

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.

Filamentous phage-derived nano-rods for applications in diagnostics and vaccines

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

Doctor of Philosophy

In

Biochemistry

at Massey University, Palmerston North

New Zealand

Sadia Sattar

2013

Dedicated to my parents

Acknowledgements

“In the name of Allah, the Most Gracious, the Most Merciful”

First of all I would like to acknowledge my supervisor Dr. Jasna Rakonjac for her continuous support and critical evaluation of my work throughout my PhD. Indeed it was due to her encouragement and innovative thinking that polished my skills and enabled me to do well. I would also like to thank my cosupervisor Dr. Kathryn Stowell for her valuable feedback. A special thanks to my lab fellows Nicholas Bennett, Wesley Wen, Carel Jobsis and Julian Spagnuolo at helipad. I would also like thank the staff at Institute of Fundamental Sciences; their cooperation and kindness will be treasured always.

I would like to extend my gratitude to my sweet friends Sophia Khanum, Shazrah Salam and Amber Faisal for their continuous support, understanding, cooperation and care; they indeed made things easier for me at the time of stress by just being around apart from giving me valuable suggestions during my research and writing this thesis.

I would like to say thanks to Sadia Tahir, Saima Ejaz and Tina Sehrish for being a sweet part of my social circle in New Zealand. Their presence was treasured and valued always.

A special thanks to my family.

My parents remained a source of contentment for me always, particularly during this course of study. My father’s continuous encouragement, support and suggestions for my study throughout these five years is remarkable, I must say **Abbu** very few people can do what you have done for me and the entire family!! You are my biggest inspiration to excel further, thank you so much for being what you are. A special thanks to my **Ammi** for letting me pursue my dream of higher education and bearing my absence with patience, I know mom it was tough for you!

Not to forget the support of my sisters Tanzeela, Adeela, Nadia, and Asia along with the continuous encouragement of my brothers Ibrahim, Ismail, Ishaq and Yaqoob. Lastly I would like to say thanks to my nephew Zaid, and my nieces Eesha, Ayesha Mariyam and cute Rania for their lovely smiles in stressful days.

Abstract

Filamentous bacteriophage, as their name indicates are filament-like bacterial viruses. The F-pilus-specific filamentous phage of *Escherichia coli*, Ff (f1, M13 and fd) are resistant to heat, pH extremes and detergents. Their structural properties and amenability to engineering using recombinant DNA technology have enabled their extensive use in modern biotechnology. For example, Ff can be functionalized by displaying up to five different proteins and peptides on their surface. Ff phage have been successfully employed in diagnostic devices. Moreover, direct use as antigen-carriers is also a subject of interest in vaccine development. However, use of Ff-phage vaccines and in the at-home diagnostic devices is controversial, mainly because of their ability to replicate in gut *E. coli*, and possibility of mobilization and horizontal gene transfer of antibiotic resistance or virulence factor-encoding genes transfer among the gut and environmental bacteria. Moreover, the large length-to-diameter ratio of the virion (1000 nm x 6 nm) impairs diffusion of filamentous phage through complex matrices and could restrict use of filamentous phage in lateral flow diagnostic devices.

To overcome both of these problems we have constructed much shorter, rod-like functionalized particles (50 nm x 6 nm), named “Ff-nano”, which do not carry any genes. The properties of these short particles were investigated, showing that they have superior resistance to heating in the presence of ionic detergent sodium dodecyl sulphate (SDS) in comparison to the full-length phage of the same virion composition. The Ff-nano particles displaying a bacterial Fibronectin-Binding (FnB) protein as fusion to virion protein pIII, localized in five copies at one of the two ends of the virion, were produced and purified. These functionalized nanorods were tested in two applications: as detector particles in a dip-stick-type lateral flow device and as antigen carrier in a vaccine trial. The FnB-displaying nanorods were able to quantitatively detect fibronectin in solution. In the vaccine trial, the Ff-nano particles elicited a weak response to the FnB displayed at a low-copy-number at the nanorod end. In contrast, the response to the major protein pVIII was strong, indicating that the multi-copy display of antigenic peptides along the rod, as fusion to the major coat protein pVIII, is required for using the Ff-nano effectively as vaccine carriers.

