Polyhydroxyalkanoate beads as a particulate vaccine against *Streptococcus pneumoniae* and *Neisseria meningitidis*

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

in

Microbiology

at Massey University, Manawatu, New Zealand.

Majela González Miró

2017

Main Supervisor: Professor Bernd Rehm

Co-supervisors: Dr Zoe Jordens, Dr Vicente Vérez-Bencomo

Abstract:

Streptococcus pneumoniae and *Neisseria meningitidis* are the major causes of pneumonia and meningitis, respectively, worldwide. Capsular polysaccharide-protein vaccines (conjugate vaccines) provide protection against these diseases but not protection against infections caused by serotypes and serogroups not included in these vaccines. Proteins have been increasingly considered as antigens for vaccine development due to their more structurally conserved composition when compared to capsular polysaccharides. Proteins subunit vaccines are safe and protective; however, they have limitations such as serotype-dependent immunity, and low immunogenicity of the proteins, requiring adjuvant to be included in these formulations or delivery systems that enhance the desired immune response. In addition, complex production procedures are required, increasing production costs and therefore market prices making these vaccines inaccessible for many people affected by these diseases. Recently, bacterial storage polymer inclusions have been developed as protein antigen carriers. Polyhydroxyalkanoate, in particular 3-polyhydroxybutyrate (PHB) inclusions have been successfully bioengineered to display antigens from pathogens like *Mycobacterium tuberculosis* and Hepatitis C virus. These particulate vaccine candidates elicited both a Th1 and Th2 immunity patterns combined with a protective immune response against *Mycobacterium bovis* in mice.

This thesis focuses on the study of polyhydroxybutyrate (PHB) beads properties as a carrier/delivery system engineered to display antigens from extracellular bacteria. The antigens Pneumococcal adhesin A, Pneumolysin (proteins) and 19F capsular polysaccharide (CPS) from *Streptococcus pneumoniae*, and Neisserial adhesin A, factor H binding protein (proteins) and serogroup C CPS from *Neisseria meningitidis* were displayed on the PHB bead surface. These antigenic proteins were produced as fusion

proteins on the PHB bead surface, while the CPS was covalently attached by chemical conjugation. Mice vaccinated with these PHB beads produced strong and antigen-specific antibody levels. In addition, splenocytes from the same mice generated both IL-17A and IFN-ɣ production.

The antibodies elicited against antigenic pneumococcal proteins were able to recognise the same protein in the context of an *Streptococcus pneumoniae* whole cell lysate from more than six different strains, while antibodies produced after vaccination with 19F CPS conjugate to PHB showed high opsonophagocytic titers against the homologous strain. In the case of *Neisseria meningitidis*, bactericidal antibodies were elicited in mice vaccinated with PHB beads displaying proteinaceous and CPS antigens.

Overall, this thesis shows that PHB as particulate vaccine candidate holds the promise of a broadly protective vaccine that can be produced cost-effectively for widespread application to prevent diseases caused by *Neisseria meningitidis* and *Streptococcus pneumoniae.*

With eternal love, gratitude and in memory of my mother

(Mercedes Miró Alonso, 1945-1993)

Acknowledgements

It has been a great experience and opportunity to work under the guidance and support of my main supervisor Professor Bernd Rehm. After hearing his oral presentation in Vaccipharma 2012, about PHA beads and their potential biomedical applications, combined with our understanding of the necessity to improve commercial vaccines against *Neisseria meningitidis* and *Streptococcus pneumoniae,* the idea to explore this platform for this purpose was born. This idea became a PhD project giving to me the possibility to enter a new field called nanotechnology. For all of these, I would like to thank Professor Bernd Rehm. In addition, I would like to thank my co-supervisors Dr Zoe Jordens and Dr Vicente Vérez-Bencomo for their support during this journey, respecting all the time their experiences, opinions and criticisms.

I thank members and ex-members of Bernd Rehm team, especially, Shuxiong Chen, Patricia Rubio, Jason Lee, Jason Smith, Jinping du, Natalie Burn, Kathryn Grage, Natalie Parlane, Andy Hollings…I learned a lot from them. The cooperation and interaction between my coworkers and myself, but also the weekly seminar allowed me to increase my scientific knowledge and my oral presentation skills.

I would like to thank my co-workers in The Finlay Vaccine Institute, Havana, Cuba, for all their professional and spiritual support, especially, Dr Dagmar García, Laura Marta Nodas, Yanet Estrada, Aylin Amador, Mildrey Fariñas, Sandra Madariaga, Dr Caridad Zayas, Neissa García, Aniuska Garces, MsC Amarilys Pérez, Leandro Camejo, Yury Valdés, Darielys Santana, Danaydis Fonseca, Ubel González, Elizabeth González, Dr Reinaldo Acevedo, Dr Reinaldo Oliva, Maria Onelia, Dr Barbara Cedré, Marilé García, MS Tamara Hernández, Alex Quintero Pérez, Dr Reinaldo Oliva Hernández, Dr Yanely Tirado, Rosmira Nicado.

I would like to thank Barry Bunn and Debra Cresswell for their support with English grammar. In addition, I would like to thank Jordan Taylor, Niki Minard and Matthew Savoian from the Manawatu Microscopy Imaging Centre for their assistance in TEM analysis. Prof Martin Hazelton and Dr Edgar Santos-Fernández are acknowledged for their assistance in statistical analysis of data in this thesis.

I am grateful for the scholarship support, by The MacDiarmid Institute for Advanced Materials and Nanotechnology and also, Massey University and The Finlay Institute for financial support.

I cannot forget to mention my friends from Cuba, New Zealand, Argentina, Mexico, Hungary, Colombia, Chile, Guatemala, Portugal, Spain, Germany, Austria, Italy, Ecuador, USA, France, The Netherlands, Paraguay, UK, Venezuela, Iran, India, Gana, Honduras thank you very much to all of them. But I would like to highlight my friend's Dr Edgar Santos-Fernández, Dr Jimena Yapura, Dr Javier Flores and Tony Reid because you have helped a lot not only to be a better scientist but to be a better human being. Thank you very much.

Last but not least, I would like to thank my family especially my father (the love of my life and my example to follow) and my sister (my friend, my confidante….). Thank you forever. I will always love you both.

Preface

This thesis is written according to the regulations of the Handbook for Doctoral Study, revised in May 2016 by the Doctoral Research Committee. This thesis complies with the format of a thesis based on publication as described in the handbook.

Chapter 1

Introduction

This chapter was written by Majela González Miró as an introductory chapter for this thesis only and is not intended for publication

Chapter 2

Self-assembled particulate PsaA as vaccine against *Streptococcus pneumoniae* infection

González-Miró, Majela[1,2], Rodríguez-Noda, Laura[1], Fariñas-Medina, Mildrey[1], García-Rivera, Dagmar[1], Vérez-Bencomo, Vicente[1], Rehm, Bernd H.A.[2]

Published: ***Heliyon*** **11 Apr 2017- Volume 3, Issue 4 Pharmaceutical Science, Biochemistry, Immunology**

G.-Miró. M: Conceived and designed the experiments; performed and supervised the experiments; analysed and interpreted the data; Wrote the paper. R.-N. L., F-M. M: Performed the experiments. G.-R. D.: Analysed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. V.-B. V: Contributed reagents, materials, analysis tools or data. R. B.H.A.: Conceived and designed the experiments; Analysed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Chapter 3

Biologically assembled polyester beads displaying pneumolysin and capsular polysaccharide induce protective immunity against *Streptococcus pneumoniae*

Majela González-Miró [1,2], Anna-Maria Radecker [2], Laura M Rodríguez-Noda [1], Mildrey Fariñas-Medina[1], Caridad Zayas-Vignier[1], Mabel Heránndez-Cedeño [1], Yohana Serrano[1], Félix Cardoso [1], Darielys Santana-Mederos[1], Dagmar García-Rivera [1], Yury Valdés-Balbín [1], Vicente Vérez-Bencomo[1], Bernd H.A. Rehm[2, 3]

M. G.-Miró, A-M. R., L.M. R.-N., M. F.-M., C. Z.-V., M. H.-C., Y.S., F.C. performed the studies and analysed the data. D. G.-R., V. V.-B., **M.G.- Miró**., Y. V.-B., D. S.-M. B.H.A. R. analysed and interpreted the data; contributed reagents, materials, analysis tools or data. **M.G.- Miró**. and B.H.A. R. conceived and designed and supervised the experiments. **M.G.- Miró**. and B.H.A. R. wrote the manuscript.

Under review by *Frontiers in Immunology*, Jan 2018

Chapter 4

Bioengineered polyester beads co-displaying protein and carbohydrate-based antigens enhance protective efficacy against bacterial infection

Majela González-Miró[1,2], Laura M Rodríguez-Noda[1], Mildrey Fariñas-Medina[1], Barbara Cedré-Marrero[1], Sandra-Madariaga-Zarza[1], Caridad Zayas-Vignier[1], Mabel Hernández-Cedeño[1], Torsten Kleffmann[3], Dagmar García-Rivera[1], Vicente Vérez-Bencomo[1], Bernd H. A. Rehm[2, 4]

M. G.- Miró, L.M. R.-N., M. F.-M., B. C.-M., S.M.-Z., C. Z.-V., M. H.-C., T.K. performed the studies and analysed the data. D.G.-R., V.V.-B., **M.G.- Miró**., B.H.A.R. analysed and interpreted the data; contributed reagents, materials, analysis tools or data. **M.G.- Miró**. and B.H.A. R. conceived and designed and supervised the experiments. **M.G.- Miró**. and B.H.A. R. wrote the manuscript.

Published: *Scientific Reports* Journal, 30 Jan 2018, Volume 8, Issue 1

Chapter 5.

General Discussion, Conclusion and Future work

This chapter was written by Majela González Miró for this thesis only and is not intended for publication.

Table of Contents

List of Figures

Chapter 4

List of Tables

Abbreviations

APCs: antigen presenting cells

APS: activate polysaccharide

BCA: Bicinchoninic acid assay

CLSM: Confocal Laser Scanning Microscope

CPS: capsular polysaccharide

CFU: colony-forming unit

CON A: Concanavalin A

DC: dendritic cell

DF: Dilution Factor

DIC: Differential interference contrast

DMEM: Dulbecco's Modified Eagle's Medium

DT: Diphtheria toxoid

ELISA: enzyme-linked immunosorbent assay

FCS: fetal calf serum

fHbp: factor H binding protein

GNA2091: genome *Neisseria* antigen 2091

GNA2091-fHbp-PhaC: genome *Neisseria* antigen 2091 fuse to factor H binding protein and PhaC

HEPES: (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

Ig: Immunoglobulin

IgG: Immunoglobulin G

IgG1, IgG2a, IgG2b and IgG3: Immunoglobulin G 1,2a,2b,3

IgM: Immunoglobulin M

IL17A: cytokine 17A

INF-γ: Interferon gamma

LB: Luria-Bertani broth (Lennox)

M. bovis: Mycobacterium bovis

N. meningitidis: Neisseria meningitidis

S. pneumoniae: Streptococcus pneumoniae

E. coli: Escherichia coli

MALDI-TOF-MS/MS: matrix assisted laser desorption ionization-time of flight mass spectrometry

MW: molecular weight

$NaBH_3CN$: Sodium Cyanoborohydride

NadA: *Neisseria* adhesin A

NadA-PhaC: NadA-PhaC fusion protein

NIBSC: National Institute for Biological Standards and Control

OVA: Ovalbumin

OPA: opsonophagocytic assay

PBS: Phosphate Buffered Saline

PCR: polymerase chain reactions

PHA: Polyhydroxyalkanoate

PhA: *β*- ketothiolase

PhaC: Polyhydroxyalkanoate synthase

PhB: Acetoacetyl-CoA reductase

PHB: Polyhydroxybutyrate

Ply: Pneumolysin

Ply-PhaC: Pneumolysin fused to PhaC

PsaA: Pneumococcal surface adhesin A

PsA-PhaC: Pneumococcal Surface protein A fused to PhaC

PspA: Pneumococcal Surface protein A

PspC: Pneumococcal Surface protein C

rpm: revolutions per minute

SBA: serum bactericidal activity

SD: standard deviation

SDS-PAGE: sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE)

SEM: standard error of the mean

TEM**:** Transmission electron microscopy.

Th17: Lymphocyte T helper 17

Triple TOF: mass spectrometry by Triple TOF

TT: Tetanus toxoid

TLR: Toll-like receptor

^0C: degrees Celsius

^1H NMR: Proton nuclear magnetic resonance

UNICEF: United Nations Children's Fund

WHO: World Health Organization

Chapter 1. Introduction

1.1 *Streptococcus pneumoniae*

Pneumonia is called by UNICEF and WHO "the forgotten killer of children"(1). *Streptococcus pneumoniae (S. pneumoniae or pneumococcus)* is one of the major causes of this disease worldwide. It is classified as a Gram-positive bacterium, behaving as a commensal in the human nasopharyngeal flora (2). However, when the environment changes, such as concomitant bacterial and/or viral infections (Influenza A) and immunosuppression, it can become pathogenic (3).

1.1.1 Pneumococcal diseases and epidemiology

S. pneumoniae is transmitted when respiratory secretions from an infected person or carrier interact with a person who does not have protective immunity. It has been reported that between 60-100% of children under five years old from developed and developing countries can carry *S. pneumoniae* without developing the disease (4). Pneumonia, meningitis and bacteraemia are classified as invasive pneumococcal diseases (IPD); however, this organism can cause another non-invasive disease such as conjunctivitis and otitis media (5, 6). The main age groups affected by IPD are young children and the elderly. Also, individuals with HIV or other immunodeficiencies are highly susceptible to IPD (6, 7). The epidemiology of IPD varies with a different range of serotypes (different capsular polysaccharide structures) found in different geographical regions. For example, serotypes 1, 5, 6B, 7F, 8, 9V, 14, 15B, 18C, 19F, and 23F are responsible for 80% of the severe pneumococcal diseases in developed countries, while serotypes 4, 6A, 6B, 9V, 14, 18C, 19F, and 23F appear as most relevant in developing countries (8, 9).

The emergence of multi-antibiotic resistant strains and the capsular polysaccharide diversity impair treatment and prevention of pneumococcal infectious diseases (7). These factors contribute to an estimated 14.5 million of serious pneumococcal diseases occurring each year (10, 11). In addition, studies by O'Brien et al (2009) estimated that 5.6% of affected individuals die before reaching five years of age. Most deaths caused by IPD worldwide occur in African and Asian countries (7).

1.1.2 Host immune defences against *Streptococcus pneumoniae*

As soon as *S. pneumoniae* enters the human body, different mechanisms from the innate immune system are activated. The first detection of the bacteria by the immune system is through non-specific interactions between pathogen recognition receptors (PRR), on phagocyte cells, and pathogen-associated molecular patterns (PAMPs) on the *S. pneumoniae* surface. Examples of these PRRs include Toll-like receptor (TLR 4 or 2), C-reactive protein, C-type lectin receptors and Nucleotide-binding oligomerization domain proteins (Nod) (12). All of these interactions lead to complement activation and phagocytosis by macrophages, resulting in pathogen clearance (13). Nevertheless, the production of many virulence factors and the development of immune system evasion mechanisms by this bacterium allow the overcoming of all these barriers and the establishment of the infection. In this stage, cooperation between acquired humoral and cellular immune response is required by the host to remove *S. pneumoniae* completely (14).

Host production of specific opsonizing molecules, like antibodies or C3b from the complement system, mediate neutrophil recruitment and their activation in alveolar lung cells (15). The production of IgA is detectable in mucosal fluid during pneumococcal infections; however, the role of this immunoglobulin in the activation

of the immune defence has not been elucidated. *In vitro* and animal model studies suggest that loss of IgA expression can increase susceptibility to pneumococcal infection (16-18). The *S. pneumoniae* can evade the defence mechanisms in lung tissue and enter the bloodstream causing bacteraemia. During this acute stage of the disease, the antibodies (IgM or IgG) play a crucial role mediating macrophage phagocytosis in organs like the liver and the spleen, but also neutralising toxins in blood circulation avoiding tissue damage (19, 20).

Cellular immunity by T-helper cell (Th) activation is critical during the colonisation process (21-23). Zhang and coworker suggested that the pneumococcal colonisation process promotes activation of Th17 lymphocytes followed with IL17-A production (24). The role of this cytokine is to enhance the neutrophils/macrophages recruitment to the colonised tissues. In addition, in this colonisation study, the authors described the production of another cytokine, MCP-1, that recruits monocytes under Th17 activation (24, 25).

1.1.3 Relevant pneumococcal virulence factors

S. pneumoniae pathogenicity is mediated by a wide range of virulence factors working together thus allowing the pathogen to adhere to mucosal tissues, evade immune surveillance and cause tissue damage. Examples of these factors include capsular polysaccharides (CPS) (26), pili (27), choline-binding proteins, such as pneumococcal surface protein A and C (PspA, PspC) (28-30), and lipoproteins like pneumococcal surface adhesin A (PsaA) and pneumolysin (Ply) (31). Virulence factors that have been considered as vaccine targets are described in further detail below.

The CPS is recognised as one of the main virulence factors, consisting of sugars mostly as homo or heteropolymers. More than 93 different chemical structures have

been identified serologically (serotypes); however, no more than 30 are considered pathogenic (6). The major role of the CPS in pathogenicity is inhibition of C3b or Fc mediated phagocytosis, although they can mediate mucosal adherence, reduce some mucus removing processes as well as increase antibiotic resistance (26, 32). It has been reported that binding of C3b to the pneumococcal surface and its degradation could be serotype specific. This may be one possible explanation for the increased prevalence of one serotype over others (33).

Sub-capsular proteins have been shown to have less variable structures than the CPS. Proteins such as PsaA and Ply are classified as major virulence factors due to their relevance in the *S. pneumoniae* pathogenicity (6, 34, 35). The PsaA is a 37 kDa highly conservated protein present in most of the pneumococcal strains (36). This protein belongs to the family called ABC-type manganese transporters (37). The biggest role ascribed to this protein is during the *S.pneumoniae* adhesion to the nasopharyngeal tissue by interaction with a mucosal receptor (E-cadherin) (36, 38). This interaction enables not only attachment but also allows passage through this mucosal epithelium to the blood circulation system. The significant reduction in nasopharyngeal colonisation by induction of antibodies in animal models after immunisation with PsaA justified its inclusion in a protein vaccine under development (39, 40).

Pneumolysin is a pore-forming protein with a molecular weight ~ 53 kDa. Ply is a sulfhydryl-mediated activation protein that, through cholesterol interaction on the surface of the human pulmonary cell, mediates pore formation (41). Experimental data from animal models showed that deletion of the *ply* gene from pneumococcal strain made it less virulent via intranasal or systemic inoculation route (42). In addition, after immunization with a detoxified Ply, the strong production of IL17a by activation of

Th 17 lymphocytes promoted neutrophil and monocyte cell recruitment increasing the phagocytosis process, mainly in alveolar cells. Although this protein is involved in tissue damage processes in the latest stage of the infection, studies have shown that small amounts of Ply can participate in the colonisation process as well (43, 44). Increased knowledge and understanding of the interplay between virulence factors, epidemiology and the role of the immune system to clear the bacteria from the body can help lead to a better vaccine design.

1.1.4 Pneumococcal vaccines

Treatments for the pneumococcal disease are based on antibiotic therapy. However, the resistance of *S. pneumoniae* to agents such as penicillin, amoxicillin and azithromycin is well known in the medical community (45). Due to this, the administration of vaccines is the most effective prevention strategy to reduce the number of pneumococcal cases.

Purified pneumococcal capsular polysaccharide vaccine (Pneumovax, PPV23) was the first vaccine recommended to prevent pneumococcal diseases in children older than two years of age. However, the low stability of these polysaccharide vaccines and the thymus-independent immune response led to the exploration of new strategies to overcome these difficulties (46). The development and implementation of pneumococcal polysaccharide-protein conjugate vaccines (PCVs) have resulted in a dramatic reduction of global pneumococcal disease by eliciting protective immunity in the targeted population (47-49). Three important PCVs currently on the market (Prevenar 7 and 13® (Wythe) and Synflorix 15® (GSK)) show a serotype dependent protective profile. Thus, after the use of these vaccines worldwide, there have been a growing number of cases caused by serotypes that are not included in these formulations, suggesting a serotype replacement. Also, the manufacturing complexity

and the high cost of production limits the availability of these vaccines to developed countries, hence new approaches are required to address these limitations (50-53).

Vaccination with pneumococcal sub-capsular proteins produced, isolated and purified from recombinant *E. coli* strains aims to generate a serotype-independent and broadly protective immune response (54). Several protein vaccines currently in the pre-clinical, as well as clinical stages of development, are shown below in Table 1.

Table 1. Targets of pneumococcal protein vaccines under study.

Protein Antigens	Virulence Mechanisms	Reference
Pneumococcal surface protein A, (PspA)	Prevention of complement system activation Lactoferrin binding protein	(55-57)
Pneumococcal surface protein C (PspC)	Inhibition of complement	(56, 58)
Pneumococcal surface antigen A (PsaA)	Bacterial adhesion (colonization) Inhibition of complement	(38, 39)
Pneumolysin (Ply)	Damaging lung epithelium Cytotoxicity	(59, 60)
PsaA, PcsB and StkP trivalent vaccine.	Bacterial adhesion (colonization) Proteins identified in pneumococcal proteome screening.	(61, 62)

1.2 *Neisseria meningitidis*

Meningitis is a significant cause of disease and mortality in children from developing and developed countries (63). *Neisseria meningitidis (N. meningitidis or* meningococcus*)* is an encapsulated Gram-negative bacterium responsible for most of the meningitis/meningoencephalitis cases reported worldwide. This microorganism has as its only niche the human nasopharyngeal tissue. It has been reported that 10% of the global population are *N. meningitidis* carriers (64, 65). This percentage can vary depent on local epidemiology and geographical factor; for example, it is reportedly higher during serogroup A outbreaks in Africa than between epidemics (66).

Neisseria meningitidis has been historically classified based on differences in capsular polysaccharide structure (serogroups), outer membrane proteins (serotypes), and lipooligosaccharides (immunotypes). Recently, multilocus sequence typing

(MLST) has created a new classification system that is being applied to study the epidemiology of meningococcal diseases (67, 68).

1.2.1 Meningococcal diseases and epidemiology

Exchanges of droplets or aerosol between infected individuals or carriers and people lacking protective immunity are the mode of spreading this microorganism. As soon as the bacteria cross the mocous membrane of the nasopharyngeal tissues, taking up position in the underlying epithelial tissues and reaching the bloodstream, complications such as meningitis or bacteraemia may result (69). Six serogroups are responsible for at least 90% of meningococcal diseases worldwide (70, 71). Meningococcal disease patterns can be endemic, hyperendemic and epidemic diseases depending on the serogroup, the geographic area and season and other factors.

In the sub-Saharan meningitis belt, A is the main serogroup causing extensive epidemics, but endemic diseases can also occur in the south of the continent. The number of cases varies from 10 to 100/100,000 inhabitants during the epidemic season (72, 73). However, after the extensive introduction of MenAfricVac (Serum Institute of India Pvt.Ltd.) vaccine (immunity against serogroup A), group W135- and X-related clinical cases increased considerably. Between December 2016 to March 2017 more than 1400 of meningitis cases were diagnosed in (Nigeria area of Africa), resulting in more than 200 deaths in the age group of 5-15 years. Interestingly, serogroup C was reported as predominantly responsible for these cases (63).

In Europe, serogroups B and C are the strains responsible for most endemic diseases with an average of 0.69/100,000 cases per annum (74). Since the introduction of the first conjugate vaccine against serogroup C, serogroup B has become the cause of the majority of cases (75, 76). In Latin America, endemic diseases prevail; however, occasional hyper-endemic periods occur in some areas. Serogroups B and C

predominate historically, but recently serogroup W135 and Y have been diagnosed in Brasil, Argentina and Mexico (77-80). In Australia and New Zealand, serogroups B and C have been reported, with the majority of the affected population being Maori and Aborigines (81).

Meningococcal diseases have been classified as devastating infectious diseases worldwide. These diseases mainly affect infants (immature immune system) but a second peak is reported in adolescent due to a higher rate of bacteria exposition and transmission (82). The global incidence of invasive meningococcal disease (IMD) has been reported as ~ 1.3 million per year with a mortality rate of 10-20%. In patients with bacteraemia, the mortality rate can increase to 40% (73, 83). About 25% of survivors of IMD suffer from permanent disabilities, such as mental retardation, deafness and neurological sequelae-neuronal afflictions (70). The common protocol to treat meningococcal diseases in hospitals is the use of antibiotics (benzylpenicillin, ceftriaxone and chloramphenicol) but the rapid onset of severe symptoms, coupled with emerging multi-antibiotic resistance, undermines this strategy. Hence, to control these diseases, prevention by vaccination is the preferred strategy.

1.2.2 Host immune defences against *Neisseria meningitidis*

As soon as this bacterium reaches the nasopharynx, the epithelial cells produce a variety of antimicrobial peptides. The role of these peptides has not been thoroughly elucidated, but it is thought that their interaction with virulence fators of the bacterium can disrupt the electrostatic-dynamic of the cellular membrane leading to an increase in membrane permeability (84). Another reported mechanism for these peptides is the cytokine-mediated recruitment of macrophages, neutrophils and monocytes which enhance oxygen and nitrogen radical activation pathways (85).

The complement system is recognised as the most important and effective destruction mechanism that helps the human immune system to clear *N. meningitidis* from the body. The deposition of C3 component on the surface of the bacterium can lead to the activation of alternative pathways of the complement system (86). Nevertheless, production of antibodies with bactericidal activity (adaptive immune response) by classical complement activation is the key to removing the *N. meningitidis* during the infection (87). To avoid all of these immune mechanisms, the bacteria produce a variety of virulent factors that allow the establishment of infection.

1.2.3 Relevant meningococcal virulence factors

Meningococcal virulence factors include CPS, lipooligosaccharide (LOS) (88), pilus proteins (89), outer membrane proteins (OMP) like PorA and PorB, and iron binding proteins (90). In addition, since elucidation of the *N. meningitidis* whole genome sequence, new and potential antigenic vaccine targets were described, for example, *Neisseria* adhesin A (NadA) (91), factor H binding protein (fHbp) (92), and Neisserial Heparin Binding Antigen (NHBA) (93). This genome-derived approach to identify vaccine targets is called reverse vaccinology (68).

The up or down-regulation of the expression of genes encoding virulence factors allow colonisation, evasion of the immune system barrier and *N. meningitidis* infection to become established. Virulence factors that have been considered as vaccine targets are described in further detail below. More than 13 serogroups have been described on the basis of the chemical difference in capsular polysaccharide structures, but only six of them (A, B, C, W135, Y and X) are responsible for the majority of meningococcal diseases. The role of the CPS in pathogenicity is diverse; for example, down-regulation of CPS is necessary in order to expose proteins from the outer membrane (opacity proteins) which allow the colonisation of the mucosal cells to proceed (66). However,

the major role of the CPS is inhibition of the membrane attack complex (MAC, from the complement system) fixation in the bacterium membrane (47, 73). Other reports show that CPS are able to protect this microorganism against desiccation and destruction by phagocyte cells (94).

Neisseria adhesin A (NadA or GNA1994) is a trimeric protein directly anchored to the outer membrane of this bacterium (91). The genes encoding NadA are present in more than 50% of pathogenic meningococcal strains. It is known that three predominant variants (NadA: allele1-3) have a highly conserved shared amino acid region among them. Immunization with NadA from allele 1 showed a cross-reactive bactericidal activity against strains from allele 2 and 3 (95). However, a fourth allele was recently identified in carrier strains (71). The main role of NadA is adhesion to the mucosal epithelium, the initial step during the colonisation process, as well as binding monocyte/macrophages during infection (96, 97). Immunologically, NadA induces bactericidal IgG antibody against a wide spectrum of strains (71). The excellent immunogenic properties showed by NadA in pre-clinical as well as clinical trials permitted its inclusion in one of the commercial vaccines against serogroup B (98, 99).

Factor H binding protein (fHbp or GNA 1870) was another protein identified by reverse vaccinology. This is a protein with a molecular weight of 29 kDa able to inhibit the alternative complement pathways (100). The fHbp can bind the human complement factor H, imitating the host and avoiding the destruction mediated by the complement system (101-105). Another function of this protein is binding to anti-microbial molecules (106). The fHbp elicited high bactericidal titers in preclinical studies, presenting an excellent protection profile against a wide spectrum of strains. Also, passive immunisation with antibodies elicited against this protein enabled

survival from meningococcal bacteraemia in a rat model. Importantly, human immunisation has shown a protective immunity profile by the production of bactericidal antibodies (107, 108). Because of all this experimental evidence, fHbp is included in both commercial vaccines currently available in the market (68, 99).

1.2.4 Meningococcal vaccines

Multivalent pure capsular polysaccharide vaccines (PSV) (Menomune®, Sanofi Pasteur) have been successfully developed to control outbreaks in adult populations by serogroups A, C, W135 and Y. These CPS are thymus-independent antigens unable to elicit an immunological memory response and showing a limited protective reaction in infants and children under two years old, who are the most susceptible group suffering from these infections (70). Furthermore, the application of *N. meningitidis* serogroup C PSV in adults and children have shown hyporesponsiveness after repeated doses (109, 110). A good memory response in the target population has been achieved by conjugation of different capsular polysaccharides to a carrier protein (conjugate vaccine, CVs), thus allowing the development of an efficient alternative to prevent these diseases. Examples of these products in the market are Menactra® (Sanofi Pasteur), Menveo® (Novartis) and Nimenrix® (GSK) (80, 111).

Serogroup B of *N. meningitidis* accounts for over 50% of all cases of meningococcal disease in the developed world, and it is the only serogroup not included in any CVs due to high antigenic similarity with polysaccharides presented in human neurological tissues (112). Consequently, vaccine research against *N. meningitidis* serogroup B has focused on surface proteins such as OMP, mainly PorA and PorB (porins), included in outer membrane vesicles (OMVs). The PorA vaccines developed by The Norway Institute of Public Health and Chiron-Novartis were successfully used to fight *N. meningitidis* serogroup B epidemics in that country.

Another example is the Cuban vaccine (VA-MENGOC-BC, Finlay Institute) which was the first vaccine containing a proteoliposome mixed with purified CPS of serogroup C and which showed 83% protection, reducing dramatically the incidence of meningitis in Cuba (113, 114). An important native OMV vaccine based on the New Zealand epidemic strain developed by Chiron-Novartis and the University of Auckland showed strain-specific bactericidal antibodies in more than 60% of infants with some cross-protection in adults. OMV and Porin vaccines were proven to be efficient, their main limitation being the strain-specific protection (115).

Reverse vaccinology approaches screening the entire genome sequence of MC58 *N. meningitidis* strain for immunodominant antigens accelerated the development of protein-based vaccines against this bacterium. More than 2000 predicted proteins were revealed (92). The antigens selected by this new technology to be included in the Novartis vaccine were ranked based on their ability to (i) induce extensive protection against a wide range of strains, as measured by serum bactericidal activity (SBA); (ii) elicit high and specific IgG antibody profiles; and (iii) elicit antibodies that conferred passive protection in rats and mice (116). The three proteins that showed the best results, based on these criteria were NadA, fHbp and NHBA. These proteins together with OMV from MeNZB make up the recently licenced vaccine 4CMenB Bexsero® (Novartis, 2013). In contrast, two variants of fHbp, a lipoprotein, make up the recently licensed vaccine Trumenba® (Pfizer, 2014) (93, 116, 117).

1.3 Adjuvants and delivery systems

The pneumococcal and meningococcal vaccines described above are called sub-unit vaccines because they contain antigens from the bacteria. Although they show a safe and effective immunity profile, the antigens included in these formulations are less immunogenic and both vaccines require adjuvant and booster inoculation. For that

reason, the addition of improved adjuvant and/or immunogenic delivery systems are required to generate the desirable protective immunity (68).

Adjuvants are compounds that combined with specific antigens can produce stronger immune responses than the antigen alone (118). The inclusion of adjuvants in vaccine formulation aims to boost the immune response against the targeted antigens. The enhanced immunogenicity properties of the targets allow for a higher amount of antibodies and longer duration of the immunity, reduced antigen dose, and regulation of the subclass and avidity of the antibodies (119).

Many efforts have focused on developing new and potent adjuvants; however, only a few examples have been used in humans, such as mineral aluminium salts (alum), MF59 and squalene-oil-water emulsion (119, 120). Adjuvants have been classified based on action mechanism as immunostimulatory on antigen presenting cells (APCs) or as delivery systems enhancing the antigen uptake (121). Particulate adjuvant preparations such as micro- and nanoparticles, liposomes and virus-like particles represent particle sizes mimicking bacteria and virus sizes. In addition, these delivery systems can include immunostimulatory molecules promoting better uptake of antigens by dendritic cells and macrophages, and their activation (119). Recently, delivery systems based on a nanocarrier such as a polylactic-co-glycolic acid (PLGA) and chitosan nanoparticles presented advantageous properties regarding induction of humoral and cellular immune response. Examples of these advantages are the nano-size of this particle enhancing the antigen uptake by APCs and its capacity to associate to peripheral lymphoid tissues thus leading to the induction of the desired protective immune response. (122).

1.4 Polyhydroxyalkanoate beads as potential particulate vaccines

Polyhydroxyalkanoates (PHAs) are bio-polyesters produced as intracellular inclusion bodies by some bacteria and archaea, during nutrient abundance (Figure 1). PHAs are used as carbon and energy reserves when the carbon sources are scarce (123).

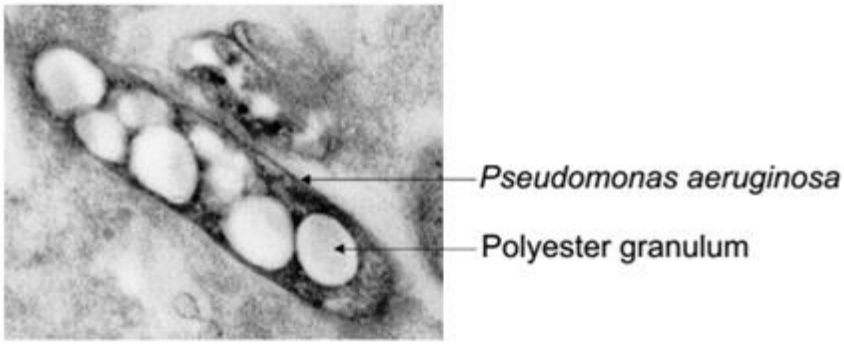


Figure 1 Electron microscopy image of Pseudomonas aeruginosa containing PHA granules. From (124).

The diversity of PHAs in nature is greater than 150 types. These polymers can present linear or branched structures, holding double or triple unsaturated bonds as well as epoxy or azido functional groups. However, the most well-known polymer is poly-3-hydroxybutyrate acid (PHB) (125).

The PHB inclusion formation pathways have been described and involve three enzyme-catalysed chemical reactions (Figure 2). The first one is catalysed by the *β*-ketothiolase (PhaA) condensing two molecules of acetyl-CoA resulting in acetoacetyl-CoA, with HS-CoA liberation. The second reaction is the acetoacetyl-CoA reduction, catalysed by acetoacetyl-CoA reductase (PhaB) with the consumption of NADPH. The 3-hydroxybutyryl-CoA molecule, a product of the previous reactions, is polymerised with another identical molecule elongating the PHB chain. The enzyme that catalyses this last reaction is polyhydroxyalkanoate synthase (PhaC) (126, 127), the main enzyme involved in bead formation. The PhaC remains covalently attached to the nascent PHB chain (Figure 3) (128). Due to the amphipathic protein/polyester

combination, they form spherical inclusion bodies with a size between 50 - 500 nm in diameter (129).

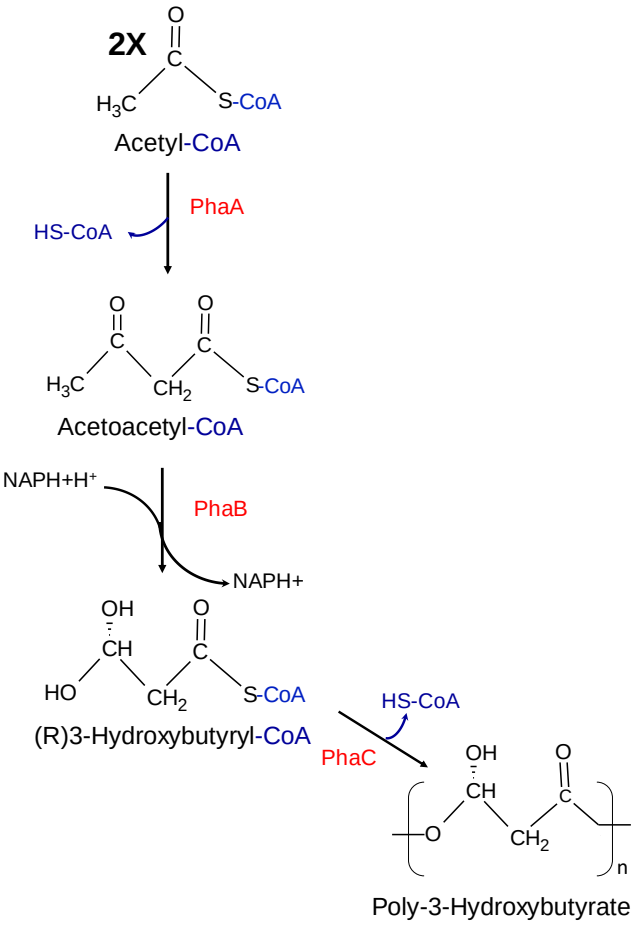


Figure 2. The biosynthetic pathway of PHB production. Modified from (124))

Four kinds of PhaC have been reported, differing in substrate specificity and subunit composition. The class I and II PhaC from *Ralstonia eutropha* and *Pseudomonas areuginosa* have one sub-unit and thioester compounds between 3-5 or 6-14 carbons as substrates, respectively (125, 130). Class III and IV, from *Allochromatium vinosum* and *Bacillus megaterium*, use a substrate with a longer chain than the previous enzymes (125, 131, 132).

The PhaC is not the only protein attached to the PHA granules; PHA depolymerase (PhaZ), in charge of polyester depolymerization, and Phasins (PhaPs) involved in inhibition of granules aggregation and non-specific protein association are

also constitutively anchored to PHA granules. Other described proteins include PhaR, PhaF, PhaI that are involved in regulation of PHA granule formation (Figure 3).

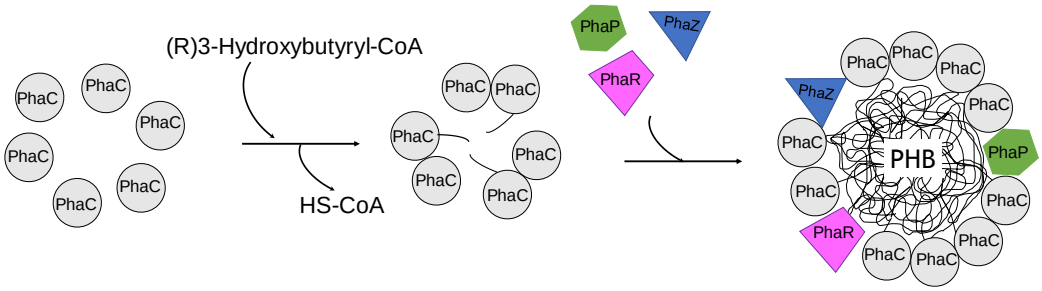


Figure 3. PHB granules Self-assembly model. Adapted from (123)

A lot is known about proteins attached to the PHB surface and there have been several reports of successful fusions of proteins with various biological properties to the PhaC or phasins, that did not disrupt the PHB bead-formation or the functionality of these fusion proteins (127). This successful functionalization of the PHB surface opened the possibility of a broad application spectrum such as subunit vaccine development, diagnostic tests, affinity purification and enzyme immobilisation (133-137). In addition, the recent introduction of PHA biosynthesis genes into heterologous expression hosts, like *ClearColi*, allows the production of PHB granules in an LPS free environment, avoiding the need for LPS removal, an essential step when using other *E.coli* hosts in vaccine production (137). These recombinant bacteria developed PHA levels approaching 80% of bacterial cell dry weight (138), suggesting this system could be a potentially efficient cell factory for vaccine production.

Future vaccines are expected to be most efficacious by including multiple antigens, and by induction of not only antibodies but also specific T lymphocytes and immunological memory. Experimental evidence has shown that protective cellular immune responses can be induced when antigens are displayed on small particles, e.g. virus-like particles (139). Bacteria such as *Lactococcus lactis* have been successfully engineered to produce intracellular PHA beads densely coated with proteins of interest.

This has been achieved by engineering of PhaC while retaining its PHA bead forming activity, as well as being tightly bound to a polyester nascent chain on the beads surface (123). Antigenic proteins from *Mycobacterium bovis* (TB) or HCV have been successfully fused to the PhaC (136, 140). The TB and HCV antigens displaying polyester beads triggered specific Th1 and Th2, and Th1 and Th17 patterns in mice, respectively. A protective immunity was obtained in mice vaccinated with TB antigen displaying beads and challenged with *Mycobacterium bovis* (140).

Recently, Lee and co-workers prepared PHA densely coated with antigenic proteins from *Pseudomonas aeruginosa*, using as production host the same bacteria (141). Mice immunisation generated Th1 immune responses, as well as strong and specific IgG1 and IgG2 antibodies response. The antibodies elicited after vaccination were able to mediate opsonophagocytic killing activity against three different *Pseudomonas aeruginosa* strains (141). Additional examples of the biomedical application of PHA beads have been published in a recent review article (127).

All previous animal vaccination experiments to date have shown a lack of adverse effects, such as no abscess or suppuration at the injection site, suggesting that polyester beads have no harmful effects (140, 141). Most importantly, these studies demonstrated, in principle, the possibility of using bacterial polyester beads as particulate protein vaccines offering unique characteristics as adjuvants toward generating a strong and specific immune response (i.e. not only antibody production but also inducing specific T lymphocytes and immunological memory).

1.5 General Hypothesis

Polyhydroxybutyrate beads can be used as a carrier/delivery system of proteinaceous and carbohydrate antigens for the development of particulate vaccines against *S. pneumoniae* and *N. meningitidis.*

1.6 Aims and scope of the thesis

The commercial sub-unit vaccines used to prevent infectious diseases present limitations that need attention. In the case of meningococcal and pneumococcal vaccines, the serotype-dependant coverage and low immunogenicity of the antigens themselves, are examples of these issues. Extensive efforts are focused on overcoming these limitations by working on alternative vaccine manufacturing procedures or improved formulations. The aim of this thesis is to explore the PHB beads platforms as a potential carrier /delivery system by displaying proteinaceous as well carbohydrate antigens from *S. pneumoniae* and *N. meningitidis* at the PHB bead surface and investigation of the immune response in animal models.

1.7 References

1. Wardlaw TM, Johansson EW, Hodge MJ. Pneumonia: the forgotten killer of children: UNICEF; 2006.
2. Watson DA, Musher DM, Jacobson JW, Verhoef J. A brief history of the pneumococcus in biomedical research: a panoply of scientific discovery. Clinical infectious diseases. 1993;17(5):913-24.
3. McDaniel LS, Swiatlo E. Pneumococcal disease: pathogenesis, treatment, and prevention. Infectious Diseases in Clinical Practice. 2004;12(2):93-8.
4. Coles CL, Kanungo R, Rahmathullah L, Thulasiraj RD, Katz J, Santosham M, et al. Pneumococcal nasopharyngeal colonization in young South Indian infants. The Pediatric infectious disease journal. 2001;20(3):289-95.
5. Crum NF, Barrozo CP, Chapman FA, Ryan MA, Russell KL. An outbreak of conjunctivitis due to a novel unencapsulated *Streptococcus pneumoniae* among military trainees. Clinical infectious diseases. 2004;39(8):1148-54.
6. Henriques-Normark B, Tuomanen EI. The pneumococcus: epidemiology, microbiology, and pathogenesis. Cold Spring Harbor perspectives in medicine. 2013;3(7):a010215.
7. O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, et al. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. The Lancet. 2009;374(9693):893-902.
8. Marimón JM, Iglesias L, Vicente D, Pérez-Trallero E. Molecular characterization of erythromycin-resistant clinical isolates of the four major antimicrobial-resistant Spanish clones of *Streptococcus pneumoniae* (Spain23F-1, Spain6B-2, Spain9V-3, and Spain14-5). Microbial Drug Resistance. 2003;9(2):133-7.
9. Overturf GD, Diseases CoI. Technical report: prevention of pneumococcal infections, including the use of pneumococcal conjugate and polysaccharide vaccines and antibiotic prophylaxis. Pediatrics. 2000;106(2):367-76.
10. Publication W. Pneumococcal vaccines WHO position paper–2012–recommendations. Vaccine. 2012;30(32):4717-8.
11. Miller E, Andrews NJ, Waight PA, Slack MP, George RC. Herd immunity and serotype replacement 4 years after seven-valent pneumococcal conjugate vaccination in England and Wales: an observational cohort study. The Lancet infectious diseases. 2011;11(10):760-8.

