyrate, indicating functionality of the polyester synthase domain in the tripartite fusion protein (data not shown). The presence of intracellular polyester inclusions was further confirmed by fluorescent microscopy using Nile Red staining as previously described (21).

The proteins associated with the Ag85A-ESAT-6 beads and the wild-type beads were separated by SDS-PAGE (Fig. 1). The beads displayed high levels of the respective overproduced protein as indicated by a prominent protein with an apparent molecular mass of 102 and 63 kDa for Ag85A-ESAT-6-PhaC and PhaC, respectively. The identity of these proteins was confirmed by tryptic peptide fingerprinting using MALDI-TOF-MS. Densitometry analysis of the gels indicated that the Ag85A-ESAT-6-PhaC protein accounted for 20% of the total protein associated with the Ag85A-ESAT-6 beads.

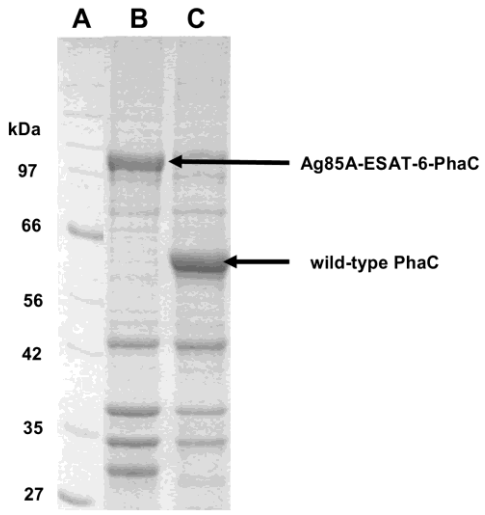

Figure 1: SDS-PAGE analysis of proteins attached to the polyester beads.
Lane A, molecular weight markers; Lane B, beads isolated from *E. coli* harboring plasmid pHAS-Ag85A-ESAT-6; Lane C, beads isolated from *E. coli* harboring plasmid pHAS. The presence of the Ag85A-ESAT-6-PhaC fusion protein (indicated with the arrow) was confirmed by tryptic peptide fingerprinting analysis using MALDI-TOF-MS.

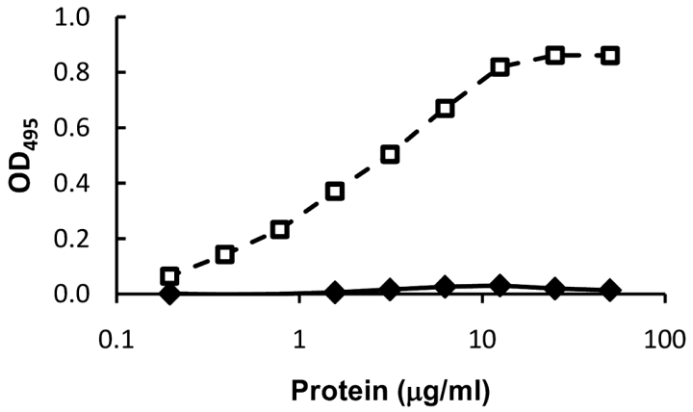

Figure 2: Display of ESAT-6 protein on the bead surface was assessed by ELISA. Beads were diluted from 0.2 – 80 μg/ml on an ELISA plate and then incubated with anti-ESAT-6 antibody. Bead bound antibody was detected using a HRP-conjugated secondary antibody. Results show that the antibody binds to Ag85A-ESAT-6 beads (- - □ - -) but not to wild-type (—♦—). Each data point represents the mean of three separate determinations and the standard deviation was less than 5% of the means.

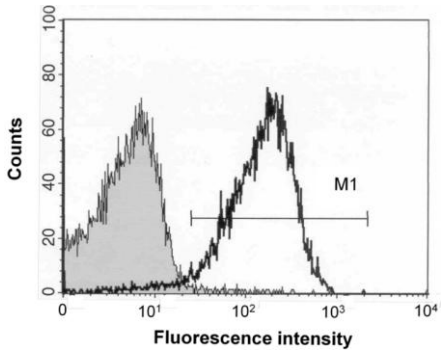

Figure 3: Flow cytometry of bioengineered beads displaying ESAT-6. Wild-type beads or Ag85A-ESAT-6 beads were incubated with anti-ESAT-6 antibody and bound antibody was detected using FITC-conjugated secondary antibody. Fluorescent events were counted and plotted on a graph comparing fluorescence intensity of wild-type beads (shaded plot) and Ag85A-ESAT-6 beads (clear plot on the right of the flow cytometry profile). Only Ag85A-ESAT-6 beads displayed ESAT-6 as indicated by the bar marker M1.

The presence of antigenic ESAT-6 at the surface of the Ag85A-ESAT-6 beads was assessed by ELISA. The results shown in Figure 2 indicated that Ag85A-ESAT-6 beads bound to the anti-ESAT-6 antibody in a dose-dependent manner. Specific binding of anti-ESAT-6 antibody to Ag85A-ESAT-6 beads was measured using flow cytometry and the results showed that >98% of Ag85A-ESAT-6 beads bound anti-ESAT-6 antibodies (Fig. 3).

2.4.2 Immunisation of mice with Ag85A-ESAT-6 beads stimulates the generation of an antigen specific humoral and cellular immune response.

Mice were immunized with wild-type beads, Ag85A-ESAT-6 beads alone, Ag85A-ESAT-6 beads formulated in Emulsigen or recombinant Ag85A-ESAT-6 protein alone by the subcutaneous route. Following immunisation, no overt toxicity was observed in any of the animals. Mouse weights did not differ significantly between groups during the time-course of the experiment, and mice in all groups gained weight (data not shown). Mice immunized with polyester beads developed small lumps (2.5 mm in diameter) at the immunisation sites but no abscess or suppuration was observed and all mice were healthy throughout the trial with normal behaviour and good quality fur (data not shown).

Immune responses were determined in immunized animals, to assess the potential of the polyester beads to act as vaccine delivery agents. A dose response experiment where mice were immunized with varying doses of beads displaying Ag85A-ESAT-6 determined that beads which display 30 μg of Ag85A-ESAT-6 is sufficient to generate both a significant antibody response (Fig. 4) and an IFN-γ (Table 2) response in mice. In addition, this dose of Ag85A-ESAT-6 beads induced significantly higher antibody titres and IFN-γ responses when compared to a 30 μg dose of recombinant Ag85A-ESAT-6 protein alone ($P<0.01$). A similar dose of Ag85A-ESAT-6 beads was used in a second experiment which included non-immunized control animals and compared beads formulated with and without the adjuvant, Emulsigen. Antigen-specific serum antibody responses were significantly higher in both vaccine groups given Ag85A-ESAT-6 beads compared to non-immunized mice ($P<0.01$) and the highest antibody responses were observed in mice immunized with Ag85A-ESAT-6 beads in Emulsigen (see Fig. 6). Antibody responses for the IgG1 isotype were stronger than responses for IgG2 in both experiments (Figs. 4 & 5).

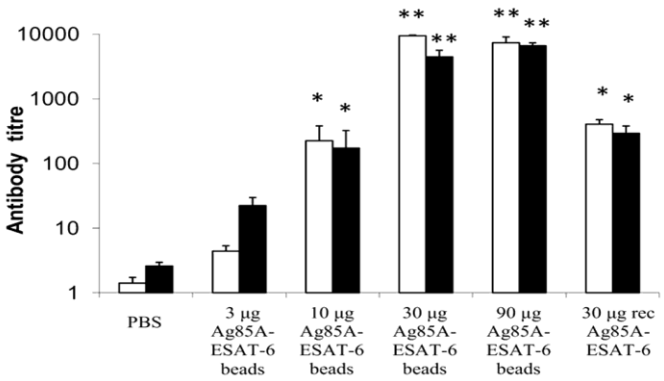

Figure 4:
Antibody responses in mice immunized 3 times with 0-90 µg beads displaying Ag85A-ESAT-6 or 30 µg recombinant Ag85A-ESAT-6. Sera were obtained 3 weeks after the final immunisation. Levels of Ag85A-ESAT-6 specific antibodies of the IgG1 isotype (white bars) or the IgG2 isotype (black bars) were measured by ELISA. Each data point represents results from 5 mice ± SEM. Results are expressed in reciprocal antibody titres, representing the dilution required to obtain half of the maximal amount of OD signal. * Significantly greater than the PBS immunized control group (P<0.01). ** Significantly greater than all other vaccine groups (P<0.01).

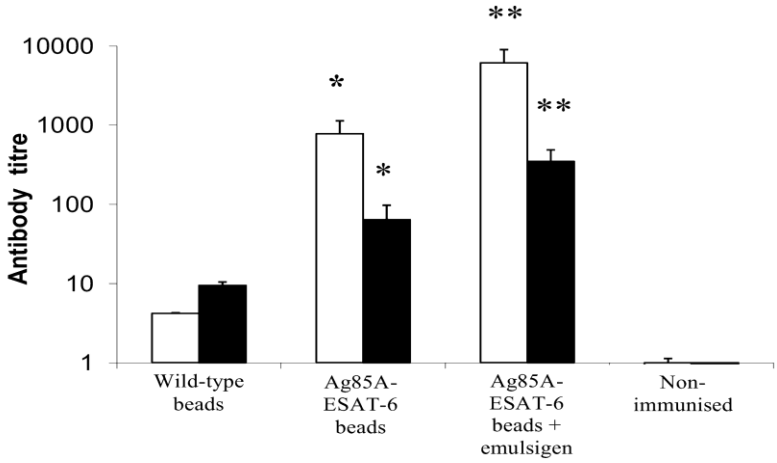

Figure 5:
Antibody responses in mice immunized 3 times with 30 µg of wild-type beads, Ag85A-ESAT-6 beads, Ag85A-ESAT-6 beads with Emulsigen or non-immunized. Sera were obtained 3 weeks after the final immunisation. Levels of Ag85A-ESAT-6 specific antibodies of the IgG1 isotype (white bars) or the IgG2 isotype (black bars) were measured by ELISA. Each data point represents results from 7 mice ± SEM. Results are expressed in reciprocal antibody titres, representing the dilution required to obtain half of the maximal amount of OD signal.
* Significantly greater than the non-immunized control group (P<0.01).
** Significantly greater than all other vaccine groups (P<0.01).

Table 2: Mean Interferon-γ responses from groups of immunized mice

Immunisation group	IFN-γ response (ng/ml +/- SEM)
PBS	0.29 +/- 0.11
3 μg Ag85A-ESAT-6 beads	2.26 +/- 0.24
10 μg Ag85A-ESAT-6 beads	4.48 +/- 0.56 *
30 μg Ag85A-ESAT-6 beads	4.60 +/-0.43 *
90 μg Ag85A-ESAT-6 beads	2.80 +/- 0.20
30 μg rec Ag85A-ESAT	1.76 +/- 0.32

Mice were immunized 3 times. Three weeks after final immunisation, splenocytes were cultured for 3 days with 5 μg Ag85A-ESAT-6 and the release of IFN-γ was measured in the supernatant by ELISA. * Significantly greater than all other vaccine groups (P<0.01).

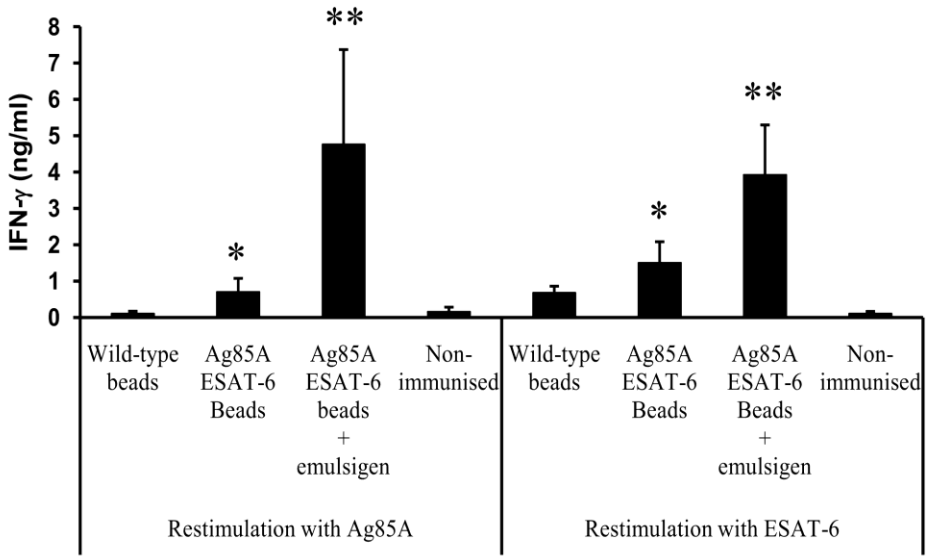

Figure 6: IFN-γ responses in mice immunized 3 times with 30 μg of wild-type beads, Ag85A-ESAT-6 beads, Ag85A-ESAT-6 beads with Emulsigen or non-immunized.

Splenocytes were cultured for 3 days with 5μg Ag85A or ESAT-6 and the release of IFN-γ was measured by ELISA. Each data point represents results from 7 mice ± SEM. * Significantly greater than the non-immunized control group (P<0.01). ** Significantly greater than all other vaccine groups (P<0.01).

A marker of the development of cell-mediated immunity was assessed by measuring the release of the key cytokine IFN-γ in splenocytes restimulated *in vitro* with proteins used for immunisation. Immunisation with Ag85A-ESAT-6 beads significantly enhanced the cell mediated immune response to Ag85A-ESAT 6 (Table 2) and to individual component antigens, namely Ag85A and ESAT-6 ($P<0.01$) (Fig. 6). This enhancement was improved by formulating the beads in Emulsigen.

2.5 Discussion

This study assessed the potential use of engineered bacterial polyester inclusions as a particulate vaccine-delivery system utilising the natural intracellular production of polyester beads by bacteria as a one-step production system, which does not require adsorption or conjugation of an antigen to a polymeric particle. A hybrid gene encoding a tripartite fusion protein Ag85A-ESAT-6-PhaC was successfully overproduced in recombinant *E. coli* mediating formation of polyester beads with the fusion protein attached to its surface. Surface display of the *M. tuberculosis* antigens Ag85A-ESAT-6 was confirmed by ELISA and flow cytometry.

Polyester beads produced in this study were injected in mice to analyse the respective humoral and cell-mediated immune response. Immunisation using beads which displayed 30 μg Ag85A-ESAT-6 were shown to induce significantly higher antibody and IFN-γ responses compared to immunisation with 30 μg recombinant Ag85A-ESAT-6 protein alone. Formulation of the beads in the adjuvant emulsigen further enhanced the specific and significant immune response. The type of immune response which develops in response to a vaccine can be crucial for various diseases. A humoral antibody response characterized by IgG1 in mice is most useful for diseases caused by extracellular pathogens whereas a cell-mediated response characterized by increased IFN-γ and IgG2 antibody is most valuable in diseases caused by intracellular pathogens, of which tuberculosis is a classic example. Tuberculosis kills approximately 2 million human deaths each year (8) and novel vaccine strategies are urgently required against this pathogen (16) because the live, attenuated *M. bovis* BCG vaccine currently used has shown highly variable protection (16). This study has shown that the use of engineered polyester beads which displayed mycobacterial antigens resulted in a specific and significant cell mediated immune response. The lack of adverse side effects such as weight loss, and no abscess or suppuration at injection site suggested that the polyester

beads are safe and non-toxic. Further studies are planned to test these vaccines for protection of mice against challenge with *M. tuberculosis*.

The versatility and potential of the bead antigen delivery system to elicit different complementary facets of the immune response could be applied to the development of multivalent vaccines (7). Furthermore, the immune responses might be enhanced by the co-display of antigen and immunostimulatory molecules on the same biopolyester bead. Immunostimulatory molecules could be used to enhance particular components of the immune response such as Toll-like receptor agonists for cellular immunity (17).

Delivery of drugs or vaccines using biocompatible particulate vehicles is an area currently gaining significant momentum. Cell-free vaccine-delivery systems are often particulate (e.g. emulsions, micro-/nano-particles and liposomes) and their dimensions compare to those of pathogens, which the immune system has evolved to inactivate (18). However such particulate systems have to be chemically synthesized and processed to enable adsorption of the separately produced and purified protein antigen (7). By comparison, this study utilizing engineered bacteria as a production host for polymeric carriers of protein subunit vaccines seems to provide an affordable and efficient alternative.

In summary, our findings showed that bioengineered polyester beads are safe and efficient delivery systems for immunisation purposes. This unique approach to design and produce these polyester beads for antigen display allows tailoring of vaccines for incorporation of specific antigens or immunostimulants without the need to produce, purify and conjugate recombinant proteins (18).

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Chapter 3 Vaccines displaying mycobacterial proteins on biopolyester beads stimulate cellular immunity and induce protection against tuberculosis

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3.1 Abstract

New improved vaccines are needed for control of both bovine and human tuberculosis. Tuberculosis protein vaccines have advantages with regards to safety and ease of manufacture, but efficacy against tuberculosis has been difficult to achieve. Protective cellular immune responses can be preferentially induced when antigens are displayed on small particles. In this study, *Escherichia coli* and *Lactococcus lactis* were engineered to produce spherical polyhydroxybutyrate (PHB) inclusions, which displayed a fusion protein of *Mycobacterium tuberculosis,* Ag85A-ESAT-6. *L. lactis* was chosen as a possible production host due its extensive use in the food industry and reduced risk of lipopolysaccharide contamination. Mice were vaccinated with PHB bead vaccines with or without displaying Ag85A-ESAT-6, recombinant Ag85A-ESAT-6 or *M. bovis* BCG. Separate groups of mice were used to measure immune responses and assess protection against an aerosol *M. bovis* challenge. Increased antigen-specific IFN-γ, IL-17A, IL-6 and TNF-α were produced from splenocytes post-vaccination, but no or minimal IL-4, IL-5 or IL-10 was produced, indicating Th1 and Th17 biased T cell responses. Decreased lung bacterial counts and less extensive foci of inflammation were observed in lungs of mice receiving BCG or PHB bead vaccines displaying Ag85A-ESAT-6 produced in either *E. coli* or *L. lactis* compared to that for the PBS control mice. No differences were observed for those receiving wild-type PHB beads or recombinant Ag85A-ESAT-6. This versatile particulate vaccine delivery system incorporates a relatively simple production process using safe bacteria and the results show that it is an effective delivery system for a tuberculosis protein vaccine.

3.2 Introduction

Mycobacterium bovis, the causative agent of bovine tuberculosis (TB), infects a wide range of hosts, including domestic livestock and wildlife, and also causes TB in humans. Bovine TB poses a public health risk particularly in regions where pasteurization of milk is not routine. This is of particular concern with more than 94% of the world's population living in such regions, in which *M. bovis* is the causative agent for up to 10% of TB cases in humans (14). Bovine TB also has considerable economic impact on the agricultural industry. The human TB vaccine, *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) is only partially effective in both cattle and humans (2, 12). Development of an effective vaccine protecting against bovine TB would provide a

cost-effective TB control strategy as well as having applicability for control of human TB caused by *Mycobacterium tuberculosis*.

A number of new TB vaccines are entering human clinical trials including recombinant BCG, virus-vectored vaccines and recombinant protein vaccines (20). One of the major constraints in developing effective recombinant protein vaccines is the difficulty of inducing the strong cellular immune responses which are required for protection against this disease. Selection of appropriate adjuvants and presentation of the proteins is critical. A number of studies have shown that antigens displayed on small particles preferentially enhance cellular immune responses to antigens (32, 47). Particles used to display antigens in vaccines include virus-like particles, liposomes, immune stimulating complexes, biological polyesters and these have been tested in a wide-range of veterinary and wild-life species (45). The particles appear to have adjuvanting effects with uptake by dendritic cells and consequential activation of the NALP-3 inflammasome (46). We have previously demonstrated that the mycobacterial fusion protein, Ag85A-ESAT-6 could be displayed on polyhydroxybutryate (PHB) polyester beads produced in *Escherichia coli* and when used as a vaccine in mice could stimulate enhanced antigen-specific interferon-γ (IFN-γ) responses compared to the recombinant protein alone (37).

