

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

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- γ 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 Amariyls 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 Yanelly 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, Laura¹, Fariñas-Medina, Mildrey¹, García-Rivera, Dagmar¹, Vérez-Bencomo, Vicente¹, 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 Radecker², Laura M Rodríguez-Noda¹, Mildrey Fariñas-Medina¹, Caridad Zayas-Vignier¹, Mabel Heránndez-Cedeño¹, Yohana Serrano¹, Félix Cardoso¹, Darielys Santana-Mederos¹, Dagmar García-Rivera¹, Yury Valdés-Balbín¹, Vicente Vérez-Bencomo¹, 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-Noda¹, Mildrey Fariñas-Medina¹, Barbara Cedré-Marrero¹, Sandra-Madariaga-Zarza¹, Caridad Zayas-Vignier¹, Mabel Hernández-Cedeño¹, Torsten Kleffmann³, Dagmar García-Rivera¹, Vicente Vérez-Bencomo¹, 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

Abstract:	i
Acknowledgements	iv
Preface.....	vi
List of Figures	xiv
List of Tables.....	xvi
Abbreviations	xvii
Chapter 1. Introduction	1
1.1 <i>Streptococcus pneumoniae</i>	1
1.1.1 Pneumococcal diseases and epidemiology.....	1
1.1.2 Host immune defences against <i>Streptococcus pneumoniae</i>	2
1.1.3 Relevant pneumococcal virulence factors.....	3
1.1.4 Pneumococcal vaccines.....	5
1.2 <i>Neisseria meningitidis</i>	6
1.2.1 Meningococcal diseases and epidemiology	7
1.2.2 Host immune defences against <i>Neisseria meningitidis</i>	8
1.2.3 Relevant meningococcal virulence factors.....	9
1.2.4 Meningococcal vaccines	11
1.3 Adjuvants and delivery systems	12
1.4 Polyhydroxyalkanoate beads as potential particulate vaccines	13
1.5 General Hypothesis	18
1.6 Aims and scope of the thesis	18
1.7 References	19
Preface to the next Chapter	33
Chapter 2. Self-assembled particulate PsaA as vaccine against <i>Streptococcus pneumoniae</i> infection.....	34
2.1 Abstract	35

2.2	Introduction	36
2.3	Materials and methods.....	37
2.3.1	Bacterial strains, oligonucleotides, plasmids and cultivation conditions.....	37
2.3.2	Construction of plasmids mediating production of PHB beads displaying PsaA39	
2.3.3	Construction of the plasmid encoding N-terminally His-tagged PsaA for production of soluble PsaA	39
2.3.4	Production, isolation and purification of PHB beads.....	39
2.3.5	Production, isolation and purification of recombinant soluble protein.....	39
2.3.6	Confirmation of the PhaC in vivo activity using transmission electron microscopy (TEM).....	40
2.3.7	Protein analysis	40
2.3.8	Measurement of the PHA bead size distribution and zeta potential	40
2.3.9	Analysis of immunological properties of PHB beads	41
2.3.10	Statistical analysis	43
2.4	Results	43
2.4.1	Construction of plasmids mediating production of the PsaA-PhaC fusion protein and His6-PsaA.....	43
2.4.2	Production and characterization of PsaA displaying PHA beads	44
2.4.3	Humoral immune response.....	50
2.5	Discussion	54
2.6	Acknowledgements	57
2.7	References	58
2.8	Supplementary material.....	63
	Preface to the next Chapter	64
	Chapter 3. Biologically assembled polyester beads displaying pneumolysin and capsular polysaccharide induce protective immunity against <i>Streptococcus pneumoniae</i>	65
3.1	Abstract	66
3.2	Introduction	67