12. Paterson GK, Mitchell TJ. Innate immunity and the pneumococcus. Microbiology. 2006;152(2):285-93.

13. Opitz B, Püschel A, Schmeck B, Hocke AC, Rosseau S, Hammerschmidt S, et al. Nucleotide-binding oligomerization domain proteins are innate immune receptors for internalized *Streptococcus pneumoniae*. Journal of Biological Chemistry. 2004;279(35):36426-32.

14. Johnston RB. The host response to invasion by *Streptococcus pneumoniae:* protection and the pathogenesis of tissue damage. Review of Infectious Diseases. 1981;3(2):282-8.

15. Winkelstein JA. The role of complement in the host's defense against *Streptococcus pneumoniae*. Review of Infectious Diseases. 1981;3(2):289-98.

16. Hiemstra PS, Biewenga J, Gorter A, Stuurman ME, Faber A, van Es LA, et al. Activation of complement by human serum IgA, secretory IgA and IgA1 fragments. Molecular immunology. 1988;25(6):527-33.

17. Richards L, Ferreira DM, Miyaji EN, Andrew PW, Kadioglu A. The immunising effect of pneumococcal nasopharyngeal colonisation; protection against future colonisation and fatal invasive disease. Immunobiology. 2010;215(4):251-63.

18. Simell B, Kilpi T, Käyhty H. Subclass distribution of natural salivary IgA antibodies against pneumococcal capsular polysaccharide of type 14 and pneumococcal surface adhesin A (PsaA) in children. Clinical & Experimental Immunology. 2006;143(3):543-9.

19. Brown EJ, Hosea SW, Hammer CH, Burch CG, Frank MM. A quantitative analysis of the interactions of antipneumococcal antibody and complement in experimental pneumococcal bacteremia. Journal of Clinical Investigation. 1982;69(1):85.

20. Zhang J-R, Mostov KE, Lamm ME, Nanno M, Shimida S-i, Ohwaki M, et al. The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells. Cell. 2000;102(6):827-37.

21. Malley R, Trzcinski K, Srivastava A, Thompson CM, Anderson PW, Lipsitch M. CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(13):4848-53.

22. McCool TL, Weiser JN. Limited role of antibody in clearance of *Streptococcus pneumoniae* in a murine model of colonization. Infection and immunity. 2004;72(10):5807-13.

23. Lipsitch M, Whitney CG, Zell E, Kaijalainen T, Dagan R, Malley R. Are anticapsular antibodies the primary mechanism of protection against invasive pneumococcal disease? PLoS Med. 2005;2(1):e15.

24. Zhang Z, Clarke TB, Weiser JN. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. The Journal of clinical investigation. 2009;119(7):1899-909.

25. Van Lookeren Campagne M, Wiesmann C, Brown EJ. Macrophage complement receptors and pathogen clearance. Cellular microbiology. 2007;9(9):2095-102.

26. Jonsson S, Musher DM, Chapman A, Goree A, Lawrence EC. Phagocytosis and killing of common bacterial pathogens of the lung by human alveolar macrophages. Journal of Infectious Diseases. 1985;152(1):4-13.

27. Barocchi M, Ries J, Zogaj X, Hemsley C, Albiger B, Kanth A, et al. A pneumococcal pilus influences virulence and host inflammatory responses. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(8):2857-62.

28. Mcdaniel LS, Yother J, Vijayakumar M, Mcgarry L, Guild WR, Briles DE. Use of insertional inactivation to facilitate studies of biological properties of pneumococcal surface protein A (PspA). Journal of Experimental Medicine. 1987;165(2):381-94.

29. Hammerschmidt S, Bethe G, Remane PH, Chhatwal GS. Identification of pneumococcal surface protein A as a lactoferrin-binding protein of *Streptococcus pneumoniae*. Infection and immunity. 1999;67(4):1683-7.

30. Kerr AR, Paterson GK, McCluskey J, Iannelli F, Oggioni MR, Pozzi G, et al. The contribution of PspC to pneumococcal virulence varies between strains and is accomplished by both complement evasion and complement-independent mechanisms. Infection and immunity. 2006;74(9):5319-24.

31. Mitchell A, Mitchell T. *Streptococcus pneumoniae*: virulence factors and variation. Clinical Microbiology and Infection. 2010;16(5):411-8.

32. van der Poll T, Opal SM. Pathogenesis, treatment, and prevention of pneumococcal pneumonia. The Lancet. 2009;374(9700):1543-56.

33. Hostetter MK. Serotypic variations among virulent pneumococci in deposition and degradation of covalently bound C3b: implications for phagocytosis and antibody production. Journal of Infectious Diseases. 1986;153(4):682-93.

34. Steel HC, Cockeran R, Anderson R, Feldman C. Overview of community-acquired pneumonia and the role of inflammatory mechanisms in the immunopathogenesis of severe pneumococcal disease. Mediators of inflammation. 2013;2013.

35. Tai SS. *Streptococcus pneumoniae* protein vaccine candidates: properties, activities and animal studies. Critical reviews in microbiology. 2006;32(3):139-53.

36. Sampson JS, O'Connor SP, Stinson AR, Tharpe JA, Russell H. Cloning and nucleotide sequence analysis of psaA, the *Streptococcus pneumoniae* gene encoding a 37-kilodalton protein homologous to previously reported *Streptococcus* sp. adhesins. Infection and immunity. 1994;62(1):319-24.

37. Dintilhac A, Alloing G, Granadel C, Claverys JP. Competence and virulence of *Streptococcus pneumoniae:* Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases. Molecular microbiology. 1997;25(4):727-39.

38. Anderton JM, Rajam G, Romero-Steiner S, Summer S, Kowalczyk AP, Carlone GM, et al. E-cadherin is a receptor for the common protein pneumococcal surface adhesin A (PsaA) of *Streptococcus pneumoniae*. Microbial Pathogenesis. 2007;42(5):225-36.

39. Rajam G, Anderton JM, Carlone GM, Sampson JS, Ades EW. Pneumococcal surface adhesin A (PsaA): a review. Critical reviews in microbiology. 2008;34(3-4):131-42.

40. Johnson SE, Dykes JK, Jue DL, Sampson JS, Carlone GM, Ades EW. Inhibition of pneumococcal carriage in mice by subcutaneous immunization with peptides from the common surface protein pneumococcal surface adhesin a. The Journal of infectious diseases. 2002;185(4):489-96.

41. Boulnois GJ. Pneumococcal proteins and the pathogenesis of disease caused by *Streptococcus pneumoniae*. Microbiology. 1992;138(2):249-59.

42. Canvin JR, Marvin AP, Sivakumaran M, Paton JC, Boulnois GJ, Andrew PW, et al. The role of pneumolysin and autolysin in the pathology of pneumonia and septicemia in mice infected with a type 2 pneumococcus. Journal of Infectious Diseases. 1995;172(1):119-23.

43. Malley R, Henneke P, Morse SC, Cieslewicz MJ, Lipsitch M, Thompson CM, et al. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. Proceedings of the National Academy of Sciences. 2003;100(4):1966-71.

44. Malley R, Srivastava A, Lipsitch M, Thompson CM, Watkins C, Tzianabos A, et al. Antibody-independent, interleukin-17A-mediated, cross-serotype immunity to pneumococci in mice immunized intranasally with the cell wall polysaccharide. Infection and immunity. 2006;74(4):2187-95.

45. Jacobs MR. *Streptococcus pneumoniae*: epidemiology and patterns of resistance. The American Journal of Medicine Supplements. 2004;117(3):3-15.

46. Teele DW, Klein JO, Bratton L, Fisch GR, Mathieu OR, Porter PJ, et al. Use of pneumococcal vaccine for prevention of recurrent acute otitis media in infants in Boston. Review of Infectious Diseases. 1981;3(Supplement 1):S113-S8.

47. Shinefield H, Black S, Ray P, Fireman B, Schwalbe J, Lewis E. Efficacy, immunogenicity and safety of heptavalent pneumococcal conjugate vaccine in low birth weight and preterm infants. The Pediatric infectious disease journal. 2002;21(3):182-6.

48. O'Brien KL, Swift AJ, Winkelstein JA, Santosham M, Stover B, Luddy R, et al. Safety and immunogenicity of heptavalent pneumococcal vaccine conjugated to CRM197 among infants with sickle cell disease. Pediatrics. 2000;106(5):965-72.

49. Hausdorff WP, Bryant J, Paradiso PR, Siber GR. Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I. Clinical Infectious Diseases. 2000;30(1):100-21.

50. Gertz RE, Li Z, Pimenta FC, Jackson D, Juni BA, Lynfield R, et al. Increased penicillin nonsusceptibility of nonvaccine-serotype invasive pneumococci other than serotypes 19A and 6A in post-7-valent conjugate vaccine era. Journal of Infectious Diseases. 2010;201(5):770-5.

51. Le C-F, Palanisamy NK, Yusof MYM, Sekaran SD. Capsular serotype and antibiotic resistance of *Streptococcus pneumoniae* isolates in Malaysia. PloS one. 2011;6(5):e19547.

52. Adam HJ, Karlowsky JA, Nichol KA, Gilmour MW, Hoban DJ, Embree J, et al. Baseline epidemiology of *Streptococcus pneumoniae* serotypes in Canada prior to the introduction of the 13-valent pneumococcal vaccine. Microbial Drug Resistance. 2012;18(2):176-82.

53. Lehmann D, Willis J, Moore HC, Giele C, Murphy D, Keil AD, et al. The changing epidemiology of invasive pneumococcal disease in aboriginal and non-aboriginal western Australians from 1997 through 2007 and emergence of nonvaccine serotypes. Clinical Infectious Diseases. 2010;50(11):1477-86.

54. Lee LH, Gu X-X, Nahm MH. Towards new broader spectrum pneumococcal vaccines: the future of pneumococcal disease prevention. Vaccines. 2014;2(1):112-28.

55. Briles DE, Hollingshead SK, King J, Swift A, Braun PA, Park MK, et al. Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. Journal of Infectious Diseases. 2000;182(6):1694-701.

56. Daniels CC, Coan P, King J, Hale J, Benton KA, Briles DE, et al. The proline-rich region of pneumococcal surface proteins A and C contains surface-accessible epitopes common to all pneumococci and elicits antibody-mediated protection against sepsis. Infection and immunity. 2010;78(5):2163-72.

57. Nabors GS, Braun PA, Herrmann DJ, Heise ML, Pyle DJ, Gravenstein S, et al. Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies to heterologous PspA molecules. Vaccine. 2000;18(17):1743-54.

58. Frey SE, Lottenbach KR, Hill H, Blevins TP, Yu Y, Zhang Y, et al. A Phase I, dose-escalation trial in adults of three recombinant attenuated Salmonella Typhi vaccine vectors producing *Streptococcus pneumoniae* surface protein antigen PspA. Vaccine. 2013;31(42):4874-80.

59. Hirst R, Kadioglu A, O'callaghan C, Andrew P. The role of pneumolysin in pneumococcal pneumonia and meningitis. Clinical & Experimental Immunology. 2004;138(2):195-201.

60. Alexander JE, Lock RA, Peeters C, Poolman JT, Andrew PW, Mitchell TJ, et al. Immunization of mice with pneumolysin toxoid confers a significant degree of protection against at least nine serotypes of *Streptococcus pneumoniae*. Infection and immunity. 1994;62(12):5683-8.

61. Giefing C, Meinke AL, Hanner M, Henics T, Minh DB, Gelbmann D, et al. Discovery of a novel class of highly conserved vaccine antigens using genomic scale antigenic fingerprinting of pneumococcus with human antibodies. Journal of Experimental Medicine. 2008;205(1):117-31.

62. Olafsdottir TA, Lingnau K, Nagy E, Jonsdottir I. Novel protein-based pneumococcal vaccines administered with the Th1-promoting adjuvant IC31 induce protective immunity against pneumococcal disease in neonatal mice. Infection and immunity. 2012;80(1):461-8.

63. WHO. http://wwwwhoint/csr/don/24-march-2017-meningococcal-disease-nigeria/en/. 2017.

64. Greenfield S, Sheehe PR, Feldman HA. Meningococcal carriage in a population of "normal" families. Journal of infectious diseases. 1971;123(1):67-73.

65. Yazdankhah SP, Caugant DA. *Neisseria meningitidis*: an overview of the carriage state. Journal of medical microbiology. 2004;53(9):821-32.

66. Stephens DS, Greenwood B, Brandtzaeg P. Epidemic meningitis, meningococcaemia, and *Neisseria meningitidis*. The Lancet. 2007;369(9580):2196-210.

67. Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, et al. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proceedings of the National Academy of Sciences. 1998;95(6):3140-5.

68. Delany I, Rappuoli R, Seib KL. Vaccines, reverse vaccinology, and bacterial pathogenesis. Cold Spring Harbor perspectives in medicine. 2013;3(5):a012476.

69. Tzeng Y-L, Stephens DS. Epidemiology and pathogenesis of *Neisseria meningitidis*. Microbes and infection. 2000;2(6):687-700.

70. Girard MP, Preziosi M-P, Aguado M-T, Kieny MP. A review of vaccine research and development: meningococcal disease. Vaccine. 2006;24(22):4692-700.

71. Capecchi B, Adu-Bobie J, Di Marcello F, Ciucchi L, Masignani V, Taddei A, et al. *Neisseria meningitidis* NadA is a new invasin which promotes bacterial adhesion to and penetration into human epithelial cells. Molecular microbiology. 2005;55(3):687-98.

72. Lapeyssonnie L. La méningite cérébro-spinale en Afrique. Organisation mondiale de la santé Geneva; 1963.

73. Pizza M, Rappuoli R. *Neisseria meningitidis*: pathogenesis and immunity. Current opinion in microbiology. 2015;23:68-72.

74.www.gov.uk/government/uploads/system/uploads/PHEw,attachment_data/file/343375/Table_1a_Invasive_meningococcal__E_W_by_capsular_, group___epi_year.pdf. Accessed January 19.

75. Greenwood B. Meningococcal meningitis in Africa. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1999;93(4):341-53.

76. Trotter C, Ramsay M, Harrison L. Introduction and epidemiology of meningococcal disease. Handbook of meningococcal disease management: Springer; 2016. p. 1-14.

77. Sadarangani M, Scheifele DW, Halperin SA, Vaudry W, Le Saux N, Tsang R, et al. The impact of the meningococcal serogroup C conjugate vaccine in Canada between 2002 and 2012. Clinical Infectious Diseases. 2014;59(9):1208-15.

78. Sáfadi M, González-Ayala S, Jäkel A, Wieffer H, Moreno C, Vyse A. The epidemiology of meningococcal disease in Latin America 1945–2010: an unpredictable and changing landscape. Epidemiology & Infection. 2013;141(3):447-58.

79. Abad R, López E, Debbag R, Vázquez J. Serogroup W meningococcal disease: global spread and current affect on the Southern Cone in Latin America. Epidemiology & Infection. 2014;142(12):2461-70.

80. Feavers I, Pollard AJ, Sadarangani M. Handbook of Meningococcal Disease Management: Springer; 2016.

81. Baker M, Martin D, Kieft C, Lennon D. A 10-year serogroup B meningococcal disease epidemic in New Zealand: Descriptive epidemiology, 1991–2000. Journal of paediatrics and child health. 2001;37(s5):13-9.

82. Campbell H, Saliba V, Borrow R, Ramsay M, Ladhani S. Targeted vaccination of teenagers following continued rapid endemic expansion of a single meningococcal group W clone (sequence type 11 clonal complex), United Kingdom 2015. Euro Surveill. 2015;20(28):21188.

83. Thompson MJ, Ninis N, Perera R, Mayon-White R, Phillips C, Bailey L, et al. Clinical recognition of meningococcal disease in children and adolescents. The lancet. 2006;367(9508):397-403.

84. Schultz H, Weiss JP. The bactericidal/permeability-increasing protein (BPI) in infection and inflammatory disease. Clinica Chimica Acta. 2007;384(1):12-23.

85. Fang FC. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nature Reviews Microbiology. 2004;2(10):820-32.

86. Schneider MC, Exley RM, Ram S, Sim RB, Tang CM. Interactions between *Neisseria meningitidis* and the complement system. Trends in microbiology. 2007;15(5):233-40.

87. Lo H, Tang CM, Exley RM. Mechanisms of avoidance of host immunity by *Neisseria meningitidis* and its effect on vaccine development. The Lancet infectious diseases. 2009;9(7):418-27.

88. Vogel U, Hammerschmidt S, Frosch M. Sialic acids of both the capsule and the sialylated lipooligosaccharide of *Neisseria meningitis* serogroup B are prerequisites for virulence of meningococci in the infant rat. Medical microbiology and immunology. 1996;185(2):81-7.

89. Stephens DS, Whitney AM, Rothbard J, Schoolnik GK. Pili of *Neisseria meningitidis*. Analysis of structure and investigation of structural and antigenic relationships to gonococcal pili. Journal of Experimental Medicine. 1985;161(6):1539-53.

90. Schryvers AB, Stojiljkovic I. Iron acquisition systems in the pathogenic *Neisseria*. Molecular microbiology. 1999;32(6):1117-23.

91. Comanducci M, Bambini S, Brunelli B, Adu-Bobie J, Aricò B, Capecchi B, et al. NadA, a novel vaccine candidate of *Neisseria meningitidis*. Journal of Experimental Medicine. 2002;195(11):1445-54.

92. Tettelin H, Saunders NJ, Heidelberg J, Jeffries AC, Nelson KE, Eisen JA, et al. Complete genome sequence of *Neisseria meningitidis* serogroup B strain MC58. Science. 2000;287(5459):1809-15.

93. Serruto D, Spadafina T, Ciucchi L, Lewis LA, Ram S, Tontini M, et al. *Neisseria meningitidis* GNA2132, a heparin-binding protein that induces protective immunity in humans. Proceedings of the National Academy of Sciences. 2010;107(8):3770-5.

94. Stephens DS, Spellman PA, Swartley JS. Effect of the (α 2→ 8)-linked polysialic acid capsule on adherence of *Neisseria meningitidis* to human mucosal cells. Journal of Infectious Diseases. 1993;167(2):475-8.

95. Malito E, Biancucci M, Faleri A, Ferlenghi I, Scarselli M, Maruggi G, et al. Structure of the meningococcal vaccine antigen NadA and epitope mapping of a bactericidal antibody. Proceedings of the National Academy of Sciences. 2014;111(48):17128-33.

96. Franzoso S, Mazzon C, Sztukowska M, Cecchini P, Kasic T, Capecchi B, et al. Human monocytes/macrophages are a target of *Neisseria meningitidis* Adhesin A (NadA). Journal of leukocyte biology. 2008;83(5):1100-10.

97. Mazzon C, Baldani-Guerra B, Cecchini P, Kasic T, Viola A, de Bernard M, et al. IFN-γ and R-848 dependent activation of human monocyte-derived dendritic cells by *Neisseria meningitidis* adhesin A. The Journal of Immunology. 2007;179(6):3904-16.

98. Jacobsson S, Mölling P, Olcén P. Seroprevalence of antibodies against fHbp and NadA, two potential vaccine antigens for *Neisseria meningitidis*. Vaccine. 2009;27(42):5755-9.

99. Giuliani MM, Pizza M, Rappuoli R. Compositions comprising *Neisseria meningitidis* antigens from serogroups B and C. Google Patents; 2017.

100. Cendron L, Veggi D, Girardi E, Zanotti G. Structure of the uncomplexed *Neisseria meningitidis* factor H-binding protein fHbp (rLP2086). Acta Crystallographica Section F: Structural Biology and Crystallization Communications. 2011;67(5):531-5.

101. Schneider MC, Prosser BE, Caesar JJ, Kugelberg E, Li S, Zhang Q, et al. *Neisseria meningitidis* recruits factor H using protein mimicry of host carbohydrates. Nature. 2009;458(7240):890-3.

102. Masignani V, Comanducci M, Giuliani MM, Bambini S, Adu-Bobie J, Aricò B, et al. Vaccination against *Neisseria meningitidis* using three variants of the lipoprotein GNA1870. Journal of Experimental Medicine. 2003;197(6):789-99.

103. Fletcher LD, Bernfield L, Barniak V, Farley JE, Howell A, Knauf M, et al. Vaccine potential of the *Neisseria meningitidis* 2086 lipoprotein. Infection and immunity. 2004;72(4):2088-100.

104. Welsch JA, Ram S, Koeberling O, Granoff DM. Complement-dependent synergistic bactericidal activity of antibodies against factor H–binding protein, a sparsely distributed meningococcal vaccine antigen. The Journal of infectious diseases. 2008;197(7):1053-61.

105. Seib K, Serruto D, Delany I, Adu-Bobie J, Veggi D, Aricò B, et al. Factor H-binding protein is important for meningococcal survival in human whole blood and serum and in the presence of the antimicrobial peptide LL-37. Infection and immunity. 2009;77(1):292-9.

106. Veggi D, Gentile MA, Cantini F, Lo Surdo P, Nardi-Dei V, Seib KL, et al. The factor H binding protein of *Neisseria meningitidis* interacts with xenosiderophores in vitro. Biochemistry. 2012;51(46):9384-93.

107. Jacobsson S, Hedberg ST, Mölling P, Unemo M, Comanducci M, Rappuoli R, et al. Prevalence and sequence variations of the genes encoding the five antigens included in the novel 5CVMB vaccine covering group B meningococcal disease. Vaccine. 2009;27(10):1579-84.

108. Findlow J, Borrow R, Snape MD, Dawson T, Holland A, John TM, et al. Multicenter, open-label, randomized phase II controlled trial of an investigational recombinant meningococcal serogroup B vaccine with and without outer membrane vesicles, administered in infancy. Clinical Infectious Diseases. 2010;51(10):1127-37.

109. O'Brien KL, Hochman M, Goldblatt D. Combined schedules of pneumococcal conjugate and polysaccharide vaccines: is hyporesponsiveness an issue? The Lancet infectious diseases. 2007;7(9):597-606.

110. Al-Mazrou Y, Khalil M, Borrow R, Balmer P, Bramwell J, Lal G, et al. Serologic responses to ACYW135 polysaccharide meningococcal vaccine in Saudi children under 5 years of age. Infection and immunity. 2005;73(5):2932-9.

111.WHO.http://www.who.int/vaccine_research/diseases/soa_bacterial/en/index1.html

112. Griffiss JM, Yamasaki R, Estabrook M, Kim JJ. Meningococcal molecular mimicry and the search for an ideal vaccine. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1991;85:32-6.

113. Sierra G, Campa H, Varcacel N, Garcia I, Izquierdo P, Sotolongo P, et al. Vaccine against group B *Neisseria meningitidis*: protection trial and mass vaccination results in Cuba. NIPH annals. 1991;14(2):195-207; discussion 8-10.

114. Feiring B, Fuglesang J, Oster P, Næss LM, Helland OS, Tilman S, et al. Persisting immune responses indicating long-term protection after booster dose with the meningococcal group B outer membrane vesicle vaccine. Clinical and Vaccine Immunology. 2006;13(7):790-6.

115. Loring BJ, Turner N, Petousis-Harris H. MeNZB™ vaccine and epidemic control: When do you stop vaccinating? Vaccine. 2008;26(47):5899-904.

116. Giuliani MM, Adu-Bobie J, Comanducci M, Aricò B, Savino S, Santini L, et al. A universal vaccine for serogroup B meningococcus. Proceedings of the National Academy of Sciences. 2006;103(29):10834-9.

117. Scarselli M, Aricò B, Brunelli B, Savino S, Di Marcello F, Palumbo E, et al. Rational design of a meningococcal antigen inducing broad protective immunity. Science translational medicine. 2011;3(91):91ra62-91ra62.

118. Ramon G. Sur I'anatoxine diphtherique. Ann de l'Inst Pasteur. 1924;36:1925.
119. O'Hagan DT, Singh M. Microparticles as vaccine adjuvants and delivery systems. Expert review of vaccines. 2003;2(2):269-83.
120. Del Giudice G, Fragapane E, Bugarini R, Hora M, Henriksson T, Palla E, et al. Vaccines with the MF59 adjuvant do not stimulate antibody responses against squalene. Clinical and vaccine immunology. 2006;13(9):1010-3.
121. Singh M, Chakrapani A, O'Hagan D. Nanoparticles and microparticles as vaccine-delivery systems. Expert review of vaccines. 2007;6(5):797-808.
122. Kim M-G, Park JY, Shon Y, Kim G, Shim G, Oh Y-K. Nanotechnology and vaccine development. Asian journal of pharmaceutical sciences. 2014;9(5):227-35.
123. Rehm BH. Bacterial polymers: biosynthesis, modifications and applications. Nature Reviews Microbiology. 2010;8(8):578-92.
124. Rehm. Biogenesis of microbial polyhydroxyalkanoate granules: a platform technology for the production of tailor-made bioparticles. Current issues in molecular biology. 2007;9(1):41.
125. Rehm. Polyester synthases: natural catalysts for plastics. Biochemical Journal. 2003;376(1):15-33.
126. Satoh Y, Tajima K, Tannai H, Munekata M. Enzyme-catalyzed poly (3-hydroxybutyrate) synthesis from acetate with CoA recycling and NADPH regeneration in vitro. Journal of bioscience and bioengineering. 2003;95(4):335-41.
127. Parlane NA, Gupta SK, Rubio-Reyes P, Chen S, Gonzalez-Miro M, Wedlock DN, et al. Self-assembled protein-coated polyhydroxyalkanoate beads: properties and biomedical applications. ACS Biomaterials Science & Engineering. 2016.
128. Rehm BH, Steinbüchel A. Biochemical and genetic analysis of PHA synthases and other proteins required for PHA synthesis. International journal of biological macromolecules. 1999;25(1):3-19.
129. Grage K, Jahns AC, Parlane N, Palanisamy R, Rasiah IA, Atwood JA, et al. Bacterial polyhydroxyalkanoate granules: biogenesis, structure, and potential use as nano-/micro-beads in biotechnological and biomedical applications. Biomacromolecules. 2009;10(4):660-9.
130. Amara AA, Bernd H. Replacement of the catalytic nucleophile cysteine-296 by serine in class II polyhydroxyalkanoate synthase from *Pseudomonas aeruginosa*-mediated synthesis of a new polyester: identification of catalytic residues. Biochemical Journal. 2003;374(2):413-21.

131. Yuan W, Jia Y, Tian J, Snell KD, Müh U, Sinskey AJ, et al. Class I and III polyhydroxyalkanoate synthases from *Ralstonia eutropha* and *Allochromatium vinosum*: characterization and substrate specificity studies. Archives of biochemistry and biophysics. 2001;394(1):87-98.

132. McCool GJ, Cannon MC. PhaC and PhaR are required for polyhydroxyalkanoic acid synthase activity in *Bacillus megaterium*. Journal of Bacteriology. 2001;183(14):4235-43.

133. Blatchford PA, Scott C, French N, Rehm BH. Immobilization of organophosphohydrolase OpdA from *Agrobacterium radiobacter* by overproduction at the surface of polyester inclusions inside engineered *Escherichia coli*. Biotechnology and bioengineering. 2012;109(5):1101-8.

134. Chen S, Parlane NA, Lee J, Wedlock DN, Buddle BM, Rehm BH. New skin test for detection of bovine tuberculosis on the basis of antigen-displaying polyester inclusions produced by recombinant *Escherichia coli*. Applied and environmental microbiology. 2014;80(8):2526-35.

135. Li J, Shang G, You M, Peng S, Wang Z, Wu H, et al. Endotoxin removing method based on lipopolysaccharide binding protein and polyhydroxyalkanoate binding protein PhaP. Biomacromolecules. 2011;12(3):602-8.

136. Parlane NA, Grage K, Lee JW, Buddle BM, Denis M, Rehm BH. Production of a particulate hepatitis C vaccine candidate by an engineered *Lactococcus lactis* strain. Applied and environmental microbiology. 2011;77(24):8516-22.

137. Reyes PR, Parlane NA, Wedlock DN, Rehm BH. Immunogenicity of antigens from *Mycobacterium tuberculosis* self-assembled as particulate vaccines. International Journal of Medical Microbiology. 2016;306(8):624-32.

138. Linko S, Vaheri H, Seppälä J. Production of poly-β-hydroxybutyrate on lactic acid by *Alcaligenes eutrophus* H16 in a 3-l bioreactor. Enzyme and Microbial Technology. 1993;15(5):401-6.

139. Rehm BH. Bioengineering towards self-assembly of particulate vaccines. Current Opinion in Biotechnology. 2017;48:42-53.

140. Parlane NA, Grage K, Mifune J, Basaraba RJ, Wedlock DN, Rehm BH, et al. Vaccines displaying mycobacterial proteins on biopolyester beads stimulate cellular immunity and induce protection against tuberculosis. Clinical and vaccine immunology. 2012;19(1):37-44.

141. Lee JW, Parlane NA, Wedlock DN, Rehm BH. Bioengineering a bacterial pathogen to assemble its own particulate vaccine capable of inducing cellular immunity. Scientific Reports. 2017;7:41607.

Preface to the next Chapter

Antigenic proteins from *Mycobacterium bovis* (TB) or HCV have been successfully fused to PhaC. These authors demonstrated a protective immunity in mice vaccinated with TB antigen displaying beads and challenged with *Mycobacterium bovis*. On the other hand, PHA coated with antigenic proteins from *Pseudomonas aeruginosa* generated Th1 immune responses, as well as strong and specific IgG1 and IgG2 antibodies response, in mice. The antibodies elicited after vaccination were able to mediate opsonophagocytic killing activity against three different *Pseudomonas aeruginosa* strains.

The studies mentioned above show the feasibility of particulate vaccine production against intracellular and extracellular bacteria. To widen the spectrum of possible biomedical application, Chapter 2 describes the first approach to producing PHB beads coated with PsaA from *S. pneumoniae* in an LPS free *E.coli*, potentially greatly simplifying the vaccine production process for these vaccines.

Chapter 2. Self-assembled particulate PsaA as vaccine against *Streptococcus pneumoniae* infection

González-Miró, Majela[1,2], Rodríguez-Noda, Laura[1], Fariñas-Medina, Mildrey[1], García-Rivera, Dagmar[1], Vérez-Bencomo, Vicente[1], Rehm, Bernd H.A.[2]

[1]Finlay Vaccine Institute. La Habana. Cuba; [2]Institute of Fundamental Sciences and MacDiarmid Institute of Advanced Materials and Nanotechnology, Massey University, Palmerston North, New Zealand

Published: ***Heliyon*** **11 Apr 2017- Volume 3, Issue 4 Pharmaceutical Science, Biochemistry, Immunology**

2.1 Abstract

S.t pneumoniae is a human pathogen responsible for the majority of childhood pneumonia and media otitis cases worldwide. The diversity of its capsular polysaccharides (CPS) results in more than 91 serotypes of which at least 23 are virulent. Various CPS conjugated to immunogenic carrier proteins are currently licensed and provide protection against the infection caused by the respective serotypes but not against new and emerging virulent serotypes. In this study, we considered the conserved protein antigen PsaA, the pneumococcal surface adhesin A, in order to overcome the limitations of CPS antigens. The PsaA was translationally fused to a polyhydroxybutyrate (PHB) synthase which mediated production of PsaA displayed on PHB inclusions in recombinant *Escherichia coli*. This suggested that the PsaA fusion to the PHB synthase did not interfere with PHB synthase activity and its ability to mediate formation of nano-sized inclusions composed of a PHB core surrounded by the PHB synthase fused to PsaA. Isolated PHB beads showed a negative surface charge. Transmission electron microscopy analysis suggested that the PsaA fusion to the PHB synthase reduced the size of PHB beads from about 500 nm to 100 nm. The integrity and antigenicity of the fusion protein attached to isolated PHB beads were confirmed by SDS-PAGE, tryptic peptide fingerprinting analysis using MALDI-TOF-MS/MS and immunoblotting using a monoclonal anti-PsaA antibody. Mice immunized with PsaA displaying PHB beads produced high and specific IgG levels dominated by IgG1 isotype. While IgG1 titer was similar between soluble and insoluble PsaA, the IgG2 titers were strongly increased upon vaccination with insoluble PsaA i.e. PsaA displayed on PHB beads. Particulate PsaA-PHB beads elicited IgG antibodies recognizing PsaA in whole cell lysates of seven different

serotypes of *S. pneumoniae.* This study suggested that PHB beads are suitable carriers for PsaA in order to induce a significant and specific Th-2-type immune response.

2.2 Introduction

Streptococcus pneumoniae is considered as a most important pathogen causing severe pneumonia, meningitis and middle ear inflammation (1). The cell surface of *S. pneumoniae* contains various proteins and capsular polysaccharides (CPS) which play an important role in pathogenicity such as attachment and immune system evasion. The diversity of CPS contributes to more than 91 serotypes, while cell surface proteins were found to be more conserved and less variable (2). Hence, *S. pneumoniae* cell surface proteins are increasingly being considered as vaccine candidate antigens.

All licensed vaccines are conjugated, CPS based and induce a specific serotype dependent protective immune response profile i.e. a strong and specific Th2-type response (3). However, the emergence of new invasive serotypes not covered by existing vaccines demands attention.

Subunit vaccines based on conserved proteins/epitopes could induce serotype independent and broader protective immunity (3). Due to their relevance for pathogenicity, cell surface proteins such as pneumococcal surface protein A (PspA), pneumococcal surface adhesin A (PsaA) and pneumolysin (Ply) are currently being considered for vaccine development (4-6). As subunit vaccines are often less immunogenic, adjuvant and/or immunogenic delivery systems are needed. Recently, polyhydroxybutyrate (PHB) beads (<1µm) displaying specific antigens have been demonstrated as effective antigen delivery system in the context of intracellular pathogens (7, 8). PHB is a polyester naturally produced by various bacteria (9). Introducing PHB biosynthesis genes into heterologous expression hosts allows the intracellular formation of discrete and spherical PHB inclusions (10). This also

resulted in PHB inclusions densely coated with proteins of interest (11-16). Translational fusion of proteins of interest to PHB synthase, PhaC, retained its PHB bead forming activity displaying the protein of interest at the PHB bead surface (13, 17). PHB beads were bioengineered to display antigens from intracellular pathogens like *Mycobacterium tuberculosis* and Hepatitis C virus. These particulate vaccine candidates elicited both Th1 and Th2 antigen-specific immune responses resulting in protective immunity (8, 18, 19). In this study, it was conceived to engineer PHB beads displaying PsaA as possible antigen delivery system to develop a particulate vaccine against the extracellular pathogen *S. pneumoniae*.

2.3 Materials and methods

2.3.1 Bacterial strains, oligonucleotides, plasmids and cultivation conditions

Bacterial strains, plasmids and primers used in this study are listed in Table 1. *E. coli* XL 1 blue was grown at 37ºC in LB in presence of ampicillin (100 µg/mL). PHB beads and recombinant soluble protein was produced in recombinant *ClearColi* (20). *ClearColi* was grown in LB Miller medium supplemented with glucose 1% (w/v), ampicillin (100 µg/mL). Chloramphenicol (50 µg/mL) was only added to media used for PHB bead production.

Table 1. Description of bacterial strains, plasmids and oligonucleotides used in this study

Strains, plasmid and primers	Characteristics	Reference
Strains		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F´ proAB lacI*^{q}*ZΔM15 Tn10* (Tet^{r})]	Stratagene
ClearColi	*F– ompT hsdSB (rB - mB -) gal dcm lon λ (DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) msbA148 ΔgutQ ΔkdsD ΔlpxL ΔlpxM ΔpagP ΔlpxP ΔeptA (*LPS negative)	(18)
Plasmids		
pET-14b	*Ap*^{R} and T7 promoter	Novagen
pET-14b-phaC	pET-14b version, holding *phaC* gene fragment	(21)
pUC57-psaA	pUC57 version, ColE1 origin, *Ap*^{R} holding *Nde*I */psaA* gene*/Spe*I.	GenScript
pET-14b-psaA-phaC	pET-14b-phaC version, holding *psaA* gene fused to 3' end of *pha*C gene	This study
pMCS69	*Cm*^{R}; T7 promoter, pBBR1MCS derivative containing *pha*A and *phaB* genes from *Ralstonia eutropha* co-downstream to lac promoter	(22)
pET14b_NanA_PhaC (reversed)	*Ap*^{R} and T7 promoter, containing *nanA* gene cloned to 3' end of *phaC* gene	(23)
pET14b- his6-psaA	*Ap*^{R} and T7 promoter, containing the *his6-psaA* gene inserted into the *Nde*I*//BamH*I sites of pET14b.	This study
Primers		
psaA fwr (*Nde*I, underline restriction site)	5'AAACATATG**CACCACCACCACCACCAC**TCGGGCAAAAAGGATACGACCTCGG3'	This study
psaA rev (*BamH*I, underline restriction site)	5' AAAGGATCCTCATTTTGCCAGACCTTCAGCGATTTTGTCCAG3'	This study

2.3.2 Construction of plasmids mediating production of PHB beads displaying PsaA

The gene encoding PsaA (amino acids 22-309) was synthesized by Genscript Corporation (USA) employing codon optimization for *E. coli* (24). This hybrid gene encoding a translational fusion of PsaA with PhaC was constructed and cloned into plasmid pET-14b as outlined in Figure 1.

2.3.3 Construction of the plasmid encoding N-terminally His-tagged PsaA for production of soluble PsaA

The gene encoding PsaA was amplified from pUC57-psaA by PCR using the corresponding primers psaA-fwr and psaA-rev. This introduced 6 histidine residues into the N terminus of PsaA in order to enable purification by immobilized Ni^{2+} affinity chromatography. The gene encoding His6-PsaA was inserted into pET-14b_NanA_PhaC using hydrolysis with *Nde*I and *BamH*I to replace *nanA*.

2.3.4 Production, isolation and purification of PHB beads

ClearColi harbouring pMCS69 was transformed with pET-14b-psaA-phaC (encoding PsaA-PhaC fusion protein for production of PsaA displaying PHB beads) and pET14b-PhaC (PhaC wild type control for the production of PHB beads). Cells were cultivated and subjected to mechanical cell disruption. Beads were isolated and sterilized as previously described (25, 26).

2.3.5 Production, isolation and purification of recombinant soluble protein

ClearColi was transformed with pET-14b-his6-psaA (encoding His6-PsaA). Cells were cultivated and lysed for purification of His6-PsaA using the Ni-NTA Fast Start Kit (Qiagen, Germany).

2.3.6 Confirmation of the PhaC in vivo activity using transmission electron microscopy (TEM)

Cells harbouring plasmid pET-14b-pasA-phaC and pET-14b-phaC, respectively, were analysed by TEM as described previously (20) to demonstrate the presence of PHB inclusions inside cells which is indicative of *in vivo* functionality of PhaC and its fusion protein variants.

2.3.7 Protein analysis

PsaA-PhaC and PhaC beads, as well as soluble His6-PsaA, were analysed by SDS-PAGE as previously described (27). Immunoblot analysis was conducted as previously described (28). A monoclonal anti-PsaA antibody (Steroid & Immunochemistry Laboratory, Canterbury Health Laboratories, Christchurch, New Zealand) was used to identify the PsaA. All images were obtained using the GEL-DOC 2000 (Bio-Rad Laboratories, USA) and analysed using Image Lab Software (Version 3.0 build 11, Bio-Rad Laboratories, USA). Proteins were further identified by MALDI-TOF/MS. To confirm the PHB bead surface display and identity of PsaA, ELISA using goat anti-mouse IgG peroxidase conjugate (Sigma-Aldrich, St. Louis, MO) as secondary antibody as well as CLSM (confocal laser scanning microscopy) using a fluorescently (Alexa Fluor 488) labelled goat anti-mouse antibody (Sigma-Aldrich, St. Louis, MO) as secondary antibody was employed as previously described (29).

2.3.8 Measurement of the PHA bead size distribution and zeta potential

Size distribution of the particles and the zeta potential were measured using the Mastersizer 3000 particle sizer (Malvern instrument, United Kingdon) and the Zetasizer Nano ZS (Malvern instrument, United Kingdon), respectively. Samples were

prepared as 0.1% (w/v) of the wet PHB beads in saline solution. The pH values were adjusted with HCl.

2.3.9 Analysis of immunological properties of PHB beads

2.3.9.1 Immunization Schedule

Four groups were prepared to each contain 6 animals between 5-6 weeks old (male, Balb/c mice). Mice were acquired from CENPALAB (Centro Nacional para la Producción de Animales de Laboratorio, La Habana, Cuba). Three doses of 4 µg of antigen plus 100 µg of alum (ALHYDROGEL, Brenntag Biosector, Denmark) were subcutaneously administrated to each animal. Animals of the placebo group received only alum. Immunizations occurred at 0, 14 and 21 d. Blood samples were collected from the retro-orbital plexus at days 0, 14, 21 and 28 d after vaccination. Group 1 (G1) received 4 µg of PsaA displayed on PHBs, Group 2 (G2) received 4 µg of His6-PsaA antigen as pure soluble protein, Group 3 (G3) received 4 µg of PhaC wild type PHB beads. Group 4 received only alum.

2.3.9.2 Assessment of anti-PsaA antibody titers in mice

Anti-PsaA and anti-PhaC IgG levels were measured using an indirect ELISA. Maxisorp 96-well plates (NUNC) were coated using 0.5 µg/ml of His6-PsaA and incubated overnight at 4°C. The plates were then washed with PBS containing 0.05% (v/v) Tween 20 (PBS-T) and blocked for 30 minutes at 37°C with PBS containing 1% (w/v) BSA. After three washes with PBS-T, plates were incubated with dilutions of serum samples from individual mice in PBS-T containing 1% (w/v) BSA and 12.6 mM of EDTA (PBS-TB), for 60 min at room temperature (RT). The initial dilution factor (DF) of all samples was 1600. After three washes with PBS-T, plates were incubated for 60 min with the secondary antibody, the goat anti-mouse IgG peroxidase conjugate

(Sigma-Aldrich, St. Louis, MO) diluted 1: 10,000 in PBS-TB. Plates were developed using the peroxidase substrate as described elsewhere (30). A serum was considered as positive if the initial absorbance at a wavelength of 570 nm was at least twofold greater than the pre-immune serum value. Results were displayed as the reciprocal antibody titres, representing the DF required to obtain half of the maximum level of absorbance.

2.3.9.3 Analysis of the isotype IgG profile in sera

The isotype IgG profile against PsaA was analyzed by using an indirect ELISA as described above. Pools of sera from all animals at time 28 d from G1 and G2 were prepared and diluted 1: 1600. To detect the various IgG isotypes, plates were incubated with the respective anti-IgG1, IgG2a, IgG2b and IgG3 antibodies (derived from goat) (Sigma-Aldrich, St. Louis, MO) using a DF of 1: 2500. Plates were developed using the anti-goat IgG peroxidase conjugate and the respective substrate as described above. The results from duplicate experiments are represented as mean values plus standard deviation (mean ± SD).

2.3.9.4 Serotype coverage of induced anti-PsaA antibodies

The specificity of sera from animals immunized with PsaA-PhaC PHB beads for PsaA in whole cell lysates of pathogenic *S. pneumoniae* serotypes 1, 3, 5, 6b, 7F, 14 and 23F (Finlay Vaccine Institute) was assessed by immunoblotting. The cell suspension was prepared in PBS (pH 7.0) and adjusted to an $OD_{620\,nm}$ 0.8-1. Then 300 µl of these suspensions were mixed with an equal volume of denaturing buffer (DF 2). Samples were incubated at 95ºC for 20 min. Twenty µl of the soluble sample were separated by SDS-PAGE. For immunoblotting pooled sera from all animals of G1 (DF: 2000) and G2 (DF: 500) were assayed.

2.3.10 Statistical analysis

Graph Pad Prism 4.03 (San Diego, USA) software was used for statistical data analysis. Results were reported as results of 6 animals and significant differences were calculated by the Kruskal–Wallis non-parametric test. When significant differences were found, the Dunn's post-test was used, considering significant differences when *p* was less than 0.05.

2.4 Results

2.4.1 Construction of plasmids mediating production of the PsaA-PhaC fusion protein and His6-PsaA

The strategy for construction of plasmids encoding PsaA from *S. pneumoniae* translationally fused to PhaC from *Ralstonia eutropha* to produce particulate PsaA is presented in Figure 1.