Contents

Chapter 1	1
Literature Review	1
1.1 Biology of Filamentous Phage: Introduction	1
1.2 Ff Filamentous Bacteriophage Genome	5
1.3 Structure of Filamentous Phage Virion	9
1.3.1 Major Coat Protein pVIII	11
1.3.2 Minor Coat Proteins	13
1.4 Morphogenetic Proteins: pI, pXI, pIV	18
1.5 DNA Metabolism Proteins: pII, pX and pV	19
1.6 Ff Phage Life Cycle	19
1.6.1 Infection	20
1.6.3 Assembly and Secretion of Filamentous Phage Virions	25
1.7 Applications of Ff Filamentous Phage.....	26
1.7.2 Application of Filamentous Phage in Diagnostics	30
1.7.3 Application as Recombinant Vaccine Carriers	31
1.8 Microphage / Nanophage.....	32
1.9 Aims of the Project.....	38
Chapter 2	40
Materials and Methods	40
2.1 Bacterial Strains and Growth Conditions.....	40
2.2 Plasmids, Oligonucleotides, Phage and Recombinant DNA Methods.....	41
2.2.1 Recombinant Methods and Construction of Recombinant Plasmids and Phage ...	42
2.2.2 Plasmid DNA Isolation.....	42
2.2.3 Construction of Rnano3FnB Phage Displaying the Fibronectin Binding (FnB) Domain of <i>Streptococcus pyogenes</i>	43
2.2.4 Construction of MBP-FnB Fusion	45
2.3 Phage Strains and General Phage Growth and Quantification Methods...45	
2.3.1 Preparation of Phage Stocks	45
2.3.2 Titration of Infectious Phage Particles	46
2.3.3 Titration of Phagemid Particles.....	46
2.3.4 Quantification of Virions by Agarose Gel Electrophoresis of Phage Particles	46

2.4 Growth, Concentration and Purification of Nanophage	47
2.4.1 Differential PEG Precipitation	47
2.4.2 Phage Preparative Agarose Gel electrophoresis.....	48
2.4.3 Electroelution of Phage Particles	48
2.4.4 Cesium Chloride Density Gradient Purification of Phage Particles.....	50
2.5 Protein Purification and Detection	50
2.5.1 Purification of MBP-FnB Fusion	50
2.5.2 Detection of Proteins by SDS-PAGE and Western Blotting.....	51
2.6 ELISA Assays	51
2.6.1 Phage ELISA Assay	51
2.6.2 MBP-FnB – Fibronectin Interactions Analyzed by ELISA.....	52
2.6.3 Evaluation of Antibody Response by ELISA.....	52
2.7 Use of Nanophage in Immunodiagnostic Devices: Dipsticks.....	53
2.7.1 Preparation of Dipsticks.....	53
2.7.2 Assay Procedure.....	54
2.7.3 Fluorescein Isothiocyanate (FITC) Labeling of Phage.....	56
2.8 Vaccination Protocols	56
2.8.1 Animals.....	56
2.8.2 Preparation of Large and Nanophage for Immunization	56
2.8.4 Challenge.....	58
2.8.5 Sample Collection	58
2.8.6 Statistical Analysis.....	58
Chapter 3	59
Investigation and Modifications of the Nanophage Production System	
.....	59
3.1 Introduction	59
3.2 Development of an Improved Nanophage Production Protocol	59
3.2.1 Purification of Nanophage	59
3.2.2 Cesium Chloride Gradient Purification	60
3.2.3 Improved Nanophage Recovery after Preparative Agarose Gel Electrophoresis	63
3.3 Stability of Nanophage to Sodium Dodecyl Sulphate (SDS) at 70 °C.....	66
3.4 Construction of a Nanophage-Display System	69
3.5 Testing a Helper Phage-Free System for the Nanophage Production.....	72

