A New Approach to Antigen Delivery

Using the Nanoparticle Templating System

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

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**Declaration**

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and the ethic procedures and guidelines has been followed.

Aya Clara Taki

Date:
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Aya
For papa and mama

パパとママへ
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<thead>
<tr>
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<tr>
<td>6xHis</td>
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<tr>
<td>°C</td>
<td>Degrees centigrade</td>
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<tr>
<td>&lt;</td>
<td>Less than</td>
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<tr>
<td>&gt;</td>
<td>Greater than</td>
</tr>
<tr>
<td>≤</td>
<td>Less than or equal to</td>
</tr>
<tr>
<td>AIDS</td>
<td>The acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>AMP</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
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<tr>
<td>APCs</td>
<td>Antigen presenting cells</td>
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<tr>
<td>APTES</td>
<td>(3-aminopropyl)triethoxysilane</td>
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<tr>
<td>BET</td>
<td>Brunauer–Emmett–Teller</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>C-terminus</td>
<td>Carboxyl terminus domain of an amino acid sequence/protein</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
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<tr>
<td>CLSM</td>
<td>Confocal laser scanning microscope</td>
</tr>
<tr>
<td>CM</td>
<td>Chloramphenocal</td>
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<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
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<td>Con A</td>
<td>Concanavalin A</td>
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<td>CpG</td>
<td>Cytosine-phosphate-guanine motif</td>
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<td>CTL</td>
<td>Cytotoxic T cell</td>
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<td>CV</td>
<td>Column volume of resin used in chromatography</td>
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<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
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<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediamine tetra acetate</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ERAAP</td>
<td>Endoplasmic reticulum aminopeptidase associated with antigen processing</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GA</td>
<td>Glutaraldehyde</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HF</td>
<td>Hydrofluoric acid</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papilloma virus</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
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<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMAC</td>
<td>Immobilised metal affinity chromatography</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kDa</td>
<td>Thousand daltons</td>
</tr>
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<td>Litre</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
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<tr>
<td>LbL</td>
<td>Layer by layer</td>
</tr>
<tr>
<td>LC</td>
<td>Loading capacity</td>
</tr>
<tr>
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<td>Loading efficiency</td>
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<td>LPS</td>
<td>Lipopolysaccharides</td>
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<td>M</td>
<td>Molarity</td>
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<tr>
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<tr>
<td>m²/g</td>
<td>Square metres per gram</td>
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<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MQH₂O</td>
<td>Milli Q water</td>
</tr>
<tr>
<td>MS</td>
<td>Mesoporous shell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MSP</td>
<td>Merozoite surface protein</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
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<td>Amino acid terminus domain of an amino acid sequence/protein</td>
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<td>Nanoparticle albumin-bound</td>
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<td>Sodium chloride</td>
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<td>Sodium hydroxide</td>
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<td>Nanogram</td>
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<td>Nickel sulphate</td>
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<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OPV</td>
<td>Oral polio live vaccine</td>
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<tr>
<td>PAH</td>
<td>poly(allylamine hydrochloride)</td>
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<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PcMSP</td>
<td>Plasmodium chabaudi adami merozoite surface protein</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
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<td>Paraformaldehyde</td>
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<td>pH</td>
<td>Negative algorithm of hydrogen ion concentration</td>
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<tr>
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<td>Isoelectric point</td>
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<td>PLGA</td>
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<td>ε-poly-L-lysine</td>
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<td>PMA/PAA</td>
<td>Poly(methacrylic acid)/Poly(acrylic acid)</td>
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<td>p-nitrophenyl phosphate substrate</td>
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<td>Poly(propylene sulfide)</td>
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<tr>
<td>PSS</td>
<td>Poly(sodium styrene sulfonate)</td>
</tr>
<tr>
<td>R</td>
<td>Resistance</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolution per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell park memorial institute</td>
</tr>
<tr>
<td>SC</td>
<td>Solid core</td>
</tr>
<tr>
<td>SC/MS</td>
<td>Solid core/mesoporous shell</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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</table>
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Summary

Vaccines are invaluable factors in our current health system for preventing pathogenic infection. However, traditional vaccines have been unsuccessful in preventing against some important infections, such as malaria, HIV and hepatitis C. Although many potential antigens have been discovered, the lack of universal protection may be due to the inability of conventional delivery methods to elicit the immune responses appropriate for a particular infectious agent. Most of the protein subunit vaccines primarily induce antibody-mediated humoral responses; therefore, there is an urgent need for a new delivery method that delivers antigen to also elicit more potent antigen-specific cellular responses.

Utilising nanotechnology in the field of medicine has gained pace in recent years. Nanoparticles such as silica, liposomes and more recently synthetic polymer particles, have been developed as vaccine/drug carriers and many are being studied comprehensively as promising candidates. These nanoparticle vehicles have been demonstrated to be efficiently taken up by dendritic cells, one of the most important cells which controls the fate of an antigen-specific immune response. These particles therefore have the ability to induce strong humoral and cellular responses. However, there have been no reports of a vaccine delivery system based on the preparation of nanoparticles derived from antigen only using a template, which would alleviate some of the disadvantages of existing nanoparticles. Through a templating system using silica nano-sized particles, proteins can be immobilised in the mesoporous shell by solution adsorption. This simple-step preparation of antigen nanoparticles has the potential to develop new generation vaccines against currently unpreventable infectious diseases.

In this study, solid core mesoporous shell (SC/MS) silica nanoparticles averaging 410 nm in diameter were fabricated as a template for the subsequent synthesis of protein-based nanocapsules, employing ovalbumin as a model protein. Firstly, the ovalbumin-based nanocapsules of approximately 500 nm were successfully taken up by murine immature dendritic cells, and processed peptides were presented on MHC class I molecules, required for the activation of cytotoxic T cells. Secondly, a murine malarial protein, the merozoite surface protein 4/5 from *Plasmodium chabaudi adami* was used to synthesise nanocapsules using the templating system, and shown to elicit both humoral and cellular responses in immunised mice. These results highlight the potential of antigen-based nanocapsules, prepared from the nanoparticle templating system, for antigen delivery.
CHAPTER 1

Literature review

This chapter provides an overview to the current vaccine technologies and explores the advances made towards next generation vaccine development, in particular using nanotechnology. This chapter also reviews one of the most prevalent global diseases - malaria. Finally, the rationale of this project is provided.
1.1 Introduction

The fight to control infectious disease has always been an endless task for humanity. With millions of deaths from infectious disease each year, it has an enormous impact and a burden on the global economy and health care system. Sanitation and prevention are key to reduce the impact of infectious disease (Nichol, 2003), rather than the use of treatments and medications. A number of infectious diseases have been prevented since the birth of vaccines practiced by Jenner and Pasteur 200 years ago; however, there are still no registered or effective vaccines for some of the most prevalent diseases in the modern era. These infectious and parasitic diseases include acquired immune deficiency syndrome (AIDS), tuberculosis, malaria, leishmaniasis and hepatitis C, which causes millions of deaths every year (data shown in Table 1.1). Recently, an outbreak of the Ebola virus was observed in the West African region (May, 2014). Absolute morbidity and mortality rate is currently low, however, the projection is set to increase if no appropriate prevention is applied.

For a vaccine to be successful, it must satisfy a number of important criteria (Table 1.2). Firstly, it must be able to elicit an immune response with a minimal number of doses (ideally a single dose), and provide long-lasting protection (Atkins et al., 2006; Beverley, 2002). Secondly, it must be totally safe and effective in all vaccinated subjects, as the vaccine will be distributed across all age groups, including infants and children.

It should also be stable and inexpensive for manufacturing (Beverley, 2002). Further to these requirements, an effective antigen delivery system must be able to deliver antigens to specific cells that play a crucial role in immune system, and initiate a specific immune response accordingly. The efficiency of the delivery system must also not be diminished by pre-existing immunity (Moron et al., 2004), or induce immune tolerance.
Table 1.1: Number of deaths caused in 2011 by infectious and parasitic diseases for which effective vaccines are not yet available.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Number of deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS (HIV)</td>
<td>1,590,952</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>975,903</td>
</tr>
<tr>
<td>Malaria</td>
<td>58,218</td>
</tr>
<tr>
<td>Leishmaniasis</td>
<td>53,675</td>
</tr>
<tr>
<td>Hepatitis C</td>
<td>39,520</td>
</tr>
<tr>
<td>Schistosomiasis</td>
<td>23,313</td>
</tr>
<tr>
<td>Trypanosomiasis</td>
<td>19,026</td>
</tr>
<tr>
<td>Ebola virus (2014 outbreak)</td>
<td>4,818 (total number of cases, 13,042)</td>
</tr>
</tbody>
</table>

(Latest data available from World Health Organization (WHO), Global health observatory data repository (World Health Organization, 2014), and the Ebola outbreak record was obtained on 2\textsuperscript{nd} Nov 2014 WHO Ebola Situation report (World Health Organization, 2014)).
Table 1.2: Properties of an ideal vaccine. (Adapted and modified from Beverley, 2002).

<table>
<thead>
<tr>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Life-long immunity</td>
</tr>
<tr>
<td>Broadly protective against all variants of an organism</td>
</tr>
<tr>
<td>Prevent disease transmission, e.g. by preventing shedding</td>
</tr>
<tr>
<td>Rapidly induce effective immunity</td>
</tr>
<tr>
<td>Effective in all vaccinated subjects, including infants and the elderly</td>
</tr>
<tr>
<td>Transmit maternal protection to the fetus</td>
</tr>
<tr>
<td>Requires few (ideally one) immunisation to induce protection</td>
</tr>
<tr>
<td>No need to be administered by injection</td>
</tr>
<tr>
<td>Cheap, stable (no requirement for cold chain), and safe</td>
</tr>
</tbody>
</table>
1.2 Types of vaccines

Since the discovery of first vaccine by Edward Jenner, which focused on using a whole-cell vaccine, different approaches of vaccine development have emerged. While they may contain killed or attenuated, or parts of pathogen, the term “vaccine” applies when the products are formulated to be introduced into the body and elicit a protective immune response, without causing disease. This section describes some of the traditional vaccine platforms used for the current vaccine scheme worldwide, and some rather newer approaches studied in more recent years. These are; inactivated vaccines, attenuated vaccines and partial component vaccines; and synthetic peptide vaccines, recombinant vector and DNA vaccines, respectively. Types of major vaccines currently distributed in the USA with formulation types are summarised in Table 1.3.

1.2.1 Inactivated/killed vaccines

Vaccines of this type are derived from the bacterium or virus grown in culture media, and killed to become non-infective by treatment, typically using heat, chemicals or radiation. This was the basis of the vaccine formulation method used in the early vaccine era. The treatments destroy the pathogens preventing their ability to replicate, while keeping antigens intact for the immune system to recognise. While the pathogen’s inability to replicate provides an advantage over attenuated vaccines since there is no risk of reversion to the virulent wild type, however, this can reduce the efficacy of the vaccine (Belshe et al., 2007). Although the majority of currently licensed vaccines are formulated as inactivated vaccines, their modest efficacy results in the need for multiple doses (Zangwill & Belshe, 2004).

1.2.2 Attenuated/live vaccines

Attenuated vaccines are also derived from organisms grown in laboratory settings. The organism is “attenuated” or weakened through a series of passages in altered conditions using culture media or typically, chicken embryos. They may be subjected to up to 200 passages in non-human cells, after which it loses the ability to replicate to a level that can cause disease in a human host. The organisms that became avirulent can still replicate at a minimal level
<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthrax</td>
<td>Inactivated</td>
</tr>
<tr>
<td>Diphtheria, Tetanus, acellular Pertussis</td>
<td>Inactivated toxin</td>
</tr>
<tr>
<td><em>Haemophilus influenza</em> type b</td>
<td>Inactivated</td>
</tr>
<tr>
<td>Hepatitis A</td>
<td>Inactivated</td>
</tr>
<tr>
<td>Hepatitis B</td>
<td>Inactivated</td>
</tr>
<tr>
<td>Human papillomavirus (bi, or quadrivalent)</td>
<td>Inactivated</td>
</tr>
<tr>
<td>Influenza, trivalent</td>
<td>Inactivated</td>
</tr>
<tr>
<td>Influenza</td>
<td>Live attenuated</td>
</tr>
<tr>
<td>Japanese encephalitis</td>
<td>Inactivated</td>
</tr>
<tr>
<td>Measles, Mumps, Rubella</td>
<td>Live attenuated</td>
</tr>
<tr>
<td>Meningococcal</td>
<td>Conjugate, polysaccharide</td>
</tr>
<tr>
<td>Pneumococcal (13, or 23-valent)</td>
<td>Conjugate, polysaccharide</td>
</tr>
<tr>
<td>Poliovirus</td>
<td>Inactivated</td>
</tr>
<tr>
<td>Rabies</td>
<td>Inactivated</td>
</tr>
<tr>
<td>Rotavirus (mono, or pentavalent)</td>
<td>Live attenuated</td>
</tr>
<tr>
<td>Typhoid Vi</td>
<td>Polysaccharide, inactivated</td>
</tr>
<tr>
<td>Typhoid</td>
<td>Live attenuated</td>
</tr>
<tr>
<td>Varicella (chickenpox)</td>
<td>Live attenuated</td>
</tr>
<tr>
<td>Yellow fever</td>
<td>Live attenuated</td>
</tr>
<tr>
<td>Zoster (Herpes zoster)</td>
<td>Live attenuated</td>
</tr>
</tbody>
</table>

(Adopted and modified from Atkinson *et al.*, 2011)
within the human host, and since the host immune system cannot distinguish between symptomatic and asymptomatic infection, an immune response is provoked. Attenuated vaccines have been shown to be much more effective, often requiring only a single dose, with protection outlasting that of inactivated vaccines (Fleming et al., 2006; Belshe et al., 2007).

However, several safety concerns are expressed about the use of attenuated vaccines in humans. Firstly, any attenuated virus vaccine may undergo spontaneous mutation that may cause the loss of attenuation and reversion to the virulent form. Although the occurrence of such an incident is quite rare, reversion to virulence has been seen with the oral polio live vaccine (OPV). Mutation in OPV resulted in the generation of a vaccine-associated polio virus which resulted in rare cases of paralytic poliomyelitis in vaccine recipients, as well as disease in unimmunised or non-adequately immunised personnel (World Health Organization, 2002; 2014).

Secondly, a double-infection of an attenuated virus and a wild strain of a non-human virus in humans may pose a serious risk. An example is influenza virus. If the wild virus is of a human origin, then it is safe to administer the vaccine at the time of infection (Youngner et al., 1994). However, if the infection was caused by a virus of non-human origin, gene reassortment between the attenuated virus from vaccine and virus of non-human can lead to the creation of a viral strain with pandemic potential. The ability of a non-human virus to infect humans has been shown in the case of avian-origin human influenza A-H5N1, in Hong Kong in 1997 (Claas et al., 2005). Gene reassortment between three host-species (including humans) was also evident with the 2009 human influenza A-H1N1, more commonly known as swine flu (Trifonov et al., 2009; Fraser et al., 2009).

Thirdly, attenuated vaccines cannot be administered to certain populations with low immunity including immunocompromised patients, as well as children and the elderly. Lastly, due to the nature of live organisms, most attenuated vaccines require cold-chain storage and transport. This may be difficult in developing countries where access to refrigeration is limited.
1.2.3 Partial component vaccines

Unlike the two previously mentioned vaccines where the entire pathogen is used, the partial component vaccine is formulated with only specific parts of the organism. It is often composed of proteins or capsular polysaccharides, which have been isolated or produced recombinantly, and shown to be immunogenic. Use of an antigen that is highly conserved may also provide protection across several species or serotypes. An example of such a vaccine is the subunit pneumococcal vaccine used in infants to prevent disease caused by *Streptococcus pneumoniae* (Rueckinger *et al.*, 2011).

The major advantage of subunit vaccines is safety. Subunit vaccines can also circumvent concerns associated with the administration of attenuated vaccine to immunocompromised patients. However, this can also become a disadvantage, as vaccine efficacy is compromised/limited in comparison to an attenuated vaccine. When polysaccharide has been used alone, it often fails to elicit T cells for long-lasting memory, and was incapable of producing highly effective IgG antibodies. Therefore a process to conjugate the polysaccharide with a protein or toxoid carrier was introduced in order to stimulate a better response. The most successful conjugate subunit vaccine is the *Haemophilus influenzae* type b (Hib) vaccine to prevent meningitis in infants and children. Since the introduction of routine Hib vaccination in the 1990s when disease was endemic, the number of cases of Hib meningitis has declined significantly (Peltola *et al.*, 1992; Georges *et al.*, 2013). However, subunit vaccines often require the addition of adjuvant in order to be effective (Aguilar & Rodriguez, 2007).

1.2.4 Synthetic peptide vaccines

As an addition to the subunit vaccines with enhanced safety, an alternative approach to the attenuated/live vaccines is the peptide vaccine. It is often a section of the whole protein that is immunogenic, and that is sufficient to elicit an immune responses. Subunit and peptide vaccines can also benefit when there might be a complication in delivering the entire organism in killed or attenuated form (e.g. parasites). By using peptides, these epitopes can be delivered in a high dose. Synthetic peptide vaccines are also economically attractive and do not require a cold-chain to maintain biological effectiveness, as they can be stored
lyophilised, and produced easily in large scale with chemically defined quality (Purcell et al., 2007).

However, similar to the subunit protein, synthetic peptides lack strong immunogenicity and require the use of an adjuvant. Peptides are more susceptible to extracellular proteases and may therefore be rapidly degraded before they are able to stimulate a T cell response. T cell tolerance or anergy may also occur if T cells are stimulated incorrectly with insufficient co-stimulatory molecules by non-professional antigen presenting cells (APCs) (Celis, 2002; Kyburz et al., 1993). Furthermore, the conformation of the peptide must be greatly considered when synthesised. Peptide vaccines usually comprise surface-exposed regions which are highly immunogenic. The conformation of these peptides plays a role in their virulence, and conformation must be maintained in order to produce antibodies. Therefore, the conformation of the peptide must closely resemble the epitope as the production of antibodies relies on the discontinuous structure (Serardic, 1993). T cells can recognise sequential or continuous epitopes of a linear peptide, however difficulties in delivering peptides to APCs hinders this process.

1.2.5 Recombinant vectors and DNA vaccines

This is a rather newer technique used for vaccines, which has developed over the past twenty years. The recombinant vector vaccine uses attenuated virus or bacteria as a vaccine vehicle, and is introduced to the host in a contained form as part of plasmid or chromosomal DNA. DNA vaccines are similar as they only consist of DNA, but they are delivered without live vectors. Recombinant vectors can produce their own heterologous protein once delivered inside the host, while DNA vaccine uses the host’s cell machinery to produce the antigen encoded in the delivered DNA. The DNA vaccine consists of a sequence encoding the antigen cloned into a plasmid, with a few other important elements for the expression of the protein within host cells. Upon administration, host cells such as dendritic cells and myocytes internalise the plasmid DNA and express the encoded antigen. The antigen is then processed and presented as endogenous antigen to induce cellular responses, or it can be secreted from cells (i.e. myocytes) as an exogenous antigen. The exogenous antigen can directly interact with B cells, which after binding the antigen with the B cell receptor, internalise it and present
peptide on MHC class II molecule to T helper (Th) cells. The subsequently activated Th cells then provide help to the B cell, enabling it to mature into a plasma cell and secrete antigen specific antibody. Lastly, the secreted exogenous antigens can also be taken up by dendritic cells and cross-presented to cytotoxic T lymphocytes (CTLs) precursors.

DNA vaccines were first discovered in 1990 by the observation of heterologous protein expression in mouse myocytes in vivo, two months after plasmid DNA was given by intramuscular injection (Wolff et al., 1990). This key discovery was further demonstrated by the induction of an immune response elicited by the marker protein encoded in the plasmid DNA (Tang et al., 1992). However, it was in 1993 that Ulmer et al. demonstrated the theory of using DNA as a vaccine, by demonstrating that protective immunity was induced in mice. Mice were immunised with DNA encoding the influenza virus A nucleoprotein and were successfully protected against challenge with homologous and heterologous viral strains, with an increased production of CTLs. This study showed that DNA vaccines are capable of eliciting potent cellular responses, which are required for many prevalent diseases.

Four DNA vaccines are currently licensed for veterinary use, however despite this success, the progress of DNA vaccines for human use has been slow. The ease of manufacture and vaccine stability at ambient temperature is a major advantage of DNA vaccines. These vaccines are also incapable of causing secondary infection in immunocompromised populations, however these advantages of DNA vaccines are often diminished by safety concerns over host gene integration and the possible spread of antibiotic resistance to pathogens (Faurez et al., 2010). However, most of all, the inability to induce appropriate levels of immune response is the major hurdle for DNA vaccines. Several strategies have been studied to improve the immunogenicity of DNA vaccines, including co-delivery of stimulatory molecules, gene optimisation, delivery in an attenuated viral or bacterial vector, and nanoparticles (reviewed in Taki et al., 2011).
1.3 Nanoparticles for vaccine delivery platform

Traditional vaccines using attenuated or killed organisms are the majority of vaccine formulations currently used in the market. Although it may be a more efficacious formulation because it can elicit sufficient humoral and cellular responses with a single dose, use of live organisms however poses many risks associated with reversion to the virulent strain, or an emergence to a new pandemic disease. Subunit vaccines offer a safer alternative to attenuated vaccines, however immunogenicity is impaired. It is very clear that a new type of vaccine is urgently required, or an efficient antigen delivery system to increase the efficacy of subunit vaccines.

Over the past decade, nano-sized materials of less than 1 µm, have presented promising potential as drug and antigen delivery systems. Nanoparticles and nanocapsules can stabilise vaccine antigens and ensure delivery to intracellular compartments to increase the vaccine immunogenicity, which subunit vaccines cannot achieve. Delivering antigens in particulate form offers several advantages over soluble antigens. Antigens are encapsulated within the nanocapsules to provide protection from extracellular protease degradation and prolong their circulation in the system. Antigens can also be adsorbed on the surface of nanoparticles, sometimes in combination with adjuvants (e.g. pathogen-associated molecular patterns (PAMPs)), which allows direct interaction of the antigens with immune cell surface receptors (i.e. Toll-like receptors (TLRs)). Particulate form also facilitates more efficient cellular uptake by the APCs, therefore capable of inducing potent antigen-specific humoral and more importantly, cellular responses by promoting a higher level of cross-presentation.

1.3.1 Materials and preparation choices for nanoparticles

A variety of materials exist from which nanoparticles can be synthesised. Some inorganic materials such as silica and iron oxide nanoparticles have shown potential as a delivery system (reviewed in Mody et al., 2013; Tang et al., 2012)(Pusic et al., 2013), however their toxicity and clearance from the body raises a few concerns (Kang & Lim, 2012; Vallhov et al., 2007; Wang et al., 2007a; Liu et al., 2011). Therefore, more biocompatible and
Biodegradable materials have gained interest as nanomedicines. Different types of nanoparticle using chemically defined materials are studied comprehensively as promising candidates.

Examples of such materials are; lipid (viral envelop or phospholipids) (Tyler et al., 2014; Slupetzky et al., 2007; Henriksen-Lacey et al., 2011; Heurtault et al., 2010), synthetic polymers such as poly(allylamine hydrochloride) (PAH) (Mauser et al., 2004; Wang et al., 2008a; 2008b; Tong et al., 2006), poly(acrylic acid) (PAA) and poly(methacrylic acid) (PMA) (Mauser et al., 2004; Zelikin et al., 2008), poly(lactide-co-glycolide) (PLGA) (Akagi et al., 2007; Yang & Hsu, 2008), and polypeptides such as poly-L-lysine (PLL) (Akiyoshi et al., 2000; Zhao & Li, 2008), natural polymer such as chitosan (Chu et al., 2012; Goethals et al., 2013; Al-Qadi et al., 2012), and protein such as albumin (Langer et al., 2008; 2003; Elzoghy et al., 2012; Altintas et al., 2013). Although the formulation of nanoparticles and preparation techniques are the same for antigen and drug delivery systems, a few examples of various types of nanoparticles studied for antigen delivery are listed in Table 1.4.

Amongst nanoparticle delivery systems, liposomes were one of the first to be studied. Liposomes are self-assembling phospholipid bilayer micelli with an aqueous core. Liposomes can be fabricated in a multilayered structure; therefore can allow the encapsulation of both hydrophilic and hydrophobic antigens between different layers. There are currently two liposome-based vaccines approved for human use, and some others in different phases of clinical trials (Watson et al., 2012). Several other liposomes are available for therapeutic drug delivery of anti-cancer agents and antimicrobials (Bhujbal et al., 2014). Several polymeric nanoparticles are also available for therapeutic use (Wang et al., 2012). PLGA is perhaps the most studied polymer material for antigen delivery due to its biodegradability.
Table 1.4: Examples of various types of nanoparticles studied for antigen delivery.

<table>
<thead>
<tr>
<th>Nanoparticle material</th>
<th>Size</th>
<th>Antigen (pathogen)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inorganic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iron (Non-degradable)</td>
<td>20-300 nm</td>
<td>MSP1 (<em>Plasmodium falciparum</em>)</td>
<td>(Pusic et al., 2013)</td>
</tr>
<tr>
<td>Silica</td>
<td></td>
<td>BSA</td>
<td>(Lim et al., 2012)</td>
</tr>
<tr>
<td><strong>Liposome</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>200 nm</td>
<td>Polysaccharides</td>
<td>(Deng et al., 2014)</td>
</tr>
<tr>
<td>Lipid (Non-viral lipids particle)</td>
<td></td>
<td>Polysaccharides (Streptococcus pneumoniae serotype 14)</td>
<td>(Deng et al., 2014)</td>
</tr>
<tr>
<td>Lipid</td>
<td></td>
<td>VMP001 (<em>Plasmodium vivax</em>)</td>
<td>(Moon et al., 2012)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RTS,S/AS01B (<em>Plasmodium falciparum</em>)</td>
<td>(Richards et al., 1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CSP + hepatitis B protein hybrid</td>
<td>(Richards et al., 1998)</td>
</tr>
<tr>
<td><strong>Virus-like particle</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viral capsid expressed in Baculovirus</td>
<td>27-60 nm</td>
<td>Capsid protein L1+L2 (HPV)</td>
<td>(Slupetzky et al., 2007)</td>
</tr>
<tr>
<td>Bacteriophage expressed in <em>E. coli</em> C41</td>
<td></td>
<td>Capsid protein L2 (HPV)</td>
<td>(Tyler et al., 2014)</td>
</tr>
<tr>
<td><strong>Polymeric</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chitosan</td>
<td>160-1000 nm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLGA</td>
<td></td>
<td>Hepatitis B</td>
<td>(Prego et al., 2010)</td>
</tr>
<tr>
<td>PLGA</td>
<td></td>
<td>Ovalbumin</td>
<td>(Shen et al., 2006)</td>
</tr>
<tr>
<td>PVPON&lt;sub&gt;Alk&lt;/sub&gt;</td>
<td></td>
<td>Tetanus toxoid</td>
<td>(Diwan et al., 2002)</td>
</tr>
<tr>
<td>γ-PGA</td>
<td></td>
<td>Ovalbumin</td>
<td>(Mintern et al., 2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gp120 (HIV-1)</td>
<td>(Wang et al., 2007b)</td>
</tr>
</tbody>
</table>
1.3.2 Disadvantages of the preparation methods

While the approval of some nanoparticles for the commercial vaccines demonstrated that the particulate delivery system is biocompatible, effective and marketable, the variability caused by the manufacturing methods adds a strict limitation on approval and success (Toh & Chiu, 2013).

Firstly, this is largely due to the requirements of harsh chemical treatment and physical stress used in the preparation process which causes degradation or possible contamination with an organic solvent (e.g. chloroform, methanol, dichloromethane) (Tamber et al., 2005). A frequently used method for encapsulating antigen is an emulsification technique, which requires the use of an organic solvent to create a water-in-oil-in-water (w/o/w) emulsion of nanoparticles. This is followed by stabilisation of the nanoparticle-antigen complex with either chemical or thermal treatment (Borchert et al., 2006; Yang & Hsu, 2008; Patil, 2003; Sundar et al., 2010).

The main issue with this encapsulation technique is the organic solvents used to dissolve polymers and lipids, as inadequate removal of surfactant and organic solvents can result in toxicity (Langer et al., 2003). The denaturation of antigens has also been observed (Sah, 1999; Panyam et al., 2003), due to the solvents and sheer stress or high temperature used in the process. Furthermore, efficiency of antigen entrapment by the encapsulation method is very low, however this method is still widely used for PLGA nanoparticles (Akagi et al., 2012).

The cationic nature of liposomes (lipid particles) and polysomes (synthetic polymer particles) also makes them difficult to use for vaccine delivery, however, there are some benefits. The positive surface charge of particles allows adsorption of negatively charged antigens (i.e. protein, DNA), and increases electrostatic interactions between the anionic cell membrane to facilitate better uptake (Kwon et al., 2005; Nam et al., 2009). However, their cytotoxic effects add a limitation to the administration dose (Lv et al., 2006).
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Nanoparticles exhibiting a strong positive electrostatic charge have greater toxic effects than strongly anionic nanoparticles, and can lead to cell death depending on the strength of the charge (Yang & Hsu, 2008; Kwon et al., 2005). A decrease in antigen presentation and reduction in metabolic activity are also accompanied with the cytotoxicity of strongly cationic particles (Kwon et al., 2005; Fischer et al., 1999). The net positive charge of a nanoparticle surface can also lead to rapid agglomeration and binding to serum proteins and erythrocytes (Khan et al., 2007; Minami et al., 2014). This may cause particle clearance from tissues where APCs reside, thus hindering the process of antigen uptake (Verma & Stellacci, 2010). Cationic nanoparticles may also cause inflammation mediated by the reactive oxygen species generated through the burst (rupture) of liposomes and PLGA particles to release the internal contents, or damage to the intracellular microenvironment from the production of acid from polymer hydrolysis during degradation, as is the case with PLGA nanoparticles (Dokka et al., 2000; Schwendeman, 2002). To overcome such issues, the addition of poly(ethylene glycol) (PEG), other hydrophilic polymers, Mg(OH)₂ or surfactants to coat the surface of cationic nanoparticles is utilised (Zhu et al., 2000; Jiang & Schwendeman, 2001). While PEG-coating is often used for liposome and polymer nanoparticles currently on the market or in clinical trial for both drug and vaccine delivery, this however reduces the efficiency of cellular uptake and inhibits endosomal escape into cytosol (Hatakeyama et al., 2013).

Other commonly used techniques to fabricate nanoparticles include; self-assembly (Xu et al., 2011; Battaglia & Ryan, 2005; Lee et al., 2006), inkjets (Hauschild et al., 2005), desolvation (Rahimnejad et al., 2009; Weber et al., 2000; Langer et al., 2003), and nanoparticle albumin-bound (nab)-technology for albumin (Desai et al., 1996; Zhang et al., 2013). While some of these methods do not require the use of organic solvents nor physical stress, the resulting nanoparticles often have high polydispersity in regard to size.

This draws attention to the importance of employing a simpler preparation method, which enables the fabrication of nanoparticles with increased homogeneity and stability. In particular, methods to utilise fewer materials for preparation with employing less cationic or amphiphilic materials to reduce cytotoxicity, is a preferred option to fabricate a safe and effective vaccine delivery platform.
1.3.3 Nanocapsule assembly using templates

A templating method has also been employed in the synthesis of polymer nanocapsules (Donath et al., 1998; Caruso et al., 1998). This approach uses adsorption of polymer to the surface of monodispersed silica nanoparticles by the electrostatic interaction, followed by the subsequent removal of the nanoparticles to form a hollow polymer nanocapsule (Yang & Hsu, 2008; Goethals et al., 2013). The templating method allows the fabrication of nanocapsules to overcome some of the issues associated with other preparation techniques.

In the non-templating system, the broad size distribution of synthesised nanocapsules can range from nanometres to micrometres. However, the use of a monodispersed, colloidal nanoparticle allows the synthesis of nanocapsules in a narrow size distribution (Wang et al., 2008a). A sacrificial template can also provide greater mechanical stability to the capsule formation during the preparation process (Cui et al., 2014). Moreover, this method does not rely on the use of organic solvents and surfactants required for the fabrication of liposome or polysome nanocapsules, as chemically cross-linking the adsorbed antigens and polymers eliminates the need for antigen encapsulation by emulsification. Finally, the properties of nanoparticle templates can provide precise control over their size, composition, colloidal stability, permeability and surface functionalisation (De Rose et al., 2008).

There are several techniques to synthesise nanocapsules, which utilise the templating method. The most commonly used technique is the layer-by-layer (LbL) assembly approach, which uses solid spherical nanoparticles as a template (shown in Figure 1.1a). More recently, a single step assembly approach employing a solid core mesoporous shell (SC/MS) nanoparticle has been demonstrated (shown in Figure 1.1b). Both methods provide the properties that are superior to the non-templating methods, however use of SC/MS nanoparticles over the LbL assembly approach offers several distinct advantages (Wang et al., 2008a). Comparison of the two different techniques is summarised below.

1.3.3.1 Layer-by-layer

In LbL assembly, a solid spherical nanoparticle is used as a sacrificial template to sequentially
1. Solid silica nanoparticle is coated with peptide (blue) by electrostatic interaction.