The advantages of using these beads for vaccine delivery are their low-cost, ease of production, mode of surface protein display and inherent biocompatibility. To produce these PHB polyester beads, *E. coli* was engineered to produce a fusion protein of PhaC polyester synthase, Ag85A and ESAT-6. The mycobacterial fusion protein, Ag85A-ESAT-6 has been successfully used in a number of experimental studies to investigate protective immunity against TB (15, 25, 48). The early secreted antigenic target 6-kDa protein (ESAT-6) is found in *M. bovis* and *Mycobacterium tuberculosis* but not in BCG (18). This antigen is recognized immunologically in TB infected humans and animals (11, 40, 41) and the virulence properties of ESAT-6 to recruit CD11c+ and T cells can be utilized to advantage for vaccine development (26). Similarly, Ag85A has long been recognised as an immunodominant antigen (22) and is incorporated in a recombinant modified vaccinia virus Ankara-expressing Ag85A vaccine currently in human trials as a BCG boosting vaccine (27, 35). Ag85 complex consists of three proteins which possess mycolyl-transferase activity and play a role in the biogenesis of mycobacterial cell wall (7).

A disadvantage of using *E. coli* as a production host for products with *in vivo* use is the contamination with lipopolysaccharide (LPS) endotoxins. However, processes used to remove LPS are costly and can destroy surface proteins and functionality of the particles (55). *Lactococcus lactis,* an organism which does not produce LPS, has been used extensively in the food industry and recently been used for recombinant protein production, delivery of therapeutic agents, vaccines and TB skin-test reagents (8, 30, 52, 56). Therefore *L. lactis* might be a suitable production host for PHB bead TB vaccines.

This paper describes engineering of the food grade bacterium *L. lactis* to produce biopolyester PHB beads displaying mycobacterial antigens, Ag85A-ESAT-6. Vaccines were prepared from PHB beads produced in *L. lactis* or *E. coli* and used to immunize mice. Immune responses were measured and following aerosol challenge with *M. bovis* the protective immunity against TB was assessed.

3.3 Materials and Methods

Construction of plasmids for production of PHB beads displaying Ag85A-ESAT-6

Plasmids used in this study are listed in the supplemental file. General cloning procedures and DNA isolation were carried out as described elsewhere (44). Biosynthesis of PHB polyester requires genes for enzymes PhaA, PhaB and PhaC. To construct pNZ-Ag85E6-CAB, for use in *L. lactis,* the gene encoding a fusion of the antigens Ag85A and ESAT-6 was synthesized by GeneScript Corporation (Piscataway, NJ). Codon usage was adapted to the codon usage bias of *L. lactis*. A fragment of pUC57-ZZ comprising part of the *nisA* promoter (P_{nisA}) was obtained by NdeI digest of pUC57-ZZ and ligated with NdeI-digested pUC57-Ag85E6 to obtain pUC57-nisAg85E6. A BstBI-BamHI fragment of pUC57-nisAg85E6 containing the section of P_{nisA} and the Ag85A-ESAT-6 gene was then inserted upstream of *phaB* at the corresponding sites of pNZ-AB, resulting in pNZ-Ag85E6-B. To introduce the *phaC* and *phaA* comprising fragment of pNZ-CAB into pNZ-Ag85E6-B, both plasmids were hydrolyzed with NheI and BamHI and the *phaCA* fragment of pNZ-CAB was inserted into pNZ-Ag85E6-B, resulting in pNZ-Ag85E6-CAB. This plasmid was electroporated into *L. lactis* (NZ9000) for subsequent production of PHB beads. For production of wild-type control PHB beads, the genes (*phaA, phaB* and *phaC*) were engineered into *E. coli* BL21(DE3) and *L. lactis* NZ9000 using methods as previously described (28, 39) and

construction of plasmids for production of Ag85A-ESAT-6 PHB beads in *E. coli* was undertaken as described elsewhere (37).

Bacterial strains, growth conditions and isolation of PHB beads

E. coli strains were grown in Luria broth (LB; Difco, Detroit, MI) supplemented with 1% (w/v) glucose, ampicillin (75 μg/ml) and chloramphenicol (30 μg/ml). *L. lactis* strains were grown in M17 medium (Merck, Darmstadt, Germany) supplemented with 0.5% glucose, 0.3% L-arginine and chloramphenicol (10 μg/ml).

PHB beads which displayed Ag85-ESAT-6 or control PHB beads alone were produced in *E. coli* and *L. lactis* as previously described (28, 37). Briefly, *E. coli* was grown at 30°C in LB, induced with 1mM isopropyl β-D-thiogalactopyranoside to produce protein and cultured for a further 48 hours to allow accumulation of beads. *L. lactis* cultures were produced in M17 broth, induced with 10 ng/ml nisin to produce protein and cultured for a further 48 hours at 30°C. Presence of PHB/polyester was determined by staining the cultures with Nile Red lipophilic dye, then using fluorescence microscopy to observe fluorescence associated with the intracellular beads (38). Bacteria were then mechanically disrupted and *E. coli* lysate was centrifuged at 4000 × *g* and *L. lactis* lysate was centrifuged at 8000 × *g* for 15 min at 4°C to sediment the polyester beads. All beads were then purified via glycerol gradient ultracentrifugation. Polyester production was determined by measuring PHB content of the granules using gas chromatography-mass spectroscopy (GC-MS) (10)

Analysis of proteins attached to the PHA beads

The concentration of proteins attached to the PHB beads was determined using the *Bio-Rad Protein* Assay (Bio-Rad; CA, USA). Proteins were separated by SDS-PAGE using NuPAGE gels (Invitrogen, Carlsbad, CA, USA) and stained with SimplyBlue Safe Stain (Invitrogen). The amount of Ag85A-ESAT-6:PhaC fusion protein relative to the amount of total proteins attached to the PHB beads was detected using a Gel DocTM XR and analyzed using Quantity One software (version 4.6.2) (Bio-Rad laboratories, Hercules, CA, USA). For Western blot analysis, proteins were separated by SDS-PAGE and transferred to nitrocellulose using an i-BLOT system (Invitrogen). A mouse monoclonal primary antibody against ESAT-6 (Abcam, Cambridge, UK) was used at a 1:800 dilution. Following incubation with rabbit anti-mouse peroxidase-conjugated immunoglobulin G (DAKO, Carpinteria, CA, USA), development was carried out using

aminoethylcarbazole. To confirm the identity of the protein of interest, a band was excised from the gel and subjected to tryptic peptide fingerprinting using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Specific activity of the Ag85A-ESAT-6 PHB beads was determined by enzyme-linked immunosorbent assay (ELISA) assay as previously described (37).

Recombinant protein antigen and peptides

Recombinant Ag85A-ESAT-6 protein (recAg85A-ESAT-6) was produced as previously described (48) with some variation. Briefly, *E. coli* BL21 Star™ (DE3)pLysS (Invitrogen) was transformed with pAg85-ESAT-6 in pET32A (a kind gift from Lynne Slobbe, Otago University, NZ) and grown in Terrific Broth. The insoluble recombinant protein in the cell culture pellet was solubilised in 6M urea and proteins separated by SDS-PAGE using NuPAGE® gels. The band of interest was excised from the gel and protein eluted using a Bio-Rad model 422 Electroeluter (Bio-Rad, CA, USA) according to manufacturer's instructions. The protein was then refolded by dialysis in decreasing concentrations of urea, desalted using a desalt column (Pierce, Rockford, IL, USA) and treated with polymyxin B-agarose (Sigma Chemicals, St. Louis, MO, USA) to remove contaminating LPS. The activity of the recAg85A-ESAT-6 was then confirmed by ELISA.

Vaccination of mice

Vaccines comprising PHB beads displaying Ag85A-ESAT-6 antigen produced in *E. coli* (EcAgE) and *L. lactis* (LcAgE) were adjusted to contain 30 μg of Ag85A-ESAT-6-PhaC protein as calculated from the densitometry profile. Similarly control wild-type vaccines produced in *E. coli* (EcWT), and *L. lactis* (LcWT), were adjusted to contain 30 μg of the PhaC alone protein. Emulsigen (MVP Laboratories, Omaha, NE, USA) adjuvant (20% v/v) was mixed with the various PHB beads, 30 μg recAg85A-ESAT-6 or PBS.

Female C57BL/6 mice aged 6 to 8 weeks (purchased from the animal breeding facility of the Malaghan Institute of Medical Research, Wellington, New Zealand) were vaccinated 3 times subcutaneously at 9 day intervals; 200 μl/injection (n=12 per group). A control group received a single dose of 10^6 colony forming units of BCG Pasteur strain 1173P2 (a kind gift from the Malaghan Institute of Medical Research). All animal experiments were approved by the AgResearch Grasslands Animal Ethics Committee (Palmerston North, New Zealand).

Cell preparations and immunological assays

Five weeks after the first vaccination half of the animals in each group were euthanized, spleens removed and a single cell suspension was prepared by passage through an 80 gauge wire mesh sieve. Spleen red blood cells were lysed using a solution of 17 mM Tris-HCl and 140 mM NH_4Cl. After washing, the cells were cultured in DMEM (Dulbecco's Modified Eagle media, Invitrogen) supplemented with 2 mM glutamine (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 5 x 10^{-5} M 2-mercaptoethanol (Sigma), 1 x non-essential amino acids (Gibco, Grand Island, NY), and 5% (w/v) foetal bovine serum (Invitrogen) in triplicate wells of flat-bottomed 96 well plates at a concentration of 5 x 10^5 cells/well in a 200 μL volume. The cells were incubated with medium alone or in medium containing a pool of Ag85A or ESAT-6 or Ag85A and ESAT-6 overlapping peptides (5 μg/ml final concentration; Auspep, Vic, Australia). Concanavalin A (5 μg/ml; Sigma) was used as a positive control. Cells were incubated at 37 ºC in an atmosphere of 10% CO_2 in air. Spleen cultures from BCG-vaccinated mice were also cultured with bovine purified protein derivative (PPD, 5 μg/ml; Prionics AG, Switzerland)

Measurement of cytokines in culture supernatants

Levels of IFN-γ and interleukin-5 (IL-5) in culture supernatants were measured by ELISA using commercial pairs of antibodies and standards (BD Biosciences, Franklin Lakes, NJ, USA). The assay used o-phenylenediamine substrate, and was read at 495nm on a VERSAmax microplate reader. Standard curves were constructed using SOFTmax PRO software and the averages of duplicate sample cytokine values were determined from the curve. A cytometric bead array (CBA; Mouse Th1-Th2 cytokine kit, BD) was used according to manufacturer's instructions to measure other cytokines: interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), tumour necrosis factor-α (TNF-α) and interleukin-17A (IL-17A). Fluorescence was measured using a FACScalibur flow cytometer (BD), and analyzed using FCAP array software (BD). Results for all cytokines were calculated as the cytokine value of the Ag85A-ESAT-6 stimulated sample minus the PBS-stimulated sample.

Mycobacterium bovis challenge and necropsy

Fifteen weeks after the first vaccination, all remaining mice (n = 6 per group) were challenged with *M. bovis* (strain 83/6235) by the aerosol route. *M. bovis* was grown from a low-passage seed lot in Tween albumin broth: Tween 80, Dubos broth base, and

oleic acid-albumin-dextrose (Difco, BD Diagnostic Systems, Sparks, MD) to early mid-log phase and aliquots of cultures were frozen at -70°C, until required. To infect mice by low-dose aerosol exposure, diluted thawed stock were administered using a Madison chamber aerosol generation device calibrated to deliver approximately 50 bacteria into the lungs. Aerosol infections, maintenance and manipulation of infected mice were performed under strict isolation conditions in a biohazard facility.

Five weeks after challenge with *M. bovis*, the mice were euthanized, and spleens and lungs removed. The right apical lung lobe was removed from the lung and preserved in 10% buffered formalin, for subsequent histological processing, followed by staining of sections with Ziehl-Neelsen stain and haematoxylin and eosin stain (H&E). The lung lesion areas were quantified relative to total lung area on randomly selected, H&E stained tissue sections. The total lung and lesion area were quantified using a stereology based method referred to as the Area Fraction Fractionator with the investigator blinded to the treatment groups. The area of inflammation relative to total lung area was estimated from sections evaluated at 200X magnification. A total of 8 to 12 fields was selected randomly by the computer and a counting frame (2000 μm^2) containing probe points with a grid spacing of 200 μm, was used to define the areas of interest. The data are expressed as the mean percentage of lung affected by lesions of all the animals within a treatment group (n=6). Lesions represented by photomicrographs are individuals that have a value closest to the mean value for the entire treatment group.

Mycobacterial culture of lungs and spleens

Spleen and remaining lung samples were mechanically homogenized in 3 ml of PBS with 0.5% Tween 80 using a Seward Stomacher® 80 (Seward, Norfolk, UK) and plated in tenfold dilutions on selective Middlebrook 7H11 agar supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment (Difco). Plates were incubated at 37°C in humidified air for 3 weeks before counting colonies.

Statistical analysis

Cytokine responses were analysed using the Kruskal-Wallis test. Data for analyses of histopathology percent lung lesion involvement were square root-transformed and bacterial counts from the *M. bovis*-challenged mice were log_{10}-

transformed and the transformed data compared by Fisher's one-way analysis of variance. The level of significance was set at $p < 0.05$.

3.4 Results

3.4.1 Production and characterization of biopolyester beads displaying Ag85A-ESAT-6 in *L. lactis* and *E. coli*

Plasmids encoding polyester synthase and Ag85A-ESAT-6 were successfully introduced into *L. lactis*, which enabled production of beads displaying Ag85A-ESAT-6. The presence of intracellular inclusions was observed by fluorescent microscopy using Nile Red staining (shown in supplemental file) and GC-MS analysis of cells confirmed the presence of the polyester, PHB (data not shown). *E. coli* was also used to produce beads displaying Ag85A-ESAT -6 as previously described (35). The size of the beads was shown to be 50-150 nm for *L. lactis* produced beads and 150-250 nm for beads produced in *E. coli*. Following purification of the beads from *L. lactis* hosts, the proteins associated with the Ag85A-ESAT-6-PhaC beads and the wild-type PhaC control beads were separated by SDS-PAGE. Proteins with molecular masses similar to the molecular mass of 102 kDa for the Ag85A-ESAT-6-PhaC fusion and 63kDa for PhaC were observed and Western blot analysis with anti-ESAT-6 antibody demonstrated a predominant band at approximately 102 kDa which corresponds to the Ag85A-ESAT-6-PhaC fusion (Fig. 1). The identity of the Ag85A-ESAT-6-PhaC band was confirmed by tryptic peptide fingerprinting using MALDI-TOF MS (shown in supplemental file). PHB beads displaying Ag85A-ESAT-6 from *E. coli* and *L. lactis* host were shown by ELISA to bind to anti-ESAT-6 antibody in a dose-dependent manner (shown in supplemental file).

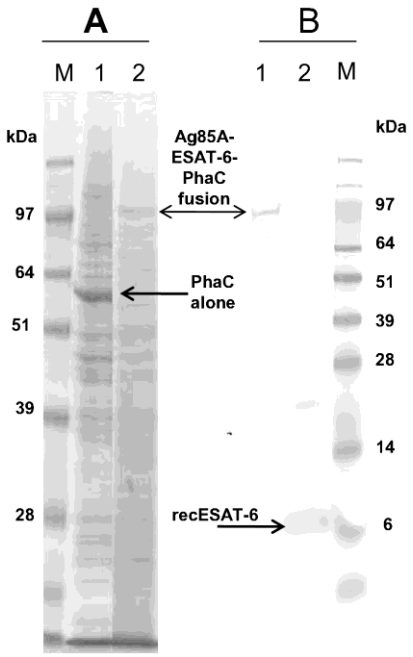

Figure 1: SDS-PAGE analysis of proteins attached to the polyester beads
(A). PHB beads isolated from *L. lactis* NZ9000 harboring the following plasmids: Lane 1, pNZ-CAB. Lane 2, pNZ-Ag85E6-CAB. Molecular weight markers are lane M. Western blot (B): reactivity of proteins to ESAT-6 antibody. Lane 1, PHB beads isolated from *L. lactis* NZ9000 harboring plasmid pNZ-Ag85E6-CAB; Lane 2: recombinant ESAT-6; Lane M, molecular weight markers (kDa)

3.4.2 Clinical and immunological responses to vaccination

Mouse weights did not differ significantly between groups during the time-course of the experiment, and mice in all groups gained weight steadily. Mice vaccinated with PHB beads developed small lumps up to 2.5 mm in diameter at the vaccination sites with no signs of abscess or suppuration. No lumps were observed in other vaccine groups.

To assess development of Th1 cell-mediated immunity, splenocytes were restimulated *in vitro* with a pool of Ag85A and ESAT-6 peptides and released cytokines measured. Vaccination of mice with PHB beads displaying Ag85A-ESAT-6 produced in both *E. coli* and *L. lactis,* stimulated the generation of an antigen-specific cellular immune response compared to PBS-vaccinated group. The vaccine groups receiving PHB beads displaying Ag85A-ESAT-6 produced in both *E. coli* and *L. lactis* produced significantly more IFN-γ, IL-2, IL-6, TNF-α and IL-17A compared to the group receiving PBS (Fig 2, $p<0.05$). The only significant increase in cytokines released from splenocytes of the recAg85A-ESAT-6 group compared to PBS control mice was for IL-2 (Fig 2B, $p<0.05$).

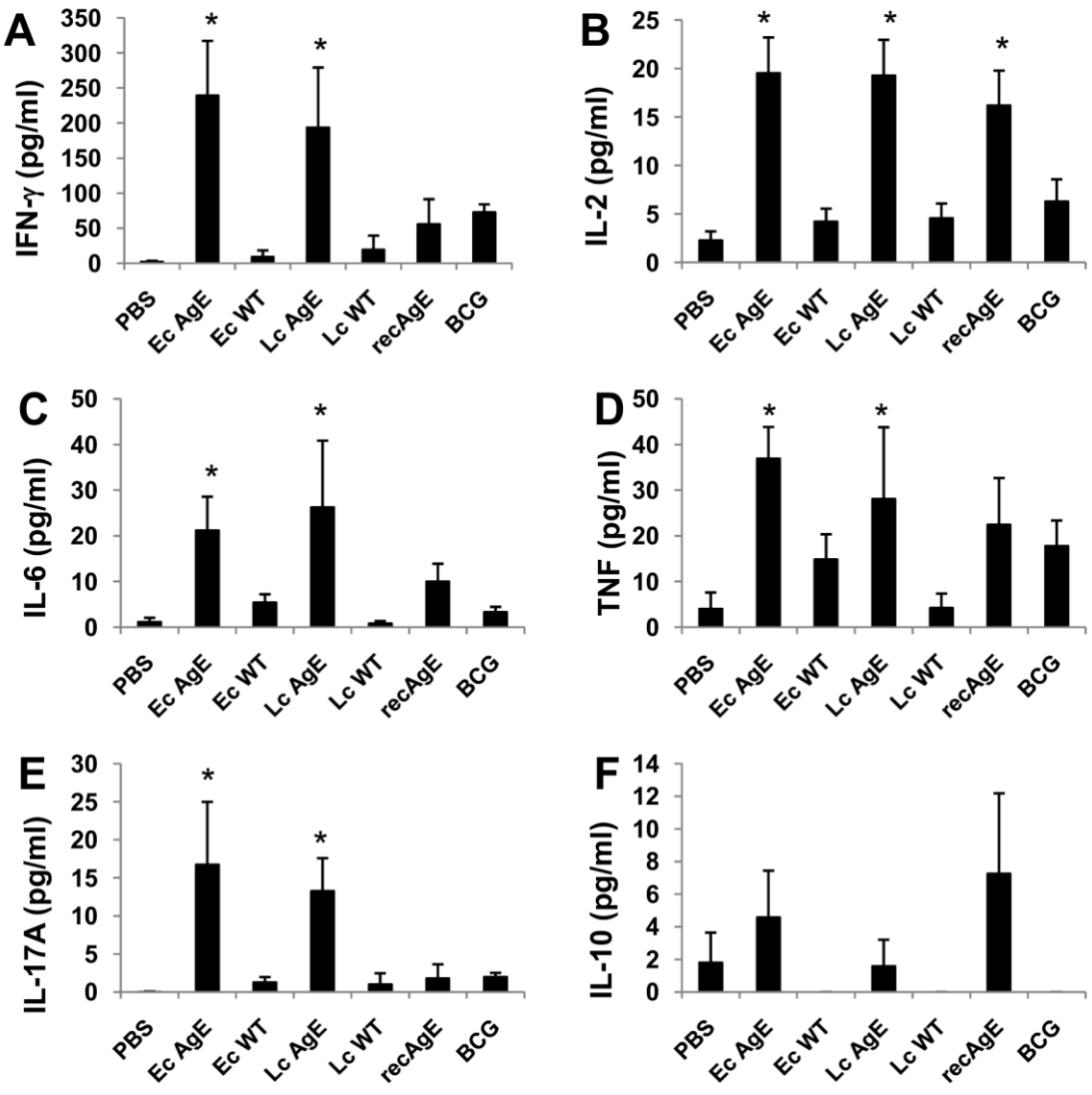

Figure 2: Cytokine responses

Cytokine responses mice vaccinated 3 times with **PBS**, PHB beads produced in *E. coli* displaying Ag85A-ESAT-6 **(EcAgE)**, control wild-type PHB beads produced in *E. coli* **(EcWT**), PHB beads displaying Ag85A-ESAT-6 produced in *L. lactis* **(LcAgE**), control wild-type PHB beads produced in *L. lactis* **(LcWT)** and recombinant Ag85A-ESAT-6 protein **(recAg85A-ESAT-6),** all in Emulsigen™ as well as mice vaccinated once with BCG vaccine **(BCG**). Three weeks after final vaccination, splenocytes were cultured for 3 days with a pool of Ag85A and ESAT-6 peptides. Release of (A) IFN-γ was measured by ELISA and other cytokines (B) IL-2, (C) IL-6, (D) TNF-α, (E) IL-17A, (F) IL-10 were measured by cytometric bead array. Results were calculated as the cytokine value of the Ag85A and ESAT-6 peptide pool stimulated sample minus the value of the PBS-stimulated sample. Each data point represents the mean for 6 mice ± SEM. * Significantly greater than the PBS-vaccinated control group ($p<0.05$).