Chapter 4	78
Applications of Nanophage in Diagnostic assays: ELISA and Dipstick	78
4.1. Introduction	78
4.2. Fibronectin-binding Activity Assay	79
4.3 Detection of Soluble Analyte in a Sample Solution Using Functionalized Nanophage and Full-length Phage Particles in a Dipstick Format	83
4.4 Comparison of FnB-displaying Nanophage with Full-length Phage in a Dip-stick Assay	87
4.5. Quantitative Measurement of Analyte Using Dipstick Competition Assay	89
4.6. Quantitative Measurement of Analyte Using Non-Competitive Assay in Dipstick Format.....	92
Chapter 5	94
Application of Nanophage in Vaccine Design	94
5.1 Introduction	94
5.2 Large-scale Purification of Nanophage and Full-length Phage Particles for Immunization.....	95
5.3 Gauging the Nano- and Full-length Particles for Vaccine Trial.....	96
5.4 Vaccination Trial Design and Immunization Schedule	96
5.5 Immune Response against Phage Proteins.....	99
5.5.1 Phage-specific Humoral Immune Response	99
5.5.2 Phage-specific IgG Antibody Subclasses	104
5.5.3 Variation in Phage-specific IgG Subclass Antibody within Each Vaccine Group ...	108
5.5.4 Phage-specific IgG Subclass Profile of Individual Mice in Each Vaccine Group	112
5.6 Immune Response Against Phage-displayed Fibronectin Binding Domain of <i>Streptococcus pyogenes</i>.....	115
5.6.1 Experimental Design	115
5.6.2 FnB Domain-specific General IgG for All Vaccination Groups	115
5.6.3 FnB Domains-specific IgG Subclass Antibody Response	120
5.6.4 Variation in FnB Domain-specific IgG Subclass Antibody Response within Each Vaccine Group.....	123
5.7 Comparison of Anti-Phage and Anti-FnB Domain General IgG Antibody Response of Individual Mice in Different Groups	128
5.8 Comparison of Antibody Response against Displayed FnB domain	132

5.9 Challenge Studies	135
Chapter 6	136
Discussion	136
6.1 Introduction	136
6.2 Improved Purification Technique for Nanophage Particles	136
6.3 Stability of Nanophage Particles	140
6.4 Helper-phage-free Production of Nanophage Particles	141
6.5 Investigation of Existing Nanophage Production System.....	142
6.6 Applications of Nanophage.....	143
6.6.1 Applications in Diagnostic Tests ELISA and Dipstick	143
6.6.2 Application as Antigen Carriers.....	145
6.7 Risk Assessment for Commercial Use of Nanophage	153
6.8 Conclusions	154
6.9 Future Directions.....	154
References.....	156

List of Figures

Figure 1.1 Map of f1 genome.	7
Figure 1.2 Structure of filamentous phage virion	10
Figure 1.3 Ribbon model of major coat protein pVIII.....	12
Figure 1.4 Schematic presentation of pIII.....	17
Figure 1.5 Schematic overview of filamentous phage life cycle.....	23
Figure 1.6 Secondary structure of filamentous phage intergenic region (IG).	24
Figure 1.7 Engineered origin of replication for microphage (nanophage) production.	35
Figure 1.8 Electron micrographs of nanophage particles.	36
Figure 2.1 Schematic presentation of construction of Rnano3FnB from Rnano.	44
Figure 2.2 Outline of the nanophage production and purification protocol.	49
Figure 2.3 Custom-made dipstick device.....	55
Figure 3.1 Separation of the nanophage and full-length helper phage by CsCl-density gradient ultracentrifugation.....	62
Figure 3.2 Gel used for quantification of electroeluted phage by agarose gel electrophoresis	64
Figure 3.3 Resistance of nanophage to heating in SDS vs. full-length (helper) phage.	68
Figure 3.4 Comparison of nanophage particles produced by Rnano3 and R408-3 as helper phage	71
Figure 3.5	77
Figure 4.1 Schematic representation of the nanophage production-display system....	80
Figure 4.2 Schematic presentation of ELISA assay setup for detecting immobilized analyte (fibronectin).....	81
Figure 4.3 Binding of FnB-domain displaying full length and nanophage particles to immobilized analyte (fibronectin) in ELISA setup.....	82
Figure 4.4 Schematic presentation of dipstick immunoassay developed to test the potential of nanophage particles in diagnostics	85
Figure 4.5 Detection of fibronectin in a test solution using a dip-stick assay	86
Figure 4.6 Dipstick assay for nanophage using collagen as fibronectin control reagent on test line	88