In this study, the amino acid sequence of PsaA, the manganese ABC transporter, a manganese-binding adhesion lipoprotein, was selected from *S. pneumoniae* CGSP14 (GenBank number: ACB90875.1). This sequence has 100% identity with orthologous proteins found in strains R6 and TIGR4, and 98-99% identity with the other orthologues of various *S. pneumoniae* strains deposited in NCBI database. The gene encoding PsaA without its predicted signal sequence (1-21 amino acid residues) was synthesized as codon–optimized for *E. coli* (GenScript, USA). This synthetic gene was used to construct pET-14b-psaA-phaC.

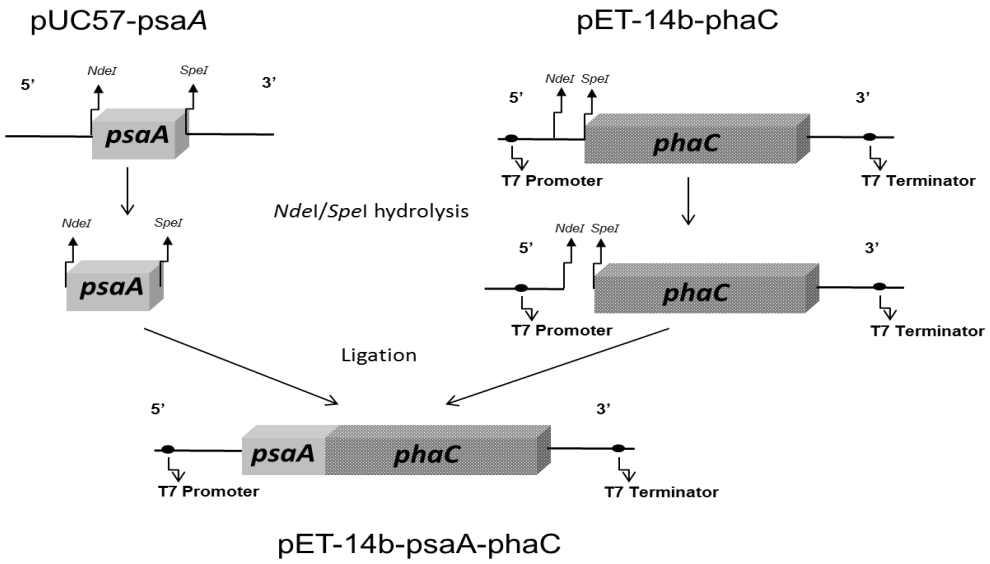


Figure 1. Schematic presentation of the construction of plasmid pET-14b-psaA-phaC encoding the PsaA-PhaC fusion protein for the formation of PHB beads in recombinant *ClearColi.*

The recombinant soluble version of PsaA was produced as N-terminally His6-tagged protein and purified Ni^{2+} affinity chromatography.

2.4.2 Production and characterization of PsaA displaying PHA beads

To produce PHB beads, *ClearColi* harboring pMCS69 (Table 1) were transformed with either pET-14b-psaA-phaC or pET-14b-phaC (non-antigen displaying negative control). To confirm the formation of PHB inclusions indicative of PhaC functionality, cells and purified beads were observed by TEM (Fig. 2). Results showed that both plasmids mediated formation of distinct inclusions. However, PHB inclusions mediated by the PsaA-PhaC were smaller and more abundant when compared to inclusions formed by only PhaC (Figure 2).

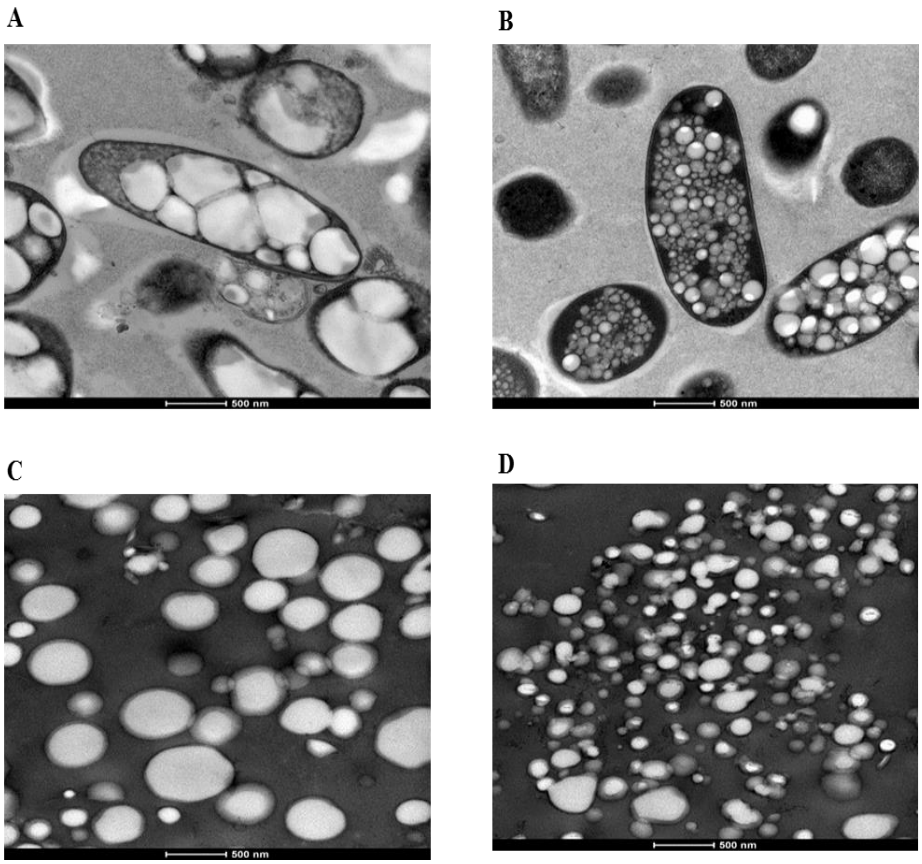


Figure 2. TEM analysis of recombinant *ClearColi* cells (pMCS69) harbouring various plasmids and respective isolated PHB beads. A, cells harboring pET-14b-phaC (PhaC wild-type PHB beads); B, cells harboring pET-14b-psaA-phaC (PsaA-PhaC PHB beads); C, PHB beads isolated from cells harboring pET-14b-phaC; D, PHB beads isolated from cells harboring pET-14b-psaA-phaC.

After isolation and purification, the protein profile of PHB beads and His6-PsaA was analyzed by SDS-PAGE and immunoblotting (Fig. 3).

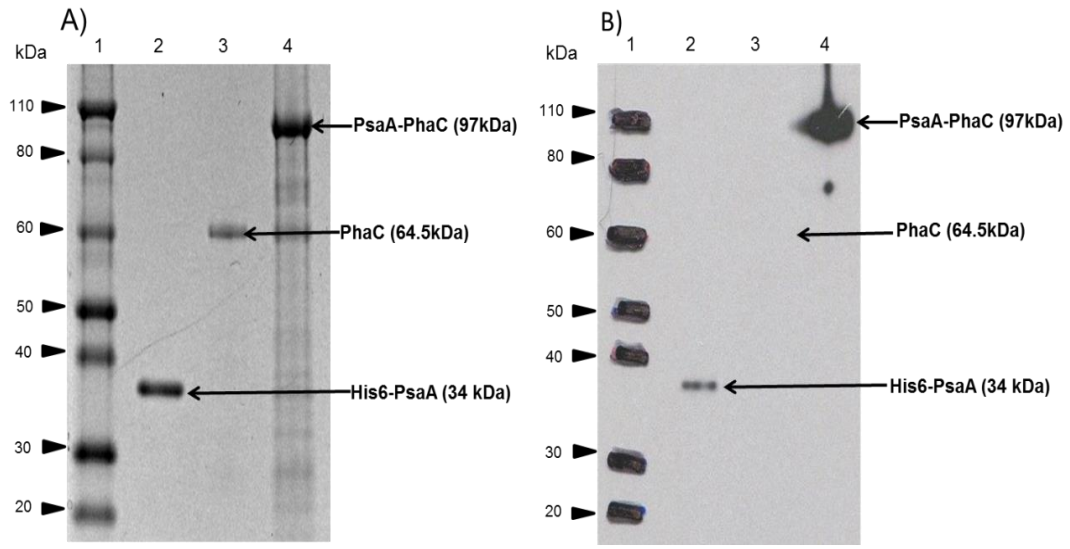


Figure 3. SDS-PAGE and immunoblot analysis of proteins attached to PHB beads and purified His6-PsaA. A, Coomassie blue stained SDS-PAGE gel; B, immunoblot of (A) developed using monoclonal anti-PsaA antibodies. Lane 1, Molecular weight (MW) standard (Novex® Sharp Pre-Stained Protein Standard, Invitrogen); Lane 2, purified His6-PsaA (MW: 34kDa) derived from cells harboring pET14b-his6-psaA; Lane 3, PHB beads isolated from cells harboring pET-14b-phaC encoding PhaC (MW: 64.5kDa); Lane 4, PHB beads isolated from cells harboring pET-14b-psaA-phaC encoding PsaA-PhaC fusion protein (MW: 97kDa). The arrows indicate the protein band of interest with the corresponding theoretical MW.

In Figure 3A dominating protein bands are observed which correspond to the theoretical molecular weights of the various proteins of interest such as His6-PsaA (34 kDa), soluble antigen control, PhaC (MW: 64.5 kDa) (mediating non-antigen displaying PHB bead formation) and PsaA-PhaC fusion protein (MW: 97 kDa) (mediating PsaA displaying PHB bead formation). The identity of proteins was further confirmed by immunoblotting using a specific anti-PsaA antibody (Figure 3B) and by tryptic peptide fingerprinting analysis using MALDI-TOF/MS (Table 2).

Table 2. Tryptic peptide fingerprinting analysis (MALDI-TOF/MS).

Protein	**Peptides fragment identified by MALDI-TOF/MS.**
PsaA-PhaC fusion protein	**N28-K58, G114-K132, V228-K243, V228-K243, T244-K267**, V311-R326, I353-R364, D368-K380, A381-R391, A381-R391, R391-R398, F392-R398, T399-R404, F405-R415, F436-R459, F436-R459, L460-R470, I485-R497, N498-K524, Y540-R553, H554-R568, N569-R589, N569-R589, D596-R625, I603-R625, E661-R676, G677-K703, L746-R768, E769-K788, F791-K808, A854-R863, A866-R873, A866-R873.

* Red bold, identified peptides belonging to the respective pneumococcal antigen

The SDS-PAGE analysis also showed a strong enrichment of the target proteins while the immunoblot confirmed identity and stability of the target protein with no obvious proteolytic truncation (Fig. 3). Densitometry suggested a purity of ≥ 95%.

In order to determine whether PsaA is displayed on the surface of the PHB beads, an ELISA and CLSM was employed which showed binding of the anti-PsaA antibody to the surface of PHB beads mediated by the PsaA-PhaC fusion protein (Fig. 4)

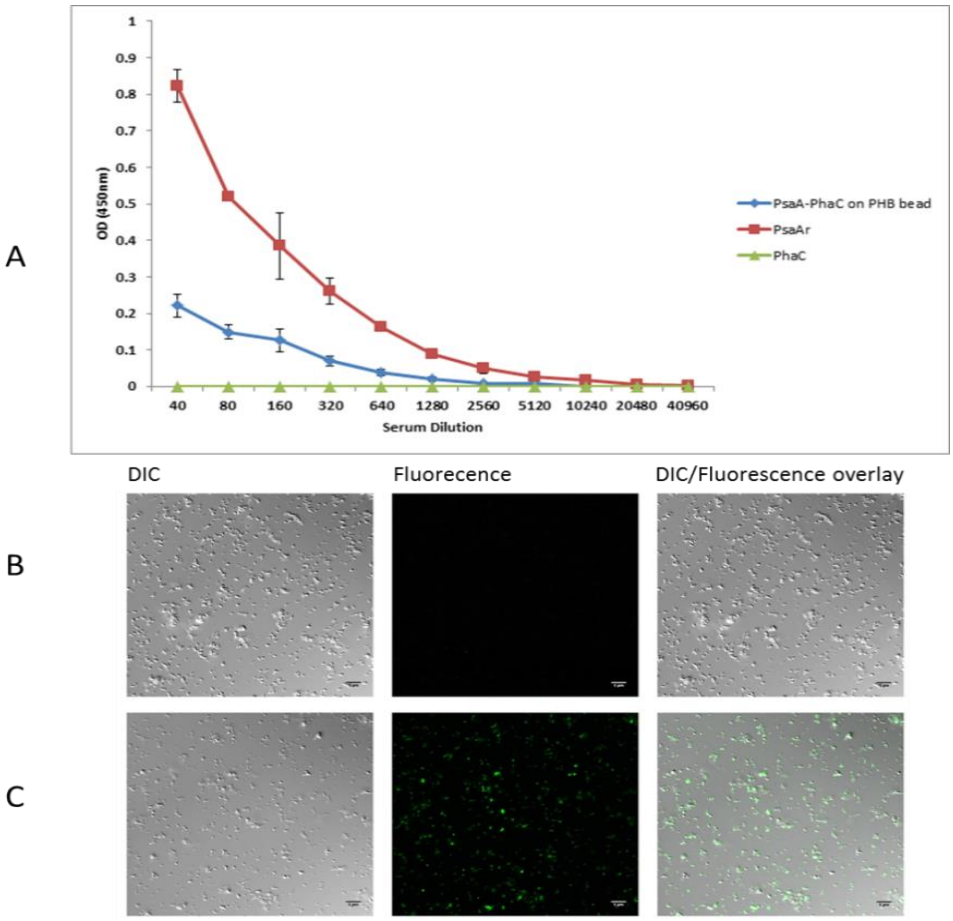


Figure 4. Immunological assessment of PsaA display on the PHB bead surface. The specific monoclonal anti-PsaA antibody (see Materials and Methods) was used to detect PsaA at the surface of PHB beads by ELISA (A) and CLSM (B and C). A, Plates were coated with PsaA-PhaC beads, PhaC beads and soluble His6-PsaA. Anti-IgG HRP-conjugated secondary antibody was used to detect the bound primary anti-PsaA antibody. Data showed that the anti-PsaA antibody bound to PsaA on PHB beads as well as soluble PsaA while PhaC bead (negative control) did not show any binding. Each data point represented the mean of two replicates ± standard deviation. CLSM was further used to demonstrated PsaA surface display by detecting bound anti-PsaA antibodies with a secondary fluorescently labelled (Alexa Fluor 488) antibody. PhaC beads (B) and PsaA-PhaC beads (C) were, after incubation with primary and secondary antibody, observed by CLSM. The fluorescent label of beads in the middle column (B, C) is indicative of PsaA display (C). DIC, differential interference contrast.

To determine the amount of PsaA-PhaC fusion protein attached to PHB beads, the SDS-PAGE analysis including the defined amount of BSA for generation of a densitometry based standard curve was applied. Densitometry using Image Analysis

Software served to deduce the amount of protein per amount of PHB bead. The amount of PsaA-PhaC or PhaC per PHB bead is shown in Table 3. These data were used to calculate the amount of PHB beads to be injected per dose.

Table 3. PHB bead yield and composition

Sample (Plasmids present in production strain)	Culture volume (L)	Wet biomass (g)	Wet PHA bead mass (g)	Antigen % in fusion protein	Antigen /wet Bead (µg/mg)
pET-14b-psaA-phaC	4	32.8	1.63	34	0.39
pET-14b-phaC	4	29.4	5.51	0	1

The Zeta potential of PHB beads was measured as a function of the pH (pH 3-7.5) suggesting a negative surface charge for both PHB beads (Fig. 5). However, display of PsaA further increased the negative surface charge.

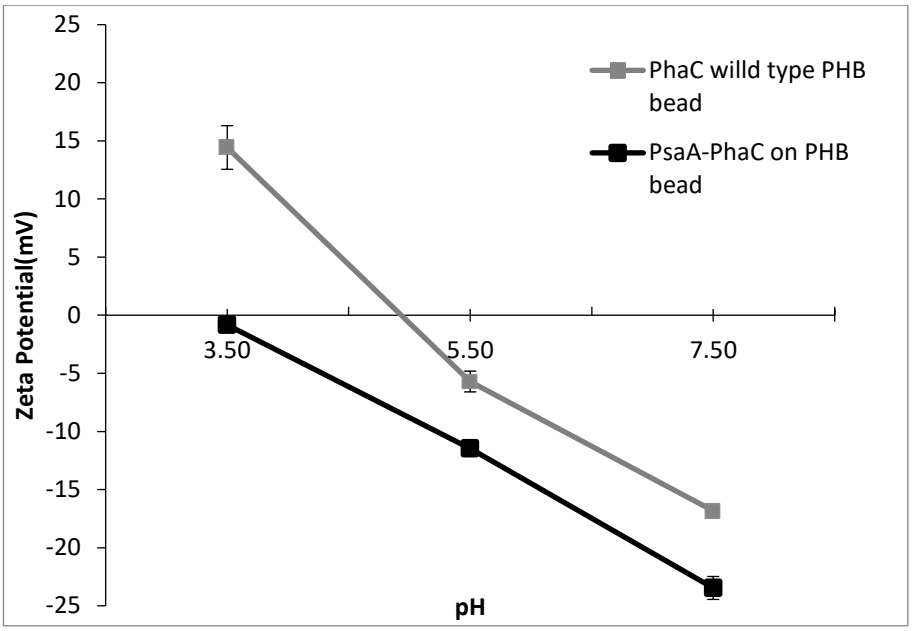


Figure 5. Correlation between zeta potential and pH of various PHB beads. Error bar represents the mean ± standard deviation of three measurements.

2.4.3 Humoral immune response

During the immunization schedule, all animals remained healthy and alive; they were gaining weight and showed no abnormal behaviour (data not shown). In the groups (G1 and G3) immunized with PsaA-PhaC and PhaC PHB beads small granulomas were detected (about 2 mm) at injection sites but no suppuration was observed. After euthanasia, the liver, lung, spleen and kidneys of the animals from groups G1, G2 and G3 showed no differences when compared with organs of animals which received the Placebo (G4).

IgG titers specific towards PsaA after each immunization dose are shown in Figure 6. The absorbance value obtained in G1 and G2 showed induction of specific anti-PsaA antibodies when compared with control groups G3 and G4. Besides, the tendency for an increase in IgG titer after each dose was evident and significant for G1 and G2 (first dose vs the third dose with ($p<0.001$)). As expected the highest titers for these groups were achieved after the third dose presenting significant differences in

comparison with G3 and G4 ((*p<0.01 and* (*p<0.05*)), respectively. No statistically significant differences were found between G1 and G2.

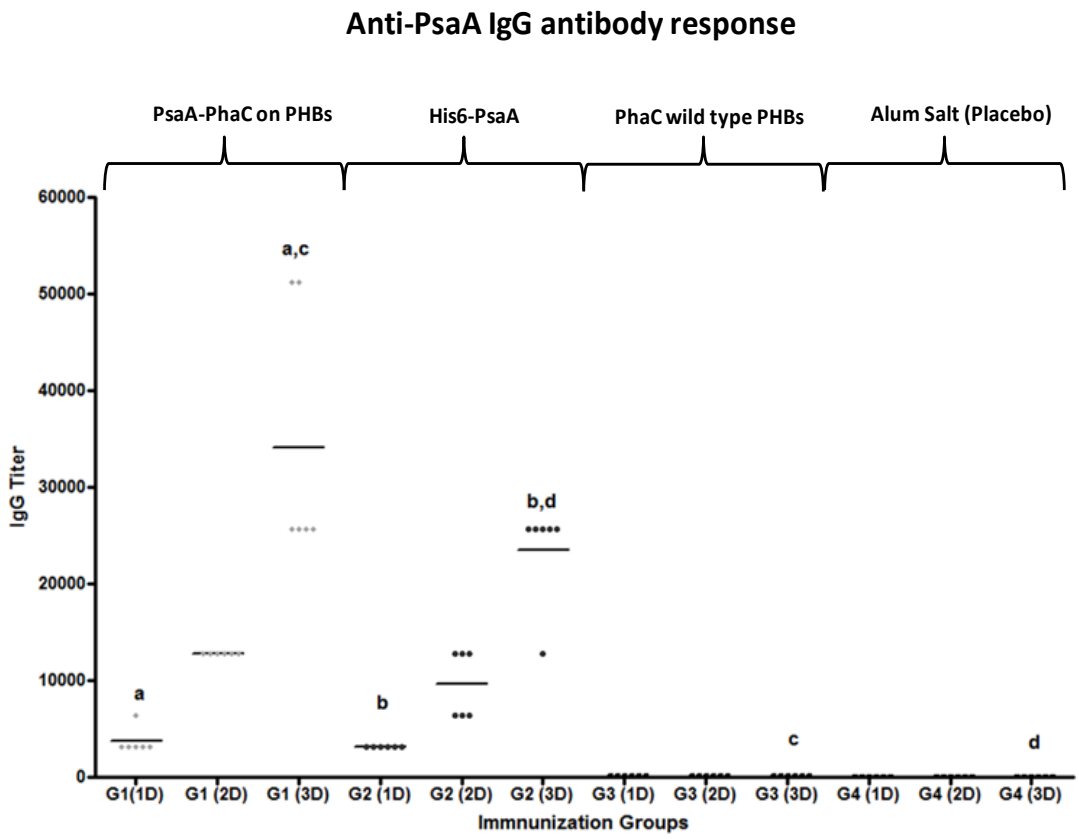


Figure 6. Anti-PsaA IgG antibody response. Anti-PsaA IgG antibody titers were obtained by ELISA. G1 (grey diamond) group immunized with 4 µg of PsaA fused to PhaC and displayed on PHA beads, G2 (black circle) group immunized with 4 µg of His6-PsaA, G3 (black triangle) group immunized with 4 µg of PhaC wild type bead (no antigen PHB bead negative control) and G4 (black square) (placebo) group immunized with only 100 µg Alum salt. All of the immunogens were extracted from *E. coli* (*ClearColi*, LPS free *E. coli* strain). Data are shown as reciprocal antibody titres, representing the dilution required to obtain half of the maximal OD value at 450/570 nm. [a,b] Statistically significant difference (p*<0.001*). [c] Significantly greater than the third dose of placebo and PhaC immunized control group (*p<0.01*), [d] Significantly greater than the third dose of placebo and PhaC immunized control group (*p<0.05*). No statistical differences were found after the third dose between G1 and G2 (*p=0.240*).

The humoral response was further studied, evaluating the IgG subclass profile in sera of animals 7 d after the last immunization. This analysis included subclasses IgG1, IgG2a, IgG2b and IgG3. IgG1 was the prevalent isotype elicited in G1 and G2. Interestingly in the group immunized with PsaA-PhaC fusion protein on PHB beads, IgG2b was the second most prevalent subtype showing higher titers than the other isotypes (Fig. 7).

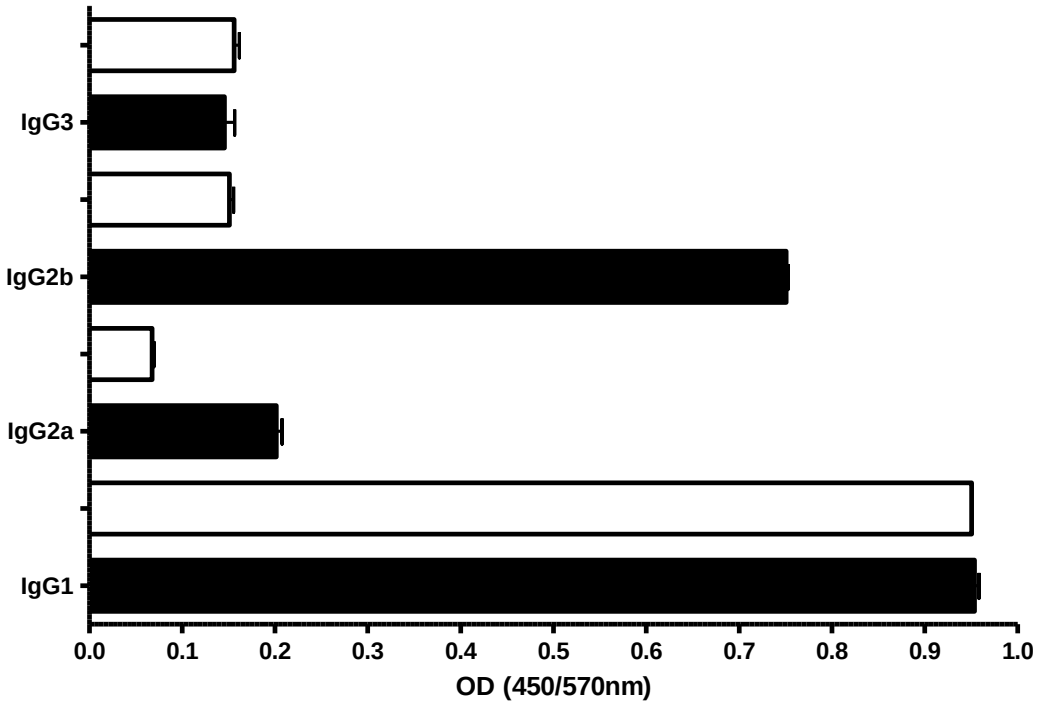


Figure 7. Isotype IgG profile evaluated by direct ELISA using ELISA plates coated with 0.5 µg of soluble His6-PsaA. The results are expressed as the average plus SD of absorbance readings at 450/570nm using pooled of sera (6 animals) from G1 and G2. The sera were collected 1 week after the third dose. The SD bars are only indicated in plus direction. G1 (black bars) group immunized with 4 µg of PsaA displayed on PHB beads, G2 (white bars) group immunized with 4 µg of soluble His6-PsaA. IgG1 is the prevalent isotype in both groups.

To assess whether induced anti-PsaA antibodies would recognize PsaA in whole cell lysates from different serotypes of *S. pneumoniae*, an immunoblot was performed using pooled sera from G1 and G2 obtained 7 d after the last immunization (Fig. 8).

Antibodies in sera of both groups G1 and G2 predominantly detected a protein with an apparent molecular weight of 37 kDa which corresponds to the theoretical molecular weight of PsaA and aligned with the purified PsaA. Interestingly, to get a visible band the sera from G2 had to be 4-fold more concentrated than from G1 suggesting the enhanced production of antibodies elicited after immunization with PsaA displayed on PHB beads versus its soluble counterpart.

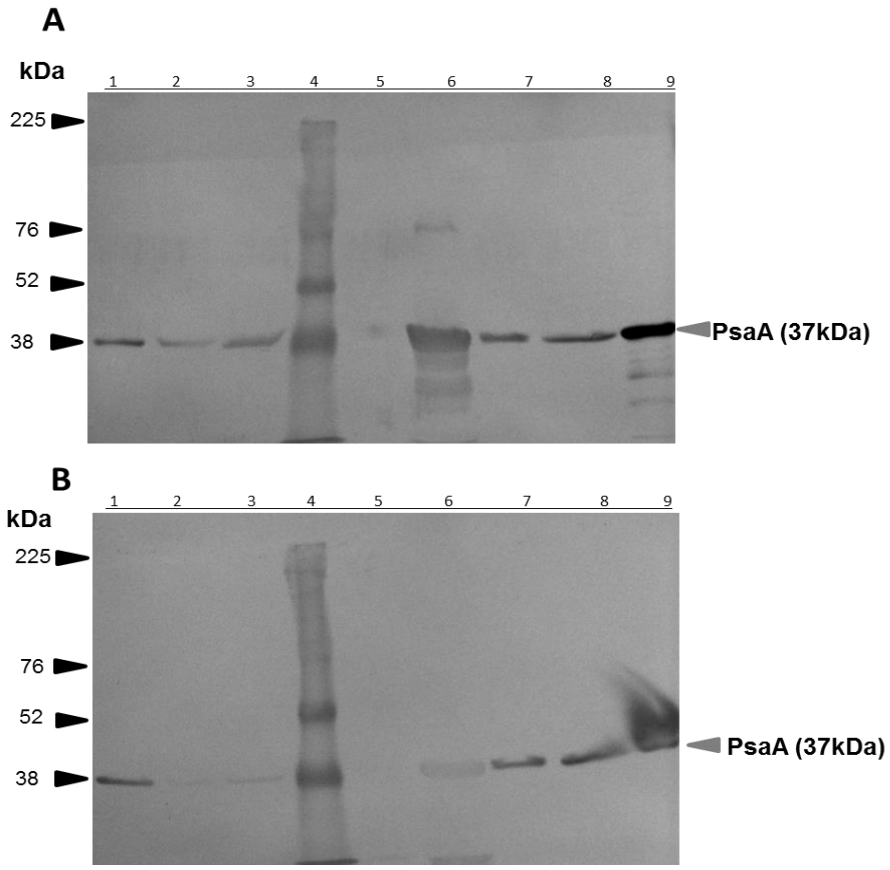


Figure 8. Recognition of PsaA in various serotypes of *S. pneumoniae* by sera from mice immunized with PsaA displayed on PHB beads or soluble PsaA. A, Immunoblots using sera (diluted 1: 2000) from mice immunized with PHB beads displaying PsaA (G1). The corresponding SDS-PAGE of whole cell lysates of various *S. pneumoniae* serotypes is provided in Supplementary Fig. 1. B, immunoblot using sera (diluted 1: 500) from mice immunized with soluble PsaA (G2). Lanes, 1, 2, 3, 5, 6, 7 and 8 correspond to cell lysates from *S. pneumoniae* serotypes: 1, 3, 5, 6b, 7F, 14, 23F, respectively; lane 4, MW standard and lane 9 soluble purified PsaA. The grey arrows indicate the protein corresponding to the molecular weight expected for PsaA.

2.5 Discussion

Current vaccines against *S. pneumoniae* infections are limited in their protection to certain serotypes and exclude important invasive pathogenic serotypes. Hence a serotype-independent broadly protective vaccine, such as a vaccine based on conserved cell surface proteins could provide an alternative approach toward vaccine development.

PsaA has been considered as one of the most important vaccine candidate antigens as it plays a critical role during pathogenesis and is conserved among virulent strains. It induces both B-cell and T-cell responses. In this study, we evaluated the concept of improving delivery and immunogenicity of PsaA by its display on PHB inclusions. Antigen displaying PHB beads (<1 μm) had been produced by engineered *E. coli* and induced both Th1 and Th2 immune responses as well as mediated protective immunity against tuberculosis and hepatitis C (8, 18, 31). Antigen PHB beads are presumably taken up by phagocytosis of antigen presenting cells (APCs) which create an antigen depot and allows cross-presentation on MHC class I and class II, which would further enhance PsaA immunogenicity (8, 29, 31).

In addition, it was shown that the co-administration of Th1-promoting adjuvants to protein-based *S. pneumoniae* vaccine candidates induced protective immunity (32). This suggested that the Th1 immune response might contribute to protective immunity against the extracellular pathogen *S. pneumoniae*. This did further justify considering particulate vaccine such as PHB beads, known to induce Th1 responses, for the formulation of an *S. pneumoniae* vaccine.

To produce PsaA displaying PHB beads, a hybrid gene was constructed (Figure 1) which encoded a single chain fusion protein of PsaA and the PHB forming enzyme, PhaC. The fusion protein-mediated production of PHB inclusions in the *ClearColi*, an

endotoxin-free mutant of *E. coli* (33) (Figure 2). Interestingly, the fusion of PsaA to PhaC resulted in the formation of smaller PHB beads (<200 nm) than observed with only PhaC suggesting an impact of PsaA on self-assembly of PHB beads inside the cell (Figure 2). Protein and immunoblot analysis confirmed that PHB beads displayed the full-length PsaA at high-copy number with an only minor indication of degradation (Fig. 3 and 4, Table 2). PHB beads as antigen carrier isolated from recombinant *E. coli* were recently demonstrated to induce only antigen-specific responses, while potentially co-purifying *E. coli* proteins did not induce detectable responses (19). As the surface charge of particulate vaccines might impact cellular uptake and antigen processing, we assessed the Zeta potential of isolated PHB beads which resulted in a negative charge at physiological pH with an increased negative Zeta potential for the PsaA-PHB beads (Figure 5). Particulate vaccine formulations which are designed for improved uptake of antigens by APCs are known to activate the NLRP3 inflammasome, which enhances the efficacy of the vaccine (34). Activation of the NLRP3 inflammasome was found to depend on the surface charge of the vaccine particle.

To compare immunological properties of soluble and particulate PsaA, the soluble PsaA was produced in *ClearColi* and purified by affinity chromatography to ≥ 95 % purity (Fig. 3). Vaccination experiments showed that IgG levels elicited by PsaA on PHB beads were significantly greater than those induced by PHB beads and within the placebo group ($p<0.01$ and $p<0.05$, respectively). Antibodies against bacterial antigens like cell surface proteins (e.g. PsaA or PspA) were shown to interfere with binding and internalization by the mucosal-nasopharyngeal cells, impairing entrance of the pathogen into blood circulation (35, 36). In particular, the results obtained by De *et al* (37) after immunization with PsaA, reinforces the above statement. *In vitro*

studies further demonstrated that anti-PsaA antibodies inhibited adherence of various *S. pneumoniae* serotypes to nasopharyngeal human carcinoma cells (38). PsaA trapped in alginate microspheres was demonstrated to mediate reduction of *S. pneumoniae* colonization resulting in protection against pneumonia and septicaemia (39).

Production of human IgG1 was related to clearance of extracellular pathogens by activating phagocytosis of pathogens by macrophages (and other phagocytic cells) through Fc receptor antibody interaction. Here immunization with PsaA displaying PHB beads induced predominantly IgG1 (Fig. 6 and 7). This result agrees with the study presented by Palaniappan *et al*, (40) where oral immunization with *S. pneumoniae* strain EF3030 promoted significant PsaA-specific titers of subclasses IgG1 and IgG2a (40). However, PsaA displayed on PHB beads induced IgG2b as the second predominant IgG subclass (Figure 7). These subclasses (IgG2a/b) of antibodies are generally identified as part of a Th1 immune response usually associated with an immune response to intracellular pathogens. However, studies on a murine pneumococcal carrier model illustrated the induction of IgG2b and IgG3 to be important against *S. pneumoniae* strain P1121 infection (41).

Due to their conserved nature, protein-based vaccines are potentially more broadly protective than against various serotypes of *S. pneumoniae* as they induce a serotype-independent immune response. To assess whether PsaA on PHB beads induced production of cross-reactive antibodies, we tested the reactivity of whole cell proteins of serotypes 1, 3, 5, 6b, 7F, 14, 23F with antibodies in pooled sera of vaccinated mice (Fig. 8). These data clearly suggested that serotype independent anti-PsaA antibodies were induced by PsaA displaying PHB beads and by soluble PsaA, regardless the difference in signal intensity. However, anti-PsaA antibody titers in response to PsaA displaying PHB were greater than antibody titers induced by soluble

PsaA. Our results agree with previous studies where PsaA was suggested to be conserved among serotypes suitable for use as an antigen for broadly protective vaccines (42-44). The PsaA displayed on the surface of PHB beads was selected from strain CGSP14 which is identical to PsaA from strains R6 and TIGR4 (Serotype 14) and shows 98-99% amino acid sequence identity with all other deposited PsaA protein sequences in the current NCBI database (24, 45). Overall, this study demonstrated that bioengineering can be used to produce immunogenic PsaA displaying PHB beads, which mediate a serotype-independent PsaA specific antibody response. Hence the PHB bead-based particulate vaccine approach holds the promise to be applicable not only for protection against intracellular pathogens but also extracellular pathogens such as *S. pneumoniae*.

2.6 Acknowledgements

The authors are also thankful for the provision the anti-PsaA antibody by John Lewis and Anja Werno (Canterbury Health Laboratories, NZ), the technical assistance by Maria Onelia González Socarras, Alex Quintero Perez, Dr Reinaldo Oliva Hernandez, Dr Juan Francisco Infante and MSC. Tamara Hernandez Salazar. We would also like to acknowledge the Manawatu Microscopy and Imaging Centre for preparation of electron microscopy sections, CLSM work, technical advice and use of their facility

2.7 References

1. O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, et al. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. Lancet. 2009;374(9693):893-902.
2. van der Poll T, Opal SM. Pathogenesis, treatment, and prevention of pneumococcal pneumonia. Lancet. 2009;374(9700):1543-56.
3. Lee LH, Gu XX, Nahm MH. Towards New Broader Spectrum Pneumococcal Vaccines: The Future of Pneumococcal Disease Prevention. Vaccines (Basel). 2014;2(1):112-28.
4. Darrieux M, Goulart C, Briles D, Leite LC. Current status and perspectives on protein-based pneumococcal vaccines. Critical Reviews in Microbiology. 2015;41(2):190-200.
5. Gor DO, Ding X, Briles DE, Jacobs MR, Greenspan NS. Relationship between surface accessibility for PpmA, PsaA, and PspA and antibody-mediated immunity to systemic infection by *Streptococcus pneumoniae*. Infection and Immunity. 2005;73(3):1304-12.
6. Nabors GS, Braun PA, Herrmann DJ, Heise ML, Pyle DJ, Gravenstein S, et al. Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies to heterologous PspA molecules. Vaccine. 2000;18(17):1743-54.
7. Parlane NA, Grage K, Lee JW, Buddle BM, Denis M, Rehm BH. Production of a particulate hepatitis C vaccine candidate by an engineered *Lactococcus lactis* strain. Applied and Environmental Microbiology. 2011;77(24):8516-22.
8. Parlane NA, Rehm BH, Wedlock DN, Buddle BM. Novel particulate vaccines utilizing polyester nanoparticles (bio-beads) for protection against *Mycobacterium bovis* infection-a review. Veterinary Immunology Immunopathology. 2014;158(1-2):8-13.
9. Rehm BH. Biogenesis of microbial polyhydroxyalkanoate granules: a platform technology for the production of tailor-made bioparticles. Current Issues Molecular Biology. 2007;9(1):41-62.
10. Lee SY, Lee KM, Chan HN, Steinbuchel A. Comparison of recombinant *Escherichia coli* strains for synthesis and accumulation of poly-(3-hydroxybutyric

acid) and morphological changes. Biotechnology Bioengineering. 1994;44(11):1337-47.

11. Hooks D, Venning-Slater M, Du J, Rehm B. Polyhydroxyalkanoate synthase fusions as a strategy for oriented enzyme immobilisation. Molecules. 2014;19(6):8629.

12. Parlane NA, Grage K, Mifune J, Basaraba RJ, Wedlock DN, Rehm BH, et al. Vaccines displaying mycobacterial proteins on biopolyester beads stimulate cellular immunity and induce protection against tuberculosis. Clinical and Vaccine Immunology. 2012;19(1):37-44.

13. Draper JL, Rehm BH. Engineering bacteria to manufacture functionalized polyester beads. Bioengineered. 2012;3(4):203-8.

14. Grage K, Peters V, Rehm BHA. Recombinant protein production by *in vivo* polymer inclusion display. Applied and Environmental Microbiology. 2011;77(18):6706-9.

15. Parlane NA, Gupta SK, Rubio Reyes P, Chen S, Gonzalez Miro M, Wedlock DN, et al. Self-assembled protein-coated polyhydroxyalkanoate beads: properties and biomedical applications. ACS Biomaterials Science & Engineering. 2016.

16. Rehm FB, Chen S, Rehm BH. Enzyme Engineering for In Situ Immobilization. Molecules. 2016;21(10).

17. Rehm BH. Bacterial polymers: biosynthesis, modifications and applications. Nature Reviews Microbiology. 2010;8(8):578-92.

18. Martinez-Donato G, Piniella B, Aguilar D, Olivera S, Perez A, Castanedo Y, et al. Protective T Cell and Antibody Immune Responses against Hepatitis C Virus Achieved Using a Biopolyester-Bead-Based Vaccine Delivery System. Clinical and Vaccine Immunology. 2016;23(4):370-8.

19. Rubio Reyes P, Parlane NA, Wedlock DN, Rehm BH. Immunogenicity of antigens from Mycobacterium tuberculosis self-assembled as particulate vaccines. Int Journal of Medical Microbiology. 2016;306(8):624-32.

20. Grage K, Rehm BH. In vivo production of scFv-displaying biopolymer beads using a self-assembly-promoting fusion partner. Bioconjugation Chemistry. 2008;19(1):254-62.

21. Peters V, Rehm BH. In vivo monitoring of PHA granule formation using GFP-labeled PHA synthases. FEMS microbiology letters. 2005;248(1):93-100.

22. Amara AA, Rehm BH. Replacement of the catalytic nucleophile cysteine-296 by serine in class II polyhydroxyalkanoate synthase from *Pseudomonas aeruginosa*-mediated synthesis of a new polyester: identification of catalytic residues. Biochemical Journal. 2003;374(Pt 2):413-21.

23. Hooks DO, Blatchford PA, Rehm BH. Bioengineering of bacterial polymer inclusions catalyzing the synthesis of N-acetylneuraminic acid. Applied Environmental Microbiology. 2013;79(9):3116-21.

24. Larentis AL, Argondizzo AP, Esteves Gods S, Jessouron E, Galler R, Medeiros MA. Cloning and optimization of induction conditions for mature PsaA (pneumococcal surface adhesin A) expression in *Escherichia coli* and recombinant protein stability during long-term storage. Protein Expression and Purification. 2011;78(1):38-47.

25. Thompson T, Rehm BH, Herbert AB, Saravolac EG. Compositions for separation methods. Google Patents; 2011.

26. Thompson T, Rehm BHA, Herbert AB, Saravolac EG, Mcdermott PB, Draper JL. Compositions for separation methods. Google Patents; 2013.

27. Hooks DO, Blatchford PA, Rehm BH. Bioengineering of bacterial polymer inclusions catalyzing the synthesis of N-acetylneuraminic acid. Applied and environmental microbiology. 2013;79(9):3116-21.

28. Hooks DO, Rehm BH. Insights into the surface topology of polyhydroxyalkanoate synthase: self-assembly of functionalized inclusions. Applied microbiology and biotechnology. 2015;99(19):8045-53.

29. Parlane NA, Wedlock DN, Buddle BM, Rehm BH. Bacterial polyester inclusions engineered to display vaccine candidate antigens for use as a novel class of safe and efficient vaccine delivery agents. Applied and Environmental Microbiology. 2009;75(24):7739-44.

30. Espinosa-Viñals C, Soroa-Millán Y, Martin-García Y, Pérez-Baños A, Nicot-Valenciano M, Rodríguez-Noda L, et al. Validación y aplicación de un ELISA para la cuantificación de anticuerpos IgG contra polisacárido capsular Vi de Salmonella Typhi. Vaccimonitor. 2015;24(1):21-32.

31. Grage K, Jahns AC, Parlane N, Palanisamy R, Rasiah IA, Atwood JA, et al. Bacterial polyhydroxyalkanoate granules: biogenesis, structure, and potential use as nano-/micro-beads in biotechnological and biomedical applications. Biomacromolecules. 2009;10(4):660-9.

32. Olafsdottir TA, Lingnau K, Nagy E, Jonsdottir I. Novel protein-based pneumococcal vaccines administered with the Th1-promoting adjuvant IC31 induce protective immunity against pneumococcal disease in neonatal mice. Infection and immunity. 2012;80(1):461-8.

33. Mamat U, Woodard RW, Wilke K, Souvignier C, Mead D, Steinmetz E, et al. Endotoxin-free protein production *ClearColi* technology. Nature Methods. 2013;10(9).

34. Neumann S, Burkert K, Kemp R, Rades T, Dunbar PR, Hook S. Activation of the NLRP3 inflammasome is not a feature of all particulate vaccine adjuvants. Immunology and cell biology. 2014;92(6):535-42.

35. Johnson SE, Dykes JK, Jue DL, Sampson JS, Carlone GM, Ades EW. Inhibition of pneumococcal carriage in mice by subcutaneous immunization with peptides from the common surface protein pneumococcal surface adhesin a. The Journal and Infectious Diseases. 2002;185(4):489-96.

36. Rajam G, Romero-Steiner, S., Johnson, S.E., Thompson, T.A., Reed, Y.M.,, Sampson JS, Carlone, G.M., and Ades, E.W. . Immunogenicity and functional activity of multi-antigenic peptides (MAPs) of pneumococcal surface adhesin A (PsaA) in a rabbit model. 105th General Meeting of ASM, ASM, Atlanta, GA. 2005.

37. De BK, Sampson JS, Ades EW, Huebner RC, Jue DL, Johnson SE, et al. Purification and characterization of *Streptococcus pneumoniae* palmitoylated pneumococcal surface adhesin A expressed in *Escherichia coli*. Vaccine. 2000;18(17):1811-21.

38. Romero-Steiner S, Pilishvili T, Sampson JS, Johnson SE, Stinson A, Carlone GM, et al. Inhibition of pneumococcal adherence to human nasopharyngeal epithelial cells by anti-PsaA antibodies. Clinical and Diagnostic Laboratory Immunology. 2003;10(2):246-51.

39. Seo JY, Seong SY, Ahn BY, Kwon IC, Chung H, Jeong SY. Cross-protective immunity of mice induced by oral immunization with pneumococcal surface adhesin a encapsulated in microspheres. Infection and Immunity. 2002;70(3):1143-9.