The wild-type control PHB groups did not show any significant increase in any of these cytokines measured compared to PBS-vaccinated mice. IL-10 responses were very low and there no significant differences between groups (Fig 2). IL-5 and IL-4 were measured but were only detected in very low amounts and only in one to two animals in any group (data not shown). The results of IFN-γ released from splenocytes stimulated with Ag85A or ESAT-6 peptides, or the combined pool of Ag85A and ESAT-6 peptides are shown in the supplemental file. Responses for the groups vaccinated with PHB beads displaying Ag85A-ESAT-6 produced in both *E. coli* and *L. lactis* were strongest for the pool of Ag85A and ESAT-6 peptides and weakest for the Ag85A peptides. BCG-vaccinated mice produced a significant increase in release of IFN-γ from splenocytes stimulated with bovine PPD compared to the PBS- vaccinated group (data not shown).

3.4.3 Histopathology

The *M. bovis*-infected lung lobes from the PBS-vaccinated mice had multiple, coalescing foci of granulomatous inflammation composed predominantly of epithelioid macrophages and lymphocytes (Fig. 3). In acid-fast (AF) stained sections, intracellular bacilli were observed in many of the macrophages that made up the lesions (not shown). The lungs of mice vaccinated with BCG or PHB beads displaying Ag85A-ESAT-6 produced in *E. coli* or *L. lactis* had loosely organized accumulations of inflammatory cells. These lesions were smaller, less extensive with fewer lymphocytes and macrophages than those from PBS-vaccinated mice and were often within the perivascular parenchyma. The lung lesions in mice vaccinated with wild-type PHB beads or recAg85A-ESAT-6 were similar to those of the PBS controls. Morphometric analysis was used to determine the percentage of normal parenchyma replaced by inflammatory lesions. BCG vaccinated mice had significantly less lung lesion involvement than those vaccinated with wild-type PHB beads produced in either *E. coli* or *L. lactis* or for the PBS-vaccinated group ($p<0.05$, Fig. 4). PHB beads displaying Ag85A-ESAT-6 produced in *E. coli* had significantly less lung lesion involvement than wild-type beads produced in *E. coli* ($p<0.05$). Although, PHB beads displaying Ag85A-ESAT-6 produced in *L. lactis* had less lung lesion involvement than wild-type beads produced in *L. lactis* this difference was not statistically significant ($p=0.11$). Differences between the various groups vaccinated with the PHB beads and the PBS control group were not significant.

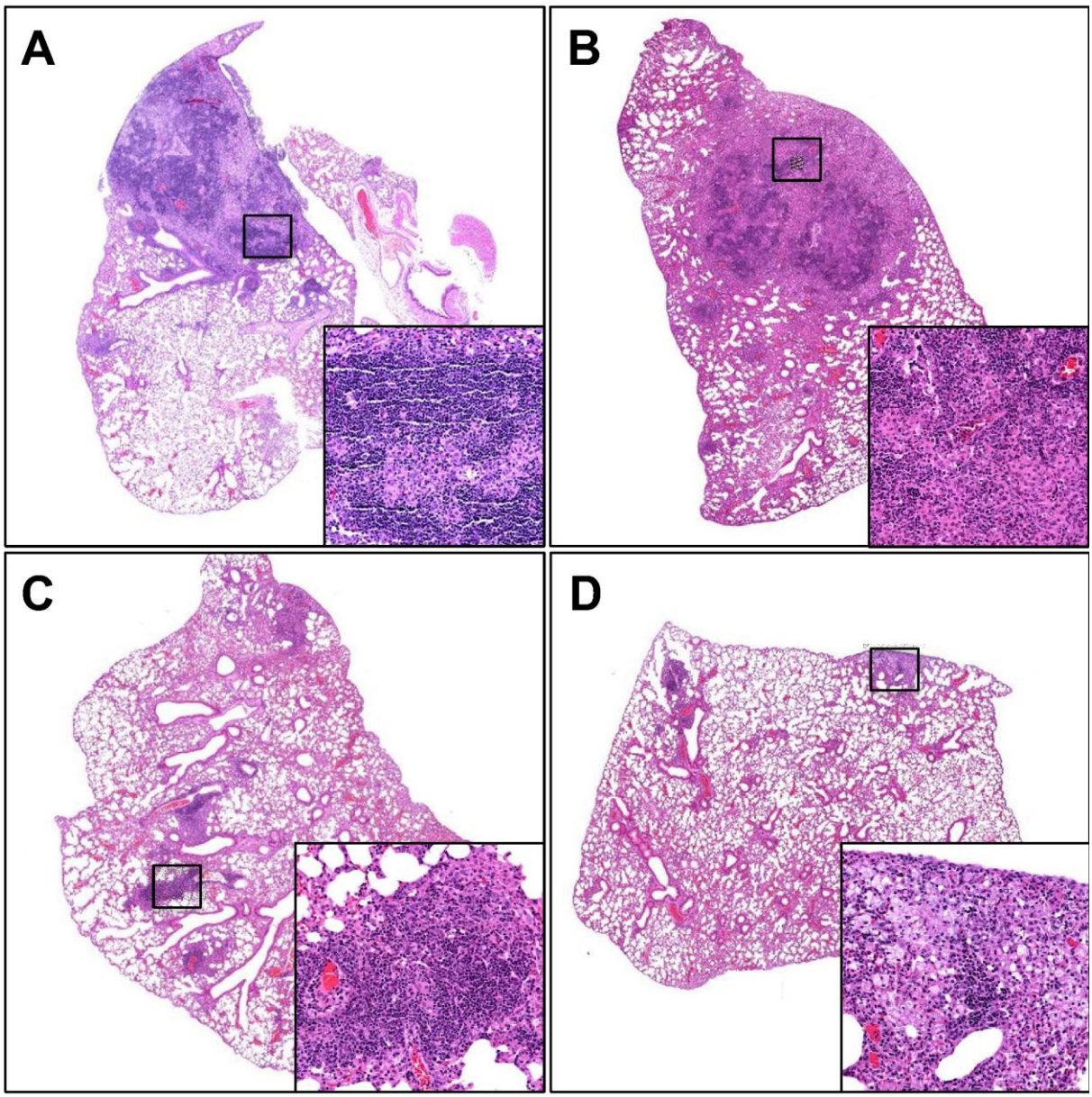

Figure 3: Histological appearance of pulmonary granulomas.
Lesions represented by photomicrographs are individuals of apical lung sections that have a value closest to the mean value for the entire treatment group from mice vaccinated 3 times at 9 day intervals with PBS (A), PHB wild-type beads produced in *L. lactis* (B), PHB beads displaying Ag85A-ESAT-6 produced *L. lactis* (C), or vaccinated once with BCG (D). All mice were challenged with *M. bovis* 15 weeks later followed by post-mortem after a further 5 weeks. Lung sections were stained with H&E stain.

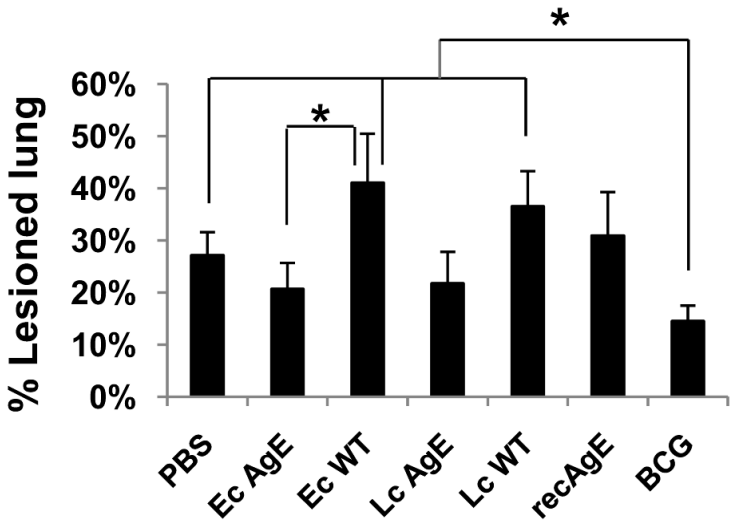

Figure 4: Morphometric analysis of *M. bovis* infected lungs.
Mice were vaccinated once with BCG or 3 times at 9 day intervals with other vaccines and then challenged with *M. bovis* 15 weeks later followed by post-mortem after a further 5 weeks. Lung sections were stained with H&E stain and then subjected to analysis using the area fraction fractionator and the data expressed as the percentage of lung affected by granulomatous inflammation. * Significantly different ($p<0.05$).

3.4.4 Mycobacterial culture

A significant reduction in the bacterial counts was observed from the lungs of animals receiving Ag85A-ESAT-6 PHB bead vaccines, produced in either *E. coli* or *L. lactis* or the BCG vaccine, compared to the PBS-vaccinated negative control group (Fig 5A, $p<0.05$). *M. bovis* culture results of spleens showed that animals vaccinated with Ag85A-ESAT-6 PHB bead vaccines produced in *E. coli* and BCG had a significant reduction in spleen bacterial counts compared to the PBS-vaccinated group (Fig. 5B, $p<0.05$). There were no significant differences between the mean lung counts for animals receiving Ag85A-ESAT-6 PHB bead vaccines compared to the BCG group while, the mean spleen counts for the Ag85A-ESAT-6 PHB bead vaccine groups were significantly higher than that for the BCG group ($p<0.05$). The group vaccinated with Ag85A-ESAT-6 PHB beads produced in *E. coli* also had significantly lower mean lung and spleen counts than those for the recAg85A-ESAT-6 group ($p<0.05$) while differences between the group vaccinated with Ag85A-ESAT-6 PHB beads produced in *L. lactis* and the recAg85A-ESAT-6 group were not significant. No significant reduction

in bacterial counts from the lungs or spleens was observed from animals vaccinated with wild-type PHB beads or recAg85A-ESAT-6.

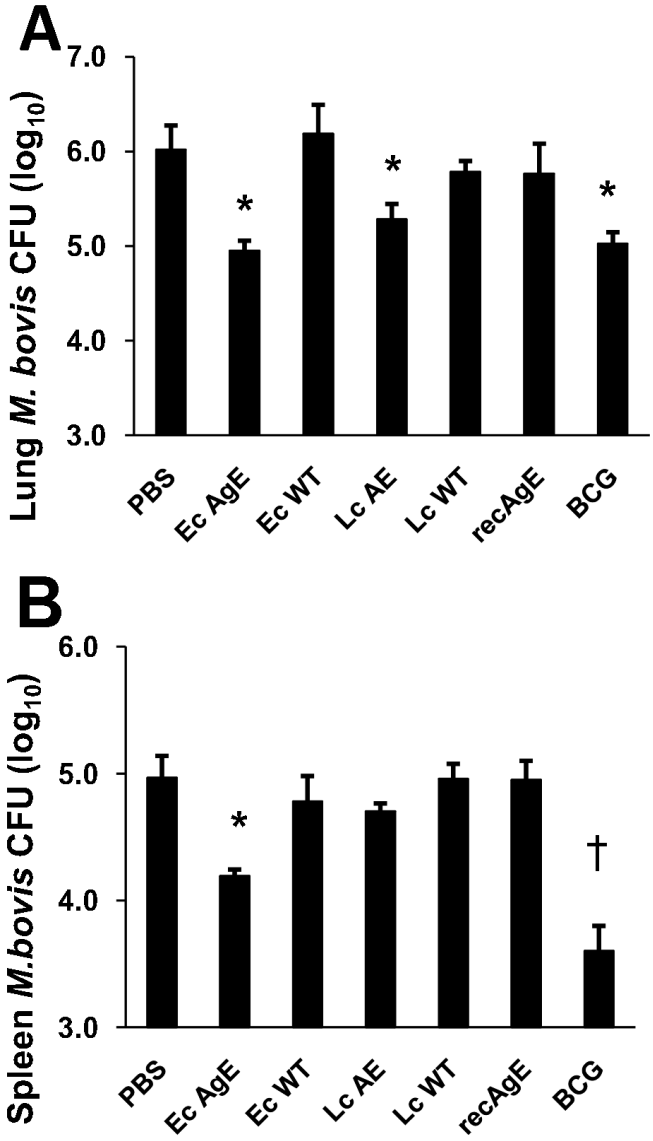

Figure 5: Lung and spleen culture results.
Lung (A) and spleen (B) culture results following vaccination of mice with PBS, PHB beads produced in *E. coli* and *L. lactis*, recAg85A-ESAT-6, all in emulsigen, or BCG. Mice were vaccinated 3 times at 9 day intervals, or once in the case of BCG, and then challenged with *M. bovis* 15 weeks later followed by post-mortem after a further 5 weeks. Each data point represents the mean for 6 mice ± SEM. * Significantly different to PBS-vaccinated group, † significantly different to all other vaccine groups (p<0.05).

3.5 Discussion

The novel vaccine delivery system based on biopolyester beads produced in *E. coli* has previously been shown to stimulate an immune response to mycobacterial antigens (37). This latest study demonstrated that the "generally regarded as safe" (GRAS) bacterium, *L. lactis*, could be used to produce vaccine beads displaying Ag85A-ESAT-6 and following vaccination with these beads similar immune responses were obtained compared to using beads from an *E. coli* production host. Furthermore, when mice vaccinated with Ag85A-ESAT-6 PHB beads produced in either *E. coli* or *L. lactis* were challenged with *M. bovis*, a significant reduction in lung bacterial counts was similar to that of the gold standard BCG vaccine. The presence of LPS endotoxin from *E. coli*-produced recombinant proteins and vaccines, limits the use of these products in humans without costly and potentially protein destructive depyrogenation processes. Major advantages of using a *L. lactis* production host are the safety record and reduced risk of LPS endotoxin contamination. As well as its long history of safe use in the food industry, *L. lactis* is now being used for a range of other *in vivo* and *in vitro* applications (5, 6) and has recently been used to produce a vaccine antigen for a human clinical malaria vaccine trial (16). Safety of the PHB polyester used in these vaccines has been recognised, with FDA approval for PHB sutures (42).

Cytokine responses in the present study were indicative of a predominantly cell–mediated immune response when animals were vaccinated with PHB beads displaying Ag85A-ESAT-6 compared to animals vaccinated with PBS or recAg85A-ESAT-6 antigen alone in emulsigen adjuvant. The antigen specific increase in IFN-γ and TNF-α with minimal IL-4, IL-5, IL-10 responses suggested Th1 type immunity rather than Th2 type immunity was induced. Increases in IL-17A indicated Th17 immunity was also stimulated. The IFN-γ response to the pool of peptides from Ag85A and ESAT-6 was greater than that for the peptides from the individual proteins. This suggested that the effect of vaccinating with the subunit vaccine and BCG could be additive compared to vaccinating with BCG alone. While it is generally accepted that IFN-γ has a significant role in protection from TB there is no single post-vaccination correlate of protection. Recent studies have shown that IFN-γ does not correlate with BCG-induced protection (29, 49) and others have determined an increase in polyfunctional T-cells, which produce IL-2, IFN-γ and TNF-α, are thought to be important possible correlates of protection (1). IL-17A appears to have a role in vaccination induced immunity against TB (21) along with IL-6 and TNF-α (17, 24), which concurs with increased levels of

these cytokines measured in the current study. Therefore, in the absence of absolute correlates of protection, challenge studies are necessary to determine vaccine efficacy.

The increased cytokine responses correlated with decreased bacterial counts and reduced pathology observed in the lungs. Histopathology results showed a distinct difference in granulomas in mice which had been vaccinated with BCG or PHB beads displaying Ag85A-ESAT-6, with these mice having smaller, less extensive foci of inflammation and fewer lymphocytes in the granulomas, compared to PBS or PHB wild-type bead vaccinated animals. The significant reduction in spleen bacterial counts from BCG and *E. coli* produced Ag85A-ESAT-6 PHB bead vaccinated animals, indicates extra-pulmonary spread of tuberculosis has been minimised. It is unclear why *L. lactis* produced Ag85A-ESAT-6 PHB bead vaccinated animals did not instigate a similar reduction. The current PHB bead formulation may prove effective as a vaccine boost following BCG priming. Cytokine responses, culture results and pathology demonstrated that mice vaccinated with recombinant Ag85A-ESAT-6 were unable to mount a protective response from *M. bovis* challenge. These results suggested that particulate vaccines were more effective than vaccines containing soluble antigens, a finding demonstrated in other vaccine studies (23, 43). The recombinant Ag85A-ESAT-6 was shown to be immunogenic as this vaccine induced an IgG response to the mycobacterial peptides (data not shown).

While these novel vaccines have shown efficacy against TB, there are modifications which could be made to further enhance their usefulness. PHB beads have previously been produced which display cytokine proteins (4) and both the N-terminus and C-terminus have been used to produce fusions for functional protein display (3). Therefore it is likely that PHB beads could be produced which display both vaccine antigens and immunomodulator proteins on one bead, allowing co-delivery of vaccine agents to dendritic cells, which has been shown to increase immune responsiveness (9, 51). Alternatively, immunomodulators could be incorporated on the PHB beads by chemical conjugation. It is also likely that increased antigen could be displayed on beads by incorporating multiple gene repeats in the bacterial production strains (31). Another strategy which utilised both N-terminus and C-terminus fusions of PhaC (19) could be applied to enable production of two different vaccine antigens on the one bead and therefore produce multi-valent vaccines. Combinations of PHB bead vaccines and BCG could result in the induction of enhanced protection against TB.

Studies in cattle have shown that concurrent administration of TB protein vaccines and BCG produced better protection against bovine TB compared with BCG alone (53, 54) and a BCG prime and TB protein boost is being advocated for use in humans (2).