3.3	Materials and methods.....	68
3.3.1	Strains and cultivation conditions	68
3.3.2	Construction of plasmids for production of soluble Ply and Ply displayed on PHB beads.....	69
3.3.3	PHB bead production	69
3.3.4	Production of soluble Ply	69
3.3.5	Conjugation of CPS to PHB beads.....	70
3.3.6	Transmission electron microscopy (TEM).....	70
3.3.7	Proteins analysis by SDS-PAGE and immunoblot	70
3.3.8	Immune response evaluation.....	71
3.3.9	Statistical analysis	75
3.4	Results	75
3.4.1	Bioengineering <i>E. coli</i> for production of Ply-PHB beads and soluble His6-Ply	75
3.4.2	Immunological properties of the various PHB beads and soluble proteins	79
3.5	Discussion	87
3.6	Acknowledgements	91
3.7	References	93
3.8	Supplementary material.....	100
	Preface to the next Chapter	101
	Chapter 4. Bioengineered polyester beads co-displaying protein and carbohydrate-based antigens enhance protective efficacy against bacterial infection	102
4.1	Abstract	103
4.2	Introduction	104
4.3	Materials and Methods	106
4.3.1	Ethics statement.....	106
4.3.2	Construction of plasmids mediating production of PHB beads	107
4.3.3	Construction of plasmids for production of soluble His-tagged proteins	107
4.3.4	Production, isolation and purification of PHB beads and soluble proteins.....	107

4.3.5	Conjugation of MenC polysaccharide (CPS) to carrier proteins.....	108
4.3.6	Transmission electron microscopy analysis (TEM).....	108
4.3.7	Measurement of PHB bead size distribution and surface charge.....	108
4.3.8	NMR spectroscopy.....	108
4.3.9	Protein analysis	109
4.3.10	Protein and carbohydrate quantification.....	109
4.3.11	Immunization schedule for proteinaceous antigens	110
4.3.12	Immunization schedule for conjugated vaccine prototypes	110
4.3.13	Assessment of anti-NadA and anti-fHbp antibody titers in mice.....	111
4.3.14	Assessment of anti-MenC antibody titers in mice	111
4.3.15	Analysis of the Ig subclass profile in sera.....	111
4.3.16	Analysis of the cytokine production.....	112
4.3.17	Serum bactericidal assay	112
4.3.18	Statistical analysis	113
4.4	Results	114
4.4.1	Bioengineering of <i>Escherichia coli</i> for production of antigen-displaying PHB inclusions and soluble antigens.....	114
4.4.2	Immunological properties of antigen displaying PHB beads versus soluble antigens.....	119
4.4.3	Chemical conjugation of capsular polysaccharides to PHB beads.	120
4.4.4	Immunological properties of antigen-coated PHB beads displaying CPS.....	123
4.5	Discussion	129
4.6	Acknowledgements	135
4.7	References	136
4.8	Supplementary material.....	144
Chapter 5. General Discussion, Conclusion, and Future work		156
5.1	General Discussion.....	156
5.2	General Conclusion	160

5.3	Future work	161
5.3.1	Gene design	161
5.3.2	New targets.....	161
5.3.3	Antigen Multivalency vaccine	162
5.3.4	Mucosal Immunity	162
5.3.5	Immunological studies	162
5.4	References	164
	Appendix	169

List of Figures

Chapter 1

- Figure 1.** Electron microscopy image of *Pseudomonas aeruginosa* containing PHA granules. 14
- Figure 2.** The biosynthetic pathway of PHB production..... 15
- Figure 3.** PHB granules Self-assembly model. 16

Chapter 2

- 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*..... 44
- Figure 2.** TEM analysis of recombinant *ClearColi* cells (pMCS69) harbouring various plasmids and respective isolated PHB beads. 45
- Figure 3.** SDS-PAGE and immunoblot analysis of proteins attached to PHB beads and purified His6-PsaA..... 46
- Figure 4.** Immunological assessment of PsaA display on the PHB bead surface. 48
- Figure 5.** Correlation between zeta potential and pH of various PHB beads..... 50
- Figure 6.** Anti-PsaA IgG antibody response. 51
- Figure 7.** Isotype IgG profile evaluated by direct ELISA using ELISA plates coated with 0.5 µg of soluble His6-PsaA. 52
- 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..... 53
- Supplementary Figure 1.** SDS-PAGE of whole cell lysates of the various *S. pneumoniae* serotypes..... 63