2. Nanocapsules are assembled by alternate deposition of polymers to create multiple layers.

3. Silica nanoparticle template is removed, leaving a peptide containing polymer capsule.

Figure 1.1: Schematic representation of a) LbL assembly process, and b) single step assembly with SC/MS silica template. (Reproduced from a) De Rose et al., 2008, and b) Wang et al., 2008a)
deposit multiple layers of polymers and antigen. The layered complex is then chemically cross-linked to immobilise the bound material, and then the template is removed (Sexton et al., 2009). The polymers and antigens usually consist of opposing charge and are adsorbed by their electrostatic force or similar interactions (Decher & Hong, 2011). The multilayer structure enables the combination of materials with different properties to be adsorbed in the one structure. The LbL approach has a greater advantage over non-templatizing techniques due to its fine control over size and composition of the nanocapsules synthesised. This is particularly important in the production of a vaccine as it minimises variability by allowing for delivery of an antigen in a measurable dose.

Polymer nanocapsules assembled with this method have shown their potential for antigen delivery. It was demonstrated that nanocapsules synthesised with a variety of polymers could bind and be internalised by blood APCs in vitro, and HIV peptide-loaded nanocapsules efficiently induced MHC class I presentation, and activated CD8+ T cells (De Rose et al., 2008; Chong et al., 2009). The efficiency of nanocapsules assembled by the LbL approach was further assessed in vivo as an antigen delivery system, and showed that ovalbumin-loaded thiol-modified PMA (PMA_SH) nanocapsules could induce high levels of humoral and cellular responses than when ovalbumin was administered alone (Sexton et al., 2009).

1.3.3.2 Single step assembly

Despite the advantages that LbL assembly can offer, sequential adsorption of materials is labour intensive and time and material-consuming. The deposition of materials is also driven by electrostatic forces or similar interaction of molecules, therefore the overall thickness of a single layer is very limited within these transient interactions (Wang et al., 2008a). This results in lower antigen loading per nanocapsule, which does not alleviate the problem associated with some of the other preparation techniques. Furthermore, this assembly approach does not result in a single component of material (i.e. polymer or antigen) in the nanocapsule structure. Therefore the control over nanocapsule behaviour in vivo becomes more complicated.

To overcome the limitations of LbL assembly, different type of nanoparticles can be
employed as a template. A mesoporous sphere is a spherical nanoparticle with a porous structure throughout. The adsorption of polymer or antigen is performed in the same principle as the LbL method, with molecules adsorbed onto the surface by the electrostatic interaction. However, molecule adsorption can be performed in a single step by infiltrating the material into the mesoporous matrix. The infiltrated matrix is then chemically cross-linked for immobilisation, then the template is removed to form a porous nanoparticle/capsule (Wang & Caruso, 2006). As the specific surface area is significantly larger with the intercalating pores, this allows a larger capacity of molecules to be loaded per nanoparticle, in a single step. A mesoporous sphere can be used to load water-insoluble compounds (Wang et al., 2010), and various types of enzymes (Wang & Caruso, 2005; 2006). However, such protein nanoparticles cannot be formed without co-loading molecules with polymers to connect the proteins (Wang & Caruso, 2006).

Another nanoparticle in this class is the SC/MS nanoparticles. The fabrication method of this type of nanoparticle has been defined since the primary method was established by Büchel and colleague in 1998. They utilised the highly monodispersed solid silica core as the inner base (Stöber et al., 1968), to build a highly robust mesoporous shell surrounding the core (Büchel et al., 1998), (Figure 1.2d). Recently, SC/MS nanoparticles were employed as a template to synthesise polymer capsules. This approach was demonstrated for the first time and produced polymer nanocapsules which were highly stable at physiological pH, yet amenable to degradation by intracellular protease (Wang et al., 2008a). In addition, the nanocapsules consisted of only a single component with a thick capsule wall resulted in the higher loading of materials per nanocapsule (Figure 1.2a). Furthermore, the structure of the polymer nanocapsule synthesised using this system were highly homogeneous (Figure 1.2b), and deformable (Figure 1.2c). Flexibility in structure is an advantage and it has been shown previously that a deformable structure like a liposome can cross the endothelium fenestration, while rigid particles of the same diameter could not pass through (Romero et al., 1999). The properties of the mesoporous layer in SC/MS nanoparticles is controllable, thus the thickness of the capsule wall and porosity can be tailored to fit the infiltrating moiety (Goethals et al., 2013). Most importantly, the fabrication of mesoporous silica nanoparticles is very simple, scalable and cost-effective (Tang et al., 2012).
Figure 1.2: Polymer nanocapsules synthesised by a single-step assembly method using a silica template. a,b) PLL nanocapsules and c) PAH nanopcapsules synthesised with d) SC/MS silica nanoparticle as a template. All images captured by TEM with a scale bar of 500 nm (a-c) and 100 nm (d). (Reproduced from a) Wang et al., 2008a, and d) Büchel et al., 1998)
1.4 Cellular fate of nanoparticles

The efficacy of a nanoparticle vaccine depends on the interaction of the particles with dendritic cells (DCs) as they are the most important cells involved in initiating an immune response. DCs are capable of inducing primary responses to infection, and the fate of subsequent responses depends on how they respond to that particular pathogen. They are also the most potent antigen processing cells which present antigen from pathogens for subsequent activation of two major T cell types. The polarity of T cell type therefore determines the immunogenicity of the vaccine delivered by nanoparticles. In this section, the basic background of DCs is explained whilst the interaction of nanoparticles is also discussed. This will be followed by an overview of three different antigen processing and presentation pathways by DCs, which nanoparticles undergo as antigen delivery system. Lastly, the subsequent T cell activation is reviewed.

1.4.1 Dendritic cells

APCs are effective at internalising, processing and presenting exogenous antigens acquired from infecting pathogens. There are three main types of APCs, dendritic cells, macrophages and B cells. Amongst these, DCs are the most potent professional APCs for the initiation of immune responses, and their ability to prime naïve T cells has been well studied (Steinman, 2007b; Villadangos & Schnorrer, 2007). The discovery of DCs by Steinman and Cohn in 1974 has profoundly changed the science of immunology (Steinman, 1974). It is now widely accepted that DCs play the central role in mediating immunity and tolerance, as well as being the link between innate and adaptive immune responses against infectious agents.

1.4.1.1 Types of DCs to target

DC populations reside in secondary lymphoid organs and most peripheral tissues such as skin, lung and intestines where they have first interaction with materials from the external environment (Banchereau et al., 2000). DCs comprise heterogeneous populations and their precursor is derived from bone marrow and their heterogeneity depends on the secondary lymphoid organs where DCs reside in a resting immature state. Different DC subsets
comprise distinct phenotypes and exhibit a variety of functional properties (Heath & Carbone, 2013).

Resident DC arise from committed progenitors which migrate from the bone marrow to lymphoid organs and finally to the blood where they reside permanently. It is only these types of DCs that are found in secondary lymphoid organs (Liu et al., 2009). Migratory DCs complete their final differentiation and development in peripheral tissues and then continuously migrate into lymph nodes (Randolph & Ochando, 2008), even in the absence of stimuli released upon binding of TLR to the pathogen (Wilson et al., 2007). Both types of DCs express high levels of cluster-of-differentiation (CD)11c, but resident DCs are characterised by the expression of CD8a (thus CD8⁺ or CD8⁻ DC), while migratory DCs are characterised by the expression of CD11b (thus CD11b⁺ or CD11b⁻ DC), along with expression of CD205 (DEC205) in all populations (Segura & Villadangos, 2009; Segura et al., 2009). DEC205 expressed on migratory DCs, especially on the dermal DCs, is an endocytosis-mediating receptor involved in exogenous antigen uptake, and which targets the late endosome or lysosome for MHC class II presentation (Mahnke et al., 2000). It is the migratory type of conventional DCs that play an essential role in antigen presentation against infections.

1.4.1.2 Immunostimulators and DC maturation

DCs remain in an immature state in the absence of infection, dedicating their function to immune surveillance by constitutively presenting self-antigens. T cell activation by these DCs is impaired and low levels of MHC class II molecules and co-stimulatory molecules such as CD40, CD80 and CD86 are expressed during this state. However, they are characterised by their high endocytic and phagocytic capacity to internalise exogenous antigens (Wilson et al., 2004). Upon infection, the expression of these molecules is up-regulated by several extracellular and intracellular stimuli (Han et al., 2009). These CD molecules can allow more intimate interaction with T cells for subsequent antigen presentation. One of the stimuli is released from the interaction with the PAMPs of pathogens, which elicit the innate immune response.
Stimuli are released when TLRs on DCs binds to the surface molecules such as PAMPs of invading pathogens, which include lipopolysaccharides (LPS) within the cell wall of gram negative bacteria, and bacterial unmethylated CpG oligodeoxynucleotides (Medzhitov, 2001). TLRs are an evolutionally conserved family of receptors and are expressed on a variety of innate immune cells (Medzhitov, 2001). PAMPs are found on non-mammalian cells, therefore TLRs have broad specificity for these conserved patterns (Underhill & Ozinsky, 2002; Kopp & Medzhitov, 2003). The ligation of PAMPs to TLRs can also stimulate cells to increase the phagocytosis of exogenous antigens (Redlich et al., 2013).

Up-regulation of surface molecule expression on DC is also induced by inflammatory cytokines including tumour necrosis factor alpha (TNFα), interleukin (IL)-1β, and IL-6 (Guermonprez et al., 2002). Using a murine immature DC line, DC2.4, He et al. (2007), demonstrated that interferon gamma (IFNγ) was also able to induce DC maturation in vitro for more efficient stimulation of CD8+ T cell responses. Upon receiving exogenous stimuli produced from PAMPs or cytokines, the intracellular trafficking of MHC molecules and antigen processing were also enhanced (Théry & Amigorena, 2001). This direct interaction with pathogens therefore induces DC to undergo a development process often referred to as maturation. The maturation process activates DCs to become efficient APCs, thus transforming them to be potent T cell activators.

1.4.1.3 Adjuvant

Improving the immunogenicity of a vaccine can be achieved by the co-delivery of stimulating molecules. Molecules such as PAMPs can induce more efficient uptake of a vaccine and ensure the activation of DCs. Adjuvants are defined compounds that are added to the vaccine to enhance antigen-specific immune responses. Adjuvants approved for human used are aluminium hydroxide mineral salts (Alum), MF59®, virus-like particles, cholera toxin, and MPL® (glycoprotein) (Reed et al., 2009). However, conventional adjuvants such as Alum have been historically shown to elicit strong humoral responses with weaker cellular responses. More potent adjuvants are available however their use in human is limited due to their high toxicity. The optimal balance between the toxicity of adjuvant and combination with the vaccine must be carefully considered.
An important feature of a particulate vaccine is the ability to directly conjugate an additional molecule on the nanoparticle surface. The conjugation of PAMPs with either protein or a DNA sequence can be achieved by adsorption by electrostatic interactions and chemical cross-linking (Hermanson, 2013; Di Marco et al., 2010). A variety of PAMPs have been studied as co-delivery molecules in a particulate delivery system, which specifically target the matching TLRs (reviewed in Demento et al., 2011), however the most commonly studied element is the unmethylated CpG motif. The CpG motif is rich in cytosine and guanine, is recognised by TLR9, which is primarily expressed in the endosome of blood APCs (Hemmi et al., 2000). The use of the CpG motif in a particulate delivery system to induce cellular responses was demonstrated using PLGA nanoparticles surface-modified with the CpG motif. This combination elicited humoral, as well as cellular responses, and provided protection against live viral infection (Demento et al., 2010). Interestingly, encapsulation of the CpG motif has also been shown to increase the cellular response much higher than the soluble antigen formulated with the CpG motif in solution, encapsulated antigen without CpG motif, and encapsulated antigen delivered with CpG in solution. The CpG motif co-encapsulated with tetanus toxoid in PLGA nanocapsules increased the level of IFNγ, IgG2b and IgG3 by Th1-biased immune response, and IgG1 by Th2-biased immune response (Diwan et al., 2002). This indicates that lower amounts of potentially toxic adjuvant can be used in conjunction with nanocapsules to induce more potent humoral and cellular immunity.

Coupling of TLR ligands and PAMPs is not the only option for targeting DCs. The surface of nanoparticles can be functionalised with DC-specific antibodies. Mintern and colleagues (2013), demonstrated that the CD11c and DEC205 DC subsets can be targeted by functionalisation of nanoparticles with monoclonal antibodies.

### 1.4.1.4 Trafficking of antigen to the lymph nodes

Once the migratory DC captures the antigen, it will migrate out from the peripheral tissues into the draining lymph nodes through afferent lymph vessels (Randolph & Ochando, 2008). It will then complete its journey for the interaction and activation of naïve T cells. The transformation of migratory DC to the mature state by encountering exogenous antigens and
Figure 1.3: Maturation and migration of conventional migratory DC. DC progenitors are formed in the bone marrow, and then migrate to various peripheral tissues (e.g. the epidermis, intestine, blood vessel wall or atherogenic plaques), and reside as immature DCs. Upon interaction with a pathogen, DC undergo maturation and migrate to lymph nodes to present the internalised antigen to naïve T cells to initiate their activation. (Reproduced from Szatmari & Nagy, 2008)
the transport to lymph nodes is presented graphically in Figure 1.3. DC migration is attributed to the changes in the expression level of surface adhesion molecules and cytoskeleton modification during the maturation process (Banchereau et al., 2000; Lamsoul et al., 2013).

After exposure to stimuli, and following the maturation process, DCs lose the ability to further endocytose and process newly countered antigens (Guernonprez et al., 2002). Therefore, the activation of T cells is limited by the presentation of specific antigen internalised by APC’s prior to maturation. The ability of migratory DC to transfer internalised antigens to the lymph node resident DC has also been documented. Allan et al. (2003), found that DCs from lymph nodes presented viral peptide after cutaneous injection of mice with human herpes simplexvirus. Initially it was thought that the skin DC, i.e. Langerhan cells migrated to lymph nodes and initiated T cell activation, however it was found to be a different DC subset. It was also shown that blocking the migration of Langerhan cells from the infected skin inhibited presentation and subsequent immune response to the viral infection (Allan et al., 2006). This finding leads to the discovery of how migratory DC can be an antigen carrier, instead of directly presenting antigen themselves and priming T cells in lymph nodes.

However, with the particulate delivery system, the resident DCs can be directly targeted for antigen uptake instead of relying on capture by the migratory DCs. Nanoparticle trafficking to draining lymph nodes has been observed, and this is thought to depend on particle size (Fifis et al., 2004; Reddy et al., 2006; Manolova et al., 2008). Nanoparticles of less than 200 nm in diameter can freely drain to the lymph nodes spontaneously by leaving the interstitial space and being transported via the interstitial flow (Nishioka & Yoshino, 2001; Reddy et al., 2006).

Reddy and colleagues (2007), assessed the feasibility of nanoparticle transport to the lymph nodes using 25 nm and 100 nm polypropylene sulphide nanoparticles loaded with ovalbumin. They found that the 25 nm nanoparticles reached the lymph nodes much more efficiently than the 100 nm nanoparticles following intradermal injection. The ovalbumin-specific IgG level subsequently induced by the 25 nm nanoparticles was equivalent to that of soluble ovalbumin formulated with adjuvant, whereas the level induced by the 100 nm was significantly lower.
(Reddy et al., 2007). This study was followed by Manolova and colleagues (2008), to demonstrate that the nanoparticles injected intracutaneously are trafficked to the draining lymph nodes in less than 2 h, while nano- and microparticles of 200 nm to 2 µm, respectively, require 8 h to enter the subcapsular sinus (Manolova et al., 2008). These findings show that smaller nanoparticles have the potential to directly target lymph nodes, and this DC-independent trafficking can decrease the time to antigen presentation (Pack, 2004).

1.4.2 Antigen processing and presentation

Exogenous antigen derived from an infectious pathogen and endogenous antigen derived from viral infection or normal cellular metabolism, are all processed within APCs via several pathways. These antigens are enzymatically digested within distinct intracellular compartments, and the processed peptides are delivered to the surface membrane in context with MHC class I and MHC class II molecules. Peptide-MHC molecule is presented to naïve CD4+ and CD8+ T cells to initiate immune responses depending on the primed T cell type. This process, called antigen presentation, is imperative for T cell activation against infection, as well as immune surveillance for tumour detection. Different intracellular processing will result in the fragmentation of antigen to be associated with either MHC molecule, thus leading to the activation of different T cells. Three pathways are known to this date; the MHC class I pathway, MHC class II pathway and the cross-presentation pathway. These pathways are presented graphically in Figure 1.4.

1.4.2.1 MHC class I pathway

Endogenous antigens such as viral proteins, tumour antigens, intracellular pathogens and defective cells are processed through the MHC class I pathway, for the activation of CD8+ T cells. MHC class I molecules are commonly expressed with fragmented endogenous self-antigens on the cell surface of all nucleated cells (Janeway, 2001). However, the presentation of peptides from intracellular pathogens and tumours will be recognised as non-self-antigens, which will induce CD8+ T cells to kill cells presenting these antigens (Belz et al., 2004; Huang et al., 1994).
Figure 1.4: The antigen presentation pathway in dendritic cells. On the left, the MHC class II presentation pathway shows where exogenous antigen or endogenous antigen secreted from other cells is taken up by endocytosis and loaded onto MHC class II molecules. On the right, the MHC class I presentation pathway is shown where endogenous antigen in the cytosol is fragmented by a multisubunit proteasome complex, and then loaded onto MHC class I. In the middle is the cross-presentation pathway where antigens are processed through the MHC class II presentation pathway but are subsequently loaded onto the MHC class I molecule. (Reproduced from Hubbell et al., 2009)
Endogenous proteins are processed in the cytosol through a multicatalytic enzyme complex called the proteasome, which consists of 28 subunits. The proteasome is expressed in all eukaryotic cells and plays a major role in the regulation of cell-cycle, proliferation, differentiation, tumour suppression and apoptosis (Zheng et al., 2004; O'Connor, 2005). Deregulation of the proteasome process can lead to malignancies (Adams, 2004). Proteasomal proteolysis produces peptides, which still require further fragmentation before loading onto MHC class I molecules. Therefore, degraded peptides are transported into the endoplasmic reticulum (ER) through a molecule termed transporter associated with antigen processing (TAP), where the peptides can be further trimmed by an aminopeptidase called ER aminopeptidase associated with antigen processing (ERAAP). TAP is localised on the ER membrane on the cytoplasmic side, binds to peptides and actively transports them from the cytosol into the lumen of the ER. The function of ERAAP was identified when blocking ERAAP expression severely reduced the presentation by MHC class I molecules on the cell surface (Serwold et al., 2002). It was further characterised by using the ERAAP-deficient mice, which showed how it selectively allows peptides to be trimmed at the N-terminus (Hammer et al., 2005). This is due to ERAAPs specific function as a molecular ruler to assess and trim peptides depending on their length (Chang et al., 2005). A peptide is loaded onto a newly synthesised MHC class I molecule in the lumen of the ER, then transported to the cell surface through the Golgi apparatus (Hewitt, 2003). The peptide presented on the MHC class I molecule is then recognised by the TCR on CD8\(^+\) T cells for subsequent induction of cellular responses.

1.4.2.2 MHC class II pathway

Exogenous antigens including extracellular pathogens and some endogenous antigens secreted from other cells are endocytosed and processed through the MHC class II pathway. Peptides processed through this pathway are presented with MHC class II molecules for the activation of CD4\(^+\) T cells. MHC class II molecules are mainly expressed by APCs, including DCs, macrophages and B cells (Janeway, 2001; Neefjes et al., 2011). Cellular proteins from APCs can also be presented on MHC class II molecules (Sant, 1994). This type of endogenous antigen is derived from some components of the endocytic pathway, plasma membrane and cytosolic proteins. Some viral proteins are also presented on MHC class II molecules (Paludan et al., 2005). Self- and pathogen-derived endogenous antigens are
however, processed through a separate pathway by autophagy. Autophagy is a pathway which separates and surrounds cytoplasmic material into an autophagosome, and delivers it to lysosomes for proteolytic degradation where it then meets MHC class II molecule for loading (Münz, 2012).

Exogenous antigens are internalised via various endocytic pathways depending on their size. Generally, antigens or particles larger than 1 µm, i.e. the size range of a bacterial pathogen, are internalised via phagocytosis. Smaller antigens (~1 µm) are internalised via macropinocytosis, and even smaller antigens (virus sized) are internalised by receptor-mediated clathrin endocytosis (~120 nm) (Daecke et al., 2005), clathrin-independent and caveolin-independent endocytosis (~90 nm) (Petros & DeSimone, 2010), or caveolae-mediated lipid rafts (Chen & Norkin, 1999) (shown in Figure 1.5).

Antigen internalised by the endocytic pathway is mediated by the plasma membrane. As the antigen localised in the endocytic vesicle cannot cross the membrane, it is not degraded by the proteasomes in the cytosol. Inactivated proteases are also encapsulated within the vesicle, which is now called the early endosome. However, acidification of the endosome increases while it egresses to the perinuclear area (late endosome stage), which results in the activation of these proteases to degrade the antigen contained within. The late endosome that carries fragmented antigen peptide fuses with lysosomes containing MHC class II molecules. The loading of peptide onto MHC class II molecules also takes place in the lysosome. MHC class II molecules are synthesised in the lumen of the ER and transported to the lysosome through the Golgi apparatus instead of binding to peptides in the ER. During the transport of the MHC class II molecule to the lysosome, the MHC class II molecule is blocked with a polypeptide called the invariant chain to inhibit the binding of other cellular peptides to the molecule (Landsverk et al., 2009). Cathepsin S, a lysosomal protease, then digests the invariant chain to allow the replacement to antigen peptide onto the MHC class II molecule for the presentation (Fernandez-Borja et al., 1996; Bania et al., 2003). The peptide-MHC class II complex is then transported to the cell surface to be recognised by CD4+ T cells.
Figure 1.5: Mode of size-dependent cellular uptake. (Adapted from Petros & DeSimone, 2010)
1.4.2.3 Cross-presentation pathway

Exogenous antigens internalised by APC are normally processed through the MHC class II pathway, however they can also be presented onto MHC class I molecules to prime CD8$^+$ T cells. Antigens are degraded in endosome/lysosome compartments, and then the peptides somehow escape the vesicle, possibly to the cytosol, where they can be loaded onto MHC class I molecules. This alternative pathway is called the cross-presentation pathway.

Evidence of cross-presentation was first observed by Bevan in 1976. Bevan reported a generation of MHC haplotype-restricted CD8$^+$ T cell responses in mice after the injection of unmatched allotype-cells (Bevan, 1976). In his experiment, mice (BALB/b x BALB/c) that expressed MHC haplotype (H-2$^{b/d}$) were immunised with the C57BL/10 (B10) mouse cells with H-2$^b$ haplotype, and B10.D2 mouse cells with H-2$^d$ haplotype. It was initially assumed that only H-2$^b$ restricted CD8$^+$ T cells would be generated, as it should only recognise H-2$^b$ on B10 cells, but not H-2$^d$ on B10.D2, due to minor histocompatibility differences. However, in addition to the H-2$^b$ priming in vivo, the H-2$^b$ restricted CD8$^+$ T cells not only recognised the H-2$^b$ B10 cell, but also H-2$^d$ B10.D2 in vitro. Since there was no H-2$^b$ expression from B10.D2 cells, it was concluded that the antigen-specific CD8$^+$ T cell recognition came from a cross-priming of B10.D2 cell-associated antigen to the mice APC with H-2$^b$, by a pathway now known as cross-presentation. The term “cross-priming” was used to describe the activation of T cells, therefore to encompass this phenomenon, the term “cross-presentation” is used in contrast to the other know effect “cross-tolerisation” of T cells.

Several mechanisms have been proposed for the cross-presentation process, mainly the cytosolic pathway and vacuolar pathway. The cytosolic pathway transfers internalised antigen to the cytosol, where it has an access to the proteasome to be degraded. This follows the MHC class I pathway, thus the fragmented peptide will then go through TAP to access MHC class I in the lumen of the ER (Delamarre et al., 2003; Rodriguez et al., 1999; Lin et al., 2008). The alternative pathway, the vacuolar or endosomal pathway is independent of the proteasome and TAP for cross-presentation. (Rock & Shen, 2005; Schirmbeck & Reimann, 2002; Lin et al., 2008). The internalised antigen is degraded by endosomal proteases and loaded onto MHC class I molecules. Preliminary studies could not address the process by which ER-derived
MHC class I molecules could bind antigen without also binding to the other cellular peptides, or recycled MHC class I molecules which have already bound peptide (Motal et al., 1993). The role of the lysosomal protease, cathepsin S has been suggested, as the level of cross-presentation was abolished in cathepsin S-deficient mice (Shen et al., 2004). The endosomal compartment is also highly acidic and internalised antigens are rapidly destroyed. This environment in the endosome might not allow sufficient time for the antigen to escape into the cytosol for cross-presentation (Segura & Villadangos, 2011). However, encapsulation of antigen in a nanocapsule was found to maintain the prolonged release of antigen within the endosome, possibly increasing the chance of antigen to escape into the cytosol (Shen et al., 2006). This indeed resulted in an increase in the cross-presentation of antigen.

More recently, another pathway for cross-presentation was proposed (Houde et al., 2003; Ackerman et al., 2003; Guermonprez et al., 2002). It has been suggested that the ER can form an endosomal vesicle known as a phagosome, to actively bring MHC class I molecules and other antigen-processing machinery from the ER to the antigen-containing endosome. It was also suggested that the early endosome or pinosomes are enough to facilitate cross-presentation (Ackerman et al., 2003). With the loading machinery transported, the antigen can be translocated into the cytosol and access the proteasome for degradation. Fragmented peptides will return to the phagosome to be loaded onto MHC class I molecules for presentation.

What determines the magnitude of cross-presentation to be induced is not fully understood. However, the endocytic pathway in which the exogenous antigen is internalised can greatly affect subsequent intracellular processing, and hence the presentation pathway (Landsverk et al., 2009). This is due to the type of endocytic vesicles made during internalisation. It was demonstrated that the mannose receptor supplied an early endosome committed to the cross-presentation on MHC class I molecules, while the scavenger receptor delivered the antigen more towards presentation on MHC class II molecules (Burgdorf et al., 2007). It has also been suggested that the cytosolic pathway is the primary pathway for efficient cross-presentation (Rock, 1996).
Certain subsets of DC can cross-present more efficiently than others. CD8⁺ resident DCs in secondary lymphoid organs have the ability to cross-present (Segura et al., 2009), while CD103⁺ migratory DCs are known to cross-present exogenous viral antigens (Waithman et al., 2013; GeurtsvanKessel et al., 2008), and apoptotic-cell antigens (Desch et al., 2011), to CD8⁺ T cells. The stimuli released upon capturing the pathogen also has an impact on cross-presentation. Wagner et al. (2013) demonstrated that TLR ligands and cytokines such as TNF-α had negative effects on cross-presentation. Furthermore, cross-presentation was strongly inhibited the most in LPS-matured DC, regardless of whether DC was pretreated with LPS or exposed to LPS and antigen simultaneously. However, Pooley et al. (2001) reported a controversial effect of LPS, as it increased the ability of CD4⁺ DC to present antigen to CD8⁺ T cells, when these DC subsets were often found to primarily prime CD4⁺ T cells. The ability of CD8⁺ T cells was also not altered by the presence of LPS.

Targeting the cross-presentation pathway for the delivery of exogenous antigen is therefore the key to develop effective vaccines against tumours, intracellular parasites, intracellular bacteria and viruses (Rock et al., 1990; Moron et al., 2004).

1.4.3 T cell activation

Following the uptake of antigen, DCs migrate to lymph nodes where naïve T cells reside. Antigen captured by DC is degraded within intracellular compartments, and the fragmented antigen is then loaded onto MHC molecules to be presented on the surface of DC by the mechanisms explained in the previous section. The antigen-MHC molecule complex is detected by the T cell receptor on naïve T cells, which causes an antigen-specific signal for the induction of T cell priming.

1.4.3.1 T helper versus cytotoxic

There are two major groups of T cells which can be activated upon interaction with DCs. These are characterised by the expression of either CD4⁺ or CD8⁺ markers on the cell surface, and referred to as Th cells and CTLs, respectively. The functional properties of CD4⁺ T cells
Figure 1.6: **Summary of the fate of CD4⁺ T helper cells.** Their functions, secreted cytokine profile and cytokines critical for determining their fate. (Image and legend adapted and modified from Zhu & Paul, 2008)
are to secrete cytokines to promote T cell proliferation, to enhance macrophage activity, recruit monocytes to the site of infection, and also to promote B cell proliferation and maturation into a plasma cell to produce antibodies specific to the pathogen. These functions are attributed by four different subsets of CD4\(^+\) T cells as summarised in Figure 1.6, which will be discussed in some depth in the subsequent section. The function of CD8\(^+\) T cells is primarily to kill cells, including self-tissue in the case of autoimmune disease. Cells which have undergone apoptosis or are virally infected are also eliminated from the body by the lytic functionality of CD8\(^+\) T cells. The control of tumour cells and transplanted organs are also targeted by this type of T cell (Zhang & Bevan, 2011).

### 1.4.3.2 Th subsets

In 1986, Mosmann and Coffman identified that naïve CD4\(^+\) T cells can differentiate into two major subsets after the activation (Mosmann et al., 1986). These subsets are named type 1 T helper (Th1) cells and type 2 T helper (Th2) cells, and are characterised by their distinct cytokine profiles. In particular, Th1 secretes its pro-inflammatory cytokine IFN\(_\gamma\), which causes macrophage activation (Paul & Seder, 1994), as well as IL-2 which promotes the proliferation of CD8\(^+\) T cells and delayed-type hypersensitivity. IL-2 produced during the initial priming of CD8\(^+\) T cells is also an important stimulating factor for antigen-specific memory CD8\(^+\) T cell expansion (Williams et al., 2006). Th1 therefore promotes cytotoxic properties by mediating the immune response against intracellular infection, such as a virus.

Th2 cell secretes an array of cytokines including IL-4, IL-5, IL-9, IL-10, IL-13 and IL-25. Most of these are associated with humoral responses, and predominantly the anti-inflammatory response. IL-4 is necessary for the proliferation and expansion of IL-4-secreting Th2 cells (Swain et al., 1990), and IL-10 is an anti-inflammatory cytokine (Berger, 2000), which inhibits macrophage activation as oppose to the Th1 cytokine. Th2 cells also mediate immune responses against extracellular parasitic infections (Mosmann & Coffman, 1989; Paul & Seder, 1994).

Both types of Th cells promote the proliferation and maturation of B cells and induce class switching of antigen-specific antibody. Under the influence of Th1 cytokines, IgM switches to
IgG2a in mice to become an opsonising antibody for mediating complement induction and cellular uptake of antigen by binding to the surface of APCs (ADCC). Cytokines secreted from Th2 cells can switch Ig class to IgG1 to become neutralising antibody against the specific antigen (Snapper & Paul, 1987; Sideras & Lindqvist, 1985).

More recently, the role of IL-17 has become clearer and the distinct Th subset that produces this cytokine was denoted Th17 cells (Steinman, 2007a). Several types of IL-17 cytokines are secreted by Th17, and other pro-inflammatory cytokines indicate that Th17 is involved in inflammatory immune responses and autoimmunity (Quesniaux et al., 2009; Steinman, 2007a), and cancer immunology (Nunez et al., 2013). IL-17a and IL-17f secreted from Th17 can also mediate immune response against bacterial and fungal infection by activating neutrophils (Weaver et al., 2006).

Additionally, another CD4+ T cell subset known as T regulatory cells was identified in the 1990s (Sakaguchi et al., 1995). Although the idea of T cell populations with a suppressing function was gaining focus in the 1970s-80s, it quickly diminished without finding any key evidence to support it (Sakaguchi et al., 2007). It is now understood that regulatory T cells are able to control the T cell repertoire by suppressing their function, therefore maintaining self-tolerance and preventing autoimmune diseases.

1.4.3.3 Nanoparticle induced immunity

Particulate antigens have been documented to enter the cross-presentation pathway in DCs (Shen et al., 1997; Sexton et al., 2009; Kaba et al., 2012; Hirosue et al., 2010; Sneh-Edri et al., 2011; Yang & Hsu, 2008; Plebanski et al., 1998; Akagi et al., 2007). Different sizes of nanoparticles can be endocytosed by various pathways; therefore it is possible that the entry pathway is mediating the intracellular fate of antigen processing.

In one study, all of the nanoparticles were found to localise to the lysosomal compartment upon internalisation into APCs, therefore the composition of material and the time it can be retained in the lysosomal compartment may influence cytosol escape for the
cross-presentation (Shen et al., 2006).

One study demonstrated that particles in the viral range (40-50 nm) are an optimal size to be trafficked to the draining lymph nodes, therefore eliciting a higher level of cellular response by activating CD8$^+$ T cells (Fifis et al., 2004). Another study showed that nano beads of 40-49 nm could activate CD4$^+$ T cells and induce Th1 biased cytokine secretion, while nano beads of 93-101 nm induced Th2 biased cytokine secretion following immunisation (Mottram et al., 2007). This suggested that the smaller nanoparticles can elicit both humoral and cellular responses by activating both CD4$^+$ and CD8$^+$ T cells. On the contrary, it was shown that the nanoparticles of much larger size (350 nm-1 µm) could also be cross-presented while inducing a robust Th1 response with predominant IFN$\gamma$ production by priming CD4$^+$ T cells (Sneh-Edri et al., 2011; Shen et al., 2006). Furthermore, nanoparticles in the larger size range could also be presented via the cross-presentation pathway in non-competent APCs (B cells) (Shen et al., 2006).