The vaccine production process described here allows for modification of the host genome so that alternative genes for vaccine antigens could be used. Most vaccines use immunodominant antigens but more recent views (13, 33, 34, 50) suggest sub-dominant antigens might be more appropriate to use for diseases for which there is no effective vaccine. PHB bead vaccines could also be developed and used for other vaccines where stimulation of cell-mediated immunity is required. Alternative antigen display has recently been demonstrated on PHB beads produced in *E. coli* and *L. lactis*. These beads displayed hepatitis C core antigen and were able to produce antigen specific immune responses following vaccination (36). In conclusion, these current results indicate vaccines based on PHB beads provide a platform for display of a range of antigens coupled with relatively simple production processes in safe bacteria.

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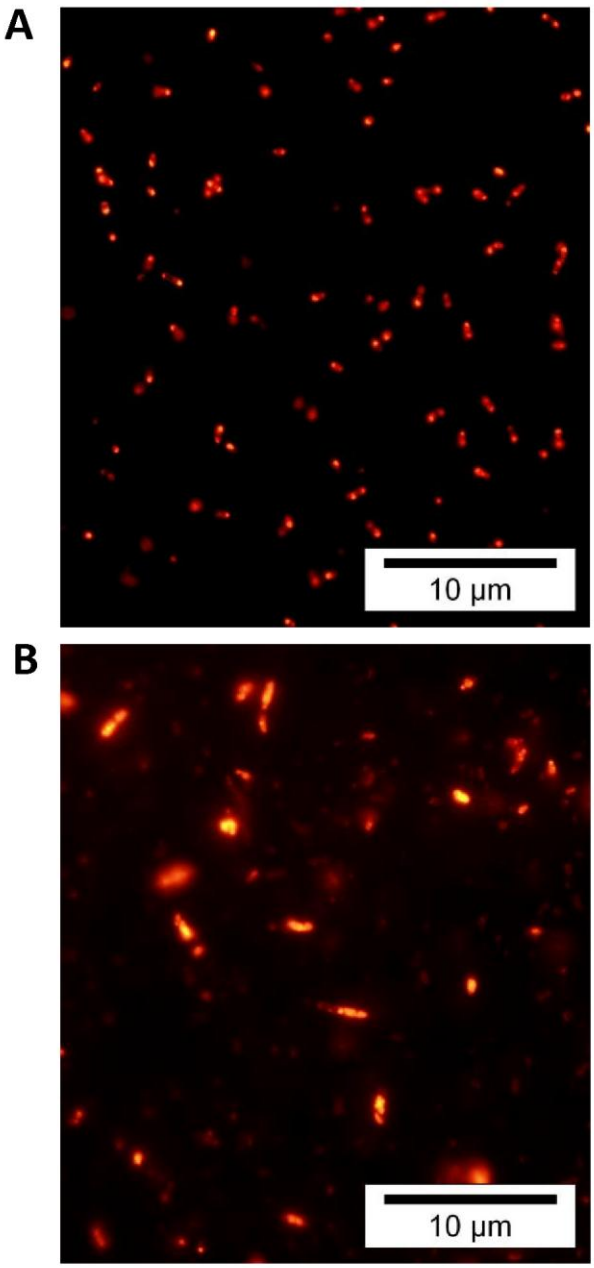
Supplemental table 1: Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference
E. coli		
BL21(DE3)	F⁻ *ompT hsdS$_B$(r_B^- m_B^-) gal dcm* (DE3)	Invitrogen
BL21 Star™ (DE3)pLysS	**F–** omp***T*** hsd***SB(rB–, mB–)*** gal dcm rne*131* (DE3) pLysS (CamR)	Invitrogen
DH5α	DH5α F⁻ *end A1 hsd R17 (r_k^-, m_k^-) sup E44 thi-1 λ^- rec A1 gyr A96 rel A1 Δ(arg F⁻ lac)*F⁻	Invitrogen
L. lactis		
MG1363	NCDO 712 derivative, plasmid and phage free strain	(2)
NZ9000	MG1363 derivative, *pepN*::*nisRK*	(3)
Plasmids		
pUC57	Cloning vector, ColE1 origin, Amp^r	Fermentas
pUC57-Ag85E6	Codon-optimized gene for Ag85A-ESAT-6 in EcoRV site of pUC57	This study
pUC57-ZZ	Codon-optimized gene for *ZZ* domain in EcoRV site of pUC57	(5)
pUC57-nisAg85E6	pUC57 derivative containing part of P_{nisA} and the gene for Ag85A-ESAT-6	This study
pNZ8148	Cm^r, pSH71 origin, P_{nisA}	(4)
pNZ-AB	pNZ8148 derivative, P_{nisA}-*phaAB*	(5)

pNZ-CAB	pNZ8148 derivative, P_{nisA}-*phaCAB*	(5)
pNZ-Ag85E6-B	pNZ8148 derivative, P_{nisA}-Ag85A-ESAT-6-*phaB*	This study
pNZ-Ag85E6-CAB	pNZ8148 derivative, P_{nisA}-Ag85A-ESAT-6-*phaC-phaAB*	This study
pMCS69	pPBR1MCS derivative containing *phaA* and *phaB* genes from *Cupriavidus necator*	(1)
pHAS	pET14b derivative containing Nde1/BamH1-inserted *phaC* from *C. necator*	(7)
pHAS-Ag85A-ESAT-6	pHAS containing Ag85A-ESAT-6 hybrid gene upstream of *phaC*	(6)



Supplemental figure 2:Nile Red staining and fluorescence microscopy was used to observe the presence of intracellular inclusions in cultures of bacteria with plasmids encoding polyester synthase and Ag85A-ESAT-6 .

A) *L. lactis* cultures

B) *E. coli* cultures

Supplemental table 2: MALDI-TOF

Matched peptides shown in Grey for Ag85A-ESAT-6-PhaC fusion

1 MFSRPGLPVE YLQVPSPSMG RDIK**VQFQSG** **GANSPALYLL** **DGLR**AQDDFS

51 GWDINTPAFE WYDQSGLSVV MPVGGQSSFY SDWYQPACGK AGCQTYK**WET**

101 **FLTSELPGWL** **QANR**HVKPTG SAVVGLSMAA SSALTLAIYH PQQFVYAGAM

151 SGLLDPSQAM GPTLIGLAMG DAGGYKASDM WGPK**EDPAWQ** **RNDPLLNVGK**

201 LIANNTR**VWV** **YCGNGKPSDL** **GGNNLPAKFL** **EGFVR**TSNIK FQDAYNAGGG

251 HNGVFDFPDS GTHSWEYWGA QLNAMKPDLQ RALGATPNTG PAPQGAGSTE

301 QQWNFAGIEA AASAIQGNVT SIHSLLDEGK QSLTKLAAAW GGSGSEAYQG

351 VQQK**WDATAT** **ELNNALQNLA** **R**TISEAGQAM ASTEGNVTGM FATSATGKGA

401 AASTQEGKSQ PFK**VTPGPFD** **PATWLEWSR**Q WQGTEGNGHA AASGIPGLDA

451 LAGVK**IAPAQ** **LGDIQQR**YMK DFSALWQAMA EGK**AEATGPL** **HDRR**FAGDAW

501 R**TNLPYRFAA** **AFYLLNARAL** **TELADAVEAD** **AK**TRQRIR**FA** **ISQWVDAMSP**

551 **ANFLATNPEA** **QRLLIESGGE** **SLR**AGVRNMM EDLTRGK**ISQ** **TDESAFEVGR**

601 **NVAVTEGAVV** **FENEYFQLLQ** **YKPLTDK**VHA RPLLMVPPCI NK**YYILDLQP**

651 **ESSLVRHVVE** **QGHTVFLVSW** **RNPDASMAGS** **TWDDYIEHAA** **IRAIEVAR**DI

701 SGQDKINVLG FCVGGTIVST ALAVLAARGE HPAASVTLLT TLLDFADTGI

751 LDVFVDEGHV QLR**EATLGGG** **AGAPCALLR**G LELANTFSFL RPNDLVWNYV

801 VDNYLKGNTP VPFDLLFWNG DATNLPGPWY CWYLR**HTYLQ** **NELK**VPGK**LT**

851 **VCGVPVDLAS** **IDVPTYIYGS** **REDHIVPWTA** **AYASTALLAN** **K**LR**FVLGASG**

901 **HIAGVINPPA** **K**NKRSHWTND ALPESPQQWL AGAIEHHGSW WPDWTAWLAG

951 QAGAKRAAPA NYGNARYR**AI** **EPAPGR**YVKA KA

Ag85A

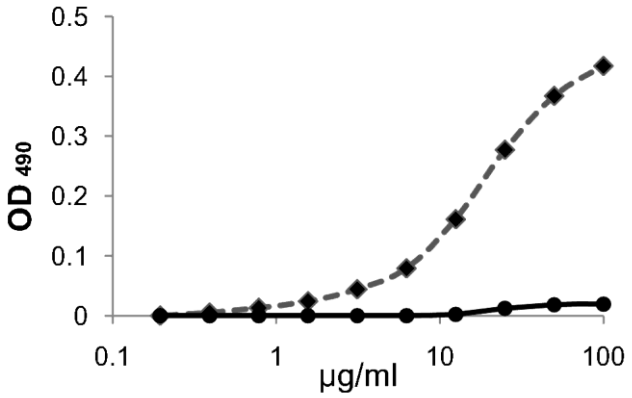
ESAT-6

PhaC

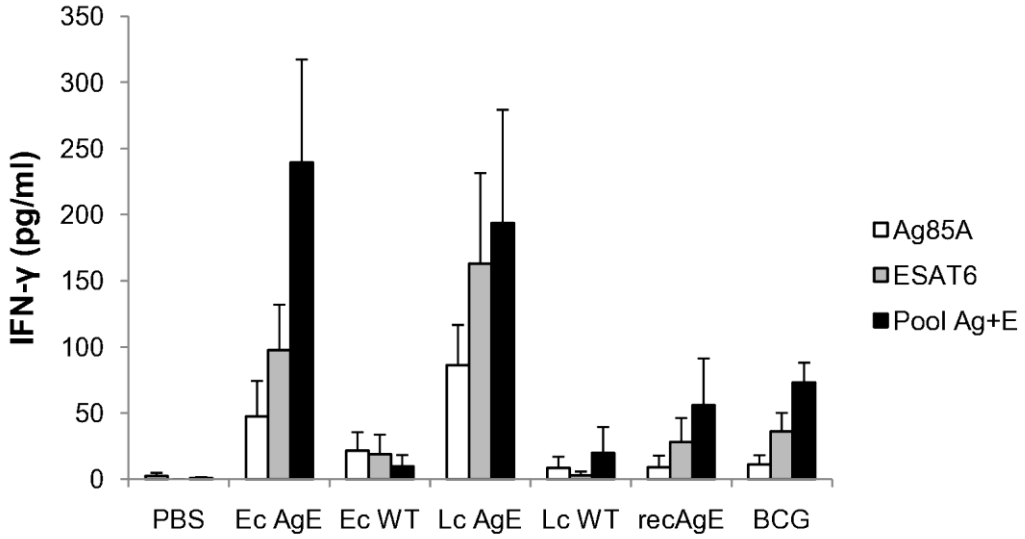
Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence	
25 - 44	2106.0803	2105.0730	2105.0905	-0.0174	0	K.VQFQSGGANSPALYLLDGLR.A	(Ions score 142)
25 - 44	2106.0886	2105.0813	2105.0905	-0.0091	0	K.VQFQSGGANSPALYLLDGLR.A	(Ions score 144)
98 - 114	2048.0039	2046.9966	2047.0163	-0.0197	0	K.WETFLTSELPGWLQANR.H	(Ions score 99)
98 - 114	2048.0151	2047.0078	2047.0163	-0.0085	0	K.WETFLTSELPGWLQANR.H	(Ions score 51)
185 - 191	901.4160	900.4087	900.4090	-0.0002	0	K.EDPAWQR.N	(Ions score 32)
192 - 200	969.5363	968.5290	968.5291	-0.0000	0	R.NDPLLNVGK.L	(Ions score 45)
208 - 228	2247.0889	2246.0816	2246.0789	0.0027	0	R.VWVYCGNGKPSDLGGNNLPAK.F	(Ions score 93)
229 - 235	867.4711	866.4638	866.4650	-0.0012	0	K.FLEGFVR.T	(Ions score 51)
355 - 371	1900.9338	1899.9265	1899.9438	-0.0173	0	K.WDATATELNNALQNLAR.T	(Ions score 126)
355 - 371	1900.9352	1899.9279	1899.9438	-0.0159	0	K.WDATATELNNALQNLAR.T	(Ions score 76)
414 - 429	1858.8944	1857.8871	1857.9050	-0.0178	0	K.VTPGPFDPATWLEWSR.Q	(Ions score 40)
456 - 467	1309.7117	1308.7044	1308.7150	-0.0105	0	K.IAPAQLGDIQQR.Y	(Ions score 97)
456 - 467	1309.7198	1308.7125	1308.7150	-0.0024	0	K.IAPAQLGDIQQR.Y	(Ions score 111)
484 - 493	1066.5281	1065.5208	1065.5203	0.0005	0	K.AEATGPLHDR.R	(Ions score 66)
484 - 494	1222.6251	1221.6178	1221.6214	-0.0036	0	K.AEATGPLHDRR.F	(Ions score 46)
502 - 507	763.4073	762.4000	762.4024	-0.0024	0	R.TNLPYR.F	(Ions score 34)
508 - 518	1256.6702	1255.6629	1255.6713	-0.0084	0	R.FAAAFYLLNAR.A	(Ions score 72)
508 - 518	1256.6715	1255.6642	1255.6713	-0.0071	0	R.FAAAFYLLNAR.A	(Ions score 83)
519 - 532	1416.6984	1415.6911	1415.7143	-0.0232	0	R.ALTELADAVEADAK.T	(Ions score 108)
519 - 532	1416.7075	1415.7002	1415.7143	-0.0141	0	R.ALTELADAVEADAK.T	(Ions score 103)
519 - 532	1416.7175	1415.7102	1415.7143	-0.0041	0	R.ALTELADAVEADAK.T	(Ions score 106)

539 - 562	**2680.2517**	**2679.2444**	**2679.2751**	**-0.0306**	**0**	**R.FAISQWVDAMSPANFLATNPEAQR.L**	(Ions score 71)
539 - 562	**2680.2725**	**2679.2652**	**2679.2751**	**-0.0098**	**0**	**R.FAISQWVDAMSPANFLATNPEAQR.L**	(Ions score 59)
563 - 573	**1173.6390**	**1172.6317**	**1172.6400**	**-0.0083**	**0**	**R.LLIESGGESLR.A**	(Ions score 79)
563 - 573	**1173.6482**	**1172.6409**	**1172.6400**	**0.0009**	**0**	**R.LLIESGGESLR.A**	(Ions score 73)
588 - 600	**1438.6779**	**1437.6706**	**1437.6735**	**-0.0029**	**0**	**K.ISQTDESAFEVGR.N**	(Ions score 84)
588 - 600	**1438.6821**	**1437.6748**	**1437.6735**	**0.0013**	**0**	**K.ISQTDESAFEVGR.N**	(Ions score 127)
601 - 627	**3115.5671**	**3114.5598**	**3114.5912**	**-0.0314**	**0**	**R.NVAVTEGAVVFENEYFQLLQYKPLTDK.V**	(Ions score 138)
601 - 627	**3115.5740**	**3114.5667**	**3114.5912**	**-0.0245**	**0**	**R.NVAVTEGAVVFENEYFQLLQYKPLTDK.V**	(Ions score 117)
643 - 656	**1695.8827**	**1694.8754**	**1694.8879**	**-0.0125**	**0**	**K.YYILDLQPESSLVR.H**	(Ions score 89)
643 - 656	**1695.8898**	**1694.8825**	**1694.8879**	**-0.0054**	**0**	**K.YYILDLQPESSLVR.H**	(Ions score 79)
657 - 671	**1793.9259**	**1792.9186**	**1792.9372**	**-0.0186**	**0**	**R.HVVEQGHTVFLVSWR.N**	(Ions score 62)
657 - 671	**1793.9296**	**1792.9223**	**1792.9372**	**-0.0149**	**0**	**R.HVVEQGHTVFLVSWR.N**	(Ions score 117)
657 - 671	**1793.9308**	**1792.9235**	**1792.9372**	**-0.0137**	**0**	**R.HVVEQGHTVFLVSWR.N**	(Ions score 62)
672 - 692	**2335.9995**	**2334.9922**	**2335.0175**	**-0.0252**	**0**	**R.NPDASMAGSTWDDYIEHAAIR.A**	(Ions score 96)
672 - 692	**2336.0085**	**2335.0012**	**2335.0175**	**-0.0162**	**0**	**R.NPDASMAGSTWDDYIEHAAIR.A**	(Ions score 52)
693 - 698	**658.3805**	**657.3732**	**657.3809**	**-0.0077**	**0**	**R.AIEVAR.D**	(Ions score 29)
764 - 779	**1513.7777**	**1512.7704**	**1512.7718**	**-0.0014**	**0**	**R.EATLGGGAGAPCALLR.G**	(Ions score 70)
764 - 779	**1513.7798**	**1512.7725**	**1512.7718**	**0.0007**	**0**	**R.EATLGGGAGAPCALLR.G**	(Ions score 117)
764 - 779	**1513.7874**	**1512.7801**	**1512.7718**	**0.0083**	**0**	**R.EATLGGGAGAPCALLR.G**	(Ions score 138)
836 - 844	**1145.5883**	**1144.5810**	**1144.5876**	**-0.0066**	**0**	**R.HTYLQNELK.V**	(Ions score 58)
836 - 844	**1145.5940**	**1144.5867**	**1144.5876**	**-0.0009**	**0**	**R.HTYLQNELK.V**	(Ions score 50)
849 - 871	**2495.2522**	**2494.2449**	**2494.2777**	**-0.0328**	**0**	**K.LTVCGVPVDLASIDVPTYIYGSR.E**	(Ions score 111)

849 - 871	**2495.2559**	**2494.2486**	**2494.2777**	**-0.0291**	**0**	**K.LTVCGVPVDLASIDVPTYIYGSR.E**	(Ions score 99)
849 - 871	**2495.2771**	**2494.2698**	**2494.2777**	**-0.0079**	**0**	**K.LTVCGVPVDLASIDVPTYIYGSR.E**	(Ions score 106)
872 - 891	**2171.0908**	**2170.0835**	**2170.1058**	**-0.0223**	**0**	**R.EDHIVPWTAAYASTALLANK.L**	(Ions score 101)
872 - 891	**2171.0952**	**2170.0879**	**2170.1058**	**-0.0179**	**0**	**R.EDHIVPWTAAYASTALLANK.L**	(Ions score 86)
894 - 911	**1747.9666**	**1746.9593**	**1746.9780**	**-0.0187**	**0**	**R.FVLGASGHIAGVINPPAK.N**	(Ions score 92)
969 - 976	**810.4534**	**809.4461**	**809.4395**	**0.0066**	**0**	**R.AIEPAPGR.Y**	(Ions score 33)



Supplemental figure 3: ELISA shows PHB beads produced in *L. lactis* and display Ag85A-ESAT-6 (- ◆ -) bind to anti-ESAT-6 in a dose-dependant manner while wild-type PHB beads (--●--) show minimal binding. Plates were coated with beads diluted from 100 µg/ml to 0.2 µg/ml



Supplemental figure 4: :Cytokine responses in mice vaccinated 3 times with PBS, control wild-type PHB beads produced in *E. coli* (EcWT), PHB beads produced in *E. coli* displaying Ag85A-ESAT-6 (EcAgE), control wild-type PHB beads produced in *L. lactis* (LcWT) and PHB beads displaying Ag85A-ESAT-6 produced in *L. lactis* (LcAgE), recombinant Ag85A-ESAT-6 protein (recAg85A-ESAT-6), all in Emulsigen™. Three weeks after final vaccination, splenocytes were cultured for 3 days with Ag85A peptides (white bars), ESAT-6 peptides (grey bars) or a pool of Ag85A and ESAT-6 peptides (black bars). Release of IFN-γ was measured by ELISA

Chapter 4 Production of a particulate hepatitis C vaccine candidate by engineered *Lactococcus lactis*

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4.1 Abstract

Vaccine delivery systems based on display of antigens on bioengineered bacterial polyester inclusions can stimulate cellular immune responses. The food grade, Gram-positive *Lactococcus lactis* was engineered to produce spherical polyhydroxybutyrate (PHB) inclusions which abundantly displayed the Hepatitis C virus core (HCc) antigen. In mice, the immune response induced by this antigen delivery system was compared to that induced by vaccination with HCc antigen displayed on PHB beads produced in *Escherichia coli,* PHB beads without antigen produced in *L. lactis* or *E. coli* or directly to the recombinant HCc protein. Vaccination site lesions were minimal in all mice vaccinated with HCc PHB beads or recombinant protein, all mixed in oil-in-water adjuvant, Emulsigen™, while vaccination with the recombinant protein in complete Freund's adjuvant produced a marked inflammatory reaction at the vaccination site. Vaccination with the PHB beads produced in *L. lactis* and displaying HCc antigen produced antigen-specific cellular immune responses with significant release of IFN-γ and IL-17A from splenocyte cultures and no significant antigen-specific serum antibody, while the PHB beads displaying HCc but produced in *E. coli* released IFN-γ and IL-17A as well as the pro-inflammatory cytokines, TNF-α and IL-6 and low levels of IgG2c antibody. In contrast, recombinant HCc antigen in Emulsigen produced a diverse cytokine response and a strong IgG1 antibody response. Overall it was shown that *L. lactis* can be used to produce immunogenic PHB beads displaying viral antigens making the beads suitable for vaccination against viral infections.