Figure 4.7 Schematic presentation of dipstick competition assay for quantitative detection of fibronectin using nanophage particles	90
Figure 4.8 Dipstick analyte competition assay for nanophage particles.....	91
Figure 4.9 Non-competitive detection of analyte in dipstick format using nanophage particles.....	93
Figure 5.1 Schematic presentation of vaccine trial design	98
Figure 5.2 Phage-specific IgG, inter-group comparison.....	101
Figure 5.3 Phage-specific IgM antibody inter-group comparisons.	102
Figure 5.4 Comparison of phage-specific IgG, IgM and IgA response.....	103
Figure 5.5 Phage-specific IgG1 antibodies	105
Figure 5.6 Phage-specific IgG2a antibody responses.....	106
Figure 5.7 Phage-specific IgG2b antibody responses.....	107
Figure 5.8 In-group comparisons of phage-specific IgG subtypes in full-length phage vaccines.....	109
Figure 5.9 In-group comparison of phage-specific IgG subtypes in the nanophage vaccine groups	110
Figure 5.10 Phage-specific IgG subtype profile in all vaccine groups	111
Figure 5.11 Phage-specific IgG subtype profile in individual animals in full-length phage vaccinated animals	113
Figure 5.12 Phage-specific IgG subtype profile in individual animals in the nanophage vaccine groups.	114
Figure 5.13 IgG antibodies (all subclasses) against FnB domain	118
Figure 5.14 Western-blotting detection of FnB-pIII fusion in the full-length phage and nanophage.	119
Figure 5.15 IgG1 antibodies against FnB domain.	121
Figure 5.16 IgG2a antibodies against phage-displayed FnB-domain.....	122
Figure 5.17 Comparison of FnB domain-specific IgG subtypes in group II.	124
Figure 5.18 Comparison of FnB domain-specific IgG subtypes in group III.....	125
Figure 5.19 FnB domain-specific IgG subtype profile in all vaccine groups.....	126
Figure 5.20 FnB domain-specific IgG subtype profile in individual animals.	127
Figure 5.21 Comparison of anti-phage IgG and anti-FnB domain IgG antibodies in individual animals in full-length phage vaccine groups.	130
Figure 5.22 Comparison of anti-phage IgG and anti-FnB domain IgG antibodies in individual animals in nanophage vaccine groups.	131

Figure 5.23 Comparison of IgG antibody responses against phage proteins in full-length and nanophage vaccine groups	133
Figure 5.24 Comparison of IgG antibodies against FnB domain in full-length and nanophage vaccine groups	134
Figure 6.1 Transmission Electron Micrographs (TEM) of purified nanophage sample EPN1	139

List of Tables

Table 1.1 Filamentous bacteriophage and their life styles.....	4
Table 1.2 Ff filamentous phage genes/proteins and their properties	8
Table 1.3 Comparison of nanophage properties with full-length Ff phage particles...37	
Table 2.1 Bacterial strains.....	40
Table 2.2 Plasmids	41
Table 2.3 Oligonucleotides used for cloning	42
Table 2.4 Phage strains	45
Table 2.5 Experimental protocol for immunization.....	57
Table 3.1 Purification of the nanophage by native agarose gel electrophoresis and electroelution.....	65