40. Palaniappan R, Singh S, Singh UP, Sakthivel SK, Ades EW, Briles DE, et al. Differential PsaA-, PspA-, PspC-, and PdB-specific immune responses in a mouse model of pneumococcal carriage. Infection and Immunity. 2005;73(2):1006-13.

41. van Rossum AM, Lysenko ES, Weiser JN. Host and bacterial factors contributing to the clearance of colonization by *Streptococcus pneumoniae* in a murine model. Infection and immunity. 2005;73(11):7718-26.

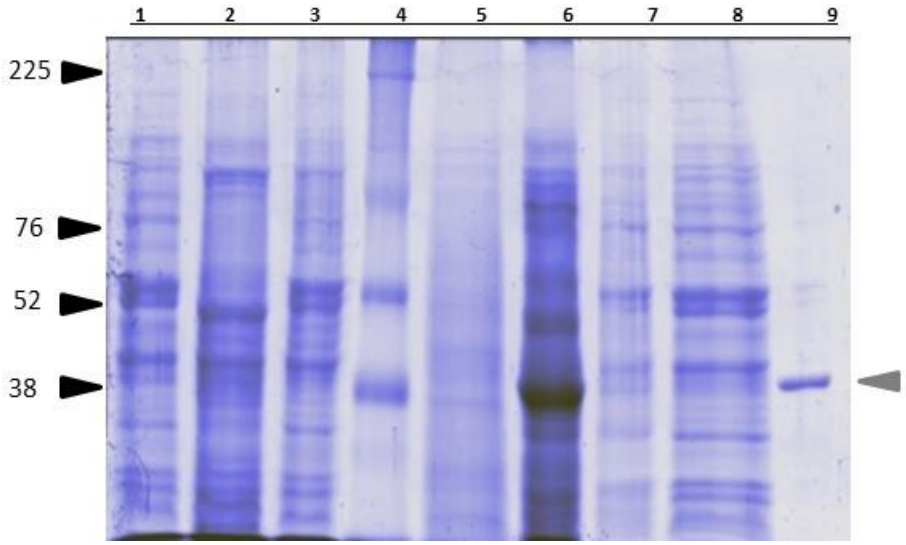
42. Ogunniyi AD, Folland RL, Briles DE, Hollingshead SK, Paton JC. Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*. Infection and Immunity. 2000;68(5):3028-33.

43. Russell H, Tharpe JA, Wells DE, White EH, Johnson JE. Monoclonal antibody recognizing a species-specific protein from *Streptococcus pneumoniae*. Journal and Clinical Microbiology. 1990;28(10):2191-5.

44. Sampson JS, O'Connor SP, Stinson AR, Tharpe JA, Russell H. Cloning and nucleotide sequence analysis of psaA, the *Streptococcus pneumoniae* gene encoding a 37-kilodalton protein homologous to previously reported *Streptococcus sp*. adhesins. Infection and Immunity. 1994;62(1):319-24.

45. Hoskins J, Alborn WE, Jr., Arnold J, Blaszczak LC, Burgett S, DeHoff BS, et al. Genome of the bacterium *Streptococcus pneumoniae* strain R6. Journal of Bacteriology. 2001;183(19):5709-17.

2.8 Supplementary material



Supplementary Figure 1. SDS-PAGE of whole cell lysates of the various *S. pneumoniae* serotypes. Lanes, 1, 2, 3, 5, 6, 7 and 8 correspond to cell lysates from *S. pneumoniae* serotypes: 1, 3, 5, 6b, 7F, 14, 23F, respectively; lane 4, MW standard and lane 9 soluble purified PsaA. The grey arrows indicate the protein corresponding to the molecular weight expected for PsaA.

Preface to the next Chapter

In Chapter 2 production, isolation and purification of the PsaA-PhaC PHB beads and His6-PsaA were achieved successfully. The mice immunization and the immune response evaluation showed the production of high and specific IgG titers against PsaA. In addition, IgG1 was the most prevalent immunoglobulin subclass followed by IgG2b in the group vaccinated with PHB beads. The recognition of the PsaA from seven different *S. pneumoniae* lysates, by the sera from mice vaccinated with PsaA-PhaC PHB beads, showed serotype independent immunity.

In this chapter, to expand the antigen repertoire and to design a more broadly protective vaccine, Ply was selected to be displayed at PHB beads surface and CPS from *S. pneumoniae* serotype 19F was chemically conjugated to PhaC wild-type PHB. Production, isolation and purification of Ply-PhaC PHB beads were successfully achieved. The humoral and cellular immune response was evaluated after mice vaccination with the new PHB beads.

Chapter 3. Biologically assembled polyester beads displaying pneumolysin and capsular polysaccharide induce protective immunity against *Streptococcus pneumoniae*

Majela González-Miró [1,2], Anna-Maria Radecker [2], Laura M Rodríguez-Noda [1], Mildrey Fariñas-Medina[1], Caridad Zayas-Vignier[1], Mabel Heránndez-Cedeño [1], Yohana Serrano[1], Félix Cardoso [1], Darielys Santana-Mederos[1], Dagmar García-Rivera [1], Yury Valdés-Balbín [1], Vicente Vérez-Bencomo[1], Bernd H.A. Rehm[2, 3]

[1]Finlay Vaccine Institute. La Habana, Cuba; [2]Institute of Fundamental Sciences, Massey University, Palmerston North, New Zealand; [3]Centre for Cell Factories and Biopolymers, Griffith Institute for Drug Discovery, Griffith University, Nathan, Queensland, Australia

Under review by *Frontiers in Immunology*, Jan 2018

3.1 Abstract

Streptococcus pneumoniae can cause life-threatening infections mostly in infants, children and elderly people. Conjugate vaccines provide serotype-dependent protection against *S. pneuomoniae* infections but fail to protect against new emerging serotypes. To overcome these limitations, pneumolysin (Ply), a conserved protein, and the capsular polysaccharide (CPS) from serotype 19F were selected to be displayed at the surface of *in vivo* self-assembled polyester beads. Ply was translationally fused to the *N*-terminus of the polyhydroxybutyrate (PHB) synthase (PhaC). Production of this fusion protein-mediated assembly of PHB inclusions in recombinant *E.coli*. Transmission electron microscopy showed that the Ply-PhaC fusion protein-mediated the formation of beads. The molecular identity of the proteins was confirmed by SDS-PAGE, peptide fingerprinting analysis and immunoblotting. The CPS was chemically conjugated to PHB beads and the soluble tetanus toxoid. Balb/c mice vaccinated with Ply-PhaC beads and 19F-PhaC beads elicited high and specific IgG levels. Additionally, restimulated splenocytes from animals vaccinated with Ply-PhaC beads produced a balanced INF-ɣ/IL-17A profile unlike animals vaccinated with soluble Ply. The induced IgG antibodies recognised Ply in whole cell lysates of six different serotypes of *S. pneumoniae*. 19F-PhaC beads induced production of antibodies showing high opsonophagocytic titers against the homologous serotype 19F strain, while purified CPS 19F mixed with PhaC beads did not elicit any immunity. This study provided insight into the design of PHB beads not only to perform as a carrier of proteinaceous antigens but also for CPS in order to induce broadly protective immune responses for the prevention of pneumococcal infections.

Keywords: Particulate vaccine, capsular polysaccharides, antigen delivery, polyhydroxybutyrate, self-assembly, pneumolysin

3.2 Introduction

Streptococcus pneumoniae is considered as one of the most important health threats during childhood. Global studies suggest that 11% of 6.6 million deaths per annum in children at the age of 1-5 year is due to pneumococcal infections (1). A considerable number of *S. pneumoniae* virulence factors are known to mediate nasopharyngeal colonisation and evasion of the human immune system. The diversity of the capsular polysaccharides (CPS) produced by *S. pneumoniae* is reflected by currently more than 91 different chemical structures (serotypes). Proteins as antigens are considered to be less variable and more conserved throughout the various virulent strains (2-4).

Polysaccharide–immunogenic carrier protein conjugate vaccines are licensed and widely used to prevent pneumococcal diseases around the world. There are three licensed vaccines in the market (Prevnar 7 or 13 and Synflorix), which include important serotypes like 1, 3, 4, 5, 6B, 7F, 9V, 19F, 23F and 18C. All of them show a safe and serotype-specific immune response profile (3, 5, 6). As the efficacy of these vaccines is limited by the CPS composition, emerging serotypes require alternative vaccination strategies (3). Proteins like the pneumococcal surface protein A (PspA), pneumococcal adhesin A (PsaA), pneumolysin (Ply) and pneumococcal surface protein C (PspC) have been shown to elicit broadly protective immune responses (7-9). Ply is a 53 kDa protein produced by all of the isolated *S. pneumoniae* strains (10). Ply belongs to a group of cytoplasmic thiol-activated lysins and plays a role in the colonisation process as well as contributing to severe lung tissue damage in later stages of infection (11-13). It also induces strong cytokine production associated with

the recruitment of immune cells, such as neutrophils, to the damaged tissue (14). Because of its properties, Ply has been considered as a vaccine candidate antigen to prevent pneumococcal diseases (15). However, as a subunit vaccine, purified soluble Ply by itself is poorly immunogenic, requiring the addition of adjuvant as part of the vaccine formulation in order to induce protective immunity.

Polyhydroxybutyrate (PHB) beads, which are naturally formed as cellular inclusions by many bacteria, have been demonstrated as antigen delivery system to induce protective immune responses against intracellular pathogens such as e.g. *Mycobacterium tuberculosis* and Hepatitis C as well as a strong and specific immune response against the extracellular pathogen *S. pneumoniae* (16-19). The introduction of three genes encoding the main enzymes involved in the PHB biosynthesis pathways in not-naturally PHB-producing bacteria, like *E.coli*, enabled the recombinant production of these PHB beads exhibiting diameters of about 500 nm-1µm (20, 21). Translational fusions of foreign proteins to the PHB synthase mediated the production of PHB inclusions coated with the respective proteins of interest (18, 19, 22-26). In this study, the aim was not only to display the antigen, Ply, on PHB beads but also to conjugate a non-proteinaceous carbohydrate antigen, the CPS from serotype 19F. The use of PHB beads as an antigen delivery system for the prevention of pneumococcal infections was investigated.

3.3 Materials and methods

3.3.1 Strains and cultivation conditions

E.coli XL1 blue was used as a plasmid propagation host and cultivated in Luria Bertani Broth (LB) containing 100 µg/mL of ampicillin at 37°C. PHB beads and

soluble His-tagged protein were produced in an endotoxin-free *E.coli* strain (*ClearColi*) (16, 27).

3.3.2 Construction of plasmids for production of soluble Ply and Ply displayed on PHB beads

The gene encoding the non-toxic Ply (470aa, from *S. pneumoniae* strain R36A with a double mutations (G293T, C428A)) was synthesised as codon optimised for expression in *E. coli* (28, 29). The Ply gene was amplified from pUC57-ply by PCR and hydrolyzed with (*Xba*I/*Spe*I) restriction enzymes. The respective Ply encoding DNA fragment was then inserted into the 5'end of the *phaC* gene. To produce soluble Ply, six CAC codons were introduced to 5' end of the gene to encode 6 histidine residues for purification by Ni^{2+} affinity chromatography. The hybrid genes encoding proteins, used in this study are outlined in Figure 1, while the oligonucleotides and plasmids are listed in Table 1. The *ply* gene sequence was checked by DNA sequencing, in the final plasmids.

3.3.3 PHB bead production

The PHB bead production, isolation, purification, and sterilization were conducted as described previously (16, 30, 31). In this study, *E. coli (ClearColi)* harbouring pMCS69 was transformed with pET14b-ply-phaC for the production of the Ply displaying PHB beads (Ply-PhaC beads) and with pET14b-phaC to produce non-antigen displaying PHB beads (PhaC beads).

3.3.4 Production of soluble Ply

The *E.coli (ClearColi)* harbouring pET-14b-his6-ply plasmid was cultivated at 25^{o}C at 200 rpm for 24 h. The cells were lysed by mechanical disruption and purified

by Ni^{2+} chromatography according to the manufacturer's protocol (HisTrap^{TM} FF crude, GE Healthcare-Bio-Sciences AB, Sweden).

3.3.5 Conjugation of CPS to PHB beads

The CPS from *S. pneumoniae* serotype 19F and tetanus toxoid (TT) were provided by the Finlay Vaccine Institute (Havana, Cuba). The chemical conjugation of the CPS to PHB beads as well as TT was performed as described above (32). Protein and carbohydrate concentration was determined by Lowry and Phenol-Sulfuric acid assays (33, 34).

3.3.6 Transmission electron microscopy (TEM)

To demonstrate production of PHB beads by *ClearColi*, cells harbouring pET14b-ply-phaC or pET14b-phaC were washed with PBS (pH 7.0) and analysed by TEM (27). The presence of spherical inclusions inside of the cells indicated functionality of PhaC. Isolated PHB beads were analysed by TEM to assess purity and size distribution.

3.3.7 Proteins analysis by SDS-PAGE and immunoblot

Proteins associated with PHB beads as well as soluble Ply were analysed by sodium dodecyl sulphate acrylamide gel electrophoresis (SDS-PAGE) (35, 36). To identify proteins tryptic peptide fingerprinting analysis using Triple TOF was used. Further identification of proteins was obtained by immunoblotting using monoclonal antibodies against Ply (PLY-7) and monospecific polyclonal anti-PhaC antibodies enabled (37, 38).

3.3.8 Immune response evaluation

3.3.8.1 Immunization schedule for Ply containing vaccine formulations

Four vaccination groups each comprising 8 animals between 5-6 weeks old (male, Balb/c mice) were prepared. Mice were acquired from CENPALAB (Centro Nacional para la Producción de Animales de Laboratorio, Havana, Cuba). Three vaccination doses of 1.5 µg of antigen plus 15 µg of alum (Alhydrogel, Brenntag Biosector, Denmark) were administered to each animal. Animals of the placebo group received only the adjuvant. Immunizations occurred at 0, 14 and 28 d. Blood samples were collected from the retro-orbital plexus at day 0 and seven days after the last vaccination (35 days). Group Ply-PhaC beads, received 1.5 µg of Ply displayed on PHB beads, Group His6-Ply received 1.5 µg of soluble His6-Ply, Group PhaC beads received 1.5 µg of PhaC on non-antigen displaying PHB beads and a Group alum received, 15 µg of alum.

These studies were carried out in accordance with the Finlay Vaccine Institute animal ethics guidelines and international recommendations. The protocols were approved by the ethics committee of the Finlay Vaccine Institute (Cuba).

To check the safety of the formulation after each immunization, lump formation, in the inoculation site, was followed in animals vaccinated with PHB beads. In addition, after euthanasia vital organs were visually checked.

3.3.8.2 Immunization schedule for CPS containing vaccine formulations

Five vaccination groups each comprising 8 animals were used as described above. Three vaccination doses of 2-10µg of antigen were administered to each animal. Animals of the placebo group received only the adjuvant. Immunizations and

blood sample collections were conducted as above. Group 19F-PhaC beads (1), received 2 µg of 19F polysaccharide conjugated to PhaC on non-antigen displaying PHB beads, Group 19F-PhaC beads (2) received 10 µg of 19F polysaccharide conjugated to PhaC on non-antigen displaying PHB beads, Group 19F-TT received 2 µg of 19F polysaccharide conjugated to TT, Group 19F + PhaC received a mix of 10 µg of CPS from serotype 19F and 30 µg of PhaC on non-antigen displaying PHB beads while Group alum, received only 0.125 µg alum.

3.3.8.3 Evaluation of anti-Ply IgG antibody levels

The anti-Ply IgG titers were evaluated as mentioned above but with minor modifications (16). Briefly, plates were coated with 0.2 µg/mL of His6-Ply in coating buffer. The serum of each mouse was diluted by 800-fold, while the pre-immune serum was diluted by 100-fold in dilution buffer. To both, a serum sample from each mouse, anti-mouse IgG peroxidase conjugate (Sigma-Aldrich, St. Louis, MO) diluted 1: 10,000 were added and plates were incubated for 90 minutes at 25ºC. The IgG titer was defined as the reciprocal value of the antibodies titer representing the dilution factor (DF) to obtain 50% of the maximum level of absorbance. The results are shown as the mean ± SEM of 8 animals.

3.3.8.4 Evaluation of anti-19F IgG antibody levels

The anti-19F CPS IgG titers were evaluated by ELISA. Briefly, plates were coated with 10 µg/mL of the CPS from serotype 19F in PBS pH 7.0. The serum of each mouse from 7 days and from 6 months after the last vaccination as well as the pre-immune serum were diluted by 100-fold and added to plates and incubated for 90 min at 25ºC. Anti-mouse IgG peroxidase conjugate (Sigma-Aldrich, St. Louis, MO) was diluted 1: 10,000 and added to the plates and incubated for 90 min at 25ºC. The

IgG titer was defined, as the reciprocal value of the antibody titer representing the dilution factor (DF) to obtain 50% of the maximum level of absorbance. The results are shown as the mean ± SEM of 8 animals.

3.3.8.5 Analysis of the IgG subclass profile

The ELISA was performed as described above and serum from each animal was diluted 200-fold. The anti-IgG1, IgG2a, IgG2b, IgG3 and IgM antibodies were derived from goat (Sigma-Aldrich, St.Louis, MO) and diluted 1: 2500. The IgG subclass levels were defined as the reciprocal value of the antibody titers representing a dilution factor (DF) 50% of the maximum absorbance. The results are shown as the mean ± SEM of 8 animals.

3.3.8.6 Cytokine assay

Mice were euthanised and spleens were removed from each mouse 7 days after the last vaccination dose and cells were recovered by perfusion and pooled for each group. Cells were recovered by perfusion and erythrocytes were then removed by osmotic shock using 0.2% (w/v) NaCl. The cells were counted, and the viability was tested by trypan blue exclusion staining. Splenocytes were adjusted to 4×10^6 cells/mL and were cultured in Dulbecco's Modified Eagle's medium supplemented with 50 µg/mL of gentamicin, 2 mmol/L of L-glutamine, 1 mmol/L of sodium pyruvate, 15 mmol/L of HEPES and 10% of inactivated Fetal calf serum (all from Sigma-Aldrich). Isolated splenocytes were stimulated *in vitro* with His6-Ply (0.2, 5 and 10 µg/mL) or Concanavalin A (positive control) for 72h. The levels of IFN-γ, IL-4, and IL-17A in the supernatant of the culture was measured by double sandwich capture ELISA (Mabtech, Sweden). Results are expressed in mean ± SD of two replicates per vaccination group.

3.3.8.7 Assessment of antibody recognition of Ply in various serotypes

The specific binding of antibodies induced by vaccination with Ply-PhaC beads or soluble His6-Ply to Ply from various *S. pneumoniae* serotypes was assayed as previously described (16). In this study, the strains assayed were from serotypes 1, 3, 5, 19F, 23F and 18C. The cell suspension was adjusted to a final concentration of 10% (w/v) and denatured by heating at 95 ^{o}C. Ten µl of the soluble fraction of these samples were separated by SDS-PAGE using a 10% (w/v) acrylamide gel. Immunoblot was conducted using sera dilutions of 1: 1750 for group Ply-PhaC and 1: 500 for group His6-Ply.

3.3.8.8 Opsonophagocytosis activity assay (OPA)

The OPA used was based on that described by Romero-Steiner (39) but using human polymorphonuclear leukocytes as effector cells. In brief, heat-inactivated mouse serum was serially diluted in twofold steps in a 96-well microtiter plate (round bottom, COSTAR) with opsono-buffer (Hank´s buffer (Sigma) containing 0.1% (w/v) gelatin) and were incubated with cells of each *S. pneumoniae* serotype (~1,000 CFU per well) for 15 min at 37 ^{o}C in a 5% CO_2 atmosphere. Complement from baby rabbit serum (Pel-Freez Biological) and peripheral polymorphonuclear leukocytes purified from human blood as effector cells were added at 4 x 10^5cell per well. The mixture was incubated at 37^{o}C for 45 min with shaking (90 rpm). The reaction was stopped by cooling on ice for 5 min. After the phagocytosis step 5 µL aliquots were removed and applied onto a tilted Todd Hewitt yeast extract agar plate, and the plate was incubated at 37^{o}C with 5% CO_2 for 18 h. OPA titers were calculated as the reciprocal of the serum dilution that caused a 50% reduction of the CFU (killing) compared to the CFU from the control wells containing all reagents except rabbit serum. The results are shown as the mean ± SEM of 8 animals.

3.3.9 Statistical analysis

Graph Pad Prism 5.00 (San Diego, USA) software was used for statistical data analysis. Statistical differences between all groups were calculated by one-way analysis of variance (ANOVA) with Kruskal–Wallis non-parametric test. When significant differences were found, the Dunn's post-test was used, considering differences to be significant when $p<0.05$. When the comparison was just between two groups the Mann-Whitney test was performed and differences were considered statistically different when $p<0.05$.

3.4 Results

3.4.1 Bioengineering *E. coli* for production of Ply-PHB beads and soluble His6-Ply

Ply is one of the most conserved proteins across pathogenic *S. pneumoniae* strains (40). The Ply amino acid sequence was selected from *S. pneumoniae* strain R36A with two mutations (G293T, C428A) to remove toxicity as described elsewhere (28, 29). A schematic representation of the genes encoding fusion proteins mediating PHB bead production and production of soluble His6-Ply are depicted in Figure 1.

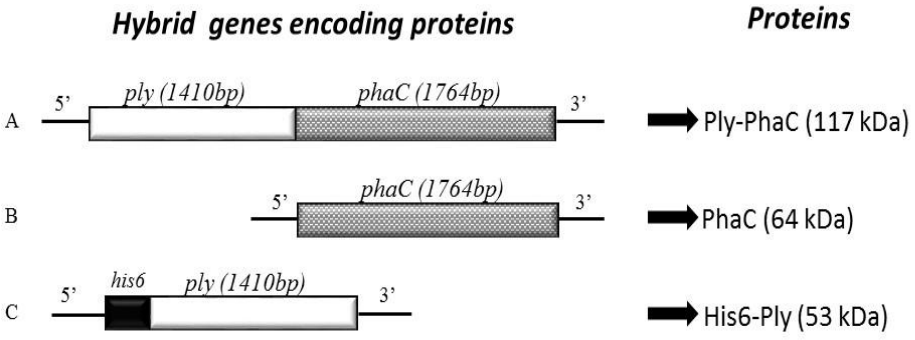


Figure 1. Schematic representation of genes encoding proteins relevant to this study. A, the hybrid gene encoding Ply-PhaC fusion protein mediating PHB bead formation; B, the gene encoding PhaC mediating non-antigen displaying PHB bead formation; C, the gene encoding a soluble His-tagged Ply.

Table 1. Characteristics of plasmids and oligonucleotides used in this study.

Plasmid and primers	Characteristics	Reference
Plasmids		
pET-14b	Ap^R and T7 promoter	Novagen
pET-14b-phaC	pET-14b version, holding *phaC* gene fragment	(41)
pUC57-ply	pUC57 version, ColE1 origin, Ap^R holding *ply* gene.	GenScript
pMCS69	Cm^R; T7 promoter, pBBR1MCS derivative containing *phaA* and *phaB* genes from *Ralstonia eutropha* co-downstream to *lac* promoter	(42)
pET14b_NanA_PhaC (reversed)	Ap^R and T7 promoter, containing *nanA* gene cloned to 3' end of *phaC*	(23)
pET-14b-ply-phaC	pET-14b-phaC version, holding *ply* gene fused to 3' end of *phaC*	This study
pET14b- his6-ply	Ap^R and T7 promoter, containing the *his6-ply* gene inserted into the *Xba*I//*BamH*I sites of pET14b.	This study
Oligonucleotides		
Ply *Xba*I fwr	5'AAATCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATCATATGGCAAATAAAGCTGTTAATGATTTTATTCTTGCTATGAATTATGAT3'	This study
Ply *Spe*I rev	5'AAAACTAGTATCATTTTCTACTTTATCTTCTACTTGAGGATACAATGTTGTTCCCC3'	This study
Ply *Xba*I Hist-tag fwr	5'AAATCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATCATATGCATCATCATCATCATCATGCAAATAAAGCTGTTAATGATTTTATTCTTGCTATGAATTATGAT3'	This study
Ply *BamH*I rev	5'CCCGGATCCTCAATCATTTTCTACTTTATCTTCTACTTGAGGATACAATGTTGTTCCCC 3'	This study

To confirm the presence of PHB inclusions in *E. coli* harbouring pET14b-ply-phaC or pET14b-phaC, the cell was subjected to TEM. Additionally, in order to determine the shape and size of the PHB beads, purified bead samples were also analysed by TEM (Fig. 2).

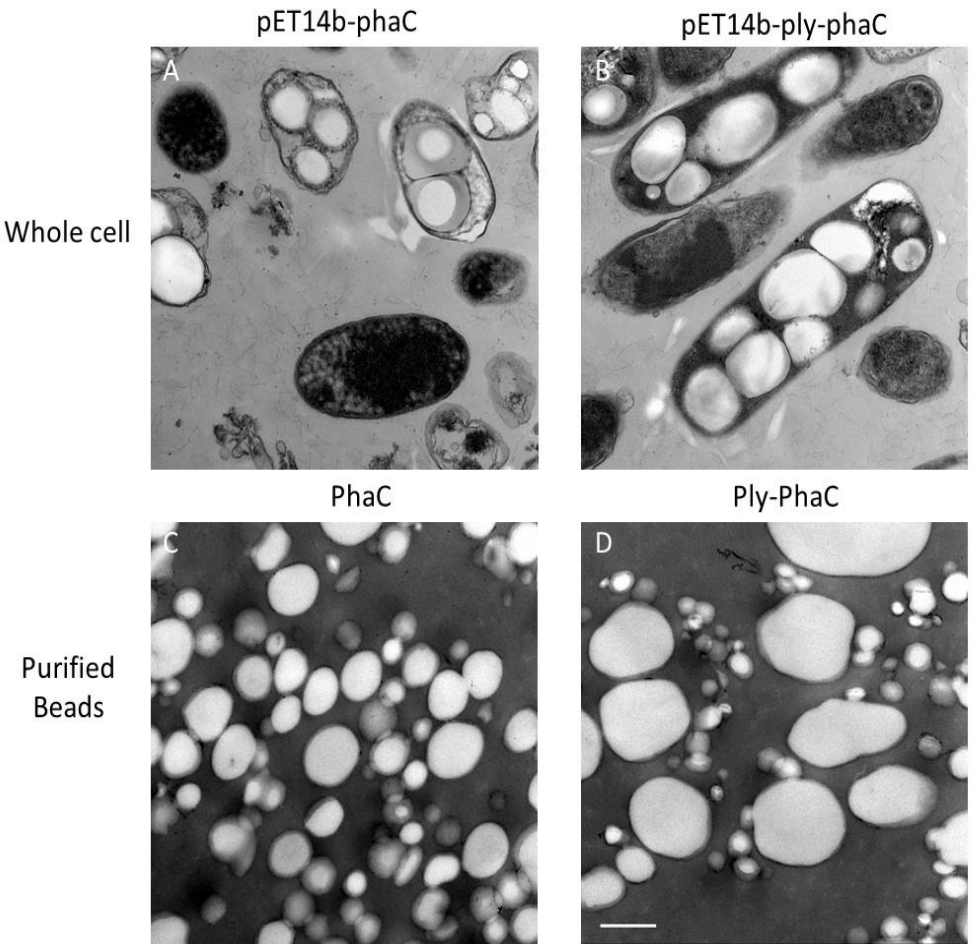


Figure 2. TEM images of *E. coli* with PHB inclusions and the corresponding purified PHB beads. A, cells harbouring pET14b-phaC (PhaC wild type, non-antigen displaying); B, cells harbouring pET14b-ply-phaC (Ply-PhaC bead); C, PhaC beads from cells harbouring pET14b-phaC; D, Ply-PhaC beads derived from cells harbouring pET14b-ply-phaC. The white bar represents 500 nm.

Figure 2A and 2B show that cells harboring either plasmid were able to produce PHB inclusions. However, after isolation and purification, beads from cells harboring pET14b-phaC appeared smaller (< 500 nm) and more homogeneously spherical than beads isolated from cells harboring pET14b-ply-phaC (Figure 2C and 2D).

Proteins associated with PHB beads as well as His6-Ply were identified by SDS-PAGE, tryptic peptide fingerprinting and immunoblotting (Fig. 3, Table 2).

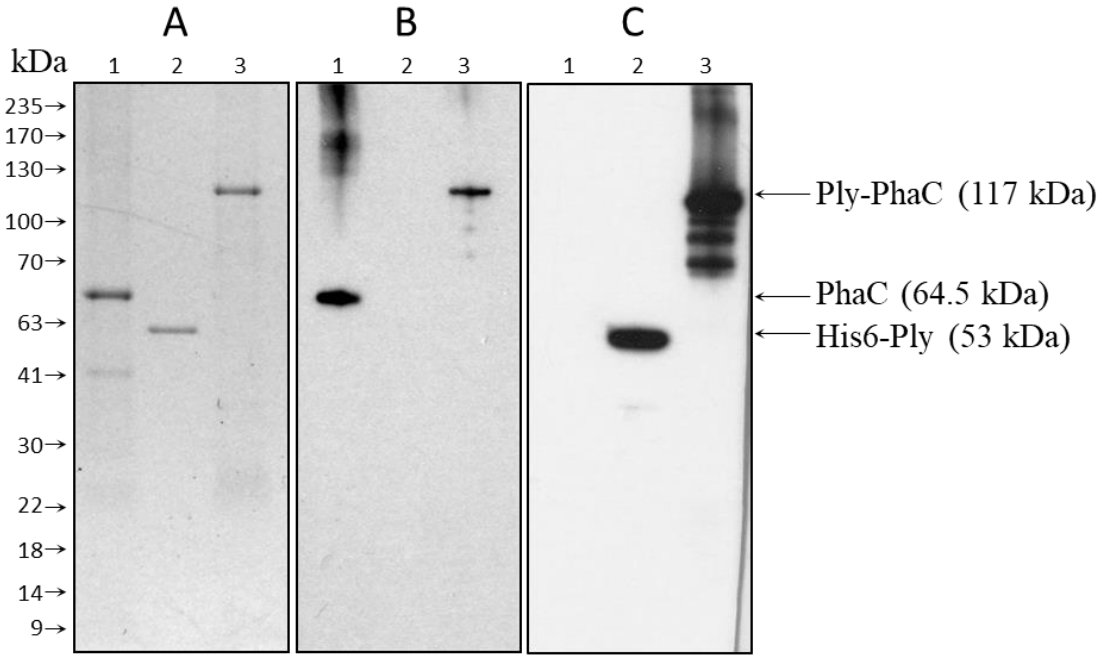


Figure 3. SDS-PAGE and immunoblot analysis of proteins attached to PHB beads as well as purified His6-Ply. (A) Coomassie blue stained SDS-PAGE gel; (B) Immunoblot using polyclonal anti-PhaC antibodies; (C) Immunoblot using monoclonal anti-PLY antibodies (PLY-7). Molecular weight (MW) standard (GangNam-Stain, iNtRON BIOTECHNOLOGY); lane 1, PHB beads isolated from cells harbouring pET-14b-phaC encoding PhaC, non-antigen displaying (64.5 kDa); lane 2, purified His6-Ply (53 kDa) derived from cells harboring pET14b-his6-ply; lane 3, Ply-PhaC beads isolated from cells harboring pET-14b-ply-phaC encoding Ply-PhaC fusion protein (117 kDa). The arrows indicate the protein bands of interest with the corresponding theoretical MW in parentheses.

In the Figure 3A, dominant bands indicated the production of the proteins of interest, all corresponding with the expected theoretical molecular weight. The molecular identity of proteins was further confirmed by tryptic peptide mass spectrometry (Table 2). In addition, immunoblotting confirmed the molecular identity of Ply as part of the PhaC fusion protein as well as its soluble counterpart (Figure 3 B and C). Densitometry analysis showed a purity of His6-Ply of > 95%.

Table 2. Tryptic peptide fingerprinting analysis (Triple TOF)

Protein	Peptides fragment identified (Triple TOF)
Ply-PhaC fusion protein	**A1-K353, N359-K388, A395-R428, I434**-K782, G807-L817, E835-G858, N876-K884, H914-Q1006, R1031-R1054
His6-**Ply**	**A11-V50, V51-R100, A101-V150, P151-N200, F201-R250, D251-G300, D301-F350, Q351-P400, K401-D450, L451-D477**

* Red bold identifies peptides belonging to the respective pneumococcal antigen

To determine the amount of antigen per mg of wet Ply-PhaC beads as well as PhaC attached to wild type PHB beads for vaccination studies, another SDS-PAGE combined with densitometry analysis was conducted including BSA standards. The results are presented in Table 3.

Table 3. PHB beads yields and the antigen/ mg of wet beads ratio

Sample	Wet biomass (g)	Wet PHA bead mass (g)	Fraction of antigen in fusion protein (%)	Antigen /wet bead (µg/mg)
PhaC beads	6.39	0.748	0	1
Ply-PhaC beads	14	1.37	45	0.142

3.4.2 Immunological properties of the various PHB beads and soluble proteins

3.4.2.1 Safety of vaccine formulations

All animals included in the immunization schedule remained alive and healthy. Small lumps at the vaccination sites with no detectable suppuration were only evident in groups immunized with PHB bead containing vaccine formulations. Additionally, organs such as lungs, liver, and kidneys showed a normal shape in all mice.

3.4.2.2 Anti-Ply IgG titers and IgG subclass profile

The IgG titers against Ply were evaluated by ELISA. The IgG titers are shown in Figure 4.

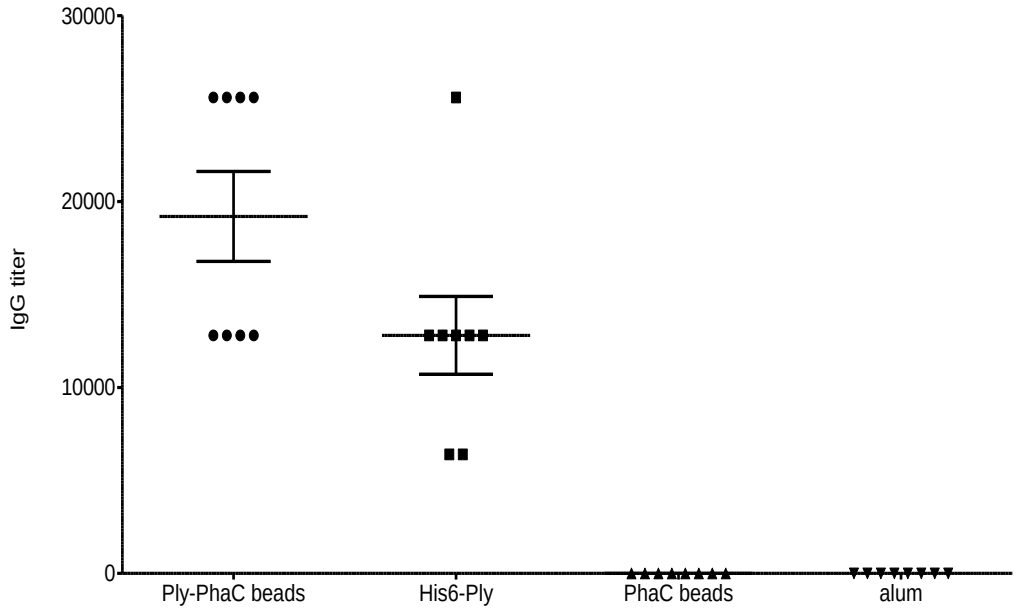


Figure 4. Analysis of induction of anti-Ply antibodies by various vaccine formulations. Black circles represent sera from mice vaccinated with 1.5 µg of Ply displayed on PHB beads; black squares represent sera from mice vaccinated with 1.5 µg of soluble His6-Ply; black upward triangles represent sera from mice vaccinated with 1.5 µg of PhaC on PHB beads (i.e. no antigen PHB bead, negative control); black downward triangles represent mice vaccinated with 15 µg of alum. All immunogens were isolated from an endotoxin-free mutant of *E. coli*. Data are shown as reciprocal antibody titers, representing the dilution required to obtain 50% of the maximal OD value at 450/570 nm. Statistical analysis showed the Ply-PhaC beads vs His6-Ply did not present statistical differences with p=0.067.

Animals from Ply-PhaC beads and His6-Ply immunization groups showed specific and high IgG titers against Ply, seven days after the last vaccination (Figure 4), while animals vaccinated with PhaC beads and alum were negative. The mean IgG titers in the Ply-PhaC beads group tended to be higher than in the His6-Ply group, but no statistical differences were found with p=0.067. Subclass IgG profiles are depicted in Figure 5. The IgG1 was the prevalent subclass in the Ply-PhaC beads and His6-Ply groups, however, the Ply-PhaC beads group presented superior titer in comparison

with the His6-Ply group with (*p<0.001*). Additionally, IgG2b titers were higher in the Ply- PhaC beads when compared to the His6-Ply group (*p<0.05*).

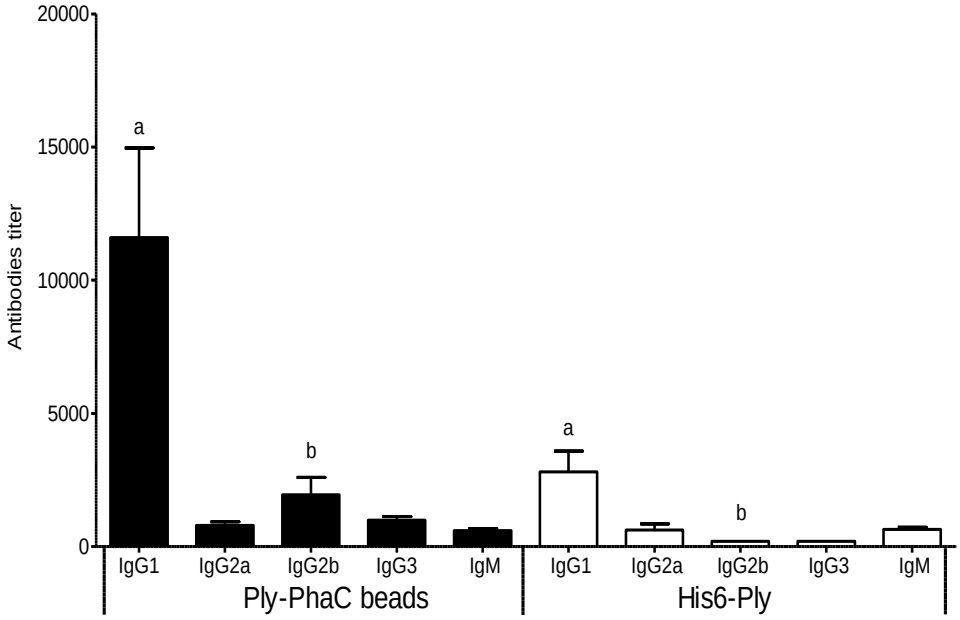


Figure 5. IgG subclass profile as assessed by ELISA. Plates were coated with 0.2 µg/mL of His6-Ply. Results are represented as the mean ± SEM of 8 animals. Statistical analysis: IgG1, a: Ply-PhaC beads group > His6-Ply group with *p<0.001*.While IgG2b, b: Ply-PhaC beads group > His6-Ply group with *p<0.05.*

3.4.2.3 Cellular immune responses

Splenocytes from the vaccinated animals were stimulated with His6-Ply, results are presented in (Figure 6).

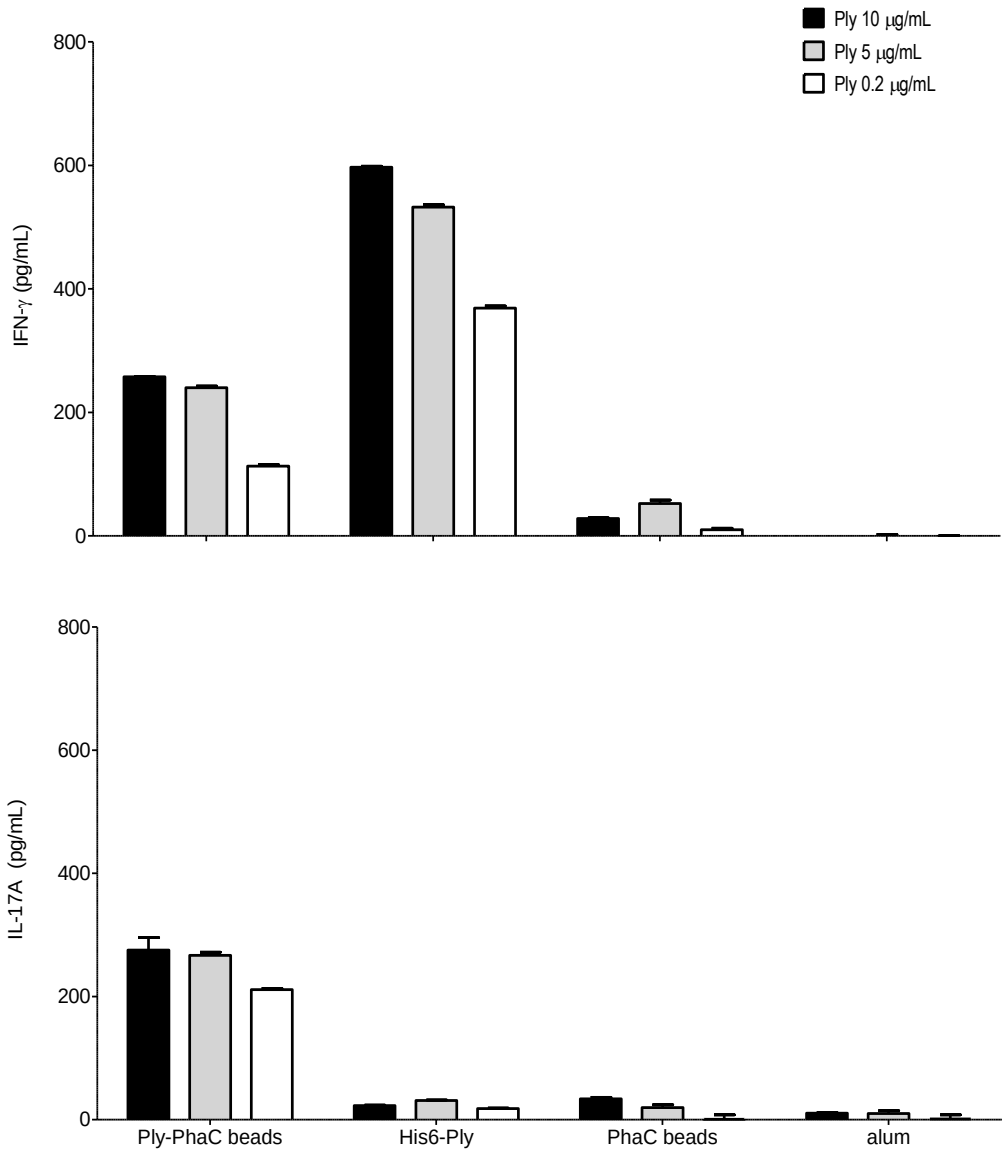


Figure 6. Cytokine profiles induced by various vaccine formulations. Splenocytes were stimulated with His6-Ply and the supernatant of splenocyte cultures was evaluated 72 h after stimulation. Splenocytes without stimulant were considered as negative controls while cells activated with ConA were considered as positive control. Results are presented as the mean ± SD of two replicates per group.

The results showed that splenocytes from animals vaccinated with Ply-PhaC beads produced high levels of IL-17A and IFN-γ. However, animals vaccinated with soluble His6-Ply produced high levels of IFN-γ, but only low levels of IL-17A were observed (Fig. 6). IL-4 was not detectable in any of the activated splenocyte cultures,

(data not shown). Additionally, animals vaccinated with PhaC beads and alum showed the lowest levels of both cytokines and were similar to the negative control.

3.4.2.4 Cross-reactivity of induced antibodies with Ply from various *S. pneumoniae* serotypes

To evaluate the binding specificity of antibodies of sera from animals vaccinated with Ply-PhaC beads and soluble His6-Ply to Ply from various *S. pneumoniae* serotypes an immunoblot was performed (Fig. 7). The results showed that in both cases, sera recognised predominantly a protein that coincided with the theoretical molecular weight of Ply. However, sera from Ply-PhaC required 3.5-fold greater dilution than sera from His6-Ply suggesting that vaccination with Ply displayed on PHB beads enhanced immunogenicity toward increased induction of anti-Ply antibodies when compared with soluble Ply.