4.2 Introduction

The food-grade Gram-positive bacterium, *Lactococcus lactis* has been increasingly considered as a production host for recombinant therapeutic proteins (6, 9, 49). The recent advances towards the development of efficient gene expression systems in *L. lactis* and the established safety profile of *L. lactis* based on long term use in dairy food processing has led to new potential applications in protein production, therapeutic drug delivery and vaccine delivery (5, 27, 30, 38).

Recently, it was shown that *L. lactis* can be engineered to produce spherical polyhydroxybutyrate (PHB) inclusions which display the *Staphylococcus aureus* protein A derived IgG binding region, the Z domain, and these can be isolated for *in vitro* use in purification of IgG (26). This was achieved by establishing the PHB biosynthesis pathway in *L. lactis* and by overproducing a Z domain-PHB synthase fusion protein which remained attached to the PHB inclusion surface. The PHB synthase represents the only essential enzyme required for PHB inclusion formation (39, 40). This strategy utilized protein engineering of the PHB synthase from *Cupriavidus necator* (previously known as *Ralstonia eutropha)* for the display of various protein based functions, such as e.g. technical enzymes, binding domains or a fluorescent protein, at the surface of PHB beads as had been previously established in recombinant *Escherichia coli* (13, 15, 34, 35, 37). The successful display of various technically relevant protein functions as well as the *in vitro* performance of the respective isolated PHB beads suggested a wide applicability of this bead display technology (12, 19, 41). Only recently have PHB beads formed by recombinant *E. coli* been considered for the display of antigens for *in vivo* use as a particulate vaccine (32). PHB beads simultaneously displaying the *Mycobacterium tuberculosis* antigens, Ag85A and ESAT-6, were produced in recombinant *E. coli*, isolated and injected into mice to assess the immune response. The respective Ag85A-ESAT-6 beads induced significantly stronger humoral and cell-mediated immune responses when compared with only the fusion protein Ag85A-ESAT-6. This antigen delivery system based on PHB beads has been considered relevant in the quest for an effective tuberculosis vaccine (31). A significant cell-mediated immune response is considered to be important for protection not only against intracellular pathogenic bacteria but also viruses (44, 45). Therefore it would be important to determine if PHB beads displaying viral antigens also demonstrate immunogenic properties making the beads suitable for vaccination against viral infections. The downside of using *E. coli* for either recombinant protein production, vaccines or other *in vivo* uses is the co-purification of lipopolysaccharide (LPS) endotoxins. LPS removal is costly and the processes can destroy surface proteins and hence functionality of the beads (50). Therefore the LPS-free *L. lactis* might be the preferred production host for antigen displaying PHB beads. The practicality of using *L. lactis* as production system for vaccine antigens is also based extensive use in the fermentation industry, abundance of genetic tools and high expression of recombinant proteins (5). Hepatitis C is a disease with world-wide distribution transmitted by blood-blood contact, often through inadequately sterilized

drug injection equipment, and co-infection with HIV is common (24). It often leads to permanent liver damage, cirrhosis and cancer. Not only is treatment limited and of variable efficacy (3) but there is no vaccine available. Research efforts have been limited because there is no cell culture system or effective small animal model, with chimpanzees being the only model in which challenge studies can be performed (46). A number of new vaccine approaches are currently being explored for control of Hepatitis C virus including recombinant protein, peptide, DNA and virus vector-based vaccines and some have reached Phase I/II human clinical trials (14). Recombinant protein hepatitis C virus vaccines have advantages of being well tolerated with low toxicity, induce cross-neutralising antibodies and proof-of-concept has been established with Hepatitis B virus vaccine, however, they suffer from the disadvantage of generally eliciting weak T cell responses. The Hepatitis C virus genome encodes three structural (Core, E1, and E2) and six non-structural (NS) proteins and vaccines are being developed which target one or several of these proteins (47).

In this study, *L. lactis* and *E. coli* were genetically engineered to produce PHB beads which displayed the Hepatitis C virus core antigen (HCc). The resulting beads were analysed and subjected to vaccination trials to determine whether a significant immune response could be generated and to what extent the production host impacts on the immunogenic properties of the PHB beads displaying HCc antigen.

4.3 Materials and methods

Plasmids, bacterial strains and growth conditions

All bacterial strains and plasmids are listed in Table 1. General cloning procedures were performed as described elsewhere (43). *E. coli* strains were grown in Luria broth (LB; Difco, Detroit, MI) supplemented with 1% (w/v) glucose, ampicillin (75 μg/ml) and chloramphenicol (30 μg/ml). *L. lactis* strains were grown in M17 medium (Merck, Darmstadt, Germany) supplemented with 0.5% glucose, 0.3% L-arginine and chloramphenicol (10 μg/ml).

Table 1: Strains and plasmids used in this study
TABLE 1

Strain/Plasmid	Description	Reference
E. coli		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsd*R17 *supE44 relA1 lac [F' proAB lacI^{q}lacZ* Δ*M15 Tn10 (Tet^r)]*	Stratagene
BL21 (DE3)	F$^-$ *ompT hsdS$_B$(r_B^- m_B^-) gal dcm* (DE3)	Novagen
L. lactis		
NZ9000	MG1363 derivative, *pepN*::*nisRK*	(18)
Plasmid		
pMCS69	pPBR1MCS derivative containing *phaA* and *phaB* genes from *C. necator*	(2)
pET-phaC	pET-14b derivative containing *phaC* gene from *C. necator*	(51)
pHAS-scFV13R4	pET-14b derivative containing the gene scFv13R4-*phaC*	(13)
pET-HCc-phaC	pET -14b derivative containing HCc-*phaC* gene	This study
pNZ8148	Cm^R, pSH71 origin, P$_{nisA}$	(25)
pNZ-AB	pNZ8148 derivative, P$_{nisA}$-*phaAB*	(26)
pNZ-CAB	pNZ8148 derivative, P$_{nisA}$-*phaCAB*	(26)
pNZ-HCc-CAB	pNZ-8148 derivative containing HCc-*phaC*, *phaA* and *phaB*	This study

Construction of plasmids for production of Hepatitis C core antigen

To display HCc on the surface of PHB beads produced *by E. coli*, the gene encoding the Hepatitis C virus core antigen (HCc) with amino acid sequence, MSTNPKPQRKTKRSTNRRPQDVKFPGGGQIVGGVYLLPRRGPRLGVRATRKS ERSQPRGRRQPIPKARQPEGRAWAQPGYPWPLYGNEGMGWAGWLLSPRGSR PSWGPTDPRRRSRNLGKVIDTLTCGFADLMGYIPLVGAPLGGAARALAHGVR VLEDGVNYATGNLPGCSFSIFLLALLSCLTIPASA, was synthesized by DNA2.0 (CA, USA), adapting it to the codon usage bias of *E. coli* and avoiding rarely used codons. A *Spe*I restriction site was inserted at the 5' end of the *HCc* gene, and a *Bst*WI restriction site and a sequence encoding five glycine residues were added at the 3' end. In order to accelerate cloning, part of *phaC* was included in the synthesis, enabling direct subcloning of the synthesized piece of DNA into pHAS-scFv13R4 (13) with *Spe*I and *Not*I, replacing the scFv gene with *HCc*. The resulting plasmid pET-HCc-phaC encodes the HCc-PhaC fusion protein under the control of the T7 promoter with HCc and PhaC connected by the pentaglycine linker. In addition to the polyester synthase gene (*phaC*), PHA biosynthesis requires the enzymes PhaA and PhaB for precursor synthesis, and these enzymes were encoded by plasmid pMCS69 which contains *phaA* and *phaB* genes. The pET-HCc-phaC and pMCS69 were transformed into *E. coli* BL21(DE3). Control PHB beads were produced using *E. coli* BL21(DE3) containing pET-phaC and pMCS69.

For display of the HCc on the surface of PHB beads produced by *L. lactis*, the gene encoding HCc with the amino acid sequence as used above for *E. coli*, was synthesized with the codon usage adapted to *L. lactis* by Genescript Corporation (USA). The *HCc* gene was designed with a small proportion of the DNA encoding the N-terminus of PhaC linked to the DNA corresponding to the antigen's C-terminus, with flanking restriction sites (*Nco*I and *Nhe*I), in order to easily subclone into a pre-existing vector pNZ-CAB (26). Plasmid pNZ-CAB harbours the codon-optimized PHB biosynthesis operon, containing *phaA, phaB* and *phaC* genes, from C. *necator* under P_{nisA} control. The *HCc* gene was ligated into pNZ-CAB downstream of the *nisA* promoter, generating an *HCc-phaC* hybrid gene, and this was transformed directly into *L. lactis* NZ9000 by electroporation.

Culture and isolation of PHB beads.

PHB beads which displayed HCc or control PHB beads alone were produced in *E. coli* and *L. lactis* as previously described (26, 32). Briefly, *E. coli* was grown at 30°C in LB, induced with 1mM isopropyl β-D-thiogalactopyranoside to produce protein and cultured for a further 48 hours at 30°C to allow accumulation of particles. *L. lactis* cultures were produced in M17 broth, induced with 10 ng/ml nisin to produce protein and cultured for a further 24 hours at 30°C. Presence of PHA/polyester was determined by staining the cultures with Nile Red lipophilic dye and observed using fluorescence microscopy. Transmission electron microscopy (TEM) was used to assess shape and size of PHB bead formation. Bacteria were then mechanically disrupted and *E. coli* lysate was centrifuged at 4000 x *g* or *L. lactis* lysate was centrifuged at 8000 x *g* for 15 min at 4°C to sediment the polyester particles. All beads were then purified via glycerol gradient ultracentrifugation as described elsewhere (15). To confirm functionality of the PhaC enzyme, PHB content of the cells was quantitatively determined by gas chromatography-mass spectroscopy (GC-MS) (7)

Analysis of proteins attached to the PHB beads.

The concentration of proteins attached to the beads was determined using the *Bio-Rad Protein* Assay, (Bio-Rad; CA, USA). Proteins were separated by SDS-PAGE using NuPAGE® Bis-Tris 4-12% gels (Invitrogen, CA, USA) and stained with SimplyBlue Safe Stain (Invitrogen). The amount of HCc-PhaC fusion protein relative to the amount of total proteins attached to the particles was detected using a Gel Doc™ XR and analysed using Quantity One software (version 4.6.2) (Bio-Rad laboratories, Hercules, CA, USA). 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). Specific activity of the HCc protein on the PHB beads was determined by enzyme-linked immunosorbent assay (ELISA) assay. Microlon high-binding plates (Greiner) plates were coated overnight at 4°C with purified PHB beads, diluted from 1 μg/ml to 60 μg/ml protein concentration using 0.2M phosphate coating buffer, pH 6.5. Plates were washed with phosphate buffered saline (PBS) containing 0.05% (v/v) Tween 20 (PBST) and blocked with 1% (w/v) bovine serum albumin (BSA) in PBS for 1hour at 25°C. Plates were then washed in PBST and incubated for 1 hour with mouse antibody to Hepatitis C core protein (Devatal, NJ, USA) diluted in 1% (w/v) BSA in PBS. Following washing with PBST, plates were

incubated for 1 hour at room temperature with biotinylated anti-mouse IgG (Sigma-Aldrich) diluted in 1% (w/v) BSA in PBS, incubated for 1 hour, washed with PBST and streptavidin-horseradish peroxidase was added. After another hours incubation, plates were washed and o-phenylenediamine (OPD) substrate (Sigma-Aldrich) was added and incubated for 30 minutes at room temperature. The reaction was stopped with 0.5 M H_2SO_4 and the absorbance was recorded at 490 nm on a VERSAmax microplate reader.

Vaccination of mice.

Vaccines comprising control wild-type PHB beads produced in *E. coli* (EcWT), control wild-type PHB beads produced in *L. lactis* (LcWT), PHB beads displaying HCc produced in *E. coli* (EcHCc) and *L. lactis* (LcHCc) were adjusted to contain 30 μg of the HCc-PhaC protein as calculated from the densitometry profile. Emulsigen (MVP Laboratories, Omaha, NE, USA) adjuvant (20% (v/v) was mixed with the various PHB beads, 30 μg recombinant hepatitis C core protein (recHCc; Devatal) or PBS. Female C57BL/6 mice aged 6 to 8 weeks were purchased from the animal breeding facility of the Malaghan Institute of Medical Research (Wellington, New Zealand) and then vaccinated 3 times sub-cutaneously at weekly intervals; 200 μl/injection (n=6 per group). A positive control group (n=6) receiving 30 μg recHCc emulsified in complete Freund's adjuvant (CFA; Sigma-Aldrich) were vaccinated once only. All animal experiments were approved by the AgResearch Grasslands Animal Ethics Committee (Palmerston North, New Zealand).

Immunological assays.

Three weeks after the last vaccination all mice were anaesthetized intra-peritoneally using 87 μg ketamine (Parnell laboratories, Alexandria, NSW, Australia) and 2.6 μg xylazine hydrochloride (Bayer, Leverkusen, Germany) per g of body weight. Blood was collected by cardiac puncture, allowed to clot and centrifuged prior to serum being collected and frozen at -20°C until assayed. Mice were euthanized, spleens removed and a single cell suspension was prepared by passage through an 80 gauge wire mesh sieve. Spleen red blood cells were lysed using a solution of 17 mM Tris-HCl and 140 mM NH_4Cl. After washing, the cells were cultured in DMEM (Dulbecco's Modified Eagle media, Invitrogen) supplemented with 2 mM glutamine (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 5 x 10^{-5} M 2-mercaptoethanol (Sigma), non-essential amino acids (Gibco, NY, USA) and 5% (v/v) FCS (Invitrogen) in triplicate wells of flat-bottomed 96 well plates at a concentration of

5 x 10^5 cells/well in a 200 μL volume. The cells were incubated with medium alone or in medium containing 5 μg/ml recHCc. Concanavalin A (ConA; Sigma, final concentration of 5 μg/ml) was used as a positive control. Cells were incubated at 37 ºC in an atmosphere of 10% CO_2 in air. Culture supernatants were removed after 4 days of incubation and frozen at -20ºC until assayed.

Measurement of cytokines.

Levels of interferon-γ (IFN-γ) in culture supernatants were measured by ELISA according to manufacturer's recommendations (BD Biosciences (BD), CA, USA). The assay used *o*-phenylenediamine substrate and was read at 495nm on a VERSAmax microplate reader. A standard curve was constructed using SOFTmax PRO software and averages of duplicate sample cytokine values were determined from the curve. Levels of other cytokines in culture supernatants were determined by cytometric bead array (Mouse Th1-Th2 cytokine kit; BD) according to manufacturer's instructions. Fluorescence was measured using a FACSCalibur flow cytometer (BD), and analysed using FCAP array software (BD). All results were calculated as the cytokine value of PBS-stimulated sample subtracted from the recHCc-stimulated sample.

Measurement of serum antibody.

Antibody in sera was measured by ELISA using Microlon high-binding plates (Greiner) coated overnight with 3 μg/ml recHCc and then blocked using 1% (w/v) BSA in PBS. After washing in PBST, dilutions of serum were added and incubated for 1 hour. Following washing with PBST, anti-mouse IgG1:HRP or IgG2c:HRP (ICL, Newberg, Oregon, USA) was added and plates incubated. Plates were washed and tetramethylbenzidine was used as a substrate prior to reading at 450 nm on a VERSAmax microplate reader. Monoclonal HCc antibody (Devatal) was used as a positive control. Results were expressed as optical density (OD) at 450 nm for sera diluted 1/250.

Statistical Analysis.

Analyses of the cytokine and antibody responses were performed by Kruskal-Wallis one-way analysis of variance.

4.4 Results

4.4.1 Microbial production and characterization of PHB beads displaying Hepatitis C core antigen.

Plasmids encoding PHB synthase with or without HCc were successfully introduced into both production strains, which enabled production of PHB beads alone or PHB beads displaying HCc. GC-MS analysis showed PHB was produced by both recombinant E. coli and L. lactis strains which in turn indicated in vivo functionality of the PHB synthase domain in the fusion protein (data not shown). The presence of intracellular polyester inclusions was further confirmed by fluorescent microscopy using Nile Red staining (data not shown) and TEM (Fig. 1). .*E. coli* pET-HCc-phaC-pMCS69 cells accumulated large numbers of PHB beads with 150 - 250 nm diameter (Fig 1 A). *L. lactis* pNZ-HCc-CAB cells produced smaller PHB beads with 50 - 150 nm diameter (Fig 1B).

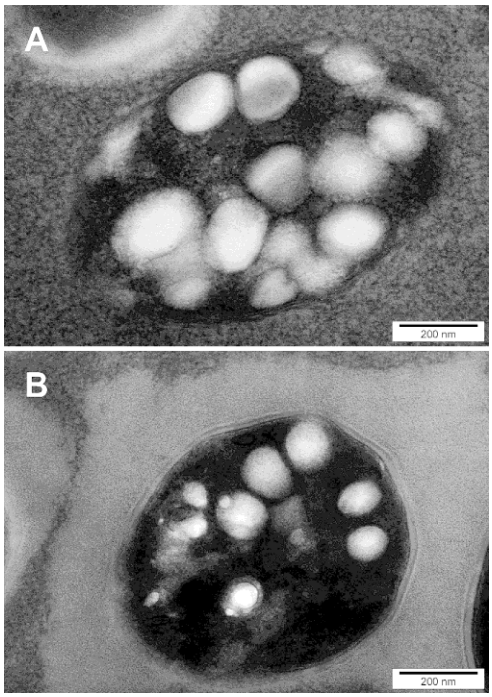

Figure 1: TEM analysis of a representative sample of bacteria accumulating PHB beads. (A) *E. coli* pET-HCc-phaC-pMCS69 cells accumulated large numbers of PHB beads (150 - 250 nm). (B) *L. lactis* pNZ-HCc-CAB cells produced smaller PHB beads (50 - 150 nm).