Chapter 3

- Figure 1.** Schematic representation of genes encoding proteins relevant to this study.. 75
- Figure 2.** TEM images of *E. coli* with PHB inclusions and the corresponding purified PHB beads. 77
- Figure 3.** SDS-PAGE and immunoblot analysis of proteins attached to PHB beads as well as purified His6-Ply. 78
- Figure 4.** Analysis of induction of anti-Ply antibodies by various vaccine formulations. 80
- Figure 5.** IgG subclass profile as assessed by ELISA. 81
- Figure 6.** Cytokine profiles induced by various vaccine formulations. 82

Figure 7. Cross-reactivity of anti-Ply antibodies with Ply from various serotypes of <i>S. pneumoniae</i>	84
Figure 8. Schematic representation of conjugation reaction between activated serotype 19F polysaccharide and PhaC on PHB beads or soluble TT.	85
Figure 9. Analysis of anti-19F CPS antibody titers.	86
Figure. 10. The opsonophagocytic activity of sera against <i>S. pneumoniae</i> serotype 19F.	87
Supplementary Figure 1. SDS-PAGE of whole cell lysates of the various <i>S. pneumoniae</i> serotypes.....	100

Chapter 4

Figure 1. Biological production and characterization of antigen coated PHB beads...	115
Figure 2. PHB bead production and their immunogenicity.	117
Figure 2. PHB bead production and their immunogenicity (continued).	118
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.....	121
Figure 4. Structural models of PhaC depicting lysine residues proposed as sites conjugated to the activated polysaccharide.....	123
Figure 5. Immunogenicity studies of the various antigens conjugated to MenC.....	125
Figure 6. Bactericidal activity of various sera.....	128
Supplementary Figure 1. FHbp confirmation of molecular identity and bead display by ELISA using a commercial monoclonal anti-fHbp antibody (JAR4, NIBCS, UK).	144
Supplementary Figure 2. ¹ H NMR monodimensional spectra of CPS.	145
Supplementary Figure 3. MenC confirmation of molecular identity by ELISA using a commercial anti-CPS (MenC) monoclonal antibody (NIBS, UK).	145
Supplementary Figure 4. Assessment of IgG subclass binding to MenC evaluated by ELISA.	146
Supplementary Figure 5. Assessment of IgG subclass binding to NadA protein evaluated by ELISA.	147

List of Tables

Chapter 1

Table 1. Pneumococcal protein vaccines under study.....	6
--	---

Chapter 2

Table 1. Description of bacterial strains, plasmids and oligonucleotides used in this study.....	38
---	----

Table 2. Tryptic peptide fingerprinting analysis (MALDI-TOF/MS).....	47
---	----

Table 3. PHB bead yield and composition.....	49
---	----

Chapter 3

Table 1. Characteristics of plasmids and oligonucleotides used in this study.....	76
--	----

Table 2. Tryptic peptide fingerprinting analysis (Triple TOF).....	79
---	----

Table 3. PHB beads yields and the antigen/ mg of wet beads ratio.....	79
--	----

Chapter 4

Supplementary Table 1. Strains, Plasmids and primers.....	148
--	-----

Supplementary Table 2. Identification of fusion proteins by peptide fingerprinting analysis (MALDI-TOF/MS).	149
--	-----

Supplementary Table 3. Correlation between Zeta potential and pH of various PHB beads.....	149
---	-----

Supplementary Table 4. Amount of neisserial antigen attached to PHB beads and immunization doses.....	150
--	-----

Supplementary Table 5. Carbohydrate/protein ratios and carbohydrate yield after conjugation and purification.	150
---	-----

Supplementary Table 6. Conjugation site analysis results by liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS).....	151
---	-----

Supplementary Table 7. IgG/IgM ratio after first (1D) and third (3D) blood collection assayed against MenC.....	155
--	-----

Supplementary Table 8. Size distribution of PHB beads in vaccine formulations (μm) as measured by dynamic laser scattering.	155
--	-----

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
NaBH₃CN: 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