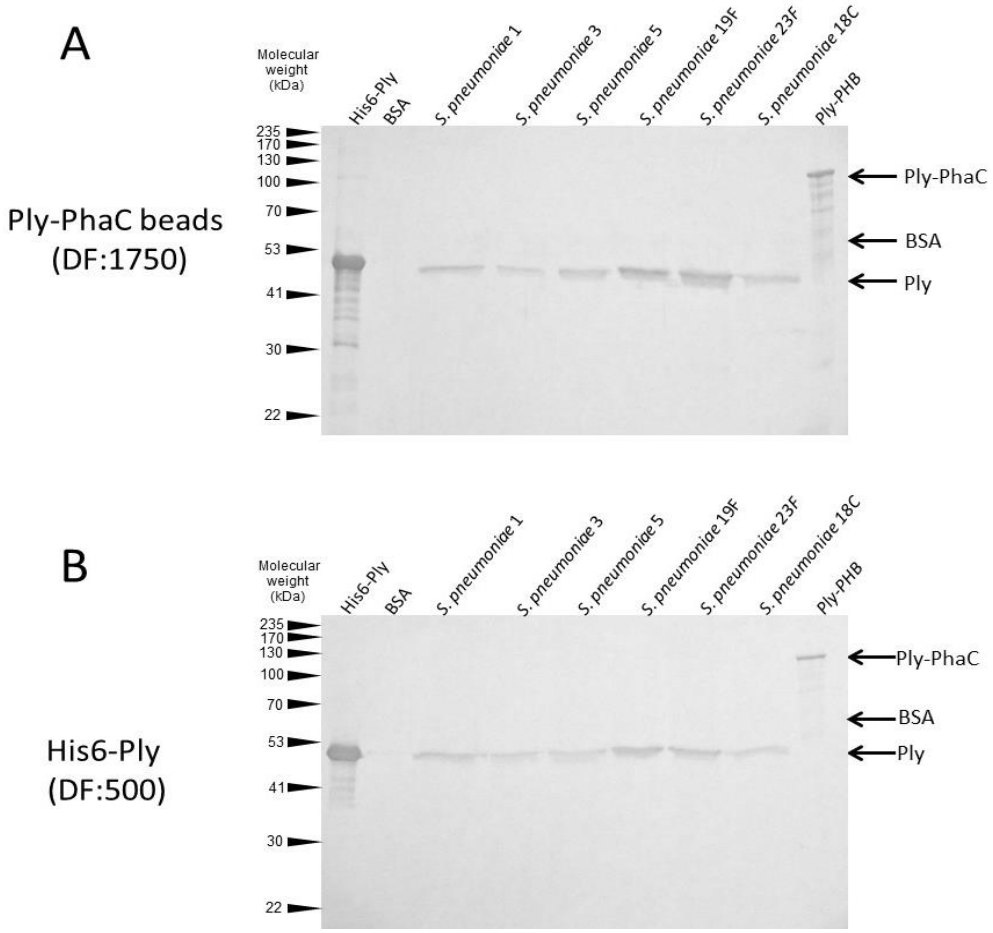


Figure 7. Cross-reactivity of anti-Ply antibodies with Ply from various serotypes of *S. pneumoniae*. A, Immunoblots using sera (diluted 1: 1750) from mice vaccinated with Ply-PhaC beads; B, immunoblot using sera (diluted 1: 500) from mice vaccinated with soluble His6-Ply. The corresponding SDS-PAGE of whole cell lysates of the various *S. pneumoniae* serotypes is provided in Supplemental Material (Supplementary Fig. 1).

3.4.2.5 Chemical conjugation of CPS from *S. pneumoniae* serotype 19F to PHB beads

Prior to conjugation, proteins from PhaC wild-type PHB beads and TT were activated with the maleimide group specifically reacting with sulfhydryl groups resulting in yields of activation in accordance with previously described results (32). The conjugation strategy and the final beads are shown in Figure 8. After conjugation

reactions, the 19F-PhaC bead conjugate was washed 5 times to remove the unbound CPS. Then, the 19F polysaccharide and protein content in the final conjugate was determined as described in Materials and Methods. The results showed that 14% of 19F polysaccharide and 75% of protein were retained after conjugation. The final ratio mg polysaccharide: mg protein was 1: 2.7.

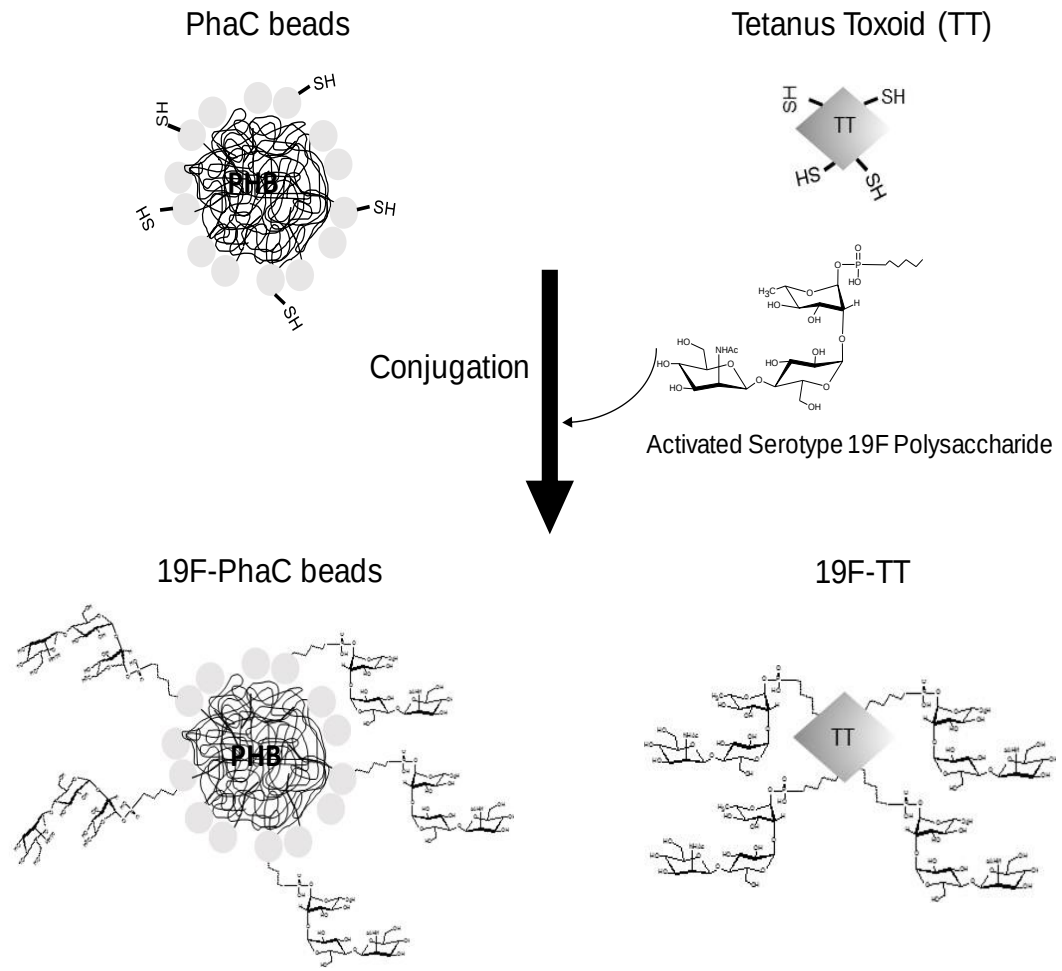


Figure 8. Schematic representation of conjugation reaction between activated serotype 19F polysaccharide and PhaC on PHB beads or soluble TT. The repetitive unit of the 19F polysaccharide is a trisaccharide of (→4)-β -D-ManNAc-(1→4)-α-D-Glcp-(1→2)-α-L-Rhap-1-PO4-) (43-45).

3.4.2.6 Anti-19F IgG titer evaluation

The IgG titers against CPS from *S. pneumoniae* serotype 19F were evaluated by ELISA. Animals vaccinated with 19F polysaccharide conjugated to PHB beads or

TT were positive 7 days after the last vaccination dose, while mice vaccinated with PhaC beads and alum were negative. The mean IgG titer from group 19F-PhaC beads (2) was statistically superior to group 19F-PhaC beads (1) and 19F-TT with $p<0.05$ and $p<0.01$, respectively (Fig. 9).

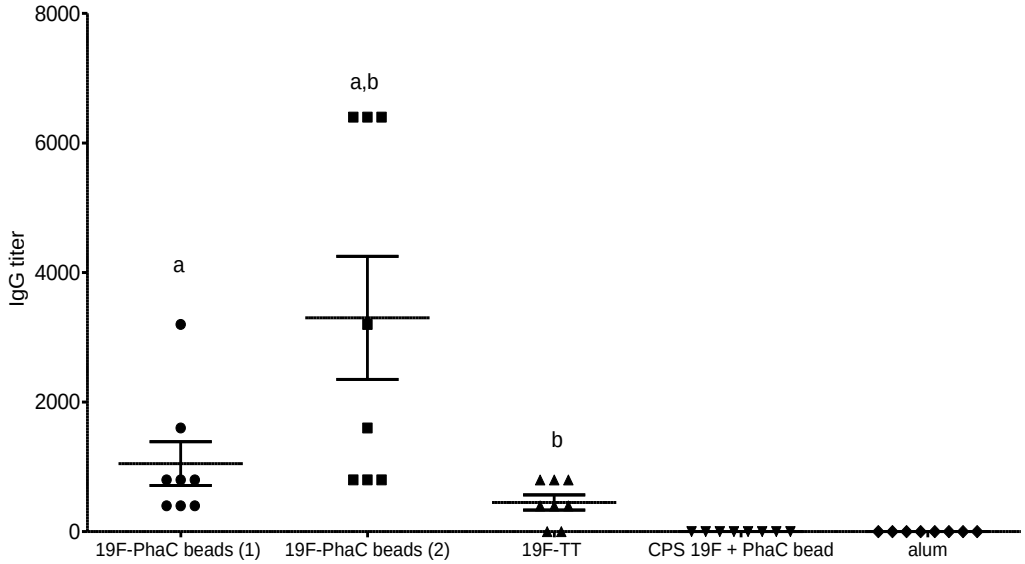


Figure 9. Analysis of anti-19F CPS antibody titers. Black circles, group vaccinated with 2 µg of 19F polysaccharide conjugated to PhaC on PHB beads; black squares, group vaccinated with 10 µg of 19F polysaccharide conjugated to PhaC on PHB beads; black upward triangles, group vaccinated with 2 µg of 19F polysaccharide conjugated to TT; black downward triangles, group vaccinated with a mix of 19F CPS (10 µg) + PhaC (30 µg) and black diamonds, group vaccinated with 0.125 µg of alum. Data are shown as reciprocal antibody titers, representing the dilution required to obtain 50% of the maximal OD value at 450/570 nm. Statistical analysis: a, group 19F-PhaC beads (2) > group 19F-PhaC beads (1) with $p<0.05$, b, group19F-PhaC beads (2) > 19F-TT with $p<0.01$.

3.4.2.7 Functionality of induced antibodies assessed by opsonophagocytosis of *S. pneumoniae* serotype 19F

To determine the functionality of the antibodies with respect to their contribution to protective immunity, an opsonophagocytic activity assay was performed (39). Animals vaccinated with both doses of 19F conjugated to PhaC

showed OPA titers greater than 512 which correlated with protective immunity. Additionally, statistical differences were found between animals vaccinated with 19F-PhaC beads and 19F-TT with $p<0.001$ (Fig. 10).

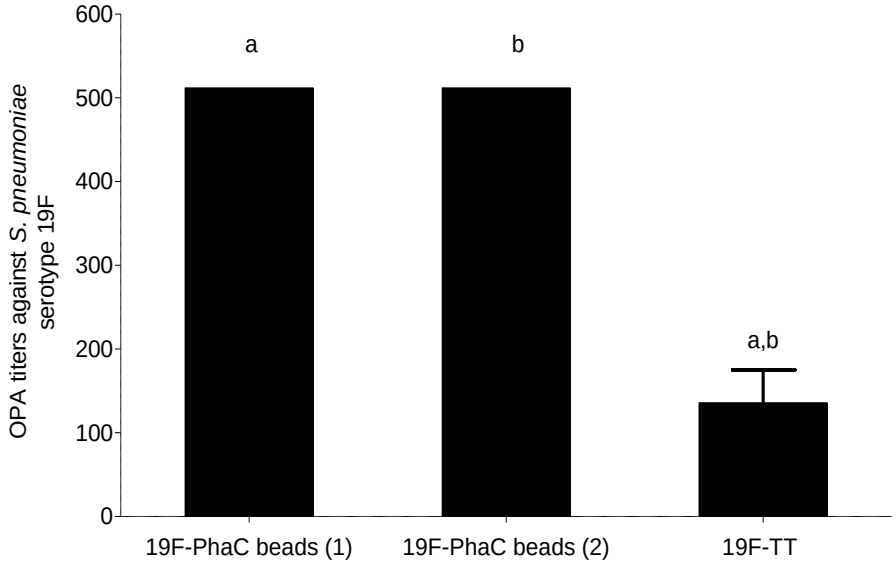


Figure. 10 The opsonophagocytic activity of sera against *S. pneumoniae* serotype 19F. 19F-PhaC beads (1), group vaccinated with 2 µg of 19F polysaccharide conjugated to PhaC wild type beads; 19F-PhaC beads (2), group vaccinated with 10 µg of 19F polysaccharide conjugated to PhaC wild type beads; 19F-TT, group vaccinated with 2 µg of 19F polysaccharide conjugated to TT. Statistical analysis: a and b, group 19F-PhaC beads (1 and 2) > 19F-TT with $p<0.001$.

3.5 Discussion

S. pneumoniae is an opportunistic human pathogen secreting virulence factors such as various CPS and proteins. More than 91 serotypes exist but only 30 are associated with the disease. CPS - protein conjugate vaccines including CPS from up to 13 serotypes are able to elicit serotype dependent protective and long-term immunity in infants. The serotype 19F is present in all of the commercial vaccines due to its epidemiological relevance. However, the serotype dependent protection

limits its use against emerging virulent serotypes for which the CPS is not included in current vaccine formulations. Alternatively, a vaccine formulation based on conserved proteins, like Ply, could provide an alternative approach to induce broadly protective (serotype-independent) immune responses overcoming limitations of existing conjugate vaccines.

Non-toxic Ply variants as part of next generation pneumococcal vaccines induced protective humoral and cellular immune responses in mice and humans (2, 46-48). However, high amounts of Ply combined with adjuvants were required to achieve this protection. Here, we explored the design of PHB beads as a potential Ply delivery system and its capacity to improve the immunogenicity of this protein.

Previous studies showed that proteinaceous antigens from intracellular pathogens like *Mycobacterium tuberculosis* and Hepatitis C virus could be displayed on PHB beads inducing protective Th1/Th2 immune responses (18, 19, 49). Particulate vaccines are inherently immunogenic often inducing both Th1 and Th2 immune responses mediating protection against pathogens (50). Hence display of Ply and the CPS 19F on PHB beads were conceived to develop a particulate vaccine for the prevention of pneumococcal infections.

Here, we engineered an endotoxin-free mutant of *E. coli* to produce Ply displaying PHB beads (Figs. 1 and 2) (51). It is not understood why Ply-PhaC fusion proteins mediated the formation of larger granules (up to 1µm) when compared with only PhaC (Fig. 2). However, these results suggested that Ply fused to the *N*-terminus of PhaC did not impact on PHB synthase activity but might have had an effect on the self-assembly process. It has been shown that vaccine particle size has an impact on uptake by antigen presenting cells (APC), as well as the subsequent antigen processing, often resulting in humoral or cellular immune responses (52, 53).

Polystyrene spheres with diameters of 0.1-1 µm were preferentially taken up by dendritic cells (DCs). In this case, spheres of 1 µm exhibiting dense positive charges were better taken up by DCs when compared with spheres showing negative surface charges. However, the uptake of spheres with a diameter of about 0.1 µm by DCs was independent of the surface charge (54). As a prerequisite for vaccine development, the production of stable Ply and its fusion protein was confirmed (Figure 3 B and C; Table 2).

To study the immunological properties of Ply-PhaC beads, mice were immunized. IgG titer evaluation showed that animals vaccinated with Ply displayed on PHB beads produced greater anti-Ply titers in comparison with the soluble protein suggesting that PHB beads enhanced immunogenicity (Fig. 4). In patients with pneumococcal bacteremia, anti-Ply-antibodies were present in about 60-80 % of the adults suggesting the importance of the Ply-specific humoral immune responses for clearance of this pathogen from the human body (55, 56). Previous vaccinations with detoxified Ply (PlyD1, double mutations, T65C, G293C) elicited high IgG titers using a vaccination dose between 0.25-1 µg of protein/animal combined with adjuvants. These antibodies inhibited hemolytic activity and reduced lung damage in mice, however, the doses mentioned above did not induce protective immunity in challenge experiments (28). In our study, IgG1 was the predominant subclass induced by Ply, which resembles the previous finding using the PdB (pneumolysoid, a mutation in W433F) protein (2, 57).

A combination of humoral and cellular immunity is necessary to clear *S. pneumoniae* in human patients (14, 58). *S. pneumoniae* mouse infection models have shown the relevance of IL-17A and IFN-ɣ secretion for the development of a protective immune response (59, 60). In the early infection state, the production of

IL-17 mediates the recruitment of macrophage and neutrophil precursors clearing bacteria from the nasopharyngeal tissue (61). In addition, several studies investigated the relevance of Ply in the cellular immunity against *S. pneumoniae*. A study which combined Ply with a TLRs like receptor agonist showed a correlation between splenocyte IL-17A, IFN-ɣ and IL-1β production and protection against infection in mice (62). Another study showed that secretion of IL-17A by γδ T lymphocytes mediated the differentiation of Th17 cells (48). Production of IFN-ɣ together with IL-18 and IL-12 were important to mediate a Th1 pattern differentiation. Here Ply displayed on PHB beads induced both IL-17A and IFN-ɣ production (Fig. 6). Hence immunization with Ply-PhaC beads might induce a cytokine profile which could promote cell-mediated protective immunity.

To demonstrate that Ply-PHB beads induced serotype-independent recognition by the sera from vaccinated animals, we performed an immunoblot with cell lysates of six *S. pneumoniae* serotypes.This showed that Ply from all serotypes was specifically recognised by all sera from Ply immunised animals (Fig. 7). Since Ply-PhaC beads induced greater titers in comparison with mice vaccinated with His6-Ply, PHB beads could be considered as particulate immunogenic antigen carrier.

As CPS, conjugated to immunogenic carrier proteins such as TT, had been previously successfully used in vaccine formulations against pneumococcal disease, they were also considered in this study. The CPS are poorly immunogenic by themselves and hence require conjugation to an immunogenic carrier protein such as TT. Here we replaced the immunogenic carrier protein by PHB beads. This was achieved by chemical conjugation of CPS serotype 19F to purified PHB beads (Fig. 8) (63). The CPS conjugation recovery was about 14%, which was less than

previously described (32). This could be due to fewer conjugation sites being accessible at the surface of the PHB beads.

In the case of the immune response against *S. pneumoniae* serotype 19F, the results showed that conjugation of CPS 19F to PHB beads induced specific and greater IgG titers when compared with 19F conjugated to TT, which was used as positive control (Fig. 7). This suggested that PHB beads can also serve as an immunogenic carrier for the delivery of carbohydrate-based antigens. In addition, the levels of antibodies induced by 19F-PhaC beads remained high up to 6 months after the last vaccination (data not shown), suggesting 19F-PhaC beads induced a strong humoral immune response including memory B cells. It is known that antibodies against CPS play a key role in protective immunity against extracellular pathogens (64). Phagocytosis by macrophages mediated by opsonic antibodies is used as an immune protection correlate to validate new pneumococcal vaccines (65, 66). 19F-PhaC beads induced the highest levels of OPA regardless the CPS doses. This last results suggested that PHB beads are immunogenic antigen carrier systems capable of inducing protective immunity (Fig. 10). Here we demonstrated the versatility of PHB beads by not only assessing the delivery of protein antigens but also by including carbohydrate-based antigens. Overall, the efficient production of the PHB bead carrier; combined with the ease of attaching antigens of interest; plus their superior immunological properties suggests that PHB beads offer an attractive alternative to existing vaccine formulations.

3.6 Acknowledgements

The authors are also thankful to Dr Anja Werno (Canterbury Health Laboratories, NZ) for the provision of anti-Ply monoclonal antibody. The technical assistance by Maria Onelia González Socarras, Alex Quintero-Perez, Dr Yanely

Tirado, Dr Reinaldo Oliva Hernández, Tamara Hernández Salazar, Sandra Madariaga, Yanet Estrada, Maryle Garcia, Elizabeth González Aznar, Rocmira Perez Nicado, Danaydis Fonseca, Abel Roscoe Fajardo Sanchez and Natalie Burr was also appreciated. We would also like to acknowledge the Manawatu Microscopy and Imaging Centre for assistance with electron microscopy analysis.

3.7 References

1. O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, et al. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. Lancet. 2009;374(9693):893-902.

2. Palaniappan R, Singh S, Singh UP, Sakthivel SKK, Ades EW, Briles DE, et al. Differential PsaA-, PspA-, PspC-, and PdB-specific immune responses in a mouse model of pneumococcal carriage. Infection and immunity. 2005;73(2):1006-13.

3. Lee LH, Gu XX, Nahm MH. Towards New Broader Spectrum Pneumococcal Vaccines: The Future of Pneumococcal Disease Prevention. Vaccines (Basel). 2014;2(1):112-28.

4. van der Poll T, Opal SM. Pathogenesis, treatment, and prevention of pneumococcal pneumonia. Lancet. 2009;374(9700):1543-56.

5. Black S, Shinefield H, Fireman B, Lewis E, Ray P, Hansen JR, et al. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. The Pediatric infectious disease journal. 2000;19(3):187-95.

6. O'Brien KL, Swift AJ, Winkelstein JA, Santosham M, Stover B, Luddy R, et al. Safety and immunogenicity of heptavalent pneumococcal vaccine conjugated to CRM197 among infants with sickle cell disease. Pediatrics. 2000;106(5):965-72.

7. Darrieux M, Goulart C, Briles D, Leite LC. Current status and perspectives on protein-based pneumococcal vaccines. Critical and Reviews in Microbioly. 2015;41(2):190-200.

8. Gor DO, Ding X, Briles DE, Jacobs MR, Greenspan NS. Relationship between surface accessibility for PpmA, PsaA, and PspA and antibody-mediated immunity to systemic infection by *Streptococcus pneumoniae*. Infection and Immunity. 2005;73(3):1304-12.

9. Nabors GS, Braun PA, Herrmann DJ, Heise ML, Pyle DJ, Gravenstein S, et al. Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies to heterologous PspA molecules. Vaccine. 2000;18(17):1743-54.

10. Paton JC, Andrew PW, Boulnois GJ, Mitchell TJ. Molecular analysis of the pathogenicity of *Streptococcus pneumoniae*: the role of pneumococcal proteins. Annual Reviews in Microbiology. 1993;47(1):89-115.

11. Kadioglu A, Taylor S, Iannelli F, Pozzi G, Mitchell TJ, Andrew PW. Upper and lower respiratory tract infection by *Streptococcus pneumoniae* is affected by pneumolysin deficiency and differences in capsule type. Infection and immunity. 2002;70(6):2886-90.

12. Rubins JB, Charboneau D, Paton JC, Mitchell TJ, Andrew PW, Janoff EN. Dual function of pneumolysin in the early pathogenesis of murine pneumococcal pneumonia. Journal of Clinical Investigation. 1995;95(1):142.

13. Heuck AP, Tweten RK, Johnson AE. Assembly and topography of the prepore complex in cholesterol-dependent cytolysins. Journal of Biological Chemistry. 2003;278(33):31218-25.

14. Kadioglu A, Gingles NA, Grattan K, Kerr A, Mitchell TJ, Andrew PW. Host cellular immune response to pneumococcal lung infection in mice. Infection and immunity. 2000;68(2):492-501.

15. Boulnois G, Paton J, Mitchell T, Andrew P. Structure and function of pneumolysin, the multifunctional, thiol-activated toxin of *Streptococcus pneumoniae*. Molecular microbiology. 1991;5(11):2611-6.

16. González-Miro M, Rodríguez-Noda L, Fariñas-Medina M, García-Rivera D, Vérez-Bencomo V, Rehm BH. Self-assembled particulate PsaA as a vaccine against Streptococcus pneumoniae infection. Heliyon. 2017;3(4):e00291.

17. Parlane NA, Gupta SK, Rubio Reyes P, Chen S, Gonzalez Miro M, Wedlock DN, et al. Self-assembled protein-coated polyhydroxyalkanoate beads: properties and biomedical applications. ACS Biomaterials Science & Engineering. 2016.

18. Parlane NA, Rehm BH, Wedlock DN, Buddle BM. Novel particulate vaccines utilizing polyester nanoparticles (bio-beads) for protection against *Mycobacterium bovis* infection- a review. Veterinary Immunoly and Immunopatholy. 2014;158(1-2):8-13.

19. Martinez-Donato G, Piniella B, Aguilar D, Olivera S, Perez A, Castanedo Y, et al. Protective T Cell and Antibody Immune Responses against Hepatitis C Virus Achieved Using a Biopolyester-Bead-Based Vaccine Delivery System. Clinical and Vaccine Immunoly. 2016;23(4):370-8.

20. Lee SY, Lee KM, Chan HN, Steinbuchel A. Comparison of recombinant *Escherichia coli* strains for synthesis and accumulation of poly-(3-hydroxybutyric acid) and morphological changes. Biotechnoly and Bioengeering. 1994;44(11):1337-47.

21. Rehm BH. Biogenesis of microbial polyhydroxyalkanoate granules: a platform technology for the production of tailor-made bioparticles. Current Issues in Molecular Biology. 2007;9(1):41-62.

22. Draper JL, Rehm BH. Engineering bacteria to manufacture functionalized polyester beads. Bioengineered. 2012;3(4):203-8.

23. Hooks DO, Blatchford PA, Rehm BH. Bioengineering of bacterial polymer inclusions catalyzing the synthesis of N-acetylneuraminic acid. Applied and Environmental Microbioly. 2013;79(9):3116-21.

24. Parlane NA, Grage K, Mifune J, Basaraba RJ, Wedlock DN, Rehm BH, et al. Vaccines displaying mycobacterial proteins on biopolyester beads stimulate cellular immunity and induce protection against tuberculosis. Clinicla and Vaccine Immunoly. 2012;19(1):37-44.

25. Parlane NA, Wedlock DN, Buddle BM, Rehm BH. Bacterial polyester inclusions engineered to display vaccine candidate antigens for use as a novel class of safe and efficient vaccine delivery agents. Applied and Environmental Microbioly. 2009;75(24):7739-44.

26. Rehm BH. Bacterial polymers: biosynthesis, modifications and applications. Nature Reviews Microbioly. 2010;8(8):578-92.

27. Grage K, Rehm BH. In vivo production of scFv-displaying biopolymer beads using a self-assembly-promoting fusion partner. Bioconjugation Chemistry. 2008;19(1):254-62.

28. Salha D, Szeto J, Myers L, Claus C, Sheung A, Tang M, et al. Neutralizing antibodies elicited by a novel detoxified pneumolysin derivative, PlyD1, provide protection against both pneumococcal infection and lung injury. Infection and immunity. 2012;80(6):2212-20.

29. Oloo EO, Yethon JA, Ochs MM, Carpick B, Oomen R. Structure-guided antigen engineering yields pneumolysin mutants suitable for vaccination against pneumococcal disease. Journal of Biological Chemistry. 2011;286(14):12133-40.

30. Thompson T, Rehm BH, Herbert AB, Saravolac EG. Compositions for separation methods. Google Patents; 2011.

31. Thompson T, Rehm B, Herbert A, Saravolac E, Mcdermott P, Draper J. Compositions for separation methods. Google Patents; 2013.

32. Fernández-Santana V, Cardoso F, Rodriguez A, Carmenate T, Peña L, Valdés Y, et al. Antigenicity and immunogenicity of a synthetic oligosaccharide-protein

conjugate vaccine against *Haemophilus influenzae* type b. Infection and immunity. 2004;72(12):7115-23.

33. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. TheJournal and biological Chemistry. 1951;193(1):265-75.

34. DuBois M, Gilles KA, Hamilton JK, Rebers Pt, Smith F. Colorimetric method for determination of sugars and related substances. Analytical chemistry. 1956;28(3):350-6.

35. Larentis AL, Argondizzo AP, Esteves Gdos S, Jessouron E, Galler R, Medeiros MA. Cloning and optimization of induction conditions for mature PsaA (pneumococcal surface adhesin A) expression in *Escherichia coli* and recombinant protein stability during long-term storage. Protein Expression and Purification. 2011;78(1):38-47.

36. Sambrook J, Fritsch E, Maniatis T. Molecular Cloning: A Laboratory Reference Manual. Cold Spring Harbor Lab. Press, Plainview, NY; 1989.

37. de los Toyos JR, Méndez FJ, Aparicio JF, Vazquez F, Suárez MdMG, Fleites A, et al. Functional analysis of pneumolysin by use of monoclonal antibodies. Infection and immunity. 1996;64(2):480-4.

38. Werno AM. Development of improved diagnostic testing methods for invasive pneumococcal disease: University of Otago; 2013.

39. Romero-Steiner S, Libutti D, Pais LB, Dykes J, Anderson P, Whitin JC, et al. Standardization of an opsonophagocytic assay for the measurement of functional antibody activity against *Streptococcus pneumoniae* using differentiated HL-60 cells. Clinical and diagnostic laboratory immunology. 1997;4(4):415-22.

40. Boulnois GJ. Pneumococcal proteins and the pathogenesis of disease caused by *Streptococcus pneumoniae*. Microbiology. 1992;138(2):249-59.

41. Peters V, Rehm BH. In vivo monitoring of PHA granule formation using GFP-labeled PHA synthases. FEMS microbiology letters. 2005;248(1):93-100.

42. Amara AA, Rehm BH. Replacement of the catalytic nucleophile cysteine-296 by serine in class II polyhydroxyalkanoate synthase from *Pseudomonas aeruginosa*-mediated synthesis of a new polyester: identification of catalytic residues. Biochemical Journal. 2003;374(Pt 2):413-21.

43. Miyazaki T, Yadomae T. Polysaccharides of type XIX Pneumococcus: Part II. the type specific polysaccharide and its chemical behaviour. Carbohydrate Research. 1971;16(1):153-9.

44. Yadomae T, Ohno N, Miyazaki T. On the phosphate linkages and the structure of a disaccharide unit of the type-specific polysaccharide of Pneumococcus type XIX. Carbohydrate research. 1979;75:191-8.

45. Jennings HJ, Rosell K-G, Carlo DJ. Structural determination of the capsular polysaccharide of *Streptococcus pneumoniae* type-19 (19F). Canadian Journal of Chemistry. 1980;58(11):1069-74.

46. Virolainen A, Jero J, Kayhty H, Karma P, Eskola J, Leinonen M. Nasopharyngeal antibodies to pneumococcal pneumolysin in children with acute otitis media. International Journal of Pediatric Otorhinolaryngology. 1996;1(36):83-4.

47. Virolainen A, Jero J, Käyhty H, Karma P, Eskola J, Leinonen M. Nasopharyngeal antibodies to pneumococcal pneumolysin in children with acute otitis media. Clinical and diagnostic laboratory immunology. 1995;2(6):704-7.

48. McNeela EA, Burke Á, Neill DR, Baxter C, Fernandes VE, Ferreira D, et al. Pneumolysin activates the NLRP3 inflammasome and promotes proinflammatory cytokines independently of TLR4. PLoS Pathog. 2010;6(11):e1001191.

49. Grage K, Jahns AC, Parlane N, Palanisamy R, Rasiah IA, Atwood JA, et al. Bacterial polyhydroxyalkanoate granules: biogenesis, structure, and potential use as nano-/micro-beads in biotechnological and biomedical applications. Biomacromolecules. 2009;10(4):660-9.

50. Rehm BH. Bioengineering towards self-assembly of particulate vaccines. Current Opinion in Biotechnology. 2017;48:42-53.

51. Mamat U, Wilke K, Bramhill D, Schromm AB, Lindner B, Kohl TA, et al. Detoxifying *Escherichia coli* for endotoxin-free production of recombinant proteins. Microbial cell factories. 2015;14(1):57.

52. Fifis T, Gamvrellis A, Crimeen-Irwin B, Pietersz GA, Li J, Mottram PL, et al. Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. The Journal of Immunology. 2004;173(5):3148-54.

53. Kanchan V, Panda AK. Interactions of antigen-loaded polylactide particles with macrophages and their correlation with the immune response. Biomaterials. 2007;28(35):5344-57.

54. Foged C, Brodin B, Frokjaer S, Sundblad A. Particle size and surface charge affect particle uptake by human dendritic cells in an in vitro model. International journal of pharmaceutics. 2005;298(2):315-22.

55. Kanclerski K, Blomquist S, Granström M, Möllby R. Serum antibodies to pneumolysin in patients with pneumonia. Journal of clinical microbiology. 1988;26(1):96-100.

56. Leinonen M, Syrjälä H, Jalonen E, Kujala P, Herva E. Demonstration of pneumolysin antibodies in circulating immune complexes-a new diagnostic method for pneumococcal pneumonia. Serodiagnosis and immunotherapy in infectious disease. 1990;4(6):451-8.

57. Ogunniyi AD, Grabowicz M, Briles DE, Cook J, Paton JC. Development of a vaccine against invasive pneumococcal disease based on combinations of virulence proteins of *Streptococcus pneumoniae*. Infection and immunity. 2007;75(1):350-7.

58. Kadioglu A, Coward W, Colston MJ, Hewitt CR, Andrew PW. CD4-T-lymphocyte interactions with pneumolysin and pneumococci suggest a crucial protective role in the host response to pneumococcal infection. Infection and immunity. 2004;72(5):2689-97.

59. Rubins JB, Pomeroy C. Role of gamma interferon in the pathogenesis of bacteremic pneumococcal pneumonia. Infection and immunity. 1997;65(7):2975-7.

60. Nakamatsu M, Yamamoto N, Hatta M, Nakasone C, Kinjo T, Miyagi K, et al. Role of interferon-γ in Vα14+ natural killer T cell-mediated host defense against *Streptococcus pneumoniae* infection in murine lungs. Microbes and infection. 2007;9(3):364-74.

61. Zhang Z, Clarke TB, Weiser JN. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. The Journal of clinical investigation. 2009;119(7):1899-909.

62. Zhu J, Paul WE. CD4 T cells: fates, functions, and faults. Blood. 2008;112(5):1557-69.

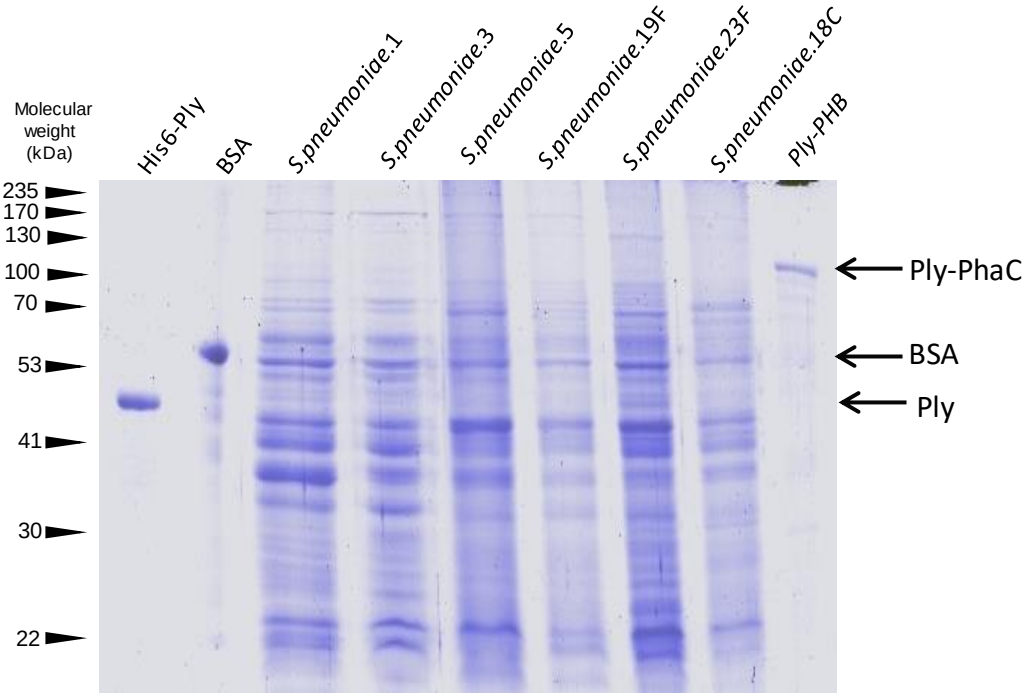
63. Jones C. Vaccines based on the cell surface carbohydrates of pathogenic bacteria. Anais da Academia Brasileira de Ciencias. 2005;77(2):293-324.

64. Plikaytis BD, Goldblatt D, Frasch CE, Blondeau C, Bybel MJ, Giebink GS, et al. An analytical model applied to a multicenter pneumococcal enzyme-linked immunosorbent assay study. Journal of clinical microbiology. 2000;38(6):2043-50.

65. Väkeväinen M, Jansen W, Saeland E, Jonsdottir I, Snippe H, Verheul A, et al. Are the opsonophagocytic activities of antibodies in infant sera measured by different pneumococcal phagocytosis assays comparable? Clinical and diagnostic laboratory immunology. 2001;8(2):363-9.

66. Organization WH. Recommendations for the production and control of pneumococcal conjugate vaccines. WHO technical report series. 2005;927:64-98.

3.8 Supplementary material



Supplementary Figure 1. SDS-PAGE of whole cell lysates of the various *S. pneumoniae* serotypes.

Preface to the next Chapter

Neisseria meningitidis is the major cause of meningitis, worldwide. Capsular polysaccharide-protein vaccines (conjugate vaccines) provide protection against these diseases but not protection against infections caused by serogroups not included in these vaccines such as serogroup B. To provide protection against serogroup B, protein-based sub-unit vaccines have been investigated extensively. However, sub-unit vaccines require a high adjuvant and booster dose to achieve the desirable protective immunity.

The efficacy of protein and carbohydrate antigens as vaccines can be improved via particulate delivery strategies. Chapters 2 and 3 present the promising results of using PHB beads as a carrier/ delivery system approach to prevent pneumococcal diseases. This chapter extends this concept by displaying meningococcal proteinaceous and carbohydrate antigens on PHB beads.

Chapter 4. Bioengineered polyester beads co-displaying protein and carbohydrate-based antigens enhance protective efficacy against bacterial infection

Majela González-Miró[1,2], Laura M Rodríguez-Noda[1], Mildrey Fariñas-Medina[1], Barbara Cedré-Marrero[1], Sandra-Madariaga-Zarza[1], Caridad Zayas-Vignier[1], Mabel Hernández-Cedeño[1], Torsten Kleffmann[3], Dagmar García-Rivera[1], Vicente Vérez-Bencomo[1], Bernd H. A. Rehm[2, 4]

[1]Finlay Vaccine Institute. La Habana, Cuba; [2]Institute of Fundamental Sciences, Massey University, Palmerston North, New Zealand; [3]Biochemistry Department, University of Otago, New Zealand; [4]Centre for Cell Factories and Biopolymers, Griffith Institute for Drug Discovery, Griffith University, Nathan, Queensland, Australia

Published: ***Scientific Reports*** **Journal, 30 Jan 2018, Volume 8, Issue 1**

4.1 Abstract

The efficacy of protein and carbohydrate antigens as vaccines can be improved via particulate delivery strategies. Here, protein and carbohydrate antigens used in formulations of vaccines against *Neisseria menigitidis* were displayed on *in vivo* assembled polyester beads using a combined bioengineering and conjugation approach. An endotoxin-free mutant of *Escherichia coli* was engineered to produce translational fusions of antigens *Neisseria* adhesin A (NadA) and factor H binding protein (fHbp), to the polyhydroxybutyrate synthase (PhaC) in order to intracellularly assemble polyester beads displaying the respective antigens. These protein antigen-coated beads, once purified, induced strong and specific humoral immune responses in mice. NadA displayed on beads showed enhanced immunogenicity compared to soluble NadA. Both soluble and particulate NadA elicited functional antibodies with bactericidal activity associated with protective immunity. To expand the antigen repertoire and to design a more broadly protective vaccine, NadA-PhaC beads were additionally conjugated to the capsular polysaccharide from serogroup C. Assessment of conjugation sites suggested that the fusion of NadA to PhaC sterically hindered access to some lysine residues while making others more accessible. Accessible lysines were also suggested to be polyester bead surface exposed informing future protein engineering approaches. Co-delivery of surface displayed NadA and the capsular polysaccharide induced a strong and specific Th1/Th17 mediated immune response associated with functional bactericidal antibodies. Our findings provide the foundation for the design of multivalent antigen coated polyester beads as suitable carriers for protein and polysaccharide antigens in order to induce protective immunity.

Keywords: Particulate vaccine, capsular polysaccharides, antigen delivery, polyhydroxybutyrate, self-assembly, *Neisseria meningitidis*, *Neisseria* adhesin A (NadA); factor H binding protein (fHbp)

4.2 Introduction

There is a growing worldwide demand for efficient and broadly protective vaccines for the prevention of infectious diseases. Although safe and specific, subunit vaccines often lack immunogenicity and, due to the limited antigen repertoire, show the impaired capacity to induce broadly protective immunity (1). Here we explored a combined bioengineering and chemical conjugation strategy to produce self-assembled particulate vaccines co-displaying protein and carbohydrate antigens in order to overcome the weaknesses of subunit vaccines.

As an example, we chose to design a vaccine against *Neisseria meningitidis* which can cause severe meningitis often resulting in permanent disability in survivors of the disease. Broadly protective and cost-effectively produced vaccines are in demand. The WHO reported a global incidence of ~ 1.3 million cases per annum with a mortality of 5-15% and more than 25% of survivors suffering from permanent disabilities (2). The pathogenicity of this bacterium is based on virulence factors such as capsular polysaccharides (CPS), outer membrane proteins (adhesins and porins) and lipopolysaccharides. Additionally, pathogenicity is enhanced by evasion of the host immune defence mechanisms via antigen mimicry and sequestration of factor H, a regulator of complement activation (3). The CPS diversity results in 13 recognised serogroups. Six serogroups A, B, C, W135, Y and X were found to be virulent causing disease (4, 5). Because the rapid onset of the disease and multiple antibiotic resistances impair treatment outcomes, prevention by vaccination is the preferred strategy.

To obtain a good memory response in the target population, conjugation of different CPS to a carrier protein (conjugate vaccine) provided an efficient strategy to prevent the disease. Examples of these serotype dependent vaccines are Menactra® (Sanofi Pasteur), Menveo® (Novartis) and Nimenrix® (GSK) (6). However, serogroup B of *N. meningitidis*, which causes more than half of the cases of meningococcal disease, is not included in any conjugate vaccine as the CPS is similar to polysaccharides presented in human neurologic tissues (7). Hence subunit vaccines against *N. meningitidis* serogroup B such as VAMENGO-BC® are focused on surface proteins such as outer membrane proteins (OMPs, PorA and PorB) (8-10). The efficiency of these vaccines is strain specific, limiting their use in many countries (11). Reverse vaccinology approaches screening the entire genome sequence of *N. meningitidis* strain MC58 for immunodominant antigens accelerated the development of protein-based vaccines against this bacterium (12, 13). Relevant proteins such as *Neisseria* adhesin (NadA), factor H binding protein (fHbp), *Neisseria* heparin binding antigen (NHBA), Genome-derived antigen (GNA) 2091 and GNA 1030 were identified by this technique (14-17). The above-mentioned proteins are included in the recently licensed vaccines 4CMenB Bexsero® (Novartis, 2015) ® and Trumenba® (Pfizer, 2014) (18). Recombinant subunit vaccines often present limitations such as the inability to elicit broadly protective immune responses and the necessity of adjuvant in the vaccine formulation in order to boost immunogenicity (19). Particulate antigen delivery systems hold the promise to overcome these hurdles (20).