Following purification of the beads, the proteins associated with the HCc and control beads from both bacterial strains were separated by SDS-PAGE (Fig. 2). Both bacterial strains demonstrated production of proteins with a molecular weight similar to the theoretical molecular weight of 85 kDa for HCc-PhaC and 63kDa for the PHB synthase (PhaC). The identity of these proteins was confirmed by tryptic peptide fingerprinting using MALDI-TOF/MS with sequence cover of 50% and ion score of >100 for *L. lactis* produced HCc-PhaC and 46% sequence cover and ion score of >100 for *E. coli* produced HCc-PhaC (Supplementary Table). Densitometry analysis of the gels indicated that the HCc-PhaC protein accounted for 25.6% of total bead protein associated with *L. lactis* HCc beads whereas this protein only accounted for 6.7% of the *E. coli* HCc beads.

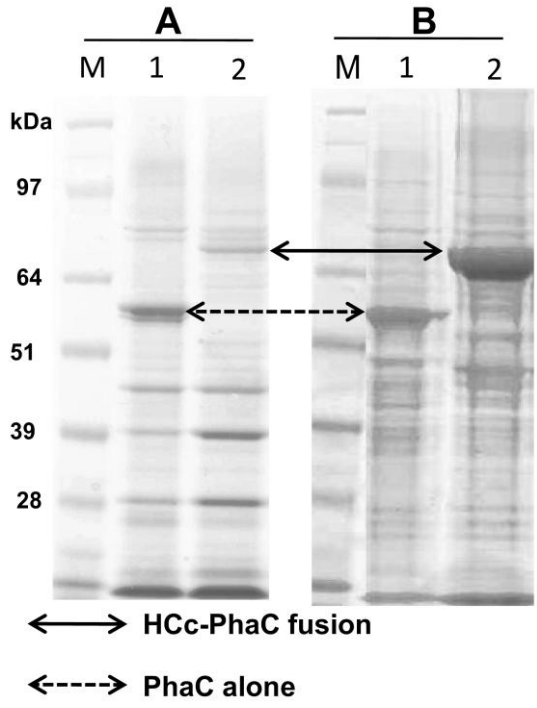

Figure 2: SDS-PAGE analysis of proteins attached to the polyester beads.
Beads isolated from (A) *E. coli* BL21 (DE3) with plasmids: lane 1, pET-phaC + pMCS69; lane 2, pET-HCc-phaC + pMCS69; (B) *L. lactis* NZ9000 with plasmids: lane 1, pNZ-CAB; lane 2, pNZ-HCc-CAB; Molecular weight markers are lane M.

Presence of HCc at the surface of *E. coli* and *L. lactis* beads was assessed by ELISA. Results indicated that HCc beads from both bacterial hosts bound to the anti-HCc antibody in a dose-dependent manner (Fig. 3).

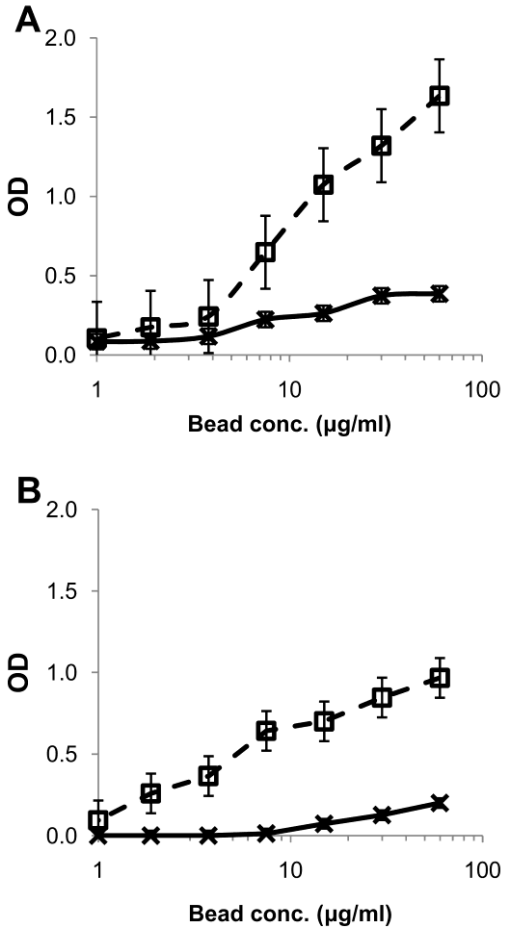

Figure 3: ELISA demonstrating HCc protein displayed on beads isolated from *E. coli* and *L. lactis* cultures.
Beads were diluted from 1– 60 µg/ml and incubated with anti-HCc antibody. Bead bound antibody was detected using biotinylated anti-mouse IgG then streptavidin-HRP conjugated secondary antibody. Results show that the antibody binds to beads which display HCc protein (- - □ - -) but not to control PhaC beads (—×—) from both *E. coli* (A) and *L. lactis* (B) cultures. These studies were replicated two times and graphs are representative of these results ±SEM.

4.4.2 Vaccination responses.

Mouse weights did not differ significantly between groups during the time-course of the experiment, and mice in all groups gained weight; an average of 2.6 grams

was gained over 5 weeks (data not shown). Mice vaccinated with PHB beads developed small lumps up to 2.5 mm in diameter at the vaccination sites with no signs of an abscess or suppuration. All mice were healthy throughout the trial and displayed normal behavior. In contrast, 3 out of 6 mice vaccinated with recHCc in CFA showed skin sloughing at the injection site.

IFN-γ is an important marker of the development of Th1 cell-mediated immunity and was assessed by measuring the release of IFN-γ in splenocytes restimulated *in vitro* with proteins used for immunization (Fig. 4A). This study showed that vaccination of mice with HCc PHB beads produced by both *L. lactis* and *E. coli* hosts stimulated the generation of a significant antigen-specific cellular immune response compared to the PBS-vaccinated group ($p<0.05$). Vaccination with recHCc in either Emulsigen™ or CFA adjuvant also induced a significant increase in IFN-γ levels ($p<0.05$). The vaccine groups receiving *E. coli* PHB beads, recHCc in Emulsigen™ and CFA produced significantly more interleukin-10 (IL-10) than the groups receiving PBS and *L. lactis* PHB beads ($p<0.05$, Fig. 4B). Tumour necrosis factor-α (TNF-α) was significantly increased in the *E. coli* HCc PHB bead-vaccinated group and CFA control group compared to PBS vaccinated mice ($p<0.05$, Fig. 4C). For the *E. coli* produced wild-type control bead vaccinated group, TNF-α values were not significantly increased although there was a positive trend. Interleukin-6 (IL-6) levels were significantly increased in both *E. coli* PHB bead-vaccinated groups and recHCc in CFA ($p<0.05$, Fig. 4D). Interleukin-17A (IL17-A) release was significantly increased in groups vaccinated with both PHB beads produced in *E. coli*, HCc PHB beads from *L. lactis* and recHCc in CFA ($p<0.05$, Fig. 4E) Interleukin-2 was measured and increased only in the control group vaccinated with recHCc in CFA (data not shown). Interleukin-4, a Th2 cytokine, was not detected from any of the groups (data not shown).

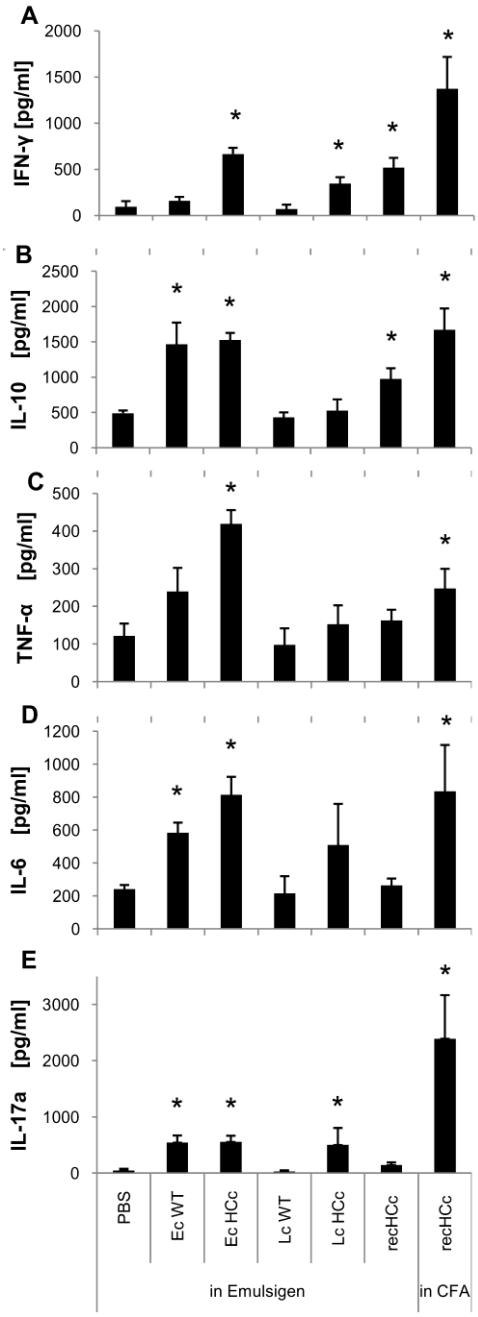

Figure 4: Cytokine responses.
Cytokine responses in mice (n=6) vaccinated 3 times with control wild-type PHB beads produced in *E. coli* (EcWT), PHB beads displaying HCc produced in *E. coli* (EcHCc), control wild-type PHB beads produced in *L. lactis* (LcWT), PHB beads displaying HCc produced in *L. lactis* (LcHCc), recombinant hepatitis C core protein (recHCc), or PBS; all in Emulsigen®. A single vaccination was used for mice vaccinated with recHCc emulsified in complete Freund's adjuvant (CFA). Three weeks after final vaccination splenocytes were cultured for 3 days with 5 μg recHCc. Release of IFN-γ (A) was measured by ELISA and all other cytokines (B) IL-10, (C) TNF-α (D) IL-6 (E) IL-17A, were measured by cytometric bead array. Results were calculated as the value of PBS-stimulated sample subtracted from the recHCc-stimulated sample. Each data point represents the mean for 6 mice ± SEM. * Significantly greater than the PBS-vaccinated control group ($p<0.05$).

Antigen-specific serum antibody levels were assessed by measuring IgG1 and IgG2 (Table 2). IgG1 results are indicative of Th2 immune responses and results indicate that antigen specific serum IgG1 to HCc was significantly increased only in the recHCc in Emulsigen vaccine group ($p<0.05$) and not increased in any vaccine groups containing PHB beads. A small but significant increase in IgG2 antibody levels to HCc antigen was detected in the *E. coli* produced HCc vaccine group and recHCc in Emulsigen™ and recHCc in CFA ($p<0.05$).

Table 2: Serum IgG1 and IgG2c antibody responses to HCc

	IgG1	IgG2c
PBS	0.001 (+/- 0.001)	0.001 (+/- 0.001)
EcWT	0.045 (+/- 0.011)	0.029 (+/- 0.004)
EcHCc	0.110 (+/- 0.065)	0.045 (+/- 0.019) *
LcWT	0.001 (+/- 0.004)	0.001 (+/- 0.002)
LcHCc	0.017 (+/- 0.003)	0.008 (+/- 0.001)
recHCc in Emulsigen	1.126 (+/- 0.079) *	0.126 (+/- 0.013) *
recHCc in CFA	0.089 (+/- 0.044)	0.036 (+/- 0.011) *

Sera were collected 5 weeks after the initial vaccination. IgG1 and IgG2c antibodies to HCc were measured by ELISA. Results were expressed as mean (+/- SEM) optical density @ 450nm for sera diluted 1/250. * Significantly greater response than PBS vaccinated control ($p<0.05$)

4.5 Discussion

Bioengineered PHB beads have previously been used to display proteins with a variety of potential end-uses. Here further evidence was provided for the versatility of bioengineered PHB beads to be used for medical applications as viral antigen displaying beads by allowing custom antigen display and the subsequent use as particulate antigen carrier systems. In this study, it was shown that the generally regarded as safe (GRAS) bacterium *L. lactis* as well as *E. coli* could be engineered as production hosts for PHB

bead based particulate vaccines which displayed HCc antigens. This antigen was used because it is a prime candidate antigen for inclusion in both therapeutic and prophylactic hepatitis C vaccines (42). However, the disadvantage of using *E. coli* as the production host for human biological products, including vaccines, is potential contamination of products with LPS. This precludes use of such products for human vaccination without costly depyrogenation, a process which may also destroy protein function (50). *L. lactis* is a Gram-positive bacterium which does not contain LPS and has been extensively used in manufacture of dairy products. More recently it has been investigated as a production host for recombinant proteins (28) and a mucosal vaccine for hepatitis B (52). The study in this paper combines both the production of recombinant protein, i.e. the viral antigen HCc, and the polymeric carrier in a one-step process.

This new vaccine delivery system has the advantage that vaccine antigens are produced on beads rather than as soluble proteins. Particulate vaccines have been shown to be more immunogenic (20) and the size of particles is likely to play a role in the type of immune response with nano-particles stimulating cell mediated immunity and larger particles stimulating antibody responses (16). The TEM images show differences in size of beads produced in *E. coli* and *L. lactis* (Fig. 1) which may account for different antibody responses being obtained.

It was demonstrated that *L. lactis* was able to produce PHB beads displaying a substantial amount HCc antigen as shown by the SDS-PAGE gel (Fig. 2). In comparison, significantly less fusion protein was seen on the surface of the PHB beads produced in *E. coli,* which indicated utilization of the nisin-controlled gene expression system by *L. lactis* enabled efficient overproduction of functional heterologous proteins (25). The strong overproduction of HCc-PhaC fusion proteins on the beads correlated with relatively fewer contaminating host proteins in the *L. lactis* produced beads. An advantage of a purer product would be the reduction in the need for extensive downstream processing for the removal of host cell proteins and hence reduced production costs.

Mice vaccinated with PHB beads produced by *L. lactis* which displayed HCc antigens were found to initiate an antigen specific Th1 immunity pattern shown by production of IFN-γ as well as an IL-17A (Fig 4). Th1 immunity has long been associated with IFN-γ production (29) and IL-17A plays a critical role in vaccine induced immunity against infectious diseases (21). Th17 cells are the major source of IL-17A and it is reported that following vaccination, Th17 cells release IL-17A which promotes the

induction of chemokines to recruit effector Th1 cells and neutrophils to control pathogens (17, 48). While it has been established that a Th1 immunity pattern is important for hepatitis C protective immunity (1), the significance of IgG1 antibodies in viral neutralizing activity remains controversial, since high titre of anti-HCc antibodies can coexist with viremia (42). Therefore the non-significant antibody responses (Table 2) measured in animals vaccinated with *L. lactis* HCc might be less relevant. The immune responses following vaccination using HCc PHB beads from the *E. coli* production host also demonstrated a Th1 immune pattern as evidenced by increased IFN-γ and serum IgG2c titres. However animals vaccinated with either wild-type control or HCc beads produced in *E. coli* also showed increased levels of the proinflammatory cytokines, TNF-α and IL-6, which can lead to tissue damage (10). It has been shown that IL-6 combined with TGF-β is a strong inducer of Th17 T cells in mice leading to the production of IL-17 and the combination of IL-6 and TGF-β induce $CD4^+$ T cells to produce both IL-17 and IL-10 (23) In addition, Lombardi et al. have shown sequential production of IL-10 and IFN-γ and eventually IL-17A by CD4+ T lymphocytes after stimulation with dendritic cells stimulated via TLR4 and TLR7/8 (22). The co-production of IL-10 is likely important in restraining the potentially destructive Th-17 cell–mediated response. The results from the current study provided evidence for co-expression of IL-6, Il-10 and Il-17A in vaccine groups receiving *E. coli*-produced PHB wild-type and HCc beads. The combination of responses seen in *E. coli*-produced bead vaccine groups may be due to LPS or contaminating *E. coli* proteins causing a non-specific adjuvant effect following vaccination. By comparison, vaccination with *L. lactis* HCc PHB beads generated a specific Th1 immune response which is needed for many diseases for which there is no effective vaccine (4, 11, 33).

The use and assessment of a suitable adjuvant is an important component of vaccine development. Adjuvants need to be assessed for each different antigen and are used to skew the immune response in the desired cell-mediated or humoral direction (8). Additionally, the presence of host cell proteins also can skew the immune response to enhance a Th1 or Th2 response (36). CFA is generally known to enhance Th1 immunity but cannot be used in humans due to severe site reactions. Vaccination with recHCc in Emulsigen™ induced a very strong IgG1 response (Table 2) associated with a Th2 immune response, and also significant induction of IFN-γ and IL-10 (Fig 4). The present study using HCc PHB beads in Emulsigen™ showed Th1 but no Th2 response which is

different to a previous tuberculosis vaccination study which showed both Th1 and Th2 immune responses after vaccination using mycobacterial antigen PHB beads in Emulsigen™ (32). Therefore it is worthwhile to investigate the use of different adjuvants and immunomodulators with PHB bead vaccines to determine the effect of adjuvant or host cell proteins on the immune response.

The vaccine production system described in this study eliminates the need for costly production, purification and conjugation of recombinant antigen to particulate carrier. Combined with the advantage of using a GRAS bacterium as production host and flexibility for antigen display, this vaccine system holds promise for future development and use.

4.6 Acknowledgements

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Chapter 5 Dendritic cell uptake of polyhydroxybutryate vaccine beads produced in *Lactococcus lactis* and *Escherichia coli*

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5.1 Abstract

Polyhydroxybutyrate beads (PHB) have been developed for a range of biomedical and laboratory uses. Recently such beads which displayed mycobacterial proteins were used successfully in mice as vaccine delivery agents to stimulate cellular immunity and induce protection against tuberculosis. An investigation into uptake of PHB beads manufactured by bacteria is presented in this paper. PHB beads were produced with and without vaccine antigens using both *Lactococcus lactis* and *Escherichia coli* hosts. PHB beads were incubated with human and mouse dendritic cells and uptake of beads was observed by electron microscopy. Maturation markers on dendritic cells were assessed using flow cytometry and cytokine release from dendritic cells was measured using ELISA. To assess the effect of lipopolysaccharide (LPS) present in beads derived from *E. coli*, antibodies against Toll-like receptor 4 and Toll-like receptor 4 knock-out mice were used. The bead uptake was found to be not mediated by LPS. Results showed rapid uptake of beads occurred independent of antigen display and continued for at least 10 days. Uptake of beads corresponded with maturation of dendritic cells and release of interleukin-12 cytokine. LPS in the bead suspension did not have an effect on bead uptake and only a minor effect on cytokine response.

5.2 Introduction

Soluble proteins are poorly immunogenic and must therefore be combined with adjuvants and delivery systems to increase their immunogenicity. Vaccine delivery systems are often of particulate nature which mimics the size of invading microorganisms and often contain immunomodulators to enhance uptake of particles and direct the type of immune response. Initially particulate antigens are taken up by dendritic cells (DC) which are the efficient antigen presenting cells (APC) and are crucial in induction of T cell responses which lead to immunological memory. Uptake occurs by phagocytosis, receptor-mediated endocytosis and macropinocytosis. Immunomodulators based on pathogen-associated molecular patterns (PAMPS) are recognized by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs). Innate TLR signalling induces up-regulation of co-stimulatory molecules B7.1 (CD80) and B7.2 (CD86) on APC. DCs are APC which trigger an adaptive immune response by capturing, processing and presenting antigens to naïve T cells. Immature DCs take up antigen and mature into cells which express high levels of CD80 and CD86, secrete cytokines such as interleukin-12 (IL-12)

and tumour necrosis factor-α (TNF-α). Another frequently used marker for matured DCs is CD83 (5). Maturation of DCs also leads to up-regulation of major compatibility complex (MHC) expression for enhanced antigen presentation (3). This occurs through binding of the T cell receptor (TCR) to an antigenic peptide bound to MHC on DC, which then leads to differentiation of naïve T cells into effector T cells. IL-12 secreted by mature DC induces CD4 T cell differentiation into Th1 cells. Hence IL-12 is often measured as an indicator of Th1-based immunity.