While the size of nanoparticles can induce different modes of cellular uptake, the differences in size may not corroborate to the type of immune response induced. Several studies demonstrated different outcomes of immune response induced by various sizes of nanoparticles.

Differences in the immune responses induced by nanoparticles may not be only limited to size, but may also depend on the density of nanoparticles (solid beads or capsules), the adjuvant effects of the materials and the administration sites for the vaccination (Yip et al., 1999). The mechanism of uptake and intracellular processing of nanoparticles may also be influenced by the surface charge or overall $\zeta$-potential, sequence or density of polymer/antigen matrices, and other formulation properties (Sneh-Edri et al., 2011). One study demonstrated that a fusion-activated virosome internalised by the receptor-mediated endocytosis, actively fused with acidified endosomal membrane to release the antigen directly into the cytosol (Arkema et al., 2000). Similarly, antigen encapsulated in the PLGA nanoparticles (compared to soluble antigen) was shown to remain longer in the endosome and increased the amount of antigen escape into the cytosol (Shen et al., 2006). The level of
antigen cross-presented and also the duration of presentation were also higher than the soluble antigen. Encapsulation of antigen was also shown to be more efficient than the antigen delivered on the surface of a solid particle. Encapsulation of soluble antigen can possibly protect them from endosomal protease degradation, while assisting the slow release of antigen over prolonged time to be processed by the cross-presentation pathway. Antigens remaining in the endosome can continue to be processed by the MHC class II pathway.

In conclusion, most of the studies demonstrated that potent cellular and humoral responses can be elicited with antigen in a particulate form, significantly higher than soluble antigen, with increased production and class switching of IgG1 and IgG2a by Th2 and Th1 cytokine secretion, respectively (Uto et al., 2013; 2009; Mohr et al., 2010).
1.5 Malaria infection

Malaria infection is one of the most prevalent diseases caused by members of the *Plasmodium* genus. Malaria causes millions of acute cases globally with more than 50,000 deaths each year (World Health Organization). However, currently there is no effective vaccine that provides protection in all vaccine recipients. One of the major difficulties in malaria vaccine development is that the parasite has the ability to undergo repeated antigenic variation in order to evade host defence immunity (Su *et al.*, 1995). Several vaccine candidates has been developed, which include the most promising candidate RTS,S vaccine currently in phase III clinical trial (Bejon *et al.*, 2013; Regules *et al.*, 2011). The RTS,S vaccine is formulated in either water-in-oil emulsion adjuvant or liposome (PATH, 2014), and consists of human malaria *Plasmodium falciparum* circumsporozite protein co-expressed with the hepatitis B antigen (Richards *et al.*, 1998).

The malaria parasite has a very complex life cycle. As depicted in Figure 1.7, the life cycle of malaria starts from the injection of sporozoites from mosquitoes into the blood stream, and it infects liver hepatocytes to proliferate to tens of thousands of haploid form before re-entering the bloodstream (Nardin & Nussenzweig, 1993). The parasite in this stage is called merozoites and it continues to infect the red blood cells during its asexual replication in the bloodstream. The time required for malaria parasites to complete one asexual cycle varies between species. *P. falciparum* completes its replication in 48 h. Within this time frame, malaria goes through several stages of life cycle within the host erythrocytes, until the reproduced merozoites are released by the rupture of the erythrocytes for further infection and replication (Tuteja, 2007). Due to the lack of expression of MHC class I molecules by the erythrocytes, the infected cells cannot display the intracellular malarial antigens for T cell recognition. It is also difficult for any antibodies to bind to malaria during the blood stage, as many of the parasites antigens are expressed only within the infected erythrocytes. The only period the parasite is exposed to the APCs in the blood stream is when the merozoite is released to infect other erythrocytes, therefore the most effective vaccine components are the proteins expressed on the surface of merozoite stage (Kedzierski *et al.*, 2001).
Figure 1.7: *Plasmodium falciparum* life cycle. (Reproduced from Regules *et al.*, 2011)
1.5.1 Merozoite surface protein 4/5

The merozoites possess many novel surface proteins are well characterised for their high potential as a vaccine candidate antigens. These proteins include merozoite Surface Protein (MSP) 1 (Cheng et al., 2013), MSP 2 (Sumari et al., 2011), MSP 4 (Marshall et al., 1997) MSP 5 (Gardner, 1998), and apical membrane antigen 1 (AMA1) (Zhu et al., 2011) from P. falciparum. MSPs play an important role in initiating the infection of erythrocytes by recognising and binding to the surface.

There are four species of rodent malaria which have been utilised extensively for vaccine development; P. yoelii, P. berghei, P. vinkei and P. chabaudi. A murine malaria species does not infect humans as malaria has very narrow host specificity, however the study of such model is very useful for understanding their immune mechanisms and vaccine potential.

A region of P. falciparum chromosome 2 has been identified which encodes for MSP2, MSP5, and MSP4 in tandem (Marshall et al., 1998). MSP4 and MSP5 encode glycosylphosphatidylinositol (GPI)-anchored proteins with observed molecular weight of 40 kDa, and both proteins contain a highly conserved region, which codes for a single epidermal growth factor (EGF)-like domain. A gene homologous to MSP4 and MSP5 was also identified in the murine malaria parasite Plasmodium chabaudi adami DS (Black et al., 1999), and hence coined its name MSP4/5. The gene has an intron-exon structure similar to those of MSP4 and MSP5 (Figure 1.8), and the encoded protein has major structural and immunochemical properties in common with MSP4 and MSP5. The same homologue was later identified in two other murine malaria species, P. yoelii and P. berghei (Kedzierski et al., 2000a). The recombinant MSP4/5 was demonstrated to elicit very strong humoral responses and IgG1 and IgG2a antibody production, as well as providing protection against lethal malaria challenge in mice (Kedzierski et al., 2000b; 2001).
Figure 1.8: Schematic representation (to scale) of the intron-exon arrangement and predicted polypeptide structure of PcMSP4/5 to *P. falciparum* MSP4 and MSP5. The position of the EGF-like domain is indicated by a hatched box. Signals for secretion and GPI attachment are represented by solid boxes. (Image and legend adopted from Black *et al.*, 1999)
Heterologous immunisation with recombinant MSP4/5 from different murine malaria species can also protect mice from the lethal strain challenge. BALB/c mice immunised with variant recombinant MSP4/5 were challenged with *P. yoelii yoelii* YM-parasitised red blood cells and the heterologous protections was observed in mice immunised with recombinant MSP4/5 from different *P. yoelii* isolates (*P. yoelii killicki* 193L, *P. yoelii nigeriensis* N67) and *P. berghei* ANKA (Goschnick *et al.*, 2004). However, there is a correlation between the protein similarity and the level of protection or the serum cross-reactivity. No serum reactivity was observed between *P. chabaudi adami* MSP4/5 to the mouse serum raised against other MSP4/5 in the above species, probably due to relatively low amino acid sequence similarities to *P. berghei* (56%) and *P. yoelii* (51%). Nonetheless, the efficacy of recombinant MSP4/5 in animal studies to confer protection is evident. This supports the potential of this protein for development of a human malaria vaccine.
1.6 Rationale of this project

Traditional vaccines are often formulated from an attenuated organism for their efficacy to elicit sufficient humoral and cellular responses with a single dose. However the use of live organisms poses many risks associated with reversion to the virulent strain. A subunit vaccine or peptide vaccine is a safer alternative to attenuated vaccines, however its immunogenicity is relatively low.

Nanoparticles and nanocapsules can stabilise vaccine antigens and ensure delivery to intracellular compartments to increase vaccine immunogenicity, therefore they are a promising antigen delivery system to increase the efficacy of subunit vaccines. Delivering antigens in particulate form offers several advantages over soluble antigens as they can provide protection from extracellular protease degradation and prolong their circulation in the system. Antigens can also be co-delivered in combination with adjuvants (i.e. PAMPs), which allow direct interaction of the antigens with immune cell surface receptors (i.e. TLRs). This facilitates more efficient cellular uptake by APCs, therefore inducing potent antigen-specific humoral and more importantly, cellular responses by promoting a higher level of cross-presentation (Shen et al., 1997; Sexton et al., 2009; Kaba et al., 2012; Hirosue et al., 2010; Sneh-Edri et al., 2011; Yang & Hsu, 2008; Plebanski et al., 1998; Akagi et al., 2007). This is very important as targeting the cross-presentation pathway is the key to develop effective vaccines against tumors, intracellular parasites, intracellular bacteria and viruses (Rock et al., 1990; Moron et al., 2004).

A variety of materials exist from which nanoparticles can be synthesised. Much research has been focused on the use of liposomes and polysomes which are biodegradable to alleviate the concerns of clearance from body. However, the preparation methods for these types of nanoparticles often introduce toxicity and causes variability for a large-scale manufacture.

A templating method has also been demonstrated for the synthesis of polymer nanocapsules (Wang et al., 2008a), employing a simpler preparation method, which enables the fabrication of nanoparticles with increased homogeneity and stability. This approach utilises a highly
monodispersed solid silica core as the inner base (Stöber et al., 1968), and builds a highly robust mesoporous shell surrounding the core (Büchel et al., 1998). Infiltration of material into the mesoporous matrix of the SC/MS silica nanoparticles allows adsorption by electrostatic interaction, then chemical cross-linking for immobilisation, followed by the subsequent removal of the nanoparticles to form a hollow polymer nanocapsule (Yang & Hsu, 2008; Goethals et al., 2013). This method produces the nanocapsules consisting of only a single component with a thick capsule wall due to the large specific surface area of SC/MS nanoparticle. This allows a larger capacity of molecules to be loaded per nanoparticle, in a single step. Most importantly, the fabrication of SC/MS nanoparticles is very simple, scalable and cost-effective (Tang et al., 2012), in addition, the properties of the MS are tailorable to fit the infiltrating molecule moiety (Goethals et al., 2013). The templating method allows the fabrication of nanocapsules to overcome some of the issues associated with other preparation techniques and therefore is attractive option to fabricate a safe and effective vaccine delivery platform.

While the templating method has demonstrated its potential for a more efficient nanocapsule preparation method, there have been no reports of nanocapsules synthesised through the templating approach without the use of polymers as an antigen delivery system. Most of the polymeric materials possess strong positive charges; therefore toxicity is a concern. Therefore, it is the focus of this study to synthesise nanocapsules derived from antigen only, by employing SC/MS nanoparticles as a template. The feasibility of utilising this templating method without the use of polymers, the ability of nanoparticle for efficient cellular uptake, particularly by DCs, and the magnitude of cross-presentation will be assessed. Furthermore, a murine malaria protein, P. chabaudi adami MSP4/5 will be applied to this templating method, to evaluate the efficacy of antigen-based nanocapsules as a new antigen delivery system in a mice model. Thus, the proposed novel preparation method could be the new generation of vaccine delivery system to provide protection from the global prevalent diseases including AIDS, malaria, tuberculosis and hepatitis C.
CHAPTER 2

Materials and suppliers

This chapter lists the general materials and suppliers used throughout this project.
2.1 General procedures

All the materials are listed below, with more specific details outlined in the appropriate chapter.

Unless otherwise stated, all solutions were prepared with reverse osmosis double distilled water (MQH₂O), obtained from the Millipore Milli-Q® water system (Millipore, USA) with the exception of media, where distilled water (dH₂O) was used.

Chemicals used were analytical or molecular grade and stored at ambient temperature unless otherwise stated.

Sterilisation of all media and glassware by autoclaving was undertaken at 121°C (15 lbf/in²) for 20 min. Samples were filter sterilised using the Acrodisc® 0.22 µm filters (Pall, USA).

The Finnpipette® digital pipette range (Thermo Fisher Scientific, USA) and the Eppendorf Research® (Eppendorf, Germany) were used to dispense solutions. The range included 0.5-10 µL, 5-50 µL, 20-200 µL, 100-1000 µL, 1-5 mL, 2-10 mL pipettes and 20-200 µL or 50-300 µL multi-channel pipettes. Volumes above 10 mL were measured with a measuring cylinder.

Volumes less than 1.5 mL underwent centrifugation with the Eppendorf microcentrifuge MiniSpin® Plus. Volumes of up to 50 mL requiring less than 5,445 × g underwent centrifugation in either the Beckman Allegra 21R centrifuge or the Heraeus Multifuge 1S-R centrifuge.

Products less than 2 g were weighed on the Mettler Toledo XS105 Dual Range top loading analytical balance. Products greater than 2 g were weighed on an ISSCO model 300 top loading balance.
2.2 Laboratory instruments and suppliers

1010 TEM
5022F Fast DLS particle sizing spectrometer
A1 confocal laser scanning microscope
AllegraTM 21R centrifuge
ASAP 2000 surface analyser
Biological safety cabinet class II, BH2000 series
BioPhotometer UV-visible spectrophotometer
CanoScan LiDE 210 scanner
Canto II cytometer
CO₂ incubator
Countess® automated cell counter
Digital sonifier
Dry heating block
EPS 3000xi power pack
GelDoc imaging system
Heraeus Multifuge 1S-R centrifuge
iBlot dry gel transfer device
iMark microplate absorbance reader
ISSCO model 300 bench top balance
IX51 inverted phase contrast microscope
Milli-Q® water filtration system
Mini gel (Mini-sub cell GT cell) DNA electrophoresis unit
Mini-PROTEAN® tetra cell PAGE unit

JEOL, Japan
ALV, Germany
Nikon, Japan
Beckman Coulter, USA
Micromeritics, USA
Clyde-Apac, Australia
Eppendorf, Germany
Canon, Japan
BD biosciences, Australia
Branson Ultrasonic, USA
Sanyo, Japan
Life Technologies, USA
Ratek, Australia
Bio-Rad Laboratories, USA
Bio-Rad Laboratories, USA
Thermo Electron Corporation, USA
Life Technologies, USA
Bio-Rad Laboratories, USA
ISSCO, Australia
Olympus, Japan
Millipore, USA
Bio-Rad Laboratories, USA
Bio-Rad Laboratories, USA
MiniSpin® Plus microcentrifuge  
Eppendorf, Germany

Nova NanoSEM  
FEI, USA

Orbital shaker incubator  
Bioline, Australia

pH meter  
Metrohm AG, Switzerland

Platform mixer  
Ratek, Australia

POLARStar Omega microplate reader  
BMG labtech, Germany

PowerPac 300 power pack  
Bio-Rad Laboratories, USA

PowerPac Basic power pack  
Bio-Rad Laboratories, USA

Pulse controller and Gene Pulser™ apparatus  
Bio-Rad Laboratories, USA

SDS-PAGE Electrophoresis unit  
Bio-Rad Laboratories, USA

Sonicator wash  
Soniclean, Australia

Suspension mixer  
Ratek, Australia

Titertek® ELISA shaker platform  
Flow Laboratories, USA

Transilluminator (UV)  
Novex, Australia

Water bath  
Ratek, Australia

XS105 Dual Range analytical balance  
Mettler Toledo, Australia
2.3 Consumables and suppliers

2.3.1 Plastic materials

Acrodisk® filter, sterile (0.22 and 0.45 µm)  
Pall, USA

Cell culture flask, filter cap (25 and 75 cm²)  
Greiner Bio-One, Germany

Cell culture plate, sterile (6 well)  
Nunc, Denmark

Cell culture plate, sterile (24 and 96 well)  
Greiner Bio-One, Germany

Centrifuge tube, sterile (10 and 15 mL)  
Sarstedt, Germany

Centrifuge tube, sterile (50 mL)  
Greiner Bio-One, Germany

Countess® chamber slide  
Life Technologies, USA

Cryovial, sterile (2 mL, star bottom)  
Iwaki, Japan

Disposable reagent reservoir, sterile  
VitaLab, USA

Flat bottom plate, non-sterile (96 well)  
Nunc, Denmark

Microfuge tube, non-sterile (1.5 mL)  
Sarstedt, Germany

Petri dishes  
Technoplast Interpath service, Australia

Polypropylene gravity flow column (1 mL)  
QIAGEN, Germany

Polystyrene round bottom tube, 12 x 75 mm  
BD biosciences, Australia

Stopcock, sterile  
BD biosciences, Australia

Syringes (1, 3, 5, 10, 20, and 60 mL)  
Terumo, Japan

UVette® (10 mm)  
Eppendorf, Germany
### 2.3.2 Non-plastic materials

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<th>Item</th>
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<tr>
<td>200-mesh holey support films, copper grid</td>
<td>ProSciTech, Australia</td>
</tr>
<tr>
<td>Aluminium SEM stub</td>
<td>ProSciTech, Australia</td>
</tr>
<tr>
<td>Cell strainer (100 µm)</td>
<td>BD biosciences, Australia</td>
</tr>
<tr>
<td>Difiltration cup</td>
<td>Sartorius-Stedim, Germany</td>
</tr>
<tr>
<td>Electroporation cuvettes (0.1 cm)</td>
<td>Molecular Bio Products, USA</td>
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<tr>
<td>Glass coverslip (22 x 22 mm)</td>
<td>HD Scientific, Australia</td>
</tr>
<tr>
<td>Glass slides (26 x 76 mm, 1 mm-1.2 mm thick)</td>
<td>Sail brand, China</td>
</tr>
<tr>
<td>Glass syringes (3 mL and 5 mL)</td>
<td>Alltech, Australia</td>
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<tr>
<td>iBlot transfer stacks (nitrocellulose)</td>
<td>Life Technologies, USA</td>
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<tr>
<td>Mini-PROTEAN® TGX™ precast gel</td>
<td>Bio-Rad Laboratories, USA</td>
</tr>
<tr>
<td>Needles (19 and 25 gauge)</td>
<td>Terumo, Australia</td>
</tr>
<tr>
<td>Vivaspin®20 (30,000 DaMWCO)</td>
<td>Sartorius-Stedim, Germany</td>
</tr>
</tbody>
</table>
Chapter 2

2.4 Chemicals, solutions and other products

2.4.1 Chemicals and solutions

(3-aminopropyl)triethoxysilane (APTES): >98% (Sigma-Aldrich, Germany).

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer: 5 mM (Gibco, USA), stored at 4°C.

4’,6-diamidino-2-phenylindole (DAPI): 5 mg/mL stock (Cell Signaling, USA), stored at −20°C.

Acqua instant stain: (Bulldog Bio Inc., USA), stored at 4°C.

Agarose: 1% (w/v) agarose, DNA grade (Bioline, England).

Ammonium fluoride: 8 M (Sigma-Aldrich, Germany).

Ammonium hydroxide: 32% (Merck, Germany).

Ampicillin (AMP): 100 mg/mL stock (CSL, Australia) in MQH₂O and filter sterilised through a 0.22 µm filter. Aliquots stored at −20°C. Final working concentration of 100 µg/mL used.

Bacteriological agar: (Oxoid, England).

Bovine serum albumin fraction V (BSA): 1 mg/mL stock (Sigma-Aldrich, Germany) in MQH₂O. Aliquots stored at −20°C.

Bradford reagent: 100 mg Coomassie Brilliant Blue G-250 (Sigma-Aldrich, Germany) dye dissolved in 50 mL 95% (v/v) ethanol (Merck, Germany), then mixed with 100 mL 85% (v/v) phosphoric acid (Chem Supply, Australia), and brought up to 1 L with dH₂O. Stored at 4°C and filtered through a 0.45 µm filter before use.

Carboxyfluorescein diacetate succinimidyl) CFSE: 5µM (Sigma-Aldrich, Germany), stored at −20°C.

Chelating Sepharose™ Fast Flow: (GE Healthcare, Sweden), charged with 0.2 M nickel sulphate (NiSO₄) (BDH, USA).
Chloramphenicol (CM): 34 mg/mL stock (Sigma-Aldrich) in 96% ethanol (Merck, Germany) and filter sterilised through a 0.22 µm filter. Aliquots stored at −20°C. Final working concentration of 34 µg/mL used.

ClearPAGE Instant Blue Stain: (C.B.S. Scientific Company, USA), stored at 4°C.

Complete Freund’s adjuvant: (Sigma-Aldrich, Germany) stored at 4°C.

Concanavalin A (Con A): 2 µg/mL (Sigma-Aldrich, Germany), stored at −20°C.

Coomassie Brilliant Blue G-250: (Sigma-Aldrich, Germany).

DNA loading buffer (11x): 10% (w/v) ficoll®-400 (BDH, USA), 50% (v/v) glycerol (BDH, USA), 0.5% (w/v) Orange G (Sigma-Aldrich, Germany), 1% (w/v) SDS (Merck, Germany), 10 mM EDTA (BDH, USA), 50 mM Tris-HCl (pH 8.0) (Merck, Germany).

Dulbecco’s phosphate buffered saline (DPBS): (Gibco, USA) stored at 4°C.

ELISA coating buffer: 4% of 84 g/L NaHCO₃ (BDH, Germany) and 8% of 106 g/L Na₂CO₃ (BDH, Germany) were mixed immediately before use. Stored at 4°C.

Ethanol: 96% (v/v), (Merck, Germany).

Ethidium bromide (EtBr): 6 mg EtBr (Sigma-Aldrich, Germany) in 2 L dH₂O.

Ethylenediamine tetra acetate (EDTA): 0.5 M EDTA (Merck, Germany).

FACS staining buffer: 3% BSA in sterile PBS, made fresh.

FACS wash buffer: Sterile PBS, 0.1% sodium azide (Sigma-Aldrich, Germany), with 10% FBS added through a 0.2 µm filter, stored at 4°C.

Fetal bovine serum (FBS): heat-inactiated, Australian-certified (Gibco, USA), stored at −20°C.

Glutaraldehyde (GA): Grade I, 5% (v/v) (Sigma-Aldrich, Germany), stored at 4°C.

Glycerol: 50% (v/v) (BDH, USA), sterile.

Histopaque-1077: (Sigma-Aldrich, Germany) stored at 4°C.

Hydrochloric acid (HCl): 32% (Merck, Germany).

Hydrofluoric acid (HF): 2M (Sigma-Aldrich, Germany).
IMAC binding buffer: 10-50 mM imidazole (Sigma-Aldrich, Germany), 0.5 M NaCl (BDH, USA), PBS (Oxoid, England)

IMAC elution buffer: 100-120 mM imidazole (Sigma-Aldrich, Germany), 0.5 M NaCl (BDH, USA), PBS (Oxoid, England)

IMAC stripping solution: 0.5 M NaCl (BDH, USA), 50 mM EDTA (Merck, Germany).

IMAC wash buffer: 40-80 mM imidazole (Sigma-Aldrich, Germany), 0.5 M NaCl (BDH, USA), PBS (Oxoid, England)

Imidazole: 2.5 M imidazole stock (Sigma-Aldrich, Germany), filtered through a 0.45 µm filter, stored at 4°C in a light-proof container.

Iminodiacetic acid Sepharose®: (Sigma-Aldrich, Germany), charged with 0.2 M nickel sulphate (NiSO₄) (BDH, USA).

Incomplete Freund’s adjuvant: (Sigma-Aldrich, Germany) stored at 4°C.

Interleukin 2 (IL-2): 20 IU/mL, mouse recombinant (Affymetrix eBioscience, USA), stored at -80°C.

Isopropyl-β-D-thiogalactopyranoside (IPTG): 1 M (Progen, Australia) filtered through a 0.22 µm filter, aliquots stored at −20°C.

Lambda (λ) DNA: (Promega, USA), stored at −20°C.

Molecular grade water (MGH₂O): DNAse and RNase free (Gibco, USA).

n-Octadecyltrimethoxysilane (TMS): 91.6% (Sigma-Aldrich, Germany).

Nickel sulfate (NiSO₄): 0.2 M NiSO₄ stock (BDH, USA), filtered through a 0.45 µm filter.

p-Nitrophenyl phosphate (pNPP) substrate: (Sigma-Aldrich, Germany) stored at 4°C.

Ovalbumin: 100 mg/mL albumin stock from chicken egg white, Grade V (Sigma-Aldrich, Germany) in MQH₂O and filter sterilised through a 0.22 µm filter. The concentration was measured and adjusted using UV-vis spectrophotometer. Solution was either used immediately or aliquots were stored at −20°C.

Paraformaldehyde (PFA): 4% stock (w/v) (Sigma-Aldrich, Germany), dissolved by heating at < 60°C in MQH₂O. Filter sterilised through a 0.22 µm filter, stored at 4°C.

PBS/Tween: PBS (Oxoid, England) with 0.05% (v/v) Tween 20® (Sigma-Aldrich,
Penicillin/Streptomycin: 100 µg/mL, 100 U/mL (Gibco, USA), stored at −20°C.

Phosphate buffered saline (PBS): 1 tablet of Dulbecco’s A PBS (Oxoid, England) in 100 mL MQH₂O (sodium chloride 0.8%, potassium chloride 0.02%, disodium hydrogen phosphate 0.115%, potassium dihydrogen phosphate 0.02%). pH 7.4 or adjusted to the desired level.

Phosphoric acid: 85% (Chem-Supply, Australia).

Phosphotungstic acid: 1% (w/v) (Sigma-Aldrich, Germany).

Precision Plus Protein™ Dual Colour standard: 10 µL per well (Bio-Rad Laboratories, USA), stored at −20°C.

Precision Plus Protein™ Unstained standard: 10 µL per well (Bio-Rad Laboratories, USA), stored at −20°C.

PrestoBlue® cell viability reagent: (Gibco, USA), stored at 4°C.

ProLong® gold antifade reagent: 20 µL per slide for mounting (Life Technologies, USA), stored at −20°C.

RPMI 1640 GlutaMAX™: (Gibco, USA), stored at 4°C.

SDS loading buffer: (5x): 60 mM Tris-HCl (Merck, Germany), 25% (v/v) glycerol (BDH, USA), 2% (w/v) SDS (Merck, Germany), 1.4 mM β-mercaptoethanol (Bio-Rad Laboratories, USA), 0.1% (w/v) bromophenol blue (Sigma-Aldrich, Germany), stored at −20°C.

SDS running buffer (10x): 3% (w/v) Tris-HCl (Merck, Germany), 14.2% (w/v) glycine (Amresco, USA), 1% (w/v) SDS (Merck, Germany).

Skim milk: powder (Diploma, Australia).

Sodium Chloride (NaCl): 0.15 M-0.5 M NaCl (BDH, USA).

Sodium dodecyl sulphate (SDS): 10% (w/v) (Merck, Germany)

Sodium Hydroxide (NaOH): 0.1-3 M NaOH (Merck, Germany).

Tetraethoxysilane (TEOS): >99% (Sigma-Aldrich, Germany).

Tetramethylbenzidine (TMB): (Life Technologies, USA), stored at 4°C.
**Tris Buffered Saline (TBS):** 25 mM Tris-HCl (pH 7.4) (Merck, Germany), 0.18 M NaCl (BDH, USA).

**Tris-Acetate-EDTA (TAE) electrophoresis buffer (1x):** 40 mM Tris-HCl (pH 8.0) (Merck, Germany), 20 mM acetic acid (Merck, Germany), 2 mM EDTA (Merck, Germany).

**Triton X-100:** 0.1% (v/v) (Sigma-Aldrich), in MQH$_2$O.

**Trypan blue stain:** 0.4% (Life Technologies, USA).

**Tryptone:** (Oxoid, England).

**Tween 20:** polyoxyethylene sorbitan monolaurate (Sigma-Aldrich, Germany).

**Western Blue® stabilized substrate for alkaline phosphatase:** (Promega, USA).

**Yeast extract:** (Oxoid, England).

**β-mercaptoethanol:** electrophoresis purity (Bio-Rad Laboratories, USA).

### 2.4.2 Bacterial culture media

**EnPresso™ Tablet cultivation set:** The cultivation set (BioSilta, Finland) included 2 EnBase® medium tablets (white bag), 1 booster tablet (black bag), Enzl'm (600 U/L) stored at 4°C, AirOtop seal flask closures (BioSilta, Finland) per 50 mL media. Prepared in MQH$_2$O.

**Luria-Bertani (LB) broth:** 1% (w/v) tryptone (Oxoid, England), 0.5% (w/v) yeast extract (Oxoid, England), 1% (w/v) NaCl (BDH, USA). Prepared in MQH$_2$O.

**Luria-Bertani (LB) agar:** 1% (w/v) tryptone (Oxoid, England), 0.5% (w/v) yeast extract (Oxoid, England), 1% (w/v) NaCl (BDH, USA), 1% (w/v) bacteriological agar (Oxoid, England). Prepared in MQH$_2$O.
2.4.3 Restriction endonucleases

Restriction enzymes were used in the supplied buffer according to the manufacturer’s instructions and stored at $-20^\circ$C.

- *BglII* New England Biolabs, USA
- *NcoI-HF®* New England Biolabs, USA
- *PstI* Promega, USA

2.4.4 Commercial kits

Materials included in the kits were stored according to the manufacturer’s guideline.

- *Bioline ISOLATE plasmid Mini kit* Bioline, Australia
- *Mouse Th1/Th2 ELISA Ready-SET-Go® kit* Affymetrix eBioscience, USA

2.4.5 Antibodies

- *Mouse anti-mouse OVA258-265 bound to H$_2$K$^b$25-D1.16 monoclonal* Affymetrix eBioscience, USA
- *DyLight 405-conjugated goat anti-mouse IgG monoclonal* Affymetrix eBioscience, USA
- *Mouse anti-6xHis monoclonal* Abcam, England
- *Alkaline phosphatase (AP)-conjugated rabbit anti-mouse IgG polyclonal* Life Technologies, USA
- *Horseradish peroxidase (HRP)-conjugated rat anti-mouse IgG1 monoclonal* Life Technologies, USA
- *Horseradish peroxidase (HRP)-conjugated rat anti-mouse IgG2a monoclonal* Life Technologies, USA
CHAPTER 3

Synthesis and characterisation of silica nanoparticle template and protein-based nanocapsules

This chapter reports the fabrication and characterisation of SC/MS nanoparticle templates and the subsequently synthesised protein-based nanocapsules using a model protein.
3.1 Introduction

Dendritic cells (DCs) play a central role in initiating primary immune responses against infectious pathogens. They are the most potent antigen presenting cells (APCs) and efficient immune stimulators. Activation of CD4+ and CD8+ T cells can be induced by the processing and presentation of antigenic peptides by DCs, leading to the potentiation of humoral and cellular responses. An effective vaccine therefore must be able to efficiently engage with DCs to facilitate the induction of an immune response (Gamvrellis et al., 2004).

Many particulate carrier systems have proven to be an effective antigen delivery vesicle, with increased levels of intracellular localisation and antigen presentation. Use of particulates in a delivery system can also induce higher immune responses. They are therefore promising candidates for a vaccine delivery system, which may be superior to the commonly used subunit vaccine technology.

Particulate carrier systems are available in a wide variety of materials including synthetic polymers, synthetic polypeptides, polysaccharides, phospholipids and proteins which are synthesised through various techniques (reviewed in Chapter 1). The use of colloidal silica particles has been employed as a sacrificial template in some nanocapsule preparations. To date, there have been no reports on the preparation of nanocapsules derived from antigenic protein alone, synthesised through a templating system utilising solid core mesoporous shell (SC/MS) silica nanoparticles for vaccine delivery system. Such templates could serve as fundamental structures that would be universally applicable to produce vaccines with antigens of any proteins and polysaccharides.

The template synthesis of protein-based nanocapsules is achievable in three simple steps; infiltration of a protein into the mesoporous shell (MS) of the particles, cross-linking infiltrated protein by covalent bonding, and then finally removing the silica nanoparticle template by acid treatment. Structures of polymer nanocapsules synthesised through this system were deformable, yet maintained high integrity. Flexibility in structure is an advantage, as the nanocapsules could possibly cross the endothelium fenestration if given by
intravenous injection, while rigid particles of the same diameter cannot (Romero et al., 1999). High integrity of the nanocapsule wall may assist in the escape from in vivo protease digestion, enabling delivery to APCs in a much higher amount than the soluble form. Another advantage of protein-based nanocapsules over polymeric nanoparticles is their susceptibility to intracellular degradation by lysosomal enzymes (Altintas et al., 2013).

The templating system using SC/MS silica nanoparticles has been successful for polymer nanocapsule synthesis in drug carrier systems (Wang et al., 2008a; Goethals et al., 2013). However, there have been no attempts to test this system with proteins for vaccine delivery. Determining the suitability of the templating system to produce protein-based nanocapsules, and its capability of enhancing immune responses must be understood. Therefore, this chapter focuses on defining the structural and cellular characteristics of nanocapsules by using ovalbumin as a model protein. SC/MS silica nanoparticles were fabricated by previously established methods (Stöber et al., 1968; Büchel et al., 1998), and ovalbumin-based nanocapsules were subsequently synthesised using the templating method (Wang et al., 2008a). The ovalbumin nanocapsules were studied for their structure, and the in vitro behaviour using murine immature DCs for their ability to localise intracellularly and induce antigen presentation.
The aim of the experiments outlined in this chapter were:

- To fabricate the SC/MS silica nanoparticles for the subsequent synthesis of ovalbumin nanocapsules.
- To observe the structural assembly under electron microscopy and by dynamic light scattering analysis.
- To determine the specific properties of the mesoporous shell.
- To evaluate the cytotoxic effect of ovalbumin nanocapsules.
- To determine the efficiency of ovalbumin nanocapsules to engage with DCs and induce cellular uptake.
- To determine the ability of ovalbumin nanocapsules to be processed and presented with MHC class I molecules on DCs by the cross-presentation pathway.
3.2 Materials and Methods

3.2.1 Nano-material protocols

This section details the methods used to fabricate the SC/MS templates and subsequent synthesis of the protein-based nanocapsules.