Polymer particles incorporating antigens as a delivery system were found to stabilise antigens, enhance uptake of antigen by antigen presenting cells (APCs) and provide an antigen depot effect for an extended display of antigens (21, 22). Naturally formed bacterial inclusions made of a polyhydroxybutyrate (PHB) core surrounded by

the PHB synthase (PhaC) have been considered for bioengineering of particulate vaccines. Translational fusions of protein antigens to PhaC enabled display of respective antigens on the surface of the PHB beads which elicited strong and specific immune responses (23-27). PHB beads were successfully bioengineered to display antigens from intracellular pathogens like *Mycobacterium tuberculosis* and the Hepatitis C virus. These particulate vaccine candidates elicited both Th1 and Th2 immune responses (24, 28, 29). The elicited immune responses mediated protective immunity against both diseases (24, 28). Antigen displaying PHB beads were also produced in other bacterial hosts such as *Mycobacterium smegmatis* and *Lactococcus lactis* (23, 30). In a recent study, the bacterial pathogens own polyhydroxyalkanoate inclusion assembly was engineered to produce antigen displaying particulate vaccines (31). The aim of this study was to design and produce a multivalent antigen delivery system co-displaying protein and carbohydrate-based antigens as safe vaccine formulations for induction of broadly protective immunity against infectious diseases such as caused by *N. meningitidis.*

4.3 Materials and Methods

4.3.1 Ethics statement

The experiments described in this study were approved by The Institutional Ethics Committee for the Care and Use of Laboratory Animals of Finlay Vaccine Institute, Havana, Cuba. The guidelines and regulations of this committee were written following the National Regulations about Principles of Good Practices of Non-Clinical Laboratory Sanitary and Environmental Safety (No. 39/04) and guidelines provided by the Canadian Council on Animal Care (32). The protocols were approved by this

ethics committee. All efforts were made to minimize animal suffering and to reduce the number of animals used.

4.3.2 Construction of plasmids mediating production of PHB beads

Genes encoding NadA, GNA2091-fHbp-G1 and fHbp-G1-G1 (14, 33-35) were synthesized by Genscript Corporation (USA) employing codon optimization for *E. coli* (12, 33, 35, 36). Hybrid genes encoding fusion proteins comprised of antigen and PhaC were generated as previously described (23). DNA sequencing confirmed construction of plasmids pET-14b-nadA-phaC, pET-14b-gna2091-fhbp-g1-phaC and pET-14b-phaC-fhbp-g1-g1 (Supplementary Table 1).

4.3.3 Construction of plasmids for production of soluble His-tagged proteins

Genes encoding NadA and GNA2091-fHbp-G1 were amplified from pUC57-nadA and pUC57-gna2091-fhbp-g1 by PCR using primers listed in Supplementary Table 1 (12, 15, 35). Six histidine residues were introduced at the C-terminus of each protein using PCR and respective oligonucleotides. Respective coding regions were cloned into pET-14b_NanA_PhaC replacing NanA_PhaC after hydrolysis with *Nde*I/*BamH*I restriction enzymes. The DNA sequence of new plasmids pET14b-nadA-his6 and pET14b-gna2091-fhbp-g1-his6 were confirmed by DNA sequencing.

4.3.4 Production, isolation and purification of PHB beads and soluble proteins

ClearColi harbouring pMCS69 was transformed with plasmids mediating PHB bead formation. Cells were cultivated (48h, 25^{o}C at 200rpm), harvested and subjected to mechanical cell disruption. Bead isolation and purification procedure were as previously described (37, 38). In the case of soluble proteins, *ClearColi* harbouring pET14b-nadA-his6 and pET14b-gna2091-fhbp-g1-his6 were cultivated (24h, 25^{0}C at

200rpm) and lysed by sonication. Proteins were purified using a Ni-NTA Fast Start Kit column (Qiagen, Germany)(39).

4.3.5 Conjugation of MenC polysaccharide (CPS) to carrier proteins

One mg of protein (PBS, pH 7.0) was conjugated to 5mg (APS) from *Neisseria meningitidis* serogroup C by reductive amination. $NaBH_3CN$ was added as a reducing agent (1/5 of the amount of carbohydrate). The reaction was stirred for 72 h at 37^{o}C. Reaction products were purified by successive washes with NaCl solution (0.9% w/v) until the absorbance in resorcinol assay (colourimetric assay for determination of sialic acid) was equal to the blank.

4.3.6 Transmission electron microscopy analysis (TEM)

E. coli (ClearColi) harbouring plasmid pET-14b-nadA-phaC, pET-14b-gna2091-fhbp-g1-phaC, pET-14b-phaC-fHbp-g1-g1 and pET-14b-phaC, as well as the respective purified PHB beads were analysed by TEM. Samples were prepared as described previously (40).

4.3.7 Measurement of PHB bead size distribution and surface charge

Size distribution of the particles and the zeta potential were measured using the Mastersizer 3000 particle sizer (Malvern Instruments, United Kingdom) and the Zetasizer Nano ZS (Malvern Instruments, United Kingdom), respectively. Samples were prepared as 0.1% (w/v) of the wet PHB beads in saline solution. The pH values were adjusted with HCl.

4.3.8 NMR spectroscopy.

^{1}H NMR spectra in deuterium oxide were recorded on a Bruker Avance 500 MHz spectrometer at 313 K using standard parameters. Spectra were referenced to the

residual HOD peak at 4.50 ppm. The results were interpreted as described elsewhere (41).

4.3.9 Protein analysis

NadA-PhaC, GNA2091-fHbp-G1-PhaC, fHbp-G1-G1-PhaC fusion proteins and PhaC wild type on PHB beads as well as NadA-His6 and GNA2091-fHbp-G1-His6 were analysed by SDS-PAGE (42). Immunoblotting was conducted as previously described (43). Anti-Meningococcal Factor H binding protein variant 1 (JAR 4) monoclonal antibody from NIBSC was used to characterize the molecular identity of fHbp in the fusion and soluble version of this protein. A monospecific polyclonal antibody was used to confirm the identity of the PhaC. All images were obtained using the GEL-DOC 2000 (Bio-Rad Laboratories, USA) and analysed using Image Lab Software (Version 3.0 build 11, Bio-Rad Laboratories, USA). Proteins were further identified using tryptic peptide analysis by matrix-assisted laser desorption ionisation time-of-flight/mass spectrometry (MALDI-TOF/MS). For conjugation site analysis tryptic and chymotryptic peptides were analysed by liquid chromatography-coupled LTQ-Orbitrap tandem mass spectrometry (LC-MS/MS).

4.3.10 Protein and carbohydrate quantification

Ratio ng of the fusion protein per mg of the wet bead was determined as mentioned above (44). The protein concentrations of NadA-His6 and GNA2091-fHbp-G1-His6 were determined by BCA colourimetric assay (Pierce™ BCA Protein Assay Kit) (45). Ratio mg of polysaccharide per mg of protein in the conjugate was determined by Lowry and the resorcinol colourimetric assay (46, 47).

4.3.11 Immunization schedule for proteinaceous antigens

Eight immunization groups each comprising 6 animals of 5-6 weeks old Balb/c mice were used in this study. Mice were purchased from CENPALAB (Centro Nacional para la Producción de Animales de Laboratorio, Havana, Cuba). Groups were organized as follows: Group NadA-PhaC received 2 µg of NadA from NadA-PhaC beads, Group GNA2091-fHbp-G1-PhaC received 7 µg of GNA2091-fHbp-G1 from GNA2091-fHbp-G1-PhaC beads, Group fHbp-G1-G1 received 1 µg of fHbp-G1-G1 from PhaC-fHbp-G1-G1 beads, Group NadA-His6 received 2 µg of soluble NadA-His6, Group GNA2091-fHbp-G1-His6 received 7 µg of soluble GNA2091-fHbp-G1-His6, Group PhaC received 7 µg of PhaC wild type beads, Group alum received 100 µg alum, Group NadA-PhaC + GNA2091-fHbp-G1-PhaC received 1 µg of NadA from NadA-PhaC beads mixed with 3.5 µg of GNA2091-fHbp-G1 from GNA2091-fHbp-G1-PhaC beads. All of the immunogens were mixed with alum (ALHYDROGEL, Brenntag Biosector, Denmark). The inoculation route was subcutaneous. Immunizations occurred at 0, 21 and 35 days. Blood samples were collected from the retro-orbital plexus at days 0, 7 (1D), 28 (2D) and 42 (3D) after vaccination.

4.3.12 Immunization schedule for conjugated vaccine prototypes

Eight immunization groups of 8 animals were prepared, from the same breeding time as previously mentioned. Groups were organized as follows: Group MenC-NadA-PhaC received 4 µg of MenC conjugated to NadA-PhaC beads, Group MenC-PhaC received 4 µg of MenC conjugated to PhaC beads, Group MenC-NadA-His6 received 4 µg of MenC conjugated to soluble NadA-His6, Group MenC-TD received 4 µg of MenC conjugated to DT, Group NadA-PhaC received 18 µg of protein from NadA-PhaC beads, Group PhaC received 14 µg of protein from PhaC beads, Group NadA-PhaC received 18 µg of protein from NadA-PhaC beads (without alum), Group alum

received just 125 µg alum (aluminium phosphate). Except in group NadA-PhaC beads (without alum), all vaccine formulations contained alum. Immunizations occurred at 0, 14 and 28 days and the blood samples were collected at days -4, 14 (1D), 28 (2D) and 35 (3D) after vaccination. Immunization and blood collection schedule was the same as mentioned above.

4.3.13 Assessment of anti-NadA and anti-fHbp antibody titers in mice

Anti-NadA and anti-GNA2091-fhbp-G1 IgG levels were measured using an indirect ELISA. Maxisorp 96-well plates (NUNC) were coated using NadA-His6 or GNA2091-fHbp-G1-His6 followed by incubation overnight at 4°C. The rest of the ELISA was performed as mentioned above (44). IgG titers were calculated as the reciprocal antibody titers, representing the Dilution Factor (DF) required to obtain half of the maximum level of absorbance, per animal. The results are expressed as the (mean ± SEM) of 6 or 8 animals.

4.3.14 Assessment of anti-MenC antibody titers in mice

Anti-MenC CPS IgG antibodies levels were measured using a standardized indirect ELISA with some modification (48). Briefly, Maxisorp 96-well plates (NUNC) were coated with a mixture of Men C Capsular polysaccharide and methylated human albumin (HSA) at final concentrations of 5 µg/ml each, followed by incubation overnight at 4°C. The rest of the ELISA was performed as mentioned above. The results are expressed as the (mean ± SEM) of 8 animals.

4.3.15 Analysis of the Ig subclass profile in sera

The Ig subclass profile against NadA, fHbp and Men C CPS was analysed by using an indirect ELISA as described above (44, 48). Plates were coated as previously described (4.3.14 and 15). To detect the various Ig subclass, plates were incubated with

the respective anti-IgG1, IgG2a, IgG2b, IgG3 and anti-IgM antibodies (derived from goat) (Sigma-Aldrich, St. Louis, MO) using a DF of 1: 2500. Plates were developed using the anti-goat IgG peroxidase conjugate and the respective substrate as described above. The results are expressed as (mean ± SD) of two replicates per subclass (see immunization schedule for proteinaceous antigens) or as (mean ± SEM) of 8 animals per subclass (see immunization schedule for conjugated vaccines).

4.3.16 Analysis of the cytokine production

Mice were euthanised and spleens were removed from each mouse 7 days after the last vaccination dose (see immunization schedule for conjugated vaccines), and cells were recovered by perfusion and pooled for each group. Erythrocytes were then removed by osmotic shock, using NaCl 0.2%. The cells were counted and the viability was tested by trypan blue exclusion staining. Spleen cells were adjusted to 4×10^6 cells/mL and were cultured in DMEM medium supplemented with 50 µg/mL of gentamycin, 2 mmol/L of L-glutamine, 1 mmol/L of sodium pyruvate, 15 mmol/L of HEPES and 10% of inactivated FCS (all from Sigma-Aldrich). Isolated spleen cells were stimulated in vitro with NadA-His6 (10 µg/mL) or Concanavalin A (positive control) for 72 h. The level of IFN-γ, IL4 and IL17A in the supernatant of the culture was measured by double sandwich capture ELISA using commercial monoclonal antibodies following manufacturer's instructions (Mabtech, Sweden). Results are expressed as (mean ± SD) of two replicas per immunization group.

4.3.17 Serum bactericidal assay

The bactericidal assays were performed following the protocol described by Borrow et al. (49). The targeted strain was *N. meningitidis* serogroup A (Strain Mk499/03), serogroup B (Strain CU385/83) and serogroup C (Strain C11) (50). Baby

rabbit complement source was used to perform the assay (51). SBA titers were expressed as mean ± SEM per group of the reciprocal value of the greatest sera dilution leading ≥ 50% of bacteria killing.

4.3.18 Statistical analysis

Graph Pad Prism 5.00 (San Diego, USA) software was used for statistical data analysis. Statistical differences between all groups were calculated by one-way analysis of variance (ANOVA) with Kruskal–Wallis non-parametric test. When significant differences were found, the Dunn's post-test was used considering significance at $p<0.05$. When the comparison was just between two groups the Mann-Whitney test was performed and statistical differences were considered significant at $p<0.05$.

4.4 Results

4.4.1 Bioengineering of *Escherichia coli* for production of antigen-displaying PHB inclusions and soluble antigens.

Five plasmids were constructed to either mediate formation of antigen displaying PHB beads or production of the respective soluble antigen (Fig. 1a).

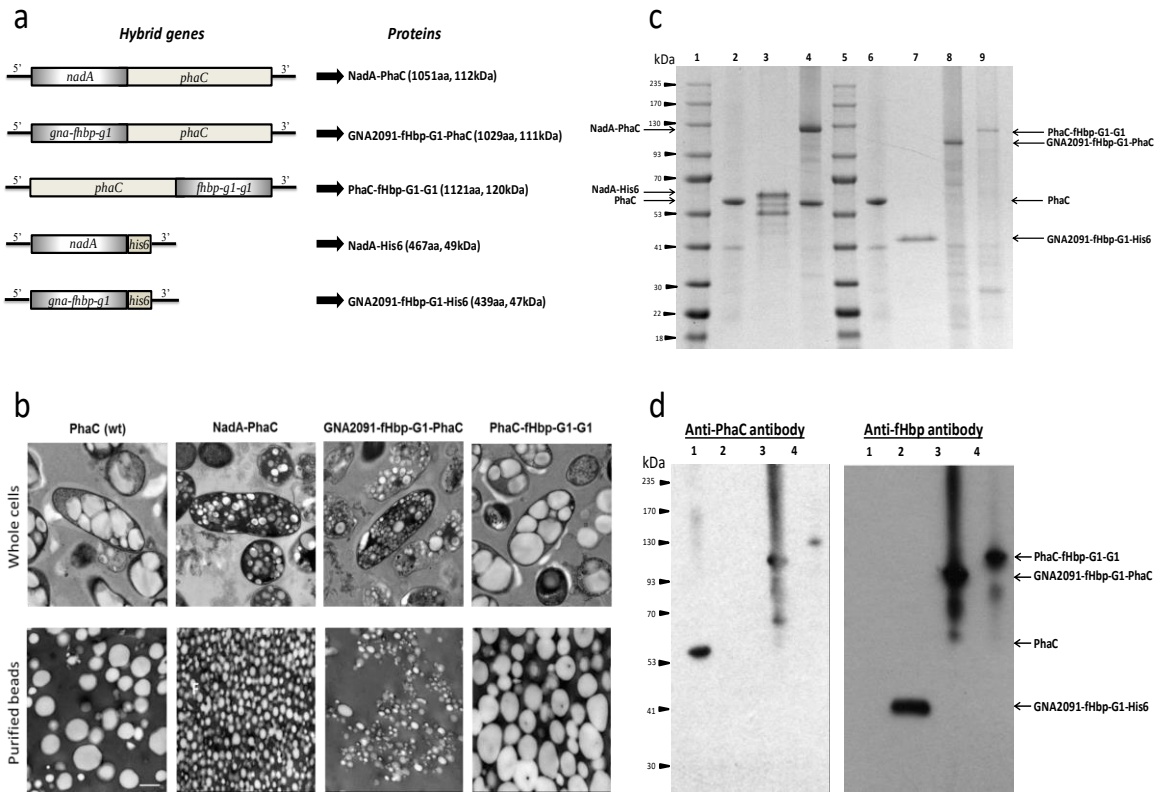


Figure 1. Biological production and characterization of antigen coated PHB beads. **a**. Schematic representation of the hybrid genes mediating formation of antigen displaying PHB beads or production of the respective soluble antigen. The amino acid sequence of the NadA was designed as a combination of the whole sequence of allele 1 (24-351 aa) (Genbank AF452481), three repeats of the immunodominant peptide (52-70 aa), two repeats of the anti-adhesion peptides (25-30aa) and (94-110aa) from the same allele 1. In the case of GNA2091-fHbp-G1 and fHbp-G1-G1 proteins, the amino acid sequence was composed as mentioned elsewhere (35). **b.** PHB inclusion production and beads size evaluation by Transmission Electron Microscopy (TEM) using whole cells of recombinant *E. coli* and the respective purified PHB beads; **c.** SDS-PAGE analysis of the protein profile of isolated PHB beads as well as the purified His-tagged proteins (selected antigens). Lane 1, MW (molecular weight) standard, (GangNam-Stain, iNtRON BIOTECHNOLOGY); Lane 2, non-antigen displaying PHB beads (PhaC ~64kDa); Lane 3, NadA-His6 protein ~50kDa; Lane 4, NadA-PhaC fusion protein (~113kDa) on PHB beads; Lane 5, MW standard, (GangNam-Stain, iNtRON BIOTECHNOLOGY); Lane 6, non-antigen displaying PHB beads (PhaC ~64kDa); Lane 7, GNA2091M-fHbp-G1-His6 protein (~47kDa); Lane 8, GNA2091-fHbp-G1-PhaC fusion protein (~111kDa) on PHB beads; Lane 9, fHbp-G1-G1-PhaC

fusion protein (~ 120kDa) on PHB beads. **d.** Immunoblotting using commercial monoclonal anti-fHbp antibodies (JAR4, NIBCS, UK) and monospecific polyclonal anti-PhaC antibodies were performed to further confirm the identity of the respective fusion proteins. Fusion proteins with the anticipated MW containing fHbp were detected. Lane 1, PhaC protein, (non-antigen displaying PHB beads); Lane 2, GNA2091-fHbp-G1-His6 protein; Lane 3, GNA2091-fHbp-G1-PhaC fusion protein on PHB bead; Lane 4 fHbp-G1-G1-PhaC fusion protein on PHB beads.

ClearColi^{TM}, an endotoxin-free mutant of *E. coli*, harbouring plasmid pMCS69 encoding PhaA and PhaB, which sequentially catalyse the synthesis of PHB precursors, was transformed with plasmids encoding PhaC fusion proteins (Supplementary Table 1). TEM images of cells harbouring plasmids encoding PhaC fusion proteins showed the presence of discrete spherical inclusions indicative of PhaC functionality (Fig. 1b). Interestingly, cells harbouring plasmids encoding NadA and GNA2091-fHbp-G1 containing PhaC fusion proteins mediated formation of smaller PHB inclusions when compared to the other PHA granule forming strains (Fig. 1b).

SDS-PAGE analysis of the protein profile of the isolated PHB beads, as well the purified His6-tagged proteins, indicated successful production of the respective proteins (Fig. 1c) which was further confirmed by immunoblotting (Fig. 1d) and tryptic peptide fingerprinting analysis using MALDI-TOF/MS (Supplementary Table 2). The PHB bead surface display of antigens was analysed by ELISA, which demonstrated the accessibility of antigens to specific antibodies (Supplementary Fig. 1).

As the surface charge of antigen beads might impact on immunogenicity, the Zeta potential of the PHB beads was determined as a function of pH (3-7.5). Negative Zeta potentials at all measured pH values suggested a negative surface charge for all beads (Supplementary Table 3).

The amount of the neisserial antigens attached to the PHB beads was determined as previously described (52) and used to adjust the amount of beads per dose to be used for the animal trial (Supplementary Table 4). Purified antigen displaying PHB beads and soluble antigens were then subjected to immunogenicity studies by vaccinating mice (Fig. 2a).

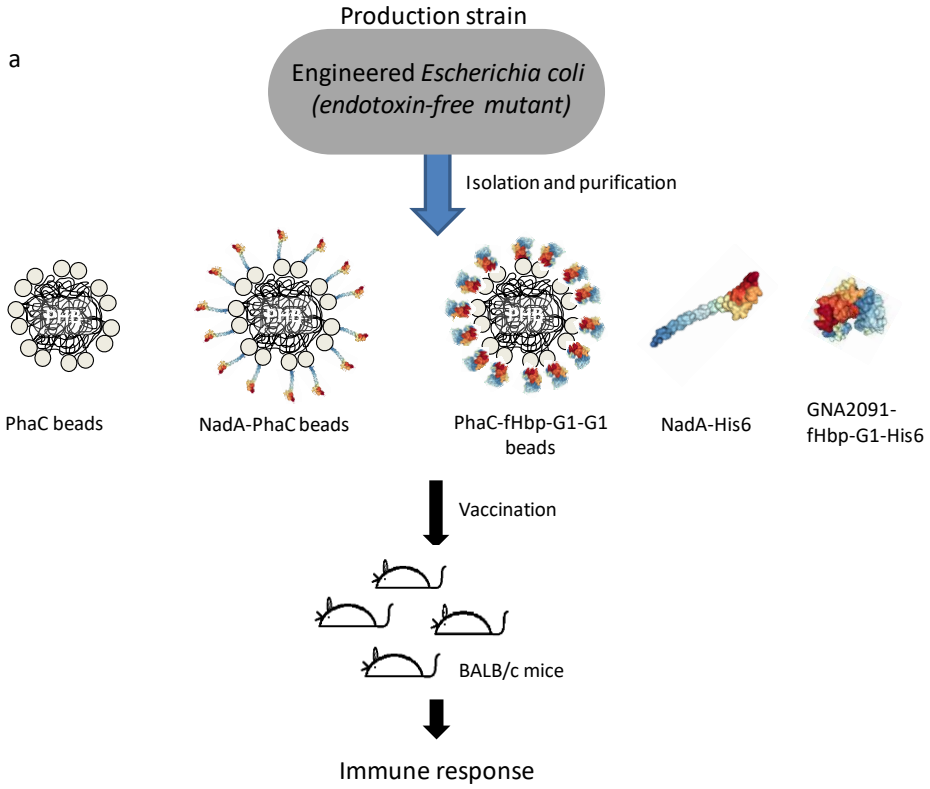


Figure 2. PHB bead production and their immunogenicity. **a.** Schematic representation of the biological production of the various PHB beads and their composition;

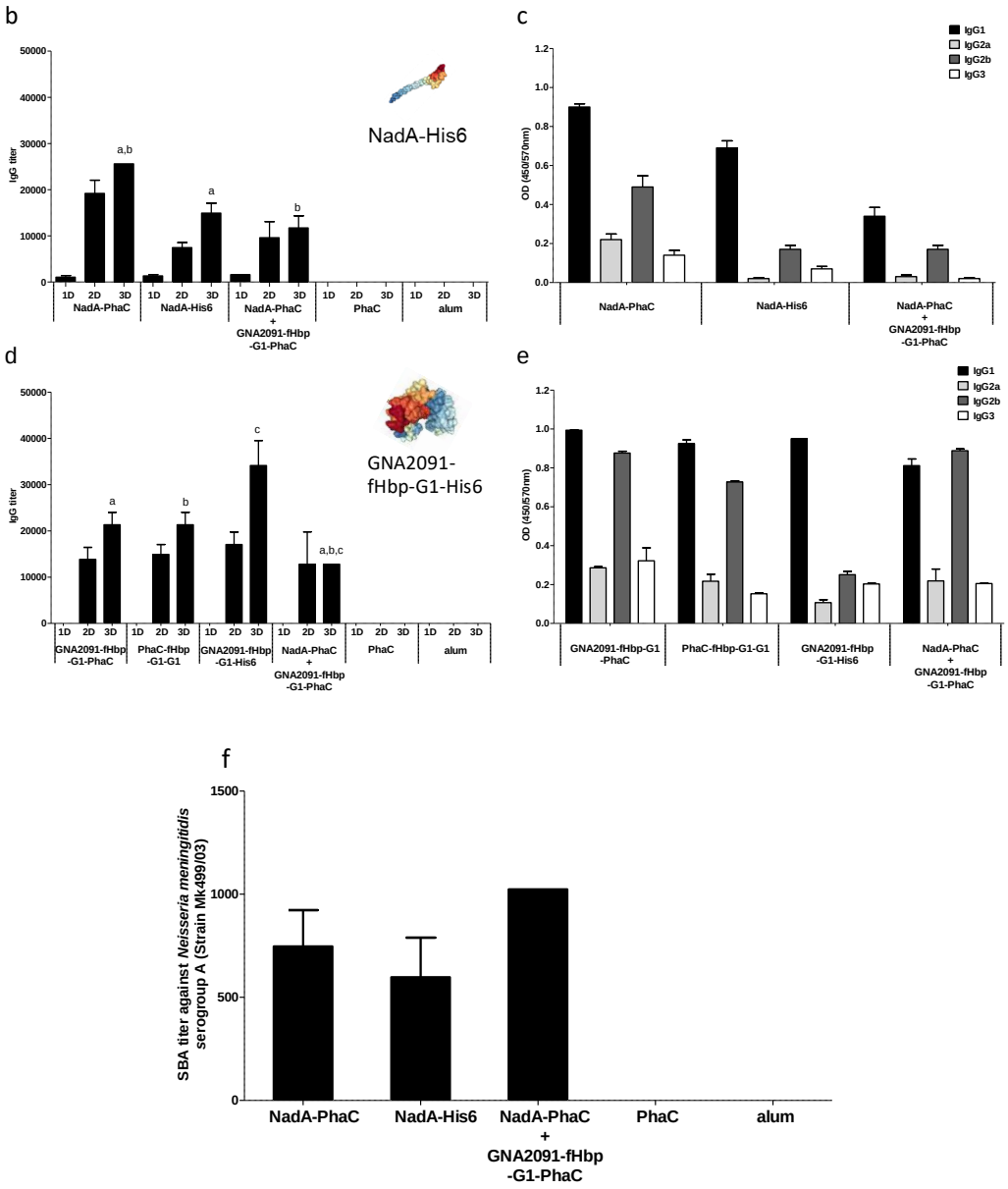


Figure 2. PHB bead production and their immunogenicity (continued). **b.** Assessment of antibodies binding to NadA; **c.** NadA-specific IgG subclass titers; **d.** Assessment of antibodies binding to GNA2091-fHbp-G1 specific; **e.** GNA2091-fHbp-G1 specific IgG subclass titers; **f.** Serum bactericidal activity (SBA) evaluation. IgG titers were evaluated by ELISA as well IgG subclass levels. IgG titers are expressed as the mean ± SEM (6 mice/group, by each blood collection time, 1D, 2D and 3D (first, second and third immunization) of the reciprocal dilution value required to reach ½ of the maximum valour of the OD signal. While the IgG subclass titers are expressed as the mean ± SD of two replicate/IgG subclass, using a pool of sera from the six animals after 3D. Bactericidal titers are expressed as mean ± SEM (6 mice each group) of the reciprocal value of the greatest sera dilution leading to ≥ 50% of the bacteria killing.

Statistically, differences between vaccination groups with $p<0.05$ were labelled with different letters. In the **Fig.2b** IgG titers after 3D, from the group, vaccinated with NadA-PhaC beads, were superior to the group vaccinated with soluble control NadA-His6, as well as the group vaccinated with PHB beads mix (a, b ($p<0.01$)), respectively. In **Fig.2d** IgG titers after 3D from groups vaccinated with GNA2091-fHbp-G1-PhaC beads, PhaC-fHbp-G1-G1 beads and GNA2091-fHbp-G1-His6 were superior to the group vaccinated with the PHB beads mix (a, b ($p<0.05$) and c ($p<0.01$)). Depicted protein structures are derived from the protein data bank as follows: NadA variant 5 (4CJD), factor H binding protein (mutant G1) (2Y7S). PHB, polyhydroxybutyrate

4.4.2 Immunological properties of antigen displaying PHB beads versus soluble antigens.

Mice were vaccinated with the various PHB bead suspensions as well as soluble antigen. All vaccinated animals remained alive and healthy during the entire study. In the groups immunized with PHB beads small granulomas were observed (up to 2 mm) at the injection site, but no suppuration was detected. Organs such as kidneys, lungs, liver and spleen from immunized mice showed no differences to those from mice that received the placebo.

The antibody responses in sera were assayed seven days after each vaccination using ELISA. Assessment of antibodies binding to NadA showed that the mean IgG titers increased for each group after each vaccination (Fig. 2b). However, as was expected, animals in the non-antigen displaying PHB bead and alum group were negative, respectively (Fig. 2b). The IgG1 subclass was predominant followed by the IgG2b subclass, while NadA displayed on PHB beads induced the strongest response, significantly different to the other groups (Fig. 2b, 2c).

Assessment of immune responses toward GNA2091-fHbp-G1 showed increasing IgG titers with each dose and was predominantly based on the IgG1

subclass followed by the IgG2b subclass (Fig. 2d, 2e). However, the soluble GNA2091-fHbp-G1-His6 showed the strongest induction of a specific IgG1 response, while the display on beads induced the strongest IgG2b response (Fig. 2e).

To evaluate the performance of the immune responses to protect against bacterial infection, the serum bactericidal assay (SBA) is a powerful tool to assess protective efficacy of these vaccine candidates. Here the functionality of induced antibodies to kill *N. meningitidis* was analysed *in vitro* by using sera from mice vaccinated with NadA-PhaC beads, NadA-PhaC beads combined with GNA2091-fHbp-G1-PhaC beads, soluble NadA-His6 and alum alone (49). The SBA titers are expressed as the mean ± SEM of the reciprocal value of the greatest dilution leading to ≥ 50% of bacteria-killing (Fig. 2f). SBA titers showed that antibodies induced by the vaccine candidates mediated killing of the bacterial pathogen (Fig. 2f).

4.4.3 Chemical conjugation of capsular polysaccharides to PHB beads.

The CPS from *N. meningitidis* serogroup C was subjected to periodic acid oxidation to introduce carbonyl groups. The molecular identity of the activated polysaccharide (APS) was evaluated by ^{1}H-NMR spectroscopy (Supplementary Figure. 2). ^{1}H-NMR analysis showed the removal of O-acetyl groups upon activation reaction as previously shown (41). Classical reductive amination was used to couple the APS to either the soluble protein or PHB bead surface proteins (53) (Fig. 3). The conjugation reaction yields were ranging from 4% to 36% (Supplementary Table 5).

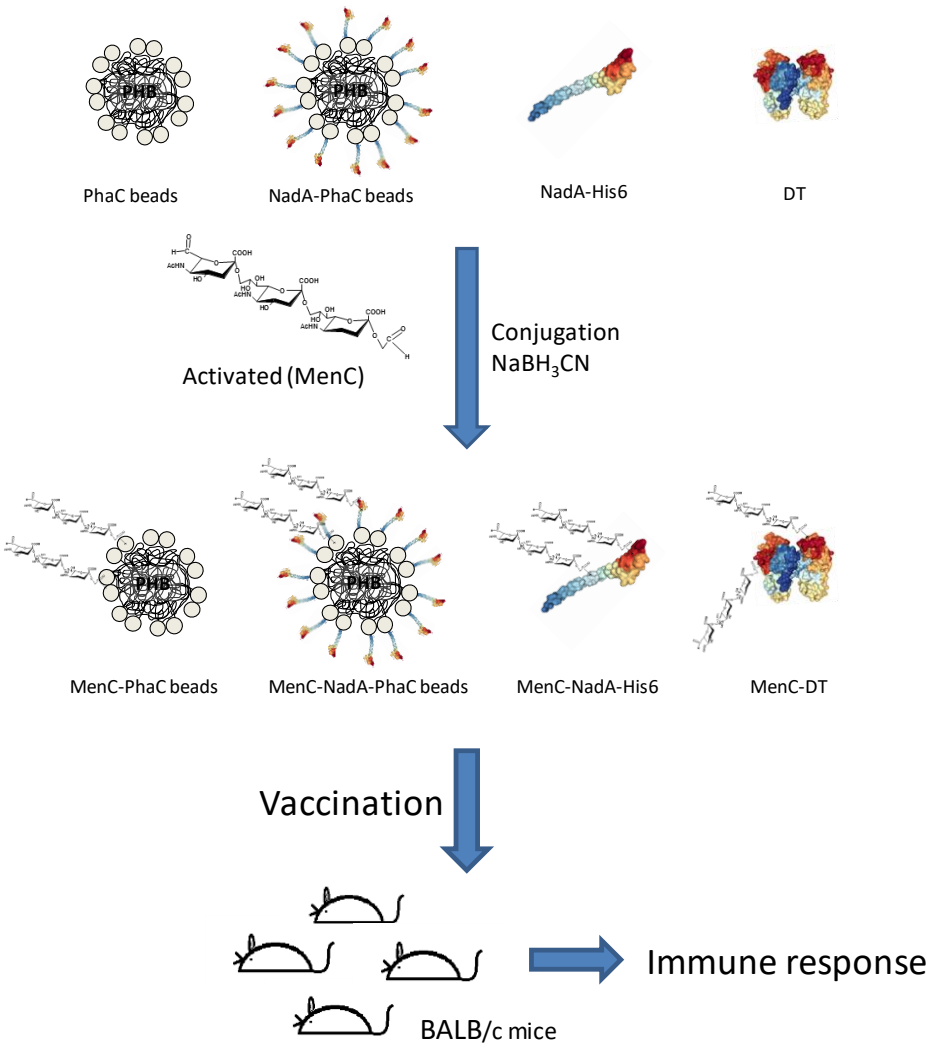


Figure 3. Schematic representation of the chemical conjugation of the CPS (MenC) to soluble and insoluble antigens displayed on PHB beads and characterization of their immunological properties. DT, diphtheria toxoid structural model was derived from protein data bank (5I82); NadA (variant 5), protein structure was derived from the protein data bank (4CJD). PHB, polyhydroxybutyrate

Immunoassays using commercial anti-CPS (MenC) monoclonal antibodies (NIBS, UK) were performed to confirm the molecular identity of the CPS after the conjugation reactions. The CPS was only detected by the monoclonal antibody when chemically conjugated to protein-coated PHB beads or soluble proteins but not without conjugation (Supplementary Fig. 3).

To locate conjugation sites in proteins coating the PHB beads, non-conjugated and conjugated PHB beads were subjected to trypsin/chymotrypsin digest and resulting peptides were analysed by liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS). The lower abundance of certain peptides upon conjugation suggested a conjugation site (lysine residue) within the peptide (Supplementary Table 6). Such identified lysine residues were depicted in the structural model of PhaC (Fig. 4). NadA fused to the N-terminus occluded 4 of the 7 sites identified without NadA. However, two new sites became accessible upon NadA fusion. Soluble NadA showed 24 conjugation sites of which 11 remained accessible after fusion to PhaC, i.e. display on PHB beads (Supplementary Table 6). NadA display on PHB beads generated 8 new accessible sites which were not detected in soluble NadA.

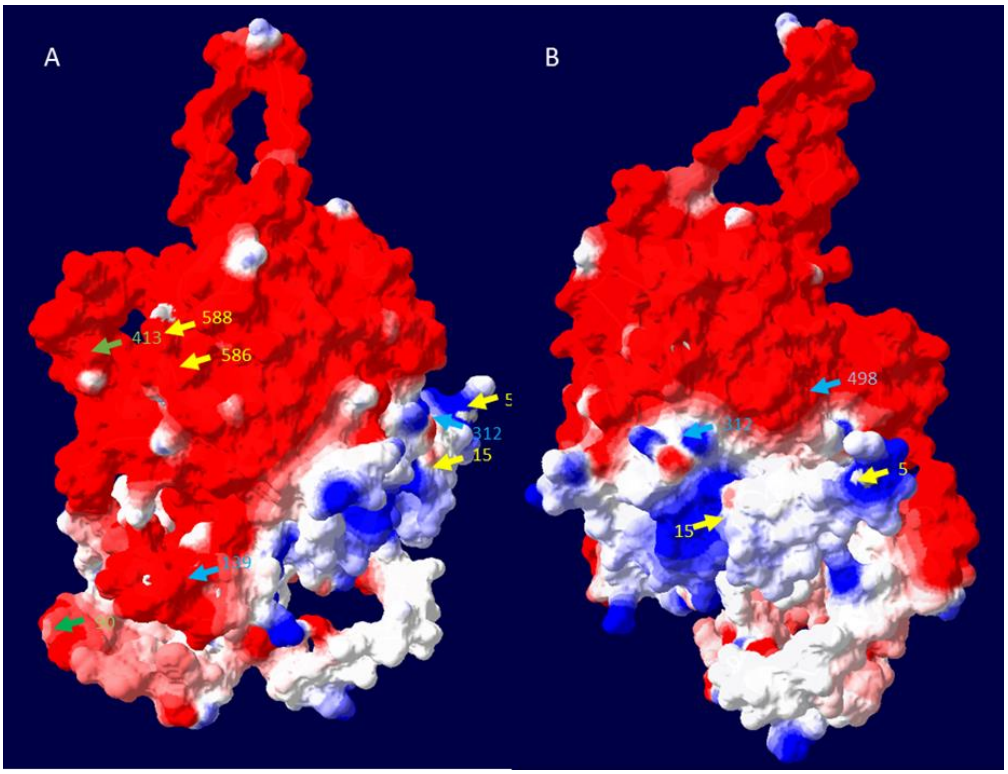


Figure 4. Structural models of PhaC depicting lysine residues proposed as sites conjugated to the activated polysaccharide. The structural model of PhaC was deduced from the crystal structure of the C-terminal region of the PHB synthase from *Ralstonia eutropha* (5HZ2) using i-Tasser server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). Resulting atom coordinates were displayed using the Swiss-PDB-Viewer (Version. 4.10) while computing the model surface using colouring based on the electrostatic potential. Arrows show the location of lysine residues and their sequence position, which were identified as possible sites for conjugation of the activated polysaccharide. Yellow and blue arrows show sites identified when only PhaC was displayed on PHB beads, while blue arrows indicate sites which remained accessible for conjugation after PhaC was fused to NadA as displayed on PHB beads. The green arrows indicate conjugation sites accessible in PhaC only upon fusion to NadA. A, horizontal 90^{o} clockwise rotation of the structural model shown in B.

4.4.4 Immunological properties of antigen-coated PHB beads displaying CPS.

The immunological properties of CPS conjugated vaccine candidates were studied by immunizing mice. All vaccinated animals remained healthy during the

entire study. The conjugation of CPS to PHB beads had no impact on anatomical features when compared to the non-conjugated vaccine candidates as described above.

The humoral immune response was evaluated with the serum from each animal at different blood collection times, as mentioned above. IgG titers against CPS increased after each vaccination dose for those vaccine candidates containing conjugated CPS (Fig 5a). Strongest anti-CPS antibody induction was observed with non-antigen coated PHB beads conjugated to CPS.

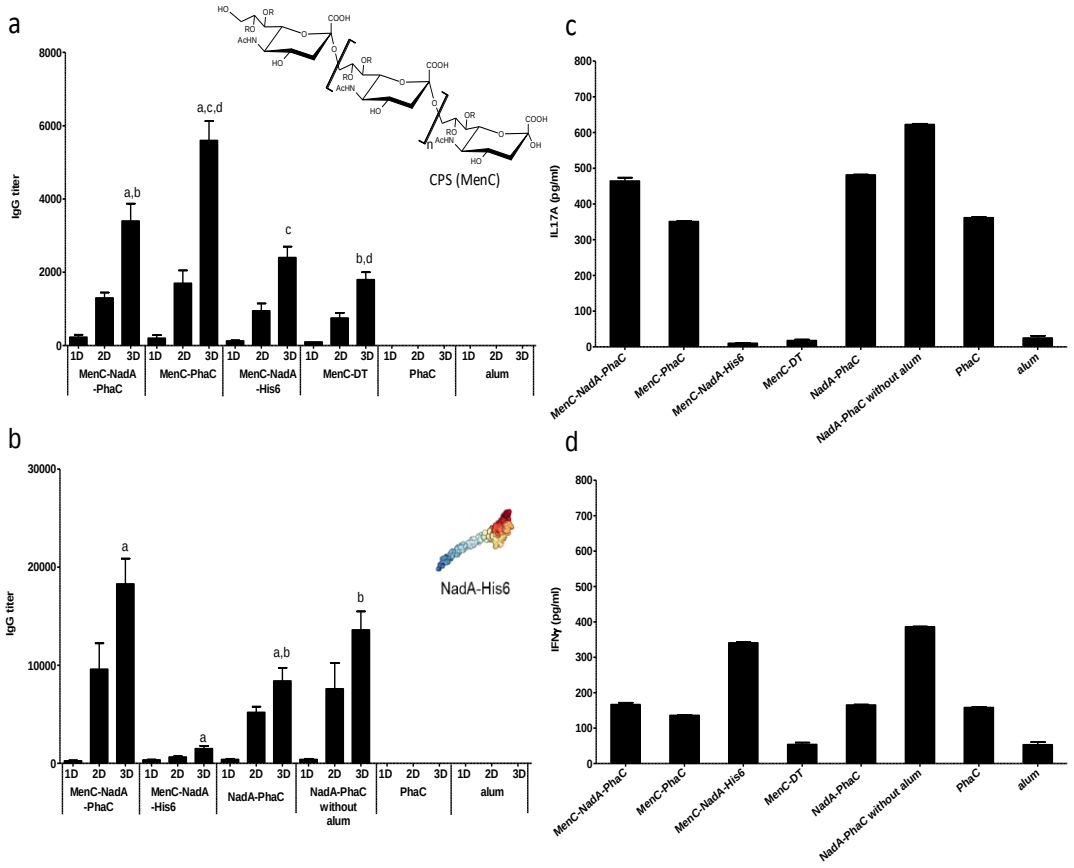


Figure 5. Immunogenicity studies of the various antigens conjugated to MenC. **a.** Assessment of antibodies binding to Men C CPS; **b.** Assessment of antibodies binding to NadA; **c.** Assessment of IL 17A cytokine levels; **d.** Assessment of IFN-ɣ cytokine levels. IgG titers were evaluated by ELISA and results are expressed as the mean ± SEM (8 mice/group, after each vaccination time, 1D, 2D and 3D (first, second and third immunization)). IgG titers were calculated as the reciprocal dilution value required to reach ½ of the maximum valour of the OD signal Figure 5a and 5b groups vaccinated with a formulation where the antigen under evaluation was not included were negative, (only control groups, PhaC and alum are shown. Cytokine production by spleen cells was evaluated by ELISA, under 10 µg/mL of NadA-His6 protein and 72h of stimulation. Results are presented as the mean ± SD of two replicas per each group. Statistically, differences between vaccination groups were labelled with letters. In **Fig5a** IgG titers after 3D, from group, vaccinated with MenC-NadA-PhaC beads, was inferior to the group vaccinated with MenC-PhaC beads and superior to the group vaccinated with Men C-DT (a, b ($p<0.01$)) while titers for group vaccinated with MenC-PhaC beads were superior to the group vaccinated with MenC-NadA-His6 and MenC-TD (c, d ($p<0.01$ and $p<0.001$ respectively)). In **Fig.4b** IgG titers after 3D from

groups vaccinated with MenC-NadA-PhaC beads were superior to group vaccinated with MenC-NadA-His6 and NadA-PhaC beads (a ($p<0.01$)) while group vaccinated with NadA-PhaC beads was inferior to the group vaccinated with NadA-PhaC beads without alum (b ($p<0.05$)). Depicted is the variant 5 NadA protein structure (4CJD) as derived from the protein data bank.

The evaluation of the IgG titers against NadA showed a trend to increase after each vaccination dose in the groups that received NadA as part of the vaccine formulation (Fig. 5b). However, as was expected animals from groups not exposed to NadA containing vaccine formulations such as e.g. CPS conjugated to diphtheria toxoid (DT) (data not shown) and non-antigen coated PHB beads were negative (Fig. 5b). The highest IgG titers were elicited after the last vaccination doses for the positive groups. To investigate whether PHB beads show adjuvant properties, alum was omitted in one NadA-PhaC bead formulation which resulted in induction of an even higher IgG titer than compared to the formulation containing alum (Fig. 5b).

In order to study the Ig subclass profile against CPS and NadA indirect ELISAs were performed using subclass-specific secondary antibodies (anti-IgG1, IgG2a, IgG2b, IgG3 and IgM). Serum from positive animals after the first and the third vaccination were assayed. Results, in the case of CPS, showed the predominant subclass elicited after vaccination was IgG1, reaching higher titers in the groups vaccinated with conjugated PHB beads than with the soluble proteins (Supplementary Fig. 4). The highest ratio of IgG/IgM was reached after the third dose, with the highest overall ratio found to be present in groups vaccinated with CPS conjugated to PHB beads (Supplementary Table 7). In the case of NadA, the IgG titer was the highest in mice immunized with NadA on PHB beads and predominantly composed of IgG1 subclass (Supplementary Fig. 5).

To study the cellular immune response and to identify the possible mode (Th1, Th2 or Th17) of response, splenocytes from 8 mice were pooled and re-stimulated with NadA-His6. The cytokines IL17A and INF-γ were measured (Fig. 5c, 5d).

Splenocytes from mice vaccinated with MenC-NadA-PhaC beads as well NadA-PhaC beads produced significantly higher levels of IL17A than mice vaccinated with soluble MenC-NadA-His6, MenC-DT and alum only (Fig. 5c). The strongest stimulation of IFN-γ production was found in splenocytes of mice vaccinated with MenC-NadA-His6 and with NadA-PhaC beads (no alum), respectively (Fig. 5d). The cytokine profile associated with antigen coated beads suggested the induction of a mixed Th1/Th17 type immune response.

The SBA is accepted as a correlate for assessing protective immunity against *N. meningitidis* infection (54, 55). Three *N. meningitidis* strains were used to investigate the functionality of the elicited antibodies. In the *N. meningitidis* serogroup C (C11 strain) only mice vaccinated with CPS containing formulations presented specific and high SBA titers (Fig. 6A). Mice vaccinated with MenC-PhaC beads showed the highest SBA titer in comparison with the rest of the positive groups. In Fig. 6B the SBA titers against *N. meningitidis* serogroup B (CU385/03, Cuban strain) showed that mice which received NadA-PhaC beads, MenC-NadA-PhaC beads and soluble MenC-NadA-His6, respectively, presented similar SBA titers. In absence of NadA, the SBA was negative. In the case of *N. meningitidis* serogroup A (Mk499/03 strain), results showed that high SBA titers against this strain were achieved when NadA was present in the vaccine formulation (Fig. 6C). Nevertheless, mice vaccinated with MenC-NadA-PhaC beads induced a significantly higher SBA titer when compared to its soluble counterpart MenC-NadA-His6. No statistically significant differences were

found between NadA-PhaC beads plus or minus alum which suggested that alum is not required to induce high SBA titer when NadA is displayed on PHB beads.

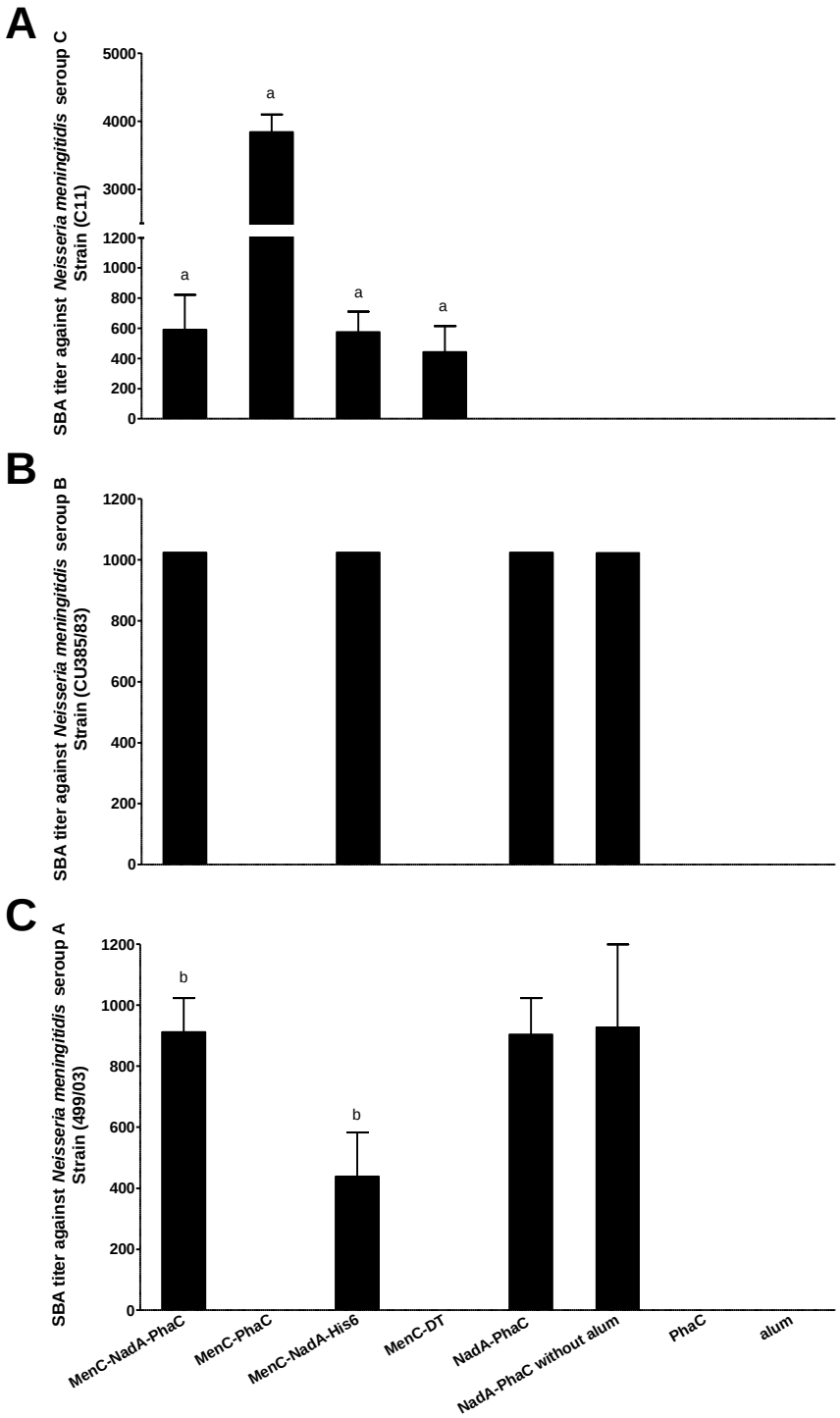


Figure 6. Bactericidal activity of various sera. A, *N. meningitidis* serogroup C (Strain C11); **B**, *N. meningitidis* serogroup B (Strain CU385/83); **C**, *N. meningitidis* serogroup

A (Strain 499/03). Bactericidal titers are expressed as mean ± SEM (8 mice in each group) of the reciprocal value of the greatest sera dilution leading to ≥ 50% of killing.

4.5 Discussion

In this study, we systematically evaluated how *in vivo* assembled PHB inclusions could be refined as an immunogenic carrier for proteinaceous and carbohydrate-based antigens in the context of a bacterial extracellular pathogen. Safe subunit vaccines composed of defined antigens are desirable, but they often lack immunogenicity and broadly protective efficacy. Here, we used bioengineering of *E. coli* to assemble antigen displaying PHB beads and extended their antigen repertoire by chemical conjugation of a vaccine candidate CPS. Carbohydrate conjugate vaccines against *N. menigitidis* are the most common vaccines used worldwide. However, these vaccines do not prevent infection by serogroup B as their CPS, polysialic acid resembles a glycan present in human tissue and hence is not suitable as antigen (56, 57). Accordingly, alternative strategies based on the proteinaceous antigens, such as PorA and PorB (outer membrane proteins), have been explored and resulted in the first vaccine generation against this serogroup (58, 59).

Although serotype independent, these vaccines faced coverage limitation due to an enormous variability of antigenic determinants in PorA and PorB (60). New serotype independent protein-based vaccines were recently licensed against serogroup B (Bexsero®, Sanofi Pasteur; Trumenba®, Pfizer). Soluble NadA and GNA2091-fHbp-G1, due to their relevance in pathogenicity and their capacity to generate a strong bactericidal antibody response, were included in these licensed vaccines (14, 17, 61). However, these antigens required the addition of adjuvants and their production is costly prohibiting their widespread use in developing and third world countries. Hence, we first explored the possibility of designing and producing a protein-based subunit

vaccine requiring less antigen and implementing a cost-effective production process. Accordingly, a particulate antigen delivery system based on bacterial PHB inclusions was designed to directly enable the production of immunogenic antigens as a particulate vaccine. Particulate vaccines are inherently immunogenic as uptake by antigen presenting cells (APCs) is facilitated (62). Bacterial PHB inclusions had been engineered to display *M. bovis* antigens and showed a strong cellular immune response resulting in protective immunity (24).

The two neisserial proteins, NadA and fHbp, were each translationally fused to the PHB bead forming enzyme, PHA synthase, and the respective fusion protein-mediated assembly of PHB beads displayed the respective antigens when produced in an endotoxin-free mutant of *E. coli* (Fig. 1). As had been previously shown some *E.coli* proteins co-purify with PHB bead isolation but had not been found to induce antibodies (63). An endotoxin free *E. coli* mutant was used as PHB bead production strain to reduce the impact of endotoxin levels on the immune response (38, 64). Since the GNA2091-fHbp-G1 fused to the N-terminus of PhaC was more abundant on PHB beads when compared fHbp-G1-G1 fused to the C-terminus of PhaC (Fig. 1), the former fusion protein and respective PHB beads were used in animal trials. Immunoblot and ELISA analysis (Supplementary Fig. 1) using a monoclonal anti-fHbp antibody confirmed the presence of linear and conformational epitopes of fHbp in GNA2091-fHbp-G1-PhaC and PhaC-fHbp-G1-G1 beads (Fig. 1d). The uptake of particulate vaccines by APCs and the respective antigen processing pathways have been found to be dependent on physical properties such as size and surface charge (65). The PHB beads produced and formulated in this study showed a mean particle size of less than 10 µm (Dx(50)). It remains unclear why PhaC beads and PhaC-fHbp-G1-G1 beads were produced at larger sizes (~500 nm) as observed by TEM when

compared with the other PHB bead variants (~100 nm) (Fig. 1b). However, upon formulation in PBS or in alum, particle sizes increased for all PHB bead variants (< 10 µm) presumably due to electrostatic or hydrophobic interactions between the beads itself and/or alum (Supplementary Table 8).

Studies with polylactic acid (PLA) beads exhibiting a range of particles sizes (<2-8 µm, 10-70 µm and 50-150 µm) loaded with tetanus toxoid (TT) elicited IgG titers against TT dependent on the particle size. The optimum particle size range to elicit the highest IgG levels against TT was between 2-8 µm whereas the reduction of the particle size to less than 2 µm resulted in decreasing IgG titers. The lowest titer was achieved by those particles with the largest particle size 50-150 µm (66). Fifis et al. (67) found that carboxylated polystyrene spheres (20 nm-2 µm) conjugated with OVA elicited a strong humoral response while the highest IgG titers were obtained with particles showing a size of about 40 nm. Additionally, the uptake of antigen by immune system cells in the draining lymph nodes of mice showed that 40 nm particles were preferentially taken up by mature/activated dendritic cells, while 1 µm particles were preferentially taken up by macrophages. This suggested that different APCs prefer different particle sizes for uptake (67). The relationship between the surface charge and uptake by dendritic cells (DCs) and the immune response is controversial. Some authors proposed for particles with sizes > 0.5 µm that positive surface charges enhanced association with DCs in comparison with particles showing negative surface charges (68). However, other studies suggested that the surface charges are less critical for particles with a size of < 0.5 µm in view of uptake efficiency by DC (65). The neisserial antigen displaying PHB beads, as well as the non-antigen displaying control PhaC beads, showed a negative surface charge at pH 7.5, which also corresponded with the theoretical isoelectric point of the PHB bead associated PhaC or its fusion

protein variant (Supplementary Table 3). How this negative surface charge contributes to uptake by APCs remains to be elucidated.

To develop an efficient broadly protective vaccine against extracellular pathogens like *N. meningitidis* the induction of a strong humoral immune response associated with the bactericidal activity is desirable and correlates with protective immunity (69). For serogroups A and C the protection correlate is 2 µg/mL of IgG antibodies with an SBA $\geq$ 4 when human complement is used as a complement source (51, 70). However, for serogroup B a correlation between SBA activity and protective immunity is not clearly defined, which further impairs the development of a vaccine against this serogroup. Immunological properties of the various PHB beads were assessed in mice (Fig. 2a) and revealed that the particulate NadA-PhaC beads induced a significantly greater humoral immune response against NadA than its soluble counterpart NadA-His6 (Fig. 2b). In contrast, GNA2091-fHbp-G1-PhaC beads or fHbp-G1-G1-PhaC beads induced high and specific IgG levels but less than obtained with the soluble GNA2091-fHbp-G1-His6 (Fig. 2d). Here the display of cell surface antigens on the PHB bead surface was intended to simulate the natural surface exposition of the antigen towards induction of an immune response associated with the production of specific functional antibodies (71). However, the particulate surface display is only one parameter while many factors, such as e.g. antigen structure, size or charge of the particles, can influence the immune response. Since it is known that IgG1 and IgG3 are the best IgG subclasses contributing to efficient bactericidal activity, the IgG subclass profile was assessed as an indicator of the functionality of the immune response (72, 73). The IgG1 subclass has been found to be predominantly involved with opsonophagocytic activity after immunisation with the meningococcal B vesicle vaccine (74, 75). In our study, the IgG1 was the predominant subclass after

immunization with PHB beads (Fig. 2c, 2e). IgG2b induction was enhanced when antigens were displayed on PHB beads. Similar results were presented by Parlane et al. (76) when antigens from *M. bovis* were displayed on PHB beads suggesting that antigen displaying PHB beads promote a mixed Th1/Th2 immune response. The immunization with NadA-PhaC beads elicited not only high IgG1 levels but in addition induced antibodies mediating killing of *N. meningitidis* serogroup A (strain MK499/03) through classical complement activation pathways (Fig. 2f). Less SBA was observed in mice immunized with soluble NadA-His6.

To further broaden protective efficacy, bioengineered antigen-displaying PHB beads were subjected to chemical conjugation of carbohydrate-based antigens (Fig. 3). The CPS of serogroup C was successfully conjugated to various PHB beads, soluble NadA-His6 or DT, respectively (Fig. 3). Assessment of conjugations sites suggested that PhaC shows an extended accessible surface area while being attached to PHB beads and that these sites might be occluded by N-terminal fusion partners (Supplementary Table 6, Fig. 4).

Two of the sites, K90 and K139, were also identified by Hooks and Rehm (43) who used biotinylation to identify surface-exposed residues. In their study further residues (K77, C382, C459, and K518) were found to be accessible which was presumably due to the smaller size of biotin. As a fusion of NadA to PhaC still enabled access to 11 of the 24 conjugation sites found in soluble NadA, plus enabled access to further 8 lysines, it is suggested that NadA is extensively exposed at the surface of the PHB beads (43).

NadA-PhaC beads were chosen as the protein antigen displaying beads for conjugation because of their superior immunological properties when compared with its soluble counterpart and the other protein antigen displaying PHB beads. All CPS

conjugate vaccine candidates induced significant IgG titers against MenC when compared with the negative controls (Fig. 5a). NadA bearing vaccine candidates co-displaying conjugated CPS also induced significant levels of anti-NadA antibodies and, when displayed on PHB beads, significantly greater levels than soluble MenC-NadA-His6 (Fig. 5b). In addition to this Th2-type immune response, cytokine analysis suggested that the MenC-NadA-PhaC and NadA-PhaC beads also induced a cell-mediated immune response, presumably of mixed Th1/Th17 pattern, while soluble antigens induced Th1 but not Th17 responses (Fig. 5c, 5d). The omission of alum in the NadA-PhaC beads formulation increased immunogenicity toward Th1-, Th2-, Th17-type immune responses suggesting a possible self-adjuvant property of the PHB bead carrier system. However, more experiments are need it to fully prove this fact.

The SBA titers against the three serogroups A, B, C confirmed the superior performance of NadA and CPS co-displayed on PHB beads when compared to their soluble counterparts (Fig. 6). While NadA on PHB beads mediated protective immunity against serogroups A and B, the co-display of MenC expanded coverage to serogroup C (Fig. 6). This suggested that combining protein and carbohydrate antigens on the surface of particulate carriers provides design space towards the development of broadly protective vaccines.

Overall, this study showed that PHB beads displaying selected neisserial antigens can be recombinantly produced in an endotoxin-free *E. coli* mutant and that these PHB beads can be further modified by chemical conjugation to co-display carbohydrate antigens. Antigen-displaying PHB beads were immunogenic mediating strong and specific humoral and cell-mediated immune responses leading to broadly protective immunity. Hence the PHB bead-based particulate vaccine approach holds

the promise to be applicable not only for protection against intracellular pathogens but also extracellular pathogens such as *N. meningitidis.*

4.6 Acknowledgements

The authors are grateful for funding provided by Massey University (New Zealand) and the Finlay Institute (Cuba). The authors are also thankful to Natalie Burr, Maria Onelia González Socarras, Yanet Estrada, Maryle Garcia, Alex Quintero Perez, Dr Reinaldo Oliva Hernández, Tamara Hernández Salazar, Dr Yanely Tirado, Dr Reinaldo Acevedo for technical assistance. The authors would like to thank Jordan Taylor, Niki Minard and Matthew Savoian from the Manawatu Microscopy Imaging Centre for their assistance in TEM analysis. Prof Martin Hazelton and Dr Edgar Santos-Fernández are acknowledged for their assistance in statistical analysis of data.

4.7 References

1. Rehm BH. Bioengineering towards self-assembly of particulate vaccines. Current Opinion in Biotechnology. 2017;48:42-53.
2. http://www.who.int/mediacentre/factsheets/fs141/en/.
3. Crum-Cianflone N, Sullivan E. Meningococcal Vaccinations. Infectious diseases and therapy. 2016:1-24.
4. Ashton FE, Ryan A, Diena B, Jennings H. A new serogroup (L) of *Neisseria meningitidis*. Journal of clinical microbiology. 1983;17(5):722-7.
5. Abio A, Neal KR, Beck CR. An epidemiological review of changes in meningococcal biology during the last 100 years. Pathogens and global health. 2013;107(7):373-80.
6. Cohn AC, MacNeil JR, Clark TA, Ortega-Sanchez IR, Briere EZ, Meissner HC, et al. Prevention and control of meningococcal disease: recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Recommendations and Reports. 2013;62(RR-2):1-28.
7. Griffiss JM, Yamasaki R, Estabrook M, Kim JJ. Meningococcal molecular mimicry and the search for an ideal vaccine. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1991;85(Supplement 1):32-6.
8. Feiring B, Fuglesang J, Oster P, Næss LM, Helland OS, Tilman S, et al. Persisting immune responses indicating long-term protection after booster dose with the meningococcal group B outer membrane vesicle vaccine. Clinical and vaccine immunology. 2006;13(7):790-6.
9. Uli L, Castellanos-Serra L, Betancourt L, Domínguez F, Barberá R, Sotolongo F, et al. Outer membrane vesicles of the VA-MENGOC-BC® vaccine against serogroup B *of Neisseria meningitidis*: Analysis of protein components by two-dimensional gel electrophoresis and mass spectrometry. Proteomics. 2006;6(11):3389-99.
10. Rodríguez AP, Dickinson F, Baly A, Martinez R. The epidemiological impact of antimeningococcal B vaccination in Cuba. Memorias do Instituto Oswaldo Cruz. 1999;94(4):433-40.
11. de Kleijn ED, de Groot R, Labadie J, Lafeber AB, van den Dobbelsteen G, van Alphen L, et al. Immunogenicity and safety of a hexavalent meningococcal outer-

membrane-vesicle vaccine in children of 2–3 and 7–8 years of age. Vaccine. 2000;18(15):1456-66.

12. Tettelin H, Saunders NJ, Heidelberg J, Jeffries AC, Nelson KE, Eisen JA, et al. Complete genome sequence of *Neisseria meningitidis* serogroup B strain MC58. Science. 2000;287(5459):1809-15.

13. Frosch M, Maiden MC. Handbook of meningococcal disease: Wiley Online Library; 2006.

14. Giuliani MM, Adu-Bobie J, Comanducci M, Aricò B, Savino S, Santini L, et al. A universal vaccine for serogroup B meningococcus. Proceedings of the National Academy of Sciences. 2006;103(29):10834-9.

15. Comanducci M, Bambini S, Caugant DA, Mora M, Brunelli B, Capecchi B, et al. NadA diversity and carriage in *Neisseria meningitidis*. Infection and immunity. 2004;72(7):4217-23.

16. Pizza M, Scarlato V, Masignani V, Giuliani MM, Arico B, Comanducci M, et al. Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. Science. 2000;287(5459):1816-20.

17. Granoff DM, Welsch JA, Ram S. Binding of complement factor H (fH) to *Neisseria meningitidis* is specific for human fH and inhibits complement activation by rat and rabbit sera. Infection and immunity. 2009;77(2):764-9.

18. Gasparini R, Amicizia D, Lai PL, Panatto D. Meningococcal B vaccination strategies and their practical application in Italy. Journal of preventive medicine and hygiene. 2015;56(3): E133.

19. Panda AK. Nanotechnology in vaccine development. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences. 2012;82(1):13-27.

20. Jiang W, Gupta RK, Deshpande MC, Schwendeman SP. Biodegradable poly (lactic-co-glycolic acid) microparticles for injectable delivery of vaccine antigens. Advanced drug delivery reviews. 2005;57(3):391-410.

21. Eyles J, Carpenter Z, Alpar H, Williamson E. Immunological aspects of polymer microsphere vaccine delivery systems. Journal of drug targeting. 2003;11(8-10):509-14.

22. Men Y, Audran R, Thomasin C, Eberl G, Demotz S, Merkle HP, et al. MHC class I and class II restricted processing and presentation of microencapsulated antigens. Vaccine. 1999;17(9):1047-56.

23. Parlane NA, Grage K, Lee JW, Buddle BM, Denis M, Rehm BH. Production of a particulate hepatitis C vaccine candidate by an engineered *Lactococcus lactis* strain. Applied Environmental Microbiology. 2011;77(24):8516-22.

24. Parlane NA, Rehm BH, Wedlock DN, Buddle BM. Novel particulate vaccines utilizing polyester nanoparticles (bio-beads) for protection against *Mycobacterium bovis* infection - a review. Veterinary Immunology and Immunopathology. 2014;158(1-2):8-13.

25. Parlane NA, Grage K, Mifune J, Basaraba RJ, Wedlock DN, Rehm BH, et al. Vaccines displaying mycobacterial proteins on biopolyester beads stimulate cellular immunity and induce protection against tuberculosis. Clinical and Vaccine Immunology. 2012;19(1):37-44.

26. Grage K, Peters V, Rehm BHA. Recombinant protein production by *in vivo* polymer inclusion display. Applied and Environmental Microbiology. 2011;77(18):6706-9.

27. Parlane NA, Gupta SK, Rubio Reyes P, Chen S, Gonzalez Miro M, Wedlock DN, et al. Self-assembled protein-coated polyhydroxyalkanoate beads: properties and biomedical applications. ACS Biomaterials Science & Engineering. 2016.

28. Martinez-Donato G, Piniella B, Aguilar D, Olivera S, Perez A, Castanedo Y, et al. Protective T Cell and Antibody Immune Responses against Hepatitis C Virus Achieved Using a Biopolyester-Bead-Based Vaccine Delivery System. Clinical and Vaccine Immunology. 2016;23(4):370-8.

29. Rubio Reyes P, Parlane NA, Wedlock DN, Rehm BH. Immunogencity of antigens from *Mycobacterium tuberculosis* self-assembled as particulate vaccines. Int Journal of Medical Microbiology. 2016.

30. Lee JW, Parlane NA, Rehm BH, Buddle BM, Heiser A. Engineering Mycobacteria for the Production of Self-Assembling Biopolyesters Displaying Mycobacterial Antigens for Use as a Tuberculosis Vaccine. Applied and environmental Microbiology. 2017;83(5).

31. Lee JW, Parlane NA, Wedlock DN, Rehm BH. Bioengineering a bacterial pathogen to assemble its own particulate vaccine capable of inducing cellular immunity. Scitific Reports. 2017;7:41607.

32. Olfert ED, Cross BM, McWilliam AA. Guide to the care and use of experimental animals: Canadian Council on Animal Care Ottawa; 1993.

33. Bambini S, De Chiara M, Muzzi A, Mora M, Lucidarme J, Brehony C, et al. *Neisseria* adhesin A variation and revised nomenclature scheme. Clinical and Vaccine Immunology. 2014;21(7):966-71.

34. Comanducci M, Bambini S, Brunelli B, Adu-Bobie J, Aricò B, Capecchi B, et al. NadA, a novel vaccine candidate of *Neisseria meningitidis*. The Journal of experimental medicine. 2002;195(11):1445-54.

35. Scarselli M, Aricò B, Brunelli B, Savino S, Di Marcello F, Palumbo E, et al. Rational design of a meningococcal antigen inducing broad protective immunity. Science translational medicine. 2011;3(91):91ra62-91ra62.

36. de Filippis I, de Lemos APS, Hostetler JB, Wollenberg K, Sacchi CT, Harrison LH, et al. Molecular epidemiology of *Neisseria meningitidis* serogroup B in Brazil. PloS one. 2012;7(3):e33016.

37. Thompson T, Rehm BH, Herbert AB, Saravolac EG. Compositions for separation methods. Google Patents; 2011.

38. Thompson T, Rehm BHA, Herbert AB, Saravolac EG, Mcdermott PB, Draper JL. Compositions For Separation Methods. Google Patents; 2013.

39. Handbook BDP. www. Qiagen. Com. Gmbh, Germany, June. 2005.

40. Grage K, Rehm BH. In vivo production of scFv-displaying biopolymer beads using a self-assembly-promoting fusion partner. Bioconjugation Chemistry. 2008;19(1):254-62.

41. Jones C, Lemercinier X. Use and validation of NMR assays for the identity and O-acetyl content of capsular polysaccharides from *Neisseria meningitidis* used in vaccine manufacture. Journal of pharmaceutical and biomedical analysis. 2002;30(4):1233-47.

42. Hooks DO, Blatchford PA, Rehm BH. Bioengineering of bacterial polymer inclusions catalyzing the synthesis of N-acetylneuraminic acid. Applied and environmental microbiology. 2013;79(9):3116-21.

43. Hooks DO, Rehm BH. Insights into the surface topology of polyhydroxyalkanoate synthase: self-assembly of functionalized inclusions. Applied microbiology and biotechnology. 2015;99(19):8045-53.

44. González-Miro M, Rodríguez-Noda L, Fariñas-Medina M, García-Rivera D, Vérez-Bencomo V, Rehm BH. Self-assembled particulate PsaA as vaccine against *Streptococcus pneumoniae* infection. Heliyon. 2017;3(4):e00291.

45. Scientific T. Pierce BCA Protein Assay Kit. Rockford, IL. 2007:1-7.

46. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. Journal of biological Chemistry. 1951;193(1):265-75.

47. Svennerholm L. Quantitive estimation of sialic acids: II. A colorimetric resorcinol-hydrochloric acid method. Biochimica et biophysica acta. 1957;24:604-11.

48. Gheesling LL, Carlone GM, Pais LB, Holder PF, Maslanka SE, Plikaytis BD, et al. Multicenter comparison of *Neisseria meningitidis* serogroup C anti-capsular polysaccharide antibody levels measured by a standardized enzyme-linked immunosorbent assay. Journal of clinical microbiology. 1994;32(6):1475-82.

49. Borrow R, Aaberge IS, Santos GF, Eudey TL, Oster P, Glennie A, et al. Interlaboratory standardization of the measurement of serum bactericidal activity by using human complement against meningococcal serogroup b, strain 44/76-SL, before and after vaccination with the Norwegian MenBvac outer membrane vesicle vaccine. Clinical and diagnostic laboratory immunology. 2005;12(8):970-6.

50. Norheim G, Rosenqvist E, Aseffa A, Yassin MA, Mengistu G, Kassu A, et al. Characterization of *Neisseria meningitidis* isolates from recent outbreaks in Ethiopia and comparison with those recovered during the epidemic of 1988 to 1989. Journal of clinical microbiology. 2006;44(3):861-71.

51. Gill C, Ram S, Welsch J, Detora L, Anemona A. Correlation between serum bactericidal activity against *Neisseria meningitidis* serogroups A, C, W-135 and Y measured using human versus rabbit serum as the complement source. Vaccine. 2011;30(1):29-34.

52. Hay ID, Hooks DO, Rehm BH. Use of Bacterial Polyhydroxyalkanoates in Protein Display Technologies. Springer; 2014.

53. Jennings HJ, Lugowski C. Immunochemistry of groups A, B, and C meningococcal polysaccharide-tetanus toxoid conjugates. The Journal of Immunology. 1981;127(3):1011-8.

54. Frasch CE, Borrow R, Donnelly J. Bactericidal antibody is the immunologic surrogate of protection against meningococcal disease. Vaccine. 2009;27:B112-B6.

55. Goldschneider I, Gotschlich EC, Artenstein MS. Human immunity to the meningococcus: II. Development of natural immunity. The Journal of experimental medicine. 1969;129(6):1327.

56. Findlow H, Borrow R. Immunogenicity and safety of a meningococcal serogroup A, C, Y and W glycoconjugate vaccine, ACWY-TT. Advances in therapy. 2013;30(5):431-58.

57. Harrison LH, Mohan N, Kirkpatrick P. Meningococcal group A, C, Y and W-135 conjugate vaccine. Nature Reviews Drug Discovery. 2010;9(6):429-30.

58. Fredriksen JH, Rosenqvist E, Wedege E, Bryn K, Bjune G, Frøholm L, et al. Production, characterization and control of MenB-vaccine" Folkehelsa": an outer membrane vesicle vaccine against group B meningococcal disease. NIPH annals. 1991;14(2):67-79; discussion -80.

59. GVG S, Campa H, Garcia I, Izquierdo P, Sotolongo P. Vaccine against group B *Neisseria meningitidis*: protection trial and mass vaccination results in Cuba. NlPH Annals 1991; 14: 195. 1991;207.

60. Panatto D, Amicizia D, Lai P, Cristina M, Domnich A, Gasparini R. New versus old meningococcal group B vaccines: how the new ones may benefit infants & toddlers. Indian Journal of Medical Research. 2013;138(6):835.

61. Shirley M, Dhillon S. Bivalent rLP2086 Vaccine (Trumenba®): A Review in Active Immunization Against Invasive Meningococcal Group B Disease in Individuals Aged 10–25 Years. BioDrugs. 2015;29(5):353-61.

62. Boraschi D, Italiani P. From antigen delivery system to adjuvanticity: The board application of nanoparticles in vaccinology. Vaccines. 2015;3(4):930-9.

63. Reyes PR, Parlane NA, Wedlock DN, Rehm BH. Immunogenicity of antigens from *Mycobacterium tuberculosis* self-assembled as particulate vaccines. International Journal of Medical Microbiology. 2016;306(8):624-32.

64. Mamat U, Woodard RW, Wilke K, Souvignier C, Mead D, Steinmetz E, et al. Endotoxin-Free Protein Production-*CleanColi*™ Technology.

65. Foged C, Brodin B, Frokjaer S, Sundblad A. Particle size and surface charge affect particle uptake by human dendritic cells in an in vitro model. International Journal Pharmaceutic. 2005;298(2):315-22.

66. Katare YK, Muthukumaran T, Panda AK. Influence of particle size, antigen load, dose and additional adjuvant on the immune response from antigen loaded PLA microparticles. International Journal of Pharmaceutic. 2005;301(1-2):149-60.

67. Fifis T, Gamvrellis A, Crimeen-Irwin B, Pietersz GA, Li J, Mottram PL, et al. Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. The Journal of Immunology. 2004;173(5):3148-54.

68. Thiele L, Merkle HP, Walter E. Phagocytosis and phagosomal fate of surface-modified microparticles in dendritic cells and macrophages. Pharmaceutical Research. 2003;20(2):221-8.

69. Goldschneider I, Gotschlich EC, Artenstein MS. Human immunity to the meningococcus. II. Development of natural immunity. The Journal of Experimental Medicine. 1969;129(6):1327-48.

70. Peltola H, Makela H, Kayhty H, Jousimies H, Herva E, Hallstrom K, et al. Clinical efficacy of meningococcus group A capsular polysaccharide vaccine in children three months to five years of age. The New England Journal of Medicine. 1977;297(13):686-91.

71. Vermont C, van den Dobbelsteen G. *Neisseria meningitidis* serogroup B: laboratory correlates of protection. FEMS Immunology and Medical Microbiology. 2002;34(2):89-96.

72. de Kleijn E, van Eijndhoven L, Vermont C, Kuipers B, van Dijken H, Rumke H, et al. Serum bactericidal activity and isotype distribution of antibodies in toddlers and schoolchildren after vaccination with RIVM hexavalent PorA vesicle vaccine. Vaccine. 2001;20(3-4):352-8.

73. Naess LM, Aarvak T, Aase A, Oftung F, Hoiby EA, Sandin R, et al. Human IgG subclass responses in relation to serum bactericidal and opsonic activities after immunization with three doses of the Norwegian serogroup B meningococcal outer membrane vesicle vaccine. Vaccine. 1999;17(7-8):754-64.

74. Vermont CL, van Dijken HH, van Limpt CJ, de Groot R, van Alphen L, van Den Dobbelsteen GP. Antibody avidity and immunoglobulin G isotype distribution following immunization with a monovalent meningococcal B outer membrane vesicle vaccine. Infection and Immunity. 2002;70(2):584-90.

75. Bowe F, Lavelle EC, McNeela EA, Hale C, Clare S, Arico B, et al. Mucosal vaccination against serogroup B meningococci: induction of bactericidal antibodies and cellular immunity following intranasal immunization with NadA of *Neisseria meningitidis* and mutants of *Escherichia coli* heat-labile enterotoxin. Infection and Immunity. 2004;72(7):4052-60.

76. Parlane NA, Wedlock DN, Buddle BM, Rehm BH. Bacterial polyester inclusions engineered to display vaccine candidate antigens for use as a novel class of safe and efficient vaccine delivery agents. Applied and Environmental Microbiology. 2009;75(24):7739-44.

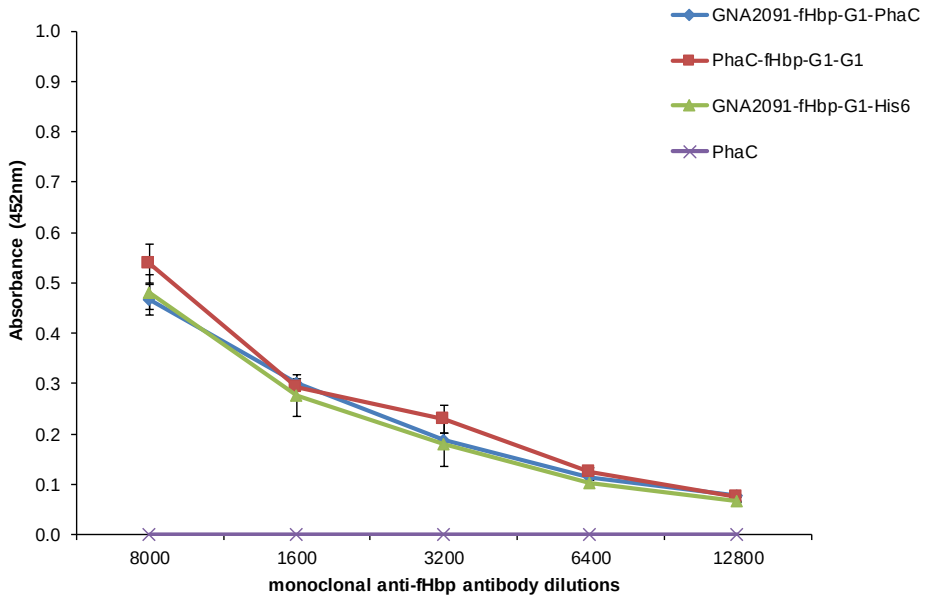
77. Grage K, Jahns AC, Parlane N, Palanisamy R, Rasiah IA, Atwood JA, et al. Bacterial polyhydroxyalkanoate granules: biogenesis, structure, and potential use as

nano-/micro-beads in biotechnological and biomedical applications. Biomacromolecules. 2009;10(4):660-9.

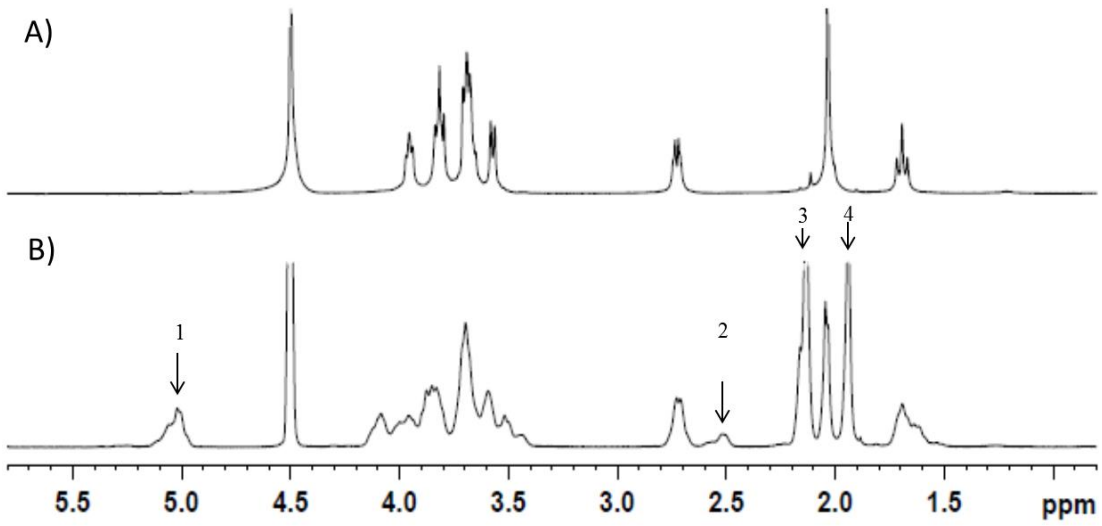
78. Peters V, Rehm BH. In vivo monitoring of PHA granule formation using GFP-labeled PHA synthases. FEMS Microbiology Letters. 2005;248(1):93-100.

79. Amara AA, Rehm BH. Replacement of the catalytic nucleophile cysteine-296 by serine in class II polyhydroxyalkanoate synthase from *Pseudomonas aeruginosa*-mediated synthesis of a new polyester: identification of catalytic residues. Biochemical Journal. 2003;374(Pt 2):413-21.

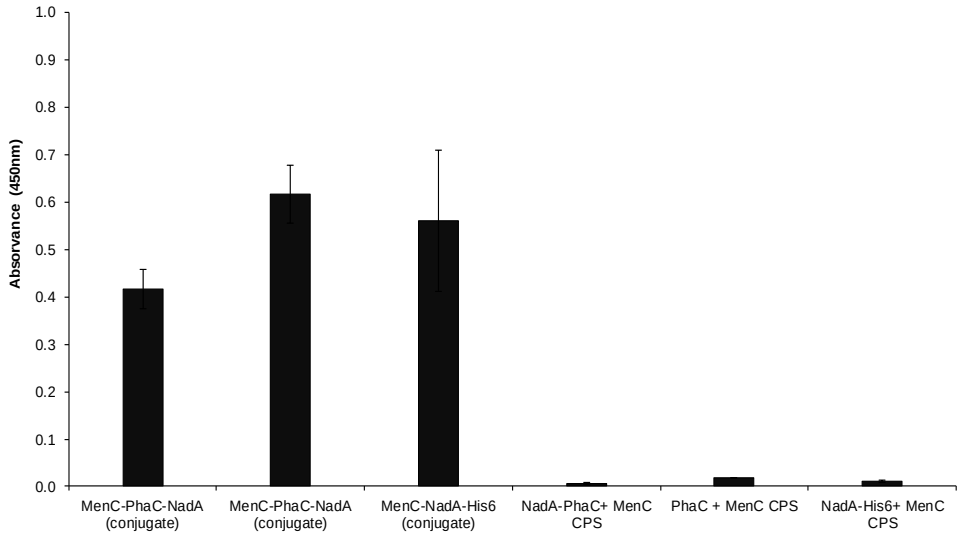
4.8 Supplementary material



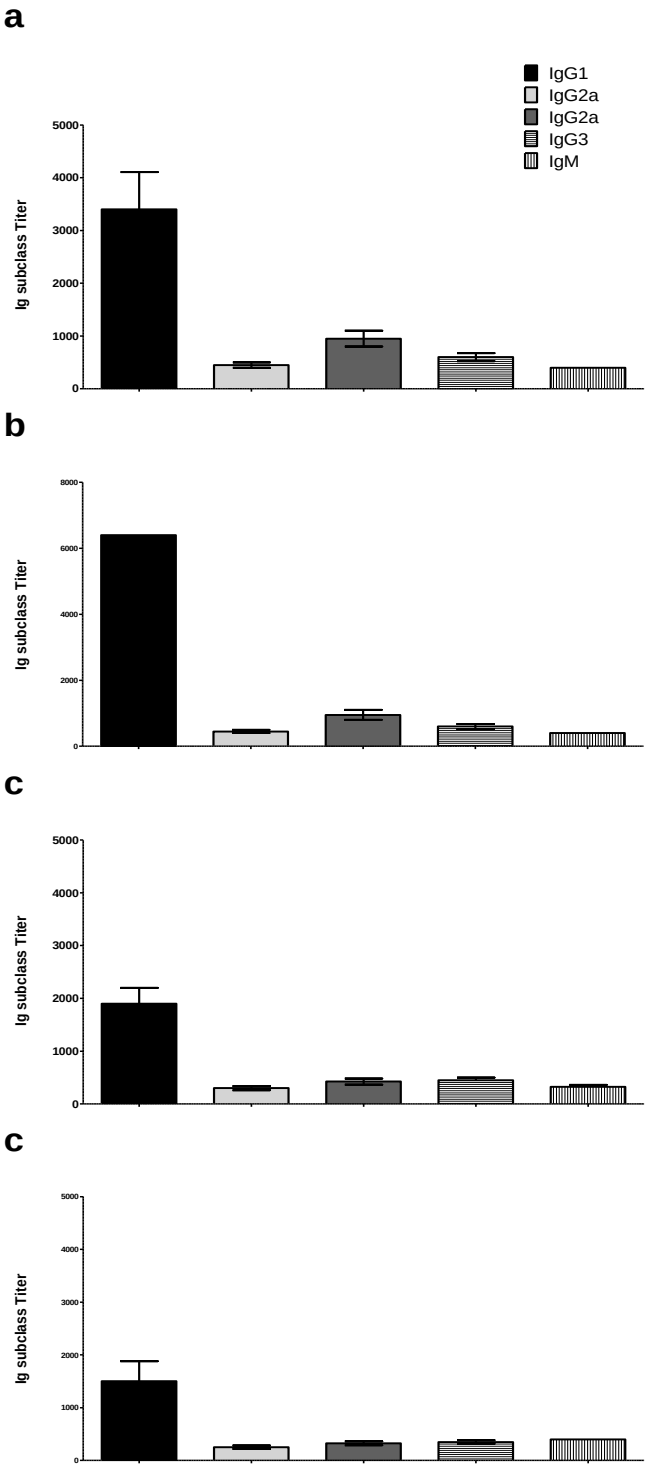
Supplementary Figure 1. FHbp confirmation of molecular identity and bead display by ELISA using a commercial monoclonal anti-fHbp antibody (JAR4, NIBCS, UK). Maxisorp 96-well plates (NUNC) were coated using 0.5 µg/ml of each protein and the rest of assay was performed as mentioned above (44). The results are expressed as the mean ± SD of two replicas per sample.



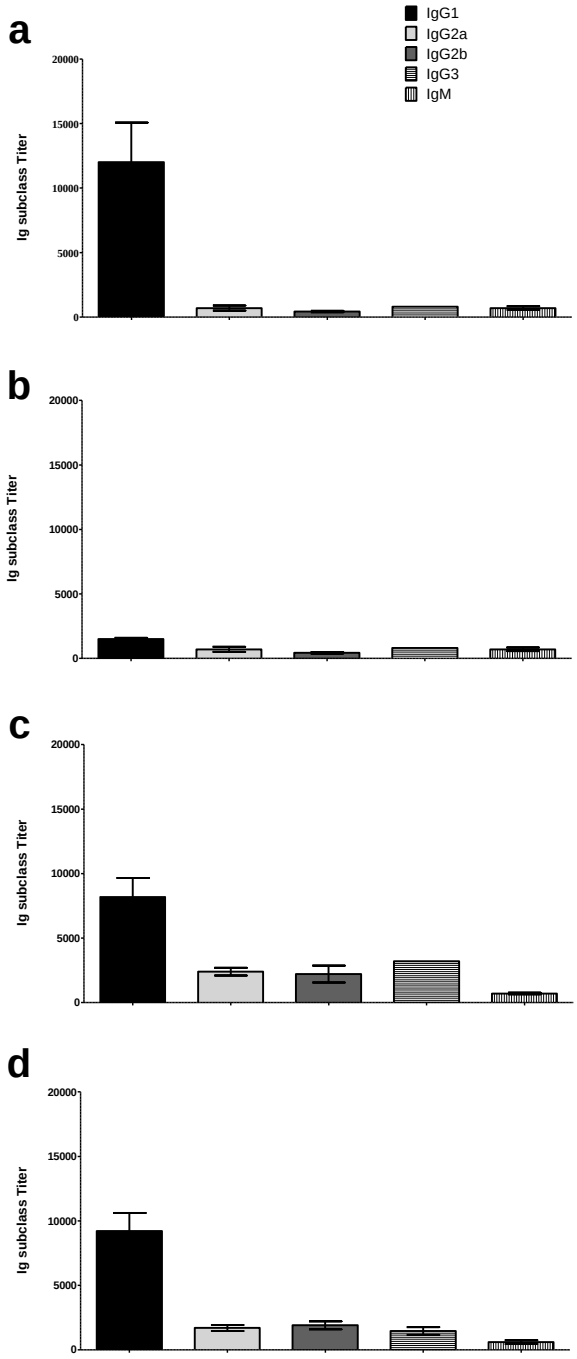
Supplementary Figure 2. 1H NMR monodimensional spectra of CPS. A) Activated polysaccharide (APS) and B) Capsular polysaccharide (CPS), both from *Neisseria meningitidis* Serogroup C. The peaks 1,2,3, and 4 are missing in the Aps spectrum because the O-Acetyl groups were removed during activation reaction, but the rest of the chemical structure is conserved after activation as mentioned elsewhere (41).



Supplementary Figure 3. MenC confirmation of molecular identity by ELISA using a commercial anti-CPS (MenC) monoclonal antibody (NIBS, UK). Maxisorp 96-well plates (NUNC) were coated with 5 µg/ml of MenC CPS conjugated or mixed NadA-PhaC beads, PhaC wild type beads or NadA-His6 the rest of assay was performed as mentioned above (44). The results are expressed as the mean ± SD of two replicas per sample.



Supplementary Figure 4. Assessment of IgG subclass binding to MenC evaluated by ELISA. Results are expressed as the mean ± SEM of 8 animals and the titer was calculated as mentioned above. a. animals vaccinated with 4 µg of MenC conjugated to NadA-PhaC beads; b. animals vaccinated with 4 µg of MenC conjugated to PhaC wild type beads; c. animals vaccinated with 4 µg of MenC conjugated to soluble NadA-His6; d. animals vaccinated with 4 µg of MenC conjugated to DT.



Supplementary Figure 5. Assessment of IgG subclass binding to NadA protein evaluated by ELISA. Results are expressed as the mean ± SEM of 8 animals and the titer was calculated as mentioned above. **a.** animals vaccinated with 4 µg of MenC conjugated to NadA-PhaC beads; **b.** animals vaccinated with 4 µg of MenC conjugated to soluble NadA-His6; **c.** animals vaccinated with 18 µg of protein from NadA-PhaC beads; **d**. animals vaccinated with 18µg of protein from NadA-PhaC beads without alum.

Supplementary Table 1. Strains, Plasmids and primers

Strains, plasmid and primers	**Features**	**Source**
Strains		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F´ proAB lacI*^{q}*ZΔM15 Tn10* (Tet^{r})]	Novagen
Clear Coli	*F– ompT hsdSB (rB - mB -) gal dcm lon λ (DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) msbA148 ΔgutQ ΔkdsD ΔlpxL ΔlpxM ΔpagP ΔlpxP ΔeptA (*LPS negative)	(77)
Plasmids		
pET-14b	*Ap^{R}* and T7 promoter	Novagen
pET-14b-phaC	pET-14b version, holding *phaC* gene fragment	(78)
pUC57-nadA	pUC57 version, ColE1 origin, *Ap^{R}* holding *Nde*I /*nadA* gene/*Spe*I.	GenScript
pUC57-gna2091-fhbp-g1	pUC57 version, ColE1 origin, *Ap^{R}* holding *Nde*I */gna2091-fhbp-g1* gene/*Spe*I.	GenScript
pUC57-fhbp-g1-g1	pUC57 version, ColE1 origin, *Ap^{R}* holding *Xho*I/ *fhbp-g1-g1*gene/ *BamH*I	GenScript
pMCS69	CmR; T7 promoter, pBBR1MCS derivative containing *phaA* and *phaB* genes from *Ralstonia eutropha* co-downstream to lac promoter	(79)
pET14b_NanA_PhaC (reversed)	Ampicillin resistance and T7 promotor, holding *nana* gene fused translationally to 3' end of phaC gene	(42)
pET-14b-nadA-phaC	pET-14b-phaC version, holding *nadA* gene fused translationally to 3' end of *phaC* gene	This study
pET-14b-gna2091-fhbp-phaC	pET-14b-phaC version, holding *gna2091-fhbp-g1* gene fused translationally to 3' end of *phaC* gene	This study
pET-14b-phaC-fhbp-g1-g1	pET-14b-phaC version, holding *fhbp-g1-g1* gene fused translationally to 5' end of *phaC* gene	This study
pET14b-nadA-hist6	Ampicillin resistance and T7 promotor, holding a *Nde*I/ *nadA hist-tag* /*BamH*I gene.	This study
pET14b-gna2091-fhbp-g1-hist6	Ampicillin resistance and T7 promotor, holding *Nde*I/ *gna2091-fhbp-g1-his-tag*/*BamH*I gene.	This study
Primers		
nadA fwr (*Nde*I, underline restriction site)	5'AAACATATGGCTACCTCAGATGATGATGTCAAAA AGGCGG3'	This study
nadA rev (*BamH*I, underline restriction site)	5'AAAGGATCCTCAGTGGTGGTGGTGGTGGTGTGCT TTCACTTTTGCGTCCACATTTTGTTTGTTTTCATTCA C3'	This study
gna2091-fhbp-g1 fwr (*Nde*I, underline restriction site)	5'AAACATATGTGCGTTTCGGCGGTTATTGGCTCAG CGGCGGTTG3'	This study
gna2091-fhbp-g1 rev (*BamH*I, underline restriction site)	5'AAAGGATCCTCAGTGGTGGTGGTGGTGGTGCTGT TTTGCCGCCAGACCAATGTGACGAATACCGTTGAC C 3'	This study

Supplementary Table 2. Identification of fusion proteins by peptide fingerprinting analysis (MALDI-TOF/MS).

Fusion proteins	**Peptides fragment identified by MALDI-TOF/MS.***
NadA-PhaC	**A11-K47, L134-K146, Q267-R309, A317-K341,** V482-R498, I535-K552, F608-641, I657-K691, Y712-K874, L928-K979, R1025-R1035
GNA2091-fHbp-G1-PhaC	**T31-R45, G52-R64, Q78-105, V170-R180, D212-R224, F259-R263, I315-R347, S388-R395, G405-R414,** V641-R476, I503-R514, A531-R565, F586-R620, I635-R647, Y690-R745, E811-R826, H873-K938, F940-K958, A1004-R1023
PhaC-**fHbp-G1-G1**	A2-W31, A59-Y65, L161-L171, T191-T218, T232-L240, V262-F273, I305-L316, A333-L346, R386-W404, L546-W554, R575-Y584, **A592-I638, T643-Y665, T725-M731, R738-Y77, K793-Y822**

* Red bold, identified peptides belonging to the respective neisserial antigen.

Supplementary Table 3. Correlation between Zeta potential and pH of various PHB beads.

PHB beads	**Theoretical pI of the respective protein***	**Zeta potential (mV) pH (3.5) ****	**Zeta potential (mV) pH (5.5) ****	**Zeta potential (mV) pH (7.5) ****
NadA-PhaC on PHB beads	5.01	5.20 ± 0.9	-13.3 ± 2.2	-20.4 ± 3.2
GNA2091-fHbp-G1-PhaC on PHB beads	6.77	16.2 ± 1.4	-8.9 ± 0.9	-16.5 ± 2.5
PhaC-fHbp-G1-G1 on PHB beads	6.21	11.1 ± 2	-8.4 ± 0.7	-17.9 ± 2
PhaC, (non-antigen displaying PHB beads)	6.08	14.4 ± 1.8	-5.7± 0.9	-16.8± 0.35

* ExPASy - Compute pI/Mw tool (theoretical isoelectric point (pI)); ** mean of 3 replicates ± SD.

Supplementary Table 4. Amount of neisserial antigen attached to PHB beads and immunization doses.

PHB beads	neisserial antigen/ wet Bead (µg/mg)	neisserial antigen immunization dose (µg)	PHB beads immunization dose (mg)
NadA-PhaC	0.254	2	10
GNA2091-fHbp-G1-PhaC	0.677	7	10
PhaC-fHbp-G1-G1	0.110	1	10
PhaC, (non-antigen displaying)	0.977	7	10

Supplementary Table 5. Carbohydrate/protein ratios and carbohydrate yield after conjugation and purification.

Conjugated	Carbohydrate/protein ratio (mg/mg)	Carbohydrate yield (%)
MenC-NadA-PhaC beads	0.20/1	4.0
MenC-PhaC beads	0.27/1	4.4
MenC-NadA-His6	2.4/1	30.2
MenC-DT	2.5/1	36.0

Supplementary Table 6. Conjugation site analysis results by liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS). Tryptic and chymotryptic digests of non-conjugated and conjugated proteins either soluble or attached to PHB beads were analysed by liquid chromatography-coupled LTQ-Orbitrap tandem mass spectrometry. Significant differences in peptide abundances between the non-conjugated and conjugated proteins were identified by comparing precursor peak intensities using the SIEVE software (ThermoScientific).

Samples	**Chymotryptic (C)/tryptic (T) peptides >3-fold more abundant in non-conjugated sample versus conjugated sample based on LC/MS-MS analysis***	**Amino acid sequence of targeted protein showing putative conjugations sites in bold**
PhaC versus conjugated PhaC displayed on PHB beads (C)	NARALTELADAVEADAKTRQRIRF GNARYRAIEPAPGRYVKAKA TGKGAAASTQEGKSQPF+Carbamidomethyl (13) Formyl (3) AAIRAIEVARDISGQDKINVL RAIEPAPGRYVKAKA	PhaC (64.2kDa) MATG**K**GAAASTQEG**K**SQPFK VTPGPFDPATWLEWSRQWQG TEGNGHAAASGIPGLDALAG VKIAPAQLGDIQQRYMKDFS ALWQAMAEGKAEATGPLHD RRFAGDAWRTNLPYRFAAAF YLLNARALTELADAVEADA**K** TRQRIRFAISQWVDAMSPANF LATNPEAQRLLIESGGESLRA GVRNMMEDLTRGKISQTDES AFEVGRNVAVTEGAVVFENE YFQLLQYKPLTDKVHARPLL MVPPCINKYYILDLQPESSLV RHVVEQGHTVFLVSWRNPDA SMAGSTWDDYIEHAAIRAIEV ARDISGQD**K**INVLGFCVGGTI VSTALAVLAARGEHPAASVT LLTTLLDFADTGILDVFVDEG HVQLREATLGGGAGAPCALL RGLELANTFSFLRPNDLVWN YVVDNYLKGNTPVPFDLLFW NGDATNLPGPWYCWYLRHT YLQNELKVPGKLTVCGVPVD LASIDVPTYIYGSREDHIVPWT AAYASTALLANKLRFVLGAS GHIAGVINPPAKNKRSHWTN DALPESPQQWLAGAIEHHGS WWPDWTAWLAGQAGAKRA APANYGNARYRAIEPAPGRY V**K**A**K**A Lysine residues (labelled bold) represent putative polysaccharide conjugations sites: **K:** 5, 15, 139, 312, 498, 586, 588
NadA-PhaC versus conjugated NadA-PhaC displayed on PHB beads (C)	AAIRAIEVARDISGQDKINVL ATSDDDVKKAATVAI ATSDDDVKKAATVAIVAA DVEADDFKGL NARALTELADAVEADAK NARALTELADAVEADAKTR	NadA (1-463)-PhaC (464-1051) fusion protein (112.8kDa) MATSDDDV**KK**AATVAIVAA YNNGQEINGFKAGETIYDIGE DGTITQKDATAADVEADDF**K** GLGLKKVVTNLT**K**TVNEN**K**Q

	TKTVNENKQNVDAKVK	NVDA**K**V**K**AAESEIEKLTTKL ADTDAALADTDAALDETTNA LNKLGENITTFAEETKTNIVKI DEKLEAVADTVDKHAEAFND IADSLDETNTKADEAVKTAN EAKQTAEETKQNVDAKVKA AETAAGKAEAAAGTANTAA DKAEAVAAKVTDIKADIATN KADIAKNSARIDSLDKNVANL RKETRQGLAEQAALSGLFQP YNVGRFNVTAAVGGYKSESA VAIGTGFRFTENFAAKAGVA VGTSSGSSAAYHVGVNYEWK AGETIYDIGEDGTITQKDKAG ETIYDIGEDGTITQKDKAGETI YDIGEDGTITQKDTSDDDVKK AATVAIVTSDDDVKKAATVA IVT**K**TVNEN**K**QNVDA**K**V**K**A T**K**TVNEN**K**QNVDA**K**V**K**ATS ATGKGAAASTQEGKSQPFKV TPGPFDPATWLEWSRQWQGT EGNGHAAASGIPGLDALAGV KIAPAQLGDIQQRYMKDFSAL WQAMAEGKAEATGPLHDRR FAGDAWRTNLPYRFAAAFYL LNARALTELADAVEADA**K**TR QRIRFAISQWVDAMSPANFLA TNPEAQRLLIESGGESLRAGV RNMMEDLTRGKISQTDESAF EVGRNVAVTEGAVVFENEYF QLLQYKPLTDKVHARPLLMV PPCINKYYILDLQPESSLVRHV VEQGHTVFLVSWRNPDASMA GSTWDDYIEHAAIRAIEVARD ISGQD**K**INVLGFCVGGTIVST ALAVLAARGEHPAASVTLLT TLLDFADTGILDVFVDEGHVQ LREATLGGGAGAPCALLRGL ELANTFSFLRPNDLVWNYVV DNYLKGNTPVPFDLLFWNGD ATNLPGPWYCWYLRHTYLQ NELKVPGKLTVCGVPVDLASI DVPTYIYGSREDHIVPWTAAY ASTALLANKLRFVLGASGHIA GVINPPAKNKRSHWTNDALP ESPQQWLAGAIEHHGSWWPD WTAWLAGQAGAKRAAPANY GNARYRAIEPAPGRYVKAKA Lysine residues (labelled in bold) represent putative polysaccharide conjugations sites: **K**: 9, 10, *60*, *73*, 79, 85, *87*, 429. 435, 441, *443*, *446*, *452*, *458*, *460*, 601 (139 in PhaC), 774 (312 in PhaC) Italics: Conjugation sites not found in soluble NadA
NadA-PhaC versus conjugated	AATVAIVAAYNNGQEINGFK+Deamidated (11) Deamidated (12)	NadA (1-463)-PhaC (464-1051) fusion protein (112.8kDa)

NadA-PhaC displayed on PHB beads (T)	AATVAIVAAYNNGQEINGFK+Deamidated (12) AATVAIVAAYNNGQEINGFK+Deamidated (12) Deamidated (17) ASMAGSTWDDYIEHAAIR CVGGTIVSTALAVLAAR+Carbamidomethyl (1) DASMAGSTWDDYIEHAAIR DFSALWQAMAEGK DFSALWQAMAEGK+Carbamidomethyl (13) DFSALWQAMAEGKAEATGPLHDR EDHIVPWTAAYASTALLAN EDHIVPWTAAYASTALLANK EDHIVPWTAAYASTALLANKLR EDHIVPWTAAYASTALLANKLR+Carbamidomethyl (20) FAISQWVDAMSPANFLATNPEAQR+Oxidation (6) FAISQWVDAMSPANFLATNPEAQR+Oxidation (6) Oxidation (10) GLELANTFSFLR GVPVDLASIDVPTYIYGSR LADTDAALADTDAALDETTNALNK LADTDAALADTDAALDETTNALNK+Deamidated (23) LDETNTKADEAVKTANEAK+Carbamidomethyl (13) Deamidated (5) Formyl (19) NMMEDLTRGKISQTDESAFEVGR PATWLEWSR PDASMAGSTWDDYIEHAAIR PNDLVWNYVVDNYLK TDAALADTDAALDETTNALNK VTPGPFDPATWLEWSR VTPGPFDPATWLEWSR+Oxidation (11)	MATSDDDVKKAATVAIVAA YNNGQEINGF**K**AGETIYDIGE DGTITQKDATAADVEADDFK GLGLKKVVTNLTKTVNENKQ NVDAKVKAAESEIEKLTTKL ADTDAALADTDAALDETTNA LN**K**LGENITTFAEETKTNIVKI DEKLEAVADTVDKHAEAFND IADSLDETNT**K**ADEAVKTAN EA**K**QTAEETKQNVDAKVKA AETAAGKAEAAAGTANTAA DKAEAVAAKVTDIKADIATN KADIAKNSARIDSLDKNVANL RKETRQGLAEQAALSGLFQP YNVGRFNVTAAVGGYKSESA VAIGTGFRFTENFAAKAGVA VGTSSGSSAAYHVGVNYEWK AGETIYDIGEDGTITQKDKAG ETIYDIGEDGTITQKDKAGETI YDIGEDGTITQKDTSDDDVKK AATVAIVTSDDDVKKAATVA IVTKTVNENKQNVDAKVKAT KTVNENKQNVDAKVKATSA TGKGAAASTQEGKSQPFKVT PGPFDPATWLEWSRQWQGTE GNGHAAASGIPGLDALAGVK IAPAQLGDIQQRYMKDFSAL WQAMAEG**K**AEATGPLHDRR FAGDAWRTNLPYRFAAAFYL LNARALTELADAVEADAKTR QRIRFAISQWVDAMSPANFLA TNPEAQRLLIESGGESLRAGV RNMMEDLTRGKISQTDESAF EVGRNVAVTEGAVVFENEYF QLLQYKPLTDKVHARPLLMV PPCINKYYILDLQPESSLVRHV VEQGHTVFLVSWRNPDASMA GSTWDDYIEHAAIRAIEVARD ISGQDKINVLGFCVGGTIVST ALAVLAARGEHPAASVTLLT TLLDFADTGILDVFVDEGHVQ LREATLGGGAGAPCALLRGL ELANTFSFLRPNDLVWNYVV DNYL**K**GNTPVPFDLLFWNGD ATNLPGPWYCWYLRHTYLQ NELKVPGKLTVCGVPVDLASI DVPTYIYGSREDHIVPWTAAY ASTALLAN**K**LRFVLGASGHIA GVINPPAKNKRSHWTNDALP ESPQQWLAGAIEHHGSWWPD WTAWLAGQAGAKRAAPANY GNARYRAIEPAPGRYVKAKA Red: Potential polysaccharide conjugation sites sterically impacting on trypsin digest (sites are not part of identified peptide) K: 482, 601, 774, 913, 917 Lysine residues (labelled in bold) represent putative polysaccharide conjugations sites:

		K: 30, 123, 173, 185, 552 (90 in PhaC), 875 (413 in PhaC), 960 (498 in PhaC)
Soluble NadA versus conjugated soluble NadA (T)	AAETAAGKAEAAAGTAN AAETAAGKAEAAAGTANTAADKAEAVAAK AATVAIVAAYNNGQEINGFK AATVAIVAAYNNGQEINGFK+Deamidated (11) AATVAIVAAYNNGQEINGFK+Deamidated (12) Deamidated (17) AATVAIVAAYNNGQEINGFK+Deamidated (14) AGETIYDIGEDGTITQKDTSDDDVKK AGETIYDIGEDGTITQKDTSDDDVKK+Deamidated (16) AVGTSSGSSAAYHVGVNYEWK HAEAFNDIADSLDETNTK HAEAFNDIADSLDETNTKADEAVK+Deamidated (16) HAEAFNDIADSLDETNTKADEAVK+Deamidated (6) LADTDAALADTDAALDETTNALNK LADTDAALADTDAALDETTNALNK+Deamidated (20) LDETNTKADEAVKTANEAK+Carbamidomethyl (13) Deamidated (5) Formyl (19) LEAVADTVDKHAEAFNDIADSLDETNTK LGENITTFAEE LGENITTFAEETKTNIVK NITTFAEETK SAAYHVGVNYEWK SGSSAAYHVGVNYEWK SSGSSAAYHVGVNYEWK TDAALADTDAALDETTNALNK TIYDIGEDGTITQK TVAIVAAYNNGQEINGFK+ Deamidated (10) Deamidated (15) TVAIVTKTVNENKQNVDAK	NadA-His6: (49.2 kDa) MATSDDDV**KK**AATVAIVAA YNNGQEINGF**K**AGETIYDIGE DGTITQ**K**DATAADVEADDFK GLGLKKVVTNLTKTVNEN**K**Q NVDA**K**VKAAESEIEKLTTKL ADTDAALADTDAALDETTNA LN**K**LGENITTFAEET**K**TNIV**K**I DEKLEAVADTVD**K**HAEAFND IADSLDETNT**K**ADEAV**K**TAN EA**K**QTAEETKQNVDAKVKA AETAAG**K**AEAAAGTANTAA D**K**AEAVAA**K**VTDIKADIATN KADIAKNSARIDSLDKNVANL RKETRQGLAEQAALSGLFQP YNVGRFNVTAAVGGYKSESA VAIGTGFRFTENFAAKAGVA VGTSSGSSAAYHVGVNYEW **K**AGETIYDIGEDGTITQ**K**DKA GETIYDIGEDGTITQ**K**DKAGE TIYDIGEDGTITQ**K**DTSDDDV **KK**AATVAIVTSDDDVKKAAT VAIVT**K**TVNEN**K**QNVDA**K**V KATKTVNENKQNVDAKVKA HHHHHH Lysine residues (labelled in bold) represent putative polysaccharide conjugations sites: **K: 9**, **10**, **30**, 47, **79**, **85**, **123**, 136, 141, 155, **173**, 179, **185**, 208, 223, 341, 358, 377, 396, 404, 405, **429**, **435**, **441** (bold number show sites identified in the NadA when fused to PhaC)

*, Samples were prepared as previously described (43)

Supplementary Table 7. IgG/IgM ratio after first (1D) and third (3D) blood collection assayed against MenC**.**

Vaccinated groups	Mean IgG (1D)	Mean IgG (3D)	Mean IgM (1D)	Mean IgM (3D)	Ratio IgG/IgM (1D)	Ratio IgG/IgM (3D)
MenC-PhaC-NadA	231	3400	325	400	0.7	8.5
MenC-PhaC	200	5600	400	400	0.5	14.0
MenC-NadA-His6	125	2400	500	325	0.25	7.3
MenC-DT	100	1800	400	400	0.25	4.5

Supplementary Table 8. Size distribution of PHB beads in vaccine formulations (µm) as measured by dynamic laser scattering. Dx represents the particle diameter corresponding to X% cumulative particle size distribution

Samples	PHB bead in PBS 1X			PHB bead in Al $(OH)_3$		
	Dx (10)	Dx (50)	Dx (90)	Dx (10)	Dx (50)	Dx (90)
NadA-PhaC beads	0.6	6.55	60.9	0.832	7.38	50.4
GNA2091-fHbp-G1-PhaC beads	3.8	9.5	68.6	3.7	9.8	70.6
PhaC-fHbp-G1-G1 beads	0.4	3.87	41.9	0.438	4.22	58.6
PhaC wild type bead	0.4	1.2	6.99	0.434	2.77	18.4

Chapter 5. General Discussion, Conclusion, and Future work

5.1 General Discussion

Polyhydroxybutyrate is a polyester naturally produced by various bacteria (1). Introducing PHB biosynthesis genes into heterologous expression hosts allows the intracellular formation of discrete and spherical PHB inclusions (2). Translational fusion of proteins of interest to PHA synthase, PhaC, retained its PHB bead forming activity displaying the protein of interest at the PHB bead surface (3-9). Previous studies showed that PHB beads were bioengineered to display antigens from intracellular pathogens like TB and HCV. The TB antigens and HCV antigen displaying polyester beads triggered specific Th1 and Th2, and Th1 and Th17 immune response patterns in mice, respectively. Protective immunity was obtained in mice vaccinated with TB antigens displaying beads and when challenged with *Mycobacterium bovis* (10).

The current pneumococcal and meningococcal vaccines are subunit vaccines and although they show a safe and effective immunity profile. However, the antigens included in these formulations are weakly immunogenic and both vaccines require adjuvant and booster inoculation. For these reasons, the addition of improved adjuvant and/or immunogenic delivery systems is required to generate the desirable protective immunity (11).

In this thesis, an endotoxin-free strain of *E. coli* was engineered to produce PHB beads displaying pneumococcal proteins (PsaA, and Ply,) as well as neisserial proteins (NadA, and fHbp). In addition, to expand the antigen repertoire and to design a more broadly protective vaccine, CPS from *S. pneumoniae* serotype 19F or *N. meningitidis* serogroup C were chemically conjugated to the PHB beads.

All genes encoding the antigenic proteins were synthesized as codon–optimized for *E. coli* and fused to the *phaC* gene. An endotoxin free *E. coli* strain harbouring pMCS69 was transformed with the new plasmids encoding the hybrid genes. The presence of inclusions in cells harbouring plasmids encoding antigen-PhaC fusion proteins showed that fusion of PsaA, Ply, NadA and fHbp to PhaC did not impact in the PHB synthase activity. However, the size of the particles was affected by the antigens fused to PhaC; for example, PsaA-PhaC, NadA-PhaC, and GNA2091-fHbp-PhaC beads were smaller than PhaC wild type beads; while Ply-PhaC and fHbp-G1-G1-PhaC beads were bigger than PhaC wild type beads (Chapter 2-4). This result suggested that fusion of these antigenic proteins can have an effect on the self-assembly process. In addition, the surface charge of the neisserial antigen displayed on PHB and PsaA-PhaC was evaluated and the results showed a negative charge for all of them. It has been shown that the vaccine particle size and the surface charge have an effect on uptake by APCs as well as the subsequent antigen processing, which could impact the magnitude of the humoral or cellular immune responses (12, 13). In this thesis, the relation between an anionic surface contribution to antigen up take by APCs and the immune response after vaccination remains to be elucidated. The molecular identity of the fusion proteins was confirmed by MALDI-TOF/MS, Triple TOF and immunoblotting using monoclonal and polyclonal antibodies. PHB beads conjugated with CPS from *N. meningitidis* serogroup C were characterized by liquid chromatography-coupled LTQ-Orbitrap tandem mass spectrometry and the conjugation sites were revealed. New and previously reported amino acids (14) were identified at the surface of the PHB beads suggesting the presence of potential modification sites able to be used to display new biomedically relevant ligands.

To develop an efficient and broadly protective vaccine against extracellular pathogens such as *N. meningitidis* and *S. pneumoniae* the induction of a strong humoral immune response associated with bactericidal and opsonophagocytosis activity is desirable and should correlate with protective immunity (15-20). Additionally, cellular immunity is also required for clearance of these bacteria from the human body (21-23). To investigate the immunological properties of the new beads developed in this thesis, mice were vaccinated. The IgG titer evaluation showed that animals vaccinated with PsaA-PhaC and Ply-PhaC elicited a high and specific titer, recognizing the PsaA and Ply in the whole cell lysate from more than 5 different *S. pneumoniae* strains, and showing the serotype-independent recognition of these antibodies (Chapter 2 and 3). However, in pneumococcal protein-based vaccines, the protection correlation evidence is controversial. For this reason, most of the vaccines being studied undergo further pre-clinical challenge trials to prove efficacy. In this thesis, the correlation between antibody titer, serotype-independent recognition of the antigenic proteins, and protection is still to be elucidated. However, for a conjugate vaccine, the protection correlated is based on antibody titer and opsonophagocytic activity (24, 25). Here, 19F-PhaC beads induced the highest levels of opsonophagocytosis activity, which further suggests that PHB beads are an immunogenic antigen carrier system capable of inducing protective immunity (Chapter 3).

S. pneumoniae mouse infection models have shown the relevance of IL-17A and IFN-ɣ secretion for the development of a protective immune response (26, 27). In the early infection state, the production of IL-17 mediates the recruitment of macrophage and neutrophil precursors clearing bacteria from the nasopharyngeal tissue (21). In addition, several studies investigated the relevance of Ply in the cellular immunity against *S. pneumoniae*. A study which combined Ply with a TLR agonist showed a

correlation between splenocyte IL-17A, IFN-ɣ and IL-1β production and protection against infection in mice (28). In this thesis, stimulated splenocytes from animals vaccinated with Ply-PhaC beads produced a balanced INF-ɣ/IL-17A profile unlike animals vaccinated with soluble Ply, suggesting the potential of Ply-PhaC to promote cell-mediated protective immunity (Chapter 3).

In the case of *Neisseria meningitidis*, IgG antibody titer evaluation showed that proteinaceous as well as polysaccharide antigens displayed on PHB beads elicited high and specific titers in mice. In the case of NadA protein, animals vaccinated with that protein displayed on PHB beads, alone or co-displayed with MenC CPS, elicited statistically superior IgG titer than the soluble counterpart. In the case of CPS from serogroup C, the IgG antibodies were superior when this CPS was conjugated to PHB beads in comparison with when it was conjugated to tetanus toxoid or soluble NadA protein. The SBA titers against the three serogroups A, B, and C confirmed the superior performance of NadA and CPS co-displayed on PHB beads when compared to their soluble counterparts (Chapter 4). While NadA on PHB beads mediated protective immunity against serogroups A and B, the co-display of MenC expanded coverage to serogroup C. This suggested that combining protein and carbohydrate antigens on the surface of particulate carriers provides design space towards the development of broadly protective vaccines. In addition to this Th2-type immune response, cytokine analysis suggested that the MenC-NadA-PhaC and NadA-PhaC beads also induced a cell-mediated immune response, presumably of mixed Th17/Th1 mode, while soluble antigens induced Th1 but not Th17 responses (Chapter 4).

5.2 General Conclusion

Overall, this thesis shows that PHB beads displaying selected neisserial and pneumococcal antigens can be recombinantly produced in an endotoxin-free *E. coli* mutant and these PHB beads can be further modified by chemical conjugation to co-display carbohydrate antigens. Antigen-displaying PHB beads were immunogenic, mediating strong and specific humoral and cellular immune responses, leading to broadly protective immunity. Hence the PHB bead-based particulate vaccine approach holds the promise of a broadly protective vaccine that can be produced cost-effectively for widespread application to prevent diseases caused by *N. meningitidis* and *S. pneumoniae.*

5.3 Future work

The application of PHB beads as a vaccine to prevent infectious diseases by *N. meningitidis* and *S. pneumoniae* is a new approach. For this reason, improvements in PHB bead's design for specific administrations as well for tailoring immune response could be made.

5.3.1 Gene design

Future studies could aim to improve production of a fusion protein by optimization of its respective amino acid sequence. Displaying immunodominant peptides instead of the whole protein could lead to the increased production of the fusion protein at the PHB beads surface. In addition, the new gene design could result in an increase in the number copies of the antigenic peptides, which could enhance the immune response after vaccination. Previous studies showed that the protein position at the N or C terminus of the PhaC impacts the production of the fusion proteins (29). In this thesis, the location of encoding genes at the N terminus of PhaC resulted in the better production of PhaC fusion proteins, however, a new study should be made after new gene design.

5.3.2 New targets

Selection of other proteins from *S. pneumoniae* and *N. meningitidis* for display at PHB beads surface may provide a broader spectrum of immunity. Other pneumococcal antigens such as PspA and PhtD could investigate further (30, 31). The PspA is involved mainly in complement system evasion mechanisms while PhtD is involved in zinc-binding pathways (32, 33). Both proteins have been shown to elicit a protective immunity after immunization in pre-clinical and clinical studies (30, 34). In the case of *N. meningitidis, Neisseria* heparin binding protein (NHBA) is classified as

a major antigen present in the Bexsero vaccine (Novartis). The role of this protein in the pathogenicity of *N. meningitidis* is not clear but experimental data shows that *N. meningitidis* increases its survival time by interaction of NHBA and glycosaminoglycans. In addition, antibodies against NHBA in humans confirm protection against this bacteria (35).

5.3.3 Antigen Multivalency vaccine

The last generation of commercial meningococcal and pneumococcal vaccines present multivalency of CPS or proteins. The fusion of protein at the N and C terminus of the PhaC offers the flexibility to display more than one protein at the PHB bead surface (29, 36). This could result in fewer vaccinations and also in the reduction of the production costs.

5.3.4 Mucosal Immunity

Mucosal immunity in pneumococcal and meningococcal disease is an important factor which has not been addressed with commercial vaccines (37, 38). Elimination of carrier would also reduce the number of cases per annum. Oral or intranasally immunization with a particulate delivery system incorporating proteins aims to achieve mucosal immunity (39). Chitosan, PLA and PLGA are examples of these particulate vaccines administered orally (40). Hence oral immunization with PHB beads such as PsaA-PhaC and NadA-PhaC would be useful for studying the potential mucosal immunity.

5.3.5 Immunological studies

Development of *in vitro* assays to demonstrate protection induced by pneumococcal conjugate vaccines is based on antibodies and opsonophagocytic titers (13). However, for protein-based subunit vaccines, a well-defined assay is not

available. Hence for proteins, complement fixation and antibody neutralization assays are used to assess protective immunity (41- 44). In addition, challenge studies are one of the most frequently used ways to demonstrate protective immunity (45). As *S. pneumoniae* is an opportunist human pathogen these studies have to be performed under appropriate biosafety conditions. In this thesis, it was not possible to demonstrate by animal challenge studies protective immunity produced by PHB beads. Future studies might encompass *in vivo* challenge experiments in order to consolidate results from *in vitro* assays conducted in this study.

5.4 References

1. Rehm BH. Biogenesis of microbial polyhydroxyalkanoate granules: a platform technology for the production of tailor-made bioparticles. Current Opinion in Biotechnology. 2007;9(1):41-62.

2. Lee SY, Lee KM, Chan HN, Steinbuchel A. Comparison of recombinant *Escherichia coli* strains for synthesis and accumulation of poly-(3-hydroxybutyric acid) and morphological changes. Biotechnology and Bioengineering. 1994;44(11):1337-47.

3. Hooks D, Venning-Slater M, Du J, Rehm B. Polyhydroxyalkanoate synthase fusions as a strategy for oriented enzyme immobilisation. Molecules. 2014;19(6):8629.

4. Parlane NA, Grage K, Mifune J, Basaraba RJ, Wedlock DN, Rehm BH, et al. Vaccines displaying mycobacterial proteins on biopolyester beads stimulate cellular immunity and induce protection against tuberculosis. Clinical and Vaccine Immunology. 2012;19(1):37-44.

5. Draper JL, Rehm BH. Engineering bacteria to manufacture functionalized polyester beads. Bioengineered. 2012;3(4):203-8.

6. Grage K, Peters V, Rehm BHA. Recombinant protein production by *in vivo* polymer inclusion display. Applied and Environmental Microbiology. 2011;77(18):6706-9.

7. Parlane NA, Gupta SK, Rubio Reyes P, Chen S, Gonzalez Miro M, Wedlock DN, et al. Self-assembled protein-coated polyhydroxyalkanoate beads: properties and biomedical applications. ACS Biomaterials Science & Engineering. 2016.

8. Rehm FB, Chen S, Rehm BH. Enzyme Engineering for In Situ Immobilization. Molecules. 2016;21(10).

9. Rehm BH. Bacterial polymers: biosynthesis, modifications and applications. Nature Reviews Microbiology. 2010;8(8):578-92.

10. Parlane NA, Grage K, Mifune J, Basaraba RJ, Wedlock DN, Rehm BH, et al. Vaccines displaying mycobacterial proteins on biopolyester beads stimulate cellular immunity and induce protection against tuberculosis. Clinical and vaccine immunology. 2012;19(1):37-44.

11. Delany I, Rappuoli R, Seib KL. Vaccines, reverse vaccinology, and bacterial pathogenesis. Cold Spring Harbor perspectives in medicine. 2013;3(5):a012476.

12. Fifis T, Gamvrellis A, Crimeen-Irwin B, Pietersz GA, Li J, Mottram PL, et al. Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. The Journal of Immunology. 2004;173(5):3148-54.

13. Kanchan V, Panda AK. Interactions of antigen-loaded polylactide particles with macrophages and their correlation with the immune response. Biomaterials. 2007;28(35):5344-57.

14. Hooks DO, Rehm BH. Insights into the surface topology of polyhydroxyalkanoate synthase: self-assembly of functionalized inclusions. Applied microbiology and biotechnology. 2015;99(19):8045-53.

15. Goldschneider I, Gotschlich EC, Artenstein MS. Human immunity to the meningococcus. II. Development of natural immunity. Journal of Experimental Medicine. 1969;129(6):1327-48.

16. Peltola H, Makela H, Kayhty H, Jousimies H, Herva E, Hallstrom K, et al. Clinical efficacy of meningococcus group A capsular polysaccharide vaccine in children three months to five years of age. The New England Journal of Medicine. 1977;297(13):686-91.

17. Gill C, Ram S, Welsch J, Detora L, Anemona A. Correlation between serum bactericidal activity against *Neisseria meningitidis* serogroups A, C, W-135 and Y measured using human versus rabbit serum as the complement source. Vaccine. 2011;30(1):29-34.

18. Malley R, Trzcinski K, Srivastava A, Thompson CM, Anderson PW, Lipsitch M. CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(13):4848-53.

19. McCool TL, Weiser JN. Limited role of antibody in clearance of *Streptococcus pneumoniae* in a murine model of colonization. Infection and immunity. 2004;72(10):5807-13.

20. Lipsitch M, Whitney CG, Zell E, Kaijalainen T, Dagan R, Malley R. Are anti-capsular antibodies the primary mechanism of protection against invasive pneumococcal disease? PLoS Med. 2005;2(1):e15.

21. Zhang Z, Clarke TB, Weiser JN. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. The Journal of clinical investigation. 2009;119(7):1899-909.

22. Van Lookeren Campagne M, Wiesmann C, Brown EJ. Macrophage complement receptors and pathogen clearance. Cellular microbiology. 2007;9(9):2095-102.

23. Fang FC. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nature Reviews Microbiology. 2004;2(10):820-32.

24. De La Sante Om. Recommendations For The Production & Control Of Pneumococcal Conjugate Vaccines.

25. Väkeväinen M, Jansen W, Saeland E, Jonsdottir I, Snippe H, Verheul A, et al. Are the opsonophagocytic activities of antibodies in infant sera measured by different pneumococcal phagocytosis assays comparable? Clinical and diagnostic laboratory immunology. 2001;8(2):363-9.

26. Rubins JB, Pomeroy C. Role of gamma interferon in the pathogenesis of bacteremic pneumococcal pneumonia. Infection and immunity. 1997;65(7):2975-7.

27. Nakamatsu M, Yamamoto N, Hatta M, Nakasone C, Kinjo T, Miyagi K, et al. Role of interferon-γ in Vα14+ natural killer T cell-mediated host defense against *Streptococcus pneumoniae* infection in murine lungs. Microbes and infection. 2007;9(3):364-74.

28. Zhu J, Paul WE. CD4 T cells: fates, functions, and faults. Blood. 2008;112(5):1557-69.

29. Jahns AC, Rehm BH. The class I polyhydroxyalkanoate synthase from *Ralstonia eutropha* tolerates translational fusions to its C terminus: A new mode of functional display. Towards a better understanding of the polyhydroxyalkanoate synthase from *Ralstonia eutropha*: Protein engineering and molecular biomimetics. 2009:84.

30. Adamou JE, Heinrichs JH, Erwin AL, Walsh W, Gayle T, Dormitzer M, et al. Identification and characterization of a novel family of pneumococcal proteins that are protective against sepsis. Infection and immunity. 2001;69(2):949-58.

31. Wizemann TM, Heinrichs JH, Adamou JE, Erwin AL, Kunsch C, Choi GH, et al. Use of a whole genome approach to identify vaccine molecules affording protection against *Streptococcus pneumoniae* infection. Infection and immunity. 2001;69(3):1593-8.

32. Crain M, Waltman W, Turner J, Yother J, Talkington D, McDaniel L, et al. Pneumococcal surface protein A (PspA) is serologically highly variable and is

expressed by all clinically important capsular serotypes of *Streptococcus pneumoniae*. Infection and immunity. 1990;58(10):3293-9.

33. Mitchell A, Mitchell T. *Streptococcus pneumoniae*: virulence factors and variation. Clinical Microbiology and Infection. 2010;16(5):411-8.

34. Wu H-Y, Nahm MH, Guo Y, Russell MW, Briles DE. Intranasal immunization of mice with PspA (pneumococcal surface protein A) can prevent intranasal carriage, pulmonary infection, and sepsis with *Streptococcus pneumoniae*. Journal of Infectious Diseases. 1997;175(4):839-46.

35. Serruto D, Spadafina T, Ciucchi L, Lewis LA, Ram S, Tontini M, et al. *Neisseria meningitidis* GNA2132, a heparin-binding protein that induces protective immunity in humans. Proceedings of the National Academy of Sciences. 2010;107(8):3770-5.

36. Jahns AC, Haverkamp RG, Rehm BH. Multifunctional inorganic-binding beads self-assembled inside engineered bacteria. Bioconjugate chemistry. 2008;19(10):2072-80.

37. Mann JF, Acevedo R, Campo Jd, Pérez O, Ferro VA. Delivery systems: a vaccine strategy for overcoming mucosal tolerance? Expert review of vaccines. 2009;8(1):103-12.

38. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nature medicine. 2005;10(4s): S45.

39. Borges O, Cordeiro-da-Silva A, Tavares J, Santarém N, de Sousa A, Borchard G, et al. Immune response by nasal delivery of hepatitis B surface antigen and co-delivery of a CpG ODN in alginate coated chitosan nanoparticles. European Journal of Pharmaceutics and Biopharmaceutics. 2008;69(2):405-16.

40. Chen F, Zhang Z-R, Yuan F, Qin X, Wang M, Huang Y. In vitro and in vivo study of N-trimethyl chitosan nanoparticles for oral protein delivery. International Journal of Pharmaceutics. 2008;349(1):226-33.

41. Plotkin SA. Correlates of protection induced by vaccination. Clinical and Vaccine Immunology. 2010;17(7):1055-65.

42. Ginsburg AS, Nahm MH, Khambaty FM, Alderson MR. Issues and challenges in the development of pneumococcal protein vaccines. Expert review of vaccines. 2012;11(3):279-85.

43. Vilaseca Méndez JC, Tamargo Martínez I, Pérez Monrrás M. Producción y purificación parcial de la hemolisina principal (Neumolisina) de *Streptococcus pneumoniae*. Revista Cubana de Medicina Tropical. 1999;51(3):160-5.

44. Harvey RM, Ogunniyi AD, Chen AY, Paton JC. Pneumolysin with low hemolytic activity confers an early growth advantage to *Streptococcus pneumoniae* in the blood. Infection and immunity. 2011;79(10):4122-30.

45. Xin W, Li Y, Mo H, Roland KL, Curtiss R. PspA family fusion proteins delivered by attenuated *Salmonella enterica serovar Typhimurium* extend and enhance protection against *Streptococcus pneumoniae*. Infection and immunity. 2009;77(10):4518-28.

Appendix

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Majela Gonzalez Miro

Name/Title of Principal Supervisor: Professor Bernd Rehm

Name of Published Research Output and full reference:

Self-assembled particulate PsaA as vaccine against Streptococcus pneumoniae infection

In which Chapter is the Published Work: Chapter 2

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  Majela González Miró conceived, designed, performed, supervised the experiments. In addition, participated in the data analysis and interpretation and wrote the manuscript.

Majela Gonzalez-Miro Digitally signed by Majela Gonzalez-Miro Date: 2017.09.26 15:48:03 +13'00'

Candidate's Signature

2017/09/26

Date

Bernd Rehm Digitally signed by Bernd Rehm Date: 2017.10.10 12:42:59 +10'00'

Principal Supervisor's signature

10/10/2017

Date

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Majela González Miró

Name/Title of Principal Supervisor: Professor Bernd Rhem

Name of Published Research Output and full reference:

Bioengineered polyester beads co-displaying protein and carbohydrate-based antigens enhance protective efficacy against bacterial infection

In which Chapter is the Published Work: Chapter 4

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  M. G.-Miró, L.M.R.-N., M.F.-M., B.C.-M., S.-M.-Z., C.Z.-V., M.H.-C., T.K. performed the studies and analysed the data. D.G.-R., V.V.-B., M.G.-Miró., B.H.A.R. analysed and interpreted the data; contributed reagents, materials, analysis tools or data. M.G.-Miró. and B.H.A.R. conceived and designed and supervised the experiments. M.G.-Miró. and B.H.A.R. wrote the manuscript.

Majela Gonzalez-Miro — Digitally signed by Majela Gonzalez-Miro Date: 2018.02.05 14:34:30 +13'00'

Candidate's Signature

2018.02.05

Date

Bernd Rehm — Digitally signed by Bernd Rehm Date: 2018.02.07 09:42:43 +10'00'

Principal Supervisor's signature

07/02/2018

Date