The biocompatible and biodegradable properties of biopolyesters make them appealing as biomaterials for applications in tissue engineering and use as drug and vaccine delivery vehicles (27). Polylactic acids (PLA) and their co-polymers are used extensively for sutures, drug delivery (1, 31) and have been studied as vaccine delivery agents (14, 23). Poly D, L-lactide-co-glycolic acids (PLGA) have been used in a variety of vaccine delivery systems (6, 16, 27). Polyhydroxybutyrate (PHB), the most common biopolyester produced by a variety of bacteria is formed naturally as spherical storage polymer granules inside the cell. Purified PHB has a wide range of uses (10) and has been recently investigated for neural tissue engineering (15) and as drug delivery agents (37). A recombinant bacterial system has recently been developed which enables the production of PHB granules displaying a protein of interest such as antigens, enzymes and binding domains (29). This approach was used to produce PHB beads displaying mycobacterial antigens and in mouse experiments these beads initiated a cell-mediated immune responses (26) and protection against tuberculosis (25). The present paper investigates the uptake of PHB beads produced by Gram-negative *Escherichia coli* or LPS-free Gram-positive *Lactococcus lactis*. *E. coli* is frequently used as a production host for recombinant proteins but removal of lipopolysaccharide (LPS) is often required whereas *L. lactis* does not contain LPS and is now being used as a production host for a range of uses: recombinant proteins (18, 21) including tuberculosis skin test reagents (4), delivery of therapeutic proteins (28) and vaccine antigens (24, 32, 38)

Understanding of the uptake mechanism of different PHB beads by DCs and its impact on maturation will inform the design of improved PHB beads toward generation of a desired immune response.

5.3 Materials and methods

Reagents and antibodies

Anti-human antibodies: CD11c-Alexa 488, CD14-PE, CD83-Alexa 488, HLA-DR-PE, CD80-Alexa 488 and CD86-PE were purchased from Biolegend (San Diego, CA, USA). Low endotoxin anti-TLR2 (MCA2484) and anti-TLR4 (MCA2061) were purchased from AbD Serotec (Oxford, UK).

Anti-mouse antibodies were purchased from a variety of companies as noted below: CD16/CD32 FC receptor block (BD), CD83-Alexa647 (Biolegend), MHCII-FITC, CD11c-PE and CD86-FITC (all from AbD Serotec), anti-TLR2 (clone 2.5, Hycult Biotech, Uden, Netherlands) and anti-TLR4 (clone MTS510, LEAF, Biolegend)

Culture and isolation of PHB beads

PHB beads which displayed a mycobacterial fusion protein Ag85A-ESAT-6, or control wild-type PHB beads alone were produced in *E. coli* and *L. lactis* as previously described (24, 26). Briefly, *E. coli* was grown at 30°C in LB, induced with 1mM isopropyl β-D-thiogalactopyranoside to produce recombinant protein and cultured for a further 48 hours at 30°C to allow accumulation of PHB beads. *L. lactis* cultures were produced in M17 broth, induced with 10 ng/ml nisin to produce protein and cultured for a further 48 hours at 30°C. Presence of intracellular inclusions was determined by staining the cultures with Nile Red lipophilic dye and observed using fluorescence microscopy. Bacteria were then mechanically disrupted and *E. coli* lysate was centrifuged at 4000 x *g* or *L. lactis* lysate was centrifuged at 8000 x *g* for 15 min at 4°C to sediment the PHB beads. All beads were then purified via glycerol gradient ultracentrifugation and washed in 50mM potassium phosphate buffer pH 7.2.

Preparation of human monocyte-derived DCs

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat preparations, from normal healthy donors, by standard density gradient centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway). PBMCs were harvested from the interface and washed three times in PBS then incubated for 15 minutes with mouse anti-human CD14 magnetic beads (Miltenyi Biotec, Auburn, CA, USA) and washed in PBS. An AutoMACS (Miltenyi Biotec) machine was used according to manufacturer's instructions to separate $CD14^+$ cells. Purity of monocytes was assessed by staining cells with anti-mouse FITC (DAKO, Denmark) and was in the range 90–95%. One ml aliquots of cells

(1 x 10^6 /ml) were incubated in 24 well plates for 7 days in at 37°C in an atmosphere of 10% CO_2 in air Media used was RPMI (Gibco, Grand Island, NY) supplemented with: 25mM HEPES and 2 mM L-glutamine, 5x10^{-5}M 2-mercaptoethanol (Sigma, St.Louis, MO), penicillin (100 U/ml), streptomycin (100μg/ml), 10% foetal calf serum (Invitrogen) and recombinant human granulocyte macrophage colony stimulating factor (GM-CSF, 100ng/ml) and interleukin-4 (IL-4, 50 ng/ml). Both cytokines were purchased from Peprotech, Rocky Hill, NJ, USA.

Preparation of murine bone marrow-derived DC

Bone marrow cells were prepared as previously described (13) from C57Bl/6 wild-type (WT) and C57BL/6 mice which lacked TLR4 (TLR4-/-). Cells were cultured in 5 ml aliquots at 1 x 10^6 /ml for 7 days in Dulbecco's Modified Eagle Media (DMEM) (Gibco, Grand Island, NY) supplemented with: 1% NEAA (Gibco, Grand Island, NY), 2 mM L-glutamine, 5x10^{-5}M 2-mercaptoethanol (Sigma, St.Louis, MO), penicillin (100 U/ml), streptomycin (100μg/ml) 5% foetal calf serum (Invitrogen), 100 U/ml of recombinant murine GM-CSF and 100 U/ml of recombinant murine IL-4. Mice and cytokines were obtained from the Malaghan Institute of Medical Research, Wellington, New Zealand.

Culture of DCs and PHB beads

After 8 days incubation, human or murine DCs (5 x 10^5 cells in 1 ml of media) were cultured with 100 μl of PHB beads at 1mg/ml or LPS (1μg/ml, Sigma). Some wells had 15 μg/ml antibodies to TLR2 or TLR4 added to cultures to block these receptors. In addition, human DCs were cultured with 100 nm latex beads (Sigma) at 1mg/ml. After further overnight or 10 day incubation, supernatants were removed and frozen for subsequent cytokine analysis and cells were stained with antibodies to determine activation markers using flow cytometry. Fluorescence was detected using a FACSCalibur flow cytometer (BD), and analyzed using CellQuestPro (BD) or Flowjo analysis software (Treestar, Ashland, OR, USA) to determine mean fluorescence intensity (MFI).

Human DCs for transmission electron microscopy (TEM) were incubated with PHB beads overnight or 10 days. DCs were assessed for viability using trypan blue (0.1% in PBS; Sigma). The samples were centrifuged at 2600 × *g* and the sediment was prepared for TEM analysis as described previously (11).

Cytokine analysis

IL-12 (p40) was measured by sandwich ELISA using OptEIA kit according to manufacturer's protocol (BD; Becton–Dickinson, San Jose, CA). The assay was read on a VERSAmax microplate reader. A standard curve was constructed using SOFTmax PRO software and averages of duplicate sample cytokine values were determined from the curve.

5.4 Results

PHB bead uptake by DCs.

To investigate the efficiency of uptake of PHB beads by DCs, a time course study was conducted incubating PHB beads with DCs. After overnight culture about 50% of the PHB beads had been taken up by DCs. Latex beads did not appear to be taken up readily. TEM shows PHB beads produced in *E. coli* and *L. lactis* that displayed Ag85A-ESAT-6 (Fig 1A). After 10 days few beads remained in the media and DCs were packed with beads (Fig 1B). Trypan blue exclusion indicated DCs were still viable at this stage (data not shown). Uptake of beads was similar for wild-type beads produced in *E. coli* and *L. lactis* (data not shown).

PHB beads stimulate maturation of DCs independent of TLR2 and TLR4.

Monocyte derived human DCs were incubated overnight with LPS, latex beads, wild-type PHB beads or PHB beads produced in *E. coli* or *L. lactis* that displayed vaccine antigen fusion of Ag85A-ESAT-6. CD11c was used as a marker for DCs and live CD11c positive cells incubated with the various PHB beads or LPS showed at least a two-fold increase in increased MFI of surface maturation markers CD86 and HLA-DR, when compared to DCs alone or cells incubated with latex beads (Fig. 2 and Table 1). CD80 and CD83 MFI showed variable increases between experiments which may reflect different donor cell preparations.

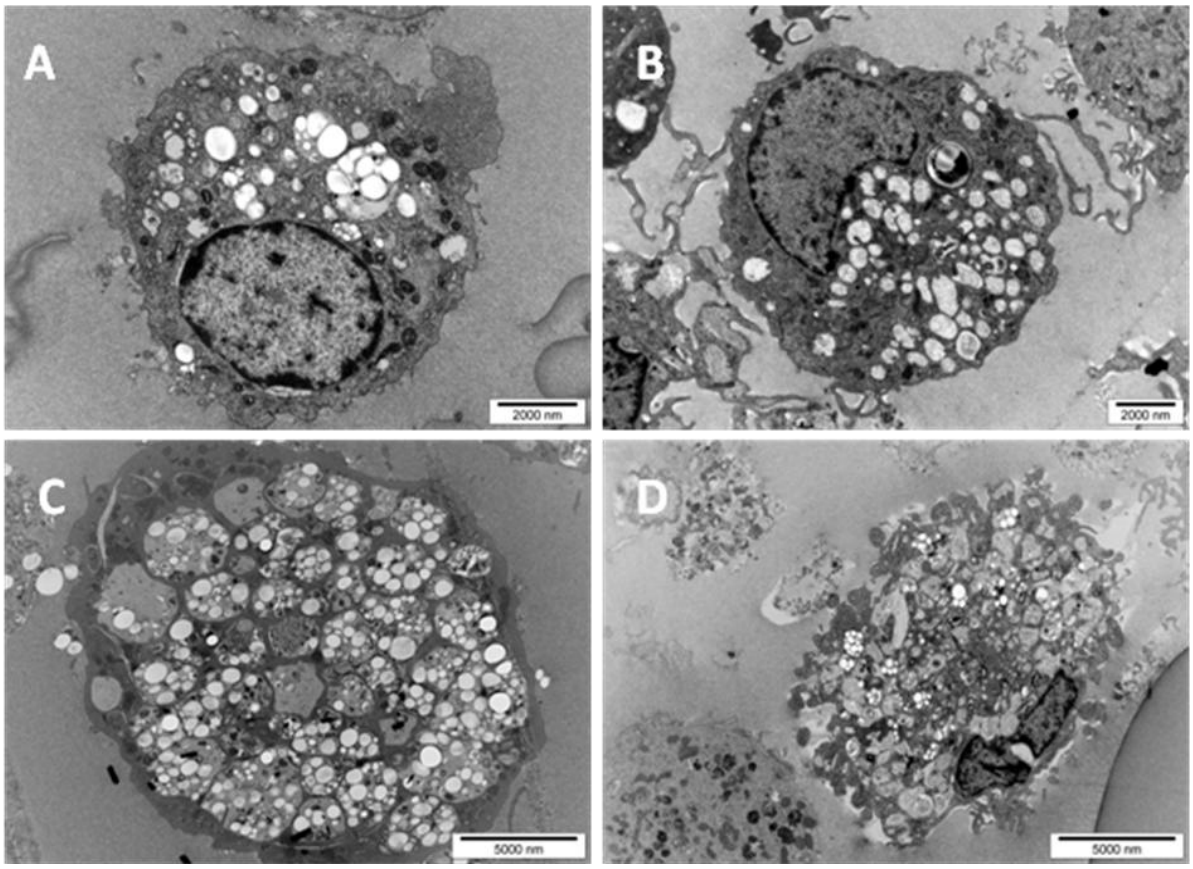
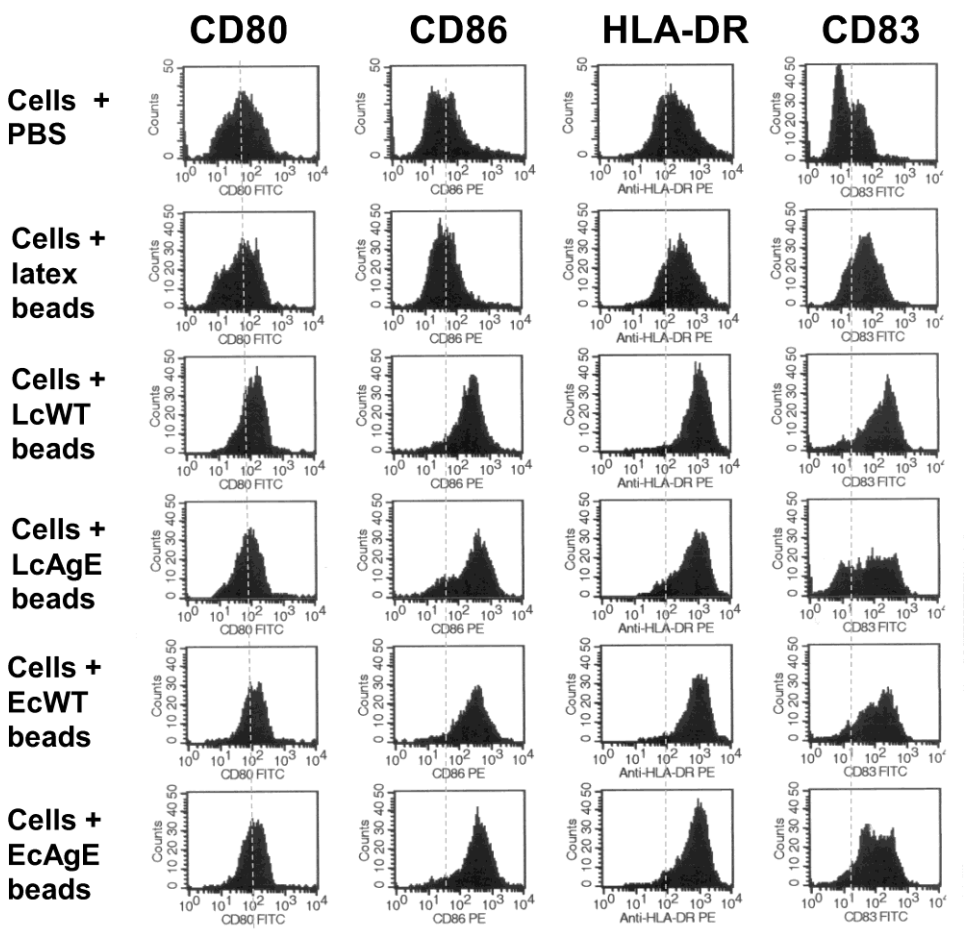


Figure 1: Transmission electron micrograph of vaccine beads inside human dendritic cells.
Monocyte derived dendritic cells (DCs) were incubated overnight with 100 µg PHB beads that displayed Ag85A-ESAT-6 and produced in A) *E. coli* or (B) *L. lactis*. Alternatively DCs were incubated for 10 days with PHB beads which had been produced in C) *E. coli* or (D) *L. lactis*.

A



B

	Mean Fluorescence Intensity (MFI)			
	CD80	CD86	HLA-DR	CD83
Cells + PBS	51.0	37.3	174.4	17.7
Cells + latex beads	49.7	39.0	251.8	50.8
Cells + LcWT beads	110.6	214.5	929.7	150.4
Cells + LcAgE beads	75.9	254.7	629.9	65.5
Cells + EcWT beads	105.8	252.3	765.0	105.2
Cells + EcAgE beads	100.9	302.8	619.6	149.4

Figure 2: Flow cytometry to assess activation markers on monocyte-derived human dendritic cells.

Cells were incubated overnight with PBS, latex beads or PHB beads produced in *L. lactis* or *E. coli,* either wild-type or displaying Ag85A-ESAT-6 (AgE) antigens. Cells were stained with antibodies to CD11c, CD80, CD86, HLR-DR and CD80. Cells were gated on live CD11c+ cells. A) histograms show relative fluorescence B) mean fluorescence intensity (MFI). Results are representative of 2 independent experiments.

Table 1: **Human dendritic cells were incubated overnight with PBS, PHB beads or LPS with and without antibodies to TLR2 and TLR4, then stained for activation markers CD80, CD86, HLA-DR and CD83.**
Beads used were: LcWT, wild-type beads produced in *L. lactis*; LcAgE, *L. lactis* produced beads displaying Ag85A-ESAT-6 antigens; EcWT, Wild-type beads produced in *E. coli*; EcAgE, *E. coli* produced beads displaying Ag85A-ESAT-6 antigens. Cells were gated on live CD11c+ cells and mean fluorescence intensity was measured. Results are representative of 2 independent experiments

	Mean fluorescence intensity			
Cells incubated with	**CD80**	**CD86**	**HLA-DR**	**CD83**
PBS	45	48	236	38
PBS + anti-TLR2	53	54	233	37
PBS + anti-TLR4	51	60	250	39
LcWT beads	68	125	390	71
LCWT beads + anti-TLR2	63	170	420	50
LCWT beads + anti-TLR4	60	185	330	54
LcAgE beads + PBS	71	272	434	66
LCAgE beads + anti-TLR2	68	281	395	61
LCAgE beads + anti-TLR4	66	319	329	47
EcWT beads + PBS	50	184	313	44
EcWT beads + anti-TLR2	52	170	319	57
EcWT beads + anti-TLR4	55	222	312	60
EcAgE beads	52	191	508	53
EcAgE beads + anti-TLR2	48	187	312	48
EcAgE beads + anti-TLR4	60	314	310	60
LPS + PBS	55	233	345	58
LPS + anti-TLR2	61	267	333	58
LPS + anti-TLR4	68	263	370	55

Furthermore, IL-12 was secreted from human DCs which had been incubated overnight with LPS or PHB beads produced in *L. lactis* or *E. coli* but not from dendritic cells alone (Fig 3A). IL-12 secretion was not changed when beads which displayed Ag85A-ESAT-6 were used but IL-12 release was greatest when cells were incubated with LPS (Fig 3A). Incubation of latex beads with human DC resulted in no increased secretion of IL-12 compared to dendritic cells alone (data not shown).

Murine DC alone did not release IL-12 but IL-12 was released when murine DCs were incubated with LPS or PHB beads. IL-12 release was greatest when incubated with LPS. The addition of antibody to TLR2 or TLR4 to the culture did not block this secretion (Fig 3B).

Incubation of anti-TLR2 or anti-TLR4 antibodies with DCs in order to block the respective receptors prior to addition of PHB beads did not show substantial changes in expression of maturation markers (Table 1).

To further investigate whether TLR4 is involved in uptake of PHB beads by DCs and their maturation, DCs were cultured from C57Bl/6 WT and C57Bl/6 TLR4-/- mice. DCs alone from WT or TLR4-/- mice did not release IL-12. However IL-12 was produced following incubation of DCs from WT mice with PHB beads or LPS (Fig 3C, white bars). DCs from TLR4-/- mice, did not produce IL-12 when incubated with LPS but these DCs secreted IL-12 following incubation with PHB beads (Fig 3C, black bars). IL-12 was secreted in similar amounts by DCs from C57BL/6 and TLR4-/- mice when cells were incubated with beads produced in *L. lactis*. However, DCs from TLR4-/- mice were able to produce moderate amounts of IL-12 when incubated with beads produced in *E. coli* although the quantity of IL-12 was reduced to that measured with C57Bl/6 mice.

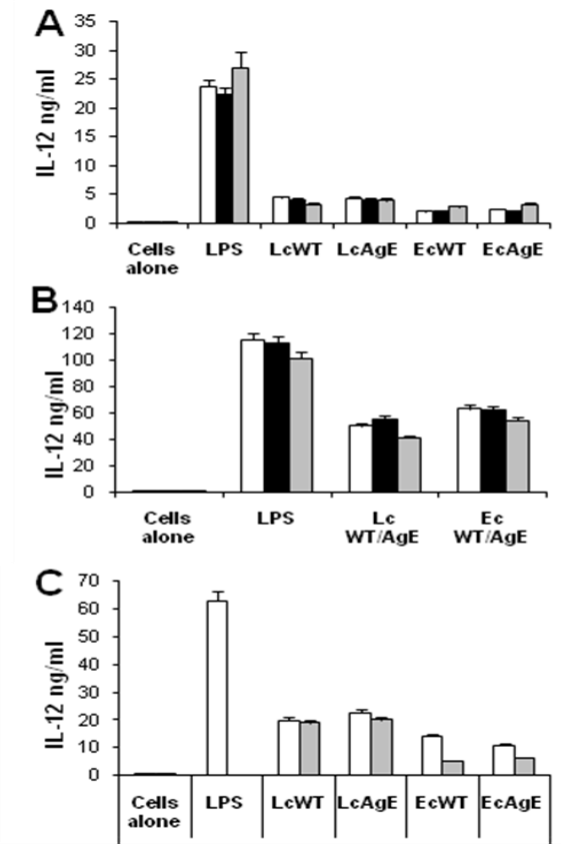


Figure 3: Interleukin-12 (IL-12) was measured by ELISA from supernatants of human or murine dendritic cells (DCs) incubated with LPS or PHB beads.