3.2.1.1 Fabrication of SC/MS silica templates

3.2.1.1.1 Preparation of solid silica particle (core)

Based on a modified version of the Stöber method (Stöber et al., 1968), the solid silica spheres were prepared through the hydrolysis and condensation of tetraethoxysilane (TEOS) in appropriate amounts of ethanol, MQH₂O and ammonia solution. More specifically, 37 mL of absolute ethanol, 5 mL of MQH₂O and 4.2 mL of 32% ammonium hydroxide (w/w) were combined in an Erlenmeyer flask and stirred vigorously using a magnetic bar while heating at 30°C. After the temperature of the solution stabilised, 2.8 mL of TEOS was added rapidly followed by immediate vortex for 5 sec to ensure the homogeneity of nanoparticles. The solution was then kept very still at room temperature while the reaction was allowed to proceed for 1 h.

3.2.1.1.2 Fabrication of SC/MS silica nanoparticle (mesoporous outer shell)

The SC/MS silica nanoparticles were prepared as reported by Büchel and co-workers (Büchel et al., 1998). These particles were synthesised by sol-gel coating of a MS on a preformed solid silica particle from Section 3.2.1.1.1. Solution containing a mixture of 2.35 mL of TEOS and 0.5 mL of 91.6% n-octadecyltrimethoxysilane (TMS) was slowly added to the solution containing solid silica particles, drop wise over a period of 20 min under stirring conditions. The solution was then incubated static for 1 h for the formation of MS, followed by centrifugation at 1,650 x g for 5 min to harvest the particles. The particles were washed three times with ethanol by centrifugation at 3,000 x g for 5 min. Following the washes, the particles were dried on a glass petri dish at 100°C overnight to completely remove ethanol.
The removal of the porogen TMS was performed through calcination of particles by heating to 550°C for 6 h. Calcinated SC/MS silica nanoparticles were kept dry in a polypropylene tube for storage. In this condition, the SC/MS silica nanoparticles could be kept indefinitely.

### 3.2.1.2 Amine-functionalisation of SC/MS nanoparticle templates

To allow the infiltration of negatively charged proteins, the surface of partially negatively charged SC/MS silica nanoparticles were converted to positive charge by the adsorption of amine groups. One hundred milligrams of SC/MS nanoparticles were resuspended in 10 mL of absolute ethanol, followed by the addition of 0.5 mL ammonium hydroxide and 2 mL (3-aminopropyl)triethoxysilane (APTES), in a 15 mL centrifuge tube. The functionalisation was allowed to process overnight at room temperature on a suspension mixer, for homogenous formation of amino groups on the particle surface. The amine-functionalised particles were harvested by centrifugation at 5,000 \( \times \) g for 2 min, and washed in 10 mL absolute ethanol to remove the unreacted APTES. The nanoparticles were washed three times and resuspended in 10 mL absolute ethanol for longer storage. Once functionalised, particles could be kept indefinitely in this condition. The SC/MS nanoparticles were washed three times with PBS before application as a template for the synthesis of protein nanocapsule.

### 3.2.1.3 Spectrophotometric quantification of ovalbumin

The concentration of ovalbumin was determined using the UV-visible (UV-vis) spectrophotometer. Absorbance was measured at a wavelength of 280 nm and the concentration of the protein sample (mg/mL), was equal to the absorbance at 280 nm (with a path length of 1 cm).
3.2.1.4 Synthesis of ovalbumin nanocapsules using the SC/MS template

The nanocapsules were synthesised using the SC/MS template based on the previously described method (Wang et al., 2008a), with minor alterations. Ten milligrams of PBS-washed SC/MS templates were added to the ovalbumin stock solution of up to 10 mg/mL in PBS, followed by an overnight incubation on a suspension mixer at 4°C. Excess protein was then removed by three cycles of centrifugation and washing with PBS at 5,000 x g for 2 min. To stabilise the infiltrated protein in the MS, particles were incubated in 1 mL of 5% (w/v) glutaraldehyde (GA) solution or 1% (w/v) paraformaldehyde (PFA) solution for a minimum of 2 h at 4°C on a suspension mixer, to form covalent links. Cross-linking agents were chosen depending on the application. GA was used in the characterization study where fluorescence emitted from GA cross-linking was required, negating the need of fluorescent tags. The SC/MS particles that cross-linked with PFA were protected from the light during the incubation. After washing with PBS three times, the SC/MS templates were removed by 500 μL of 2 M hydrofluoric acid (HF) treatment in an 8 M ammonium fluoride buffer, pH 5 to obtain the protein-based nanocapsules (Yu et al., 2005). Each infiltration reaction was performed in 1 mL to ensure the proper removal of silica by HF. HF treatment required 5 min incubation with occasional inversion of tube at room temperature, followed by three washes with sterilised PBS and centrifugation at 6,500 x g for 5 min. The synthesised nanocapsules were resuspended in 100 μL of sterilised PBS, followed by five cycles of 1 min sonication to separate aggregated nanocapsules.

All washes were kept at 4°C and the concentration of excess ovalbumin was determined by UV-vis spectrophotometry (Section 3.2.1.3). The protein solution was briefly centrifuged to precipitate any remaining SC/MS nanoparticle templates for the concentration determination as silica could interfere with UV absorbance measurement.

**HF is highly corrosive and toxic. The above work was performed only under the supervision of trained personnel in a HF filter fitted fume hood, with an adequate supply of calcium gluconate gel.
3.2.1.5 Optimisation of protein infiltration into SC/MS template

To measure the amount of ovalbumin load within the MS of the SC/MS template, the concentration of ovalbumin was measured before and after the infiltration process. This was used to determine the percentage loading efficiency and capacity per gram of nanoparticles. Influence of the protein:template concentration ratio of 1:1, 1:2 and 1:10, and the solvent pH ranging 5.5, 7.4 and 8.5, were tested. In a single infiltration reaction, ovalbumin and the SC/MS templates were incubated overnight, in a total volume of 1 mL. The ovalbumin-loaded templates were washed three times with PBS, and then the concentration of ovalbumin in each wash was measured using the UV-vis spectrophotometer (Section 3.2.1.3).

The percentage of protein infiltration loading efficiency (LE), and loading capacity (LC) per gram of nanoparticles was calculated using the equation below (Al-Qadi et al., 2012). Infiltration %LE was determined to measure the efficiency of protein loading in the MS of SC/MS template, while LC expressed the amount of protein loaded per gram of template.

\[
\text{%LE} = \frac{\text{(Total amount of protein)} - \text{(Free amount of protein)}}{\text{(Total amount of protein)}} \times 100\%
\]

\[
\text{LC (mg of protein/g of template)} = \frac{\text{(Total amount of protein)} - \text{(Free amount of protein)}}{\text{(Weight of nanoparticle)}}
\]
3.2.1.6 Quantification of synthesised nanocapsules

Concentration of synthesised nanocapsules was determined by the UV-vis spectrophotometry (Section 3.2.1.3). The concentration of PFA cross-linked ovalbumin nanocapsules was also estimated using SDS-PAGE.

3.2.1.6.1 SDS-PAGE

One-dimensional SDS-PAGE was performed using a discontinuous buffer system (Lane, 1991). PFA cross-linked nanocapsules were mixed with SDS loading buffer and heated to 100°C in a dry heating block for 30 min. The proteins were separated using a Bio-Rad electrophoresis unit containing SDS running buffer, by electrophoresis at 150 V for 50 min or until the dye front reached the bottom of the gel. Mini-PROTEAN® TGX™ precast 4-15% gradient gels were used. The Precision Plus Protein™ Unstained standard was used for the determination of relative protein mass (Appendix 2).

3.2.1.6.2 Instant stain

Instant stain was used in place of Coomassie staining. ClearPAGE Instant Blue stain was used for staining the SDS-PAGE gel after electrophoresis. The SDS-PAGE gel was immersed in instant stain for up to 1 h until bands were appropriately resolved. Gels were rinsed in dH$_2$O until the background cleared.
3.2.2 Protocols for structural characterisation of nano-materials

This section details the methods used to characterise the physical properties of SC/MS templates and synthesised ovalbumin nanocapsules.

3.2.2.1 Surface characterisation of SC/MS template by nitrogen adsorption measurement

The specific surface area, pore diameter and pore volume of the SC/MS templates were measured using the Brunauer–Emmett–Teller (BET) method (Brunauer et al., 1938), on the Micromeritics ASAP 2000 surface analyser. Nitrogen gas was used for the adsorption step.

3.2.2.2 Scanning Electron Microscopy (SEM) imaging of SC/MS nanoparticle templates

The homogeneity and the diameter of dispersed SC/MS templates were examined by SEM. Approximately 50 µL of SC/MS templates suspended in 96% ethanol were placed directly onto an aluminium SEM stub and allowed to air-dry. The dried SEM stub was observed under the FEI Nova NanoSEM, operating at an accelerating voltage of 10-15 kV, with a spot size of 3.5 nm and a working distance of 5-8.2 mm. The diameter of 100 nanoparticles was measured to determine the average size of particles from the representative electron micrograph images. The relative standard deviation was calculated using Microsoft Office Excel.

3.2.2.3 Transmission Electron Microscopy (TEM) of SC/MS template and synthesised protein nanocapsules

The thickness of the MS on SC/MS template and the size of synthesised protein nanocapsules were determined from TEM images. For TEM measurements, approximately 10 µL of nanoparticles were placed on top of a 200-mesh holey support film copper grid and allowed to air-dry. The excess moisture was removed by placing the copper grid on a tissue. The copper grid loaded with the protein nanocapsules was negatively stained with 1% phosphotungstic acid and air-dried before imaging. The dried copper grid was observed under the JEOL1010
TEM at an accelerating voltage of 100 kV.

3.2.2.4 Particle size distribution of SC/MS template by Dynamic Light Scattering (DLS)

To determine the size distribution of fabricated SC/MS template, a batch-mode DLS measurement was conducted in the ALV 5022F Fast DLS particle sizing spectrometer, at 22°C with a fixed angle of 90° on highly aqueous solutions. SC/MS templates were suspended in 96% ethanol.
3.2.3 Cellular and immunoassay protocols

This section details the methods used to characterise the in vitro effects of synthesised ovalbumin nanocapsules.

3.2.3.1 Cell line

Murine immature DCs, DC2.4 were used for all in vitro assays and immunoassays. This cell line was kindly donated by Dr Dodie Pouniotis from the School of Medical Sciences, RMIT University, Australia.

3.2.3.2 Culture condition

DC2.4 cells were cultured in RPMI 1640 GlutaMAX™ media supplemented with GlutaMAX™ and 5 mM HEPES buffer with 10% heat inactivated foetal bovine serum added through a 0.22 µm filter. To avoid bacterial contamination, 100 U/mL of penicillin and 100 µg/mL streptomycin were added to the media for the cellular assays; however the cell passage was maintained with no antibiotics. Cells were maintained at 37°C in a 5% CO₂ humidified atmosphere. Cells were passaged every 3 to 4 days and used for experimentation at 70-90% confluence.

3.2.3.3 Peptide

Synthesised ovalbumin-derived peptide OVA258-265 (or SIINFEKL) was purchased from Auspep, Australia. The lyophilised peptide was reconstituted in PBS and stored in aliquots at −80°C.

3.2.3.4 Cell counting

The number of cells were counted using a Countess® Automated Cell Counter. Ten microlitres of either neat or diluted cell sample was mixed with 10 µL of 0.4% Trypan Blue Dye in a sterile tube, and then 10 µL of mixture was transferred into a Countess® chamber.
slide. All samples were processed and read within 2 min. The actual volume counted was 0.4 µL, the same as counting four (1 mm x 1 mm) squares in a standard haemocytometer.

3.2.3.5 Evaluation of the cytotoxicity of ovalbumin nanocapsules

The cytotoxicity of GA cross-linked ovalbumin nanocapsules was analysed at different concentrations using PrestoBlue® Cell Viability reagent. DC2.4 cells were seeded in a 96 well tissue culture plate at a density of 5 x 10^4 cells per well, in RPMI 1640 GlutaMAX™ medium supplemented with 10% heat inactivated FBS and incubated at 37°C in a 5% CO_2 humidified atmosphere, overnight. Following incubation, ovalbumin nanocapsules suspended in 20 µL of sterile PBS were added to a final concentrations of 3.125, 6.25, 12.5, 25 and 50 µg/mL in 120 µL per well. Replicate plates for different time points were made and were further incubated for up to 72 h with the nanocapsules in the same condition. At each time point, 12 µL of PrestoBlue® Cell Viability reagent was added to each well and the absorbance read at 570 nm with 600 nm reference wavelength for normalisation after 60 min incubation at 37°C in the dark. Absorbance readings were measured using the POLARstar Omega microplate reader.

Culture media and 0.1% Triton X-100 served as an internal negative and positive control, respectively. Percentage cell viability was calculated by setting the negative control to 100% after non-live cell dependent colour conversion (background and positive control), was eliminated from all treatment groups.

Statistical variables (mean, standard deviation, and standard error), were calculated for all data and presented graphically. Comparisons between groups were accessed by two-way ANOVA followed by a Dunnett’s multiple comparison test to determined significant differences in cell viabilities against the negative control (media alone) group. GraphPad Prism 6 software was used to perform the statistical analysis.
3.2.3.6 Cellular uptake of ovalbumin nanocapsules by DCs

To determine the cellular uptake of synthesised nanocapsules over time, DC2.4 cells were seeded onto 25 mm glass coverslips placed in a 6 well tissue culture plate at a density of 2 x 10^5 cells per well, and incubated at 37°C in a 5% CO₂ humidified atmosphere for 24 h. Approximately 30-50 µg of GA cross-linked ovalbumin nanocapsules were added to each well, suspended in RPMI 1640 GlutaMAX™ medium with 10% heat inactivated FBS and incubated at 37°C for up to 90 min to allow cellular uptake of the nanocapsules. A replicate plate was made and incubated at 4°C to serve as a negative control with no cellular uptake, but inducing only cell surface attachment. Following incubation, the unbound nanocapsules from both plates were removed by gentle washing with cold PBS three times. Cells were then fixed onto the coverslips by adding 4% PFA for 15-20 min at room temperature followed by three washes with PBS. The cellular nucleus was counter-stained with 4',6-diamidino-2-phenylindole (DAPI) (1:2000), at room temperature for 5 min, followed by three washes with PBS. The coverslips were carefully mounted and sealed onto a glass slide with gold antifade reagent. The ovalbumin nanocapsules were highly autofluorescent due to GA cross-linking, thus no fluorescent labelling was required. The confocal microscopic images of the cells were obtained using a Nikon A1 confocal laser scanning microscope (CLSM).

3.2.3.7 Antigen presentation assay

Presentation of ovalbumin-derived peptide OVA\textsubscript{(258-265)} (or commonly known as SIINFEKL) by MHC class I molecule (K\textsuperscript{b}), by APCs was measured by an immunoassay.

DC2.4 cells were seeded in a 24 well tissue culture plate at a density of 1 x 10^6 cells per well, in RPMI 1640 GlutaMAX™ medium supplemented with 10% heat inactivated FBS and incubated at 37°C in a 5% CO₂ humidified atmosphere overnight. Following incubation, either PFA cross-linked ovalbumin nanocapsules or soluble ovalbumin suspended in media, was added to the test wells to a final concentration of approximately 50 µg/mL in 1 mL per well. Internal controls were set up as listed in Table 3.1. Cells treated as the SIINFEKL control were pulsed with 40 µM SIINFEKL peptide for 2 h. All other cells were incubated for 6 h with soluble or capsulated ovalbumin, to allow the uptake and presentation of the proteins.
Table 3.1: List of conditions and internal control settings.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Primary antibody staining</th>
<th>Secondary antibody staining</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstained</td>
<td>✗</td>
<td>✗</td>
<td>No antibody labelling</td>
</tr>
<tr>
<td>Unstimulated</td>
<td>✓</td>
<td>✓</td>
<td>Unstimulated control</td>
</tr>
<tr>
<td>SIINFEKL positive control</td>
<td>✓</td>
<td>✓</td>
<td>Positive control</td>
</tr>
<tr>
<td>SIINFEKL negative control</td>
<td>✗</td>
<td>✓</td>
<td>Secondary antibody control</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>✓</td>
<td>✓</td>
<td>Ovalbumin test</td>
</tr>
<tr>
<td>Ovalbumin nanocapsule</td>
<td>✓</td>
<td>✓</td>
<td>Nanocapsule test</td>
</tr>
<tr>
<td>Ovalbumin nanocapsule control</td>
<td>✗</td>
<td>✗</td>
<td>Autofluorescence control</td>
</tr>
</tbody>
</table>

*✓ = YES, ✗ = NO.*
Following incubation, cells were harvested and excess protein was removed by washing three times with ice-chilled FACS wash buffer, with centrifugation at 400 x g for 5 min at 4°C. The cells were then resuspended in 100 µL FACS staining buffer for surface immunostaining. First, SIINFEKL in context with the MHC class I was detected with the 25-D1.16 monoclonal antibody (mouse anti-mouse OVA\textsubscript{(258-265)} bound to H\textsubscript{2}K\textsuperscript{b}) (1:200), followed by 1 h incubation at 4°C. After the incubation, stained cells were washed three times with chilled FACS wash buffer, and labelled with DyLight 405-conjugated goat anti-mouse IgG (1:200), in 100 µL FACS staining buffer for 1 h at 4°C in the dark. Following incubation, cells were washed three times with chilled FACS wash buffer and fixed in 1% PFA until analysis by a FACS Canto II cytometer.
3.3 Results

3.3.1 Structural characterisation of fabricated SC/MS silica nanoparticle templates

3.3.1.1 Surface characterisation of SC/MS template by nitrogen adsorption measurement

SC silica nanoparticles were prepared through the hydrolysis and condensation of TEOS in a mixture of ethanol, ammonia and water. Subsequently, a mesoporous silica shell was built upon the SC by sol-gel coating of TEOS and TMS. Three batches of fabricated SC/MS nanoparticles were analysed for their specific surface area, pore size and pore volume in their MS.

Nitrogen adsorption analysis indicated that the SC/MS nanoparticles had an average surface area of 209.5 m$^2$/g and an average pore size of 3.22 nm in diameter, with an average pore volume of 0.17 mL/g. The nanoparticles with the largest surface area of 219.7 m$^2$/g, and a pore size and volume of 3.07 nm and 0.17 mL/g, respectively, were chosen for the subsequent ovalbumin-based (Section 3.2.1.4), and malarial protein-based (Section 4.2.2.3) nanocapsule synthesis.

3.3.1.2 Determination of particle size and distribution by SEM and DLS

SEM was used to observe the morphology and measure the size of the fabricated SC/MS nanoparticle template. Fifty microlitres of the nanoparticles were placed on an aluminium SEM stub and air-dried. Aggregates of dried nanoparticles were observed under low magnification (3147 x), as shown in Figure 3.1A. This was possibly due to the electro-static interactions occurring between the silica particles, as this was also seen when dried on a glass petri dish during the fabrication process. However, it was not clearly determined under SEM observations whether these particles were in fact aggregated or fused together.
Figure 3.1: Observation of the SC/MS silica nanoparticles under SEM.

Magnification of A) 3147 x, B) 42,216 x, and C) 103,685 x with the scale bars shown at the bottom at the length of 50, 3 and 1 µm, respectively. The red arrow indicates the diameter (di).
At higher magnification, it was revealed that the fabricated SC/MS nanoparticles were spherical and homogeneous in structure (Figure 3.1B and C), with an average diameter of 516 ± 20 nm. The diameter of 100 nanoparticles was measured from the representative electron micrograph in the SEM imaging program (Figure 3.1C), however, it was difficult to observe the edge of MS, therefore more precise measurements were performed.

DLS was used to measure the size and distribution of nanoparticles for better precision. DLS measurement determined the size of particles by detecting the random changes in the intensity of light scattered by the particles in a solution. The even peaks shown in Figure 3.2A indicate that the SC/MS nanoparticles were fabricated with no aggregation, and an average diameter of 410 nm (shown in radius in Figure 3.2B). Although some particles of larger size were detected, they were found to be relatively low in polydispersity.

3.3.1.3 Observation of MS by TEM

The detail of the MS was observed under TEM. As the transmitted electrons allowed the visualisation of the MS, it was able to distinguish it from the solid silica core (SC). The SC appeared to be a round object of high density, surrounded by an even coating of porous formations. The overlap of nanoparticles indicated that the aggregation observed in SEM analysis was in fact due to the electro-static interaction caused by the dried particles, rather than agglomeration of fused particles (Figure 3.3A). The thickness of the MS was recorded by measuring the distance from the edge to the SC of the particle (as indicated in Figure 3.3B). The average thickness was determined to be 62.5 nm.
Figure 3.2: Particle size and distribution measurement by DLS. A) The graph indicates minimal aggregation of SC/MS silica nanoparticles, and in B) the size distribution.
Figure 3.3: Observation of the SC/MS silica nanoparticles under TEM. A magnification of A) 12,000 x, and, B) 75,000 x, with the scale bars shown at the bottom of lengths of 500 nm and 100 nm, respectively. The red arrow indicates the regions measured to determine the thickness of MS.
3.3.2 Synthesis of ovalbumin nanocapsules using the SC/MS template

3.3.2.1 Optimisation of protein infiltration into the SC/MS template

The SC/MS silica nanoparticle underwent functionalisation of the particle surface with amine groups, in order to gain the positive charge to allow the negatively charged protein to infiltrate. Albumin from chicken egg white (ovalbumin), was employed as the model protein to synthesise the protein-based nanocapsules. The infiltration load of protein in the MS was optimised by measuring the effect of different conditions such as the protein:template ratio and pH of the solvent where the infiltration reaction occur.

In the first optimisation experiment, the influence of the protein:template concentration ratio was tested. In a single infiltration reaction, 10 mg of the SC/MS nanoparticles were used as a template. An ovalbumin concentration of 10 mg, 5 mg and 1 mg were incubated with nanoparticles to give a protein:template concentration ratio of 1:1, 1:2 and 1:10, respectively. This was performed to determine the optimal concentration of ovalbumin required for subsequent nanocapsule synthesis, with minimal adverse effects, which may be caused by the saturation of protein. The efficiency of protein loading was found to be the highest when the amount of template was double that of the protein (1:2). This method achieved a loading efficiency of 6.23%, while 1:1 and 1:10 ratios had an efficiency of 3-4%. While the differences in the loading efficiency between the 1:1 and 1:10 ratio was much lower, there was a 10-fold increase in the amount of protein loaded per gram of template in the 1:1 ratio, compared to the ratio of 1:10 (Table 3.2). This showed that the loading efficiency may not necessary translate to the actual amount of ovalbumin loaded into the template. The loading capacity was proportional to the amount of ovalbumin used for infiltrating reaction; therefore an increase in the amount of synthesised nanocapsule was also expected at higher concentration of protein. The results also suggested the there was no adverse effects in the rate of infiltration by applying high concentrations of protein to the SC/MS template.
Table 3.2: The influence of protein:template ratio on the infiltration %LE and LC.

<table>
<thead>
<tr>
<th>Protein (mg):Template (mg)</th>
<th>%LE</th>
<th>LC (mg of protein/g of template)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 : 10 (1:1 ratio)</td>
<td>3.99</td>
<td>38.7</td>
</tr>
<tr>
<td>5 : 10 (1:2 ratio)</td>
<td>6.23</td>
<td>15.05</td>
</tr>
<tr>
<td>1 : 10 (1:10 ratio)</td>
<td>3</td>
<td>0.36</td>
</tr>
</tbody>
</table>
In the second optimisation experiment, the influence of solvent pH was studied. The optimal pH was determined at which the greatest electro-static attractions could form between the negatively charged ovalbumin, to the positively charged SC/MS templates. This attraction could improve when the isoelectric point (pI) of the infiltrating protein was lower than the pH of the buffer used for the reaction. The PBS used to prepare the ovalbumin stock, and in all wash steps was originally adjusted to pH 7.4. This was the pH commonly used to maintain proteins, however, PBS of pH 5.5 and 8.5 was also introduced to determine the effect on ovalbumin infiltration load at different pH. In a single infiltration reaction, 10 mg of the SC/MS nanoparticles were used as templates. The ovalbumin concentration of 10 mg was chosen for this optimisation study, as this concentration was determined to have the best infiltration loading capacity (described in Table 3.2). The effect of pH was quite clear. At pH 5.5, ovalbumin did not show any capacity to infiltrate the template, while both the loading efficiency and capacity significantly increased as the pH increased (Table 3.3). An increase was also seen from pH 7.4 to 8.5. As the theoretical pI of ovalbumin was 4.6, this result confirmed that the higher pH could promote the infiltration of protein with a low pI. By observing the results from these optimisation studies, it was decided that subsequent synthesis of ovalbumin nanocapsules were to be carried out with 10 mg of ovalbumin to infiltrate 10 mg of template, in PBS at pH 8.5.
Table 3.3: The influence of solvent pH on the infiltration %LE and LC.

<table>
<thead>
<tr>
<th>pH</th>
<th>%LE</th>
<th>LC (mg of protein/g of template)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7.4</td>
<td>4.28</td>
<td>42.8</td>
</tr>
<tr>
<td>8.5</td>
<td>6.52</td>
<td>65.2</td>
</tr>
</tbody>
</table>
3.3.2.2 Quantification of synthesised nanocapsules

Ovalbumin nanocapsules were synthesised by cross-linking the infiltrated ovalbumin with either 5% (w/w) GA, or 1% (w/v) PFA. The cross-linking agent was chosen based on their application in the subsequent in vitro analysis. The GA cross-linked nanocapsules possessed autofluorescence due to the formation of Schiff bases between aldehyde groups from the cross-linking reaction (Collins & Goldsmith, 1981; Lee et al., 2013). This autofluorescence caused by GA cross-linking was known to excite maximally at 540 nm, giving highest emission peak at 560 nm (Collins & Goldsmith, 1981). Therefore, GA cross-linking was used in instances where the autofluorescence could aid in microscopic visualisation, negating the need to add a fluorescent tag to the nanocapsules. The PFA cross-linked nanocapsules also emitted fluorescence, but at much lower level. The PFA cross-linked nanocapsules were used for the in vitro study where fluorescent tags were used for minimal interference.

The synthesised nanocapsules were initially quantified by the UV-vis spectrophotometry, however it was difficult to obtain an accurate concentration for GA cross-linked nanocapsules due to the autofluorescence. The concentration could not be estimated based on the infiltration load, despite extensive washes given after HF treatment. Loss of some synthesised nanocapsules was observed during this process. Finally, attempts were made to quantify the concentration of the GA cross-linked nanocapsules, based on the concentration obtained for the PFA cross-linked nanocapsules.

A single reaction using the same amount of ovalbumin, was used to synthesise both types of nanocapsules, therefore the final nanocapsule concentration was predicted to be very similar for both methods. The concentration of the PFA cross-linked nanocapsules was first measured by the UV-vis spectrophotometer, and 1 µg of nanocapsule was subjected to SDS-PAGE for concentration estimation. Formalin cross-links made by PFA was reversible by a previously reported method (Klockenbusch & Kast, 2010), with modification to boiling the nanocapsules at 99°C for 30 min in SDS-PAGE sample buffer and successfully entered the SDS-PAGE gel (Figure 3.4, lane 4). However, the GA cross-linked nanocapsules did not enter the gel (Figure 3.4, lane 3). Smearing was seen in the lane where the PFA cross-linked nanocapsules had migrated. This was probably due to some residual cross-linking remaining after the treatment.
Figure 3.4: Protein estimation of the cross-linked ovalbumin nanocapsules, visualised on an instant stained SDS-PAGE gel. Lane 1: protein molecular marker, Lane 2: soluble ovalbumin, Lane 3: GA cross-linked ovalbumin capsule, and Lane 4: PFA cross-linked ovalbumin nanocapsules. An estimate of 1 µg was run in lane 2-4.
One microgram of soluble ovalbumin was also run to make a comparison (Figure 3.4, lane 2).

Both soluble ovalbumin and the PFA cross-linked nanocapsules appeared to be present in the same amount, therefore the concentration determined by the UV-vis spectrophotometry for the PFA cross-linked nanocapsules was applied to the GA cross-linked nanocapsules.
3.3.2.3 Observation of synthesised protein-based nanocapsules by TEM

Successfully synthesised ovalbumin nanocapsules were observed under TEM for their morphology. TEM analysis revealed that the individual nanocapsules were approximately 500 nm in diameter (Figure 3.5A). Part of the nanocapsules was creased and slight invaginations were visible (Figure 3.5B and C). Aggregation was also observed (Figure 3.5B), probably due to the drying process. It was difficult to measure the thickness of the nanocapsule wall, but it appeared that it had some level of rigidity in the structure.

When nanocapsules were synthesised using protein of a different molecular weight, the structural morphology changed from that of the ovalbumin nanocapsules. A 14 kDa recombinant viral protein, C27, was expressed in pET45b vector with 6xHis tag in *E. coli* BL21(DE3)pLysS in a separate study (Van, T., Personal communication, 2010), and was used to synthesise nanocapsules using the SC/MS templates. C27 forms a hinge region of the haemagglutinin protein from influenza virus H1N1 (A/PC/8/34). Purified C27 maintained in PBS was applied to SC/MS template at pH 7.4 for the infiltration study. A C27-based nanocapsule exhibited a distinctively thicker wall, which may provide higher integrity to the structure (Figure 3.6B), as compared to the collapsed ovalbumin nanocapsule (Figure 3.6A). A greater amount of protein was required to synthesise and produce the same amount of nanocapsules, yet much lower infiltration efficiency was recorded (data not shown). The theoretical pI of C27 is 6.81, therefore the infiltration of C27 performed in PBS at pH 7.4 was probably deterred by having a low electro-static attraction to the SC/MS templates.
Figure 3.5: Observation of negatively stained ovalbumin nanocapsules under TEM.
Figure 3.6: Observation of negatively stained A) ovalbumin-based, and B) C27-based nanocapsules under TEM.
3.3.3 In vitro characterisation of synthesised ovalbumin nanocapsules

3.3.3.1 Cytotoxicity of ovalbumin nanocapsules

The cytotoxic effect of the ovalbumin nanocapsules was evaluated using DC2.4 cells. Different concentrations of ovalbumin nanocapsules were applied to 5 x 10^4 cells and exposed for up to 72 h. Cell viability of 100% was represented by cells which were incubated with culture medium alone, and complete cell death was achieved by 0.1% Triton X-100. The relationship between the nanocapsule concentration (µg/mL) and cell viability (% of control) of up to 72 h of exposure time is presented in Figure 3.7A. The same conditions were applied to cells incubated with the soluble ovalbumin solution which was the precursor ovalbumin solution of synthesise nanocapsules, and the results are shown in Figure 3.7B. Each experimental curve represents the mean of three experiments.

The cell viability of cells treated with soluble ovalbumin was maintained above 100% for up to 72 h, and the differences in ovalbumin concentration did not change the level of viability. The cells treated with ovalbumin nanocapsule, however, showed a decrease in cell viability at all times points as nanocapsule concentration increased. The effect of the nanocapsules on cell viability became apparent at 25 µg/mL, with viability reduced to 73% when treated with 50 µg/mL nanocapsules for 24 h. A discrepancy in cell viability was observed as the cells treated with 25 µg/mL of ovalbumin nanocapsules for 48 h displayed higher toxicity than the cells treated for 72 h. When cells were treated with 50 µg/mL, cell viability was significantly lowered to 55% at both 24 h and 72 h time points (p≤0.05) (Figure 3.8), indicating that the toxicity of the ovalbumin nanocapsule was dose dependent.
Figure 3.7: Cell viability curve of DC2.4 cells against protein concentration of A) ovalbumin nanocapsules, and B) soluble ovalbumin over 72 h.
Figure 3.8: Cell viability of DC2.4 cells over different time points against different concentration of ovalbumin materials. (Statistical significance against 100% viable control group is represented in asterisk. * is p≤0.05)
3.3.3.2 *In vitro* uptake of ovalbumin nanocapsules by DCs

GA was used to cross-link ovalbumin in this study in order to utilise the autofluorescence in the cellular imaging using CLSM, without the need to label the nanocapsules with a fluorescent tag. DC2.4 murine DCs were incubated with the GA cross-linked nanocapsules for up to 90 min, at 4°C or 37°C.

The *in vitro* uptake of ovalbumin nanocapsules was observed at various time points, and the images were taken at three different wavelengths. The fluorescence of the GA cross-linked nanocapsules enabled observation in red at 561 nm, while the nuclei were counterstained with DAPI and seen in blue at 405 nm. Transmitted light channel (TD) was recorded to capture the entire cell morphology merged with the fluorescent signals.

The *in vitro* uptake of ovalbumin nanocapsules was successfully demonstrated, as shown in Figure 3.9. There was no indication of immediate nanocapsule uptake at 0 min and 5 min, however it became apparent after 15 min of incubation. The level of internalised ovalbumin nanocapsules significantly increased at 30 min, and was maintained for up to 90 min. Most of the cells exhibited a large number of internalised ovalbumin nanocapsules by 60 min, indicated by the red fluorescence displayed adjacent to the blue-stained nuclei.

A negative control was set up to confirm the internalisation of the ovalbumin nanocapsules, as the 2D images could not differentiate from the membrane-adhered nanocapsules, which may be viewed as overlaid in the image. As expected, incubation at 4°C did not promote the uptake of nanocapsules, but allowed surface attachment of the ovalbumin nanocapsules (Figure 3.10). This was clearly indicated by the differences seen in the location of the ovalbumin nanocapsules at 90 min (Figure 3.11). When incubated at 37°C, ovalbumin nanocapsules localised adjacent to the nucleus, while those incubated at 4°C were located nonadjacent/away from to the nucleus and outside the cell surface. The fluorescent images captured could only show the distance of the ovalbumin nanocapsules from the nucleus, however, the merged TD images showed that these nanocapsules were in fact adhered to the membrane, and not internalised.
Figure 3.9: Cellular uptake of ovalbumin nanocapsules (red) in DCs over 90 min incubation at 37°C. The top row represents the TD image merged with fluorescent signals, the second row indicates the DAPI-stained nucleus in blue, the third row indicates the fluorescing ovalbumin nanocapsules in red, and the bottom row represents the merged fluorescent images of nucleus and ovalbumin nanocapsules.
Figure 3.10: Surface attachment of ovalbumin nanocapsules (red) in DCs over 90 min incubation at 4°C. The top row represents the TD image merged with fluorescent signals, the second row indicates the DAPI-stained nucleus in blue, the third row indicates the fluorescing ovalbumin nanocapsules in red, and the bottom row represents the merged fluorescent images of nucleus and ovalbumin nanocapsules.
Figure 3.11: Ovalbumin nanocapsule (red) uptake at 37°C, and surface attachment at 4°C. Ovalbumin nanocapsules are located adjacent to the nucleus (blue) at 37°C indicating internalisation, while bound to the cell surface at 4°C. Images showing A) multiple cells, and B) single cells at higher magnification.
These observations confirmed that the ovalbumin nanocapsules of 500 nm, synthesised by the templating system could be endocytosed by APCs, such as the DCs used in this study.

Single cell imaging indicated a more precise localisation of internalised ovalbumin nanocapsules (Figure 3.12). The ovalbumin nanocapsules were transported and located adjacent to the nucleus in the cytoplasm, however the exact pathway of how the nanocapsules were internalised and transported to this location could not be determined from this image. An attempt was made to label the lysosomal compartment, as it was the presumed intracellular destination of the nanocapsules. However, a large amount of autofluorescence was emitted from the GA cross-linked ovalbumin nanocapsule at the same wavelength where the lysosome staining was visualised. As the fluorescent signal from ovalbumin nanocapsules was detected over a wide range of wavelengths, this limited the number of fluorescent labels that could be used for staining the lysosomal compartment. For this reason, determining the co-localisation of ovalbumin nanocapsules within the intracellular compartment was not possible in this study.
Figure 3.12: Single cell image of a DC with ovalbumin nanocapsules. The internalised ovalbumin nanocapsules are visualized in red.
3.3.3.3 Ovalbumin processing and presentation by DCs

To test for antigen presentation, DC2.4 cells were incubated with the ovalbumin nanocapsules for 6 h for cellular uptake and antigen processing. Ovalbumin nanocapsules were used at a concentration of 50 µg/mL, to achieve a measurable presentation level from DC2.4 cells with minimal toxicity (as determined in Section 3.3.3.1).

The level of cross-presentation of ovalbumin was measured by detecting the amount of presented ovalbumin-derived peptide. An ovalbumin-derived peptide OVA\textsubscript{(258-265)}, commonly known as SIINFEKL, in association with an MHC class I molecule (K\textsuperscript{b}) produced by DC2.4 was detected with the 25-D1.16 monoclonal antibody (Porgador et al., 1997). Several internal controls were deployed, to eliminate the influence of non-specific binding of detection antibodies, along with the autofluorescence of nanocapsules.

Firstly, a population of DC2.4 with forward scatter ranging from 50,000 to 250,000 was gated (Figure 3.13A). Secondly, cells pulsed with SIINFEKL peptides, but not labelled with the 25-D1.16 antibody, were used as the baseline to compare the levels from test groups. A shift in the fluorescence intensity of samples incubated with soluble and nanocapsule ovalbumin was used to evaluate the relative percentage of cross-presented peptide.

The cells treated with the positive control SIINFEKL peptide displayed the highest levels SIINFEKL/K\textsuperscript{b} complex at 94.11%, as the peptides were directly bound to the K\textsuperscript{b} molecule expressed on the surface of DC2.4 (Figure 3.13D). SIINFEKL from soluble ovalbumin could be presented at low levels by cross-presentation (3.23%, Figure 3.13C), however the level significantly increased for ovalbumin nanocapsules (15.61%, Figure 3.13B) (p≤0.0001%). Two small populations of DC2.4 presenting SIINFEKL from ovalbumin nanocapsules were also found, as indicated by their stronger fluorescence intensity. This indicated that these cells were expressing higher levels of MHC class I molecules with SIINFEKL presentation. The increased level of cross-presentation in these populations may be due to the inconsistent loading of ovalbumin per cell. This may have happened by internalising different numbers of nanocapsules, or internalising larger nanocapsules thus higher amount of infiltrated ovalbumin in such capsules, resulting in the higher ovalbumin load.
Figure 3.13: Cross-presentation by DC2.4 cells. A) DC2.4 population was gated as shown. The level of SIINFEKL in context with K\textsuperscript{b} molecule was measured from the cells incubated with B) ovalbumin nanocapsules, C) soluble ovalbumin, and D) SIINFEKL. The negative control is represented by the red open histogram, while the blue shaded histogram represents the samples. The Y-axis indicates 100% of the total cell number within each group, achieved by normalising to the peak height at the mode of distribution. The X-axis is the level of relative fluorescence intensity.
3.4 Discussion

3.4.1 Structural characterisation

3.4.1.1 Examination of SC/MS silica nanoparticle template

The SC/MS silica nanoparticles were fabricated and SEM analysis revealed that they were homogeneous and spherical in structure. These particles had a MS thickness of 62.5 nm, and the detail of the porous structure was evident from TEM images. The average diameter was measured by counting 100 particles from the representative SEM micrograph, and was found to be $516 \pm 20$ nm. A more accurate measurement using DLS revealed a diameter of 410 nm. The use of DLS has been widely employed for measuring the size of colloidal silica nanoparticles (Feifel & Lisdat, 2011; Karpo et al., 2009), and mesoporous silica nanoparticles (Tang et al., 2012; Chen et al., 2009).

DLS analysis provides a more precise measurement by calculating the hydrodynamic radius on an ensemble average of the particles with the same diffusion coefficient, in a solution with specific viscosity (Berne & Pecora, 2000). Although the image magnification may be directly proportional to the scanning length in SEM, DLS analysis measures on a statistically larger number of particles, thus providing better accuracy. A diameter of 420 nm was actually observed when the SC/MS silica nanoparticles were fabricated using the same method with a similar MS thickness of 60-75 nm (Wang et al., 2008a; Büchel et al., 1998). Therefore, the size measurement obtained by DLS analysis was probably more true to the actual size, despite the difference between two measurements being larger than 100 nm.

Nitrogen adsorption data indicated that the SC/MS nanoparticles used for the subsequent synthesis of protein-based nanocapsules had a surface area of 219.7 m$^2$/g, and a pore diameter and volume of 3.07 nm and 0.17 mL/g, respectively. These nanoparticles allowed infiltration of up to 65.2 mg of ovalbumin/g of nanoparticle template. A previous study where the SC/MS silica nanoparticles were fabricated with a similar diameter and MS wall thickness, reported 65 mg/g and 35 mg/g loading of 15 kDa and 70 kDa poly(allylamine hydrochloride) (PAH),
respectively (Wang et al., 2008a). It was also reported that the use of smaller PAH of 5 kDa resulted in an increase in polymer loading (110 mg/g). The nanoparticles used in the study by Wang and colleagues (2008) possessed a larger surface area (390 m²/g), porosity (4.5 nm) and volume (0.28 mL/g) of MS. In consideration with the molecular weight of ovalbumin being 44 kDa, the loading capacity was therefore relatively high for the nanoparticles fabricated in this study.

The influence of the wall thickness and the porosity of MS on chitosan loading was investigated by Goethals and colleagues (2013), and it was demonstrated that the infiltration load increased proportionally to the porosity, pore volume and thickness of the MS, as well as an increase in the amount of drug load which was encapsulated within the chitosan nanocapsule. The effect of pore size on protein or polymer immobilisation is due to molecule adsorption being restricted to the nanoparticle surface, including the MS (Wang & Caruso, 2005). The method to control the properties of the MS is well established (Büchel et al., 1998; Goethals et al., 2013; Chiang et al., 2011), therefore the amount of protein loading can be tailored to the optimal level required for specific applications. For advanced specifications, the orientation and the shape of pores in the MS can be also modified (Yoon et al., 2007). In the same study, use of templates with various properties influenced the degree of integrity in the resulting nanocapsules. This indicated the possibility of controlling the degradability of the protein-based nanocapsules to target intracellular localisation in different stages of acidic endosomes, as well as prolong the presentation period. This may allow the induction of an antigen presentation pathway appropriate for a specific pathogen (Sieling et al., 2007).

### 3.4.1.2 Influence of the physicochemical property of protein on infiltration

The loading capacity of SC/MS templates can change based on the physicochemical properties of the infiltrating molecules. The influence of molecular weight was evident in several previous studies where higher loading of smaller molecular weight molecules was observed (Wang et al., 2008a; Wang & Caruso, 2005). Accordingly, a decrease in the surface area for pristine nanoparticles was reported, indicating that more dense structure was formed in the MS. In the present study, C27, a recombinant H1N1 viral protein was used for nanocapsule synthesis using this templating system, and it was found that the capsule
structure was different to that of the synthesised ovalbumin nanocapsule. From the TEM micrograph observation, a C27-based nanocapsule showed a much thicker capsule wall structure compared to that of the ovalbumin nanocapsule, and a higher protein density, as indicated by the negative staining from the TEM micrograph. The protein-based nanocapsules exhibited a folded shape when observed under TEM due to the drying process during the sample preparation. Creasing in the ovalbumin nanocapsule was much more obvious than that present in the C27 nanocapsules. The structural rigidity of nanocapsules synthesised with a higher amount of protein was in line with the capsules synthesised using the template with a larger pore volume, which allowed larger numbers of molecules to infiltrate (Goethals et al., 2013).

The pI of an infiltrating molecule is also an important factor for determining infiltration load. Protein immobilisation increases when the pI of the infiltrating protein is lower than the physiological pH of buffer in which the reaction takes place. At several pH units away, the electrostatic attraction between the positively charged silica template and the protein increases, thereby forcing protein to immobilise at a higher rate. An increase in the ovalbumin infiltration load was evident from the optimisation study when the pH of PBS increased from 5.5 to 8.5. This was due to the ovalbumin possessing a stronger negative charge at pH 8.5 as the ovalbumin pI is 4.6. In contrast, C27 has a pI of 6.81, which resulted in much lower infiltration efficiency in PBS at pH 7.4 than ovalbumin for nanocapsule synthesis (data not shown). This may be due to the weaker electrostatic interaction induced by having two similarly charged materials. This highlights that the conditions for protein infiltration need to be altered according to the molecule applied for the synthesis.

3.4.1.3 Quantification of synthesised ovalbumin nanocapsules

The amount of immobilised protein was quantified by measuring the protein concentration in the washes produced from the infiltration and cross-linking procedure. However, this may not accurately translate to the amount of protein in the synthesised nanocapsules, as none of the washes after HF treatment were assessed for ovalbumin quantification due to safety reasons. Therefore, the protein estimation was made by subjecting nanocapsules to SDS-PAGE analysis. PFA cross-linked ovalbumin nanocapsules were able to enter the SDS-PAGE gel as
the formalin-induced cross-link was reversed by boiling the nanocapsules. However, cross-links induced by GA seemed irreversible after boiling. The aldehyde group from GA and PFA react with nitrogen in protein (mainly with the ε-amino group of lysine), and form methylene bridge (-CH$_2$-) by reacting between two proteins. GA therefore formed stronger cross-linking due to the presence of two aldehyde groups per molecule.

For protein-based nanocapsules to be used in a vaccine delivery system, it is crucial that the cross-linked proteins can be degraded by the intracellular enzyme for the presentation of peptides. It has previously been reported that the degradation of human serum albumin (HSA) nanoparticles, cross-linked by GA is achievable by enzymatic digestion. HSA nanoparticles were shown to be degraded in situ by cathepsin B (Langer et al., 2008), which often localised in the lysosomes (Tanaka et al., 2000; Mukherjee et al., 1997). In vitro lysosomal digestion of GA cross-linked HSA nanoparticles was also demonstrated using a head and neck squamous cell carcinoma cell line (Altintas et al., 2013). These findings suggested that the other GA cross-linked protein-based nanocapsules, including ovalbumin capsules, would most likely be degraded by intracellular proteases.

While the protein-based nanocapsules must be easily degraded by intracellular proteases, it is equally important that the nanocapsules can maintain integrity prior to vaccination. An ideal vaccine should be very stable, preferably with no requirement for a cold chain, to be able to be distributed to countries and rural regions that may require a longer transportation period. Therefore, the stability of protein-based nanocapsules at different temperatures (i.e. 4°C, and room temperature) needs to be evaluated for different periods of time (i.e. hours to days) in future work.
3.4.2 In vitro characterisation

3.4.2.1 Cytotoxicity of ovalbumin nanocapsules

The use of DC2.4 has been reported in numerous publications (Sneh-Edri et al., 2011; Carney et al., 2012; Okada et al., 2001; Shen et al., 1997). DC2.4 cells are immature DCs therefore they are suitable for the uptake and presentation studies using extracellular antigens, as the uptake efficiency can be reduced in matured DCs (reviewed in Chapter 1).

The ovalbumin nanocapsules were tested for their cytotoxicity by measuring the level of DC2.4 cell viability after 24, 48 and 72 h exposure. No significant reduction in cell viability was observed until 48 h by cells treated with 50 µg/mL of ovalbumin nanocapsules. The same concentration of ovalbumin nanocapsules showed a similar cytotoxic effect after 72 h; however the cell viability was well maintained at other concentrations, even after 72 h of exposure.

Commercially available ovalbumin that was purchased from Sigma-Aldrich (details in Section 2.4.1) in this study is known to contain on average of 40 ng endotoxin/µg of ovalbumin (Mac Sharry et al., 2014). Endotoxins often include bacterial LPS, and are stimulating molecules (reviewed in Chapter 1). It could be possible that the results observed for the ovalbumin nanocapsules were influenced by the non-specific stimulation caused by the endotoxin in ovalbumin. However, it is highly unlikely that it had a major cytotoxic effect on cells as the cells incubated with soluble ovalbumin were able to maintain a high cell viability over 72 h.

One main advantage of the templating system for vaccine delivery is that the resulting nanocapsules only consists of negatively charged proteins. Many polymeric nanoparticles and liposome are increasingly being used in delivery systems, however these polymers and lipids often bear a positive charge and possess cytotoxic effects due to their nature (Lv et al., 2006). The cytotoxicity of cationic nanoparticles can be much higher with increasing charge, in comparison to anionic nanoparticles with an opposite, but similarly strong charge (Yang &
Hsu, 2008). These cationic nanoparticles have also shown to decrease subsequent antigen presentation by DCs (Kwon et al., 2005). Several polycationic polymers have even significantly higher cytotoxicity (Fischer et al., 2003). Due to these cytotoxic effects, the amount of cationic nanoparticles used for vaccine administration may have to be kept to a minimal amounts, therefore protein-based nanocapsules (anionic) may be probably more suitable for antigen delivery.

3.4.2.2 Cellular uptake of ovalbumin nanocapsules by DCs

A qualitative study using the CLSM analysis was performed to determine the efficiency of uptake of ovalbumin nanocapsules by DC2.4 cells. The result revealed a high level of internalisation of ovalbumin nanocapsules in less than 30 min by DC2.4 cells. By 60 min, most of the cells had successfully internalised ovalbumin nanocapsules of approximately 500 nm in size. The cellular uptake of nanoparticles synthesised with various materials has been well documented. Nanoparticles consisting of polystyrene beads (Mottram et al., 2007; Foged et al., 2005), human serum albumin (Altintas et al., 2013; Langer et al., 2003), PLGA (Shen et al., 2006; Lutsiak et al., 2002), PPS (Hirosue et al., 2010), chitosan (Namazi & Phillips, 2010; Goethals et al., 2013), lecithin (Sloat et al., 2010), and silica (Huang et al., 2010), are just some examples of a wide range of materials tested as nano-carrier systems. However, regardless of the nanoparticle materials used and the method of either encapsulation or adsorption techniques to create the carrier system, many reported on the increased load of drug and antigen delivered to intracellular components when delivered in a particulate form.

To confirm the ovalbumin nanocapsules were in fact internalised, a control was set up at 4°C. At low temperature, where energy-dependent cellular uptake was blocked, the ovalbumin nanocapsules were only able to bind to the membrane surface (Mintern et al., 2013). After 90 min, a predominant population of cells indicated ovalbumin nanocapsule internalisation, while cells incubated at 4°C showed no indication of internalisation.

The uptake mechanism of nanoparticles may vary depending on the particle properties, however larger particles (>200 nm), in the size range of a bacterial pathogen, often enter DCs via phagocytosis, while smaller particles (<200 nm), exhibit virus-size enter via
receptor-mediated clathrin-coated pits (Daecke et al., 2005), or caveolae lipid rafts (Chen & Norkin, 1999). In the present study, the pathway which ovalbumin nanocapsules took to enter DC2.4 was not investigated. This was largely due to the excessive amount of fluorescence from the GA cross-linking interfering with other fluorescence detection at various wavelengths. This hindered the fluorescent staining of different cell organelles to determine exactly where the ovalbumin nanocapsules co-localised. Such a study could possibly indicate the endocytic pathway the 500 nm sized-ovalbumin nanoparticle had taken, hence possibly obtaining clues to the fate of antigen processing and presentation by the two MHC pathways.

Investigation of the endocytosis pathway using chemical inhibitors is not uncommon in studies of a particulate delivery system. Wortmannin is an inhibitor of phosphatidylinositol-3 kinase, an extracellular signal-regulated kinase, and therefore blocks Fc receptor-mediated phagocytosis (Garcia-Garcia et al., 2002; Wymann et al., 1996; Manna & Aggarwal, 2000). Cytochalasin B blocks actin polymerisation, and therefore inhibits actin-mediated membrane rearrangement for phagocytosis (Torii et al., 2001). Amiloride directly affects clathrin-independent pinocytosis by blocking the Na\(^+\)-H\(^+\) exchange within cells (Tujulin et al., 1998; Bakker-Grunwald et al., 1986). Investigation using these inhibitors determined that the PLGA nano- and microparticles ranging from 500 nm to 4 µm in diameter were internalised via phagocytosis by DCs and macrophages (Lutsiak et al., 2002; Yang & Hsu, 2008). However, a combination of inhibitors such as chlorpromazine and filipin III, which inhibits clathrin-mediated endocytosis and caveolae formation, respectively, indicated that more than one pathways were involved in the uptake of 350 nm chitosan nanoparticles (Nam et al., 2009). It is commonly known that smaller particles than this are preferred for non-phagocytic pathways, however chitosan nanoparticles possessed a positive charge, which may influence the unusual uptake pathways for a particle this size. Anionic ovalbumin nanoparticles of approximately 500 nm, developed in this study, might therefore be endocytosed by the phagocytic pathway.

While polystyrene microparticles of up to 15 µm in diameter can be taken up by DCs (Foged et al., 2005), smaller size particles of <500 nm are more efficiently taken up. It was also reported that nanoparticles in the viral range (40-50 nm), can induce higher localisation in DCs within draining lymph nodes, and subsequently elicit a higher immune response (Fifis et
A number of studies have demonstrated the correlation between particle uptake with a range of sizes using near monodispersed spherical particles, however the shape of the particle may result in different uptake efficiency by nonspecific cellular uptake. Huang et al., (2010), investigated the effect of the shape of nanoparticles on cellular uptake and found that 450 nm long rod-shaped nanoparticles were internalised at a much faster rate than 100 nm spherical nanoparticles (Huang et al., 2010). It was suggested that the differences in contact area of particles with the cell membrane attachment might have assisted in the uptake efficiency, as the longitudinal axis of rod shaped-particles was much larger in surface area. Whether the rod shaped-particle can mimic bacilli bacteria for more efficient uptake is not known, however these particles had increased cytotoxic effects. These investigations were performed using dense core beads or mesoporous silica particles, therefore the size- and shape-dependent uptake efficiency may not apply to hollow core nanocapsules. Nanocapsules used in the present study should exhibit flexibility, therefore size reduction by folding or creating different shape by flattening may be possible.

Surface charge of nanoparticles may possibly a key factor for the uptake efficiency. Alteration of surface charge to synthesise cationic nanoparticle can increase the electrostatic interactions between the anionic cell membrane to facilitate better uptake (Kwon et al., 2005; Nam et al., 2009). This is especially important for larger particles of 1 μm size, in order to reach a similar degree of cellular association as 100 nm nanoparticles could provide (Foged et al., 2005). A higher surface charge can bind strongly to the cell surface, however this results in non-specific interaction with many types of cells. In order to increase the antigen loading to APCs, the use of smaller sized negatively charged nanoparticles might therefore be more superior to the cationic particles in a vaccine delivery system.

### 3.4.2.3 Antigen presentation of ovalbumin nanocapsule peptides

Effective activation of DCs is important for a vaccine delivery system, as they are the most potent APCs capable of inducing primary immune responses, and thus activation of naïve T cells (Marland et al., 1996). The ability of a vaccine delivery system to appropriately induce the cross-presentation pathway is often studied using ovalbumin (Mottram et al., 2007; Kwon et al., 2005; Hirosue et al., 2010; Segura et al., 2009; Shen et al., 1997; 2006). This system is
widely employed because of the well characterised ovalbumin-derived peptide (OVA\textsubscript{258-265}). SIINFEKL is known to be a MHC class I molecule-restricted peptide, and the amount of SIINFEKL presented on MHC class I molecules (K\textsuperscript{b}) translates to the level of cross-presentation (Shastri & Gonzalez, 1993). DC2.4 cells are immature DCs with a low amount of CD80 and no MHC class II and CD40 expressed, while the expression of MHC class I molecules (K\textsuperscript{b}) is present. These have been shown to successfully induce OVA-specific CD8\textsuperscript{+} T cell responses (He et al., 2007).

The level of cross-presented ovalbumin delivered by either soluble or particulate form was detected as a shift in the fluorescence intensity from the level induced by the SIINFEKL peptide with incomplete staining. Flow cytometry analysis showed that the cross-presentation of soluble ovalbumin was significantly less efficient than particulate form. The results also indicated that the level of cross-presentation was increased at a higher level in two separate DC2.4 populations. Due to the nature of SC/MS templates being slightly polydispersed, different sizes of ovalbumin nanocapsules were most likely synthesised. Therefore, larger ovalbumin nanocapsules might have delivered an increased amount of ovalbumin, possibly resulting in a higher level of presentation. However, it is understood that smaller nanoparticles can be taken up more efficiently, as discussed in the previous section. Therefore, it is possible that smaller ovalbumin nanocapsules synthesised due to polydispersity of the template, which might have been internalised at a better rate/more efficiently and caused increased nanocapsule localisation per cell, thus possibly increasing the level of presentation. The ovalbumin nanocapsules were often seen aggregated after the template removal by HF treatment. Prior to applying the ovalbumin nanocapsules to cells, they were sonicated to dissociate the aggregation. However, it is possible that some aggregations were still present and internalised by the cells. Mant et al. (2012) have investigated the effect of nanoparticle aggregation on cross-presentation in DC2.4 cells. It was observed that the aggregated ovalbumin-bound nanoparticles without the sonication treatment produced a different magnitude of cross-presentation in DC2.4 cells. The nanoparticles with sonication treatment also produced various level of cross-presentation, but with much less variability. This indicates that various levels of cross-presentation seen in DC2.4 cells might be due to the uptake of aggregated ovalbumin nanocapsules.
Nevertheless, the increased level of cross-presentation induced by the nanocapsules was in line with other studies. Delivery of an exogenous antigen in PLGA nanoparticles was shown to be much more effective at inducing cross-presentation (Shen et al., 2006). In their study, the presentation level of SIINFEKL induced by ovalbumin-encapsulating PLGA nanoparticles was significantly higher than the soluble ovalbumin alone, it would require a 1000-fold higher concentration of soluble ovalbumin to induce the same level of presentation. The subsequent activation of CD8+ T cells by the PLGA nanoparticle was much higher than soluble ovalbumin, demonstrating that nanoparticles were much more effective than soluble antigen for eliciting immune responses also. Interestingly, these nanoparticles were also capable of inducing cross-presentation in B cells, which are naturally not very competent cross-presenting cells. Soluble ovalbumin and ovalbumin-coated latex beads were used as comparisons, however neither resulted in presentation, as expected. This is a great indication that delivery of antigen in a particulate system is sufficient to elicit an immune response at a much lower dose of administration.

The fate of exogenous antigens can often be indicated by the intracellular location for the processing and presentation. Exogenous antigens internalised by phagocytosis are often trafficked to acidic early endosomes, then eventually to late endosomes or lysosomes where the antigens are degraded by proteases. This is where the peptides meet MHC class II molecules. In contrast, endogenous antigen or viral proteins are processed by the proteasome within the cytosol and bind to MHC class I molecules in the endoplasmic reticulum (Villadangos & Schnorrer, 2007). The link between the two pathways for the exogenous antigens to be cross-presented is escape into the cytosol from the lysosomal compartment. In a study where biodegradable PPS nanoparticles were subjected to mouse bone marrow-derived DCs; while the majority of nanoparticles co-localised with LAMP-1 (lysosomal associated membrane protein-1), and other intracellular MHC class II compartments after 90 min, a small number of particles were also found to co-localise with calreticulin, a protein found in the endoplasmic reticulum for MHC class I molecule loading (Hirosue et al., 2010). This finding indicated the nanoparticles could lead the loaded antigens to escape from the endosome. Encapsulation of ovalbumin in PLGA nanoparticles could also increase escape into the cytosol by 30-fold more than the soluble ovalbumin (Shen et al., 2006). Efficient cytosol escape might be attributed to the prolonged release of antigen within
endosomes and lysosomes, as these PLGA nanoparticles maintained the sustained level of antigen beyond the period which soluble antigen could remain. Supported by these findings, a particulate antigen delivery system is most likely more superior to a subunit vaccine due to prolonged antigen presentation that in turn can induce higher immune responses.
3.5 Conclusion

Ovalbumin nanocapsules were synthesised by employing the SC/MS silica nanoparticles as a template. The properties of resulting nanocapsules can easily be modified by alteration of the silica template moiety, providing great flexibility in antigen loading and for \textit{in vivo} processing and presentation. Ovalbumin nanocapsules were synthesised using minimal steps, with final particles consisting of only the cross-linked protein. Negatively charged ovalbumin nanocapsules produced minimal toxicity to cells, alleviating the cytotoxic effect that cationic polymer particles might possess. These ovalbumin nanocapsules were efficiently internalised by immature DCs in less than 30 min, and induced a significantly higher level of ovalbumin peptide to be processed via the cross-presentation pathway than soluble ovalbumin. Increased levels of peptide presentation with MHC class I molecules will lead to the efficient induction of a cellular response by activating CD8$^+$ T cells.

In conclusion, SC/MS nanoparticles are suitable for synthesising protein-based nanocapsules, which could be tailored for different pathogenic antigens. Protein-based nanocapsules can be efficiently taken up by APCs, and increase the level of antigen presentation greater than that of soluble protein. The use of such a templating system is therefore suitable for an antigen delivery system.
This chapter reports the expression and purification of an antigenic malarial protein and the subsequent synthesise of the protein-based nanocapsules for \textit{in vivo} study.
4.1 Introduction

In order to investigate the potential of protein-based nanocapsules as a new vaccine delivery system, it is best to compare the immunogenicity with differently formulated vaccines in an animal model. To achieve comparable results between a free sub-unit and a nanocapsule vaccine and elucidate their efficacy, an immunogenic antigen is the preferred choice to formulate the nanocapsules. The use of an immunogenic antigen, which has been shown to elicit an immune response in an animal model, will ensure that a measurable immune response will be elicited. The antigen of choice must also have suitable physiochemical properties for the nanocapsule synthesis, that is, a low pI and a moderately large molecular weight. For those reasons, a murine malarial protein, the merozoite surface protein 4/5 (MSP4/5) was chosen for this study as it is a very efficacious vaccine antigen candidate, which also possesses very similar properties to that of ovalbumin described in the previous chapter.

In 1999, Black et al. (Black et al., 1999) identified a gene in murine malaria Plasmodium chabaudi adami homologous to the MSP2, MSP4 and MSP5 genes found in P. falciparum. Despite having low sequence similarity, the gene is structurally similar in that it has an intron, and the encoded protein also has common properties to MSP4 and MSP5 in P. falciparum, with a well-conserved region of epidermal growth factor (EGF)-like domain and glucosylphosphatidylinositol (GPI) anchor. Homologues were also identified in two other murine malaria species, P. berghei and P. yoelii in 2000 (Kedzierski et al., 2000a), and the recombinant MSP4/5 was shown to provide protection against lethal malaria challenges in mice (Kedzierski et al., 2000b; 2001).

The complete sequence of the P. chabaudi adami MSP4/5 (PcMSP4/5) gene is available from GenBank (accession number: AF080446) (Black et al., 1999) and in silico protein characterisation revealed that its predicted pI is 4.19. The expressed recombinant protein and the synthesised nanocapsule will be used in an animal model, therefore all solvents have to be at a physiological pH of 7.4. As described in Chapter 3, lower protein pI can promote a higher infiltration load of protein into a mesoporous layer of positively charged SC/MS templates in
PBS at pH 7.4. Having a similar pI to ovalbumin (pI = 4.6), PcMSP4/5 is an ideal protein model for nanocapsule vaccine study.

To fully understand the efficacy of nanocapsules as a delivery system, the recombinant protein for both nanocapsule and the free sub-unit vaccine must be produced in the same expression system. *Escherichia coli* is a widely used expression host and all recombinant MSP4/5 from previous studies have been expressed in this system. The expression vector was obtained from the group who identified the PcMSP4/5 gene. This plasmid was used to express the recombinant PcMSP4/5 in this part of the study.

The cloning strategy used to construct this plasmid in the previous study (Black *et al.*, 1999) was very extensive. This chapter explains the cloning process used to build this construct, and subsequently DNA analysis to confirm the gene orientation and absence of mutation. Recombinant PcMSP4/5 was expressed in *E. coli* and purified by immobilised metal affinity chromatography (IMAC). Purified protein was then subjected to nanocapsule synthesis reactions and *in vitro* cytotoxic effects were assessed in murine dendritic cells (DCs) before application as a vaccine in the animal model.
The aim of the experiments outlined in this chapter were:

- To understand the cloning strategy for building a pTrcHis-A vector construct with PcMSP4/5 gene from the previous study (Black et al., 1999). This plasmid was named pT.MSP4/5 in this study.
- To confirm the integrity of the PcMSP4/5 gene, pT.MSP4/5 vector was subjected to the restriction endonuclease analysis and nucleotide sequencing.
- To express PcMSP4/5 as a recombinant 6xHis-tagged protein in *E. coli* BL21(DE3)pLysS.
- To assess the suitability of PcMSP4/5 for nanocapsules made with the SC/MS nanoparticle templating system, and determine any *in vitro* cytotoxic effects.
4.2 Materials and Methods

4.2.1 General protocols

This section details the general materials, methods and techniques applied in the experimental methods.

4.2.1.1 Bacterial strain

Plasmid clones were introduced into *E. coli* strain DH5α and BL21(DE3)pLysS and were used for storage and recombinant protein expression, respectively. The description of all the bacterial strains used throughout this study is listed in Table 4.1.

4.2.1.2 Plasmid

A previously constructed pT.MSP4/5 clone was used in this study (Black *et al.*, 1999). The plasmid vector pTrcHis-A ligated with the *P. chabaudi adami* MSP4/5 gene, lacking the predicted hydrophobic signal and GPI anchor sequences, was kindly provided by Dr Casilda Black from Monash University, Clayton, Australia. pTrcHis-A utilised in constructing pT.MSP4/5 was obtained from Invitrogen, USA.

4.2.1.3 Storage condition

Both transformed *E. coli* DH5α and BL21(DE3)pLysS strains with pT.MSP4/5 were stored at −80°C in 50% glycerol LB broth in 2 mL cryovials.
Table 4.1: Bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Genotype/Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>F' endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK⁻ mK⁺), λ⁻</td>
<td>(Hanahan et al., 1983)</td>
</tr>
<tr>
<td>BL21(DE3)pLysS</td>
<td>F' ompT hsdS₈(λB⁻ mB⁻) gal dcm (DE3) pLysS (Cam⁷⁺)</td>
<td>Invitrogen Corporation, USA</td>
</tr>
</tbody>
</table>
4.2.1.4 Culture condition

All *E. coli* strains, grown in broth or on solid microbiological media were incubated under aerobic conditions at 37°C for 16-20 h. *Escherichia coli* DH5α containing pT.MSP4/5 plasmid was grown in media with 100 µg/mL AMP and *E. coli* BL21(DE3)pLysS containing pT.MSP4/5 was grown in 100 µg/mL AMP and 34 µg/mL CM, while not containing plasmid was grown with 34 µg/mL CM for the maintenance of pLysS plasmid. Broths were shaken at approximately 200 rpm on a Bioline shaking incubator unless otherwise specified.

4.2.1.5 Preparation of electrocompetent cells

All steps were performed aseptically. Bacterial cells were grown in 10 mL LB broth on a shaker at 37°C overnight. Two millilitres of this culture was used to inoculate 200 mL of LB broth in a 1 L Erlenmeyer flask and shaken vigorously at 37°C until an optical density at 600 nm of 0.4 (early to mid-log phase), was obtained. The culture was then placed on ice to chill for 30 min, followed by centrifugation at 4,000 x g for 15 min at 4°C to harvest the cells. The supernatant was completely removed and the cells were resuspended and washed in 200 mL of sterile ice-cold MQH₂O. The centrifugation process was repeated twice, with the cells firstly resuspended in 100 mL of sterile ice-cold MQH₂O, then 10 mL of sterile ice-cold 10% glycerol. The cells were resuspended in a final volume of 400 µL of sterile ice-cold 10% glycerol and aliquots of 40 µL were stored at −80°C immediately.

4.2.1.6 Transformation of electrocompetent cells

The method of electro-transformation was designed according to the Gene Pulser apparatus user manual with minor alterations. Frozen electrocompetent cells were thawed on ice for 5 min and mixed with 1 µL of 100 ng/µL pT.MSP4/5. After sitting on ice for another 1 min, the mixture was transferred into a chilled electroporation cuvette with a 0.1 cm gap. The cells were pulsed with settings of 2.2 kV, 25 µF and 200 Ω. After the pulse, cells were gently resuspended in 1 mL of LB broth and incubated at 37°C for 1 h in a fresh microfuge tube. Fifty microlitres of transformed cells were then plated onto LB agar containing the appropriate antibiotics and grown overnight at 37°C.
4.2.1.7 Plasmid extraction (miniprep)

pT.MSP4/5 was purified using the Bioline ISOLATE Plasmid Mini kit as per the manufacturer’s instructions. Five millilitres of *E. coli* BL21(DE3)pLysS transformed with pT.MSP4/5 were grown overnight in LB broth supplemented with AMP and CM was used for the isolation of plasmid.

4.2.1.8 Restriction endonuclease digestion of DNA

Restriction enzyme used in this study was purchased from Promega USA. Restriction enzyme reaction condition was as specified by the manufacturer. Up to 150 ng of DNA was digested with 10 U of enzyme, in 1× buffer in a total volume of 20 µL at 37°C for 3 h. Acetylated BSA was added to a concentration of 100 µg/mL in every reaction. Enzymes used in the previous and present studies are listed in Table 4.2.
Table 4.2: List of restriction endonuclease used in this study.

<table>
<thead>
<tr>
<th>Restriction endonuclease</th>
<th>Recognition sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bgl</em>II</td>
<td><em>Ar</em>GATCT</td>
</tr>
<tr>
<td><em>Bsp</em>HII</td>
<td><em>Cr</em>CATGG</td>
</tr>
<tr>
<td><em>Nco</em>I</td>
<td><em>Cr</em>CATGG</td>
</tr>
<tr>
<td><em>Pst</em>I</td>
<td>CTGCAR*G</td>
</tr>
</tbody>
</table>

Where *r* represents the point of cleavage.
4.2.1.9 Quantification of DNA

Two methods were used in the quantification of nucleic acids. For the exact quantification of well-purified DNA, a UV-vis spectrophotometer was used, and for approximate quantification of DNA, DNA was separated on an agarose gel and compared to known standards of λ-PstI DNA ladder.

4.2.1.9.1 Spectrophotometric quantification of DNA

The concentration of DNA and purity were determined using a BioPhotometer UV-vis spectrophotometer. The absorbance was determined at wavelengths of 260 and 280 nm. The concentration was calculated from the absorbance at 260 nm while the purity was checked using the absorbance ratio from 260/280 nm. One absorbance unit at 260 nm was considered equal to 50 µg of double-stranded DNA. A ratio between 1.7-2.0 was considered acceptable for DNA and RNA purity (Sambrook & Russell, 2001). The purity of the DNA was also checked visually by electrophoresis on an agarose gel.

4.2.1.9.2 Agarose gel DNA electrophoresis

4.2.1.9.2.1 Preparation of λ-DNA marker

A solution of 0.1 µg/µL digested λ-DNA was prepared by incubating 20 µg λ-DNA with 10 U PstI in 1× Buffer H and MQH₂O made up to 120 µL, at 37°C overnight. Twenty microlitres of 11× DNA loading buffer was then added to stop the digest, and MQH₂O was added to make the total volume 200 µL. The marker was stored at −20°C, with 10 µL used on a DNA agarose gel. A diagram of PstI digested λ-DNA ladder is located in Appendix 1.

4.2.1.9.2.2 DNA electrophoresis

One percent agarose gels were used to separate and view DNA fragments. DNA samples were mixed with 11x loading buffer and underwent electrophoresis against an appropriate DNA marker in an adjacent lane. Gels were run in 1x TAE electrophoresis buffer at 100 V. Gels
were stained with ethidium bromide (3 µg/ml) before destaining in running tap water. The DNA products were visualised with an UV illuminator and photographed using a Biorad Geldoc imaging system running Quantity One software.

4.2.1.9.2.3 DNA electrophoresis quantification

λ-DNA previously digested with _PstI_ was separated on an agarose gel along with sample DNA fragments. The quantity of DNA within each digested fragment of λ-DNA (Appendix 1) was compared with intensities of unknown DNA samples.

4.2.1.10 Protein determination

4.2.1.10.1 Bradford assay

Protein content determination in samples containing no traces of detergent was performed using the Bradford method (Bradford, 1976; Lane, 1991). One millilitre of Bradford reagent was added to 100 µL of protein, or standard, mixed by inversion and left to stand for 2 min. When required, protein and standards were diluted in 0.15 M NaCl. Bovine serum albumin fraction V (BSA) was used as the standard protein solution. Two hundred microlitres of processed protein was added to the wells of a 96-well microtiter plate. The absorbance was measured using the iMark™ Microplate absorbance reader at 595 nm, and a standard curve constructed by plotting the concentrations of the BSA standards (µg) versus absorbance, was used to determine the protein content of samples.

4.2.1.10.2 Spectrophotometric quantification of protein

The concentration of protein was determined using a UV-vis spectrophotometer. The absorbance was measured at a wavelength of 280 nm. The concentration of the protein sample (mg/mL) was equal to the absorbance at 280 nm (with a path length of 1 cm).
4.2.1.11 Protein visualisation

4.2.1.11.1 SDS-PAGE

One-dimensional SDS-PAGE was performed using a discontinuous buffer system (Lane, 1991). Proteins were mixed with SDS loading buffer and heated to 100ºC in a dry heating block for 10 min. The proteins were separated in the BioRad electrophoresis unit containing SDS running buffer, by electrophoresis at 150 V for 50 min or until the dye front reached the bottom of the gel.

Gels where then transferred onto nitrocellulose membranes for immunoblotting, or were stained using instant stains. Mini-PROTEAN® TGX™ precast AnykD™ or 4-15% gradient gels were used. The Precision Plus Protein™ Unstained standard or Precision Plus Protein™ Dual color was used for the determination of relative protein mass (Appendix 2).

4.2.1.11.2 Instant stain

Instant stain was used in place of Coomassie staining. ClearPAGE Instant Blue Stain, or Acqua Stain was used for staining SDS-PAGE gels after electrophoresis. SDS-PAGE gels were immersed in instant stain for up to 1 h until bands were appropriately resolved. Gels were rinsed in dH₂O until the background cleared.
4.2.1.12 Immunoblotting

4.2.1.12.1 Transfer of protein to nitrocellulose membrane

Proteins resolved by SDS-PAGE were transferred by Western blotting onto a nitrocellulose membrane using an iBlot® Gel transfer device as per the manufacturer’s instructions.

4.2.1.12.2 Development of nitrocellulose membrane

Immunoblot was performed using based on the method of Sambrook and Russell (Sambrook & Russell, 2001), with all incubations and washes performed on a orbital shaker. After immunotransfer, the nitrocellulose membrane was placed in TBS/skim milk (5% w/v) for 1 h at room temperature. The membrane was washed twice in TBS for 5 min, then incubated overnight at 4°C in mouse anti-6xHis antibody diluted (1:5000) in TBS/Skim milk (1% w/v). The membrane was washed three times in TBS for 5 min, and then incubated with an alkaline phosphatase (AP)-conjugated rabbit anti-mouse IgG antibody diluted (1:5000) in TBS/Skim milk (1% w/v) for 2 h at room temperature. The membrane was washed three times for 5 min in TBS/Tween and the developed in darkness using the Western Blue® Stabilized Substrate for alkaline phosphatase for up to 30 min. The reaction was stopped by washing in dH₂O and the membrane was then air dried.
4.2.2 Experimental protocols

4.2.2.1 pT.MSP4/5 construct

This section describes the cloning strategy used in constructing the pT.MSP4/5 plasmid vector in the previous study (Black et al., 1999), the subsequent gene analysis by endonuclease digestion, and nucleotide sequencing to confirm the orientation of gene and to determine any mutations which may have occurred during amplification. This work was included to ensure that the recombinant PcMSP4/5 could be successfully expressed in *E. coli* BL21(DE3)pLysS. *In silico* characterisation of PcMSP4/5 was performed to evaluate the suitability of the protein for subsequent nanocapsule synthesis.

4.2.2.1.1 Background on the construct (pT.MSP4/5) development

Early study on the design and development of the *P. chabaudi adami* DS MSP4/5 construct was carried out at Monash University, Clayton, Australia. They identified a region of the *P. chabaudi adami* genome in chromosome 2 as the MSP4/5 gene, a homologue to *P. falciparum* MSP4 and MSP5 (Black et al., 1999). The MSP homologue was identified by detecting a highly conserved adenylosuccinate lyase (ASL) gene that was closely located to the region which contained MSP4 and MSP5 genes. A chromosome walking analysis using various primers were performed, and the resulting sequence was published (GenBank AF080446) and was identified to be 713 bp in length with a 83 bp intron (Figure 4.1, top sequence).

A pUC18 cDNA construct (pMC346) was derived from a total RNA isolated from *P. chabaudi adami* DS ring-stage parasites using primers corresponding to the predicted 5’ and 3’ ends of the PcMSP4/5 gene from the sequenced identified. A plasmid construct with a spliced version of PcMSP4/5 gene lacking the 83 bp intron was made to express the recombinant PcMSP4/5 protein in an *E. coli* system. A region containing the PcMSP4/5 gene with no predicted hydrophobic signal or GPI anchor sequence was amplified from pMC346. A forward primer added a *Bsp*HI restriction site and an ATG translation initiation codon, and a reverse primer added a *Bgl*II site, a termination codon and a C-terminal 6xHis-tag on both ends of gene. A C-terminal 6xHis-tag was introduced downstream of the gene (Figure 4.1, bottom sequence).
Figure 4.1: A schematic diagram of *P. chabaudi adami* MSP4/5 gene modified for protein expression in *E. coli* system. Top sequence indicates the genomic sequence (published sequence) including the 5’ end signal sequence, an 83 bp intron and 3’ end GPI anchor sequence. cDNA was derived from the total RNA with the same sequence as this top sequence of the figure to amplify the bottom sequence. Bottom sequence indicates the amplified sequence used for PcMSP4/5 expression with the addition of an ATG translation initiation codon, a termination codon and 6xHis sequence for purification, with two restriction endonuclease sites flanking the sequence to allow the ligation into the plasmid vector. (Numbers indicate the base pair positions in the genomic sequence)
The amplified PCR product was sub-cloned into the plasmid vector pTrcHis-A (Invitrogen, USA), of which the original N-terminal 6xHis tag, ATG translation codon, Xpress™ epitope and enterokinase cleavage site were removed by restriction endonuclease digestion with NcoI and BglII (Figure 4.2). Although the restriction endonuclease used to design one of the primers was different to those from the spliced sub-cloning vector, both endonucleases (BspHI on PCR products and NcoI on spliced sub-cloning vector) produced the compatible ends, enabling the ligation of PCR products into the sub-cloning vector (detailed in Table 4.3). The original N-terminal 6xHis-tag from the vector was removed as a C-terminal 6xHis-tag was found to favour purification of full-length product of *P. falciparum* MSP4 with the same vector (Wang *et al.*, 1999).

The resulting construct, pT.MSP4/5 (Figure 4.3) was used for the expression of *P. chabaudi adami* MSP4/5.

### 4.2.2.1.2 Storage of plasmid construct

Upon receiving the construct, 1 µL of pT.MSP4/5 was used to electro-transform 40 µL of the prepared electro-competent *E. coli* DH5α for the long term storage (Section 4.2.1.6). Clones that grew on LB agar with AMP were selected and used to grow an overnight culture of 5 mL of LB broth with AMP. An overnight culture was stored at −80°C in 50% glycerol LB broth in 2 mL cryovials (Section 4.2.1.3).
Figure 4.2: Diagrammatic representation of the introduction of manipulated PcMSP4/5 gene into pTrcHis-A plasmid vector. Adopted from pTrcHis vector manual (Invitrogen, U.S.A)
### Table 4.3: Ligation strategy for pT.MSP4/5 construct.

<table>
<thead>
<tr>
<th>Restriction endonuclease</th>
<th>Recognized sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bsp</em>H<em>I</em></td>
<td>TRCATGA</td>
</tr>
<tr>
<td></td>
<td>GGTAC*C</td>
</tr>
<tr>
<td><em>Nco</em>I</td>
<td>CATGG</td>
</tr>
<tr>
<td></td>
<td>AGTAG*T</td>
</tr>
<tr>
<td>(Ligated product)</td>
<td>CCATGA</td>
</tr>
<tr>
<td></td>
<td>GGTACT</td>
</tr>
</tbody>
</table>

Where *r* represents the point of cleave; and **black** are recognized sequences in PCR product, and **red** are recognized sequences in the sub-cloning vector pTrcHis-A.
Figure 4.3: Vector map of pTrcHis-A plasmid construct with PcMSP4/5 gene.
4.2.2.1.3 Screening of the PcMSP4/5 gene in the donated plasmid

A step to transforming *E. coli* BL21(DE3)pLysS with the donated plasmid was included in order to obtain a fresh stock of plasmid for further DNA analysis. Upon receiving the plasmid construct, 1 µL of pT.MSP4/5 was used to electro-transform 40 µL of electrocompetent *E. coli* BL21(DE3)pLysS (Section 4.2.1.6). Clones that grew on LB agar with AMP and CM were chosen for screening of the PcMSP4/5 gene.

A broth culture of transformed *E. coli* BL21(DE3)pLysS was grown overnight from a selected colony on LB agar and plasmids were isolated using the Bioline miniprep kit as per manufacture’s instruction (Section 4.2.1.7). Freshly isolated plasmids were visualised on a 1% agarose gel by DNA electrophoresis (Section 4.2.1.9.2) and the concentration was determined using a UV-vis spectrophotometer (Section 4.2.1.9.1). The isolated plasmids were subjected to an endonuclease digestion to confirm the presence of the PcMSP4/5 gene. A single digestion using either *Pst*I was performed instead to measure the size of linearised plasmid. In a reaction, 120 ng of DNA was digested over 3 h in the presence of 10 U of *Pst*I in restriction enzyme buffer H (Promega) (Section 4.2.1.8) with a negative control sample excluding the endonuclease. Twenty microlitres of each reaction was visualised on a 1% agarose gel (Section 4.2.1.9.2), and a single band indicating for the linearised plasmid vector with insert was determined. Once the presence of the insert was indicated by DNA electrophoresis, 1000 ng of isolated plasmid was sent for DNA sequencing.

4.2.2.1.4 Nucleotide sequencing analysis

4.2.2.1.4.1 Primer design

Primers were designed with the aid of Primer Designer 5, version 5.11 within the Sci Ed Central Program. When possible, primers were designed to have guanine/cytosine (GC) content between 40-60% and a melting temperature (Tm°C) greater than 50°C. Primers were obtained as lyophilised samples from Geneworks. List of primers are shown in Table 4.5.
4.2.2.1.4.2 Sequencing

One thousand nanograms of purified plasmid DNA was combined with 9.6 pmol of primers (0.8 pmol/µL) in a single microfuge tube according to the sequencing facility’s protocol. DNA was sequenced using the Life Technologies AB3730xl 96-capillary sequencer at the Australian Genome Research Facility Ltd, Victoria, Australia.

4.2.2.1.5 *In silico* protein characterisation

To analyse sequencing data, nucleotide sequence alignments were performed using the program SE Central. Basic Local Alignment Search Tool (BLAST®) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was also used to confirm identity to the registered *P. chabaudi* *adami* DS MSP4/5 sequence on GenBank (accession number: AF080446.1) (Black *et al.*, 1999).

The nucleotide sequence was translated into a protein sequence using Translate (http://web.expasy.org/translate/) in the Expasy suite of programs (http://au.expasy.org/tools/). The most suitable open reading frame was selected for the prediction of the protein sequence.

The program Compute pI/MW (Bjellqvist *et al.*, 1993; Bjellqvist *et al.*, 1994; Gasteiger *et al.*, 2005) (http://web.expasy.org/compute_pi/) was used to compute the theoretical molecular weight and the isoelectric point of PcMSP4/5. The web-based program ProtParam (Gasteiger *et al.*, 2005) (http://web.expasy.org/protparam/) was also used to calculate the theoretical molecular weight and isoelectric point of PcMSP4/5, and other theoretical and chemical properties including the instability index (Guruprasad *et al.*, 1990) and the charges of amino acids in the protein sequence.
Table 4.4: Primers used for the sequencing analysis.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ ➔ 3’)</th>
<th>Description</th>
<th>Tm°C*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSP45-Fwd</td>
<td>GCGCCGATTAATAAGGAGG</td>
<td>Forward primer with GC extension, position 389</td>
<td>52</td>
</tr>
<tr>
<td>MSP45-Rev</td>
<td>ACAGCCAAGCTTCGAATTCC</td>
<td>Reverse primer, position 990</td>
<td>52</td>
</tr>
</tbody>
</table>

*Calculated by Geneworks.
4.2.2.2 Protein expression and IMAC purification

This section describes the methods to obtain *E. coli*-expressed recombinant PcMSP4/5. Recombinant PcMSP4/5 was expressed in *E. coli* BL21(DE3)pLysS grown in EnPresso™ cultivation system and purified by IMAC.

4.2.2.2.1 Preparation of expression culture

To ensure the proper expression of PcMSP4/5, as well as avoiding mutations in the plasmid sequence, *E. coli* BL21(DE3)pLysS were electrotransformed for each protein expression. One microlitre of 100 ng/µL pT.MSP4/5 was used to electro-transform 40 µL of *E. coli* BL21(DE3)pLysS (Section 4.2.1.6) and LB agar with AMP and CM was used for colony selection to ensure only recombinant clones were present. A single colony was selected to inoculate 1 mL LB broth with AMP and CM and incubated at 37°C for 6 h on a shaking incubator at 200 rpm.

4.2.2.2 Protein expression

All protein expression was performed using EnPresso™ Tablet Cultivation Set. EnBase® Medium tablets were dissolved in 50 mL of sterile MQH₂O in a sterile 500 mL Erlenmeyer flask, and inoculated with 500 µL of previously prepared recombinant *E. coli* culture after the addition of AMP, CM and 50 µL of EnZI’m. The inoculated culture was sealed with AirOtop seal and incubated at 30°C on a shaking incubator at 200 rpm overnight. Culture was supplemented with a Booster tablet after an overnight incubation and protein expression was induced with 1 mM IPTG via the trc (trp-lac) promoter (Amann *et al.*, 1983). Protein expression was to proceed for 8 h in the same culture conditions, and the cells were then harvested.
4.2.2.2.3 Protein purification by Immobilised metal affinity chromatography (IMAC)

4.2.2.2.3.1 Cell lysis

Cells were harvested by centrifugation at 4,000 x g for 15 min at 4°C and resuspended in chilled IMAC lysis buffer with 10-50 mM imidazole added to the final volume of 10-20 mL. The cell suspension was submitted to sonication in an ice bath for 6 pulse cycles of 15 sec and intercalated by 30 sec without sonication, at amplitude of 27% using a Branson Ultrasonic Digital sonifier. The lysate was clarified by centrifugation at 5,000 x g for 20 min to remove cellular debris and the supernatant was aspirated for purification.

4.2.2.2.3.2 IMAC column preparation

Chelating sepharose fast flow or Iminodiacetic acid Sepharose® (depending on the availability), was prepared in 1 mL gravity-flow column. Once packed and washed with MQH₂O, the resin was charged using half the column volume (CV) of 0.45 μm filtered 0.2 M nickel sulphate (NiSO₄) solution. The column was washed with at least 10 CV of MQH₂O and equilibrated with 5 CV of IMAC binding buffer.

4.2.2.2.3.3 Purification optimisation by imidazole gradient

Cells were lysed in IMAC lysis buffer with 10-50 mM imidazole and clarified by centrifugation (Section 4.2.2.2.3.1). Increasing concentrations of imidazole were used in IMAC wash buffer to obtain the optimal concentration required to purify the PcMSP4/5 with minimal contamination by non-PcMSP4/5 proteins. An increment of 20 mM in imidazole concentration ranging from 20 mM to 240 mM was applied to the column to determine the elution profile of the proteins. Clarified sample was passed through a 0.22 μm filter to remove any particles capable of blocking the IMAC column. Five millilitres of sample was applied to the charged nickel column which was equilibrated with 10 mM imidazole. Ten millilitres of IMAC wash buffer with 10 mM imidazole was first applied to the column, then subsequently 1 mL of IMAC wash buffers with increasing imidazole concentration were added. One millilitre fractions from each concentrations were collected and visualised by SDS-PAGE (Section 4.2.1.11) and western immunoblotting. The nitrocellulose membrane was probed
with mouse anti-6xHis antibodies and secondary antibodies (Section 4.2.1.12).

4.2.2.3.4 IMAC purification

Clarified sample was passed through a 0.22 µm filter to remove any particles capable of blocking the IMAC column. Up to 10 mL of sample was applied to the column followed by 1 h incubation on a rotary shaker at low speed for binding at 4°C. The column was washed with series of 10 CV of IMAC wash buffers at different imidazole concentrations, then the protein eluted with 10 CV of IMAC elution buffer. All washes were collected and 10 µL of each was visualised by SDS-PAGE (Section 4.2.1.2.4). Samples containing the recombinant PcMSP4/5 were pooled, concentrated and buffer exchanged before storing at −20°C (Section 4.2.2.2.4).

4.2.2.3.5 IMAC column cleaning and regeneration

Gravity flow columns were stripped and cleaned between each use to remove contaminants and were recycled up to 3 times. The Ni\(^{2+}\) was stripped with 2 CV of 50 mM EDTA, 0.5 M NaCl, pH 7.0. Cleaning involved the addition of 10 CV of 2 M NaCl, followed by 10 CV of 1 M NaOH, and finally 10 CV of 70% ethanol (v/v). Columns were washed with MQH\(_2\)O between each solution then finally stored in 20% ethanol solution at 4°C.

4.2.2.4 Buffer exchange and concentration

After purification, appropriate protein fractions containing the recombinant PcMSP4/5 were pooled then concentrated using a Vivaspin®20 centrifugal concentrator with a 30 kDa cut-off membrane, according to the manufacturer’s instructions. Vivaspin®20 devices were centrifuged at 5,000 x g at 4°C in a swing bucket rotor. Once the protein samples were concentrated to 1 mL, buffer exchange to PBS was performed in the same device. Any remaining salts and metals were removed by continual dilution of the sample using the Sartorius™ Diafiltration Cups by applying a total of 20 mL PBS. The protein concentration of final product was quantified by Bradford assay and protein was stored at −20°C. One microgram of protein was visualised using SDS-PAGE (Section 4.2.1.2.4).
4.2.2.3 Synthesis of PcMSP4/5 nanocapsules

This section describes the process taken to synthesise the PcMSP4/5 nanocapsules using SC/MS templates fabricated in Chapter 3. Successfully expressed and purified recombinant PcMSP4/5 from the previous section was utilised. To determine its safety for the use as a vaccine in the later in vivo study, the cytotoxicity of the synthesised PcMSP4/5 nanocapsule was tested using DC2.4 cell, a murine dendritic cell.

4.2.2.3.1 Synthesis of PcMSP4/5 nanocapsules using SC/MS nanoparticle templates

The nanocapsule synthesis using the SC/MS template was based on the method of (Wang et al., 2008a) with minor alterations. Ten milligrams of PBS-washed amine functionalised SC/MS templates were added to the processed PcMSP4/5 (up to 1 mg/mL in PBS), followed by an overnight incubation on a suspension mixer at 4°C. Excess protein was then removed by one cycle of centrifugation at 5,000 x g for 2 min, and kept for the loading efficiency analysis. To stabilise the infiltrated protein in the mesoporous shell, particles were incubated in 1 mL of 5% (w/w) glutaraldehyde (GA) solution or 1% (w/v) paraformaldehyde (PFA) solution for minimum of 2 h at 4°C on a suspension mixer, to form covalent link. After washing with PBS three times, the SC/MS templates were removed by 500 µL of 2 M hydrofluoric acid (HF) treatment in an 8 M ammonium fluoride buffer at pH 5 to obtain the PcMSP4/5 nanocapsules (Shimizu et al., 1998). HF treatment required 5 min incubation with occasional inversion of the tube at room temperature, followed by three washes by centrifugation with sterilised PBS at 6,500 x g for 5 min. Each infiltration reaction was performed in 1 mL to ensure the proper removal of silica by HF. The synthesised nanocapsules were resuspended in 100 µL of sterilised PBS, followed by five cycles of 1 min sonication to separate aggregated nanocapsules before combining several batches together.

All the washes were kept at 4°C and the concentration of PcMSP4/5 in each solution was determined by both UV-vis spectrophotometry and Bradford assay (Section 4.2.1.10.1). Before each assay, the protein solution was briefly centrifuged to precipitate any remaining SC/MS nanoparticle templates for the concentration determination as silica could interfere with UV absorbance measurement.
**HF** is highly corrosive and toxic. The above work was performed only under the supervision of trained personnel in a HF filter fitted fume hood, with an adequate supply of calcium gluconate gel.

4.2.2.3.2 Quantification of synthesised PcMSP4/5 nanocapsules

Quantification was first attempted by UV-vis spectrophotometry (Section 4.2.1.10.2), Bradford assay (Section 4.2.1.10.1), and by SDS-PAGE (Section 4.2.1.11). Finally, the concentrations of synthesised nanocapsules were determined by a simplified dot-blot method. Two microlitres of PcMSP4/5 nanocapsule in various dilutions (1/100, 1/10, 1/2, neat and supernatant) were spotted onto a nitrocellulose membrane with known concentrations of serially diluted BSA ranging from 0.078 mg/mL to 10 mg/mL. The spotted nitrocellulose membrane was stained with instant stain (Section 4.2.1.11.2) until spots developed and then rinsed with tap water. The concentration was estimated by comparison of size and colour intensity to the BSA standards. As a recombinant protein standard, 0.45 mg/mL of recombinant PcMSP4/5 was also spotted.

4.2.2.3.3 Stability check for the recycled PcMSP4/5

In order to maximise the use of processed PcMSP4/5, first washes from the post-infiltration centrifugation were recycled in the repeated nanocapsule synthesis. All washes were stored at 4°C immediately after washing, and the integrity of PcMSP4/5 was determined using SDS-PAGE to ensure its usability for the repeated synthesis. Five microlitres of each wash was measured by SDS-PAGE under reducing conditions as described in Section 4.2.1.11.
4.2.2.3.4 The dose-dependent cytotoxicity of PcMSP4/5 nanocapsules

The cytotoxicity of nanocapsules was analysed at different concentrations using PrestoBlue® Cell Viability reagent. DC2.4 cells were seeded in a 96 well tissue culture plate at a density of 5 x 10^4 cells per well, in RPMI GlutaMAX™ 1640 medium supplemented with 10% heat inactivated FBS and incubated at 37°C in a 5% CO₂ humidified atmosphere overnight. Following the incubation, PcMSP4/5 nanocapsules suspended in 20 µL of sterile PBS were added to give a concentration of 3.125, 6.25, 12.5, 25 and 50 µg/mL in a final volume of 120 µL per well. Replica plates for different time points were made and were further incubated for up to 96 h with the nanocapsules in the same conditions. At each time point, 12 µL of PrestoBlue® Cell Viability reagent was added to wells and the absorbance read at 570 nm with 600 nm reference wavelength for normalisation after 60 min incubation at 37°C in the dark. Absorbance readings were measured using the POLARstar Omega microplate reader.

Culture media and 0.1% triton X-100 served as an internal negative and positive control, respectively. Percentage cell viability was calculated by setting the negative control to 100% after non-live cell dependent colour conversion (background and positive control) was eliminated from all the treatment groups.

Statistical variables (mean, standard deviation, and standard error) were calculated for all data and presented graphically. Comparisons between groups were accessed by two-way ANOVA followed by a Dunnett’s multiple comparison test to determine significant differences in cell viabilities against the negative control (media alone) group. GraphPad Prism 6 software was used to perform the statistical analysis.
4.3 Results

4.3.1 Endonuclease digestion and sequencing analysis

4.3.1.1 Transformation of *E. coli* BL21(DE3)pLysS and plasmid isolation

The donated stock of pT.MSP4/5 plasmid was successfully introduced into the *E. coli* BL21(DE3)pLysS which allowed the isolation of new plasmid stocks from an overnight grown culture. This freshly isolated plasmid was used for restriction endonuclease digestion and sequence analysis.

4.3.1.2 Restriction endonuclease digestion analysis

A single endonuclease digestion was performed using *Pst*I, to determine the presence of the PcMSP4/5 gene by measuring the size of linearised plasmid with inserted gene. The enzyme was chosen based on the computer analysis that recognise only one site in pT.MSP4/5. *Pst*I successfully linearised plasmid and the DNA fragment was visualised on 1% agarose gel. This was the expected size of approximately 4834 bp indicated that the PcMSP4/5 gene was indeed present in the vector (Figure 4.4). pT.MSP4/5 incubated without enzyme in the same conditions was included as an internal negative control. It was further confirmed by nucleotide sequencing to ensure that there were no mutations in the target gene.

4.3.1.3 Nucleotide sequencing analysis

Nucleotide sequencing of pT.MSP4/5 was performed and produced a sequence. This was aligned with the PcMSP4/5 gene sequence used to build a vector map. This sequence was predicted based on GenBank data, with no intron, signal or GPI anchor sequences, with added 6xHis tag. Sequence alignment revealed no mutations in the gene as shown in Figure 4.5 and further analysis showed that the PcMSP4/5 gene was placed in the correct orientation, in frame with the start codon and encoded a 159 amino acid product. The C-terminal 6xHis-tag sequence required for downstream processing was also present directly upstream of the stop codon. The identity of the sequence to the published PcMSP4/5 was confirmed by BLAST®.
Figure 4.4: *PstI* digestion of pT.MSP4/5 on 1% agarose gel. Lane 1: λ-*PstI* ladder, Lane 2: undigested negative control, and Lane 3: complete digestion of pT.MSP4/5. The red arrow indicates the predicted base pair size of linearised plasmid.
### Figure 4.5: Sequence alignment with the predicted PcMSP4/5 gene.

Top sequence is the predicted sequence from the vector map, while the bottom sequence is from nucleotide sequencing. ATG start codon is highlighted in **yellow**, 6xHis tag is in **magenta**, and termination codon is highlighted in **cyan blue**. This shows 100% match of two sequences within the PcMSP4/5 gene. Dots indicates the matching nucleotides.
4.3.2 Recombinant PcMSP4/5 protein purification

4.3.2.1 Expression of recombinant PcMSP4/5 in *E. coli* BL2(DE3)pLysS

Recombinant PcMSP4/5 was successfully expressed as a soluble protein in *E. coli* with the EnPresso™ system by induction with 1 mM IPTG. The EnPresso™ cultivation condition was already optimised for the controlled growth and metabolism for protein expression for between 6-24 h (BioSilta, Finland). Recombinant PcMSP4/5 was produced for 8 h.

4.3.2.2 Optimisation of imidazole concentration and purification of PcMSP4/5

Recombinant PcMSP4/5 was isolated by utilising a C-terminal 6xHis-tag, allowing purification by affinity binding using an IMAC column charged with nickel ions. Following the protein expression, the clarified *E. coli* lysate was applied to an IMAC column and washed with series of wash buffers containing an increasing concentration of imidazole to purify PcMPS4/5 from *E. coli* proteins. Eluates from each wash were subjected to SDS-PAGE. The concentration of imidazole that eliminated most of the *E. coli* proteins with minimal loss of PcMSP4/5 was selected for repeat purifications.

A protein of approximately 37 kDa and two smaller proteins of 20 and 23 kDa were separated on a SDS-PAGE gel from elutions with a higher imidazole concentration (Figure 4.6A, Lane 4-6). One of these proteins was suspected as a 6xHis tag recombinant protein. Interestingly, the theoretical molecular weight of a recombinant PcMSP4/5 was predicted to be 16.7995 kDa, which is much lower than any of these proteins detected using SDS-PAGE. To confirm that one of these observed protein was the recombinant PcMSP4/5, Western blotting was performed. A nitrocellulose membrane was probed with anti-6xHis antibodies and it showed strong reactivity to the protein size of 37 kDa (Figure 4.6B). This observation revealed that the recombinant PcMSP4/5 has extremely slow mobility on SDS-PAGE under reducing condition.
Figure 4.6: Purification optimisation by imidazole washes visualised on A) an instant stained SDS-PAGE gel, and B) a nitrocellulose membrane probed with anti-6xHis antibody. Concentrations of imidazole increasing by 20 mM increment. Lane 1-2: From 80 mM to 100 mM, Lane 3: Molecular weight marker, and Lane 4-7: From 120 mM to 180 mM. Samples loaded on lane 1 in both figures were run on a separate gel/membrane, and merged as one image by aligning the molecular weight marker.
Optimisation of wash buffer with an imidazole gradient ranging from 20 to 240 mM showed that the recombinant PcMSP4/5 was eluted from the nickel charged column at 100 mM imidazole. Wash buffers of up to 80 mM imidazole removed most of the contaminants, however some proteins at several different molecular weights were co-eluting and contaminating the PcMSP4/5 fractions in 100 mM and above. Proteins of around 23 and 27 kDa were also present in the 80 mM wash, suggesting that more stringent washes were required for higher PcMSP4/5 purity.

From optimisation of imidazole, 5 CV of 80 mM imidazole wash was initially chosen to purify PcMSP4/5 from E. coli proteins. It was observed that the proteins with molecular weights of 23 and 27 kDa were still contaminating PcMSP4/5 (Figure 4.7, left), showing strong binding capacity much the same as the recombinant protein. Although most of the contaminating proteins were removed with 5 CV of 80 mM imidazole wash, PcMSP4/5 was also partially eluted from the column as indicated on an immunoblot probed with anti-6xHis antibody with a single band at 37 kDa (Figure 4.7, right). More pure PcMSP4/5 was obtained from 120 mM imidazole buffer, however the loss of product had to be avoided due to a low expression level found, albeit with the use of EnPresso™ system. Protein expression in LB broth showed almost no protein yield (data not shown).

Clarified E. coli lysate was bound to the IMAC column with 10 mM imidazole buffer, but this was changed to 50 mM to increase the PcMSP4/5 competition against the initial binding of the 23 and 27 kDa contaminants to the column. However, no obvious changes were observed in the final elution product (data not shown). Finally the concentration of imidazole in the binding buffer was adjusted to 20 mM and wash buffers were decreased to 60 mM with the addition of 40 mM wash prior to avoid protein loss (Figure 4.8). Contaminants at 23 and 27 kDa were still present after this purification method, as confirmed by both SDS-PAGE and Western blot analysis, however this method gave the highest PcMSP4/5 yield of up to 0.5 mg (with >70% purity), per 50 mL EnPresso™ culture. In some experiments, an extra protein approximately 70 kDa was observed. This was most likely a dimer complex of recombinant proteins as it reacted with anti-6xHis antibodies (Figure 4.9).
Figure 4.7: Purification of recombinant PcMSP4/5 with 80 mM imidazole wash visualised on (left) an instant stained SDS-PAGE gel, and (right) a nitrocellulose membrane probed with anti-6xHis antibody. Lane 1: Molecular weight marker, Lane 2: 80 mM wash, second CV, Lane 3: 80 mM wash, last CV, and Lane 4: 120 mM final elution. The red arrow indicates recombinant PcMSP4/5.
Figure 4.8: Purification of recombinant PcMSP4/5 with 40 and 60 mM imidazole wash visualised on an instant stained SDS-PAGE gel. Lane 1: protein molecular marker, Lane 2: 100 mM final elution concentrated and buffer exchanged, estimated 1 µg. The red arrow indicates recombinant PcMSP4/5.
Figure 4.9: Purification of recombinant PcMSP4/5 with dimer complexes visualised on (left) an instant stained SDS-PAGE gel, and (right) a nitrocellulose membrane probed with anti-6xHis antibody. Lane 1: protein molecular marker, Lane 2: concentrated and buffer exchanged PcMSP4/5, estimated 1 µg. The red arrows indicate monomer and dimmer recombinant PcMSP4/5.
4.3.2.3 Protein processing and storage

Concentration of the final elution containing PcMSP4/5 and the buffer exchange was carried out in a Vivaspin®20 filtration device with 30 kDa cut-off membrane. Despite the size of the filter membrane being bigger than the 23 and 27 kDa, these contaminants were still in the filtered PcMSP4/5 solution. Flow-through collected from the filtration was also subjected to SDS-PAGE but showed neither of contaminants was filtered through (Figure 4.10).

These contaminating proteins were taken into consideration for the preparation of PcMSP4/5 in the vaccine trial. Processed PcMSP4/5 was stored at −20°C for nanocapsule synthesis. The concentration of protein was determined for pre- and post-storage of up to 6 months. There was no loss in the protein amount and no breakdown.
Figure 4.10. Samples from buffer exchange visualised on an instant stained SDS-PAGE gel. Lane 1: protein molecular marker, Lane 2: flow through from the filtration, Lane 3: filtered PcMSP4/5 with 23 and 27 kDa proteins.
4.3.3 PcMSP4/5 nanocapsule synthesis

4.3.3.1 Recombinant PcMSP4/5 for nanocapsule synthesis

The surface of SC/MS nanoparticle templates was modified by amine functionalisation to gain positive charges. This step was necessary to allow the negatively charged protein to infiltrate into the mesoporous layer of the SC/MS nanoparticle template. The net charge of recombinant PcMSP4/5 was estimated to be negative by the number of charged amino acids it possesses. The total number of negatively charged residues (aspartic acid and glutamic acid) were calculated to be 35, while the total number of positively charged residues (arginine and lysine), was 9.

An infiltration load efficiency of PcMSP4/5 in PBS pH 7.4 was mostly above 25%, and as high as 40.6%. This was greatly higher than ovalbumin, however, the loading capacity translated to 32.96 mg/g SC/MS template. This is equivalent to the ovalbumin loading capacity in PBS at pH 7.4. This was expected as ovalbumin and the recombinant PcMSP4/5 had very close pI value of 4.6 and 4.19 (discussed in Section 3.4.1.2), respectively. The attraction between the positively charged silica template and the negatively charged protein increases when the pI of the infiltrating molecule is lower than the physiological pH of the buffer where the reaction takes place. An increase in the filtration load was evident in Chapter 3, where the ovalbumin infiltration load into SC/MS template was tested against different pH of PBS (Section 3.3.2.1).

To increase the integrity of the nanocapsule in order to avoid in vivo degradation before reaching immune cells, GA was selected as a cross-linking agent for PcMSP4/5 nanocapsule synthesis. GA was favoured based on the stronger covalent bonds it can provide as each molecular possess two aldehyde groups compared to those of formalin, made from PFA with only one aldehyde group (Hopwood & Path, 1976). GA is often used to cross-link nanoparticles of different formulas (Langer et al., 2003; Coester et al., 2006; Wang et al., 2010; Altintas et al., 2013), as it provides strong bonding. GA cross-linked nanoparticles can be degraded by a simple enzymatic digestion by proteases, such as α-chymotrypsin and trypsin (Leo et al., 1997; Lin et al., 2001). This was particularly important, as a nanocapsule
must be easily degraded in vivo to enable antigen presentation.

4.3.3.2 Quantification of synthesised recombinant PcMSP4/5 nanocapsules

Although it is the preferred choice of cross-linking agent, using GA compromised the accurate quantification of synthesised nanocapsules. During cross-linking of specific amine residues, Schiff bases are formed between the aldehyde groups, causing autofluorescence of the nanocapsule (Collins & Goldsmith, 1981; Lee et al., 2013). Although attempted, it was suspected to have interfered with quantifying an accurate concentration of nanocapsules when measured by UV absorbance reading at 280 nm. Autofluorescence of synthesised ovalbumin nanocapsule was also observed (Section 3.3.2.2) but a broad estimation was made by UV-vis spectroscopy and SDS-PAGE as such estimation was suitable for the purpose of in vitro studies. However, more accurate quantification of PcMSP4/5 nanocapsules was necessary in order to use the material for a vaccine trial.

Bradford assay was used next to measure the concentration of PcMSP4/5 nanocapsules however; it too was unsuccessful due to false readings given by the insoluble nanocapsules in solution. Synthesised nanocapsules were sonicated extensively before assays were carried out in an attempt to dissociate and solubilise the aggregated capsules, but strongly cross-linked structure hindered solublisation of the cross-linked material. Bradford reagent binds to amino acids in protein to give a colorimetric reaction (Bradford, 1976), and some blue-stained particulates were seen in the solution, however this was not detectable at 595 nm. Microscopic observations confirmed that these particulates were in fact the stained nanocapsules remained intact, which suggested that solublisation was not achievable by sonication. The concentration determined for nanocapsules by the Bradford assay was as low as 50 µg/mL, despite the values obtained by an infiltration load estimation of up to 330 µg/mL.

Formalin cross-links made by PFA are reversible by boiling at 99°C for 15 min in SDS-PAGE sample buffer (Klockenbusch & Kast, 2010). Therefore, the same treatment with a longer incubation time of 30 min was applied to nanocapsules to estimate concentration on a SDS-PAGE. To confirm protein was changed to a reduced state, a batch of PFA cross-linked
nanocapsules were also treated. As expected, reduced PFA cross-linked PcMSP4/5 nanocapsules and also PFA cross-linked ovalbumin nanocapsules were able to enter the SDS-PAGE gel (Figure 4.11A, Lane 2), however GA cross-linked PcMSP4/5 remained intact in the well (Figure 4.11B, lane 2-4).

Protein concentration was finally estimated by a simple dot-blot method by marking spots of nanocapsule solution onto a nitrocellulose membrane. A serially diluted BSA of known concentration was also applied onto the membrane as concentration standards. The estimated concentration of PcMSP4/5 nanocapsules was then compared to the values obtained from UV-vis spectrophotometry. With considerations of the affect of autofluorescence possibly increasing the spectrophotometric measurement, the concentration of PcMSP4/5 in synthesised nanocapsule was finally estimated to be 0.7 mg/mL.

4.3.3.3 Protein stability for repeated use for nanocapsule synthesis

In order to maximise the use of purified recombinant PcMSP4/5, protein samples were recycled for consecutive synthesis reactions. It was evident from the infiltration load optimisation study in Chapter 3 that a large amount of protein did not infiltrate into SC/MS templates. There were large amounts of unbound protein that could be reused for subsequent infiltration experiments. The washes obtained by the initial centrifugation step were collected from each infiltration reaction. To prevent the nanocapsules being synthesised with degraded protein, each batch of washes were visually audited on SDS-PAGE gels for its integrity before applying them to an infiltration experiment for the next synthesis batch.

No obvious degradation was seen up to four uses of the same protein sample (Figure 4.12). This was probably aided by maintaining the storage and protein infiltration condition at 4°C. The computed protein instability index showed that the recombinant PcMSP4/5 is stable. This also supported the stability of recombinant PcMSP4/5 for multiple uses in consecutive experiments over a week. A protein of approximately 32 kDa was seen in the wash after the fourth synthesis reaction indicating possible protein degradation, therefore the protein was recycled for no more than four uses.
Figure 4.11: PcMSP4/5 nanocapsules after boiling treatment at 99°C for 15 min. A) PcMSP4/5 nanoparticles cross-linked with PFA were able to enter the gel, while B) cross-linked GA remained intact in the wells. A) Lane 1: protein molecular marker, Lane 2: 1 µg PFA cross-linked PcMSP4/5 nanocapsules. B) Lane 1: protein molecular marker, Lane 2: 1 µL, Lane 3: 5 µL, Lane 4: 10 µL of GA cross-linked PcMSP4/5 nanocapsules. The red box indicates the presumed intact nanocapsules remaining in the well.
Figure 4.12: PcMSP4/5 collected after the initial washes from nanocapsule synthesis. Lane number corresponds to the number of synthesis reactions taken. Lane 1: wash collected after the 1\textsuperscript{st} synthesis reaction, Lane 2: wash collected after the 2\textsuperscript{nd} synthesis reaction, Lane 3: wash collected after the 3\textsuperscript{rd} synthesis reaction, Lane 4: wash collected after the 4\textsuperscript{th} synthesis reaction, and Lane 5: Molecular weight marker. 5 µL of proteins at different concentrations were separated.
4.3.3.4 Cytotoxicity of PcMSP4/5 Nanocapsules

The cytotoxic effect of recombinant PcMSP4/5 nanocapsules was evaluated using DC2.4 murine DCs. Different concentrations of PcMSP4/5 nanocapsules were applied to $5 \times 10^4$ cells and exposed for up to 96 h. Cell viability of 100% was represented by those of which were incubated with culture medium alone and complete cell death was achieved by inhibition with 0.1% Triton X-100. An internal PBS control was also included to exclude the effect caused by the solvent which maintained nanocapsules. The relationship between the nanocapsule concentration ($\mu$g/mL) and cell viability (% of control) of up to 96 h of exposure time is presented in Figure 4.13A. The same conditions were applied to cells with the purified PcMSP4/5 used to synthesise nanocapsules and the results are shown in Figure 4.13B. Each experimental curve represents the mean of three experiments.

The cell viability of PcMSP4/5 nanocapsule applied cells was maintained at 100% for even the highest concentration tested across different time points measured. Differences in nanocapsule concentration did not change the level of viability and also showed a slight increase after 96 h. Purified PcMSP4/5 had a reverse effect on cell viability. An immediate response indicated after 24 h was especially noticeable by a significant decrease in the viability to 75% in the lowest concentration of $3.13 \mu$g/mL ($p \leq 0.001$). Cell viability was significantly lowered in all time points at $12.5 \mu$g/mL ($p \leq 0.0001$). Although it was slightly less effective at lower concentrations with longer incubations, cell viability reached below 50% with $25 \mu$g/mL of PcMSP4/5 (Figure 4.14).

The trend seen in the effect of soluble or nanocapsule proteins on DC2.4 cells was completely contradictory to the finding in the ovalbumin study. The soluble ovalbumin had minimal toxicity effect across all the concentration tested while ovalbumin nanocapsules showed also minimal, but increasing toxicity after $25 \mu$g/mL (Section 3.3.3.1). On the contrary, the soluble PcMSP4/5 showed nearly dose-dependent cytotoxic effects, while the nanocapsule showed no effects.
Figure 4.13: Cell viability curve of DC2.4 cells against protein concentration of A) PcMSP4/5 nanocapsules, and B) purified PcMSP4/5 over 96 h.
Figure 4.14: Cell viability of DC2.4 cells over different time points against different concentration of PcMSP4/5 materials. (Statistical significance against 100% viable control group is represented in asterisks. * is $p \leq 0.05$, ** is $p \leq 0.01$, *** is $p \leq 0.001$ and **** is $p \leq 0.0001$)
4.4 Discussion

4.4.1 Endonuclease digestion and sequencing analysis of pT.MSP4/5 construct

The purpose of this study was to confirm the integrity of the PcMSP4/5 gene in pT.MSP4/5 including no mutation has been caused before receipt of the plasmid. Nucleotide sequencing of pT.MSP4/5 produced a sequence that aligned with a predicted PcMSP4/5 sequence. Alignments of these two sequences showed 100% match in the gene and showed no introduction of mutations. It also showed a sequence encoding for six cysteines was preserved in the C-terminal region, which encodes an EGF-like domain.

The second exon in the full length PcMSP4/5 gene contains a region that is highly similar to the EGF-like domains present in the *P. falciparum* MSP4 and MSP5 genes (Marshall et al., 1997; Black et al., 1999). The EGF-like domain on the C-terminal end of the protein is also present in MSP4/5 from other murine malaria species including *P. berghei* ANKA, *P. yoelii. yoelii* YM (Kedzierski et al., 2000a), *P. yoelii. killicki* 193L, *P. yoelii. nigeriensis* N63 (Goschnick et al., 2004) and in many *P. falciparum* MSP’s (Kaslow et al., 1988; Black et al., 2001; 2003; Wu et al., 1999). This conservation indicates that the EGF-like domain may have potential as vaccine candidate in preventing malaria infections. Immunisation with MSP with the EGF-like domain or the domain alone is capable of eliciting antibodies to induce protective immunity (Calvo et al., 1996; Kedzierski et al., 2000b; James et al., 2006), and inhibits the parasite invasion of host erythrocytes (Blackman, 1990; Woehlbier et al., 2010).

The conformation of the epitope is crucial as the antigenicity of MSP is possibly dependent on the correct folding of the EGF-like domain (Wang et al., 1999). The conformation of the protein may influence vaccine efficacy, as either reduced or alkylated MSP protein and the parasite lysate decreased the reactivity of anti-MSP4/5 antibody binding (de Silva et al., 2011; Wang et al., 1999). However, if the level of antibody production is very high, the recognition of native MSP4/5 may not be abolished (Kedzierski et al., 2000b). This needs to be taken into consideration when the PcMSP4/5 nanocapsules are synthesised for the vaccine trial. As the
covalent cross-linking of proteins may hinder the exposure of EGF-like domain in PcMSP4/5, it may possibly decrease the efficacy of the vaccine by reducing conformation-specific antibody production, if protective humoral immunity is desired.
4.4.2 Recombinant PcMSP4/5 protein expression in *E. coli* BL2(DE3)pLysS and purification by IMAC

4.4.2.1 Expression of recombinant PcMSP4/5 in *E. coli* BL2(DE3)pLysS

It was previously demonstrated that the expression of recombinant PcMSP4/5 was successful in *E. coli* BL21(DE3) and purified by IMAC for the purpose of raising antisera in animals (Black *et al.*, 1999). The same plasmid construct with the PcMSP4/5 gene was used in this study. pLysS is a non-cloning vector that encodes a T7 phage lysozyme to inhibit transcription initiation from T7 promoter (Duberndorff & Studier, 1991). It has benefits by providing tight regulation of basal expression by the T7 promoter, however it was thought that pLysS had no effect on the expression of PcMSP4/5 as the introduced pTrcHis-A vector did not have a T7 promoter region.

Regulation of PcMSP4/5 expression was still met by the repression gene in the pTrcHis-A vector. The pTrcHis-A vector is capable of expressing proteins through the *trc* (*trp-lac*) promoter (Amann *et al.*, 1983). The *trc* promoter contains regions of the *trp* and *lac* promoter (Brosius *et al.*, 1985; Mulligan *et al.*, 1985), and higher transcriptional regulation of the inserted gene in any *E. coli* strain is achievable with the repression protein produced by the *lacI* gene. The expression of gene can be induced by the addition of a synthetic lactose analogue, such as IPTG to inhibit repression of the *trc* promoter.

Difficulties were encountered when expressing PcMSP4/5 in *E. coli* BL21(DE3) grown in LB broth, and no expression was detected for up to 5 h after an induction with 1 mM IPTG. There was no obvious reduction in the amount of *E. coli* cells between 0 and 5 h post-induction, however it could possibly be due to a toxic effect of expressed recombinant PcMSP4/5 inducing *E. coli* cell death. The EnPresso™ cultivation system was therefore used instead of LB broth as this system supports more difficult protein expression with slow protein synthesis by controlled cell growth. With the use of the EnPresso™ system, recombinant PcMSP4/5 was successfully expressed in *E. coli* BL21(DE3)pLyS.
4.4.2.2 Purification of recombinant PcMSP4/5 by IMAC

Successfully expressed recombinant PcMSP4/5 was purified using a nickel charged IMAC column. A series of wash buffers with an increasing concentration of imidazole were applied to the column of which the clarified *E. coli* cells have been passed through. Fractions of wash buffers from each imidazole concentration were collected and the proteins were separated and visualised on a SDS-PAGE gel. Several proteins were detected to have a high affinity to the nickel column but none of them match the predicted molecular weight of recombinant PcMSP4/5. It was revealed from the immunoblotting with anti-6xHis antibody that the protein of 37 kDa on SDS-PAGE gel was in fact the recombinant PcMSP4/5, despite its predicted molecular weight of 16.8 kDa.

Slow mobility of other murine malarial recombinant MSP4/5s on SDS-PAGE has been observed previously (Black *et al.*, 1999; Goschnick *et al.*, 2004; Kedzierski *et al.*, 2000a; 2000b). These recombinant MSP4/5s were also expressed without N-terminal signal and C-terminal GPI anchor sequences, yet all migrated as approximately 36 kDa protein on SDS-PAGE under reducing conditions despite their predicted molecular weight being much lower. Furthermore, a full-length PcMSP4/5 from a *P. chabaudi adami* DS parasite lysate also had slow mobility of approximately 36 kDa under reducing condition (Black *et al.*, 1999) while the molecular weight for a full-length PcMSP4/5 is only 22.3 kDa. This apparent slow mobility is also typical for many *P. falciparum* asexual-stage proteins, including MSP4 (Wang *et al.*, 1999; Anders *et al.*, 1988).

It was difficult to obtain PcMSP4/5 at higher purity due to two unknown proteins co-eluting, even in stringent imidazole washes. Several optimisation studies were performed by changing the imidazole concentrations, however the protein of approximately 23 and 27 kDa could not be eliminated. Neither of these proteins nor PcMSP4/5 filtered through the 30 kDa cut-off membrane when concentrating the sample, it was speculated that all three proteins were somehow bound together in the non-reduced state. Interestingly, a similar purification profile was seen in a study where the same expression vector was used to express murine malarial MSP4/5s (Goschnick *et al.*, 2004; Kedzierski *et al.*, 2000a; Black *et al.*, 1999) and *P. falciparum* MSP4 (Wang *et al.*, 1999) in *E. coli*. These proteins did not react with the
anti-6xHis antibody, indicating that they are either C-terminally truncated products which have lost 6xHis tag, or *E. coli* contaminants. As these were always co-purified through a cobalt-charged IMAC, but not present in lysates purified through amylose resin or glutathione agarose resin (Kedzierski *et al.*, 2000a; Wang *et al.*, 1999), or when the protein was expressed in yeast system (Kedzierski *et al.*, 2000a), it was suggested that these unknown proteins of 23 and 27 kDa were in fact co-purified *E. coli* contaminants.

Since these contaminants were *E. coli* proteins, a yeast expression system was considered. It was thought that the six cysteine residues in the EGF-like domain of PcMSP4/5 was binding to these *E. coli* proteins, therefore expression in a yeast system may eliminate these *E. coli* proteins by producing recombinant protein in a form closer to the native conformation of PcMSP4/5. Upon reduction by β-mercaptoethanol, these proteins separate from PcMSP4/5 as the reducing agent breaks the disulphide bonds within the protein. However, the *E. coli* derived MSP4/5 can provoke better protection than yeast derived MSP4/5 in mice (Kedzierski *et al.*, 2001), therefore expression of PcMSP4/5 in *E. coli* expression was preferred for the purpose of the *in vivo* trial performed in study.
4.4.3 PcMSP4/5 nanocapsule synthesis

4.4.3.1 Recombinant PcMSP4/5 for nanocapsule synthesis

This section describes the synthesis of recombinant PcMSP4/5 nanocapsule. Successfully expressed and purified recombinant PcMSP4/5 from the previous section was used to infiltrate the SC/MS templates. Recombinant PcMSP4/5 has a relatively low pI, allowing better electrostatic attraction to the positively charged SC/MS templates than proteins of higher pI. Ovalbumin and recombinant PcMSP4/5 share a similar pI, therefore a high loading efficiency of PcMSP4/5 PBS was expected.

The theoretical molecular weight of recombinant PcMSP4/5 was predicted to be 16.8 kDa, which is less than half of what was detected on SDS-PAGE when purified protein was separated. Although it was due to its slow mobility on the SDS-PAGE gel, PcMSP4/5 was most likely bound to two *E. coli* proteins in their native form; therefore the total molecular mass was expected to be much higher than 16.8 kDa. This made the PcMSP4/5-complex sizing close to that of ovalbumin (44 kDa), which might have also aided in increasing the infiltration efficiency. It was shown in Chapter 3, that the ovalbumin formed a complete capsulated structure when observed under TEM, therefore a capsule structure with similar wall thickness was expected for PcMSP4/5 nanocapsules.

4.4.3.2 Quantification of synthesised recombinant PcMSP4/5 nanocapsules

Difficulties were encountered with the quantification of synthesised nanocapsules. The concentration was unable to be determined using commonly used protein determination methods such as UV-vis spectrophotometry or Bradford assay. Estimation by SDS-PAGE was also not possible due to the large molecular mass of the nanocapsules. GA cross-linked nanocapsules retained their structural integrity despite treatment with reducing agents. Nanocapsules were unable to enter the SDS-PAGE gel and remained intact in the well. GA cross-linked nanoparticles can be digested with simple enzymes (Leo et al., 1997; Lin et al., 2001), with the exception of cross-linked nanoparticles which may be too rigid (Babin et al., 2013). This may reduce the level of the peptide’s availability by cellular proteases for presentation in antigen presenting cells. Therefore, a balance between nanocapsule integrity
and ease of digestion by an optimal GA concentration needs to be made.

4.4.3.3 Cytotoxicity of MSP4/5 nanocapsules

The cytotoxicity of synthesised PcMSP4/5 nanocapsules was evaluated using DC2.4, a murine dendritic cell. Surprisingly, the soluble PcMSP4/5 had a significantly higher toxicity level compared to the PcMSP4/5 nanocapsules. This was totally opposite to the effects observed with ovalbumin nanocapsules, which displayed more toxic effect on cells while the soluble ovalbumin had none. A higher toxicity level of soluble PcMSP4/5 may be due to the presence of *E. coli* endotoxin in the purified product. Lipid A is the hydrophobic lipid region of bacterial LPS and is responsible for its toxicity (Rietschel *et al.*, 1994). Lipid A may be infiltrated and cross-linked within the protein layer of nanocapsule, causing it to have less exposure to the cells compared to the level in soluble state. However, this is highly unlikely due to the extensive washes given during the purification process.

To eliminate any possible cytotoxic effects of endotoxin in the synthesised protein nanocapsules to use as an antigen delivery system, the removal of endotoxin from the final product purified from *E. coli* must be considered.
4.5 Conclusion

Findings from this work determined that the SC/MS nanoparticle templating system is suitable for proteins, therefore an antigenic protein was used to synthesise nanocapsules in this section of study, in order to prepare for *in vivo* immunisation study. Firstly, *P. chabaudi adami* MSP4/5 (PcMSP4/5) was successfully expressed as a soluble protein in *E. coli* BL21(DE3)pLysS and purified using IMAC.

The purified recombinant PcMSP4/5 co-eluted with two *E. coli* proteins, resulting >70% purity. However, the purpose of the next phase of study is to make a comparison between two different delivery systems, rather than determining the efficacy of the antigen. Therefore, it was decided that PcMSP4/5 with this level of purity was sufficient to continue.

Secondly, PcMSP4/5 was applied to the templating system, and subsequently synthesised PcMSP4/5 nanocapsules showed minimal toxicity effects on DCs. Therefore PcMSP4/5 nanocapsules were deemed suitable for the use in an animal model in the next phase of study.
CHAPTER 5

Evaluation of protein-based nanocapsules as a delivery system in an animal model

This chapter reports the comparative study of the in vivo effect of the subunit and nanocapsule vaccines in immunised mice.
5.1 Introduction

Following the *in vitro* characterisation of cellular uptake and presentation of the ovalbumin-based nanoparticles using murine dendritic cells (DCs) as described in Chapter 3, this study outlines the final experiments performed to investigate the potential of protein-based nanocapsules as a new antigen delivery system.

As shown in Chapter 3, the ovalbumin-based nanocapsule synthesised using the SC/MS silica template successfully localised intracellularly in less than 30 min of incubation with DCs. Accordingly, these endocytosed ovalbumin-based nanocapsules were processed and the ovalbumin peptides were presented in context with the MHC I molecule on DCs via the cross-presentation pathway. This indicated that the protein-based nanocapsules are possibly capable of activating CD8$^+$ T cells (Segura et al., 2009). Primed CD4$^+$ T cells can divide into two different subsets, known as T helper 1 (Th1) and T helper 2 (Th2) cells (Mosmann et al., 1986; Constant & Bottomly, 1997). Each subset can effectively influence immune responses by secreting the cytokines associated with cellular and humoral responses, respectively. Up-regulation in Th1 cells can indicate induction of the cellular responses. In order to surpass the subunit vaccine, which primarily induces a humoral response, the induction of such cellular responses from the protein-based nanocapsule vaccine may be observed.

The activation of cellular responses by different types of nanoparticles either used as a vaccine or adjuvant has been previously documented in many reports (Emeny et al., 2002; Uto et al., 2009; Keller et al., 2014; Chong et al., 2005), and increased secretion of IFN$\gamma$ was evident. While various nanoparticles were shown to be phagocytosed by DCs and macrophages (Lutsiak et al., 2002; Choi et al., 2010; Carney et al., 2012; Akagi et al., 2007; Uto et al., 2013), the influence of particle size on the type of induced immune response is debatable (Gutierro et al., 2002; Reddy et al., 2006; Kanchan & Panda, 2007).

The study described in this chapter compared the immunogenicity of two different delivery systems using the recombinant PcMSP4/5 and the recombinant PcMSP4/5 nanocapsules, obtained as described in Chapter 4. It also determined the efficacy of two differently
formulated nanocapsule vaccines, by using the recombinant PcMSP4/5 nanocapsules formulated with the presence or absence of complete Freund’s adjuvant, as vaccines. The magnitude of humoral and cellular response was measured by detecting the level of antigen-specific antibodies and cytokines, as well as the population of induced memory cells.
The aim of the experiments outlined in this chapter were:

- To use the recombinant PcMSP4/5 and the recombinant PcMSP4/5 nanocapsules as vaccines in the C57BL/6 mouse strain.
- To determine the level of serum antigen-specific antibodies, more specifically IgG and IgG1, by indirect enzyme-linked immunosorbent assay (ELISA).
- To determine the concentration of antigen-specific cytokines, more specifically IFNγ, IL-2, IL-4 and IL-10 from the restimulated mouse splenocytes, by sandwich ELISA.
- To determine the number of proliferated antigen-specific memory cells within the restimulated mouse splenocytes using flow cytometry.
5.2 Materials and Methods

All experiments involving animals were carried out with the approval of the RMIT Animal Ethics Committee (AEC permit number 1319).

5.2.1 Immunisation protocols

This section details the immunisation studies in mice to evaluate the efficacy of different types of vaccine delivery systems.

5.2.1.1 Animal model

Six week old female C57BL/6 mice were used. Four mice per group were maintained in a single housing unit. In total, 16 mice were immunised. Mice were bred and sourced from the Animal Resource Centre, Canningvale, Western Australia.

5.2.1.2 Immunisation groups

Four mice were randomly allocated per immunisation group. The detail of vaccine formulation is listed in Table 5.1.

5.2.1.3 Protein preparation

The antigenic protein, recombinant PcMSP4/5 was purified as described in Section 4.2.2.2.

5.2.1.4 Nanocapsule preparation

The recombinant PcMSP4/5 nanocapsule was synthesised as described in Section 4.2.2.3.
Table 5.1: Immunisation groups and the vaccine formulation used.

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccine formulation</th>
<th>Adjuvant</th>
<th>Number of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PBS</td>
<td>✓</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Soluble recombinant PcMSP4/5</td>
<td>✓</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>PcMSP4/5 nanocapsules</td>
<td>✓</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>PcMSP4/5 nanocapsules</td>
<td>✗</td>
<td>4</td>
</tr>
</tbody>
</table>

*✓ = added, ✗ = not added.*
5.2.1.5 Preparation of vaccines

Sterile PBS (negative control), and the PcMSP4/5 or the PcMSP4/5 nanocapsules in PBS were used. PBS, PcMSP4/5 or PcMSP4/5 nanocapsules for three separate groups were combined with an equal volume of Freund’s adjuvant. PcMSP4/5 nanocapsules for one group was maintained in PBS without adjuvant. Each vaccine was made up to a total volume of 100 µL per mouse. To minimise the loss of prepared vaccine, a volume equivalent to double the number of doses required was prepared, e.g. if 4x100 µL doses were needed, 800 µL of vaccine was prepared. Vaccines were prepared in a biosafety cabinet class II using sterile materials. An emulsion was made by mixing the adjuvant and protein/PBS using two glass syringes connected with a sterile plastic stopcock, using around 300 mixing repetitions. The emulsion was tested by injecting a single drop of the mixture into a beaker of tap water for the formation of a stable ball.

Mice were immunised twice, the first immunisation was emulsified in complete Freund’s adjuvant and the subsequent immunisation was emulsified in incomplete Freund’s adjuvant.

5.2.1.6 Vaccination

Mice were given an intraperitoneal injection of a total vaccine volume of 100 µL containing 15 µg of protein, using a 25-gauge needle. The animals were monitored following the injection for at least 4 h. Two vaccinations were timed two weeks apart.

5.2.1.7 Termination of experiment

Mice were killed by cervical dislocation and organs were collected immediately for immunological assays.

5.2.1.8 Serum collection

Once mice were killed, an incision was made in the abdomen and diaphragm to visualise the heart, and blood was collected from the left ventricle using a 19-gauge needle. Blood was
centrifuged at maximum speed for 3 min and the sera supernatants were transferred to a clean tube. Serum was stored at 4°C for immediate use, otherwise at −20°C for longer storage.
5.2.2 Immunoassay protocols

This section describes the post-trial analysis. Collected serum samples were subjected to ELISA assays to measure humoral antibody responses. Spleens were removed and the cellular cytokine response from the isolated lymphocytes was quantitatively analysed. Proliferation of splenocytes in response to PcMSP4/5 was also assessed.

5.2.2.1 Measurement of humoral responses by serum antibody detection

5.2.2.1.1 Indirect ELISA

Indirect ELISA was performed to measure antibody production. This protocol was used to measure the level of IgG and IgG subtype - IgG1.

Ninety-six-well flat-bottomed sterile plates were coated with 100 µL of 2 µg/mL purified PcMSP4/5 in ELISA Coating buffer and incubated at 4°C overnight. Coating buffer alone was added to two wells to serve as a blank. Two wells of the first row of the plate were used for each of the following controls: no antigen (coating buffer only), no blocking, no antisera, and no secondary antibody. All other ELISA steps were performed on each of the control wells. All samples were tested in duplicate. Coating buffer and unbound protein were removed by inverting the plate with three washes in PBS/Tween and patted dry on absorbent paper towelling, then blocked with 200 µL blocking buffer for 1 h at 37°C. After incubation, the plate was washed and patted dry as above, and 100 µL of two-fold serially diluted antisera was added to the blocked wells. Vaccine trial antisera were diluted in PBS/Tween with skim milk (1% w/v) in doubling dilutions performed in a separate 96-well plate. The plate was incubated at 37°C on an ELISA shaker platform for 2 h. Unbound serum was removed and the plate was washed as above and patted dry. Detection antibody conjugated to enzyme was diluted in PBS/Tween with skim milk (1% w/v) and 100 µL was added to each well. Following 1 h incubation at 37°C on a shaking platform, the plate was washed four times with PBS/Tween followed by two washes with PBS, then patted dry. Substrate was added in 100 µL to each well and the colour development was allowed to proceed for up to 30 min and the reaction was stopped by the addition of 50 µL of substrate-specific reagent. Absorbance was recorded at the wavelength appropriate for each assay using the POLARstar Omega
microplate reader.

5.2.2.1.2 IgG indirect ELISA

Alkaline phosphatase (AP)-conjugated rabbit anti-mouse IgG polyclonal antibody was used to detect the level of IgG in the collected serum. Working dilution used for detection was 1:5000. p-nitrophenyl phosphate (pNPP) was used as a substrate and the reaction was stopped with the addition of 3M NaOH. Absorbance at 405 nm was recorded.

5.2.2.1.3 IgG1 indirect ELISA

Horseradish peroxidase (HRP)-conjugated rat anti-mouse IgG1 monoclonal antibody was used to detect IgG subtype level in the collected serum. The working dilution used for detection was 1:750. 3,3′,5,5′-tetramethylbenzidine (TMB) was used as a substrate and the reaction was stopped with the addition of 1M HCl. Absorbance at 450 nm was recorded.

5.2.2.2 Measurement of cellular responses by ex vivo restimulation

5.2.2.2.1 Counting cell number using an automated machine

The number of cells was counted using a Countess® Automated Cell Counter. Ten microlitres of either neat, or diluted cell sample was mixed with 10 µL of 0.4% Trypan Blue Dye in a sterile tube, then 10 µL of mixture was transferred into a Countess® chamber slide. All samples were processed and read within 2 min. The actual volume counted was 0.4 µL, the same as counting four (1 mm x 1 mm) squares in a standard haemocytometer.

5.2.2.2.2 Collection of spleen cells

Mice were sacrificed and their spleens collected in sterile RPMI GlutaMAX™ 1640 with 10% heat inactivated FBS, 100 U/mL of penicillin and 100 µg/mL streptomycin. Spleens were placed in petri dishes, crushed with the end of a 3 mL syringe and resuspended in a final volume of 3 mL of the same media, and filtered through a 100 µm cell strainer.
5.2.2.3 Isolation of splenocytes from buffy coats by Ficoll density centrifugation

Splenocytes were isolated from the spleen cell suspension by a standard Ficoll density separation method, as per the manufacturer’s instructions. The filtered cell suspension was slowly added to a tube containing 3 mL of Histopaque-1077 with minimal disturbance. Following centrifugation at 400 x g for 20 min at room temperature without the break stop, the upper layer was carefully removed with a sterile Pasteur pipette to within 0.5 cm of the opaque interface containing splenocytes. The opaque interface was transferred to a clean tube with a fresh Pasteur pipette and washed in 10 mL of sterile DPBS solution by centrifugation at 250 x g for 10 min at 4°C. This process was repeated once more under the same conditions and resuspended in a final volume of 1 mL of DPBS. Recovered cells were counted using a Countess® Automated Cell Counter.

5.2.2.4 Labelling of lymphocytes with carboxyfluorescein diacetate succinimidyl ester (CFSE)

The number of splenocytes were counted and adjusted to 1.2 x 10^7 cells/mL in a final volume of 3 mL and warmed briefly in a 37°C water bath. Three millilitres of freshly prepared 5 µM CFSE staining solution in DPBS was also warmed and combined with the 3 mL of warm cell suspension, immediately followed by gentle mixing by inverting the tube. The mixture was incubated in a CO₂ incubator at 37°C for 15 min with occasional mixing to ensure proper staining. The staining mixture was washed twice in 10 mL DPBS by centrifugation at 400 x g for 5 min at room temperature and resuspended in 3 mL of media.

5.2.2.5 Restimulation of splenocytes

CFSE-stained splenocytes were re-counted and seeded in 24 well plates at the final cell density of 1 x 10^6 cell per well. Cells from each mouse were seeded in quadruplicate in a final volume of 1 mL and restimulated in vitro in the presence or absence of recombinant PcMSP4/5. The first well was restimulated with 2 µg/mL Concanavalin A (Con A) to elicit a positive response. The second and third wells were restimulated with 10 µg/mL of the purified recombinant PcMSP4/5 prepared as described in Section 4.2.2.2, and the fourth well
was unstimulated and served as an internal control. Splenocytes were incubated for 3 days at 37°C in a 5% CO₂ humidified atmosphere. Following 65 h incubation, culture supernatants were collected for cytokine level measurement. Freshly prepared media with 20 IU/mL recombinant mouse IL-2 was added and incubated for a further 2 days.

5.2.2.2.6 Cytokine profiling - ELISA

Collected culture supernatants were subjected to ELISA to quantify the level of IFNγ, IL-2, IL-4 and IL-10 using the Affymetrix eBioscience Mouse Th1/Th2 ELISA Ready-SET-Go® kit, as per the manufacturer’s instructions. Mean values were expressed in pg/mL derived from standard curves performed with the corresponding murine recombinant cytokine standards.

5.2.2.2.7 Splenocyte proliferation assay

The ability of memory cells to induce cell proliferation was assessed in response to in vitro restimulation. CFSE-stained splenocytes were restimulated in presence or absence of recombinant PcMSP4/5 as described in Section 5.2.2.2.5. After 5 days of culture (3 days with mitogens and 2 days with IL-2 supplement), cells were harvested by centrifugation at 300 x g for 5 min at room temperature. Cells were washed once in a 96 well plate with 200 µL of chilled FACS wash buffer. Resuspended cells were fixed with 1% PFA and analysed for proliferation using the FACS Canto II cytomter. The level of relative fluorescence intensity of CFSE was measured at 490 nm. The cytometer setting for detection of the FITC label was used for the wavelength settings.

5.2.2.2.8 Statistical analysis

Statistical variables (mean, standard deviation, and standard error), were calculated for all data and presented graphically. Comparisons between groups were accessed by one-way ANOVA followed by a Tukey’s multiple comparison test to determine significant differences between groups. GraphPad Prism 6 software was used to perform the statistical analysis.
For cytokine levels, a Dunnett’s multiple comparison test was used to determined the significant differences between the immunised groups against the control (PBS with adjuvant) group. A P value of 0.05 was used to determine the significance.
5.3 Results

5.3.1 Humoral responses against the PcMSP4/5 vaccines

5.3.1.1 Levels of antigen-specific IgG responses

Mice were immunised by i.p. injection with 15 µg of PcMSP4/5 in different formulations as listed in Table 5.1. One group was given subunit PcMSP4/5 adjuvated with Freund’s adjuvant, while two other groups were given PcMSP4/5 nanocapsules in the presence or absence of Freund’s adjuvant. Serum samples were collected three weeks after the final vaccination and tested for humoral immune responses by detecting the levels of MSP4/5-specific IgG antibodies.

Antigen-specific IgG titres for individual mice were determined by indirect ELISA. Sera obtained from immunised mice were used as the primary antibody, which bound to PcMSP4/5 pre-coated plates. Enzyme-conjugated detection antibodies were used to give a colorimetric reaction to obtain absorbance readings in order to interpolate the titre. The titre cut-off point was determined based on the mean and 3 standard deviations from the negative control readings. IgG levels were expressed as a logarithm of the titre.

Serum analysis from individual mice is shown in Figure 5.1. It revealed that immunisation with the subunit vaccine induced very high levels of PcMSP4/5-specific IgG in 75% of the group, exceeding a titre of 60,000, with the highest titre measured at 128,000. PcMSP4/5-specific IgG production was also detected in groups immunised with PcMSP4/5 nanocapsules. It was detected in 100% of the group vaccinated without adjuvant with the highest titre being 3,200, however only 25% produced IgG in the group vaccinated with adjuvant, with a titre of 400. The result depicted in Figure 5.1 indicates that humoral responses were preferably induced by adjuvated subunit vaccine or nanocapsules alone.
Figure 5.1: Titre of IgG in individual immunised mice. PcMSP4/5 specific-IgG level from individual mice was tested from collected serum. Measurable responses were observed in the groups in which PcMSP4/5 was delivered. No response was observed in the PBS control group.
The mean titre for each group was calculated and presented in Figure 5.2. While there was no significant difference between the two nanocapsule groups, IgG levels in both groups were significantly lower when compared to those of the subunit vaccine group (p<0.05). The mean titre of the subunit vaccine group was 64,250, 640-fold higher than the adjuvated nanocapsule group (titre = 100), and 45-fold higher than non-adjuvated nanocapsule group (titre = 1,400).

5.3.1.2 Levels of antigen-specific IgG subtype composition

To determine whether the production of IgG was influenced by Th2 response, the level of specific IgG1 in individual mice sera was determined and the mean titre was correlated as shown in Figure 5.3. High levels of IgG1 were detected in mice immunised with the subunit vaccine, and some level of IgG1 detected in mice immunised with nanocapsules without adjuvant. Subunit vaccine and the non-adjuvated nanocapsule groups had an average titre of 25,600 and 1700, respectively. A significantly high level of IgG1 from the subunit vaccine group, compared to the PBS control, was expected from the result observed for IgG (p≤0.0001). Statistical differences were also observed in the levels of IgG1 between the non-adjuvated nanocapsule groups and the PBS control group (p≤0.001).

Low level of IgG1 was also detected in the adjuvated nanocapsules group, however this may be due to the non-specific binding of anti-IgG1 antibodies to the saturated serum, tested at a 1:20 dilution. Similar titre was also observed in the PBS control group. As the mice in PBS control group never received PcMSP4/5, non-specific binding of anti-IgG1 antibodies was suspected.

IgG2a level was also determined, however no detectable levels were measured by ELISA analysis (data not shown).
Figure 5.2: Titre of IgG in immunised groups. Mean titre for PcMSP4/5-specific IgG in each group was determined. Statistical differences were analysed by a Tukey’s test. (Statistical significance against 100% viable control group is represented in asterisks. * is p≤0.05, ns = no significant difference).
Figure 5.3: Mean titre of IgG1 in immunised groups. Mean titre for PcMSP4/5 specific-IgG1 in each group was determined. Statistical differences were analysed by a Tukey’s test. (Statistical significance against PBS group is represented in asterisks. ★★ is p≤0.01, ★★★ is p≤0.001 and ★★★★ is p≤0.0001).
5.3.2 Cellular responses against the PcMSP4/5 vaccines

5.3.2.1 Levels of antigen-specific cytokine production

The production of four different cytokines by restimulated splenocytes was measured in the culture supernatant by sandwich ELISA. The concentrations of IFN\(_\gamma\), IL-2, IL-4 and IL-10 in each immunised group were assessed, and the mean values were expressed in pg/100 \(\mu\)L, derived from standard curves performed with the corresponding mice recombinant cytokine standards. The concentrations depicted in the figures were expressed per 100 \(\mu\)L, as this was the volume of culture supernatant subjected to ELISA.

An elevated IFN\(_\gamma\) level was observed in all groups given PcMSP4/5 (Figure 5.4). Although the mean value for the non-adjuvated nanocapsule group was lower and the distribution of detected concentration was very wide, one mouse actually produced the highest amount of IFN\(_\gamma\) in the whole experiment (1120 pg/100 \(\mu\)L). The statistically significant difference to the PBS control group was found with the subunit and adjuvated nanocapsule groups (\(p \leq 0.05\)), with a mean concentration of 878 pg/100 \(\mu\)L and 843 pg/100 \(\mu\)L, respectively.

The levels of IL-2 between all four immunised groups were significantly different (\(p=0.0002\)). The production of IL-2 in the groups immunised with any form of PcMSP4/5 was elevated in a way similar to that of IFN\(_\gamma\). As such, a significantly higher level of IL-2 compared to the PBS control group was detected in both subunit and adjuvated nanocapsule groups (\(p \leq 0.01\) and \(p \leq 0.001\), respectively), and an increase was also observed in the non-adjuvated nanocapsule group (Figure 5.5). The mean concentration of IL-2 detected in the adjuvated nanocapsule group was 80.8 pg/100 \(\mu\)L, a somewhat higher level than in the subunit group at 62.5 pg/100 \(\mu\)L. The level of IL-2 was significantly lower when nanocapsules were given without the use of adjuvant (\(p \leq 0.01\)).
Figure 5.4: IFNγ production by immunised groups. Supernatant concentrations of IFNγ, produced by splenocytes restimulated *in vitro* in the presence or absence of recombinant PcMSP4/5. Statistical differences were analysed by a Dunnett’s test. (Statistical significance against PBS group is represented in asterisks. * is p≤0.05).
Figure 5.5: IL-2 production by immunised groups. Supernatant concentrations of IL-2, produced by splenocytes restimulated in vitro in presence or absence of recombinant PcMSP4/5. Statistical differences were analysed by a Dunnett’s test. (Statistical significance against PBS group is represented in asterisks. * is p≤0.05, ** is p≤0.01 and *** is p≤0.001).
Despite the strong responses observed in the other cytokines, no measurable level of IL-4 was detected in any group (Figure 5.6). A high concentration of IL-4 was measured from the Con A positive group; therefore it showed that the assay system was valid to use.

While the level of the Con A positive control was very low, the PBS negative control group indicated an increased level of IL-10 concentration (Figure 5.7). The mean concentration of PBS, subunit and the adjuvated nanocapsule groups were very similar, and were 1,989 pg/100 μL, 2,559 pg/100 μL and 2,339 pg/100 μL, respectively. In comparison to the level of IL-10 detected in the subunit group, which was the highest amongst all groups, the non-adjuvated nanocapsule group produced IL-10 at a significantly lower level (p<0.05). However the mean concentration of IL-10 in this group was 1,059 pg/100 μL, which was almost double that produced in the Con A-stimulated cells (564.3 pg/100 μL).
Figure 5.6: IL-4 production by immunised groups. Supernatant concentrations of IL-4, produced by splenocytes restimulated *in vitro* in presence or absence of recombinant PcMSP4/5.
**Figure 5.7: IL-10 production by immunised groups.** Supernatant concentrations of IL-10, produced by splenocytes restimulated *in vitro* in presence or absence of recombinant PcMSP4/5. Statistical differences were analysed by a Dunnett’s test. (Statistical significance against PBS group is represented in asterisks. ✻ is $p \leq 0.05$).
5.3.2.2 Levels of antigen-specific cell proliferation

The induction of memory T cells was assessed in vitro by measuring the proliferation of splenocytes in response to recombinant PcMSP4/5. Isolated splenocytes were stained with CFSE, which was retained within the cells to monitor cell division by measuring the progressive halving of the CFSE fluorescence within daughter cells. The stained cells were restimulated in vitro for 3 days, with or without the presence of the recombinant PcMSP4/5, and incubated in fresh media containing the recombinant mouse IL-2 for a further 2 days to allow continuous cell division.

Firstly, a population of splenocytes with forward scatter ranging from 30,000 to 240,000 was gated (Figure 5.8A). Secondly, the level of relative fluorescence intensity of CSFE was measured at 490 nm. The cytometer setting for the detection of the FITC label was used for the wavelength settings, as CFSE fluoresced at the same wavelength. The number of cells at different fluorescence intensity was displayed in a histogram. The unstimulated cells were set as a baseline (red), to make comparisons to the stimulated cells (blue) (Figure 5.8B-F). The percentage of the proliferated cell population was evaluated and is shown in Figure 5.9.

Proliferation was observed when splenocytes were restimulated in vitro with the recombinant PcMSP4/5, while unstimulated cells produced no proliferating cells as expected. While Con A-stimulated cells produced a large number of proliferated cells (Figure 5.8B), the splenocytes isolated from the subunit vaccine group also had a notably large population (Figure 5.8D). The percentage population of proliferated cells within the subunit group was 8.8%, which was very close to that of Con A-stimulated cells, with 11.1% (Figure 5.9). The group immunised with nanocapsule with adjuvant showed increased proliferated population of 7.6% (Figure 5.8E, and Figure 5.9), however, the same population was also observed in the PBS control group (Figure 5.8C). The PBS control group never received the recombinant PcMSP4/5, yet 6.3% of splenocytes were observed to have proliferated (Figure 5.9). In contrast, the group immunised with nanocapsule without adjuvant, had the smallest population of proliferated cells at 3.7% (Figure 5.8F), while cells from the PBS control group induced higher levels of proliferation. This was unexpected, as PcMSP4/5 was never introduced to mice in the PBS control group.
Figure 5.8: Proliferation of mouse splenocytes, restimulated *in vitro* with PcMSP4/5.

A) Lymphocyte population was gated. The percentages of proliferated lymphocytes in each vaccination groups (B-F) are represented in the blue open histogram, while the red open histogram represents unstimulated cells. The Y-axis represents the number of cells detected for each group. The X-axis represents the level of relative fluorescence intensity of CFSE measured at 490 nm. The cytometer setting for detection of FITC label was used for the wavelength settings.
Figure 5.9: Percentage of proliferated mouse splenocytes, restimulated *in vitro* with PcMSP4/5. Percentage of population calculated based on the unstimulated cells.
5.4 Discussion

Significantly high levels of IgG were detected in mice immunised with the subunit PcMSP4/5 emulsified with Freund’s adjuvant, or when immunised with the PcMSP4/5 nanocapsules with no adjuvant. The ability of recombinant PcMSP4/5 to elevate antibody production was expected, since in previous studies the recombinant *P. yoelii* MSP4/5 expressed in *E. coli* showed a significant increase in the level of antibodies in mice (Kedzierski *et al.*, 2000b; 2001). High level of IgG1 was detected in these two groups, while no measurable level of IgG2a was detected in either group (data not shown).

It has been found that IgG2a cannot be detected in C57BL/6 mouse sera, as C57BL/6 mice express a different IgG subtype due to deletion of the allele for IgG2a gene (Martin & Lew, 1998; Martin *et al.*, 1998). The corresponding antibody for IgG2a in C57BL/6 mice is IgG2c, however ELISA analysis was still attempted using anti-IgG2a. The detection of IgG2c was unsuccessful, as might have been expected. The differences in amino acid sequence between IgG2a and IgG2c is 16%, therefore the commonly available anti-IgG2a sera probably could not cross-react with IgG2c.

Previously reported BALB/c mouse immunisation trial showed that *P. yoelli* MSP4/5 induced higher level of IgG2a production compared to IgG1, when MSP4/5 was given i.p. vaccine with Freund’s adjuvant (Kedzierski *et al.*, 2001). Given that a similar protein was used for immunisation in the same formula and vaccination method, IgG2c may have been increased in mice from the subunit group. Contradictory to their result, higher level of IgG1 production than IgG2a was recorded in another study, when *P. falciparum* MSP4 and MSP5 were given subcutaneously in the same vaccine formulation (Bracho *et al.*, 2009). However, the site of vaccine administration may have influenced the immune response (Yip *et al.*, 1999).

Antibody class switches from the initially produced IgM to different Ig and IgG subtypes upon maturation of B cells (Coffman *et al.*, 1993), and this can be influenced by the cytokines secreted from CD4⁺ T cells (Stavnezer & Amemiya, 2004). Th1 cells secreting IFNγ can induce an immunoglobulin class switch to IgG2a, while IL-4 secreted from Th2 cells induces
an IgG1 class switch (Snapper & Paul, 1987; Sideras & Lindqvist, 1985). More recently, it was documented that IFNγ secreted by the activated CD8⁺ T cells can also induce Ig class switching to IgG2a (Mohr et al., 2010). Therefore, the secretion of IFNγ and IL-4 is an indication of either Th1 or Th2 responses elicited in mice, respectively (Constant & Bottomly, 1997; Mosmann et al., 1986).

Although the production of IgG2c could not be determined, a high level of IgG1 was detected in mice from the subunit group and the adjuvated nanocapsule group. This suggested that there were some degree of Th2 bias in the immune response, therefore an elevated level of IL-4 was anticipated for the cytokine ELISA analysis in these groups. However, no production of IL-4 was found in any of the immunised groups. The animal used for this trial was mouse C57B/6 strain, which is known to preferentially produce Th1 cytokines with high IFNγ and low IL-4 (Mills et al., 2000). The use of this Th1-biased mouse could possibly be the reason that no detectable level of IL-4 was produced (Diwan et al., 2002). IL-10 is another cytokine secreted by Th2 cells, which can also induce IgG class switching to IgG1 (Malisan et al., 1996). Increased level of IL-10 in subunit group and nanocapsule group without adjuvant was in line with the high IgG1 level detected, however no conclusion could be made to indicate the influenced of Th2 cells, due to the high amount of IL-10 observed in PBS control group.

In contrast, the nanocapsules emulsified with Freund’s adjuvant did not produce any IgG or IgG subtype, but produced significantly higher levels of IFNγ and IL-2. Furthermore, the IL-2 production was elevated at much higher level than that of the subunit group, and also significantly higher than the non-adjuvated nanocapsules (p≤0.01). In consideration with the IgG level found and the elevation in IFNγ and IL-2 levels, it was suggested that the immune response induced in mice from the adjuvated nanocapsule group was most likely mediated by the cellular response involving Th1 cells.

The differences in the use of adjuvant with nanocapsules were contrary. The complete Freund’s adjuvant used for this experiment was composed of inactivated mycobacteria, which is known to promote the proliferation of CD4⁺ T cells and skew to Th1 responses by various
mechanisms (Reviewed in Billiau & Matthys, 2001). The mycobacteria in the adjuvant contains several active components including LPS and the unmethylated CpG oligodeoxynucleotides, which are pathogen-associated molecular patterns (PAMPs). PAMPs are found on non-mammalian cells and recognition by TLRs can provoke innate immunity to mediate against the invasion of a microbial pathogen (Underhill & Ozinsky, 2002; Kopp & Medzhitov, 2003). The ligation of PAMPs to TLR can stimulate cells to increase the phagocytosis of exogeneous antigens (Redlich et al., 2013), in this case the nanocapsules, thereby the phagocytosis of nanocapsules enhanced and leads to T cell differentiation to Th1 cells. Therefore, the elevated level of IFNγ and IL-2 in the adjuvated nanocapsule group may be explained by the functionality of the complete Freund’s adjuvant. However, the elevation in IFNγ was probably not due to the adjuvant components alone, but rather a combination of adjuvant effect with the nanocapsules, resulting in the enhancement of the cellular response. This was supported by the significantly low level of IFNγ detected in the PBS control group.

The observation of IL-10 production between the immunised groups was very interesting. High level of IL-10 was detected in all groups, including the PBS negative control group, which was not expected, as this group did not receive any form of PcMSP4/5. The mean concentration of IL-10 from the PBS control group was as high as the other groups given the adjuvant. It was also higher than the concentration measured in the non-adjuvated nanocapsule group. PBS was given to mice with the addition of Freund’s adjuvant; therefore this could possibly be the reason for such a response. IL-10 is anti-inflammatory cytokine (Berger, 2000), therefore IL-10 may have been produced to counteract the inflammation possibly caused by the adjuvant. On the contrary, this hypothesis did not explain the low level of IL-10 indicated in the unstimulated control. The cells used for this control type were isolated from mice in the PBS control group, however no such elevation was observed. This also applied to the Con A control, which also exhibited low levels of IL-10. IL-10 functions to suppress inflammation caused by the Th1 response, by blocking IFNγ secretion. If high levels of IL-10 were secreted in response to the adjuvant, the level of IFNγ would also be expected to rise. However, a very low level of IFNγ was detected in the PBS control group. Furthermore, the production of cytokines in this assay was induced by the specific antigen used for immunisation. Therefore, any increase in the level of these cytokines should be PcMSP4/5-specific, and not due to the adjuvant.
A similar observation was made for cell proliferation, where the PBS control group had a high population of proliferated cells. Memory cells produced from immunisation were restimulated to proliferate specifically against the recombinant PcMSP4/5 in this assay. All groups given the adjuvant achieved a larger proliferated population, while the non-adjuvated nanocapsule group had half that detected in the PBS group. However, the unstimulated cells isolated from the PBS group mice showed differences in the proliferated cell population to the PBS group. It is unlikely that the increase of IL-10 and the proliferation of memory cells was solely attributed to the effect of the adjuvant. The mechanism behinds these results are not fully understood.
5.5 Conclusion

Immune response can be elicited by the antigen-based nanocapsules given by i.p. vaccine, while the humoral or cellular response is induced with or without the use of adjuvant. Significantly high level of IgG, possibly influenced by Th2 cells is achievable by immunisation with the antigen-based nanocapsules alone. While the strong cellular response with significantly high levels of IFNγ and IL-2 can be elicited by the antigen-based nanocapsules, in combination with adjuvant.

These findings suggest that the antigen-based nanocapsules may also be capable of promoting a stronger cellular response than the subunit vaccine when formulated with the adjuvant, by inducing a higher level of Th1-biased cytokine secretion.
Chapter 6

Concluding remarks
An ideal vaccine should be able to elicit strong humoral and cellular responses with a single dose without posing any risks associated with the attenuated vaccines reverting to being virulent (World Health Organization, 2014; Youngner et al., 1994). For this reason, subunit protein or peptide vaccines are a safer alternative to attenuated vaccines and many are considered to have potential as vaccine candidates (Rueckinger et al., 2011; Peltola et al., 1992; Georges et al., 2013; Purcell et al., 2007), however the immunogenicity is often suboptimal (Aguilar & Rodriguez, 2007). Therefore, novel antigen delivery methods are being investigated.

It has been demonstrated that the nanocapsule is a promising antigen delivery system to increase the efficacy of subunit vaccines, as they can stabilise the antigens and ensure delivery to intracellular compartments. Antigen delivered in a particulate form has been shown to facilitate more efficient cellular uptake by the APCs, and induced potent antigen-specific humoral response, in particular cellular responses by promoting a higher level of cross-presentation (Shen et al., 1997; Sexton et al., 2009; Kaba et al., 2012; Hirosue et al., 2010; Sneh-Edri et al., 2011; Yang & Hsu, 2008; Plebanski et al., 1998; Akagi et al., 2007).

A sacrificial template can provide greater mechanical stability to capsule formation during the preparation process (Cui et al., 2014). Highly monodispersed SC/MS silica nanoparticles are suitable as a nanocapsule template because of the unique properties of the MS. The structure of resulting nanocapsules can be tailored with minimal alteration to the structure of the MS, such as porosity and size (Goethals et al., 2013), and the fabrication of mesoporous silica nanoparticles is very simple, scalable and cost-effective (Tang et al., 2012). Well-defined surface chemistry can also functionalise the silica template to accommodate almost any antigen to be infiltrated to form the antigen nanocapsule. Most importantly, this templating approach eliminates the need of polymeric or lipid materials often used in the other nanoparticle system. The particles consisting of a single component (antigenic protein) is an attractive feature as a delivery system.

In this study, a templating method was utilised to synthesise antigen-based nanocapsules. This
method enabled the synthesis of nanocapsules with high homogeneity and stability by employing highly monodispersed SC/MS silica nanoparticles with an average diameter of 410 nm as a template (Stöber et al., 1968; Büchel et al., 1998). Ovalbumin, C27 H1N1 protein, \textit{P. chabaudi adami} MSP4/5-based nanocapsules were synthesised using minimal chemical complexity, with final particles consisting of only the cross-linked protein (Wang et al., 2008a). Negatively charged protein nanocapsules induced minimal toxicity to cells, alleviating the cytotoxic effects often associated with the cationic polymer particles. This method allowed a larger capacity of antigen to be loaded per nanoparticle in a single step compared to the currently existing nanocapsule preparation techniques, resulting with a thicker capsule wall fabricated using the large specific surface area of SC/MS nanoparticle (Yang & Hsu, 2008; Goethals et al., 2013).

DCs are the most potent professional APCs for the initiation of immune responses, and their ability to prime naïve T cells has been well studied (Steinman, 2007b; Villadangos & Schnorrer, 2007). It was shown that the synthesised ovalbumin nanocapsules were efficiently internalised by immature DCs in less than 30 min, and induced a significantly higher level of ovalbumin peptide to be processed via the cross-presentation pathway than incubation with soluble ovalbumin. This is a crucial finding, as targeting the cross-presentation pathway is the key to develop effective vaccines against tumors, intracellular parasites, intracellular bacteria and viruses (Rock et al., 1990; Moron et al., 2004). Increased levels of peptide presentation with MHC class I leads to the efficient induction of a cellular response by activating CD8$^+$ T cells (Sneh-Edri et al., 2011; Shen et al., 2006). In addition, internalised antigen may be also presented by MHC class II molecules to prime CD4$^+$ T cells to promote Th1 and Th2 biased cytokine secretion (Paul & Seder, 1994; Swain et al., 1990; Berger, 2000; Uto et al., 2013; 2009; Mohr et al., 2010).

The application of antigen-based nanocapsules as delivery system was further investigated in the immunization trial using a mouse model. Synthesised PcMSP4/5 nanocapsules were administered by i.p. injection twice with a two week interval. The PcMSP4/5 nanocapsule was able to produce significantly high levels of IgG without being formulated with adjuvant. The Ig class switch to IgG1 indicated that there was a Th2 influence in the immune response. A strong cellular response was also achievable with the PcMSP4/5 nanocapsules in

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combination with adjuvant, promoting significantly high levels of IFN\(\gamma\) and IL-2 compared to vaccination with soluble antigen formulated with adjuvant. This latter result suggests that the antigen-based nanocapsules may be capable of promoting a stronger cellular response than the subunit vaccine when formulated with the adjuvant, by inducing a higher level of Th1-biased cytokine secretion.

Conventional adjuvants such as Alum have been approved for human use however it is historically shown to elicit strong humoral responses with weaker cellular responses. More potent adjuvants that can induce stronger cellular responses are available, however their use in human is limited due to their high toxicity. This study showed that the antigen in a particulate form could potentially elicit strong cellular responses, possibly mediated by primed Th1 cells. Therefore, the inability of Alum to induce cellular responses may be overcome when co-delivered with antigen-based nanocapsules. The conjugation of various immunostimulating molecules such as PAMPs to the surface of antigen-based nanoparticle can be achieved by adsorption, chemical cross-linking or encapsulation (Diwan et al., 2002; Hermanson, 2013; Di Marco et al., 2010), to induce more efficient uptake and APC activation (Demento et al., 2011; Redlich et al., 2013). Particular subsets of DCs can be targeted by surface modification of nanocapsule to immobilise monoclonal antibodies (Mintern et al., 2013). The mechanical stability of the particles allows the conjugation of almost any materials to be co-delivered, and the possibility is broad.

Some pathogens such as malaria are difficult to vaccinate against due to antigenic variability during their complex life cycle (Su et al., 1995). This enables the pathogen to evade the host antigen-specific immune response. One approach to overcome this is to formulate vaccines with multiple antigens expressed by the pathogen. Multivalent vaccines are shown to be effective across different serotypes of Streptococcus pneumoniae and several viruses (Rueckinger et al., 2011; Atkinson et al., 2011). Employing this templating method can most likely cater the formation of multivalent nanocapsules by multiple antigens infiltration into the MS of nanoparticle template. This should be an area of future research.

The work conducted in this study demonstrated that the templating approach using the
monodispersed SC/MS silica nanoparticle is suitable to synthesise various types of antigen-based nanocapsules. The antigen-based nanocapsules were also able to be internalised and cross-presented on DCs with minimal toxicity, and induce both humoral and cellular responses in an animal model. Therefore this study highlighted the potential of the templating approach for the nanocapsule preparation technique to be used for the antigen delivery.

The factors that determine the type of immune response and the level of cross-presentation remain unclear in the field of particulate delivery system. Therefore, further studies need to be conducted to assess the effect of nanoparticle size on antigen presentation and the protective efficacy of the vaccines *in vitro* and *in vivo*, by fabricating various sizes of SC/MS templates. More precisely, ovalbumin nanocapsules at 50 nm (viral size range) and 500 nm (bacterial size range) should be tested in a mouse study, and pathogenic antigen-based nanocapsules to be used in studies where the immunised mice are challenged with an infectious agent.

The future work should also comprise further characterisation of protein-based nanocapsules. Nanocapsule stability at various temperatures is crucial to use as a vaccine. The degradation of protein-based nanocapsules incubated at different temperatures (i.e. -20°C, 4°C and room temperature) should be monitored over time (hours to days). Aggregation behaviour of nanocapsules should also be observed. In this way the potential of these particles as stable, efficacious vaccines can be assessed.
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8.1 Appendix 1

Figure A1: *PstI*-digested lambda DNA ladder. *PstI* digested-DNA separated by agarose gel electrophoresis with size and quantity of individual DNA fragments specified. Reproduced from Lambda DNA/*PstI* marker certificate of analysis (Fermentas).
8.2 Appendix 2

Figure A2: Protein molecular markers. A) Precision Plus Protein™ standard (unstained). Reproduced from Precision Plus Protein™ standard product specifications sheet (BioRad). B) Precision Plus Protein™ Dual Colour Standard. Reproduced from Precision Plus Protein™ Dual Color standard product specifications sheet (BioRad).