A) Human DCs were incubated with PBS (white bars), anti-TLR2 antibody (black bars) and anti-TLR4 antibody (grey bars). PHB beads were then added and incubated overnight. Beads used were: LcWT, wild-type beads produced in *L. lactis*; LcAgE, *L. lactis* produced beads displaying Ag85A-ESAT-6 antigens; EcWT, Wild-type beads produced in *E. coli*; EcAgE, *E. coli* produced beads displaying Ag85A-ESAT-6 antigens. IL-12 in supernatants was measured by ELISA. Data represents 3 independent experiments. Bars indicate standard deviation of mean of replicate samples.

B) DCs from C57Bl/6 mice were mixed with PBS (white bars), anti-TLR2 (black bars) and anti-TLR4 (grey bars) antibodies then incubated overnight with LPS or PHB beads. Beads contained a mixture of wild-type beads and beads displaying Ag85A-ESAT6 antigens and were produced in *L. lactis* (LcWT/AgE) and *E. coli* (EcWT/AgE). IL-12 in supernatants was measured by ELISA. Data represents 2 independent experiments. Bars indicate standard deviation of mean of replicate samples.

C) DCs from C57Bl/6 mice (white bars) and TLR4-/- mice (grey bars) were incubated overnight with LPS or PHB beads: LcWT, wild-type beads produced in *L. lactis*; LcAgE, *L. lactis* produced beads displaying Ag85A-ESAT-6 antigens; EcWT, Wild-type beads produced in *E. coli*; EcAgE, *E. coli* produced beads displaying Ag85A-ESAT-6 antigens. IL-12 in supernatants was measured by ELISA. Data is representative of two independent experiments. Bars indicate standard deviation of mean of replicate samples.

5.5 Discussion

PHB beads displaying different antigens have previously been used in vaccination studies and were shown to initiate specific immune responses either alone or mixed with Emulsigen® (24, 26). A further study showed antigen specific Th1 immune responses were detected and protection from tuberculosis was observed in mice previously vaccinated with PHB beads displaying mycobacterial antigens (25). In the present study the uptake of PHB beads produced in *E. coli* and *L. lactis* hosts by DCs was investigated to gain insight into the mechanism of induction of the protective immune response.

Beads produced in both *E. coli and L. lactis* were shown to be taken up readily by DCs (Fig. 1). Such uptake is important as the first step in initiating an immune response. Uptake of beads from both bacterial hosts was shown to be independent of antigen display. This suggests general bead properties excluding the display of specific surface proteins are sufficient for uptake by DCs. Factors such as bead size, structure and generic surface display of PAMPs may be important factors for innate responses.

DCs have evolved to take up particulate infectious bacteria or viruses and therefore the design of vaccines as particulate structures is desirable. In the present study, PHB bead vaccines were 50-500 nm in size which represents particulate dimensions suitable for uptake by DCs (36). Previous studies have shown that size of particle vaccines can affect the type of immune response; consequently if a cell-mediated immune response is required then particles should be less than 1 μm (7, 14, 22). Maturation of DCs was observed when PHB beads from either production host were used and this suggested PAMP recognition. The role of IL-12 is important in promoting a Th1 response (17) and results in the present study showed secretion of IL-12 was increased in cells exposed to PHB beads which is consistent with Th1 responses detected in vaccination studies using PHB beads (24, 26).

The PHB by itself could act as an adjuvant and act through one or more pattern recognition receptors (2, 34). During production and purification, LPS has been shown to co-purify with PHB (19) so it had been hypothesized that uptake of these beads would result in signalling through the LPS receptor, TLR4 and lead to IL-12 release. Initially mouse and human antibodies to TLR4 were used to investigate the role of TLR4 signalling. These did not block maturation of DC and there was only a small decrease in IL-12 release when anti-TLR4 antibody was used. Therefore DCs from TLR4-/- mice

were used to definitively determine signalling through TLR4 and the role of LPS associated with PHB beads in uptake by DCs and their maturation. If TLR4 was the sole receptor involved in initiating IL-12 responses, then complete abrogation of IL-12 responses would have been observed in DCs from TLR4-/- mice when incubated with PHB beads. However, there was only a modest decrease in IL-12 secreted from these mouse cells incubated with *E. coli* produced beads which confirmed TLR4 signalling played a minor part in DC maturation and release of IL-12. Therefore other receptors or mechanism were involved with induction of this cytokines release. It had generally been thought that lipoteichoic acid and peptidoglycan from Gram-positive bacterial cell walls act as TLR2 agonists (30) so blocking antibodies were used to determine the role of TLR2 signalling in DC maturation and IL-12 release. DC maturation was observed in cells exposed to PHB beads produced in the LPS-free Gram- positive host *L. lactis* but blocking DC with antibody to TLR2 did not result in decreased maturation or IL-12 release by DCs. This suggested TLR2 signalling is not associated with IL-12 release from DCs. Recent reports indicate lipoteichoic acid can induce cytokines independently of TLR2 (8, 12) and NOD-like receptors (NLRs), which recognise intracellular PAMPs have been suggested as alternative receptors (8) for lipoteichoic acid.

The synthase proteins which are used as anchors for the display of antigen have been shown to be located on the outer surface of PHB beads (9) and it is therefore possible that following uptake, the bead proteins are rapidly processed and intracellular receptor signalling pathways could be activated. TLR9 signalling is a possible intracellular pathway which could be involved in PHB bead responses by DCs. TLR9 is expressed intracellularly, within the endosomal compartments and binds to DNA rich in unmethylated CpG motifs from bacteria. TLR9 signals leads to activation of DCs initiating pro-inflammatory reactions that result in the production of cytokines such as IL-12. Various studies have added CpG oligonucleotides to vaccines to act as adjuvants which induce this signalling pathway (20, 33, 35). The results from the present study indicated the need to further investigate signalling through alternative recognition molecules such as TLR9 and NOD-like receptors (NLRs) which recognise intracellular PAMPs. A range of methodologies, cytokine array measurement and use of TLR and NLR knock-out mouse models would be useful. Such studies could determine if PHB beads act synergistically through a range of receptors. Further analysis of the signalling pathways associated with the use of PHB beads as particulate vaccines will provide

insight on how to rationally design the PHB beads to promote and enhance specific interactions between PHB beads and DCs.

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Chapter 6 Summary

6.1 Summary

This thesis described the development of a novel vaccine delivery system utilizing PHB beads. PHB beads have been developed and used previously for a range of *in vitro* applications but *in vivo* uses had not been investigated. The thesis chapters cover the process of vaccine bead design, production and validation to enable their use in vaccine trials. Different vaccine antigens and alternative production hosts were investigated. Immune responses were measured in mice vaccinated with PHB vaccine beads and tuberculosis protection studies in mice were completed. In addition to the relevant specific discussions in each chapter, some additional comments are presented here.

The initial aim of the project was to determine if PHB beads which displayed vaccine antigens could be produced by bacteria. PHB biosynthesis genes and vaccine antigen genes were able to be successfully incorporated into the *E. coli* and *L. lactis* production strains as assessed by *in vitro* analyses (Chapters 2, 3 and 4).

Following insertion of genes for different vaccine antigens into both bacterial hosts, PHB beads were produced which displayed Ag85A-ESAT-6 fusion protein (Chapter 2-3) and hepatitis C core antigen (Chapter 4). Beads were purified, used for vaccination and immune responses were measured.

Cytokine responses: An immune response of antigen specific IFN-γ release by restimulated spleen cells was detected following vaccination with PHB beads. IFN-γ is regarded as a marker of a Th1 response in CMI. In addition IL-17A antigen specific responses were detected following vaccination with Ag85-ESAT-6 PHB beads (Chapter 3). IL-17A appears to be important in vaccine induced immunity against TB (22) but pathological effects induced by IL-17A must be finely balanced against its protective effects (12). Both Th1 and Th17 responses are needed for protective immunity from tuberculosis. Griffiths *et al* (13) detected IL17+ cells producing IFN-γ appeared at the same time as maximal antigenic responses to Ag85A which may be a result of NLRP-3 inflammasome activation.

IL-17A was produced following vaccination with hepatitis C core antigen PHB beads produced in *L. lactis* (Chapter 4). The release of IL-17A may inhibit viral replication in

hepatitis B (44). In the same experiment, there were non-specific increases in IL-10, IL-6 and IL-17A from animals vaccinated with beads produced in *E. coli*. This highlights the advantage of using *L. lactis* as a production host instead of *E. coli*.

Antibody and Th2 responses: It is interesting to note antibody responses were different following vaccination with Ag85A-ESAT-6 beads and hepatitis C beads.

A strong antigen specific antibody response of IgG1 and IgG2c isotypes were detected following vaccination with Ag85A-ESAT-6 beads (Chapter 2) suggesting a mixed Th1 and Th2 immune response.

In contrast, Hepatitis C beads produced in *E. coli* stimulated a small IgG2c response which supports Th1 immunity (Chapter 4). *L. lactis* produced hepatitis C beads did not show any significant antibody responses.

The reasons for this difference in Th2 immunity could be due to several reasons. Different epitopes on the vaccines may preferentially stimulate Th1 versus Th2 responses. Suppression and synergism by different Th1 and Th2 epitopes have been described previously (46). In the present studies, the Hepatitis C vaccine used the whole core protein and it is therefore likely that this mixture of epitopes were able to suppress Th2 responses. This strengthens the case for using this versatile PHB bead vaccine system so that gene sequences for specific epitopes can be used in the future. The presence of contaminating proteins on PHB beads could also account for differences in immune responses. The strong over-production of hepatitis C vaccine antigen on *L. lactis* produced PHB beads indicates less space for non-specific binding proteins and conversely *E. coli* produced beads had greater amounts of contaminating proteins. These proteins could signal through different receptors than the predominant hepatitis C antigens and therefore moderate the Th1/Th2 bias. Variations in immune response could be due to size of particles because it has been shown that uptake of particulate antigens is related to size (20). In Chapter 4, electron microscopy images of hepatitis C PHB beads show all to be less than 500nm. *L. lactis* produced beads were 50-150 nm and beads produced in *E. coli* were 150-250 nm. These size variations may account for the small variations measured in antibody responses. In contrast, electron microscopy images in Chapter 5 uptake studies indicated a wider range of bead sizes with *E. coli* produced beads being up to 1mm in size while *L. lactis* produced beads were consistently smaller. This could explain the mixed Th1 and Th2 immune responses

obtained in Chapter 2 and confirm immune responses are influenced by particle size. PHB bead size is influenced by factors including culture conditions and incubation time, so control of this could add further flexibility to vaccines.

Studies were undertaken to gain insight into the mechanism of uptake of PHB beads (Chapter 5). These preliminary studies suggested PHB beads are taken up readily by DCs and stimulate maturation of DCs independent of antigen display. The range of receptors involved with maturation and Il-12 release were not determined so further investigations are needed so that design and production of beads could be focused to enable optimal immune responses.

6.2 Future Outlook

This is a new vaccine technology so there are developments which could be made to the bead biosynthesis, vaccine formulations and production processes.

6.2.1 Optimization of genes

To aid in the production of PHB polyester, it was important PHB biosynthesis genes were optimized for codon usage for the respective bacterial hosts to avoid rarely used codons. Antigen display on the beads may be increased/enhanced through use of multiple gene repeats of the required proteins, similarly to that done by Mullaney and Rehm (28). For such an approach, inclusion of a linker molecule may be necessary, particularly if C terminus fusions are used (18).

Results revealed in this thesis also demonstrated the added advantage of a very strong over-production of hepatitis C core antigen on beads produced using the nisin expression system (Figure 2, Chapter 4). Such over-expression was not observed with beads displaying Ag85A-ESAT-6 so this is an area which could be further optimized, possibly through multiple gene repeats or simple changes to culture conditions.

6.2.2 Host

The United States Pharmacopeial Convention (USP) sets world-wide standards for the quality, purity, identity, and strength of medicines, drugs and ingestible products. USP have set the maximal endotoxin limit of parenteral delivered drugs at 5 EU/kg, but vaccines are excluded from these requirements. Indeed, human vaccine endotoxin levels vary considerably; toxoid and live vaccines are likely to have high endotoxin levels,

whereas purified recombinant protein vaccines often have low levels of endotoxin (10). It is sensible to produce vaccines by a method which reduces the likelihood of endotoxin contamination. *E. coli* production systems are inevitably contaminated with LPS, which then requires extensive and usually costly removal treatment. There are a range of methods to remove LPS including Polymixin B (35), sodium hydroxide (24), and Triton-X 114 (21, 26). All the references agree that removal needs to be tailored to the specific product and removal may not be easily achieved. Gram negative bacteria are often used for the production of PHAs but LPS co-purifies with PHA (41). Therefore Gram positive hosts are an LPS-free alternative for PHA production. *Streptomyces sp.* and *Bacillus sp.* produce natural PHAs and Valappil suggests using these hosts for the production of PHA which could be used for medical applications (41). Recently, *Corynebacterium glutamicum* has been used as a recombinant host to produce a PHA co-polymer (27). The present study used two different bacterial hosts for production of PHB vaccine beads. Initially *E. coli* was used successfully as a "proof of concept" host (Chapter 2) and results showed immune responses had been initiated which then prompted the use of an alternative production host, *L. lactis* (Chapters 3-4). *L. lactis* was selected as an alternative host because it has had prior extensive safe use in foods and more recent use for vaccines and recombinant protein production, largely due to it being seen as a safe LPS free host.

6.2.3 Alternative antigens

It was very encouraging to find PHB beads displaying mycobacterial antigens were able to protect mice from tuberculosis (Chapter 3). Mice vaccinated with beads produced in *L. lactis* did not show that extra-pulmonary spread of TB had been arrested but it is a field for future development, possibly through the use of alternative antigens, immunomodulators or use of PHB beads as a boost vaccine to follow BCG priming.

A feature of using bio-engineered PHA beads as antigen delivery vehicles, is the scope for developing a range of vaccines. This thesis used two different vaccine antigens aimed at diseases for which there is no effective vaccine despite much research. Results demonstrated the versatility of the system, simply requiring a selected gene sequence along with PHB biosynthesis genes. The antigens chosen had been shown in earlier studies to be immunodominant and have been used by other researchers in vaccination

studies (6, 15, 19, 38). However sub-dominant antigens and/or dominant antigens incorporated in vaccines is an alternative approach (1, 16, 32, 39)

6.2.4 Adjuvants and immunomodulators

There are many adjuvants and immunomodulators available for research and the type of immune response depends on the antigen used and animal model (2, 31). Emulsigen® adjuvant has been used successfully by our group in past cattle experiments (42). This oil-in-water adjuvant is practical to use because it is stored at room temperature and mixes easily with antigen. By comparison, water-in-oil adjuvants are messy, require refrigeration and preparation can be time-consuming and fickle to achieve the desired consistency. In Chapter 2, initial vaccination experiments using *E. coli* produced PHB beads displaying Ag85A-ESAT-6 showed enhancement using Emulsigen® (Chapter 2). Therefore this adjuvant was used in all further vaccination experiments. Future experiments could include the use of alternative adjuvants to specifically tailor the vaccine to the desired immune response.

Immunomodulators can be added to vaccines to activate PRRs and are often based on TLR agonists such as flagellin, CpG oligonucleotides, LPS or detoxified LPS derived monophosphoryl lipid A (MPL®). Cytokines, such as interleukin-2 (IL-2) and granulocyte monocyte colony stimulating factor (GM-CSF), stimulate innate and adaptive immune cells and are also used as vaccine immunomodulators (43). Co-delivery of vaccine antigen and immunomodulatory agents to dendritic cells has been shown to increase immune responsiveness (9, 40). Previously, PHB beads have been produced which display IL-2 (4) and both the N-terminus and C-terminus of PhaC have been used to produce fusions for functional protein display (3). Therefore further development to produce PHB beads which display both vaccine antigens and immunomodulator proteins on one bead could increase vaccine efficacy.

Streptavidin PHB beads have been produced using a C-terminus-PhaC fusion (34) and these genes could be combined with an N-terminus-PhaC vaccine antigen fusion, such as the Ag85A-ESAT-6, to produce beads. Subsequently a biotinylated adjuvant such as phosphatidyl inositol di-mannoside (PIM2) (33) could be added, resulting in beads displaying Ag85A-ESAT6-PhaC-streptavidin–biotinylated PIM2 which would then be used to deliver antigen and immunomodulator to the same cells. Alternatively, co-

delivery could be achieved by chemical conjugation of immunomodulators to antigen-displaying PHB vaccine beads.

6.2.5 Multi-valent vaccines

Multivalent vaccines are already in use for both human e.g. diphtheria-pertussis-tetanus vaccine, and animal vaccines e.g. multi-component clostridial vaccine, Ultravac® 7 in 1 (Pfizer Animal Health, NZ). Their use is desirable to reduce the number of vaccinations needed and hence reduce the distress which can be associated with vaccination and also reduce operational costs (7). This new PHB bead technology could be applied to the development of multivalent vaccines by utilizing both N-terminus and C-terminus fusions (17) and would have the added advantage of reducing overall production costs.

6.2.6 Mucosal immunity

Sub-cutaneous administration is acceptable for laboratory animals and many veterinary animals but mucosal immunisation of humans and animals would be a more efficient and safe way to administer vaccines (30). Mucosal vaccines have been developed which use a range of polymeric particles such as chitosan (8, 11) PLGA and PLA (14, 23, 29, 37). Furthermore, there has been increased interest in mucosal vaccination using live recombinant *L. lactis* (5, 25, 45) and non-living non-genetically modified *L. lactis* particle vaccines (36). Therefore, it would be useful to investigate vaccine delivery via the mucosal route using PHB beads produced in *L. lactis*.

6.2.7 Optimization of production

The production system described in this thesis could be optimized further to advance from a basic research laboratory to scaled-up systems and finally commercial production. Some enhancements to production processes are noted below.

Bioreactor: Use of a bioreactor which is able to monitor growth conditions oxygen, glucose and pH would enable fine control of growth so that optimal time for induction and harvesting of cultures could be determined. It is likely that such optimization and standardization would increase the PHB yield and therefore improve cost-effectiveness and enhanced commercial appeal.

Lysis: In these studies mechanical lysis was used to release beads. Chemical lysis is possible using protein extraction reagents such as B-PER® or BugBuster®, and therefore

chemical lysis could be investigated. However use of such chemicals vastly increases costs and introduces further chances of new contaminants. Alternative mechanical disruption is available through the use of microfluidizers and homogenizers to ensure uniform shear that is repeatable and scalable. Under optimized conditions, microfluidizer technology can result in maximal recovery of intracellular beads and ensuing destruction of viable organisms.

Purification: glycerol gradient and ultra-centrifugation were used in these studies but this is not practical for large-scale production. Therefore it is likely an alternative purification process would be used such as crossflow filtration (tangential flow filtration).

6.3 Conclusion

In conclusion, these novel PHB beads have been shown to be useful in the field of vaccine delivery due to the ease of creating a diverse antigen repertoire and they can be produced commercially by efficient cost-effective processes. Of prime importance is their ability to initiate immune responses, particularly cell mediated responses and when mycobacterial antigen PHB beads were used, protection against tuberculosis was elicited. It would therefore be worthwhile to further develop PHB beads for delivery of vaccine antigens and immunomodulators.

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Appendix:

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATION